PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ANALYSIS OF WHEAT CULTIVARS UNDER BORON TREATMENTS

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ABSTRACT

PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ANALYSIS OF WHEAT CULTIVARS UNDER BORON TREATMENTS

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Many studies have described the physiological, biochemical and molecular responses to boron (B) toxicity and deficiency individually in plants. However, overall pattern of gene expression changes and their physiological and biochemical significances after high B, low B and supplementing artificial B-enriched fertilizer (Tarımbor) application have not been proposed up till now. In the present study, we assessed global changes in gene expression of two wheat cultivars Bolal-2973 and Atay-85 differing in their B responses by using Affymetrix wheat GeneChip under B-toxicity, deficiency and Tarımbor applications. Another objective of this study was to clarify physiological and biochemical changes that are mainly related to growth, photosynthesis, antioxidants, antioxidant enzymes, water status and oxidative damage under same conditions. The visual symptoms of B-toxicity, regarding chlorosis and necrosis, were only seen in leaf tips of Atay. Coordinately, B accumulates higher in Atay leaves than Bolal at the end of the B-toxicity. However, all B conditions did not cause a cessation of vegetative growth in leaf and root of both wheat cultivars. Genes for oxidative stress and detoxification of ROS were not

significantly regulated after all B conditions. This result was in accordance with the measurements of antioxidant enzyme activities. Genes related protein degradation were induced more under all B conditions in Atay than Bolal. These results considering programmed cell death in senescing leaves were confirmed by the visual phenotype of the plants. Our findings may introduce new targets for breeding researches aimed at improving B tolerance of crop plants.

Keywords: Wheat, Boron, Microarray analysis, Oxidative stress, Tarımbor.

ÖZ

BOR UYGULAMALARI ALTINDA BUĞDAY ÇEŞİTLERİNİN FİZYOLOJİK, BİYOKİMYASAL ve MOLEKÜLER ANALİZLERİ

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Bor (B) toksisitesi ve eksikliğine karşı fizyolojik, biyokimyasal ve moleküler cevapların ayrı ayrı açıklandığı birçok çalışma mevcuttur. Buna karşın, bitkilerde yüksek B, düşük B ve suni B-zengini gübre (Tarımbor) uygulamaları sonrasında gen ekspresyon değişimlerinin ve bu değişimlerin fizyolojik ve biyokimyasal anlamlarını kapsayan bir yaklaşım şimdiye kadar sunulmamıştır. Bu çalışmada ilk olarak biz, B toksisitesi, eksikliği ve Tarımbor uygulaması koşulları atında, B cevapları farklı olan Bolal-2973 ile Atay-85 buğday çeşitlerinin gen ekspresyonlarındaki global değişimleri Affymetrix buğday GeneChip kullanarak değerlendirdik. Bu çalışmanın bir diğer amacı, aynı koşullar altında başlıca büyüme, fotosentez, antioksidanlar, antioksidan enzimler, su durumu ve oksidatif zarara bağlı fizyolojik ve biyokimyasal değişimleri aydınlatmaktı. B toksisitesinin görsel belirtileri olan klorosis ve nekroz sadece Atay yaprak uçlarında görülmüştür. Bununla uyumlu olarak, B toksisitesi sonrasında Atay yapraklarında Bolal çeşidine göre daha fazla B birikmiştir. Bununla birlikte, tüm B koşulları her iki buğday çeşidine ait yaprak ve köklerin vejetatif büyümesinin durmasına yol açmamıştır. Tüm B koşullarını takiben, oksidatif stres ve

reaktif oksijen türlerinin (ROS) detoksifikasyonu ile ilgili genler anlamlı bir şekilde düzenlenmemiştir. Bu sonuç, antioksidan enzim aktivite ölçümleri ile uyumlu bulunmuştur. Atay çeşidinde protein degradasyonu ile ilgili genler tüm B koşulları altında Bolal çeşidine göre daha fazla uyarılmıştır. Senesens geçiren yapraklardaki programlı hücre ölümü ile ilişkilendirilen bu sonuçlar, bitkilerin görsel fenotipi ile doğrulanmıştır. Bulgularımız, tahıl bikilerinin B toleransını geliştirmeyi amaçlayan çaprazlama çalışmaları için yeni hedefler sunabilir.

Anahtar kelimeler: Buğday, Bor, Mikroarray analizi, Oksidatif stress, Tarımbor

To my wife Doğa and my mom

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APX	Ascorbate peroxidase
aRNA	Amplified RNA
ATP	Adenosine triphosphate
B	Boron
H_3BO_3	Boric acid
C	Control
Ca	Chlorophyll a
CAT	Catalase
Cb	Chlorophyll b
cDNA	Complementary DNA
Cx+c	Carotenoid
CYP	Cytochrome P450
D	B-deficiency
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
ETR	Electron transport rates
F_M	Maximum fluorescence
F_o	Minimum fluorescence
GA	Gibberellic acid
GDH	Glutamate dehydrogenase
GFP	Green fluorescent protein
GO	Gene ontology
GR	Glutathione reductase
GST	Glutathione S-transferase
JA	Jasmonate
MDA	Malondialdehyde
MIP	Major intrinsic protein

NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NIP	Nodulin 26-like intrinsic protein
NPQ	Non-photochemical quenching
NR	Nitrate reductase
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
RG-II	Rhamnogalacturonan II
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
RWC	Relative water content
SEA	Singular enrichment analysis
T	B-toxicity
T.F	Transcription factors
Tb+D	Tarımbor+Deficiency

CHAPTER 1

INTRODUCTION

1.1. Wheat

Wheat (*Triticum* L.) is the world's most widely grown crop (Feldman *et al*., 1995). It is an annual plant that belongs to the order Poales (Glumiflorae), the grass family Poaceae, tribe Triticeae and subtribe Triticineae. Wheat is one of the first domesticated crop plants more than 10,000 years ago in the Middle East and subsequently spread over the Old World (Lev-Yadun *et al*., 2000). The genus *Triticum* exists as a three-level ploidy series; diploid (2n = 2x = 14), tetraploid (2n =4x = 28), and hexaploid (2n = 6x = 42) (Provan *et al*., 2004).

Diploid wheats are either wild such as *T. urartu tumanian* ex Gandilyan and *T. boeoticum*, or else domesticated directly from the latter (Einkorn wheat, *T. monococcum ssp. monococcum* L.). The tetraploids contain wild forms such as T. *araraticum jakubz* and *T. turgidum* L. ssp. dicoccoides but comprise a wider range of domesticated wheats such as durum or macaroni wheat (*T. turgidum ssp. durum* L.) (Huang *et al*., 2002). The hexaploid wheats are dominated by cultivated forms such as *T. zhukovsky* Menabde and Ericzjan, spelt wheat (*T. spelta* L.), club wheat (*T. compactum* Host), and common or bread wheat (*T. aestivum* L.) (Haider, 2013).

Of the many species of wheat, one of the most important modern wheat cultivars is hexaploid bread or common wheat (*T. aestivum*) that is widely cultivated and used for human food. The others are tetraploid Durum wheat (*T. durum*), and hexaploid Spelt wheat (*T. spelta*) and the tetraploid *T. polonicum* (Miller and Pike 2002).

Hybridization within Triticeae resulted in polyploids in the form of amphiploids. *T. aestivum* is a one of the good example for this hybridization process (Kihara, 1924). It consists of three homologous genomes, A, B and D, each of which contributes seven pairs of chromosomes to wheat total genome. They are derived from its early ancestors from which modern wheat evolved around 8,000 B.P. (McFadden and Sears, 1946). In other words, this species is believed to have originated from spontaneous hybridization of *T. turgidum* L., AABB genomes, with *Aegilops tauschii*, DD genomes (Kihara, 1944).

Common wheat (*Triticum aestivum* L., 2n=6X=42), world's most commercially grown, is an allohexaploid with the AABBDD genomes. The 21 pairs of chromosomes are grouped into 7 homologous groups and each chromosome contains 2 homologues in the other 2 genomes (Sears, 1954; 1966). Common wheat possesses large and complex genome with a size of ~16000Mb, which consists of 90% repeated sequences (Li *et al*., 2004). In other words, the genetic structure of wheat is more complicated than the other domesticated species.

Hexaploid wheat behaves as a diploid organism during meiosis because of the Ph1 (pairing homologous) gene on the long arm of chromosome 5B. It prevents pairing between homologous chromosomes (Riley and Chapman, 1958). Because the mutation of Ph1 induces homologous pairing, its manipulation has been a main approach for transferring desirable genes from related wheat species to common wheat.

1.1.1. Wheat Production and Uses

Wheat is one of most important cereal crops with more than 600 million tones being harvested annually. For instance, wheat production in the world for years 2006, 2007 and 2008 was 622.0, 594.0, and 610.2 million metric tons, respectively (http://www.ndwheat.com). For the same years, the wheat utilization of these years was 624.4, 621.0, and 620.1 million metric tons, respectively.

According to FAOSTAT, United States, China, and Russia are the great wheat producing countries of the world. On the other hand, Turkey is one of the top ten producers in the world. For example, total annual wheat production is estimated at 20.1 million tons in 2012 (http://faostat.fao.org).

About 95% of the wheat grown in the world is hexaploid bread wheat, the rest 5% is tetraploid durum wheat that is grown more than bread wheat in Mediterranean climate. Durum wheat is called pasta wheat to reflect its major end-use (Shewry, 2009). Low amounts of wheat species such as einkorn, emmer, spelt are grown in Spain, Turkey, the Balkans, and the Indian subcontinent.

Human food and animal feed are two main uses of wheat in the world. However, it can be fractionated into starch, gluten, and oil (grain components), straw (non-grain components) (Bergthaller, 1997). Many functional products including paper, adhesives, plastic films, sweeteners, thickeners, cosmetic powders and creams, packaging materials, and foams are produced by modification or hydrolyzation of wheat starch (Maningat and Seib, 1997). Production of bio-fuel by yeast fermentation of starch and industrial alcohol are popular interest in the world (Maningat and Seib, 1997).

Wheat straw is rich in fibrous materials. It is used for making textiles, filters, sorbents, structural composites, molded products, and packaging materials as well as animal feed. Also, it might be used as a relatively clean energy source since its gas emissions are low (Culshaw, 1997).

Flour from hard wheats has a high amount of gluten and is used for making bread and cakes. The hardest-kerneled wheat is durum (*T. durum*). The flour of this type is mainly used to make macaroni, spaghetti, and other pasta products. White- and soft-wheat varieties are paler and have starchy kernels. The flour of these varieties is used for piecrust, biscuits.

1.1.2. Spring and Winter Wheats

Wheat has three different growth habits depending on the distinct growing seasons. Winter wheat is primarily grown during the winter months and harvested in the spring or summer. It needs vernalization to flower, and resists prolonged periods of freezing temperatures. Dominant vernalization gene Vrn1 controls the spring growth habit of common wheat (Yan *et al*., 2003).

Facultative wheat is sown primarily during the winter months in mild climates. It may or may not need vernalization to flower and can not resist prolonged periods of freezing temperatures.

Lastly, spring wheat is primarily planted during the spring months and harvested in late summer or early fall. It does not need vernalization to flower and can not withstand moderate periods of below freezing temperatures.

1.2. Boron

Boron (B) is indispensable for plants and animals. It is classed as a micronutrient due to requirement of small amounts. It was discovered by Joseph Gay-Lussac and Louis Thénard and independently by Sir Humphry Davy in 1808.

B does not occur in nature as free element. It is associated with clay and other impurities in nature as a mineral. There are more than 200 naturally occurring B containing minerals and the most commercially important minerals are tincal, colemanite, ulexite and kernite. These minerals have different percentage of B_2O_3 in their structures. This amount is essential factor for using industry. In fact, one B mineral can be trade competitor the other one because of B_2O_3 in their structures.

Turkey is the largest producer of B in the world. Also, United States of America (USA) and Russia contain the important B mines. Turkey has a share of %72.20 and USA is %6.8 based on total reserve basis. Total world B reserves on the basis of B_2O_3 content are 369 million tons proven. Turkey has 227 million tons proven B reserves and 624 million probable possible boron reserves on the basis of B_2O_3 content (http://en.etimaden.gov.tr).

B minerals can be used as crude minerals for industry application. However, their applications after refining and end-products are wider than crude ones. The primary markets are glass, ceramics, frit, detergents, soaps and agriculture.

B is used for glass and its various forms since it is a powerful flux beucause of lowering melting point, viscosity, thermal expanding coefficient and increasing breakage index, transparency, brightness and heat resistance.

High amounts of borates (e.g. 25%) are used for formulations of frit and glaze with 13% of the global borate demand since they ensure a good fit between the glazes and cover material and increasing the chemical and mechanical strength.

B is used for production of detergents and soaps as a bleaching and cleaning agent. B controls alkalinity of soaps and synthetic detergents, balances active oxygen, softens water, reduces the time and heat of the washing and prohibits the corrosion of the metal and machine (http://en.etimaden.gov.tr).

B is widely used in cleaning industry for diverse purposes such as germicide and bleaching. Borax decahydrate is used for soaps and detergents due to its water softening and germicide properties. Sodium perborate is used for powder detergents as bleaching agent because it is an active oxygen source.

B is generally used in metallurgy such as in abrasives, cutting tools, magnets and soldering in order to reduce melting temperature, increase fluidity, increase strength of the steel and lower the corrosion of the refractory material in the furnace.

Boric acid and sodium borates can be used as wood preservatives since they are water soluble compounds that are absorbed by the wood surface. Furthermore, they are good fire retardants. Boric acid and its mixtures with sodium borates are particularly effective in lowering the flammability of cellulose materials. Therefore, they are used as fire retardants in wood products and cellulose insulation.

Finally, in small concentrations B compounds are used for plant growth as micronutrients in fertilizers. For this porpose, borax decahydrate and borax pentahydrate are widely used in fertilizer. Sodium borates can be used into the soil or by spraying onto plants due to their good solubility. Disodium octaborate tetrahydrate (Etidot-67) is the most preferable boron product in agricultural application because it has better solubility when compared to the other B products such as borax decahydrate and borax pentahydrate.

Besides the small amount, B compounds are also used as herbicides, algaecides and other pesticides in large concentrations. B for herbicides is produced from borax and boric acid and they are widely mixed with sodium chloride (http://en.etimaden.gov.tr).

1.2.1. B Chemistry

B is a member of metalloid family elements from Group IIIA of the periodic table and has properties intermediate between metals and non-metals. B atom has one less (three) valence electron. This property causes an electron deficiency that has a dominant effect on the behavior of B in chemical processes. Elements of

this type usually have metallic bonding, however, the small size and high ionization energies of B lead to covalent rather than metallic bonding (Kot, 2009). B has unique property among elements with structural complexity of its allotropic modifications. Many organic compounds including B-O are known (Thompson, 1980; Bowser and Fehlner, 1989). There are also a binary B sulphides and B-sulphur anions. They might form chains, rings and networks (Greenwood and Earnshaw, 1997). Simple alcohols react with boric acid to give esters $B(OR)_3$. For instance, polyhydric alcohols form cyclic esters with boric acid (Steinberg, 1964).

B is not abundant element in spite of its importance because it is bypassed in the normal chain of thermonuclear reactions in stars (Reeves, 1974). In addition, B may also be produced during explosions of massive stars (Crosswell, 1992). Although it is low natural abundance, B is widely distributed in both the lithosphere and hydrosphere (Morgan, 1980). B concentration averages about 10-20 mg B kg^{-1} in rocks. In sea water it can range from ca. 1-10 mg B kg^{-1} while its concentration in river water is about 1/350 that of sea water (Power and Woods, 1997). Soils can be categorized as low B content (<10mg B kg^{-1}) or high B content (10-100mg B kg^{-1}).

Borates are mainly natural occurring form, however, less often form is boric acid and much more rarely one is BF^-_4 ion. The formation of $B(OH)_3$ interaction in aqueous solution is easily understood. $B(OH)_3$ is the main compound exists at physiological pH. It behaves as a weak Lewis acid (Ka =$6x10^{-10}$, pK_a 9.1) according to the equilibrium in Equation (1). Thus, boric acid mainly exists as the undissociated acid $B(OH)_3$ and low amount of borate anion $B(OH)^-_4$ in aqueous solution at physiological pH (Bolanos *et al*., 2004).

$$B(OH)_3+H_2O \leftrightarrow B(OH)^-_4+ H^+ \quad (1)$$

Both $B(OH)_3$ and $B(OH)^-_4$ readily form complexes with a wide variety of sugars and other biomolecules containing cis-hydroxyl groups (Loomis and Durst, 1992) (Figure 1.1). Examples of biomolecules reacting with $B(OH)_3$ are ribose, apiose, sorbitol, as well as phenolics and amino acids such as serine (Tate and Meister, 1978). Binding reactions of $B(OH)_3$ and $B(OH)^-_4$ with hydroxyl groups of diverse biomolecules is critical to understand physiological and other possible roles of B in plants (Brown *et al*., 2002; Bolanos *et al*., 2004).

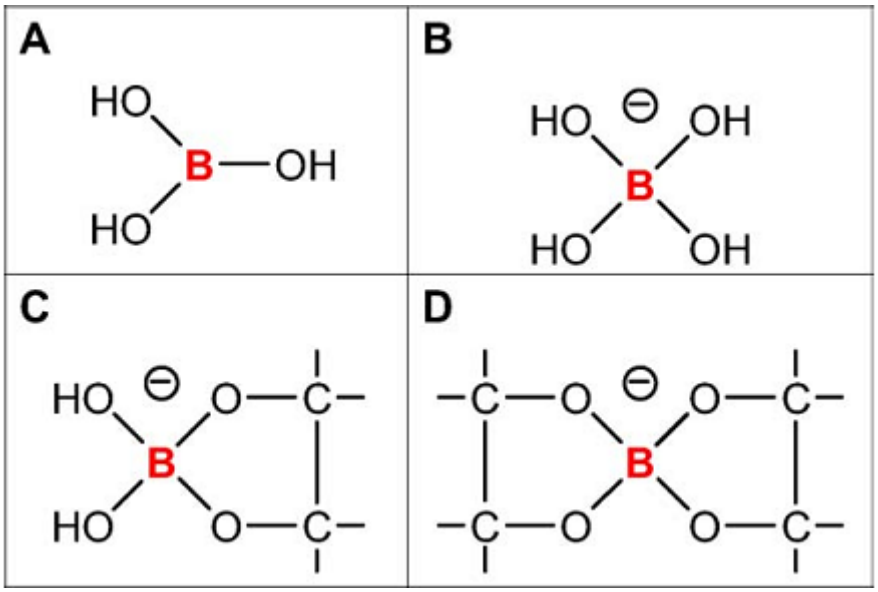


Figure 1.1. Chemical structures of boric acid (A), borate anion (B) and their diol esters (C, D).

1.2.2. The Function of B in Plants

The essentiality of B for vascular plants was first shown in *Vicia faba* (Warington, 1923). Since that time, B has been established as an essential micronutrient for plant growth. Whereas great improvement has been made in recent years, the primary role of B remains unclear since clarifying of roles of B in plants require consideration of the physical and chemical characteristics of B

and the processes that govern its efflux and influx (Brown *et al*., 2002). For this purpose, improving techniques for new methodology are critical to further progress for understanding B functions. Stable isotopes (e.g. ^{10}B and ^{11}B), development of markers binding cis-diols like boronic acids, B-auto inducers are extremely useful methodologies for B studies.

Today there is little doubt that B has a primary role in cell wall biosynthesis since a close relationship between the primary cell walls and B nutrition have been found by many researchers over the years. Loomis and Durst (1992) suggest that apiose can be the key sugar moiety for borate-crosslinking complex from driselase-treated radish root cell walls. Furthermore, a correlation between pectin sugars and B content in cell walls of tobacco and squash was reported (Hu and Brown, 1994). Eventually, Kobayashi *et al*. (1996) purified the pectin fraction from cell walls of radish root and isolated the first B-containing pectic polysaccharide complex (rhamnogalacturonan II (RG-II)-B) from plants. They also demonstrated that removal of B from the RG-II-B complex lowered the molecular weight of the complex by half. RG-II is a complex polysaccharide of the pectic fraction of cell walls. Its apiose residue is responsible for binding of B to the polysaccharide chains. Recently, Fleischer *et al*. (1998) found that B deficiency rapidly increased cell wall pore size in suspension-cultured *Chenopodium album* L cells. Moreover, the larger pore size in B deficient cells correlated with dB-RG-II and after B-was supplied again, pore size was lowered (Fleischer, 1999). These results suggest that the formation of dB-RG-II also influences plant growth, metabolism and physiologically important processes such as cell wall modification.

Under B deficiencies, there are rapid changes in membrane function such as membrane transport processes and the composition of the cell membrane. Potassium uptake by plants does not occur in the absence of B (Schon *et al*., 1991). Leakage of K^+ from sunflower (*Helianthus annuus*) leaves was 35 times higher in B-deficient than in control plants (Cakmak *et al*., 1995). B is also stated

to play an important role in phosphate transport across membranes (Loughman, 1977). Heyes *et al*. (1991) suggest that the membrane capacity is under the influence and probable control of the low levels of B in the cell due to B maintaining a preferred conformation of the active protein in the membrane.

Membrane potential measurements show that the proton gradient was affected by B (Blaser-Grill *et al*., 1989). In fact, glycoprotein complexation with B at the membrane surface creates additional negative charges which can influence the electrostatics across the membrane. Beside the glycoproteins, both glycolipids in the bilayer, as well as transmembrane and surface glycoproteins (Alberts *et al*., 1994), have oligosaccharide side chains capable of forming borate complexes. This interaction can change the membrane permeability, surface charge and rigidity.

In contrast to primary cell wall structure and membrane function, possible role of B in plant metabolism is still less well studied. In plants, B deficiency inhibits glucose-6-phosphate dehydrogenases, leading to increased production of phenols (Gomez-Rodriguez *et al*., 1987; Heyes *et al*., 1991). Borate is inhibitor for alcohol dehydrogenase (Weser, 1968; Smith and Johnson, 1976). These competitive inhibitions likely contain complexation with the ribityl hydroxyls of the coenzyme NAD (nicotinamide adenine dinucleotide), slowing its conversion to NADH. Blevins and Lukaszewski (1994) have demonstrated that boric acid inhibits allantoate amidohydrolase, a manganese-containing enzyme. Similarly, the activity of indolyacetic acid oxidase depended on B nutrition in squash root apices (Blevins and Lukaszewski, 1998).

B can be involved in a number of metabolic pathways due to the formation of complexes with a variety of hydroxylated molecules. Berger (1949) suggested that B plays an important role in the translocation of sugars. Shortly after, Gauch and Dugger (1953) showed that B speeds up the uptake and transport of sugars in normal versus B deficient plants. Eventually, Hu *et al*. (1997) characterized

soluble sorbitol-B-sorbitol complexes from the floral nectar of peach and mannitol-B-mannitol complexes from phloem sap of celery as first identified B transport molecules. They suggest that the sugar or polyol transport molecules affect B movement in phloem.

1.2.3. B Uptake and Allocation in Plants

The most plentiful form of B in soil solution is the soluble undissociated boric acid H_3BO_3. Plants take up B from soil in this chemical form. There are three different molecular mechanisms for B uptake by roots depending on B availability. These are passive diffusion across lipid bilayer, facilitated transport by major intrinsic protein (MIP) channel and an energy-dependent high affinity transport system by means of BOR transporters (Tanaka and Fujiwara, 2008).

Under normal and toxic B conditions, passive process that comprises mostly B diffusion across the lipid bilayer carries on the boric acid absorption in roots (Brown *et al*., 2002; Tanaka and Fujiwara, 2008). In fact, the lipid permeability coefficient of boric acid was calculated theoretically (Raven, 1980) and experimentally from isolated membrane vesicles (Dordas *et al*., 2000; Stangoulis *et al*., 2001). As a result, it can pass membranes by a passive process in order to satisfy B requirement in plants (Brown *et al*., 2002).

Recent studies state that B uptake might be performed by channels mediated membrane transport in addition to passive diffusion. Dordas *et al*. (2000) demonstrated that maize PIP1 (a member of the MIP family) expression in *Xenopus laevis* oocytes leading to an increase of B absorption. Lately, AtNIP5;1 has been identified as a novel boric acid channel in *Arabidopsis* that belongs to the nodulin 26-like intrinsic proteins (NIP) subfamily of the MIPs family (Takano *et al*., 2006). The expression of AtNIP5;1 is up-regulated in roots under B-deficiency. Two independent T-DNA insertion lines of NIP5;1 showed lower biomass production and elevated sensitivity of root and shoot development to B

limitation. These data suggests that it is crucial for B import into root cells under B deficiency (Takano *et al*., 2006). OsNIP3;1 has also been identified as a boric acid channel required for efficient growth under B limitation in rice (Hanaoka and Fujiwara, 2007).

When B has been absorbed by root cells, B must be loaded into xylem. An energy-dependent high-affinity transport system mediated by BOR transporters is mainly responsible for export B towards xylem under B-deficient conditions. BOR1 was identified as the first efflux-type boron transporters for xylem loading in *Arabidopsis* under B limiting conditions since the mutant plants showed a lowered transport of B to the shoot under B limitation (Takano *et al*., 2002). Afterwards, another BOR-1 like gene has been identified in rice (OsBOR1). It has role in both xylem loading of B and its absorption into the root cells under B deficiency (Nakagawa *et al*., 2007).

B is transported through vascular system to shoot tissue mediated by transpiration stream when B is into xylem (Raven, 1980; Shelp *et al*., 1995). Also, B can be transported by phloem to reproductive and vegetative tissues (Shelp *et al*., 1995; Matoh and Ochiai, 2005), though this capacity changes among species (Brown and Shelp, 1997). The formation of B-diol complexes is one of the suggested mechanisms for phloem transport of B (Brown and Hu, 1996; Hu *et al*., 1997).

In fact, B can bind to cis-hydroxyl groups of sugar alcohols such as mannitol and sorbitol. This allows B to be transported through phloem. For example, B-polyol complexes were characterized from the phloem sap in *Apium graveolens* (Hu *et al*., 1997). Additionally, it was shown that transgenic tobacco with elevated sorbitol levels had higher capacity to transport B by phloem and enhanced tolerance to B limitation (Bellaloui *et al*., 1999; Brown *et al*., 1999). However, B transport by means of phloem also occurs in plants that are not able to produce these types of complexes, especially to young tissues. Importantly, this translocation is not as efficient as phloem transport via sugar alcohols (Stangoulis

et al., 2001a; Takano *et al*., 2001; Matoh and Ochiai, 2005). Still, the molecular mechanism that supports this type of transport remains unclear. Recently, Tanaka *et al*. (2008) identified a boric acid channel (NIP6;1) in *Arabidopsis thaliana*. They suggest that it is responsible for B transfer from xylem towards phloem in young shoot tissues under B limitation.

1.2.4. B Deficiency

As mentioned above, B is an essential microelement for plant growth and is constantly required for throughout the plant life. B deficiency is a wide-spread problem in agricultural areas in the world, because B in soil solution exists primarily as boric acid $B(OH)_3$, which can be easily leached under high rainfall conditions resulting in deficiencies in plants that grow there (Shorrocks, 1997; Yan *et al*., 2006). B deficiency has been reported in over 80 countries and for 132 crops more than the last 60 years (Shorrocks, 1997).

One of the most obvious symptoms of B deficiency is death of the growing meristem due to its function in strengthening components of the cell wall (O'Neill *et al*., 2001). In fact, these are a result of the role of B in cross-linking of the cell wall RG-II and pectin assembly (O'Neill *et al*., 2004). Another reason for this symptom is that B is immobile in plants since B has restricted mobility in most plant species once it has accumulated in a particular organ. Thus, its deficiency symptoms are more severe on young leaves with marginal, dull yellow chlorosis at the tip of young leaves (Goyal, 2012). Moreover, irregular shaped cells with thicker cell walls due to B deprivation caused a poor differentiation of xylem and phloem elements and then this could be associated with disturbances in auxin metabolism (Mattson *et al*., 2003), caused by the death of the apical meristem as a primary effect of B limitation (Hajiboland *et al*., 2012).

Furthermore, it has recently been demonstrated that the disturbances of ethylene and auxin in B limitation condition induced root growth inhibition (Martín-Rejano *et al*., 2011). In fact, the growth of root is more sensitive to B limitation than the growth of shoot (Dell and Huang, 1997).

Besides B function in cell wall, several studies have shown possible roles of B in cytoskeleton structure and related processes (Yu *et al*., 2001; 2003; Bassil *et al*., 2004). Therefore, the levels of actin and tubulin proteins were increased under B deprivation in *Arabidopsis* roots (Yu *et al*., 2001) and altered the cytoskeletal polymerization patterns in the cells of maize root apices (Yu *et al*., 2003). This accumulation of cytoskeletal proteins may be related to an adaptive response to contribute to mechanical reinforcement of root periphery cells under B limitation (Yu *et al*., 2003).

B may require for the maintenance of the structure and functions of membranes by modulating the hydration and fluidity of lipid bilayers (Verstraeten *et al*., 2005) due to the ability of B for binding to hydroxyl-containing constituents, such as phosphoinositides, glycoproteins, glycolipids (Bonilla *et al*., 1997; Brown *et al*., 2002). For instance, B limitation changed the membrane potential in *Helianthus annuus* (Ferrol and Donaire, 1992) and the permeability for ions and other solutes (Cakmak *et al*., 1995; Wang *et al*., 1999). Recently, it leads to a decrease in the expression of several arabinogalactan (AGP) genes in *Arabidopsis* roots (Camacho-Cristobal *et al*., 2008). Thus, B might have important functions in membranes by stabilizing membrane molecules with cis-diol groups (Bolanos *et al*., 2004a) and by regulating the expression of genes involved in membrane function and structure.

B may play a crucial role for nitrogen assimilation. For example, a lowered nitrate reductase (NR) activity and increased accumulation of nitrate have been shown in B-deficient plants (Kastori and Petrovic, 1989; Shen *et al*., 1993), Also, tobacco plants subjected to B limitation had a significant decrease in leaf NR

activity and nitrate concentrations (Camacho-Cristobal and Gonzalez-Fontes, 1999). This decrement was attributable to the lower net nitrate uptake rate in B-deficient plants, probably as a result of the decrease H^+-ATPase (PMA2) expression level in root plasma membrane after B-deficieny (Camacho-Cristobal and Gonzalez-Fontes, 2007).

Photosynthesis has been shown to be affected by B deficiency due to reducing efficiency of photosystem II and the photosynthetic oxygen evolution rate (Kastori *et al*., 1995; El-Shintinawy, 1999). Leaves of sweet orange seedlings subject to B deprivation had lower photosynthetic enzymes activities by excess hexoses, which led to a decrease in growth (Han *et al*., 2008). Furthermore, accumulation of soluble sugars in B deficient leaves could inhibit net photosynthesis (Dugger, 1983; Dell and Huang, 1997). However, Goldbach and Wimmer (2007) stated that the effects of boron deficiency on photosynthesis are secondary in nature because the mechanism for a primary role of B in photosynthesis is unknown. They also suggested that this secondary effect on chloroplasts and growth inhibition could indirectly cause a lowered sink activity and an oversaturation of the electron acceptors of photosystems. These possible effects might increase photo-oxidative damage in response to further stresses.

The imbalance between photosynthetic production of carbohydrates and their use in growth caused to be used less of the absorbed photon energy captured by the light harvesting system in CO_2 assimilation. Thus, the photosystem electron transport chain becomes over-reduced. This results in increased accumulation of reactive oxygen species (ROS) (Han *et al*., 2008). The accumulation of ROS in cells could be responsible for the impairment of membrane functions (Cakmak and Römheld, 1997). The addition of lipophilic antioxidants suppressed the death of B-deficient cells. This suggested that oxidative damage is the major cause of cell death under low B supply (Koshiba *et al*., 2009).

1.2.5. B Toxicity

B toxicity is not as widespread as B deficiency in nature. Three main conditions can cause high concentration of B in soils. These are soils inherently high in B (Severson and Gouch, 1983), over fertilization with minerals high in B (Gupta *et al*., 1976) and through the use of irrigation waters high in B leading to B accumulation and concentration in soil (Branson, 1976). It has been recognized as an important problem limiting crop production in soils of arid and semi-arid environments throughout the world.

The typical visible symptom caused by B toxicity is leaf burn (chlorotic and/or necrotic patches) progressing along the older leaves tip (Bennett, 1993; Bergmann, 1992) in contrast to the deficiency symptoms. Tanaka and Fujiwara (2008) suggest that toxicity effects of B appear to be loosely correlated with the accumulation of high concentrations of B in old leaves, especially at the margin of leaves since B introduced into the transpiration stream accumulates at the end of this stream. The chlorotic/necrotic patches have greatly elevated B concentrations compared with the other surrounding leaf tissues (Oertli and Roth, 1969). There is no general perception in the characteristic of leaf burn in all species under B toxicity. For instance, in plants in which B is phloem mobile (e.g. Prunus, Malus, Pyrus), in which B accumulates in developing sinks rather than at the end of the transpiration stream, these symptoms are fruit disorders (gummy nuts, internal necrosis), bark necrosis and stem die back (Brown and Hu, 1996). Visible symptoms caused by B toxicity do not appear to develop in roots (Nable *et al*., 1997). B concentrations in the roots remain relatively low compared to those in leaves in spite of toxic B levels (Nable, 1988; Oertli and Roth, 1969). Thus, it was suggested that toxic concentrations of B do not occur in root tissues.

Although the physiological basis for B toxicity remains unclear, three main causes have been proposed relating to the ability of B to bind compounds with two hydroxyl groups in the cis-configuration. These are the changing of cell wall structure, metabolic disruption by binding to the ribose moieties of molecules such as adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) and disruption of cell division and development by binding to ribose, either as the free sugar or within ribonucleic acid (RNA) (Reid *et al*., 2004).

Metabolic disruption was shown by using nuclear magnetic resonance technique that demonstrated complexation of ATP, NADH or NADPH with B (Hunt, 2002). Reid *et al*. (2004) demonstrated an increase in Km with increasing B under high B concentrations (more than 5 mM B) by using malate dehydrogenase and isocitrate dehyrogenase *in vitro* experiments since the substrate of these enzymes are NADH and NADPH, respectively. These results could be related to reduction of the available substrate concentration due to complexation. Vmax in these reactions was much less sensitive to B. This result suggests that B targets the substrate rather than the enzyme itself. Reid *et al*. (2004) supported this view with acid phosphatase. The substrates of this enzyme have low affinity to B and Km and Vmax was not significantly affected by up to 50 mM B (Reid *et al*., 2004). These data from *in vitro* experiments are not consistent with the consequences of this binding *in vivo*. For instance, it was shown that neither photosynthesis, respiration or protein synthesis was particularly sensitive to B (Reid *et al*., 2004). Accordingly, photosynthesis was not affected after 50 mM B treatment in barley leaves and partially inhibited by 23% after 100 mM B treatment. On the other hand, Respiration was reduced by 37% at 50 mM B and by 60% at 100 mM B.

The physiological effects of B toxicity may be lowered root cell division (Liu *et al*., 2000), reduced leaf and root growth (Lovatt and Bates, 1984: Nable *et al*., 1990), inhibition of photosynthesis, lower stomatal conductance (Lovatt and

Bates, 1984). Furthermore, Karabal *et al*. (2003) suggest elevated membrane leakiness, peroxidation of lipids and altered antioxidant enzyme activities at the end of the B toxicity. Reid *et al*. (2004) also focused the possibility that the toxic B accumulation in leaves may lead to osmotic problems, however, deduced from measurements of total leaf ion concentrations that there was no evidence to support this possibility that toxicity in leaves is due to osmotic stress induced by toxic B accumulation.

Though growth was rapidly inhibited by internal B concentrations (1-5 mM), this inhibition was not attributable to effects of B on either energy supplies or inhibition of protein synthesis but the toxicity to mature tissues was rather (Reid *et al*., 2004). Thus, there is a greater dependence on translation and deoxyribonucleic acid (DNA) replication. Deoxyribose in DNA does not contain cis-diol groups to bind B. For this reason, blocking of DNA replication by B is not feasible. Likewise, one of the hydroxyl groups of ribose in RNA is used to link the nucleotide bases together. Also, this is not available for cis-diol bonding to B. However, both of the hydroxyl groups of ribose are exposed at the 3' end of RNA molecules. This region might or might not be important in either transcription or translation. Critical information in terms of B binding at the 3' end is that RNA undergoes splicing, during which ribose is transiently exposed to B in plants and animals (Reid, 2007). In fact, Shomron and Ast (2003) have demonstrated that B prohibits one step of *in vitro* pre-mRNA splicing reaction. Interestingly, several genes that encode transcription factors or ribosomal proteins provided B tolerance from Lupinus and *Arabidopsis* in yeast (Nozawa *et al*., 2006; Reid, 2007b). Thus, these proteins might act as splicing sites protectors from B at splice sites of mRNA, which would be one of the mechanisms to confer B tolerance (Reid, 2007b).

B toxicity inhibits photosynthesis although the effect of B on photosynthetic process is poorly understood due to contradictory experimental data. For instance, some authors suggest that the reduction in photosynthetic rate under B

toxicity was accompanied by an increase in intercellular CO_2 concentration; however, stomatal conductance remained unchanged (Sotiropoulos *et al*., 2002). On the contrary, other authors found a reduction in stomatal conductance (Lovatt and Bates, 1984; Papadakis *et al*., 2004a). Also, Pereira *et al*. (2000) suggested that the structural damage of thylakoids at the end of the B toxicity was one of the possible reasons for the reduction of photosynthesis. Thus, this damage might alter the rate of electron transport and influence CO_2 photo assimilation that can also be limited by stomatal reduction. On the other hand, decrease in photosynthetic enzymatic activities, oxidative load, and an impaired electron transport rate might account for the reduction in CO_2 assimilation under B toxicity (Han *et al*., 2009). However, the mechanisms involved in the alteration of photosynthesis by B stress have not been determined yet.

Uronic acid contents in barley and wheat were investigated under B toxicity in order to determine the possible function of uronic acid in B tolerance mechanism (Mahboobi *et al*., 2001). Uronic acid is a structural component of pectins in cell wall. However, uronic acid amount was not significantly changed under B toxicity. Also, no significant difference between tolerant and sensitive cultivars was detected. Therefore, Mahboobi *et al*. (2001) suggested that cell wall uronic acid content does not contribute to detoxification of high B in wheat and barley.

Toxic B concentrations lead to significant changes in various enzymes activities in higher plants. Importantly, Mahboobi *et al*. (2000) focused the changes in protein profiles of barley cultivars after B toxicity. Accordingly, in root tissues abundance of 3 proteins and 7 proteins in leaf tissues were induced in tolerant barley cultivar; however, these were unchanged in sensitive barley cultivar. Also, it was suggested that B caused an alteration of the metabolism of nitrogen compounds (Bonilla *et al*., 1980; Kastori and Petrovic, 1989). For this reason, NR and glutamate dehydrogenase (GDH) activities in barley and wheat were assessed at the end of the B toxicity (Mahboobi *et al*., 2002). NR activity was significantly decreased with 16% in leaves and roots of tolerant and sensitive

barley cultivars. On the other hand, the GDH activity was increased by 30% in leaves and 81% in roots of two both cultivars. It could be an adaptive mechanism under B stress in barley and wheat (Mahboobi *et al*., 2002).

1.2.6. B Tolerance

The ability of plants to grow in soils that have toxic B levels is widely variable from species to species and even among varieties of the same species. Crop varieties that can cope with toxic B concentrations in soils have been identified by means of screening of genotypes in order to determine these differences in B tolerance. It was primarily established that B tolerance is related to the ability to restrict B uptake into the plant (Nable, 1988; Paull *et al*., 1988). Afterwards, this was demonstrated to be a common feature in other plant species (Paull *et al*., 1992). However, studies over the past few years have shed new light on the phenomenon. The new approach was to clarify how B enters cells in order to understand how some species are able to restrict toxic B accumulation.

Importantly, Stangoulis *et al*. (2001) measured B permeability across the plasma membrane of an intact plant cell. Accordingly, entry of B into cells was very rapid. The equilibrium of B between the internal and external phases occurred in these cells within a few hours. This equilibrium would be expected to be more rapid in smaller cells such as those of roots with a much higher surface area. This meant that it would be futile for B uptake to be mediated by a transport protein. Hayes and Reid (2004) also verified this equilibration in the roots of B-sensitive barley cultivars. In addition, they demonstrated that after B was added to the solution around the roots, B permeability was similar in both sensitive and tolerant genotypes. However, at equilibrium, the B concentration in the sensitive cultivar was similar to that in the solution, whereas the internal concentration was much lower in the tolerant cultivar.

The consequence from these data is that B is actively pumped from the cells in the tolerant genotypes. In other words, the ability to restrict B accumulation is associated with B in both roots and shoots (Reid, 2007a). In this sense, the activity of boric acid transporters must be tightly regulated in order to maintain the rate of radial transport of B within an acceptable range (Takano *et al*., 2008).

The first B transporter which catalyzes the loading of B from xylem parenchyma cells into the xylem was identified as BOR1 in *A. thaliana* (Takano *et al*., 2002). It is also accumulated in the plasma membrane of pericycle cells under B limitation. However, Takano *et al*. (2005) have demonstrated that the Bor1 protein is degraded via endocytosis in the presence of B toxic levels. Furthermore, overexpression of the BOR1 gene does not result in a better plant growth at high B (Miwa *et al*., 2006). Thus, it is not involved in B tolerance.

BOR4, another efflux-type borate transporter, is one of the six BOR1 paralogs in the Arabidopsis genome. This gene is not degraded at the posttranslational under toxic B condition (Miwa *et al*., 2007). Moreover, by means of Green Fluorescent Protein (GFP) fluorescence, BOR4 protein was strongly detected in the plasma membranes of the distal sides of epidermal cells in the root elongation zone of *Arabidopsis* transgenic lines. They suggest that distal localization is important for the directional export of B content to the soil

Bot1, the barley 4H QTL (Quantitative Trait Locus) gene, has been identified as the gene responsible for the B-toxicity tolerance in the barley landrace Sahara 3771 (Reid 2007; Sutton *et al*., 2007). This gene shows 58% identity to the orthologous *Arabidopsis* B transporter BOR1 (Takano *et al*., 2002). Importantly, in Sahara, 4H-derived tolerance mechanism identified is naturally occuring derived from an increase in Bot1 gene copy number and mRNA transcript abundance (Sutton *et al*., 2007). Sahara contains 3.8 times more Bot1 gene copies than Clipper. In Sahara, Bot1 expression levels are about 160-fold and 18-fold higher in roots and leaves, respectively when compared to Clipper (Sutton *et al*.,

2007). In addition, they are consistent with a lower net entry of B into the barley root and in a higher B efflux from leaf guttation through hydathodes (Sutton *et al*., 2007). Thus, the ability to sustain lower shoot B concentration in Sahara cultivar is at least due to a mechanism of active B efflux from the root. As a result, Bot1 encodes a functional B efflux transporter involved in B tolerance.

At the same time, Reid (2007) cloned this transporter from wheat (*Triticum aestivum*; TaBOR2) and both TaBOR2 and Bot1 reduce B concentration in roots of tolerant cultivars. A positive correlation between expression levels of both genes and the degree of tolerance of different cultivars was established (Reid, 2007, Sutton *et al*., 2007).

A second class of proteins capable of B transport belongs to the superfamily of MIPs or aquaporins. They are transmembrane channel proteins, which facilitate the passive and bidirectional diffusion of water and a variety of small and non-charged compounds across biological membranes under physiological conditions (Ma *et al*., 2006; Takano *et al*., 2006; Choi and Roberts, 2007). Dordas et al. (2000), Fitzpatrick and Reid (2009) suggested that aquaporins might have a role in B transport in higher plants. For instance, AtNIP5;1, a specific NIP subclass aquaporin gene in *Arabidopsis thaliana*, is required for normal growth under B deficiency (Takano *et al*., 2006, Tanaka *et al*., 2008). HvNIP2;1, the barley orthologue of the rice gene Lsi1 (OsNIP2;1) was functionally characterized in heterologous expression systems. Accordingly, the protein of HvNIP2 can transport B, as well as two other metalloid elements, arsenic and germanium (Schnurbusch *et al*., 2010). Additionally, quantitative real-time RT-PCR (Reverse Transcription Polymerase Chain Reaction) data show that its expression level is lower in roots of Sahara compared to Clipper. Reduced expression of HvNIP2;1 at distal side of epidermal and cortical cells in older sections of Sahara roots should inhibit entry of B into the stele in regions of the root (Schnurbusch *et al*., 2010).

Finally, they suggest that a combination of lower expression level of HvNIP2;1 and higher expression level of Bot1 of in Sahara roots should result in reduced B uptake and less transfer of B to the shoot.

Transcriptomics, gene expression analysis by mRNA profiling, is thought to be the most prominent and powerful tool for functional genomics (Öktem *et al*., 2008). It often provides useful information about the responses of various plant mechanisms that facilitate the improvement of plant growth. Firstly, low B induced genes have been described in tobacco BY-2 cultured cells by means of a cDNA differential subtraction method (Kobayashi *et al*., 2004). In fact, the expression of NIP5;1 induced by B deficiency was found by using microarray analyses in *Arabidopsis thaliana* roots (Takano *et al*., 2006). Furthermore, Kasajima and Fujiwara (2007) performed transcriptome analysis under toxic B condition as well as low B supply in *Arabidopsis thaliana*. They identified novel high B-induced genes involved in a heat shock protein and multidrug and toxic compound extrusion (MATE) family transporter genes. The characterization of these genes may help to clarify the novel physiological response pathways to B toxicity in plants. In spite of these studies, there are no gene expression profiling studies of wheat cultivars under B toxicity or deficiency in order to determine B-stress related genes and -tolerance mechanisms.

1.3. Aim of the Study

Structural and functional integrities with cell wall are the only well-defined primary function of B due to cross-linking two (RG-II monomers. However, this function can not satisfactorily explain the other physiological and biochemical changes. Recently, several successful studies on gene expression in response to B stress have been reported. These studies have provided some valuable insights into B stress responses and their linkage with other biological pathways. Furthermore, this knowledge at molecular level suggests critical mechanisms for B-uptake and distribution in plants. In fact, manipulating transporters is one of

the possible ways to improve plant growth at high or low B conditions. However, it is clear that much is unknown about B regulation mechanisms. Therefore, identifying genes that contribute to B-stress response and checking whether the up or down regulations of these genes are critical to improve the tolerance of plants to B deficiency or toxicity. Although a few studies have suggested integrative view of plant responses to B deficiency stress at the transcriptional level, too few genes that induced by high B have been reported to date. More importantly, overall patterns of gene expression changes and their physiological and biochemical significances at high or low B have not been proposed up till now. In addition, there is no report for global expression profiles and their linkages to physiological and biochemical changes after supplementing artificial B-enriched fertilizer to B deficiency condition.

In the present study, the main objective is to assess global changes in gene expressions of wheat plants under B deficiency, toxicity and supplemented fertilizer (Tarımbor) to B deficiency conditions. For this purpose, Affymetrix wheat GeneChip was used to elucidate the overall pattern of gene expression changes in leaf and root tissues of two wheat cultivars, Bolal-2973 and Atay-85 differing in their B responses. Thereby, the molecular responses were comparatively analyzed in sensitive and tolerant wheat cultivars and in leaf and root tissues of these cultivars in response to B toxicity, deficiency and Tarımbor conditions.

Another objective of this study was to clarify physiological and biochemical changes that are mainly related to growth, photosynthesis, antioxidants, antioxidant enzymes, water status and oxidative damage under same conditions. Subsequently, these data were used to compare the transcriptional changes in order to determine the regulation mechanisms and their involvement in B-tolerance in plants.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

In this study, Turkish wheat (*Triticum* aestivum) cultivars Bolal-2973 and Atay-85 were used as B-tolerant and B-sensitive, respectively. The seeds of wheat cultivars were obtained from the Turkish Ministry of Agriculture and Rural Affairs, Central Research Institute for Field Crops, Ankara.

2.1.2. Plant Growth Media

Seedlings were grown by using half-strength Hoagland's solution (Hoagland and Arnon, 1950). The compositions of these nutrient solutions are listed in Appendix A. The pH of the solution was adjusted to 5.7 before sterilization. Boric acid (H_3BO_3) solution was used for sufficient, excess and low concentrations of B in growth solution.

2.1.3. Chemicals, Reagents and Kits

In this study, the chemicals and reagents were obtained from Sigma-Aldrich Corporation (St. Louis, MO, US), Merck KGaA (Darmstadt, Germany), Fermentas (Thermo Fisher Scientific Inc; Ontario, Canada), AppliChem GmbH (Darmstadt, Germany), Invitrogen Corporation (Carlsbad, CA, US) and Affymetrix (Santa Clara, CA, US), Tarımbor (Kayseri, Turkey).

Total RNA was isolated using Trizol reagent from Invitrogen. cDNAs (complementary DNA) were synthesized from RNAs following the manual of RevertAid™ First Strand cDNA Synthesis Kit from Fermentas. GeneChip Wheat Genome Arrays from Affymetrix were used for genome-wide expression profiling. Validation of microarray data via real-time RT-PCR was done by using Qiagen RT-PCR Kits.

2.2. Methods

2.2.1. Growth of Plants

The seeds of wheat were surface sterilized with 40% sodium hypochloride solution by shaking for 20 minutes. Then, they were rinsed with distilled water for three times. Surface sterilized seeds were transferred to plastic tubes immersed half-way into half strength Hoagland's solution (pH 5.8) (Hoagland and Arnon, 1950) Seedlings were grown for 8 days at 21 ± 2 °C with 16 h light (400 μmol m^{-2} s^{-1}) and 8 h dark photocycle with 70% relative humidity supplemented with 10 μM H_3BO_3.

2.2.2. B Toxicity, Deficiency and Tarımbor Treatments

At the 8th day of growth, half strength Hoagland's solutions were refreshed with another Hoagland's solutions containing 5000 μM $B(OH)_3$, 0,02 μM $B(OH)_3$, Tarımbor (B-enriched fertilizer) treatments (10 mM $B(OH)_3$ + 24 mg/L Tarımbor, 0,02 μM $B(OH)_3$ + 24 mg/L Tarımbor) for 5 days. The detail information in Tarımbor was given in Appendix B. Control groups were maintained in fresh Hoagland's solutions containing 10 μM H_3BO_3. In detail, experimental set-up was shown in below.

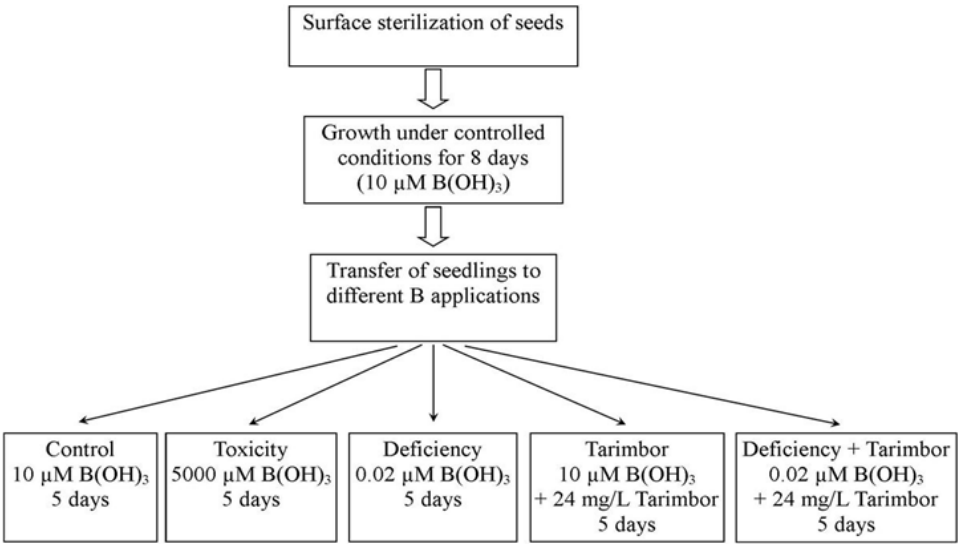


Figure 2.1. Experimental design for different B treatments.

2.2.3. Physiological Experiments

2.2.3.1. B Contents

After the period of B and Tarımbor treatments was complete, the seedlings were rinsed three times in deionized water, and then the shoot and root tissues were detached and dried at 80 °C for 48 h. The dried samples were ashed at 500°C for 5 h and dissolved in 0.1 N HNO_3. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used to measure B concentrations in dissolved samples expressed as ppm.

2.2.3.2. Growth measurements

After the period of B stress and Tarımbor treatments was complete, the seedlings were taken from each pot and shoot and root parts were separated immediately. Subsequently, the length of shoot and root parts was measured and weighed immediately to obtain their wet weights. These plants were then dried in an oven for

24 h at 60°C. Afterwards, dry weights were measured and recorded. 15-20 seedlings were used for each treatment. Also, the experiments were repeated at least five times.

2.2.3.3. Measurement of Relative Water Content

Relative water content (RWC) was measured as described by Smart and Bingham (1974). Fresh detached leaves and roots were weighed immediately to record the fresh weight (WW), followed by dipping them in deionized water for 24 h. They were then blotted dry and weighed to record the turgid weight (TW), and subjected to oven drying at 60 °C for 24 h to measure the dry weight (DW).
The RWC was calculated using the equation:
RWC (%) = (WW-DW)/(TW-DW)*100

2.2.3.4. Electrolyte Leakage

Electrolyte leakage was measured for leaf and root tissues following the procedure described earlier (Nanjo *et al*., 1999). For measuring the electrical conductivity, samples were shaken gently in 5 ml of 0.4 M mannitol solution for 3 h at room temperature and then their electrical conductance were measured (C1). Afterwards, tthey were incubated at 100ºC for 10 min to kill the tissue completely and the second conductance (C2) was measured to determine the ion concentration at the end of the membrane disintegration. Relative electrolyte leakage expressed as a percentage of the total conductivity was calculated according to the equation:
Relative electrolyte leakage = (C1/C2)*100.

2.2.3.5. Pigment Analysis

Total chlorophylls (Chla+b), chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids (Car) were determined spectrophotometrically using 80% acetone as a solvent (Lichtenthaler, 1987). Concentrations of these pigments were calculated by equations of Lichtenthaler, (1987) as follows:

Chl a = 11.24 * A661.6 - 2.04 * A644.8;

Chl b = 21.13 * A644.8 - 4.19 * A661.6;

Chla+b = 18.09 * A644.8 + 7.05 * A661.6;

Car = (1000 * A470 - 1.90 * Chl a - 63.14 * Chl b) / 214

2.2.3.6. Chlorophyll Fluorescence

Chlorophyll a fluorescence measurements were performed with a portable, modulated fluorescence monitoring system, (OS5-FL Modulated), on randomly selected leaves of wheat in a growth cabinet at 24ºC. The minimum chlorophyll a fluorescence (F_o) was determined using a measuring beam of 0.2 µmole m^{-2} s^{-1} intensity after 30 min dark adaptation. A saturation pulse (1 s white light 7500µmol m^{-2} s^{-1}) was used to measure the maximum fluorescence (F_M) in dark-adapted state. The quantum efficiency of PS II photochemistry (Fv/Fm) in dark adapted seedlings (ΦP_o) was calculated from $F_V/F_M = (F_M-F_o)/F_M$.

Light induced changes in chlorophyll a fluorescence after actinic illumination (300 µmole m^{-2} s^{-1}) were measured as minimum chlorophyll a fluorescence in light saturated state ($F_{o'}$) and maximum fluorescence in light saturated stage ($F_{M'}$). The quantum efficiency of PS II open centres light adapted state, referred to as $\Phi P_{S'}$ was calculated from $F_{M'}- F_S/ F_{M'}$. The electron transport rate (ETR) was calculated as $[(F_{M'}- F_S/ F_{M'})$ x PAR x 0.84 x 0.5)] by multiplying the quantum efficiency by incident photon flux density and average factor of 0.84 for leaf absorbance, and dividing by a factor 2 to account for the sharing of absorbed photons between two photosystems (PSI and PSII).

2.2.3.7. Water Potential

Randomly selected five B-treated and control leaves were used to estimate leaf water potential (LWP). The measurements of LWP were carried out by Sholander pressure chamber (PMS Instrument Co., Corvallis, OR, USA).

2.2.4. Biochemical Experiments

2.2.4.1. Estimation of Lipid Peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) according to Hodges *et al*. (1999). The samples (0.5 g) were homogenized in a mortar with 80% ethanol and then further diluted with 80% ethanol. The homogenate was centrifuged at 3000 x g for 10 min at 4°C. ~ 650 mL aliquot of the sample was added to a test tube with an equal volume of either a solution comprised of 20% TCA (Trichloroacetic acid) and 0.5mM EDTA (Ethylenediaminetetraacetic acid) (solution named as "-TBA (Thiobarbituric acid)" or a solution of 20% TCA (w/v), 0.5mM EDTA and 0.65 % TBA (w/v) (solution named as "+TBA"). Samples were mixed vigorously and then heated at 95°C in a block heater for 25 min, cooled and centrifuged at 3000 x g for 10 min. Absorbance was read at 440 nm, 532 nm, and 600 nm. Malondialdehyde equivalents were calculated following equation:

$A = [(Abs\ 532_{+TBA}) - (Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs600_{-TBA})]$

$B = [(Abs\ 440_{+TBA} - Abs\ 600_{+TBA})\ 0.0571]$

$MDA\ (nmol.\ ml^{-1}) = [(A-B)/157\ 000)]\ x10^{+6}$

2.2.4.2. Determination of Proline Content

The concentration of the proline was determined by ninhydrin method (Bates *et al*., 1973). The leaf and root tissues (0.2 g) were homogenized in a mortar with 1 mL 3% sulphosalicilic acid. The extract was centrifuged at 14,000 rpm for 5 min at 4ºC. 0.2 mL acid ninhydrin, 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 shaken and incubated at 96°C for 1 h. 1 mL toluene was then added into the tubes. Subsequently, they were centrifuged at 14,000 rpm for 5 min at 4ºC. Absorbance of the the upper red phase of the centrifuged samples was read at 520 nm.

2.2.4.3. Glycine Betaine Content

The amount of glycine betaine was estimated following the method of Grieve and Grattan (1983). The plant tissue (2 g) was shaken with 20 ml of deionized water for 48 h at 25°C. They were then filtered, and the filtrates were diluted 1:1 with 2 N H_2SO_4. Aliquots (0.5 ml) were transferred to centrifuge tubes and incubated in ice water for 1 h. 2 ml of cold potassium KI-I_2 reagent was added and then stored at 48°C for 16 h. After centrifugation at 10 000 x g for 15 min at 0°C, the supernatant was carefully aspirated with a 1-ml tube. The periodide crystals were dissolved in 9.0 ml of 1,2-dichloroethane, and the absorbance was recorded spectrophotometrically at a wavelength of 365 nm. The reference standards of glycine betaine (50–200 $\mu g ml^{-1}$) were prepared using 1 N H_2SO_4.

2.2.4.4. The Activities of Antioxidant Enzymes

0.5 g leaf and root samples were homogenized with liquid nitrogen and suspended in 50mM potassium phosphate buffer (pH 7.5) containing 2% (w/v) polyvinylpyrrolidone, 1mM EDTA and 1mM ascorbate. The homogenate was centrifuged at 15000 *g* for 20 min at 4° C. The supernatant was used for protein quantitation and further enzyme activities. The soluble protein concentration was determined in leaf and root crude extracts following to Bradford method (Bradford, 1976).

2.2.4.4.1. Catalase Activity

Catalase activity was determined following the method of Chance and Maehly (1955). Assay solution contained 50 mM potassium phosphate buffer (pH 7.0), 100 mM H_2O_2, and the enzyme extract containing 50 µg proteins. The decrease in absorbance was recorded at 240 nm for 90 s (Extinction coefficient of H_2O_2 = 40 $mM^{-1}\ cm^{-1}$). One enzyme unit was defined as µmole ml^{-1} H_2O_2 destroyed per min.

2.2.4.4.2. Ascorbate Peroxidase Activity

APX activity was determined following the method of Wang *et al.* (1991). Assay medium included 50 mM potassium phosphate buffer (pH 6.6), 0.25 mM ascorbate, 10 mM H_2O_2 and enzyme extract containing 100 µg proteins. The decrease in ascorbate concentration was recorded at 290 nm for 90 s (Extinction coefficient of ascorbate = 2.8 $mM^{-1}cm^{-1}$ at 290 nm). One enzyme unit was defined as µmol ml^{-1} ascorbate oxidized per min.

2.2.4.4.3. Glutathione Reductase Activity

Glutathione reductase activity was determined following the method of Sgherri *et al.* (1994). The assay solution included 200 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.50 mM GSSG, 50 µM NADPH and enzyme extract containing 100 µg protein. The oxidation of NADPH was recorded by reading the absorbance at 340 nm continuously for 90 s. (Extinction coefficient of NADPH = 6.2 $mM^{-1}cm^{-1}$). One enzyme unit was defined as µmol ml^{-1} GSSG oxidized per min.

2.2.5. Microarray Experiment

2.2.5.1. Preparation of RNA Isolation and Total RNA Extraction

Total RNAs were isolated from leaf and root tissues of wheat seedlings using TRIzol reagent (Chomczynski and Sacchi, 1987). According to this protocol: Approximately, 0.5 g samples from control and B-treated plants were ground with liquid nitrogen and were transferred 100 mg of the ground tissues to precooled 2 ml-tubes. Subsequently, 1 ml of TRIzol reagent was added and vortexed for 15 minutes in a block shaker at room temperatures, and then centrifuged in eppendorf centrifuge for 5 minutes at room temperatures at maximum speed. 900 µl of the supernatant was transferred to a new tube and added 180 µl chloroform and incubated at room

temperature for 6 minutes. At the end of the incubation, it was centrifuged for 15 minutes at 4°C at maximum speed. 450 µl from the upper phase was transferred into a new tube, and 200 µl chloroform was added, and then incubated at room temperature for 3 minutes. It was then spinned for 5 minutes at room temperature at maximum speed and pipetted 400 µl from the upper phase and added 1 volume isopropanol, and then incubated for 10 minutes at room temperature. Tube was centrifuged for 10 minutes at room temperatures at maximum speed and supernatant was removed. The pellet was washed with 1 ml 75% ethanol and left for 3 minutes at room temperatures and spin down for 5 minutes at room temperature at maximum speed, and removed the supernatant. In order to remove ethanol, it was centrifuged for an additional 15 seconds and air dried for 10 minutes. Finally, 50 µl DEPC (Diethylpyrocarbonate)-water added and it was incubated for 15 minutes at 65°C to dissolve pellet.

2.2.5.2. Determination of RNA Quality and Quantity

Quantitation of RNA was performed by using Quant-iTTM RiboGreen® RNA Assay Kit (Invitrogen) including RiboGreen as a sensitive fluorescent dye. Diluted RNA samples were incubated with Quant-iT RiboGreen RNA reagent for 5 min at room temperature and the fluorescence was then recorded with NanoDrop 3300 Fluorospectrometer (Thermo Scientific). RNA concentrations of the samples were determined according to the standard curve generated using a ribosomal RNA (rRNA) standard. Agarose gel electrophoresis was first used to check the quality and integrity of the RNA. It resolved cytosolic, chloroplastic and mitochondrial rRNA species from total RNA. RNA exhibited 2:1 ratio of 28S to 18S rRNA bands. This means that the mRNA in the sample is likely to be mostly full-length. Secondly, the integrity of RNA preparations was confirmed by using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with an RNA 6000 Nano Kit following the manufacturer's protocols. Electropherograms with sharp peaks of rRNA demonstrated integrity of RNA samples.

2.2.5.3. Microarray Analysis

Microarray analysis was performed using the Affymetrix (Santa Clara, CA) Wheat GeneChips Genome Array in order to compare the gene expression profiles of leaf and root tissues of Bolal-2973 and Atay-85 cultivars under Deficiency+Tarımbor, B-toxicity and B-deficiency conditions. Experimental design in microarray analysis did not contain Tarımbor condition ((10 mM $B(OH)_3$ + 24 mg/L Tarımbor) because we found that physiological and biochemical changes in this condition were negligible. The number of biological replicates was three for each treatment. Thus, 48 GeneChip® Wheat Genome Arrays were used for microarray analysis.

2.2.5.3.1. Target Labeling Process

Target preparation and labeling process were performed according to GeneChip® 3' IVT Express Kit User Manual. This process was summarized in Figure 2.2. Eukaryotic GeneChip probe array has probe sets for several *Bacillus subtilis* genes which are absent in eukaryotic samples (lys, phe, thr, and dap). These poly-A RNA controls are in vitro synthesized, and the polyadenylated transcripts for the B. subtilis genes are premixed at staggered concentrations. Thus, the concentrated Poly-A RNA Controls were diluted with the Poly-A Control Dil Buffer and then spiked directly into RNA samples in order to monitor the entire target labeling process. A set of poly-A RNA controls is supplied in the GeneChip® 3' IVT Express Kit. These controls are amplified and then labeled together with the total RNA samples. The hybridization intensities of these controls on GeneChip arrays help to monitor the labeling process independently from the quality of the starting RNA samples.

Total RNA/poly-A Control Mixture was used to synthesize first strand cDNA and immediately preceded to Second-Strand cDNA synthesis. These double-stranded cDNA was used as a template for *In Vitro* Transcription in order to synthesize Biotin labeled amplified RNA (aRNA). After synthesis, the aRNA was purified to remove enzymes, salts, and unincorporated nucleotides. Purified aRNA can be stored at ≤ –

20 °C for up to 1 year. The concentration of aRNA was determined by using RiboGreen fluorescence-based assay. Subsequently, aRNA was fragmented with Array Fragmentation Buffer before hybridization onto GeneChip probe arrays. An Agilent RNA 6000 Nano Kit was used to determine the size of the fragmentation reaction products. It was detected that the reaction produced a distribution of 35–200 nucleotide (nt) aRNA fragments with a peak at approximately 100-120 nt.

2.2.5.3.2. Target Hybridization, Washing, Staining and Scanning

Fragmented and labeled aRNA (12.5 μg) was used to prepare hybridization cocktail for probe array. The components of this cocktail and the necessary amount of aRNA required for the specific probe array format used were shown in Table 2.1. The hybridization cocktail was then incubated at 99°C for 5 min and at 45°C for 5 min in a heat block and subsequently centrifuged at maximum speed for 5 min to collect any insoluble material from the hybridization mixture. Meanwhile the array, equilibrated to room temperature, was filled with Pre-Hybridization Mix and incubated at 45°C for 10 min with rotation. The Pre-Hybridization Mix was then extracted from the array with a micropipettor and 200 μL of hybridization cocktail was refilled to the array avoiding any insoluble matter at the bottom of the tube. Samples were hybridized for 16 h to Wheat Genome Array in Hybridization Oven 640 (Affymetrix) at 45 °C and 60 rpm.

Table 2.1. Hybridization cocktail for single probe array (standard array format).

Component	**Amount**	**Final Dilution**
Fragmented and Labeled aRNA	12.5 µg (33.3 µL)	0.05 µg/µL
Control Oligonucleotide B2 (3 nM)	4.2 µL	50 pM
20X Hybridization Controls (*bio*C, *bio*C, *bio*D, *cre*)	12.5 µL	1.5 pM
2X Hybridization Mix	125 µL	1X
DMSO	25 µL	10 %
Nuclease-free water	50 µL	
Total Volume	**250 µL**	

Fluidics Station 450 (Affymetrix) was used to wash and stain the Wheat Genome Array according to standard protocol. The staining was performed with streptavidin-phycoerythrin (Invitrogen) and biotinylated antistreptavidin antibody (Sigma) according to the standard protocol for Affymetrix 49 format wheat array. Fluidics Station 450 was controlled by GeneChip Operating Software 1.4 (GCOS; Affymetrix). After the period of hybridization was complete, arrays were immediately scanned with GeneChip Scanner 3000 (Affymetrix) following the manufacturer's protocol. Array scanning at a specific wavelength provided signal intensities both probes and probe sets. GeneChip Operating Software 1.4 was used to obtain the microarray data from GeneChip images. Hybridization reaction, scanning and preliminary data analysis were performed at Middle East Technical University Central Laboratory.

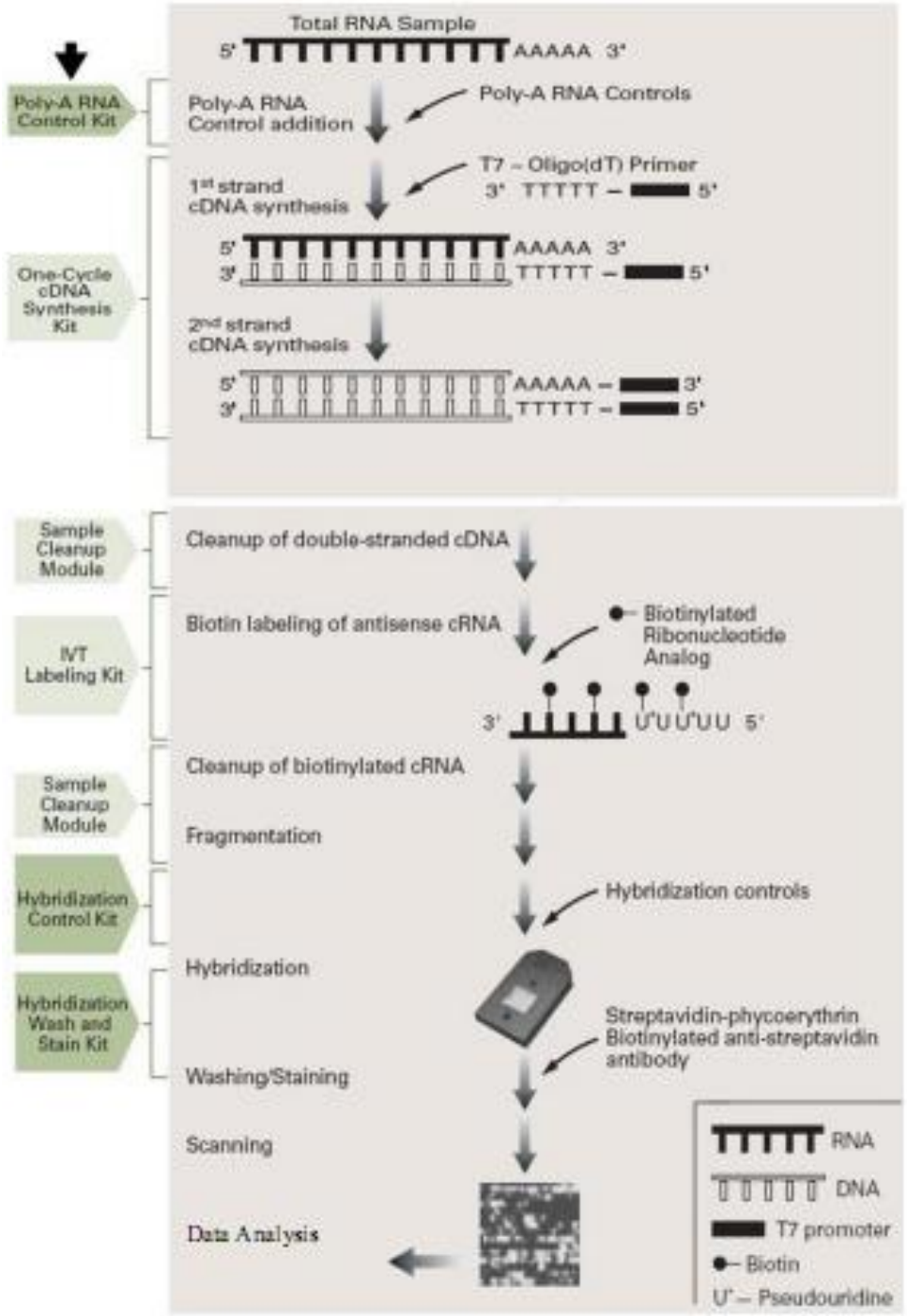


Figure 2.2. Experimental process used in global expression profiling of wheat cultivars under different B treatments. (GeneChip Expression Analysis Manual, Affymetrix).

2.2.5.4. Microarray Data Analysis

The raw data or signal intensity values imported as .CEL files were normalized with Robust Multiarray Analysis (RMA) by using GeneSpring GX 12.5 (Agilent Technologies, Santa Clara CA) software programme. RMA uses only perfect match probes (PM) and contains probe-specific background correction, normalization across all arrays, and median polishing (Irizarry *et al*., 2003).

The expression level of thousands of genes across different genotypes or conditions increases the complexity of the microarray data. This exponential complexity becomes impossible to derive the relationship between genes and conditions. Therefore, the dimensionality of the microarray data should be reduced into 2 or 3 to visualize trends or relations. Principal Components Analysis (PCA) is most widely used method that reduces the data dimensionality by performing a covariance analysis between factors.

After RMA preprocessing was complete, initial filtering was used to lower the number of probe sets. Probe sets that have higher intensity values than the 20^{th} percentile in at least one out of all hybridizations were retained. The normalized signal intensities of the filtered probe sets were used for statistical analyses. Analysis of variance (ANOVA) was used for statistical analysis of microarray data. Benjamini Hochberg false discovery rate (FDR) multiple testing corrections were used for correcting P-values (Benjamini and Hochberg, 1995). A gene was indicated as differentially expressed if the P-value was smaller than 0.05 and fold change is larger than at least 2. Comparisons were performed between data of control groups and treated groups. Hierarchical clustering on genes and treatments were performed with euclidean similarity measure and centroid linkage rule.

Target sequences of probe sets were obtained from NetAffx™ Analysis Center (Affymetrix). HarvEST:Barley (version 1.59, assembly XW; http://harvest.ucr.edu) was used to annotate the differentially expressed probe sets. MapMan was used to display all expression data on the diagrams of metabolic pathways and other processes (Thimm *et al*., 2004). The program MapMan was originally developed to display *Arabidopsis thaliana* gene expression in a functional context by classifying genes into hierarchical categories (called 'BINs'). Gene Ontology enrichment analysis was performed at AGRIGO website (http://bioinfo.cau.edu.cn/agriGO/).

2.2.6. Validation of Microarray Data Using Two-Step Real-Time RT-PCR

Two-step (reverse transcription carried out in a separate reaction) real-time reverse transcriptase PCR (RT-PCR) was used to validate the expression profiles of microarray analyses. In fact, this method involves creating cDNA first by means of a separate reverse transcription reaction and then adding the cDNA to the PCR reaction. Single-stranded cDNA synthesis was performed using QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's protocols. Significantly changed five genes across treatments in the microarray results were chosen for validation by RT-PCR. Vector NTI was used to design the primer pairs to amplify a region of target sequence. Real-time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) with Rotor-Gene Q (Qiagen) real-time cycler. Sequences of primer pairs and sizes of amplicons and amplification conditions in RT-PCR were given in Appendix C. Glyceraldehyde phosphate dehydrogenase gene (*TaGAPDH*) was used as house-keeping controls.

CHAPTER 3

RESULTS

In this study, we analyzed the changes in gene expression profiling in response to low, excess B and supplementing B-specific fertilizer (Tarımbor) to B-deficient condition in two wheat cultivars by using genomic approach via microarray. These changes were juxtaposed with measurements of physiological and biochemical traits following the same conditions. This multidisciplinary analysis may lead to the identification of novel mechanisms that might be useful for the development of wheat cultivars capable of maintaining yield under low or high B conditions. In addition, the understanding of the biochemical and molecular responses to B-specific fertilizer supplementation to B deficiency stress can be important for the improvement of selection strategies to overcome the productive limitations due to low B availability.

3.1. Phenotypic Responses of Wheat Cultivars to B-toxicity, deficiency and Tarımbor Applications

After the process of B-toxicity, deficiency and Tarımbor applications was complete, chlorosis and necrotic lesions were not observed under all conditions in Bolal leaves (Figure 3.1). On the other hand, B-toxicity stress for 5 days led to chlorosis and necrotic lesions in Atay leaves, however, B-deficiency and supplementary Tarımbor to control (Tarımbor) and deficient conditions (Deficiency+Tarımbor) did not cause any symptoms in these plants (Figure 3.2).

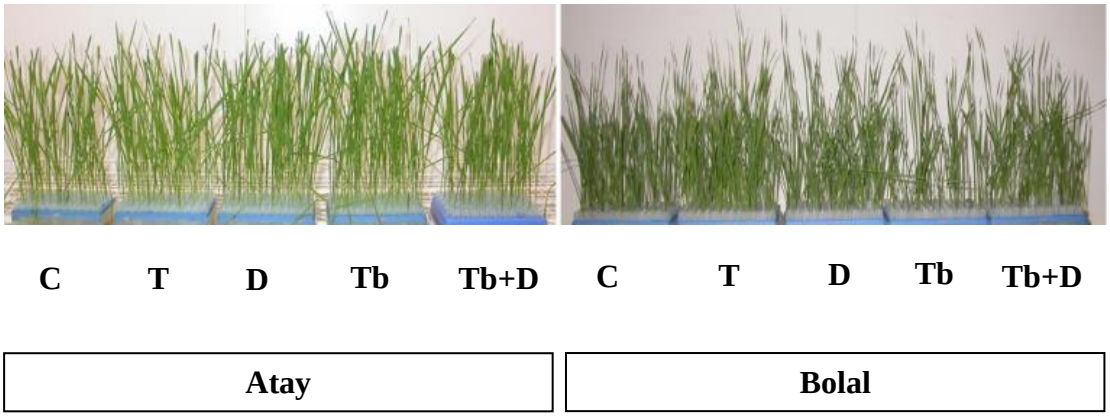


Figure 3.1. The seedlings of Atay and Bolal cultivars under B-toxic, deficient and two different Tarımbor conditions. C: Control, T: B-toxicity, D: deficiency, Tb: Tarımbor and Tb+D: B-deficiency+Tarımbor. Photographs were taken at the end of 5 days of growth under different treatments.

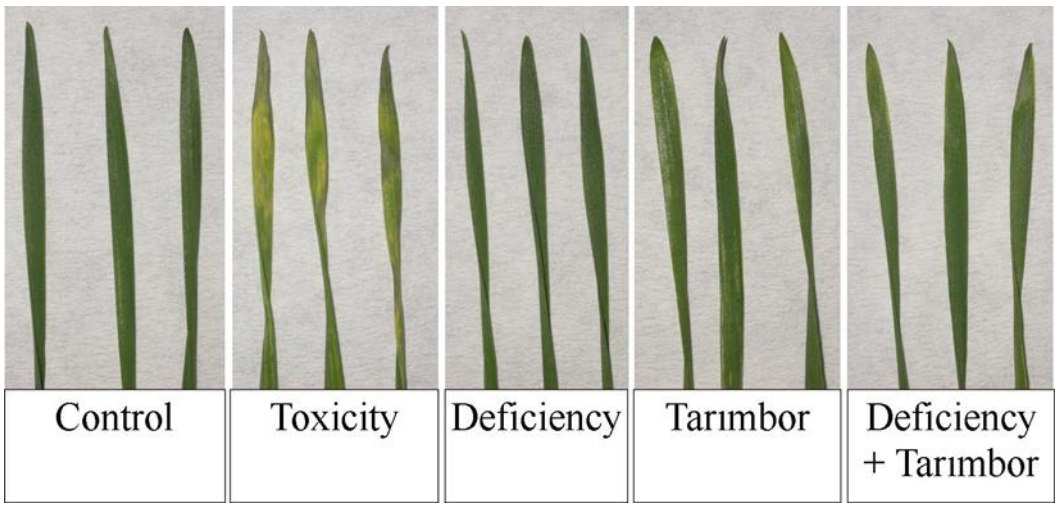


Figure 3.2. Phenotypic responses to B-toxicity, deficiency and two Tarımbor applications in Atay leaves. Photographs were taken at the end of the treatments.

3.2. B Contents of Wheat Cultivars under B-toxicity, deficiency and Tarımbor Applications

To quantify the effect of B-toxicity, deficiency and Tarımbor applications on both cultivars in more detail, B levels were determined using inductively coupled plasma spectrometry (ICP). Accordingly, B content dramatically increased in the leaves of both cultivars under B-toxicity condition (Figure 3.3). In fact, B accumulates higher in Atay leaves than Bolal under this condition. Furthermore, compared to control and B-deficiency, B content significantly increased during Tarımbor and B-deficiency+Tarımbor applications in both cultivars (Figure 3.3).

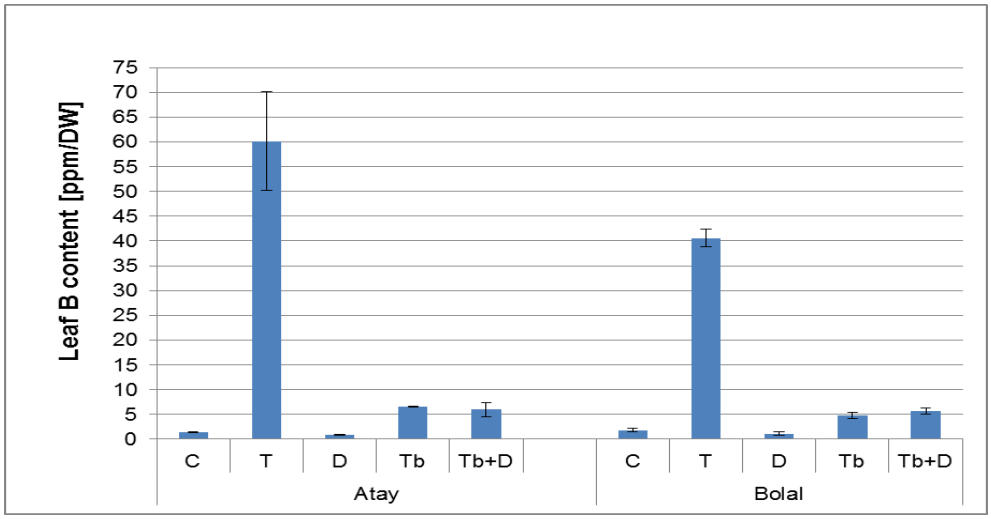


Figure 3.3. Leaf B content in Atay and Bolal cultivars exposed to control (C), B-toxicity (T), deficiency (D), Tarımbor (Tb) and Deficiency +Tarımbor (Tb+D) conditions. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

Figure 3.4 shows B content of root tissues of both cultivars. According to this figure, in 5 mM B treated roots of both cultivars, B-content increased almost eight-fold compared to the control. Moreover, Tarımbor and B-deficiency+Tarımbor applications did not cause a remarkable change in root B-content in both cutivars. In fact, these applications did not have a toxic effect on root tissues (Figure 3.4).

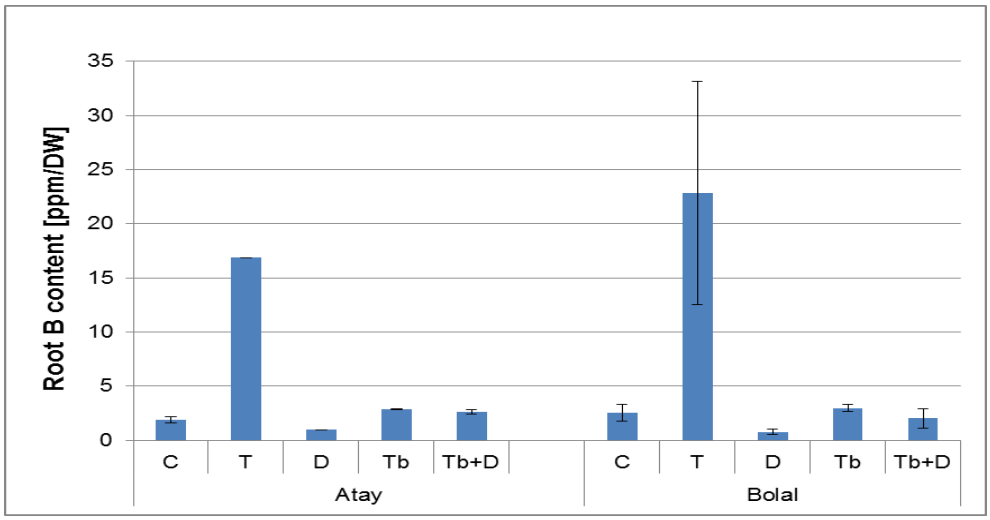


Figure 3.4. Root B content in Atay and Bolal cultivars exposed to control (C), B-toxicity (T), deficiency (D), Tarımbor (Tb) and Deficiency +Tarımbor (Tb+D) conditions. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

3.3. Physiological Responses of Wheat Cultivars to B-toxicity, deficiency and Tarımbor Applications

3.3.1. Leaf and Root Lengths

The changes in leaf and root lengths after B-toxicity, deficiency and Tarımbor applications in two wheat cultivars were shown in Figure 3.5 and 3.6, respectively. Length values of Bolal leaves had longer than Atay leaves under B-toxicity,

deficiency and Tarımbor applications (Figure 3.5). However, all B and Tarımbor applications did not lead to any significant changes in lengths of Atay leaves. Likewise, length was not sensitive to B-toxicity, deficiency and Tarımbor conditions in Bolal leaves. However, length values of Bolal leaves in B-deficient+Tarımbor were only slightly lowered when compared with Tarımbor samples (Figure 3.5).

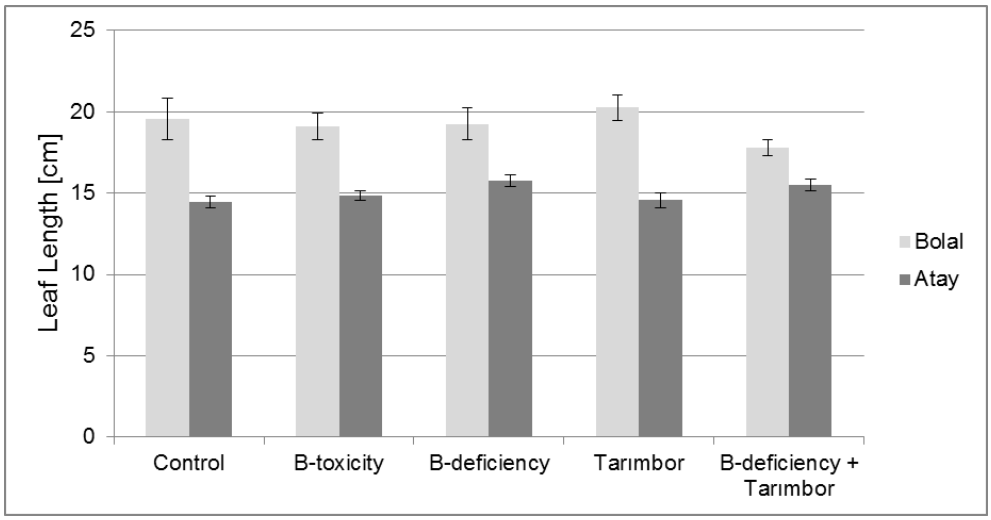


Figure 3.5. Mean values for leaf length in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

B-toxicity, deficiency, Tarımbor and B-deficiency+Tarımbor applications led to decrease in length values in Bolal roots, however, the decreases in B-toxic and Tarımbor applications were only statistically significant when compared to controls (Figure 3.6).

On the other hand, all treatments did not affect the root lengths in Atay cultivar. After B-deficiency+Tarımbor application, root lengths were significantly higher compared with corresponding Tarımbor samples in both cultivars (Figure 3.6).

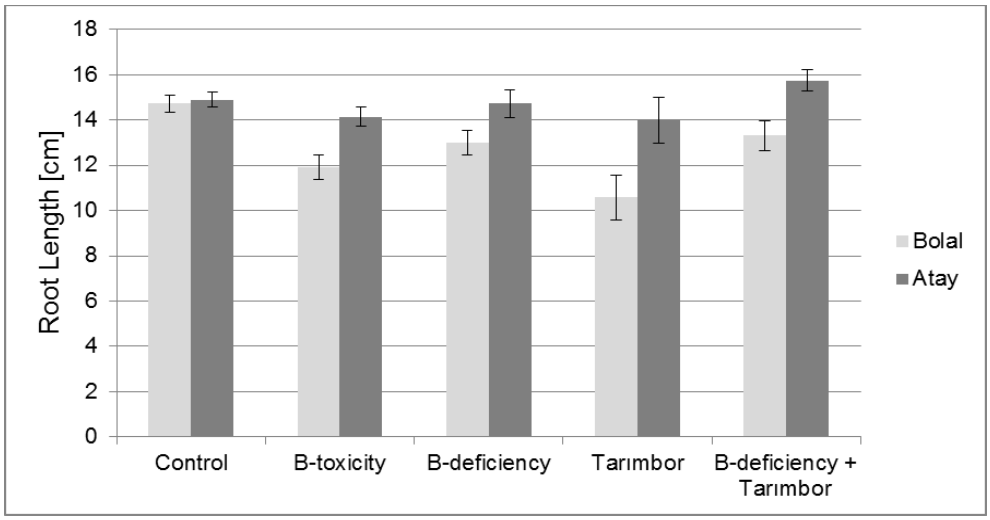


Figure 3.6. Mean values for root length in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

3.3.2. Wet and Dry Weights

B-toxicity, deficiency, Tarımbor and B-deficiency+Tarımbor applications did not alter the wet weight values in leaves of both cultivars when compared to control plants (Figure 3.7). However, wet weight values of Bolal leaves in B-deficient+Tarımbor were only slightly lowered when compared with corresponding Tarımbor plants as verified length values of Bolal leaves (Figure 3.7).

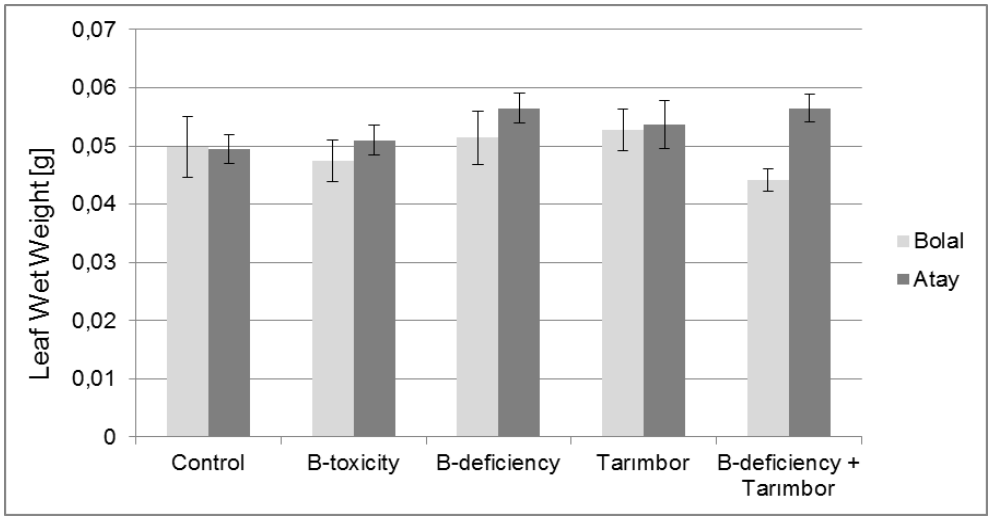


Figure 3.7. Mean values for leaf wet weight in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

In Bolal roots, all B and Tarımbor applications led to decrease in wet weights, however, the changes under B-toxic and Tarımbor applications were only statistically significant compared with control plants (Figure 3.8). Likewise, in Tarımbor applications, wet weights of Atay roots were significantly lower than other B treatments. Furthermore, when wet weight values were used to compare with two different cultivars, Atay roots had higher values than Bolal under all B applications (Figure 3.8).

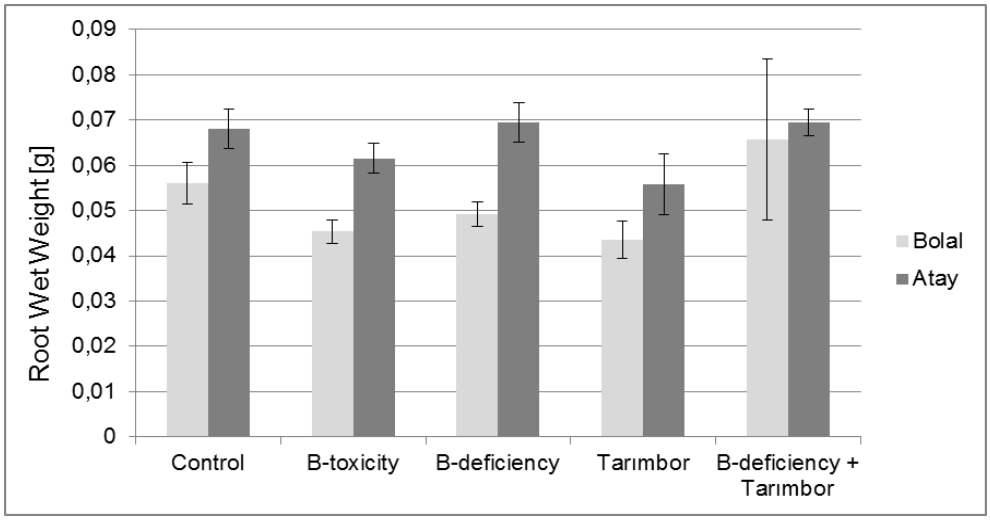


Figure 3.8. Mean values for root wet weight in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

B-toxicity, deficiency and B-deficient+Tarımbor applications did not cause any remarkable changes in the dry weight values of Atay leaves in comparison with control leaves (Figure 3.9). However, it was significantly increased at the end of the Tarımbor application in Atay leaves. On the other hand, in Bolal leaves this value did not significantly change after exposure to B-toxicity, deficiency and Tarımbor applications. However, B-deficient+Tarımbor application led to decrease in dry weights of Bolal leaves when compared to B-toxicity and Tarımbor applications (Figure 3.9).

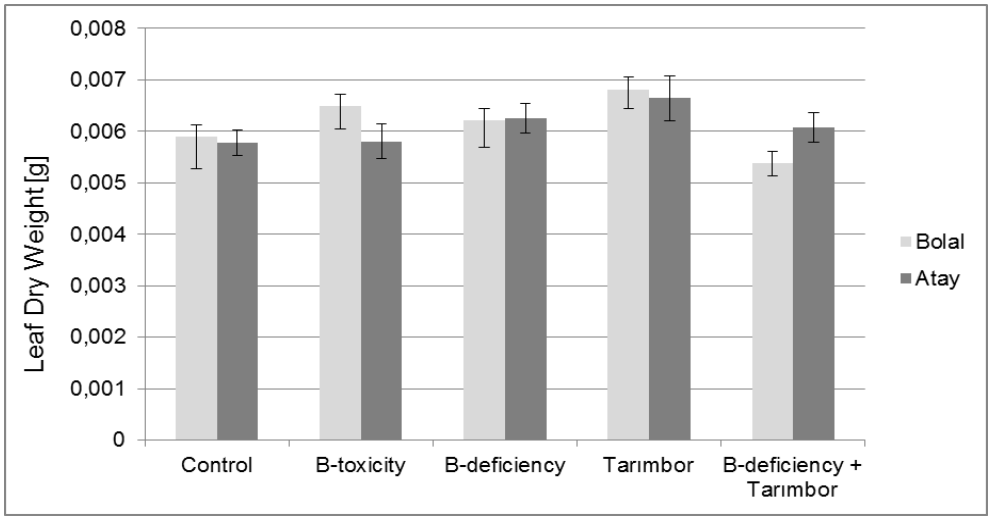


Figure 3.9. Mean values for leaf dry weight in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

In Atay roots dry weight values did not change under all B and Tarımbor applications. Likewise, B-toxicity, deficiency and B-deficient+Tarımbor applications did not lead to any significant changes in these values in Bolal roots. However, they were significantly lowered at the end of the 5 d Tarımbor applications when compared to controls (Figure 3.10).

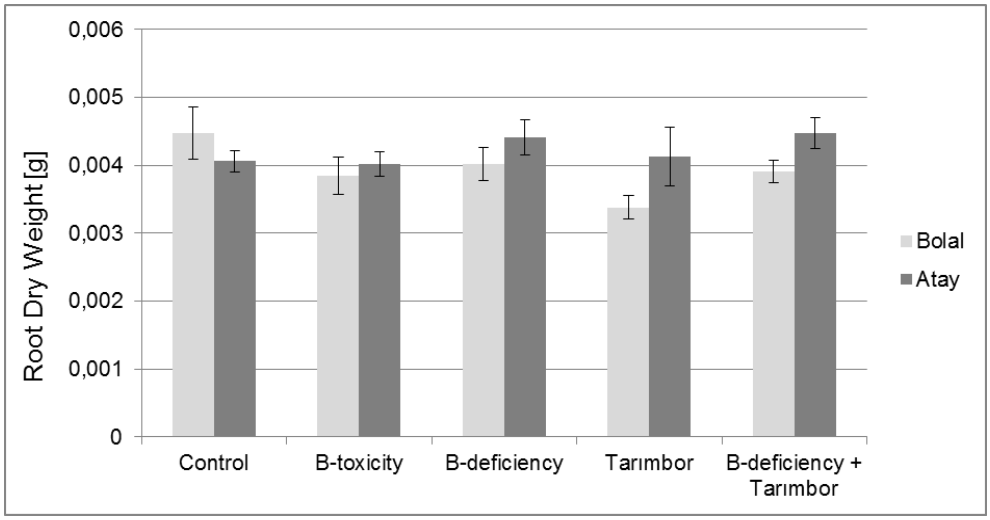


Figure 3.10. Mean values for root dry weight in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

3.3.3. Ion Leakage

Mean values of leaf and root ion leakage were shown in Figure 3.11 and 3.12, respectively in order to determine the possible B induced damage in B-stresses and Tarımbor treated wheats. Accordingly, B-toxicity, deficiency and Tarımbor application did not alter the ion leakage in Bolal leaves; however, B-deficient+Tarımbor caused a decrease in ion leakage with respect to control plants (Figure 3.11). On the other hand, this decrement was only observed in Tarımbor application in Atay leaves. Also, the values of ion leakage did not change significantly under B-toxicity, deficiency and B-deficient+Tarımbor application compared with corresponding control leaves (Figure 3.11).

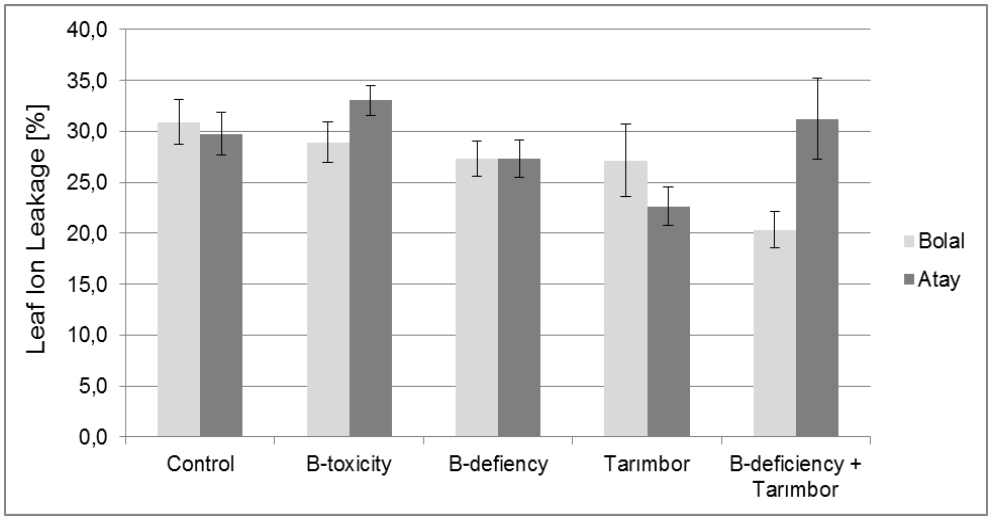


Figure 3.11. Mean values for leaf ion leakage in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

In root tissues of Bolal cultivars B stresses and two Tarımbor applications did not lead to any significant change in the values of ion leakage (Figure 3.12). On the other hand, it was slightly induced under B-toxicity in root tissues of Atay cultivar. However, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications did not cause any remarkable change in the values of root ion leakage in Atay cultivar (Figure 3.12).

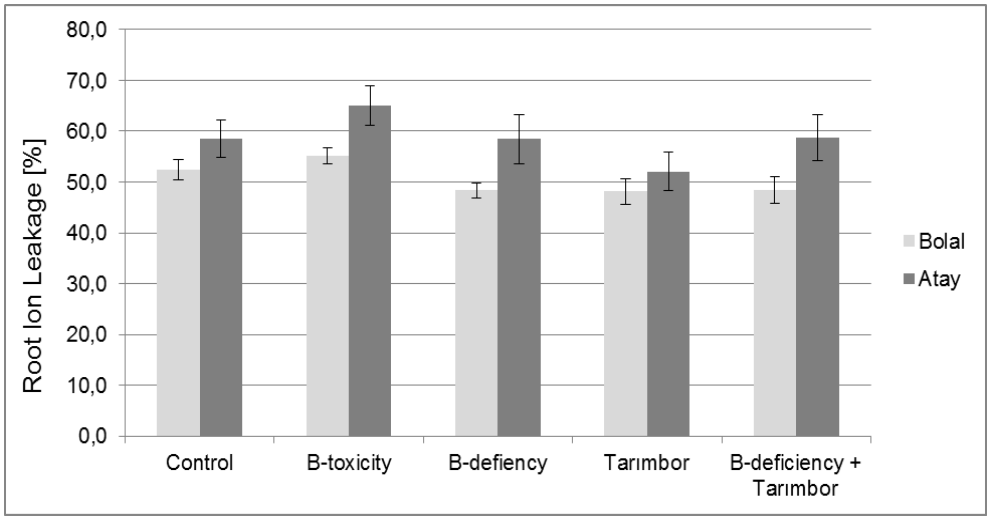


Figure 3.12. Mean values for root ion leakage in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 20 replications each. Values represent means ± SE ($P < 0.05$).

3.3.4. Relative Water Content

Relative Water Content (RWC), represented a useful indicator of the state of water balance of a plant, was used to evaluate the possible changes of water status under B-stress and Tarımbor applications. This content was only measured by using leaf tissues of two cultivars since plants were grown with Hoagland solution and root tissues had maximum capacity of water retention. In Bolal, RWC increased slightly after Tarımbor application and maintained an increased level in B-deficiency+Tarımbor application when compared to control leaves (Figure 3.13). Similarly, this content increased slightly at the end of the B-toxicity and deficiency in Atay leaves. However, Atay leaves subjected to Tarımbor and B-deficiency+Tarımbor applications showed RWC values as high as control leaves (Figure 3.13).

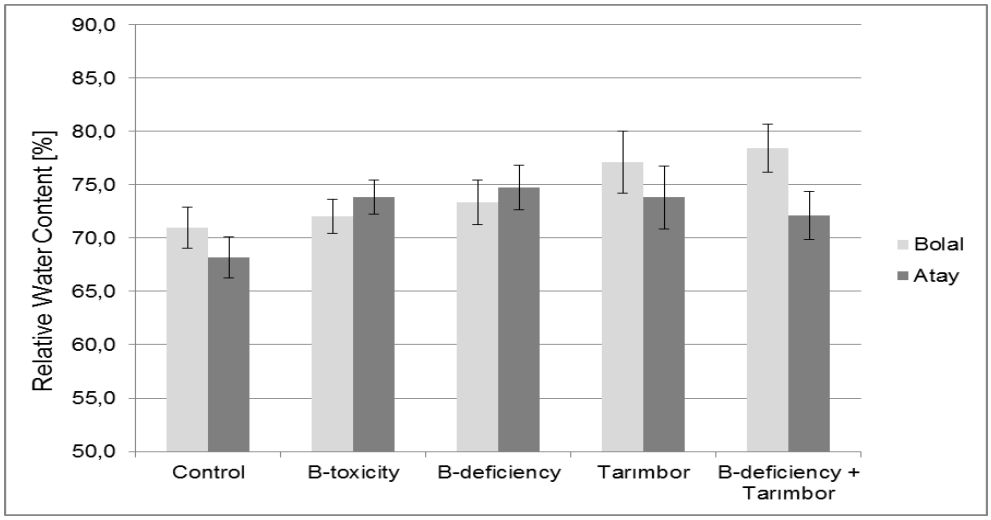


Figure 3.13. Leaf relative water content (RWC) (%) in Atay (black column) and Bolal (gray column) cultivars under B-toxicity, deficiency, Tarımbor (supplementary Tarımbor to control condition) and B-deficiency+Tarımbor conditions. Measurements were immediately conducted 5 d after B-treatments initiation. Mean values of three independent experiments with 24 replications each. Values represent means ± SE ($P < 0.05$).

3.3.5. Chlorophyll a Fluorescence Measurements

The photosynthetic response to B-toxicity, deficiency and Tarımbor applications were determined by analyzing several fluorescence parameters under dark-adapted and steady state conditions (Figure 3.14 and 3.15). F_o values decreased under B-toxicity and deficiency conditions in both cultivars, however, they were not statistically significant (Figure 3.14). On the other hand, Tarımbor and B-deficiency+Tarımbor applications led to decrease in values of minumum flourescence (F_o) compared with control leaves. Similar to F_o, F_M values did not significantly alter at low and high B conditions and decreased after Tarımbor and B-deficiency+Tarımbor applications in both cultivars (Figure 3.14). The values of the quantum efficiency of open PSII reaction centers in the dark-adapted state (F_V/F_M) were approximately 0.80–0.82 in control leaves of both cultivars. These values

slightly decreased after B-toxicity and increased under B-deficiency in both cultivars. However, they were not statistically significant. On the other hand, two Tarımbor applications did not cause any significant changes in F_V/F_M in both cultivars (Figure 3.14). All B and Tarımbor applications did not result in the efficiency of excitation energy trapping of PSII reaction centers (F_V'/F_M'), the quantum yield of PSII electron transport (ϕPSII), the non-photochemical energy dissipation (NPQ) and the electron transport rates (ETR) in Bolal cultivars (Figure 3.15). On the other hand, in Atay F_V'/F_M' values increased slightly under all B and Tarımbor applications when compared to corresponding control leaves (Figure 3.15). Furthermore, there was no significant change in ϕPSII and ETR under all conditions; however, B-toxicity and B-deficiency+Tarımbor application caused a significant decrease in NPQ in Atay leaves (Figure 3.15).

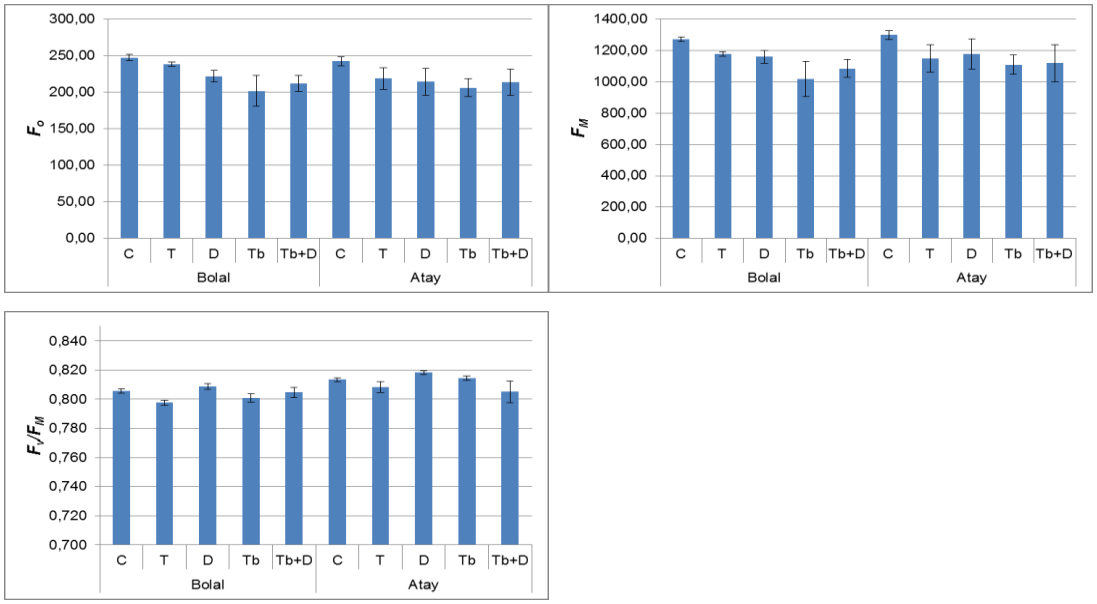


Figure 3.14. Chlorophyll fluorescence responses of Atay and Bolal leaves to exposed B stress and Tarımbor applications. F_o: minimum fluorescence F_M: maximum fluorescence F_V/F_M: the quantum efficiency of PSII in dark adapted state. Mean values of three independent experiments with 10 replications each. Values represent means ± SE ($P < 0.05$).

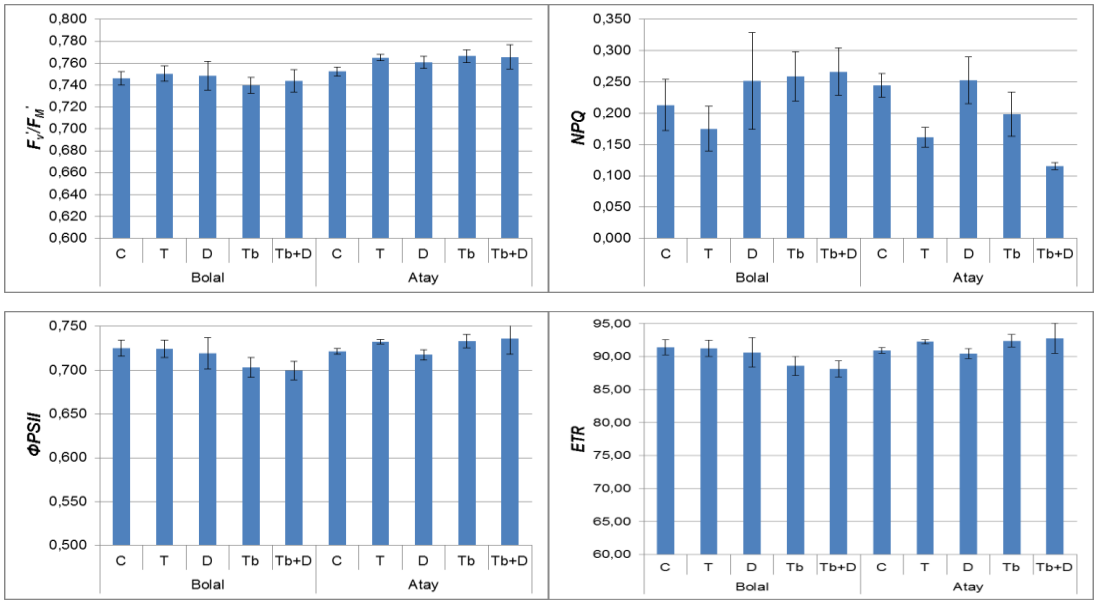


Figure 3.15. Chlorophyll fluorescence responses of Atay and Bolal leaves to exposed B stress and Tarımbor applications. F_V'/F_M': the quantum efficiency of excitation energy trapping of PSII in light-adapted state, NPQ: non-photochemical quenching, ΦPSII: the quantum efficiency of PSII in light adapted state, ETR: Electron transport rates. Mean values of three independent experiments with 10 replications each. Values represent means ± SE ($P < 0.05$).

3.3.6. Pigment Analyses

B-toxicity imposed at the early seedling stage caused a significant decrease in chlorophyll a (Ca), chlorophyll b (Cb) and carotenoid (Cx+c) contents in Atay cultivar (Figure 3.16A); however, it induced a significant increase in the Ca/Cb ratio in Atay leaves (Figure 3.16B). On the other hand, these contents did not significantly change after exposure to B-toxicity in Bolal leaves. Under B-deficiency condition, the values of Ca, Cb and Cx+c were significantly lower in Atay than the values of control leaves, whereas the Ca/Cb ratio was significantly higher than respective control leaves. Tarımbor and B-deficiency+Tarımbor applications caused a less decrease in these contents when compared to B-deficiency plants.

Also, these applications did not cause any significant change in the Ca/Cb ratio in leaf tissues of Atay. On the other hand, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications led to significant increase in the contents of Ca, Cb, and Cx+c compared with control leaves in Bolal cultivar; however, the Ca/Cb ratio was not significantly changed under all B treatments in leaf tissues of Bolal cultivar (Figure 3.16A, B).

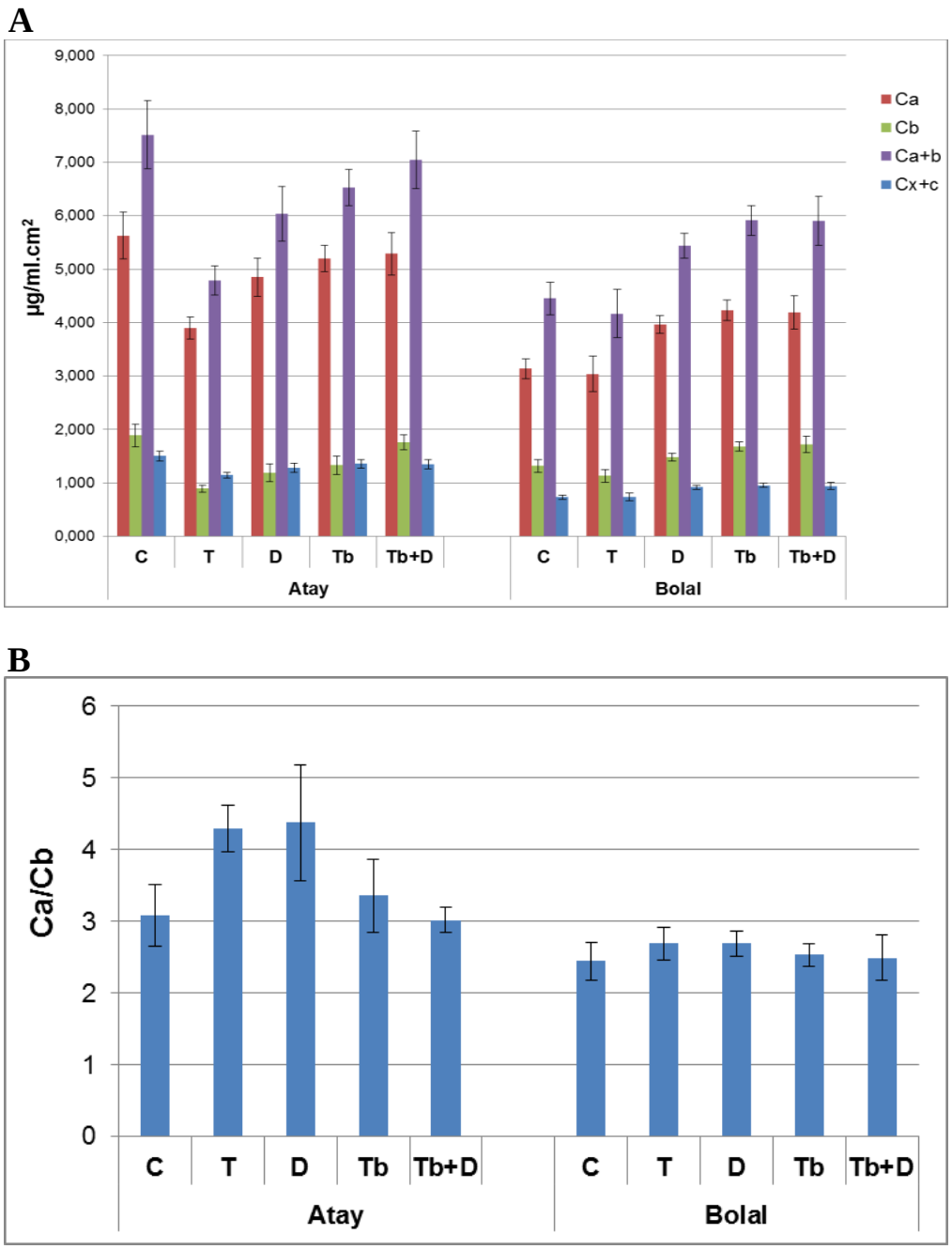


Figure 3.16. Changes in foliar concentration of chlorophyll a (Ca), chlorophyll b (Cb), total carotenoids (Cx+c) (A) and Ca/Cb ratio (B) in two wheat cultivars (Atay and Bolal) subjected to control (C) B-toxicity (T), deficiency (D), Tarımbor (Tb) and Deficiency+Tarımbor (Tb+D) conditions. Mean values of three independent experiments with 10 replications each. Values represent means ± SE ($P < 0.05$).

3.3.7. Leaf Water Potential

B-toxicity caused a significant decrease in leaf water potential of both Atay and Bolal wheat plants (Figure 3.17). On the other hand, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications did not lead to any significant changes in water potential in both cultivars when compared to control leaves. In other words, the values of water potential in these conditions reached to control values in both cultivars. As a conclusion, leaf water potential showed lowest values after B-toxicity treatments in both cultivars. However, there were no differences in these values between Atay and Bolal plants during B-stresses and two Tarımbor applications (Figure 3.17).

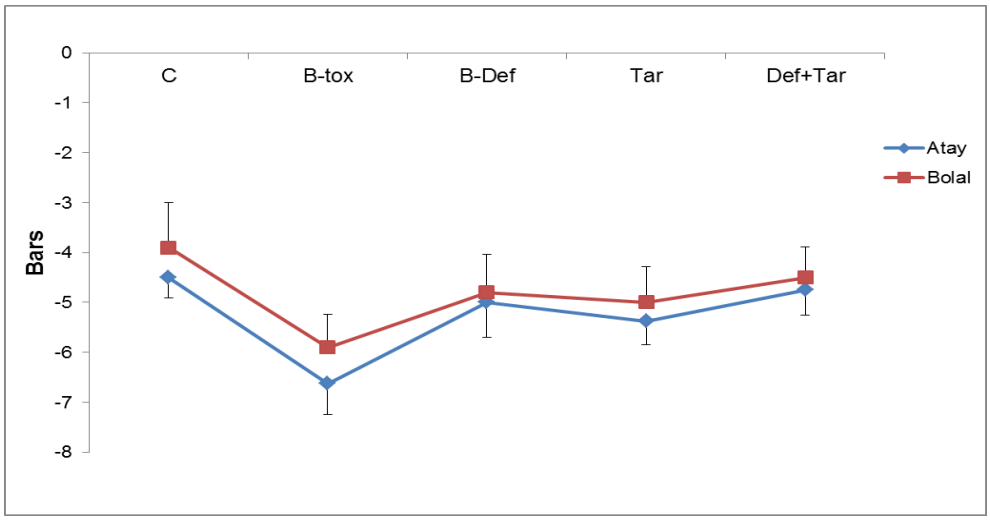


Figure 3.17. Changes in leaf water potential of wheat cultivars Atay and Bolal exposed to control (C), B-toxicity (B-tox), B-deficiency (B-Def), Tarımbor (Tar) and Deficiency+Tarımbor (Def+Tar) applications. Mean values of three independent experiments with 9 replications each. Values represent means ± SE ($P < 0.05$).

3.4. Biochemical Responses of Wheat Cultivars to B-toxicity, deficiency and Tarımbor Applications

3.4.1. Lipid Peroxidation

In order to evaluate the oxidative damage in wheat cultivars, lipid peroxidation was estimated by measuring the accumulation of MDA. The leaves and roots MDA content under different B and Tarımbor applications were shown in Figure 3.18 and 19, respectively. B-toxicity, deficiency and two Tarımbor applications did not cause any significant changes in MDA level in Atay leaves, however, B-toxicity increased MDA level in Bolal leaves compared with their respective controls (Figure 3.18). Similar to Atay leaves, there were no change in MDA levels under B-deficiency, Tarımbor and B-deficiency+Tarımbor applications (Figure 3.18).

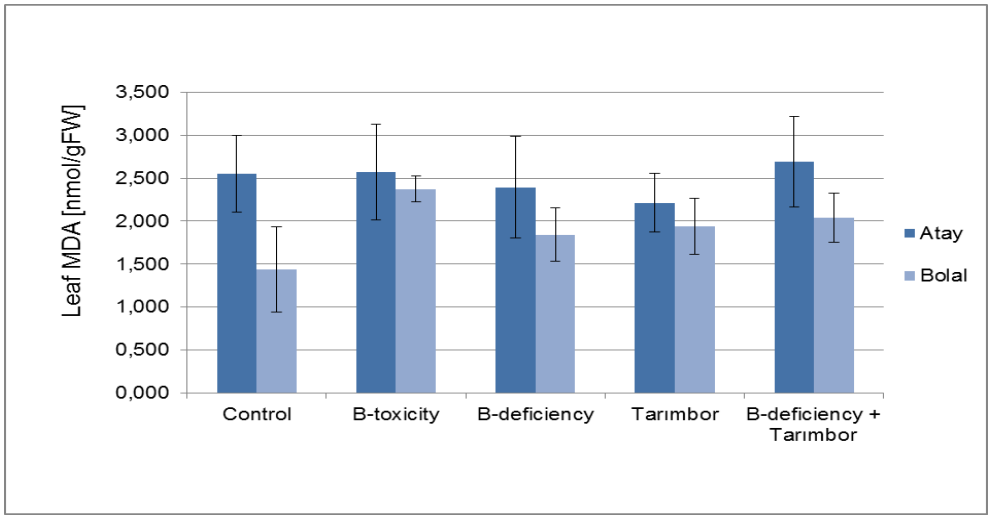


Figure 3.18. Lipid peroxidation evaluated by MDA concentrations in leaves of wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of four independent experiments with 4 replications each. Values represent means ± SE ($P < 0.05$).

Compared with controls, the MDA content declined significantly in Atay roots under B-toxicity (Figure 3.19). On the contrary, it did not change significantly after B-toxicity treatments in Bolal roots. The root MDA was not significantly changed in both cultivars under B-deficiency, Tarımbor and B-deficiency+Tarımbor applications (Figure 3.19).

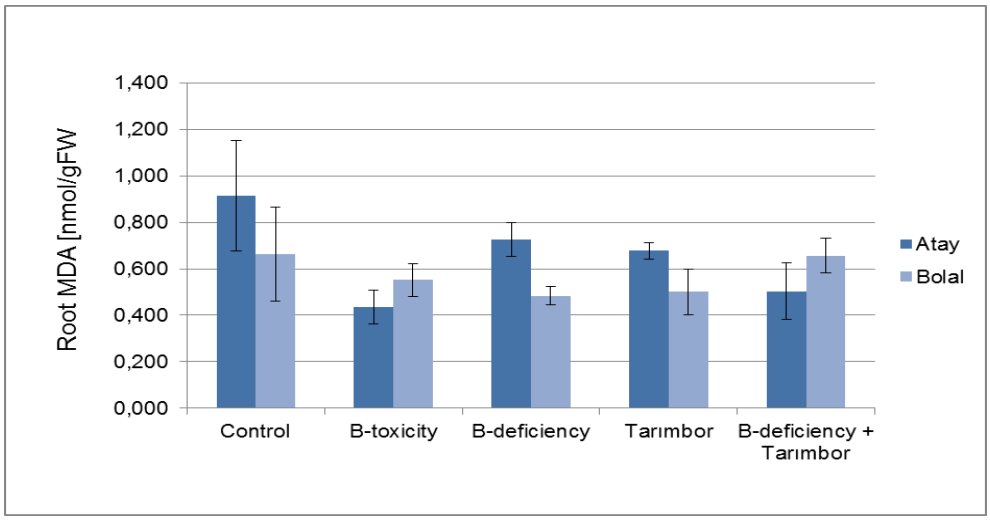


Figure 3.19. Lipid peroxidation evaluated by MDA concentrations in roots of wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of four independent experiments with 4 replications each. Values represent means ± SE ($P < 0.05$).

3.4.2. Proline Content

All B and Tarımbor applications did not lead to a significant change in leaf proline content in Atay (Figure 3.20). On the other hand, this content was lowered after B-deficiency, Tarımbor and B-deficiency+Tarımbor applications in Bolal leaves when compared to controls as well as having no change under B-toxicity (Figure 3.20).

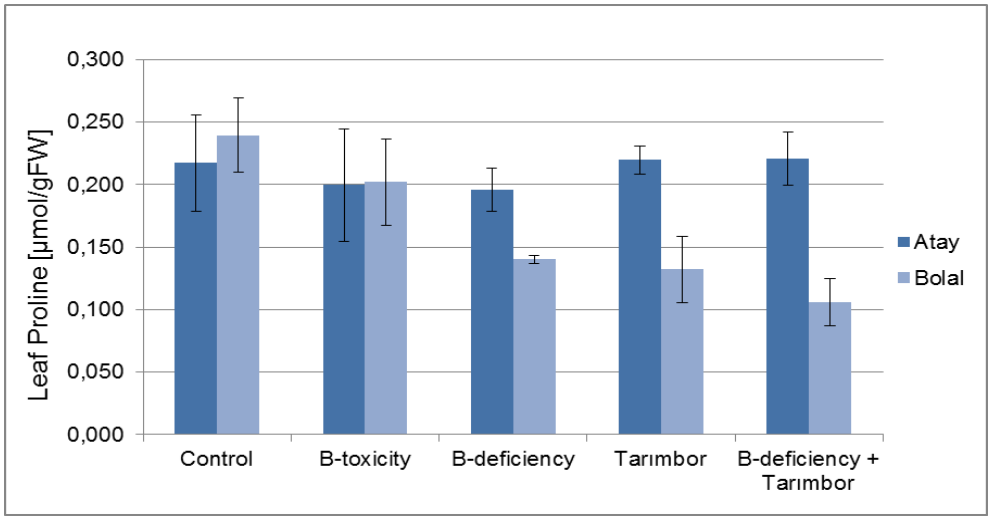


Figure 3.20. Changes in leaf proline content of wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of four independent experiments with 4 replications each. Values represent means ± SE ($P < 0.05$).

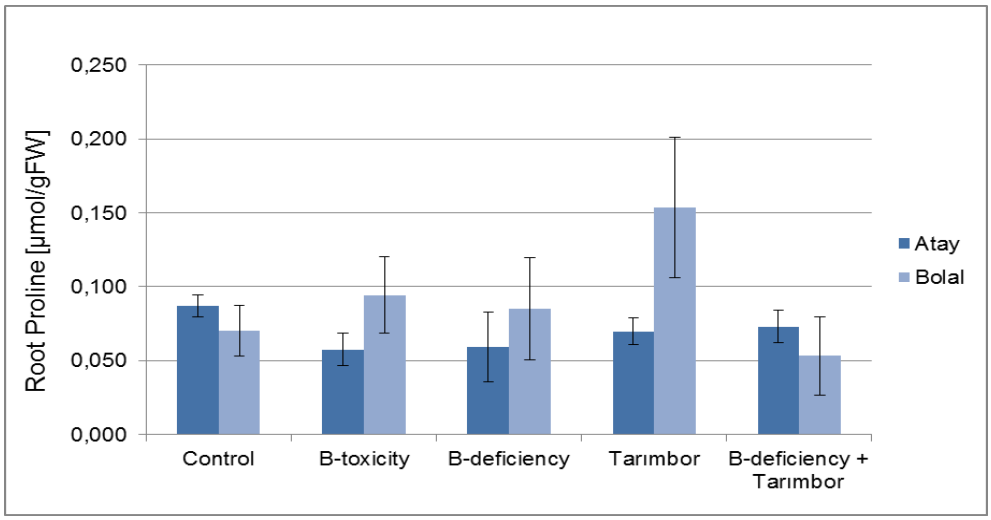


Figure 3.21. Changes in root proline content of wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of four independent experiments with 4 replications each. Values represent means ± SE ($P < 0.05$).

In root tissues, B-toxicity increased slightly the accumulation of proline content in Bolal but it was slightly decreased in Atay, however, these differences were found insignificant (Figure 3.21). Furhermore, similar to previous results, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications did not alter the root proline accumulation in Atay, however, accumulation of proline was higher after Tarımbor application than control and other conditions in Bolal (Figure 3.21).

3.4.3. Glycine Betaine Content

In leaf tissues of both cultivars, the accumulations of glycine betaine were lower at the end of the B-toxicity, deficiency, Tarımbor and B-deficiency+Tarımbor applications when compared to controls but they were not significant (except for Tarımbor application in Bolal) (Figure 3.22).

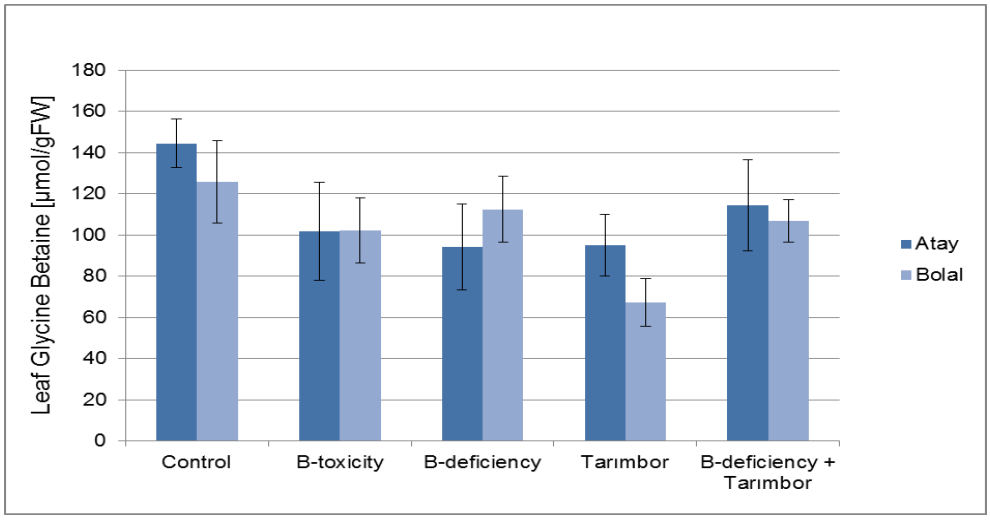


Figure 3.22. Changes in leaf glycine betaine content of wheat cultivars (‘Atay’ dark blue bars and ‘Bolal’ light blue bars) exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of four independent experiments with 4 replications each. Values represent means ± SE ($P < 0.05$).

B-toxicity induced slightly the accumulation of glycine betaine in root tissues of Atay; however, there were no significant change after B-deficiency and other two Tarımbor applications (Figure 3.23). On the other hand, this accumulation was significantly higher under B-deficiency+Tarımbor application as compared to respective control, however, it remained unchanged after B-toxicity, deficiency and Tarımbor application in Bolal roots (Figure 3.23).

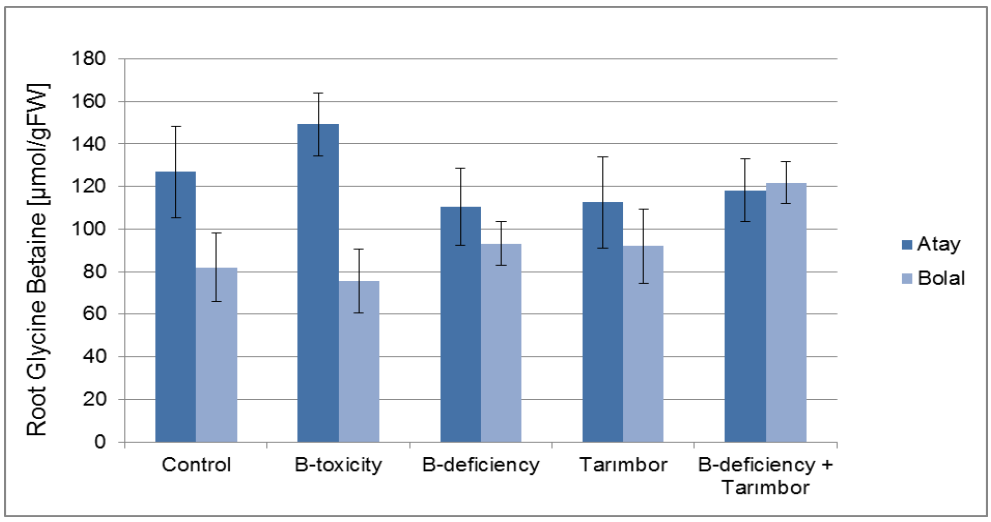


Figure 3.23. Changes in root glycine betaine content of wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of four independent experiments with 4 replications each. Values represent means ± SE ($P < 0.05$).

3.4.4. Antioxidant Enzymes

Activities of APX, CAT and GR activities were measured after exposure to B toxicity, deficiency and two Tarımbor applications to investigate changes in H_2O_2 scavenging enzymes. The changes in the activities of these enzymes in leaf and root tissues of both cultivars were shown in Figure 3.24-29.

3.4.4.1. Ascorbate Peroxidase (APX)

In both cultivars, the leaf APX activity increased significantly following B-toxicity treatment when compared to control (Figure 3.24). Furthermore, Tarımbor and B-deficiency+Tarımbor applications induced a slight increase in the leaf APX activity in both cultivars. It increased significantly under B-deficiency in Atay and did not change in Bolal (Figure 3.24).

In comparison with control, all B and Tarımbor applications did not lead to any significant change in the root APX activity in Bolal cultivar (Figure 3.25). On the other hand, APX activity in roots of Atay decreased only after exposure to B-toxicity as well as having no change under B-deficiency and two Tarımbor applications (Figure 3.25).

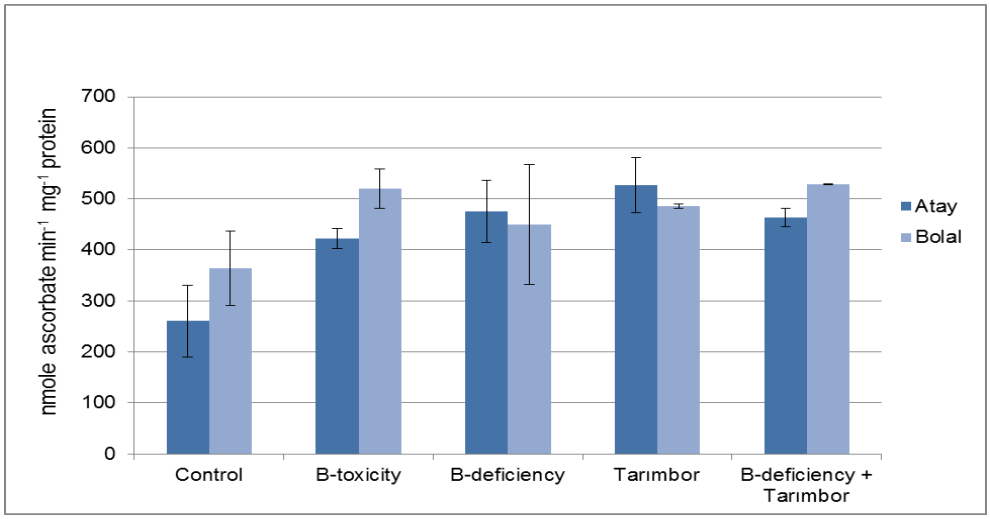


Figure 3.24. Ascorbate Peroxidase (APX) activity in leaves of two wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) subjected to exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

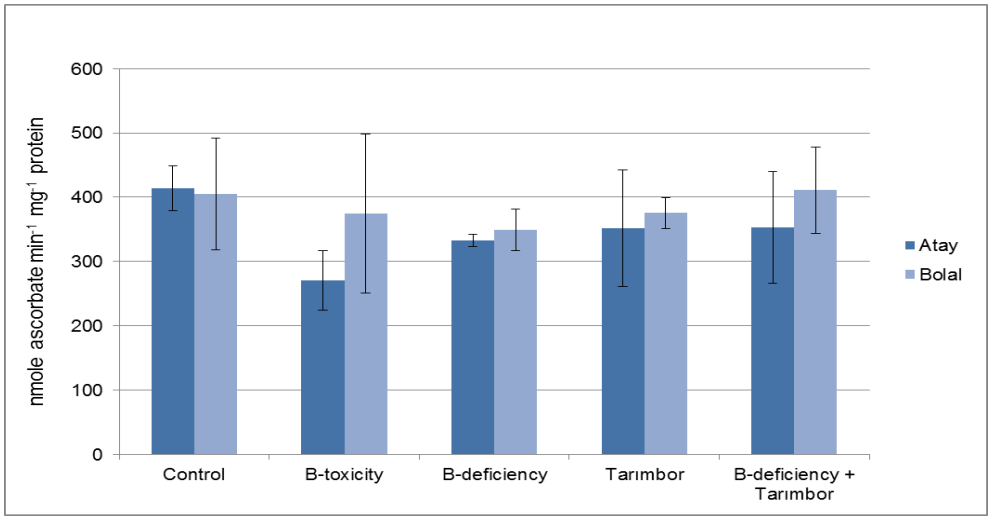


Figure 3.25. Ascorbate Peroxidase (APX) activity in roots of two wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) subjected to exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

3.4.4.2. Catalase (CAT)

In all conditions no significant change in leaf CAT activity was detected in both cultivars compared to respective controls (Figure 3.26). Likewise, the root CAT activity did not significantly change under all B and Tarımbor conditions in Atay and increased significantly at the end of the B-toxicity, deficiency and Tarımbor application in Bolal (Figure 3.27).

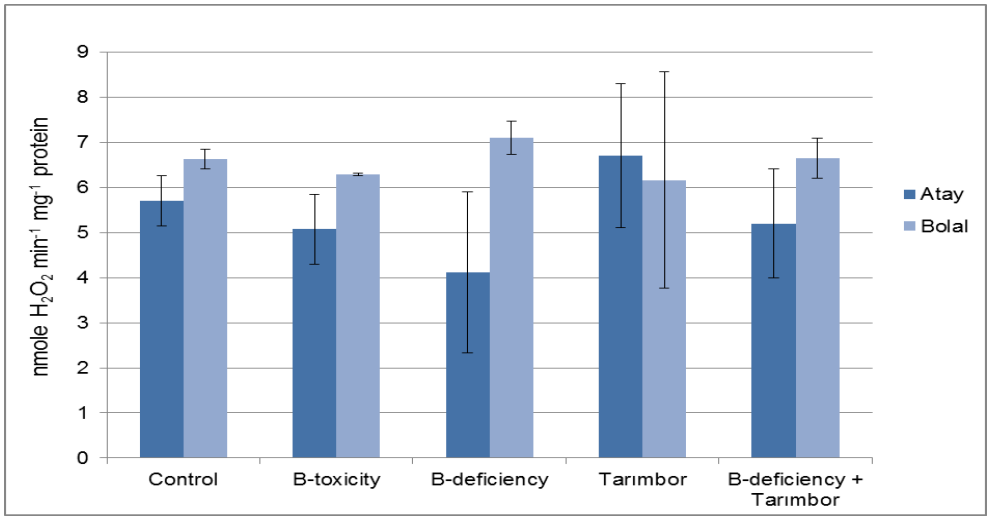


Figure 3.26. Catalase (CAT) activity in leaves of two wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) subjected to exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

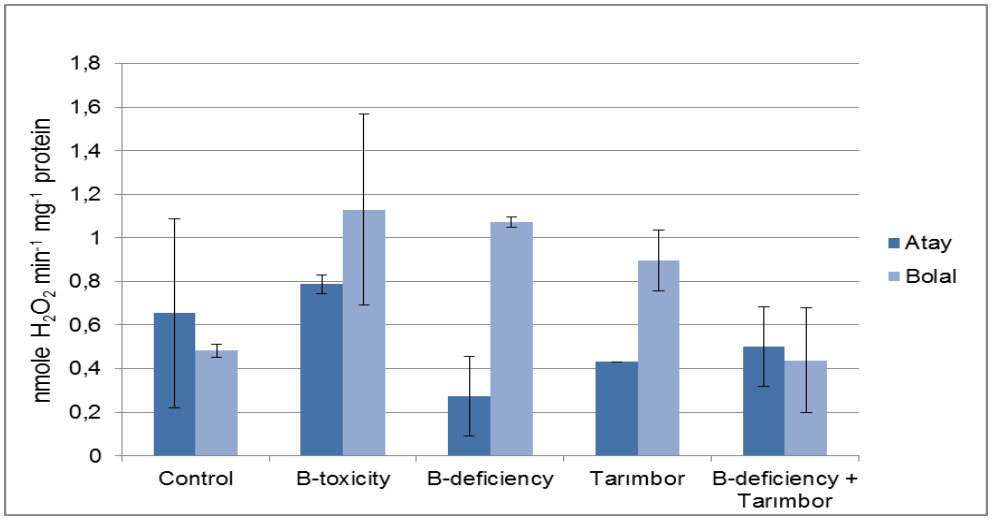


Figure 3.27. Catalase (CAT) activity in roots of two wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) subjected to exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

3.4.4.3. Glutathione Reductase (GR)

The leaf GR activity was significantly higher in B-toxicity and Tarımbor application than control and did not change in B-deficiency and B-deficiency+Tarımbor application in Atay (Figure 3.28). On the other hand, all treatments resulted in a slight decrease in the leaf GR activity in Bolal (Figure 3.28).

All B and Tarımbor applications did not cause any remarkable change in the root GR activity in Atay; however, B-toxicity induced a significant increase in GR activity in root tissues of Bolal (Figure 3.29). On the contrary, it was not significantly changed in B-deficiency and two Tarımbor applications in Bolal with respect to control (Figure 3.29).

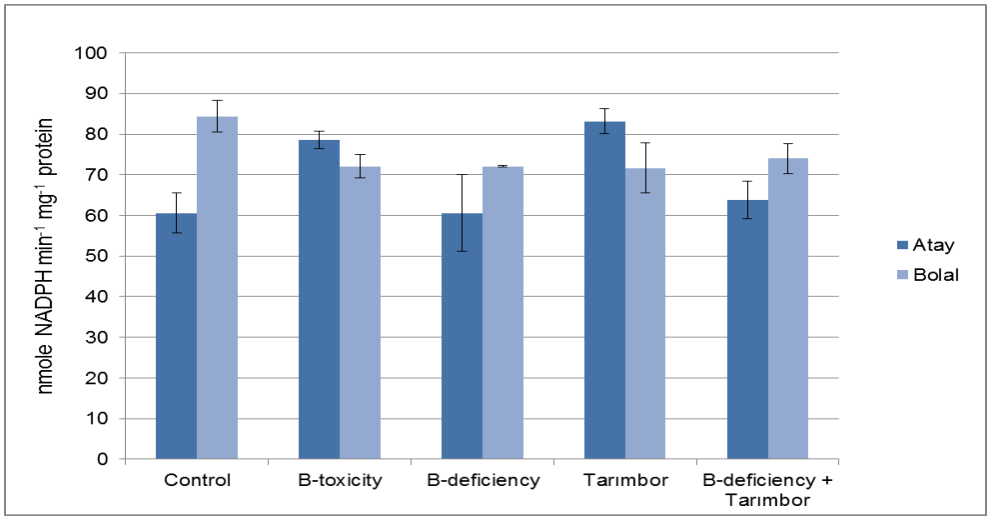


Figure 3.28. Glutathione reductase (GR) activity in leaves of two wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) subjected to exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

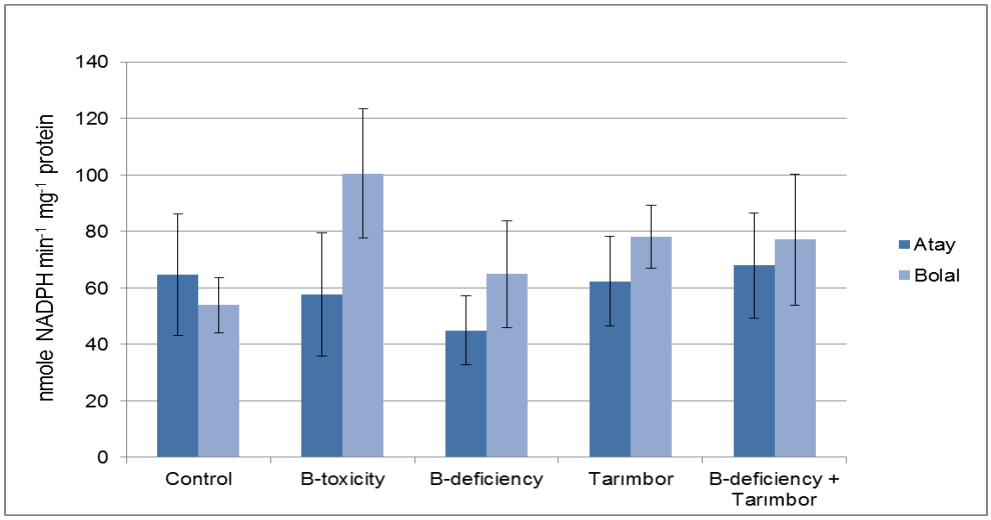


Figure 3.29. Glutathione reductase (GR) activity in roots of two wheat cultivars ('Atay' dark blue bars and 'Bolal' light blue bars) subjected to exposed to control, B-toxicity, B-deficiency, Tarımbor and B-deficiency+Tarımbor applications. Mean values of three independent experiments with 3 replications each. Values represent means ± SE ($P < 0.05$).

3.5. Differential Gene Expression in Response to B-toxicity, deficiency and Tarımbor Application in Wheat Cultivars

In this part, unlike the physiological and biochemical studies, leaf and root tissues of wheat cultivars were subjected to four different treatments: control, B-toxicity, B-deficiency and addition of soluble Tarımbor to the B-deficient condition. 48 microarrays from three biological replicates were used to assess the effects of these conditions on global expression profiling.

3.5.1. Quality and Quantity Control of RNA Samples

In the present study, total RNA was isolated from the leaf and root tissues of two wheat cultivars exposed to control, B-toxicity, deficiency, Tarımbor and B-deficiency+Tarımbor application (Tarımbor RNAs were not used for microarray experiments). For gel profiling, 3 µg of isolated RNA was loaded on 1% agarose gel. The sharpness and intensity of the bands of 28S and 18S rRNA transcript in agarose gel electrophoresis were shown in Figure 3.30 and indicated no degradation of isolated total RNAs.

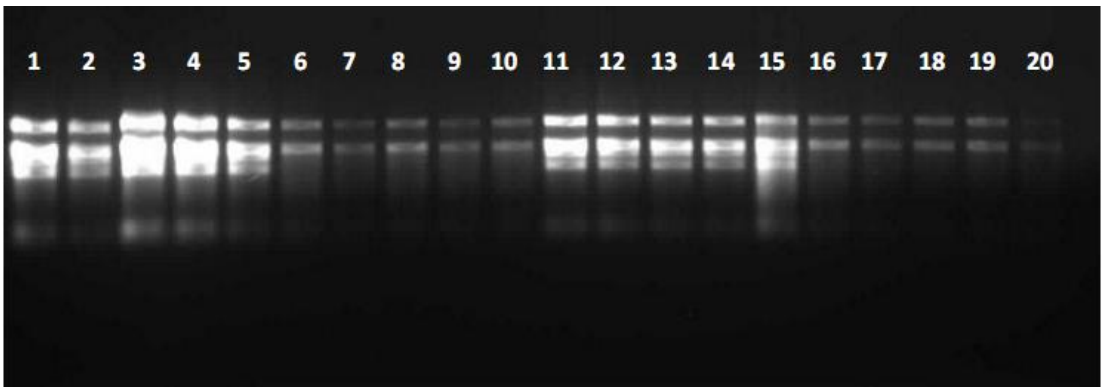


Figure 3.30. Agarose gel profile of isolated total RNA by TRIzol method. 1; Atay Control Leaf, 2; Atay B-toxicity Leaf, 3; Atay B-deficiency Leaf, 4; Atay Tarımbor Leaf, 5; Atay B-deficiency+Tarımbor Leaf, 6; Atay Control Root, 7; Atay B-toxicity Root, 8; Atay B-deficiency Root, 9; Atay Tarımbor Root, 10; Atay B-deficiency+Tarımbor Root, 11; Bolal Control Leaf, 12; Bolal B-toxicity Leaf, 13; Bolal B-deficiency Leaf, 14; Bolal Tarımbor Leaf, 15; Bolal B-deficiency+Tarımbor Leaf, 16; Bolal Control Root, 17; Bolal B-toxicity Root, 18; Bolal B-deficiency Root, 19; Bolal Tarımbor Root, 20; Bolal B-deficiency+Tarımbor Root.

For confirmation of the results of agarose gel-based assay, the samples were run on Agilent 2100 Bioanalyzer with RNA 6000 nano kit. The bioanalyzer software generates electropherogram and displays RNA concentration, its ribosomal ratio and visual assessment of the quality of an RNA sample. RNA quality was determined by measuring 28S/18S rRNA ratios and calculating the respective RNA integrity number (RIN). The RIN software algorithm is used for the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. These RINs and their representative electropherograms were shown in Figure 3.31 and 3.32. In the electropherograms, no degraded RNAs were observed in all samples and their concentrations were measured by using florescent dye (Ribogreen) in spectrofluorometer. The changes in RNA concentrations were shown in Appendix D. They were used for further microarray analysis.

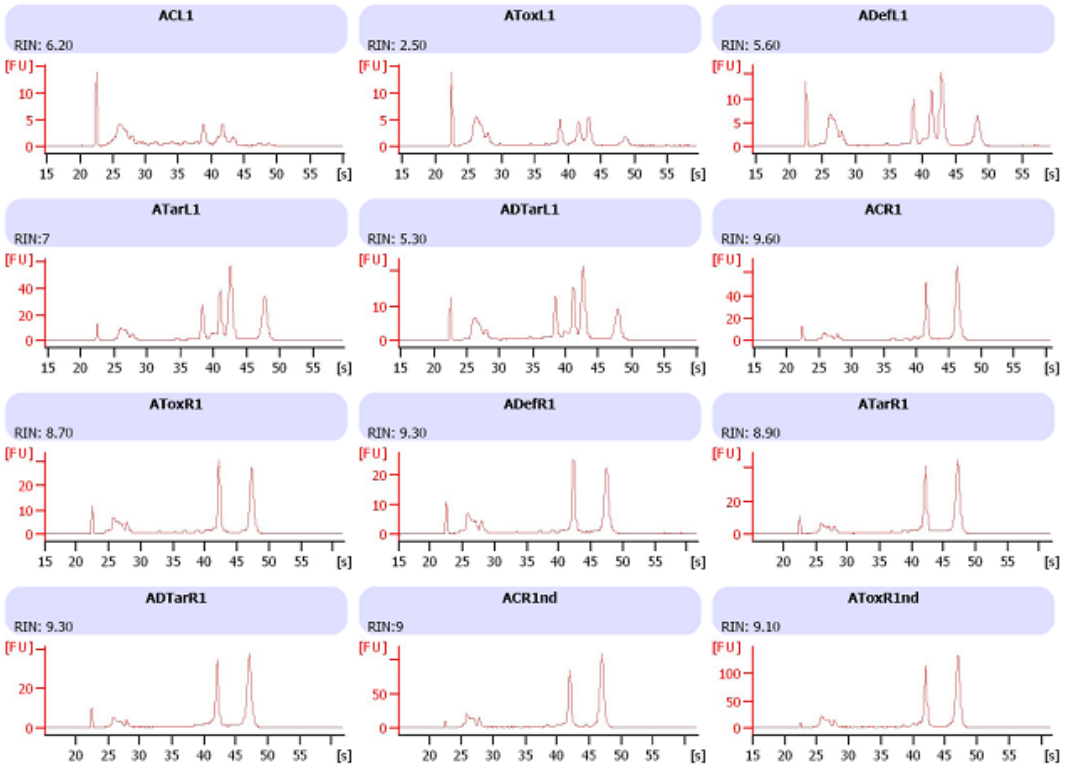


Figure 3.31. Bioanalyzer profile (electropherogram) of isolated RNAs from leaf and root tissues of Atay cultivar. ACL; Atay Control Leaf, AToxL; Atay Toxicity Leaf, ADefL; Atay Deficiency Leaf, ATarL; Atay Tarımbor Leaf, ADTarL; Atay Deficiency+Tarımbor Leaf, ACR; Atay Control Root, AToxR; Atay Toxicity Root, ADefR; Atay Deficiency Root, ATarR; Atay Tarımbor Root ADTarR; Atay Deficiency+Tarımbor Root.

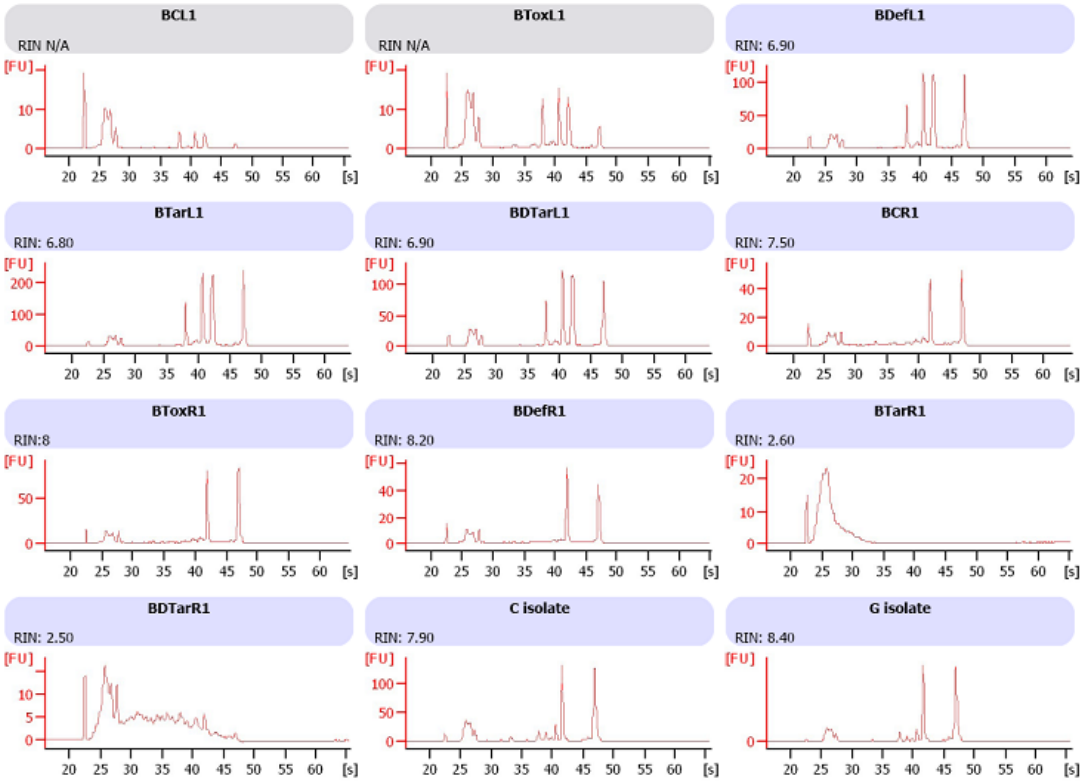


Figure 3.32. Bioanalyzer profile (electropherogram) of isolated RNAs from leaf and root tissues of Bolal cultivar. BCL; Bolal Control Leaf, BToxL; Bolal Toxicity Leaf, BDefL; Bolal Deficiency Leaf, BTarL; Bolal Tarımbor Leaf, BDTarL; Bolal Deficiency+Tarımbor Leaf. BCR; Bolal Control Root, BToxR; Bolal Toxicity Root, BDefR; Bolal Deficiency Root, BTarR; Bolal Tarımbor Root, BDTarR; Bolal Deficiency+Tarımbor Root.

3.5.2. Quality Control of Microarray Hybridization

In microarray experiments quality control measures were considered before performing the statistical analysis. These measurements give the idea of the efficiency of the labelling reaction and hybridization performance with the following parameters: the polyA spike in controls and the prokaryotic control. All these graphs were given in Appendix E.

3.5.3. Principal Component Analysis

Principal Component Analysis (PCA) is an exploratory multivariate statistical technique in order to simplify the complex data sets. Also, the aim of the PCA is to reduce the dimensionality of the microarray data matrix. When all the arrays were represented as data points on a PCA scatter plot, biological replicates in same tissues of same cultivar clustered together (Figure 3.33). In other words, the arrays including Atay and Bolal cultivars were separated with 14%. However, main source of variance were the tissue differences with 77.5%. Afterwards, our hypothesis was based on the grouping of cultivar and treatment for second PCA (Figure 3.34). Accordingly, likewise the first PCA, the tissue differences comprised the main variation (77.15 %), however, 14.3 % of the all variations were originated from the cultivar (Figure 3.34). To sum up, all the PCA results showed that the main sources of variance were due to the tissue and cultivar differences.

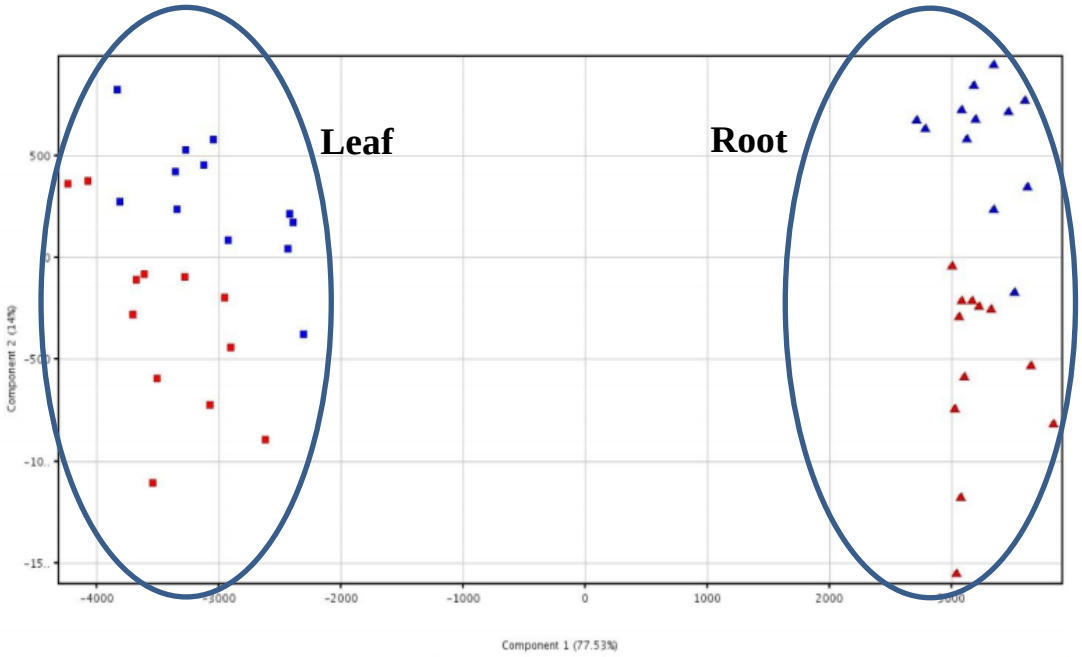
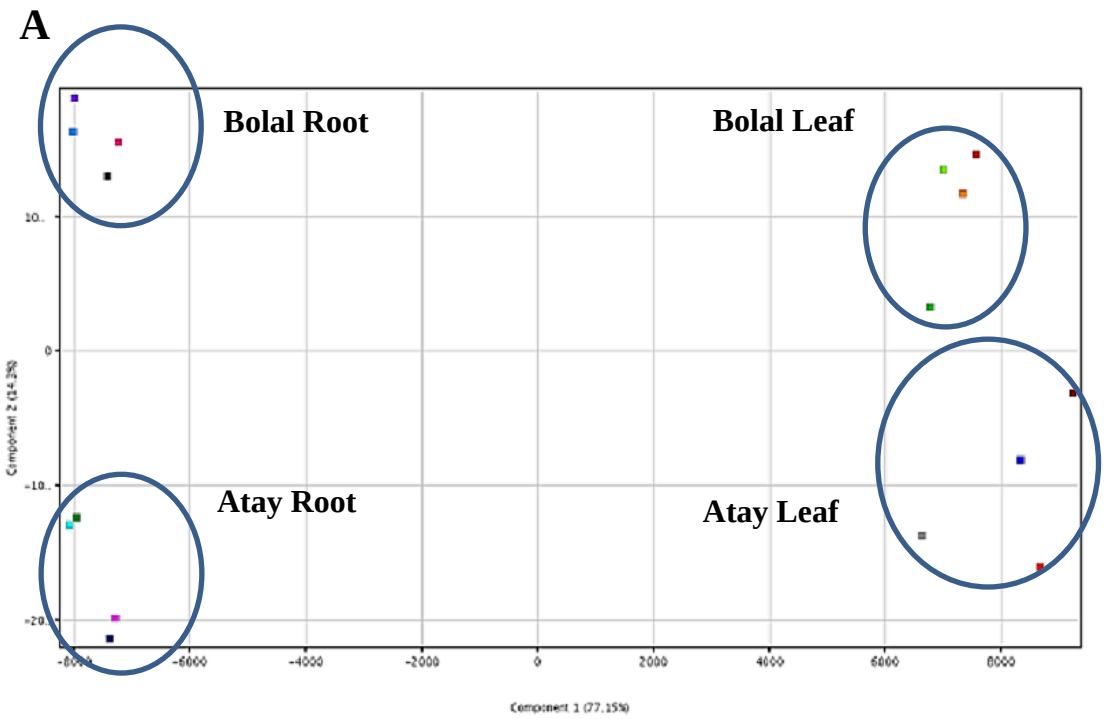


Figure 3.33. Principal Component Analysis of all microarray hybridizations conducted for leaf and root samples of Atay and Bolal cultivars. Variance is indicated by percentages. Biological replicates of a condition are indicated with the same color. (■; leaf, ▲: root. Red: Atay, Blue: Bolal)



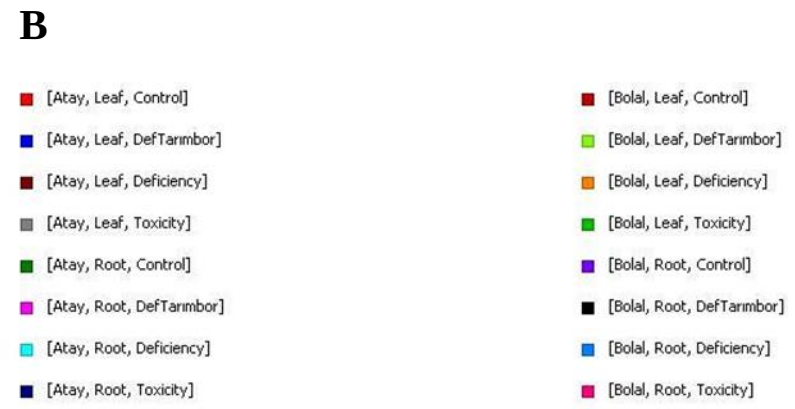


Figure 3.34. Principal Component Analysis (A) of all microarray hybridizations conducted for leaf and root samples of Atay and Bolal cultivars under control, B-toxicity, B-deficiency and B-deficiency+Tarımbor application. Variance is indicated by percentages. Colored boxes indicate different treatment (B).

3.5.4. Overall Assessment of Gene Expression Profiling under B-toxicity, deficiency and B-deficiency+Tarımbor Application in Wheat Cultivars

The GeneChip® Wheat Genome Array, contained 61127 probe sets representing 55052 transcripts for all 42 chromosomes in the wheat genome, was used for expression profiling under different B conditions in two wheat cultivars. The probe-level data from all probe sets were summarized with Robust Multi-array Average (RMA) and then 59997 probe sets were used for further data analysis since their normalized intensity values had higher than 20^{th} percentile at least one hybridization.

3.5.4.1. Differentially Expressed Transcripts in Leaves of Both Cultivars

Using the filtering criteria, in Atay leaves 1698 and 1349 and 1664 genes were differentially expressed at least two-fold when compared to respective control after B-toxic, deficiency and B-def+Tarımbor application respectively. Among these, 995, 978, and 1137 genes were significantly expressed after B-toxicity, deficiency and B-deficiency+Tarımbor application, respectively ($p<0.05$) (Table 3.1). Of these, 528 were down-regulated and 467 were up-regulated under B-toxic condition, and 524 were down-regulated and 545 were up-regulated under B-deficient condition. When Tarımbor was applied to B-deficient condition, 674 genes were down-regulated and 463 were up-regulated in Atay leaves (Table 3.1).

Table 3.1. Significantly regulated transcript numbers in leaves of Atay and Bolal cultivars ($p<0,05$). ↑: up-regulation ↓: down-regulation

	Atay	**Bolal**
B-toxicity	995 (528↑ 467↓)	892 (399↑ 493↓)
B-deficiency	978 (454↑ 524↓)	915 (482↑ 433↓)
B-deficiency+Tarımbor	1137 (463↑ 674↓)	1138 (521↑ 617↓)

For Bolal leaf, 1393, 1219 and 1592 genes were differentially expressed at least two-fold and 892, 915 and 1138 genes were significantly expressed under B-toxic, B-deficient and B-Def+Tar application, respectively ($p<0,05$). Among the significantly expressed genes, 493 were down-regulated and 399 were up-regulated under B-toxic condition (Table 3.1). 433 and 482 were down-regulated and up-regulated, respectively, in response to B-deficiency. After supplementing Tarımbor to B-deficient condition, 617 genes were down-regulated and 521 genes were up-regulated in Bolal leaves (Table 3.1). In leaf tissues, among the significantly expressed probesets, 396 and 423 were common to all treatments in Atay and Bolal cultivars, respectively.

Among the 995 significantly expressed probe sets in response to B-toxicity, 247 were specifically induced and 222 were specifically repressed in leaves of Atay (Figure 3.35). However, in leaves of Bolal, 179 and 187 probe sets were up- and down-regulated under B-toxicity, respectively. 220 and 306 genes were commonly induced and repressed in both cultivars, respectively. On the other hand, of the 978 significantly expressed probe sets after B-deficiency in leaves of Atay, 139 and 166 were specifically up- and down-regulated, respectively, however, in leaves of Bolal, 166 and 76 were specifically up- and down-regulated, respectively (Figure 3.35). Common up- and down-regulated genes in both cultivars were 315 and 358, respectively. When Tarımbor were applied to B-deficient condition in leaves as a B-specific fertilizer, 139 and 197 genes were specifically induced and 156 and 99 genes

were specifically repressed in Atay and Bolal, respectively. On the other hand, 324 genes were commonly up-regulated and 518 genes were down-regulated in both cultivars ((Figure 3.35).

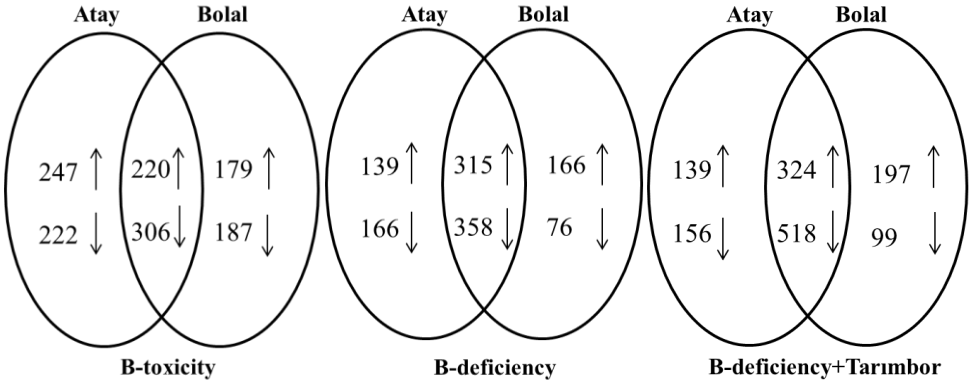


Figure 3.35. Specifically induced and common transcript numbers in leaves of Atay and Bolal cultivars under B and Tarımbor application. ($p<0,05$). ↑: up-regulation ↓: down-regulation.

3.5.4.2. Differentially Expressed Transcripts in Roots of Both Cultivars

Filtering on expression values showed that 1480 and 425 and 621 genes were differentially affected at least two-fold when compared to their corresponding control in response to B-toxicity, deficiency and B-def+Tarımbor application in Atay root, respectively (Table 3.2). Among those probe sets showing differential expression, 1248, 354, and 496 were significantly expressed under B-toxic, B-deficient and B-deficiency+Tarımbor application in Atay root, respectively. Of these, 736 were down-regulated and 512 were up-regulated under B-toxic condition, and 311 were down-regulated and 43 were up-regulated under B-deficient condition. At the end of the B-deficiency+Tarımbor application, 423 genes were repressed and 73 were induced in Atay roots (Table 3.2).

Table 3.2. Significantly regulated transcript numbers in roots of Atay and Bolal cultivars (p<0,05). ↑: up-regulation ↓: down-regulation

	Atay	**Bolal**
B-toxicity	1248 (512↑ 736↓)	957 (454↑ 503↓)
B-deficiency	354 (43↑ 311↓)	286 (92↑ 194↓)
B-deficiency+Tarımbor	496 (73↑ 423↓)	516 (266↑ 250↓)

For Bolal root, 1172, 377 and 1005 genes were differentially expressed at least two-fold and 957, 286 and 516 genes were significantly expressed after B-toxicity, deficiency and B-deficiency+Tarımbor application, respectively (Table 3.2). Of these, 503 were down-regulated and 454 were up-regulated after B-toxic condition, and 194 were down-regulated and 92 were up-regulated under B-deficient condition. When the period of B-deficiency+Tarımbor was complete, 250 genes were down-regulated and 266 were up-regulated in Bolal roots (Table 3.2). In root tissues, among the significantly expressed probesets, 189, 61 were common to all treatments in Atay and Bolal cultivars, respectively.

In roots of Atay, among the 1248 significantly expressed probe sets in response to B-toxicity, 229 were specifically induced and 397 were specifically repressed (Figure 3.36). However, in leaves of Bolal, 171 and 164 probe sets were specifically up- and down-regulated at the end of the B-toxicity, respectively. 283 and 339 genes were commonly induced and repressed in both cultivars, respectively. On the other hand, of the 354 significantly expressed probe sets after B-deficiency in leaves of Atay, 29 and 175 were specifically up- and down-regulated, respectively, however, in leaves of Bolal, 79 and 57 were specifically up- and down-regulated, respectively (Figure 3.36). Common up- and down-regulated genes in both cultivars were 14 and 136 under B-deficiency, respectively. When Tarımbor were applied to B-deficient condition as a B-specific fertilizer, 37 and 226 genes were specifically induced and 303 and 134 genes were specifically repressed in root tissues of Atay and Bolal,

respectively. However, 36 genes were commonly up-regulated and 120 genes were down-regulated in both cultivars (Figure 3.36).

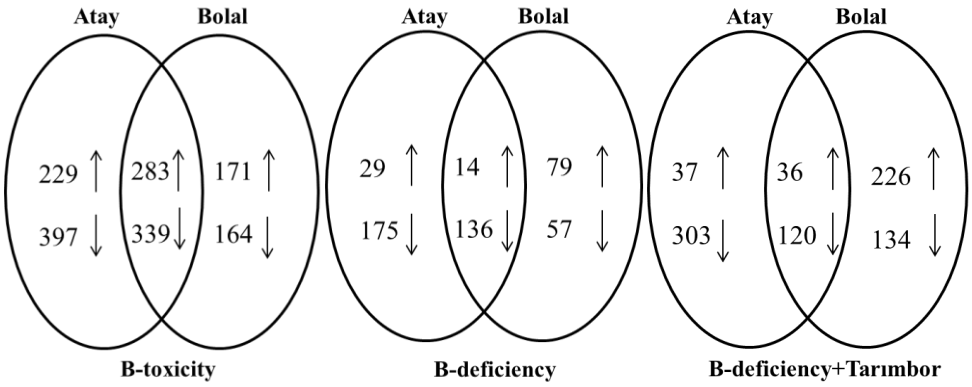


Figure 3.36. Specifically induced and common transcript numbers in roots of Atay and Bolal cultivars under B and Tarımbor application. ($p<0,05$). ↑: up-regulation ↓: down-regulation.

3.5.4.3. Singular Enrichment Analysis (SEA) of B-related Genes in Both Cultivars

The numbers of specifically induced and common genes in leaf and root tissues of both cultivars under different B treatments were separately shown in above. In this analysis, each subgroup in these tables was set out to determine the biological significance of B-related genes by using induced probe sets at least one B condition.

Accordingly, 2275 and 2088 probe sets were significantly changed at least one B condition in leaf and root tissues of both cultivars, respectively. These probe sets were subjected to singular enrichment analysis (SEA) by using agriGO to identify enriched GOs. Finding enriched GO terms corresponds to finding enriched biological facts. Also, term enrichment is evaluated by comparing query list to a background population from which the query list is derived. Figure 3.37 and 3.38 show GO

descriptions that contain biological process of B-related genes for leaf and root tissues, respectively. Each box includes the number of GO term, GO term and the p-value in parenthesis. The first pair of numerals represents the number of genes in the input list related to that GO term and the number of genes in the input list. The second pair of numerals represents the number of genes related to the particular GO term in the wheat database and the total number of wheat genes with GO annotations in the wheat database. The color scale from yellow to red represents increasing levels of statistical significance. Accordingly, among the B-related genes in leaf tissues of both cultivars, GOs associated with cellular nitrogen compound metabolic process, jasmonic acid metabolic and biosynthetic processes were significant (Figure 3.37). On the other hand, in root tissues of both cultivars, several GO categories were significant among the genes that were induced at least one B condition. These included protein-DNA complex assembly, nucleosome organization and assembly, chromatin assembly and disassembly, DNA packaging, amine catabolic process (Figure 3.38). Also, GOs for cellular nitrogen compound metabolic process, response to wounding and external stimulus were over-represented (Figure 3.38).

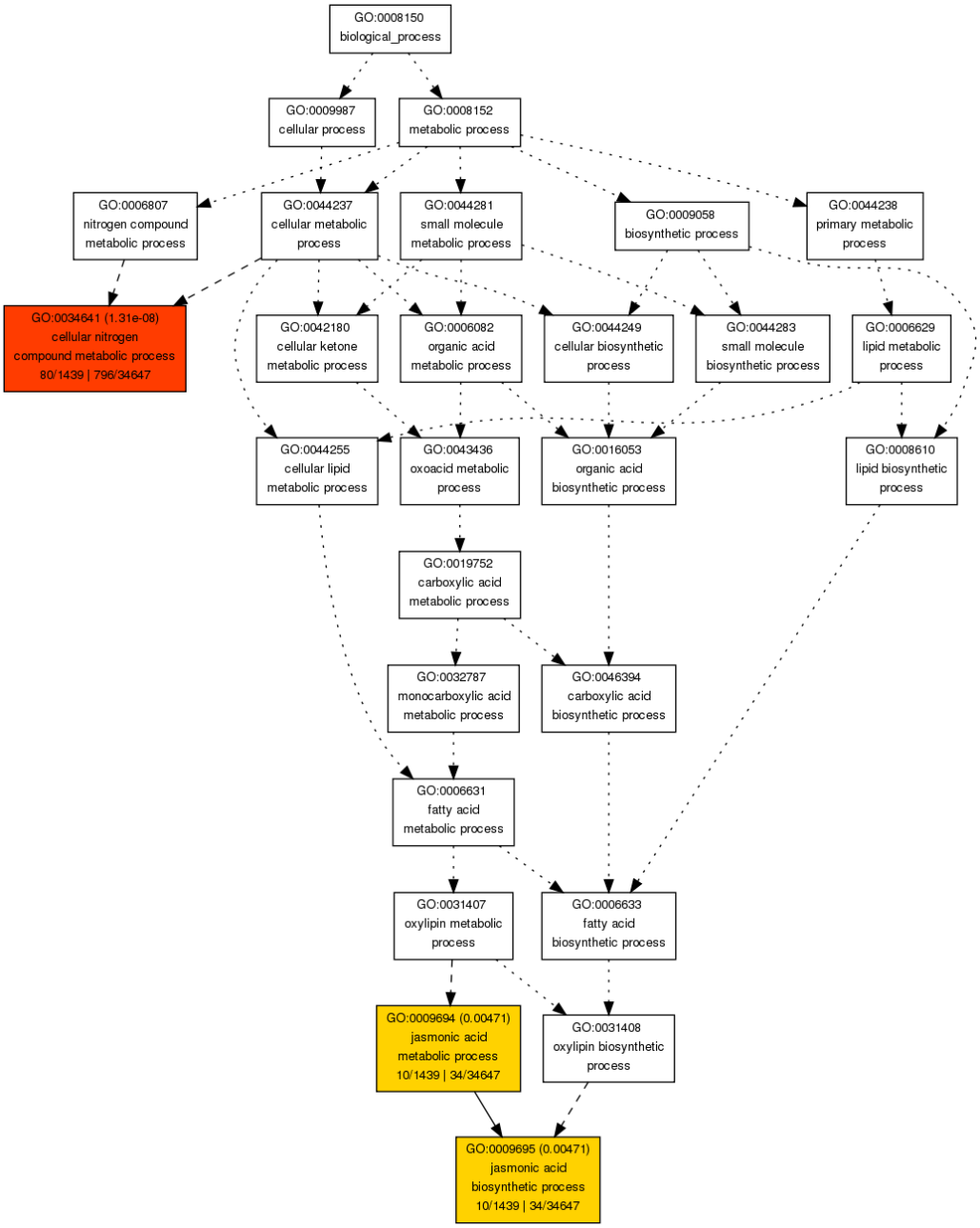


Figure 3.37. Gene Ontology (GO) analysis of B-related genes in leaves of Atay and Bolal cultivars using agriGO.

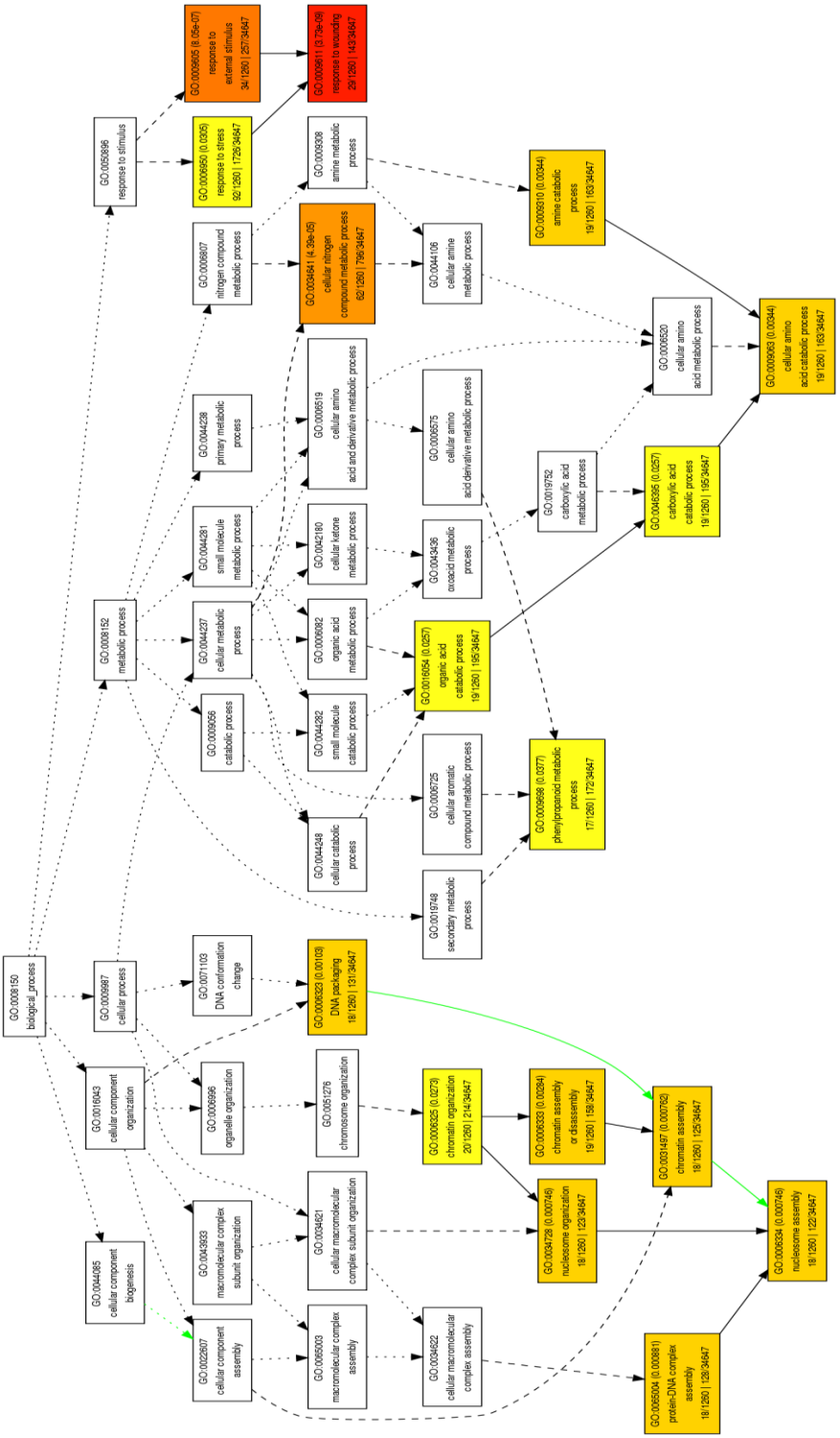


Figure 3.38. Gene Ontology (GO) analysis of B-related genes in roots of Atay and Bolal cultivars using agriGO.

3.5.5. Functional Categories of Differentially Expressed Genes

In this part, the probe sets that were specifically or commonly up and down-regulated under B-toxicity, deficiency and B-deficiency+Tarımbor application compared to respective controls in Atay and Bolal cultivars were functionally categorized to determine the possible candidate genes for B-regulated mechanisms. For this purpose, common genes between Atay and Bolal cultivars were subjected to Singular Enrichment Analysis by using agriGO in order to determine the possible basal B mechanism between tolerant and sensitive wheat cultivars. On the other hand, MapMan analysis was used for specifically induced (up or down) genes in only Atay or Bolal cultivars in order to determine the possible B-tolerance mechanism.

3.5.5.1. Singular Enrichment Analysis of Common Genes between Atay and Bolal Cultivars

3.5.5.1.1. Singular Enrichment Analysis of Common Genes under B-toxicity between Atay and Bolal Cultivars

As shown in Figure 3.35, at the end of the B-toxicity, 220 and 306 probe sets were commonly up-regulated and repressed in leaves of both cultivars, respectively. These probe sets were subjected to Singular Enrichment Analysis to investigate the GO assignments by using GO group such as molecular function. The well-represented molecular functions were calcium ion binding, transcription regulator and factor activity for common down-regulated genes under B-toxicity in leaves of both cultivars; however, it was catalytic activity for up-regulated genes (Figure 3.39). On the other hand, in roots of both cultivars, regarding molecular function, genes involved in transcription factor and regulator activity were highly represented and GO related to hydrolase activity, DNA binding, peptidase and endopeptidase inhibitor activity were significant for common down-regulated genes under B-toxicity (Figure 3.40). Similarly, peptidase and endopeptidase inhibitor activity were significant for up-regulated genes in roots of both cultivars (Figure 3.40).

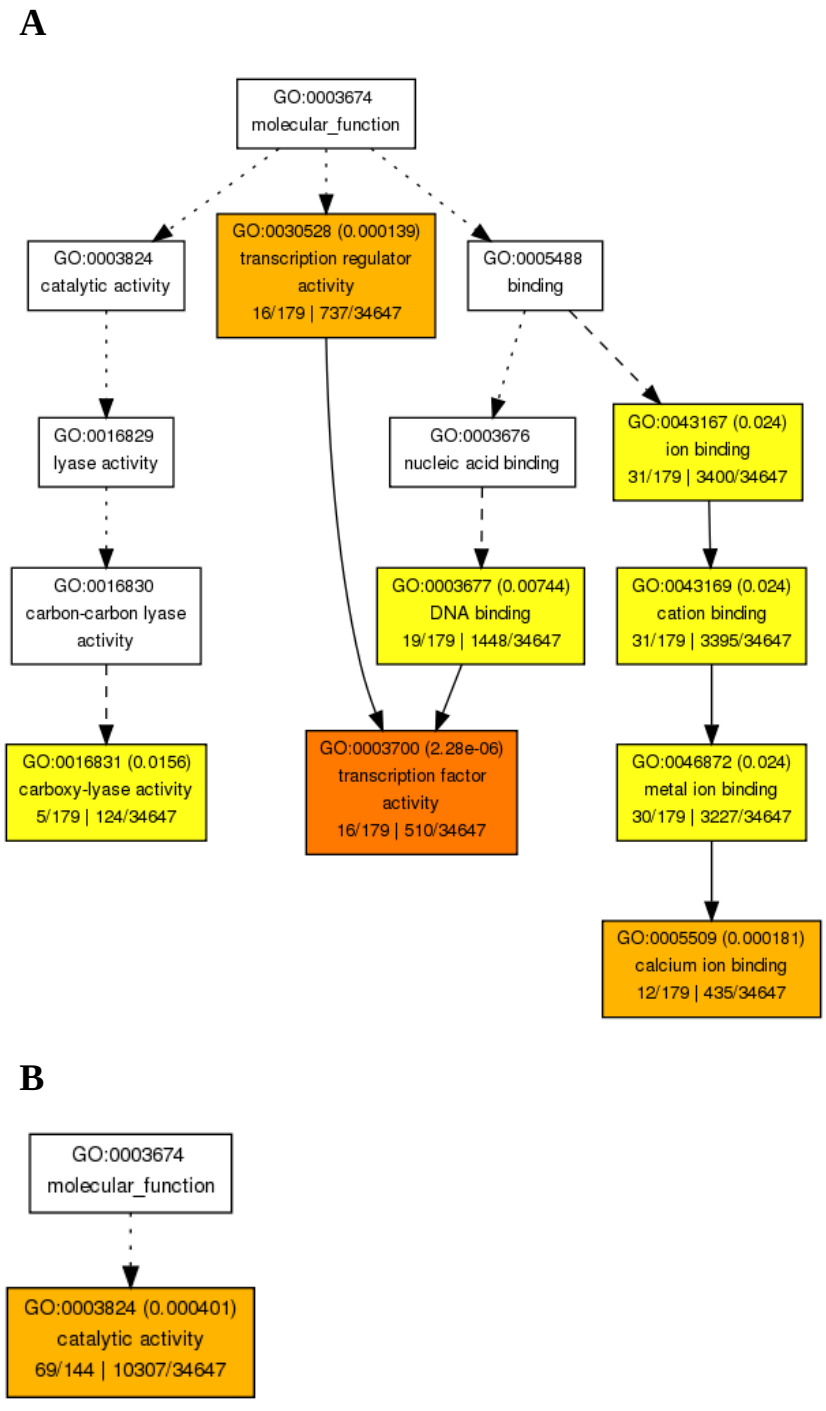


Figure 3.39. Gene Ontology (GO) classification (molecular function) of the common down- (A) and up- (B) regulated genes under B-toxicity in leaves of both cultivars.

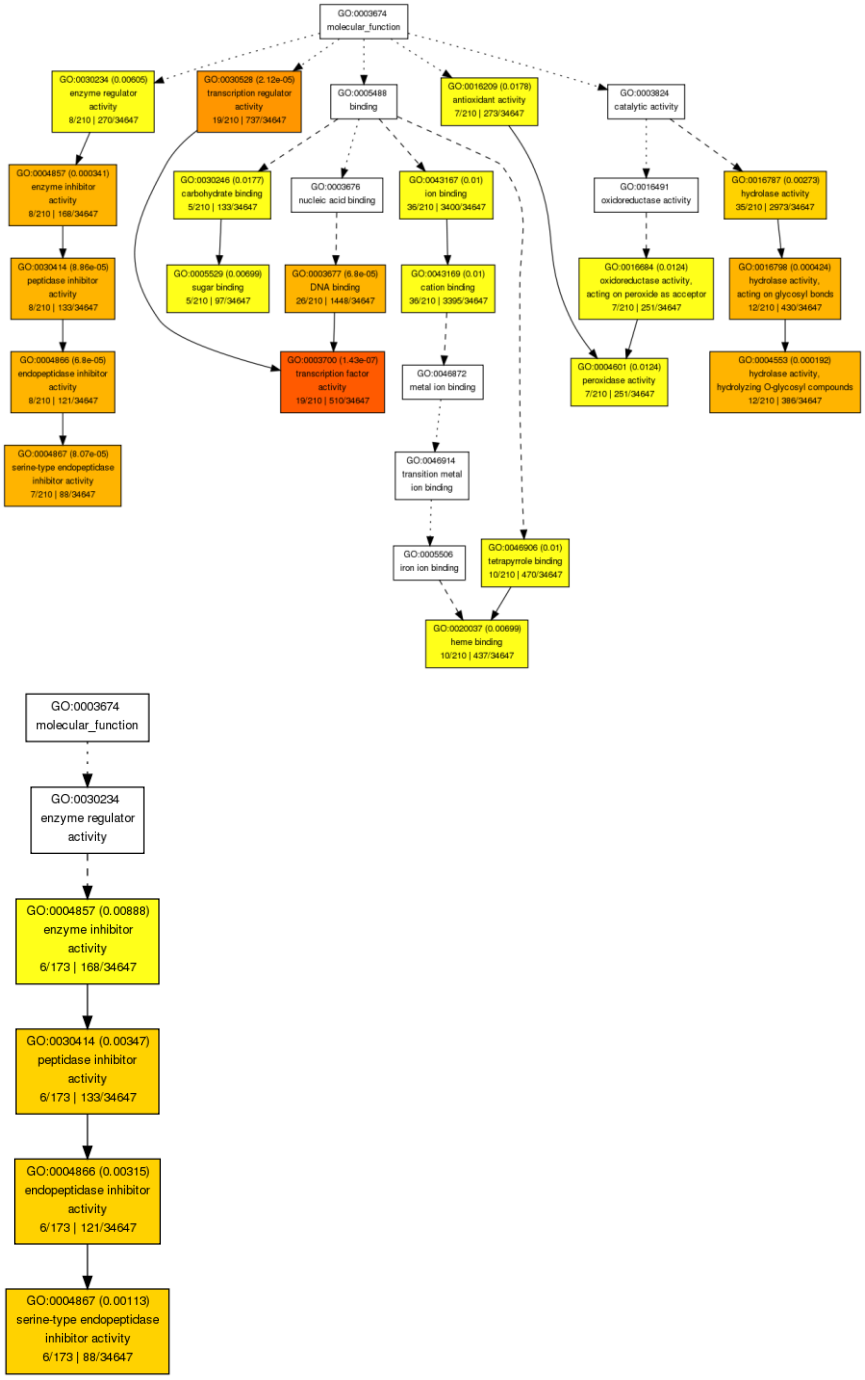


Figure 3.40. Gene Ontology (GO) classification (molecular function) of the common down- (above figure) and up- (below figure) regulated genes under B-toxicity in roots of both cultivars.

3.5.5.1.2. Singular Enrichment Analysis of Common Genes under B-deficiency between Atay and Bolal Cultivars

In B-deficient leaves of both cultivars, 315 and 358 probe sets were used for Singular Enrichment Analysis as common down- and up-regulated genes, respectively. Similar to B-toxicity, GO associated with transcription regulator and factor activity were well-represented and GO categories including DNA binding, lyase and ligase activities were significant for common down-regulated genes in leaves of both cultivars (Figure 3.41). On the other hand, significant GOs were transferase and kinase activities for common up-regulated genes in leaves of both cultivars (Figure 3.42).

Figure 3.41. Gene Ontology (GO) classification (molecular function) of the common down- regulated genes under B-deficiency in leaves of both cultivars.

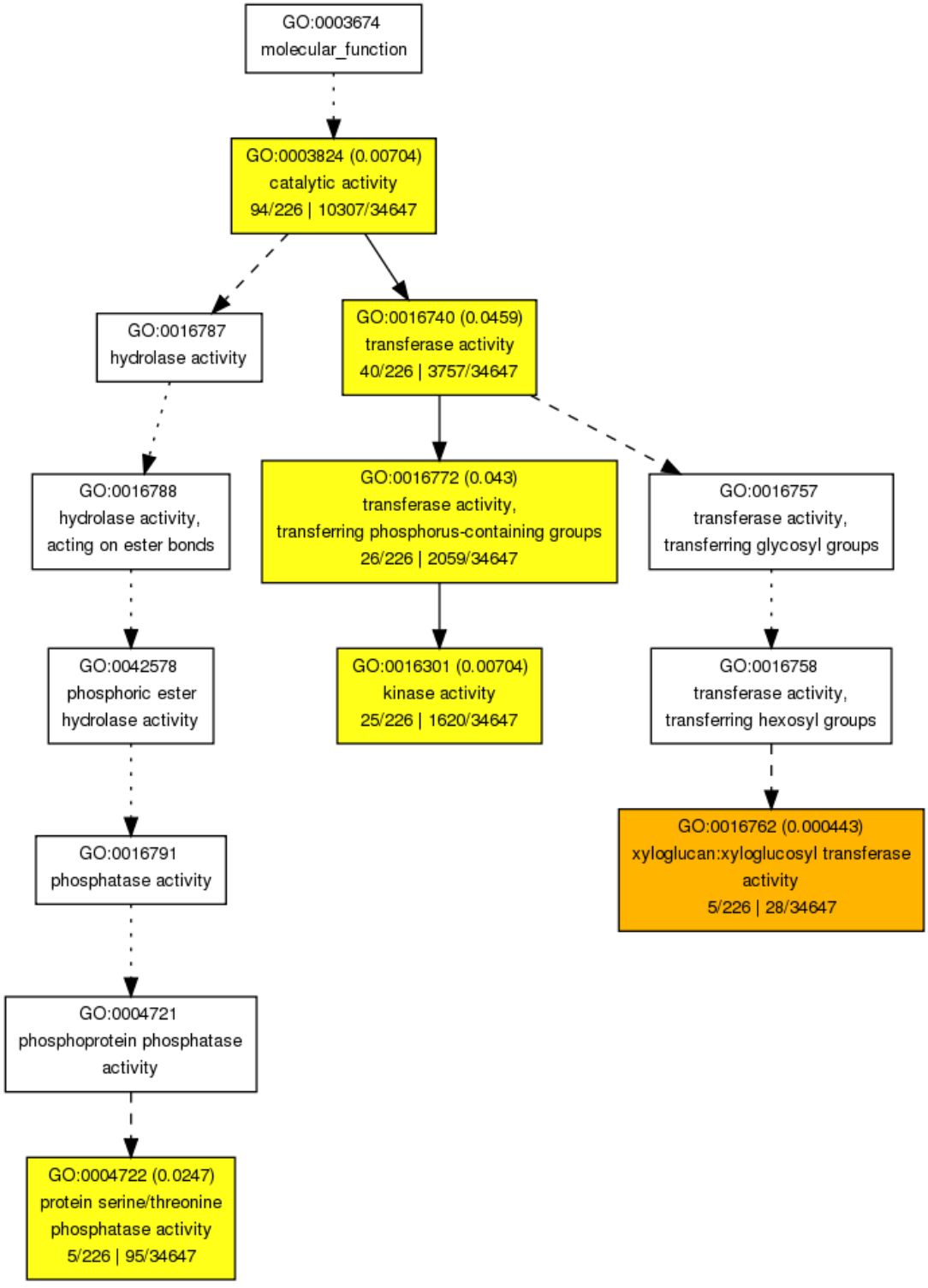


Figure 3.42. Gene Ontology (GO) classification (molecular function) of the common up- regulated genes under B-deficiency in leaves of both cultivars.

In B-deficient roots of both cultivars, several GO categories were significant for common down-regulated genes. These included transcription regulator and factor activity, DNA binding, nucleic acid binding and calcium ion binding (Figure 3.43). On the other hand, significant GOs were not found by using common up-regulated genes in root tissues of both cultivars.

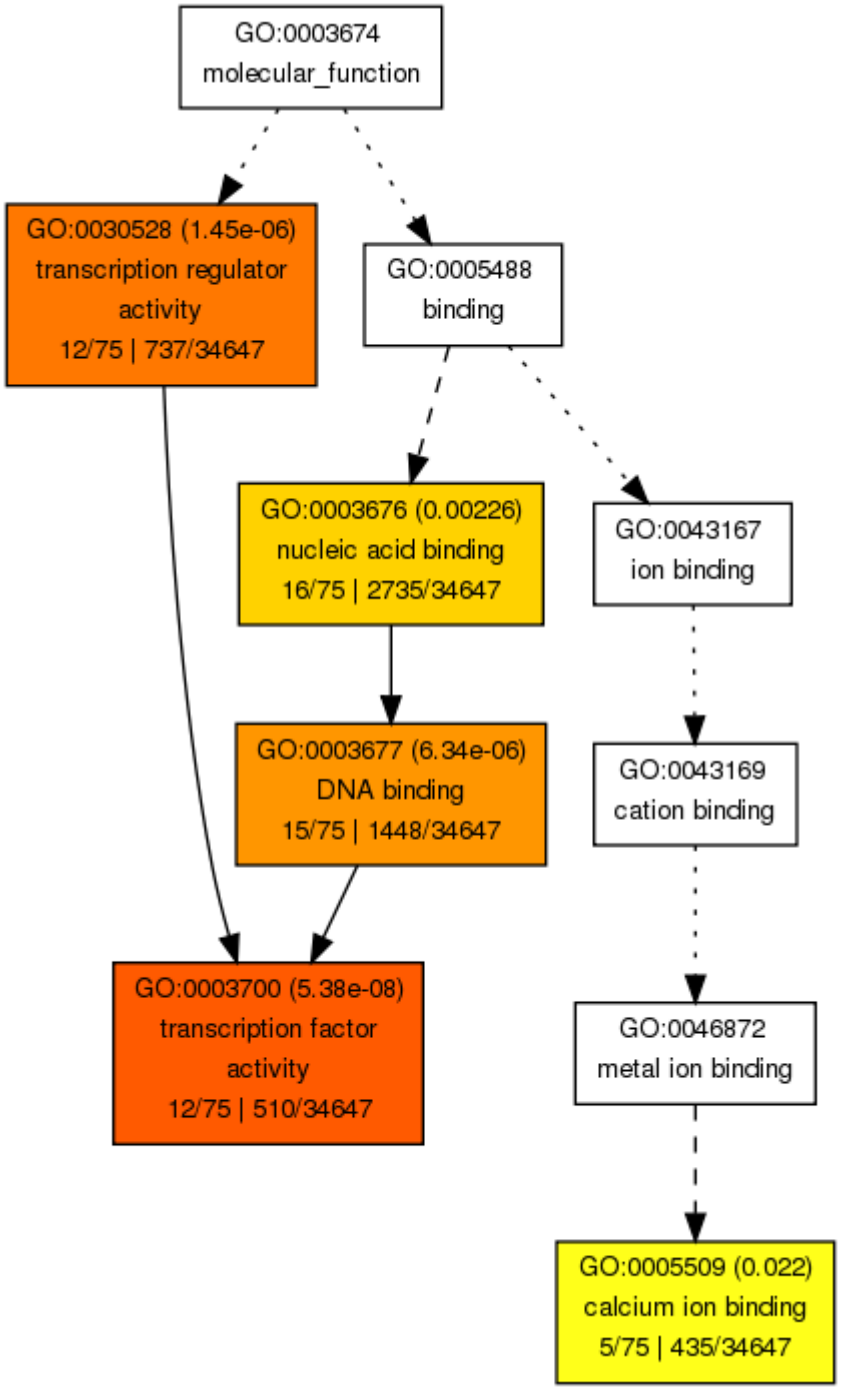


Figure 3.43. Gene Ontology (GO) classification (molecular function) of the common down-regulated genes under B-deficiency in roots of both cultivars.

3.5.5.1.3. Singular Enrichment Analysis of Common Genes under B-deficiency+Tarımbor Application between Atay and Bolal Cultivars

Several GOs were significant for common down-regulated genes in leaves of both cultivars under B-deficiency+Tarımbor application. These were transcription regulator and factor activity, calcium ion binding, liyase, kinase and tranferase activities (Figure 3.44). On the other hand, for common up-regulated genes, GOs with oxidoreductase and xyloglucan: xyloglucosyl transferase activities were highly represented (Figure 3.45).

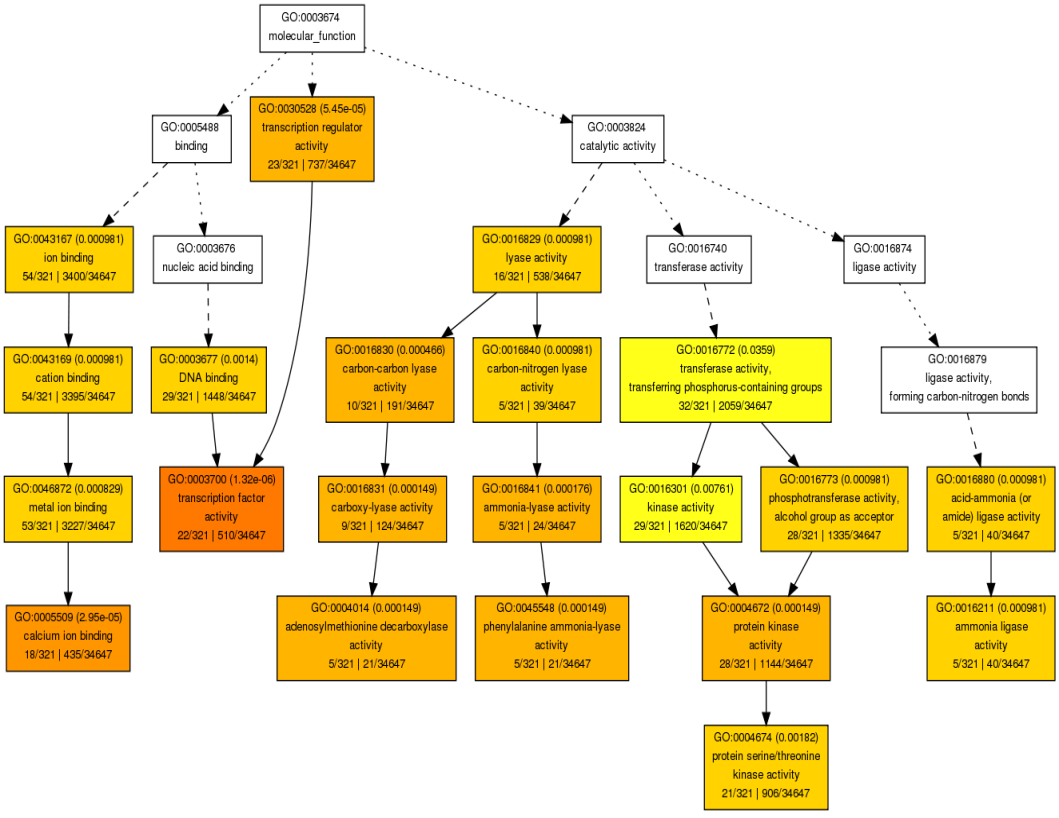


Figure 3.44. Gene Ontology (GO) classification (molecular function) of the common down-regulated genes under B-deficiency+Tarımbor in leaves of both cultivars.

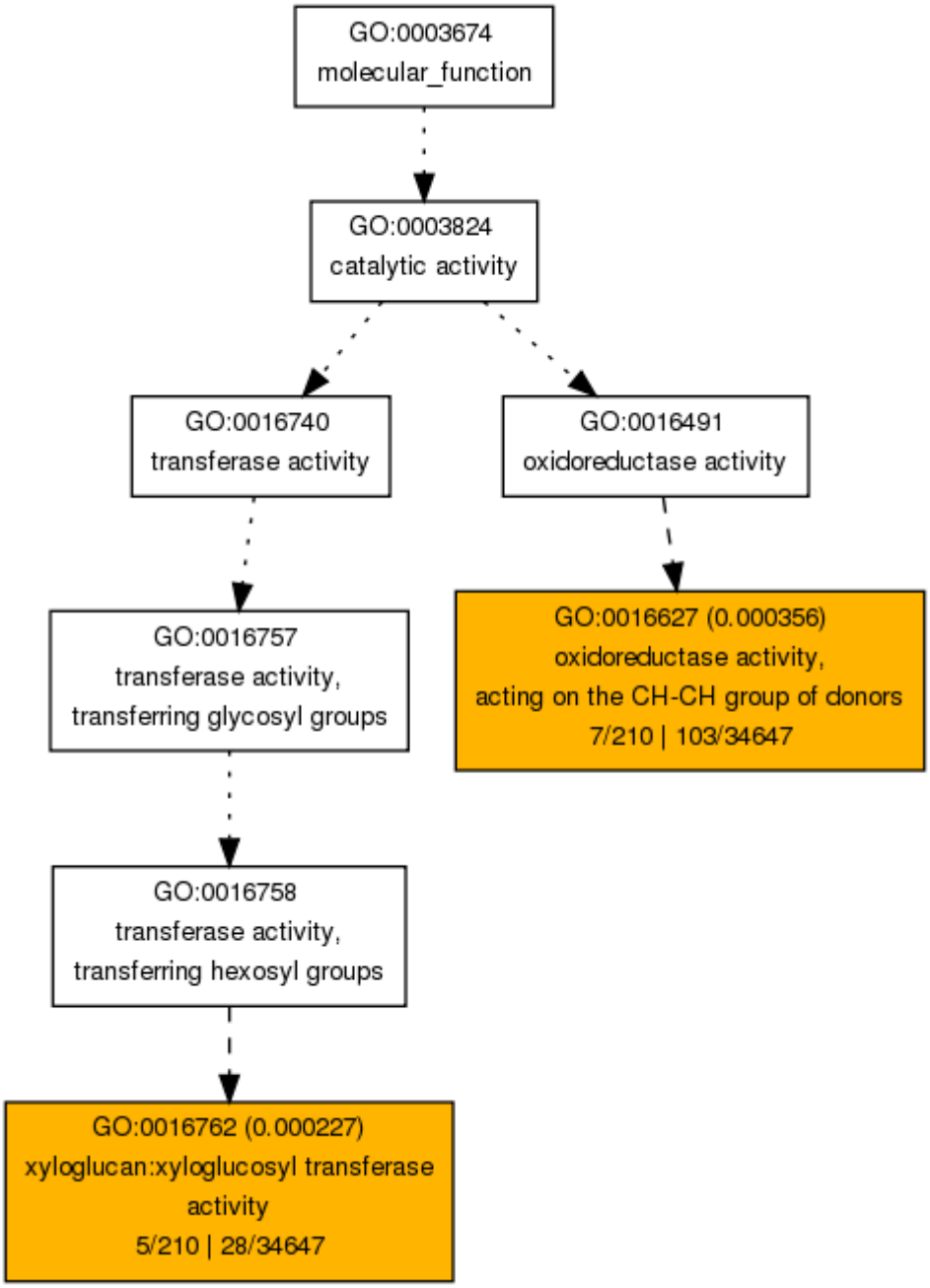


Figure 3.45. Gene Ontology (GO) classification (molecular function) of the common up-regulated genes under B-deficiency+Tarımbor in leaves of both cultivars.

After Tarımbor was applied to B-deficiency condition, 120 and 36 probe sets were down- and up-regulated commonly in root tissues of both cultivars, respectively. Highly represented GOs were calcium ion binding lyase, and ligase activities for common down-regulated genes (Figure 3.46). On the other hand, significantly represented GOs were not found for up-regulated gene in root tissues of both cultivars under B-deficiency+Tarımbor condition.

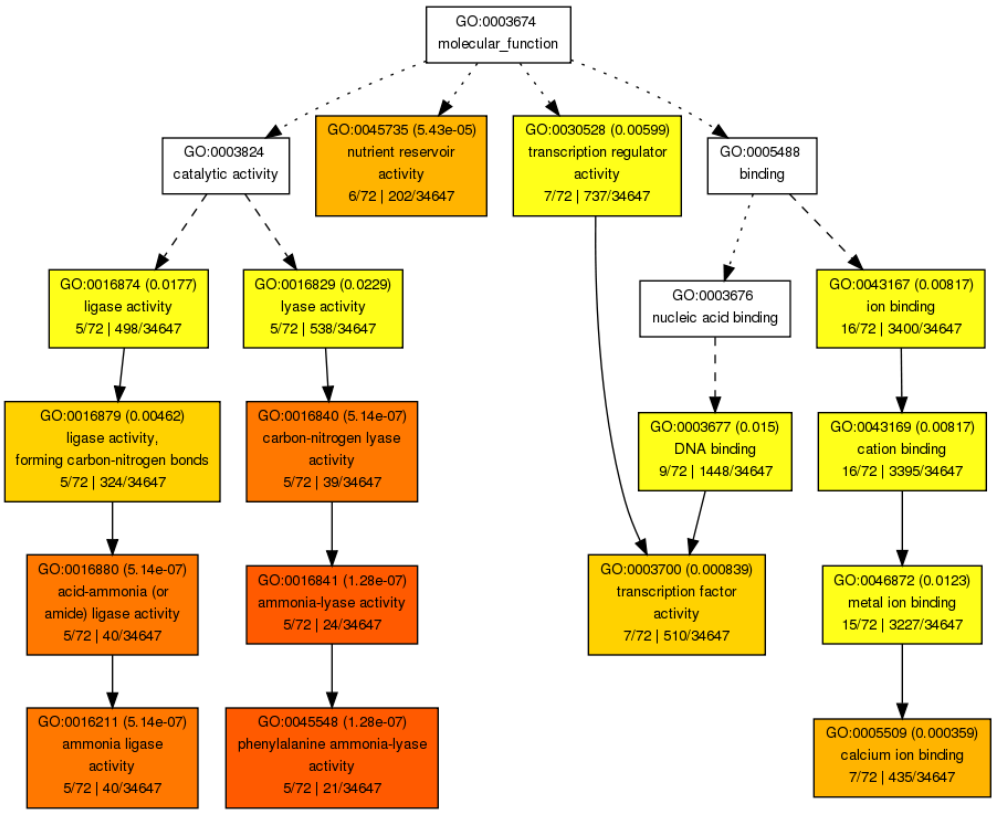


Figure 3.46. Gene Ontology (GO) classification (molecular function) of the common down-regulated genes under B-deficiency+Tarımbor in roots of both cultivars.

3.5.5.2. Metabolic Pathways of Specifically Induced Genes in Atay and Bolal Cultivars

3.5.5.2.1. Metabolic Pathways of Specifically Induced Genes under B-toxicity in Atay and Bolal Cultivars

At the end of B-toxicity, expression levels of specifically induced genes categorized into each subBINs were visualized as shown in Figure 3.47 and 3.48 for leaf and root tissues of both cultivars, respectively. In leaves of Atay, genes encoding cell wall precursor and modification, isoprenoid synthesis, MYB related transcription factor, WRKY domain transcription factor and 5 kinase and 9 protein post translational modification genes were down-regulated; however, genes for glutathione S transferases (GSTs), ribosomal proteins, amino acid metabolisms, protein degradation and jasmonate metabolism were up-regulated (Figure 3.47). On the other hand, in leaves of Bolal, 5 genes encoding lignin biosynthesis, 3 genes related abiotic stress and 3 genes for peroxidases were down-regulated; but 3 genes for cell wall modification, 4 genes encoding ribosomal proteins, 9 genes related protein degradation and 5 genes for post translational modification were up-regulated (Figure 3.47).

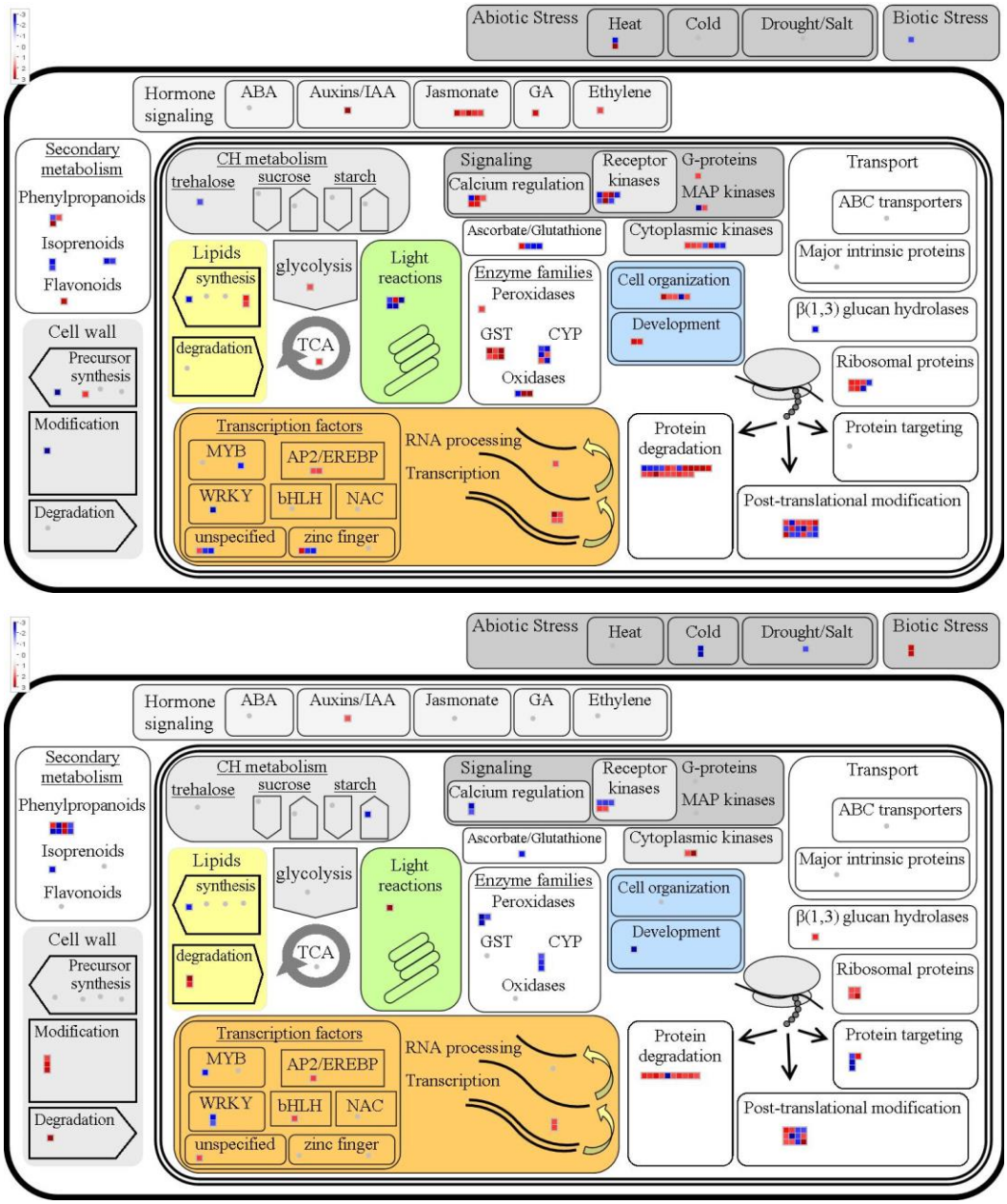


Figure 3.47. Overview of metabolism pathways of specifically induced genes under B-toxicity in leaves of Atay (above figure) and Bolal (below figure) cultivars. The MapMan tool was used to map on various metabolic pathways. Small red, blue and dark gray squares within each metabolism pathway indicate up-regulation, down-regulation and no detection of mapped genes, respectively.

In root tissues of Atay, 12 genes for cell wall modification, 5 genes related abiotic stress, 12 genes encoding peroxidases, 7 genes for kinases, 10 genes related calcium regulation and 38 genes for DNA synthesis were down-regulated; however, 3 genes encoding abiotic stress, 2 genes for jasmonate metabolism, 3 genes related ribosomal proteins and 8 genes for protein degradation were up-regulated (Figure 3.48). In roots of Bolal, 3 genes for cell wall modification and for lignin degradation, 7 genes related hormone metabolism and 7 genes encoding peroxidases were down regulated; but 5 genes related light reaction in photosynthesis, 3 genes related abiotic stress, 3 genes for kinases and 4 genes for ribosomal proteins and for post-translational modification were up-regulated (Figure 3.48).

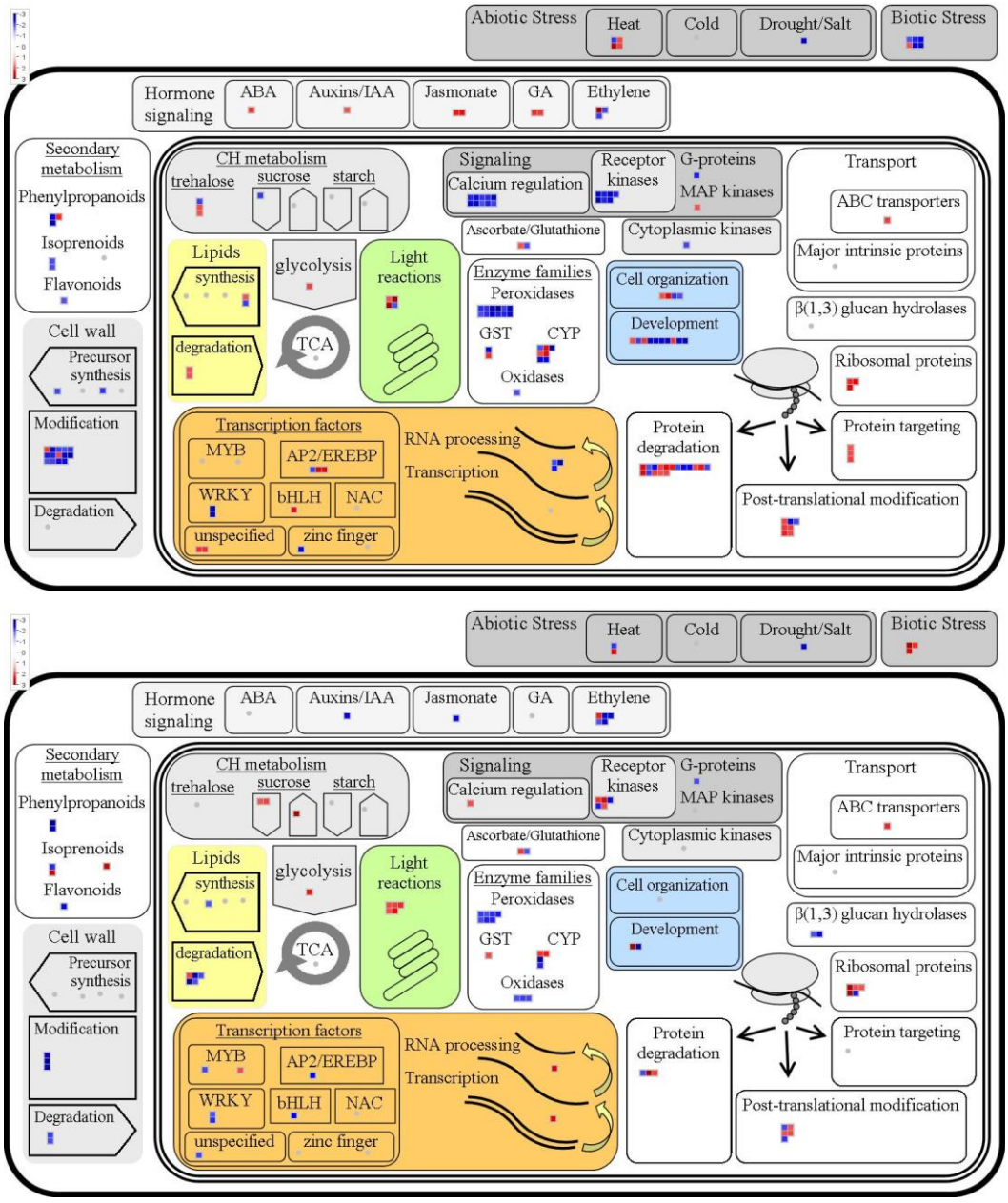


Figure 3.48. Overview of metabolism pathways of specifically induced genes under B-toxicity in roots of Atay (above figure) and Bolal (below figure) cultivars. The MapMan tool was used to map on various metabolic pathways. Small red, blue and dark gray squares within each metabolism pathway indicate up-regulation, down-regulation and no detection of mapped genes, respectively.

3.5.5.2.2. Metabolic Pathways of Specifically Induced Genes under B-deficiency in Atay and Bolal Cultivars

After B-deficiency, in Atay leaves, 1 gene for WRKY and for MYB domain transcription factors, 8 genes encoding kinases and 9 genes related post-translational modification were down-regulated; however, 9 genes related hormone metabolism such as auxin, ethylene, gibberellin, 2 genes for peroxidases, 9 genes for protein degradation, 2 genes related lignin synthesis and 2 genes for AP2 transcription factor were up-regulated (Figure 3.49). On the other hand, in Bolal leaves, 1 gene encoding GST, 1 gene for lignin biosynthesis and for C2H2 zinc finger family were down-regulated; but genes related cell wall modification, protein degradation, post-translational modification, kinases, biotic stress, calcium regulation, peroxidases, cell organization and cell vesicle transport were down-regulated (Figure 3.49).

In Atay roots, 4 genes for WRKY, C2H2 transcription factors, 3 genes for MYB transcription factor, 6 genes related lignin biosynthesis, 3 genes related biotic stress, 4 genes for protein degradation and for post-translational modification were down-regulated (Figure 3.50). However, genes for lipid synthesis and degradation and major intrinsic proteins were up-regulated. On the other hand, in Bolal roots, 4 genes related carbohydrate metabolism, peroxidases and post-translational mechanism were down-regulated; but 2 genes for abiotic stress and cell wall modification, cell development and secondary metabolism were up-regulated (Figure 3.50).

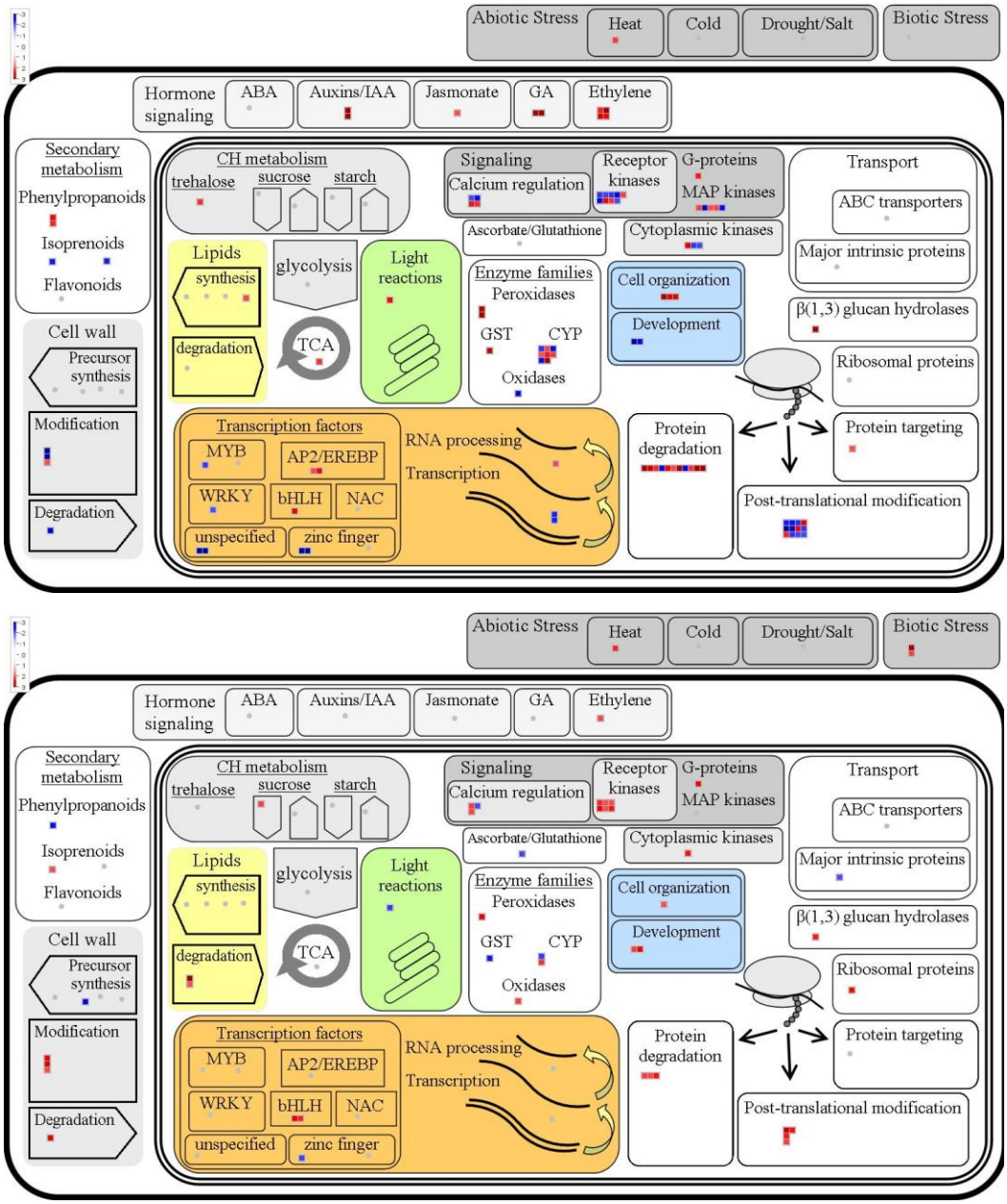


Figure 3.49. Overviews of metabolism pathways of specifically induced genes under B-deficiency in leaves of Atay (above figure) and Bolal (below figure) cultivars. The MapMan tool was used to map on various metabolic pathways. Small red, blue and dark gray squares within each metabolism pathway indicate up-regulation, down-regulation and no detection of mapped genes, respectively.

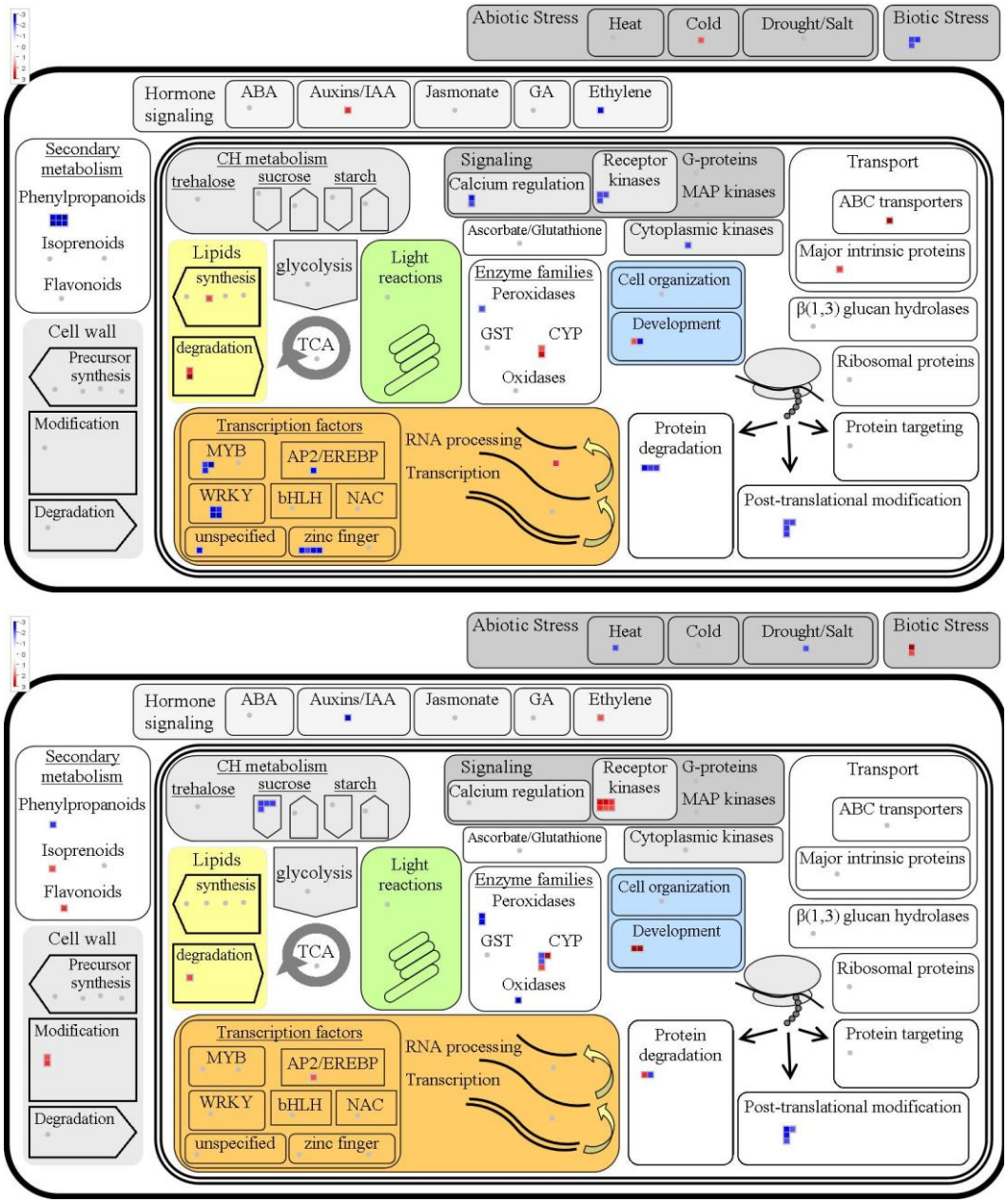


Figure 3.50. Overviews of metabolism pathways of specifically induced genes under B-deficiency in roots of Atay (above figure) and Bolal (below figure) cultivars. The MapMan tool was used to map on various metabolic pathways. Small red, blue and dark gray squares within each metabolism pathway indicate up-regulation, down-regulation and no detection of mapped genes, respectively.

3.5.5.2.3. Metabolic Pathways of Specifically Induced Genes under B-deficiency+Tarımbor in Atay and Bolal Cultivars

After Tarımbor was applied to B-deficient condition, in Atay leaves, genes related kinases, calcium regulation, jasmonate metabolism and ribosomal proteins were down-regulated. However, genes for abiotic and biotic stresses, genes related hormone metabolism such as auxin, ethylene, gibberelic acid and genes for cell organization and development were up-regulated (Figure 3.51). On the other hand, in Bolal leaves, 2 genes for cellulose synthase and cytoplasmic kinases and cell organization and development and 1 gene related WRKY and C2H2 transcription factors were down-regulated, however, 4 genes for ethylene metabolism, 4 genes related secondary metabolism, 5 genes encoding peroxidases and 6 genes related protein degradation were up-regulated (Figure 3.51).

In Atay roots, many genes for WRKY, MYB and C2H2 transcription factors and 5 genes related calcium regulation, kinases and biotic stress and genes for post-translational modification, protein degradation and secondary metabolism were down-regulated; however, genes for lipid synthesis and degradation were only up-regulated (Figure 3.52). On the other hand, in Bolal roots, 5 genes related cell wall modification, 4 genes encoding β (1,3) glucan hydrolases and 3 genes for secondary metabolism were down-regulated; but 7 genes related secondary metabolism, 5 genes for cell development, protein degradation, post-translational modification and 3 genes related kinases and biotic stress were up-regulated (Figure 3.52).

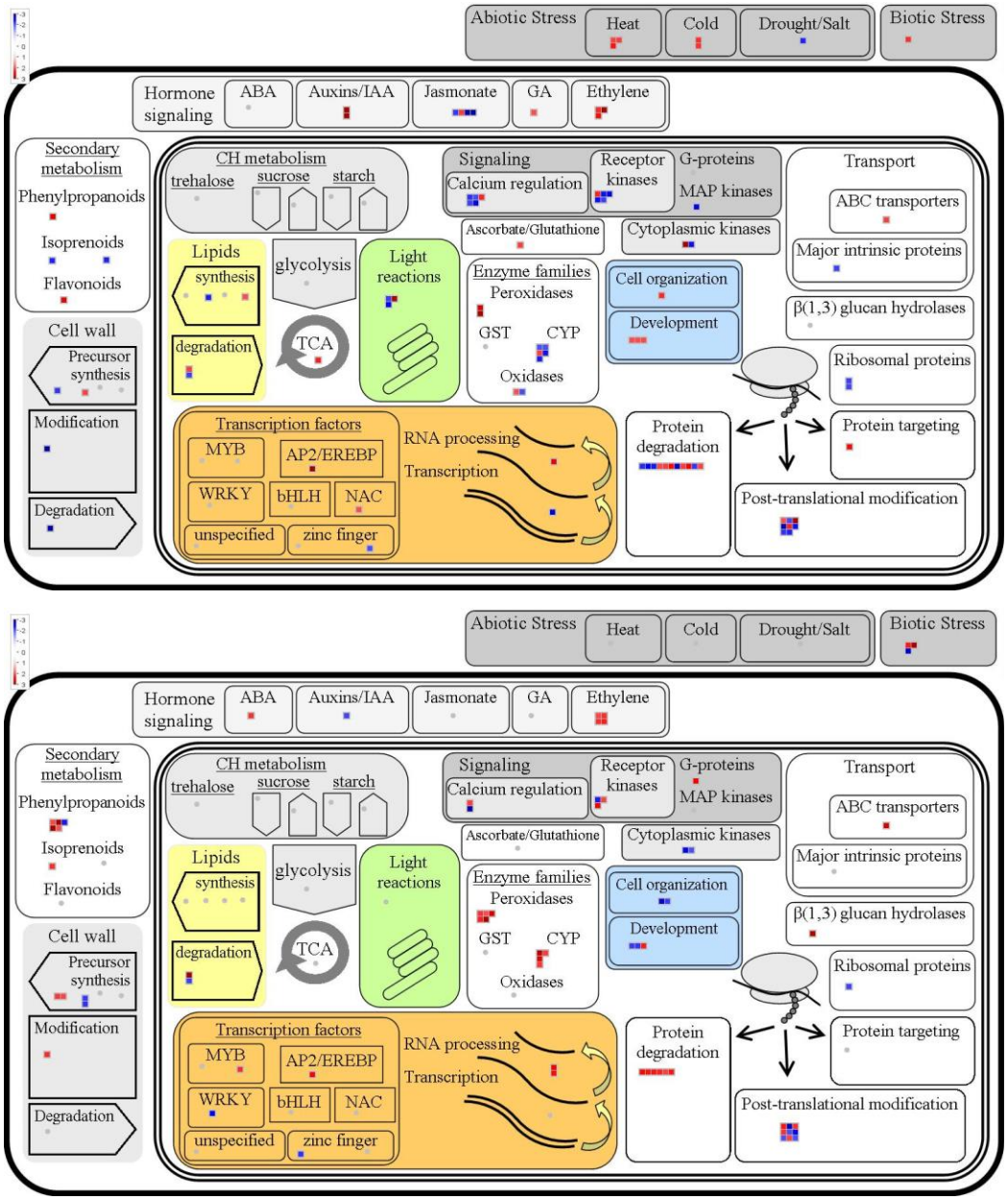


Figure 3.51. Overviews of metabolism pathways of specifically induced genes under B-deficiency+Tarımbor application in leaves of Atay (above figure) and Bolal (belove figure) cultivars. The MapMan tool was used to map on various metabolic pathways. Small red, blue and dark gray squares within each metabolism pathway indicate up-regulation, down-regulation and no detection of mapped genes, respectively.

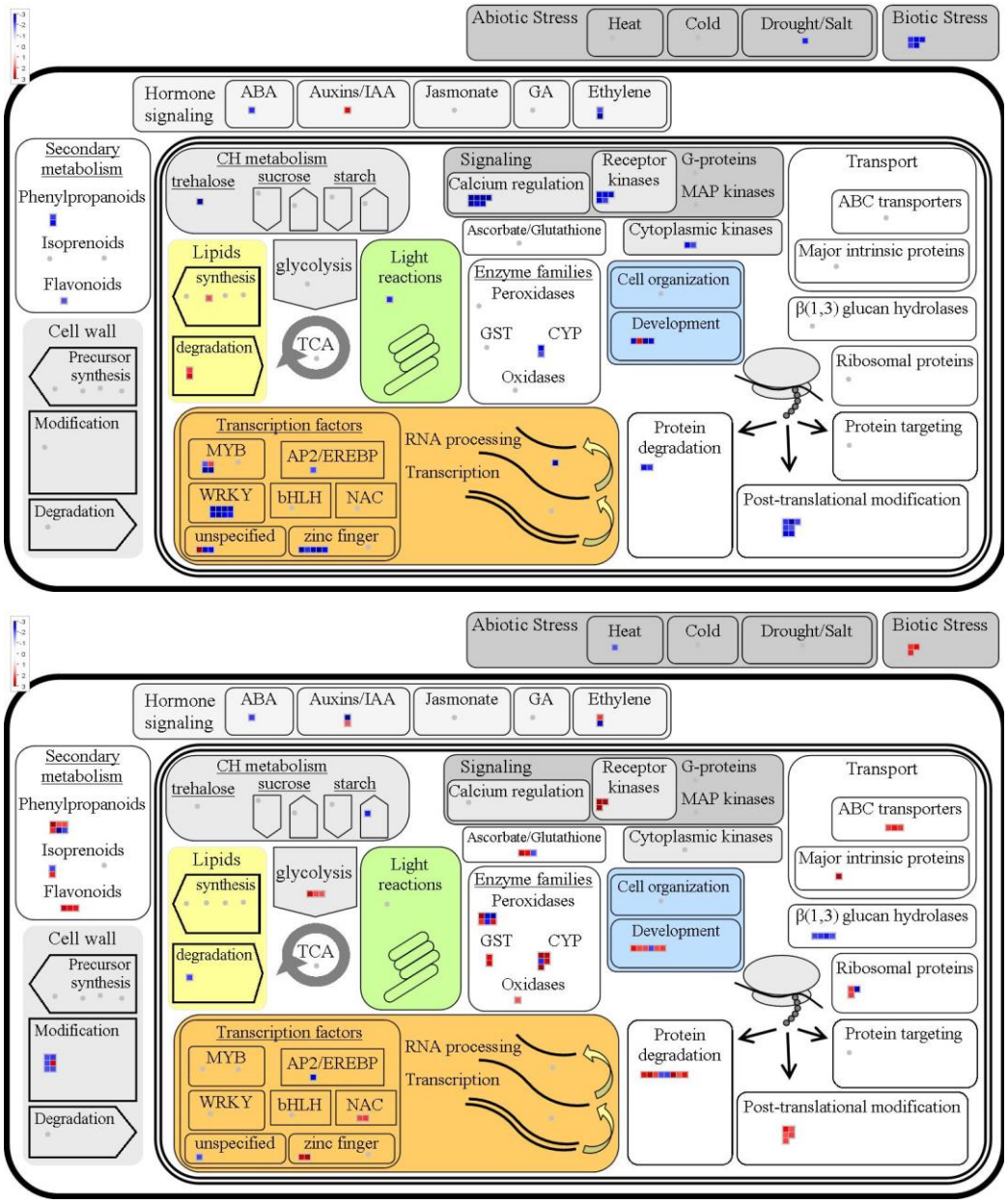


Figure 3.52. Overviews of metabolism pathways of specifically induced genes under B-deficiency+Tarımbor application in roots of Atay (above figure) and Bolal (below figure) cultivars. The MapMan tool was used to map on various metabolic pathways. Small red, blue and dark gray squares within each metabolism pathway indicate up-regulation, down-regulation and no detection of mapped genes, respectively.

3.5.5.3. Specifically Regulated Genes Involved in Transcription Factors under B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal Cultivars

As mentioned above, our hypothesis in microarray experiment was based on determining the genes that showed a specific response to B-stress in the B-tolerant cultivar Bolal-2973 when compared to B-sensitive cultivar Atay-85 since this analysis may help to clarify the B tolerance mechanism. In order to explore the transcriptional regulation in these contrasting wheat cultivars under B-toxicity, deficiency and B-deficiency+Tarımbor conditions, significantly regulated genes related to transcription factors were assessed. Accordingly, 14 genes in Atay and 10 genes in Bolal involved in transcription factors following B-toxicity were identified (Table 3.3). Among these, any gene related to C2H2 transcription factor was not significantly expressed in leaf and root tissues of Bolal. On the other hand, 2 genes related to C2H2 transcription factor were down-regulated (ta.7991.3.s1_x_at, ta.7991.3.s1_at) and 2 genes were up-regulated (ta.14101.1.s1_at, taaffx.98394.1.s1_at) in leaf tissues of Atay. Similarly, ta.7991.1.s1_x_at was significantly down-regulated gene for C2H2 in Atay root. ta.27337.1.s1_at and taaffx.81130.1.s1_at were specifically down-regulated genes related to MYB transcription factor under B-toxicity in Atay and Bolal leaves, respectively. Moreover, 3 genes in Atay (ta.25837.1.s1_at, taaffx.109191.1.s1_at, taaffx.109191.1.s1_x_at) and 3 genes (ta.8614.1.s1_at, taaffx.128870.1.s1_at, ta.4678.2.s1_at) in Bolal involved in WRKY transcription factor were identified under same treatment and all of them were down-regulated in leaf and root tissues of both cultivars. Furthermore, 5 genes related to AP2 transcription factor in Atay (ta.27316.1.s1_at; taaffx.80154.2.s1_at, ta.14000.1.s1_at, ta.13336.1.s1_at, ta.27316.1.s1_at) and 2 genes in Bolal (ta.21124.1.s1_x_at, ta.22338.2.s1_a_at) were specifically regulated after B-toxicity (Table 3.3).

Table 3.3. Specifically regulated genes involved in transcription factors under B-toxicity in Atay and Bolal cultivars. T.F: Transcription factors, ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

T.F.	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
C2H2	ta.7991.3.s1_x_at (2.2); ta.7991.3.s1_at (2.4);↓	-	ta.7991.1.s1_x_at (2.5); ↓	-
	ta.14101.1.s1_at; taaffx.98394.1.s1_at;↑			
MYB	ta.27337.1.s1_at (2.0);↓	taaffx.81130.1.s1_at (2.4);↓	-	ta.5405.1.s1_x_at (2.1);↓
				ta.8661.1.s1_at (2.0);↑
WRKY	ta.25837.1.s1_at (3.5);↓	ta.8614.1.s1_at (2.4); taaffx.128870.1.s1_at (2.0); ↓	taaffx.109191.1.s1_at (3.0); taaffx.109191.1.s1_x_at (2.9); ↓	ta.4678.2.s1_at (2.2);↓
AP2	ta.27316.1.s1_at (2.0); taaffx.80154.2.s1_at (2.1); ↑	ta.21124.1.s1_x_at (2.1);↑	ta.14000.1.s1_at (2.1); ↓	ta.22338.2.s1_a_at (2.6);↓
			ta.13336.1.s1_at (2.6); ta.27316.1.s1_at (2.6); ↑	
bHLH	-	ta.5856.1.s1_at (2.1);↑	ta.7389.3.s1_x_at (2.5);↑	ta.19622.1.s1_at (2.6); ↓

Similar to B-toxicity, under B-deficiency, ta.7991.3.s1_x_at and ta.7991.3.s1_at were significantly down-regulated genes related C2H2 transcription factor in Atay leaf and root (Table 3.4). Moreover, in leaf and root tissues of Atay, many genes involved in other transcription factors following B-deficiency were significantly regulated. These were 3 MYB family genes (ta.25268.1.s1_at, ta.5405.1.s1_x_at, taaffx.81130.1.s1_at), 4 WRKY family genes (ta.4725.1.s1_at, taaffx.109191.1.s1_at, taaffx.78545.2.s1_s_at, taaffx.109191.1.s1_x_at) and 1 AP2 family gene (ta.14000.1.s1_at) in Atay root. All of them were down-regulated. In leaf and root tissues of Bolal, there were not any significantly expressed genes related to MYB and WRKY transcription factors after B-deficiency treatment (Table 3.4).

Table 3.4. Specifically regulated genes involved in transcription factors under B-deficiency in Atay and Bolal cultivars. T.F: Transcription factors, ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

T.F.	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
C2H2	ta.7991.3.s1_x_at (3.0); ta.7991.3.s1_at (3.7); ↓ taaffx.87976.1.s1_at (2.2); ↑	taaffx.98394.1.s1_at (2.1); ↓	ta.7991.1.s1_x_at (2.4); ta.29449.1.s1_s_at (2.1); taaffx.98394.1.s1_at (2.4); ta.7991.3.s1_x_at (2.5); ↓	-
MYB	taaffx.34241.1.s1_at (2.1); ↓	-	ta.25268.1.s1_at (2.2); ta.5405.1.s1_x_at (2.3); taaffx.81130.1.s1_at (3.0); ↓	-
WRKY	ta.4678.1.s1_x_at (2.0); ↓	-	ta.4725.1.s1_at (2.6); taaffx.109191.1.s1_at (2.8); taaffx.78545.2.s1_s_at (2.3); taaffx.109191.1.s1_x_at (2.7); ↓	-
AP2	ta.28294.1.s1_x_at (2.0); taaffx.80154.2.s1_at (2.4); ↑	-	ta.14000.1.s1_at (2.5); ↓	ta.2781.1.s1_at (2.0); ↑
bHLH	ta.9648.3.s1_at (2.5); ↑	ta.5856.1.s1_at (2.5); ta.25394.1.s1_at (2.3); ↑	-	-

Interestingly, when Tarımbor was applied to B-deficient condition, significantly expressed genes involved MYB, WRKY, AP2 and bHLH transcription factors were not identified in Atay leaves (Table 3.5). Instead, in Atay root, 5 C2H2 family genes (ta.7991.1.s1_x_at, ta.29449.1.s1_s_at, ta.7991.3.s1_x_at, ta.103.1.s1_at, and ta.7991.3.s1_at), 3 MYB family genes (ta.25268.1.s1_at, ta.5405.1.s1_x_at, taaffx.81130.1.s1_at), 8 WRKY family genes (ta.16082.1.a1_at, ta.4725.1.s1_at, ta.4678.2.s1_at, taaffx.109191.1.s1_at, taaffx.78545.2.s1_s_at, ta.4678.1.s1_x_at, taaffx.109191.1.s1_x_at, ta.4678.1.s1_at) and 1 AP2 family gene (ta.14000.1.s1_at) were significantly down-regulated under B-deficiency+Tarımbor application. On the other hand, genes involved in MYB and WRKY transcription factors were not significantly regulated under B-deficiency+Tarımbor application in Bolal root (Table 3.5).

Table 3.5. Specifically regulated genes involved in transcription factors under B-deficiency+Tarımbor in Atay and Bolal cultivars. T.F: Transcription factors, ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

T.F.	**Probe sets significantly expressed**			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
C2H2	ta.9252.1.s1_x_at (2.0); ↓	taaffx.98394.1.s1_at (2.2); ↓	ta.7991.1.s1_x_at (3.0); ta.29449.1.s1_s_at (2.2); ta.7991.3.s1_x_at (2.7); ta.103.1.s1_at (3.3); ta.7991.3.s1_at (2.4); ↓	ta.1897.1.s1_x_at (2.7); ta.1897.1.s1_at (2.9); ↑
MYB	-	taaffx.118164.1.s1_x_at (2.2); ↑	ta.25268.1.s1_at (2.0); ta.5405.1.s1_x_at (6.3); taaffx.81130.1.s1_at (5.8); ↓	-
			ta.12834.1.s1_s_at (2.1); ↑	
WRKY	-	ta.4678.1.s1_x_at (2.5); ↓	ta.16082.1.a1_at (2.9); ta.4725.1.s1_at (2.7); ta.4678.2.s1_at (2.9); taaffx.109191.1.s1_at (3.4); taaffx.78545.2.s1_s_at (3.0); ta.4678.1.s1_x_at (2.4); taaffx.109191.1.s1_x_at (3.5); ta.4678.1.s1_at (2.4); ↓	-
AP2	-	ta.27316.1.s1_at (2.4); ↑	ta.14000.1.s1_at ; (2.1) ↓	ta.22338.2.s1_a_at (2.8); ↓
bHLH	-	-	-	-

3.5.5.4. Specifically Regulated Genes Involved in Translational and Post-Translational Modification under B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal Cultivars

The expression profiles of genes involved in regulating gene expression from transcriptional to post-translational controls were monitored by using microarray technique. In previous part, in leaf and root tissues of two contrasting cultivars Atay and Bolal, RNA metabolism including transcription factors was mentioned in detail. Hereby, differentially regulated genes involved in protein degradation, post-translational modification and ribosomal proteins in Atay and Bolal were identified under B-toxicity, deficiency and B-deficiency+Tarımbor. The majority of genes related to protein metabolism following B-toxicity were involved in protein degradation (Table 3.6). In fact, the numbers of specifically regulated genes related to protein degradation were higher in Atay leaf and root than those in Bolal leaf and root under B-toxicity. Most of these genes were up-regulated in leaf and root tissues of both cultivars. Moreover, 6 and 5 genes involved in post-translational modification were down-regulated in Atay and Bolal leaves, respectively (Table 3.6). On the other hand, taaffx.46790.1.s1_at, ta.12348.1.a1_at, ta.12876.1.s1_a_at, ta.2820.1.s1_at, ta.3786.1.a1_at in Atay leaf and ta.3840.1.s1_at, ta.4936.1.s1_at, taaffx.7979.1.s1_at, taaffx.2963.1.s1_at, ta.7585.1.s1_at in Bolal leaf were specifically up-regulated genes related to post-translational modification at the end of the B-toxicity. 1 (ta.28353.1.s1_x_at) and 2 genes (ta.18507.2.s1_x_at, taaffx.113419.1.s1_at) involved in post-translational modification under B-toxicity were down-regulated in Atay and Bolal roots, respectively (Table 3.6). Also, 4 and 2 genes related to post-translational modification were specifically up-regulated in Atay and Bolal roots, respectively. On the other hand, 7 genes in leaf tissue and 3 genes in root tissues involved in ribosomal proteins following B-toxicity were identified in Atay, however, 4 genes in leaf tissues and 5 genes in root tissues for ribosomal proteins were determined in Bolal. Most of these genes were up-regulated in both cultivars (Table 3.6).

Table 3.6. Specifically regulated genes involved in translational and post-translational modification under B-toxicity in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Protein Degradation	ta.537.2.a1_at (2.4); ta.693.3.s1_a_at (2.3); ta.693.1.s1_at (2.3); ta.4188.1.a1_at (2.1); ta.10814.1.s1_at (2.2); ↓	ta.23252.1.s1_at (3.4); ↓	ta.30933.1.a1_at (2.0); ta.5431.1.s1_x_at (2.4); taaffx.5381.1.s1_at (2.1); ta.8579.1.a1_at (2.4); ta.9445.1.a1_at (2.4); taaffx.92973.1.a1_at (2.1); taaffx.6798.1.s1_at (2.1); ↓	taaffx.18815.1.s1_at (2.1); ↓
	ta.541.1.s1_at (2.3); ta.541.1.s1_x_at (2.0); ta.23673.1.s1_a_at (2.7); ta.14733.2.s1_a_at (2.9); taaffx.81871.1.s1_at (2.7); ta.3902.1.s1_at (2.9); ta.7458.1.s1_at (2.5); ta.11015.1.s1_at (2.1); ta.27816.1.a1_at (2.3); ta.23317.2.s1_at (3.0); ta.18494.1.a1_at (2.1); ta.2514.1.s1_at (2.1); ta.6883.1.s1_at (2.1); ta.7011.2.s1_a_at (2.3); taaffx.55762.1.s1_x_at (2.0); ↑	ta.2854.2.s1_at (2.3); ta.13399.1.s1_at (2.2); taaffx.28669.1.a1_at (2.5); taaffx.97978.1.s1_at (2.0); ta.7944.1.s1_at (2.0); ta.11560.1.s1_x_at (2.3); taaffx.288.1.a1_at (2.1); taaffx.109719.1.s1_at (2.2);↑	taaffx.121677.1.s1_at (2.5); ta.29794.1.a1_x_at (2.1); ta.4898.1.a1_s_at (2.5); ta.119.1.s1_x_at (2.3); ta.6979.1.s1_s_at (2.1); taaffx.9425.1.s1_at (2.4); ta.7944.1.s1_at (2.5); ta.6322.2.s1_a_at (2.2); ↑	ta.23673.1.s1_a_at (4.0); ta.14475.1.s1_at (2.1); ↑
Post-Translational Modifications	ta.6553.1.s1_at (2.1); ta.1389.1.s1_at (2.6); ta.29444.1.s1_x_at (2.1); taaffx.71395.1.s1_at (2.2); ta.13268.1.s1_at (2.2); ta.29444.2.s1_s_at (3.8); ↓	ta.8400.2.s1_at (3.3); taaffx.121297.1.s1_at (2.1); ta.9542.2.s1_x_at (2.1); taaffx.53429.1.s1_at (2.3); ta.8375.1.s1_at (2.1); ↓	ta.28353.1.s1_x_at (2.6); ↓	ta.18507.2.s1_x_at (2.1); taaffx.113419.1.s1_at (2.2); ↓
	taaffx.46790.1.s1_at (2.2); ta.12348.1.a1_at (2.8); ta.12876.1.s1_a_at (2.3); ta.2820.1.s1_at (2.1); ta.3786.1.a1_at (2.2); ↑	ta.3840.1.s1_at (2.4); ta.4936.1.s1_at (2.1); taaffx.7979.1.s1_at (2.0); taaffx.2963.1.s1_at (2.2); ta.7585.1.s1_at (2.0); ↑	taaffx.129233.1.s1_at (2.1); ta.12876.1.s1_a_at (2.2); taaffx.7979.1.s1_at (2.3); ta.25418.2.s1_a_at (2.1); ↑	taaffx.128466.1.s1_at (2.0); ta.9810.1.s1_at (2.0); ↑

Table 3.6. (continued)

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Ribosomal Proteins	ta.9212.1.s1_at (2.7); ta.10577.1.s1_at (2.3); ↓	taaffx.128896.2.s1_at (2.1); taaffx.3720.3.s1_at (2.1); taaffx.3720.3.s1_at (2.1); taaffx.129824.2.s1_at (2.8); ↑	taaffx.15128.1.s1_at (2.3); ta.29371.2.s1_a_at (2.6); ta.14621.2.a1_x_at (2.5); ↑	ta.28514.1.a1_at (2.3);↓
	taaffx.129824.8.s1_at (2.3); taaffx.128896.15.s1_s_at (2.2); ta.14225.1.s1_x_at (2.3); ta.14225.1.s1_at (2.3); ta.4432.1.s1_s_at (2.1); ↑			taaffx.129824.9.s1_x_at (2.8); taaffx.129824.4.s1_x_at (3.0); ↑

Similar to B-toxicity, after B-deficiency treatment, 9 genes related to protein degradation were specifically up-regulated in Atay leaf; however, 3 genes in Bolal leaf involved in protein degradation were up-regulated (Table 3.7). In addition, B-deficiency affected genes involved in post-translational modification in leaf and root tissues of Atay and Bolal cultivars. In fact, 11 genes in Atay and 7 genes in Bolal involved in post-translational modification were specifically regulated after B-deficiency. On the other hand, genes related to ribosomal protein were not affected by B-deficiency in leaf and root tissues of Atay and Bolal with exception of taaffx.129824.2.s1_at in Bolal leaf (Table 3.7). Furthermore, supplementing Tarımbor to B-deficient condition specifically altered the expression levels of genes associated with protein metabolism involved in protein degradation, post-translational modification and ribosomal proteins in both Atay and Bolal cultivars. Approximately, same number of genes was significantly regulated after B-deficiency+Tarımbor application in both cultivars (Table 3.8).

Table 3.7. Specifically regulated genes involved in translational and post-translational modification under B-deficiency in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Protein Degradation	ta.10814.1.s1_at (2.4); ta.9720.1.a1_at (2.5); ↓	ta.21131.1.s1_x_at (2.1); taaffx.288.1.a1_at (2.1); taaffx.109719.1.s1_at (2.4); ↑	ta.4014.2.s1_at (2.5); ta.19463.1.s1_at (2.2); taaffx.6798.1.s1_at (2.2); ↓	ta.30884.1.a1_at (2.2); ↓
	ta.9405.1.s1_x_at (3.0); ta.26151.1.a1_at (2.7); taaffx.134015.1.s1_x_at (2.2); taaffx.134015.1.s1_s_at (2.5); ta.18900.1.s1_at (2.1); ta.23317.2.s1_at (3.0); ta.13279.2.s1_a_at (2.2); ta.3827.2.a1_at (3.2); taaffx.24090.1.s1_at (3.8); ↑			ta.18805.1.a1_a_at (2.2); ↑
Post Translational Modifications	ta.10236.2.s1_a_at (2.3); taaffx.54530.2.a1_x_at (3.0); ta.1389.1.s1_at (2.3); taaffx.60976.1.s1_at (2.7); ta.13268.1.s1_at (2.2); ta.25921.1.a1_at (2.2); ↓	taaffx.102420.1.s1_at (2.8); ta.3840.1.s1_at (2.3); ta.11603.1.s1_at (2.1); ↑	ta.28353.1.s1_at (2.1); ta.29444.1.s1_x_at (2.2); ta.29444.2.s1_s_at (2.2); ↓	ta.28002.2.a1_a_at (2.5); ta.28002.1.a1_at (2.3); ta.28353.1.s1_x_at (2.0); ta.28353.2.s1_at (2.0); ↓
	taaffx.65739.1.s1_s_at (2.3); taaffx.83758.1.s1_s_at (2.5); ↑			
Ribosomal Proteins	-	taaffx.129824.2.s1_at (2.4); ↑	-	-

Table 3.8. Specifically regulated genes involved in translational and post-translational modification under B-deficiency+Tarımbor in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Protein Degradation	ta.537.2.a1_at (2.2); ta.7470.1.s1_at (2.4); ta.12543.1.s1_at (2.3); ta.5392.1.a1_at (2.7); taaffx.3070.1.s1_at (2.2); ↓	taaffx.131906.1.s1_at (2.4); ta.25330.1.a1_at (2.3); ta.25578.2.s1_x_at (2.3); ta.13279.1.s1_a_at (2.3); taaffx.109719.1.s1_at (2.1); ta.13279.2.s1_a_at (2.3); ↑	ta.747.1.s1_at (2.4); ta.19463.1.s1_at (2.2); ↓	taaffx.18815.1.s1_at (2.1); taaffx.9559.1.s1_at (2.1); ↓
	ta.25320.2.a1_at (2.1); taaffx.134015.1.s1_x_at (2.1); taaffx.134015.1.s1_s_at (2.4); taaffx.1109.1.s1_at (2.1); ta.23317.2.s1_at (2.4); ta.7011.2.s1_a_at (2.1); ↑			ta.13399.1.s1_at (2.4); ta.5202.1.s1_at (2.9); ta.30933.1.a1_at (2.1); ↑
Post Translational Modifications	taaffx.54530.2.a1_x_at (3.0); ta.1389.1.s1_at (2.2); ta.451.1.s1_at (2.1); ta.9542.2.s1_x_at (2.3); ↓	taaffx.66712.1.s1_at (2.0); ta.8400.2.s1_at (2.8); taaffx.121297.1.s1_at (2.0); ↓	ta.8590.1.s1_at (2.2); ta.8590.1.s1_s_at (2.2); taaffx.29193.1.a1_at (2.6); ta.5408.1.s1_at (2.9); ta.29444.2.s1_a_at (2.0); ↓	ta.3840.1.s1_at (2.5); taaffx.129233.1.s1_at (2.0); ta.5214.1.a1_at (2.1); ta.28353.2.s1_at (2.0); ta.9810.1.s1_at (2.1); ↑
	ta.6207.2.s1_a_at (2.0); ta.11603.2.s1_a_at (2.3); ↑	taaffx.102420.1.s1_at (2.3); ta.3840.1.s1_at (2.2); taaffx.83758.1.s1_s_at (2.1); ↑		
Ribosomal Proteins	ta.4749.1.a1_at (2.1); ta.9212.1.s1_at (2.1); ↓	taaffx.56158.1.s1_at (2.1); ↓	-	ta.28514.1.a1_at (2.8); ↓
				taaffx.129824.5.s1_x_at (2.2); ↑

3.5.5.5. Specifically Regulated Genes Involved in Abiotic and Biotic Stress Under B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal Cultivars

Specifically regulated genes including abiotic and biotic stress responses in Atay and Bolal upon exposure to B-toxicity, deficiency and B-deficiency+Tarımbor compared to respective controls are displayed in Table 3.9-11. The number of genes related to biotic stress response was higher than abiotic stress response under all B conditions in leaf and root tissues of both cultivars. In fact, the root tissues of sensitive cultivar Atay had highest number of specifically regulated genes for biotic stress response under all B conditions. ta.4479.2.s1_x_at was common down-regulated gene related to biotic stress under all B conditions in Atay roots (Table 3.9-11).

Table 3.9. Specifically regulated genes involved in abiotic and biotic stress under B-toxicity in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Abiotic Stress/Heat	ta.28758.1.s1_at (2.6); ↓	-	ta.14573.1.s1_at (2.2); ↓	ta.21335.1.s1_at (2.2); ↓
	ta.24154.1.s1_s_at (4.1); ↑		ta.1459.1.s1_at (2.9); ta.15800.1.s1_at (2.1); ta.12773.1.s1_at (2.1); ↑	taaffx.37294.1.s1_at (2.6); ↑
Abiotic Stress/Cold	-	taaffx.87119.1.s1_at (2.8); taaffx.87119.1.s1_x_at (2.9); ↓	-	-
Abiotic Stress/ Drought/Salt	-	taaffx.131223.1.s1_at (2.1); ↓	ta.22764.1.s1_x_at (2.7); ↓	ta.5458.1.s1_x_at (2.6); ↓
Biotic Stress	ta.25384.1.s1_at (2.1); ↓	ta.2002.1.s1_at (2.8); ta.13785.1.s1_at (2.8); ↑	ta.30526.1.a1_at (2.0); ta.25774.1.s1_at (2.3); ta.22687.1.a1_at (2.5); ta.4479.2.s1_a_at (2.5); ta.4479.2.s1_x_at (2.7); ↓	ta.231.1.s1_x_at (3.0); ta.27275.1.s1_at (2.2); ↑
			ta.1037.1.s1_at (2.0); ↑	

Table 3.10. Specifically regulated genes involved in abiotic and biotic stress under B-deficiency in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Abiotic stress/heat	ta.11133.1.s1_at (2.1); ↑	ta.1459.1.s1_at (2.2); ↑	-	ta.11133.1.s1_at (2.1); ↓
Abiotic stress/cold	-	-	ta.27725.1.s1_at (2.0); ↑	-
Abiotic stress/ drought/salt	-	-	-	ta.16236.1.s1_at (2.1); ↓
Biotic stress	-	ta.23322.2.s1_at (3.0); taaffx.63989.1.a1_at (2.1); ↑	ta.13315.1.a1_s_at (2.1); ta.4479.2.s1_a_at (2.1); ta.4479.2.s1_x_at (2.2); ↓	ta.25774.1.s1_at (3.6); ta.27275.1.s1_at (2.1); ↑

Table 3.11. Specifically regulated genes involved in abiotic and biotic stress under B-deficiency+Tarımbor in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Abiotic stress/heat	taaffx.120716.1.s1_at (2.1); ta.1459.1.s1_at (2.3); ta.30772.1.s1_a_at (2.1); ↑	-	-	ta.12773.1.s1_at (2.0); ↓
Abiotic stress/cold	ta.19248.1.s1_x_at (2.2); ta.19248.1.s1_at (2.2); ↑	-	-	-
Abiotic stress/ drought/salt	-	-	ta.11100.1.s1_at (2.3); ↓	-
Biotic stress	taaffx.115796.1.s1_at (2.2); ↑	ta.25834.1.a1_at (2.7); ↓ ta.23322.2.s1_at (2.3); ta.25384.1.s1_at (3.0); ↑	ta.13315.1.a1_s_at (2.2); ta.13315.1.a1_x_at (2.1); ta.25774.1.s1_at (2.5); ta.4479.2.s1_a_at (2.7); ta.4479.2.s1_x_at (2.4); ↓	ta.23322.2.s1_at (2.2); taaffx.12180.1.s1_at (2.3); ta.27275.1.s1_at (2.4); ↑

3.5.5.6. Specifically Regulated Genes Involved in Signaling under B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal Cultivars

Genes related to hormone signaling were specifically up- or down-regulated during B-toxicity including auxin, jasmonate (JA), gibberellic acid (GA) and ethylene in leaf and root tissues of Atay and Bolal cultivars (Table 3.12). B-toxicity induced the expression level of genes involved in auxin in leaf tissues of Atay (ta.26922.1.s1_at) and Bolal (ta.23215.1.s1_at). Moreover, in root tissues, 1 gene (taaffx.38763.1.s1_at) for auxin was up-regulated in Atay and 1 gene (ta.22220.1.s1_at) was down-regulated in Bolal (Table 3.12). At the end of the B-toxicity, 5 genes involved in JA metabolism were specifically up-regulated in leaf tissues of Atay; however, there were no any specifically regulated genes for JA in Bolal leaf (Table 3.12). On the other hand, ta.27016.1.a1_s_at, ta.27016.1.a1_x_at were specifically up-regulated genes related to JA in Atay root, however, ta.526.1.s1_x_at was specifically down-regulated in Bolal root. Furthermore, B-toxicity induced 3 genes involved in GA under B-toxicity in Atay leaf (taaffx.12175.1.s1_at) and root (ta.24934.3.s1_at, ta.16799.1.s1_at). However, it did not cause any significant change in the expression level of genes for GA in leaf and root tissues of Bolal (Table 3.12). Also, ethylene metabolism was mainly affected by B-toxicity in root tissues of both cultivars. Genes involved in calcium mediated signal pathway were mainly regulated in leaf and root tissues of sensitive cultivar Atay. Most of these were down-regulated in Atay root. In addition, genes encoding the receptor kinases were differentially regulated in leaf and root tissues of Atay and Bolal after B-toxicity, however, differentially regulated genes related to cytoplasmic kinases were not identified in root tissues of both cultivars (Table 3.12).

Table 3.12. Specifically regulated genes involved in signaling under B-toxicity in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Hormone signaling/auxin	ta.26922.1.s1_at (3.6); ↑	ta.23215.1.s1_at (2.0); ↑	taaffx.38763.1.s1_at (2.0); ↑	ta.22220.1.s1_at (2.5); ↓
Hormone signaling/JA	ta.7830.1.s1_at (2.7); ta.7703.1.s1_x_at (2.1); ta.1207.1.s1_x_at (2.8); ta.1207.1.s1_at (2.3); ta.1207.1.s1_s_at (2.2); ↑	-	ta.27016.1.a1_s_at (2.3); ta.27016.1.a1_x_at (2.4); ↑	ta.526.1.s1_x_at (2.4); ↓
Hormone signaling/GA	taaffx.12175.1.s1_at (2.6); ↑	-	ta.24934.3.s1_at (2.2); ta.16799.1.s1_at (2.2); ↑	-
Hormone signaling/ethylene	taaffx.80154.2.s1_at (2.1); ↑	-	ta.21049.2.s1_at (2.1); ta.14000.1.s1_at (2.1); ↓	ta.9107.1.s1_x_at (2.0); ta.22475.1.s1_at (2.6); ta.22338.2.s1_a_at (2.6); ta.9939.1.s1_at (2.5); ↓
			ta.25763.1.s1_at (5.5); ↑	ta.6153.2.s1_at (2.3); ↑
Calcium Regulation	ta.320.2.s1_a_at (2.5); ↓	ta.2882.1.s1_s_at (2.8); taaffx.104457.1.s1_at (2.0); ↓	ta.7568.2.s1_at (2.3); ta.13803.1.s1_x_at (2.5); taaffx.59339.1.a1_at (2.4); ta.26330.1.a1_at (2.5); ta.7568.2.s1_x_at (2.2); ta.9099.3.s1_x_at (2.0); ta.7568.1.s1_x_at (2.2); ta.13803.1.s1_s_at (2.3); ta.320.2.s1_a_at (2.9); ta.2882.1.s1_s_at (2.1); ↓	-
	taaffx.107222.1.s1_at (2.5); ta.4855.1.s1_at (2.7); ta.9178.1.s1_at (2.4); ta.7364.1.a1_at 2.0); ↑			

Table 3.12. (continued)

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Receptor Kinases	ta.25825.1.a1_at (2.5); taaffx.84014.1.s1_at (2.0); taaffx.83027.1.s1_at (2.2); ta.12205.1.a1_at (2.3); ↓	ta.7017.1.s1_at (2.1); taaffx.27775.1.s1_at (2.2); ta.7017.1.s1_at (2.1); ↓	ta.25821.1.a1_at (2.6); ta.9228.1.s1_at (2.9); ta.27859.1.a1_at (2.3); taaffx.57030.1.s1_at (2.3); ta.5425.1.s1_at (2.8); ta.25821.1.a1_at (2.6); taaffx.57030.1.s1_at (2.3); ↓	taaffx.59304.1.a1_at (2.3); taaffx.113891.2.s1_s_at (2.5); ↓
	ta.941.1.a1_at (2.4); taaffx.129414.2.s1_x_at (3.7); taaffx.129414.2.s1_at (4.9); ↑	ta.14561.1.s1_at (2.2); ta.25461.1.s1_a_at (2.1); ↑		ta.27258.1.s1_at (2.2); ta.3850.1.s1_at (2.6); ta.25461.1.s1_a_at (2.1); ↑
Cytoplasmic Kinases	taaffx.84014.1.s1_at (2.0); ta.4736.1.a1_at (2.3); taaffx.98753.1.s1_at (2.3); ↓	ta.7443.1.s1_at (2.1); ta.29629.1.s1_s_at (3.4); ↑	-	-
	ta.12499.2.a1_a_at (2.2); ta.7742.1.s1_at (2.2); ta.11488.1.s1_s_at (2.1); ta.12499.2.a1_at (2.6); ↑			

At the same time, B-deficiency caused a significant change in the expression level of genes involved in some hormone signaling pathways and kinases in leaf and root tissues of both cultivars (Table 3.13). In fact, genes related to JA and GA metabolisms were not significantly changed after B-deficiency in leaf and root tissues of both cultivars with the exception of 2 genes in Atay leaves. However, 5 genes in Atay and 2 genes in Bolal related to ethylene metabolism were specifically regulated under same B-deficient condition (Table 3.13). Similar to B-toxicity, differentially regulated genes for calcium regulation caused by B-deficiency were not identified in Bolal root. Also, the large number of genes involved in receptor kinases was significantly up- or down-regulated after B-deficiency in leaf and root tissues of both cultivars (Table 3.13). Interestingly, after supplementing Tarımbor to B-

deficient condition, expression profile of differentially regulated genes related to hormone and kinases signaling pathways were similar to the changes in those under B-deficiency in leaf and root tissues of both cultivars (Table 3.14). For instance, differentially regulated genes for JA and GA metabolisms were not identified after B-deficiency+Tarımbor in Bolal leaf and root tissues of both cultivars. Similar to B-toxicity and deficiency, differentially regulated genes for calcium regulation under B-deficiency+Tarımbor were not determined in Bolal root. 5 genes in Atay and 6 genes in Bolal involved in ethylene metabolism were specifically regulated under same B-deficient+Tarımbor condition (Table 3.14). The large number of genes including receptor kinases was specifically regulated after B-deficiency+Tarımbor in leaf and root tissues of both cultivars similar to B-toxicity and deficiency (Table 3.14).

Table 3.13. Specifically regulated genes involved in signaling under B-deficiency in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Hormone signaling/auxin	taaffx.108287.1.s1_at (3.6); taaffx.108287.1.s1_x_at (3.6); ↑	-	taaffx.38763.1.s1_at (2.3); ↑	ta.5171.1.s1_at (2.7); ↓
Hormone signaling/JA	ta.8433.2.s1_a_at (2.0); ↑	-	-	-
Hormone signaling/GA	taaffx.12175.1.s1_at (3.1); ta.16799.1.s1_at (4.0); ↑	-	-	-
Hormone signaling/ethylene	ta.9107.1.s1_x_at (2.3); ta.9107.2.s1_a_at (2.7); ta.9107.2.s1_at (3.0); taaffx.80154.2.s1_at (2.4); ↑	ta.22475.1.s1_at (2.1); ↑	ta.14000.1.s1_at (2.6); ↓	ta.2781.1.s1_at (2.0); ↑
Calcium regulation	ta.6564.1.s1_x_at (2.1); ta.320.2.s1_a_at (2.4); ↓	ta.4580.2.s1_at (2.1); ↓	ta.2882.1.s1_s_at (2.1); ↓	-
	ta.6853.1.a1_at (2.3); taaffx.104457.1.s1_at (2.2); ↑	taaffx.71016.1.a1_at (2.1); ta.13245.1.s1_x_at (2.1); ↑		
Receptor kinases	ta.8590.2.s1_a_at (2.2); taaffx.31626.1.s1_at (2.4); taaffx.4501.1.a1_at (2.1); taaffx.84014.1.s1_at (2.2); ta.8054.1.a1_at (2.4); taaffx.30760.1.s1_s_at (2.1); ↓	ta.1441.1.s1_at (2.2); ta.14303.1.s1_at (2.5); ta.7081.1.s1_at (2.0); taaffx.128570.1.s1_at (2.0); ta.7081.1.s1_at (2.0); taaffx.12194.1.s1_at (2.4); ↑	ta.7017.1.s1_at (2.1); ta.3850.2.s1_at (2.1); ta.7017.1.s1_at (2.1); ↓	taaffx.110222.1.s1_x_at (2.6); ta.25821.1.a1_at (2.3); ta.5331.1.a1_x_at (2.6); taaffx.57030.1.s1_at (2.1); ta.25821.1.a1_at (2.3); taaffx.57030.1.s1_at (2.1); ↑
	taaffx.94232.1.s1_at (2.5); taaffx.129414.2.s1_x_at (2.2); taaffx.12166.1.a1_at (2.1); ↑			
Cytoplasmic kinases	taaffx.77051.1.a1_s_at (2.1); taaffx.84014.1.s1_at (2.2); taaffx.98753.1.s1_at (2.1); ↓	ta.9766.1.s1_at (2.4); ↑	ta.15863.1.a1_at (2.1); ↓	-
	ta.3054.2.s1_at (2.6); ↑			

Table 3.14. Specifically regulated genes involved in signaling under B-deficiency+Tarımbor in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Hormone signaling/auxin	taaffx.108287.1.s1_at (3.2); taaffx.108287.1.s1_x_at (3.3); ↑	taaffx.78729.2.s1_at (2.1); ↓	taaffx.38763.1.s1_at (2.5); ↑	ta.5171.1.s1_at (4.2); ↓ taaffx.77892.1.s1_at (2.0); ↑
Hormone signaling/JA	ta.1207.1.s1_x_at (2.2); ta.1207.1.s1_at (3.5); ta.1207.1.s1_s_at (3.7); ↓ ta.8433.2.s1_a_at (2.1); ↑	-	-	-
Hormone signaling/GA	taaffx.12175.1.s1_at (2.0); ↑	-	-	-
Hormone signaling/ethylene	taaffx.59867.1.s1_at (2.2); ta.425.3.a1_at (2.4); taaffx.80154.2.s1_at (3.3); ↑	ta.9107.1.s1_x_at (2.1); ta.9107.2.s1_at (2.2); taaffx.22824.1.s1_at (2.3); ↑	ta.14000.1.s1_at (2.1); ta.17378.1.s1_at (3.2); ↓	ta.22338.2.s1_a_at (2.8); ↓ ta.7223.2.s1_at (2.2); ↑
Calcium regulation	taaffx.71016.1.a1_at (2.2); ta.8389.1.a1_at (2.1); ta.6564.3.s1_x_at (2.0); ta.320.2.s1_a_at (2.5); ↓ ta.13245.1.s1_x_at (2.2); ↑	ta.7568.1.s1_x_at (3.1); ↓ ta.320.1.s1_at (2.1); ↑	ta.13803.1.s1_x_at (3.5); taaffx.59339.1.a1_at (4.7); ta.9099.3.s1_x_at (2.4); ta.13803.1.s1_s_at (3.6); ta.2882.1.s1_s_at (2.7); ta.6564.1.s1_at (3.7); ↓	-
Receptor kinases	taaffx.84014.1.s1_at (2.4); ta.8054.1.a1_at (2.3); taaffx.30760.1.s1_s_at (2.1); taaffx.83027.1.s1_at (2.5); ↓ taaffx.94232.1.s1_at (2.3); ↑	ta.8590.2.s1_at (2.3); ↓ ta.14303.1.s1_at (2.5); taaffx.12194.1.s1_at (2.1); ↑	ta.8590.2.s1_a_at (2.6); ta.7017.1.s1_at (2.6); ta.9228.1.s1_at (2.5); ta.12007.2.s1_at (2.1); ta.7017.1.s1_at (2.6); ↓	ta.27258.1.s1_at (5.4); ta.5331.1.a1_a_at (3.1); ta.5331.1.a1_x_at (2.9); ↑
Cytoplasmic kinases	taaffx.84014.1.s1_at (2.4); ↓ ta.3054.2.s1_at (3.2); ↑	taaffx.98753.2.s1_s_at (2.5); ta.15863.1.a1_at (2.0); ↓	ta.15863.1.a1_at (2.4); ta.4736.1.a1_at (2.1); ↓	-

3.5.5.7. Specifically Regulated Genes Involved in Secondary Metabolism under B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal Cultivars

Another important functional group of differentially expressed genes following B-toxicity stress were secondary and cell wall metabolisms in Atay and Bolal cultivars (Table 3.15). 10 genes in Bolal and 6 genes in Atay involved in phenylpropanoids were identified as specifically regulated under B-toxicity. Genes related to isoprenoids were mostly down-regulated after B-toxicity in leaf and root tissues of both cultivars. Among the secondary metabolism related genes, large number of genes involved in cell wall modification following B-toxicity were identified in both cultivars. In fact, 3 genes for cell wall modification were down-regulated in tolerant cultivar Bolal, however, 12 genes were down-regulated in sensitive cultivar Atay (Table 3.15). At the end of the B-deficiency, genes involved in phenylpropanoids were specifically up- or down-regulated in leaf and root tissues of both cultivars (Table 3.16). However, B-deficiency did not lead to any significant change in the expression level of genes related to flavonoids and precursor synthesis of cell wall in leaf and root tissues of both cultivars (Table 3.16). When Tarımbor was applied to B-deficient condition, the number of differentially regulated genes related to secondary metabolism and cell wall were higher than those in B-deficiency (Table 3.17). Most of these were specifically regulated following B-deficiency+Tarımbor application in root tissues of tolerant cultivar Bolal.

Table 3.15. Specifically regulated genes involved in secondary metabolism and cell wall under B-toxicity in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Secondary metabolism/ phenylpropanoids	taaffx.106960.2.s1_at (2.1); ↓	ta.7022.1.s1_s_at (2.8); taaffx.45277.1.s1_x_at (3.0); ta.7022.1.s1_x_at (2.4); taaffx.128482.1.s1_x_at (2.1); ta.24122.2.s1_at (2.3); ↓	ta.7022.1.s1_at (2.5); ta.7022.1.s1_x_at (2.7); ↓	ta.25383.1.a1_at (3.6); ta.7828.1.a1_at (2.7); ↓
	ta.2659.1.s1_at (3.9); ta.13798.1.s1_at (2.1); ↑	ta.9498.1.s1_x_at (2.3); ta.9498.3.s1_at (2.7); ta.9498.3.s1_x_at (2.6); ↑	ta.5491.1.a1_at (2.2); ↑	
Secondary metabolism/ isoprenoids	ta.12691.1.a1_at (2.5); ↓	taaffx.5829.2.a1_at (2.6); ↓	ta.17243.1.s1_at (2.0); ta.28293.1.s1_at (2.0); ↓	taaffx.86828.1.s1_s_at (2.2); ↓
				ta.8484.1.a1_at (2.7); ↑
Secondary metabolism/ flavonoids	ta.1727.1.a1_at (2.7); ↑	-	ta.14339.1.a1_at (2.0); ↓	ta.6822.1.s1_at (2.4); ↓
Cell wall/precursor synthesis	ta.25823.1.a1_at (3.0); ↓	-	ta.2657.1.s1_x_at (2.1); taaffx.6317.1.a1_at (2.2); ↓	-
	ta.4084.1.s1_at (2.2); ↑			

Table 3.15. (continued).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Cell wall/modification	ta.3274.1.s1_at (3.4); ↓	ta.25481.2.s1_s_at (2.1); ta.29462.1.a1_a_at (2.6); ta.29462.1.a1_x_at (2.7); ↑	taaffx.86321.1.s1_at (2.3); ta.30668.1.s1_at (2.0); ta.26458.1.a1_at (2.6); taaffx.56776.1.s1_at (2.3); ta.11809.2.s1_x_at (2.1); ta.30706.1.s1_at (2.1); taaffx.84102.1.s1_at (2.4); ta.8785.1.a1_at (2.0); ta.18703.1.s1_at (2.7); ta.20771.1.s1_at (2.4); ta.11809.2.s1_at (2.2); ta.2969.1.a1_x_at (3.0); ↓	ta.19273.1.s1_at (2.7); ta.10186.1.s1_at (3.3); ta.24423.1.s1_s_at (2.8); ↓
			ta.14995.1.s1_x_at (2.2); ta.6316.1.s1_at (2.1); ↑	
Cell wall/ degradation	-	ta.27751.5.s1_at (3.0); ↑	-	taaffx.124202.1.s1_at (2.1); ta.14588.1.s1_x_at (2.1); ↓

Table 3.16. Specifically regulated genes involved in secondary metabolism and cell wall under B-deficiency in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Secondary metabolism/ phenylpropanoids	ta.16968.1.a1_at (2.4); ta.9434.1.a1_at (2.3); ↑	ta.24122.2.s1_at (2.4); ↓	ta.7022.1.s1_at (2.5); ta.7022.1.s1_s_at (3.1); ta.7022.2.s1_at (2.8); ta.7022.2.s1_x_at (2.7)); taaffx.45277.1.s1_x_at (3.3); ta.7022.1.s1_x_at (2.7); ↓	taaffx.113333.1.s1_at (2.2); ↓
Secondary metabolism/ isoprenoids	ta.12691.1.a1_at (2.3); ↓	ta.1921.1.a1_at (2.0); ↑	-	taaffx.258.1.a1_at (2.1); ↑
Secondary metabolism/ flavonoids	-	-	-	ta.27359.1.s1_at (2.2); ↑
Cell wall/ precursor synthesis	-	-	-	-
Cell wall/ modification	ta.24120.1.s1_at (3.1); ta.3274.1.s1_at (3.6); ↓ ta.653.1.s1_at (2.0); ↑	ta.25481.2.s1_at (2.4); ta.25481.2.s1_x_at (2.7); ta.25481.1.s1_at (2.0); ↑	-	ta.18703.1.s1_at (2.0); ↑
Cell wall/ degradation	ta.11160.1.s1_s_at (2.6); ↓	ta.27751.5.s1_at (2.6); ↑	-	-

Table 3.17. Specifically regulated genes involved in secondary metabolism and cell wall under B-deficiency+Tarımbor in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Secondary metabolism/ phenylpropanoids	ta.9434.1.a1_at (2.6); ↑	ta.1856.1.s1_at (2.0); ta.9717.1.a1_x_at (3.0); ta.9717.1.a1_a_at (3.0); ta.9498.3.s1_at (2.0); ↑	ta.28046.1.a1_at (2.2); taaffx.131379.1.a1_at (2.4); ↓	taaffx.78987.1.s1_at (3.0); taaffx.115378.1.s1_at (2.1); ↓
				ta.14333.1.a1_at (3.5); ta.14165.1.s1_at (2.3); ta.3369.1.s1_at (2.1); ta.25173.1.s1_at (2.1); ↑
Secondary metabolism/ isoprenoids	ta.12691.1.a1_at (2.3); ↓	taaffx.128629.3.s1_s_at (2.2); ↑	-	ta.6247.1.a1_x_at (2.1); ↓
				ta.26208.1.a1_at (2.3); ↑
Secondary metabolism/ flavonoids	ta.1727.1.a1_at (2.6); ↑	-	taaffx.128729.1.s1_at (2.0); ↓	ta.27359.1.s1_at (2.8); ta.3448.3.a1_at (2.4); ta.25529.1.s1_at (2.4); ↑
Cell wall/ precursor synthesis	ta.6794.2.a1_at (2.2); ↓	ta.9118.1.s1_at (2.1); ta.4084.1.s1_at (2.2); ↓	-	-
		ta.22605.1.s1_at (2.1); ta.22605.1.s1_x_at (2.1); ↑		
Cell wall/ modification	ta.24120.1.s1_at (8.4); ↓	ta.28244.1.a1_s_at (2.1); ↑	-	ta.10186.1.s1_at (2.1); ta.11809.2.s1_x_at (2.1); taaffx.105595.1.s1_at (2.0); ta.11809.2.s1_at (2.2); ta.24423.1.s1_s_at (2.1); ↓

3.5.5.8. Specifically Regulated Genes Involved in Enzyme Families under B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal Cultivars

Most of the genes including detoxification of ROS were identified after 5 d in response to B-toxicity, deficiency and B-deficiency+Tarımbor in Atay and Bolal cultivars. Among these, genes related to GST, peroxidases, cytochrome P450 (CYP) and oxidases after B-toxicity in leaf and root tissues of both cultivars were shown in Table 3.18. Accordingly, 6 genes encoding GSTs were specifically up-regulated under B-toxicity in Atay leaf (ta.14483.1.s1_x_at, ta.30726.1.s1_at, ta.25377.1.s1_at, ta.5629.1.s1_x_at, ta.23704.1.s1_x_at, taaffx.42385.1.s1_at) while differentially regulated genes were not determined in Bolal leaf. On the other hand, 2 (ta.12936.1.s1_at, taaffx.86924.2.s1_at) and 1 gene (taaffx.78864.1.s1_at) were specifically regulated in Atay and Bolal roots, respectively. Genes encoding peroxidases were also affected by B-toxicity in leaf and root tissues of both cultivars (Table 3.18). The number of differentially regulated genes related to peroxidases was higher in root tissues of both cultivars than those in leaf tissues. In fact, 12 genes in Atay root and 7 genes involved in peroxidases in Bolal root were specifically regulated after B-toxicity. All of them were up-regulated. Furthermore, among the genes coding for CYP, 4 (ta.3381.1.s1_at, ta.4986.1.s1_at, taaffx.16125.1.s1_at, ta.27781.1.s1_at) were down-regulated and 2 (ta.10696.1.a1_at, ta.13841.1.s1_at) were up-regulated in Atay leaf, however, 3 (ta.20127.1.s1_at, ta.25587.1.s1_at, taaffx.76521.1.s1_at) were down-regulated in Bolal leaf. Also, B-toxicity altered the expression level of many genes for CYP in root tissues of both cultivars (Table 3.18). On the other hand, it did not cause any significant change in expression level of genes encoding for oxidases following B-toxicity in Bolal leaf, however, 2 (ta.8405.1.a1_at, ta.16981.1.s1_at) genes were up-regulated and 1 (ta.14103.1.s1_at) genes was down-regulated in Atay leaf (Table 3.18).

Table 3.18. Specifically regulated genes involved in enzyme families under B-toxicity in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Enzyme Families/ GST	ta.14483.1.s1_x_at (4.8); ta.30726.1.s1_at (2.0); ta.25377.1.s1_at (2.1); ta.5629.1.s1_x_at (2.3); ta.23704.1.s1_x_at (3.1); taaffx.42385.1.s1_at (4.7); ↑	-	ta.12936.1.s1_at (2.4); ↓ taaffx.86924.2.s1_at (2.2); ↑	taaffx.78864.1.s1_at (2.1); ↑
Enzyme Families/ PEROXIDASES	taaffx.1268.1.a1_s_at (2.1); ↑	ta.21505.1.s1_at (3.4); ta.30697.1.s1_at (2.9); ta.21307.2.s1_x_at (2.1); ↓	taaffx.86680.1.s1_at (2.2); ta.962.1.a1_at (2.0); taaffx.119224.1.a1_x_at (2.2); ta.952.1.s1_s_at (2.2); taaffx.37365.1.a1_at (2.8); ta.18757.1.s1_at (2.5); ta.8955.1.s1_at (3.6); taaffx.119224.1.a1_at (2.3); ta.952.2.s1_a_at (2.1); ta.5406.1.s1_at (2.3); taaffx.37365.2.s1_at (2.7); ta.5334.1.a1_at (2.6); ↓	ta.1588.1.a1_at (2.1); ta.21115.2.s1_x_at (2.1); ta.24687.1.s1_at (2.0); ta.25629.1.s1_at (2.3); ta.9334.1.s1_s_at (2.2); ta.1807.1.s1_at (2.3); ta.1807.1.s1_x_at (2.3); ↓

Table 3.18. (continued).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Enzyme Families/ CYP	ta.3381.1.s1_at (2.2); ta.4986.1.s1_at (2.6); taaffx.16125.1.s1_at (2.5); ta.27781.1.s1_at (2.6); ↓	ta.20127.1.s1_at (2.0); ta.25587.1.s1_at (2.3); taaffx.76521.1.s1_at (2.3); ↓	ta.174.1.s1_at (2.0); ta.20127.1.s1_at (2.6); ta.9332.3.s1_x_at (2.5); taaffx.111067.1.s1_x_at (5.1); ↓	ta.21061.1.s1_x_at (3.9); taaffx.125755.1.a1_at (2.3); ↓
	ta.10696.1.a1_at (2.1); ta.13841.1.s1_at (2.0); ↑		ta.1875.1.s1_at (2.1); taaffx.48690.1.s1_at (2.5); ta.5658.1.a1_at (2.4); ↑	ta.26216.1.a1_at (2.2); taaffx.195311.1.a1_x_at (2.3); ↑
Enzyme Families/ OXIDASES	ta.14103.1.s1_at (2.4); ↓	-	ta.16981.1.s1_at (2.0); ↓	ta.9135.2.s1_at (2.0); ta.9135.1.s1_x_at (2.1); ta.9135.1.s1_at (2.1); ↓
	ta.8405.1.a1_at (2.9); ta.16981.1.s1_at (4.7); ↑			

Following B-deficiency stress, differentially regulated genes encoding GSTs were not identified in root tissues of both cultivars, while 1 gene (ta.25779.1.s1_at) was up-regulated in Atay leaf and 1 gene (ta.14632.1.s1_at) down-regulated in Bolal leaf (Table 3.19). B-deficiency also affected the regulation of genes encoding enzymes involved in peroxidases, CYP and oxidases in leaf and root tissues both cultivars. Most of these were related to CYP enzyme families in both cultivars. In particular, 3 genes (ta.5724.1.s1_at, ta.4986.1.s1_at ta.8859.1.s1_at) were down-regulated and 5 genes (taaffx.64667.1.s1_at, taaffx.19531.1.a1_s_at taaffx.76521.1.s1_at, ta.5479.1.a1_x_at, taaffx.19531.1.a1_x_at) were up-regulated after B-deficiency in leaf tissues of sensitive cultivar Atay (Table 3.19).

Table 3.19. Specifically regulated genes involved in enzyme families under B-deficiency in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Enzyme Families/ GST	ta.25779.1.s1_at (2.8); ↑	ta.14632.1.s1_at (2.3); ↓	-	-
Enzyme Families/ PEROXIDASES	ta.21505.1.s1_at (2.9); taaffx.6050.1.s1_at (3.6); ↑	ta.5406.1.s1_at (2.5); ↑	ta.23366.2.s1_x_at (2.1); ↓	taaffx.16965.1.s1_at (2.6); ta.17325.1.s1_x_at (2.4); ↓
Enzyme Families/ CYP	ta.5724.1.s1_at (2.2); ta.4986.1.s1_at (2.4); ta.8859.1.s1_at (2.5); ↓	ta.25587.1.s1_at (2.1); ↓	taaffx.48690.1.s1_at (2.0); ta.5479.1.a1_x_at (2.7); ↑	ta.3381.1.s1_at (2.1); ta.25587.1.s1_at (2.0); ↓
	taaffx.64667.1.s1_at (2.0); taaffx.19531.1.a1_s_at (2.1); taaffx.76521.1.s1_at (2.3); ta.5479.1.a1_x_at (3.1); taaffx.19531.1.a1_x_at (2.1); ↑	taaffx.50125.2.s1_at (2.2); ↑		taaffx.19531.1.a1_x_at (2.1); ta.13971.1.a1_at (2.9); ↑
Enzyme Families/ OXIDASES	ta.10587.1.a1_at (2.5); ↓	ta.4062.2.a1_at (2.1); ↑	-	ta.339.2.s1_at (2.8); ↓

Table 3.20. Specifically regulated genes involved in enzyme families under B-deficiency+Tarımbor in Atay and Bolal cultivars. ↑: up-regulation, ↓: down-regulation. The numerals in parentheses represent fold changes (P<0.05).

	Probe sets significantly expressed			
	Atay leaf	**Bolal leaf**	**Atay root**	**Bolal root**
Enzyme Families/ GST	-	-	-	taaffx.64766.1.s1_at (2.5); ta.25382.1.s1_at (2.7); ↑
Enzyme Families/ PEROXIDASES	ta.24106.1.s1_x_at (2.7); taaffx.6050.1.s1_at (3.1); ↑	taaffx.1268.1.a1_s_at (2.2); ta.18261.2.s1_x_at (2.3); ta.23366.3.a1_at (2.0); ta.5406.1.s1_at (3.5); ta.22593.1.a1_at (2.6); ↑	-	taaffx.56560.1.s1_at (2.5); ta.4876.1.a1_x_at (2.3); taaffx.134950.1.s1_at (3.1); ↓
				ta.26230.2.s1_at (3.0); ta.26230.1.s1_at (2.2); ta.26230.1.s1_x_at (2.4); ↑
Enzyme Families/ CYP	ta.1875.1.s1_at (2.0); ta.20127.1.s1_at (2.3); ta.8859.1.s1_at (2.0); taaffx.16125.1.s1_at (2.4); ↓	ta.27570.1.s1_at (2.6); ta.11257.1.a1_at (2.2); ta.8399.1.s1_at (2.1); ↑	taaffx.63970.1.a1_at (2.6); ta.9332.3.s1_x_at (2.1); ↓	ta.3381.1.s1_at (2.3); ↓
	taaffx.64667.1.s1_at (2.1); ↑			ta.8447.1.s1_x_at (6.7); ta.8447.1.s1_a_at (9.4); ta.13971.1.a1_at (2.8); ta.27576.1.a1_at (2.7); ↑
Enzyme Families/ OXIDASES	ta.10587.1.a1_at (2.0); ↓	-	-	ta.21293.2.s1_at (2.0); ↑
	ta.1522.1.s1_at (2.0); ↑			

When Tarımbor was supplemented to B-deficient condition, genes encoding for GSTs were not specifically regulated in leaf and root tissues of both cultivars with exception of 2 genes in Bolal root (Table 3.20). Also, 6 genes encoding peroxidases were differentially regulated after B-deficiency+Tarımbor application in Bolal root; however, differentially regulated genes for peroxidases were not identified in Atay root. Furthermore, similar to B-toxicity and deficiency, large number of differentially regulated genes encoding for CYP were identified at the end of the B-deficiency+Tarımbor in both cultivars, while too few genes encoding oxidases were specifically regulated in Atay and Bolal cultivars (Table 3.20).

Lastly, as mentioned above, specifically regulated genes in Atay and Bolal cultivars after B-treatments were applied to MapMan program in order to determine the possible B responding genes in B tolerance mechanism; however, many genes were not functionally identified in this tool. For this reason, some genes that had high fold change after B-treatments compared to controls were chosen for blastx analysis and then their tentative annotations were determined and shown in Table 3.21. Accordingly, the expression levels of cold responsive protein WCOR14c, putative glutathione S-transferase GSTU6, 3-hydroxybenzoate 6-hydroxylase, receptor-like kinase were markedly increased at the end of the B-toxicity in Atay leaves; however, expression levels of jasmonate induced protein and short-chain type dehydrogenase/reductase were dramatically decreased under same condition in Bolal leaves (Table 3.21). Furthermore, jasmonate-induced protein and WRKY19-b transcription factor expressions were significantly decreased after B-toxicity in Atay root, however, Subtilisin-chymotrypsin inhibitor-2A and Bowman-Birk type trypsin inhibitor expressions were markedly decreased in Bolal root. On the other hand, putative glutathione S-transferase GSTU6 and WIR1 expressions were dramatically increased under B-toxicity in Bolal root (Table 3.21).

After B-deficiency treatment, the expression level of F-box protein SKIP27 and two-component response regulator ARR8 were significantly decreased in Atay leaves; however, disease resistance protein RPM1 and putative transcription factor X1 expressions were significantly decreased in Bolal leaves. The expression levels of WRKY19-b transcription factor and thionin-like peptide were markedly decreased under B-deficiency in Atay root; however, jasmonate-induced protein and senescence-associated protein expressions were dramatically increased under same condition in Bolal root (Table 3.21).

Table 3.21. Specifically regulated genes identified by using blastx analysis in Atay and Bolal cultivars. F.C: Fold change, +: up-regulation, -: down-regulation.

Probe Set IDs	Tentative Annotation	Cultivar-Tissue	Treatment	F.C	Representative GenBank
Ta.245.1.S1_at	cold responsive protein WCOR14c	Atay-Leaf	B-toxicity	+ 7.4	AF491837.1
Ta.30843.1.S1_at	LRR receptor-like serine/threonine-protein kinase	Atay-Leaf	B-toxicity	-5.1	EMT20914.1
TaAffx.129414.2.S1_at	receptor-like kinase	Atay-Leaf	B-toxicity	+4.9	AAD43962.1
Ta.14483.1.S1_x_at	putative glutathione S-transferase GSTU6	Atay-Leaf	B-toxicity	+4.8	EMS59368.1
Ta.16981.1.S1_at	3-hydroxybenzoate 6-hydroxylase	Atay-Leaf	B-toxicity	+4.7	EMS68723.1
TaAffx.103568.1.S1_at	jasmonate induced protein	Bolal-Leaf	B-toxicity	-17.6	CAA58110.1
Ta.12469.1.A1_at	Anthranilate N-benzoyltransferase protein 1	Bolal-Leaf	B-toxicity	+5.9	EMS65855.1
Ta.418.1.S1_at	Short-chain type dehydrogenase/reductase	Bolal-Leaf	B-toxicity	-5.3	EMT26243.1
TaAffx.52653.1.A1_s_at	Protein WAX2	Bolal-Leaf	B-toxicity	+4.7	EMS68717.1
Ta.5252.1.S1_at	jasmonate-induced protein	Atay-root	B-toxicity	-9.9	ACG37700.1
Ta.5810.1.S1_at	wali6	Atay-root	B-toxicity	+7.5	AAC37417.1
Ta.5456.1.A1_at	WRKY19-b transcription factor	Atay-root	B-toxicity	-5.8	ABO15545.1
Ta.25763.1.S1_at	putative 2-oxoglutarate/Fe(II)-dependent dioxygenase	Atay-root	B-toxicity	+5.5	EMS47228.1
Ta.24535.1.S1_at	Subtilisin-chymotrypsin inhibitor-2A	Bolal-Root	B-toxicity	-11.3	EMS48067.1
Ta.30922.1.S1_at	putative glutathione S-transferase GSTU6	Bolal-Root	B-toxicity	+8.2	EMS59368.1
Ta.20928.1.S1_at	Bowman-Birk type trypsin inhibitor	Bolal-Root	B-toxicity	-8.0	EMS60285.1
Ta.15072.1.A1_at	WIR1	Bolal-Root	B-toxicity	+5.7	CAA61018.1

Table 3.21. (continued)

Probe Set IDs	Tentative Annotation	Cultivar-Tissue	Treatment	Fold Change	Representative GenBank
Ta.7015.1.S1_at	F-box protein SKIP27	Atay-Leaf	B-deficiency	-4.3	EMS57773.1
Ta.25635.3.S1_a_at	Annexin D4	Atay-Leaf	B-deficiency	+4.2	EMS49319.1
TaAffx.119928.1.A1_s_at	Two-component response regulator ARR8	Atay-Leaf	B-deficiency	-4.0	EMS50922.1
TaAffx.92191.1.A1_at	Disease resistance protein RPM1	Bolal-Leaf	B-deficiency	-5.1	EMS45741.1
TaAffx.128836.1.S1_at	putative transcription factor X1	Bolal-Leaf	B-deficiency	-5.1	AAL35831.2
Ta.7740.2.A1_at	Phospholipase A1-II 7	Bolal-Leaf	B-deficiency	+4.6	EMS68153.1
Ta.5456.1.A1_at	WRKY19-b transcription factor	Atay-Root	B-deficiency	-5.3	ABO15545.1
Ta.5503.1.S1_s_at	thionin-like peptide	Atay-Root	B-deficiency	-4.8	AAX19516.1
Ta.5252.1.S1_at	jasmonate-induced protein	Bolal-Root	B-deficiency	+7.3	ACG37700.1
Ta.20928.1.S1_at	Bowman-Birk type trypsin inhibitor	Bolal-Root	B-deficiency	-7.1	EMS60285.1
TaAffx.499.1.A1_at	senescence-associated protein	Bolal-Root	B-deficiency	+5.5	AAX13288.1

3.6. Validation of Microarray Results by Real-Time RT-PCR

In order to validate our microarray data of B effects on global expression profiling, real-time RT-PCR assays were carried out by using selected genes including Ta.428.1.S1_at, Ta.16082.1.A1_at, Ta.24954.1.S1_at and Ta.103.1.S1_at. Our criterion in verification of microarray data was to randomly select the genes that had unchanged, increased and decreased expression levels after B-treatments. Expression changes obtained by Real-time RT-PCR were in accordance with the trend as detected by microarray (Figure 3.53- 54). The putative annotations of Ta.428.1.S1_at, Ta.16082.1.A1_at, Ta.24954.1.S1_at and Ta.103.1.S1_at were NOD26-like intrinsic protein 4;1, WRKY41 transcription factor, unknown hypothetical protein and Arabidopsis zinc-finger protein 2, respectively.

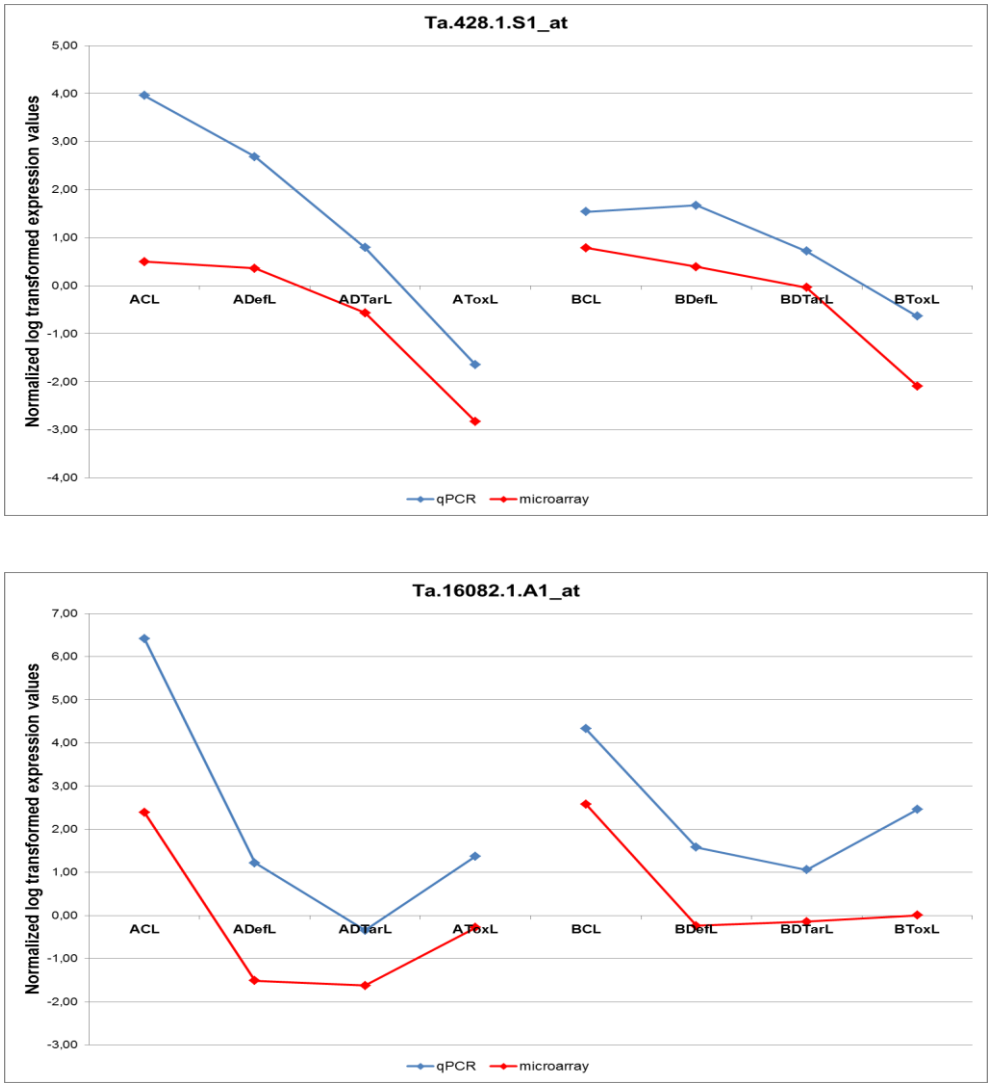


Figure 3.53. Verification of microarray results of selected probe sets (Ta.428.1.S1_at, Ta.16082.1.A1_at) by from real-time RT-PCR analyses. ACL; Atay Control Leaf, AToxL; Atay Toxicity Leaf, ADefL; Atay Deficiency Leaf, ADTarL; Atay Deficiency+Tarımbor Leaf, BCL; Bolal Control Leaf, BToxL; Bolal Toxicity Leaf, BDefL; Bolal Deficiency Leaf, BDTarL; Bolal Deficiency+Tarımbor Leaf. Red lines show expression values from microarray and blue lines show expression values from real-time RT-PCR. Three biological replicates were used for real-time RT-PCR.

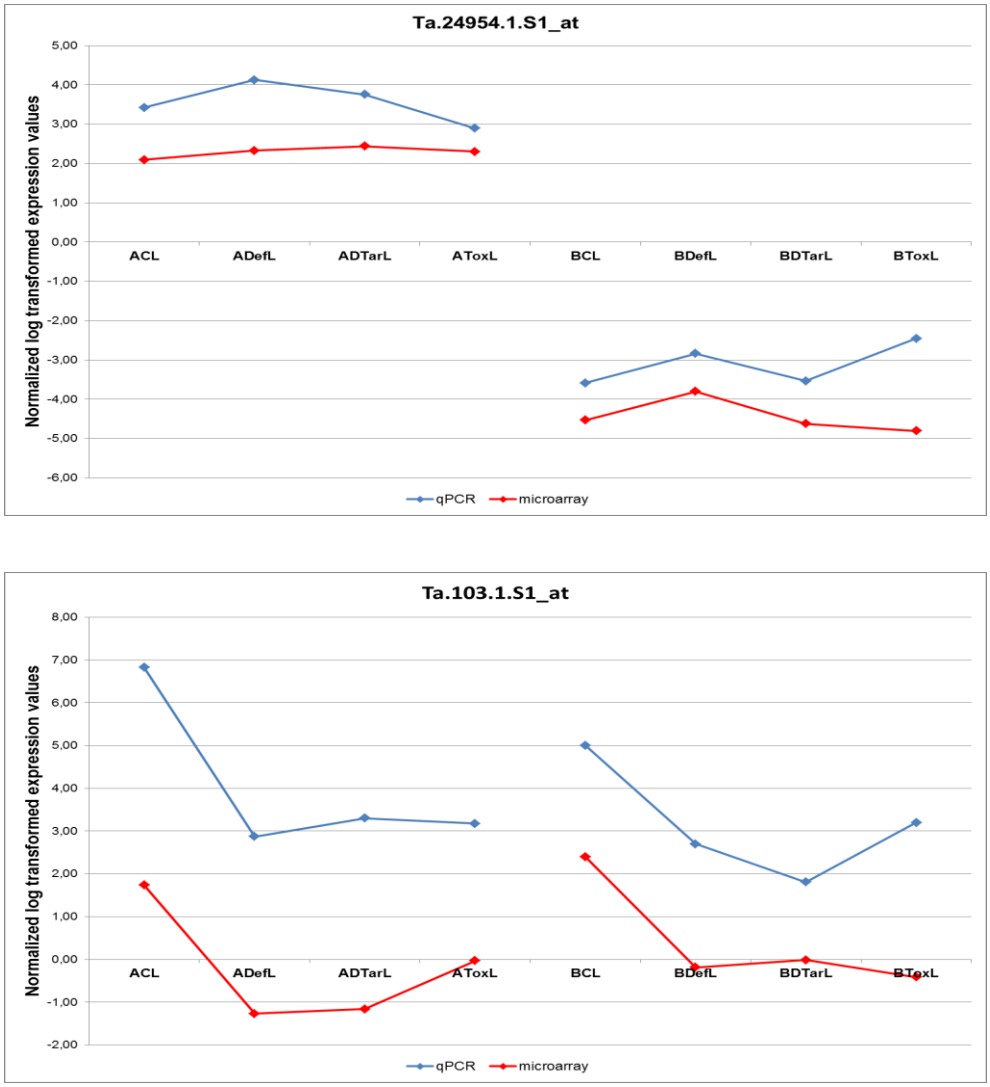


Figure 3.54. Verification of microarray results of selected probe sets (Ta.24954.1.S1_at and Ta.103.1.S1_at) by real-time RT-PCR analyses. ACL; Atay Control Leaf, AToxL; Atay Toxicity Leaf, ADefL; Atay Deficiency Leaf, ADTarL; Atay Deficiency+Tarımbor Leaf, BCL; Bolal Control Leaf, BToxL; Bolal Toxicity Leaf, BDefL; Bolal Deficiency Leaf, BDTarL; Bolal Deficiency+Tarımbor Leaf. Red lines show expression values from microarray and blue lines show expression values from real-time RT-PCR. Three biological replicates were used for real-time RT-PCR.

CHAPTER 4

DISCUSSION

4.1. Physiological and Biochemical Responses of Wheat to B-toxicity

Although B is an essential micronutrient for plant growth, B toxicity affects a wide variety of plants growing in soils naturally containing high B levels or irrigation water including excessive B (Stangoulis and Reid, 2002). The typical visible symptoms of B-toxicity are chlorotic and necrotic patches on leaf margins or leaf tips of older leaves (Bennett, 1993; Bergmann, 1992). In the present work, they were not seen after B-toxicity in Bolal; but these symptoms appeared in Atay leaves. Furthermore, B accumulates higher in Atay leaves than Bolal at the end of the B-toxicity. These results suggest that higher accumulation of B in Atay leaves was responsible for chlorotic and necrotic lesions. Likewise, Nable *et al*. (1997) suggest that chlorotic and necrotic patches reflect the distribution of B in most plants with B accumulating at the end of the transpiration stream. On the other hand, any visible symptom caused by B-toxicity was not observed in root tissues of both cultivars. Also, in both cultivars, B concentrations in the roots were relatively low compared to those in leaves. These expected results have already been suggested by Nable (1988); Oertli and Roth (1969). Although a reduced growth of shoots and roots is typical of plants exposed to toxic B levels (Nable *et al*., 1990), delayed development and reduced vigour were not observed in leaf and root tissues of Atay and Bolal cultivars because lengths, wet and dry weights were not markedly changed after B-toxicity. Furthermore, B toxicity mediated membrane damage was not observed in leaf and root tissues of both cultivars due to stable level of ion leakage.

Chlorophyll fluorescence is non-invasive method that gives the opportunity to measure the photosynthetic performance (Oxborough, 2004). In this study, in Bolal leaves all parameters of chlorophyll florescence and chlorophyll contents did not significantly change after exposure to B-toxicity; however, in Atay leaves, chlorophyll a (Ca), chlorophyll b (Cb), total chlorophyll (Ca+b) and carotenoid (Cx+c) contents were significantly decreased; however, all chlorophyll florescence parameters were not remarkably changed (except for NPQ). These results suggest that decreasing of chlorophyll content might be related to chlorophyll degradation due to chlorophyll synthesis deficiency or changes of thylakoid membrane structure; however, this may lead to an inhibition of the photosynthetic performance and imbalance between the light capture and its utilization since plants may rapidly adapt to B-toxic condition.

In order to evaluate the oxidative damage in wheat cultivars, lipid peroxidation was estimated by measuring the accumulation of MDA since it is accepted to be one of the simplest signs of oxidative stress in organisms (Zhang and Kirkham, 1996; Mittler, 2002). In this study, B-toxicity only caused a slight increase in MDA level in Bolal leaves and did not cause any significant change in these levels in Atay leaves and root tissues of both cultivars. In other words, MDA levels fluctuated with applying excess B in wheat plants. This fluctuation can be explained by the instant metabolite and pathway shifts of enzymatic and non-enzymatic detoxification mechanisms (Ayvaz *et al*., 2013).

Protection of cell membrane may be a major factor in plant tolerance against oxidative stress. Proline plays an important role in osmoregulation, protection of enzyme denaturation and scavenging ROS in plant tissues as a hydroxyl radical scavenger (Yoshiba *et al*., 1997). The proline accumulation under stress has been correlated with stress tolerance in plants (Ashraf and Foolad, 2007). Similar to proline, glycine-betaine is osmoprotectant synthesized by many plants in response to various stresses (Delauney and Verma, 1993).

In our experiment, proline and glycine-betaine contents were not significantly changed in leaf and root tissues of both cultivars at the end of the B-toxicity. This may be reason for stable photosynthetic activity and MDA content in B-treated plants.

Besides osmoprotectans such as proline and glycine-betaine, scavenging of ROS is achieved by antioxidative defense enzymes. Bowler *et al*. (1992) suggest that higher scavenging activity may correlate with enhanced abiotic stress tolerance of the plants. Unfortunately, in this study, activities of APX, CAT and GR in leaf and root tissues of both cultivars under B-toxicity showed an increase, fluctuation, and decrease in comparison with the respective controls. Therefore, total antioxidant enzyme activities in wheat plants may not involve in B toxicity tolerance mechanism. Likewise, Karabal *et al*. (2003) suggest that toxic B concentrations lead to membrane damage in an oxygen free radical independent manner and apparently antioxidant enzymes do not play a role in B tolerance mechanism against toxic B level in plants.

4.2. Physiological and Biochemical Response of Wheat to B-deficiency

B is needed as a constant supply by plants to maintain growth. B deficiency is a widespread problem in agricultural areas in the world and sudden occurrences of B deficiency or inconsistent effects of foliar B application cause a challenge the management of B nutrition (Wimmer and Eichert, 2013). For this purpose, we mentioned physiological and biochemical responses to B-deficiency and supplementary Tarımbor as a B-enriched fertilizer to B-deficient condition. In leaf tissues of cultivars, the chlorosis and necrosis were not observed after B-deficiency since wheat (*Triticum aestivum* L.) has generally been considered to have a low requirement for B (Marten and Westermann, 1991). Furthermore, B-deficiency did not lead to any significant change in the values of length, wet weight, dry weight in leaf and root tissues of both cultivars. Normally, the most rapid response to B limitation is the cessation of root elongation in higher plants (Dugger, 1983; Marschner, 1995). This cessation is related to the death of the growing meristem,

which is in line with the function of B in structurally strengthening components of the cell wall, however, it has rarely been seen in wheat (Rerkasem and Jamjod, 2004). B deficiency in early vegetative growth is much less readily inducible in wheat than in dicotyledons. Similarly, the young wheat plants grew well and were free of B deficiency symptoms under low B supply (Asad *et al*., 2001).

Although B has also function on plasma membrane and this includes the physical stabilization of membranes via formation of B cross-links with diol containing molecules, the incorporation of diol-containing molecules into membranes, or a direct effect on membrane-localized enzyme activities (Wimmer *et al*., 2009), the values of ion leakage in leaf and root tissues of both cultivars did not significantly change at the end of the B-deficiency treatment. These results may be verified with stable MDA level under B-deficiency in leaf and root tissues of both cultivars. Furthermore, B-deficiency did not alter the water status in leaf tissues of both cultivars since leaf and root growth was not severely inhibited by B-deficiency, which directly reduced the available surface for water and nutrient uptake (Wimmer and Eichert, 2013).

B deficiency can impair photosynthesis by a reduction of chlorophyll concentrations in leaves (Hajiboland and Faranghi, 2010; Tewari *et al*., 2010) and of the photosynthetic capacity of photosystem II, as indicated by analysis of chlorophyl fluorescence parameters. There is no evidence of a direct role for B in photosynthesis (Dugger, 1983; Shelp, 1993). In this study, there were no correlation between chlorophyll contents and chlorophyll fluorescence measurements in leaves of both cultivars similar to uncorrelated photosynthetic measurements on excess B-treated plants since this may be reason for unchanged ion leakage and MDA contents. Correlatively, authors suggest that the possible negative effect of B deficiency on photosynthesis can be explained by membrane damage because of increased levels of ROS (Tewari *et al*., 2010; El-Shintinawi, 1999).

Similar to B-toxicity, proline and glycine-betaine contents were not markedly changed under B-deficiency in leaf and root tissues of both cultivars since stable level of MDA, ion leakage and water potential. Furthermore, fluctuations in APX, CAT and GR activities were also observed in leaf and root tissues of both cultivars under B-deficiency condition. Thus, antioxidant enzyme activities in wheat plants may not involve in B-deficiency tolerance mechanism similar to B-toxicity tolerance in wheat plants. Several studies focused the effect of deficient, appropriate and excessive b on activity of antioxidant enzymes (Cakmak and Römheld, 1997; Garcia *et al*., 2001, Karabal *et al*., 2003); however, their results were not consistent in the changes of antioxidant enzyme. In other words, it is likely that changes of antioxidant enzymes in different plants in response to B deficiency or excess are inconsistent (Liu *et al*., 2005).

4.3. Physiological and Biochemical Responses of Wheat to Supplementary Tarımbor to B-deficient Condition

Although wheat (*Triticum aestivum* L.) has generally been accepted to have a low requirement for B, it is estimated that currently in the central southern and eastern Anatolia regions of Turkey about 30% of the soils are B deficient (Gezgin and Hamurcu, 2006). Therefore, the management of fertilization is crucial to overcome the negative effect of B deficiency on wheat plants. In this study, we investigated physiological and biochemical responses to Tarımbor application to control and B-deficient condition in two wheat cultivars. Accordingly, after the process of Tarımbor applications was complete, necrotic and chlorotic lesions were not observed in leaf tissues of both cultivars. This means that although tissue B content increased 5-fold after Tarımbor applications in leaves of both cultivars, Tarımbor applications did not cause injury of plant cells. Furthermore, when Tarımbor was applied to B-deficiency and control conditions, values of lengths, wet and dry weights and water potential were not markedly changed in leaf and root tissues both cultivars. These results suggest that Tarımbor applications did not affect the vegetative growth in wheat plants since B-deficiency stress can be easily tolerated by

both wheat cultivars. Also, these applications did not cause membrane damage due to stable level of ion leakage in both cultivars. This result was verified by constant MDA levels in leaf and root tissues of both cultivars.

Similar to B-toxicity and deficiency, there were no correlation between chlorophyll contents and chlorophyll fluorescence measurements in both cultivars. In fact, Tarımbor application did not affect the net photosynthetic activity in spite of variable chlorophyll contents. Also, Tarımbor applications did not alter significantly the proline and glycine and betaine content and activities of CAT, APX, and GR in leaf and root tissues of both cultivars since oxidative damage due to overproduction of ROS in B-deficient plants was not occurred. As a result, Tarımbor applications did not significantly affect the vegetative growth and activate the osmoprotection because both wheat cultivars may have low B requirement.

4.4. Effect of B-toxicity, deficiency and B-deficiency+Tarımbor Application on Gene Expression in Wheat Cultivars

In this section, B-induced molecular changes were analyzed both wheat cultivars by investigating gene expression profiles under B-toxicity, deficiency and Tarımbor application to B-deficient condition. For this purpose, we performed PCA in order to unravel cultivar, tissue and stress specific patterns. As mentioned above, PCA converts large microarray data sets into a few numbers which can be represented as a measure of distance between the samples. Therefore, the closer two samples are in the PCA, the closer the similarity of the overall transcriptional expression. Accordingly, tissue and cultivar differences are main source of variation. In other words, the expression profile of wheat induced by B-stress is not stress specific since the responses of wheat to B-stress contain mainly cultivar and tissue specific elements.

The number of responsive genes identified from leaf tissues was higher than that of root tissues in both cultivars under B-deficiency and B-deficiency+Tarımbor application. On the contrary, B-toxicity induced more genes in root tissues of both wheat cultivars when compared to leaf tissues. These results may be explained by the tissue specific nature of the stress response. Cotsatis *et al*. (2011) and Walia *et al*. (2005) found the similar results in rice under salt stress condition.

At least one B condition, the number of GO categories was higher in roots than that of leaves. Also, cellular nitrogen compound metabolic process was commonly regulated between leaf and root tissues; however, jasmonic acid metabolic and biosynthetic processes, and response to wounding and external stimulus were specifically represented in leaf and root tissues, respectively at least one B condition.

Comparison of gene expression profiles between B-tolerant and sensitive cultivars under B-stress might be essential for the elucidation of the B response networks in wheat. For this purpose, we first evaluated the GO categories for common genes between Atay and Bolal cultivars under B-toxicity, deficiency and B-deficiency+Tarımbor conditions. Accordingly, transcription factor activity and transcription regulator activity for common down-regulated genes between Atay and Bolal were over-represented under B-toxicity. In fact, this category is common in all down-regulated genes under B-toxicity, deficiency and B-deficiency+Tarımbor conditions in leaf and root tissues of both cultivars. To sum up, transcription factor activity and transcription regulator activity might be a crucial part of the down-regulation mechanism under all B condition. At the same time, in leaf and root tissues, catalytic and enzymatic activities were common for up-regulated genes in both cultivars under B-toxicity. However, in root tissues, significant GO categories were not determined under B-deficiency and B-deficiency+Tarımbor for common up-regulated genes in both cultivars since low number of genes were significantly up-regulated. On the other hand, in leaf tissues, kinase and tansferase activity were common categories for up-regulated genes under B-deficiency and B-deficiency+Tarımbor condition.

Secondly, we evaluated genes involved in transcription factors, translation processes, abiotic and biotic stress, hormone signaling, secondary metabolism and enzyme families that showed differences in expression between Atay and Bolal cultivars in order to determine genes that may be relevant special process of B tolerance mechanism.

Transcription factors are transcriptional regulators that contain DNA-binding domains interacting with cis-elements in the promoter or enhancer regions of a gene and therefore induce or repress the mRNA synthesis resulting in regulating many biological processes in plants (Agarwal and Jha, 2010). Expression profiling studies suggest that stress tolerance or susceptibility is controlled at the transcriptional level via stress-related gene induction (Chen *et al*., 2012; Singh *et al*., 2002). Approximately 1500 transcription factors in *Arabidopsis thaliana* genome have been recognized and recent analyses reported that about more than 2000 genes encoded transcription factors based on sequence similarities with known DNA-binding domain (Davuluri *et al*., 2003; Guo *et al*., 2005; Riano-Pachon *et al*., 2007). In this study, differentially expressed transcription factors in Atay and Bolal cultivars following different B conditions were assessed. Accordingly, genes encoding C2H2 transcription factors were not differentially expressed under B-toxicity in leaf and root tissues of Bolal cultivar. On the other hand, 5 genes related to C2H2 were up- or down-regulated in leaf and root tissues of sensitive cultivar Atay. Likewise, transcriptome analyses demonstrated that low temperature, salt, drought, osmotic and oxidative stress caused an elevated the transcript level of many C2H2-type zinc finger proteins (Kiełbowicz-Matuk, 2012). In fact, overexpression of C2H2 related genes resulted in both the activation of some stress-related genes and enhanced tolerance to various stresses (Sugano *et al*., 2003 and Huang *et al*., 2007). Furthermore, in both cultivars, differentially regulated genes encoding WRKY, MYB, AP2 and bHLH transcription factors after B-toxicity were identified. It has been reported that WRKY transcription factors have been suggested to play important roles in the regulation of transcriptional reprogramming related to various abiotic stress responses (Chen *et al*., 2012). Also, it was shown that the AP2/EREBP

transcription factors mediated distinct responses to various abiotic stresses by hormone-dependent gene expression (Kizis *et al*., 2001). Furthermore, Zhou *et al*. (2009) and Kim and Kim (2006) suggest that the bHLH transcription factors have an important role in the abscisic acid-mediated signal transduction pathway and in secondary metabolism, which regulates the plant adaptation to abiotic stresses.

On the other hand, differentially regulated genes encoding MYB, WRKY, C2H2 and AP2 transcription factors following B-deficiency were not identified in tolerant cultivar Bolal, however, some genes related to these transcription factors were significantly regulated in sensitive cultivar Atay under B-deficiency, especially in Atay root. Of these, WRKY and MYB transcription factors may participate mainly in the B-deficiency response signaling pathways in root tissues of sensitive cultivar. Likewise, in *Arabidopsis thaliana*, AtWRKY75 was the first WRKY family member involving in regulating phosphate starvation (Devaiah *et al*., 2007). Interestingly, when Tarımbor as a B-specific fertilizer was applied to B-deficient condition, too few transcription factors were differentially regulated in leaf tissues of both cultivars. Thus, transcriptional regulation may not be required for leaf tissues of two wheat cultivars in recovered B condition after B-deficiency.

A large number of genes related protein degradation was identified under B-toxicity in leaf and root tissues of both cultivars. These genes are related to the ubiquitin/proteasome system that target proteins and result in their degradation. The ubiquitin-dependent protein degradation pathway is involved in photomorphogenesis, hormone regulation, floral homeosis, senescence, and pathogen defense (Suzuki *et al*., 2002; Xie *et al*., 2002; Hellmann and Estelle, 2002; Devoto *et al*., 2003). Also, ubiquitination has a significant role in stress responses in plants. Interestingly, Zhou *et al*. (2010) overexpressed soybean ubiquitin-conjugating enzyme gene GmUBC2 in *Arabidopsis* and suggested that this gene was involved in the regulation of ion homeostasis, osmolyte synthesis, and oxidative stress responses. In this study, more genes related for protein degradation were induced by B toxicity in leaf and root tissues of Atay than those in Bolal. Differentially regulated genes for

protein degradation may be associated with programmed cell death in senescing leaves (Degenkolbe *et al*., 2009). The visual phenotype of the plants confirmed this interpretation since Atay showed yellowing and partial leaf death under B-toxicity, whereas the leaves of Bolal remained green. Similar to B-toxicity, number of genes related to protein degradation following B-deficiency was higher in Atay leaf than those in Bolal leaf, but B deficiency symptoms were not clearly seen in leaf tissues of sensitive cultivar Atay.

As well, controlling protein folding and protein turn-over/degradation by differentially regulated genes contribute to regulate protein synthesis in order to prevent severe cellular damage imposed by B-toxicity condition (Ambrosone *et al*., 2013). In both cultivars, remarkable number of genes related to ribosomal proteins was significantly regulated following B-toxicity. Ribosomal proteins are essential for protein synthesis and thus have an important role in metabolism, cell division, and growth. Recently, Wang *et al*. (2013) suggest that ribosomal proteins are involved in protein translation and the response of plants to nutrient deficiency, possibly changing the composition of ribosomes in Pi- or Fe-deficient roots. However, in this study, unlike B-toxicity, expression levels of genes related to ribosomal proteins were not affected by B-deficiency in leaf and root tissues of Atay and Bolal (except of taaffx.129824.2.s1_at in Bolal leaf). Likewise, the composition of ribosomes is changed in a stress-specific manner (Wang *et al*., 2013). Interestingly, when Tarımbor was applied to B-deficient condition, more genes related to ribosomal proteins were regulated in both cultivars compared to B-deficiency condition. This may be related to remodeling of composition of ribosomes after supplementing B to deficient condition.

The expression of genes involved in abiotic and biotic stress was mainly changed after B-toxicity, while only a few genes were affected following B-deficiency and B-deficiency+Tarımbor application in both cultivars. In fact, among the differentially regulated genes related to abiotic stress response, genes encoding for heat shock family proteins were mostly regulated under all B conditions in leaf and root tissues

of both cultivars. Wang *et al*. (2004) suggest that these proteins were expressed not only in high temperature but also in other abiotic stresses, such as oxidative stress, osmotic, salinity, cold and water stress. Very interestingly, some pathogen-related genes involved in biotic stress were differentially regulated in response to all B conditions in mainly root tissues of sensitive cultivar Atay. This result might be explained that they have possible roles in the crosstalk between biotic and abiotic stresses. Kalemtas (2011) similarly found that genes encoding for chitinases and disease resistance proteins involved in biotic stress responses were cold-responsive in wild type and transgenic lines of potato. Also, WRKY transcription factors which have important role in response to pathogens were shown to be cold responsive in *Arabidopsis* (Lee *et al*., 2005).

Interestingly, following B-toxicity stress, in only Atay leaves, the genes related to JA, GA and ethylene metabolisms were specifically up-regulated; however, there were no any differentially regulated genes for these metabolisms in Bolal leaf. Similar to B-toxicity, under B-deficiency and B-def+Tar conditions, the genes for hormone signaling such as auxin, JA, GA and ethylene were specifically regulated in only Atay leaves in comparison with Bolal leaves. To sum up, the genes related to phytohormone metabolism were specifically induced in sensitive cultivar Atay under all B conditions. Similarly, in *Arabidopsis* gene expression analysis showed that JA biosynthesis were induced as a prominent response to low K+ (Armengaud *et al*., 2004). Öz *et al*. (2008) also found up-regulation of genes involved in JA biosynthesis under high B treatment. Likewise, up-regulation of genes encoding JA-responsive proteins under drought stress in barley was reported by Ozturk *et al*. (2002). JA might be involved in ion homeostasis in the plants, regarding its induction in response to a nutritional deficiency (Walia *et al*., 2006). Furthermore, JA acts in coordination with other plant hormones such as ethylene (Walia *et al*., 2006). JA and ethylene regulate the protective responses of plants against abiotic stresses by synergistic and antagonistic actions (Fujita *et al*., 2006).

Also, detecting of hormonal regulation in only leaves of sensitive cultivar Atay might be related to senescence mechanism after especially B-toxicity because ethylene has an important role in regulating senescence of leaves (Grbic and Bleecker, 1995).

Many genes related to calcium ions (Ca^{2+})-mediated signaling mechanisms were down-regulated in only Atay roots under B-toxicity. The Ca^{2+} ion is an important cellular signaling component, and transient increases in the levels of cytoplasmic Ca^{2+} are evident in the response to many stresses (Knight *et al*., 1997). However, down-regulation of genes related to Ca^{2+} signaling might restrict intracellular B accumulation after exposure to high B in roots of sensitive cultivar Atay. Similarly, P2B phosphatase calcineurin (CaN), a key component of Ca^{2+} dependent signal transduction pathway, has function to restrict intracellular Na^{+} accumulation to mediate salt adaptation in plants (Pardo *et al*., 1998). Furthermore, in leaf and root tissues of both cultivars, large number of genes involved in receptor kinases were differentially regulated under B-toxicity, deficiency and B-deficiency+Tarımbor conditions. They have important roles in plant development, hormone perception. Also, these genes are responsible for earliest response of biotic or abiotic stresses in plants (Ouyang *et al*., 2010).

Similar to calcium regulation in Atay root, large number of genes involved in cell wall modification at high B were down-regulated in only Atay root. These genes in wheat are highly similar to Expansin (EXP) gene family in *Arabidopsis thaliana*. EXPs are a family of cell wall loosening proteins and have vital role in the regulation of plant adaptation to environmental stress by regulating cell growth and expansion (Padmalatha *et al*., 2012). Thus, down-regulation of these genes suggests that B-toxicity might cause a decreased cell wall extensibility resulting in a decreased ability of cells to expand. Likewise, down-regulation of EXP gene family was observed in *Arabidopsis* under water deficit condition (Bray, 2004).

On the other hand, secondary metabolites such as phenylpropanoids, isoprenoids, flavonoids following B-toxicity, deficiency and B-deficiency+Tarımbor application were differentially up- or down-regulated in leaf and root tissues of both cultivars. These metabolites play a protective role in response to biotic and abiotic stresses (Mazid *et al*., 2011). However, the exact roles of them are not clarified at present due to contradictory results. For instance, the flavonoid biosynthesis pathway was identified in salt-sensitive rice genotypes but not in salt-tolerant rice genotypes (Walia *et al*., 2005). However, it was induced not only in the salt-tolerant barley but also in the salt-sensitive barley.

Similar to calcium regulation and cell wall modification as mentioned above, many genes encoding peroxidases were down-regulated after B-toxicity in only Atay root. Peroxidases play a key role in ROS scavenging mechanism under stress conditions. Remarkably, they are also associated with cell elongation processes and with inhibition reactions of cell elongation (Passardi *et al*., 2004). By this way, the availability of H_2O_2 in the cell wall can be controlled, which is a prerequisite for the cross-linking of phenolic groups, to inhibit cell elongation (Passardi *et al*., 2004). This process is catalyzed by peroxidases in response to environmental stresses or developmental processes from germination to senescence and peroxidase-mediated cross-linking of several compounds lead to cell wall rigidification (Passardi *et al*., 2004). Finally, down-regulation of genes encoding peroxidases and cell wall modification may be related to control B influx under B-toxicity.

Furthermore, in only Atay leaves, 6 genes encoding for GSTs were specifically up-regulated following B-toxicity. These enzymes help to protect cells from oxidative damage (Kumari *et al*., 2008). However, this regulation in only leaves of sensitive cultivar Atay might be more related to the response of B toxicity rather than B tolerance mechanism. Likewise, Chandran *et al*. (2008a) suggest that oxidative stress gene expression is more of a manifestation of Al toxicity rather than a tolerance mechanism.

In both cultivars, limited genes encoding for GST, peroxidases, CYP and oxidases after B-deficiency and B-deficiency+Tarımbor application were differentially regulated. In other words, only a small proportion of ROS related genes regulated their transcript abundances under these conditions. This result was in accordance with the measurements of antioxidant enzyme activities. Therefore, regulation of gene expression might initiate a transient change in redox state that might be used as signal for activation of other stress responsive genes.

Finally, B toxicity or deficiency induce perturbation in cellular processes and thus cause changes in gene expression in both B-tolerance and –stress related genes mechanisms. For distinguishing of two mechanisms, specifically expressed genes after B treatments in B-tolerant and sensitive cultivars were assessed. This analysis for the first time provides a global insight about the transcriptomic responses to B treatments in wheat cultivars. However, a large number of genes with unknown biological functions were found to be regulated differentially. Some of these genes were tentatively annotated by comparing their EST sequences using blastx analysis. Besides genes as mentioned above, these genes may need further study for their roles in the B tolerance mechanism in plants.

CONCLUSION

Both B-toxicity and deficiency did not cause a cessation of vegetative growth in leaf and root tissues of both wheat cultivars because wheat (*Triticum aestivum* L.) has generally been considered to have a low requirement for B and it may be easily tolerate toxic B concentrations with B efflux mechanism.

There was no correlation between chlorophyll contents and chlorophyll fluorescence measurements in leaves of both cultivars under all B conditions. Also, a few genes related photosynthesis were significantly changed under same conditions. These results show that the net photosynthetic activity may not be affected after exposure to B-toxicity and deficiency.

Tissue and cultivar differences are main source of variation in microarray experiments according to PCA results.

The genes related to protein degradation were induced more in Atay than Bolal under all B conditions. These results considering programmed cell death in senescing leaves were confirmed by the visual phenotype of the plants because Atay showed yellowing and partial leaf death after B-toxicity, however, the leaves of Bolal remained green.

The expression of genes involved in oxidative stress and detoxification of ROS was not significantly regulated after B-toxicity and deficiency. This result was in accordance with the measurements of antioxidant enzyme activities. Therefore, the majority of genes that were induced by B stress might be related to senescence rather than to stress tolerance mechanisms.

Large number of specifically regulated genes involved in cell wall modification, peroxidases and Ca^{2+}-mediated signaling mechanisms identified in roots of sensitive cultivar Atay in response to B-toxicity may help to regulate H_2O_2 availability and thus control B influx in roots.

Tarımbor applications did not markedly alter the physiological, biochemical and molecular responses in two wheat cultivars since the symptoms of B-deficiency were not clearly seen and low requirement for B in both wheat cultivars.

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APPENDIX A

HOAGLAND'S MEDIUM PREPARATION

Table A.1. Preparation of Macronutrient Stock Solution.

COMPOSITION	**STOCK SOLUTION**	**USE (ml/ 1 L) for full-strength**
$MgSO_4.7H_2O$	24.6 g/100ml	1.0 ml
$Ca(NO_3)_2.4H_2O$	23.6 g/100ml	2.3 ml
KH_2PO_4	13.6 g/100ml	0.5 ml
KNO_3	10.1 g/100ml	2.5 ml
Micronutrients	See table B	0.5 ml
FeEDTA	See table C	20.0 ml
B as H_3BO_3	See table D	

Table A.2. Preparation of Micronutrient Stock Solution.

MICRONUTRIENTS	**STOCK SOLUTION**
H_3BO_3	-
$MnCl_2.4H_2O$	0.182 g / 100 mL
$ZnSO_4.7H_2O$	0.022g / 100 mL
$Na_2MoO_4.2H_2O$	0.009 g / 100 mL
$CuSO_4.5H_2O$	0.009 g / 100 mL

Table A.3. Preparation of FeEDTA Stock Solution.

	STOCK SOLUTION
$FeCl_3.6H_2O$	0.121 g / 250 ml
EDTA	0.375 g / 250 ml

Table A.4. Boric Acid Stock Solution.

	STOCK SOLUTION	**proper dilutions for final concentrations of**
H_3BO_3	0.5 M (500 mM) in 100 mL	control (10 µM) deficiency (0.02 µM) toxicity (5000 µM)

APPENDIX B

TARIMBOR ($(Na_2O.5B_2O_3.10H_2O)$)

Table B.1. Chemical properties of Tarımbor.

% B	% B_2O_3	% Na_2O	Bulk Density (kg/m^3)	pH	
				1 %	10 %
18-19	58-60	11	740-760	8,1	7,2

Table B.2. The effect of Tarımbor on plant productivity (www.bmbor.com)

Crop Name	Yield Increase
Garlic	**% 22**
Clover	**% 28**
Wheat	**% 6**
Durum wheat	**% 23**
Strawberry	**% 26**
Tomato	**% 11**
Cucumber	**% 6**
Sugar beet	**% 18**
Lettuce	**% 11**
Hazelnut	**% 19**
Brussel sprout	**% 31**

APPENDIX C

PROBE SETS USED IN REAL TIME RT-PCR FOR VALIDATION OF MICROARRAY DATA

Table C.1. The gene-specific primers used for Real Time RT-PCR.

Probe Set ID	Primer Orientation	Primers
GAPDh (internal control)	Forward	5'TTCAACATCATTCCAAGCAGCA
	Reverse	5'CGTAACCCAAAATGCCCTTG
Ta.428.1.S1_at (NOD26-like intrinsic protein 4;1)	Forward	5'GAGCGAGCTCTGTGCCAAAG
	Reverse	5'TTATGCAACCTCTGACGCAG
Ta.16082.1.A1_at (WRKY41 transcription factor)	Forward	5'CTTCTACACATTCCAGTCCGATTG
	Reverse	5'TGTCCTAAATTTCAGACCTGCAC
Ta.24954.1.S1_at (unknown hypothetical protein)	Forward	5'AATGGACAAGAGATACCCGG
	Reverse	5'CATATTTTTCCAACCCTCCC
Ta.103.1.S1_at (Arabidopsis zinc-finger protein 2)	Forward	5'TAGGCAGTAAGTTGTAACG
	Reverse	5'CGGTACTGCTAGTTCATG

Table C.2. Amplicon size and amplification conditions of the gene specific primers.

Probe Set ID	Amplicon Size (base pair)	Amplification Conditions	
Ta.428.1.S1_at	202	95ºC	15 min
		94 ºC	15 sec
		56 ºC	30 sec
		72 ºC	30 sec
		45 cycle	
Ta.16082.1.A1_at	240	95 ºC	15 min
		94 ºC	15 sec
		56 ºC	30 sec
		72 ºC	30 sec
		45 cycle	
Ta.24954.1.S1_at	208	95 ºC	15 min
		94 ºC	15 sec
		52 ºC	30 sec
		72 ºC	30 sec
		45 cycle	
Ta.103.1.S1_at	130	95 ºC	15 min
		94 ºC	15 sec
		52 ºC	30 sec
		72 ºC	30 sec
		50 cycle	

APPENDIX D

CONCENTRATION OF ISOLATED RNAs

Table D.1. Isolated RNAs from leaf and root tissues of Atay and Bolal cultivars. BR: Biological Replicates, DF: Dilution Factor, C: Concentration, *: low amount.

Samples	BR	DF	C(µg/µl)	Samples	BR	DF	C(µg/µl)
Atay Control Leaf	1	200	1,809	Bolal Control Leaf	1	200	0,902
Atay Toxicity Leaf	1	200	1,767	Bolal Toxicity Leaf	1	200	1,432
Atay Deficiency Leaf	1	200	1,717	Bolal Deficiency Leaf	1	200	0,743
Atay Tarımbor Leaf	1	200	1,435	Bolal Tarımbor Leaf	1	200	1,329
Atay Def+Tar Leaf	1	200	1,188	Bolal Def+Tar Leaf	1	200	0,954
Atay Control Root	1	200	0,600	Bolal Control Root	1	200	0,252
Atay Toxicity Root	1	200	0,333	Bolal Toxicity Root	1	200	0,266
Atay Deficiency Root	1	200	0,437	Bolal Deficiency Root	1	200	0,242
Atay Tarımbor Root	1	200	0,216	Bolal Tarımbor Root	1	200	0,351
Atay Def+Tar Root	1	200	0,298	Bolal Def+Tar Root	1	200	0,297
Atay Control Leaf	2	400	2,142	Bolal Control Leaf	2	*	*
Atay Toxicity Leaf	2	200	1,730	Bolal Toxicity Leaf	2	*	*
Atay Deficiency Leaf	2	200	0,932	Bolal Deficiency Leaf	2	200	1,342
Atay Tarımbor Leaf	2	200	1,931	Bolal Tarımbor Leaf	2	200	1,682
Atay Def+Tar Leaf	2	200	1,496	Bolal Def+Tar Leaf	2	200	1,446
Atay Control Root	2	200	0,277	Bolal Control Root	2	200	0,477
Atay Toxicity Root	2	200	0,388	Bolal Toxicity Root	2	200	0,509
Atay Deficiency Root	2	200	0,529	Bolal Deficiency Root	2	200	0,461
Atay Tarımbor Root	2	200	0,307	Bolal Tarımbor Root	2	200	0,363
Atay Def+Tar Root	2	200	0,317	Bolal Def+Tar Root	2	200	0,378
Atay Control Leaf	3	200	1,143	Bolal Control Leaf	3	200	1,560
Atay Toxicity Leaf	3	200	0,993	Bolal Toxicity Leaf	3	200	1,621
Atay Deficiency Leaf	3	200	1,285	Bolal Deficiency Leaf	3	400	2,277
Atay Tarımbor Leaf	3	200	1,990	Bolal Tarımbor Leaf	3	200	1,375
Atay Def+Tar Leaf	3	200	1,825	Bolal Def+Tar Leaf	3	200	1,815
Atay Control Root	3	200	0,189	Bolal Control Root	3	200	0,598
Atay Toxicity Root	3	200	0,177	Bolal Toxicity Root	3	200	0,747
Atay Deficiency Root	3	200	0,194	Bolal Deficiency Root	3	200	0,242
Atay Tarımbor Root	3	200	0,194	Bolal Tarımbor Root	3	200	0,322
Atay Def+Tar Root	3	200	0,173	Bolal Def+Tar Root	3	200	1,219
Atay Control Leaf	4	400	2,661	Bolal Control Leaf	4	200	1,531
Atay Toxicity Leaf	4	200	1,281	Bolal Toxicity Leaf	4	200	1,506
Atay Deficiency Leaf	4	400	2,597	Bolal Deficiency Leaf	4	200	1,329
Atay Tarımbor Leaf	4	400	1,055	Bolal Tarımbor Leaf	4	200	1,415
Atay Def+Tar Leaf	4	200	1,450	Bolal Def+Tar Leaf	4	200	1,581
Atay Control Root	4	200	0,794	Bolal Control Root	4	200	0,625
Atay Toxicity Root	4	200	0,184	Bolal Toxicity Root	4	200	0,331
Atay Deficiency Root	4	200	0,456	Bolal Deficiency Root	4	200	0,562
Atay Tarımbor Root	4	200	0,324	Bolal Tarımbor Root	4	200	0,647
Atay Def+Tar Root	4	200	0,535	Bolal Def+Tar Root	4	200	0,174

APPENDIX E

MICROARRAY HYBRIDIZATION CONTROLS

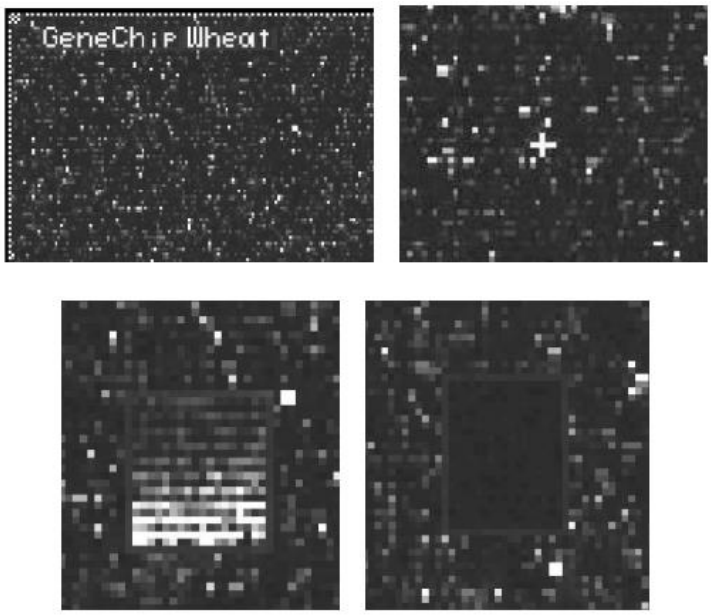
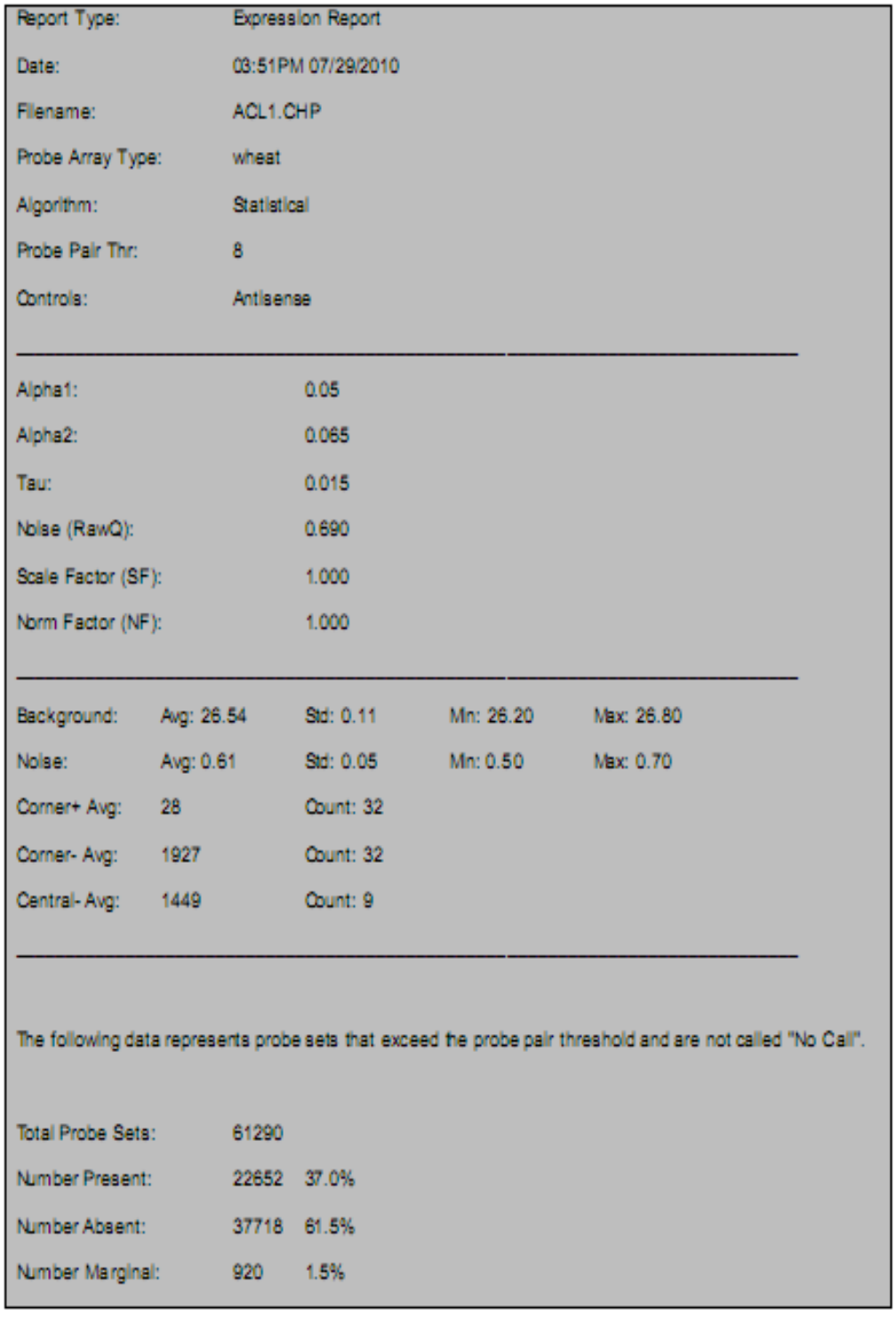


Figure E.1. The view of chip name and hybridization controls after scanning.



Report Type:	Expression Report			
Date:	03:51PM 07/29/2010			
Filename:	ACL1.CHP			
Probe Array Type:	wheat			
Algorithm:	Statistical			
Probe Pair Thr:	8			
Controls:	Antisense			
Alpha1:	0.05			
Alpha2:	0.065			
Tau:	0.015			
Noise (RawQ):	0.690			
Scale Factor (SF):	1.000			
Norm Factor (NF):	1.000			
Background:	Avg: 26.54	Std: 0.11	Min: 26.20	Max: 26.80
Noise:	Avg: 0.61	Std: 0.05	Min: 0.50	Max: 0.70
Corner+ Avg:	28	Count: 32		
Corner- Avg:	1927	Count: 32		
Central- Avg:	1449	Count: 9		

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	61290	
Number Present:	22652	37.0%
Number Absent:	37718	61.5%
Number Marginal:	920	1.5%

Figure E.2. Example of report from pre-analysis of microarray data by using GCOS.

Average Signal (P):	84.5
Average Signal (A):	1.6
Average Signal (M):	6.0
Average Signal (All):	32.3

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	18.5	P	26.6	P	23.4	P	22.84	1.26
AFFX-BIOC	70.0	P			94.7	P	82.36	1.35
AFFX-BIOD	208.1	P			423.2	P	315.68	2.03
AFFX-CRE	1709.3	P			1782.5	P	1745.92	1.04
AFFX-DAP	24.2	P	106.9	P	243.7	P	124.93	10.05
AFFX-LYS	16.6	P	17.9	P	74.4	P	36.29	4.47
AFFX-PHE	26.8	P	26.3	P	41.6	P	31.58	1.55
AFFX-THR	6.4	P	25.9	P	69.2	P	33.85	10.77
AFFX-TRP	2.7	A	0.1	A	0.8	A	1.22	0.32
AFFX-R2-EC-BIOB	29.3	P	39.8	P	34.4	P	34.51	1.18
AFFX-R2-EC-BIOC	79.9	P			123.4	P	101.66	1.54
AFFX-R2-EC-BIOD	509.3	P			749.7	P	629.48	1.47
AFFX-R2-P1-CRE	2129.5	P			2930.7	P	2530.12	1.38
AFFX-R2-BS-DAP	36.0	P	127.4	P	376.0	P	179.81	10.44
AFFX-R2-BS-LYS	28.9	P	21.9	P	40.9	P	30.56	1.42
AFFX-R2-BS-PHE	32.4	P	29.8	P	63.1	P	41.73	1.95
AFFX-R2-BS-THR	8.5	P	33.1	P	149.3	P	63.63	17.54

Figure E.2. (continued)

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Kayıhan C, Eyidoğan F, Afsar N, Oktem HA, Yücel M (2012) Cu/Zn superoxide dismutase activity and respective gene expression during cold acclimation and freezing stress in barley cultivars. Biologia Plantarum 56: 693-698.

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