ELUCIDATION OF BORON STRESS SIGNALING PATHWAYS IN YEAST

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in Molecular Biology and Genetics

by
İrem ULUIŞIK

December 2015
İZMİR

We approve the thesis of **İrem ULUIŞIK**

Examining Committee Members:

_____

Prof. Dr. Ahmet KOÇ
Department of Molecular Biology and Genetics, İzmir Institute of Technology

_____

Assoc. Prof. Dr. H. Çağlar KARAKAYA
Department of Molecular Biology and Genetics, İzmir Institute of Technology

_____

Assist. Prof. Dr. Bekir ÇÖL
Department of Biology, Muğla Sıtkı Koçman University

_____

Prof. Dr. Anne FRARY
Department of Molecular Biology and Genetics, İzmir Institute of Technology

_____

Assist. Prof. Dr. Yavuz OKTAY
İzmir Biomedicine and Genome Institute, Dokuz Eylül University

21 December 2015

_____

Prof. Dr. Ahmet KOÇ
Supervisor, Department of Molecular Biology and Genetics
İzmir Institute of Technology

_____ _____

Prof. Dr. Ahmet KOÇ
Head of the Department of Molecular Biology and Genetics

Prof. Dr. Bilge KARAÇALI
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor Prof. Dr. Ahmet KOÇ for opening my mind, for his valuable guidance, encouragement, support, and patience during my graduate research. I feel lucky to be working with such a special mentor.

I would also like to thank my committee members Assoc. Prof. Dr. H. Çağlar KARAKAYA, Assist. Prof. Dr. Bekir ÇÖL, Prof. Dr. Anne Frary, and Assist. Prof. Dr. Yavuz OKTAY for their advices, cooperation and significant contributions.

I am thankful to The Scientific and Technological Research Council of Turkey (TUBITAK) for financial support for this Project (110T917).

Also, I am grateful to faculty members and all of my friends in the Molecular Biology and Genetics Department for providing such a motivated atmosphere. I would like to thank all members of Biotechnology and Bioengineering Central Research Laboratories for their helps with some of the experiments. Additionally, the assistance of my dear friends Esra ŞAHİN and Melda GÜRAY is essential for the completion of my experimental work. It is always very supportive knowing that you are always there when I needed you. I also wish to express my thanks all my co-workers for their friendship. To start the day with you was very nice and motivating.

My deepest gratitude is for my family members Cengiz ULUIŞIK, Nurhayat ULUIŞIK, Gizem ULUIŞIK. Your all kinds of support were essential to my success. Thank you so much for always being with me in all conditions, for your endless trust and love. I feel privileged to have such a great family. Finally, I would like to dedicate my thesis to the memories of my grandfathers Mehmet AYVALI and Celil ULUIŞIK.

ABSTRACT

ELUCIDATION OF BORON STRESS SIGNALING PATHWAYS IN YEAST

Boron is an essential micronutrient not only for plants but also for many other organisms. The excess of boron causes toxicity and the mechanism of this toxicity is not known. The yeast *Saccharomyces cerevisiae* was used as a model system in this study. In order to reveal boron metabolism related genes, a genome-wide screen has been conducted. Among the identified mutants, six boron resistant and eight boron sensitive mutants were chosen for further investigation to understand how cells cope with boron stress. Boron resistant mutants were found to have increased levels of boron efflux pump *ATR1* and its transcription activator Gcn4. The sensitive mutants were lacking the genes that are involved in different cellular pathways. They were found to accumulate higher amounts of boron inside the cells upon boron treatment. To reveal how boron stress is conducted to Gcn4 transcription factor, the deletion mutants of transcription factors that are known to regulate *GCN4* were investigated in terms of their effects on Gcn4 and *ATR1* expression. Additionally, signaling cascades that converge on Gcn4 transcription factor such as TOR, PKA, and SNF1 pathways were analyzed for their roles in boron stress response mechanism. We found that the Gcn system is activated by the uncharged tRNA stress in response to boron treatment and that *GCN1,* which plays a role in transferring uncharged tRNAs to Gcn2, was necessary for the kinase activity of Gcn2. Additionally, boron treatment caused the phosphorylation of eIF2α in mammalian cells, in a similar manner to that of yeast cells, which suggested that boron toxicity and tolerance mechanisms were conserved between yeast and mammals

ÖZET

MAYADA BOR STRESİ SİNYAL YOLAKLARININ ARAŞTIRILMASI

Bor sadece bitkiler için değil pek çok organizma için esansiyel bir mikrobesindir. Bor fazlalığı toksisite oluşturmaktadır ve bu toksisitenin mekanizması bilinmemektedir. Maya *Saccharomyces cerevisiae* çalışmada model system olarak kullanılmıştır. Bor metabolizması ile ilgili genlerin bulunması amacıyla maya genomu taranmıştır. Bulunan mutantların arasından altı bor dirençli ve sekiz bor duyarlı mutant seçilmiş ve hücrelerin bor stresiyle nasıl başa çıktıklarını anlamak amacıyla daha ileri araştırmalar yapılmıştır. Bor dirençli mutantlarda bir bor pompası olan *ATR1* ve aktivatörü Gcn4 seviyelerinin arttığı bulunmuştur. Bor duyarlı mutantların ise farklı hücresel yolaklarda yer alan genleri eksiktir ve bor muamelesi ile hücre içerisinde yüksek miktarlarda bor biriktirdikleri bulunmuştur. Bor stresinin Gcn4 transkripsiyon faktörüne nasıl iletildiğini anlamak için, *GCN4'*ü regüle ettiği bilinen transkripsiyon faktörlerinin delesyon mutantları Gcn4 ve *ATR1* ifadelenmelerine etkileri yönünden incelenmiştir. Ek olarak, Gcn4 transkripsiyon faktöründe birleşen TOR, PKA ve SNF1 gibi sinyal yolakları bor stress yanıt mekanizmasındaki rolleri yönünden analiz edilmiştir. Bor muamelesine yanıt olarak Gcn sisteminin, yüksüz tRNA stresi tarafından aktive edildiği bulunmuştur ve yüksüz tRNA'ların Gcn2'ye transferinde rol oynayan *GCN1*, Gcn2'nin kinaz aktivitesi için gerekmektedir. Aynı zamanda bor muamelesi, memeli hücrelerinde maya hücrelerine benzer şekilde eIF2α'nın fosfatlanmasına neden olmaktadır. Bu da bor toksisite ve tolerans mekanizmalarının maya ve memelilerde korunmuş olduğunu göstermektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table **Page**

CHAPTER 1

INTRODUCTION

1.1. Biochemical Characteristics of Boron

Boron is a metalloid located on group 13 in the periodic table with atomic number 5, atomic mass 10.811 g/mol and chemical symbol B. Although boron compounds have been known for centuries, it was discovered as an element by Sir Humphry Davy and by Gay-Lussac and Thenard in 1808 (Bolanos et al. 2004).

Boron has different isotopes among which ^{10}B and ^{11}B are the most stable ones. Boron is not present in an elemental form in nature, rather it forms organoboron complexes with sodium and oxygen, which are the physiologically important forms of boron in organisms (Hunt 2003). Although boron has low abundance when compared with hydrogen, carbon, nitrogen, or oxygen, it is common in the environment and naturally found in rocks, soil, and water (Woods 1994).

Organoboron complexes, which contain B-O or B-N bonds like orthoborates, are important in biological systems. These organoboron complexes are formed in plant, animal, and human tissues. In an organism, during normal physiological conditions nearly 96% of the boron is present as an uncharged boric acid $B(OH)_3$ and as a small amount of borate anion $B(OH)_4^-$ (Bolanos et al. 2004, Devirian and Volpe 2003, Hunt 2003). Boric acid is a weak Lewis acid with a p*K*a of 9.25 and it accepts a hydroxyl ion, rather than being a proton donor. This pKa value decreases when boron forms covalent bonds with its ligands, and it becomes a negatively charged tetrahedral borate anion $B(OH)_4^-$ (Hunt 2003).

Both boric acid and borate make reversible interactions with biomolecules which contain adjacent cis-hydroxyl groups such as riboflavin, adenosine monophosphate, pyridoxine, pyrimidine nucleotides, ascorbic acid, and five carbon sugar molecules such as ribose, apiose, and polysaccharides. Boron also binds NAD^+ in animals and affects its functions (Barranco et al. 2009, Hunt 1998b, Kim et al. 2003). When hydroxyl groups are next to each other and on the same side of the molecules, there is an interaction

between boron and its ligands via ester bonds (Hunt 2003). Such an affinity may be responsible for some biological effects of boric acid (Bolanos et al. 2004). Boric acid and the sodium salts of boron (borax, disodium tetraborate) are commonly used as antiseptics, bactericides, cleaning agents such as soaps and detergents, preservatives, fire retardants, fertilizers, insecticides, herbicides, and in cosmetic products. Boron is also used in many industrial applications including glass production, fiberglass insulation, porcelain enamel, ceramic glazes, and metal alloys (Moore et al. 1997, Woods 1994).

Major world boron deposits are located in Turkey and California, and are also found in Argentina, Chile, Russia, China, Peru, Egypt, Iraq, Libya, Morocco and Syria (Tanaka and Fujiwara 2008).

1.2. Essentiality of Boron

Boron is found mostly as boric acid in biological systems and plays vital roles in many mechanisms and is essential especially for plants (Tanaka and Fujiwara 2008). Additionally, people consume many products containing boron in their daily life; one can consume 1-7 mg boron daily. It exists abundantly in vegetables, fruits, nuts, and their products (Baker, Tomsho, and Benkovic 2011, Pahl et al. 2001).

The first natural compound found to contain boron is the antibiotic Boromycin (produced by *Streptomyces antibioticus*) acting on gram-positive bacteria (Hutter et al., 1967). In addition, boron is present in the bacterial antibiotics tartrolons (produced by *Sorangium cellulosum*) (Irschik et al. 1995, Schummer et al. 1996), borophisin, aplasmomycin (produced by *Streptomyces griseus*) (Hunt 2003, Rezanka and Sigler 2008); the bacterial quorum sensing molecule autoinducer AI-2 (Chen et al. 2002); a boron containing siderophore vibrioferrin which is produced by a marine bacterium (Amin et al. 2007); and in borolithochromes which is responsible for the pink color of the algae *Solenopora jurassica* (Wolkenstein, Gross, and Falk 2010).

Apart from these natural compounds, boron has important roles in physiological and metabolic activities of animals and microbial systems (Tanaka and Fujiwara 2008). It is required for bacterial nitrogen fixation in *Azotobacter* (Anderson and Jordan 1961). *Bacillus boroniphilus* requires boron for its growth and it can tolerate more than 450 mM boron (Ahmed, Yokota, and Fujiwara 2007). Heterocystous cyanobacteria and

actinomycetes of the genus *Frankia* also require boron for both growth and nitrogen fixation. The blue - green alga Cyanobacteria predominated in the Middle Pre-Cambrian Period. Some species of the phylum Cyanobacteria contain heterocysts which are specific cells for nitrogen fixation. Boron was suggested to interact and stabilize the glycolipids of the heterocysts. The requirement of Cyanobacteria for boron indicates that it has been an essential element since the early evolution of life (Bolanos et al. 2004, Bonilla, Garciagonzalez, and Mateo 1990, Hunt 2003).

Boron is also required for several marine species, brown algae, diatoms and algal flagellates (Hunt 2003). In addition it is necessary during embryonic development of zebra fish (*Danio rerio*) (Rowe and Eckhert 1999), rainbow trout (*Oncorhynchus mykiss*) (Eckhert 1998) and frogs (*Xenopus laevis*) (Fort et al. 1998). The levels of boron required are different among these organisms. Boron deprivation affects the reproduction and development in organisms including frogs, zebrafish, rat, and mice (Eckhert 1998, Fort et al. 1998, Lanoue et al. 1998). Additionally, the yeast *Saccharomyces cerevisiae,* which is a unicellular eukaryote, needs boron for its growth. Bennett (1999) found that yeast cells could not divide and arrested in the cell cycle under boron deficiency. When boron was added back to the media, yeast cells proliferated normally, however when the boron levels increased in the environment they could not grow well (Bennett et al. 1999). It has been also revealed that yeast can survive up to 80 mM boric acid and has been considered as tolerant to boron. Yeast is a suitable model for investigating the molecular biology of boron effects (Kaya et al. 2009, Nozawa et al. 2006).

In animals boron affects various mechanisms which include carbohydrate, mineral metabolism, energy consumption, regulation of several enzyme activities and embryonic development. However molecular mechanisms of boron function especially in animals are not well understood (Tanaka and Fujiwara 2008). An essentiality of boron in humans has not been reported so far but it has beneficial effects. It has roles in steroid hormone metabolism, healthy bone development, and cell membrane maintenance (Cui et al. 2004, Tanaka and Fujiwara 2008).

According to Warington's report in 1923, growth of *Vicia faba* (field bean) was reduced in a medium that lacked boron but when the medium was supplied with only boron, the growth was rescued (Warington 1923). Since that time boron has been accepted as a necessary and beneficial microelement for various organisms. In plants, boron has important roles in nucleic acid metabolism, carbohydrate and protein

metabolism, cell wall synthesis, cell wall structure, membrane integrity and function, and phenol metabolism. However, the molecular mechanisms of these roles are mostly unknown (Goldbach and Wimmer 2007, Miwa et al. 2007).

A primary function of boron at the molecular level is that it cross-links pectins in plant cell walls. Pectins are complex polysaccharides and are important components of plant cell wall structure. Boron forms a cross-link with apiose residues of pectin rhamnogalacturonanII (RG-II), which is essential for cell wall stability and normal leaf extension (Kobayashi, Matoh, and Azuma 1996, O'Neill et al. 2004). RG-II is a pectic polysaccharide which covalently binds to borate esters with cis-diol bounds (York et al. 1985). Borate crosslinking in this pectic system strengthens cell wall physically and plays important roles in cell adhesion (Ishii and Matsunaga 2003, Scorei 2012). In plants, boron has important roles in expanding organs, for this reason, the symptoms of boron deficiency can be mainly observed in expanding organs. Boron deficiency results in the formation of abnormal cell wall, altered cytoskeletal polymerization, and changes in plasma membrane permeability. In addition leaf expansion, root elongation, flower and fruit development are inhibited (Dell and Huang 1997). Boron deprivation interferes with many physiological processes and can drive cells even to death. It can affect sugar metabolism, carbohydrate metabolism, RNA metabolism, phenol metabolism, and cell wall structure (Parr and Loughman 1983, Kot 2009). In 2004, it was reported that the expression of glutathione S-transferase and glucosyltransferase was induced in tobacco (*Nicotiana tabacum*) BY-2 cells under boron limitation (Kobayashi, Mutoh, and Matoh 2004). In 2009, another study with the same cells showed that oxidative damage is directly involved in cell death under boron deprivation and it is not likely a typical programmed cell death (Koshiba, Kobayashi, and Matoh 2009).

1.3. Toxicity of Boron

Metalloids lead to toxicity by affecting different cellular mechanisms in biological systems. The causes of metalloid toxicity can include the increase in oxidative system, DNA damage, the impairment of DNA repair systems and membrane functions, and the inhibition of protein folding, protein function and activities. Cells develop various mechanisms to cope with the toxicity caused by metalloids. Some

examples of such mechanisms include export of the metalloid out of the cell, storage of the metalloid in intracellular organelles, decreased import of the metalloid by metalloid binding proteins and peptides (Lemire, Harrison, and Turner 2013, Verbruggen, Hermans, and Schat 2009).

Boron is an important nutrient for plants and animals. It is necessary especially for growth and development of many organisms. When present at high concentrations, boron can also have toxic effects. In contrast to deficiency, boron toxicity symptoms are observed especially in margins of mature leaves in plants. It causes reduced root cell division, lower photosynthetic rates, chlorosis or necrosis of old leaves. However the physiological basis of boron toxicity is not clear (Camacho-Cristobal, Rexach, and Gonzalez-Fontes 2008).

Studies carried out to understand the molecular mechanisms of boron toxicity have revealed that there is a relation between the expression of *ATR1* gene which is the major boron efflux pump and is involved in boron detoxification in yeast and the expression of amino acid biosynthesis genes (Kaya, et al. 2009). This mechanism is strictly regulated by the transcription factor Gcn4 in response to boron. In regulating protein synthesis eIF2 plays a central role. It provides the delivery of charged methionyl initiator tRNA to the initiation codon and forms a ternary complex with GTP. It is a multimeric protein and is phoshorylated on serine-51 of its α subunit in response to viral infection, amino acid limitation, apoptosis, nutrient deprivation, heme deprivation, and other stresses (Kimball 1999). Boron was also shown to induce the phosphorylation of eIF2α in a Gcn2 protein kinase dependent manner, interferes with the initiation of translation, and as a result protein synthesis is inhibited (Uluisik, Kaya, Fomenko, et al. 2011).

In addition, to find the genes that play roles in boron metabolism, a haploid yeast deletion collection was screened for the genes that confer boron resistance or sensitivity to yeast cells. A total of 21 boron sensitive and 6 boron resistant mutants were found. Additionally proteomic analyses showed that there are proteins whose expression increases in the presence of toxic levels of boric acid. All these genes and proteins indicate different pathways within the cell, which need further investigation (Uluisik, Kaya, Unlu, et al. 2011).

Boron may also be toxic for humans. Humans consume boron daily, mostly from fruits and vegetables. When consumed at high doses it can cause developmental and reproductive abnormalities in animals (Pahl et al. 2001). A study on rats revealed that

the lowest observed adverse effect level (LOAEL) of boron for developmental toxicity was 13.3 mg/kg body weight/day and the no observable adverse effect level (NOAEL) of boron was 9.6 mg/kg body weight/day (Pahl et al. 2001, Price et al. 1996). In humans and animals, boron deprivation results in impairment of growth, abnormal bone development, decrease in blood steroid hormone levels, increase in urinary calcium excretion, and changes in macromineral status (Murray 1995). It was also found that mice and frogs have developmental disorders arising from boron deficiency (Heindel et al. 1992). Excessive boron intake causes neurological effects, kidney damage, diarrhea, anorexia, weight loss, and testicular atrophy in mice, rats, and dogs. It also causes decrease in fetal body weight and increase in skeletal malformation and cardiovascular defects in pregnant female animals (Yazbeck et al. 2005). High concentrations of boron are generally used to control bacterial and fungal infections. The toxic activity of boric acid is suggested to be especially on mitochondria and mitochondrial enzymes in *Saprolegnia* which is a fungus and causes saprolegniasis in fish and fish eggs (Ali et al. 2014). Despite its importance, little is known about the mechanisms of boron actions in humans.

1.4. Uptake and Transport of Boron

Both boron deficiency and toxicity may have negative outcomes for the metabolism of plants and animals. (Yazbeck et al. 2005, Camacho-Cristobal, Rexach, and Gonzalez-Fontes 2008). Therefore, to maintain the boron homeostasis, boron levels that are transported inside or outside of the cell should be strictly regulated by control mechanisms.

The effects of boron are observed primarily in plants since they are exposed to boron directly from the soil. Thus, many plant genes have been identified related to boron transport and tolerance. Plants absorb boron from soil in the form of boric acid. It is taken up by roots and is loaded to xylem for transport to shoots. During this long distance transport, boron follows the transpiration stream and accumulates at margins of mature leaves since the transpiration rate is high in these regions (Brown and Shelp 1997). For the uptake of boron from the soil there is evidence for both passive and active transport in plants. Boron is a small uncharged molecule and its volume (71.5 A) is nearly the same as urea (75.3 A) (Hu and Brown 1997, Nable, Banuelos, and Paull

1997). The studies conducted with squash root (*Cucurbita pepo*) and charopyte alga (*Chara corallina*) (Stangoulis et al. 2001) cells have revealed that the permeability coefficient of boric acid for lipid bilayer is 8×10^{-6} cm/s (Dordas and Brown 2000, Raven 1980). Thus, under high boron supply, passive diffusion represents a significant way for boron to pass the root epidermis.

When the available level of boron is low in the environment, there is a need for membrane proteins, which indicate an active transport mechanism for boron. Many plant genes, which are involved in boron transport and tolerance, have been found so far (Kato et al. 2009, Takano, Miwa, and Fujiwara 2008). It was found that boric acid is transported against its concentration gradient in sunflower (*Helianthus annuus*) in low boron conditions (Dannel, Pfeffer, and Romheld 1998). Similar to sunflower, *Arabidopsis thaliana* accumulates boron in the xylem against a concentration gradient under boron limitation. *BOR1*, a boron efflux transporter is responsible for this xylem loading (Takano, Noguchi, Yasumori, et al. 2002). *A. thaliana bor1-1* mutant cannot translocate boron from roots to shoots thereby shoot growth can be inhibited under low boron conditions (Miwa, Takano, and Fujiwara 2006). *BOR1* is degraded in the vacuole following endocytosis when plants are exposed to high levels of boron (Takano et al. 2005). *BOR1* belongs to animal bicarbonate transporter superfamily (SLC4) and its homologs have been found in many organisms such as rice OsBOR1, yeast *YNL275w*, and mammals NaBC1 (Nakagawa et al. 2007, Park et al. 2004, Takano, Noguchi, Hayashi, et al. 2002).

Yeast has been used as a model organism for the characterization of many plant boron tolerant genes so far. Yeast BOR1 is localized to the plasma membrane and has a role in boric acid efflux (Takano et al. 2007). In addition to *BOR1*, *DUR3* and *FPS1* were found to be involved in boron tolerance in yeast. However the functions of these transporters in boron detoxification are not clear (Nozawa et al. 2006). *ATR1* was found as a boron tolerance gene in yeast (Kaya, et al. 2009). It is composed of 14 membrane spanning domains and is a member of the DHA2 family of drug-H+ antiporters (Gbelska, Krijger, and Breunig 2006). It reduces the intracellular boron levels and provides extreme resistance to boron (Kaya et al. 2009). Bioinformatics analyses showed that *YMR279C* and *YOR378W* were two paralogs of ATR1 gene. The expression of YMR279C decreased the intracellular boron levels and provided remarkable boron resistance to the yeast cells (Bozdag et al. 2011). NaBC1 is a voltage regulated bicarbonate sodium coupled borate cotransporter and it was identified as one

of the BOR1 homologs in mammalian system. This gene improves cell proliferation when boron concentrations are low (Henderson et al. 2009, Park et al. 2004). *AtBOR1* has six homologs in the *A. thaliana* genome and only *AtBOR4* shows tolerance to boron when overexpressed in *A. thaliana* under high boron conditions through boron efflux (Miwa et al. 2007). Besides *A. thaliana, VvBOR1* and *CmBOR1* have been characterized as boron transporters in *Vitis vinifera* and *Citrus macrophylla* (Canon et al. 2013, Perez-Castro et al. 2012). In crop plants, *BOT1,* which is a *BOR1* ortholog provides boron tolerance to barley (Sutton et al. 2007).

The membrane transport of boric acid is also facilitated by the special channel protein, NIP5:1. NIP5:1 belongs to the plant-specific nodulin 26-like intrinsic proteins (NIP) subfamily and has been identified as a boric acid channel that is upregulated in *A. thaliana* roots under boron deficiency (Takano et al. 2006). It is also a member of major intrinsic protein family (MIP) which are integral membrane proteins and serve as channels for water and small uncharged molecules in mammals, amphibians, yeast, bacteria and plants (Wallace, Choi, and Roberts 2006). However it is unclear whether other MIPs are involved in boric acid transport in animals. Vacuolar compartmentation of boron has been also reported in *A. thaliana* and *Citrus macrophylla* by the activity of *TIP5,* an aquaporin family member localized in cell tonoplast membrane (Martinez-Cuenca et al. 2015, Pang et al. 2010).

Compared to what is known in plants, the information about physiology and molecular biology of boron uptake in humans and animals is limited. Boron is ingested from the diet or inhaled in the form of boric acid and is not metabolized in humans and animals (Moseman 1994). It does not tend to accumulate in tissues. However bone, nails, and hair have been found to have higher boron levels whereas fat tissue has low boron levels (Ku et al. 1991). Boron is rapidly absorbed from the human intestine and then is excreted completely in the urine (Gallardo-Williams et al. 2003, Hunt 1996, Pahl et al. 2001). The half-life of boron is about 21 h (Pahl et al. 2001). There are not many cases of boron toxicity for humans. Although it has been reported that people living near high boron containing water supplies or borate ores have high boron intake daily (27 mg/day), there are no observable effects (Barr et al. 1993, Pahl et al. 2001, Sayli, Tuccar, and Elhan 1998). One boric acid poisoning case was recorded in humans in 1954 and symptoms included capillary fragility and anemia (Hunt 1998a).

1.5. Boron in Medicine

Boron and its compounds have been known for a while as beneficial for the metabolism of humans and animals. Especially, dietary boron plays regulatory roles in physiological processes such as immune response of cells. High amounts of boron are reported to interfere with the development of some organs of chicken and rat immune system (Hu et al. 2014). It is shown that boron is involved in inflammatory response by suppressing the activities of specific enzymes in some inflammatory diseases (joint swelling). 6-phosphogluconate, which is a pentose phosphate pathway constituent, forms a complex with boron and subsequently inhibits 6-phosphogluconate dehydrogenase enzyme in the same pathway. In the conditions of boron deficiency, more phenolic compounds were shown to be synthesized due to the action of pentose phosphate pathway which then resulted in plant death (Lovatt and Bates 1984).

Natural (i.e. sodium borate) and synthetic (i.e. peptide boronic acid) boron compounds have been shown to inhibit serine proteases such as chymotrypsin, cathepsin G, elastase in a reversible manner in vitro (Hunt 1998b, Bauer and Petterss.G 1974, Kettner and Shenvi 1984).

Boron was shown to regulate extracellular matrix by directly affecting the activities of some enzymes such as elastase, trypsin-like enzymes, and collagenase and induces the release of TNF-α in fibroblasts (Benderdour et al. 2000). Gene expression levels of some of the bone tissue related extracellular matrix proteins such as collagen type I (COLI), osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP) were increased by boron treatment (Dzondo-Gadet et al. 2002, Hakki, Bozkurt, and Hakki 2010). By the action of boron on extracellular matrix, it has been also suggested that boron may also improve wound healing (Nzietchueng et al. 2002). In one study, it was reported that an antimicrobial hydrogel which contains boron was found to be successful in the healing of burn wounds in vitro (Demirci et al., 2015).

Boron is beneficial for bone formation and composition especially in regulating the hormones that support bone growth (Hakki, Bozkurt, and Hakki 2010, Nielsen 2000). A study conducted with postmenopausal women showed that more boron intake caused more plasma estrogen and testosterone and less calcium excretion. In the postmenopausal period, bone loss was observed in women due to reduced estrogen levels. Gallardo-Williams (2003) suggested that an increase of boron in diet resulted in an enhancement of plasma 17β-estradiol and testosterone levels and an impediment of

calcium excretion in urine (Gallardo-Williams et al. 2003). There are also supportive results from animal studies. For example, dietary supplementation of boron in pigs, chicken, and rats has been shown to promote bone strength (Armstrong et al. 2002, Chapin et al. 1998, Gallardo-Williams et al. 2003, Hunt and Nielsen 1987).

There are several experimental and epidemiological studies showing the effect of boric acid on human prostate cancer cells (Barranco and Eckhert 2006, Barranco et al. 2009, Cui et al. 2004). When boric acid was supplemented in the diet of mice having impaired immune system, the human prostate cancer tumors that were transplanted to mice showed decreased growth (Gallardo-Williams et al. 2004). It also inhibits the growth and proliferation of prostate cancer cell lines DU-145 and LNCaP in a dose dependent manner and without causing any shift in the stages of cell cycle (Barranco and Eckhert 2004, Rowe and Eckhert 1999). There is also evidence that the intake of boron with the diet reduces the risks of lung cancer and breast cancer in women (Mahabir et al. 2008, Scorei 2012). Boric acid is effectively used in vaginal yeast infections especially caused by the dimorphic fungus *Candida albicans* (Iavazzo et al. 2011).

Boron nitride nanotubes can be used in biomedical applications in addition to carbon nanotubes although their structures are physically and chemically different from each other. The biocompatibility of boron nitride nanotubes has also been confirmed by different studies in vitro (Ciofani et al. 2013, Ciofani et al. 2008, Ishigami, Aloni, and Zettl 2003, Zhi et al. 2010). However the usage of boron nitride nanotubes in nanobiotechnology needs to be further investigated and improved. Boron neutron capture therapy (BNCT) is primarily used to treat brain cancer, head and neck cancer, lung and liver tumors (Kouri et al. 2004, Suzuki et al. 2007, Wittig et al. 2008, Zhu et al. 2010). However the employment of in vivo and clinical studies is necessary to develop this system.

1.6. Yeast As a Model

Yeast *Saccharomyces cerevisiae* has been widely used to study signaling pathways and to understand the mechanisms by which cells respond to various stresses including copper, iron, and zinc metal stresses. Other properties like being genomically characterized, existing in both haploid and diploid forms, and the ease of manipulation

also make yeast a useful model. Many selectable markers are also available for yeast. Importantly many of the signaling pathways are conserved between yeast and higher eukaryotes. Due to the availability of strain libraries and having a well-characterized genome and proteome, yeast has been used extensively as a research tool. Many researchers have used yeast as a model to characterize plant boron tolerance genes (Nozawa et al. 2006, Sutton et al. 2007, Takano et al. 2007, Takano et al. 2006). Genome-wide screening studies in yeast also revealed some genes related to boron metabolism (Kaya et al. 2009, Uluisik, Kaya, Unlu, et al. 2011).

1.7. Aim of the Study

Turkey is one of the natural reservoirs of boron and contains 73% of world boron deposits (National Boron Research Institute). The essentiality of boron for plants and animals has been known for many years. However there are several negative outcomes of both boron deficiency and toxicity to these organisms. To cope with the outcomes in question, molecular signaling mechanisms underlying boron biology need to be known. The main objective of this study is to discover the boron stress response and toxicity mechanisms in yeast.

With the studies carried on so far, it has been suggested that Atr1 is a boron exporter and plays essential role in boron stress tolerance in yeast. Gcn4 is the major transcription factor that regulates the expression of *ATR1*. General amino acid control system is activated and blocks protein synthesis in boron stress. However it is still unclear whether there are other transcription factors that play roles in boron stress response, how boron induces Gcn4, and what the sensing and signaling mechanisms of boron stress are. Since translation initiation mechanisms are conserved among eukaryotes, the knowledge obtained from these detailed studies will be of great importance. Within the scope of this study we aim to investigate the biochemical and molecular reasons of boron stress and the cellular response mechanisms to boron in a detailed manner using the power of the yeast model system.

CHAPTER 2

MATERIALS AND METHODS

2.1. Yeast Growth and Media

WT strain BY4741 (*MATa* his3Δ leu2Δ met15Δ ura3Δ) and its isogenic deletion mutants were obtained from the yeast deletion library (Invitrogen). Cells were grown in either YPD (2% glucose, 1% yeast extract, 2% peptone and 2% agar for solid media) or YNB (yeast nitrogen base) (2% glucose, 0.67% yeast nitrogen base without amino acids and 2% agar) media supplemented with required amino acids and bases.

2.2. Yeast Transformation with LiAc Method

Yeast transformations were performed using the LiAc method (Gietz and Schiestl 1995). 5 ml of liquid YPD was inoculated and incubated overnight with shaking at 30˚C. Logarithmically grown yeast cells were harvested by centrifugation and washed with sterile distilled water. Cell pellets were resuspended in 0,1M LiAc and transferred to 1.5 ml microcentrifuge tubes. After top speed centrifugation pellet was resuspended in 240 µl PEG (50 % w/v), 36 µl (1M) LiAc, 5 µl salmon sperm single stranded carrier DNA (10 mg/ml), 20 µl plasmid DNA, 10 µl dH_2O respectively, vortexed for 1 minute and incubated for 30 minutes at 30˚C. Then heat shocked at 42°C for 20-25 minutes. Transformed cells were spread on selective YNB media. Cells were allowed to grow for 3-4 days obtained colonies were used for further experiments.

2.3. Determination of Boron Tolerances of Mutants

Boric acid (AppliChem) was used as a boron source in the assays. YPD or YNB agar plates with different concentrations of boric acid were prepared. Overnight cultures of mutants and wild type yeast were diluted to an optical density of 0.2 at 600 nm and

serially diluted to 0.02, 0.002, and 0.002. A 5 µl aliquot of each dilution was spotted on these plates and incubated for 3 days at 30°C, and then plates were photographed.

2.4. Cloning of the Genes That Cause Boron Sensitivity

Gateway Cloning System (Invitrogen) was used for the cloning experiments. Wild type alleles of related genes were amplified from yeast genomic DNA using High Fidelity Enzyme (Fermentas). The primers used for cloning are indicated in Table 1. The amplified fragments were first cloned into the pDONR vector using BP ClonaseTM II enzyme (Invitrogen) and then moved to LR clonase reaction. This reaction was performed with isolated entry clones, pAG426GPD expression vector, and LR ClonaseTM II enzyme mixture (Invitrogen). Isolated plasmids were verified by sequence analyses, and then yeast cells were transformed with the empty vector or with a gene expression construct, and selected for uracil prototrophy.

Table 1. The list of primers used in cloning experiments.

Genes	**Primer Sequences**	**The length of amplified region (base pair)**
HOM6	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTAGATAGTATCATCAATCGAAT3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTTGTCTGTTTACTGATCTA3'	1071 bp
ERG4	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTAGTGTACATAGATTAGCATCGC3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTACATAAGGAATAAAGACGTAAGGGC3'	1422 bp
RPE1	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTAAGAGCGAGGTAAACACACAAGAAA3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTGCCGCATATGTACAACTAATC3'	717 bp
OCH1	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTGAAAGCAAGTAAAAGAAAGAAGAG3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTAGCCTCGTTATTTATGACCTGC3'	1443 bp

(cont. on next page)

Table 1. (cont.)

SLA1	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTGTATGACTGTGTTTCTGGGCA3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTAATCTAGAATCCAAACGGATTTGA3'	3735 bp
YGR283C	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGTAGCATAATAGGGGAAGA3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTATTTATTTCAACTTGCCCAACGCTG3'	1026 bp
CCS1	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTGCACAAAAATGACCACGAACGATAC3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTGGATGTTCTATTTGATGTTGTTGGC3'	750 bp
YMR310C	Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTTAGTTTGATGAGCAGCACG3' Reverse: 5'GGGGACCACTTTGTACAAGAAAGCTGGGTTCGTACTTATCCATTGCATGAAGG3'	954 bp

2.5. Overexpression of *SNF1* Gene

Gateway Cloning System (Invitrogen) was used for the cloning experiments. Wild type allele of SNF1 gene was amplified by High Fidelity Enzyme (Fermentas) from yeast genomic DNA using the primers SNF1F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTCCCTTAATAAAGTCAACATGAG-3') and SNF1R (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTTTTACGTTCCACCATCAA-3'). The amplified fragment was first cloned into the pDONR vector using BP ClonaseTM II enzyme (Invitrogen) and then moved to LR clonase reaction. This reaction was performed with isolated entry clones, pAG426GPD expression vector, and LR ClonaseTM II enzyme mixture (Invitrogen). Isolated plasmids were verified by sequence analyses, and then yeast cells were transformed with the empty vector or with a gene expression construct, and selected for uracil prototrophy.

2.6. Determination of Intracellular Boron Concentrations

Exponentially growing boron sensitive mutant cells were treated with 50 mM boric acid or not for 1 hour and harvested by centrifugation. The cells were washed with distilled water and disrupted by using lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl pH: 7.6, 1mM EDTA), glass beads, and vortex. The supernatants were used for further analyses. The boron concentrations of diluted cell extracts were determined using the Boron Cell Test Kit (Merck). The method determines photometrically the color change in the environment and has high measurement sensitivity (0.05 mg boron/liter). The obtained absorbance values were normalized according to the values of wild type cells. The experiments were repeated at least three times.

2.7. RNA Isolation and Real Time PCR Analyses

Total RNA was isolated from boron-treated and untreated samples using RNA Isolation Kit (Invitrogen). Genomic DNA traces were removed by DNAse treatment (Fermentas). Complementary DNA (cDNA) from total mRNA was obtained using the First Strand cDNA Synthesis Kit (Fermentas). The cDNA was used as a template for amplifying a 186 bp fragment for the *ATR1* gene, and a 103 bp fragment for the internal control *ACT1* gene. Triplicate real-time PCR analyses were performed with the IQ5 Real-Time PCR System (Bio-Rad) using Fermentas MaximaTM SYBR Green qPCR Master Mix (2X). The primers used for the amplification of *ATR1* were ATR1F (5'-ACGCGTATAGCATAGCCGCTTTCA-3') and ATR1B (5'-TGTAAGCCTGGTTCCAACCCGATA-3'), the primers used for the amplification of *ACT1* were ACT1F (5'-ACGTTCCAGCCTTCTACGTTTCCA-3') and ACT1B (5'-ACGTGAGTAACACCATCACCGGAA-3'). The conditions of PCR amplification for *ATR1* were as follows: 40 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s.

2.8. β-galactosidase Activity Assays

Wild type and related yeast mutant cells were transformed with the p180 plasmid which carries the GCN4-lacZ gene (Hinnebusch 1985). Overnight cultures of

wild type cells in liquid YNB-URA were diluted and after two hours of incubation, cultures were split into two flasks with or without 50 mM boric acid. After 3 hours growth in the presence of boron, 500 µl of cell solution, at an optical density between 0.2 and 0.6 at 600 nm, was taken and homogenized in 400 µl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol and 0.2% N-lauryl sarcosine). After 30 min preincubation at 30°C, 150 µl of Z-buffer with 4 mg/ml, ONPG (o-nitrophenyl-b-D galactopyranoside) (Sigma) was added to the suspensions and the resulting solutions were incubated at 30°C for half an hour. The reaction was stopped by adding 400 µl 1.5 M Na_2CO_3. After centrifugation for 1 min, supernatants were taken and their absorbances were assayed at 420 nm. The obtained absorbance values were normalized according to the OD values of cells.

2.9. Western Blot Analyses

Wild type, and the isogenic *gcn1Δ* and *gcn20Δ* strains were grown to logarithmic phase in YPD media, with or without boron treatment and equal numbers of cells were harvested by centrifugation for each strain. Cell extracts were resuspended in sample buffer (0.06 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue) and heated at 95°C for 5 minutes. Samples were separated in 5–15% SDS-PAGE and transferred to a PVDF (Sigma) membrane. HeLa cells (5000 cells/well) were used for the mammalian cell culture experiments. After cells were grown in DMEM media containing 0, 50, 100, 250, 500, and 1000 µM boric acid for 5 days, they were lysed with lysis buffer (10 mM Tris pH:7,5, 1 mM EDTA, %0,1 Triton X-100, 1X protease inhibitor, 1 mM DTT, 1 mM Na_3VO_4, 50 mM NaF). Bradford assay was performed using obtained supernatants and protein amounts were adjusted for each sample. 26 µg protein/sample was used for Western blot analyses. Phospho (Ser51) specific eIF2α (US Biological) was used as primary antibodies. As secondary antibody, antirabbit IgG conjugated to horseradish peroxidase (Sigma) was used. The membranes were first treated with SuperSignal West Pico chemiluminescent substrate solutions (Pierce), then they were stripped and reprobed with antibodies specific for yeast phosphoglycerate kinase (Invitrogen) and mammalian ɣ-tubulin (Sigma) as an internal control. The blots were visualized using the VersaDoc Imaging System (Bio-Rad).

2.10. Northern Blot Analyses

Total RNA was isolated from yeast by using hot phenol method. 10 μg of total RNA was run on 12.5% polyacrylamide gel containing 8M urea (10X TBE (0.9 M Tris base, 0.9 M Boric acid, 20 mM EDTA pH 7), 40% Acrylamide). To transfer RNAs separated on denaturing gel to positively charged Nylon membrane (Amersham), Thermo Pierce G2 Fast Blotter was used (25 V, 0,5 A, 25 min.). The transferred RNAs were cross-linked by UV crosslinker (UVP HL-2000 Hybrilinker) (40.000 μjoule/cm^2). The membrane was then incubated at 37°C in a hybridization oven (UVP HL-2000 Hybrilinker) overnight with the hybridization buffer which contains biotin labeled RNA probes. Following the washing and blocking steps (the membrane blocking was done with 2% BSA), the membrane was incubated with 1 μl of HRP-conjugated Streptavidin (0,5 μg/ml) (Pierce). The signals of RNA probes were obtained and visualized by using SuperSignal West Femto Chemiluminescent Substrate solutions (Pierce) and VersaDoc Imaging System (Bio-Rad). The biotin-labelled methionine, histidine and threonine tRNA probes were commercially synthesized and were used in the experiments. The sequences of the tRNA probes were as follows:

5'-CTCCTAGAATCGAACCAGGG-3' (3'-$tRNA^{His}_{GTC}$) –tRNA/Histidine

5'- GCGCCGCTCGGTTTCGATCC-3' (3'-$tRNA^{Met}_{CAT}$) –tRNA/Methionine

2.11. Mass Spectrometry Analyses

200 μM of stock solution of NADH (Sigma) was prepared and was analysed with WAT (water-acetonitrile-triethylamine) as solvent. Additionally boric acid was added to the same mixture and the sample was analysed by Applied Biosystems/MDS SCIEX – 4000 QTRAP LC-MS/MS immediately or after 1 h of incubation. Final concentrations of boric acid and NADH in the solvent were 500 μM and 100 μM, respectively. The procedure was the same for ATP (Sigma), as well. In addition, another sample which contains a commercial tRNA mixture of yeast (Sigma) was analysed by negative ion ESI mass spectrometer. The solvent used in this analysis was obtained by mixing isopropanol and DEPC-dH_2O (1:1) and the final concentration of tRNA mixture in solvent was 15 pmol/μl. To get a mass spectrum of tRNA mixture ammonium acetate precipitation method was also used (Limbach, Crain, and McCloskey 1995). According

to this method 40-70 µl of stock RNA solution was mixed with 1/3 volume of sterile 10 M ammonium acetate. Cold absolute ethanol (2.5 volumes) was then added to precipitate the RNAs. These solutions were stored at -20 °C for 3 h and then centrifuged for 15 min at 12,500 rpm. Supernatant was removed from each tube and the pellet was again washed with about 400 µl cold 70% ethanol to reduce the ammonium acetate content. These solutions were stored about 2 h at -20 °C and then centrifuged at 12,500 rpm. Supernatant was removed and the pellet was dried in Speed-Vac centrifuge for 20 min. The pellet was dissolved in sterile DEPC treated dH_2O and was used for the analyses. Final concentrations of RNA solutions after precipitation were 300 pmol/µl in H_20. Multiple precipitations were performed with the same method. For the mass spectrometry analyses of 10-mer tRNA, an oligomer solution that had 10 pmol/ul final concentration in isopropanol- DEPC-dH_2O (1:1) was prepared and was analysed by negative mode option of Applied Biosystems 4000 Q-trap mass spectrometer. Dionex Ultimate 3000 HPLC system and Thermo LTQ XL ion trap mass spectrometer were used for the LC-MS analyses of 10-mer tRNA. Kromasil C18 HPLC column (2,1x10 mm) was used to obtain a pure oligomer. 1,5 µg of the sample was loaded to the column and was eluted by the solvent gradient between A channel (8 mM ammonium acetate solution- dH_2O) and B channel (8 mM ammonium acetate solution- methanol). Absorbance was measured at 260 nm with the help of the detector. The flow at the end of the column was sent to the mass spectrometry in order to save the chromatogram and mass spectrum of the sample simultaneously. The HPLC fraction that belongs to oligomer was then collected and after the addition of boric acid to this fraction, the analysis was repeated by using ion trap mass spectrometer.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Investigation of Tolerance Mechanisms in Yeast Mutants Resistant to Boron

It is known that wild type yeast can grow in the presence of 75 mM boric acid (Kaya et al. 2009, Nozawa et al. 2006). As the result of a previous genome-wide screen, *elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ mutants have been found resistant to boric acid (Uluisik, Kaya, Unlu, et al. 2011). They can tolerate up to 150 mM boric acid. To find out whether the overexpression of the genes whose absence provide boron resistance affects the boron tolerances of wild type yeast, the genes were first cloned and overexpressed in wild type (BY4741) cells. The expression of these genes from a high copy plasmid did not provided extra boron resistance to the wild type yeast (Figure 1A and Figure 1B). When the genes were complemented and overexpressed in their own mutants, the expression of *ELP3* gene resulted in a sensitivity to boron in *elp3Δ* mutant and they did not grow in the presence of 100mM boric acid. Elp3 is one of the subunits of elongator complex and involved in its histone acetyltransferase activity (Winkler et al. 2002). It is also found that the mutants lacking genes of elongator subunit genes including *ELP1, ELP3, ELP6,* and *KTI12* exhibited different phenotypes such as resistance to *Kluyveromyces lactis* killer toxin (Frohloff et al. 2001, Huang, Johansson, and Bystrom 2005). The expression of *NCS2* gene in *ncs2Δ* provided transformants to grow on media containing 125mM boric acid whereas the other transformants showed similar growths on boric acid and tolerated 100mM boric acid (Figure 1C).

Atr1 is known as a boron efflux transporter in yeast and when the gene is overexpressed, yeast cells can tolerate 225mM boric acid in the environment (Kaya et al. 2009). To test if extra boron resistance can be provided to the yeast cells, *ATR1* gene was overexpressed in boron resistant mutants. It was found that *ATR1* overexpressing *elp3Δ* and *ncs6Δ* transformants can tolerate 350mM boric acid, while the other transformants showed wild type-like growths (Figure 1D).

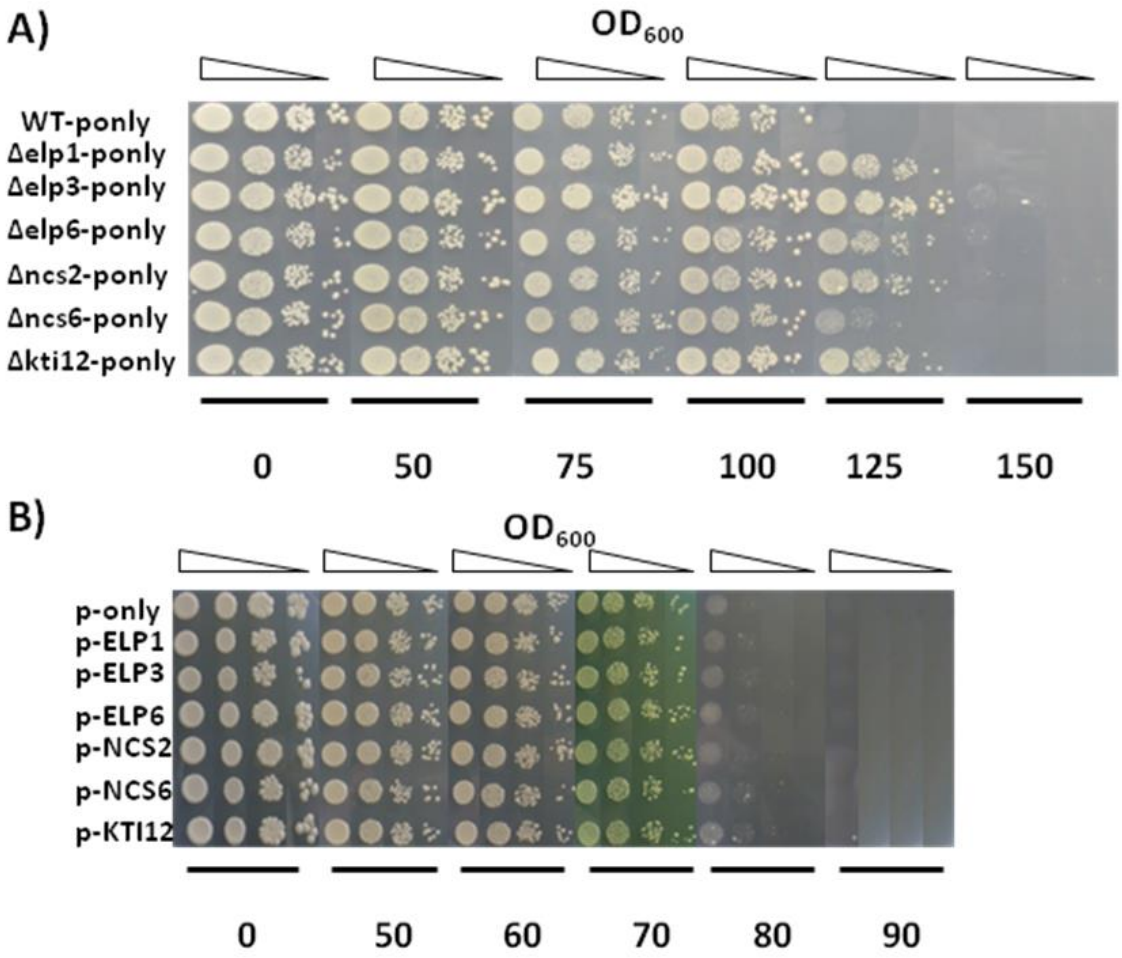


Figure 1. Boric acid tolerances of the yeast cells overexpressing boron resistance genes. (A) Wild type cells and boron resistant mutants were transformed with an empty vector (p426). (B) Wild type cells were transformed with the plasmids containing *ELP1, ELP3, ELP6, NCS2, NCS6, KTI12* genes. (C) Boron resistant *elp1Δ*, *elp3Δ*, *elp6Δ*, *ncs2Δ*, *ncs6Δ*, and *kti12Δ* mutants were transformed with the plasmids containing *ELP1, ELP3, ELP6, NCS2, NCS6, KTI12* genes. (D) Wild type cells and boron resistant mutants were transformed with the vector containing *ATR1* gene. The transformants obtained from these different experimental setups were grown to logarithmic phase and serially diluted to an optical density of 0.2, 0.02, 0.002, and 0.0002 at 600 nm. 5 µl of each dilution was spotted onto YNB-Ura media containing indicated amount of boric acid. The plates were incubated at 30°C for 3 days and photographed.

(cont. on next page)

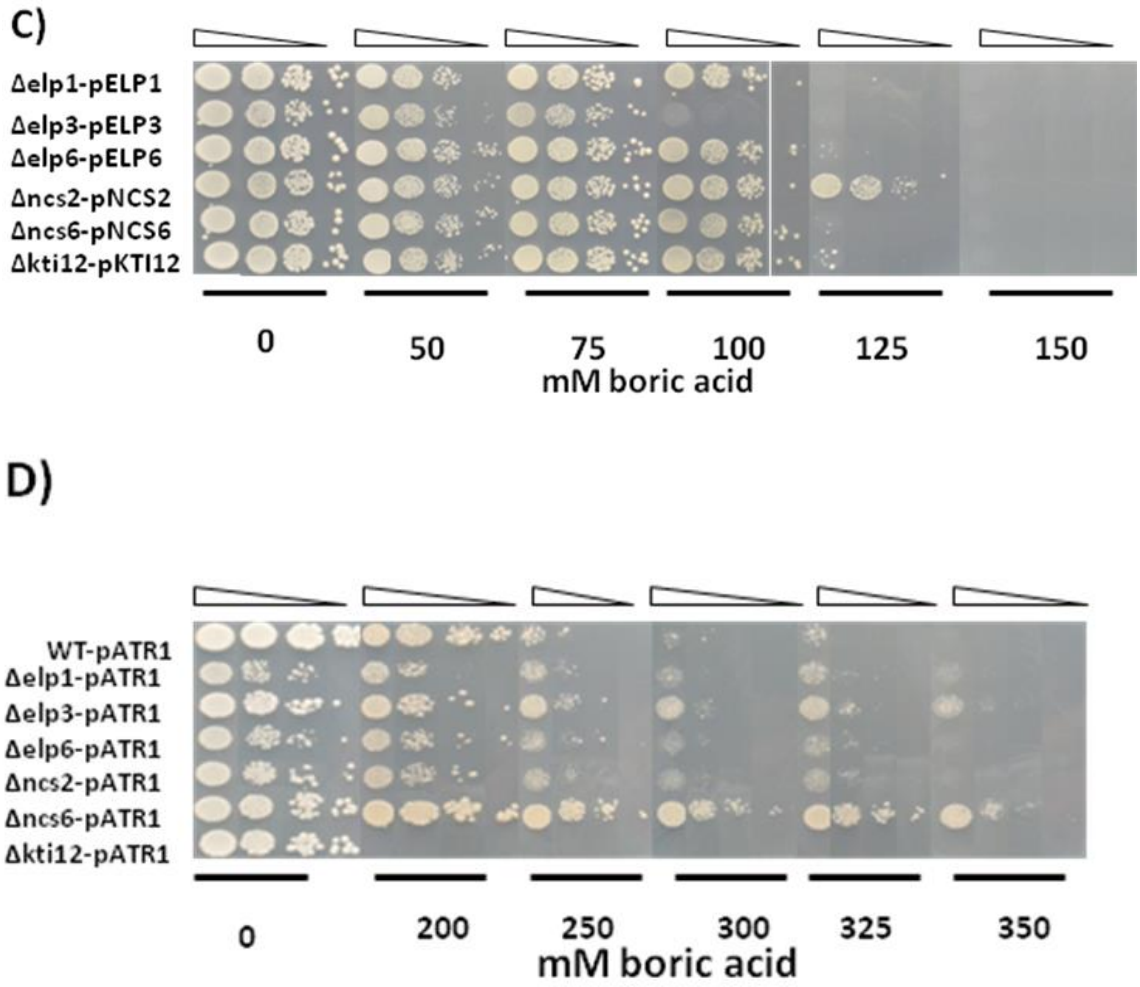


Figure 1 (cont.)

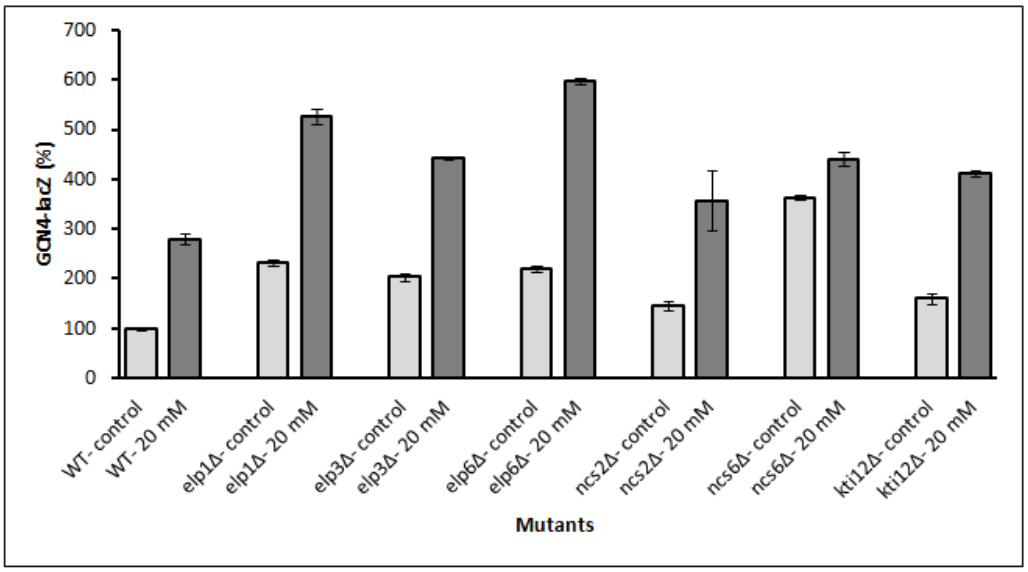
The mutants that are boron resistant are lacking functions related to tRNA wobble base modifications (Table 2) (Uluisik, Kaya, Unlu, et al. 2011). The problems with these modifications were shown to induce Gcn4 transcription factor (Huang, Lu, and Bystroem 2008). Therefore we speculated that the basal levels of Gcn4 might be higher in these mutants. To test this, *elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ, *kti12*Δ mutants and wild type cells were firstly transformed with pGcn4-lacZ plasmid and Gcn4 levels were determined by β-galactosidase analyses. As the result of the β-galactosidase analyses consisting of multiple repeats, Gcn4-lacZ expression levels of mutants which were treated with 20mM boric acid or not were higher than that of wild type (Figure 2A). This result indicates that Gcn4 transcription factor is involved in the resistance mechanism of boron-resistant yeast mutants.

Table 2. The functions of genes whose deficiency confer boron resistance
(Source: Saccharomyces Genome Database)

Common name	Systematic name	Function
ELP1	YLR384C	Subunit of Elongator complex which is required for modification of wobble nucleosides in tRNA; maintains structural integrity of Elongator.
ELP3	YPL086C	Subunit of Elongator complex which is required for modification of wobble nucleosides in tRNA; exhibits histone acetyltransferase activity.
ELP6	YMR312W	Subunit of hexameric RecA-like ATPase Elp456 Elongator subcomplex which is required for modification of wobble nucleosides in tRNA; required for Elongator structural integrity.
NCS2	YNL119W	Protein required for the thiolation of uridine at the wobble position of Lys(UUU) and Glu(UUC) tRNAs; has a role in urmylation and in invasive and pseudohyphal growth.
NCS6	YGL211W	Protein required for the thiolation of uridine at the wobble position of Gln, Lys, and Glu tRNAs; has a role in urmylation and in invasive and pseudohyphal growth.
KTI12	YKL110C	Protein that plays role in tRNA wobble nucleoside modification with Elongator complex; involved in sensitivity to G1 arrest induced by zymocin; interacts with chromatin throughout the genome.

Gcn4 was also found to be one of the main regulators of the gene expressions including *ATR1* in boron stress (Uluisik, Kaya, Fomenko, et al. 2011). Therefore the high Gcn4 levels in boron resistant mutants might induce the expression of *ATR1*, and consequently the mutants become resistant to boric acid. *ATR1* gene encodes for a boron efflux pump which has important functions in providing boron resistance in yeast (Kaya et al. 2009). Thus, it is expected that *ATR1* is active and functional in *ncs2Δ, ncs6Δ, kti12Δ, elp1Δ, elp3Δ, elp6Δ* mutants. To test this part of the hypothesis, *ATR1* mRNA expression levels were monitored by real time PCR analyses. *ATR1* mRNA expression levels were found to be increased considerably compared to the levels of wild type cells as indicated in Figure 2B. This might be the reason for the boric acid tolerance of corresponding mutants. The high expression levels of *ATR1* in these mutants can be resulted in the efflux of high amounts of boric acid so that the mutants might have low levels of boron in the cell.

A)



B)

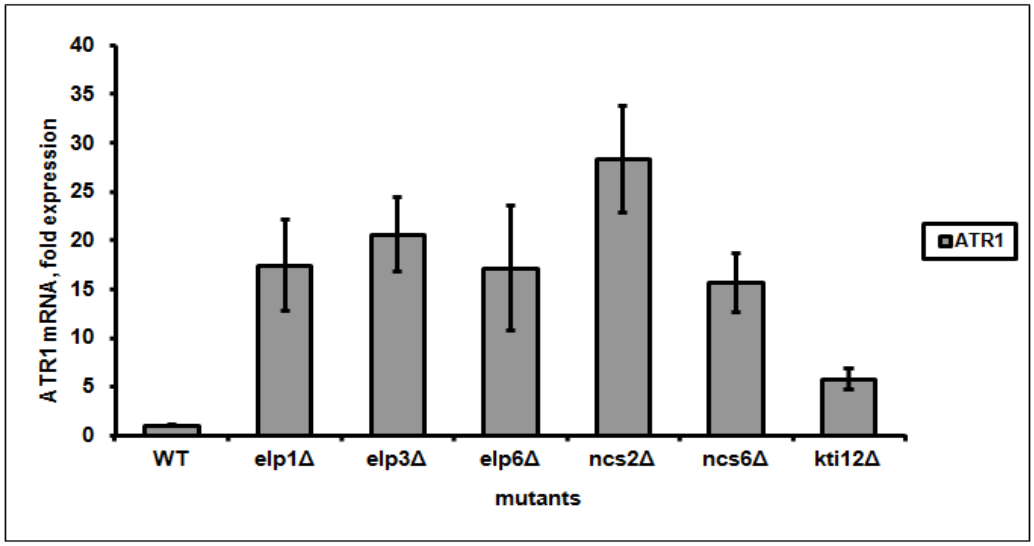


Figure 2. Gcn4-LacZ and *ATR1* expressions in boron resistant mutants. A) Wild type and all boron resistant yeast mutants were transformed with a plasmid containing *GCN4-LacZ* gene. Logarithmically growing cultures were treated with 20mM boric acid for 3 hours or not. B- galactosidase assay were performed as indicated in Materials and Methods part. B) *ATR1* gene expression was determined by real time PCR analyses in wild type and boron resistant mutants. Yeast actin gene was used as the internal control.

3.2. Investigation of Sensitivity Mechanisms in Yeast Mutants Sensitive to Boron

Boron-sensitive mutants were identified by yeast deletion library screenings (Uluisik, Kaya, Unlu, et al. 2011). To elucidate the mechanism of sensitivity, 8 of the most boron sensitive mutants (*ymr310c*Δ*, sla1*Δ*, erg4*Δ*, ygr283c*Δ*, rpe1*Δ*, hom6*Δ*, ccs1*Δ*, and och1*Δ) were selected for further characterization. According to the known data the genes that are absent in these mutants take part in different metabolisms such as amino acid metabolism, vesicular transport, cell cycle, and carbohydrate metabolism in the cell. The genes whose absence confers boron sensitivity to yeast cells were cloned by using Gateway cloning system. Wild type and boron sensitive mutant yeast cells were transformed with the destination vectors carrying the genes. Boron tolerances of wild type and mutants overexpressing related genes were determined by spotting assays. It was previously shown that wild type yeast cells can tolerate 75 mM boric acid (Kaya et al. 2009) and as seen in Figure 3B, WT cells carrying empty plasmids confirmed this discovery. The overexpression of the genes whose absence can cause extreme boric acid sensitivity did not provide any extra sensitivity or resistance to WT cells.

Boron tolerances of some of the boron sensitive mutants were changed by complementing the corresponding genes. When *HOM6*, *RPE1*, *YGR283C*, *CCS1*, *OCH1*, *YMR310C* genes were cloned and expressed in their mutants, the transformants exhibited similar boric acid tolerance to that of wild type cells and could grow in the presence of 75 mM boric acid. Thus it indicates that the complementation tests were performed successfully in these mutants. *erg4Δ*, and *rpe1Δ* mutants carrying *ERG4* and *RPE1* genes respectively did not gain extra resistance and tolerated 50 mM boric acid (Figure 3A).

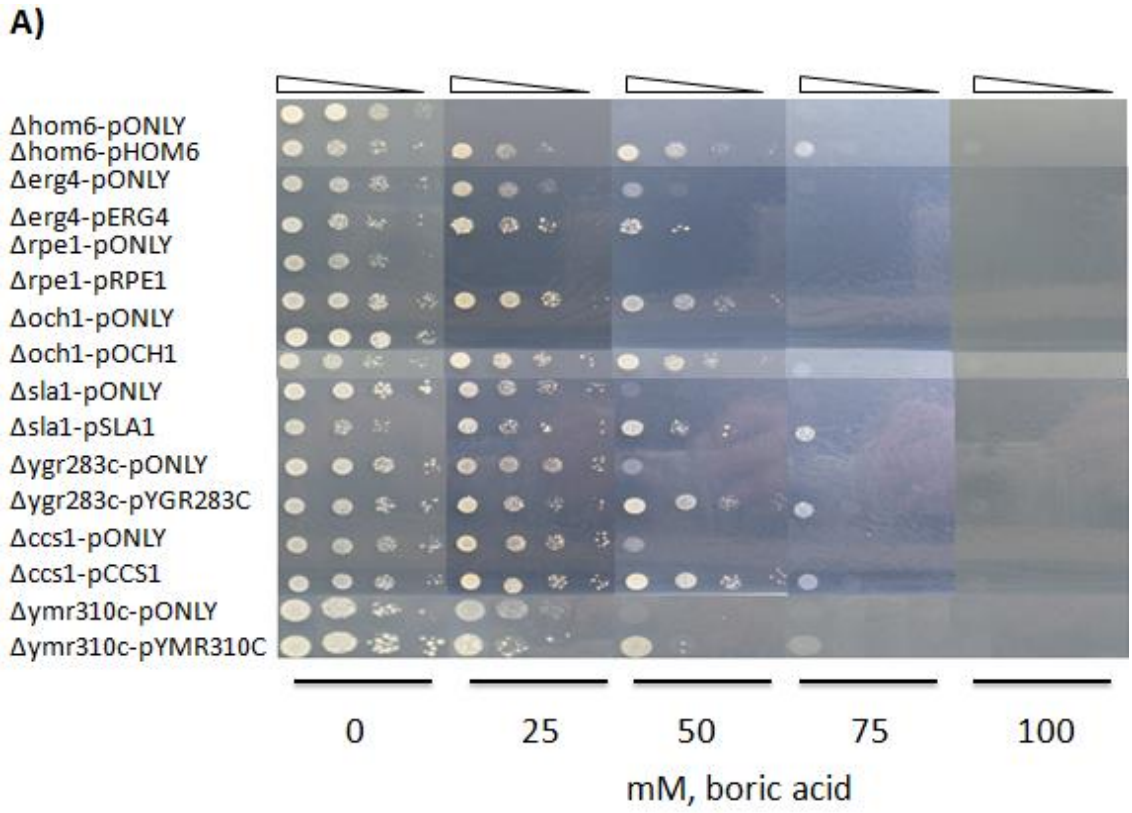


Figure 3. Boric acid tolerances of the yeast cells overexpressing boron sensitivity genes. (A) Boron sensitive mutants were transformed with either an empty vector (pAG426GPD) or with the plasmids containing *HOM6*, *ERG4*, *RPE1*, *OCH1*, *SLA1*, *YGR283C*, *CCS1*, *YMR310C* genes. (B) Wild type cells were transformed with the plasmids containing *HOM6*, *ERG4*, *RPE1*, *OCH1*, *SLA1*, *YGR283C*, *CCS1*, *YMR310C* genes. The obtained transformants were grown to logarithmic phase and serially diluted to an optical density of 0.2, 0.02, 0.002, and 0.0002 at 600 nm. 5 µl of each dilution was spotted onto YNB-Ura media containing indicated amount of boric acid. The plates were incubated at 30°C for 3 days and photographed.

(cont. on next page)

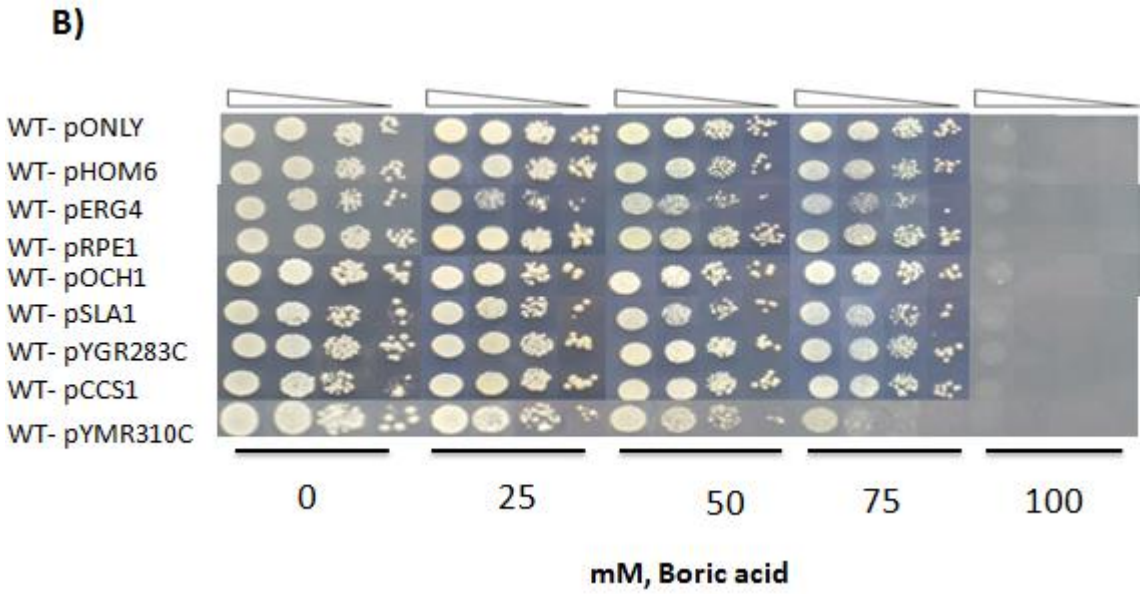


Figure 3 (cont.)

To find out if the Gcn4 trancription factor is active in these mutants or not, Gcn4-lacZ expression levels were determined. Almost all mutants had lower basal levels of Gcn4-lacZ than wild type cells. Boron treatment caused an increase in Gcn4-lacZ levels in wild type cells as expected. However Gcn4-lacZ levels decreased in *hom6Δ*, *rpe1Δ*, *sla1Δ*, *ygr283cΔ*, *ymr310cΔ* mutants whereas an increase was observed in *erg4Δ*, *och1Δ*, and *ccs1Δ* mutants (Figure 4A).

In addition, intracellular boron levels were determined in mutants and in wild type cells overexpressing related genes after boron treatment. As indicated in Figure 4B, the intracellular boron levels in the control groups of cells which did not have boric acid treatment were found to be very close to each other. However after boric acid treatment some mutants (*rpe1Δ, och1Δ, sla1Δ, ygr283cΔ*) had higher levels of boric acid within the cell when compared to the wild type. It is known that boric acid is toxic when present in excess. Therefore the boric acid sensitivity of these mutants might arise from the relatively high amounts of boric acid in the cells. *erg4Δ* mutant cells had lower intracellular boron after treatment. Interestingly; *hom6Δ, ccs1Δ,* and *ymr310cΔ* mutants exhibited similar levels of intracellular boron as wild type cells after boric acid treatment.

The overexpression of the genes might eliminate the boron sensitivity arising from their absence. However as shown in Figure 4C, intracellular boron levels in WT cells expressing the plasmids containing these genes were higher than in WT cells

expressing empty plasmid. In the absence of these genes, cells became more sensitive to boric acid whereas the overexpression of the genes prevented the boric acid sensitivity (Figure 3B) but did not provide a decrease in intracellular boron levels (Figure 4C).

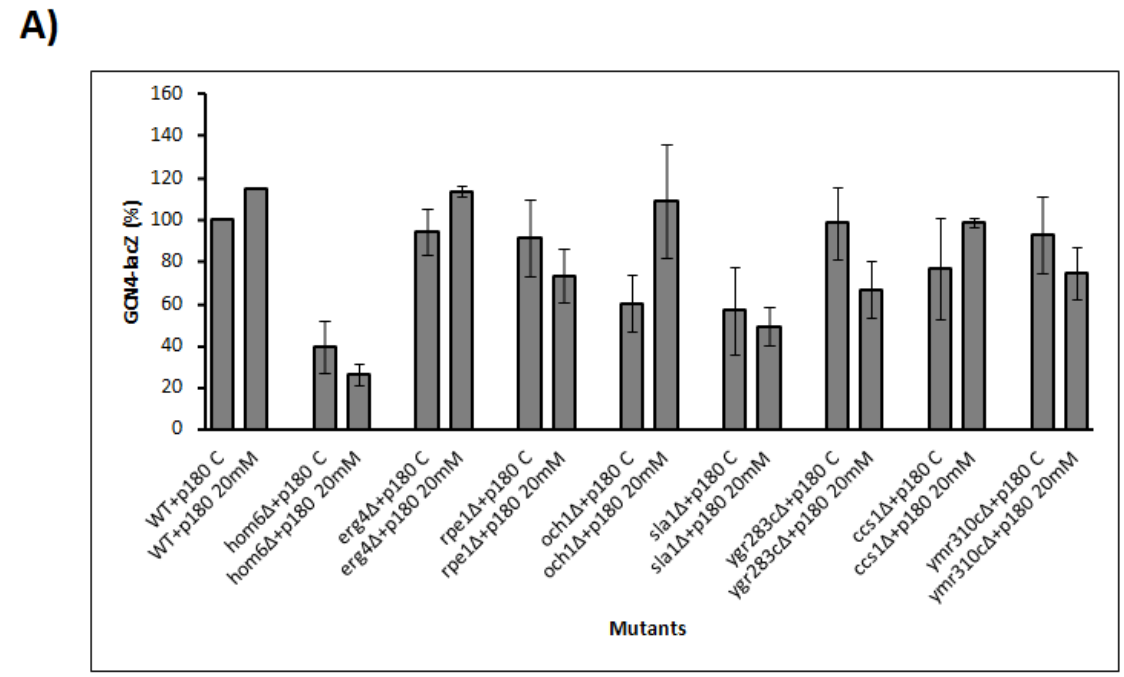
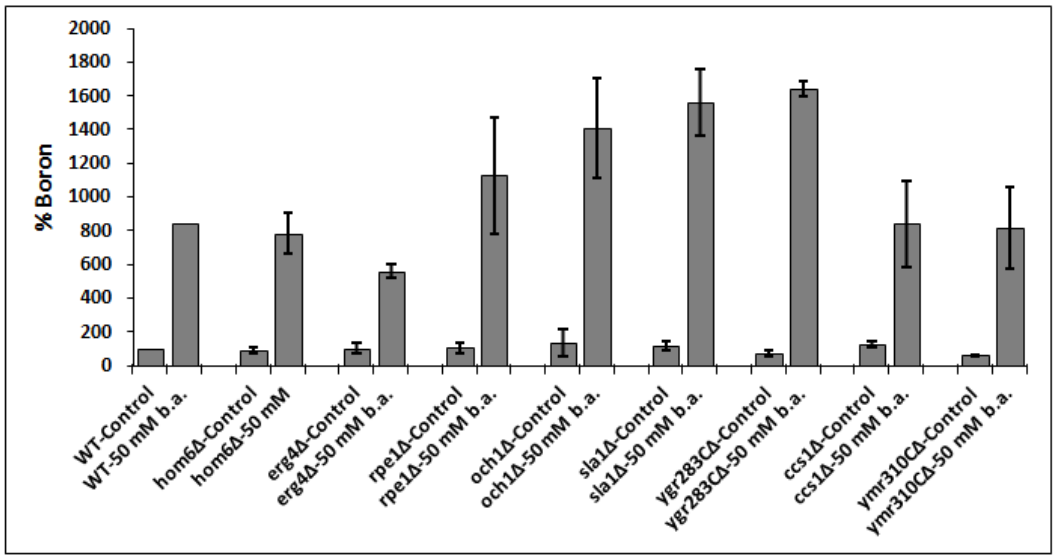


Figure 4. GCN4-LacZ expressions and intracellular boron measurements in boron sensitive mutants upon boron treatment. A) Wild type and all boron sensitive yeast mutants were transformed with a plasmid containing GCN4-LacZ gene. Logarithmically growing cultures were treated with 20mM boric acid for 3 hours or not. B- galactosidase assay were performed as indicated in Materials and Methods part. B) Intracellular boron levels of wild type and boron sensitive mutant cells that were exposed to 50mM boric acid for 1 h or not. C) Intracellular boron levels of wild type and boron sensitive mutant cells overexpressing the genes whose absence causes boron sensitivity. The transformants were exposed to 50mM boric acid for 1 h or not. Boron levels were measured using Boron Cell Test Kit as indicated in Materials and Methods. Error bars represent separate measurements.

(cont. on next page)

B)



C)

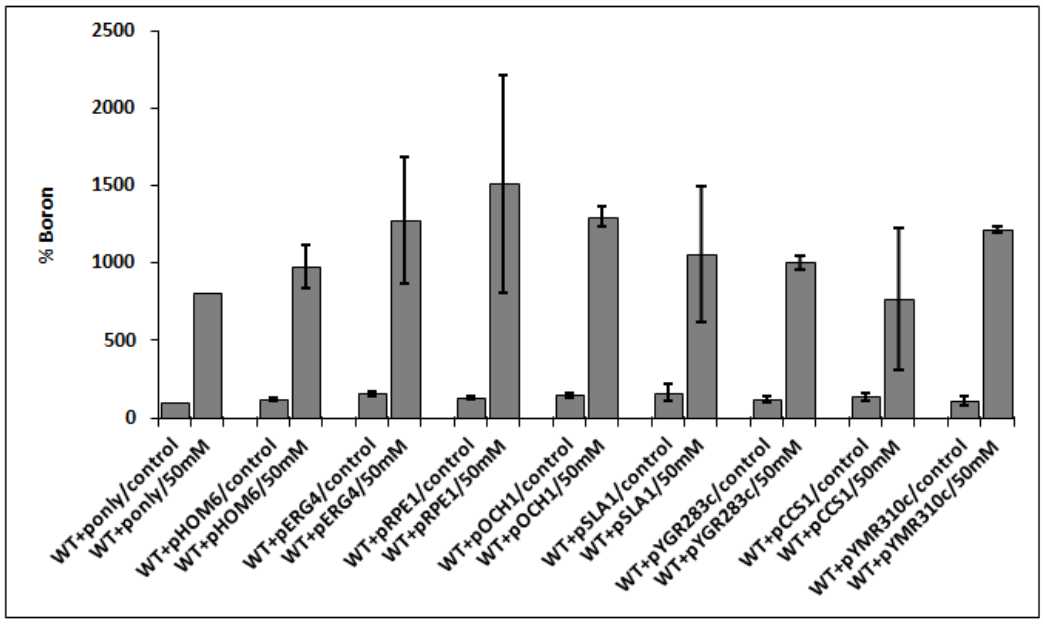


Figure 4 (cont.)

3.3. Uncovering the Transcription Factor(s) That Could Control Gcn4 in Boron Stress

Gcn4 is a major transcriptional regulator related to boron toxicity (Uluisik, Kaya, Fomenko, et al. 2011). However it is not known how boron stress induces the transcription of *GCN4*. There are several transcription factors that can induce or inhibit

the expression of *GCN4*. To find out how boron stress is transmitted to Gcn4, and which transcription factor(s) have roles in boron stress response; the transcription factor(s) that can bind to *GCN4* promoter were obtained by using YEASTRACT database (Teixeira et al. 2006). Boron tolerances of the 12-determined transcription factor mutants which are *fhl1*Δ, *flo8*Δ, gln3Δ, *hap2*Δ, *hap4*Δ, *mga1*Δ, *rap1*Δ, *sok2*Δ, *ste12*Δ, *stp1*Δ, *tec1*Δ, *yap1*Δ was monitored by drop tests on YPD media containing different concentrations of boric acid. As seen in Figure 5, *tec1Δ, mga1Δ*, and *sok2Δ* mutants exhibited wild type-like growth whereas other mutants were found to be relatively sensitive to boric acid.

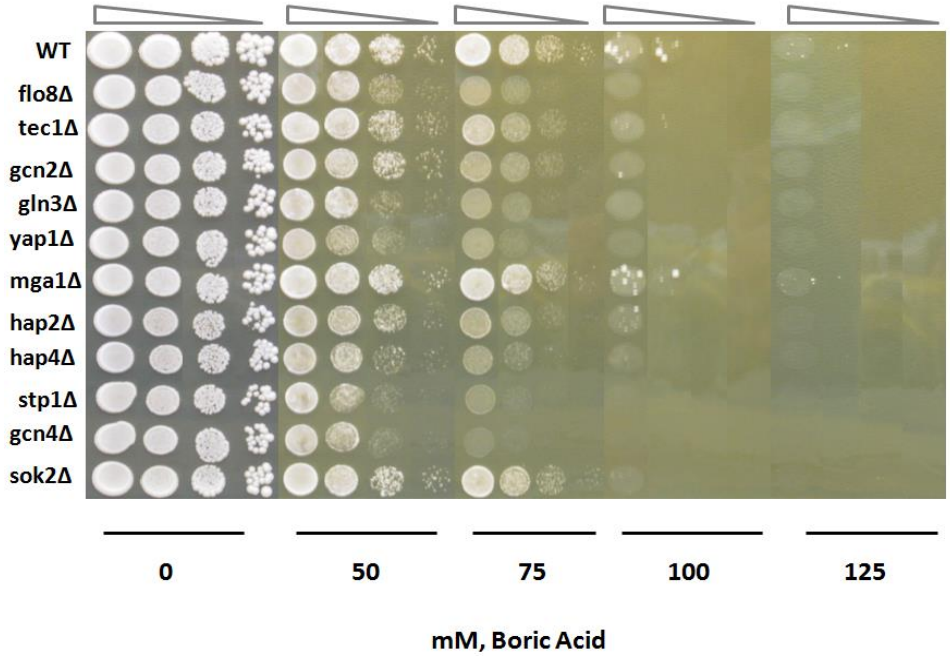


Figure 5. Boron tolerances of transcription factors that bind to *GCN4* promoter.

Deletion mutants that lack these transcription factor genes were transformed with pGCN4-lacZ plasmid and Gcn4 levels were determined by β-galactosidase analyses. The expression of Gcn4-lacZ decreased in *mga1Δ*, *stp1Δ*, *gln3Δ*, *yap1Δ*, and *flo8Δ* mutants; whereas it increased in *hap4Δ, gcn2Δ,* sok2*Δ, gcn4Δ,* and *tec1Δ* mutants.

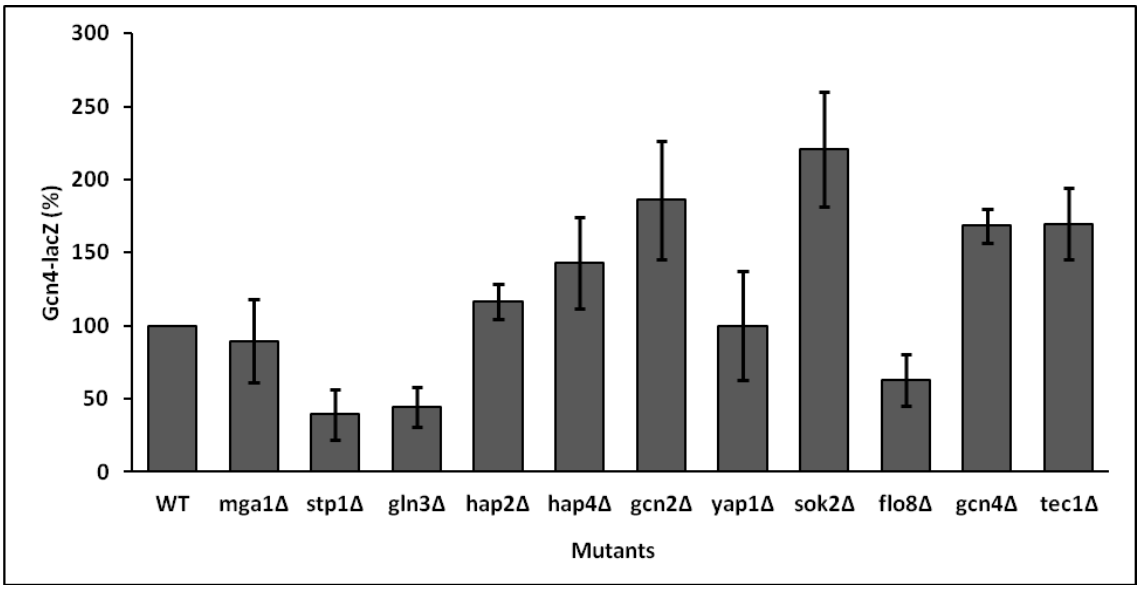


Figure 6. Gcn4-lacZ expression in transcription factor mutants that bind to *GCN4* promoter.

Mutants that had low levels of Gcn4-lacZ which were *mga1Δ, stp1Δ, gln3Δ, yap1Δ, and flo8Δ* (Figure 6) were used for real time PCR analyses. If these transcription factors have roles in the activation of *GCN4*, they might have roles indirectly in the activation of *ATR1* as well. Therefore *ATR1* mRNA expression profiles were examined by real time PCR. However in the absence of the genes in question, a decrease in the expression of *ATR1* was not observed (Figure 7). These transcription factor genes have roles in different pathways in the cell in addition to the regulation of Gcn4. The deletion of these genes might affect other mechanisms within the cell, thus, the levels of *ATR1* expression did not drop directly or *GCN4*-dependent manner.

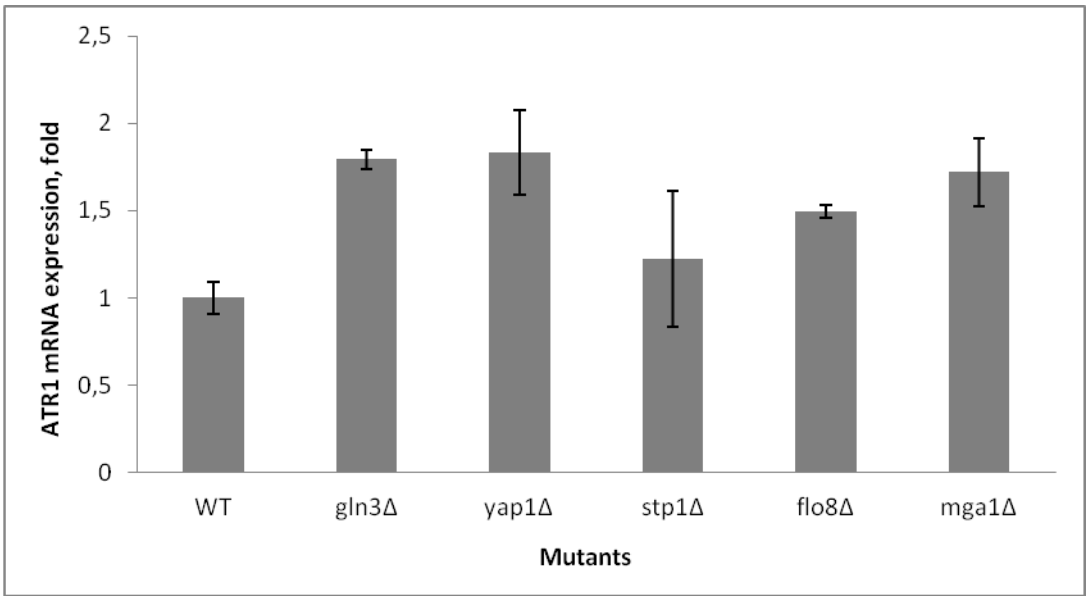


Figure 7. *ATR1* mRNA expression levels in WT and transcription factor mutants. Normalizations were done based on the Ct values of *ACT1* and *ATR1* mRNAs.

3.4. Investigation of the Relationship Between Boron Stress and TOR Kinase Pathway

To reveal the effects of boron stress on intracellular signal transduction, Tor1 kinase pathway was first investigated. It has been known that TOR is a target molecule of rapamycin which is used as an immunosuppressive drug for organ transplantation and for some cancer types. *TOR* is an essential serine/threonine kinase and its structure and function are conserved among eukaryotes. It is composed of Tor1 and Tor2 complexes. Tor1 which has significant regulatory roles on cell division and cell metabolism is inhibited by rapamycin. Tor2 is responsible for the regulation of cell polarity and is not inhibited by rapamycin (De Virgilio and Loewith 2006, Loewith and Hall 2011). TOR pathway regulates gene expressions especially if there is nutrient starvation or environmental stress. It helps translation to start by increasing the initiation factor eIF4F amount which induces mRNA to bind to 40S ribosomal subunit. Additionally, it phosphorylates 4E-BP proteins that are bound to eIF4E so that eIF4E becomes released. eIF4E is necessary for the formation of eIF4F in translation initiation. It has been found that TOR pathway decreases the phosphorylation of eIF2α by Gcn2 kinase in translation initiation step (Cherkasova and Hinnebusch 2003).

Gcn2 is a protein kinase that phosphorylates eIF2α and increases the *GCN4* translation in amino acid starvation conditions. The activation of Gcn2 as a protein kinase depends on the binding of uncharged tRNAs to the histidiyl tRNA synthetase region on *GCN2*. The phosphorylation of Ser577 on GCN2 inhibits the function of GCN2 by decreasing the tRNA binding affinity. Rapamycin inhibits the Tor pathway whereas it activates Gcn2 directly by decreasing the phosphorylation of Ser577. As indicated the mechanisms about Tor pathway are more active especially in translation initiation. It is known that Gcn2 is activated by boron stress, consequently activates Gcn4 and protein synthesis is inhibited (Uluisik, Kaya, Fomenko, et al. 2011). Therefore there may be a relation between Tor pathway and boron stress.

If there are limited sources of nitrogen, the expression of the genes that code for the enzymes responsible for the catabolism of nitrogeneous compounds is increased in yeast. Gln3 and Gat1 transcription factors are responsible for the regulation of these events. Yeast Tor pathway also has roles in the regulation of the genes that are involved in the catabolism of limited nitrogen sources. It causes Gln3 to interact with the cytoplasmic protein Ure2 and is transferred outside of the nucleus, thus transcriptional activation is inhibited (Valenzuela, Aranda, and Gonzalez 2001). In addition Tor proteins provide an interaction between *SIT4* and *TAP42* and this interaction results in the inactivation of *SIT4*. It causes GLN3 to be then inactivated and thus the expression of nitrogen catabolism genes is repressed. Rapamycin reverses this effect and activates *GLN3* by inhibiting *TOR* and causes *TAP42/SIT4* complex to separate (Cherkasova and Hinnebusch 2003). Some of the target genes of Gcn4 are regulated by both GCN4 and GLN3. *tor1Δ* and *gln3Δ* mutants have basic roles in Tor pathway and were chosen for the experiments. Initially pGCN4-lacZ plasmid was transformed to the related mutants and the changes in Gcn4-lacZ expression levels were followed by β-galactosidase assay in the presence or absence of boron (Figure 8).

According to the results the expression of Gcn4-lacZ was found to be lower in control cells which were not treated with boric acid than in wild type cells. Since the genes have roles in GAAC pathway, it is an expected result that the deletion of these genes can possibly result in a decrease in the expression of Gcn4-lacZ. Boric acid treatment generally resulted in a decrease in Gcn4-lacZ expression except 5 mM and 10 mM treatment of boric acid. Both genes have significant regulatory roles in cell metabolism therefore the deletion of these genes may cause interaction of different molecules in the cell and activation of different pathways.

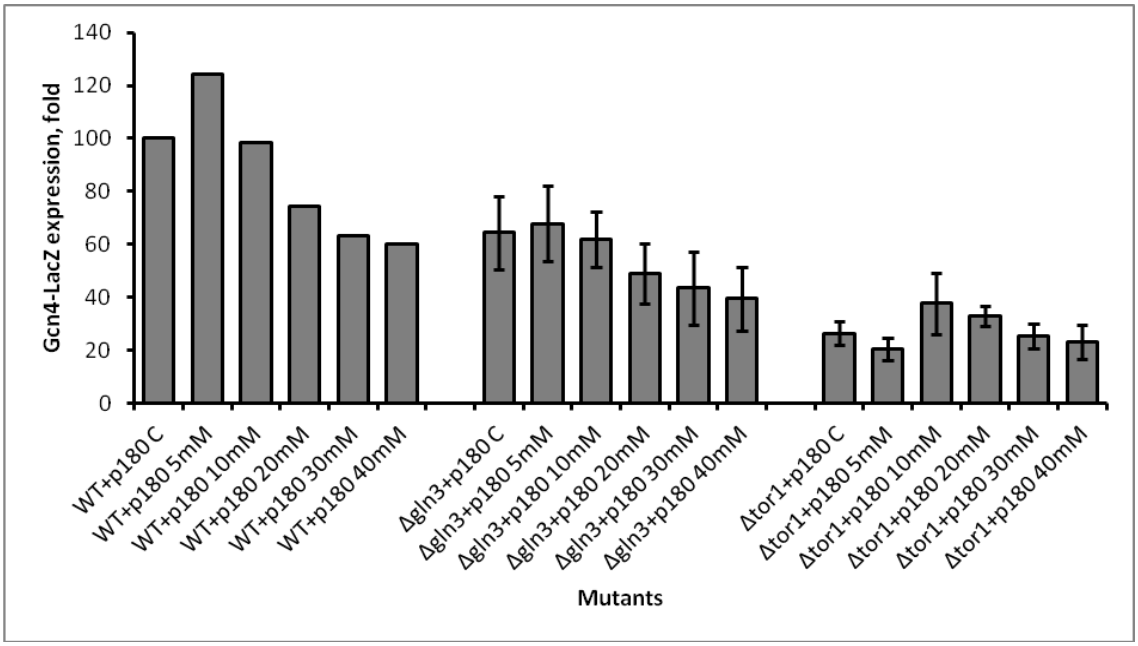


Figure 8. The changes of Gcn4-lacZ expression in p180 plasmid carrying WT, *gln3Δ* and *tor1Δ* mutants. The cells were treated with boric acid or not. The experiment was repeated at least three times and in each experiment, mutant and wild-type yeast cells were at least three replicates. Normalization was performed in accordance with wild-type yeast cells that were not treated with boric acid. 5mM, 10mM, 20mM, 30mM and 40mM were used as the boric acid concentrations for treatments. Control group (C) indicates the cells that were not treated with boric acid.

Gln3 is a transcriptional activator that regulates most of the genes in nitrogen catabolite repression (NCR) metabolism. If there is nitrogen starvation, Gln3 is moved to the nucleus and activates the expression of NCR genes. If there is no nitrogen starvation in the environment Gln3 is phosphorylated and is localized in the cytoplasm resulting in minimal expression of NCR genes. Gln3 can be phosphorylated by various kinases and Tor kinases are in the first place that phosphorylate Gln3 (Beck and Hall 1999). It is also known that Snf1 has roles in the regulation of Gln3 (Bertram et al. 2002). Since *TOR2* is an essential gene, *tor1Δ* mutant was used in the experiments. It is shown that Gln3 plays role in Gcn4 regulation. If these genes play roles Gcn4 regulation dependent or Atr1 regulation dependent manner in boron stress, *ATR1* mRNA transcript levels were expected to be low in the mutants of these genes. The low expression of *ATR1* was observed in *tor1Δ* and *gln3Δ* mutants (Figure 9). Even if *ATR1* expression increased after 1h of 20 mM and 50 mM boric acid treatment when compared to the

untreated control cells, the expression was quite low when compared to the WT cells indicating *TOR1* and *GLN3* play active roles in boron stress.

ATR1 mRNA expression levels were analysed by real time PCR. The details of the analysis were indicated in materials and methods part. Wild type, *tor1Δ* and *gln3Δ* mutants were grown in rich media and they were separated into three in their logarithmic growth phase. One group was not treated with boron and was used as the control group, the second group was treated with 20 mM boric acid for 1 h, and the third group was treated with 50 mM boric acid for 1 h. After obtaining total RNAs from these cells, real time PCR analyses were performed. Each analysis had at least three replicas and was performed in different times.

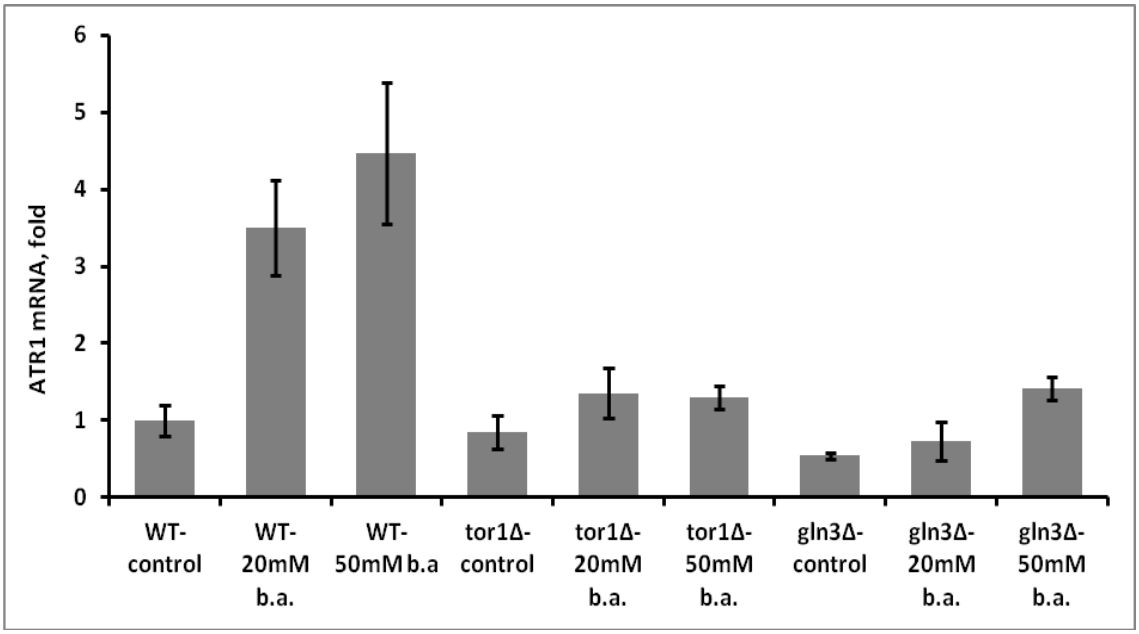


Figure 9. *ATR1* mRNA expression levels in boric acid treated or non-treated WT, *tor1Δ* and *gln3Δ* mutants. Normalizations were done according to the Ct values of *ATR1* and *ACT1* mRNAs (b.a. = boric acid)

3.5. Investigation of the Relationship Between Boron Stress and SNF1 Pathway

Snf1 is a protein kinase and is named as cytoplasmic glucose sensor. The expression of the genes that play role in the transport or in the catabolism of other carbon sources is inhibited in the presence of excess glucose in the environment. This

process is known as glucose repression (Carlson 1999). Snf1 is activated when glucose levels are low in the environment and by inducing gluconeogenesis it provides cells to use alternative carbon sources. The glucose levels in the medium were shown to influence the activity of Gln3 transcription factor (Bertram et al. 2002). It is also known that Gcn4 transcription factor can be activated by Gln3 (Harbison et al. 2004, Lee et al. 2002). A potential mechanism that is responsible for the indirect activation of Gcn4 is the activation of Gln3 by Snf1 in glucose starvation conditions. Another study showed that *SNF1* deletion increases Gcn4 protein levels by the help of the activities of Gcn2 and Gcn20. It is thought that Snf1 functions previous to Gcn20 and regulates the translation of *GCN4* in *S. cerevisiae* (Shirra et al. 2008). To find out whether Snf1 kinase plays roles in boron stress response pathway, the effects of the deletion and overexpression of *SNF1* gene on Gcn4-lacZ and *ATR1* mRNA levels in cells have been studied. *SNF1* was first cloned using Gateway cloning system, then the plasmids containing *SNF1* gene were transformed into WT and *snf1Δ* mutants. the boric acid sensitivities of the transformants was shown by the drop tests in which each transformant was spotted onto media containing different concentrations of boric acid. Additionally boric acid tolerance of *snf1Δ* mutant was determined using drop test on YPD media (Figure 10). *snf1Δ* mutant was found to be sensitive to boric acid. However there was no difference between overexpression or complementation of *SNF1* in *snf1Δ* mutant (Figure 11).

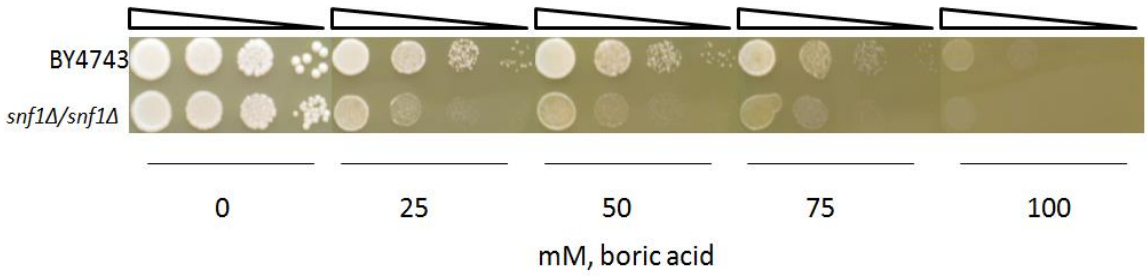


Figure 10. Boric acid tolerance of *snf1Δ* mutant

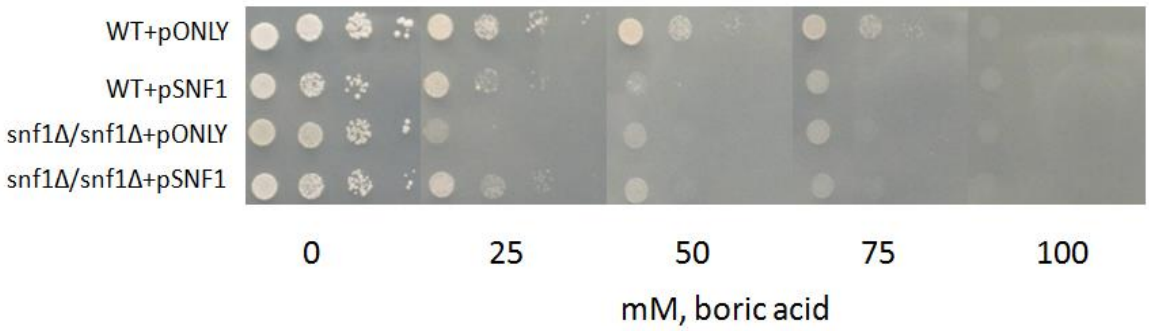


Figure 11. Boric acid tolerances of WT and *snf1Δ* mutants overexpressing *SNF1* gene

The effects of the deletion and overexpression of *SNF1* gene on the activity of Gcn4-lacZ in cells were determined by β-galactosidase assays (Figure 12 and Figure 13). WT and *snf1Δ* mutant cells were transformed with an empty plasmid or *SNF1*-carrying plasmids. Obtained transformants were then transformed Gcn4-lacZ plasmids and spread onto media that lack both uracil and histidine amino acids. Resulting transformants were used for the β-galactosidase assays. As shown in figure 12, when *SNF1* is deleted Gcn4 expression levels increased when compared to the wild type cells and overexpression of *SNF1* in WT and *snf1Δ* also increased the levels of Gcn4 (Figure 13). Based on these data *SNF1* can be considered to be involved in the regulation of Gcn4 expression.

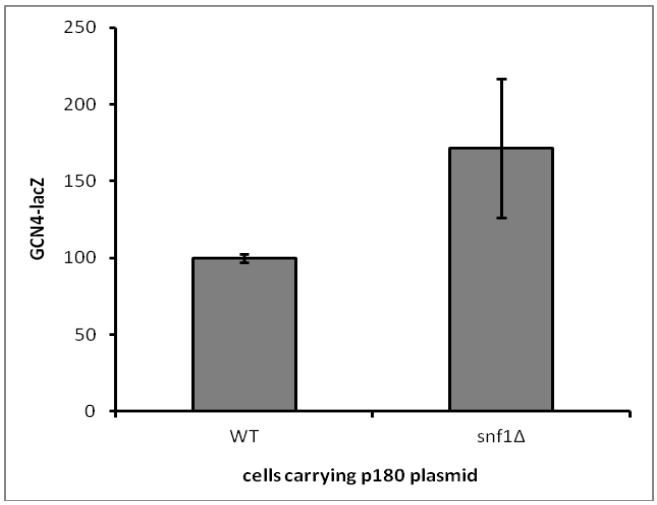


Figure 12. The changes in Gcn4-lacZ levels in WT and *Δsnf1* mutants carrying p180 plasmid

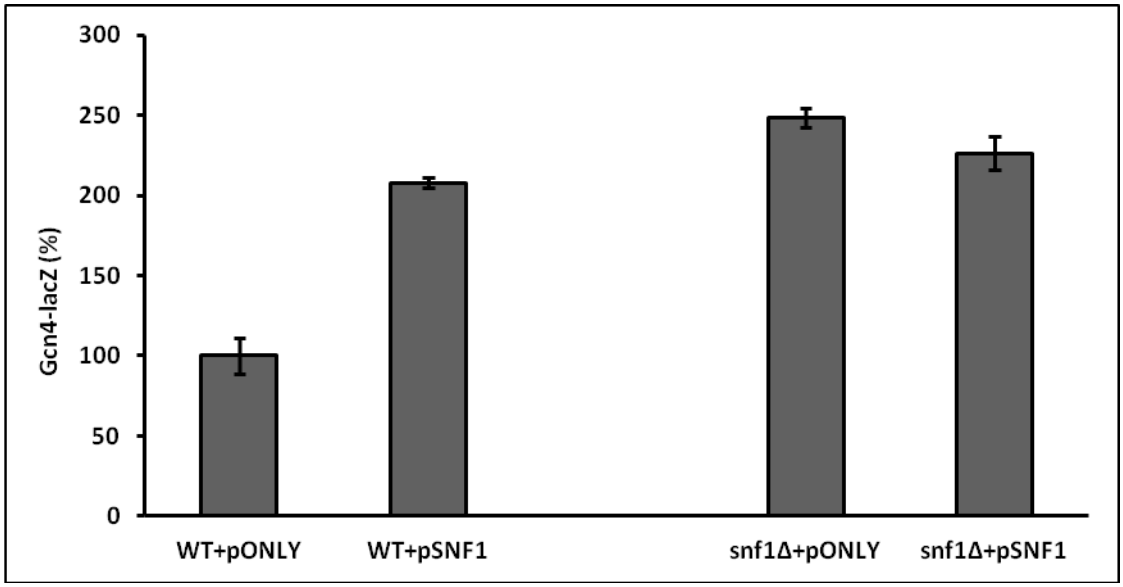


Figure 13. Gcn4-lacZ expression levels in WT and *snf1Δ* mutants overexpressing SNF1 gene. WT and *snf1Δ* mutants carrying empty plasmid was used as a control.

In addition WT and *snf1Δ* cells that overexpressed *SNF1* gene were used and total RNA was isolated from them. *ATR1* expression levels were determined by real time PCR both in the presence and absence of boron. As indicated in Figure 14, *ATR1* expression was low in *snf1Δ* mutant compared to the WT whereas the expression of the gene increased approximately five fold compared to control with boron treatment.

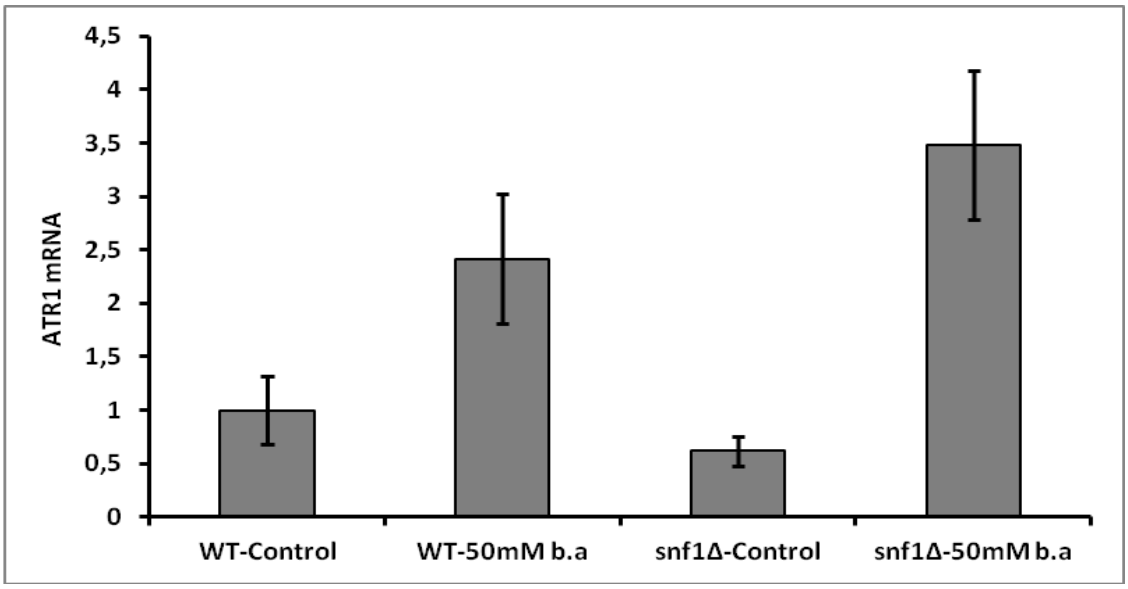


Figure 14. Real time PCR analyses of *ATR1* mRNA in WT and *snf1Δ* cells. The cells were treated with 50 mM boric acid for 1h, the cells used as control were not treated. Yeast *ACT1* gene was used as experimental control. Normalizations were based on Ct values of *ACT1* and *ATR1* mRNAs.

SNF1 gene was cloned and overexpressed in WT and *snf1Δ* mutants. Total RNA was isolated from these transformants and real time PCR analyses were performed. Overexpression of *SNF1* had no dramatic difference between WT and *snf1Δ* mutant in terms of *ATR1* mRNA expression levels (Figure 15).

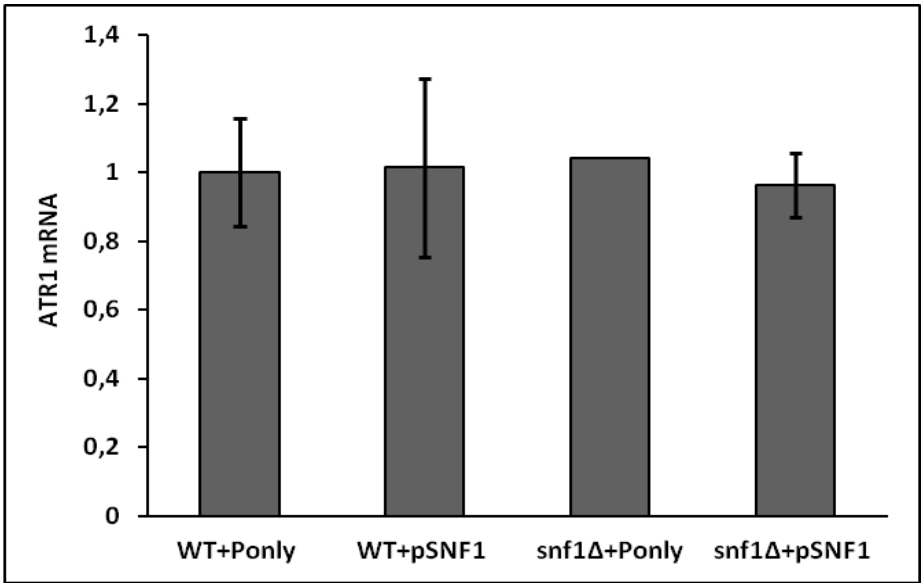


Figure 15. Real time PCR analyses of *ATR1* mRNA in WT and *snf1Δ* mutant overexpressing *SNF1* gene. Yeast actin gene was used as experimental control. Normalizations were based on the Ct values of *ACT1* and *ATR1* mRNAs.

3.6. Investigation of the Relationship Between Boron Stress and PKA Pathway

cAMP/PKA pathway in *S. cerevisiae* play roles in glucose signaling pathway and responsible for the regulation of some metabolic responses such as resistance to stress, glycolysis, gluconeogenesis. In general, PKA is found in the cell as an inactive tetramer that has two regulatory and two catalytic subunits. Adenylyl cyclase is activated by the environmental signals such as glucose levels, cAMP levels then increases and a conformational change occurs and catalytic subunits of PKA become activated by the binding of cAMP to the regulatory subunits of PKA (Zurita-Martinez and Cardenas 2005). Pka1 and Pka2 are cAMP-dependent catalytic subunits of protein kinase in PKA pathway. It has been shown that UV stress induces the transcription and translation of Gcn4 through PKA signaling so that the target genes of *GCN4* are activated. The increase in *GCN4* translation was suggested to occur independently from the pathway including Gcn2 protein kinase. Additionaly this mechanism differs from the DNA repair mechanisms (Engelberg et al. 1994). To investigate if there is a possibility that this pathway can activate Gcn4 in the presence of boron or not, the

changes in Gcn4-lacZ levels in boric acid treated *pka1Δ* and *pka2Δ* mutants were observed by β- galactosidase analyses. *pka1Δ* and *pka2Δ* mutants were transformed with pGCN4-lacZ (p180) plasmid. The transformants that grew on YNB-Ura selective media were chosen and used in further analyses. Wild type yeast cells that were treated with boric acid in the same way as mutants were used as control groups. Analyses were repeated at least three times with three replicas of each sample. Normalizations were based on the Gcn4-lacZ levels of boric acid-nontreated wild type yeast cells.

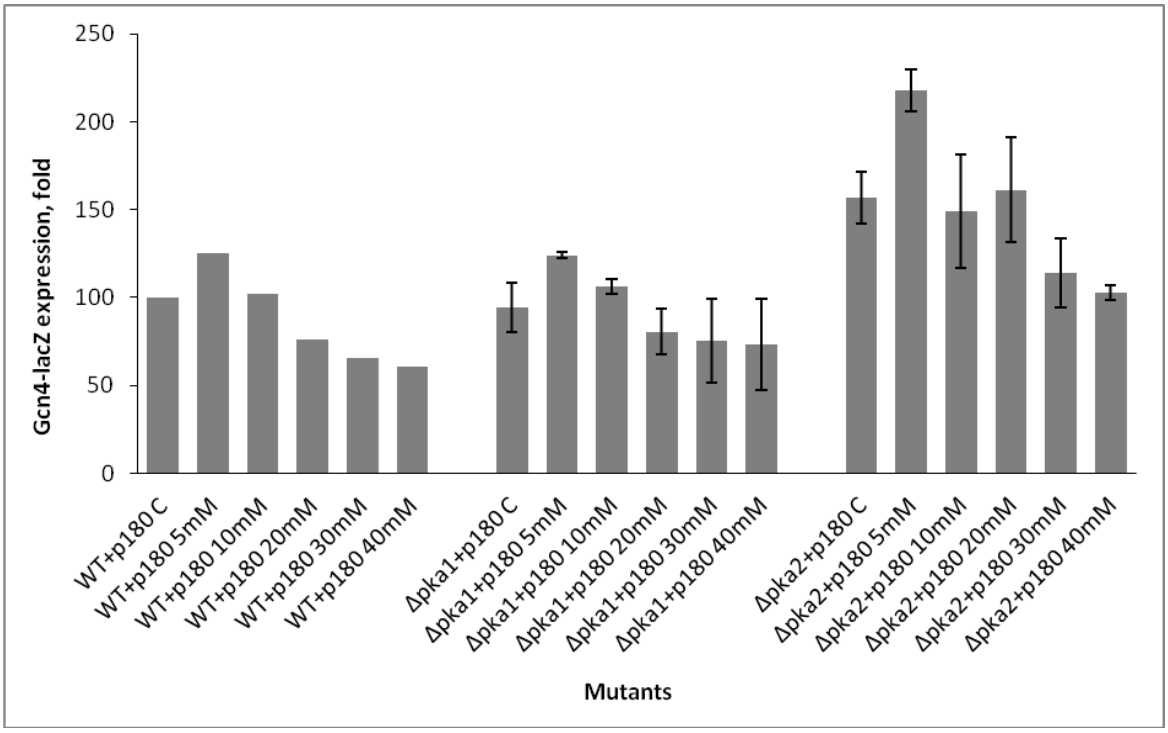


Figure 16. The changes in Gcn4-lacZ levels in WT, *Δpka1* and *Δpka2* mutants carrying p180 plasmid and treated with different concentrations of boric acid. The boric acid concentrations used in the analyses were 5mM, 10mM, 20mM, 30mM and 40mM and C indicates the boric acid nontreated control group.

As shown in Figure 16, when control gorups of WT, *Δpka1* and *Δpka2* mutants are compared to each other, the expression of Gcn4-lacZ is found to be same in *pka1Δ* cells but it increases approximately 50% in *pka2Δ* cells. After boric acid treatment wild type-like patterns of Gcn4-lacZ expressions were shown in the graph. If a pathway that can be activated by UV stress is similarly activated by boron stress, positive regulation of PKAs and the induction of Gcn4 can be evaluated as expected results. The deletion of PKA genes in the pathway can be expected to reduce the expression of Gcn4.

However a sharp decrease was not observed in Gcn4 expression levels according to β-galactosidase analyses. *ATR1* is one of the target genes of Gcn4 in boron stress response. To investigate the effects of Ras/cAMP (PKA) pathway on boron stress and on Gcn4 levels, *ATR1* mRNA expression levels in *Δpka1* and *Δpka2* mutants were observed by real time PCR analyses. The analyses were repeatedly performed at different time points using different cDNAs with at least three replicas of each sample. As a result, *ATR1* expression was similar in control cells that were not treated with boric acid whereas upon boron treatment there was an increase in *ATR1* expression in *pka1Δ* mutant (aprx 13 folds) and *pka2Δ* mutant (aprx 7 folds) (Figure 17). In this case, yeast PKAs can be considered to have regulatory roles on Atr1 in boron stress in Gcn4-dependent or –independent manner.

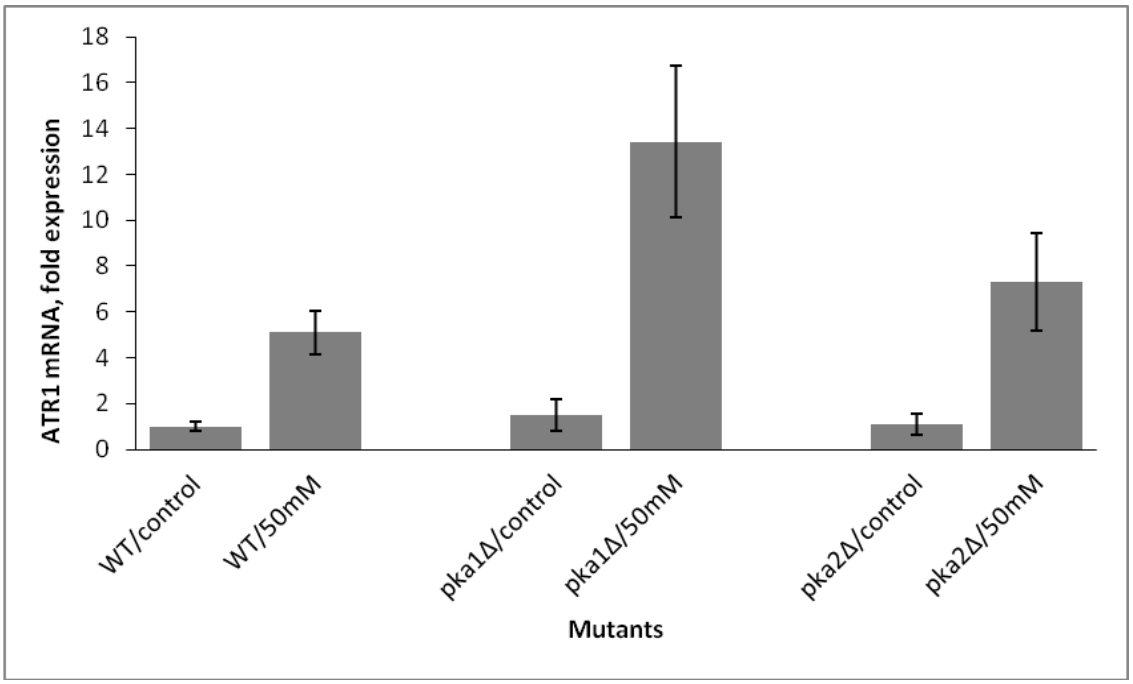


Figure 17. *ATR1* mRNA expression levels in WT, *pka1Δ*, and *pka2Δ* mutants. Normalizations were based on the Ct values of *ACT1* and *ATR1* mRNAs.

3.7. Investigation of the Activation of Uncharged tRNA Signaling Mechanism by Boron using Mass Spectrometry

Boric acid is known to activate Gcn2 kinase by creating an uncharged tRNA signal, however it is not known how this signal is created by boric acid (Uluisik, Kaya,

Fomenko, et al. 2011). It has been suggested that boron has an affinity to compounds containing cis-diol groups. Nucleotides that contain ribose cis-diol group are metabolically significant molecules and are widespread. It has been shown that boric acid can bind to ribose sugar via ester bound (Reid et al. 2004). The regions containing cis-diol on tRNAs are 2' and 3' OH groups of ribose at the acceptor end to where amino acids bind. Boric acid may bind to these groups on tRNAs so that it may inhibit the binding of amino acids. If boric acid binds to tRNAs, there will be a mass change and this change can be monitored by mass spectrometry analyses. Basic characteristics of borate-nucleotide complexes were revealed by a study in 2004 (Kim et al. 2004). By considering the information in this article, NADH which is a dinucleotide and boric acid complexes were first analysed by mass spectrometry. Electrospray ionization (ESI) mass spectrum of NADH can be observed at Figure 18. When the spectrum of NADH (Figure 18A) is compared with the spectrum of NADH-boric acid mixture (Figure 18B), in Figure 18A, a signal which belongs to NADH at 664, and signals which belong to NADH-Na and NADH-K compounds at 686 and 702, respectively can be observed. In Figure 18B, in addition to these signals another signal at around 690 which belongs to NADH-boric acid complex can be clearly seen. Thus the data from *Kim et al.* were also confirmed by these analyses (Kim et al. 2004). This confirmation is important in terms of the chemicals, method and mass spectrometer were used effectively.

A)

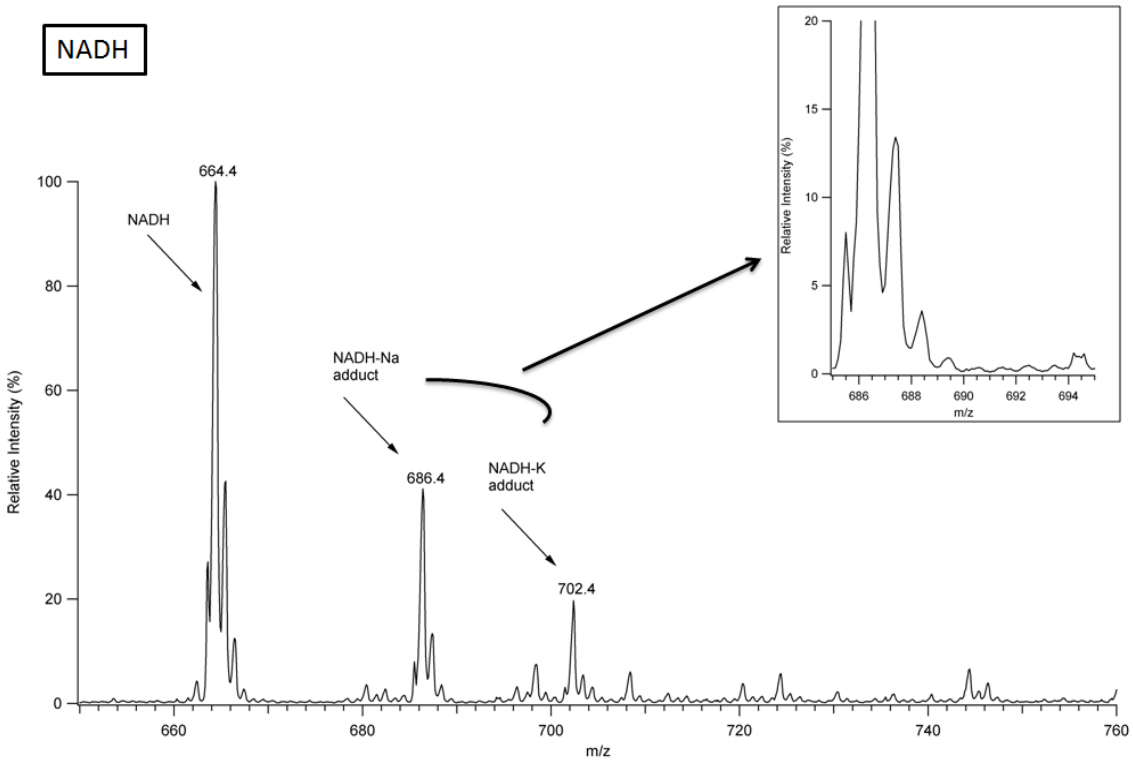


Figure 18. Mass spectrometry analyses of NADH and NADH- boric acid mixture. A) Negative ion ESI mass spectrum of NADH. B) Negative ion ESI mass spectrum of NADH-boric acid mixture.

(cont. on next page)

B)

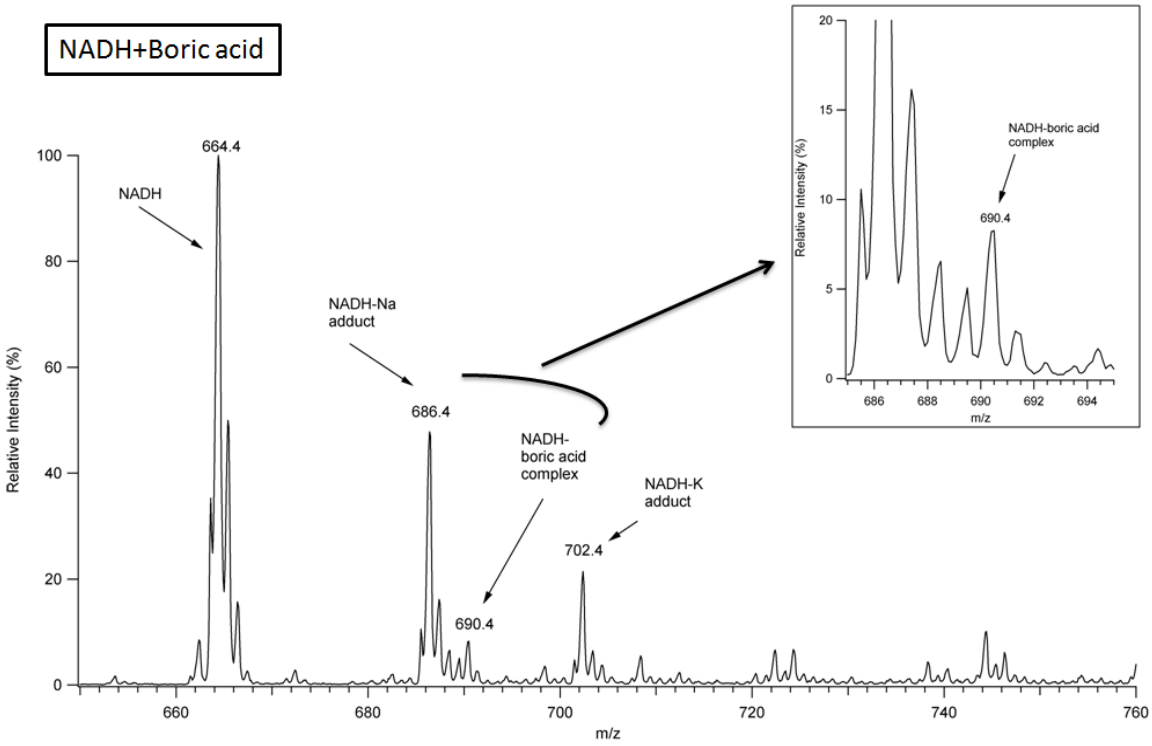


Figure 18 (cont.)

ATP or ATP-boric acid mixture was used for the ESI mass spectrometry analyses of the complex that is composed of boric acid and adenine nucleotides. The spectra in Figure 19 easily showed the signals of ATP, ATP-Na, and ATP-K compounds. However there was no additional signal which belongs to the ATP-boric acid complex (Figure 19B). *Kim et al.* observed the signals of boric acid-AMP and boric acid-ADP clearly, but a signal of boric acid-ATP was not observed. Thus it was thought that the phosphate groups on nucleotide effects the formation of the complex between boric acid and nucleotides (Kim et al. 2004). Therefore, our results seem to confirm the data of the article in question. To find out the effect of timing on analyses, ATP was first mixed with boric acid and the mixture was then incubated for 1h. However after 1h there was again no signal belonging to ATP-boric acid complex in the spectrum. To reveal whether boric acid binds to tRNAs and forms a complex with them, a commercial yeast tRNA mixture was analysed by ESI mass spectrometer. The mass spectrum of solvent and tRNA mixture can be seen in Figure 20. However the spectrum in Figure 20B is very complicated and it does not allow accurate mass measurements. Although oligonucleotides can be observed clearly by mass spectrometer, it is hard to

get a sharp signal when molecular weight or the chain length of the molecule increases. Additionally the mass spectrometry analyses of oligonucleotides and nucleic acids are blocked by cation adduct problem which arise from the phosphodiester backbone of these molecules. To obtain a good mass spectrum of nucleic acids, the cation adducts should be removed from the environment. Different methods have been developed for the elimination of the cation adducts. Ammonium acetate precipitation is one of these methods (Limbach, Crain, and McCloskey 1995).

A)

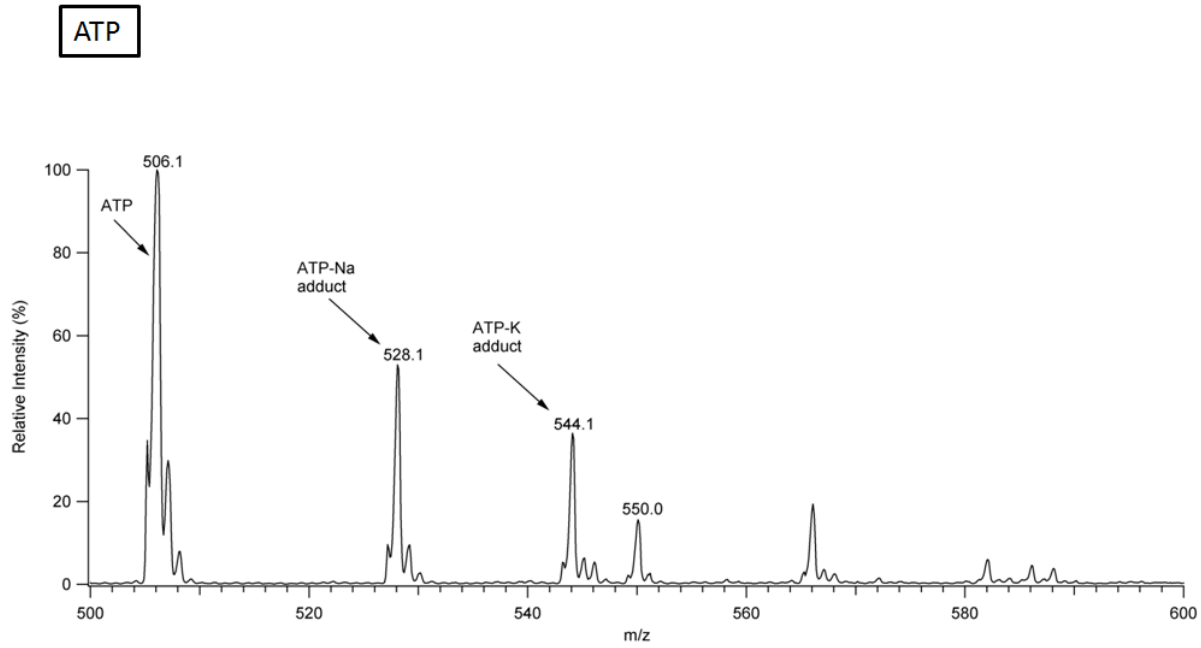


Figure 19. Mass spectrometry analyses of ATP and ATP- boric acid mixture A) Negative ion ESI mass spectrum of ATP. B) Negative ion ESI mass spectrum of ATP-boric acid mixture.

(cont. on next page)

B)

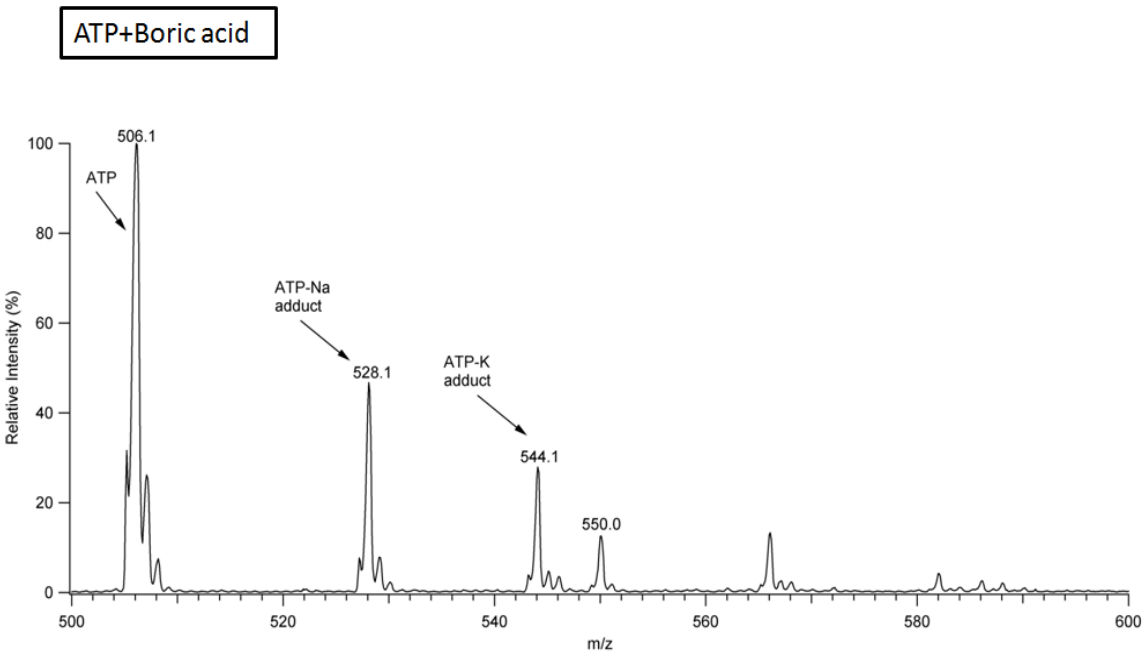


Figure 19 (cont.)

A)

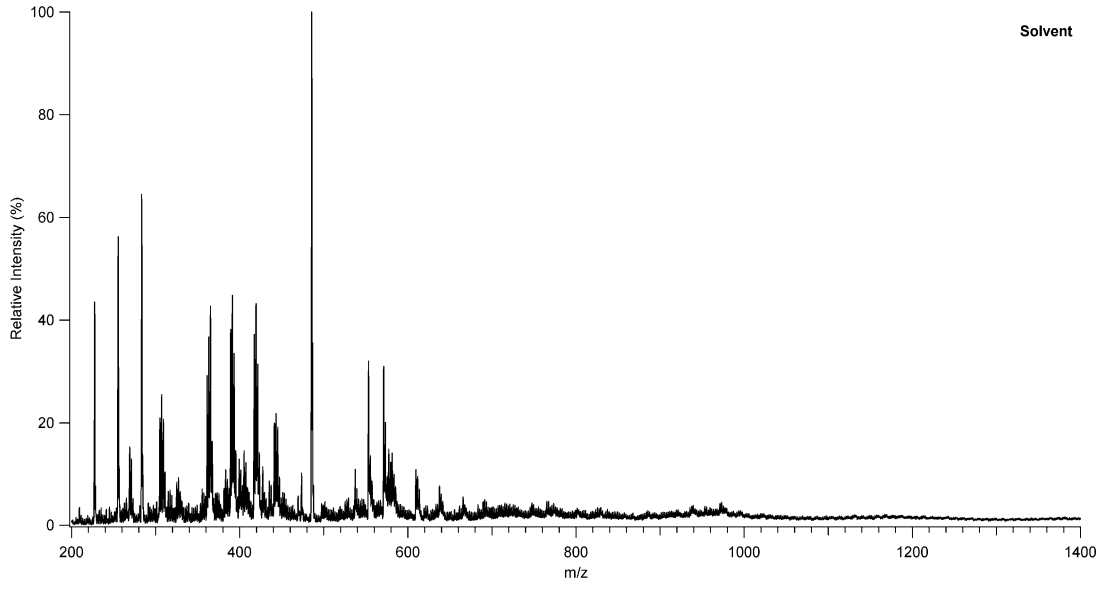


Figure 20. Mass spectrometry analyses of solvent and tRNA mixture. A) Electrospray mass spectrum of solvent. B) Electrospray mass spectrum of tRNA mixture.

(cont. on next page)

B)

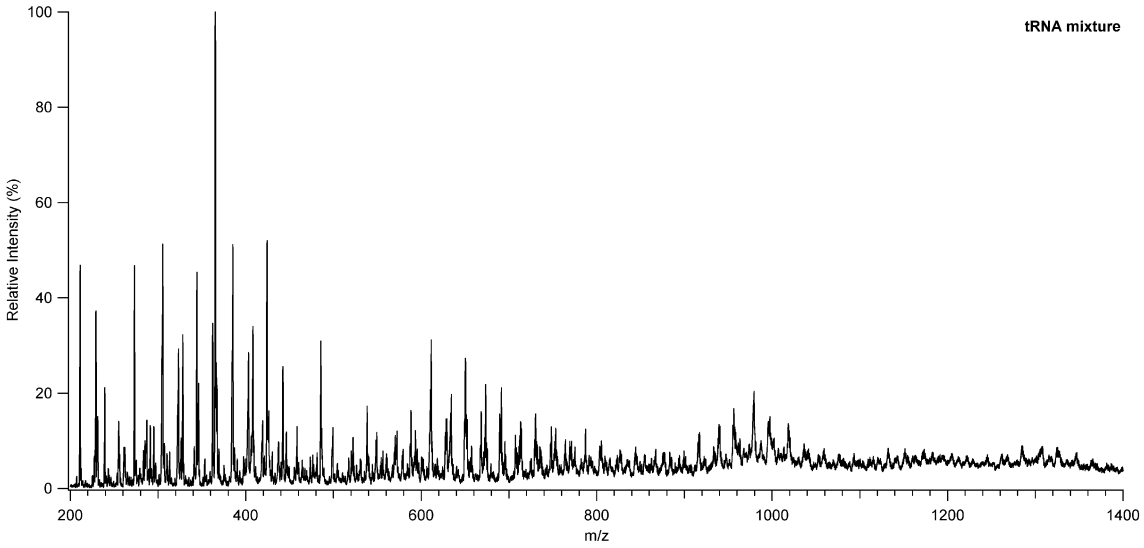


Figure 20 (cont.)

To obtain a high quality mass spectrum of tRNA mixture ammonium acetate precipitation method was used. The mass spectrum of tRNA was obtained after first, second, and third ammonium acetate precipitation. After each precipitation step, signals in spectra were developed and there was a decrease in signal to noise ratio. However the spectra have not been still useful for the correct mass measurements.

After third ammonium acetate precipitation, the resulting tRNA sample was mixed with 500 μM of boric acid and analysed directly. However, as can be seen in Figure 21, a signal related to the complex that was formed by binding of boric acid to one or more tRNA cannot be distinguished. There could be many reasons of this situation. The sample was not pure; it is composed of tRNA mixtures. The changes in signals that occur by binding of boric acid to this mixture may be monitored using an even higher resolution device. The ions in the mixture can inhibit the signal change that occurs by binding of boric acid due to their charges. For these reasons to improve the spectrum, a tRNA primer (10-mer in legnth) containing tRNA 3'-CCA tail sequence was designed and synthesized. (RNA Primer: 5' AACCGCACCA3')

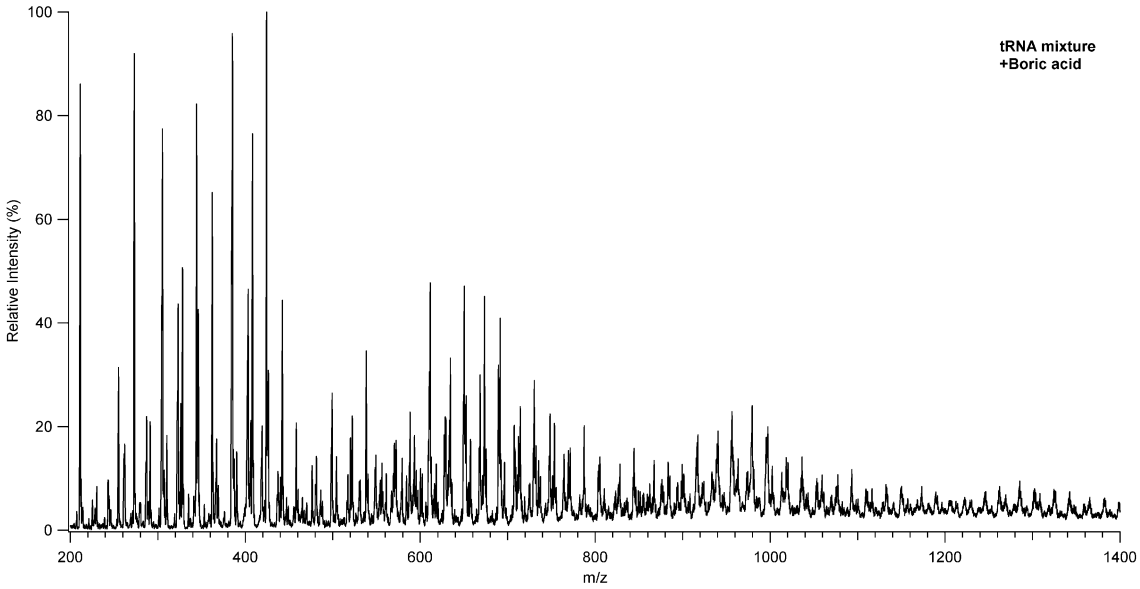


Figure 21. ESI mass spectrum of tRNA-boric acid complex after third ammonium acetate precipitation.

By using this primer Q-trap mass spectrometry analyses were performed, however a pure spectrum was not obtained. Any impurities remaining in the sample during the synthesis of primer may be suppressed or prevented the ionisation of the oligomer. Additionally, cation adduct problem (especially Na-adduct) may prevent to obtain a good spectrum of the molecule. Then it was thought that HPLC application before mass spectrometry can give more reliable results. There is an HPLC chromatogram of RNA oligomer in Figure 22. Fraction 13th was collected then treated with boric acid and used for further analyses.

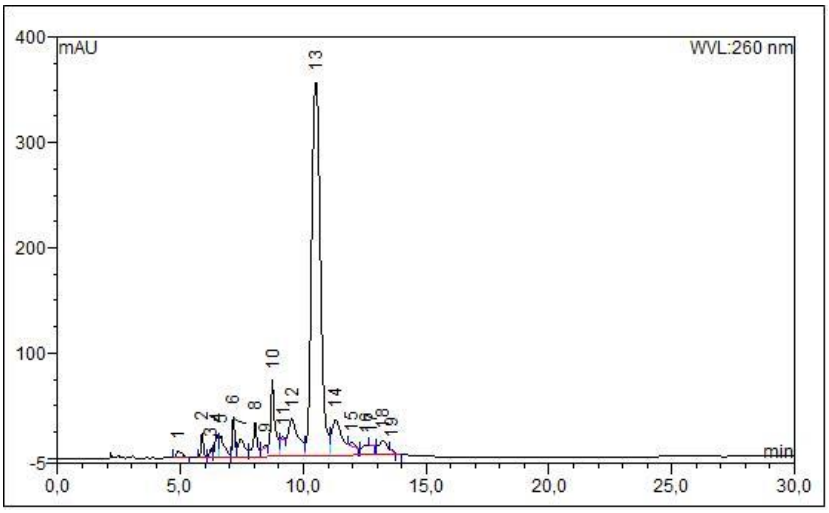
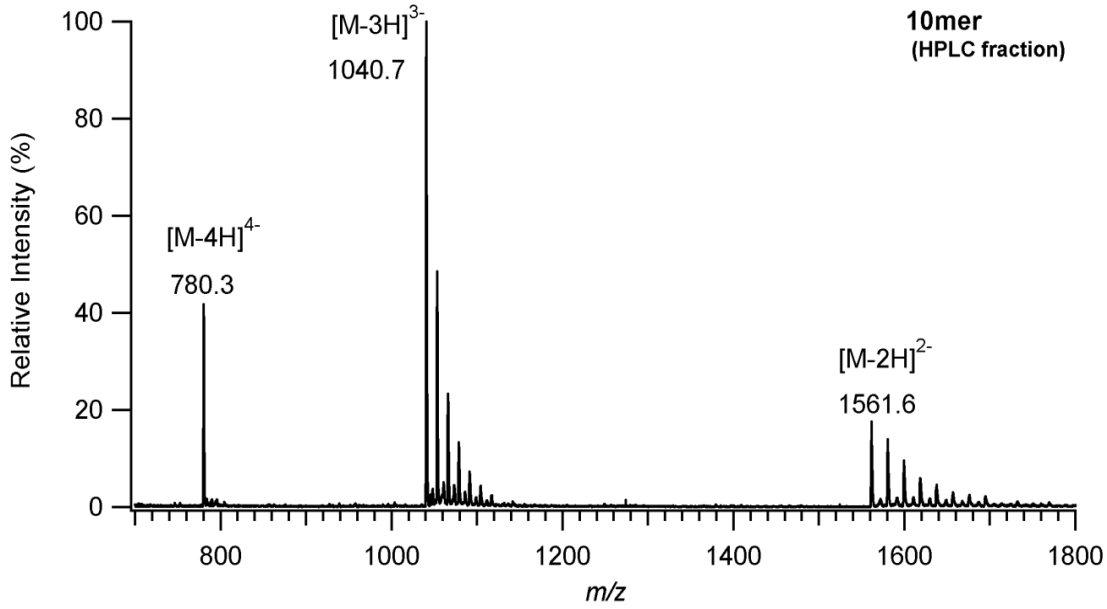


Figure 22. HPLC chromatogram of the oligomer.

In Figure 23A, ion-trap mass spectrum of the fraction belonging to the oligomer sample. During ionisation two, three, and four protons lost states of the molecule can be observed in the spectrum. Boric acid (final concentration 500 μM) was then added to this fraction and the mixture was analysed again with mass spectrometer. As can be seen in Figure 23B, the signal densities of doubly and triply charged states of the molecule were changed. This density difference in doubly and triply charged states of the molecule before and after boric acid treatment is a result of the presence of molecule in multiple charges due to adding boric acid to the environment may cause a pH change in solvent medium. However after boric acid treatment there were no signals that indicate the binding of boric acid to 10-mer RNA primer.

A)



B)

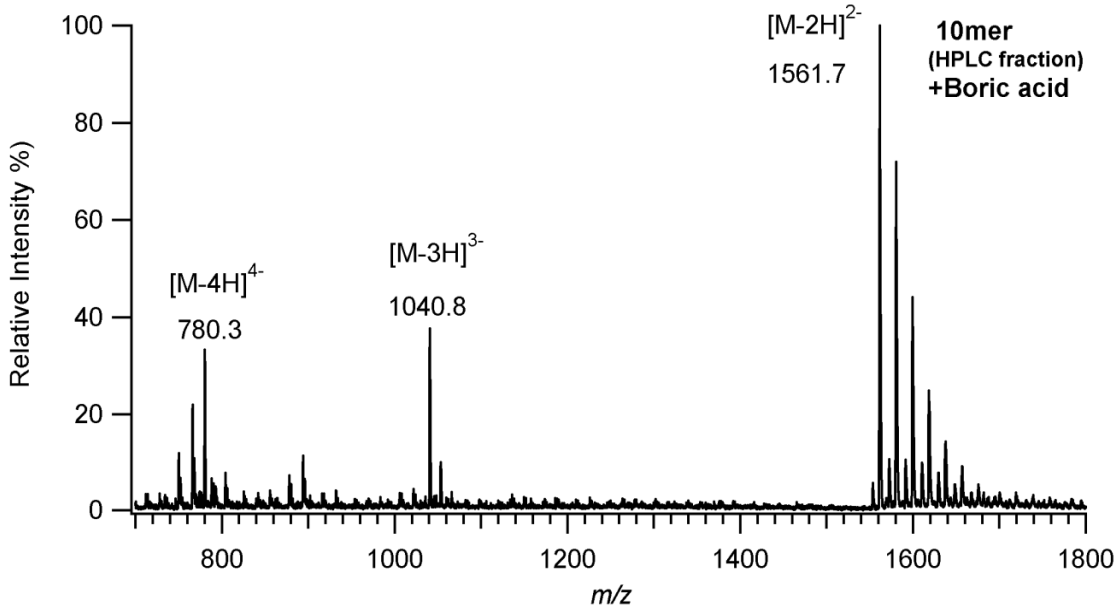


Figure 23. The analysis of purified fraction of RNA primer in ion-trap mass spectrometer. A) The mass spectrum of the fraction 13 that was collected by HPLC. B) The same fraction was treated with boric acid, then analysed, and mass spectrum was obtained.

3.8. Investigation of the Activation of Uncharged tRNA Signaling Mechanism by Boron using Northern Blot Method

Boric acid causes the activation of Gcn2 kinase in yeast cells and the phosphorylation of eIF2α accordingly, and then protein synthesis is inhibited. For Gcn2 kinase to become activated by boron, it should contain a histidyl tRNA-synthetase (m2) domain to which uncharged tRNAs bind. Some physical interactions occur between the regions on GCN2 and as a result Gcn2 becomes activated and functions as a protein kinase. If m2 domain is removed Gcn2 has lost its kinase activity in the presence of boron. Boron may activate uncharged tRNA signaling by increasing the uncharged tRNAs in the cell. However it is not known how boron creates this signaling and activates Gcn2 kinase. In certain stress conditions, it was found that anticodon regions of tRNAs were cleaved and degraded. In such conditions, Gcn2 kinase can become activated (Thompson et al. 2008). A similar mechanism may play a role in the presence of boron and tRNAs may be cleaved by Rny1 which is a specific Rnase in yeast. Thus protein synthesis will be blocked and cell will enter metabolically inactive phase and this process will allow time for recovery of conditions. To investigate whether boron stress results in the cleavage of tRNAs, RNA isolation was done from WT and *rny1*Δ mutant that were treated with boric acid and northern blot experiments were performed using tRNA-histidine and tRNA-methionine probes.

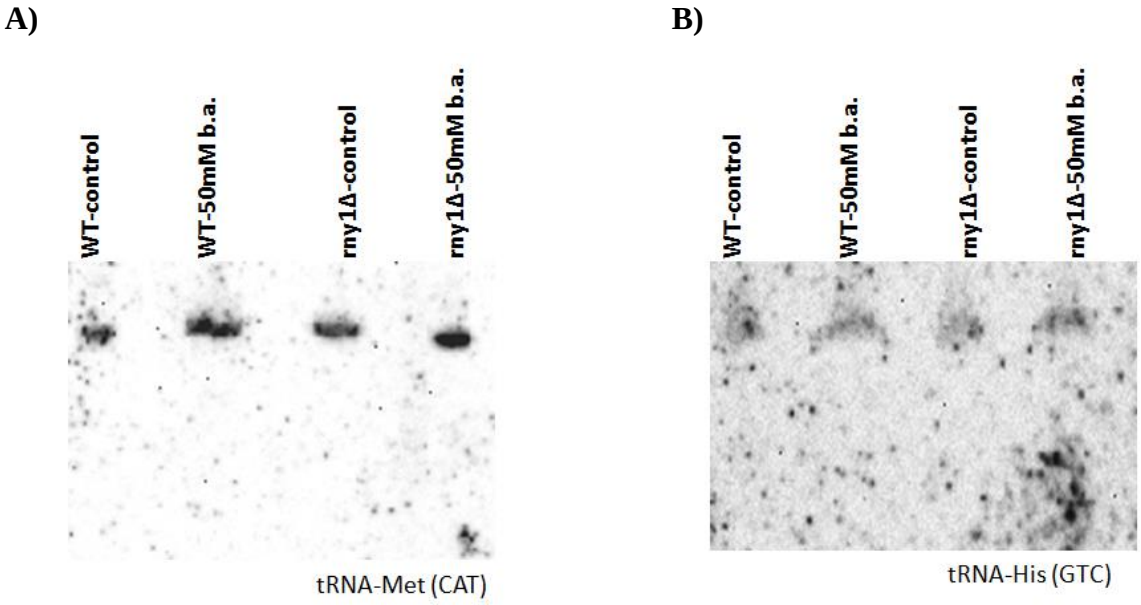


Figure 24. Northern blot analyses of total yeast RNA. Total RNA was isolated from boric acid non-treated or 50 mM 1h boric acid treated WT and rny1Δ mutant. The obtained RNAs were used in northern blot experiments. A) tRNA-Methionine (CAT) probe B) tRNA-Histidine (GTC) probe.

According to the northern blot analyses there was not a sign of tRNA cleavage in boric acid stress (Figure 24). Boron stress and oxidative stress may not have similar outcomes in this sense. Alternatively, different tRNA probes can be designed and cleavage may be examined in different tRNA species.

3.9. Investigation of the Roles of Gcn1/Gcn20 Complex in Boron Stress

Gcn1 and Gcn20 proteins are the subunits of a protein complex that activates Gcn2 kinase in amino acid starvation conditions. Gcn20 is 85 kDA and one of the members of the ABC protein family. Gcn1 is however 297 kDa protein composed of multiple segments. It is known that Gcn1 and Gcn20 interact with each other in vivo. Gcn1/Gcn20 complex also physically interact with Gcn2 by binding to the N-terminal end of the protein. This binding is necessary for the Gcn2 activation by uncharged tRNAs (Garcia-Barrio et al. 2000, Marton et al. 1997, Vazquez de Aldana, Marton, and Hinnebusch 1995). The only known eIF2α kinase in yeast is Gcn2 (Hinnebusch 1997). To investigate the possibility if boron stress is transferred to Gcn2 through *GCN1* and

GCN20 genes, Gcn2 kinase activity was examined by eIF2α phosphorylation in the mutants that lack *GCN1* and *GCN20* genes.

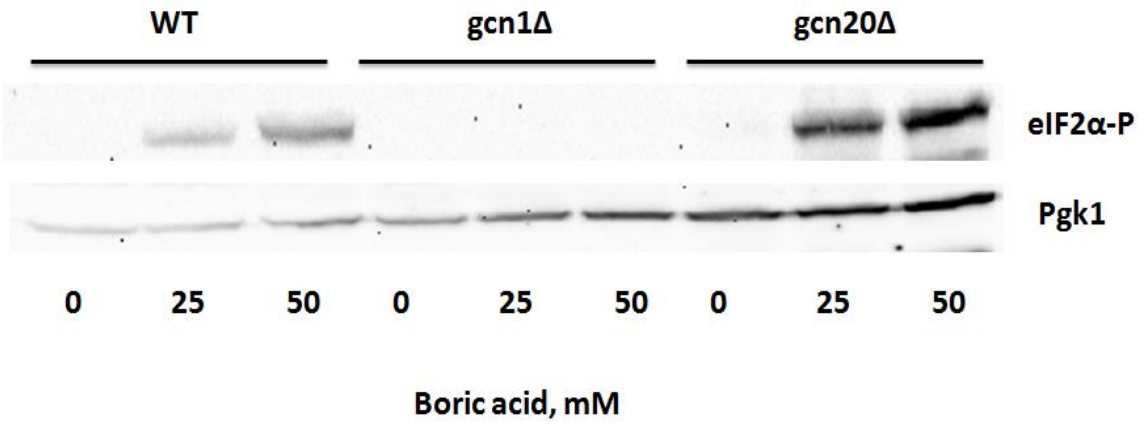


Figure 25. eIF2α phosphorylation was determined by western blot analyses. WT, *gcn1Δ* and *gcn20Δ* cells were grown and then separated into three. One group has not boric acid treatment, the other group was treated with 25 mM boric acid and another group was treated with 50 mM boric acid. The phosphorylation of eIF2α was shown by Western blot analyses using phospho-Ser51 antibody. The membrane was stripped and was treated again with the yeast Pgk1 antibody as an experimental control.

As seen in Figure 25, eIF2α was not phosphorylated in WT control cells, whereas it was phosphorylated by boron stress. The pattern was similar for g*cn20Δ* mutant; however the deletion of *GCN1* prevented eIF2α phosphorylation. It is known that *GCN1* is a positive regulator of Gcn2 kinase activity. The interaction between N-terminal region of *GCN2* and C-terminal region of *GCN1* is essential for *GCN2* function and general amino acid control mechanism. *GCN1* and *GCN2* are bound to the ribosome as *GCN1/GCN20/GCN2* complex and *GCN1* in this complex is responsible for the transfer of uncharged tRNAs from the ribosomal A site to the tRNA binding region on *GCN2* and thus it provides *GCN2* kinase becomes activated (Sattlegger and Hinnebusch 2000). According to the western blot results, *GCN1* gene is necessary for the activation of Gcn2 in boron stress.

3.10. Discovering Whether the Mechanisms of Action of Boron is Specific to Yeast Cells

To elucidate how general the mechanisms of action of boron, eIF2α phosphorylation was shown using mammalian cell culture, as well. It is known that the conditions that induce GAAC system have similar effects on yeast and mammalian cells (Hinnebusch 2005). However it is not clear whether this situation is the same for boron toxicity. The primary structure of mammalian and yeast eIF2α was similar, especially at the region of Ser51. HeLa cells which are human cervix epithelium cells were used in the experiments. Previous studies have shown that DU-145 prostate cancer cell line was found to be highly sensitive to boric acid. Their proliferation decreased 98% at day 8 by 1000 μM of boric acid treatment (Barranco and Eckhert 2004). Considering this inhibition, 0, 50, 100, 250, 500, and 1000 μM concentrations of boric acid were chosen for western blot analyses. As seen in Figure 26, eIF2α phosphorylation occurred in mammalian cells as a result of boric acid treatment. Therefore the mechanisms of action of boron toxicity are not specific to only yeast cells on the contrary it indicates in the presence of a more general mechanism.

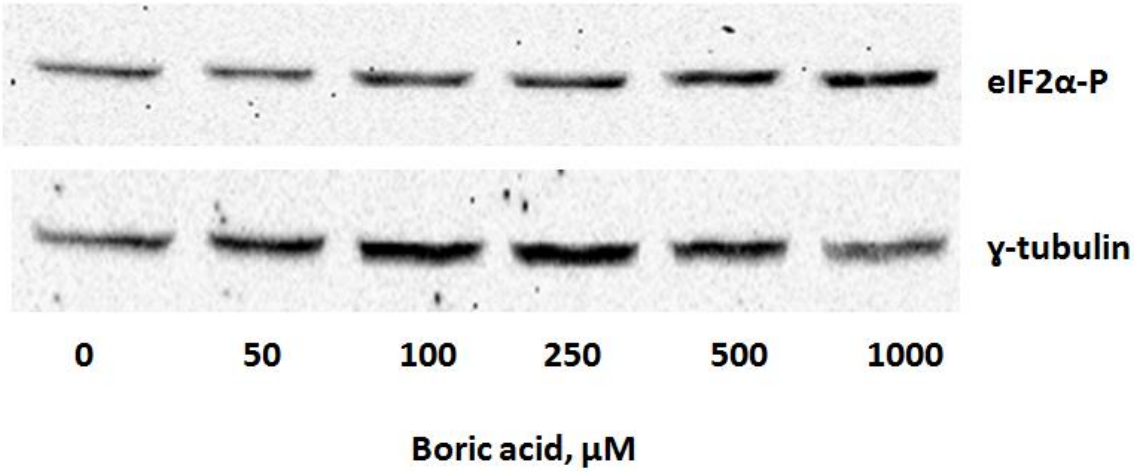


Figure 26. eIF2α is phosphorylated in HeLa cells upon boron treatment. HeLa cells were treated with indicated amounts of boric acid for 5 days. Protein amounts were normalized by Bradford analysis and same amount of protein was used for western blot experiments. The phosphorylation of eIF2α was determined by using phospho-Ser51 antibody. The membrane was then stripped and incubated with γ-tubulin antibody as control.

Figure 27 shows the bar graph obtained from the band densities of western blot of HeLa cells. eIF2α phosphorylation increased severely with the treatment of 1000μM boric acid.

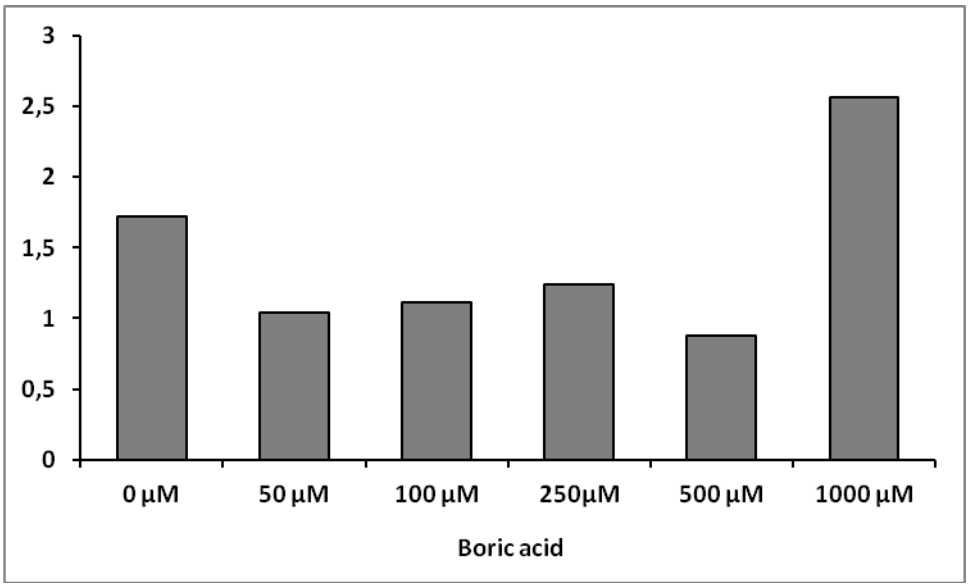


Figure 27. The normalizations of band densities that indicate the protein expression levels in HeLa cells.

CHAPTER 4

CONCLUSIONS

Boron is required for the metabolism of almost all living organisms. However it has toxic effects when present at high concentrations and the reasons of this toxicity are not known yet. To establish possible links between boron toxicity and boron stress response a genome-wide screen was conducted and some boron resistant and boron sensitive mutants were obtained that point out different pathways (Uluisik, Kaya, Unlu, et al. 2011). In this study, first of all, further investigations were carried out by using these mutants.

It is known that the genes whose absence provides boron resistance to yeast cells have roles in wobble base modifications. These modifications are necessary for efficient translation (Huang, Johansson, and Bystrom 2005). Therefore the mutants (*elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ) lacking these genes may have defects in their metabolism. It is known that Gcn4 is the major transcription factor that regulates the expression of many genes playing roles in response to various stresses. It is also known that one of the genes that are regulated by Gcn4 is *ATR1* boron efflux transporter (Uluisik, Kaya, Fomenko, et al. 2011). In this sense the upregulation of Gcn4-lacZ and *ATR1* mRNA in *elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ mutants is logical. This upregulation facilitates the efflux of boric acid, so the resistance of these mutants to boron might arise from the low boron accumulation inside the cell. However, to fully uncover the mechanisms of resistance in these mutants, the relationship between boron stress and tRNA modifications should be further investigated.

Wild type and atr1Δ cells expressing *ATR1* were shown to tolerate 225mM boric acid (Kaya et al. 2009). *elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ mutants can tolerate 150mM boric acid (Uluisik, Kaya, Unlu, et al. 2011). Interestingly the overexpression of *ATR1* in these mutants provided very strong boron resistance and superresistant mutants that can survive in the presence of 350mM boric acid were obtained. Getting such information will provide valuable outcomes from the point of view of medicine and agriculture.

When the data from yeast deletion collection screenings are interpreted, eight boron sensitive mutants were further analyzed. The cloning and overexpression of the genes whose absence makes cells extremely sensitive to boron did not provide an additional boron sensitivity or resistance to wild type and related mutants. Intracellular boron measurements showed that some of the mutants accumulate more boron inside the cells when compared with the wild type yeast whereas some of them had lower boron than wild type yeast treated with boric acid. High boron accumulation in boron sensitive mutants is an expected result and it may be one of the reasons related to boron sensitivity of the mutants. However more detailed investigation need to be carried on understanding the sensitivity mechanisms of mutants that have low amount of boron in the cell. The observations pointed out different metabolic pathways and indicate that somehow these pathways might interact with each other in boron stress response. However detailed mechanisms and factors playing roles in the response pathway still remain unclear. The link between these pathways and boron toxicity pathway need to be further investigated.

GCN4 is the main metabolic regulator in general amino acid control mechanism (GAAC) and it is known to play a role in response to boron stress (Uluisik, Kaya, Fomenko, et al. 2011). However it is not known yet which factors affect the activity of Gcn4 in boron stress. The studies with the transcription factors which can bind to GCN4 promoter showed that Gcn4-LacZ levels were reduced in mutants lacking *MGA1, STP1, GLN3, YAP1, FLO8* genes. This result indicates that these transcription factors function by activating Gcn4. However, unexpectedly, a decrease in ATR1 expression levels was not observed in these mutants. The transcription factors in question have regulatory roles in many different signaling pathways within the cell. Therefore, the absence of these genes in cells may directly affect the regulation of other signaling pathways.

To reveal the relationship between intracellular signal transduction and boron stress, we focused on TOR, SNF1 and PKA pathways. The genes analyzed in these pathways have roles in important metabolic events in the cell. Therefore the deletion of focused genes may affect not only the boron stress pathway but also the interaction of other pathways with each other. Gcn4 expression was reduced in the absence of especially TOR1 gene by boric acid treatment. Likewise ATR1 mRNA expression levels were found to be lower compared to the levels in wild-type cells and in tor1Δ and gln3Δ mutants. Therefore, these genes are considered to be important players in the boron stress response mechanism. In snf1Δ mutant Gcn4 levels were higher than wild

type whereas ATR1 expression has been found lower than wild type. In PKA pathway after boron treatment, Gcn4 levels were found to be high in pka2Δ mutant and ATR1 levels were high in pka1Δ mutant. PKA genes may be responsible for the regulation of Atr1 either Gcn4 dependent or independent in boron stress. However, to prove the truth of this assumption there is a need for additional tests.

Northern blot and mass spectrometry methods were used to investigate the uncharged tRNA signal created by boron in cells. The mass spectrometer is widely used for proteomic analysis. The attempts that have been made with nucleic acids are limited thus there are still difficulties in the optimization of this kind of trial. An observation which verifies that boric acid binds to tRNAs was not obtained according to the mass spectrometry analyses of different tRNA samples. It is known that the fragmentation of some tRNAs occurs in the adaptation of cells to different environmental stress conditions (Thompson et al. 2008). To determine whether the same situation occurs in boron stress, RNA samples were obtained from the cells treated with boric acid and northern blot experiment was performed. However, a disruption in tRNAs was not observed for the used probes in trials. Therefore, it is still unknown how boron creates an uncharged tRNA signal in the cell.

It was found that Gcn2 protein kinase requires GCN1 gene for its function in boron stress response. Additionally it was shown that eIF2α is phosphorylated in HeLa mammalian cell line by boric acid treatment. Thus, the mechanism of action of boron toxicity is not specific to yeast cells.

Boron occurs naturally in the earth, in rocks and soil and is present at high concentrations in certain regions of the World. Our country is a natural boron deposit and it has an approximately 73% boron reserves of the World (National Boron Institute, 2012). Boron has been widely used in agriculture in terms of the importance in plant growth and development as well as being used in various industrial applications. In particular, the plant growth disorders which are resulted from the lack or the excess of boron cause serious economic and agricultural losses. Thus organisms have to get sufficient amounts of boron to protect themselves from the toxic effects of it. The primary roles of boron in the organisms and the molecular mechanisms of boron toxicity are not fully clarified yet. To understand how organisms balance the boron metabolism and to develop new techniques for it, there is need for the information obtained from the molecular studies. Enlightening the boron toxicity mechanisms is of great importance especially for agricultural and medical studies. However

understanding the molecular mechanisms of the metal toxicity is a complex process since many pathways within the cell can interact with each other and they can suppress or activate one another. For this reason, it is not yet possible to reveal the cellular response to boron stress clearly. However, data obtained from this study will lead to further and detailed investigations in order to understand the molecular mechanisms underlying boron toxicity.

REFERENCES

Ahmed, I., A. Yokota, and T. Fujiwara. 2007. "A novel highly boron tolerant bacterium, Bacillus boroniphilus sp nov., isolated from soil, that requires boron for its growth." *Extremophiles* 11**:**217-224. doi: 10.1007/s00792-006-0027-0.

Ali, Shimaa E., Even Thoen, Oystein Evensen, Jannicke Wiik-Nielsen, Amr A. A. Gamil, and Ida Skaar. 2014. "Mitochondrial Dysfunction Is Involved in the Toxic Activity of Boric Acid against Saprolegnia." *Plos One* no. 9 (10). doi: 10.1371/journal.pone.0110343.

Amin, Shady A., Frithjof C. Kupper, David H. Green, Wesley R. Harris, and Carl J. Carrano. 2007. "Boron binding by a siderophore isolated from marine bacteria associated with the toxic dinoflagellate Gymnodinium catenatum." *Journal of the American Chemical Society* no. 129 (3):478-479. doi: 10.1021/ja067369u.

Anderson, G.R., and Jordan, J.V. 1961. "Boron: A non-essential growth factor for Azotobacter chroococcum." *Soil Science* 92 (2):113-116.

Armstrong, T. A., W. L. Flowers, J. W. Spears, and F. H. Nielsen. 2002. "Long-term effects of boron supplementation on reproductive characteristics and bone mechanical properties in gilts." *Journal of Animal Science* no. 80 (1):154-161.

Baker, Stephen J., John W. Tomsho, and Stephen J. Benkovic. 2011. "Boron-containing inhibitors of synthetases." *Chemical Society Reviews* no. 40 (8):4279-4285. doi: 10.1039/c0cs00131g.

Barr, R. D., W. B. Clarke, R. M. Clarke, J. Venturelli, G. R. Norman, and R. G. Downing. 1993. "Regulation of lithium and boron levels in normal human blood- environmental and genetic considerations." *Journal of Laboratory and Clinical Medicine* no. 121 (4):614-619.

Barranco, W. T., and C. D. Eckhert. 2004. "Boric acid inhibits human prostate cancer cell proliferation." *Cancer Letters* no. 216 (1):21-29. doi: 10.1016/j.canlet.2004.06.001.

Barranco, W. T., and C. D. Eckhert. 2006. "Cellular changes in boric acid-treated DU-145 prostate cancer cells." *British Journal of Cancer* no. 94 (6):884-890. doi: 10.1038/sj.bjc.6603009.

Barranco, Wade T., Danny H. Kim, Salvatore L. Stella, Jr., and Curtis D. Eckhert. 2009. "Boric acid inhibits stored Ca2+ release in DU-145 prostate cancer cells." *Cell Biology and Toxicology* no. 25 (4):309-320. doi: 10.1007/s10565-008-9085-7.

Bauer, C. A., and Petterss.G. 1974. "Effect of boric-acid on catalytic activity of Streptomyces griseus protease 3." *European Journal of Biochemistry* no. 45 (2):473-477. doi: 10.1111/j.1432-1033.1974.tb03572.x.

Beck, T., and M. N. Hall. 1999. "The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors." *Nature* no. 402 (6762):689-692.

Benderdour, M., T. Van Bui, K. Hess, A. Dicko, F. Belleville, and B. Dousset. 2000. "Effects of boron derivatives on extracellular matrix formation." *Journal of Trace Elements in Medicine and Biology* no. 14 (3):168-173. doi: 10.1016/s0946-672x(00)80006-1.

Bennett, A., R. I. Rowe, N. Soch, and C. D. Eckhert. 1999. "Boron stimulates yeast (Saccharomyces cerevisiae) growth." *Journal of Nutrition* no. 129 (12):2236-2238.

Bertram, P. G., J. H. Choi, J. Carvalho, T. F. Chan, W. D. Ai, and X. F. S. Zheng. 2002. "Convergence of TOR-nitrogen and Snf1-glucose signaling pathways onto Gln3." *Molecular and Cellular Biology* no. 22 (4):1246-1252. doi: 10.1128/mcb.22.4.1246-1252.2002.

Bolanos, L., K. Lukaszewski, I. Bonilla, and D. Blevins. 2004. "Why boron?" *Plant Physiology and Biochemistry* no. 42 (11):907-912. doi: 10.1016/j.plaphy.2004.11.002.

Bonilla, I., M. Garciagonzalez, and P. Mateo. 1990. "Boron requirement in Cyanobacteria- its possible role in the early evolution of photosynthetic organisms." *Plant Physiology* no. 94 (4):1554-1560. doi: 10.1104/pp.94.4.1554.

Bozdag, Gonensin Ozan, Irem Uluisik, Gulce Sila Gulculer, Huseyin C. Karakaya, and Ahmet Koc. 2011. "Roles of ATR1 paralogs YMR279c and YOR378w in boron stress tolerance." *Biochemical and Biophysical Research Communications* no. 409 (4):748-751. doi: 10.1016/j.bbrc.2011.05.080.

Brown, P. H., and B. J. Shelp. 1997. "Boron mobility in plants." *Plant and Soil* no. 193 (1-2):85-101. doi: 10.1023/a:1004211925160.

Camacho-Cristobal, J. J., J. Rexach, and A. Gonzalez-Fontes. 2008. "Boron in plants: Deficiency and toxicity." *Journal of Integrative Plant Biology* no. 50 (10):1247-1255. doi: 10.1111/j.1744-7909.2008.00742.x.

Canon, Paola, Felipe Aquea, Amparo Rodriguez-Hoces de la Guardia, and Patricio Arce-Johnson. 2013. "Functional characterization of Citrus macrophylla BOR1 as a boron transporter." *Physiologia Plantarum* no. 149 (3):329-339. doi: 10.1111/ppl.12037.

Carlson, M. 1999. "Glucose repression in yeast." *Current Opinion in Microbiology* no. 2 (2):202-207. doi: 10.1016/s1369-5274(99)80035-6.

Chapin, R. E., W. W. Ku, M. A. Kenney, and H. McCoy. 1998. "The effects of dietary boric acid on bone strength in rats." *Biological Trace Element Research* no. 66 (1-3):395-399. doi: 10.1007/bf02783150.

Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson. 2002. "Structural identification of a bacterial quorum-sensing signal containing boron." *Nature* no. 415 (6871):545-549.

Cherkasova, V. A., and A. G. Hinnebusch. 2003. "Translational control by TOR and TAP42 through dephosphorylation of eIF2 alpha kinase GCN2." *Genes & Development* no. 17 (7):859-872. doi: 10.1101/gad.1069003.

Ciofani, Gianni, Serena Danti, Simone Nitti, Barbara Mazzolai, Virgilio Mattoli, and Mario Giorgi. 2013. "Biocompatibility of boron nitride nanotubes: An up-date of in vivo toxicological investigation." *International Journal of Pharmaceutics* no. 444 (1-2):85-88. doi: 10.1016/j.ijpharm.2013.01.037.

Ciofani, Gianni, Vittoria Raffa, Arianna Menciassi, and Alfred Cuschieri. 2008. "Cytocompatibility, Interactions, and Uptake of Polyethyleneimine-Coated Boron Nitride Nanotubes by Living Cells: Confirmation of Their Potential for Biomedical Applications." *Biotechnology and Bioengineering* no. 101 (4):850-858. doi: 10.1002/bit.21952.

Cui, Y., M. I. Winton, Z. F. Zhang, C. Rainey, J. Marshall, J. B. De Kernion, and C. D. Eckhert. 2004. "Dietary boron intake and prostate cancer risk." *Oncology Reports* no. 11 (4):887-892.

Dannel, F., H. Pfeffer, and V. Romheld. 1998. "Compartmentation of boron in roots and leaves of sunflower as affected by boron supply." *Journal of Plant Physiology* no. 153 (5-6):615-622.

De Virgilio, Claudio, and Robbie Loewith. 2006. "The TOR signalling network from yeast to man." *International Journal of Biochemistry & Cell Biology* no. 38 (9):1476-1481. doi: 10.1016/j.biocel.2006.02.013.

Dell, B., and L. B. Huang. 1997. "Physiological response of plants to low boron." *Plant and Soil* no. 193 (1-2):103-120. doi: 10.1023/a:1004264009230.

Demirci, S., Dogan, A., Karakus, E., Halici, Z., Topcu, A., Demirci, E., and Sahin, F. 2015. "Boron and poloxamer (F68 and F127) containing hydrogel formulation for burn wound healing." *Biological Trace Element Research*, 1-12. doi: 10.1007/s12011-015-0338-z.

Devirian, T. A., and S. L. Volpe. 2003. "The physiological effects of dietary boron." *Critical Reviews in Food Science and Nutrition* no. 43 (2):219-231. doi: 10.1080/10408690390826491.

Dordas, C., and P. H. Brown. 2000. "Permeability of boric acid across lipid bilayers and factors affecting it." *Journal of Membrane Biology* no. 175 (2):95-105. doi: 10.1007/s002320001058.

Dzondo-Gadet, M., R. Mayap-Nzietchueng, K. Hess, P. Nabet, F. Belleville, and B. Dousset. 2002. "Action of boron at the molecular level - Effects on transcription and translation in an acellular system." *Biological Trace Element Research* no. 85 (1):23-33. doi: 10.1385/bter:85:1:23.

Eckhert, C. D. 1998. "Boron stimulates embryonic trout Growth." *Journal of Nutrition* no. 128 (12):2488-2493.

Engelberg, D., C. Klein, H. Martinetto, K. Struhl, and M. Karin. 1994. "The UV response involving the Ras signaling pathway ans AP-1 transcription factors is conserved between yeast and mammals." *Cell* no. 77 (3):381-390.

Fort, D. J., T. L. Propst, E. L. Stover, P. L. Strong, and F. J. Murray. 1998. "Adverse reproductive and developmental effects in Xenopus from insufficient boron." *Biological Trace Element Research* no. 66 (1-3):237-259. doi: 10.1007/bf02783141.

Frohloff, F., L. Fichtner, D. Jablonowski, K. D. Breunig, and R. Schaffrath. 2001. "Saccharomyces cerevisiae Elongator mutations confer resistance to the Kluyveromyces lactis zymocin." *Embo Journal* no. 20 (8):1993-2003. doi: 10.1093/emboj/20.8.1993.

Gallardo-Williams, M. T., R. E. Chapin, P. E. King, G. J. Moser, T. L. Goldsworthy, J. P. Morrison, and R. R. Maronpot. 2004. "Boron supplementation inhibits the growth and local expression of IGF-1 in human prostate adenocarcinoma (LNCaP) tumors in nude mice." *Toxicologic Pathology* no. 32 (1):73-78. doi: 10.1080/01926230490260899.

Gallardo-Williams, M. T., R. R. Maronpot, C. H. Turner, C. S. Johnson, M. W. Harris, M. J. Jayo, and R. E. Chapin. 2003. "Effects of boric acid supplementation on bone histomorphometry, metabolism, and biomechanical properties in aged female F-344 rats." *Biological Trace Element Research* no. 93 (1-3):155-169. doi: 10.1385/bter:93:1-3:155.

Garcia-Barrio, M., J. S. Dong, S. Ufano, and A. G. Hinnebusch. 2000. "Association of GCN1-GCN20 regulatory complex with the N-terminus of elF2 alpha kinase GCN2 is required for GCN2 activation." *Embo Journal* no. 19 (8):1887-1899. doi: 10.1093/emboj/19.8.1887.

Gbelska, Y., J. J. Krijger, and K. D. Breunig. 2006. "Evolution of gene families: the multidrug resistance transporter genes in five related yeast species." *Fems Yeast Research* no. 6 (3):345-355. doi: 10.1111/j.1567-1364.2006.00058.x.

Gietz, R. D., and R. H. Schiestl. 1995. "Transforming yeast with DNA." *Methods in Molecular and Cellular Biology* no. 5 (5):255-269.

Goldbach, H. E., and M. A. Wimmer. 2007. "Boron in plants and animals: Is there a role beyond cell-wall structure?" *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* no. 170 (1):39-48. doi: 10.1002/jpln.200625161.

Hakki, Sema S., Buket S. Bozkurt, and Erdogan E. Hakki. 2010. "Boron regulates mineralized tissue-associated proteins in osteoblasts (MC3T3-E1)." *Journal of Trace Elements in Medicine and Biology* no. 24 (4):243-250. doi: 10.1016/j.jtemb.2010.03.003.

Harbison, C. T., D. B. Gordon, T. I. Lee, N. J. Rinaldi, K. D. Macisaac, T. W. Danford, N. M. Hannett, J. B. Tagne, D. B. Reynolds, J. Yoo, E. G. Jennings, J. Zeitlinger, D. K. Pokholok, M. Kellis, P. A. Rolfe, K. T. Takusagawa, E. S. Lander, D. K. Gifford, E. Fraenkel, and R. A. Young. 2004. "Transcriptional regulatory code of a eukaryotic genome." *Nature* no. 431 (7004):99-104. doi: 10.1038/nature02800.

Heindel, J. J., C. J. Price, E. A. Field, M. C. Marr, C. B. Myers, R. E. Morrissey, and B. A. Schwetz. 1992. "Developmental toxicity of boric-acid in mice and rats." *Fundamental and Applied Toxicology* no. 18 (2):266-277. doi: 10.1016/0272-0590(92)90055-m.

Henderson, Kimberly, Salvatore L. Stella, Jr., Sarah Kobylewski, and Curtis D. Eckhert. 2009. "Receptor Activated Ca2+ Release Is Inhibited by Boric Acid in Prostate Cancer Cells." *Plos One* no. 4 (6). doi: 10.1371/journal.pone.0006009.

Hinnebusch, A. G. 1985. "A hierarchy of trans-acting factors modulates translation of an activator of amino acid biosynthetic genes in Saccharomyces cerevisiae." *Mol Cell Biol* no. 5 (9):2349-60.

Hinnebusch, A. G. 1997. "Translational regulation of yeast GCN4 - A window on factors that control initiator-tRNA binding to the ribosome." *Journal of Biological Chemistry* no. 272 (35):21661-21664.

Hinnebusch, A. G. 2005. "Translational regulation of GCN4 and the general amino acid control of yeast." *Annual Review of Microbiology* no. 59:407-450. doi: 10.1146/annurev.micro.59.031805.133833.

Hu, H. N., and P. H. Brown. 1997. "Absorption of boron by plant roots." *Plant and Soil* no. 193 (1-2):49-58. doi: 10.1023/a:1004255707413.

Hu, Qianqian, Shenghe Li, Enmei Qiao, Zhongtao Tang, Erhui Jin, Guangming Jin, and Youfang Gu. 2014. "Effects of Boron on Structure and Antioxidative Activities of Spleen in Rats." *Biological Trace Element Research* no. 158 (1):73-80. doi: 10.1007/s12011-014-9899-5.

Huang, B., M. J. O. Johansson, and A. S. Bystrom. 2005. "An early step in wobble uridine tRNA modification requires the Elongator complex." *Rna-a Publication of the Rna Society* no. 11 (4):424-436. doi: 10.1261/rna.7247705.

Huang, Bo, Jian Lu, and Anders S. Bystroem. 2008. "A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in Saccharomyces cerevisiae." *Rna-a Publication of the Rna Society* no. 14 (10):2183-2194. doi: 10.1261/rna.1184108.

Hunt, C. D. 1996. "Biochemical effects of physiological amounts of dietary boron." *Journal of Trace Elements in Experimental Medicine* no. 9 (4):185-213. doi: 10.1002/(sici)1520-670x(1996)9:4<185::aid-jtra6>3.0.co;2-q.

Hunt, C. D. 1998a. "Dietary boron enhances the beneficial effects of exercise training on bone calcium, phosphorus, and magnesium concentrations in the rat." *Faseb Journal* no. 12 (4):A205-A205.

Hunt, C. D. 1998b. "Regulation of enzymatic activity - One possible role of dietary boron in higher animals and humans." *Biological Trace Element Research* no. 66 (1-3):205-225. doi: 10.1007/bf02783139.

Hunt, C. D. 2003. "Dietary boron: An overview of the evidence for its role in immune function." *Journal of Trace Elements in Experimental Medicine* no. 16 (4):291-306. doi: 10.1002/jtra.10041.

Hunt, C. D., and F. H. Nielsen. 1987. "Effects of dietary boron on bone calcification in magnesium and cholecalciferol deficient chicks." *Federation Proceedings* no. 46 (3):754-754.

Hutter, R., Keller-Schien, W., Knusel, F., Prelog, V., Rodgers jr., G.C., Suter, P., Vogel, G., Voser, W., and Zahner, H. 1967. "Stoffwechselprodukte von Mikroorganismen. 57. Mitteilung. Boromycin." *Helvetica Chimica Acta*, 50 (6):1533-1539. doi: 10.1002/hlca.19670500612.

Iavazzo, Christos, Ioannis D. Gkegkes, Ioanna M. Zarkada, and Matthew E. Falagas. 2011. "Boric Acid for Recurrent Vulvovaginal Candidiasis: The Clinical Evidence." *Journal of Womens Health* no. 20 (8):1245-1255. doi: 10.1089/jwh.2010.2708.

Irschik, H., D. Schummer, K. Gerth, G. Hofle, and H. Reichenbach. 1995. "The tartrolons, new boron-containing antibiotics from a Myxobacterium, Sorangium cellulosum." *Journal of Antibiotics* no. 48 (1):26-30.

Ishigami, M., S. Aloni, and A. Zettl. 2003. Properties of boron nitride nanotubes. Paper read at 12th International Conference on Scanning Tunneling Microscopy/Spectroscopy and Related Techniques, 2003 Jul 21-25, at Eindhoven, Netherlands.

Ishii, T., and T. Matsunaga. 2003. "Chemical structure and function of the plant cell wall polysaccharide rhamnogalacturonan II." *Mokuzai Gakkaishi* no. 49 (3):153-160.

Kato, Y., K. Miwa, J. Takano, M. Wada, and T. Fujiwara. 2009. "Highly Boron Deficiency-Tolerant Plants Generated by Enhanced Expression of NIP5;1, a Boric Acid Channel." *Plant and Cell Physiology* no. 50 (1):58-66. doi: 10.1093/pcp/pcn168.

Kaya, A., H. C. Karakaya, D. E. Fomenko, V. N. Gladyshev, and A. Koc. 2009. "Identification of a Novel System for Boron Transport: Atr1 Is a Main Boron Exporter in Yeast." *Molecular and Cellular Biology* no. 29 (13):3665-3674. doi: 10.1128/mcb.01646-08.

Kettner, C. A., and A. B. Shenvi. 1984. "Inhibition of the serine proteases leukocyte elastase, pancreatic elastase, cathepsin-G, and chymotrypsin by peptide boronic acids." *Journal of Biological Chemistry* no. 259 (24):5106-5114.

Kim, D. H., K. F. Faull, A. J. Norris, and C. D. Eckhert. 2004. "Borate-nucleotide complex formation depends on charge and phosphorylation state." *Journal of Mass Spectrometry* no. 39 (7):743-751. doi: 10.1002/jms.645.

Kim, D. H., B. N. Marbois, K. F. Faull, and C. D. Eckhert. 2003. "Esterification of borate with NAD(+) and NADH as studied by electrospray ionization mass spectrometry and B-11 NMR spectroscopy." *Journal of Mass Spectrometry* no. 38 (6):632-640. doi: 10.1002/jms.476.

Kimbal, S.R. 1999. "Eukaryotic initiation factor eIF2." *The International Journal of Biochemistry & Cell Biology* 31(1):25-29. doi:10.1016/S1357-2725(98)00128-9.

Kobayashi, M., T. Matoh, and J. Azuma. 1996. "Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls." *Plant Physiology* no. 110 (3):1017-1020.

Kobayashi, M., T. Mutoh, and T. Matoh. 2004. "Boron nutrition of cultured tobacco BY-2 cells. IV. Genes induced under low boron supply." *Journal of Experimental Botany* no. 55 (401):1441-1443. doi: 10.1093/jxb/erh142.

Koshiba, Taichi, Masaru Kobayashi, and Toru Matoh. 2009. "Boron Nutrition of Tobacco BY-2 Cells. V. Oxidative Damage is the Major Cause of Cell Death Induced by Boron Deprivation." *Plant and Cell Physiology* no. 50 (1):26-36. doi: 10.1093/pcp/pcn184.

Kot, F.S. 2009. "Boron sources, speciation and its potential impact ob health." *Reviews in Environmental Science and Bio/Technology* 8(1):3-28. doi: 10.1007/s11157-008-9140-0.

Kouri, M., L. Kankaanranta, T. Seppala, L. Tervo, M. Rasilainen, H. Minn, I. Eskola, J. Vahatalo, A. Paetau, S. Savolainen, I. Auterinen, J. Jaaskelainen, and H. Joensuu. 2004. "Undifferentiated sinonasal carcinoma may respond to single-fraction boron neutron capture therapy." *Radiotherapy and Oncology* no. 72 (1):83-85. doi: 10.1016/j.radonc.2004.03.016.

Ku, W. W., R. E. Chapin, R. F. Moseman, R. E. Brink, K. D. Pierce, and K. Y. Adams. 1991. "Tissue disposition of boron in male Fischer rats." *Toxicology and Applied Pharmacology* no. 111 (1):145-151. doi: 10.1016/0041-008x(91)90143-3.

Lanoue, L., M. W. Taubeneck, J. Muniz, L. A. Hanna, P. L. Strong, F. J. Murray, F. H. Nielsen, C. D. Hunt, and C. L. Keen. 1998. "Assessing the effects of low boron diets on embryonic and fetal development in rodents using in vitro and in vivo model systems." *Biological Trace Element Research* no. 66 (1-3):271-298. doi: 10.1007/bf02783143.

Lee, T. I., N. J. Rinaldi, F. Robert, D. T. Odom, Z. Bar-Joseph, G. K. Gerber, N. M. Hannett, C. T. Harbison, C. M. Thompson, I. Simon, J. Zeitlinger, E. G. Jennings, H. L. Murray, D. B. Gordon, B. Ren, J. J. Wyrick, J. B. Tagne, T. L. Volkert, E. Fraenkel, D. K. Gifford, and R. A. Young. 2002. "Transcriptional regulatory networks in Saccharomyces cerevisiae." *Science* no. 298 (5594):799-804. doi: 10.1126/science.1075090.

Lemire, Joseph A., Joe J. Harrison, and Raymond J. Turner. 2013. "Antimicrobial activity of metals: mechanisms, molecular targets and applications." *Nature Reviews Microbiology* no. 11 (6):371-384. doi: 10.1038/nrmicro3028.

Limbach, P. A., P. F. Crain, and J. A. McCloskey. 1995. "Molecular-mass measurement of intact ribonucleic-acids via electrospray-ionization quadrupole mass-spectrometry." *Journal of the American Society for Mass Spectrometry* no. 6 (1):27-39. doi: 10.1016/1044-0305(94)00086-f.

Loewith, Robbie, and Michael N. Hall. 2011. "Target of Rapamycin (TOR) in Nutrient Signaling and Growth Control." *Genetics* no. 189 (4):1177-1201. doi: 10.1534/genetics.111.133363.

Lovatt, C. J., and L. M. Bates. 1984. "Early effects of excess boron on photosynthesis and growth of Cucurbito pepo." *Journal of Experimental Botany* no. 35 (152):297-305. doi: 10.1093/jxb/35.3.297.

Mahabir, S., M. R. Spitz, S. L. Barrera, Y. Q. Dong, C. Eastham, and M. R. Forman. 2008. "Dietary boron and hormone replacement therapy as risk factors for lung cancer in women." *American Journal of Epidemiology* no. 167 (9):1070-1080. doi: 10.1093/aje/kwn021.

Martinez-Cuenca, Mary-Rus, Belen Martinez-Alcantara, Ana Quiones, Marta Ruiz, Domingo J. Iglesias, Eduardo Primo-Millo, and M. Angeles Forner-Giner. 2015. "Physiological and Molecular Responses to Excess Boron in Citrus macrophylla W." *Plos One* no. 10 (7). doi: 10.1371/journal.pone.0134372.

Marton, M. J., C. R. V. deAldana, H. F. Qiu, K. Chakraburtty, and A. G. Hinnebusch. 1997. "Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2 alpha kinase GCN2." *Molecular and Cellular Biology* no. 17 (8):4474-4489.

Miwa, K., J. Takano, and T. Fujiwara. 2006. "Improvement of seed yields under boron-limiting conditions through overexpression of BOR1, a boron transporter for xylem loading, in Arabidopsis thaliana." *Plant Journal* no. 46 (6):1084-1091. doi: 10.1111/j.1365-313X.2006.02763.x.

Miwa, K., J. Takano, H. Omori, M. Seki, K. Shinozaki, and T. Fujiwara. 2007. "Plants tolerant of high boron levels." *Science* no. 318 (5855):1417-1417. doi: 10.1126/science.1146634.

Moore, J. A., M. Callahan, R. Chapin, G. P. Daston, D. Erickson, E. Faustman, P. Foster, J. M. Friedman, L. Goldman, M. Golub, C. Hughes, R. J. Kavlock, C. A. Kimmel, J. C. Lamb, S. C. Lewis, C. Lunchick, S. Morseth, B. K. Mortensen, E. J. Oflaherty, A. K. Palmer, J. Ramlow, P. M. Rodier, K. Rudo, L. Ryan, B. A. Schwetz, A. Scialli, S. Selevan, R. Tyl, M. Campbell, E. Carney, W. Faber, J. Hellwig, S. R. Murphy, M. A. Smith, P. L. Strong, and M. Weiner. 1997. "An assessment of boric acid and borax using the IEHR Evaluative Process for Assessing Human Developmental and Reproductive Toxicity of Agents." *Reproductive Toxicology* no. 11 (1):123-160. doi: 10.1016/s0890-6238(96)00204-3.

Moseman, R. F. 1994. "Chemical disposition of boron in animals and humans." *Environmental Health Perspectives* no. 102:113-117. doi: 10.2307/3431973.

Murray, F. J. 1995. "A human health risk assessment of boron (boric acid and borax) in drinking water." *Regulatory Toxicology and Pharmacology* no. 22 (3):221-230. doi: 10.1006/rtph.1995.0004.

Nable, R. O., G. S. Banuelos, and J. G. Paull. 1997. "Boron toxicity." *Plant and Soil* no. 193 (1-2):181-198. doi: 10.1023/a:1004272227886.

Nakagawa, Y., H. Hanaoka, M. Kobayashi, K. Miyoshi, K. Miwa, and T. Fujiwara. 2007. "Cell-type specificity of the expression of os BOR1, a rice efflux boron transporter gene, is regulated in response to boron availability for efficient boron uptake and xylem loading." *Plant Cell* no. 19 (8):2624-2635. doi: 10.1105/tpc.106.049015.

National Boron Research Institute. http://www.boren.gov.tr/en/boron/reserves.

Nielsen, F. H. 2000. "The emergence of boron as nutritionally important throughout the life cycle." *Nutrition* no. 16 (7-8):512-514. doi: 10.1016/s0899-9007(00)00324-5.

Nozawa, A., J. Takano, M. Kobayashi, N. von Wiren, and T. Fujiwara. 2006. "Roles of BOR1, DUR3, and FPS1 in boron transport and tolerance in Saccharomyces cerevisiae." *Fems Microbiology Letters* no. 262 (2):216-222. doi: 10.1111/j.1574-6968.2006.00395.x.

Nzietchueng, R. M., B. Dousset, P. Franck, M. Benderdour, P. Nabet, and K. Hess. 2002. "Mechanisms implicated in the effects of boron on wound healing." *Journal of Trace Elements in Medicine and Biology* no. 16 (4):239-244. doi: 10.1016/s0946-672x(02)80051-7.

O'Neill, M. A., T. Ishii, P. Albersheim, and A. G. Darvill. 2004. "Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide." *Annual Review of Plant Biology* no. 55:109-139. doi: 10.1146/annurev.arplant.55.031903.141750.

Pahl, M. V., B. D. Culver, P. L. Strong, F. J. Murray, and N. D. Vaziri. 2001. "The effect of pregnancy on renal clearance of boron in humans: A study based on normal dietary intake of boron." *Toxicological Sciences* no. 60 (2):252-256. doi: 10.1093/toxsci/60.2.252.

Pang, Yongqi, Lijuan Li, Fei Ren, Pingli Lu, Pengcheng Wei, Jinghui Cai, Lingguo Xin, Juan Zhang, Jia Chen, and Xuechen Wang. 2010. "Overexpression of the tonoplast aquaporin AtTIP5;1 conferred tolerance to boron toxicity in Arabidopsis." *Journal of Genetics and Genomics* no. 37 (6):389-U52. doi: 10.1016/s1673-8527(09)60057-6.

Park, M., Q. Li, N. Shcheynikov, W. Z. Zeng, and S. Muallem. 2004. "NaBC1 is a ubiquitous electrogenic Na+-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation." *Molecular Cell* no. 16 (3):331-341.

Parr, A.J., and Loughman, B.C. 1983. "Boron and membrane function in plants." *Metals and Micronutrients: Uptake and utilization by plants / Edited by D.A. Robb and W.S. Pierpoint*, 87-107.

Perez-Castro, Ramon, Koji Kasai, Felipe Gainza-Cortes, Simon Ruiz-Lara, Jose A. Casaretto, Hugo Pena-Cortes, Jaime Tapia, Toru Fujiwara, and Enrique Gonzalez. 2012. "VvBOR1, the Grapevine Ortholog of AtBOR1, Encodes an Efflux Boron Transporter That is Differentially Expressed Throughout Reproductive Development of Vitis vinifera L." *Plant and Cell Physiology* no. 53 (2):485-494. doi: 10.1093/pcp/pcs001.

Price, C. J., M. C. Marr, C. B. Myers, J. C. Seely, J. J. Heindel, and B. A. Schwetz. 1996. "The developmental toxicity of boric acid in rabbits." *Fundamental and Applied Toxicology* no. 34 (2):176-187. doi: 10.1006/faat.1996.0188.

Raven, J. A. 1980. "Short-distance and long-distance transport of boric-acid in plants." *New Phytologist* no. 84 (2):231-249. doi: 10.1111/j.1469-8137.1980.tb04424.x.

Reid, R. J., J. E. Hayes, A. Post, J. C. R. Stangoulis, and R. D. Graham. 2004. "A critical analysis of the causes of boron toxicity in plants." *Plant Cell and Environment* no. 27 (11):1405-1414.

Rezanka, Tomas, and Karel Sigler. 2008. "Biologically active compounds of semi-metals." *Phytochemistry* no. 69 (3):585-606. doi: 10.1016/j.phytochem.2007.09.018.

Rowe, R. I., and C. D. Eckhert. 1999. "Boron is required for zebrafish embryogenesis." *Journal of Experimental Biology* no. 202 (12):1649-1654.

Sattlegger, E., and A. G. Hinnebusch. 2000. "Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells." *Embo Journal* no. 19 (23):6622-6633. doi: 10.1093/emboj/19.23.6622.

Sayli, B. S., E. Tuccar, and A. H. Elhan. 1998. "An assessment of fertility in boron-exposed Turkish subpopulations." *Reproductive Toxicology* no. 12 (3):297-304. doi: 10.1016/s0890-6238(98)00013-6.

Schummer, D., D. Schomburg, H. Irschik, H. Reichenbach, and G. Hofle. 1996. "Antibiotics from gliding bacteria .75. Absolute configuration and biosynthesis of tartrolon B, a boron-containing macrodiolide from Sorangium cellulosum." *Liebigs Annalen* (6):965-969.

Scorei, Romulus. 2012. "Is Boron a Prebiotic Element? A Mini-review of the Essentiality of Boron for the Appearance of Life on Earth." *Origins of Life and Evolution of Biospheres* no. 42 (1):3-17. doi: 10.1007/s11084-012-9269-2.

Shirra, Margaret K., Rhonda R. McCartney, Chao Zhang, Kevan M. Shokat, Martin C. Schmidt, and Karen M. Arndt. 2008. "A Chemical Genomics Study Identifies Snf1 as a Repressor of GCN4 Translation." *Journal of Biological Chemistry* no. 283 (51):35889-35898. doi: 10.1074/jbc.M805325200.

Stangoulis, J. C. R., R. J. Reid, P. H. Brown, and R. D. Graham. 2001. "Kinetic analysis of boron transport in Chara." *Planta* no. 213 (1):142-146. doi: 10.1007/s004250000484.

Sutton, T., U. Baumann, J. Hayes, N. C. Collins, B. J. Shi, T. Schnurbusch, A. Hay, G. Mayo, M. Pallotta, M. Tester, and P. Langridge. 2007. "Boron-toxicity tolerance in barley arising from efflux transporter amplification." *Science* no. 318 (5855):1446-1449. doi: 10.1126/science.1146853.

Suzuki, Minoru, Yoshinori Sakurai, Satoru Hagiwara, Shinichiro Masunaga, Yuko Kinashi, Kenji Nagata, Akira Maruhashi, Masatoshi Kudo, and Koji Ono. 2007. "First attempt of boron neutron capture therapy (BNCT) for hepatocellular carcinoma." *Japanese Journal of Clinical Oncology* no. 37 (5):376-381. doi: 10.1093/jjco/hym039.

Takano, J., M. Kobayashi, Y. Noda, and T. Fujiwara. 2007. "Saccharomyces cerevisiae Bor1p is a boron exporter and a key determinant of boron tolerance." *Fems Microbiology Letters* no. 267 (2):230-235. doi: 10.1111/j.1574-6968.2006.00556.x.

Takano, J., K. Miwa, and T. Fujiwara. 2008. "Boron transport mechanisms: collaboration of channels and transporters." *Trends in Plant Science* no. 13 (8):451-457. doi: 10.1016/j.tplants.2008.05.007.

Takano, J., K. Miwa, L. X. Yuan, N. von Wiren, and T. Fujiwara. 2005. "Endocytosis and degradation of BOR1, a boron transporter of Arabidopsis thaliana, regulated by boron availability." *Proceedings of the National Academy of Sciences of the United States of America* no. 102 (34):12276-12281. doi: 10.1073/pnas.0502060102.

Takano, J., K. Noguchi, H. Hayashi, and T. Fujiwara. 2002. *Arabidopsis thaliana mutant bor1-1 is defective in boron translocation*. Edited by H. E. Goldbach, B. Rerkasem, M. A. Wimmer, P. H. Brown, M. Thellier and R. W. Bell, *Boron in Plant and Animal Nutrition*.

Takano, J., K. Noguchi, M. Yasumori, M. Kobayashi, Z. Gajdos, K. Miwa, H. Hayashi, T. Yoneyama, and T. Fujiwara. 2002. "Arabidopsis boron transporter for xylem loading." *Nature* no. 420 (6913):337-340. doi: 10.1038/nature01139.

Takano, J., M. Wada, U. Ludewig, G. Schaaf, N. von Wiren, and T. Fujiwara. 2006. "The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation." *Plant Cell* no. 18 (6):1498-1509. doi: 10.1105/tpc.106.041640.

Tanaka, M., and T. Fujiwara. 2008. "Physiological roles and transport mechanisms of boron: perspectives from plants." *Pflugers Archiv-European Journal of Physiology* no. 456 (4):671-677. doi: 10.1007/s00424-007-0370-8.

Teixeira, Miguel C., Pedro Monteiro, Pooja Jain, Sandra Tenreiro, Alexandra R. Fernandes, Nuno P. Mira, Marta Alenquer, Ana T. Freitas, Arlindo L. Oliveira, and Isabel Sa-Correia. 2006. "The YEASTRACT database: a tool for the analysis of transcription regulatory associations in Saccharomyces cerevisiae." *Nucleic Acids Research* no. 34:D446-D451. doi: 10.1093/nar/gkj013.

Thompson, D. M., C. Lu, P. J. Green, and R. Parker. 2008. "tRNA cleavage is a conserved response to oxidative stress in eukaryotes." *Rna-a Publication of the Rna Society* no. 14 (10):2095-2103. doi: 10.1261/rna.1232808.

Uluisik, I., A. Kaya, D. E. Fomenko, H. C. Karakaya, B. A. Carlson, V. N. Gladyshev, and A. Koc. 2011. "Boron Stress Activates the General Amino Acid Control Mechanism and Inhibits Protein Synthesis." *Plos One* no. 6 (11). doi: 10.1371/journal.pone.0027772.

Uluisik, Irem, Alaattin Kaya, Ercan S. Unlu, Kadir Avsar, Huseyin C. Karakaya, Talat Yalcin, and Ahmet Koc. 2011. "Genome-wide identification of genes that play a role in boron stress response in yeast." *Genomics* no. 97 (2):106-111. doi: 10.1016/j.ygeno.2010.10.006.

Valenzuela, L., C. Aranda, and A. Gonzalez. 2001. "TOR modulates GCN4-dependent expression of genes turned on by nitrogen limitation." *Journal of Bacteriology* no. 183 (7):2331-2334. doi: 10.1128/jb.183.7.2331-2334.2001.

Vazquez de Aldana, C. R., M. J. Marton, and A. G. Hinnebusch. 1995. "GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the eIF-2 alpha kinase GCN2 in amino acid-starved cells." *EMBO J* no. 14 (13):3184-99.

Verbruggen, Nathalie, Christian Hermans, and Henk Schat. 2009. "Mechanisms to cope with arsenic or cadmium excess in plants." *Current Opinion in Plant Biology* no. 12 (3):364-372. doi: 10.1016/j.pbi.2009.05.001.

Wallace, Ian S., Won-Gyu Choi, and Daniel M. Roberts. 2006. "The structure, function and regulation of the nodulin 26-like intrinsic protein family of plant aquaglyceroporins." *Biochimica Et Biophysica Acta-Biomembranes* no. 1758 (8):1165-1175. doi: 10.1016/j.bbamem.2006.03.024.

Warington, Katherine. 1923. The Effect of Boric Acid and Borax on the Broad Bean and certain other Plants. Oxford University Press.

Winkler, G. S., A. Kristjuhan, H. Erdjument-Bromage, P. Tempst, and J. Q. Svejstrup. 2002. "Elongator is a histone H3 and H4 acetyltransferase important for normal histone acetylation levels in vivo." *Proceedings of the National Academy of Sciences of the United States of America* no. 99 (6):3517-3522. doi: 10.1073/pnas.022042899.

Wittig, Andrea, Jean Michel, Raymond L. Moss, Finn Stecher-Rasmussen, Heinrich F. Arlinghaus, Peter Bendel, Pier Luigi Mauri, Saverio Altieri, Ralf Hilger, Piero A. Salvadori, Luca Menichetti, Robert Zamenhof, and Wolfgang A. G. Sauerwein. 2008. "Boron analysis and boron imaging in biological materials for Boron Neutron Capture Therapy (BNCT)." *Critical Reviews in Oncology Hematology* no. 68 (1):66-90. doi: 10.1016/j.critrevonc.2008.03.004.

Wolkenstein, Klaus, Juergen H. Gross, and Heinz Falk. 2010. "Boron-containing organic pigments from a Jurassic red alga." *Proceedings of the National Academy of Sciences of the United States of America* no. 107 (45):19374-19378. doi: 10.1073/pnas.1007973107.

Woods, W. G. 1994. "An introduction to boron- history, sources, uses, and chemistry." *Environmental Health Perspectives* no. 102:5-11. doi: 10.2307/3431956.

Yazbeck, C., W. Kloppmann, R. Cottier, J. Sahuquillo, G. Debotte, and G. Huel. 2005. "Health impact evaluation of boron in drinking water: a geographical risk assessment in Northern France." *Environmental Geochemistry and Health* no. 27 (5-6):419-427. doi: 10.1007/s10653-005-1796-6.

York, W. S., A. G. Darvill, M. McNeil, and P. Albersheim. 1985. "Structure of plant-cell walls .16. 3-deoxy-D-manno-2-octulosonic acid (KDO) is a component of rhamnogalacturonan-II, a pectic polysaccharide in the primary-cell walls of plants." *Carbohydrate Research* no. 138 (1):109-126. doi: 10.1016/0008-6215(85)85228-9.

Zhi, Chunyi, Yoshio Bando, Chengchun Tang, and Dmitri Golberg. 2010. "Boron nitride nanotubes." *Materials Science & Engineering R-Reports* no. 70 (3-6):92-111. doi: 10.1016/j.mser.2010.06.004.

Zhu, Yinghuai, Yongxiang Lin, Yi Zhun Zhu, Jia Lu, John A. Maguire, and Narayan S. Hosmane. 2010. "Boron Drug Delivery via Encapsulated Magnetic Nanocomposites: A New Approach for BNCT in Cancer Treatment." *Journal of Nanomaterials*. doi: 10.1155/2010/409320.

Zurita-Martinez, S. A., and M. E. Cardenas. 2005. "Tor and cyclic AMP-protein kinase A: Two parallel pathways regulating expression of genes required for cell growth." *Eukaryotic Cell* no. 4 (1):63-71. doi: 10.1128/ec.4.1.63-71.2005.

VITA

Name: İrem ULUIŞIK
Date and Place of Birth: January 10, 1985/ Muğla, Turkey

Education:

- **BSc:** Ege University, Department of Biology (2002-2007); Anadolu University, Department of Business Administration (2004-2011)
- **MSc:** İzmir Institute of Technology, Department of Molecular Biology and Genetics (2007-2010)
- **PhD:** İzmir Institute of Technology, Department of Molecular Biology and Genetics (2010-2015)

Publications:

- Genome-Wide Identification of Genes That Play Role in Boron Stress Response in Yeast**.** Irem Uluisik, Alaattin Kaya, Ercan S. Unlu, Kadir Avsar, Huseyin C. Karakaya, Talat Yalcin and Ahmet Koc, *Genomics*. 2011 Feb;97(2):106-11.
- Roles of *ATR1* paralogs *YMR279c* and *YOR378w* in boron stress tolerance. Gonensin Ozan Bozdag, Irem Uluisik, Gulce Sila Gulculer, Huseyin C. Karakaya, Ahmet Koc, 2011, *Biochem & Biophy Res Com*. 2011,409(4): 748-51.
- Boron stress activates the general amino acid control mechanism and inhibits protein synthesis. Uluisik İ., Kaya A., Fomenko DE., Karakaya HC., Carlson BA., Gladyshev V., Koç A. *PLoS One*. 2011;6(11):e27772.
- Boron, in point of view of biological spectrum. Irem Uluisik, Huseyin Caglar Karakaya, Ahmet Koc. *Frontiers in Cell and Developmental Biology, Cellular Biochemistry*. 2015 (in revision)
- Elucidation of the mechanisms of boron sensitivity and boron tolerance in yeast mutants lacking genes, which play possible roles in boron stress response. Irem Uluisik, H. Caglar Karakaya, Ahmet Koç (*manuscript in preparation*)
- Transcription in Eukaryotes. Ahmet Koç, İrem Uluışık, Gözde Bekki. Molecular Biology Book, Chapter 9, ISBN: 978-605-88012-7-1, Nobel Akademik Yayıncılık Eğitim Danışmanlık Tic. Ltd. Şti. 2012 *(in Turkish).*
- Atr1 as a boron exporter in yeast. Ahmet Koc and İrem Uluisik. Encyclopedia of Metalloproteins, DOI 10.1007/978-1-4614-1533-6, # Springer Science+Business Media, LLC 2012.
- Boron Stress Tolerance, YMR279c and YOR378w. Ahmet Koc and İrem Uluisik. Encyclopedia of Metalloproteins, DOI 10.1007/978-1-4614-1533-6, # Springer Science+Business Media, LLC 2012.

Awards and Honors:

- Stipend from Scientific and Technological Research Council of Turkey (TUBITAK) during Master study, 2008-2010
- Erasmus Placement Grant, University of Gothenburg, 2012-2013 (Spring Term)

Teaching and Professional Experience:

- Research and Teaching Assistant (2013- 2015), İzmir Institute of Technology, Department of Molecular Biology and Genetics, İzmir, Turkey
- Erasmus PhD Exchange Student (March-June 2013), University of Gothenburg, Department of Cellular and Molecular Biology, Gothenburg, Sweden