MOLECULAR MECHANISMS OF BORON TOXICITY AND IDENTIFICATION OF BORON METABOLISM GENES

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

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
İrem ULUIŞIK

June 2010
İZMİR

We approve the thesis of **İrem ULUIŞIK**

Assoc. Prof. Dr. Ahmet KOÇ
Supervisor

Assist. Prof. Dr. Bünyamin AKGÜL
Committee Member

Assoc. Prof. Dr. Talat YALÇIN
Committee Member

25 June 2010

Assoc. Prof. Dr. Sami DOĞANLAR
Head of the Department of Molecular Biology and Genetics

Assoc. Prof. Dr. Talat YALÇIN
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGEMENTS

My special thanks are due to Assoc. Prof. Dr. Ahmet KOÇ, who has been a true mentor for my professional and personal growth. Your guidance, encouragement, support and patience during my thesis studies gave me strength and will always be very valuable. There are still many things that I will learn from you.

I would also like to thank Assist. Prof. Dr. H. Çağlar KARAKAYA for his comments, criticism, and continuous support throughout my research.

I wish to acknowledge my committee members Assoc. Prof. Dr. Talat YALÇIN and Assist. Prof. Dr. Bünyamin AKGÜL for their suggestions and significant contributions to my thesis.

Many thanks go to my co-workers Beren ATAÇ, Elise HACIOĞLU, Melda Z. GÜRAY, A. Banu DEMİR, G. Ozan BOZDAĞ, Gözde BEKKİ, Işıl ESMER and Kadir AVŞAR. To start the day with you was very nice and motivating. Besides them I wish to thank İsmail CAN and Yıldız KELAHMETOĞLU for their sincere help offers. You are wonderful. I am also thankful to Özge TÜNCEL and Hatice YİĞİT for sharing their experiences with me and for their invaluable friendship. I also wish to express my thanks all my other friends working at İYTE.

Also, I am grateful to faculty members of the Molecular Biology and Genetics Department for giving me the chance to do an MSc in such a motivating atmosphere. I would like to thank all members of Biotechnology and Bioengineering Central Research Laboratories for their helps with some of the experiments.

Finally, my deepest gratitude goes to my family members Cengiz ULUIŞIK, Nurhayat ULUIŞIK, Gizem ULUIŞIK, and my brother-like friend Necati ALTINDİŞ, who have been the source of my inspiration and motivation. Your love, faith and support helped me to come to this point. You will be always in my heart.

ABSTRACT

MOLECULAR MECHANISMS OF BORON TOXICITY AND IDENTIFICATION OF BORON METABOLISM GENES

Boron is an essential micronutrient for plants and it is either necessary or beneficial for animals. Studies identified only few genes related to boron metabolism thus far and details of how boron is imported into cells and used in cell metabolism are largely unknown. In this study, in order to identify genes that play role in boron metabolism, the entire set of yeast haploid deletion mutants was screened and 6 boron resistant and 21 boron sensitive mutants were identified. Boron treatment activated the expression of *ATR1* and many genes that are regulated by genereal amino acid control in a *GCN4* dependent manner. Polysome analyses and 35-S methionine labelling assays suggested that boron inhibits protein synthesis. Inhibition of protein synthesis was also confirmed by the phosphorylation of translation factor eIF2α in boron treated cells, and phosphorylation of eIF2α was totally dependent on the uncharged tRNA binding domain of Gcn2 kinase. Overall, our results revealed many genes and pathways related to boron stress response and suggest a possible link between boron toxicity and translational control.

ÖZET

BOR TOKSİSİTESİNİN MOLEKÜLER MEKANİZMALARI VE BOR METABOLİZMASI GENLERİNİN TANIMLANMASI

Bor bitkiler için esansiyel bir mikrobesin olmasının yanında hayvanlar için de yararlı ya da gerekli olabilmektedir. Bugüne kadarki çalışmalar bor metabolizması ile ilgili çok az geni ortaya çıkarmıştır ve borun hücre içerisine nasıl taşındığı ve hücresel metabolizmada nasıl kullanıldığı hala bilinmemektedir. Bu çalışmada bor metabolizmasında rol oynayan genlerin bulunması amacıyla maya haploid delesyon mutantlarından oluşan bir set taranmış ve 6 mutant bor dirençli ve 21 mutant da bora karşı duyarlı bulunmuştur. Bor muamelesi *ATR1* ifadelenmesini ve *GCN4*'e bağlı olarak genel amino asit kontrol mekanizması tarafından düzenlenen çoğu genin ifadelenmesini aktive etmiştir. Polizom analizleri ve 35-S metiyonin işaretlemesi denemeleri borun protein sentezini inhibe ettiğini göstermektedir. Protein sentezinin inhibisyonu aynı zamanda, eIF2α translasyon faktörünün bor ile muamele edilmiş hücrelerde fosfatlanmasıyla doğrulanmıştır ve eIF2α'nın fosforilasyonunun Gcn2 kinazının yüksüz tRNA bağlanma bölgesi ile ilişkili olduğu gösterilmiştir. Sonuç olarak, bor metabolizması ile alakalı pek çok gen ve yolak aydınlatılmıştır ve bor toksisitesi ile translasyonel kontrol arasında bir bağ olabileceği öne sürülmüştür.

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CHAPTER 1

INTRODUCTION

1.1. Boron Biochemistry

Boron is an element which has intermediate properties between metals and non-metals (Bolanos, et al. 2004). It is a naturally occuring substance but is never found as a single element (GreenFacts 2004). In nature it forms compounds such as borax, borates and boric acid with combinations of sodium and oxygen (Expert Group on Vitamins and Minerals 2003). However only 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 are present as boric acid $B(OH)_3$ and as a small amount of borate anion $B(OH)_4^-$ (Hunt 2003, Bolanos, et al. 2004). Boric acid is a Lewis acid with a p*K*a of 9.25 and it accepts a hydroxyl ion, rather than donating protons, to form the tetrahedral anion $B(OH)_4^-$.

$$B(OH)_3 + 2H_2O \longleftrightarrow H_3O^+ + B(OH)_4^- \quad pKa = 9.25\ (25^\circ C) \quad (1.1)$$

(Source: Greenwood and Earnshaw 1984)

Both boric acid and borate make strong interactions with biomolecules, especially which containing cis-hydroxyl groups such as riboflavin, adenosine monophosphate, pyridoxine, ascorbic acid, sugar molecules and polysaccharides. When the 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).

Boric acid and borates have been commonly used as an antiseptic, bactericide, cleaning agent such as soaps and detergents, preservatives for leathers and wood, fire retardants, fertilizers, insecticides, and herbicides, cosmetic products; also used for many industrial purposes including manufacture of glass, fiberglass insulation, porcelain enamel, ceramic glazes, and metal alloys (Woods 1994, Moore 1997, EPA 2004).

Major world boron deposits are located in Turkey and California, and also found in Argentina, Chile, Russia, China, Peru, Egypt, Iraq, Libya, Morocco, Syria (Green Facts 2004, Tanaka and Fujiwara 2008).

1.2. Necessity and Function of Boron in Living Things

The first report which proposes essentiality of boron for plants came in the year 1923. In that year Warington found the 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 then boron has been established as an essential micronutrient not only for plants but also for animals. As a result of accumulating evidence boron has been thought to play roles in many physiological and metabolic processes in microbial systems, plants and animals. However it is toxic to all these organisms when present at high concentrations (Nielsen 1997, Tanaka and Fujiwara 2008).

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. Nevertheless, molecular mechanisms of these roles are mostly unknown (Goldbach, et al. 2001, Miwa, et al. 2007). Recently a primary function of boron was found at molecular level. It cross-links pectins in 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 rhamnogalacturonan II (RG-II) and this cross-linking is essential for normal leaf extension (Kobayashi, et al. 1996, O'Neill, et al. 2004).

Boron has also roles in microbial growth. It is required for bacterial nitrogen fixation in *Azotobacter*, although it is not essential for growth (Anderson and Jordan 1961). However the species *Bacillus boroniphilus* require boron for its growth and can tolerate more than 450 mM boron (Ahmed, et al. 2007). Heterocystous cyanobacteria and actinomycetes of the genus *Frankia* also require boron for both growth and nitrogen fixation. It was suggested that boron stabilizes the glycolipid layer of the heterocysts through interacting with their hydroxyl groups. This requirement of boron for Cyanobacteria indicates it was an essential element during early evolution of life since Cyanobacteria were dominant organisms in Pre-Cambrian age (Mateo, et al. 1986,

Bonilla, et al. 1990, Bolanos, et al. 2002). Newly a boron containing molecule which mediates quorum sensing in bacteria was isolated (Chen, et al. 2002, Bolanos, et al. 2004). Boromycin and tartrolon A and B are boron containing antibiotics synthesized by bacteria (Irschik, et al. 1995, Kohno, et al. 1996).

Boron is also required for several marine species, diatoms and algal flagellates. In addition it was found necessary during embryonic development of zebra fish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) and frogs (*Xenopus laevis*) (Rowe and Eckhert 1999). The levels of boron required are different among these organisms. In animals boron affects various mechanisms. These include carbohydrate, mineral metabolism, energy consumption, regulation of several enzyme activities and embryonic development. However molecular mechanisms of boron function in animals are not well understood (Tanaka and Fujiwara 2008).

Human beings are exposed to boron through their diet, from drinking water and from consumer products such as soaps, detergents, cosmetic, deodorant, pesticides, and preservatives. An essentiality of boron has not been reported to date in human but it has beneficial effects. It has roles in steroid hormone metabolism, healthy bone development, cell membrane maintenance. Additionally, clinical experiments showed when boron was taken with foods, the risk of prostate cancer significantly reduced. Therefore molecular basis of these functions should be uncovered (Green Facts 2004, Cui, et al. 2004, Tanaka and Fujiwara 2008).

1.3. Boron Deficiency and Toxicity

Boron is an important nutrient for plants and animals. Although the necessity has been known for growth and development of these organisms, boron is also toxic when present at high concentrations. In plants boron deficiency symptoms are seen mainly expanding organs. Boron deficiency results in the formation of abnormal cell wall, cytoskeletal polymerization changes and alteration of plasma membrane permeability for ions. In addition to these, leaf expansion, root elongation, flower and fruit development are inhibited. At the cellular level boron restriction harms many physiological processes and can drive cells even to death. In 2004, it was reported that the expression of glutathione S-transferase and glucosyltransferase induced in tobacco (*Nicotiana tabacum*) BY-2 cells under boron limitation and in 2009, another study with

these tobacco cells showed that oxidative damage is directly involved in cell death under boron deprivation and it is not likely a typical programmed cell death. In contrast, boron toxicity symptoms are seen margins of mature leaves. 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 (Kobayashi, et al. 2004, Camacho-Cristobal, et al. 2008, Koshiba, et al. 2009).

In human and animals, as a result of boron deprivation growth impairs, bone development becomes abnormal, blood steroid hormone levels decrease, urinary calcium excretion increases and macromineral status changes. It is also found that mice and frogs have developmental disorders arising from boron deficiency. 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). Despite its importance little is known about the mechanisms of these boron actions.

1.4. Boron Uptake and Transport in Plants and Animals

In metabolism of essential micronutrients there are generally special carrier systems that carry the nutrient into the cell and several factors that modulate the regulation of these nutrients in the cell.

Boron is also an essential nutrient for many organisms. Plants acquire 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 boron follows the transpiration streams and accumulates at margins of mature leaves since transpiration rate is high in these regions (Brown and Shelp 1997). In human and animal, there is limited information about the physiology and molecular biology of boron uptake. Fruits and vegetables are the primary sources of boron. After absorbed from the gastrointestinal and respiratory tracts more than 90% of boron are rapidly excreted in the urine. Boron concentrations in tissues are held constant by kidney-regulated homeostasis. However the mechanism of this homeostasis is not known now (Devirian and Volpe 2003).

The essentiality of boron for higher plants has been known for many years and boron deficiency is an agriculturally important problem in many parts of the world.

Therefore an optimum supply of boron is necessary for normal growth. To solve this major problem in boron deficient regions boron is supplied as fertilizer, but this usage should be also tightly controlled because boron is toxic when present in excess. There is a narrow range between boron deficiency and toxicity. Thus a need arises to understand the mechanisms that regulate boron homeostasis at molecular level. To achieve this, many studies related to boron have used plants as a model and to date many plant genes which are involved in boron transport and tolerance have been found (Takano, et al. 2008, Kato, et al. 2009).

It has long been believed that the passive diffusion of boric acid is the only and major transport mechanism of boron through membrane. However recent studies revealed three mechanisms for membrane transport of boric acid. The first is passive diffusion. First experiments showed that the theoretical permeability coefficient of boric acid for lipid bilayer was 8×10^{-6}cm s^{-1} (Raven 1980). In 2000, experiments with squash root (*Cucurbita pepo*) and charopyte alga (*Chara corallina*) cells also revealed a similar permeability coefficient of boric acid to calculated value by Raven (Dordas, et al. 2000, Stangoulis, et al. 2001). According to these results passive diffusion represents a significant way for passing lipid bilayer when there is high boron supply, but under low boron conditions there is a need for membrane proteins.

Recent studies suggest that in addition to passive transport there are also active transport mechanisms for boric acid. Using tracer boric acid (^{10}B) it was found that in sunflower (*Helianthus annuus*) growing under low boron, the transport of boric acid takes place against concentration gradient (Dannel, et al. 2000). Similar to the sunflowers *Arabidopsis thaliana* accumulates boron in the xylem against a concentration gradient under boron limitation. In this study *BOR1* gene was identified as the first boron efflux transporter for xylem loading (Takano, et al. 2002). Shoot growth inhibition and reduced fertility were shown in *A.thaliana bor1-1* mutant under low boron conditions and this mutant can not translocate boron from roots to shoots. When *BOR1* was overexpressed, improved shoot growth and fertility were observed (Miwa, et al. 2006). However *BOR1* gets internalized via endocytosis and degraded in the vacuole when plants are exposed to high levels of boron (Takano, et al. 2005). *BOR1* belongs to animal bicarbonate transporter superfamily (SLC4) and *BOR1* homologs were found in many organisms (Figure 1.1). For example OsBOR1 was identified in rice and has roles in boron efflux transport for boron uptake and xylem loading (Nakagawa, et al. 2007). In *Saccharomyces cerevisiae YNL275w* was also

characterized as one of the *BOR1* homologs and is responsible for transporting boron in plasma membrane (Takano, et al. 2002). *BOR1* also shows a significant homology to mammalian NaBC1. In 2004, NaBC1 was shown as a Na^+-coupled borate transporter and it is essential for boron homeostasis of mammalian cells (Park, et al. 2004). *BOR1* has six paralogs. One of them, *BOR4*, provides tolerance to plants against high levels of boron whereas *BOR1* can not (Miwa, et al. 2007). Additionally, *BOT1* is a *BOR1* ortholog and provided boron tolerance to barley (Sutton, et al. 2007).

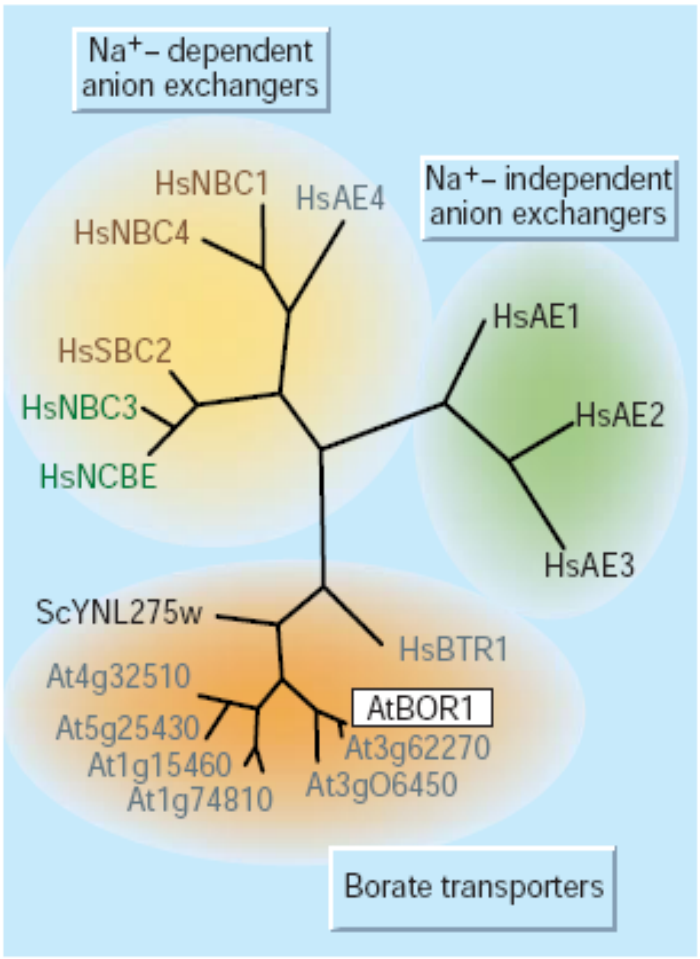


Figure 1.1. Phylogenetic tree of the SLC4 anion-exchanger superfamily. Na^+-bicarbonate transporters are shown in brown, Na^+-dependent anion exchangers in green and proteins of unknown function in grey. Hs: Human, Sc: Yeast, At: Arabidopsis (Source: Frommer and von Wiren 2002).

As a third mechanism for the membrane transport of boric acid, facilitated transport by NIP channels represents new aspects. NIP5:1 is a member of major intrinsic protein family (MIP) **(**Figure 1.2). MIPs are integral membrane proteins and serve as channels for water and small uncharged molecules in mammals, amphibians, yeast, bacteria and plants. MIPs are divided into four subgroups: the plasma membrane intrinsic proteins (PIP), the tonoplast intrinsic proteins (TIP), the nodulin 26-like intrinsic proteins (NIP), and the small basic intrinsic proteins (SIP) (Wallace, et al. 2006). NIP5:1 belongs to plant-specific NIP subfamily and has been identified as a boric acid channel that upregulated in *A. thaliana* roots under boron deficiency (Takano, et al. 2006). However it is unclear whether other MIPs are involved in boric acid transport in animals. Thus there is a need for determining other channels and the molecular mechanisms of boric acid transport by these transporters and channels. It is also inevitable to investigate the sensing mechanisms behind the boron toxicity.

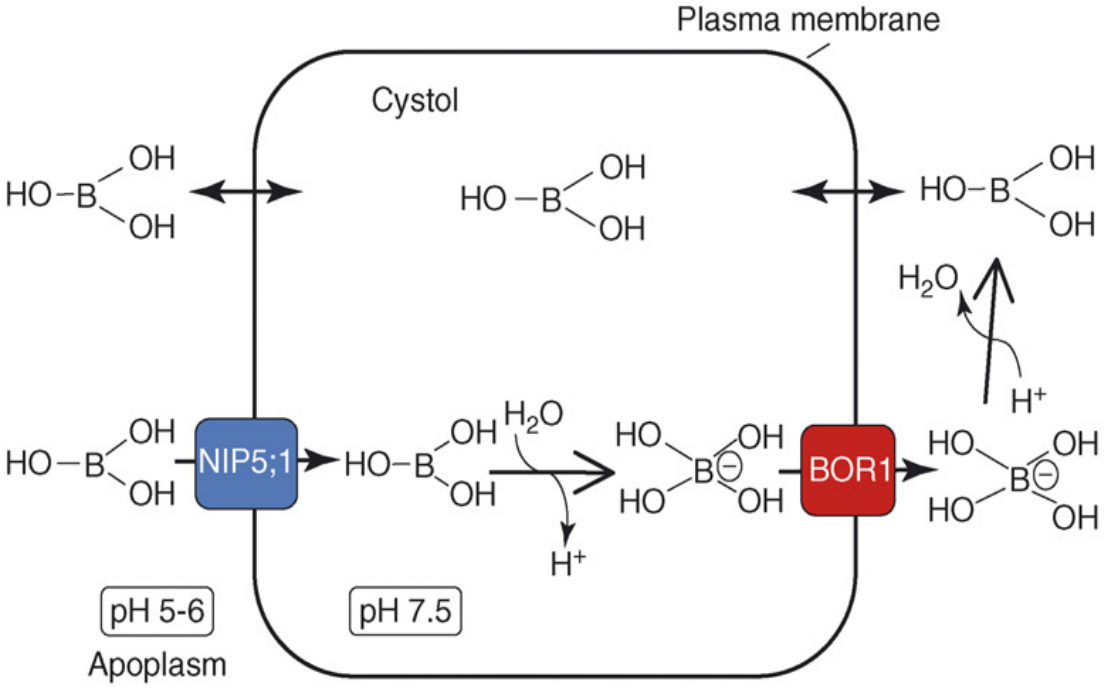


Figure 1.2. Boron transport mechanism of NIP5:1 and BOR1 in plants. In addition to the passive diffusion NIP5:1 facilitates the boric acid transport into cells following the concentration gradient. It is predicted that BOR1 exports borate [$B(OH)_4^-$)] which is converted from boric acid in the plant cytosol where the pH is high (Source: Takano, et al. 2008).

1.5. Yeast and Boron Relationship

Previous studies have shown that unicellular eukaryotic organism *Saccharomyces cerevisiae* is an excellent model to search the molecular trafficking of several metals such as copper, iron and zinc and to understand the mechanisms by which cells respond to various stress conditions (Lin, et al. 1997, Jamieson 1998, Culotta, et al. 1999). As a result of a study which also used yeast cells, it was found that cells could not divide and cell cycle stopped under boron deficiency. However when boron added to media, yeast cells proliferated normally and when exposed to high levels of boron, cells were poisoned. This boron dependent growth has suggested yeast can be used as a suitable model for boron molecular biology researches (Bennett, et al. 1999). In addition, the properties like growing rapidly in liquid and solid medias, being genomically characterized, the availability of haploid and diploid deletion sets, simple expression systems for extrachromosomal genes, the applicability of many techniques in molecular genetics and evolutionary conservation between yeast and higher eukaryotes make yeast a convenient system to get information related to fundamental cellular processes in higher organisms.

Yeast has been also used for characterization of plant boron tolerance genes so far. It is known as a boron tolerant organism because it can grow in the presence of 80 mM boric acid (Nozawa, et al. 2006, Kaya, et al. 2009). Yeast *BOR1* was characterized in detail. This protein is localized to plasma membrane and has a role in boric acid efflux and therefore tolerance of boron toxicity (Takano, et al. 2007). In addition to *BOR1*, *DUR3* and *FPS1* were found to be involved in boron tolerance in yeast. Dur3 is a urea transporter and Fps1 is a member of the MIP family and transports glycerol. Mutants that lack *DUR3* and *FPS1* showed decreased accumulation of boron in cells suggesting these genes are involved in boron import. However it is not clear which functions have these transporters in boron detoxification (Nozawa, et al. 2006).

In another study with yeast which was conducted by our group, *ATR1* has been found as a boron tolerance gene (Kaya, et al. 2009). ATR1 was characterized as a suppressor of the 3-amino-1,2,4-triazole sensitivity of *gcn4Δ* mutants for the first time (Kanazawa, et al. 1988). Apart from that it conferred resistance to 4-nitroquinoline-N-oxide in a genetic screen (Mack, et al. 1988). Yeast Atr1 is composed of 14 membrane-spanning segments and a member of the DHA2 family of drug-H^+ antiporters (Gbelska,

et al. 2006). Yeast genomic DNA library screen revealed expression of ATR1 provided extreme resistance to boron and reduced the intracellular boron levels. Mutants that lack ATR1 gene were found sensitive to boron treatment and had high intracellular boron. Taken together it can be concluded that Atr1 functions as a boron efflux pump and therefore provides tolerance (Kaya, et al. 2009).

CHAPTER 2

MATERIALS AND METHODS

2.1. Yeast Growth and Media

WT strain BY4741 (*MATa* his3 leu2 met15 ura3) and its isogenic deletion mutants were obtained from the yeast deletion library (Invitrogen) and diploid strain BY4743 (*MATa/α* his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0) and its isogenic deletion mutants were obtained from EUROSCARF. YPD medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar for solid media) was used for cell growth and selective YNB (yeast nitrogen base) medium with required amino acids and bases was used for plasmid carrying cells.

2.2. Screening of Yeast Deletion Collection Set

To identify mutants that were boron resistant, haploid yeast deletion collection strains were inoculated into 200 µl of liquid YPD media in microtiter plates using a home made 96 pin-replicator. After overnight incubation at 30 ºC, cells were replicated onto YPD agar plates supplemented with 100 mM boric acid. Cells were incubated at 30 ºC for 3 days. Mutants that were resistant to 100 mM boric acid were picked and tested on 150 mM boric acid. Similar analyses were performed for homozygous diploid cells. In order to identify mutants that were sensitive to boron, cells were replicated onto YPD plates containing 10-70 mM boric acid. Related diploid mutants were also tested in a similar manner. Both resistant and sensitive mutants were tested on different boron concentrations several times.

2.3. Cloning of the *YAP1*, *GCN2* and *GCN4* Genes with Gateway Technology® and Plasmids Used for Yeast Transformation

The wild type alleles of the *YAP1*, *GCN2* and *GCN4* was amplified by either Long PCR Enzyme or High Fidelity enzyme (Fermentas) from yeast genomic DNA using the primers

YAP1F

(5′GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTAAACCATGAGTGTGTCT-3′)

and

YAP1B

(5′GGGGACCACACTTTGTACAAGAAAGCTGGGTGTTCCCGCTTTAGTTCATAT-3′)

for *YAP1* gene,

GCN2F

(5′GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCATTGAGTCATCTCAC-3′)

and

GCN2B

(5′GGGGACCACTTTGTACAAGAAAGCTGGGTGCCTACCTCTGTAAATCGAT-3′)

for *GCN2* gene,

GCN4F

(5′GGGGACAAGTTTGTACAAAAAAGCAGGCTAATGTCCGAATATCAGCCAA-3′)

and

GCN4B

(5′GGGGACCACTTTGTACAAGAAAGCTGGGTATGAAATCAGCGTTCGCCAA-3′)

for *GCN4* gene. The amplified fragments was first cloned into PDONR vector using 2 µl BP Clonase^{TM} II enzyme (Invitrogen) with an overnight incubation at 25^0C and then moved to LR clonase reaction. This reaction was performed with 2 µl of isolated entry clones, 1.5 µl pAG423GPD expression vector, 3 µl TE buffer and 2 µl LR Clonase^{TM} II enzyme mixture (Invitrogen). Isolated plasmids were confirmed with sequence analyses and then yeast cells were transformed with the empty vector or with gene expression construct and selected for histidine prototrophy. Plasmids used for translational regulation of eIF2α phosphorylation in *gcn2Δ* cells were provided from Alan G. Hinnebusch (Laboratory of Gene Regulation and Development, National Institutes of Health). These include p4384 (expressing a functional, flag epitope-tagged form of *GCN2*), p4055 (containing flag epitope-tagged *GCN2* with nonphosphorylatable S577-

Alanine mutation), p4056 (containing flag epitope-tagged *GCN2* with substitutions in the m2 motif of the HisRS-like domain), p4057 (containing flag epitope-tagged *GCN2* with substitutions both in m2 motif and S577A). All four plasmids contain *URA3* region for selection.

2.4. Yeast Transformation with LiAc Method

Yeast transformations were performed by LiAc method (Gietz 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. Pelleted cells 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), 10 µl DTT (0,3mM), 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 spreaded on YNB-Ura or YNB-His media. Cells were allowed to grow for 3-4 days to form a colony. The colonies obtained were used for further experiments.

2.5. Determination of Boron Tolerance of Mutants

Boric acid was used as a source of boron and spotting assays on solid media were performed to determine boron tolerances of mutants and wild type yeast. For spotting assays, overnight cultures were diluted to an optical density of 0.2 at 600 nm initially and to 0.2, 0.02, 0.0002 by serial dilution. 5 µl of each dilution was spotted on YPD or YNB agar plates containing different amounts of boric acid. Plates were incubated 3 days at 30˚C.

2.6. DNA Microarray Analysis

BY4741 wild type, *yap1Δ*, *gcn2Δ*, *gcn4Δ* mutant cells were grown to an optical density of 0,2-0,3 at 600 nm in 200 ml YPD medium separately, with or without treatment for 1 hour with 20 mM boric acid. Then, cells were harvested by

centrifugation. Total RNA was isolated by using Ambion RiboPure™-Yeast Kit. The Agilent Two-Color Low RNA Input Linear Amplification Kit was used to generate fluorescently labeled cRNA for two-color microarray hybridizations. Fluorescently labeled, cRNA molecules were purified from the reaction mixture using the Qiagen RNeasy mini kit. cRNA samples (825 ng each) were combined with Agilent Hi-RPM hybridization buffer. Microarray hybridizations were performed using Agilent SureHyb hybridization chambers. The hybridization chambers were loaded onto a rotisserie in an Agilent Hybridization oven and were incubated at 65˚C for 17 hours with a rotational speed of 10 rpm. Following incubation, the microarray slides were washed for 1 minute each in Gene Expression wash buffer 1 (6×SSPE, 0.005% N-lauroylsarcosine at room temperature) and Gene Expression Wash Buffer 2 (0.06×SSPE, 0.005% N-lauroylsarcosine at 31˚C) for 1 minute each. The microarray slides were briefly dipped in a solution of acetonitrile, dried and scanned in an Agilent Technologies G2505B microarray scanner at 5 μm resolution.

2.7. RNA Isolation and Real Time PCR Analyses

Total RNA was isolated using the 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. Real time PCR analyses were performed with the IQ5 real-time PCR system (Bio-Rad) via using Fermentas Maxima^{TM} SYBR Green qPCR Master Mix (2X) (Fermentas) for determining quantitave expression levels. The primers used for amplification of *ATR1* were ATR1F (5′-ACGCGTATAGCATAGCCGCTTTCA-3′) and ATR1B (5′-TGTAAGCCTGGTTCCAACCCGATA-3′); and those used for amplification of *ACT1* were ACT1F (5′-ACGTTCCAGCCTTCTACGTTTCCA-3′) and ACT1B (5′-ACGTGAGTAACACCATCACCGGAA-3′). The conditions for PCR amplification were as follows: 30 cycles at 94 ˚C for 30 s, 58 ˚C for 30 s and 72 ˚C for 30 s.

2. 8. Polysome Profiling

Density gradient centrifugation system is used in order to separate total RNAs based on their molecular weight. This experiment can be analyzed in three parts as sucrose density gradient, sample homogenization and fraction collection. First of all 5% and 70% sucrose solutions [RiboLock™ RNase inhibitor (Fermentas), 100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES] were prepared and thereafter sucrose gradients were prepared by using ISCO systems. BY4741 cells were grown to an optical density of 0,7 to 0,9 at 600 nm in 30 ml YPD medium. These cells which exposed to 0 mM, 50 mM and 100 mM boric acid for 1 hour harvested by centrifugation, washed with two times sterile distilled water and were frozen at -80˚C as pellets. The day of trial, yeast pellets were homogenized in 5 ml lysis buffer [100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES, 40 u/µl RiboLock™ RNase inhibitor (Fermentas), 1% Triton-X 100, 1% sodium deoxycolate (NaDOC), 10 mg/ml cycloheximide (Sigma)] to obtain total RNA with the help of glass beads and vortexing. Followed by 8.minutes incubation on ice, the homogenate was centrifuged for 10 minutes at 12.000 x g at 4^0C. The supernatant was spreaded onto prepared 5%-70% (w/v) sucrose gradients and sedimented for 2.55 h, at 27.000 rpm, 4^0C in SW28 rotor (Beckman Coulter (Optima™ L-XP Ultracentrifuge System). In fraction collection step, 40 fractions were collected from the top of the gradient by using ISCO Tris pump while monitoring absorbance at 254 nm with an ISCO UA-6 monitor. The RNP populations were grouped according to their sedimentation rate: mRNP, 40S, monosome and polyribosome fractions. Following the addition of 10% SDS and 200 mM NaCl to fractions, phenol- chloroform extraction was applied twice in order to remove proteins. Aqueous phase was recovered after vortexing and centrifuging at 3000 rpm, at 24^0C, for 5 minutes. The aqueous parts containing RNA were ethanol precipitated in 1/10 NaOAC, 2X 96% ethanol and stored at -20^0C overnight. Ethanol-precipitated RNA was centrifuged at 12.000 rpm at 4^0C for 20 minutes. The RNA pellet was washed twice with %70 ice cold ethanol and centrifuged at 12.000 rpm at 4^0C for 5 minutes. Finally the RNA pellet was resuspunded in 200-300 µl DEPC water. RNA concentration was measured by NanoDrop spectrophotometer. Samples were aliquoted and stored at -80˚C.

2.9. Metabolic Labeling

BY4741 yeast cells were grown in 200 ml of YNB medium to OD_{600}= 0.5, splitted to 100 ml aliquots and one aliquot was treated with 20mM of boric acid. After 1 hour of incubation both aliquots were metabolically labeled with 0.5 mCi of EXPRE35S35S Protein Labeling Mix (Perkin-Elmer) for 30 minutes. Cells were harvested by centrifugation, washed three times with PBS buffer and disrupted with glass beads during 10 minutes in PBS buffer. The resulting cellular lysates were normalized according to protein concentration and 10µg of each lysate were subjected to SDS-PAGE. Resolved proteins were transferred to the polyvinylidene difluoride (PVDF) membrane. ^{35}S-labeled proteins were visualized with a Storm PhosphorImager system (Amersham Biosciences) after 2 days of exposure.

2.10. Western Blot Analyses

Wild type cells, and the isogenic *GCN4-MYC*, *ATR1-GFP* and *gcn2Δ* strains were grown to mid-log phase in either YPD or selective YNB-Ura media, with or without boron treatment and equal number of cells were harvested by centrifugation for each strain. Cell extracts were resuspended with 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 after vortexing vigorously. For analysis, cell extracts were resolved by 5%-15% SDS-PAGE and blotted to PVDF membrane. Primary antibodies that used in these experiments were anti-c-myc antibody produced in rabbit for GCN4-MYC (Sigma), anti-gfp, N-terminal, antibody produced in rabbit for ATR1-GFP (Sigma), rabbit anti-eukaryotic translation initiation factor 2, alpha, phosphorylated (Ser52) antibody (USBiological). The blots were stripped and reprobed with antibodies against yeast phosphoglycerate kinase monoclonal antibody (Invitrogen) as an internal control. As secondary antibodies anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) or goat anti mouse IgG horseradish peroxidase conjugated was used. The membranes were treated with enhanced chemiluminescence system (Bio-Rad) and were visualized using VersaDoc Imaging System (Bio-Rad).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Identification of Boron Resistant Yeast Deletion Mutants

It is known that wild type yeast can tolerate up to 80 mM boric acid (Kaya, et al. 2009). To identify genes whose deficiency confer resistance to high boron levels, all mutants of yeast haploid deletion set (approximately 4700 mutants) were screened via using solid media containing 100 mM boric acid, and 24 resistant mutants were identified. These resistant mutants, then, were retested on higher boron concentrations and 6 of them mutants (*ncs2*Δ, *ncs6*Δ, *kti12*Δ, *elp1*Δ, *elp3*Δ, *elp6*Δ) could grow on media which contained 150 mM boric acid (Figure 3.1). Since many strains in the yeast deletion set could have suppressor mutations or aneuploidy, these mutants were also verified in a different background in terms of boron resistance. As seen in (Figure 3.2), homozygous diploid cells lacking the same genes were also boron resistant. Among them Elp1, Elp3 and Elp6 are subunits of the elongator complex which has six members (Elp1-Elp6) and play role in formation of the 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5) groups on wobble uridines in several tRNAs, in transcriptional elongation by RNA Polymerase II and in exocytosis. Also Kti12 plays a role in modification of the wobble base together with the elongator complex. Ncs2 and Ncs6 are required for thiolation of the uridine at the wobble position of Lys, Glu, Gln tRNAs and have roles in urmylation and in invasive and pseudohyphal growth. Since the formation of modifications in the wobble position of tRNAs negated the deficiency in exocytosis and transcriptional activation, it seems to be an important function of the elongator complex. Thus, resistance of *ncs2*Δ, *ncs6*Δ, *kti12*Δ, *elp1*Δ, *elp3*Δ and *elp6*Δ mutants to boron could be related to the absence of uridine modifications at the wobble base of tRNAs which are needed for efficient decoding of lysine and glutamine codons in mRNAs during translation, however mechanisms how absence of tRNA modifications could cause boron resistance need to be investigated (Goehring, et al. 2003, Esberg, et al. 2006, Huang, et al. 2008).

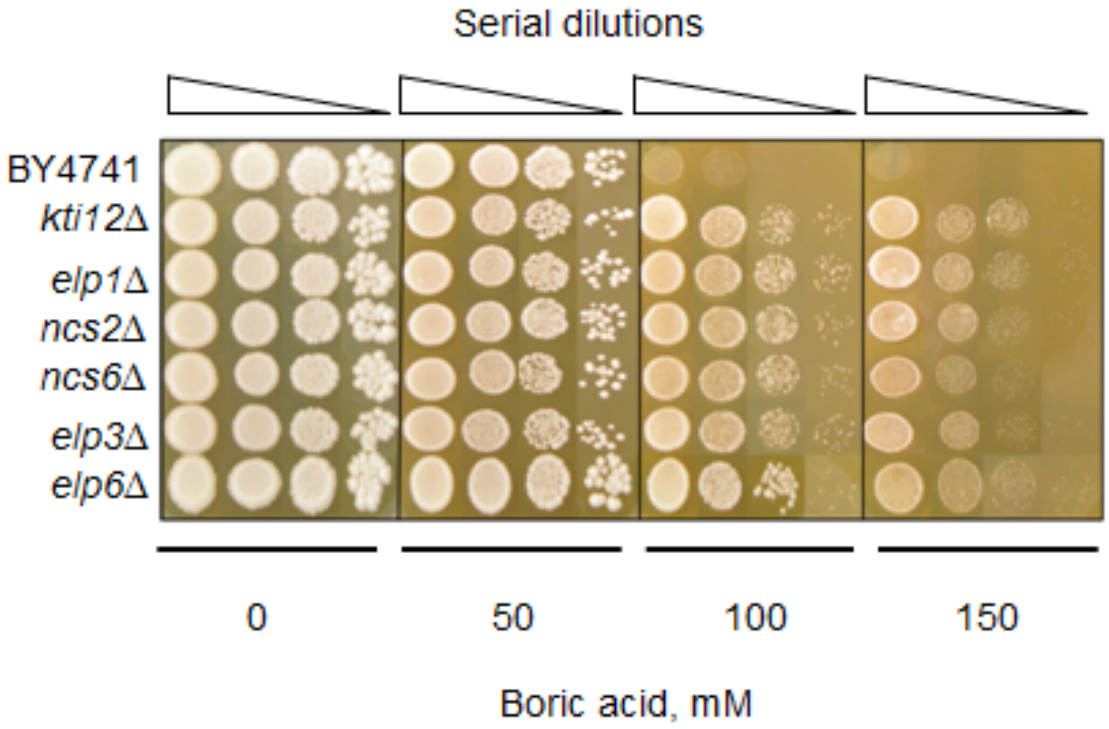


Figure 3.1. Haploid boron resistant mutants

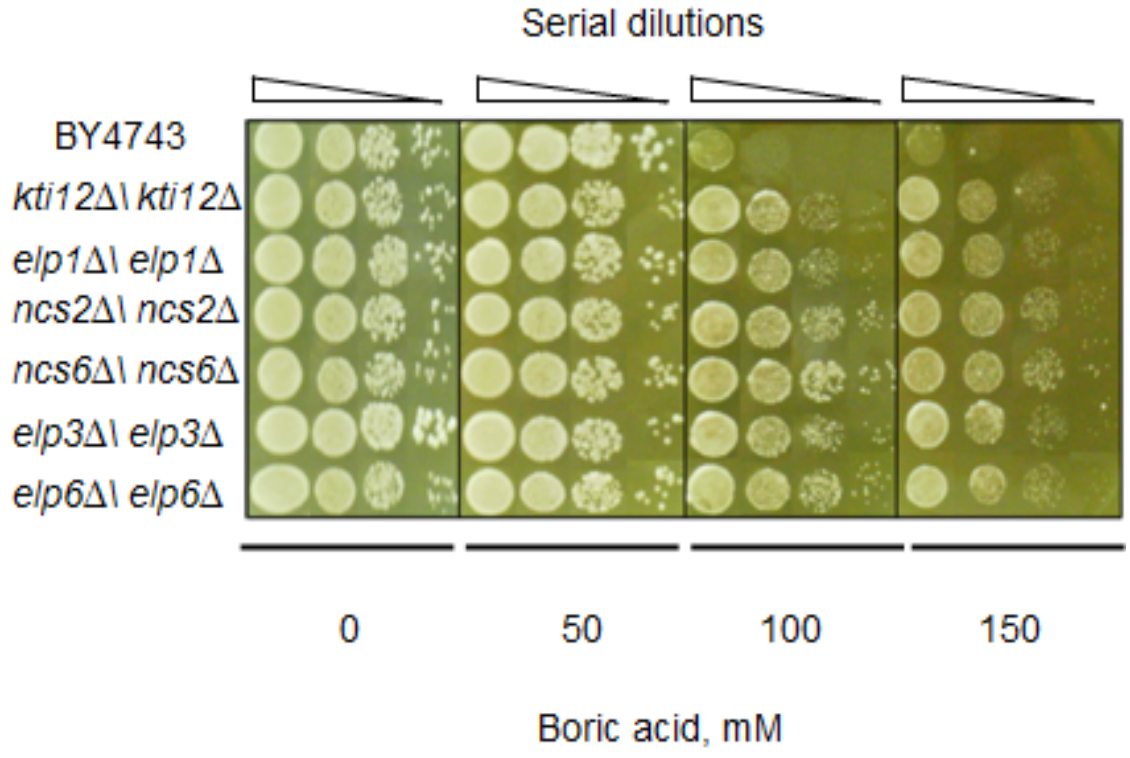


Figure 3.2. Boron resistant homozygous diploid mutants

3.2. Identification of Boron Sensitive Mutants

To find genes whose absence sensitizes cells for boron, the yeast haploid deletion set was screened for the mutants that can not tolerate boron levels that are not toxic. 21 mutants that can not tolerate boron concentrations over 40 mM were identified through this screening (Figure 3.3). Absence of the same genes in homozygous diploid background also caused sensitivity to boron (Figure 3.4). As seen in Table 3.1 these mutants were lacking genes that play role in many different metabolic pathways. For example, absence of two genes (*THR1* and *HOM6*) related to amino acid metabolism resulted in boron sensitivity. *THR1* is a homoserine kinase which involves in threonine, serine, methionine and isoleucine metabolism, and regulated by general amino acid control mechanism (Mannhaupt, et al. 1990). *HOM6* encodes for homoserine dehydrogenase which plays role in theronine, serine, methionine and lysine metabolism. Also cells lacking *VPS16, BRE5, UBP3, BRO1* and *SAC1*genes were found sensitive to boron. These genes play roles in vesicular and vacuolar transport systems. Sac1 has phosphatase activity and is localized to Golgi and endoplasmic reticulum. Bro1 plays a role in multivesicular body pathway (Odorizzi, et al. 2003). *UBP3* and *BRE5* work together to regulate trafficking between Golgi and ER (Cohen, et al. 2003). *PHO85* gene encodes for a cyclin-dependent kinase (CDK). This CDK plays role depending on the cyclin partner, in cellular responses to nutrients, environmental stress, and in regulation transcription during the cell cycle (Huang, et al. 2007). *CCS1* has been known as an antioxidant protein since it plays role as a copper chaperone for superoxide dismutases I (Sod1) (Schmidt, et al. 1999). Also there were sensitive mutants deficient in carbohydrate metabolism (*rpe1*Δ, *reg1*Δ, *och1*Δ). Apart from these, absence of *ERG4* which is a sterol reductase and plays role in ergosterol synthesis and ADK1 which has roles in purine metabolism also provided boron sensitivity.

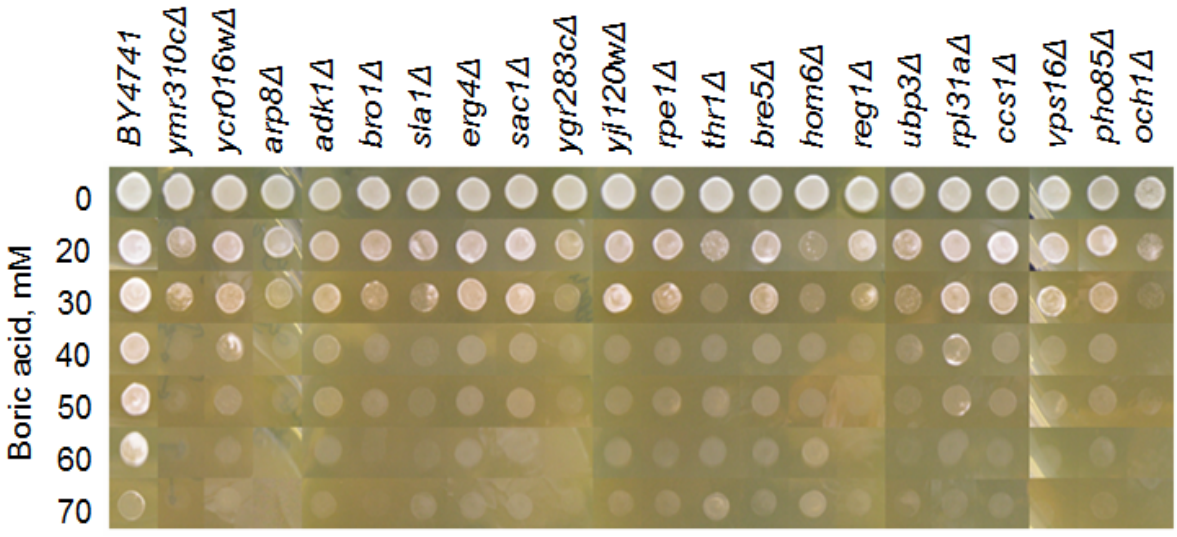


Figure 3.3. Haploid boron sensitive mutants

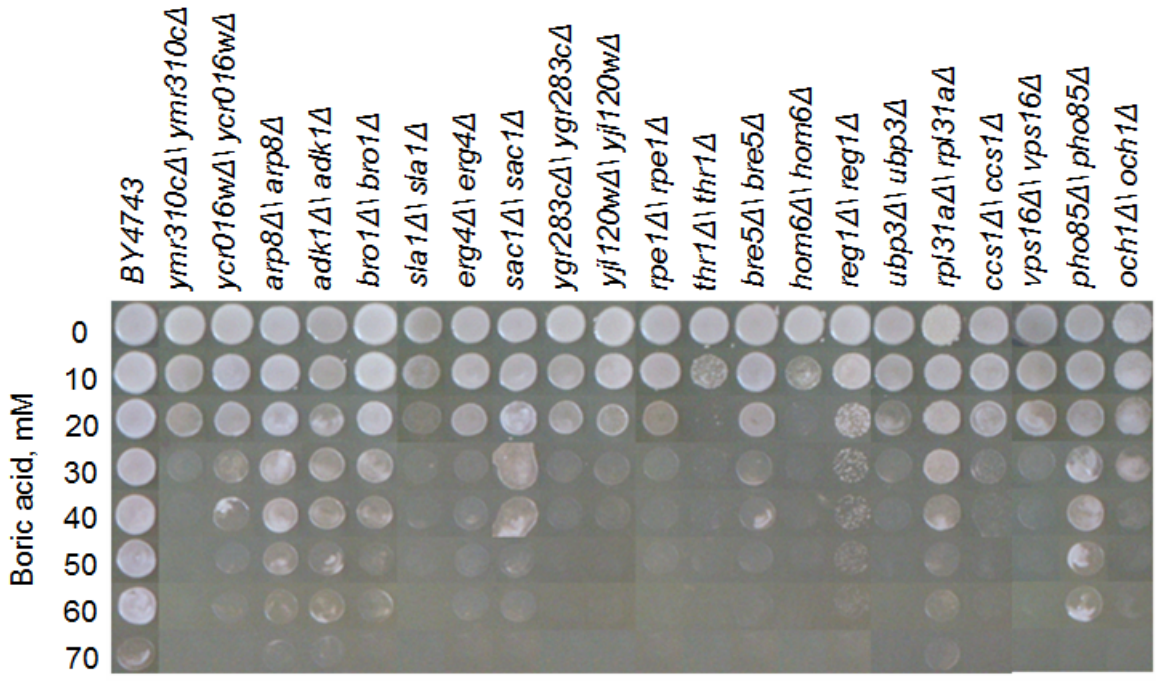


Figure 3.4. Boron sensitive homozygous diploid mutants

Table 3.1. List of the mutants that show boron sensitivity. Data extracted from Saccharomyces Genome Database

Systematic Name	Common Name	Function
YBL007C	SLA1	Cytoskeletal protein binding protein
YCR016W		Putative protein of unknown function
YDL075W	RPL31A	Protein component of the large (60S) ribosomal subunit
YDR028C	REG1	Regulatory subunit of type 1 protein phosphatase Glc7p
YDR226W	ADK1	Adenylate kinase
YER151C	UBP3	Ubiquitin-specific protease
YGL012W	ERG4	C–24(28) sterol reductase
YGL038C	OCH1	Mannosyltransferase of the cis-Golgi apparatus
YGR283C		Protein of unknown function
YHR025W	THR1	Homoserine kinase
YJL120W		Dubious open reading frame
YJL121C	RPE1	D-ribulose-5-phosphate 3-epimerase
YJR139C	HOM6	Homoserine dehydrogenase
YKL212W	SAC1	Phosphatidylinositol phosphate (PtdInsP) phosphatase
YMR038C	CCS1	Copper chaperone for superoxide dismutase Sod1p
YMR310C		Putative protein of unknown function
YNR051C	BRE5	Ubiquitin protease cofactor
YOR141C	ARP8	Nuclear actin-related protein
YPL031C	PHO85	Cyclin-dependent kinase
YPL045W	VPS16	Subunit of the vacuole fusion and protein sorting HOPS complex and the CORVET tethering complex
YPL084W	BRO1	Cytoplasmic class E vacuolar protein sorting (VPS) factor

3.3. Transcriptional Regulation as an Answer to Boron Treatment

Recently our group found that *ATR1* is responsible for high boron tolerance in *S. cerevisiae* via screening a genomic DNA library. *ATR1* codes for a multidrug resistance transport protein of the major facilitator superfamily. Deletion of *ATR1* caused increased sensitivity to high boron in the environment and its expression provided a strong tolerance. It was localized to the cell membrane and was a boron efflux pump. It is widely distributed among bacteria and fungi. This study also showed that a group of genes involved in amino acid biosynthesis and membrane transport were expressed as a result of boron treatment which were presented with DNA microarrays (Kaya, et al. 2009). Since boric acid treatment induced *ATR1* expression over threefold which was the greatest increase among transporter genes, we further analyzed the expression of *ATR1* gene to understand molecular mechanisms between the *ATR1* gene and boron treatment. A previous study showed *GCN4* and *YAP1* transcription factors play roles in *ATR1* induction (Coleman, et al. 1997). Yap1 as a well known transcription factor controls the oxidative stress response genes and is likely to take a role in activation of *ATR1* (Lee, et al. 1999). Gcn4 is a major regulator of gene expression during amino acid starvation in yeast and also under conditions of nutrient limitation and other forms of cell stress. Gcn2 is a protein kinase and upregulates the expression of *GCN4* mRNA translation under starvation conditions and accumulation of high levels of uncharged tRNA seems to be an important factor that induces Gcn2 activity (Natarajan, et al. 2001). When considering all these information, we reasoned that boron treatment may transcriptionaly activate ATR1 in a GCN-system dependent manner. To test this hypothesis, firstly spotting assays were conducted in terms of examining the boron sensitivity of the mutant cells that lack *YAP1*, *GCN2* and *GCN4* respectively. As seen in Figure 3.5 these mutants were all sensitive to boron.

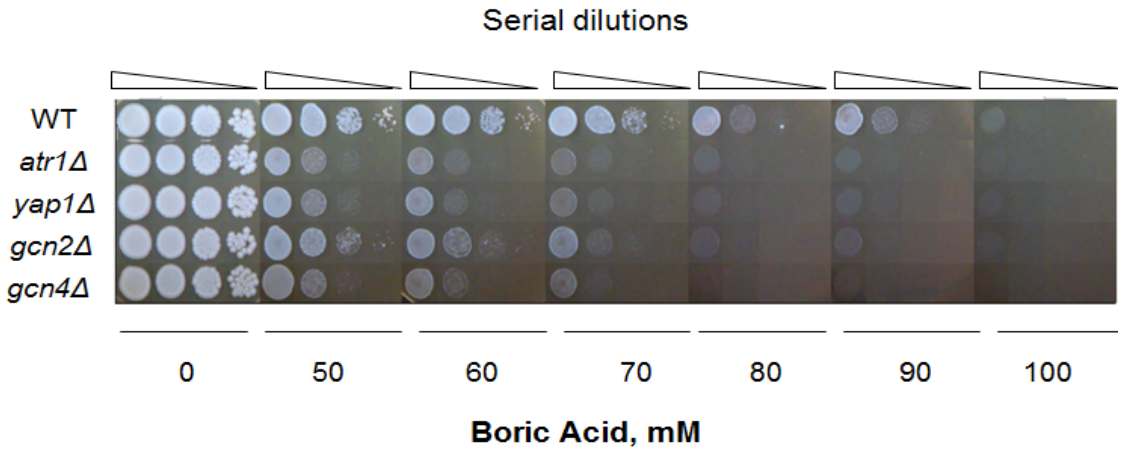
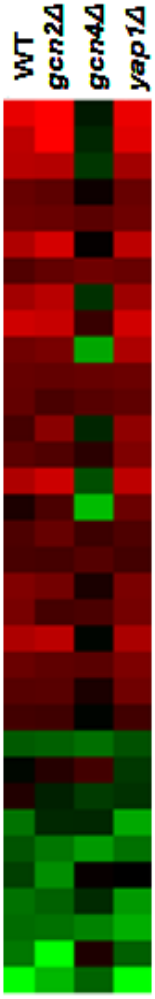


Figure 3.5. Comparison of boron sensitivity of haploid transcription factor mutants

To reveal which transcription factor regulates the expression of ATR1 in response to boron DNA microarray experiments were conducted. In these experiments the induction of *ATR1*, amino acid biosynthesis genes and transporter genes were analyzed in wild type, *gcn2*Δ, *gcn4*Δ, *yap1*Δ mutant cells after a 20 mM boric acid treatment for one hour. As shown in Figure 3.6, *ATR1* was not upregulated in only *gcn4*Δ mutant cells in response to boron. Deletion of *YAP1* and *GCN2* did not alter the expression profile of *ATR1*. Thus, transcription factor Gcn4 is required for *ATR1* expression in response to boron. Besides ATR1, upregulation of amino acid biosynthesis genes were also inhibited in cells lacking *GCN4* gene.



		Biological function, Molecular process, Cellular component
ATR1	YML116W	Multidrug transport, Multidrug efflux pump, Plasma membrane
YMC2	YBR104W	Transport, Not yet annotated, Not yet annotated
YMC1	YPR058W	Transport, Not yet annotated, Not yet annotated
ESBP6	YNL125C	Unknown, Unknown, Not yet annotated
DUR3	YHL016C	Transport, Not yet annotated, Not yet annotated
BAP2	YBR068C	Amino acid transport, Amino acid transporter, Plasma membrane
GIT1	YCR098C	Unknown, Unknown, Not yet annotated
MCH1	YDL054C	Unknown, Unknown, Not yet annotated
-	YGL186C	Unknown, Unknown, Not yet annotated
SSU1	YPL092W	Sulfite transport, Sulfite transporter, Plasma membrane
-	YIL023C	Unknown, Unknown, Not yet annotated
AGP3	YFL055W	Transport, General amino acid permease, Not yet annotated
BAP3	YDR046C	Amino acid transport, Amino acid transporter, Plasma membrane
PXA2	YKL188C	Transport, Not yet annotated, Not yet annotated
OAC1	YKL120W	Sulfate transport, Oxaloacetate carrier, Mitochondrial inner membrane
AGP1	YCL025C	Amino acid transport, Amino acid transporter, Plasma membrane
YBT1	YLL048C	Transport, Not yet annotated, Not yet annotated
PHO87	YCR037C	Transport, Not yet annotated, Not yet annotated
MUP1	YGR055W	Sulfur amino acid transport, L-methionine porter, Integral plasma membrane protein
ENB1	YOL158C	Ferric-enterobactin transport, Ferric-enterobactin transporter, Integral membrane
AQR2	YBR043C	Unknown, Not yet annotated, Not yet annotated
-	YBR293W	Unknown, Not yet annotated, Not yet annotated
PDR12	YPL058C	Transport, Xenobiotic-transporting ATPase, Not yet annotated
HXT5	YHR096C	Hexose transport, Glucose transporter, Not yet annotated
ACB1	YGR037C	Fatty acid metabolism, Acyl-CoA binding, Unknown
HXT6	YDR343C	Hexose transport, Glucose transporter, Not yet annotated
FUI1	YBL042C	Transport, Not yet annotated, Not yet annotated
HXT3	YDR345C	Hexose transport, Glucose transporter, Not yet annotated
GSP2	YOR185C	Nuclear organization and biogenesis, RAN small monomeric GTPase, Nucleus
CTR3	YLR411W	Transport, Not yet annotated, Not yet annotated
HXT2	YMR011W	Hexose transport, Glucose transporter, Not yet annotated
SSA1	YAL005C	Protein folding, Chaperone, Cytoplasm
CTR1	YPR124W	Transport, Not yet annotated, Not yet annotated
HXT4	YHR092C	Hexose transport, Glucose transporter, Not yet annotated

Figure 3.6. The changes in the expression levels of transporter genes in transcription factor mutants by boron treatment

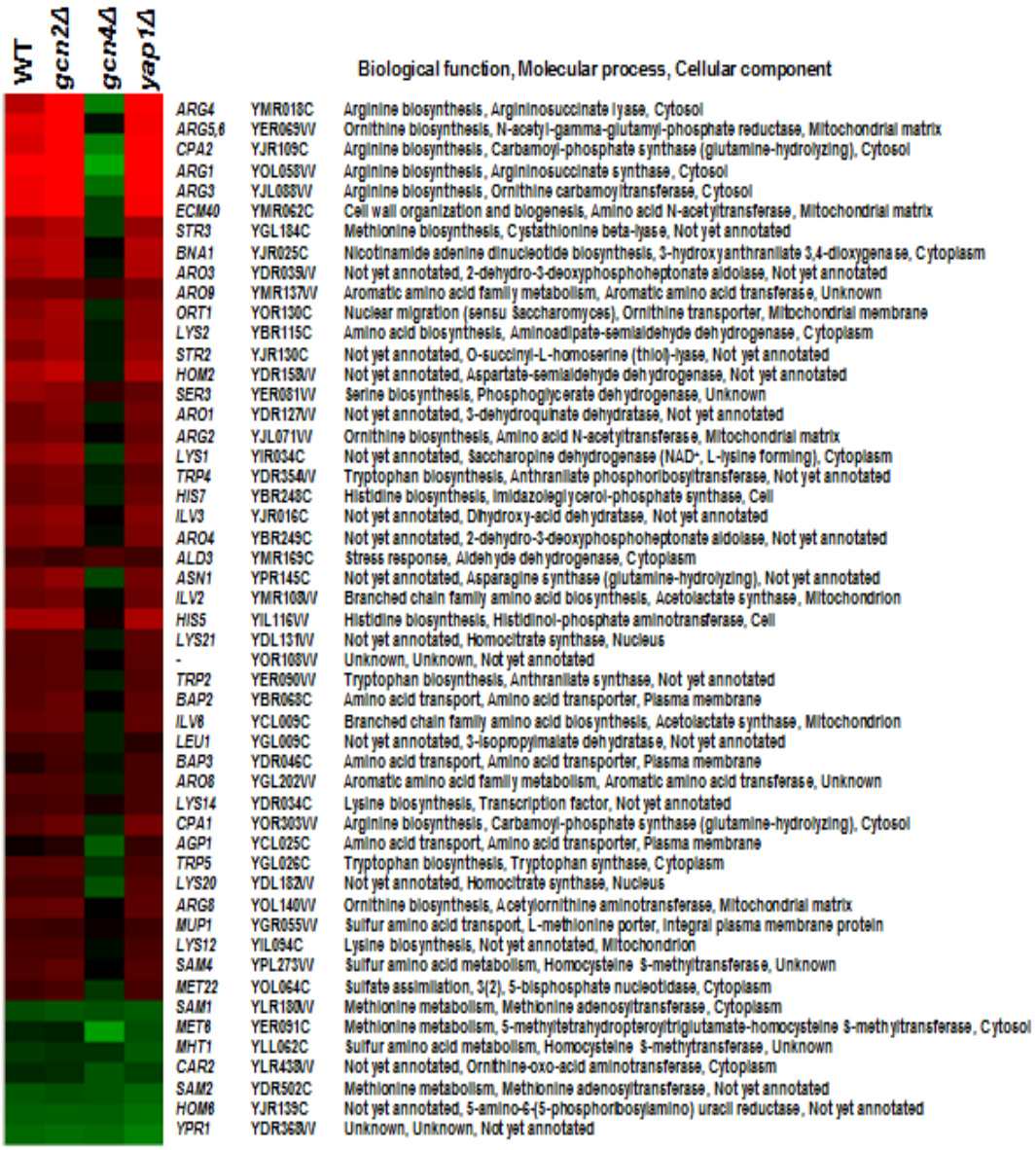


Figure 3.7. The changes in the expression levels of amino acid biosynthetic genes in transcription factor mutants by boron treatment

To confirm that *ATR1* is not upregulated in *gcn4*Δ mutants, we checked the mRNA level of ATR1 in a similar manner by a Real Time PCR approach. It was found that *ATR1* mRNA levels were similar in untreated cells, but upon boron treatment *ATR1* upregulation was not observed in *gcn4*Δ cells which confirmed the microarray results. However, deletion of *YAP1* and *GCN2* partially prevented ATR1 upregulation in response to boron.

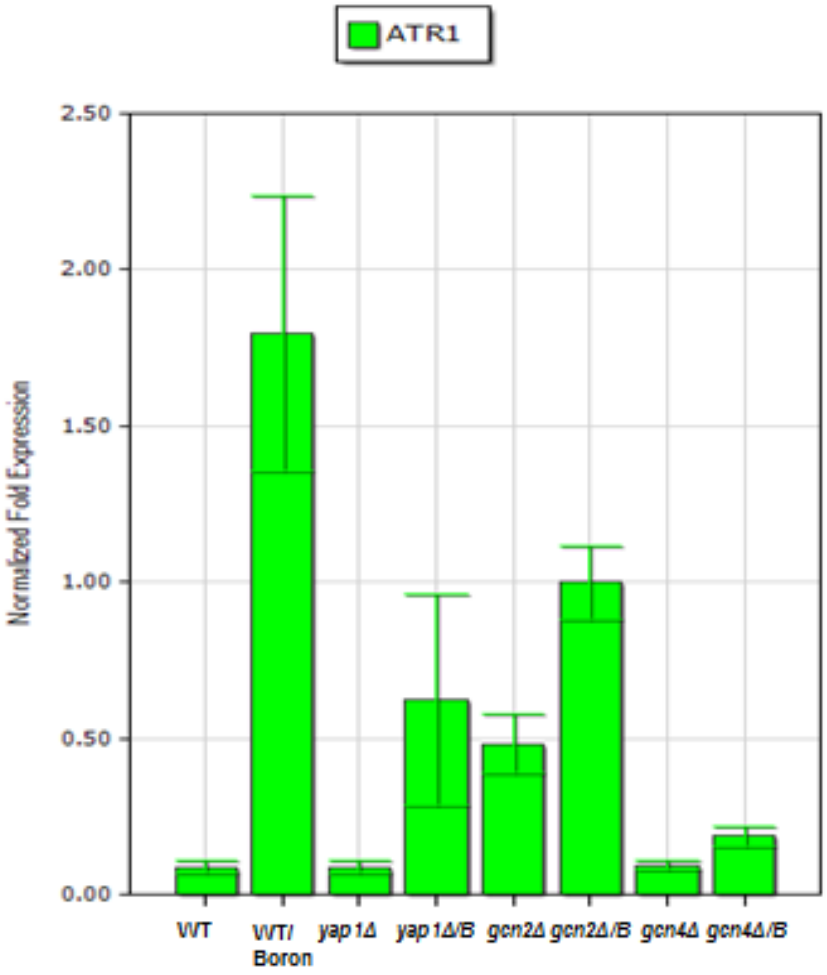


Figure 3.8. Real time PCR transcriptional analyses of *ATR1*

Moreover, we cloned and overexpressed *GCN2*, *GCN4* and *YAP1* genes to see whether they provide resistance to boron. Interestingly only YAP1 overexpressing cells are viable in the presence of 150 mM boric acid (Figure 3.9 and Figure 3.10).

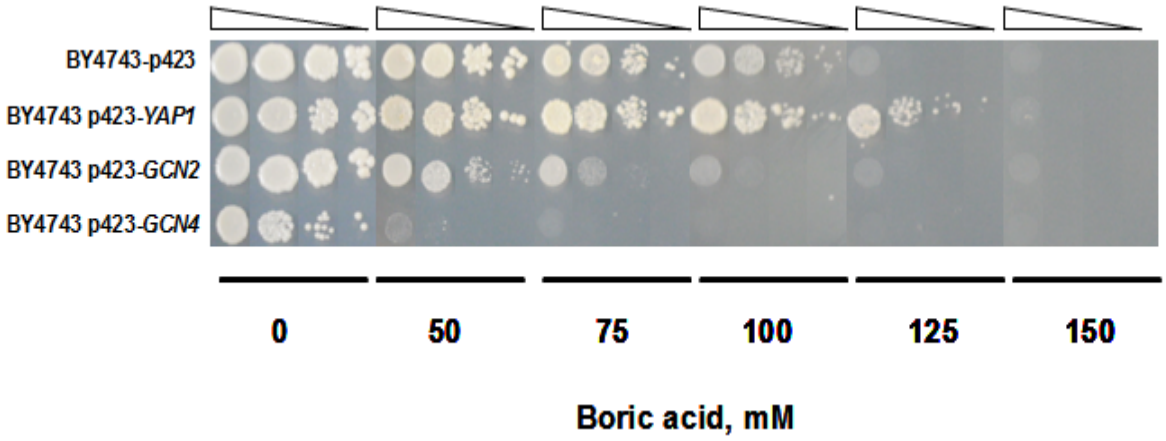


Figure 3.9. Wild type cells overexpressing *GCN2*, *GCN4*, and *YAP1* genes

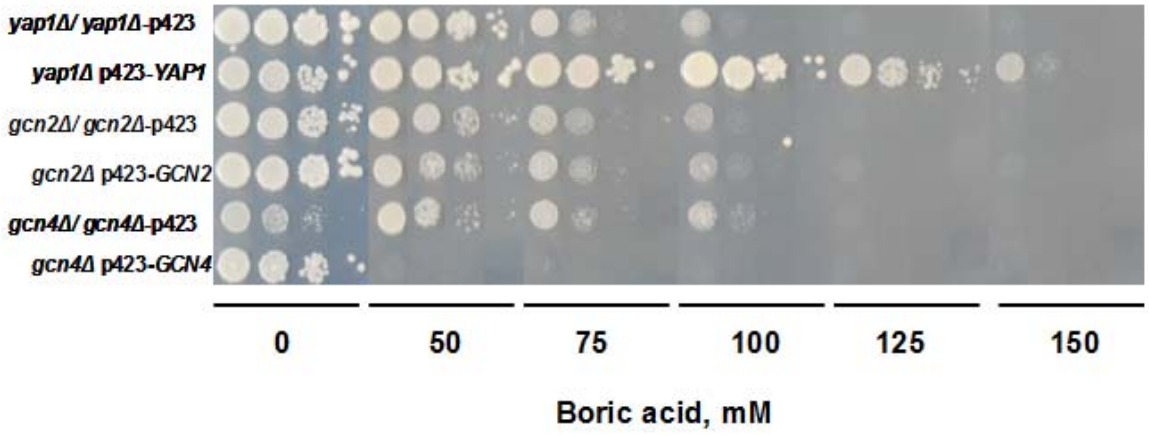


Figure 3.10. Isogenic mutants overexpressing *GCN2*, *GCN4*, and *YAP1* gene

3.4. Translational Response to Boron Treatment

In order to understand molecular mechanisms that lay behind boron toxicity, it should be known what boron does in the cell and in which direction it changes transcriptional and translational machineries of cells for survival. First of all, polysome profiling was carried on to reveal how cells respond to boron translationally and then the entry of ^{35}S-labeled methionine to newly synthesizing proteins was followed.

Polysomes are a cluster of ribosomes bound to an mRNA molecule and actively translate the message in the process of protein synthesis. And metabolic labeling techniques, such as 35-S methionine labelling, are widely used to study the biosynthesis properties of proteins. As seen in Figure 3.11, boron treatment decreased the number of polysomes in samples separated by sucrose gradient centrifugation. Similarly, the ratio of 35S labelled methionine incorporation into newly synthesized proteins was lower than that of wild type cells (Figure 3.12). Both of these assays suggest that protein synthesis is impaired under boron stress.

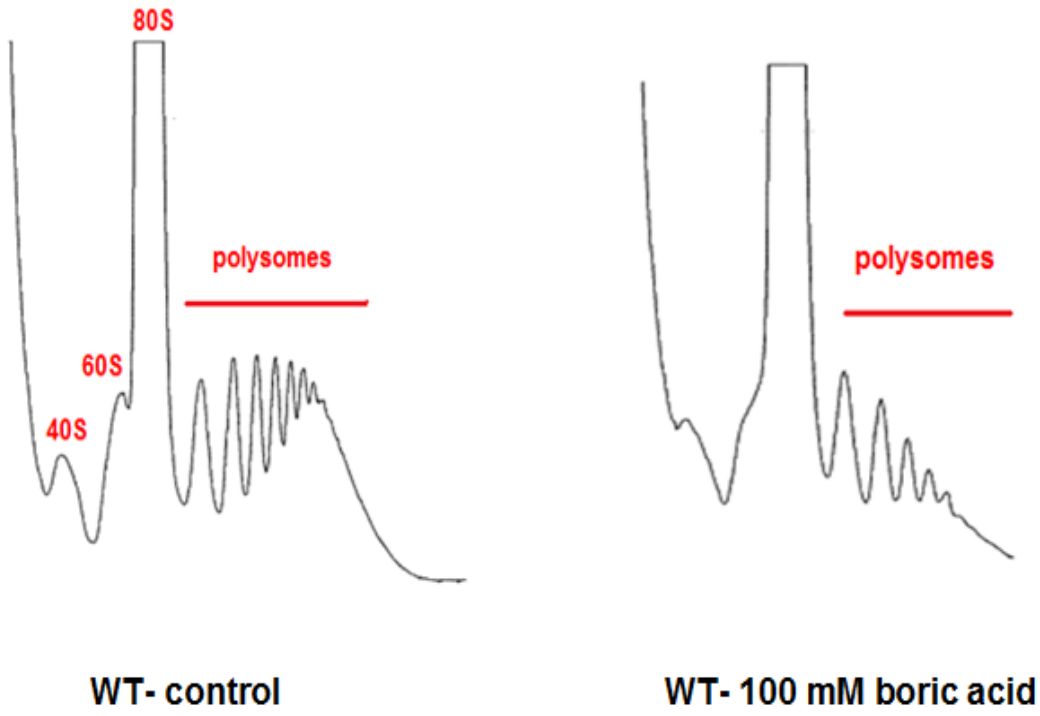


Figure 3.11. Polysome profiles of wild type yeast cells which were exposed either 0 mM or 100 mM boric acid

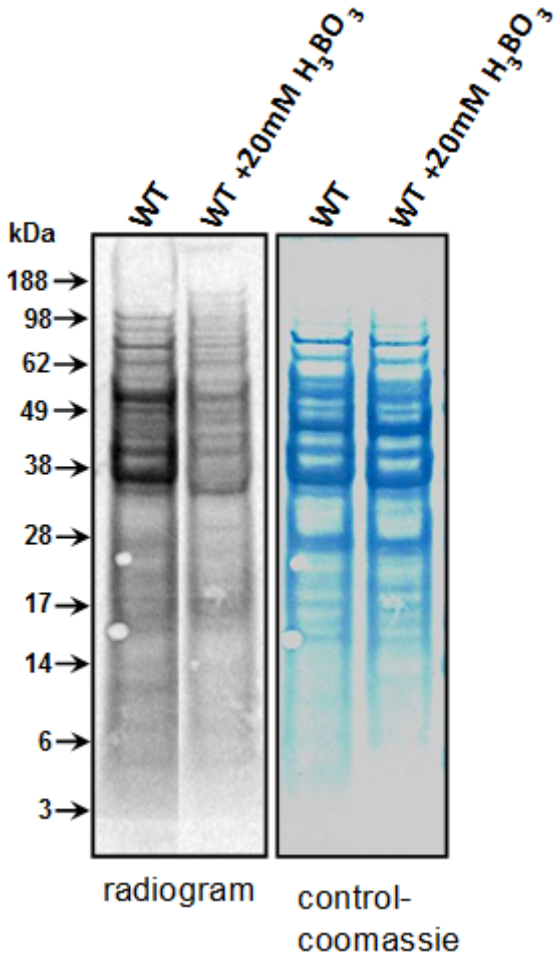


Figure 3.12. Radioactive methionine enters into newly synthesized proteins

When considering polysome profiles and metabolic labeling, it was shown that boron plays role in translation initiation. Therefore it is necessary to review the basic principles of translation in eukaryotic cells. Protein translation can be analyzed in three different stages. Firstly, translation initiation modulates the positioning of the 80S ribosome at the AUG start codon correctly. Secondly, translation elongation makes sure the addition of the amino acids to the polypeptide chain which is growing and lastly, translation termination leads to the release of the protein when faced with a stop codon. Following the release, newly synthesized proteins are subject to cotranslational and posttranslational quality control mechanisms.

Protein synthesis is basicly regulated at translation initiation level as a result of extracellular and intracellular signals, and stress conditions. Sequestration of cap-binding eIF4 by eIF4E-binding proteins under nutrient deprivation and as opposite, phosphorylation of eIF4E-binding proteins by TOR kinase when presence of excess

nutrient. Apart from that under starvation and stress conditions eIF2α becomes an inhibitor of eIF2B with the phosphorylation by Gcn2. These are just two mechanisms that control translation initiation. Although *GCN2*-dependent regulation inhibits general translation, because of reduced ternary complex levels, it also activates some stress related proteins such as mammalian *ATF4* and yeast *GCN4* (Figure 3.13). The expression of more than 500 target genes most of which consisted of amino acid biosynthesis genes is controlled by Gcn4. Besides amino acid deprivation, the conditions of starvation or stress also cause an upregulation in the Gcn4 protein levels (Deplazes, et al. 2009). This regulatory response has been known as general amino acid control (GAAC).

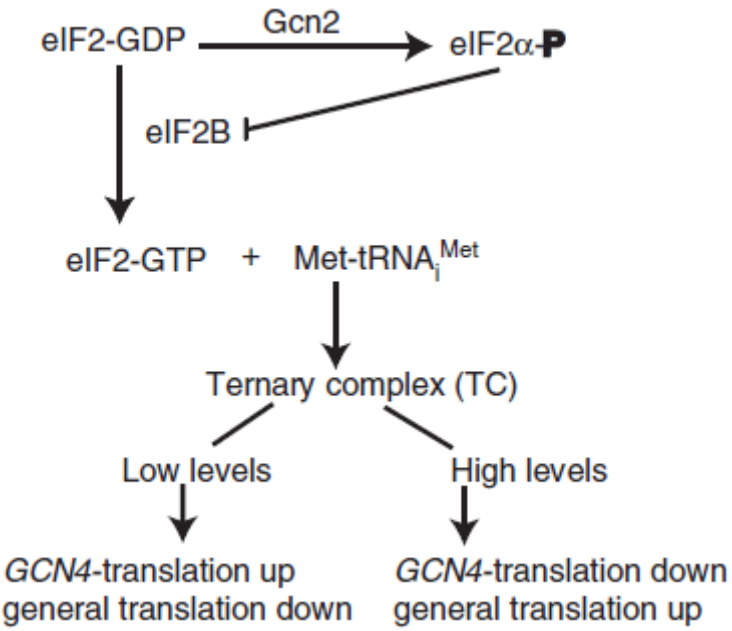


Figure 3.13. Regulation of *GCN4* translation and general translation by ternary complex levels (Source: Deplazes, et al. 2009)

As a best characterized mechanism for 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 that activate the kinases heme-regulated inhibitor (HRI), double-stranded RNA-activated

protein kinase (PKR), pancreatic protein kinase activated by endoplasmic reticulum stress (PERK/PEK), and nutrient-regulated protein kinase (Gcn2) (Kimball 1999).

As indicated in Figure 3.14 yeast protein kinase *GCN2* contains a histidyl tRNA synthetase-related domain (HisRS) which includes conserved motif 2 sequence that interacts with the acceptor stem of tRNA. According with the idea that amino acid starvation activates Gcn2, it has been found that Gcn2 has affinity for different uncharged tRNAs (Dong, et al. 2000). Binding of uncharged tRNA to the HisRS-like domain would produce a conformational change in Gcn2 and this activates its kinase function. Also autophosphorylation of threonines 882 and 887 in protein kinase domain is essential for Gcn2 function. However under nonstarvation conditions phoshorylation of serine 577 inhibits Gcn2 function by decreasing the affinity of Gcn2 for uncharged tRNA (Cherkasova and Hinnebush 2003, Hinnebusch 2005).

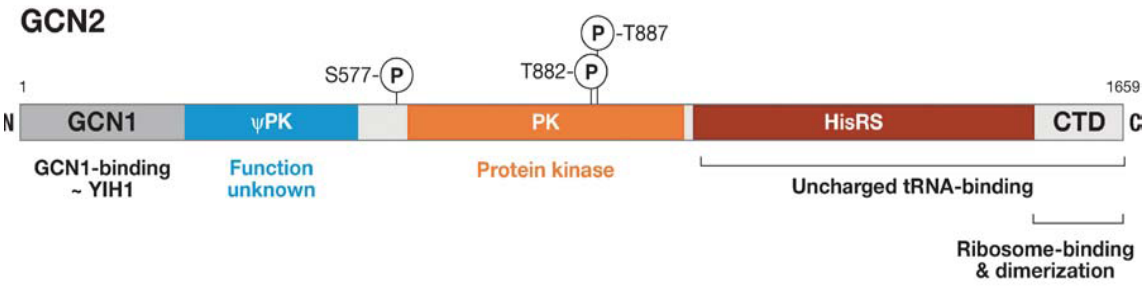


Figure 3.14. Functional domains and phosphorylation sites of Gcn2
(Source: Hinnebusch, 2005)

Since boron stress is mediated by Gcn4 transcription factor, we tested whether Gcn4 protein level is upregulated by boron stress. To do so we used a yeast strain whose chromosomal *GCN4* was tagged with Myc epitope. Using Myc antibodies, expression of Gcn4 was shown to be increased in boron treated cells (Figure 3.15).

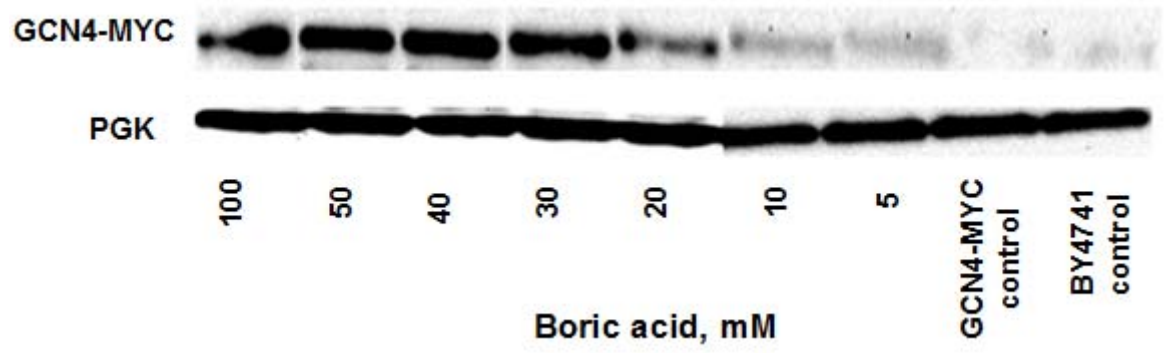


Figure 3.15. Western blot analysis for *GCN4*-MYC. PGK is phosphoglycerate kinase and used as internal control

It was interesting to see that boron inhibited protein syntesis, but synthesis of Gcn4-Myc was not affected from this inhibition. Previously it was shown that in the case of starvation, Gcn2 kinase play role in inhibition of general protein synthesis, but on the other hand specifically activate the translation of Gcn4 (Hinnebusch 2005). Being a boron tolerance gene, protein level of Atr1 was not significantly induced in response to boron. Low doses of boron induced the expression of chromosomally expressed Atr1-GFP fusion protein as detected by anti-GFP antibodies, however higher doses of boron blocked the increase in protein level of Atr1-GFP because of the inhibitory effect of boron on protein synthesis (Figure 3.16).

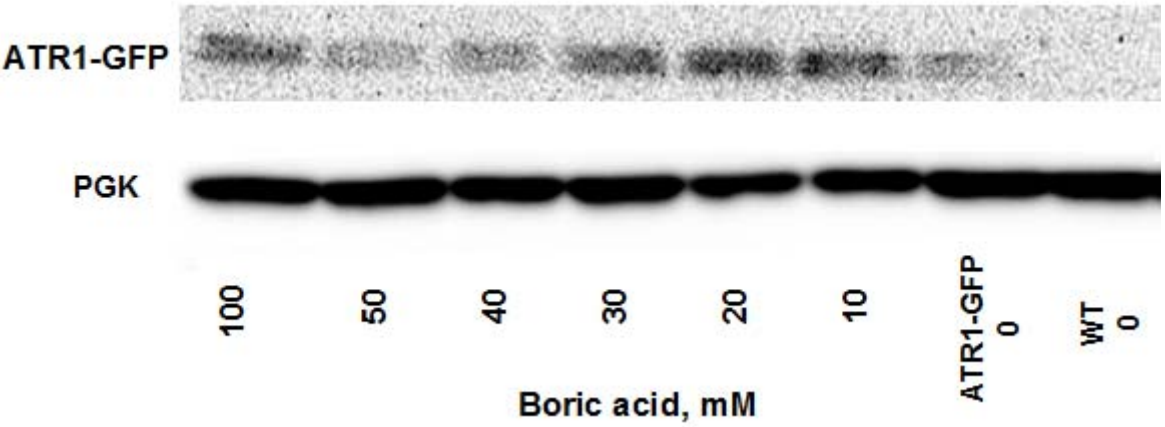


Figure 3.16. Western blot analysis for *ATR1*-GFP. PGK is phosphoglycerate kinase and used as internal control

Additionaly, to see if boron treatment causes phosphorylation of eIF2α and if this was modulated by Gcn2 kinase, a western blot assay was performed with an antibody which specifically recognize the Ser-51 phosphorylated form of yeast eIF2α. As expected, boron treatment caused eIF2α phosphorylation mediated by Gcn2 kinase, because this phosphorylation did not occur in cells lacking the *GCN2* gene (Figure 3.17).

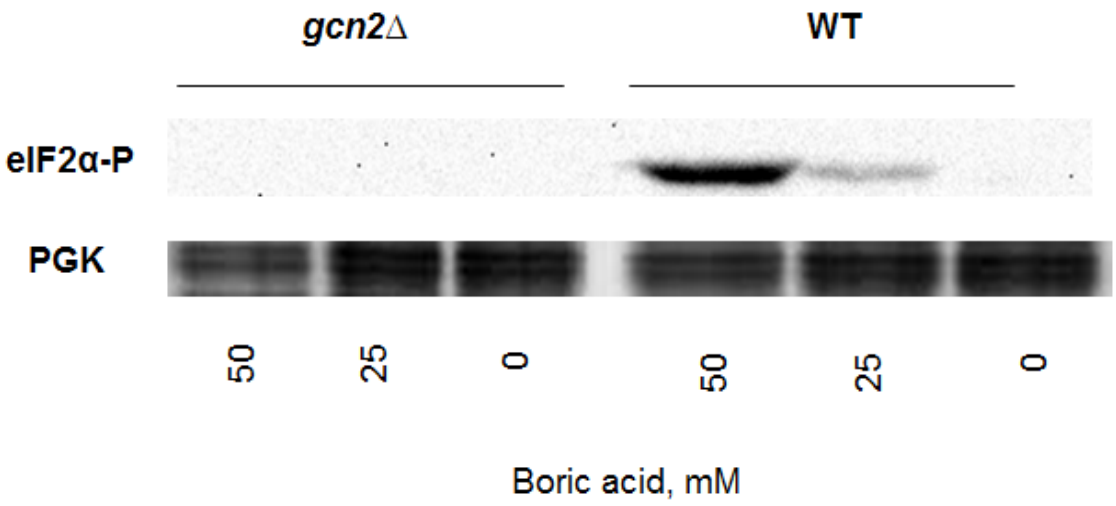


Figure 3.17. Western blot analysis for eIF2α-P

To determine whether Gcn2 activation by boric acid requires binding of uncharged tRNAs to tRNA binding site of Gcn2 (m2 domain) or phosporylation of serine 577, plasmids containing wild type *GCN2* and mutated form of *GCN2* (*GCN2* without m2 domain and *GCN2* with S577A or combination of both) were transferred to *gcn2Δ* mutant cells and their ability to phosphorylate eIF2α was investigated in boron treated cells. As seen in Figure 3.18, S577A substitution in Gcn2 did not prevent eIF2α phosphorylation showing that boron effect is mediated independently of regulatory serine 577 status. However phosphorylation of eIF2α did not occur without the m2 domain of Gcn2 in the presense of boron which suggest that Gcn2 kinase activation requires uncharged tRNA binding to an intact HisRS-like (m2) domain in Gcn2.

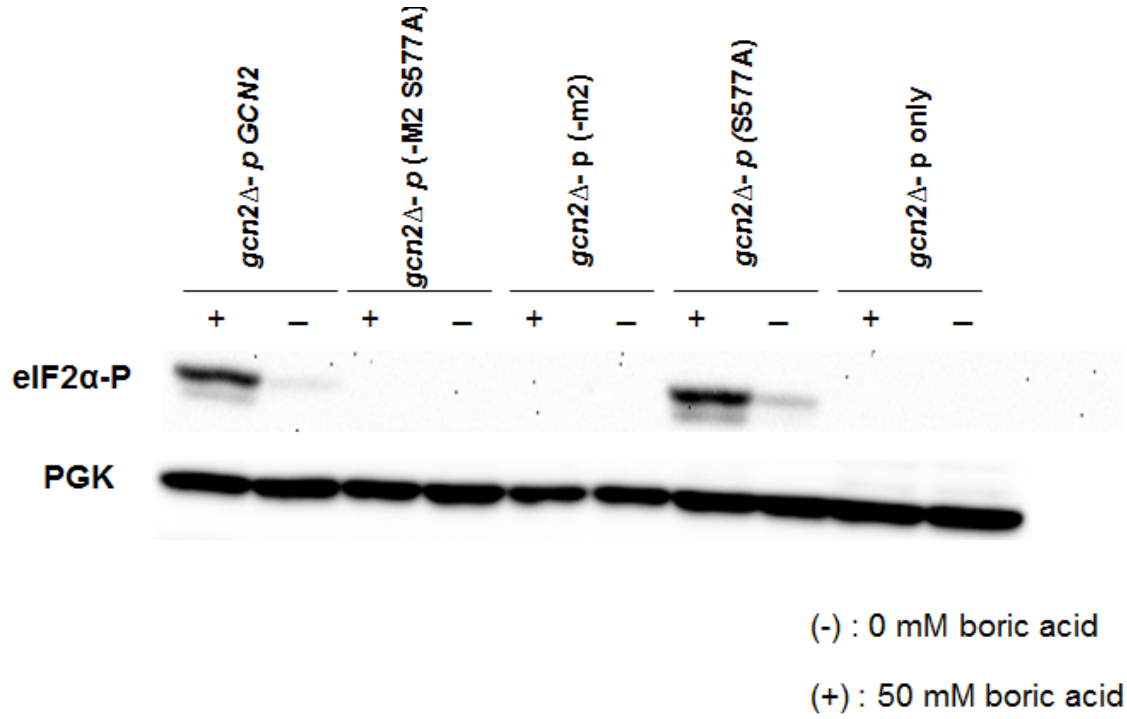


Figure 3.18. Western analysis showing the effect of boric acid on eIF2α phosphorylation

CHAPTER 4

CONCLUSIONS

Boron is an important micronutrient in plants and animals and has many structural and physiological functions in these cells. However these specific functions are not yet clear. In addition, boron is toxic to cells when present excess in the environment. To survive against the toxic effects of boron, it is vital for cells to develop strategies. These strategies which provide low intracellular boric acid content can include removing this toxic element from the cytosol either by active transport or by sequestration in an internal organelle, and controlling the influx. Several genes associated with boron transport and tolerance have been identified in plants so far. For the characterization of these genes the yeast *Saccharomyces cerevisiae* has been widely used as a model system. Recently, *ATR1* has been found as a boron tolerance gene by screening a yeast genomic DNA library. Overexpression of *ATR1* conferred high resistance to boron treatment whereas deletion of this gene caused hypersensitivity. Also it was shown that *ATR1* expression was regulated by boron (Kaya, et al. 2009).

In this study, we investigated the transcriptional regulation of *ATR1* in response to boron treatment and found that the transcription factor Gcn4, which is a very well known activator of amino acid biosynthesis genes, mediates the boron stress and induces the *ATR1* efflux pump along with the amino acid biosynthesis genes. Gcn2, which is an activator of Gcn4, but an inhibitor of translation did not play role in *ATR1* regulation, however Gcn2 played a role in inhibition of translation in response to boron treatment. The M2 domain of Gcn2, where uncharged tRNAs bind to, was required for Gcn2 to phosphorylate eIF2α and inhibit translation. Thus boron treatment causes accumulation of tRNAs carrying no amino acids and triggers uncharged tRNA response. However, the details of how boron affects the tRNAs are not known and it needs to be characterized.

In addition to mechanisms of ATR1 regulation, we also found several genes that play role in boron tolerance by screening a yeast deletion library and many distinct genes and pathways were found to be important in boron stress response. All of the boron resistant mutants identified by the genetic screen lack the genes that play roles in

the wobble base modifications of tRNAs during the translation. Boron sensitive mutants were lacking genes that play role in different metabolic pathways such as amino acid metabolism, lipid metabolism, vacuolar transport, oxidative stress response.

Most of the genes identified in this study have homologs in animals and plants. Therefore this study may shed light on the mechanisms of boron transport and toxicity in higher organisms. Our results may also provide clues about the unknown metabolism of other transporter and metal tolerance genes.

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