IDENTIFICATION OF GENES THAT PLAY ROLES IN BORON METABOLISM

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 Biotechnology

by
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July 2008

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ACKNOWLEDGMENTS

First of all, I would like to thank my supervisor Assist. Prof. Dr. Ahmet KOÇ who give me the chance to study in this delightful project, his guidance, beneficial suggestions, his support and help not only directly related with the project, but also with all the troubles I have met, and for always being there for me whenever I needed him.

I would like to thank Assist. Prof. Dr. Çağlar KARAKAYA for his guidance and moral support during my studies and also his trustworthy friendship.

I also would like to thank Assoc. Prof. Dr. Talat YALÇIN, Assist. Prof. Dr. Alper ARSLANOGLU and Assoc. Prof. Dr. Anne FRARY for their guidance and help.

I would like to thank my friends and lab mates Ercan Selçuk ÜNLÜ, Ersin AKINCI, Ahmet Emin ATİK, Elise HACIOĞLU, Hasan Cihad TEKEDAR and Erhan BAL for their sincere help and their good friendship. I also wish to express my thanks to all my other friends working in the Molecular Genetic Laboratory.

Finally, I would like to thank my family for both the financial and moral support they showed in every stage of my life. They will always be in my heart.

I also would like to thank The Scientific and Technological Research Council of Turkey (TUBITAK) for their financial support, it was important for my success to have TUBITAK's scholarship.

ABSTRACT

IDENTIFICATION OF GENES THAT PLAY ROLES IN BORON METABOLISM

Boron, is an essential microelement that plays a role in plant and animal development. However, an excess amount of boron is toxic for both types of organisms. The yeast *Saccharomyces cerevisiae* is an ideal model organism for describing the mechanism and regulation of metal ion transport in eukaryotes. We screened a yeast genomic DNA library to find genes that confer boron resistance to wild-type cells.Thirty transformants were isolated that were able to grow in the presence of a toxic amount of boron and all of them contained the multidrug efflux transporter gene *ATR1* (YML116w) in the expression cassette. Our subsequent analysis revealed that *ATR1* deletion mutants (Δ*atr1*) were sensitive to boron treatment and hyper-accumulated boron inside cells, whereas wild-type cells overexpressing the *ATR1* gene were resistant to boron and hypoaccumulated boron in cells. We also analysed the global gene expression pattern in response to boron treatment and found that expression of *ATR1* is upregulated along with many other transporter and amino acid biosynthesis genes. Our data suggest that the *ATR1* gene functions as a boron efflux pump and is reqired for boron tolerance in yeast cells.

ÖZET

MAYA DA BOR METABOLİZMASINDA ROL OYNAYAN GENLERİN BULUNMASI

Bor bitki ve hayvan gelişiminde rol oynayan temel mikroelementlerdendir. Yine de dokularda ki yüksek konsantrasyonu iki organizma grubu içinde toksik etki göstermektedir. Maya mantarı (*Saccharomyces cerevisiae)* ökaryotlar da metal iyon transport mekanizmalarının çalışılması açısından ideal bir model organizmadır. Bu çalışma da maya genomik DNA' sı taranarak yabani tip maya hücreside bor dirençliliği sağlayan genler bulunmaya çalışıldı. Çalışma sonunda otuz maya transformantının yüksek miktarda toksik etki yapan bor mineralinin oldugu ortamda büyümeyi sürdürdüğü gözlemlenmiş ve analizler sonucunda tüm transformantların multidrug efflux transporter - *ATR1* (YML116w) gen bölgesini tasıyan DNA kasetini içerdiği bulunmuştur. Sonraki çalışmalarımız da *ATR1* mutantı (Δ*atr1*) hücrelerin bor stresine duyarlı oldukları ve hücre içerisinde yüksek miktarda bor biriktirdikleri gözlemlenirken, *ATR1* geninin yüksek oranda ifadelendiği yabani tip maya hücrelerinin bor stresine karşı dirençli hale geldikleri ve borun hücreden büyük oranda uzaklaştırıldığı gözlemlenmiştir. Bir diğer çalışmamız da ise bor stresi altındaki tüm maya genlerinin ifadelenmeleri analiz edilmiş ve *ATR1* geninin diğer transporter genlerin arasından daha fazla oranda ifadelendiği bulunmuştur. Çalışmalarımız sonun da bulduğumuz tüm bu veriler bize *ATR1* geninin maya hücresinde bir bor transporter'ı olarak görev yaptığını ve maya hücresinin borun toksik etkisinden korunmasında rol oynayan önemli bir gen olduğunu göstermiştir.

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CHAPTER 1

INTRODUCTION

1.1. Boron

Boron is the only non-metal element of Group III in the periodic table whose atomic number is 5 and an atomic mass of 10.81 g/mol with the chemical symbol B. Boron is not a widespread element in rocks, soil and water. However; it is found in every part of the environment and it is likely that life evolved in the presence of this element (Woods 1992). The various inorganic compounds of boron are found widely at low concentrations throughout nature. The boron concentration in rocks varies from 5 mg/kg in basalts to 100 mg/kg in shales and in the ocean, is approximately 4.5 mg/liter (Woods 1992). Boron enters the environment mainly through the weatering of rocks, borax mining and processing, geothermal releases, boric acid volatilization from seawater, and volcanic activity (Versar Inc 1975, Anderson, et al. 1994, Larsen 1988). The major world boron deposits are found in Turkey, USA, Argentina, Russia, Chile, China, and Peru (Culver, et al. 1994).

The degree of adsorbtion of boron onto the surfaces of soil particles depends on the type of soil, pH, salinity, organic matter content, iron and aluminium oxide content, iron- and aluminium-hydroxy content, and clay content (Sprague 1972). According to Whetstone et al. (1942), boron concentrations in soils range from 10 to 300 mg/kg (average 30 mg/kg), depending on the physical and chemical characteristics of the soil under consideration. (Biggar and Fireman 1960). It was shown that the fixation of boron in soils occurs by one of three mechanisms: physical binding (molecular), in which the boron is adsorbed to the surface of the soil by van der Waals bonds; anion exchange; or chemical precipitation. Chemical adsorption occurs in two ways: ionic and covalent bonding. (Biggar and Fireman 1960)

In nature, the element boron does not exist by itself. It is combined with other common elements such as sodium to make salts like borax and with oxygen to make boric acid (H_3BO_3). Borate, is the general term associated with boron-containing minerals such as borax and boric acid. Borates are a fundamental part of daily life and

boric acid is one of the most widely used borate compounds. The most important commercial boron compounds and minerals are borax pentahydrate, borax, sodium perborate, boric acid, colemanite, and ulexite. Boric acid is one of the important boron compounds naturally occuring in rocks, soil and water (Woods 1994). Boric acid is a combination of boron, oxygen, and hydrogen (H_3BO_3). It is a very weak acid, with a p K_a of 9.15. Boric acid and borates, have been widely used in medicine as antiseptic, bactericide, and fungicide, since the 1860s (Quarles 2001).

Other boron compounds are found in consumer products such as soaps and detergents (as a bleaching agent), preservatives, adhesives, porcelain, enamel, leathers, carpets, artificial gems, high-contrast photographic materials, wicks, electric condensers, fertilizers, insecticides, and herbicides (Moore, et al. 1997). Sodium borate and boric acid are important boron compounds which are commonly used in numerous cosmetic products, including makeup, skin and hair care preparations, deodorants, moisturizing creams, breath fresheners, and shaving creams, with concentrations up to 5% (US FDA 1981, Beyer, et al.1983). In 1981, the boric acid concentrations in consumer products was determined by the US FDA to 5% (US FDA 1981) .

The elemental form of boron has a tendency to form complexes with many biomolecules (Hunt 2007). Organoboron is one of the boron complex compounds that contains B-O bonds, i.e., the orthoborates ($B(OR)_3$ (Greenwood and Earnshaw 1984). The only boron compounds that are apparent in biological systems are organoboron compounds and they are the result of combination with O, H. Amine groups can also be added into organoboron complexes (Hunt 1991). As is shown in the below reaction, organoboron complexes occur with biomolecules both in plant and animal tissues. The form of dietary boron after ingestion and hydrolysis is boric acid (orthoboric acid) $B(OH)_3$ (Greenwood and Earnshaw 1984). Boric acid consists of boron and hydroxyl ion (a Lewis acid) in the form of the tetrahedral anion $B(OH)_4$ (Greenwood 1973).

$$B(OH)_3 + 2H_2O \longleftrightarrow H_3O^+ + B(OH)_4^-$$

Boric acid interacts with many organic substances found in the body, such as carbohydrates (sugars and polysaccharides), nucleotides (adenosine monophosphate and niacinamide adenine dinucleotide) and vitamins (ascorbic acid, pyridoxine and riboflavin). The interactions occur via esters bonds with hydroxyl groups found in many

of these compounds. This formation can be observed when the hydroxyl groups are next to each other and on the same side of the molecules.

Boron (in the form of boric acid) has been determined to function in a variety of metabolic and physiological systems in animals, plants and microbes but in high concentrations it is toxic to all organisms (Nielsen 1997). Boron is an essential micronutritient for plants and some boric acid products are added to plant growth media (Woods 1994). Boric acid also plays roles in the reproduction of molds and fungi (EPA 1993). It has been shown that boron is required for embryonic development in frogs and mice (Fort, et al. 1998, Lanoue, et al. 1998). According to Anderson et al. (1994) the daily intake of boron through the diet is nearly 1.21 mg/day per person (Bennett et al. 1999). Despite its importance, the biochemical mechanisms and genetic regulation of boron metabolism are not known.

The amount of boron found in various foods has been reported by Hunt et al. (1991) and Anderson et al. (Anderson 1994). The richest boron-containing foods are fruits, vegetables, pulses, legumes, and nuts (Table 1). Dairy products such as fish, meats, and most grains contain less boron than fruits and vegetables (Greenfacts 2004).

Table 1.1 The boron concentration of various foods

(Source:Hunt et, al. 1991, Anderson, et al. 1994)

Food	Boron concentration in mg/kg, fresh weight bais	
	Hunt et al. (1991)	**Anderson et al. (1994)**
Fruits		
Apple	2.73	2.38
Apple juice	1.88	2.41
Banana, raw	-	3.72
Cherries, dark	1.47	0.92
Grape juice	2.02	2.06
Orange juice	0.41	1.59
Vegetables		
Beans, green	0.46	1.56
Broccoli, stalks	0.89	-
Nuts		
Almonds	23	-
Hazelnuts	16	-
Peanuts	18	13.8
Meats		
Beef, round	<0.015	<0.05
Chicken breast	<0.015	0.09
Turkey breast	<0.015	-

1.2. Necessity, Function and Deficiency of Boron

Boron was first described as an essential micronutrient for plant growth and development by Warington in 1923 (Warington 1923). From the first findings that boron is essential for plants, many experiments were designed in order to determinethe effect of boron on different species. Most of our previous knowledge about boron comes from these plant experiments. Findings from these experiments led scientists to identify the physiological effects of boron on animals and humans. Subsequent studies have shown that boron is also an essential micronutrient for animals (Ford, et al. 1998, Rowe, et al. 1998, Park, et al. 2004).

Boron enters plants mainly as boric acid from the soil via roots (Greenwood 1973, Hu and Brown 1997, Powers and Wood 1997). Studies identified three pathways for the transmembrane transport mechanism of boron in plants. For a long time, the passive diffusion of boron across the plasma membrane had been thought to be the only

pathway for boron transport (Hu and Brown 1997). Major intrinsic proteins (MIPs), which normally shuttle water or other small uncharged molecules across the lipid bilayer, are the second method of boron transportation (Dordas et al. 2000). Recently Takano et al. (2002) showed that the BOR1 gene is required for boron export in root cells in *Arabidopsis thaliana* (Takano, et al. 2002).

In plants, boron plays important roles in carbohydrate metabolism, sugar translocation, optimum growth, pollen germination and in the structure of the pectic polysaccharide, rhamnogalacturonan II. (O'Neill et al. 2001, Iwai et al. 2002, Lovatt and Dugger 1984). Boron deficiency causes sterility and flower malformation in a wide variety of both monocots and dicots and many secondary effects including anatomical, physiological, and biochemical changes. Other than vascular plants, boron is also essential for diatoms, marine algal flagellates, and *Cyanobacteria*.

Boron requirements have not yet been clearly determined for humans but according to Nielsen et al. (2006) boron may be beneficial, especially for transportation and regulation of some ions. Ford et al. showed that boron deficiency in *Xenopus laevis*, caused abnormal embryo-larval development (Ford et al. 1998). Boron is also necessary for the normal proliferation of yeast cells (Bennett et al. 1999).

After the determination that boron is essential for plants in 1923 by Warington, studies have been based on animal experiments in order to show that boron is also essential for animals (Warington 1923). The first result from animal experiments was reported in 1945 and indicated that an excess boron level in the diet (from 100 to 1000 g/1 diet), increased survival, and total body fat and glycogen concentration in the liver in potassium deficient rats (Skinner and McHargue 1945).

Studies showed that boron is also required for the normal development of *Xenopus* embryos (Fort, et al. 1999). In addition, boron supplementation plays an important role in the growth of trout and zebrafish (Rowe, et al. 1998, Rowe and Eckhert 1999).

1.3. Toxicity of Boron

Agriculturally accessible excess concentrations of micronutritients cause toxicity which affects cultivated crops in some parts of the world (Nable and Paull 1991, Gupta 1993), Boron rich soils cause boron toxicity and decrease crop yields in different

regions of the world. Different plant species show variable boron tolerance because of differences in membrane permability to boric acid. (Huang and Graham, 1990, Hu and Brown 1997).

The toxic effect of boron can be observed when dietery boron exceeds 100 μg g^1 in animals. Toxicity of borate to early life stages of fish has been documented for several species (Birge and Black 1977, Black et al. 1993). The World Health Organisation (WHO/FAO/IAEA, 1996) indicated that a safe boron intake is a maximum of (?) 13 mg/day for humans. However, further studies should be performed as there is not enough studies on the toxic affects of boron for humans.

1.4. Yeast and Boron

The yeast *Saccharomyces cerevisiae* has been used as a model organism for the characterization of plant boron tolerance genes (Sutton 2007, Takano 2006, Nozawa 2006, Takano 2007). Yeast can grow at very high concentrations of boron and is considered as a high boron-tolerant organism (Nozawa 2006). The yeast *BOR1* gene was characterized in detail. It is localized to the plasma membrane (Zhao 2001) and functions as a boric acid exporter across the cell membrane (Takano 2007). The Δ*bor1* yeast strain overaccumulates boron inside cells (Takano 2002, Nozawa 2006). Cells that overexpress *BOR1* have lower intracellular boron and show resistance to boron treatment (Nozawa 2006). In addition to *BOR1*, two other yeast transporter genes, *DUR3* and *FPS1*, seem to play roles in boron tolerance, but their significance is not clear. Overexpression of *FPS1* and *DUR3* have opposite effects on cellular boron levels, while *FPS1* expression lowers the protoplasmic boron concentrations, *DUR3* expression leads to a small increase (Nozawa 2006).

The yeast *ATR1* gene was first identified in a genetic screen as a high copy suppressor of 3-amino-1,2,4-triazole (3-AT) sensitivity of Δ*gcn4* mutants (Kanazawa 1988). *ATR1* was also identified as conferring resistance to the DNA damaging agent 4-nitroquinoline-N-oxide (4-NQO) in a different genetic screen (Mack 1988). Expression of *ATR1* was shown to be regulated by binding of Yap1 and Gcn4 transcription factors to the same element in the ATR1 promoter (Coleman 2007). The *ATR1* gene seems to provide resistance to 3-AT and 4-NQO, but its cellular function is not known.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used chemicals, buffers, solutions and their compositions are presented in Appendix A.

2.2. Methods

2.2.1. Yeast Growth and Media

The *S.cerevisiae* strain used in this study was BY4741 (MATα *his3 leu2 met15 ura3*). Mutant yeast strains were purchased from ATCC. YPD rich medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) was used for yeast growth. Plasmids carrying cells were grown on YNB selective media with required amino acids and bases.

2.2.2. DNA Microarray Analysis

Cells were grown to 0.2-0.3 OD_{600} in 200 ml of YPD medium, treated for one hour with 20 mM boric acid, harvested by centrifugation, and kept at –80°C. Total RNA was isolated by Ambion RiboPure™-Yeast Kit according to the manufacturer's instructions. 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. 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 (6X SSPE, 0.005% N-lauroylsarcosine; room temperature) and Gene Expression Wash Buffer (0.06X SSPE, 0.005% N-lauroylsarcosine; 31˚C) for 1 minute each. Microarray slides were briefly dipped in a solution of acetonitrile and dried and were scanned in an Agilent Technologies G2505B Microarray Scanner at 5 μm resolution.

2.2.3. Yeast Genomic Library Screening

In a preliminary experiment the level of tolerance of wild type yeast (BY4741) to boron was determined. In this experiment, the wild type strain was grown on YPD medium which contained different boron concentrations (figure 3.1). Ypd plates were incubated at 30 ˚C overnight. It was observed that 80mM boric acid was a toxic level for wild type yeast cells.

A yeast genomic DNA library was obtained commercially from ATCC (no.37323). Library plasmids were purified from *E.coli* cells with a maxi plasmid preparation kit (Invitrogen) after incubation on LB plates at 37 ˚C overnight. Wild type yeast (BY4741) was transformed with purified library plasmids using the standart LiAc method (Ito et al. 1983). Following transformation, cells were grown on YNB (-his) medium which contained different boric acid concentrations (higher than 70mM). Plates were incubated at 30 ˚C for five days. Thirty boron resistant colonies, which were able to grow in the presence of 100 mM boric acid, were observed. These colonies were selected and re-grown with higher concentrations of boron (125 mM to 250 mM) to make sure transformants were boron tolerant. Purification of boron resistant plasmids from yeast cells was carried out with methods of dirty plasmid isolation, which is based on transformation of *E.coli* cells with total yeast extracts (Cold Spring Harbor laboratory course manual, 2005). After plasmids were isolated from yeast cells and transferred into bacterial cells, the normal bacterial mini prep plasmid isolation procedure was carried out according to the manufacturer's instructions (Invitrogen). Finally purified plasmids were sequenced with vector (Yep13) specific primers to identify genes that conferred boron resistance. Primers used in sequencing were; YEP13F- CTTGGAGCCACTATCGACTACG and YEP13B-GTGATGTCGGCGATATAGGCG.

2.2.4. Overexpression of Libray Cassette Genes (*ATR1* and *VAN1)*

Wild-type alleles of the *ATR1* and *VAN1* genes were amplified by Pfu polymerase (Fermentas) from yeast genomic DNA using primers ATR1F; TGGATCCGAGGCAATTAGAGAATCTCAAACAGG and ATR1B; ACTCGAGCTTTCCGCTAAGCCACAGTGCAATCG for *ATR1* and VAN1F; GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGTCATTTGAGCTTGTTGACC TAC and VAN1B; GGGGACCACTTTGTACAAGAAAGTGGGTGTCATGATTCTTA for *VAN1*. These primers contain BamHI and XhoI restriction sites, respectively. Amplified fragments were first cloned into the Sma1 site of pBS II KS + (Stratagene) and then moved into BamHI/Xho1 sites of the yeast high copy expression vector p426GPD. Yeast cells were transformed with empty p426, p426-ATR1 and p426-VAN1 with LiAc method and transformants were selected for uracil prototrophy.

2.2.5. Determination of Intracellular Boron Concentrations

Exponentially growing cells were treated with 50 mM boric acid for one hour and harvested by centrifugation. Cells were washed with fresh water three times and either kept at -80 C or disrupted in 500 µl lysis buffer (20 mM Tris pH 7.6, 100 NaCl, 1 mM EDTA pH 8, 2 % Triton X-100, 1% SDS, 10 mg/ml protease inhibitor cocktail 500 µl glass beads) using a vortex. Supernatants were transferred to new tubes and protein concentrations were determined by Bradford protein detection assay (Bradford, 1976). Boron concentrations of extracts were determined by boron cell test kit [Merck, City, Country] as described in the manufacturer's instructions. Boron levels were confirmed by the ICP-MS method (Takano 2006).

2.2.6. RNA Isolation and Real Time-PCR Analyses

Total RNA isolation was performed from samples with the Rnaesy kit mini prep (QIAGEN). Traces of genomic DNA were removed by DNAse treatment (DNAse RQ1, Promega). Complementary DNA (cDNA) from total mRNA was obtained using the First strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s

instructions. The cDNA was used as template to amplify a 186 bp fragment for the *ATR1* gene and a 103 bp fragment for the internal control *ACT1* gene. Real time PCR assays were performed with the IQ5 real-time PCR system (BIO-RAD) and with PCR mix which contained SYBERGREEN dye for optical measurement of expression levels (Sigma). Primers used for amplification of *ATR1* gene were ATR1F-ACGCGTATAGCATAGCCGCTTTCA and ATR1B-TGTAAGCCTGGTTCCAACCCGATA and for amplification of ACT1: ACT1F-ACGTTCCAGCCTTCTACGTTTCCA and ACT1B-ACGTGAGTAACACCATCACCGGAA. The conditions for PCR amplification were as follows : 30 cycles at 94 ˚C for 30 s, 62 ˚C for 30 s and 72 ˚C for 1 minute.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Identification of The *ATR1* Gene As a Boron Tolerance Gene

Although it is known that boric acid is toxic for yeast growth and the level of boric acid tolerance of wild-type yeast was determined previously (Sutton 2007, Takano 2006, Nozawa 2006, Takano 2007), we tested our wild-type strain and observed that yeast growth was sensitive to boric acid treatment (Figure 3.1). Cells could not grow in the presence of more than 80 mM boric acid (Figure 3.2).

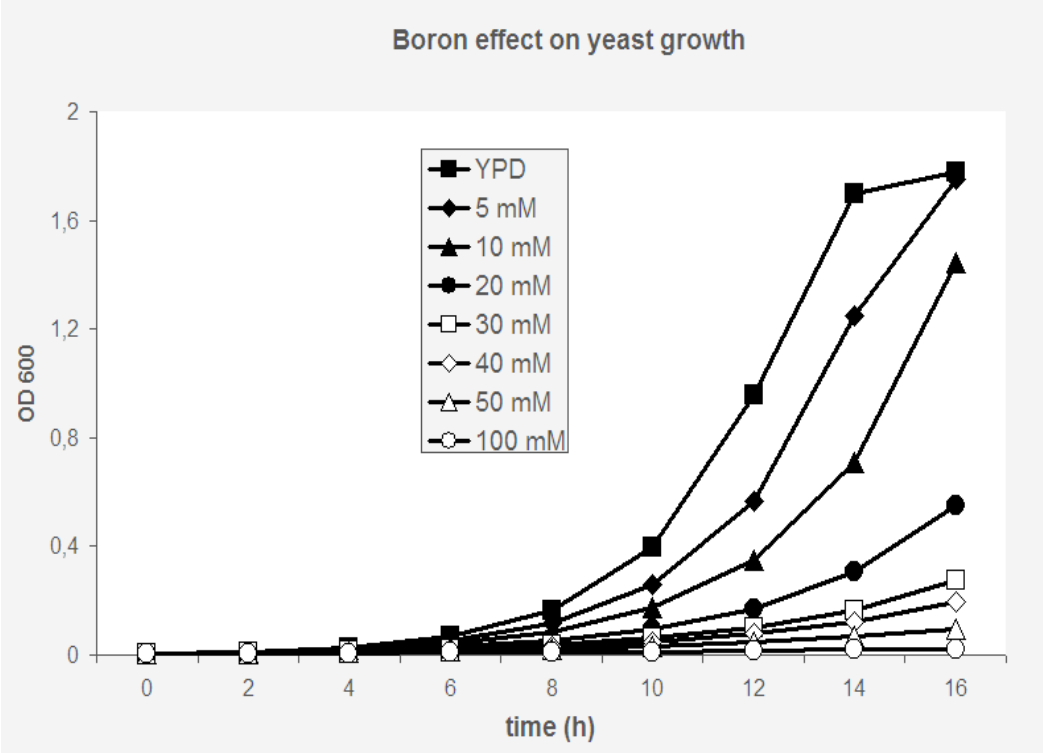

Figure 3.1.Effect of boric acid on yeast growth

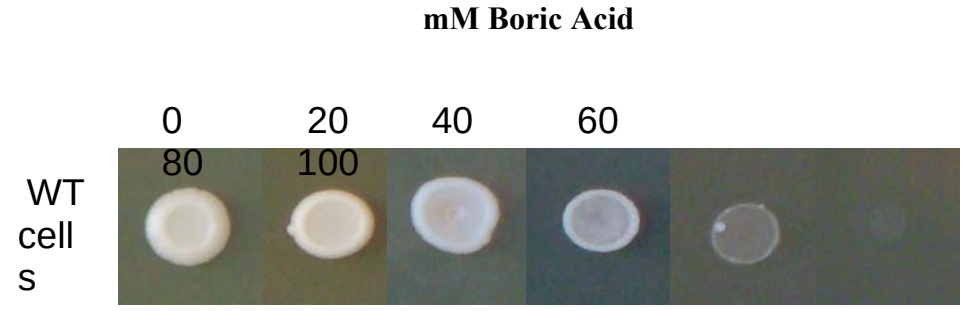

Figure 3.2.Boric acid tolerance level of wild-type yeast; Wild type cells are not viable above 80 mm boric acid stress

After determining the toxic concentration of boron, we screened a high copy yeast genomic DNA library to identify genes that confer boron resistance to wild-type cells. Transformed wild-type cells were exposed to 100 mM boric acid for 5 days. Thirty colonies that could grow in the presence of 100 mM boric acid were detected and cells from these colonies were picked and replica plated to a series of plates containing higher amounts of boric acid. All selected yeast colonies were able to grow in the presence of 200 mM boric acid (Figure 3.3).

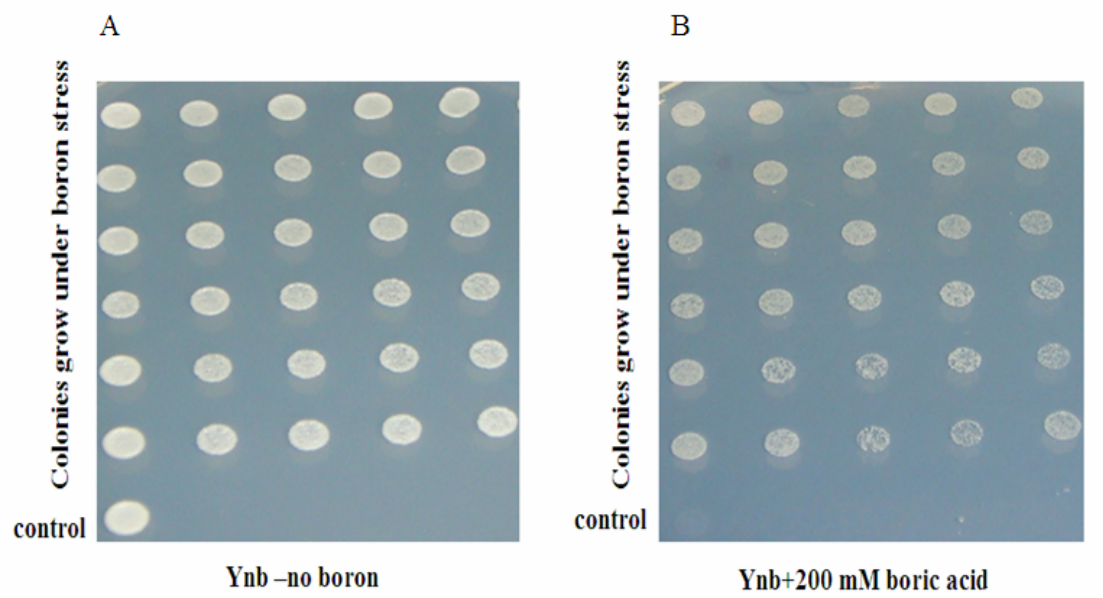

Figure 3.3.Genomic DNA library transformed wild-type cells could grow in the presence of 200 mM boric acid. A) Control plate with Ynb – histidine medium contains no boron and B) Ynb -histidine medium containing 200 mM boric acid

We recovered plasmids from five randomly chosen colonies and sequenced the inserts using a pair of vector based primers. Blast results of sequenced data revealed that all of the inserts came from yeast chromosome XIII, spanning a region that contained the intact *VAN1*(YML115c) and *ATR1*(YML116w) genes. Both *VAN1* and *ATR1* genes have been previously studied. *VAN1* plays a role in mannan synthesis (Stolz 2002) and in vanadium tolerance (Kanik-Ennulat 1990), and *ATR1* is a characterized multidrug efflux pump (Kanazawa 1988).

To identify whether *VAN1* or *ATR1* conferred boron resistance to cells, both genes were separately cloned into yeast high copy expression vector p426GPD and expressed in wild-type cells. Overexpression of only the *ATR1* gene provided boron resistance (Figure 3.4) which showed that the *ATR1* gene, not *VAN1,* was responsible for boron tolerance.

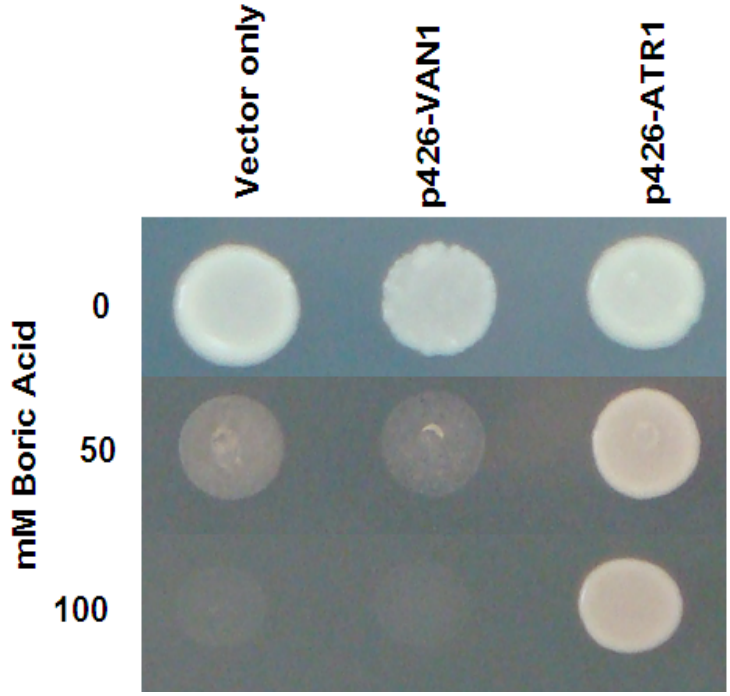

Figure 3.4.Overexpression of only the *ATR1* gene provided boron resistance. In this figure *atr1* overexpressed wild type cells provide more boron resistant than *van1* gene

Next, we tested whether deletion of *ATR1* creates any boron sensitivity. As seen in Figure 3.5, Δ*atr1* mutants and wild-type cells were transferred to plates containing different concentrations of boric acid. The Δ*atr1* mutants could not grow in the presence

of more than 50 mM boric acid, whereas wild-type cells were able to tolerate up to 80 mM. Overexpression of *ATR1* in the Δ*atr1* mutant complemented the deficiency and enabled cells to tolerate higher concentrations of boron. In fact, *ATR1* overexpression provided very strong boron resistance to both Δ*atr1* and wild-type cells and they were able to grow in the presence of 225 mM boric acid (Figure 3.5).

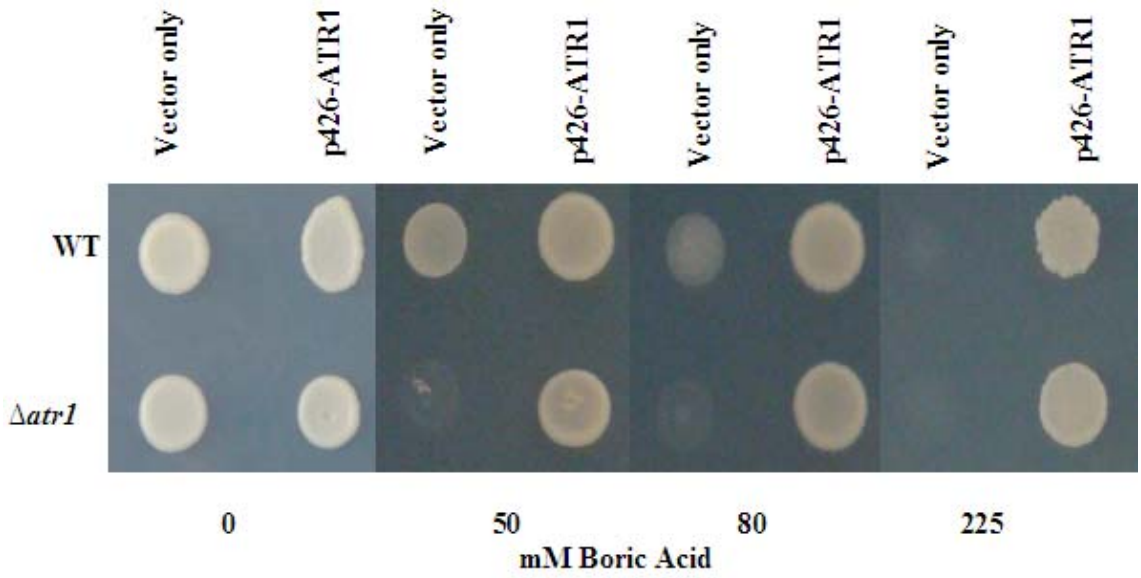

Figure 3.5.*ATR1* overexpression provides super boron resistance to both Δ*atr1* and wild-type cells

In addition, we tested yeast deletion mutants *Δ*bor1, *Δdur3* and *Δfps1* for their boron sensitivity along with the *Δatr1 mutants.* These three genes, BOR1, DUR3, and FPS1, were previously thought to be associated with boron tolerance in yeast (Nazowa, et al. 2006). We compared *Δatr1* mutant cells to *Δbor1, Δdur3* and *Δfps1* mutants to determine which gene provides the highest sensitivity when deleted. As seen in Fig 3.6, only cells lacking the *ATR1* gene were sensitive and other mutants were not sensitive to boron treatment.

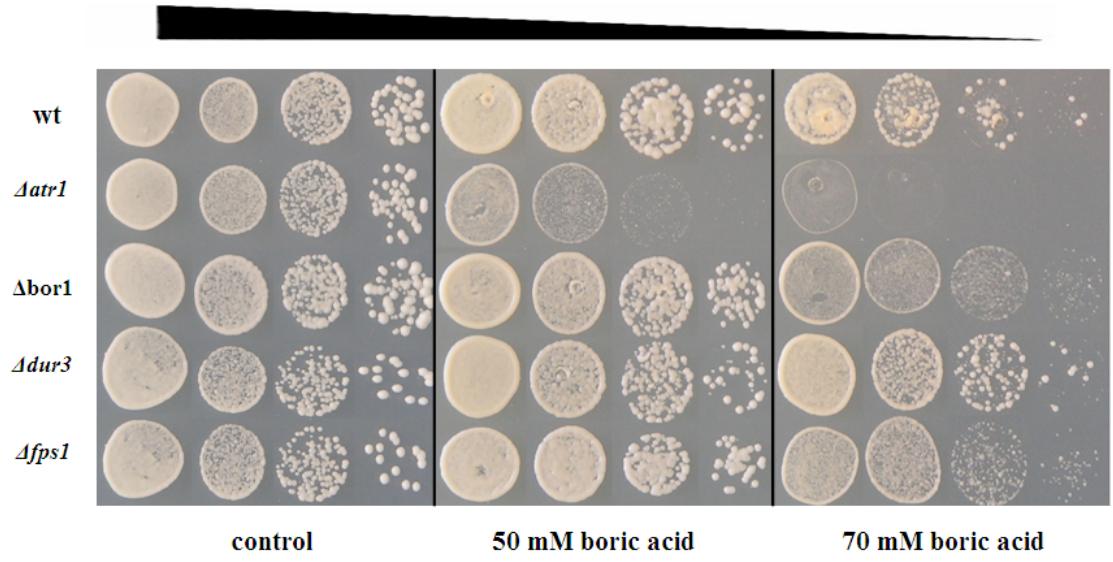

Figure 3.6.Comparison of *Δatr1* deletant strains with *Δbor1, Δdur3* and *Δfps1* deletants

3.2. *ATR1* Is an Efflux Transporter

To elucidate boron efflux activity of the Atr1 protein, we determined intracellular boron concentrations of wild-type and Δ*atr1* cells in parallel. Exponentially growing cells were treated with 50 mM boric acid for one hour and then their intracellular boron levels were determined. As shown in Figure 3.7, Δ*atr1* cells contained 21 % higher levels of boron than the wild-type cells. Overexpression of *ATR1* significantly lowered intracellular boron concentration by 25 % (p=0.024) in wild-type cells and by 47 % (p=0.0001) in Δ*atr1*cells. Thus, absence of the *ATR1* gene caused accumulation of boron inside the cells, whereas, overexpression of *ATR1* led to exclusion of boron from the protoplasm (Figure 3.6).

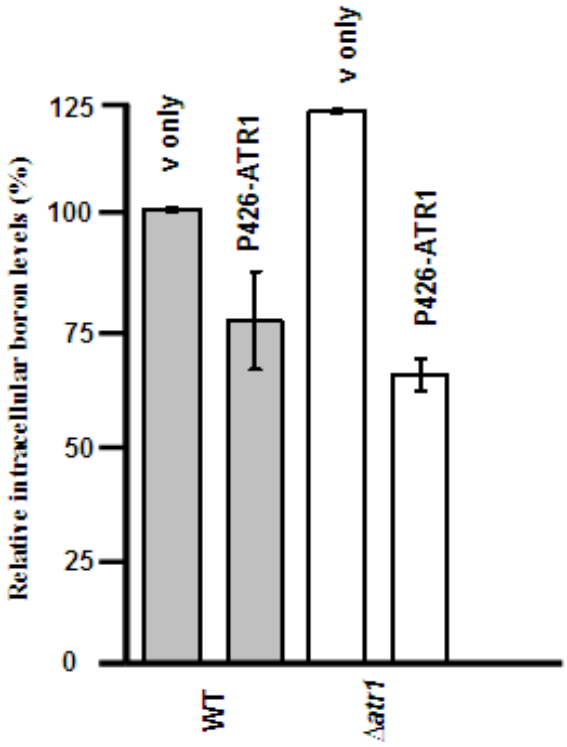

Figure 3.7. Overexpression of *ATR1* lowers intracelular boron concentrations by 25 % inwild-type cells and by 47 % in Δ*atr1*cells

3.3. Transcriptional Response to Boron Treatment

Upon exposure to unfavorable conditions, cells induce genes that help cells to tolerate the stress and increase their survival. We performed a DNA microarray assay for indentification of genes which are differentially expressed in response to boric acid. Wild-type yeast was grown in the presence or absence of 20 mM boric acid for one hour and gene expression profiles of treated and untreated cells were compared. Genes that showed 3 or more fold change in their expression levels were determined. Boron led to 3-fold upregulation of 171 genes and 3-fold downregulation of 43 genes in the treated samples. Many of these genes had unknown functions and were not associated with any pathway in yeast genomic databases, however, some of the genes that are involved in amino acid biosynthesis and membrane transport including *ATR1* were differentially expressed (Figure 3.8). Among the activated transporter genes, *ATR1* located at the top of the cluster with more than 3-fold upregulation (Figure 3.9). Moreover microarray results showed that while expression of the ATR1 gene was upregulated in response to

boron, expression of the *BOR1, DUR3 and FPS1* genes were either not affected or showed only slight changes (Figure 3.10).

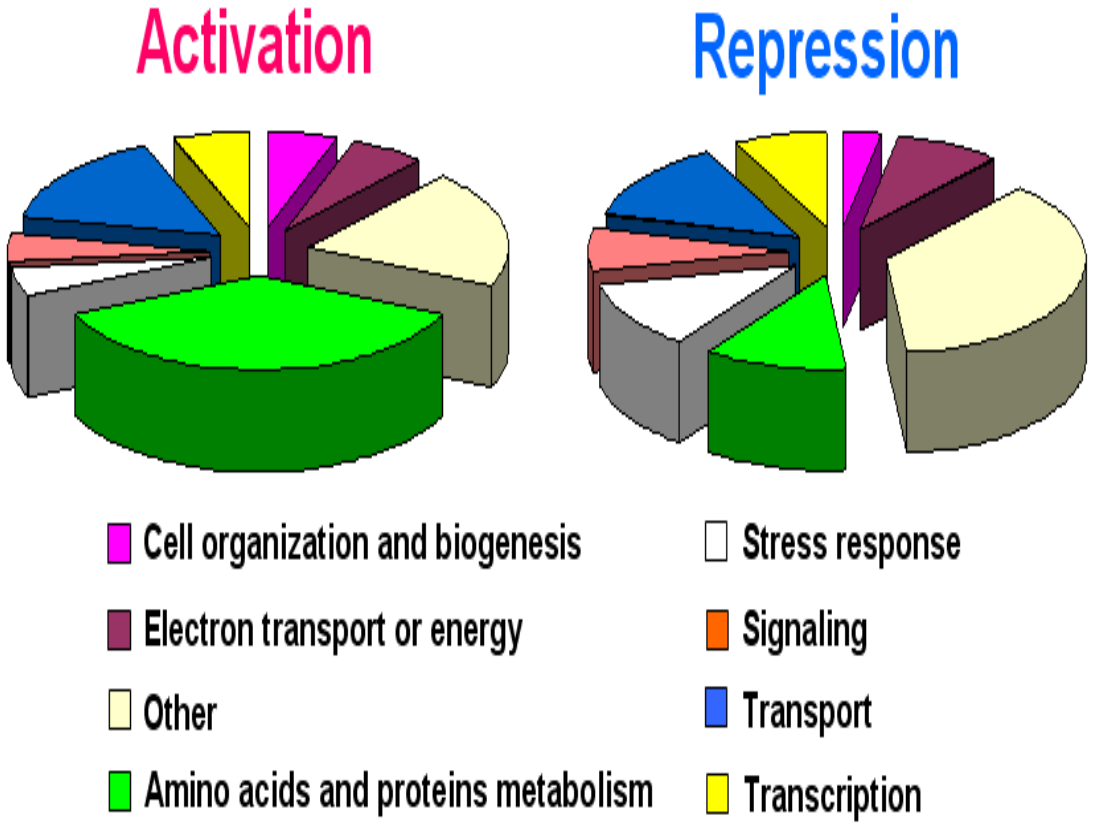

Figure 3.8.Results of DNA microarray assay for indentification of genes which are differentially expressed in response to boric acid

YML116W	Multidrug efflux pump
YBR104W	Mitochondrial inner membrane transporter
YPR058W	Mitochondrial inner membrane transporter
YNL125C	Transport of monocarboxylates
YHL016C	Transporter for urea and polyamines
YBR068C	High-affinity leucine permease
YCR098C	Plasma membrane permease
YDL054C	Monocarboxylate permeases
YGL186C	Pyridoxine (vitamin B6) transporter
YPL092W	Plasma membrane sulfite pump
YIL023C	Zinc transporter
YFL055W	Low-affinity amino acid permease
YDR046C	Amino acid permease
YKL188C	Subunit of a ATP-binding transporter complex
YKL120W	Mitochondrial inner membrane transporter
YCL025C	Low-affinity amino acid permease
YLL048C	Transporter of the ATP-binding cassette (ABC)
YCR037C	Low-affinity inorganic phosphate (Pi) transporter
YGR055W	High affinity methionine permease
YOL158C	Endosomal ferric enterobactin transporter
YBR043C	Multidrug transporter
YBR293W	Permease of basic amino acids
YPL058C	ATP-binding cassette (ABC) transporter
YHR096C	Hexose transporter
YGR037C	Transports acyl-CoA esters
YDR343C	High-affinity glucose transporter
YBL042C	High affinity uridine permease
YDR345C	Low affinity glucose transporter
YOR185C	RNA processing and transport
YLR411W	High-affinity copper transporter
YMR011W	High-affinity glucose transporter
YAL005C	(NLS)-directed nuclear transport
YPR124W	High-affinity copper transporter
YHR092C	High-affinity glucose transporter

Figure 3.9.Transporter genes that are up or down regulated by boron treatment

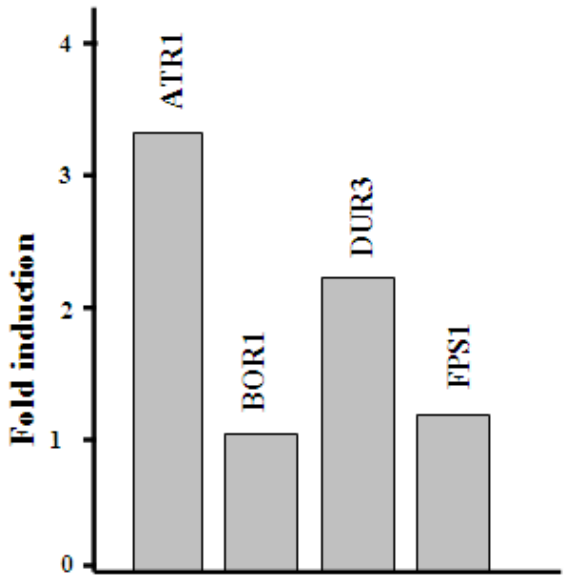

Figure 3.10. Microarray results showing the expression level of the *ATR1, BOR1, DUR3,* and *FPS1* genes under boron stress

Upregulation of the *ATR1* gene in response to boron treatment was also observed by a Real-Time PCR assay. *ATR1* transcript level was 4-fold higher in cells exposed to 50 mM boric acid for one hour.Thus, our expression analyses showed that the *ATR1* gene is upregulated in the presence of boron (Figure 3.11).

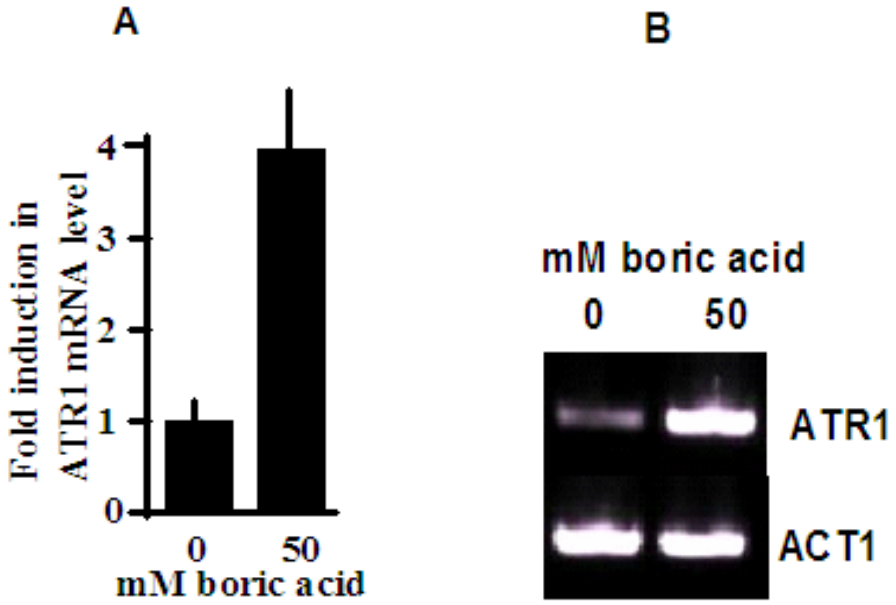

Figure 3.11.Real Time PCR analysis of ATR1 mRNA level. A) Real time plots. B) 1 % agarose gel analysis of PCR fragments. Actin-1 (ACT1) was used as the internal control

3.4. ATR1 in Other Species

We analysed the pattern of occurrence of the *ATR1* gene in all major domains of life (Figure 3.12). Its homologs are present in Fungi, Bacteria, Archea and Mycetozoa species but not in multicellular animals and plants which makes *ATR1* a boron tolerance gene in lower eukaryotes and prokaryotes. We also looked at the presence of other boron related genes in different life domains. *FPS1* homologs are present in all organisms, *BOR1* is mainly found in eukaryotes and *DUR3* is present in both prokaryotes and multicellular eukaryotes.

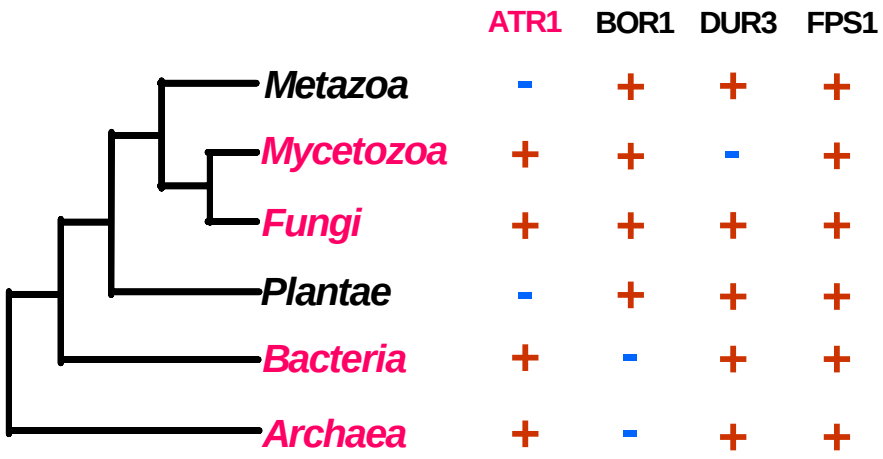

Figure 3.12..*ATR1* gene in all major domains of life

CHAPTER 4

CONCLUSION

We screened a yeast genomic DNA library to identify genes that support growth of yeast cells in the presence of high concentrations of boric acid and identified *ATR1* as a major boron resistance gene in yeast. We found that, in boron containing medium, the intracellular boron concentration of Δ*atr1* mutants was higher than that of wild-type cells, whereas overexpression of the *ATR1* gene reduced intracellular boron. These data indicate that *ATR1* functions as a boron exporter in yeast.

Only a few genes have been shown to have a role in boron resistance thus far. Among these, the efficiencies of the products of *DUR3* and *FPS1* genes in boron export are not clear. Mutants of the *DUR3* and *FPS1* genes did not have elevated levels of intracellular boron. Even though *BOR1* seems to be more effective in boron tolerance than *DUR3* and *FPS1* in yeast (Nozawa 2006), its expression is not regulated in the presence of boron (Jennings 2007).

Our microarray data showed that expression of the *BOR1* and *FPS1* genes did not change in response to boron-mediated stress, while *DUR3* was upregulated 2.5-fold (Fig. 3.10). We also compared the growth rates of Δ*atr1*, Δ*bor1,* Δ*fps1* and Δ*dur3* mutants in the presence of boron and found that only the *ATR1* gene deletion created boron hypersensitivity (Fig. 3.6). Our work suggests that *ATR1* has a more critical function in eliminating the toxic effects of boron. The Δ*atr1* mutant was hypersensitive to boron treatment and cells overexpressing *ATR1* survived in the presence of 225 mM boric acid, which is the highest amount of boron shown to be tolerated by a eukaryotic system. In addition, transcriptional regulation of *ATR1* by boron makes it a physiologically-relevant boron tolerance gene.

Currently, the mechanisms of boron-mediated *ATR1* transcriptional regulation are not known. To better understand regulation of the *ATR1*, *BOR1*, *DUR3* and *FPS1* genes, we analyzed expression profiles of these genes from previously published microarray datasets (Gasch, 2000). Expression of ATR1 shows significant changes in response to hydrogen peroxide, diamide and menadione. Expression of other boron transporters was not changed. DTT-mediated reductive stress did not change expression levels of all four boron transporters. Transcriptional control of *ATR1* has not

been studied in detail, but it is known that *Gcn4* and *Yap1* transcription factors play roles in *ATR1* induction (Coleman 1997). Oxidative stress control of the *ATR1* gene could be explained by a Yap1-dependent mechanism. Yap1 is a transcription factor which plays a role in activation of oxidative stress response genes (Lee 1999). Expression patterns of both the *ATR1* and *DUR3* genes in response to amino acid starvation are similar. *Gcn4* is known to activate the transcription of amino acid biosynthesis genes (Natarajan 2001). Involvement of *Gcn4* in activation of both *ATR1* and amino acid synthesis genes is likely in response to boron treatment, however, neither *GCN4* transcript level increased in response to boron treatment nor Δ*gcn4* mutants had lower sensitivity to boron in our analyses. It is also unlikely that *ATR1* is playing a direct role in transcriptional control of amino acid synthesis genes since it is a transmembrane protein with no transcription factor features (Kanazawa 1988). Overexpression of amino acid biosynthesis genes might be evidence for amino acid starvation in response to boron treatment. Boric acid might block utilization of amino acids resulting in activation of their biosynthesis genes. In this case, one would expect that supplementation of cells with higher levels of amino acids may result in increased resistance to boron. However, cells supplemented with 10-fold higher concentration of amino acids did not gain any resistance to boric acid.This suggests that under boron stress, the purpose of induction of amino acid synthesis genes is not to supply cells with amino acids. It is likely that signal transduction pathways that play roles in boron resistance and amino acid biosynthesis may share a common activator.

ATR1 could be considered as a stress tolerance gene in lower eukaryotes and prokaryotes. Its substrate spectrum is not clear at present. In addition to 3-AT and 4 NQO resistance which were shown previously we showed that ATR1 is also required for boron resistance in yeast.

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APPENDIX A

COMPOSITION of BUFFERS AND STOCK SOLUTIONS

1. Boric Acid Stock Solution, 0.5M
    - 30.915 g Boric acid in 1L dH_2O

2. 50x TAE Electrophoresis Buffer, per 1 L
    - 242 g Tris base
    - 37.2 g Na_2EDTA (H_20)
    - 57.1 ml Glacial acetic acid
    - dH_2O to 1 L

3. Ethidium Bromide (10 mg/ml)
    - 0.2 g Ethidium bromide in 20 ml dH_2O

4. 10mM dNTP
    - 10mM each dATP, dTTP, dCTP, dGTP

5. LB Broth, per liter
    - 10 g Tryptone
    - 5 g Yeast Extract
    - 5 g NaCl
    - dH_2O up to 1 L

6. SOC Medium, per 100 ml
    - 2 g Tryptone
    - 0.5 g Yeast Extract
    - 1 ml 1M NaCL
    - 0.25 ml 1M KCL
    - 1 ml 2M Mg^{2+} Stock
    - 1 ml 2m Glucose

- dH_2O up to 100 ml

8. YPD Media, per liter
   - 20 g Peptone
   - 10 g Yeast Extract
   - 20 g Glucose
   -20 g Agar for solid media

9. YNB Media,per liter
   - 6.7 g Yeast Nitrogen Base with ammonium sulfate
   - 20 g Glucose
   - Appropriate nutritional supplements;
     Uracil 0.2 g/100 ml
     Leucine 1 g/100 ml
     Methionine 1 g/100 ml
     Histidine 1 g/100 ml

10. Amphicilin (100 mg/ml)
    - 0.1 g amphicilin in 1 ml dH_2O

11. X-GAL Stock Solution (30mg/ml)
    - 3% x-gal in di-methyl formimide

12. IPTG Stock Solution (1M)
    - Dissolve 2.83 g IPTG in 8 mL dH_2O. Bring to 10 mL with dH_2O.

APPENDIX B

SEQUENCES of YML116w AND YML115c

1.YML116w *(atr1)* 1628bp:

5’ATGGGCAATCAGTCATTAGTTGTGCTTACGGAAAGTAAGGGTGAGTAT
GAGAATGAGACAGAACTACCTGTTAAAAAATCGTCACGAGATAATAATA
TCGGAGAATCTTTAACAGCAACAGCTTTTACGCAGTCTGAAGATGAAAT
GGTAGATAGCAATCAGAAATGGCAGAACCCAAACTATTTTAAATATGCA
TGGCAAGAATATCTTTTTATATTCACATGCATGATAAGTCAGCTTCTAAA
TCAAGCAGGCACTACACAGACTCTTTCGATCATGAATATTCTTTCGGACA
GTTTTGGCTCAGAAGGAAACTCAAAGTCATGGCTGATGGCATCTTTTCCG
CTAGTTTCAGGCTCATTTATTTTGATTAGTGGCAGACTAGGTGACATATA
CGGATTAAAAAAAATGTTGTTAGTAGGATATGTTCTGGTTATTATATGGT
CTTTGATTTGTGGGATTACCAAGTATTCTGGTAGCGATACTTTTTTTATTA
TTAGTAGAGCCTTCCAAGGGCTAGGGATTGCATTTGTTTTACCTAATGTG
CTGGGAATAATTGGTAATATATATGTAGGTGGTACTTTTCGTAAAAACAT
CGTGATTAGTTTTGTTGGTGCGATGGCCCCTATTGGAGCAACTTTAGGTT
GTCTTTTTGCAGGACTGATCGGTACCGAGGACCCAAAACAATGGCCATG
GGCATTCTACGCGTATAGCATAGCCGCTTTCATTAATTTTGTGCTCTCCA
TATATGCCATTCCGAGTACTATACCAACAAATATTCATCATTTTTCTATG
GATTGGATTGGTTCTGTTTTGGGCGTGATAGGTCTCATTTTATTAAATTTT
GTGTGGAACCAAGCTCCTATATCGGGTTGGAACCAGGCTTACATCATCG
TAATTTTAATCATTTCTGTGATTTTTCTTGTCGTTTTCATCATTTATGAGA
TTCGATTTGCCAAGACTCCACTATTGCCGCGCGCAGTTATAAAGGATCGT
CATATGATTCAAATTATGCTGGCTTTATTCTTTGGATGGGGCTCTTTTGG
CATCTTTACGTTTTATTATTTCCAATTTCAATTAAATATAAGGCAGTACA
CGGCATTATGGGCTGGTGGAACTTACTTTATGTTTTTAATTTGGGGTATT
ATTGCCGCCTTACTGGTAGGATTTACTATCAAGAATGTGTCTCCATCAGT
GTTTTTGTTCTTTTCTATGGTAGCATTCAATGTGGGCTCAATAATGGCAA
GTGTTACACCGGTTCACGAGACATACTTTCGTACTCAGTTAGGAACGAT
GATAATTTTAAGTTTTGGGATGGATCTTTCATTTCCTGCTTCTTCCATTAT

CTTTAGTGATAATTTACCGATGGAGTACCAAGGCATGGCTGGGTCATTG
GTGAATACTGTTGTCAATTACTCCATGTCCTTGTGTCTCGGTATGGGTGC
CACAGTAGAGACACAGGTCAATTCAGACGGAAAGCATCTTTTGAAAGGC
TATAGAGGTGCTCAGTACCTTGGGATAGGATTGGCAAGTTTAGCATGCA
TGATTAGCGGGCTTTACATGGTCGAAAGCTTCATAAAAGGCCGCAGGGC
AAGAGCTGCTGCAGAATACGATTGCACTGTGGCTTAG3`

2. YML115c *(van1)* 1607bp:

5’ATGGGCATGTTTTTTAATTTAAGGTCAAATATAAAGAAGAAAGCCATG
GACAATGGACTAAGCCTGCCCATTTCAAGGAACGGTAGCTCGAACAACA
TCAAGGACAAACGCTCAGAGCATAACTCCAACTCATTAAAGGGCAAATA
CAGGTACCAGCCGCGCTCCACACCGTCTAAATTCCAGCTTACGGTGAGT
ATCACATCTCTTATTATTATCGCCGTTCTGTCGTTATATCTCTTTATATCA
TTTCTCTCCGGAATGGGCATTGGTGTATCCACGCAAAATGGTAGGTCGTT
GTTGGGTTCCTCAAAATCCTCCGAAAATTACAAGACTATCGACCTAGAA
GATGAAGAATATTACGACTATGATTTTGAGGATATCGATCCTGAAGTGA
TTTCAAAATTTGATGATGGTGTGCAACATTATCTAATATCACAATTTGGT
TCAGAAGTGTTGACTCCCAAGGATGATGAAAAATACCAAAGGGAACTCA
ACATGCTTTTTGATTCCACTGTTGAGGAGTACGACCTGTCGAATTTTGAA
GGTGCTCCGAATGGATTGGAAACACGTGATCACATTCTTTTATGCATTCC
ACTAAGAAACGCTGCGGATGTATTGCCATTAATGTTCAAGCATTTAATG
AACCTAACCTATCCACACGAGCTGATTGATCTGGCCTTTTTAGTCAGTGA
TTGCTCAGAAGGTGACACTACGTTGGATGCTTTAATAGCGTATTCTAGGC
ACTTACAAAATGGCACGTTGTCTCAGATTTTTCAAGAGATTGACGCTGTC
ATTGATTCGCAAACAAAAGGCACCGATAAATTATATCTTAAATATATGG
ACGAAGGTTATATCAACCGTGTCCACCAGGCATTTTCACCACCATTCCAT
GAAAATTATGACAAGCCATTTAGATCAGTACAAATTTTCCAAAAGGATT
TTGGCCAAGTAATTGGACAAGGTTTTAGTGACAGACATGCCGTTAAGGT
TCAAGGTATAAGACGAAAACTAATGGGGAGGGCAAGAAATTGGTTAAC
TGCCAATGCTTTGAAACCTTACCACTCATGGGTTTATTGGAGAGATGCTG
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CAAAGAAAATGGGGTACAGGGTTGGTGGTTTACCGCATTATACTATTTG
GCATATTTATGAACCGAGCGATGACGATTTGAAGGAAATTGCGTCAAGG
GAAAGAGAGAAGAGAAGACAATCAGAGTAA3`