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AKDENIZ UNIVERSITY

DETERMINATION OF ABIOTIC STRESS TOLERANCE GENES IN *Gypsophila perfoliata*

Nurefşan CIRIK

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This thesis on 18/05/2023 Unanimously accepted by the jury.

Assoc. Prof. Dr. Mehmet Aydın AKBUDAK

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Assoc. Prof. Dr. Aysun ÖZÇELİK

ÖZET

Gypsophila perfoliata'da ABİYOTİK STRES TOLERANS GENLERİNİN BELİRLENMESİ

Nurefşan CIRIK

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı

Danışman: Doç. Dr. M. Aydın AKBUDAK

Mayıs 2023; 47 sayfa

Bor toksisitesi Türkiye'de yerel bir mikro besin sorunu olup yapraklarda sararma ve nekroza, meyvelerde sayı, boyut ve ağırlıkta azalmaya neden olarak ürün verimini düşürmektedir.

Bor hiperakümülatör türlerinde toksik bor stresi ile ilgili genlerin tanımlanması, bitkilerde bor tolerans mekanizmalarını aydınlatacağı düşünülmektedir. Bu düşünce göz önünde bulundurularak, bor stresi verilmiş *G. perfoliata* yaprak ve kök cDNA kütüphaneleri oluşturulmuştur. cDNA kütüphaneleri maya hücrelerine transforme edilip yüksek konsantrasyondaki H_3BO_3 varlığında taramaları gerçekleştirilmiştir. Bor toleransı gösteren 10 transformant arasından 1 transgenik maya plasmidi seçilip yeni nesil dizileme yapıldıktan sonra gen özellikleri BLASTX kullanılarak açıklanmıştır. Sonuçlar, tanımlanan *GpEEFA1a* geninin *Saccharomyces cerevisiae*'da stres sinyali metabolizmasını geliştirdiğini göstermektedir. Maya hücrelerinin seçilen plazmit ile retransformasyonu, hücrelerde bor toleransı sağlamıştır. Bu stresle ilişkili genin ekspresyon seviyeleri qPCR ile *G. perfoliata* yapraklarında kontrol edilmiştir. Bu çalışmada sonuç olarak *G. perfoliata*'da hafif bor toleransı sağlayan bir gen belirlenmiştir.

ANAHTAR KELİMELER: *G. perfoliata,* bor toksisitesi, functional screening

JÜRİ: Doç. Dr. M. Aydın AKBUDAK

Prof. Dr. Muhammet ŞAKİROĞLU

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ABSTRACT

DETERMINATION OF ABIOTIC STRESS TOLERANCE GENES IN *Gypsophila perfoliate*

Nurefsan CIRIK

MSc Thesis in The Department of Agricultural Biotechnology

Supervisor: Assoc. Prof. Dr. M. Aydın AKBUDAK

May 2023; 47 pages

Identification of genes related to toxic boron stress in boron hyperaccumulator species is thought to illuminate boron tolerance mechanisms in plants. With this in mind, toxic amount boron treated *G. perfoliata* leaf and root cDNA libraries were created. cDNA libraries were transformed into yeast cells and screened in the presence of high concentration of H_3BO_3. After selecting 1 transgenic yeast plasmid among 10 boron-tolerant transformants and next generation sequencing, gene properties were annotated using BLASTX. The results show that the identified *GpEEFA1a* gene enhances stress signal metabolism in *Saccharomyces cerevisiae*. Retransformation of yeast cells with the selected plasmid provided boron tolerance in the cells. Expression levels of this stress-related gene were checked by qPCR in *G. perfoliata* leaves. In this study, a gene providing slightly boron tolerance was identified in *G. perfoliata*.

KEYWORDS: *G. perfoliata,* boron toxicity, functional screening

COMMITTEE: Assoc. Prof. Dr. M. Aydın AKBUDAK

Prof. Dr. Muhammet ŞAKİROĞLU

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Words cannot express my gratitude to my advisor and chair of my committee for him invaluable patience and feedback. I also could not have undertaken this journey without my defense committee, who generously provided knowledge and expertise. I am also grateful to my laboratory members, especially my office mates, for their editing help, late-night feedback sessions, and moral support. Thanks should also go to the librarians, research assistants, and study participants from the university, who impacted and inspired me. I would be remiss in not mentioning my family. Their belief in me has kept my spirits and motivation high during this process.

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AKADEMİK BEYAN

Yüksek Lisans Tezi olarak sunduğum "Determination of Abiotic Stress Tolerance Genes in *Gypsophila perfoliata*" adlı bu çalışmanın, akademik kurallar ve etik değerlere uygun olarak yazıldığını belirtir, bu tez çalışmasında bana ait olmayan tüm bilgilerin kaynağını gösterdiğimi beyan ederim.

18/05/2023

Nurefşan CIRIK

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

µL : microliter
bp : basepair
g : gram
mb : megabase
sec : second
min : minute
ml : milliliter
ng : nanogram
kb : kilobase
kv : kilovolt
ºC : centigrade degree
V : volt
M : molarity
l : liter
mm : millimeter
cm : centimeter

Abbreviations

DNA : Deoxyribonucleic acid
RNA : Ribonucleic acid
PCR : Polymerase Chain Reaction
LB : Luria-Bertani
OD : Optic Density
Rpm : Revolutions per minute
MS : Murashige & Skoog

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1. INTRODUCTION

Boron

Boron, which has an atomic number of 5 and is denoted by the symbol B in the periodic table. Due to it belongs to group 3A of the periodic table, boron has semi-metallic and semiconductor properties. The elemental form of boron is never found in free form in nature; it forms various compounds like boron minerals with various metal or non-metal elements. Boron minerals are natural compounds containing boron oxide (B_2O_3) in different proportions. There are 230 divergent boron minerals are situated in the nature. The most important boron minerals are: boric acid, tincal, colemanite, kernite, ulexite and pandermite. As of 2021, Turkey has earned the title of the world's largest reserves (approximately 75%) with an estimated 1.2 billion metric tons of boron reserves. Important boron reserves in Turkey are located in the northwestern parts of the country which are Balıkesir, Kütahya and Eskişehir provinces (Öztürk et al., 2010). On the other hand, The USA and Russia share the 2nd place in the ranking of the largest boron reserves with only 40 million metric tons. Thanks to this feature, Turkey is the country extracts the most boron minerals in the world. Approximately 40% of the world boric acid market is met by the Eti Maden, the world boron leader. Boric acid, borax pentahydrate, calcine and tincal are produced at the facilities in the operating directorates of Eti Maden. These main boron minerals are transformed into high value-added products, then they are supplied to domestic and foreign markets.

Necessity, Function and Deficiency of Boron

Boron (B) is an essential micronutrient for plant growth and development, and involved in nucleic acid, carbohydrate, protein and indole acetic acid metabolisms (Warrington et al., 1923). Lewis (1980) theorized that the necessity of boron emerged with the evolution of vascular plants and played a primary role in lignin synthesis. That is why boron is important in cell wall synthesis and stability through cis-diol groups and ester formation. 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*. The range between deficiency and toxicity of boron in the soil is very narrow. Boron deficiency in plants

occurs when the amount of boron in the soil is less than 0.5 mg/kg, if it is more than 5.0 mg/kg, boron toxicity occurs (Ryan and Rashid, 2006). This narrow range of boron concentrations causes deficiency or toxicity carries the plant boron nutrition to a significant level when it is compared with other nutrients (Goldberg, 1997). Boron transport through the phloem is quite difficult than the other elements, consequently, boron transport from old tissues to young tissues is limited. For this reason, the symptoms of boron deficiency first appear in the young tissues of the plants with low boron mobility.

Toxicity of Boron

Boron is widely distributed both in the lithosphere and hydrosphere. Boron concentration in rocks is approximately 10-20 mg B/kg. Soils are divided into two in terms of the boron concentration they contain as low (<10 mg B/kg) and high (10-100 mg B/kg) boron-containing soils. Low amount of boron has seen in most of the soil types. In despite of other countries, most of the soils in Turkey are classified as high boron containing soils. Boron toxicity is a local micronutrient problem in Turkey, about a quarter of our soils are classified as high or very high in terms of boron content (Seferoğlu et al., 2020). Boron toxicity, especially seen in arid and semi-arid soils, is due to the high boron level in the soil, unconscious fertilization in agricultural activities (Nable et al., Chapter 12) and also the lack of drainage in saline soils.

The toxic level of dissolved boron in the soil causes toxicity by carried to other organs and tissues by some proteins expressed in the plant root cells. Boron toxicity causes chlorosis and necrosis in leaves, and decrease in number, size and weight in fruits (Paull et al., 1992; Camacho-crist et al., 1997). Boron toxicity also causes deterioration of cell metabolism by changing the structure of the cell development and division (Öztürk et al., 2010).

Boron Uptake Mechanism and Tolerance Genes

Boron enters plants mainly as boric acid from the soil via roots. When boron amount is sufficient in the soil, the boron requirement of the plant can be satisfied by passive diffusion of boric acid. However, when the availability of boric acid is limited in the soil, plants use boric acid channels of the major intrinsic protein (MIP) family and the BOR protein families for boron transport. 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). In addition, plants under high boron stress need BOR borate carriers to remove the excess boron amount from tissues. Studies identified three pathways for the transmembrane transport mechanism of boron in plants. First mechanism is thought to be restricted boron uptake or boron leakage from plant root cells to the environment. This causes limited accumulation of boron in plant root tissues. The second hypothesis is restricted boron transport from roots to shoots. The third one is assumed that to be increased tissue tolerance against high boron concentrations (Sutton T. 2007). In addition to boron toxicity stress, other abiotic stress factors that negatively affect plant growth and crop yield are drought and salinity. Especially all stages of growth and development are affected in plants exposed to abiotic stresses such as drought, salinity and extremely high temperature. The stages which plants are most sensitive to abiotic stress are seed germination, seedling development and flowering stages (Patade et al., 2011; Partheeban et al., 2017). Abiotic stresses, which reduce the average crop yield in crop plants by more than 50%, are known to be the major cause of crop yield loss worldwide (Wang et al., 2004). Some preliminary studies have been carried out by various research groups in order to find salt, drought and toxic boron tolerance genes and to reveal the tolerance mechanism in *Gypsophila*. However, the studies carried out have been limited to transcriptome analysis, proteomic and physiological analysis, and tolerance genes have not yet been identified. It is clear that additional studies are needed to better understand both the physiological and genetic mechanisms of boron tolerance and accumulation. The main purpose of this thesis is to identify the genes conferring tolerance to toxic boron amounts in *Gypsophila* (Babaoğlu et al., 2004) which is adapted to areas containing toxic levels of boron (8900 mg B/kg).

It is thought that the determination of the mentioned tolerance genes in the *Gypsophila* will give a significant idea about the ways of coping with abiotic stress factors, especially boron toxicity, in agriculturally important crops such as potato, soybean and tomato. It is also thought that if the tolerance genes that are expected to be characterized as a result of the study are transferred to different plant systems through genetic engineering and breeding, it will allow the prevention of yield losses due to high boron in crops grown in areas classified as high and very high boron content.

Gypsophila and Boron Tolerance

Babaoglu et al. (2004) identified *Gypsophila sphaerocephala, Gypsophila perfoliata* L., *Elymus elongatus* and *Puccinellia distans* species growing in a boron mine region in Turkey. In their study, it was determined that the mentioned species can naturally grow and develop in soils with toxic level of boron content (8900 mg kg^{-1}). It was also reported that the green parts of *G. sphaerocephala* contain higher boron compared to the root and vegetative organs of other species. In addition to the aforementioned study, Babaoğlu et al. (2004) reported that *G. sphaerocephala* does not only grow in soils contaminated with excess boron but can also accumulate boron at unexpectedly high concentrations. In this way, it is thought that studies on *G. perfoliata* will enable the identification of the gene(s) providing boron resistance.

There are 63 *Gypsophila* species in Turkey (Özçelik and Muca, 2010), which is the gene center for the *Gypsophila* genus, which has a high economic value due to the saponins it contains. Özçelik and Özgökçe (2021) reported that 41 of them are endemic species (Özçelik and Özgökçe, 2021). According to IUCN (International Union for Conservation of Nature), *G. pilulifera* species is in the CR (very endangered) category (Ekim et al., 2000). The use of species belonging to the genus *Gypsophila* for both medicinal and aromatic and economic purposes such as pharmaceutical raw material causes the extinction of these plants (Özçelik and Yıldırım, 2011). It is very important to determine the optimum growing conditions of endemic species and to investigate their natural abiotic stress tolerance. For example, Üstüner et al., (2021) investigated the drought and salinity stress tolerance of *G. pilulifera* in *in vitro* conditions in their latest study. In this way, it is predicted that this species will contribute to the protection of natural populations (Üstüner et al., 2021).

Yeast and Boron

The budding yeast *Saccharomyces cerevisiae* is an ambidextrous and powerful model system of eukaryotic genetics. Since it is a domesticated microorganism and a sexual eukaryote, it is the most used industrial yeast and emerged as a significantly acquiescing eukaryotic model system. Budding yeast has similar cellular structure and primary life cycle with multicellular eukaryotes like plant and animals. They can be easily

manipulated and cultured in laboratory conditions as they are nonpathogenic and motile microorganisms. Hence, it is significantly easier to study gene analysis and cloning on this organism whether than more complex eukaryotic organisms. There are approximately 6000 genes on the 12 mega base pair DNA located on 16 chromosomes in the baker's yeast own genome. Although this unicellular eukaryotic organism is considerably simpler, it uses variations of the same mechanisms found in higher eukaryotes to make developmental decisions and differentiate into various cell types.

Saccharomyces cerevisiae can grow at very high boron concentrations, hence thought to be a boron tolerant organism. The boron tolerance mechanism in yeast is centered on the export of boron from the cell. In yeast, some transporter proteins such as BOR1, DUR3 and FSP1 were studied in detail, and these proteins are located in the plasma membrane. The other membrane protein is Atr1 has been characterized as boron efflux transporter. Atr1 deficient cells are sensitive to boron and accumulates high amount of boron. On the other hand, Atr1 overexpressing cells are tolerant to toxic amount of boron and contain less amount of boron. Yeast Atr1 belongs to 10 membered DHA2 drug family. This transporter family has been conserved among the yeast species.

2. LITERATURE REVIEW

There are several studies on the understanding the molecular mechanism of boron uptake, export and transport in plants. *AtBor1* is the first identified plant boron transporter gene (Takano vd., 2002). Then, the six more genes that paralogs of *AtBor1* gene were identified (Miwa vd, 2007). Boric acid transporters were also identified in other plant species and organisms than Arabidopsis. These are *HvBot1* (barley), *OsBOR1* (rice), *NaBC1* (human) and *Atr1* and *Bor1* genes in *Saccharomyces cerevisiae* (Nakagawa et al., 2007; Frommer and von Wiren, 2002; Kaya et al., 2009; Takano et al., 2007). Nable R. et al., (1988) and Paull J. et al., (1988) were identified high boron tolerant wheat and barley varieties. According to their study, boron tolerance is associated with the less boron amount in the plant tissues (Nable R. et al., 1988, Paull J. et al., 1988). Moreover, the overexpression of boron transporters in Arabidopsis roots increases the boron tolerance was reported by Miwa K. (2007). Besides, there are some transcriptome studies in barley that found in the nature as boron tolerant to understand the boron tolerance mechanisms (Tombuloglu vd, 2013, 2015, 2016). With the help of these studies, it is thought to be that the further studies on boron uptake, transport and accumulation in plants need to be continued extensionally. For this reason, it is very important to examine plants, that have a high adaptability to stress conditions in the natural flora, are closely related to cultivated plants, or can be brought into agriculture due to their different plant characteristics such as phytamination. In this respect, it is necessary to examine plants tolerant to boron toxicity and to understand the mechanisms of that.

Babaoglu et al. (2004) identified *Gypsophila sphaerocephala, Gypsophila perfoliata* L.*, Elymus elongatus* and *Puccinellia distans* species tolerant to toxic amount of boron that growing in a boron mine region in Turkey. In their study, it was determined that the mentioned species naturally grow and develop in soils with a toxic level of boron content (8900 mg kg-1). It was also reported that the green parts of *G. sphaerocephala* contain higher boron amount when it is compared to the root and vegetative organs of other species. In addition to the aforementioned study, Babaoğlu et al. (2004) reported that *G. sphaerocephala* not only grows in soils contaminated with excess boron, but can also accumulate boron at unexpectedly high concentrations. In this way, it is thought that studies on *G. sphaerocephala* will enable the identification of the gene(s) providing boron

tolerance. Turkey is the position of gene center for the *Gypsophila* genus, and 63 individual *Gypsophila* species were discovered. These species have high economic value due to the saponins they contain (Özçelik and Muca, 2010). Özçelik and Özgökçe (2021) reported that 41 of these *Gypsophila* plants are endemic species (Özçelik and Özgökçe, 2021). According to the IUCN (International Union for Conservation of Nature), *Gypsophila* species are categorized under the CR (very endangered) category (Ekim et al., 2000). The use of *Gypsophila* species without culturing for economic purposes such as medicine, aromatic and pharmaceutical raw materials cause the extinction of these endemic plants (Özçelik and Yıldırım, 2011). The areas that *Gypsopilla* species cultured are open to antropogenic effects. This situation could be cause decreasing the population density of specific species and finally to the extinction of the species. It is quite important to investigate the abiotic stress conditions and determine the optimum living conditions for endemic species like *Gypsophila spp*. For example, in the latest study of Üstüner et al., (2021), drought and salinity stress tolerance of *G. piluliferea* in invitro conditions were studied.

As a consequence, it is predicted that the result of the study has a great contribution on preserving the natural population of this species (Üstüner vd., 2021).

3. MATERIALS AND METHODS

3.1. Yeast Media and Growth

The *S. cerevisiae* strain used in this study was *atr1Δ*. 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.

3.1.1. Preparation of *atr1Δ* (WT) Yeast Culture

The *atr1Δ* strain used in the study was taken from the glycerol stock and spread in solid YPD nutrient medium, then the petri dish was turned upside down and left to develop at 30 °C for 3 days. The yeast culture growing in the solid nutrient media were parafilmed the next day and continue to incubate at 30 °C.

3.2. Determination of Toxic Boron Concentration in *atr1Δ* Yeast Strain

To determine the toxic boron concentration prior to the electroporation step, five independent boric acid concentrations (0-50-60-70-80-100 mM) were tested along with the YPD plate as a control. Nutrient media containing 0-50-60-70-80-100 mM boric acid were prepared as one repeat. After preparation of nutrient media, the grown liquid *atr1Δ* yeast culture was taken and spread to the solid YPD nutrient medium containing different concentrations of boric acid as defined above and left to develop at 30 °C for 3 days.

3.3. Transformation of EV, GY (*Gypsophila perfoliata* leaf) and GK (*Gypsophila perfoliata* root) cDNA Libraries to *atr1Δ* Yeast Strain

Yeast *atr1Δ* strain containing pAG426GPD-cDNA (Empty vector: EV) (Figure 3.1.) expression vector was used for yeast transformation.

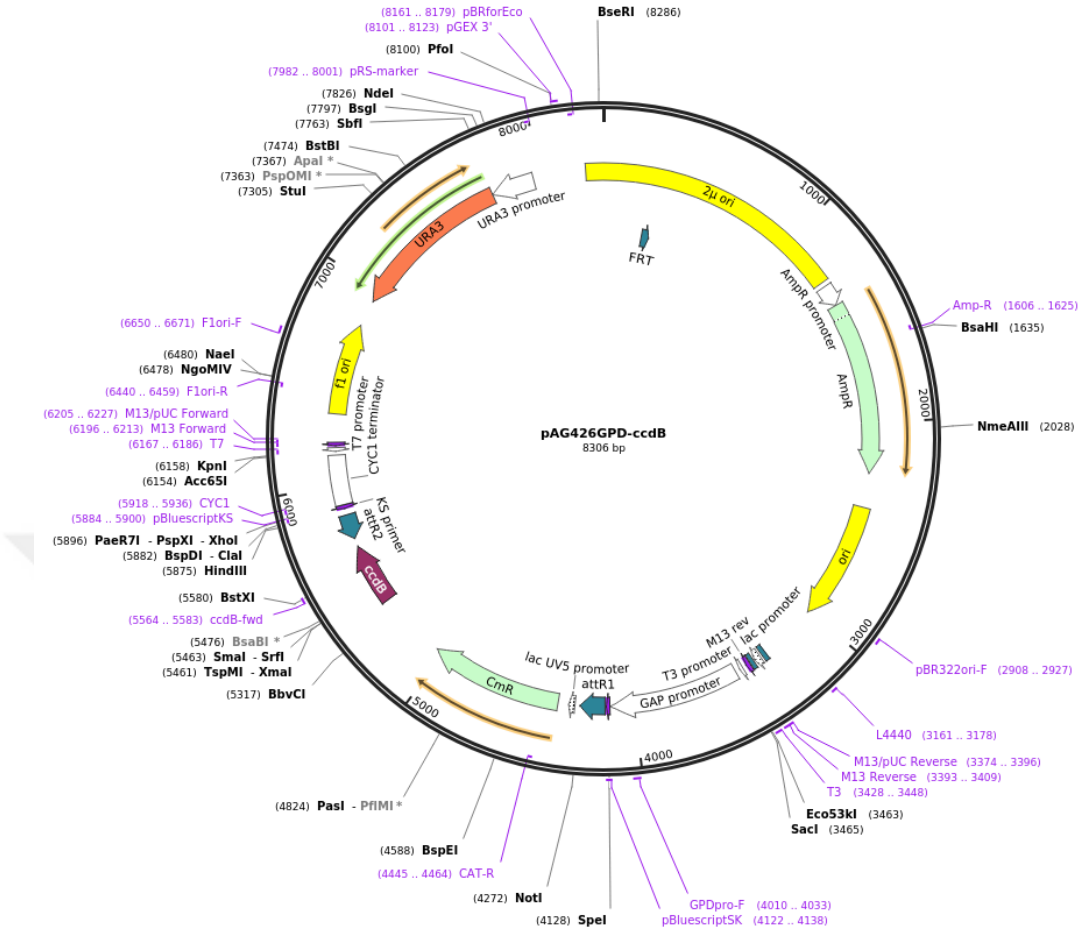


Fig 3.1. Map of pAG426GPD-cDNA (Empty vector: EV) expression vector

Besides empty vector, the *Gypsophila* leaf (GY) and *Gypsophila* root (GK) cDNA libraries that inserted into the pAG426GPD-cDNA yeast expression vector were also used. The yeast expression vector, pAG426GPD-cDNA were transferred to *atr1Δ* strain with using electroporation device (Eppendorf Eporator® for Bacteria and Yeast #4309000027). After 3 days of spreading the WT cell culture on solid YPD medium, a single WT colony was selected and re-suspended in 5 ml YPD liquid nutrient medium, then grown overnight at 30 °C in a shaking incubator with 150 rpm to stationary phase (OD_{600} to or about 3). After incubation of almost 18 hours, the number of cells in 1 ml of culture was determined by measuring the OD_{600} value with the help of a spectrophotometer (0.1 value in OD_{600} for yeast cells corresponds to a concentration of $1x10^6$ cells/ml). Then

aliquot amount of the overnight culture was inoculated into 50 ml of YPD broth at an initiate 0.2 OD_{600} and placed into the shaker again. It was allowed to grow the cells until they reached a concentration of OD_{600} is about 1.2 (approximately 5 hours). Cells were collected by centrifugation at 3000 rpm for 3 minutes and washed the cell pellet twice with 50 ml ice cold sterile distilled water and once by 50 ml ice cold electroporation buffer (1 M Sorbitol/1 mM $CaCl_2$). The cell pellet dissolved in 10 ml 0.1M LiAc/10mM DTT solution and shook at 225 rpm in a culture flask for 30 minutes at 30 °C. Then the cells were collected by centrifugation at 3000 rpm for 3 minutes and washed the cell pellet twice with 50 ml ice cold electroporation buffer, then the cell pellet was re-suspended in 200 µl electroporation buffer to reach a final volume of 2 ml. The cells were kept on ice until electroporation. 200 µl electrocompetent cells and 5 µl DNA were gently mixed and transferred to a pre-chilled Bio-Rad GenePulser cuvette (0.2 cm electrode gap). The cells were electroporated at 2.5 kV and 25 µF. typical time constant ranges from 3.0 to 4.5 milliseconds. Electroporated cells were transferred from each cuvette into 8 ml of 1:1 mix of 1M Sorbitol: YPD media then incubated on a shaker at 225 rpm and 30 °C for 1 hour. After incubation period, 200 µl of culture was taken and spread on solid YNB nutrient medium, along with YPD nutrient medium as a positive control, besides, empty electrocompetent *atr1Δ* cells were spread on both YPD and YNB nutrient media as a positive control to check the cells' survival ability. Transformation was carried out in 3 separate processes using our 2 cDNA libraries, GY, GK plasmids and an empty vector (EV) as a control. For each treatment, 20 YNB-URA agar selective media were inoculated into petri dishes (thanks to the URA3 gene in the pAG426 yeast expression vector, transformed yeast cells will be able to grow on CSM-URA medium, selection provides in this way). The media was incubated for 3 days at 30 °C. After electroporation, a large number of colonies were observed on plate. Several of the colonies were randomly selected and screened by yeast colony PCR method to confirm the presence and see the diversity of the GY and GK cDNA libraries in *atr1Δ* yeast cells. Yeast Colony PCR was performed using forward (GPD pro_F) and reverse (CYC-1 R) primers (Table 3.1.) by taking tiny pieces from selected colonies. After PCR scanning, the PCR products were run on 1% agarose gel and visualized.

Table 3.1. The Sequence of Primers Used in PCR Studies

GPD pro-F (forward primer)	5′-CGGTAGGTATTGATTGTAATTCTG-3′
CYC1 (reverse primer)	5′-GCGTGAATGTAAGCGTGAC-3′

3.4. Screening of GY and GK Yeast Libraries at Toxic Level of Boric Acid

At the end of 3 days of growth period after electroporation, all of the colonies on YNB media were scrapped and collected in falcon tubes containing 20 ml liquid YNB-URA medium. Thus, GY and GK cDNA libraries were created to be used for stress tolerance determination studies in boric acid-containing media. 15 µl of the libraries were spread on YNB media supplemented with 80 mM H_3BO_3 for the purpose of give high boron stress to yeast cells containing libraries (Figure 3.2.). The colonies were developed after 3 days. Putative tolerant colonies were obtained on media containing 80 mM boric acid from the transformation of the *atr1Δ* GY cDNA library. Approximately 10 possible tolerant colonies observed after transfer of the GY yeast library after incubation were selected one by one and labelled by coding with the name and sequence number of the cDNA library from which they were created. The codes of the selected colonies are given: GY1, GY2, GY4, GY4, GY5, GY6, GY7, GY8, GY9, GY10.

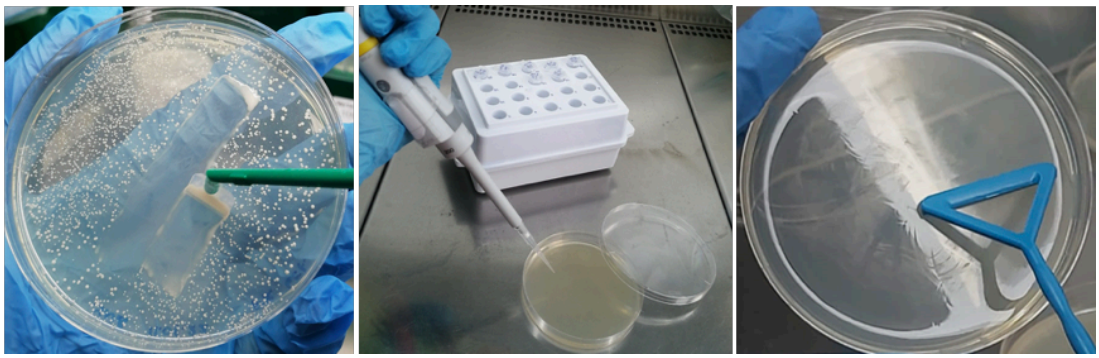


Fig 3.2. Screening of GY and GK Yeast Libraries

3.4.1. Spot Test of Possible Tolerant *atr1Δ* GY Colonies

Ten *atr1Δ* GY colonies were individually selected and cultured in 500µl of liquid YNB nutrient media in 1.5 ml Eppendorf tubes and then incubated on a shaker at 150 rpm and 30 °C for 18 hours. The number of cells in 1 ml of culture was determined by measuring the OD_{600} value with the help of a spectrophotometer, and diluted to a concentration of 0.2 OD_{600}. Spot test was performed by 5µl of colony cultures were spotted on YNB solid media containing different concentrations of H_3BO_3 (0 mM, 80 mM, 100 mM and 150 mM) (Figure 3.3.). The media was incubated for 3 days at 30 °C. 40% glycerol stocks of 10 colonies individually were prepared and they kept at -80 °C for further studies.

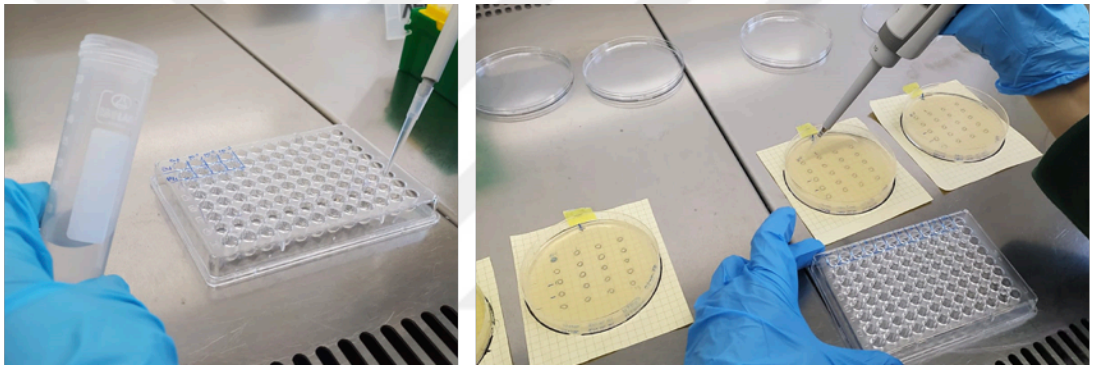


Fig 3.3. Spot Test of Possible Tolerant *atr1Δ* GY Colonies

3.4.2. Serial Dilution Test of Possible Tolerant *atr1Δ* GY Colonies

After spot test, serial dilution test was performed. The colonies were taken from the glycerol stock and streaked them onto solid YNB nutrient media, then incubated for 3 days at 30 °C. Then the single colonies formed from possible tolerant colonies, then a single colony was taken and put in 1.5 ml sterile Eppendorf tube with 500 µl liquid YNB nutrient medium, then grown overnight at 30 °C in an incubator (150 rpm). The number of cells in 1 ml of culture was determined by measuring the OD_{600} value with the help of a spectrophotometer, and diluted to a concentration of 0.2 OD_{600}. 10-fold serial dilutions (OD 0.2, 0.02, 0.002, 0.0002) of these colonies were prepared and spotted 5µl, then their growth was observed in YNB-URA media containing 0 mM, 55 mM, 80 mM and 125

mM boric acid. After serial dilution of possible boron tolerant colonies, they were screened by yeast colony PCR to confirm the presence and the diversity of the GY libraries in *atr1Δ* yeast cells.

3.5. Yeast Plasmid Isolation of Possible Tolerant *atr1Δ* GY Colonies

The possible tolerant *atr1Δ* GY colonies were taken from the glycerol stock kept at -80 °C and put in 50 ml sterile falcon tube with 5 ml liquid YNB nutrient medium, then grown overnight at 30 °C in a shaking incubator with 150 rpm. Plasmids were isolated by Zymoprep Yeast Plasmid Miniprep I (Cat # D2001) according to the manufacturer's instructions. The isolated plasmids were run on the 1% agarose gel to see to ensure the sizes of them. Then PCR assay was performed to see if the size of the plasmids is different from each other or not. The reaction mixture is shown in Table 3.2. PCR conditions are seen in Table 3.3. PCR results were analyzed by gel electrophoresis using 1% agarose gel. After controlling the size of the plasmids, each of the plasmids was transformed into *E. coli* DH5α cells by heat shock method. After heat shock transformation process, the cells were taken into 1.5 ml Eppendorf tubes, 1 ml of liquid LB medium was added, and incubated for 2 hours by shaking at 37 °C and 200 rpm. 200 µl of bacteria was taken and added to solid LB medium containing ampicillin (100 µg/ml) and spread out. The culture medium was incubated overnight at 37 ° C. A large number of colonies were observed on it and several of the colonies were randomly selected and screened by colony PCR method to confirm the presence of any tolerance genes gene in *E. coli* DH5α bacteria. Positive colonies were inoculated at 37 °C in liquid LB medium containing 100 µg/ml ampicillin and incubated overnight at 200 rpm. After confirming the size of plasmids, the plasmids were also confirmed by restriction enzyme digestion to see their size's difference. Digestion was performed by two restriction enzymes: *XhoI (Xanthomonas vasicola) (5896th bp on* pAG426GPD-cDNA vector) and *SpeI (Sphaerotilus natans) (4128th bp on (*pAG426GPD-cDNA vector). 1000 ng plasmids were used for each digestion. After 1 hour incubation at 37 °C, the cut and uncut plasmids were run on 1% agarose gel and visualized. The band size of the restriction enzyme digestion did not show any difference. That is why the experiment was repeated by only changing the restriction enzyme: *Bsa*I (*Bacillus stearothermophilus*) (2109th, 5601st and 7063rd bps on pAG426GPD-cDNA vector).

Table 3.2. PCR Reaxion Mixture

DreamTaq Green PCR Master Mix (2X)	12 µl
Forward primer (GPDpro-F)	1 µl
Reverse primer (CYC1)	1 µl
Plasmid DNA	3 µl
Nuclease-free H_2O	8 µl
Total	25 µl

Table 3.3. PCR Conditions

Temperature	Time	Cycle
95 °C	5 min	1
95 °C	3 min	39
58 °C	45 sec	
72 °C	1.30 min	
72 °C	7 min	1

3.6. Retransformation of Possible Tolerant Gene Including Plasmids to Yeast *atr1Δ* Cells

The yeast *atr1Δ* cells used in the study was taken from the glycerol stock and streaked on fresh YPD plate and incubated at 30 °C. After the cells grown, a single colony was selected and put into liquid YPD medium and incubate overnight on a shaker at 150 rpm and 30 °C. After 16 hours of growth, the number of cells in 1 ml of culture was determined by measuring the OD_{600} value with the help of a spectrophotometer, and diluted to a concentration of 0.4 OD_{600} (A suspension containing $1x10^6$ cells ml^{-1} will give an OD_{600} of 0.1). Then add $2.5x10^8$ cells to pre-warmed 2X YPD medium. The amount of cell of this solution should be $5x10^6$ cells ml^{-1}. Incubate the flask in the shaking incubator at 150 rpm and 30 °C till the cell titer at least $2x10^7$ cells ml^{-1}. Carrier DNA was denatured

in boiling water for 5 min and chills immediately. Cells were harvested by centrifugation at 3.000xg for 5 min and resuspended the pellet and centrifugation at 3.000g for 5 min to pellet the cells. This washing step was repeated three times. Then the cells were resuspended again and take 100µl of samples containing 10^8 cells for each transformation. The sample was centrifuged and the supernatant was removed. After this step, the transformation mix was added to each transformation tube along with negative control and resuspend the cells by vertexing. Then the tubes were placed in a water bath at 42 °C and incubated for 40 min. The tubes were centrifuged and the supernatant was removed. The cell suspension was plated onto YNB and YPD media. Finally, plates were incubated at 30 °C. After retransformation of 3 boron tolerant colonies, they were screened by colony PCR to confirm the presence and the diversity of the *atr1Δ* yeast cells. After colony formation observed as a result of retransformation, the colonies were taken and cultured. The number of cells in 1 ml of culture was determined by measuring the OD_{600} value with the help of a spectrophotometer, and diluted to a concentration of 0.2 OD_{600}. 10-fold serial dilutions (OD 0.2, 0.02, 0.002, 0.0002) of these colonies were prepared and spotted 5µl, then their growth was observed in YNB-URA media containing 0 mM, 55 mM, 80 mM and 125 mM boric acid and incubated at 30 °C.

3.7. Sequencing and Phylogenetic Analysis

The plasmid (GY-5) derived from *E. coli* DH5α cells was sent to New Generation Sequencing.

3.8. Plant Material and Boron Application to Plants

In this study, we used *G. perfoliata* plant as a plant material. The seeds of *G. perfoliata* were sterilized by treating with 70% EtOH and 5% NaOCl for 2 and 10 minutes, respectively. Then the seeds were rinsed with distilled water 3 times (Figure 3.4). Seeds were sown in Murashige and Skoog (MS) medium were and allowed to grow at 24 °C 16/8 long day conditions (140 µmol m^{-2} $s^{-1)}$.

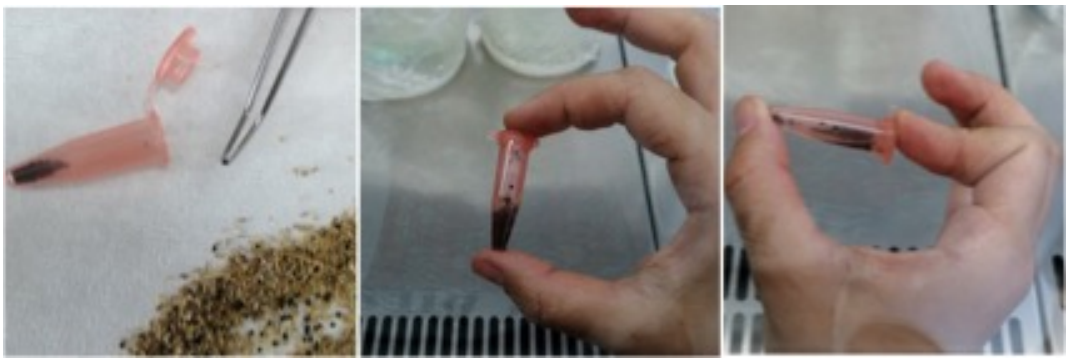


Fig 3.4. *G. perfoliata* Seed Surface Sterilization

The 2-month-old plants were transferred to hydroponic system containing ½ Hoagland solution (Figure 3.5.). The pH of the solutions was adjusted to 5.7. The Hoagland solution was changed every week during acclimatization.

Fig 3.5. *G. perfoliata* Development in Hydroponic System

G. perfoliata plants were exposed to stress with ½ Hoagland solution containing 0, 200 and 400 mg/L boron, respectively. The amount of boron at the toxic dose to be given to the plants in the study was determined as 500 mg/L B in a previous study (unpublished datum). Therefore, the boron concentration determined as stress was given as 200 and 400 mg/L B. Boron was added in the form of boric acid during the preparation of the hoagland solution. The experiment was set up in 10-11 plants for each stress application. After the boron stress application, the roots were washed with water to remove the contamination of boron and other elements originating from the cultivation process. Leaves and roots were harvested after 12 and 24 hours and stored at -80 °C.

3.9. qPCR analysis of Determined Tolerance Gene

Leaf and root RNA isolations of plants were performed using the RNA Plant Mini Kit (Qiagen). RNA amounts were determined using the Qubit Fluorometer (Invitrogen). DNA impurities were cleaned with RQ1 RNase-Free DNase (Promega, WI, USA) and the samples were made ready for qPCR. qPCR studies were performed on a Light Cycler 96 (Roche). The expression of the determined gene was calculated using Luna® Universal One-Step RT-qPCR Kit (NEB) with DNase treated 10 ng RNA. Primers (Table 3.4.) used in RT-qPCR analyzes were designed with the help of Primer 3 Input program (http://bioinfo.ut.ee/primer3-0.4.0/). The reaction was set up as given in the Table 3.5.

Table 3.4. The qPCR Primers Designed for *G. perfoliata*

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Gy5	AATGGCGGGAATCAAGCTTC	TGTTCGTCGTCTAAGGTGCT
GHK (actin)	TTTGACTCAACACGGGGAAA	CAGACAAATCGCTCCACCAA

Table 3.5. The qPCR Reaction Mix

Luna Universal One-Step Reaction Mix (2X)	2,5 µl
Luna WarmStart® RT Enzyme Mix (20X)	0,25 µl
Forward Primer (10 µM)	0,4 µl
Reverse Primer (10 µM)	0,4 µl
Template RNA (5 ng/µl)	2 µl
Nuclease Free H_2O	1,45 µl
Total Volume	7 µl

4. FINDINGS

4.1. Determination of Toxic Boron Concentration in *atr1Δ* Yeast Strain

In order to determine the boron tolerance of *Saccharomyces cerevisiae atr1Δ* yeast strain (WT- wild type), images were taken after the cells were cultured at different boron concentrations are as follows (Figure 4.1.).

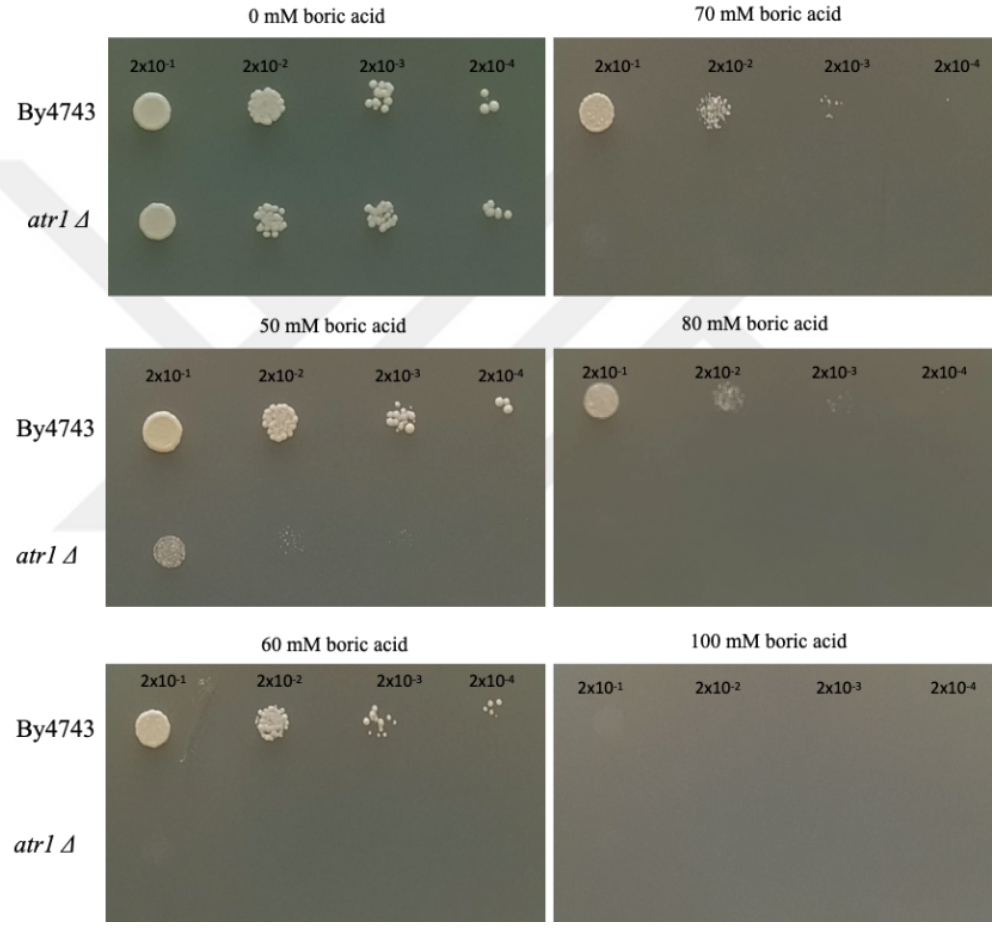


Fig 4.1. Determination of Toxic Boron Concentration in *atr1Δ* Yeast Strain

Wild *atr1Δ* (WT) yeast cells have previously been shown to be resistant to 55 mM boric acid concentration (Figure 4.1.). Since the boron tolerance of the yeast strain tested was like the one in this study, it was decided to use the medium containing 55 mM boric acid as a selective medium after transformation.

4.2. Transformation of EV, GY (*Gypsopila* leaf) and GK (*Gypsopilla* root) cDNA Libraries to *atr1Δ* Yeast Strain

After incubation of the transformed cells at 30 ºC for 3 days, the formation of many colonies in each petri dish showed that the transformation processes were successful (Figure 4.2. and 4.3.).

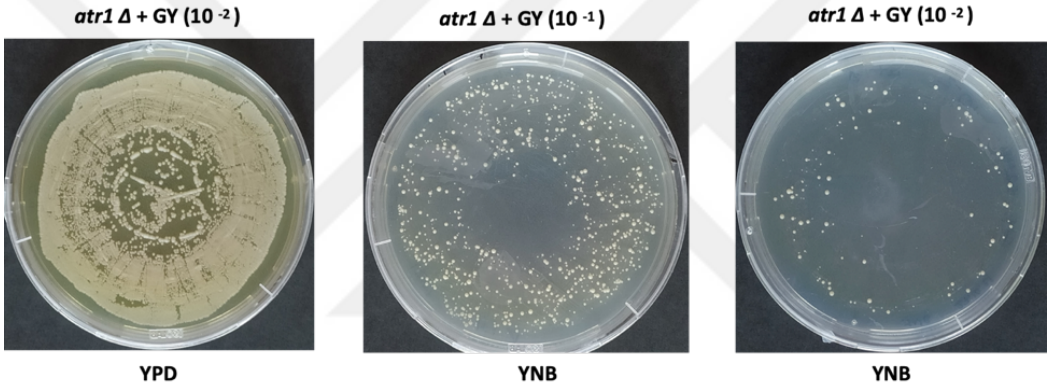


Fig 4.2. Transformation of EV, GY (*Gypsopilla* leaf) cDNA Library to *atr1Δ* Yeast Strain

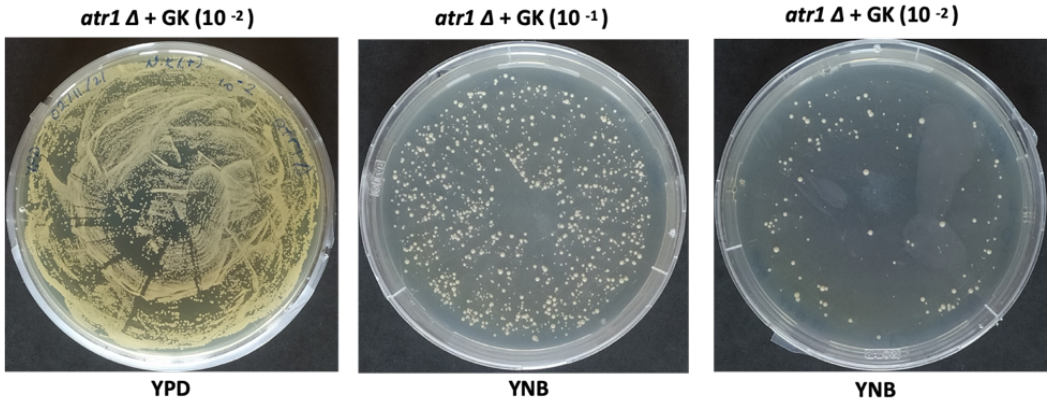


Fig 4.3. Transformation of EV, GK (*Gypsopilla* root) cDNA Library to *atr1Δ* Yeast Strain

Colonies formed were stripped from petri dishes and collected in falcon tubes containing 25 ml liquid YNB-URA medium. Thus, EV, GY and GK yeast libraries were created from EV, GY and GK cDNA libraries to be used for tolerance studies in boron-containing media. In addition, it is seen that the band sizes are different from each other as a result of colony PCR performed to understand whether the colonies contain cDNAs. This shows the diversity of cDNA libraries. The reason why we can't see the difference between the bands very clearly in the figure 4.4 may be since the samples after colony PCR were not run sufficiently in agarose gel. However, in the figure 4.5. the variation between band sizes can be seen more clearly. This may be due to the samples being run for sufficient time after colony PCR. (Figure 4.4. and 4.5.).

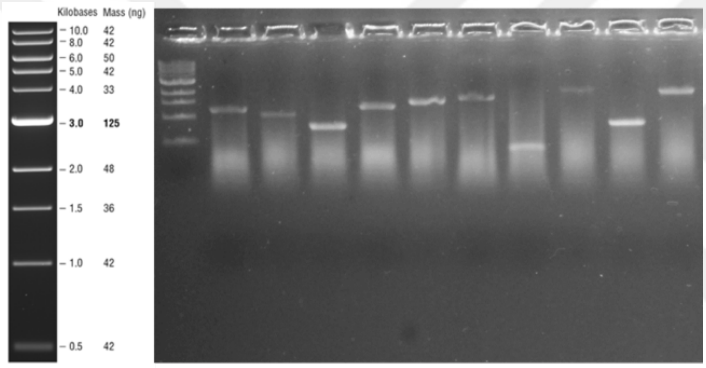


Fig 4.4. Agarose Gel Image of Colony PCR of EV and GY (*Gypsopilla* leaf) cDNA Library in *atr1Δ* Yeast Strain. Ladder: 1 kb DNA Ladder

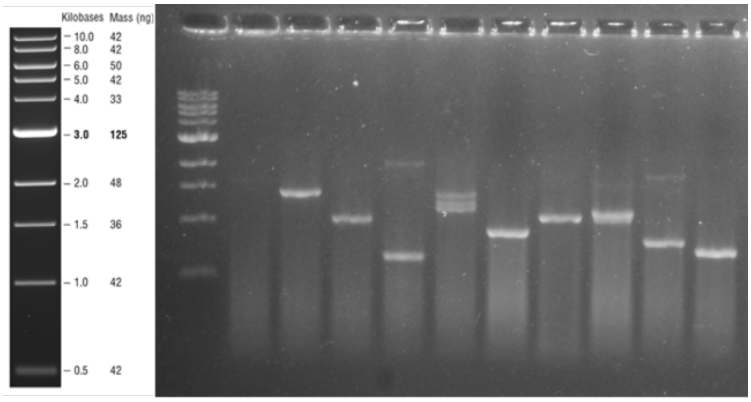


Fig 4.5. Agarose Gel Image of Colony PCR of EV and GK (*Gypsopilla* root) cDNA Library in *atr1Δ* Yeast Strain. Ladder: 1 kb DNA Ladder

4.3. Screening of GY and GK Yeast Libraries at Toxic Level of Boric Acid

After determining the toxic concentration of boron, we screened 2 yeast cDNA libraries which are GY and GK to identify genes that confer boron tolerance to wild-type cells. In our previous experiment, determination of toxic amount of boric acid, it was decided to use the medium containing 55 mM boric acid as a selective medium after transformation. However, after transformation, the EV could grow at 55 mM boric acid. Hence, the experiment for determining the toxic boric acid concentration was repeated, and it was observed that EV could not grow on medium containing 80 mM boric acid. therefore, it was decided to use 80 mM boric acid instead of 55 mM boric acid in future experiments.

Transformed cells were exposed to 80 mM boric acid for 3 days. 10 colonies that could grow in the presence of 80 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. While GY yeast libraries showed possible tolerant colony formation at 80 mM boric acid containing YNB plate, GK yeast libraries did not show any colonies despite of the repeated attempts (Figure 4.7.). Hence, after this step, the remaining studies were carried out with GY cDNA yeast library.

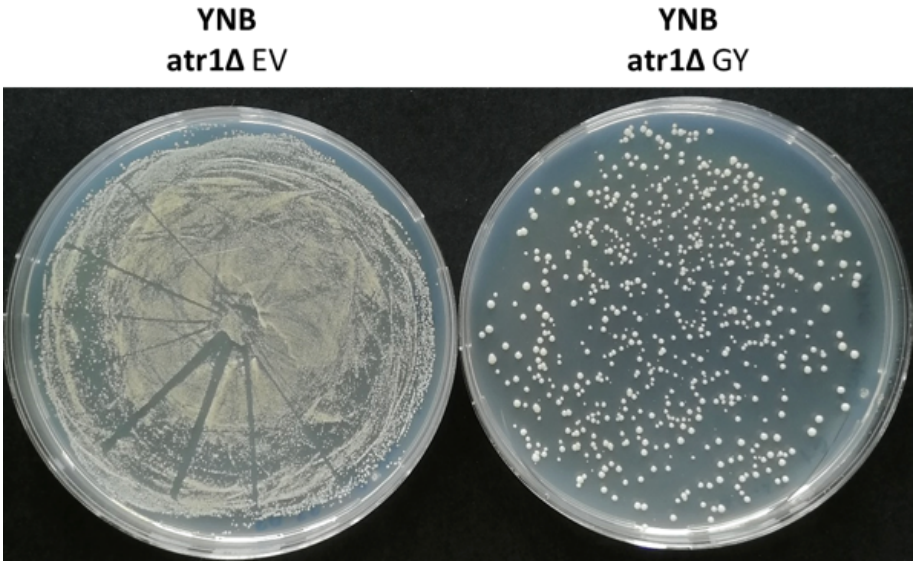


Fig 4.6. Growing Single Colonies Before Screening

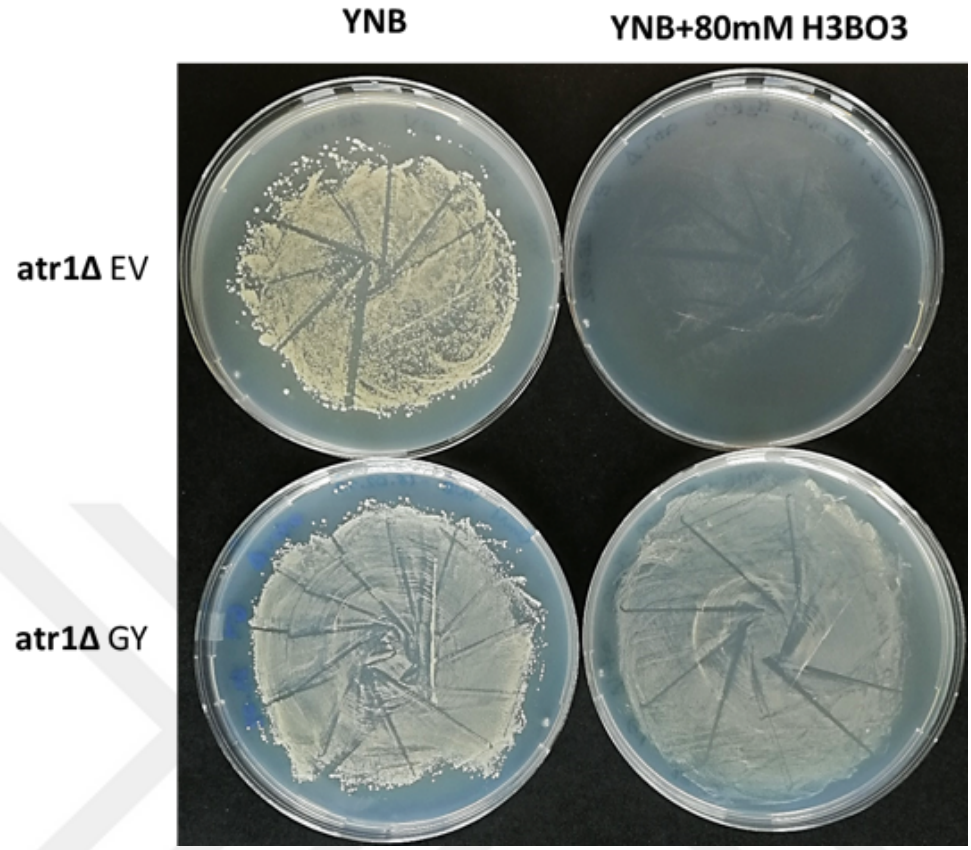


Fig 4.7. Screening of GY Yeast Library at Boric Acid Containing YNB Media

4.3.1. Spot Test of Possible Tolerant *atr1Δ* GY Colonies

After spotting the selected colonies at YNB media containing different concentrations of boric acid (0, 80, 100 and 150 mM), all selected yeast colonies were not able to grow in the presence and above the concentration of 100 mM boric acid. As seen in Figure 4.8., the *Δatr1* EV could not grow in the presence of 80 mM boric acid, whereas some of the GY *Δatr1* cells were able to tolerate up to 80 mM.

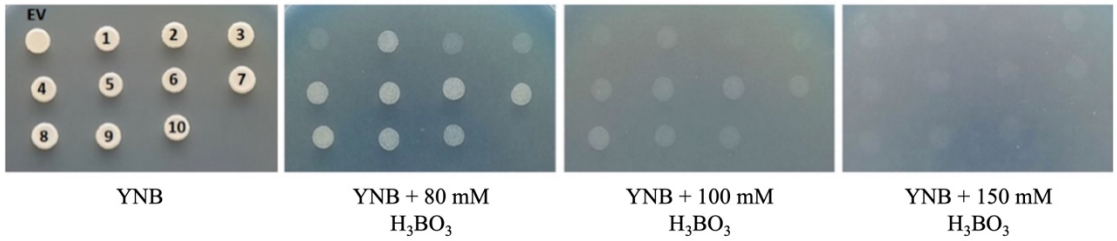


Fig 4.8. Spot Test of Possible Tolerant *atr1Δ* GY Colonies

4.3.2. Serial Dilution Test of Possible Tolerant *atr1Δ* GY Colonies

All of the possible tolerant *atr1Δ* GY colonies were cultured on liquid YNB media and the number of cells in 1 ml of culture was determined by measuring the OD_{600} value, and diluted to a concentration of 0.2 OD_{600}. 10-fold serial dilutions (OD 0.2, 0.02, 0.002, 0.0002) of these colonies were prepared and spotted 5 µl. Their growth was observed in YNB-URA media containing 0 mM, 55 mM, 80 mM and 125 mM boric acid. The toxic boric acid concentration was again changed in here again (55 mM). In Figure 4.9., it can be seen that 7 of the colonies could survive in the presence of 55 mM boric acid concentration.

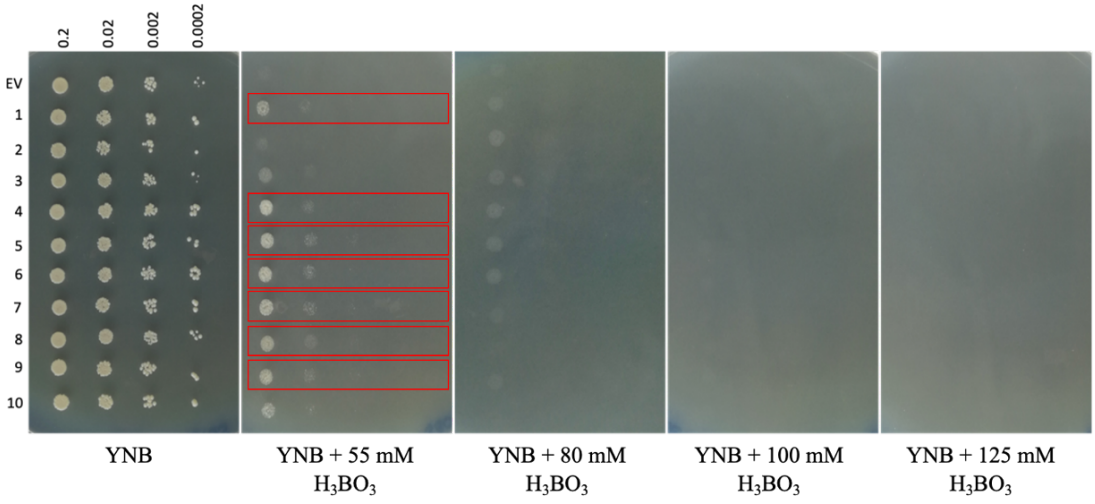


Fig 4.9. Serial Dilution Test of Possible Tolerant *atr1Δ* GY Colonies

After serial dilution of possible boron tolerant colonies, they were screened by yeast colony PCR to confirm the presence and the diversity of the GY libraries in *atr1Δ* yeast cells. Colony PCR was performed using forward (GPD pro_F) and reverse (CYC-1 R) primers. After PCR scanning, the PCR product was run on 1% agarose gel and visualized. As a result of yeast colony PCR assay, 3 of the colonies (Colony 4, 5 and 6) showed any band (Figure 4.10.). Since performing the yeast colony PCR is challenging, even though the bands were quite weak, the studies were continued with these 3 colonies.

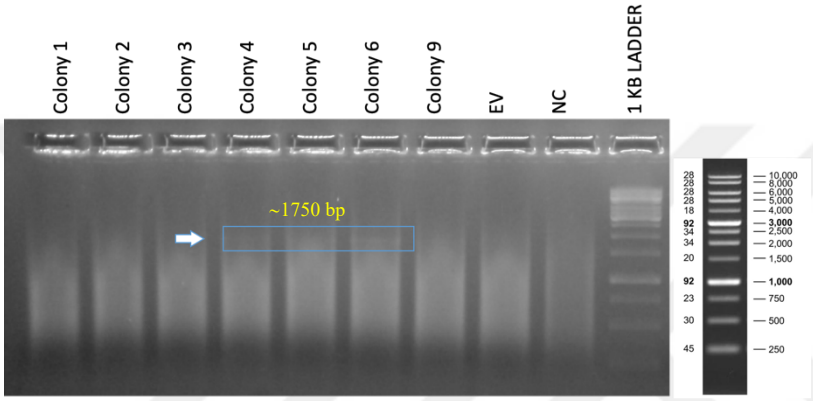


Fig 4.10. Colony PCR Results of Possible Tolerant *atr1Δ* GY Colonies. Ladder: 1 kb DNA Ladder

4.4. Yeast Plasmid Isolation of Possible Tolerant *atr1Δ* GY Colonies

Plasmids were isolated by Zymoprep Yeast Plasmid Miniprep I (Cat # D2001) according to the manufacturer's instructions. The isolated plasmids were run on the 1% agarose gel to see to ensure the size of them. Then PCR assay was performed to see if the size of the plasmids is different from each other or not. All the PCR products were almost similar to each other (~1750 bp) but different from the EV PCR product (Figure 4.11.).

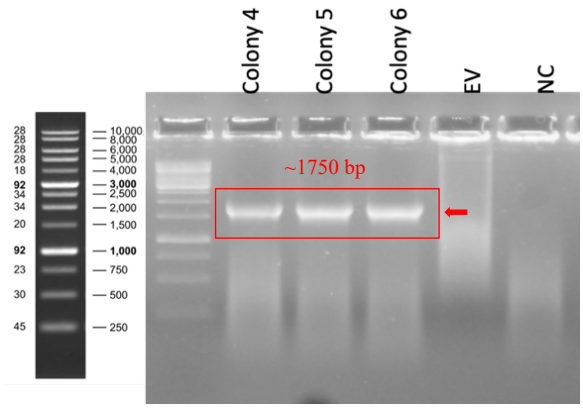


Fig 4.11. PCR Result of Possible Tolerant *atr1Δ* GY Plasmids. Ladder: 1 kb DNA Ladder

After confirming the yeast plasmids have any tolerance gene, each of the plasmids was transformed into *E. coli* DH5α cells by heat shock method. All of the possible tolerance gene containing gene transformed cell samples showed colony formation on LB+amp100 plate while DH5 α cells did not (Figure 4.12.).

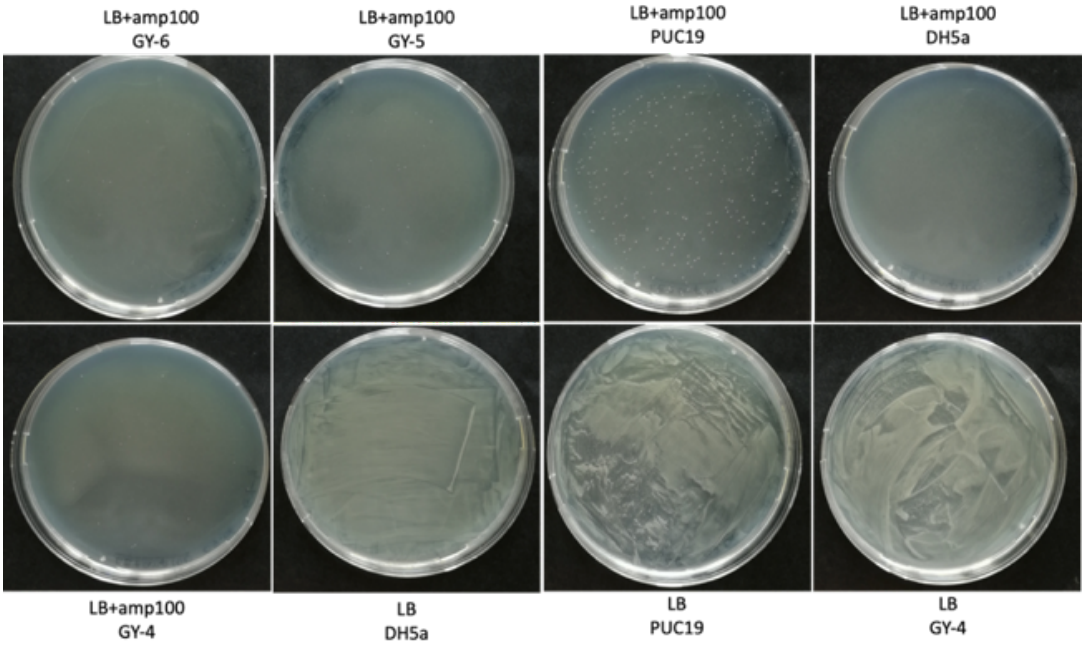


Fig 4.12. Heat shock of Possible Tolerant Plasmids to *E. coli* DH5α Cells

After formation of colonies of LB+amp100 plates, randomly 2 colonies were selected for each sample and *E. coli* colony PCR assay were performed. As similar to previous yeast colony PCR result, the band sizes were about ~1750 bp (Figure 4.13.).

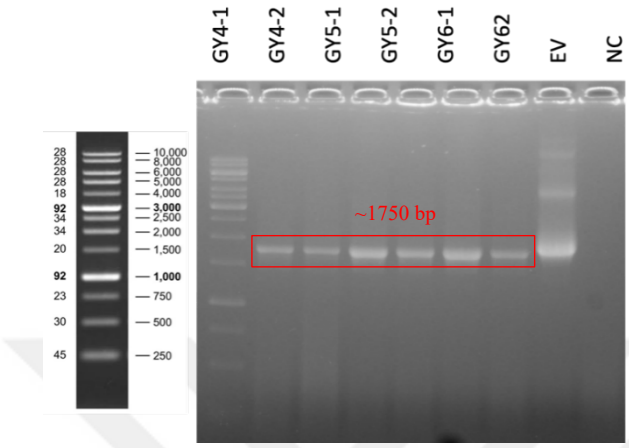


Fig 4.13. Colony PCR Result of *E. coli* DH5α Cells Containing the Plasmids

After PCR, restriction enzyme digestion was performed to verify the plasmids and prove that they are different from each other by *XhoI* and *SpeI.* The expected band sizes of cut plasmids were: 1768 bp and 6538 bp. According to this result, 4th, 5th and 6th plasmids showed bands of the same size with each other and EV (Figure 4.14.).

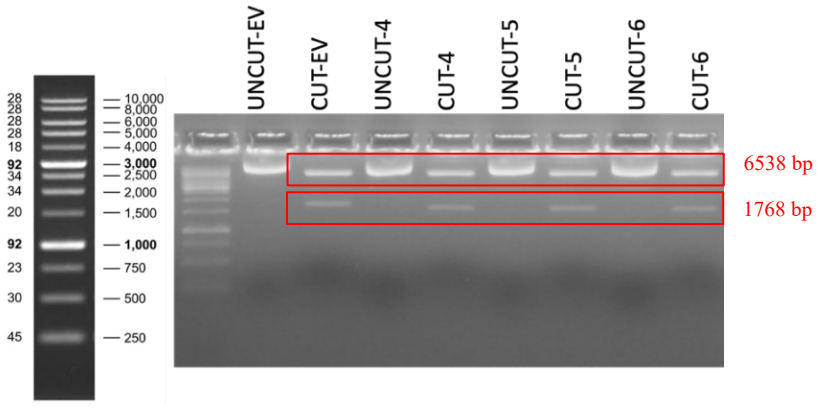


Fig 4.14. Restriction Enzyme Digestion Result of the Isolated Plasmids (*XhoI* and *SpeI)*

The band size of the restriction enzyme digestion by *XhoI* and *SpeI* did not show any difference. That is why the experiment was repeated by only changing the restriction enzyme: *Bsa*I (Figure 4.15.). The expected band sizes of BsaI enzyme in EV were: 1462 bp, 3352 bp and 3492 bp.

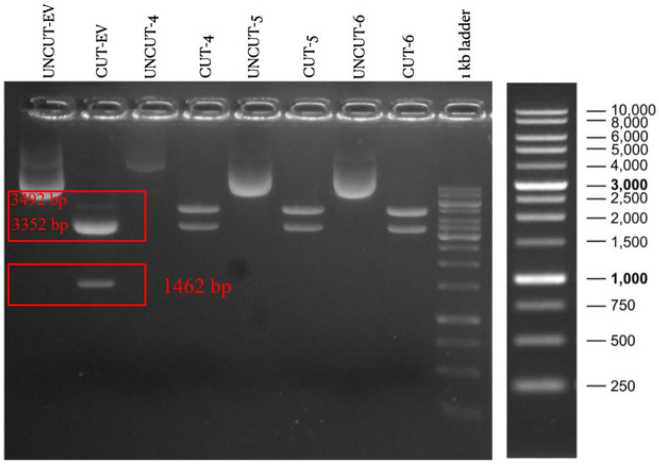


Fig 4.15. Restriction Enzyme Digestion Result of the Isolated Plasmids (*BsaI)*

4.5. Retransformation of Plasmids *atr1Δ* Yeast Strain

After all validation procedures for plasmids, *E. coli* plasmids were retransformed into *atr1Δ* yeast strain using the LiAc method. After 3 days of incubation, the colony observation has seen in all of the YNB media means the retransformation was successful (Figure 4.16.).

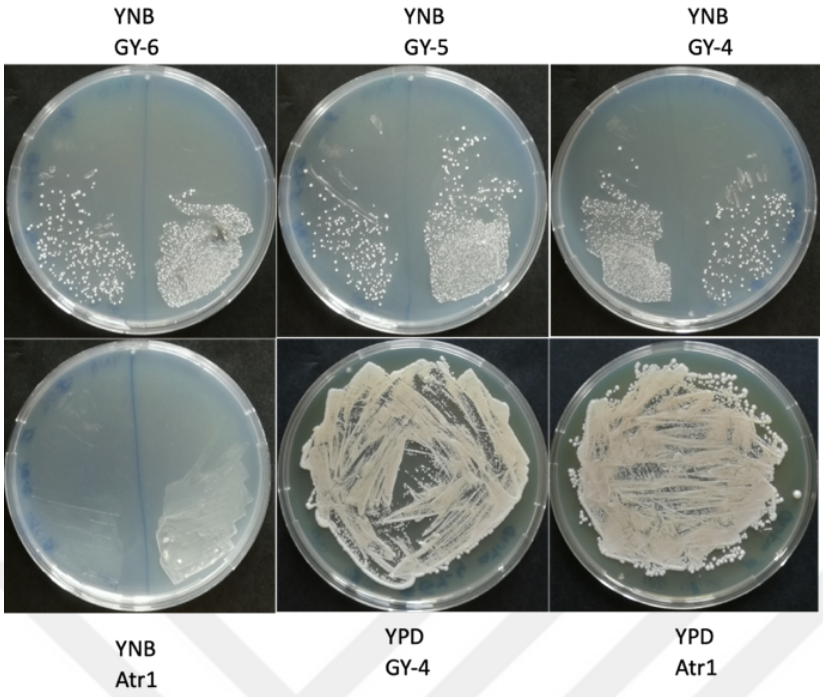


Fig 4.16. Retransformation of Plasmids *atr1Δ* Yeast Strain

The transformed cells were subjected to colony PCR after 3 days of incubation. then colony PCR was performed with the developing colonies and confirmed. As a result of colony PCR, it is seen that the bands are weak because of the challenge of yeast colony PCR. The band sizes of colony 4, 5 and 6 are similar to each other while they are bigger than EV plasmid (Figure 4.17.).

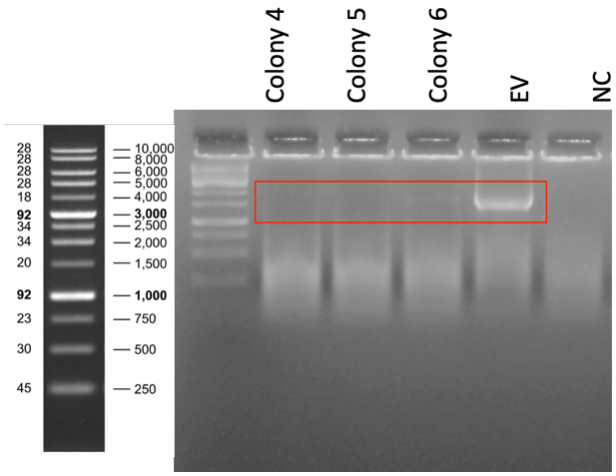


Fig 4.17. Colony PCR Results After Retransformation of Plasmids *atr1Δ* Yeast Strain

Serial dilution was made following the yeast colony PCR, and it was observed that yeast cells, into which 3 of the 4th, 5th and 6th plasmids were transferred, showed tolerance to the toxic dose of boron which is 80 mM (Figure 4.18.).

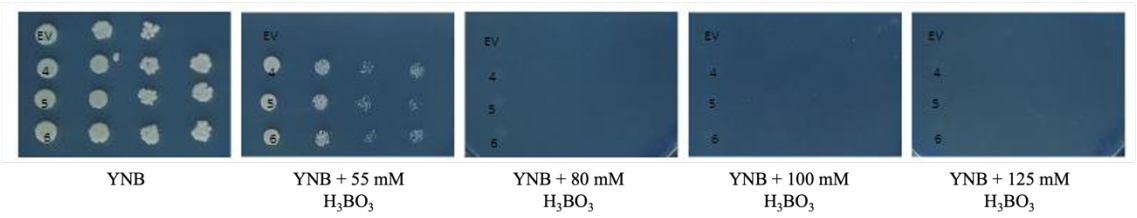
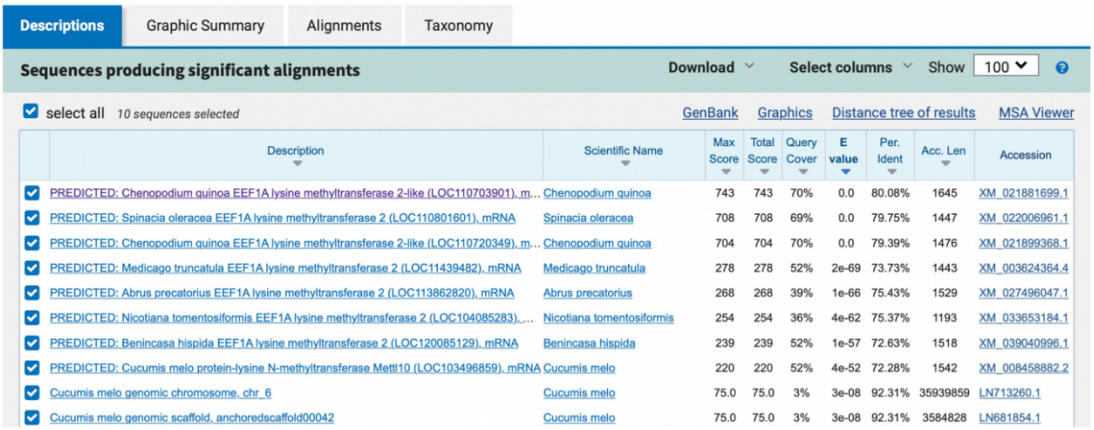


Fig 4.18. Serial Dilution of Tolerant *atr1Δ* GY Colonies

4.6. Sequencing and Phylogenetic Analysis

After NGS results reach to us, we analyzed the sequence in NCBI program by blasting. According to the blast results, the sequence is showing 80% similarity with *Chenopodium quinoa* EEF1A lysine methyltransferase-2-like gene. When we look at *Chenopodium quinoa* and *Spinacia oleracea* EEF1A genes, it is seen that the E value is 0. The expected value (E) describes the number of hits expected by chance when searching a database for a particular sequence. As this value (S) increases, the similarity decreases exponentially. Basically, the E value describes random background noise. In this case, it indicates that the gene identified is EEF1A, but not previously expressed in G. perfoliata. Looking at the identity values, there is actually not much similarity (80%). This indicates that a new gene has been discovered. Besides, the determined gene is showing less similarity with the mentioned genes of *Spinacia oleracea, Benincasa hispida, Medicago trunculata, Abrus precatorius*, respectively (Figure 4.19. and 4.20.).



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *10 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
PREDICTED: Chenopodium quinoa EEF1A lysine methyltransferase 2-like (LOC110703901), m...	Chenopodium quinoa	743	743	70%	0.0	80.08%	1645	XM_021881699.1
PREDICTED: Spinacia oleracea EEF1A lysine methyltransferase 2 (LOC110801601), mRNA	Spinacia oleracea	708	708	69%	0.0	79.75%	1447	XM_022006961.1
PREDICTED: Chenopodium quinoa EEF1A lysine methyltransferase 2-like (LOC110720349), m...	Chenopodium quinoa	704	704	70%	0.0	79.39%	1476	XM_021899368.1
PREDICTED: Medicago truncatula EEF1A lysine methyltransferase 2 (LOC11439482), mRNA	Medicago truncatula	278	278	52%	2e-69	73.73%	1443	XM_003624364.4
PREDICTED: Abrus precatorius EEF1A lysine methyltransferase 2 (LOC113862820), mRNA	Abrus precatorius	268	268	39%	1e-66	75.43%	1529	XM_027496047.1
PREDICTED: Nicotiana tomentosiformis EEF1A lysine methyltransferase 2 (LOC104085283), ...	Nicotiana tomentosiformis	254	254	36%	4e-62	75.37%	1193	XM_033653184.1
PREDICTED: Benincasa hispida EEF1A lysine methyltransferase 2 (LOC120085129), mRNA	Benincasa hispida	239	239	52%	1e-57	72.63%	1518	XM_039040996.1
PREDICTED: Cucumis melo protein-lysine N-methyltransferase Mettl10 (LOC103496859), mRNA	Cucumis melo	220	220	52%	4e-52	72.28%	1542	XM_008458882.2
Cucumis melo genomic chromosome, chr_6	Cucumis melo	75.0	75.0	3%	3e-08	92.31%	35939859	LN713260.1
Cucumis melo genomic scaffold, anchoredscaffold00042	Cucumis melo	75.0	75.0	3%	3e-08	92.31%	3584828	LN681854.1

Fig 4.19. NCBI Blast Result of *GpEEF1A* Gene

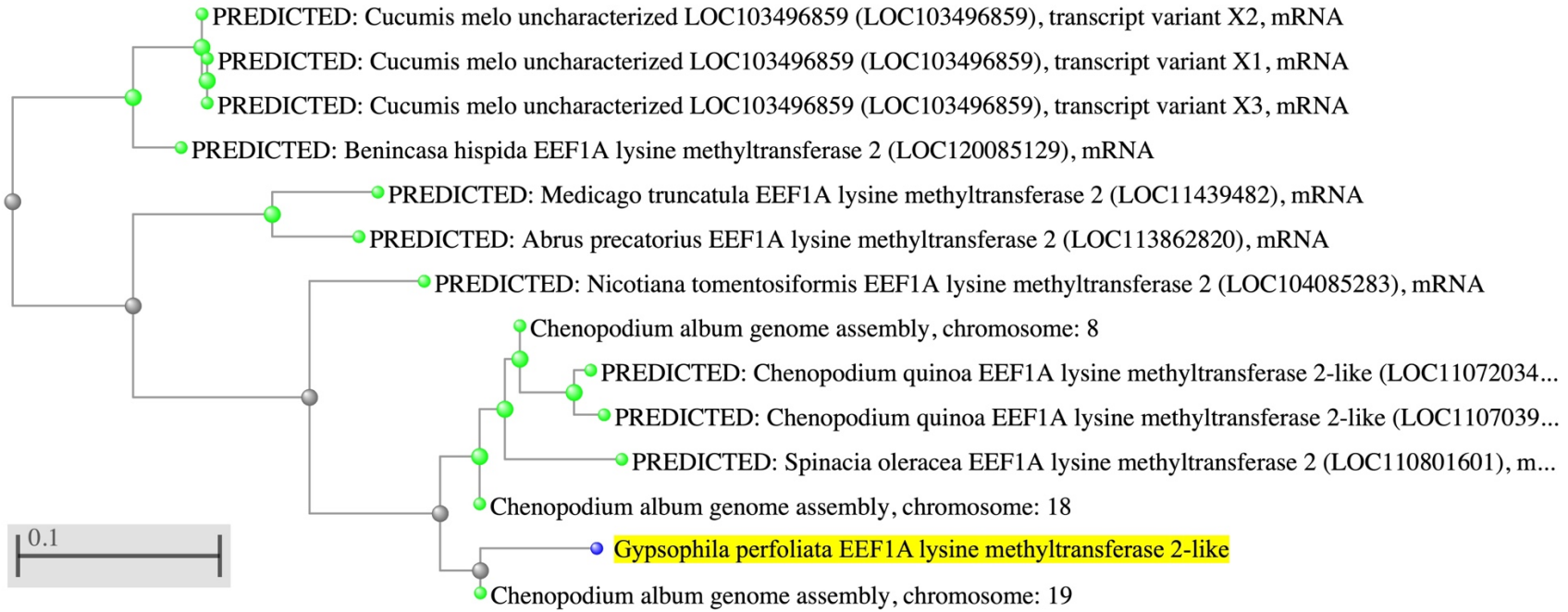


Fig 4.20. The Relationship Between the *GpEEF1A* Gene and Its Orthologs

After the phylogenetic analysis, a restriction enzyme has chosen: *HindIII (Haemophilus influenzae)* (this enzyme only cuts the EV at one place: 5875th bp) that cut in the insert in two different places, but cuts only one place on EV to ensure the other tolerant plasmids are same with each other or not. As seen in the figure 4.21., while the other three plasmids were cut into exactly to the same size. EV plasmid was only cut at one place (5875th bp) by the *HindIII*.

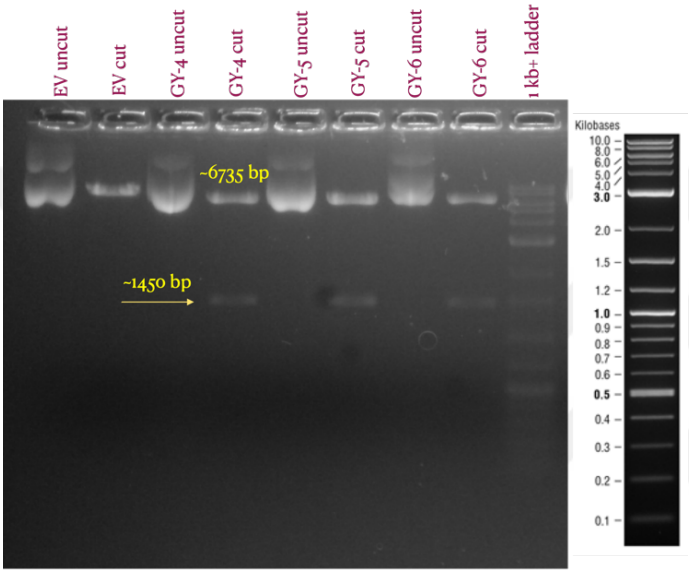


Fig 4.21. Restriction Digestion (*HindIII)* Result of the Tolerant Plasmids

The restriction digestion experiment was repeated by changing the regions cut by the enzyme: *EcoRI (Escherichia coli BS5)* (cuts the EV at 4592nd and 5863rd bp) *and NcoI (Nocardia corallina)* (cuts the EV at 4893rd and 7532nd bp)

Here, the enzyme would cut the EV at 4 different sites, while it would cut the plasmids containing the insert at 2 sites. The results confirmed this: when cut samples were run on agarose gel, 4 different bands were observed in the EV, while 2 bands were observed in the plasmids containing the insert. The cut bands of the plasmids containing the insert were identical to each other (Figure 4.22.). We understand that these are the same inserts. This could be the reason of the multiple copy of the gene.

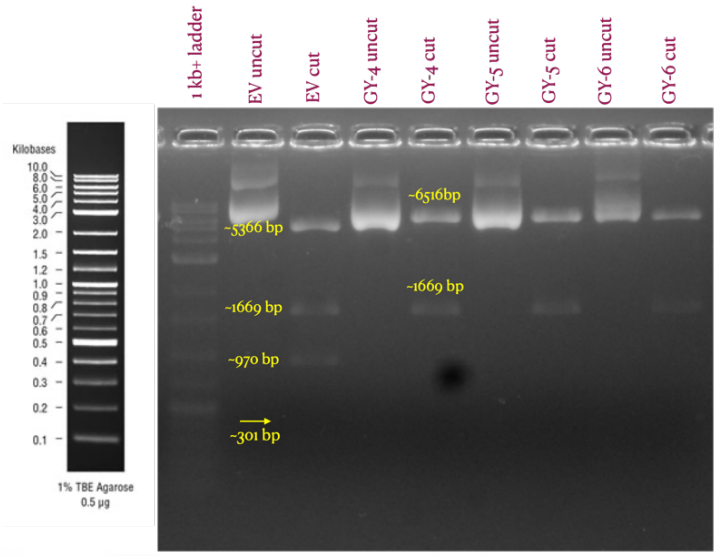


Fig 4.22. Restriction Digestion (*EcoRI and NcoI)* Result of the Tolerant Plasmids

4.7. Plant Material and Boron Application to Plants

The 200 and 400 mg/L B treatments provided the high boron stress application in the hydroponic experiment. Plants were harvested after 12 and 24 hours of boron application to plants in hydroponic system. No signs of B toxicity were observed in the form of necrotic spots along the leaf margins and leaf tips and/or roots. However, symptoms such as chlorosis in leaves and shortening of root length appeared within 24 hours, not significantly after 12 hours of high Boron application both in 200 and 400 mg/L B (Figure 4.23. and 4.24.).

Figure 4.23. After 12 hours of Boron Application (control, 200 and 400 mg/L B Respectively) to Plants in Hydroponic System

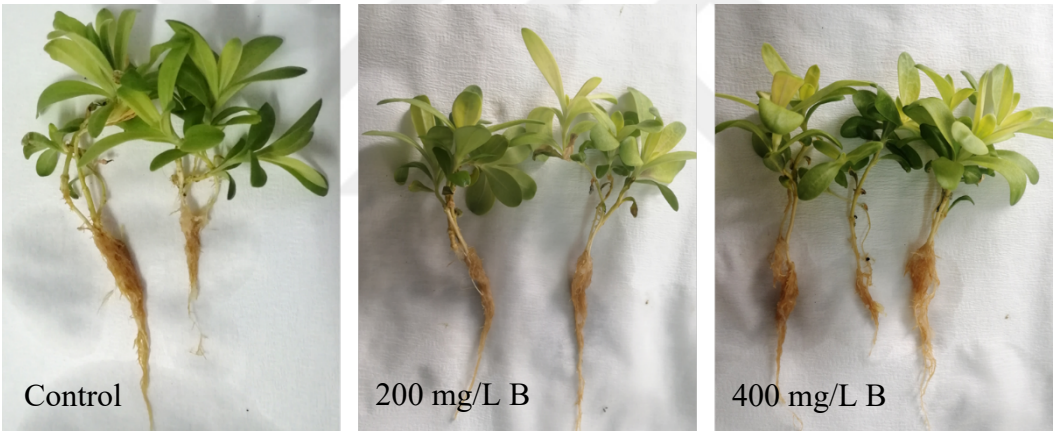


Figure 4.24. After 24 hours of Boron Application (control, 200 and 400 mg/L B Respectively) to Plants in Hydroponic System

4.8. qPCR Analysis of Determined Tolerance Gene

Melting-curve analysis confirmed the absence of multiple products and primer dimers. The expression levels of the analyzed genes were normalized via comparison to the expression of the internal reference gene which is Actin as the reference gene. The standard error was calculated from the standard deviation and the variation coefficient of

the reference gene and of the gene under assessment. For statistical analysis, we compared stress condition plants with its own control. Data represent means ± SD of 3 independent experiments performed in triplicate. Fold changes were calculated using 2−ΔΔCt method. CT values for individual variants were compared to CT values for a reference control for all treated samples to assess the fold change in gene expression. The results show single peak as can be seen in Figure 4.25. The blue curve shows the negative control (NTC). This peak was different from the others due to there is no amplicon in this sample.

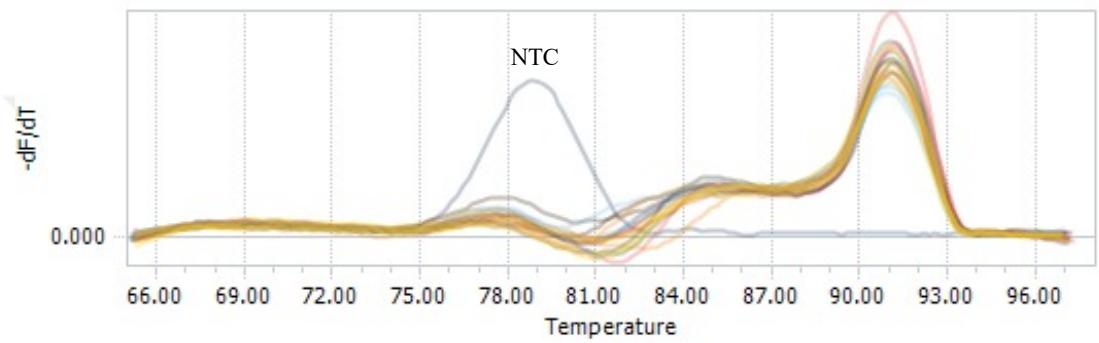


Figure 4.25. Melting Peaks of Amplified Gene After qPCR.

As can be seen in the graph below (Figure 4.26.), in the application of 200 mg/L boron, a 53% decrease in gene expression was observed in the first 12 hours, and a 60% decrease in the following 24 hours. But this situation was reversed when we applied 400 mg/L boron stress. In the first 12 hours, 2.5-fold increase was observed compared to the gene expression in 200 mg/L boron application, which is a serious increase. When compared to control plants, 19% increase in gene expression in the first 12 hours and 20% increase in the following 24 hours were observed. The result we deduced from this is that while gene expression decreases at lower concentrations such as 200 mg/L, a significant increase is observed in gene expression when the amount of applied stress increases. This increase in gene expression may provide boron tolerance in *G. perfoliata*.

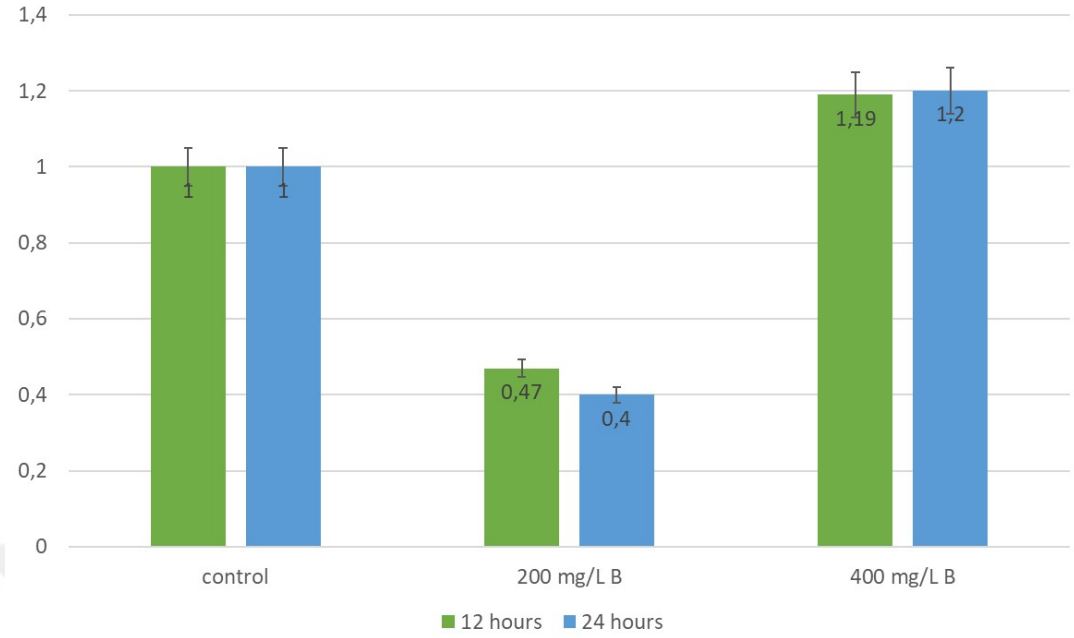


Figure 4.26. Expression analysis of *GpEEF1A* gene in leaves of G. *perfoliata*. Actin was used for normalization of gene expression. Error bars show standard deviation.

5. DISCUSSION

Boron stress limits plant growth and development, leading to productivity loss. It is very important to examine plants that have a high adaptability to stress conditions in the natural flora, are closely related to cultivated plants, or can be brought into agriculture due to their different plant characteristics such as phytamination. In this respect, it is necessary to examine plants resistant to boron toxicity and to understand the mechanism.

The Caryophyllaceae species, are thought to be bushes for their tolerance to drought (Üstüner et al., 2021), heavy metals (Muszyńska, E et al., 2018) and extreme temperature (de Luis M et al., 2019) and are considered to serve as a potential candidate for the boron-responsive genes. A few reports identified transcripts from *Gypsophila* genus (Unver T. et al., 2008; Padmanabhan, P et al., 2012; Tombuloglu, H).

Babaoglu et al. (2004) identified *Gypsophila sphaerocephala, Gypsophila perfoliata* L., *Elymus elongatus* and *Puccinellia distans* species growing in a boron mine region in Turkey and with high bora tolerance. In this study, it was determined that the mentioned species naturally grow and develop in soils with a toxic level of boron content (8900 mg kg-1). They reported that *G. sphaerocephala* not only grows in soils contaminated with excess boron, but can also accumulate pipes in unexpectedly high concentrations. In this way, it is thought that studies on *G. sphaerocephala* will enable the identification of the gene(s) providing boron tolerance.

Various studies have been carried out to elucidate the molecular mechanism of boron uptake, transport and excretion in plants. *AtBor1* is the first gene identified as a boron transporter in plants (Takano et al., 2002). Subsequently, six more genes (*AtBOR2-7*) that are paralogs of the *AtBor1* gene have been identified (Miwa et al, 2007). Boric acid transporters have also been identified in other plant species and organisms besides Arabidopsis. These; It appears as *HvBot1* in barley, *OsBOR1* in rice, *NaBC1* in humans, and *Atr1* and *Bor1* genes in *Saccharomyces cerevisiae* (Nakagawa et al., 2007; Frommer and von Wiren, 2002; Kaya et al., 2009; Takano et al., 2007). Nable R. et al. 1988 and Paull J. et al. 1988 defined boron-tolerant wheat and barley varieties as a result of their studies. Based on their studies, they reported that boron tolerance was associated with a lower amount of boron in plant tissues (Nable R. et al. 1988, Paull J. et al. 1988). In

addition, it was reported by Miwa K. (2007) that overexpression of the boron flux transporter in Arabidopsis roots increases boron tolerance. However, in order to understand the boron tolerance mechanisms, transcriptome and proteome-wide studies have been carried out (Tombuloglu et al., 2013,2015,2016), but not any of the tolerance genes have been identified yet.

Based on all these, *G. perfoliata* presents a new plant genotype to describe the boron hyperaccumulation mechanism. It is thought that studies on *G. perfoliata* will enable the identification of the gene(s) providing boron tolerance.

The main purpose of this thesis is to identify genes conferring boron resistance in *G. perfoliata* adapted to areas containing toxic levels of boron (8900 mg B/kg).

Yeast functional screening expressing heterologous cDNA libraries is an authoritative tool to identify genes, regardless of the regulation of their expression. Screening of yeast-expressing plant cDNAs has been used successfully to identify genes involved in enhanced stress tolerance.

In the present study, we cloned the cDNA library into a yeast expression system, followed by yeast *S. cerevisiae atr1Δ* transformation. Yeast expression system was proved to be a rapid and robust assay system for large-scale screening of genes for abiotic stress tolerance with attractive features like rapid growth in culture, eukaryotic expression and conserved signaling and regulatory pathways, and post-translational modification process with the advantage of facile genetic manipulation.

In this master thesis, cDNA libraries were screened in media containing toxic amounts of boric acid after they were transferred into the pAG426GPD-ccdb yeast expression vector by electroporation. The transformed yeasts were stressed with 80mM H3BO3. While GY yeast libraries showed possible tolerant colony formation at 80 mM boric acid containing YNB plate, GK yeast libraries did not show any colonies despite of the repeated attempts. The reason for that might be the identified gene does not express in the roots. After retransformation, the possible tolerant colony number decreased to 3 which may be because yeast cells cannot transmit the plasmids to the next generation after retransformation (Ding et al., 2003).

One of the isolated total 10 genes recapture the boron tolerance in re-transformed yeasts, correlated to the expression profiles demonstrated by qRT-PCR after 200- and 400-mM boron treatment in *G. perfoliata* for that gene. qRT-PCR data revealed that the tested gene responded to boron stress rapidly by downregulating in 200 mg/L B and upregulating in 400 mg/L B, (within 12 h) and subsequently possibly translated into corresponding proteins, functioning somewhat to maintain homeostasis.

However, how the boron tolerance-related genes take use of the host yeast signaling pathways to play functions leading to enhanced yeast cell survival rates upon boron treatment is still unknown. Taken together, these studies indicate that elongation factor 1 proteins might play secondary roles in abiotic stress tolerance in plants. However, the underlying functions and mechanisms of those proteins in plant abiotic stress tolerance remains to be determined.

EEF1A lysine methyltransferase-2-like gene belonging the homologous translational GTPase family protein were isolated in our work. Recent studies demonstrated that this multifunctional protein play crucial role in the development of abiotic stress tolerance in plants by creating a complex with guanosine triphosphate, they mediate the amino acylated tRNA to bind the free space of ribosome. EEF1A is a cytosolic multifunctional protein and plays central role in the elongation phase of translation. Besides its role in translation elongation phase, EEF1A plays crucial roles in many cellular processes. For example, EEF1A participates the degradation of N-terminal blocked proteins. It interacts with actin filaments and microtubules both in *in vitro* and *in vivo*. Thus, it regulates the organization of cellular skeleton and morphology. It has also been found that EEF1A binds to several kinases. Chaperone activity of elongation factors could be important to protect the plants under abiotic stress factors that cause to protein degradation. The roles of EEF1A in protecting the plants from abiotic stresses have been described in many studies. It is found that the early gene induction of EEF1A showed up as a response to water deficiency. Under osmotic and salinity stresses, EEF1A was significantly accumulated. By activating the heat shock transcription factor1, EEF1A helps the cell to respond to heat stress and regulates the heat shock protein expression.

Exposure to heat stress resulted in increased expression of cytosolic protein elongation factor EF-1a in mature spring wheat. Heat-induced accumulation of EF-1a has been previously reported in fish (Buckley et al. 2006). In rice, Li and Chen (1999) observed that heat shock (37°C) induced accumulation of EF-1a transcripts, but the level of EF-1a protein remained unknown. Chung, E. S., (2009) investigated EEF1A RNA expression level in soybean plants treated with abiotic stresses. EEF1A RNA level showed maximum after 6 h by low temperature stress and its RNA level was reduced after that. EEF1A RNA was strongly induced by high salt, ABA, or drought stress. After 24 h of drought stress, it seems that RNA got degraded to smaller sizes (Chung, E. S. Et al., 2009). There are evidences proving that EEF1A gene expression is induced by environmental stresses in plants (Dunn et al. 1993; Morelli et al. 1994; Berberich et al. 1995; Vayada et al. 1995). It was reported that increases in protein synthesis are accompanied by an accumulation of EEF1A protein and transcript and that the expression of EEF1A may serve as an indicator of translational stimulation (Morelli et al. 1994).

In this study, cDNA libraries were screened in media containing toxic amounts of boric acid after they were transferred into the pAG426GPD-ccdb yeast expression vector by electroporation. After the plasmids were obtained by retransformation to the possible tolerant yeast colonies obtained, they were sent to NGS and the expression of the identified gene under toxic level of boron stress was determined.

We screened 2 yeast cDNA libraries which are GY and GK to identify genes that confer boron tolerance. Transformed cells were exposed to toxic amount of boric acid. While GY yeast libraries showed possible tolerant colony formation at 80 mM boric acid containing YNB plate, GK yeast libraries did not show any colonies despite of the repeated attempts. The reason for that might be the identified gene does not express in the roots. 10 GY colonies could grow in the presence of 80 mM boric acid. After retransformation, the possible tolerant colony number decreased to 3 which may be because yeast cells cannot transmit the plasmids to the next generation after retransformation (Ding et al., 2003).

Babaoglu et al. (2004) identified *Gypsophila sphaerocephala*, *Gypsophila perfoliata* L., *Elymus elongatus* and *Puccinellia distans* species growing in a boron mine region in

Turkey and with high bora tolerance. In this study, it was determined that the mentioned species naturally grow and develop in soils with a toxic level of boron content (8900 mg kg-1). They reported that *G. sphaerocephala* not only grows in soils contaminated with excess boron, but can also accumulate pipes in unexpectedly high concentrations. In this way, it is thought that studies on *G. sphaerocephala* will enable the identification of the gene(s) providing boron tolerance.

Various studies have been carried out to elucidate the molecular mechanism of boron uptake, transport and excretion in plants. *AtBor1* is the first gene identified as a boron transporter in plants (Takano et al., 2002). Subsequently, six more genes (*AtBOR2-7*) that are paralogs of the AtBor1 gene have been identified (Miwa et al, 2007). Boric acid transporters have also been identified in other plant species and organisms besides Arabidopsis. These; It appears as *HvBot1* in barley, *OsBOR1* in rice, *NaBC1* in humans, and *Atr1* and *Bor1p* genes in *Saccharomyces cerevisiae* (Nakagawa et al., 2007; Frommer and von Wiren, 2002; Kaya et al., 2009; Takano et al., 2007). Nable R. et al. 1988 and Paull J. et al. 1988 defined boron-tolerant wheat and barley varieties as a result of their studies. Based on their studies, they reported that boron tolerance was associated with a lower amount of boron in plant tissues (Nable R. et al. 1988, Paull J. et al. 1988). In addition, it was reported by Miwa K. (2007) that overexpression of the boron flux transporter in Arabidopsis roots increases boron tolerance. However, in order to understand the boron tolerance mechanisms, transcriptome and proteome-wide studies have been carried out (Tombuloglu et al., 2013,2015,2016), but not any of the tolerance genes have been identified yet.

Studies identified three pathways for the transmembrane transport mechanism of boron in tolerant plants. First mechanism is thought to be restricted boron uptake or boron leakage from plant root cells to the environment. This causes limited accumulation of boron in plant root tissues. The second hypothesis is restricted boron transport from roots to shoots. The third one is assumed that to be increased tissue tolerance against high boron concentrations (Sutton T. 2007). The identified gene is thought to be included in the third of the hypotheses. In subcellular studies, it was found that the EEF1A protein is located in the cytoplasm (Wang, S. et al., 2017). According to this study, since the EEF1A is not found in the cell membrane like exporter/efflux proteins, it is thought that it cannot be

responsible for the uptake or excretion of boron into the cell. EEF1A in the cytoplasm may help translate vital proteins as a result of stress when toxic amounts of boron are taken into the cell.

Based on all these, *G. perfoliata* presents a new plant genotype to describe the boron hyperaccumulation mechanism. It is thought that studies on *G. perfoliata* will enable the identification of the gene(s) providing boron tolerance.

6. CONCLUSION

In this study, we transformed two cDNA libraries (GY and GK) were transferred to *atr1Δ* cells. Then both of the yeast libraries were screened with 80 mM boric acid. Putative tolerant colonies were obtained on media containing 80 mM boric acid from the transformation of the *atr1Δ* GY cDNA library. Then we performed spot test and serial dilution assay to be sure the tolerance of the colonies to toxic amount of boric acid. After these tests, we transformed the plasmids to *E. coli* DH5α cells. After confirming the plasmids with PCR and restriction enzyme digestion, one out of three colonies was randomly selected and and sent for new generation sequencing for confirmation. After NGS results reach to us, we analyzed the sequence in NCBI program by blasting. According to the blast results, the sequence is showing 80% similarity with *Chenopodium quinoa* EEF1A lysine methyltransferase-2-like gene. Besides, the determined gene is showing less similarity with the mentioned genes of *Spinacia oleracea, Benincasa hispida, Medicago trunculata, Abrus precatorius*, respectively. After BLAST analysis, qPCR primers were designed to see the expression level of the determined gene in *G. perfoliata* under boron stress conditions. For this purpose, the plants were grown in hydroponic conditions, and the increasing boron stress (200 mg/L and mg/L B) were given to the plants for 12 and 24 hours. After harvesting the plants, RNA samples were isolated and subjected to qPCR with designed primers. The results shown that the expression of the determined gene is increasing with the increased amount of boric acid.

Although the gene we identified confers slightly tolerance to a toxic amount of boron, this gene does not provide any tolerance at increasing concentrations in yeast cells. However, the gene can provide boron tolerance in plants. It is thought that this may be due to the fact that this gene is not involved in the expression of any protein involved in boron uptake or exportation.

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