MICROARRAY BASED EXPRESSION PROFILING OF BARLEY UNDER BORON STRESS AND CLONING OF 3H BORON TOLERANCE GENE

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

MICROARRAY BASED EXPRESSION PROFILING OF BARLEY UNDER BORON STRESS AND CLONING OF 3H BORON TOLERANCE GENE

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Both deficiency and toxicity of the essential micronutrient boron (B) lead to reduced crop yield in agriculture. However, our understanding of the molecular responses of plants under B stresses to tackle the yield loss is limited. Therefore, in the present study, transcriptional alterations in sensitive and tolerant barley cultivars under B deficiency and toxicity were investigated in order to reveal the molecular responses. Transcriptomes were monitored at seedling stage by global expression profiling using oligonucleotide microarrays.

In the context of the study, we have determined that response to B toxicity in barley involved jasmonic acid and various components of biotic stress responses. Examination of expression profiles indicated that B toxicity and deficiency resulted in significant global changes in the transcriptomes of leaf and root tissues, respectively. Inter-varietal comparison of sensitive and tolerant genotypes of barley revealed that a combinatorial effect of transcription factors on regulation could alter the gene expression patterns in tolerant cultivar and provide B toxicity tolerance. Furthermore, mechanisms of vacuolar sorting or efflux by transporters and aquaporins might be contributing to the tolerance to B stresses in barley according to the results of this study.

Additionally, we have identified and cloned the *HvBor1a* gene encoding a putative B transporter in barley using candidate gene approach and functionally characterized its roles in the tolerance to B stresses. The full length coding sequence and also the non-coding regions of the gene were identified. It was demonstrated that the protein product of *HvBor1a* was localized to the plasma membrane and it displayed B transporter activity. High transcript abundances in leaf tissues of barley suggested a role for HvBor1a in re-distribution of B within the plant tissues. Interestingly, examination of last intron of *HvBor1a* has led to the identification of an alternatively spliced variant in certain cultivars of barley. Furthermore, interval mapping and positional cloning was performed to locate the *HvBor1a* on 3H B tolerance QTL and a novel CAPS marker was developed to narrow the genetic distances at the locus.

As a conclusion, this work presents, for the first time, the transcriptome profiling of a member of *Triticeae* under B toxicity and deficiency. The data generated should enlighten succeeding studies to unravel molecular mechanisms and signaling networks of tolerance to B stresses especially in crops like barley and wheat. The results of the study will provide novel tools and genes for conventional and biotechnological approaches for the reduction of yield loss due to B toxicity or deficiency.

Keywords: Barley, Boron, Toxicity, Deficiency, Microarray, Boron transporter, Tolerance, Cloning, Mapping.

ÖZ

BOR STRESİ ALTINDA ARPA GEN İFADE PROFİLLERİNİN MİKRODİZİ ANALİZİ İLE BELİRLENMESİ VE 3H BOR TOLERANS GENİNİN KLONLANMASI

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Temel bir mikro besin olan bor (B) elementinin hem eksikliği hem de toksisitesi tarımda düşük ürün verimine neden olmaktadır. Bununla beraber bitkilerin, B stresleri altında verdikleri moleküler yanıtlar ile ilgili, ürün kaybının üstesinden gelmek için gerekli bilgimiz sınırlıdır. Dolayısıyla bu çalışmada, hassas ve dayanıklı arpa çeşitlerinin B eksikliği ve toksisitesi altında gösterdikleri transkripsiyonel değişimler, moleküler cevapları ortaya koymak amacıyla incelenmiştir. Fide aşamasında transkriptomlar, oligonükleotid mikrodiziler kullanılarak, global gen ifade profillerinin belirlenmesi ile izlenmiştir.

Çalışma kapsamında, arpada B toksisitesine yanıtın, jasmonik asit ve biyotik strese cevabın çeşitli unsurlarını içerdiği belirlenmiştir. Gen ifade profillerinin incelenmesi, B toksisite ve eksikliğinin sırasıyla yaprak ve kök dokusu transkriptomlarında önemli global değişikliklere neden olduğunu göstermiştir. Arpaya ait hassas ve dayanıklı genotiplerin çeşitler arası karşılaştırılması, transkripsiyon faktörlerinin regülasyon üstüne olan bütünleşik etkisinin, dayanıklı çeşitte gen ifade desenlerini değiştirebildiğini ve B toksisitesine toleransı sağlayabildiğini ortaya koymuştur. Ayrıca bu çalışmanın sonuçlarına göre kofula yönlendirme veya taşıyıcılar ve aquaporinler ile dışarı atım mekanizmaları arpada B streslerine toleransa katkı sağlıyor olabilir.

Bunlara ek olarak, arpada B taşıyıcı olduğu varsayılan bir proteini kodlayan *HvBor1a* geni belirlenmiş, aday gen yaklaşımı kullanılarak klonlanmış ve genin B streslerine toleranstaki rolleri işlevsel olarak karakterize edilmiştir. Genin tüm kodlayan dizisi ve kodlamayan bölgeleri belirlenmiştir. *HvBor1a* genine ait protein ürününün plazma zarına yerleştiği ve B taşıyıcı aktiviteye sahip olduğu gösterilmiştir. Arpa yaprak dokularındaki yüksek transkript seviyeleri, bitki dokularındaki B elementi dağılımında HvBor1a proteininin bir rolü olduğunu önermiştir. Ayrıca *HvBor1a* genine ait son intronun incelenmesi, arpanın belirli genotiplerinde alternatif olarak intronları çıkarılan bir varyantın tanımlanmasını sağlamıştır. Bunların yanısıra *HvBor1a* geninin 3H B tolerans QTL üzerinde konumlandırılmasına yönelik olarak haritalama ve konumsal klonlama gerçekleştirilmiş ve lokustaki genetik uzaklıkların daraltılması için yeni bir CAPS markörü geliştirilmiştir.

Sonuç olarak, B toksisitesi ve eksikliği altında bir *Triticeae* üyesinin transkriptomlarının belirlenmesi ilk kez bu çalışmada sunulmaktadır. Bulunan veriler, özellikle arpa ve buğday gibi ekinlerde B streslerine toleransın moleküler mekanizmalarını ve sinyal ağlarını çözmek üzere yapılacak çalışmaları aydınlatacaktır. Çalışmanın sonuçları B toksisitesi ve eksikliğinden kaynaklanan verim kayıplarını azaltmaya yönelik geleneksel ve biyoteknolojik yaklaşımlar için yeni araç ve genler sağlayacaktır.

Anahtar kelimeler: Arpa, Bor, Toksisite, Eksiklik, Mikrodizi, Bor taşıyıcı, Tolerans, Klonlama, Haritalama.

To my mom, my sister Meltem, and my lovely niece İrem

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ABC	ATP-binding cassette
Acetosyringone	3',5'-Dimethoxy-4'-hydroxyacetophenone
ANOVA	Analysis of variance
aRNA	Amplified RNA
B	Boron
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
C	Control
CAPS	Cleaved amplified polymorphic sequence
cDNA	Complementary DNA
CDS	Coding sequence
C_T	Threshold cycle
CTAB	Cetyltrimethyl-ammonium bromide
cv	Cultivated variety
D	Deficiency
DEPC	Diethylpyrocarbonate
DH	Doubled haploid
DMSO	Dimethyl sulfoxide
e	Exon
EDTA	Ethylenediamine tetra acetic acid
EST	Expressed sequence tag
FC	Fold change
GFP	Green fluorescent protein
GOI	Gene of interest
GW	Genome walking
GW-MDA	Genome walking using multiple displacement amplification
GW-RED	Genome walking using restriction enzyme digestion

H_3BO_3	Boric acid
i	Intron
JA	Jasmonic acid
LOD	Logarithm of odds
MES	2-(*N*-morpholino)ethanesulfonic acid
MIAME	Minimum information about a microarray experiment
NIP	NOD26-like intrinsic protein
NTC	No-template control
OD	Optical density
ORF	Open reading frame
PCA	Principle component analysis
PCR	Polymerase chain reaction
QTL	Quantitative trait locus
RACE	Rapid amplification of cDNA ends
RG-II	Rhamnogalacturonan II
RMA	Robust multiarray analysis
RRL	Relative root length
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SSC	Saline sodium citrate
ssp	Subspecies
SSR	Simple sequence repeat
T	Toxicity
TAE	Tris-Acetic acid-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TF	Transcription factor
Tris	Tris(hydroxymethyl)aminomethane
UTR	Untranslated region

CHAPTER 1

INTRODUCTION

1.1. Barley

Cultivated barley (*Hordeum vulgare* ssp. *vulgare* L.), a major cereal grain, is a member of the Poaceae family. Domesticated about 10,000 years ago in the Fertile Crescent, barley is one of the eight founder crops of agriculture (Abbo *et al.*, 2010). The wild ancestor of domesticated barley, *H. vulgare* ssp. *spontaneum*, is still common in grasslands and woodlands throughout the aforementioned region. Barley is a monocotyledonous, self-pollinating, and diploid species with 14 chromosomes. Barley is probably one of the most genetically diverse cereal grains. It is classified as spring or winter types, two-rowed or six-rowed, and hulled or hulless. Barley is used as major animal fodder, as a component of various foods and bread, and as malt for production of beer and certain distilled beverages like whiskey (Baik and Ullrich, 2008).

1.1.1. The Barley Crop

As a member of grasses, barley plant has erect and stout stems with 60 – 110 cm height. Alternating leaves might be up to 25 – 30 cm long. Barley inflorescence is composed of triplets of spikelets, one central and two lateral. Triplets are arranged alternately at rachis nodes forming a spike. All three spikelets of the six-rowed barley cultivars are fully fertile and able to develop into grains, but the lateral spikelets of two-rowed barley are sterile and reduced in size. Recently, positional cloning has been used to isolate *Vrs1* (*HvHox1*) which encodes a homeodomain-leucine zipper transcription factor and is mapped to 2HL.

The recessive loss-of-function mutation of the gene is responsible for the transition from two to six rows of spikelets in barley (Komatsuda *et al.*, 2007). All cultivated barleys have non-brittle spikes whereas wild ancestors have brittle spikes that facilitate seed dispersal. In wild barley, the brittle rachis is specified by the two complementary dominant genes *Btr1* and *Btr2*, which are tightly linked to each other and mapped to 3HS (Komatsuda and Mano 2002). However, in cultivated barley, one or the other of these genes has been lost by mutation during domestication (Sakuma *et al.*, 2011).

Barleys of different classes often differ widely in physical and compositional characteristics. Based on grain composition, barley is further classified as normal, waxy or high amylose types, high lysine, high β-glucan, and proanthocyanidin-free. Barley grain is clean, bright yellow-white, plump, thin hulled, medium hard and uniform in size. The grain is comprised of the caryopsis and the enclosing hull. The caryopsis consists of the pericarp, integuments, aleurone layer, endosperm, and embryo (Jadhav *et al.*, 1998). Chemical composition of barley grain includes 60 – 64% starch, 4.4 – 7.8% arabinoxylans, 3.6 – 6.1% β-glucans, 1.4 – 5.1% cellulose, 0.6 – 4.6% simple carbohydrates, 8 – 15% proteins, and 2 – 3% lipids in dry weight (MacGregor, 1993).

Barley ranks fourth among the cereals after wheat, maize, and rice in worldwide production. It is grown for many purposes, but the majority is used for livestock feeding, malting or human consumption. High protein barleys are generally valued for food and feeding, and starchy barley for malting. Nowadays, the use of barley in human foods is very limited. Although small quantities are used in breakfast cereals, soups, bakery blends, and for baby foods, efforts are being made in order to increase utilization of barley grain for human foods (Jadhav *et al.*, 1998).

Besides agronomic and economic importance, barley is considered as a model species for genetic and physiological research on *Triticeae* (Koornneef *et al.*, 1997) owing to its wide diversity in genetics, morphology, and physiology and comparably less complex diploid genome. A great body of information and resources for barley genetics and genomics has been developed over the last decade. These include expressed sequence tag (EST) datasets, bacterial artificial chromosome (BAC) libraries, genetic and transcript maps, single nucleotide polymorphism (SNP) databases, a GeneChip® Barley Genome Array, publicly

available substantial amount of microarray data and many more. Though genome of barley has not been sequenced yet, International Barley Sequencing Consortium (IBSC) consisting of member laboratories in Australia, Japan, Finland, Germany, UK and US is currently working on sequencing and physically mapping of barley genome. The estimates indicate the number of unique genes to be approximately 50,000 for barley, based on assemblies of about 580,000 and 370,000 ESTs from wheat and barley, respectively. Large sets of several thousands of EST-based unigenes have been mapped to genetic and physical maps in barley (Nasuda *et al.*, 2005; Cho *et al.*, 2006). Based on the size of barley genome (~5,300 Mb) and proposed number of genes, physical distances between randomly distributed genes would be around 100 – 160 kb (Stein, 2007).

1.1.2. Barley Production and Factors Affecting the Yield

Barley is one of the most widely adapted cereal crops. Genetic variation has made it possible to be cultivated in diverse environments ranging from the sub-arctic to the sub-tropic. It is grown in a range of extreme environments that vary from northern Scandinavia to the Himalayan Mountains to monsoon paddies. Its annual world production in 2009 is approximately 150 million metric tons (MT). Russian Federation is the top barley producer in the world. France and Germany are the leading European producers and Turkey is the seventh country globally with 7.3 million MT of production (Table 1.1; FAOSTAT, 2009).

Table 1.1. Top 10 barley producing countries. The countries are ranked according to production in metric tons (MT). (Source: FAOSTAT, 2009).

Rank	Country	Production (MT)	Production (International $1000)
1	Russian Federation	17,880,800	680,672
2	France	12,875,800	1,115,551
3	Germany	12,288,100	694,628
4	Ukraine	11,833,100	789,219
5	Canada	9,517,200	182,627
6	Australia	8,098,000	657,851
7	Turkey	7,300,000	126,507
8	United Kingdom	6,769,000	369,915
9	United States of America	4,949,370	450,126
10	Poland	3,983,900	108,737

Barley has a wide geographic and ecological range compared to any other cereal because it is probably the most adaptable among grasses. Though it can tolerate many diverse environments and show tolerance to cold, drought and salinity, there are factors especially viral, bacterial or fungal diseases affecting the yield of barley. Serious diseases of barley include powdery mildew, caused by *Blumeria graminis* f.sp. *hordei*, leaf scald, caused by *Rhynchosporium secalis*, barley rust, caused by *Puccinia hordei*, head blight caused by certain species of *Fusarium*, and various diseases caused by *Cochliobolus sativus*. Barley plant is susceptible to bacterial blight caused by the bacterial pathogen *Xanthomonas campestris* pv. *translucens*. Barley is also susceptible to diseases caused by certain plant viruses like barley mild mosaic bymovirus (BaMMV) and barley stripe mosaic hordeivirus (BSMV). Some causative agents of barley diseases not only decrease yield but also reduce grain quality and contaminate it with mycotoxins.

Tolerance of barley to abiotic stresses including boron (B) deficiency and toxicity is dependent on many factors including developmental stage, severity of stress and the variety being considered. Barley is extremely vulnerable to abiotic stresses such as cold and drought during specific developmental stages like early reproductive development and the young microspore stage of pollen development (Dolferus *et al.*, 2011). Among cereals barley is considered the most tolerant against salinity (Munns and Tester, 2008). On the other hand salinity tolerance during germination, vegetative growth and reproductive stage might differ considerably (Yamaguchi and Blumwald, 2005).

Barley is regarded as moderately tolerant to B toxicity (Mengel and Kirkby, 2001). However, a large variation in tolerance has been reported among cultivated barley in Australia and Turkey (Cartwright *et al.*, 1984; Torun *et al.*, 2003). A substantial variation in tolerance based on evaluation of traits such as severity of toxicity symptoms on leaves, levels of shoot dry matter production, and grain yield was reported among ten cultivated barley genotypes from Turkey. According to differences in these traits Hamidiye and Bülbül were identified as the most sensitive, and Anadolu and Tarm-92 as the most tolerant cultivars. On the other hand, Tokak, Cumhuriyet, Erginel, Obruk, Yesevi, and Yea-1868 were classified as moderately tolerant (Torun *et al.*, 2003).

Mineral and nutrient stresses caused by excess amounts of aluminum, manganese, copper, and B impose large losses on crop yield. Approximately a quarter of agricultural land worldwide is suffering from some form of mineral stress (Clark, 1982). Majority of these stresses are not easily reversible by conventional agricultural practices. Though unfeasible some amelioration methods proposed for reclaiming high B-containing soils include leaching and liming (Nable *et al.*, 1997). Alternative approaches including vegetation and water management, and phytoremediation have been suggested to remove excess B from agricultural land (Banuelos *et al.*, 1995).

Genes and quantitative trait loci (QTLs) related with tolerance to abiotic stresses have been identified in wild progenitor species of wheat and barley as well as cultivated varieties of the *Triticeae* (Cattivelli *et al.*, 2002; Nevo and Chen, 2010). Advances in genetics, understanding of molecular basis of stress responses, and determination of genes and QTLs related to stress tolerance have increased the possibility for breeding B-tolerant plant cultivars or engineering crops for B tolerance (Cattivelli *et al.*, 2002; Nable *et al.*, 1997).

1.2. Boron as an Essential Micronutrient

Most of soil B is found combined in borax, colemanite, ulexite, tourmaline, and borate minerals and is inaccessible to plants. The available and water soluble boric acid (H_3BO_3) which behaves as a weak monobasic acid (pKa 9.24) is un-dissociated under most soil pH conditions. Thus, B is unique among all other essential nutrients which are present in soil solution in ionized form.

Naturally occurring compounds of B, the borate minerals, are mined industrially. Borax is used in various household laundry and cleaning products. Polymers of B play specialized roles as high-strength lightweight structural and refractory materials. The repeating polymer and semi-crystalline structure of B carbide gives it great structural strength. It is used in tank armor, bulletproof vests, airplane coating, nuclear power plant shielding, and various other structural applications. Boron compounds are used in silica-based glasses and ceramics to give them resistance to thermal shock. Several B compounds are known for their extreme hardness and toughness. Implantation of B ions into metals and alloys results in a spectacular increase in surface resistance and hardness. These borides are an alternative to

diamond coated tools. Sodium borates are fire retarding additives to plastics and rubber. Boron nitride forms nanotubular structures with high strength, high chemical stability, and high thermal conductivity.

Boron can easily be adsorbed on surfaces of soil constituents such as aluminum and iron oxides, organic matter, calcium carbonate, magnesium hydroxide, and clay minerals. On the other hand, B can easily be leached from soil since H_3BO_3 is found un-dissociated in soil solution. Therefore B can accumulate to toxic concentrations in the upper soil layer in arid and semi arid regions (Nable *et al.*, 1997). Addition of B via irrigation water, as well as lack of drainage, results in excessive concentrations of B in agricultural soil (Goldberg, 1997). Physical factors such as pH, soil texture and moisture, temperature, and clay mineralogy affect availability of B to plants (Goldberg, 1997; Mengel and Kirkby, 2001). Increasing soil solution pH, in the range of 4 – 9, increases adsorption which in turn decreases availability of B. Similarly increasing clay content increases B adsorption (Goldberg, 1997). These factors are generally not independent of each other during partitioning of B between soil solution and the surfaces of soil constituents. There are interactive effects of soil temperature, moisture, pH and soil content on adsorption and availability of B.

Although B is essential for plant growth and development, the range between levels of toxicity and deficiency is narrow compared to the range for any other nutrients. Moreover, B requirements of plants vary tremendously among species and among genotypes within a species. Graminaceous monocots such as barley and wheat, with the lowest demand, require 4 – 10 µg B /g dry weight whereas dicots require 20 – 55 µg B /g dry weight (Hu *et al.*, 1996; Mengel and Kirkby, 2001). It has been suggested that variability in B requirements of most species is correlated with amount of cell wall pectin (Hu *et al.*, 1996) or amounts of sugars and polyphenols (Mengel and Kirkby, 2001).

Responses to B deficiency or positive responses to B application in field have been used to determine the B requirements of crops (Shorrocks, 1997). Some dicotyledenous members of the Brassicaceae such as cabbage, turnips and cauliflower are highly sensitive to B deficiency and most responsive to B application. Some other crops sensitive to B deficiency include celery, coffee, cotton, sunflower, olive, and grapes. Some legumes such as alfalfa, peas and beans also have a high B requirement (Mengel and Kirkby, 2001). In most plant species the B

requirement for reproductive growth is much higher than for vegetative growth. Impairment of fruit formation in grapes, sunflower, and apples has been reported (Shorrocks, 1997) and the critical role of B for pollen tube growth, pollen germination, and pollen viability has been reviewed (Dugger, 1983; Blevins and Lukaszewski, 1998).

1.2.1. Chemistry and Complexes of B

Being a member of metalloid family of elements, B shows properties of metals and electronegative non-metals. Like carbon (C), B has a tendency to form stable covalent bonds. It tends to form anionic complexes with covalent bonds due to its high ionization potential (Power and Woods, 1997). In the context of biology, B forms complexes with a large variety of compounds containing hydroxyl groups and the chemistry of B is dominated by B-oxygen (O) compounds. Naturally occurring B is found exclusively bound to O as borates, less often as H_3BO_3. Under physiological pH, and in the absence of interaction with biological molecules, B exists mainly as H_3BO_3. Since H_3BO_3 is a weak acid (pKa 9.24), at the pH found in cytoplasm (pH 7.5) approximately 98% of B exists in the form of free H_3BO_3, and less than 2% as borate anion, $H_4BO_4^-$ (Woods, 1996). At pH values found in apoplast (pH 5.5), greater than 99.95% of B exists as H_3BO_3, and less than 0.05% as $H_4BO_4^-$ (Equation 1.1).

$$H_3BO_3 + H_2O \rightleftharpoons H_4BO_4^- + H^+; \quad (Ka = 5.8 \times 10^{-10} \text{ mol/L; pKa} = 9.24) \qquad (1.1)$$

Both H_3BO_3 and $H_4BO_4^-$ can readily react with various kinds of biological molecules. Boric acid forms borate esters with a wide variety of organic compounds. It is well known that H_3BO_3 and $H_4BO_4^-$ can form cyclic borate esters with polyols such as glycerol or mannitol. Formation of cyclic borate esters is shown schematically in Figure 1.1. In general, the most stable borate esters are formed when B reacts with compounds that have *cis*-diols on a furanoid ring. Ribose and apiose among the biological compounds found in plants have the aforementioned configuration (Loomis and Durst, 1992). The stability of *cis*-diol borate complexes depend on several factors such as pH and presence of cations (Kobayashi *et al.*, 1997). The capacity of H_3BO_3 and $H_4BO_4^-$ to react with hydroxyl groups of various compounds is considered the key for understanding functional roles of B in plants (Bolanos *et al.*, 2004).

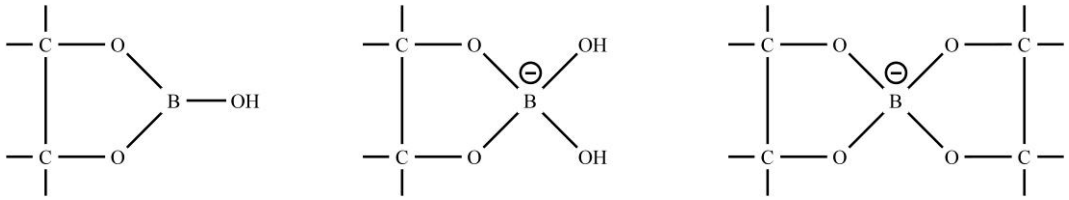

Figure 1.1. Schematic representation of cyclic borate esters. Displayed left to right are neutral *cis*-diol monoborate ester, anionic monoborate complex, and anionic bis(diol) borate complex. (Adapted from Power and Woods, 1997)

1.2.2. Biology and Functional Roles of B in Plants

It is over 80 years since B was shown to be an essential micronutrient for vascular plants (Warington, 1923; Sommer and Lipman, 1926). However the role of B in plant nutrition is still least well understood of all the nutrients. Assessing the primary role of B in plants is difficult since proposed roles were determined according to rapid and diverse symptoms that occurred upon withholding B or re-supplying it after deficiency.

The suggested functions of B in plant growth and development are long lists including sugar transport, carbohydrate metabolism, cell wall synthesis and structural maintenance, membrane structural maintenance, lignification, respiration, RNA metabolism, phenol metabolism, nitrogen fixation, and ascorbate metabolism (Parr and Loughman, 1983). Some of these postulated roles were suggested to arise primarily not because of lack of B but because of secondary effects. Therefore timing and nature of the symptom developed after deficiency is critical to assign primary functions to B in plant cells. Moreover, most of the proposed roles can be explained by the confirmed role of B as a component of cell wall pectin (O'Neill *et al.*, 2004).

Functional role of B in cell wall organization has been demonstrated with isolation and characterization of a B-polysaccharide complex from radish root cell walls (Matoh *et al.*, 1993). Analysis of glycosyl composition and linkage revealed that polysaccharide component of the complex is rhamnogalacturonan II (RG-II) which exists as a dimer cross-linked by a borate diol ester (Kobayashi *et al.*, 1996). Moreover, RG-II was isolated from suspension-

cultured sycamore cells and etiolated pea shoots and it was shown that covalently cross-linked RG-II is required for plant growth and development (O'Neill *et al.*, 1996; 2001). RG-II is a complex polysaccharide of the pectic fraction of cell walls and contains apiose which is responsible for covalent binding of B to the polysaccharide chains (Figure 1.2a). Since borate atom is chiral, two diasteroisomers can form (Figure 1.2b). However, it is not known which of the isomers is found naturally in plant cell wall.

Another compound containing *cis*-diols on a furanoid ring is ribose which is abundantly present in various biological molecules such as ribonucleotides, adenosine 5'-triphosphate (ATP), and nicotinamide adenosine dinucleotide (NAD^+). It was shown that ribose moiety of nucleotides bind to B (Landers *et al.*, 1992). NAD^+ has a high B binding affinity (K_d = 14.4 mM) among ribose containing biomolecules (Hunt, 2002). According to *in vitro* analysis of kinetics of some metabolic enzymes such as malate dehydrogenase with B binding substrates, it was concluded that B has the ability to disrupt metabolism by forming complexes with NAD^+ (Reid *et al.*, 2004).

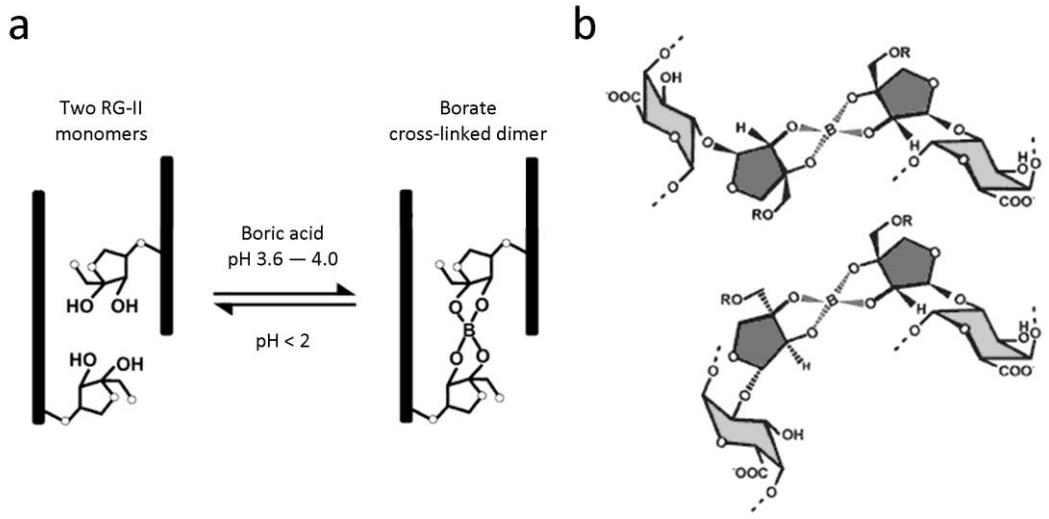

Figure 1.2. Schematic representation of rhamnogalacturonan II (RG-II) cross-linked by borate diol ester. Formation of the borate ester cross-link between apiose of each monomer (a) and configuration of two diasteroisomers of RG-II dimer formed (b) are displayed.

Structurally similar to NAD^+, diadenosine phosphates (Ap_nA) have two adenosine thus, two ribose moieties that can bind B (Barnes *et al.*, 1985). Moreover couple of B binding biomolecules structurally resembling each other such as NAD^+, Ap_nA, and S-adenosylmethionine (SAM) was shown to bind B *in vitro* using capillary electrophoresis (Ralston and Hunt, 2001). Additionally formation of stable complexes of pentoses with B has been suggested (Ricardo *et al.*, 2004).

Biological molecules other than apiose and ribose that react strongly with B include sorbitol and other sugar alcohols, phenols, and amino acids such as serine (Brown *et al.*, 2002). In celery and peach, the isolated soluble B-polyol complexes have been characterized using mass spectrometry (Hu *et al.*, 1997). Moreover, it was proposed that B mobility in phloem in certain higher plants is achieved by means of these soluble B complexes. Functional significance of putative complexes of B other than RG-II has not been determined. Though demonstrated to form *in vitro*, complexes of B with biological molecules such as sugar alcohols or phenols containing *cis*-diols support the possibility of B possessing functional roles in regulatory or signaling processes. Another evidence for possible involvement of B complexes in signaling in plants comes from the discovery of the autoinducer-2 (AI-2) a B-containing signal molecule that induces quorum sensing in marine bacterium *Vibrio harveyi* by interacting with a specific membrane receptor (Chen *et al.*, 2002).

A particular role for B on maintenance of integrity of plasma membrane has been suggested (Cakmak and Römheld, 1997) depending on alterations in ion fluxes in root tips of maize (Pollard *et al.*, 1977) and sunflower (Schon *et al.*, 1990) after B deficiency or B re-supply. Moreover, lower activities of plasma membrane bound H^+ pumping ATPases were demonstrated in B deficient cells or roots (Pollard *et al.*, 1977; Blaser-Grill *et al.*, 1989). Restoration of ATPase activity after B re-supply was indicated by hyperpolarization of membrane potential and a net excretion of protons. Therefore effects of B on ion fluxes are probably mediated directly or indirectly by membrane bound ATPases. It was suggested that the effects of B are primarily on plasma membrane rather than direct effects on ATPase activity (Cakmak and Römheld, 1997). Possible binding of B to hydroxyl groups of glycoproteins and glycolipids in plasma membranes has been proposed as the major reason for stimulatory effects of B on membrane bound ATPases and for alteration of membrane permeability. It was suggested that B complexes of glycoproteins and glycolipids provide

stabilization of membrane structure which in turn keeps enzymes and channels within membranes at optimum conformation (Cakmak *et al.*, 1995; Cakmak and Römheld, 1997; Brown *et al.*, 2002; Goldbach and Wimmer, 2007).

A structural function for B in the cytoskeleton have been proposed depending on the observation that boronic acids, which compete with H_3BO_3 for binding to *cis*-diols, caused disruption of cytoplasmic strands in cultured tobacco cells (Bassil *et al.*, 2004). Moreover, putative B-binding proteins from root microsomes of *Arabidopsis thaliana* and maize have been isolated using phenylboronate affinity chromatography (Wimmer *et al.*, 2009). Molecular mechanisms or significance underlying these functional roles of B remain to be unraveled.

Most of the postulated functional roles of B in plants can be explained by structural roles of B in organization of cell wall and membrane. On the other hand, effects of B on plant reproduction (Dell and Huang, 1997), premature flower and fruit drop in tree crops (Hanson *et al.*, 1985), abortion of flower initials in cauliflower (Gauch and Dugger, 1954), male sterility in wheat (Rawson, 1996; Dell and Huang, 1997), impairment of male gametogenesis in cereals (Rerkasem and Loneragan, 1994), and pistil sterility in maize (Agarwala *et al.*, 1981) under B deficient conditions cannot be explained solely by structural roles of B in cell wall. Moreover, evidence for stimulatory effects of B on yeast growth (Bennett *et al.*, 1999), requirement of B in embryonic development of various organisms such as zebrafish (Rowe and Eckhert, 1999) and mouse (Lanoue *et al.*, 2000), requirement of a B-containing siderophore, vibrioferrin, for growth of dinoflagellate *Gymnodinium catenatum* (Amin *et al.*, 2007), and nutritional importance of B for animals and human (Nielsen, 1997) suggest functional or metabolic roles for B beyond cell wall.

1.2.3. Absorption by Plant Roots

Roots take up B from soil solution as soluble un-dissociated H_3BO_3 which is about 10% of the total soil B and is permeable to plant cell membranes (Power and Woods, 1997). Passive uptake of B with no indication of saturation kinetics or effects of metabolic inhibition was demonstrated in a study where uptake by roots of sunflower and squash as well as cultured tobacco cells was investigated over a wide concentration range (Brown and Hu, 1994).Based

on theoretical considerations of membrane permeability data, it was suggested that uptake of B is achieved with passive diffusion which adequately satisfies the B requirement of plants (Raven, 1980).

Physiological studies and direct measurement of membrane permeability have revealed the presence of channel-mediated facilitated diffusion as well as passive diffusion in B transport systems (Stangoulis *et al.*, 2001). Furthermore it was shown that expression of the plasma membrane intrinsic protein 1 (PIP1), a member of major intrinsic protein (MIP) superfamily, in *Xenopus laevis* oocytes resulted in a 30% increase in the B permeability of the oocytes (Dordas *et al.*, 2000). However, the assertion of passive diffusion coupled with facilitated diffusion can neither address B uptake under B-limiting conditions (Brown *et al.*, 2002) nor explain reported differences in B uptake among plant species or genotypes (Nable, 1988).

Evidence for carrier mediated transport of B has been provided by molecular genetic studies in *A. thaliana*. Two types of B transporters, AtNIP5;1 and AtBOR1, both of which are important for efficient transport of B across the plasma membrane under B limitation have been identified (Takano *et al.*, 2002; 2006). In *A. thaliana*, the radial transport of B from the root surface to the xylem (xylem loading) is achieved by these two B transporters (Figure 1.3a). AtBOR1 mediates B export from pericycle cells into root xylem against a concentration gradient under low external B supply whereas AtNIP5;1 is crucial for B import into cells in root elongation and root hair zones. Involvement of other members of MIP family, such as AtNIP6;1 (Tanaka *et al.*, 2008) in B transport has been suggested. It has also been demonstrated that expression of *H. vulgare* PIP1;3 (HvPIP1;3) and HvPIP1;4 in yeast cells provided increased sensitivity to B (Fitzpatrick and Reid, 2009).

Among the *AtBOR1*-like genes in rice (*Oryza sativa*) genome, one was identified as *OsBOR1* using phylogenetic analysis (Nakagawa *et al.*, 2007). It was demonstrated that OsBOR1 was expressed in exodermal and endodermal cells of elongation zone of rice roots (Figure 1.3b) and predicted to function in both uptake and xylem loading of B under B-limiting conditions (Nakagawa *et al.*, 2007).

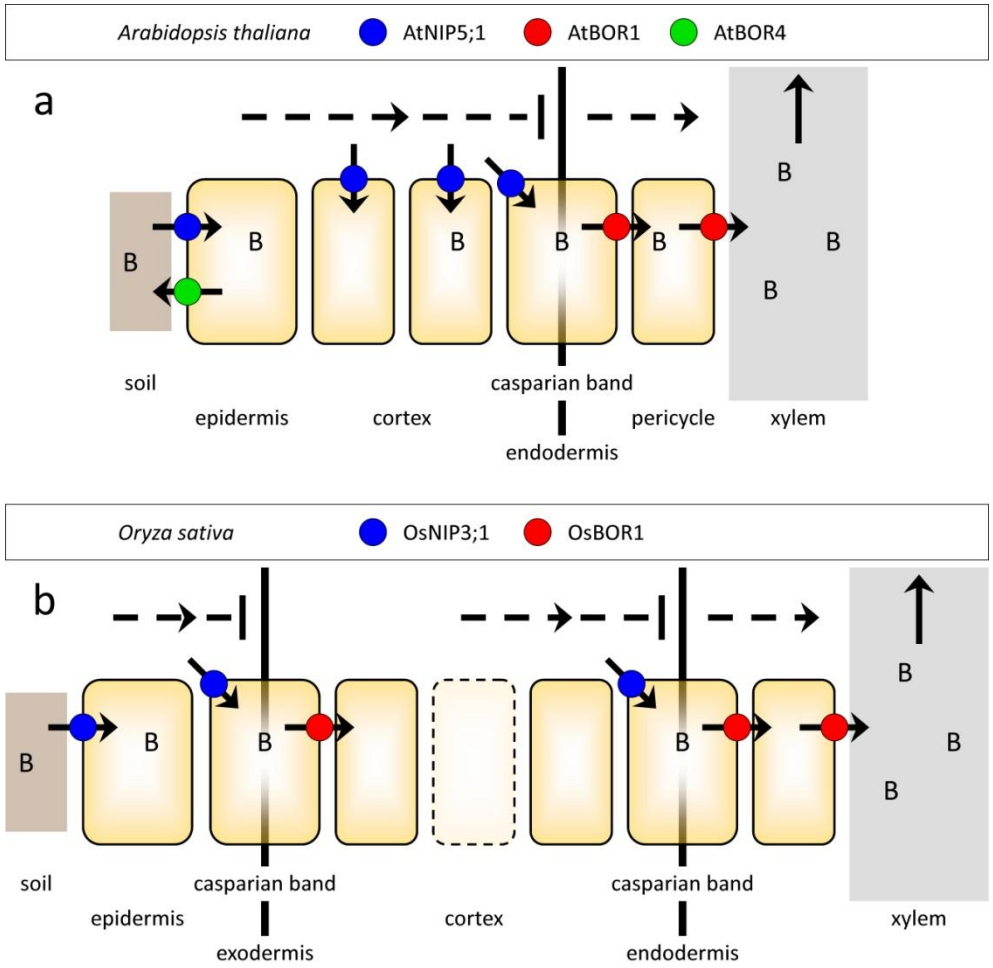

Figure 1.3. Boron uptake in root tissues of plants. Radial B transport in root tissues of *Arabidopsis thaliana* (a) and *Oryza sativa* (b) are displayed. Apoplastic flow, which is blocked by casparian band, is indicated with dashed arrows. (Adapted from Takano *et al.*, 2008)

Functional roles of identified B transporters were mainly verified in yeast or *X. laevis* oocyte expression systems, whereas subcellular localization was performed by transient expression of gene-of-interest (GOI) fused to gene encoding green fluorescent protein (GFP). Moreover, it was shown that expression and/or accumulation of B transporters in plants were tightly regulated in response to internal and external concentrations of B (Miwa and Fujiwara, 2010). Transport of B through cellular membranes is now believed to be mediated by passive or facilitated diffusion or by energy-dependent transport against concentration gradients (Miwa and Fujiwara, 2010).

1.2.4. Mobility in Plant Tissues

Although mobility of B in plant tissues predominantly in the transpiration stream is widely accepted, movement of B is extremely divergent in different plant species (Mengel and Kirkby, 2001). For the majority of plant species, B displays limited phloem mobility. On the other hand, preferential distribution of B to developing shoot tissues has been reported in plant species, such as *A. thaliana* (Takano *et al.*, 2001), wheat (Huang *et al.*, 2001), and sunflower (Matoh and Ochiai, 2005). It was suggested that AtBOR1 and AtNIP6;1 might be involved in transfer of B from xylem to phloem in shoot tissues of *A. thaliana* on the basis of demonstrated expression in shoots (Miwa and Fujiwara, 2010). Plasma membrane localized AtNIP6;1 was shown to facilitate B uptake into *X. laevis* oocytes and a strong *AtNIP6;1* promoter activity was observed in phloem region at nodes of the stem (Tanaka *et al.*, 2008). Thus, B transporters might be regulating preferential distribution of B into growing young tissues.

Within a leaf, higher concentrations of B were observed at the leaf tip and leaf margin as a result of transpirational flow through the leaf. Moreover, some plant species such as barley might have the ability to secret B out of leaves in guttation fluid (Oertli, 1962; Sutton *et al.*, 2007). Recently it was hypothesized that re-distribution of B in leaf tissues of wheat and barley might be achieved by efflux B transporters moving B from symplastic compartments into leaf apoplast (Reid and Fitzpatrick, 2009).

Phloem mobility of B in plants that produce and transport sugar alcohols has been demonstrated with isolation and characterization of B-polyol complexes. Complexes of B with mannitol isolated from phloem sap of celery and complexes with sorbitol and fructose isolated from extrafloral nectar of peach provided evidence for rapid and significant mobility of B in phloem (Hu *et al.*, 1997; Brown and Hu, 1996). Moreover, transgenic tobacco engineered to synthesize sorbitol displayed increased re-translocation of B within plant tissues, increased plant growth and yield, and enhanced tolerance to transient B deficiency in the soil (Brown *et al.*, 1999; Bellaloui *et al.*, 1999). It was also reported that enhancement of sorbitol synthesis in rice via genetic engineering facilitated B mobility in phloem (Bellaloui *et al.*, 2003). However, there are no reports of phloem mobility of B in wheat and barley and

these species are not known to produce polyols. Analysis of tissue specific expression and promoter regions of B transporters in wheat and barley should shed light on understanding of preferential distribution of B in shoot tissues as well as tolerance mechanisms in these grasses.

1.2.5. Boron Deficiency

Symptoms of B deficiency relate closely to the mobility of B within plants. In many plant species in which B is relatively immobile, deficiency symptoms appear as abnormal and retarded growth at apical meristems. The youngest leaves are irregularly shaped and wrinkled. Progression of deficiency leads to death of terminal apical growing points, reduction of plant growth, and inhibition of formation of flower and fruit (Mengel and Kirkby, 2001). In plant species in which B is phloem mobile relatively high concentrations of B are found in younger leaves and growing parts. Therefore, stated symptoms of deficiency are not observed. Most of the symptoms were proposed to be associated with requirement of B in structure of cell wall (Matoh *et al.*, 1993; Kobayashi *et al.*, 1996).

Moreover, requirement of B for pollen tube growth, pollen germination, and pollen viability (Blevins and Lukaszewski, 1998) have been used to explain the impairment of fruit formation under B deficiency in certain plant species such as grapes. Higher requirement of B for reproductive growth compared to vegetative growth (Dell and Huang, 1997) leads to impairments in crop production under B deficiency. Besides male sterility, pistil sterility has been reported due to B deficiency in maize (Agarwala *et al.*, 1981).

It is known that B deficiency inhibits elongation of both roots and shoots. Additionally, root growth is more sensitive to B deficiency than shoot growth (Dell and Huang, 1997). Loss of cell wall plasticity is the primary effect of B deficiency. Inhibition of cell division was proposed as the second important effect of B limitation on root meristematic tissues. It was also proposed that hormonal effects of B deficiency might be responsible for the reduction in growth and apical dominance (Li *et al.*, 2001). Studies were conducted to revert hormonal alterations caused by B deficiency. Exogenous application of B restored endogenous levels of cytokinins and indole acetic acid and partly restored apical dominance in pea plants (Wang *et al.*, 2006).

Effects of B deficiency on plant nutrition have been evaluated and reviewed previously (Blevins and Lukaszewski, 1998). Inhibition of root growth in squash plants under B deficiency was correlated with decreased concentrations of ascorbate in root apices (Lukaszewski and Blevins, 1996). It was shown that under B deficiency, root tips of squash and alfalfa contained high concentrations of iron, with majority in the ferric form and accumulated in the root cell wall. It was proposed that the low levels of ascorbate caused by B deficiency might prevent the reduction of iron to the ferrous form that plants can use. Though this did not cause an iron deficiency, it was suggested that ferric iron might precipitate phosphorus and form a type of iron plaque that could in general inhibit root uptake efficiencies (Blevins and Lukaszewski, 1998).

Several studies pointed out that B availability regulates the expression level of genes involved in several physiological processes (Camacho-Cristobal *et al.*, 2011). Genes regulated differentially under B limitation in cultured tobacco BY-2 cells (Kobayashi *et al.*, 2004) and *Arabidopsis* (Camacho-Cristobal *et al.*, 2008; Kasajima *et al.*, 2010) have been reported. It was proposed that activities of B transporters and aquaporins are tightly regulated to avoid B deficiency and toxicity and to maintain the rate of radial transport of B within an acceptable range (Takano *et al.*, 2008). In *Arabidopsis*, it was shown that regulation of AtNIP5;1 and AtBOR1 at the level of transcription or posttranslation is employed under B limitation to regulate B uptake (Takano *et al.*, 2005; Takano *et al.*, 2006). Similarly, the channel OsNIP3;1, similar to *Arabidopsis* AtNIP5;1, was shown to be required for efficient B uptake in rice (Takano *et al.*, 2008).

1.2.6. Boron Toxicity

High concentration of soil B is a serious constraint on agricultural production in arid and semi-arid regions throughout the world including Turkey, South Australia, California and Chile (Nable *et al.*, 1997; Cartwright *et al.*, 1984). Most land associated with recent volcanic activity and receiving low rainfall cause B toxicity in crops (Power and Woods, 1997). Sources of high B in soil are groundwater, B-loaded irrigation water, mining practices, fertilizers, and industrial products such as detergents (Nable *et al.*, 1997). Leach from rocks and soil containing B compounds is the main source of B-loaded groundwater and rivers. Toxicity is

frequently associated with saline soils and results from lack of sufficient drainage (Goldberg, 1997). Some amendments such as leaching and liming have been proposed for detoxification of B-loaded agricultural soil (Nable *et al.*, 1997). Alternative approaches to remove excess B from soil include phytoremediation and management of water and vegetation (Banuelos *et al.*, 1995; Nable *et al.*, 1997).

Turkey and the United States are the largest producers of B. Turkey has more than 70% of world's B reserves and largest sodium borate deposits which are located in Central and Western Anatolia including the provinces of Balıkesir, Kütahya and Eskişehir (Kar *et al.*, 2006). Borate mines are located north of the town of Bigadiç and along the Simav River. The amount of B in the Simav River varies between 4 to 7 mg B/L due to pollution from borate mines. The normal value for B content of Simav River before reach to the mining area is less than 0.5 mg B/L.

In Turkey, occurrences of B toxicity and resultant decreases in yield of cereals such as wheat and barley have been reported (Kalayci *et al.*, 1998; Torun *et al.*, 2001; 2003). Similarly in South Australia, significant yield reduction up to 17% in barley has been reported to result from B toxicity (Cartwright *et al.*, 1984). On the other hand, a large variation in tolerance to B toxicity has been observed among barley cultivars grown in Australia (Cartwright *et al.*, 1984) and Turkey (Torun *et al.*, 2003).

Accumulated B is toxic to all organisms including plants (Nable *et al.*, 1997). In human and animals B can affect the metabolism or utilization of various vital substances including macronutrients, energy substrates such as triglycerides and glucose, nitrogen containing molecules such as amino acids and proteins, reactive oxygen species, and estrogen. Low dietary B results in altered bone development, brain function, immune response, and insulin secretion. Boron has low toxicity when administered orally. Toxicity signs in animals generally occur only after dietary B exceeds 100 μg/g. Homeostasis of B is maintained by rapid excretion in the urine. Thus B does not accumulate in tissues, and is maintained in a relatively narrow range of concentrations in blood of healthy individuals (Nielsen, 1997). The primary health effects associated with inhalation exposure of humans to B are acute irritation of respiratory tract and eyes.

It is widely accepted that crop plants suffer from toxicity when the hot water-soluble B in the soil exceeds 5.0 mg B/L (Mengel and Kirkby, 2001). However, this value extremely varies with the crop species, physical growing conditions, and irrigation water. The fraction of B available to plants is the principal indicator of potential to induce B toxicity in crops (Nable *et al.*, 1997). The form of B in soil, composition and texture of soil, temperature, and pH greatly affect B availability to plants (Goldberg, 1997). Moreover, extraction techniques might not be reliable in predicting the available B since the mentioned physical and chemical properties of soil vary extremely and determine the partitioning of B between soil solution and adsorbing surfaces. Certain techniques attack certain components of soil and release different fractions of B. Therefore, it is widely believed that soil analysis can provide little information and can be a general risk assessment for B toxicity (Nable *et al.*, 1997).

Similarly, diagnosis of B toxicity in plants using visible symptoms or tissue B contents has limited applicability. High concentrations of B reduce growth of roots and shoots, and cause necrosis at the leaf tip and margins of mature leaves (Nable *et al.*, 1997; Reid *et al.*, 2004; Reid and Fitzpatrick, 2009). This is hypothesized to result from the accumulation of B transported through the transpiration stream. As phloem mobility and re-translocation of B is negligible in many plant species, B tends to accumulate in old leaves (Marschner, 1995).

Although development of necrotic zones is considered the typical visible symptom of B toxicity, it shows distribution of B in tissues and accumulation at the end of transpiration stream. High concentrations of B result in chlorosis followed by necrosis at the tip of a leaf. The symptoms then spread between the lateral veins towards the midrib (Mengel and Kirkby, 2001). Grasses such as wheat and barley show characteristic patterns of necrosis for different genotypes. Thus, leaf burn and necrosis have been extensively used for evaluation of toxicity in barley and wheat (Sutton *et al.*, 2007; Brennan and Adcock, 2004; Torun *et al.*, 2003; Jefferies *et al.*, 1999; Nable, 1988; Kluge and Podlesak, 1985). On the other hand, symptoms of toxicity in plant species in which phloem mobility of B has been demonstrated (Brown and Hu, 1996) include fruit disorders, bark necrosis and stem dieback rather than leaf burn and necrosis (Nable *et al.*, 1997). Another specific visible symptom of B toxicity, leaf cupping, has been observed in certain plant species and suggested to result from disturbance of cell wall cross-linking and expansion (Loomis and Durst, 1992).

Diagnosis of B toxicity has been extensively done by tissue B content and foliar analysis of genotypes. Moreover, critical values of tissue B concentrations for toxicity have been established in many plant species (Nable *et al.*, 1997; Marschner, 1995). Leaf B concentrations of sensitive and tolerant species have been reported to vary extremely, up to ten-folds (Furlani *et al.*, 2003). Additionally, crop species as well as cultivars in a species have varying ranges at which B is considered adequate. These ranges lie in the narrow differences between the critical values for B deficiency and toxicity. Concentrations of B also vary greatly within a plant among different parts or tissues of plant and at different developmental stages. In barley and wheat critical values have been reported to range between 10 and 130 mg B/kg dry weight (Kludge and Podlesak, 1985; Riley *et al.*, 1994; Mengel and Kirkby, 2001). On the other hand, critical leaf B concentrations for wheat as high as 700 mg B/kg dry weight has been reported (Ayars *et al.*, 1993).

Crops most sensitive to B toxicity include apple, peach, grapes, beans, and figs. Semi-tolerant plants are barley, peas, maize, potato, tobacco, and tomato while the most tolerant crops are sugarbeet, turnips, carrot, alfalfa, and cotton (Mengel and Kirkby, 2001).

There has been extensive research on interactive effects of B toxicity and salinity (Wimmer *et al.*, 2005; Yermiyahu *et al.*, 2008; Smith *et al.*, 2010a; 2010b). However, the reports on interactions of these two abiotic stresses as they affect plant growth and performance are contradictory. When both stresses occur together, studies have shown that salinity may reduce or induce toxic effects of B. For instance salinity increased B-related toxic effects in crops such as tomato, cucumber, and wheat (Grieve and Poss, 2000; Alpaslan and Gunes, 2001; Wimmer *et al.*, 2003). Conversely, increases in salinity decreased B toxicity in various vegetables, wheat, and chickpea (Ferreyra *et al.*, 1997; Yadav *et al.*, 1989).

1.3. Protein and Metabolite Profiling under B Stress

Stress-induced alterations in synthesis or activity of proteins and enzymes in plants, animals, and microorganisms have been reported. Levels of B above the optimum range cause significant changes in activity of various enzymes, consequently the metabolism of higher plants. Changes in total protein profiles of barley cultivars under B toxicity have been investigated using two-dimensional gel electrophoresis (Mahboobi *et al.*, 2000). Toxic

concentrations of B resulted in increases or decreases in abundance of a number of proteins in root and leaf tissues. As a result of B treatment, one newly synthesized protein with relative molecular weight of 35.0 kDa and pI value of 7.8 was detected in root profiles of the tolerant barley cultivar. Moreover, abundance of 3 proteins in roots and 7 in leaf tissues were induced in tolerant cultivar but were unchanged in sensitive cultivar of barley. A number of proteins were detected to decrease in amount while a group of others were completely disappeared in the sensitive cultivar (Mahboobi *et al.*, 2000).

In a recent study, two-dimensional gel electrophoresis was used to screen differentially expressed proteins in tolerant barley cultivar under B toxicity (Atik *et al.*, 2011). Seven proteins were determined to be up-regulated. They were ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) large chain, a thaumatin-like protein (TLP5), a basic pathogenesis-related protein (PR5), an RNase S-like protein, a PSI type III chlorophyll a/b-binding protein, a light-harvesting complex I (LHC I), and the vacuolar proton-translocating ATPase (V-ATPase) subunit E protein. Moreover, heterologous expression of the gene coding barley V-ATPase subunit E in yeast provided B resistance to yeast cells (Atik *et al.*, 2011). In wheat it was demonstrated using denaturing polyacrylamide gel electrophoresis that B toxicity increased intra- and inter-cellular soluble protein concentrations (Wimmer *et al.*, 2003). A change in protein profile of inter-cellular fluid was determined upon B stress treatment. This included an increase in abundance of a 19 kDa protein and decreases in abundances of proteins with molecular weights of 58 and 51 kDa (Wimmer *et al.*, 2003).

It was also reported that B affects the metabolism of nitrogen compounds (Bonilla *et al.*, 1980; Kastori and Petrovic, 1989). Nitrate reductase (NR) and glutamate dehydrogenase (GDH) activities in leaf and root tissues of barley and wheat were investigated under B toxicity (Mahboobi *et al.*, 2002). An average of 16% of reduction in NR activity was determined in leaf and root tissues of both tolerant and sensitive cultivars. On the other hand, the activity of GDH was increased by 30% in leaf and 81% in root tissues of both tolerant and sensitive cultivars. It was proposed that the increase in activity of GDH could be an adaptive mechanism in wheat and barley under B stress conditions (Mahboobi *et al.*, 2002). In another study response of nitrogen metabolism to B toxicity was investigated in tomato (Cervilla *et al.*, 2009). Activities of glutamine synthase, glutamate synthetase, and

GDH were increased under B toxicity. It was concluded that B toxicity causes inhibition of nitrate reduction and increases ammonium assimilation in tomato (Cervilla *et al.*, 2009).

A comparative proteomic technique was employed to investigate the abundances of proteins from tolerant and sensitive barley plants from a Clipper X Sahara doubled haploid (DH) population (Patterson *et al.*, 2007). Three enzymes that are involved in siderophore production were determined to be elevated in abundance in the B-tolerant plants. The enzymes were iron deficiency sensitive2 (IDS2), IDS3, and a methylthio-ribose kinase. A potential link between iron, B, and the siderophore hydroxymugineic acid was proposed (Patterson *et al.*, 2007).

Alterations in levels of sucrose or various carbohydrates are among the plant responses to environmental stresses such as salinity, drought, infection, and extreme temperatures (Rosa *et al.*, 2009). In a large body of research B has been shown to have variable effects on biosynthesis of plant glycosides including sucrose (Dugger and Humphreys, 1960).The rate of replenishing the uridine diphosphate glucose (UDP-glucose) pool and particularly its differential utilization by different biosynthetic reactions are affected by B stress (Avigad, 1982). Since sucrose synthesis and degradation are closely associated with UDP-glucose pool, it is not surprising that levels and utilization of UDP-glucose are significantly influenced by B deficiency (Dugger and Palmer, 1980). It has been reported that B inhibit the formation of starch from sugar. Reducing sugars have been found to increase in the root tip when soil B concentrations are high (Marschner, 1995).

A metabolomics approach was used to compare metabolite profiles in root and leaf tissues of a sensitive barley cultivar Clipper and a tolerant landrace Sahara (Roessner *et al.*, 2006). Leaf metabolite profiles of two cultivars were determined to be similar in the early stages of development. The only striking difference was that in young leaves the polyamine, putrescine, was only detected in Sahara in control conditions, whereas it was only detected in Clipper leaves after 3 weeks of growth following treatment with 1 mM B for 2 weeks. However, it was concluded that none of the analyzed metabolites in the study seemed sufficient to explain the cellular tolerance mechanism in the Sahara cultivar (Roessner *et al.*, 2006).

Uronic acid contents of sensitive and tolerant cultivars of barley and wheat were investigated under B toxicity (Mahboobi *et al.*, 2001) to unravel the possible function of uronic acid in tolerance mechanisms. Uronic acid is a significant structural component of cell wall pectins. However, no significant change in amount of uronic acid under B toxicity and no significant difference between tolerant and sensitive cultivars were determined. Thus, it was concluded that cell wall uronic acid content does not contribute to detoxification of excess B in wheat and barley (Mahboobi *et al.*, 2001).

Another study has investigated the involvement of antioxidative metabolites and enzymes in mechanism of tolerance to B toxicity in barley (Karabal *et al.*, 2003). Boric acid treatment did not cause significant changes in proline contents of both leaf and root tissues of both sensitive and tolerant cultivars. Though changes in activities of some antioxidant enzymes were detected in leaf or root tissues, it was concluded that B toxicity induced membrane damage in barley leaves, do not involve active oxygen species and antioxidant enzyme activity is not a critical factor in B toxicity tolerance mechanism in barley (Karabal *et al.*, 2003). On the other hand, it has been suggested that antioxidants and antioxidant enzymes might reduce B toxicity in some plants such as grapevine, tomato, chickpea, and *Artemisia annua* L (Gunes *et al.*, 2006; Cervilla *et al.*, 2007; Ardic *et al.*, 2009; Aftab *et al.*, 2010).

1.4. Mechanisms of Tolerance

The mechanisms, associated with tolerance to B toxicity or deficiency, are not well understood. Absorption of B from soil, mobility within a plant, accumulation at the end of transpiration stream, concentration gradient observed within a leaf, toxicity symptoms, tissue B contents, and variation in tolerance to B toxicity existing among species and cultivars have all been gathered for proposal of tolerance mechanisms in vascular plants (Reid *et al.*, 2004). One model assumes the existence of compounds binding B once it accumulates to toxic concentrations within the cell (Reid *et al.*, 2004), a second one proposes compartmentation of B, and another one describes an active efflux of B by a transporter (Hayes and Reid, 2004).

Taken together research on tolerance to B toxicity has led to proposal of a mechanism of tolerance involving an ability to maintain low concentrations of B in plant tissues. The

mechanism involves reduction of B uptake or active efflux of B – at least partly – from the roots (Hayes and Reid, 2004). Moreover, accumulation of lower concentrations of B in tolerant compared to sensitive cultivars suggests exclusion rather than internal tolerance mechanisms such as binding of B in complexes or compartmentation in vacuoles.

Recently, an important number of transporters have been identified in *Arabidopsis*, rice, barley, and wheat (Miwa and Fujiwara, 2010). Involvement of these transporters as well as aquaporins in tolerance mechanisms has been proposed. *Arabidopsis* AtBOR1 and AtNIP5;1 are required for efficient B uptake under B limiting conditions (Takano *et al.*, 2002; 2006). It was also shown that B uptake is regulated under B toxicity mainly through the transcriptional regulation of *AtNIP5;1* (Takano *et al.*, 2006) or endocytosis and degradation of AtBOR1 (Takano *et al.*, 2005). Recently it was reported that B dependent degradation of *AtNIP5;1* mRNA under excess B is controlled by the 5' untranslated region (UTR) of *AtNIP5;1* (Tanaka *et al.*, 2011).

Moreover, another B transporter – AtBOR4 – having functional roles under B toxicity was characterized in *Arabidopsis*. It was demonstrated that AtBOR4 shows B efflux activity in yeast cells and localizes in a polar manner to the plasma membrane of the distal side of root epidermal cells. Overexpression in *A. thaliana* resulted in AtBOR4 accumulation and significant growth improvement under high B conditions. The growth enhancement was attributed to the reduced concentration of B in roots and shoots due to the efficient efflux of B from the roots (Miwa *et al.*, 2007). Additionally, it was suggested that AtBOR1 and AtNIP6;1 might be involved in transfer of B from xylem to phloem in shoot tissues and regulating preferential distribution of B into growing young tissues in *A. thaliana* (Tanaka *et al.*, 2008).

It was proposed in rice that OsNIP3;1 imports boric acid into cells and OsBOR1 exports boric acid or borate from the exodermal and endodermal cells under low B conditions (Takano *et al.*, 2008). Recently, a gene encoding a NAC-like transcription factor was identified using recombinant inbred lines (RILs) in rice (Ochiai *et al.*, 2011). It was demonstrated that suppression of expression of the transcript provides tolerance to B toxicity in rice.

In barley, physiological comparisons of a highly B-tolerant cultivar and a sensitive cultivar revealed that exclusion of B from the roots is a major mechanism of tolerance to B toxicity (Hayes and Reid, 2004). Mapping of QTL on 4H was used to determine an *AtBOR1*-like gene, *HvBot1* in barley (Sutton *et al.*, 2007). It was the first B toxicity tolerance gene identified in plants. It was demonstrated that tolerance mechanism identified in Sahara is derived from an increase in copy number of *HvBot1* gene and abundance of mRNA transcript (Sutton *et al.*, 2007). Moreover, a gene coding a NIP-like aquaporin – *HvNIP2;1* – was identified in barley and genetically mapped to B tolerance QTL on 6H (Schnurbusch *et al.*, 2010b). It was proposed that reduced expression of HvNIP2;1 as well as increased expression of HvBot1 confers tolerance to B toxicity. Additionally, combinatorial effect of root and shoot efflux transporters, rain, and guttation to alleviate B toxicity was proposed as a mechanism of tolerance in barley (Reid and Fitzpatrick, 2009).

Although physiological basis for tolerance to B toxicity in wheat was proposed to be associated with limitation of B accumulation within plant, less is known about the molecular basis for tolerance (Schnurbusch *et al.*, 2010a). A gene coding for a B transporter – *TaBor2* – has been identified and cloned from cDNA prepared from roots of wheat grown under B toxic conditions (Reid, 2007). Besides efflux or exclusion of B, internal mechanisms such as adsorption to cell walls, binding of B in complexes, and compartmentation in vacuoles have been proposed to be functioning in wheat for tolerance. Results of a study conducted using 70 durum wheat genotypes indicated involvement of internal mechanisms rather than B exclusion mechanism in differential expression of B tolerance within durum wheat or at least within 70 genotypes tested in the study (Torun *et al.*, 2006).

1.5. Genetics of Tolerance to B Stress

Wide genetic variation in tolerance to B toxicity exists in wheat and barley (Nable, 1988; Torun *et al.*, 2001; 2003). It was reported that durum wheat is much less tolerant to high soil B concentration compared to bread wheat based on leaf symptom score, shoot B content, and grain yield (Kalayci *et al.*, 1998; Yau *et al.*, 1997). Barley is a semi-tolerant species with a large variation in tolerance to B toxicity among cultivars within the species (Cartwright *et al.*, 1984; Torun *et al.*, 2003; Mengel and Kirkby, 2001). Interval regression mapping in both

wheat and barley has identified the chromosomal locations of several major QTL (Jefferies *et al.*, 1999; 2000).

Studies of B toxicity tolerance in a Clipper X Sahara F1-derived doubled haploid (DH) mapping population of barley identified B tolerance alleles at four genetic loci on chromosomes 2H, 3H, 4H, and 6H (Jefferies *et al.*, 1999). The traits used in identification of loci included shoot B concentration, leaf symptom score, relative root length, and dry matter response. In all cases the favorable alleles in the mapping population were derived from the B tolerant landrace Sahara. The major QTL on 4H was associated to all four traits. On the other hand, QTL on 2H, 3H, and 6H were associated to leaf symptom score, relative root length, and shoot B concentration, respectively. The QTL on 3H was identified to control relative root length at toxic B concentrations having less effect than that of QTL on 4H and acting in additive manner with the QTL on 4H (Jefferies *et al.*, 1999).

Research directed at identification of the genes underlying these QTL resulted in characterization of a B transporter and an aquaporin. Barley 4H QTL gene *HvBot1* was identified using an approach combining positional cloning and reverse genetics (Sutton *et al.*, 2007). Furthermore, an aquaporin gene named *HvNIP2;1* – ortholog of the rice *OsNIP2;1* – was genetically mapped to the region containing the B tolerance QTL on 6H (Schnurbusch *et al.*, 2010b).

In bread wheat, tolerance to B toxicity is controlled by at least three unlinked genes *Bo1*, *Bo2*, and *Bo3* on chromosomal groups 4 and 7 (Paull *et al.*, 1991; 1992). Loci have been mapped to 4A, 7B, and 7D (Paull *et al.*, 1992; Jefferies *et al.*, 2000). Though the molecular basis and genes underlying these QTL have not yet been identified, it was proposed that the 7B locus is the main determinant of yield in cultivars which perform well under B toxic conditions (Nable *et al.*, 1997).

Breeding efforts to increase tolerance to B toxicity are focused on introgression of one or two tolerance QTL from tolerant cultivars to sensitive high yielding backgrounds in barley and wheat (Schnurbusch *et al.*, 2010b). However, no yield advantage was determined in the tested backcross families in a study evaluating the combination of presence or absence of Sahara alleles on 2H and 4H in near isogenic lines (NILs) of barley (McDonald *et al.*, 2009).

Thus, it was concluded that the inconsistent and varying yield effects might stem from a complex interaction of environmental factors such as site-specific or seasonal B toxicity effects, and other subsoil constraints including salinity and water shortage. For barley, these results suggested that yield advantages from selection based solely on B exclusion and leaf symptom expression might be small (McDonald *et al.*, 2009; Reid, 2010).

1.6. Genomic Approaches to Reveal Tolerance

Environmental stresses including toxic or deficient concentrations of B in soil contribute significantly to reduced yields (Cartwright *et al.*, 1984; Kalayci *et al.*, 1998; Torun *et al.*, 2001; 2003). Plants dramatically alter their gene expression patterns to cope with a variety of environmental stresses. These transcriptional changes result in either successful adaptations leading to tolerance or failure to adapt to the new environment leading to sensitivity (Hazen *et al.*, 2005). Upon stress imposition, a cascade of events including signal perception and transduction lead to alterations in expression and defense responses at the cellular level. However, improvement of plant tolerance to environmental stresses remained limited due to lack of insight and understanding of inherent complexity of stress signaling, adaptation processes, and signal transduction (Cushman and Bohnert, 2000) as well as multigenic nature of stress tolerance (Habash *et al.*, 2009). Insight into molecular basis of stress tolerance should suggest novel strategies for crop improvement. Requirement for the intended insight is to gather information on gene regulation and signal transduction pathways involved in stress response. Measurement of gene expression can provide information on cellular processes, biochemical pathways, regulatory mechanisms, and stress responses (Clarke and Zhu, 2006).

Complete genome sequence information from model organisms and key plant species including *A. thaliana*, *O. sativa* and *Populus trichocarpa* have been available for a while. This genomic information has yielded the ability to perform high-throughput, genome-wide screens of gene function and has boosted application of a range of new technologies to functional plant gene analysis (Holtorf *et al.*, 2002). Such technologies allow analysis of different constituents namely the transcripts, proteins and metabolites of a cell that help to deduce gene function. This is where genomics turns out to be functional genomics. Functional genomics, which includes transcriptomics, proteomics and metabolomics, aims to

determine the biological functions of genes and their products. The goal is not simply to provide a catalogue of all the genes, gene products and information about their functions, but to understand how the components work together to comprise functioning cells and organisms (Lockhart and Winzeler, 2000). Transcriptomics, defining gene expression analysis by mRNA profiling, is considered to be the most prominent and powerful tool for functional genomics (Öktem *et al.*, 2008).

Qualitative and quantitative determination of transcript profiles of a genome and identification of differentially regulated genes are of great importance. Specific metabolic or morphogenetic functions of genes might be assigned by comparing the concentration of individual mRNAs present in samples originating from different genotypes, tissues, developmental stages, growth or environmental conditions. Analyses of transcript patterns are valuable in assessing roles of novel sequences in an organism, since similarity of expression patterns of sequences of unknown function with those of known genes might indicate functional homology (Kuhn 2001). For instance, similarly regulated genes might be involved in same or similar pathways or responses providing information on how cells function and components of cell work together. Moreover, expression under specific conditions allows the comparison of the promoter or regulatory sequences of genes. Common *cis*-elements might be localized within a genome and their presence or activity might be correlated with specific features of the expression profile of corresponding gene, gene groups or genomes.

Advances and technical developments in bioinformatics and functional genomics have offered the opportunity to gain a more complete understanding of total set of genes that become integrated to create tolerance to abiotic stresses such as B stress. Thus it is now possible to address the complexity of a stress response on a large scale through genome wide expression profiling using microarrays (Schena *et al.*, 1995; Lockhart *et al.*, 1996). Genes and regulators identified by expression profiling can be explored in succeeding studies for their specific roles in tolerance or sensitivity to stress. Furthermore, identified novel genes might be used as genetic markers for determination of diversity in germplasm and as candidates for genetic modification of crop plants for elevated stress tolerance.

Barley is one of the crop species for which both cDNA- and oligonucleotide-based microarray platforms have been developed. A cDNA microarray was used to monitor expression changes in leaves of barley under dehydration shock and drought stress (Talame *et al.*, 2007). Approximately 10% of all transcripts profiled were declared up- or down-regulated in at least one of the conditions tested, namely, dehydration shock for 6 h, slow drying for 7 or 11 days, and rehydration (Talame *et al.*, 2007). Up-regulated expression levels for transcripts encoding proteins involved in response to abiotic stimulus and stress, such as jasmonic acid-responsive proteins, allene oxide synthase, late embryogenesis abundant (LEA) proteins, and osmoprotectant biosynthesis related proteins like arginine decarboxylase and P5CS were reported.

In another study, inductions in expression levels of genes involved in methionine cycle in both Zn- and Fe-deficient barley roots, to meet the demand for methionine, were reported (Suzuki *et al.*, 2006). Methionine is a precursor in the synthesis of metal chelators that are produced in graminaceous plants in response to Fe deficiency. Early responses of barley genes to salinity stress at seedling stage were investigated using GeneChip Barley Genome Array (Walia *et al.*, 2006). A large number of abiotic stress related genes were found to be responsive to salinity stress. On the other hand, genes involved in jasmonic acid biosynthesis and genes known to respond to jasmonic acid treatment were reported to be induced. In another study, variation in gene expression patterns of barley embryos during the first 4 days of germination was investigated using a cDNA microarray (Watson and Henry, 2005).

In one of the first studies involving microarrays for barley, a cDNA array was used to monitor large-scale changes in transcript abundance under drought- and salt-stress (Ozturk *et al.*, 2002). Transcripts that showed significant up-regulation under drought stress are exemplified by jasmonate-responsive, metallothionein-like, late embryogenesis abundant (LEA) and ABA-responsive proteins. Most drastic down-regulation was observed for photosynthesis-related processes. Up-regulation under both drought and salt stress was restricted to ESTs for metallothionein-like and LEA proteins, while increases in ubiquitin-related transcripts characterized salt stress (Ozturk *et al.*, 2002).

Though a large body of research conducted on transcriptional analyses of barley under various environmental conditions or developmental stages using microarrays is available, there are no investigations reporting transcriptome changes upon B toxicity or deficiency.

1.7. Aim of the Study

Mechanisms for tolerance to B toxicity, function of several B transporters, and a structural role of B in plants have been proposed. However, various cellular responses such as signaling, modulation of transcriptome, alteration of metabolism, and key cellular components in these responses are unknown. Moreover, transcriptomes of cereals under B stresses have not been investigated at whole genome level. Therefore, this study concentrated mainly on determination of transcriptional regulation upon B toxicity and deficiency.

The main objective of this study was to investigate and comparatively analyze the molecular responses of sensitive and tolerant barley cultivars to B toxicity and deficiency. Additionally, determination of novel genes, molecular mechanisms, and networks of signaling and regulation in barley for tolerance to B toxicity or deficiency was intended.

Another purpose of this study was to isolate and functionally characterize a putative B transporter gene from barley and examine its possible roles in tolerance to B toxicity. Additionally development of novel genetic markers was aimed for positional cloning of the B transporter gene.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material and Growth Media

Throughout microarray and gene cloning studies of this work, cultivated varieties of barley (*Hordeum vulgare* L.) and a landrace, which were previously screened for tolerance to boron (B) toxicity in field studies, were used. Turkish cultivars Tarm-92 and Hamidiye were designated as B-tolerant and B-sensitive, respectively (Torun *et al.*, 2003). The Algerian landrace Sahara-3771 was identified as B-tolerant owing to its ability to exclude excess B, whereas the Australian malting variety Clipper was identified as B-sensitive (Jefferies *et al.*, 1999). The seeds of Turkish cultivars were obtained from Central Research Institute for Field Crops (Ankara, Turkey) while seeds of Clipper and Sahara were obtained from germplasm collection maintained at Waite Agricultural Research Institute (Adelaide, SA, Australia).

Defined half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) and basal growth solution which was formulated at Waite Agricultural Research Institute were used for seedling growth where appropriate. The compositions of these nutrient solutions are provided in Appendix A. Solution of boric acid (H_3BO_3) which is used for application of B stress was added at adequate amounts to provide sufficient, toxic or deficient concentrations of B in growth solution. Both growth media were prepared with distilled water and sterilized by autoclaving at 121°C for 20 min. The pH of the media was adjusted to 5.6 – 5.8 prior to sterilization.

2.1.2. Bacterial Strains and Media

During gene cloning and nucleotide sequencing, chemically competent *Escherichia coli* strain TOP10 (Invitrogen) was utilized. TOP10 was cultured in Luria Bertani (LB) medium supplemented with appropriate antibiotics. S.O.C. medium was used to aid fast recovery during grow-out period after introduction of plasmids into competent TOP10 cells. *Agrobacterium tumefaciens* strain C58C1 was utilized for transient transformation of onion epidermal cells. Yeast Extract Broth (YEB) medium with proper antibiotics was used to culture *A. tumefaciens* C58C1. The compositions of bacterial growth media are given in Appendix B.

Culture media were prepared with distilled water and pH was adjusted to 7.0 – 7.2. Media were solidified with addition of 1.5% (w/v) agar when required. Sterilization was done by autoclaving at 121°C for 20 min. Selective agents and antibiotic solutions were filter sterilized with 0.2 µm pore-sized filters and added freshly to the sterilized and cooled media.

2.1.3. Yeast Strain and Culture Media

Yeast (*Saccharomyces cerevisiae*) strain INVSc2 (*his3, ura3*; Invitrogen), which requires histidine and uracil for growth, was utilized for heterologous expression. Yeast extract Peptone Dextrose (YPD) medium was used for routine culturing of yeast, whereas double-strength YPD medium was used during transformation. Synthetic Dextrose (SD) minimal medium containing glucose (2% w/v) was also used during transformation of yeast. Glucose in SD medium was replaced with galactose (2% w/v) to induce transcription of gene-of-interest (GOI). The compositions of yeast media are given in Appendix C.

Culture media were prepared with ultrapure water (18.2 MΩ cm) and pH was adjusted to 7.0 – 7.2. YPD and SD media were solidified with addition of 2% (w/v) agar to prepare media for petri plates. Sterilization was done by autoclaving at 121°C for 20 min. Volume of media were measured after autoclaving and it was completed to required amount using sterile ultrapure water. Stock solutions of glucose, galactose, histidine and uracil were filter sterilized with 0.2 µm pore-sized filters and added freshly to the sterilized and cooled media.

2.1.4. Plasmids

Throughout this study, commercially available linearized plasmids pCR®8/GW/TOPO® and pENTR™/D-TOPO® (Invitrogen) were utilized for cloning of fragments of DNA or complementary DNA (cDNA). Both vectors were used to create entry clones for Gateway® (Invitrogen) cloning system with the aid of Topoisomerase I covalently bond to the 3' ends of linearized vectors.

Gateway-compatible destination vectors pYES-DEST52 (Invitrogen), pIPKb004 (Himmelbach *et al.*, 2007) and pEarleyGate100 (Earley *et al.*, 2006) were used for heterologous expression of GOI. pYES-DEST52 provided galactose-inducible expression of GOI under the control of *GAL1* promoter in yeast. pIPKb004, kindly provided by The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK; Gatersleben, Germany), was used for expression of GOI fused to the gene encoding modified green fluorescent protein 5 (mGFP5) under the control of doubled enhanced CaMV35S promoter in plant cells. Similarly, pEarleyGate100, obtained from The Arabidopsis Biological Resource Center (ABRC; Columbus, OH, US) through The Arabidopsis Information Resource (TAIR; Stanford, CA, US), was used for expression of GOI-mGFP5 fusion under the control of CaMV35S promoter in plant cells. Maps and features of the vectors are provided in Appendix D.

2.1.5. Culture Conditions

Plant hydroponic cultures were maintained in growth chambers at 22±2°C with 70% relative humidity and with 16 h light (300 µmol m^{-2} s^{-1}) and 8 h dark photo-cycle. Cultures of *E. coli* TOP10, *A. tumefaciens* C58C1 and *S. cerevisiae* INVSc2 were incubated at 37±1°C, 28±1°C and 30±1°C, respectively, unless otherwise stated. Liquid cultures of bacteria or yeast were mixed and aerated at 180 – 200 revolution per min (rpm) in an orbital shaker.

2.1.6. GeneChip Barley Genome Array

Genome-wide expression profiling via DNA microarrays was carried out using GeneChip® Barley Genome Array (Affymetrix), which contains 22,840 probe sets representing

transcripts expressed by *H. vulgare* genome (Close *et al.*, 2004). Eleven pairs of oligonucleotide probes that constitute a probe set were used to measure the relative expression level of each transcript represented. Members of these pairs, perfect match (PM) and mismatch (MM) probes, were exactly same in sequence with a single nucleotide difference for the 13th nucleotide in the middle of the 25 bp long-oligonucleotide probe.

GeneChip Barley Genome Array was produced by a worldwide cooperation of international barley community, United States Department of Agriculture Initiative for Future Agricultural and Food Systems (USDA-IFAFS) Triticeae Improvement Group and Affymetrix. Approximately 400,000 raw barley Expressed Sequence Tags (ESTs) from 84 cDNA libraries were utilized for array design. Quality pruning and stringent CAP3 clustering resulted in 53,030 unigenes including 26,634 contigs and 26,396 singletons. Probes were designed using unigenes with complete 3' ends (approximately 25,500) which included all 1,145 known barley genes from GenBank® (National Center for Biotechnology Information, NCBI) non-redundant database.

Moreover control sequences for hybridization and target preparation, and barley house-keeping genes were included during array design. Hybridization controls are *bioB*, *bioC*, *bioD* from *E. coli*, and *cre* from P1 Bacteriophage. Target preparation controls, also known as poly-A controls, are *dap*, *lys*, *phe*, *thr*, and *trp* from *Bacillus subtilis*. Barley house-keeping genes are ones encoding for actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ubiquitin, tubulin alpha subunit, and translation initiation factor 5A. Arrays were manufactured in the 49 format with 18 µm feature size.

2.1.7. Chemicals, Reagents and Kits

The chemicals and reagents used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, MO, US), Merck KGaA (Darmstadt, Germany), Fermentas (Thermo Fisher Scientific Inc; Ontario, Canada), AppliChem GmbH (Darmstadt, Germany), New England BioLabs Inc. (NEB; Ipswich, MA, US), Qiagen N.V. (Venlo, Netherlands), Invitrogen Corporation (Carlsbad, CA, US), Ambion (Austin, TX, US) and Affymetrix (Santa Clara, CA, US).

Chemicals, enzymes, oligonucleotides and kits for molecular biology studies such as electrophoresis, polymerase chain reaction (PCR), nucleic acid purification and handling, expression profiling, enzymatic digestion, and sequencing were purchased mainly from Qiagen, Invitrogen, NEB and Affymetrix. All of the media and solutions were prepared using distilled or ultrapure water.

2.1.8. Instruments

Plant growth under controlled conditions was performed in SGC1700 plant growth chamber (Weiss Gallenkamp Ltd; Loughborough, UK) maintained at 22±2°C and 70% relative humidity with diurnal cycle of 16 h light (300 µmol m^{-2} s^{-1}) and 8 h dark. Cultures of bacteria and yeast were incubated in orbital shakers or incubators manufactured by Gerhardt GmbH (Königswinter, Germany), Heidolph Instruments GmbH (Schwabach, Germany), Weiss Gallenkamp Ltd (Loughborough, UK) and Nüve Ltd (Ankara, Turkey).

Instruments used during microarray analysis included GeneChip Scanner 3000, Fluidics Station 450 and Hybridization Oven 640 which were manufactured by Affymetrix (Santa Clara, CA, US). Real-time PCR was performed using Rotor-Gene Q (Qiagen N.V.; Venlo, Netherlands). Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, US) was used for microfluidic separation of biological macromolecules. Capillary separation for sequencing was done on the ABI 3730*xl* sequencing platform (Applied Biosystems Inc; Foster City, CA, US) at Australian Genome Research Facility Ltd (AGRF; Adelaide, SA, Australia).

Centrifugations in techniques of molecular biology were done using 3K30 and 3-16PK centrifuges (Sigma GmbH; Osterode, Germany) and centrifuges manufactured by Beckman Coulter Ltd (Brea, CA, US), MPW Med Instruments (Warsaw, Poland) and Thermo Fisher Scientific (Waltham, MA, US). All incubations during molecular studies were done in thermal cyclers manufactured by Bio-Rad Laboratories Inc (Hercules, CA, US), G-Storm Ltd (Somerset, UK) and Applied Biosystems Inc (Foster City, CA, US). Gel electrophoresis was performed using systems manufactured by Bio-Rad Laboratories Inc (Hercules, CA, US) and C.B.S. Scientific Company Inc (Del Mar, CA, US). Gel documentation was done using GelDoc-It Imaging System (UVP Ltd; Cambridge, UK).

2.2. Experimental Strategy

This study comprises three main parts, investigation of global expression profiles of barley under B toxicity or deficiency using microarrays, cloning and functional characterization of a gene encoding a putative B transporter, and mapping on 3H for B tolerance (Figure 2.1).

Plant responses to B stress, like other abiotic stresses, appears to be multigenic and controlled temporally and spatially. Global monitoring of gene expression at specific developmental stage or in specific tissues is therefore crucial to gain insight into such multigenic responses. In this study, transcriptional responses of barley to B toxicity or deficiency at seedling stage were investigated using leaf and root tissues of hydroponically grown seedlings.

Seedlings were subjected to shock treatments of B toxicity or deficiency under aseptic and controlled environmental conditions. Though field conditions differ extremely from laboratory conditions and B stress that plants are exposed to in the field is mild, in order to reveal responses associated solely with B toxicity or deficiency, transcriptomes were profiled under controlled physical conditions and extreme B treatments. Each set of experiment, with a completely randomized design, was repeated 3 times and used as independent biological replicates. Inter- and intra-varietal differences as well as B responsive changes in transcriptomes were examined in microarray analysis. Moreover, expression levels were validated with an independent gene expression profiling method (Figure 2.1).

In the second part of the study, a gene encoding a putative B transporter was cloned and examined for its role in tolerance to B toxicity in barley (Figure 2.1). A candidate gene approach was employed for cloning of the B transporter gene which was hypothesized to be located on 3H. Cloned coding sequence (CDS) of the gene was transiently expressed in yeast and plant cells. Yeast expression system was used for assessment of B transporting activity of the protein product whereas plant epidermal cells were used for determination of subcellular localization. Moreover, changes in transcript abundance in leaf and root tissues of barley under B toxicity were determined using real-time reverse transcriptase PCR (RT-PCR). Gene expression profiles were further validated with northern blotting.

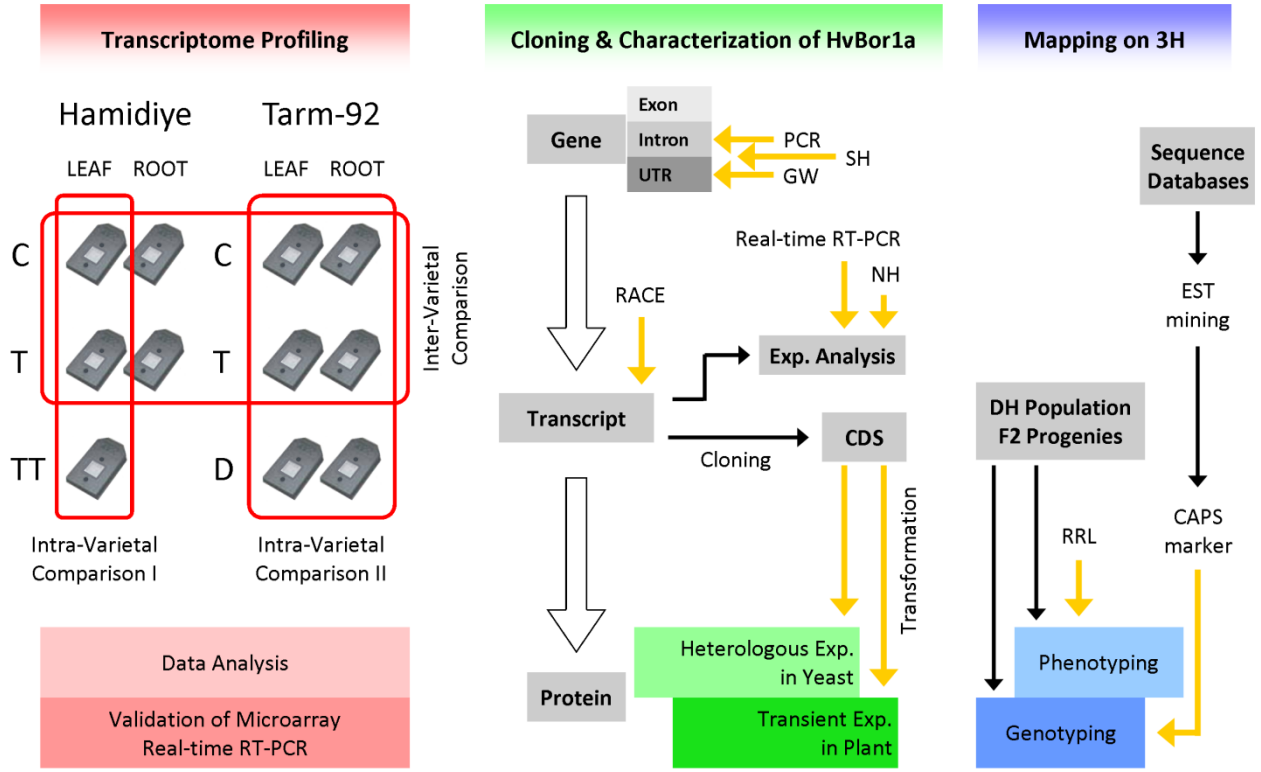

Figure 2.1. Overview of experimental strategies and methods considered in this study for transcriptome profiling, cloning of *HvBor1a*, and mapping on 3H. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment; D: Deficiency applied as 0.02 µM H_3BO_3 treatment; CDS: Coding sequence; UTR: Untranslated region; RACE: Rapid amplification of cDNA ends; PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase PCR; GW: Genome walking; SH: Southern hybridization; NH: Northern hybridization; EST: Expressed sequence tag; CAPS: Cleaved amplified polymorphic sequence; DH: doubled haploid; RRL: Relative root length)

Introns and untranslated regions (UTRs) of the cloned gene were amplified from genomic DNA of various cultivars of barley using conventional PCR and genome walking (GW). The amplified regions were sequenced for determination of variations in sequence with an intention to develop markers for tolerance to B toxicity.

In the third part of the study, genetic mapping on 3H was performed for positional cloning of the B transporter gene (Figure 2.1). A novel genetic marker for the quantitative trait locus (QTL) associated with tolerance to B toxicity on 3H was developed. Doubled haploid (DH) population and F2 progenies were genetically screened with the marker developed. Moreover, the individuals from these populations were phenotyped for toxicity tolerance using relative root length (RRL) as the trait since the locus was associated with RRL under B toxicity.

2.3. General Methods

2.3.1. Preparation of Plant Material

Leaf and root tissues of barley seedlings were used as plant material to isolate RNA and DNA for microarray, gene cloning and other molecular biology studies. Harvested tissues were frozen in liquid nitrogen and stored at -80°C for further use.

2.3.1.1. Seed Surface Sterilization and Germination

The seeds of barley were surface sterilized in 3% (w/v) sodium hypochlorite including 0.05% (v/v) Tween-20 for 20 min with gentle continuous mixing on an orbital shaker at room temperature. Then they were rinsed in sterile distilled water at least five times, each lasting for 4 to 5 min. The seeds were then blotted dry on sterile filter papers. Dried seeds were placed individually in plastic tubes immersed half-way into growth solution.

2.3.1.2. Hydroponic Culture

After sowing, seedlings were hydroponically grown for 8 days in half-strength Hoagland's solution supplemented with 10 µM H_3BO_3 which is sufficient for barley. Seedlings were grown in plastic boxes filled with growth solution. Boxes were covered with black sheets of paper or plastic to provide a dark environment in the root zone. The lids of boxes were fitted with plastic tubes which were suspended through holes in the lid and immersed in solution (Figure 2.2). The tubes had narrow openings at the bottom to allow root growth into the solution. The seeds and seedlings in the tubes were supported by the semi-solid media at the tip of the tubes. The semi-solid media was prepared with distilled water and 0.6% (w/v) agar and solidified prior to setting up the boxes for hydroponic culture and seed sowing.

The lid together with tubes and seedlings was uncovered to supply fresh growth solution or apply B stress. The solution was refreshed every third day. B stress was applied at the end of 8 days of growth.

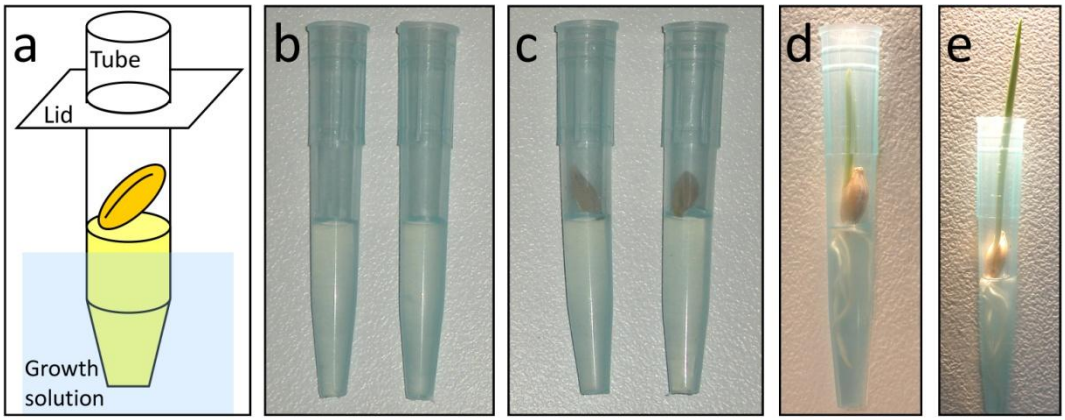

Figure 2.2. Hydroponic culturing system used for plant growth. Schematic representation of the system (a), tubes with supportive solid media (b), tubes with sown seeds (c), seedlings 3 and 4 days after seed sowing (d, e) are displayed.

2.3.1.3. Application of B Stress

Eight-day-old seedlings were subjected to treatments of B toxicity or deficiency for 5 days. Nutrient solutions were replaced with half-strength Hoagland's solutions containing either 5 mM H_3BO_3 for application of B toxicity or 0.02 µM H_3BO_3 for application of B deficiency. Control groups were maintained in fresh solutions containing 10 µM H_3BO_3.

2.3.1.4. Collection of Plant Material

Leaf tissues of control and treated seedlings were excised using scissors cleaned with 70% (v/v) ethanol or RNaseZap (Ambion). Root tissues were blotted dry before excision. Samples from 7 to 9 seedlings were pooled to minimize variation between individuals. Plant materials harvested at the end of B treatments were frozen immediately in liquid nitrogen and preserved at -80°C until used for RNA and DNA isolation.

2.3.2. RNA Preparation and Handling

2.3.2.1. Decontamination

All solutions, equipments, glass- and plastic-ware that come in contact with RNA samples or are used during isolation should be free of any contaminating RNase. Decontamination of the equipments was performed by immersion into distilled water containing 0.1% (v/v) Diethylpyrocarbonate (DEPC). The equipments in water were incubated overnight in a vertical laminar flow and autoclaved at 121°C for 20 min. The solutions were prepared with DEPC-treated, autoclaved and cooled water and were autoclaved once more if necessary. Surfaces of the benches, gloves and heat-labile equipments or materials were cleaned with RNaseZap and then wiped with tissue paper.

2.3.2.2. Total RNA Extraction

RNA isolation from plant material was performed using TRIzol reagent (Invitrogen) according to a single-step method described previously (Chomczynski and Sacchi, 1987). All incubation and centrifugation steps were performed at room temperature unless otherwise stated. Leaf

and root tissues approximately 200 – 250 mg in weight were ground to powder in pre-cooled mortars using pestles and liquid nitrogen. Powdered tissue samples were immediately transferred to pre-cooled 2 mL-tubes and suspended in 1 mL TRIzol reagent. The tubes were shaken vigorously for 15 min in a vortex mixer mounted with a block platform to permit complete dissociation of nucleoprotein complexes. Samples were then centrifuged at 21,000*g* for 5 min to precipitate insoluble material. Approximately 900 µL of the supernatant were transferred to clean 1.5 mL-tubes and 200 µL of chloroform were added on top. The mixtures were vortexed vigorously for 15 sec for complete mixing and incubated standing in a rack for 3 min. Samples were then centrifuged at 21,000*g* for 15 min at 4°C in a refrigerated centrifuge for phase separation. Approximately 450 – 500 µL of the upper aqueous phase were transferred to clean 1.5 mL-tubes and phase separation was performed once more by adding 200 µL of chloroform. The mixtures were vortexed vigorously for 15 sec and then incubated for 3 min. Samples were then centrifuged at 21,000*g* for 5 min at 4°C in a refrigerated centrifuge.

After phase separation, 400 µL of the upper phase were transferred to clean 1.5 mL-tubes. RNA precipitation was performed by adding one-volume of pre-chilled isopropanol and gentle mixing. After incubation at room temperature for 10 min, the samples were centrifuged at 21,000*g* for 10 min. The supernatant was discarded carefully and RNA often visible as a white pellet was washed with 1 mL 75% (v/v) ethanol. Samples were mixed briefly and centrifuged at 21,000*g* for 5 min. The supernatant was discarded and leftover ethanol was totally removed by brief air drying for 5 – 10 min. The pellet was not dried completely as this greatly decreased RNA solubility. Finally the RNA pellet was dissolved in 35 µL of DEPC-treated ultrapure water.

2.3.2.3. RNA Clean-up and DNase I Treatment

DNA contamination in RNA samples was removed using RNase-free DNase I (Fermentas) according to instructions of the manufacturer. In a total reaction volume of 10 µL, approximately 1 µg of total RNA was incubated at 37°C for 30 min in the presence of 1 U DNase I. The reaction was buffered with 1 µL of 10X reaction buffer (supplied by manufacturer) providing a final concentration of 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The reaction was stopped by addition of 1 µL 50 mM EDTA and subsequent

incubation at 65°C for 10 min. The reaction volumes were scaled up in case higher amounts of RNA were treated.

Ethanol-sodium acetate precipitation was performed immediately after DNase I treatment to remove impurities and concentrate the RNA samples. RNase-free solution of 3 M sodium acetate (pH 5.2) was added to RNA samples to obtain a final concentration of 0.3 M. Then three-volumes of pre-chilled molecular biology-grade pure ethanol were added. The mixtures were vortexed thoroughly and incubated overnight at -20°C for precipitation. The samples were centrifuged at 21,000*g* for 30 min at 4°C in a refrigerated centrifuge. The supernatant was discarded and RNA pellet was washed twice with 1 mL of pre-cooled 80% (v/v) ethanol. Pellet was recovered after each step of washing by centrifugation at 21,000*g* for 5 min at 4°C. The supernatant was discarded and leftover ethanol was totally removed by brief air drying for 5 – 10 min. Finally the RNA pellet was dissolved in appropriate volumes of DEPC-treated ultrapure water.

2.3.2.4. Determination of RNA Quality and Quantity

The RNA concentration was determined by recording the absorbance of diluted samples at 260 nm on a single beam spectrophotometer (UVmini 1240; Shimadzu). An optical density (OD) of 1.0 at 260 nm corresponds to a concentration of 40 µg/mL for single-stranded RNA. Alternatively, Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen), which employs a sensitive fluorescent dye, was used for quantitation of RNA in solution according to instructions of the manufacturer. Dilutions of RNA samples were incubated with Quant-iT RiboGreen RNA reagent for 5 min at room temperature and the fluorescence was recorded with NanoDrop 3300 Fluorospectrometer (Thermo Scientific). RNA concentrations of the samples were determined according to the standard curve generated using at least 5 different dilutions (1 – 10 µg/mL) of ribosomal RNA (rRNA) standard.

The ratio of OD at 260 and 280 nm (OD_{260}/OD_{280}) was used to assess the purity of RNA samples. Pure RNA preparations have ratio values of ~2.0 for OD_{260}/OD_{280}. Quality and integrity of the total RNA was checked using agarose gel electrophoresis (Section 2.3.5) which in turn was used to separate and visualize major rRNA species. Highly intact total RNA resolved into discrete bands of cytosolic, chloroplastic and mitochondrial rRNA species with

no smearing below each band. Alternatively, microfluidic analysis using the Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent) was employed to assess the integrity of RNA preparations according to instructions of the manufacturer. Electropherograms with sharp and discrete peaks of rRNA species and no additional peaks between them demonstrated integrity of total RNA samples.

2.3.3. Synthesis of Single-Stranded cDNA

Synthesis of cDNA from total RNA samples was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The kit employs an engineered version of the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT) with low RNase H activity. M-MuLV RT synthesizes cDNA at sites determined by the type of the primer used, either random hexamer primer, oligo$(dT)_{18}$ or sequence specific primers. Choice of primer type was determined according to the downstream analysis where cDNA would be used.

Reaction mixture containing 2.5 µg of total RNA and 1 – 2 µL of appropriate primer in a total volume of 12 µL was incubated at 70°C for 5 min. After chilling on ice, 4 µL of 5X reaction buffer (supplied by manufacturer), 1 µL of RiboLock™ Ribonuclease Inhibitor (20 U/µL) and 2 µL of dNTP mix (10 mM) was added to the reaction mixture. Following a brief incubation at 37°C for 5 min, 1 µL of M-MuLV RT (200 U/µL) was added making a 20 µL-mixture. The reaction mixture was incubated in a thermal cycler at 42°C for 60 min. The reaction was stopped by incubation at 70°C for 10 min. The mixture, chilled on ice, was stored at -20°C and used for downstream purposes like PCR and cloning.

2.3.4. DNA Preparation and Handling

2.3.4.1. Decontamination

All solutions and equipments that are used during isolation should be free of any contaminating DNase. Decontamination was performed by autoclaving at 121°C for 20 min. Surfaces of the benches, gloves and heat-labile equipments or materials were cleaned with 70% (v/v) ethanol and then wiped with tissue paper.

2.3.4.2. DNA Extraction Using an Anionic Detergent

Harvested leaf tissues were placed into collection tubes and left at -80°C overnight. Subsequently, plant materials were freeze-dried overnight. Tissue homogenization was done using a mixer mill MM 300 (Retsch GmbH; Haan, Germany). Incubations and centrifugations were performed at room temperature unless otherwise stated. One 3 mm stainless steel bead was placed into each tube and tissues were ground in mixer mill for 5 min at a frequency of 25 Hz (1500 min^{-1}). Steel beads were removed by slightly tilting the tubes.

Ground material was suspended in 600 µL of extraction buffer containing 0.1 M Tris-HCl (pH 7.5), 50 mM EDTA and 1.25% (w/v) SDS. The tubes were shaken thoroughly and incubated at 65°C for 30 min. Afterwards tubes were left to stand in a fridge (~4°C) for 15 min and cooled down to room temperature. After addition of 300 µL of pre-chilled 6 M ammonium acetate, samples were mixed vigorously and then placed again in a fridge for 15 min. Precipitated proteins, insoluble plant material and cell debris were removed by centrifugation at 4,000*g* for 15 min at 4°C in a refrigerated centrifuge. In a clean new tube 360 µL of isopropanol was placed and 600 µL of the supernatant from the centrifugation was added on top. The tubes were mixed thoroughly but gently and were left to stand at room temperature for 5 min to allow DNA to precipitate. Afterwards, centrifugation at 4,000*g* for 15 min was performed in order to pellet DNA. Supernatant was discarded and pellet was washed with 400 µL of 70% (v/v) ethanol. DNA pellet was recovered by centrifugation at 4,000*g* for 15 min and supernatant was discarded. After brief air drying for 10 min, the DNA pellet was suspended in 400 µL of ultrapure water. The tubes were placed in a fridge overnight to let DNA dissolve. Subsequently centrifugation at 4,000*g* for 15 min was performed to remove any insoluble material. Supernatant containing genomic DNA was transferred to clean tubes and used as template in PCR.

2.3.4.3. DNA Extraction Using a Cationic Detergent

Genomic DNA isolation from fresh leaf tissues was performed using cetyltrimethyl-ammonium bromide (CTAB) according to a previously reported method (Murray and Thompson, 1980) with minor modifications. Approximately 200 – 250 mg plant material was

ground to powder using liquid nitrogen, mortar and pestle. Ground samples were immediately transferred to pre-cooled 1.5 mL-tubes and suspended in 1 mL of pre-heated extraction buffer containing 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 0.2 % (v/v) β-mercaptoethanol and 2% (w/v) CTAB. After complete suspension of material in solution, 10 µL of RNase A (10 mg/mL) was added. The mixture was incubated at 65°C for 45 min in a circulating water bath with occasional shaking. After centrifugation at 10,000*g* for 10 min at 4°C in a refrigerated centrifuge, the supernatant was transferred to a clean 2 mL-tube and an equal volume of chloroform:isoamylalcohol (24:1, v:v) was added. After thorough mixing, phase separation was performed by centrifugation at 10,000*g* for 10 min at 4°C. The aqueous upper phase was transferred to a clean tube and mixed with an equal volume of pre-chilled isopropanol to precipitate DNA. The tubes were gently inverted couple of times and left to stand at -20°C overnight. DNA was collected by centrifugation at 5,000*g* for 5 min at 4°C. Subsequently supernatant was discarded and DNA pellet was washed with 1 mL of 70% (v/v) ethanol. DNA was recovered once again by centrifugation at 5,000*g* for 5 min at 4°C and supernatant is discarded. After air drying at room temperature for 5 – 10 min, DNA was suspended in ultrapure water or Tris-EDTA (TE) buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The isolated DNA was stored at -20°C for further use.

2.3.4.4. Quantitation of DNA

Concentrations of DNA preparations were determined using UVmini 1240 (Shimadzu), a single beam spectrophotometer or NanoDrop 1000 (Thermo Scientific), a micro-volume spectrophotometer. The absorbance at 260 nm was used to calculate concentration. An OD of 1.0 at 260 nm corresponds to a concentration of 50 µg/mL for double-stranded DNA. The ratio of OD_{260}/OD_{280} was used to assess the purity of DNA samples. Pure DNA preparations have ratio values of ~1.8. DNA quality was checked using agarose gel electrophoresis (Section 2.3.5).

2.3.5. Electrophoretic Separation of Nucleic Acid Fragments

Electrophoresis was performed to separate or isolate DNA, total RNA, PCR amplicons or nucleic acid fragments. Visualization was done with ethidium bromide, an intercalating dye, under UV illumination using a gel documentation system. Depending on the size of the

fragments to be visualized, 1 – 2% (w/v) agarose gel was prepared in 0.5X Tris-Borate-EDTA (TBE) buffer containing final working concentrations of 45 mM Tris base (pH 8.0), 45 mM Boric acid and 0.001 mM EDTA. Total RNA samples were separated in a gel prepared with 1.5% (w/v) agarose and 0.5X TBE buffer which in turn was prepared with DEPC-treated water. Ethidium bromide at a final concentration of 0.4 µg/mL (2 µL of 10 mg/mL stock solution of ethidium bromide for 100 mL of gel) was added to microwaved and subsequently cooled gel solution. Samples (2 – 10 µL) were mixed with appropriate amounts of 6X loading dye (Fermentas) and loaded to wells of solidified gel. Appropriate nucleic acid size markers (either 50 – 1,000 bp or 250 – 10,000 bp; Fermentas) were included for comparison. Electrophoresis was run at ~5V/cm for ~30 min in 0.5X TBE buffer and terminated when fragments were resolved properly. Finally image of the gel was recorded using a documentation system.

2.3.6. Recovery of DNA Fragments from Agarose Gels

Bands of fragments were excised using a clean scalpel blade on a trans-illuminator set to long-wavelength UV. The gel-slice was placed in a 1.5 mL-tube and extraction of DNA fragments was performed with QIAquick® Gel Extraction Kit (Qiagen) or NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturers' instructions. The kits employ spin-columns and centrifugations at room temperature. At the end of the procedure, the DNA retained in the column was eluted with 30 – 50 µL of either TE buffer (pH 8.0), 5 or 10 mM Tris-HCl (pH 8.5) or ultrapure water (pH 7.5 – 8.5) depending on the downstream uses of DNA.

2.3.7. Plasmid Preparation and Handling

2.3.7.1. Competent Cell Preparation

Chemically competent *E. coli* TOP10 cells were prepared using Rubidium chloride (RbCl). A single colony of TOP10 was cultured in 5 mL of liquid LB overnight at 37°C with continuous shaking at 180 rpm. This initial culture was used to inoculate 200 mL of liquid LB. Culturing at 37°C and 180 rpm was done until the bacterial suspension reached to an OD of ~0.5 at 600 nm. Bacterial cells were then chilled and strictly maintained cold on ice during all steps

of the procedure. After incubation on ice for 15 min, the bacterial solution was centrifuged at 4,000*g* for 10 min at 4°C in a refrigerated centrifuge. The bacterial pellet was re-suspended in 40 mL of pre-chilled, filter-sterilized buffer (pH 5.8) containing 100 mM RbCl, 50 mM Manganese (II) chloride, 30 mM Potassium acetate, 10 mM Calcium chloride and 15% (v/v) glycerol. After a second step of incubation on ice for 15 min, bacterial cells were collected once more as indicated above. The bacterial pellet was re-suspended in 8 mL of pre-chilled, filter-sterilized buffer (pH 6.8) containing 10 mM RbCl, 10 mM MOPS, 75 mM Calcium chloride and 15% (v/v) glycerol. After a final incubation on ice for 15 min, 100 µL-aliquots were dispensed into clean 1.5 mL-tubes and frozen immediately in liquid nitrogen. The aliquots of competent TOP10 cells were stored at -80°C for further use.

For preparation of electroporation-competent cells of *A. tumefaciens* C58C1, a single colony was cultured at 28°C and 180 rpm for 2 days in 5 mL of liquid YEB supplemented with rifampicin (100 µg/mL) and ampicillin (100 µg/mL). This starter culture was used to inoculate 400 mL of YEB medium containing mentioned antibiotics. Batch culture was incubated at 28°C and 180 rpm. When culture reached an OD of ~0.6 at 600 nm, whole suspension was chilled on ice. Bacterial culture was maintained cold on ice during the procedure. To wash the cells and get rid of all the media and salts, bacterial cells were pelleted by centrifugation at 4,000*g* for 10 min at 4°C in a pre-cooled centrifuge. Then the supernatant was discarded and pellet was completely re-suspended in 100 mL of ice-cold ultrapure water. This washing step was repeated two more times with ice-cold ultrapure water. Finally cells were collected by centrifugation at 4,000*g* for 10 min at 4°C and supernatant was removed totally. Cells were re-suspended in 5 mL of 10% (v/v) pre-chilled sterile glycerol which was prepared with ultrapure water. Competent cells of C58C1 were dispensed as 50 µL-aliquots into sterile 1.5 mL-tubes, frozen immediately in liquid nitrogen and stored at -80°C-freezer.

2.3.7.2. Introduction of Plasmids into Bacterial Cells

Transformation of chemically-competent cells of *E. coli* TOP10 in a solution containing monovalent and divalent cations was achieved by exposure to a pulse of heat-shock. Competent cells of *A. tumefaciens* C58C1 were transformed with electroporation using Gene Pulser® II Electroporation System (Bio-Rad).

An aliquot of competent TOP10 was thawed on ice. When the bacterial suspension was still icy, 10 – 50 ng (6 – 12 µL) of the plasmid to be introduced was added to suspension. The mixture was tapped gently with a finger and incubated on ice for 30 min. Then the cells were heat-shocked at 42°C for 45 sec in a water bath without shaking and immediately returned to ice and incubated for 1 min. After addition of 500 µL of S.O.C. medium, the cells were incubated at 37°C and 180 rpm for 1 h for recovery. Then 100 µL of the transformation mixture was spread on pre-warmed solid LB containing appropriate antibiotics. The plates were incubated overnight at 37°C and positive clones were selected according to colony PCR (Section 2.3.10) where fragments of the plasmid were amplified.

An aliquot of electroporation-competent C58C1 cells were thawed on ice. Approximately 1 µg (4 – 8 µL) of water eluted-plasmid DNA was added to bacterial suspension. The mixture was tapped gently with a finger, incubated on ice for 5 min and then loaded to pre-cooled 0.1 cm-electroporation cuvettes (Bio-Rad). Gene Pulser II electroporation system was set to produce pulses of 25 µF, 2.4 kV and 200 Ω with 5 msec pulse length. After pulsing, 1 mL of liquid YEB medium was added to electroporation cuvette and the mixture was immediately transferred to a 2 mL-tube. Recovery was performed by culturing at 28°C and 180 rpm for 4 h. Then cells were collected by centrifugation at 3,000*g* for 10 min and re-suspended in 100 µL of media which was subsequently spread on pre-warmed solid YEB containing appropriate antibiotics. The plates were incubated at 28°C for 2 – 3 days and positive clones were selected according to colony PCR (Section 2.3.10).

2.3.7.3. Plasmid Isolation from Bacterial Cells

Plasmids were isolated from *E. coli* and *A. tumefaciens* cells using QIAprep® Spin Miniprep Kit (Qiagen) according to procedures provided by the manufacturer. At the end of the procedure the plasmid DNA retained in the column was eluted with 30 – 50 µL of Buffer EB (10 mM Tris-HCl, pH 8.5; provided by the supplier) or ultrapure water.

2.3.8. Introduction of DNA Fragments into Plasmids Using Gateway Cloning System

Fragments amplified from DNA or cDNA were introduced into appropriate entry vectors using Gateway cloning system. Gateway cloning employs Topoisomerase I which is covalently attached at a tyrosyl residue (Tyr-274) to the 3' phosphate of a deoxythymidine through a phosphotyrosyl bond between linearized vector DNA and enzyme. Free 5' hydroxyl group of DNA fragments to be cloned attack phosphotyrosyl bond, breaking it and releasing the enzyme. Simultaneously DNA fragment and the vector are covalently linked by a phosphodiester bond which is formed by Topoisomerase I.

Entry vectors used in this study included pCR8/GW/TOPO and pENTR/D-TOPO, which use TA and directional cloning, respectively. Cloning reactions were performed according to the procedures supplied by the manufacturer. Briefly, 6 µL of reaction mixture containing 4 µL of fresh, purified PCR amplicon, 1 µL of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$; provided by the supplier) and 1 µL of linearized vector was incubated at room temperature for 30 min. Afterwards total reaction mixture was chilled on ice and used for transformation of *E. coli* TOP10 (Section 2.3.7.2). Transformed cells were selected on solid LB media containing appropriate antibiotic determined according to the resistance gene present on the entry vector. Additionally positive clones were verified with colony PCR (Section 2.3.10). Recombinant entry vectors carrying GOI was isolated from TOP10 (Section 2.3.7.3) and sequenced (Section 2.3.11) for verification of sequence of the insert. Subsequently entry vectors were used in recombination reactions to move GOI into destination vectors (Section 2.3.9).

TA cloning required fragment amplification using *Taq* polymerase, which introduced 3' adenine (A) overhangs to both strands in a final extension step of PCR. When *Pfu* polymerase, a proofreading polymerase, was used for fragment amplification, the amplicon would have blunt ends. To make the amplicon suitable for TA cloning, addition of A to blunt-ended fragments was performed using *Taq* polymerase. Initially, the product from *Pfu* polymerase directed-PCR was purified with QIAquick® PCR Purification Kit (Qiagen). Subsequently, 7.1 µL of purified PCR product was mixed with 0.8 µL of 2.5 mM dATP, 1 µL of 10X *Taq* buffer (provided with *Taq* polymerase), 0.6 µL of 25 mM $MgCl_2$ and 0.5 µL of *Taq*

polymerase (5 U/µL; Fermentas) in a 0.2 mL-tube. The mixture was incubated in a thermal cycler at 94°C for 2 min and then at 72°C for 20 min. The product was used for TA cloning.

Directional cloning required inclusion of a 4 bp-sequence (CACC) at the 5' end of the forward primer used for amplification of DNA fragment. Moreover, *Pfu* polymerase was used in preceding PCR.

2.3.9. *In Vitro* Recombination Using Gateway Cloning System

Gateway cloning system employs site specific *in vitro* recombination between *att* sites to move GOI from entry vector into destination vector. Expression clone is generated by LR recombination between *att*L sites on the entry vector and *att*R sites on the destination vector. Briefly, 1 µL of entry clone (~150 ng/µL), 1 µL of destination vector (~150 ng/µL), 6 µL of TE buffer (pH 8.0) and 2 µL of LR Clonase™ II Enzyme Mix (Invitrogen) were added to a 0.2 mL-tube. The reaction mixture was incubated in a thermal cycler at 25°C for 1 h. To terminate the reaction 1 µL of Proteinase K solution (2 µg/µL; provided by the supplier) was added to reaction mixture and samples were incubated at 37°C for 10 min.

The reaction mixture was chilled on ice and used directly for transformation of TOP10 cells (Section 2.3.7.2). Positive selection was done on solid LB media containing appropriate antibiotic determined according to the resistance gene present on the destination vector. Negative selection was done on solid LB media containing 12.5 µg/mL chloramphenicol. True recombinant clones could not confer resistance to chloramphenicol since resistance gene for chloramphenicol was replaced by GOI during recombination. Moreover, positive clones were verified with colony PCR (Section 2.3.10). Recombinant expression vectors carrying GOI was isolated from TOP10 (Section 2.3.7.3) and subsequently introduced into *A. tumefaciens* C58C1 (Section 2.3.7.2) for plant transformation and expression of GOI in plant cells or introduced into *S. cerevisiae* INVSc2 (Section 2.8.1.3) for heterologous expression in yeast.

2.3.10. Conventional PCR and Variants

PCR and its variants were used in this study for various purposes including gene cloning, amplification of DNA or cDNA fragments, generating hybridization probes, dye-terminator sequencing, screening transformed bacterial colonies, genome walking and mapping with genetic markers.

The reagents used in PCR and their final concentrations, unless otherwise stated, are provided in Table 2.1. DNA polymerase used in PCR varied depending on the purpose. Gene cloning was performed with *Pfu* polymerase (Fermentas), whereas routine fragment amplification was carried out with *Taq* polymerase (Fermentas) or Immolase™ (Bioline; London, UK). Choice of buffer was determined according to the DNA polymerase used. Solution of $MgCl_2$ was replaced with $MgSO_4$ when *Pfu* polymerase was employed.

Thermal cycling conditions, unless otherwise stated, are presented in Table 2.2. Duration for initial denaturation was increased to 8 min when Immolase was employed. Annealing temperature was set to 2 – 3°C below the melting temperature (Tm) of the primers used. The Tm values of primers were calculated using Vector NTI® Software (Invitrogen) which was also used for primer design. Duration for extension step was determined according to the length of the amplicon to be amplified in PCR. Typically 1,000 bp are polymerized in 1 min by DNA polymerases.

Transformed bacterial cells were screened with colony PCR, where a fragment of plasmid DNA was amplified in 20 µL-reactions with volumes of ingredients reduced proportionally. Initially an individual colony, picked with a sterile pipette tip, was re-suspended in 50 µL of ultrapure water. Then the suspension was used as template in PCR.

Products from PCR were separated and visualized using agarose gel electrophoresis (Section 2.3.5). Amplicons were isolated either by recovery from agarose gel (Section 2.3.6) or by direct purification of PCR products. PCR clean-up was performed using QIAquick® PCR Purification Kit (Qiagen) or NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturers' instructions.

Table 2.1. Reagents used in PCR.

Reagent	Stock concentration	Final concentration	Volume (µL)
GS primer I *	5 µM	0.25 µM	2.5
GS primer II *	5 µM	0.25 µM	2.5
dNTP mix	2.5 mM	0.2 mM	4
Buffer	10 X	1 X	5
$MgCl_2$	25 mM	1.5 mM	3
DNA polymerase **	5 U/µL	0.05 U/µL	0.5
DMSO	100 %	3 %	1.5
Template	~5 ng/µL	variable	2
Ultrapure water			29
Total			**50**

* Gene-specific (GS) primers I and II were sense and antisense (forward and reverse) primers for the fragment to be amplified.

** DNA polymerase used was either *Taq* polymerase, *Pfu* polymerase or Immolase.

Table 2.2. Thermal cycling conditions used in PCR.

		Temperature (°C)	Duration	Number of cycles
Initial denaturation		96	3 – 8 min	1
Amplification	Denaturation	94	20 sec	30 – 35
	Annealing	50 – 64	20 sec	
	Extension	72	1 – 3 min	
Final extension		72	10 min	1

2.3.11. Sequencing Reactions

Nucleotide sequencing was performed when appropriate during gene cloning, heterologous expression, mapping and other molecular studies. Chain-termination based sequencing reactions (Sanger *et al.*, 1977) comprised of cycle-sequencing (cycling) and capillary electrophoresis. PCR amplicons and plasmids, after purification (Section 2.3.6) and quantitation (Section 2.3.4.4), were used as template in cycling reactions. Template amount varied depending on the concentration and size of the fragment to be sequenced. For PCR amplicons of length 100 – 1,000 bp, 1 – 10 ng of fragment (1 ng/100 bp) were used as template, whereas for double-stranded plasmids 200 – 400 ng of DNA were used. In 0.2 mL-

tubes or wells of a 96-well plate, template was mixed with 1 µL of 5 µM primer, 1 µL of BigDye® Terminator (BDT) v3.1 dye (Applied Biosystems) and 2 µL of 5X BDT v3.1 sequencing buffer in a final volume of 10 µL. Tubes or plates were incubated in a thermal cycler for cycle-sequencing. Thermal cycling conditions are provided in Table 2.3. Extension products were purified using $MgSO_4$ solution containing 0.2 mM $MgSO_4$ and 70% (v/v) ethanol. Tubes or plates from cycling were spun to collect the contents at the bottom and 75 µL of freshly prepared $MgSO_4$ solution was added to each tube or well. The mixtures were vortexed thoroughly and allowed to sit in dark at room temperature for 15 min. After precipitation in dark, centrifugation at room temperature was performed to collect labeled extension products. Tubes were centrifuged at 18,000*g*, whereas plates were centrifuged at 2,000*g* for 15 min in a bench-top centrifuge. After discarding the supernatant by simply inverting, plates were centrifuged upside down on clean tissue paper for 1 min at 400*g*. Tubes were tipped on tissue paper. The tubes and plates were dried at 37°C for 5 min in dark and sent to AGRF for capillary separation on ABI 3730*xl* sequencing platform (Applied Biosystems). Sequence reads were handled with ContigExpress and AlignX modules of Vector NTI.

Table 2.3. Thermal cycling conditions used in cycle-sequencing.

		Temperature (°C)	Duration	Number of cycles
Initial denaturation		96	1 min	1
Amplification	Denaturation	96	10 sec	30
	Annealing	50	20 sec	
	Extension	60	4 min	

2.4. Microarray Analysis

Global profiling of barley transcriptome under B toxicity or deficiency at seedling stage was performed using leaf and root tissues of Turkish cultivars Tarm-92 and Hamidiye, which were reported as tolerant and sensitive to B toxicity, respectively. GeneChip Barley Genome Array was utilized for determination of changes in expression levels associated with B toxicity or deficiency.

2.4.1. Experimental Design

Seedlings were grown in hydroponic cultures (Section 2.3.1.2) for 8 days with a supplement of 10 µM H_3BO_3 which is sufficient for barley. At the end of 8 days, nutrient solutions were replaced with solutions containing either 5 or 10 mM H_3BO_3 for application of B toxicity or 0.02 µM H_3BO_3 for B deficiency. Treatments of B lasted for another 5 days. Control groups were maintained in fresh solutions containing sufficient amounts of H_3BO_3. Leaf and root tissues harvested at the end of B treatments were used to isolate total RNA (Section 2.3.2.2), which in turn was used as starting material for microarray experiments.

Microarray analysis was designed to investigate both inter- and intra-varietal differences in gene expression of Tarm-92 and Hamidiye under B toxicity and deficiency. The treatments applied and comparisons made are summarized in Table 2.4. Intra-varietal comparisons included investigations of expression differences in Hamidiye under B toxicity at two toxic levels of B, with application of 5 and 10 mM H_3BO_3. Gene expression levels were examined in Tarm-92 under B deficiency and toxicity to make a second intra-varietal comparison. Inter-varietal comparison, on the other hand, was performed to reveal differences of expression between Tarm-92 and Hamidiye under B toxicity applied as 5 mM H_3BO_3.

Table 2.4. Experimental design and comparisons made in microarray analysis.

Cultivar	Treatment	H_3BO_3	Abbreviation
Intra-varietal comparison of B toxicity			
Hamidiye	Control	10 µM	Ha-C
	B toxicity	5 mM	Ha-T
	B toxicity	10 mM	Ha-TT
Intra-varietal comparison of B deficiency and toxicity			
Tarm-92	Control	10 µM	Ta-C
	B deficiency	0.02 µM	Ta-D
	B toxicity	5 mM	Ta-T
Inter-varietal comparison of cultivars under B toxicity			
Hamidiye	Control	10 µM	Ha-C
	B toxicity	5 mM	Ha-T
Tarm-92	Control	10 µM	Ta-C
	B toxicity	5 mM	Ta-T

2.4.2. Target Preparation and Labeling

Target preparation for GeneChip arrays, single channel-oligonucleotide arrays, includes series of molecular tools. Total RNA isolated from cells, tissues or organs of samples is first reverse transcribed using T7 promoter-oligo(dT) primer fusion in the first strand cDNA synthesis reaction. Second strand cDNA synthesis generates double-stranded cDNA carrying a transcriptional start site for T7 RNA polymerase. During *in vitro* transcription double-stranded cDNA is used as a template and biotin labeled nucleotides are incorporated into the newly synthesized RNA molecules. Resulting biotin labeled RNA from each target sample is hybridized to a separate array. Sample processing used in GeneChip platform is summarized in Figure 2.3.

Total RNA preparations, precipitated with ethanol and sodium acetate to remove impurities, were used for target preparation. Amplified RNA (aRNA) synthesis and labeling was performed according to GeneChip Expression Analysis Technical Manual (Affymetrix). Exactly 15 µg of total RNA were used to generate double-stranded cDNA by reverse transcription, using One-Cycle cDNA Synthesis Kit (Affymetrix). Initially poly-A control mixture (provided by the supplier) was spiked in at a defined dilution to each sample to monitor efficiency of target preparation and labeling. Subsequently, mixture of total RNA and poly-A control was used in first strand cDNA synthesis which was driven by SuperScript II at 42°C for 1 h. Second strand cDNA was synthesized by *E. coli* DNA polymerase I with the aid of *E. coli* DNA ligase and RNase H at 16°C for 2 h. End filling and pruning was performed with T4 DNA polymerase at 16°C for 5 min. The reaction was terminated with addition of 10 µL of 0.5 M EDTA. After clean-up of double-stranded cDNA using spin-columns of Sample Clean-up Module (Affymetrix), *in vitro* transcription (IVT) reaction was employed to generate labeled aRNA.

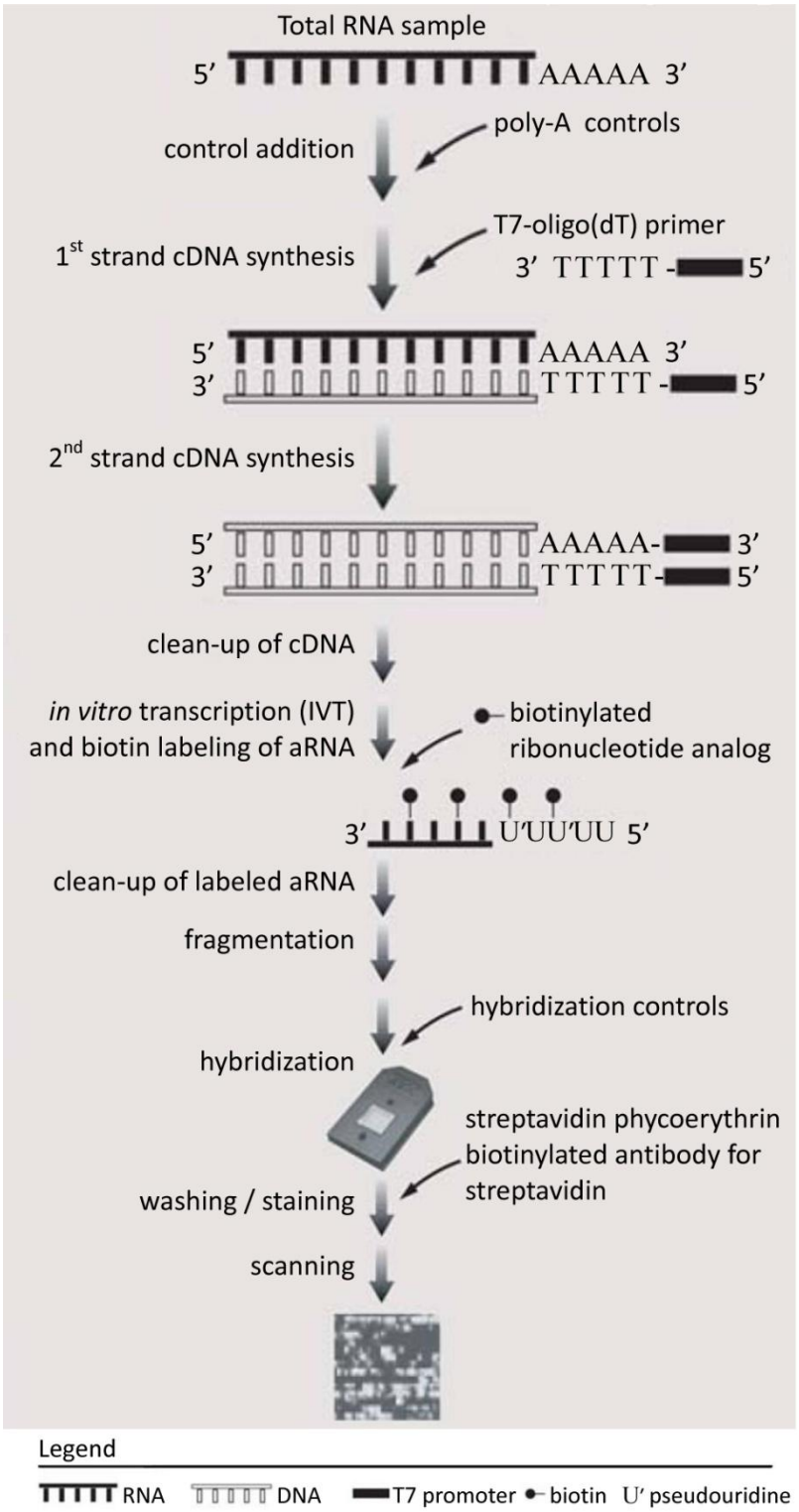

Figure 2.3. Sample processing used in global expression profiling. Technical steps such as target preparation, hybridization and scanning are displayed. (Adapted from GeneChip Expression Analysis Technical Manual)

RNA amplification and simultaneous biotin labeling was performed using GeneChip IVT Labeling Kit (Affymetrix) in a thermal cycler at 37°C for 16 h. Enzyme mix used at this step contained T7 RNA polymerase which directed transcription of cDNA *in vitro*. Then biotin-labeled aRNA was purified using spin-columns of Sample Clean-up Module. Yield and quantity of labeled aRNA was determined with spectrophotometric analysis (Section 2.3.2.4). Size distribution of labeled target was estimated using agarose gel electrophoresis (Section 2.3.5) or microfluidics using Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit according to instructions defined by manufacturer. Labeled aRNA sample showed a wide distribution of sizes from 250 – 5500 nucleotides (nt) with most of the population having sizes between 600 – 1200 nt.

Following quantitation and quality-check with electrophoresis or microfluidics, 20 µg of labeled aRNA was fragmented in a total volume of 40 µL by metal-induced hydrolysis at 94°C for 35 min using a Fragmentation Buffer obtained from Affymetrix. The efficiency of the fragmentation was checked by analysis of sizes of the fragments on an agarose gel (Section 2.3.5) or on a microfluidics chip. Fragmented aRNA sample showed a distribution of fragments that are sized between 35 – 200 nt with an accumulation around 100 – 120 nt.

2.4.3. Array Hybridization, Washing and Staining

Fragmented aRNA sample (15 µg) was used to prepare 300 µL of hybridization cocktail (Table 2.5). The hybridization cocktail was then incubated at 99°C for 5 min and at 45°C for 10 min and subsequently centrifuged at 18,000*g* for 5 min to remove any insoluble material. Meanwhile the array, equilibrated to room temperature, was filled with 1X hybridization buffer (Table 2.5) and incubated at 45°C for 10 min with rotation. Afterwards the buffer was removed from the array, and 200 µL of hybridization cocktail was loaded to array. Samples were hybridized for 16 h to Barley Genome Array in Hybridization Oven 640 (Affymetrix) at 45 °C and 60 rpm.

Table 2.5. Hybridization cocktail prepared for GeneChip Barley Genome Array.

Reagent	Stock concentration		Final concentration		Volume (µL)
Fragmented aRNA (15 µg)	0.5	µg/µL	0.05	µg/µL	30
Control oligonucleotide B2	3	nM	50	pM	5
Herring sperm DNA	10	mg/mL	0.1	mg/mL	3
BSA	50	mg/mL	0.5	mg/mL	3
DMSO	100	%	10	%	30
Eukaryotic hybridization controls *	20	X	1	X	15
Hybridization buffer **	2	X	1	X	150
Ultra pure water					64
Total					**300**
* Eukaryotic hybridization controls					
bioB	30	pM	1.5	pM	
bioC	100	pM	5	pM	
bioD	500	pM	25	pM	
cre	2	nM	100	pM	
** Hybridization buffer					
MES	200	mM	100	mM	
NaCl	2	M	1	M	
EDTA	40	mM	20	mM	
Tween-20	0.02	%	0.01	%	

Target binding to probes on the array was detected by streptavidin-conjugated phycoerythrin (SAPE) after series of washing and staining steps. Post-hybridization washes were followed by staining of target aRNA hybridized to probes with SAPE. The signal was amplified by a second stain using biotinylated antibody for streptavidin, followed by a third staining step with SAPE. Washing and staining was performed in Fluidics Station 450 (Affymetrix) according to standard protocol for Barley Genome Array. Fluidics Station 450 was controlled by GeneChip Operating Software 1.4 (GCOS; Affymetrix). At the end of the run, arrays were checked visually for large air bubbles. Arrays with no bubbles were immediately scanned. In case bubbles were present, the holding buffer was removed and replaced with fresh buffer either manually or in Fluidics Station.

2.4.4. Array Scan

Arrays were scanned with GeneChip Scanner 3000 (Affymetrix) according to instructions of the manufacturer. GeneChip Scanner 3000 was run by GCOS. Scanning of the array at a specific preset wavelength provided signal intensities for probes and probe sets. Ratios of signal intensities for a single probe set from two different arrays, calculated *in silico*, provided relative mRNA abundance for the genes represented on the array. Hybridization, wash and array scan were performed at the Middle East Technical University Central Laboratory.

2.4.5. Quality Control of Array Data and Masking

Besides instrument control, GCOS was also used for initial analysis and quality control of data. The software was employed to calculate signal intensity values for probe sets using algorithms specific for GeneChip arrays. MAS 5.0 probe set algorithms integrated into GCOS automatically assigned flags (calls) such as present (P), absent (A) or marginal (M) to probe sets according to signal intensities of PM and MM probes of the probe set.

Data quality was evaluated according to report files (.RPT files) generated by GCOS based on MAS 5.0. Reports summarized critical metrics such as noise, signal values for background, average signal for probe sets flagged P, A, or M, signal values for spiked-in poly-A controls and spiked-in hybridization controls, scaling factor, and few others. Averages of background signal values were less than 100 and were lower than the average signals for probe sets flagged P. Average values of noise were from 1 – 5 and similar for different array scans. Percentages of probe sets with P calls were 40 – 70% which indicated a good quality target, highly efficient target preparation and high quality array scan. All spike-in controls *bioB*, *bioC*, *bioD* and *cre* had P calls, which indicated successful hybridizations. Similarly, poly-A controls *dap*, *lys*, *phe*, *thr*, and *trp* had P calls, which indicated efficient target preparation. Moreover, scaling factor values were less than 3 and similar among hybridizations within the project. Scaling factor and percentage of probe sets with P calls were considered important quality control criteria (Hoffman *et al.*, 2004). Though these values were not set in stone, they presented an unbiased evaluation of quality of array data.

After evaluation of metrics in .RPT files for quality, scanned images were visually inspected. The border around the array, checkerboard corners, central plus sign, the GeneChip array name, and control regions in the center was checked for successful, high quality hybridization. Spiked-in control oligonucleotide B2 hybridized probes located in these areas to form the patterns. All images displayed expected patterns. Moreover, any defects, areas of high background or areas of low signal intensity were checked for hybridization uniformity. Small defects, caused by foreign insoluble material or air bubbles, were masked since they were less than 1% of the total probes for the array. Masking was done using GCOS. Signal intensities from defected area were excluded as outliers without affecting the quality of data generated. After quality control, all measured signal intensities of probes from hybridizations (.CEL files) were used for data analysis.

2.5. Microarray Data Analysis

Data analysis comprises two main steps, generation of normalized signal intensities for each transcript on array and subsequent statistical analysis of differences between arrays. In the first step algorithms use signal intensities from all, or just PM, probes within a probe set and derive a single signal which provides relative mRNA abundance for the transcript represented on the array. The second step involves use of statistical and bioinformatics methods to reveal and identify subsets of data from all arrays. Finally tools for data visualization are used to interpret biologically important data.

2.5.1. Data Processing and Normalization

Signal intensity values computed as .CEL files were processed and normalized using Robust Multiarray Analysis (RMA), integrated into GeneSpring GX 11.0 (Agilent). RMA is a summarization algorithm performing quantile normalization over multiple arrays using only PM probes of probe sets. The algorithm includes probe-specific background correction, normalization across all arrays in a project, and median polishing (Irizarry *et al.*, 2003). Baseline was transformed to median of all hybridizations. After signal derivation for probe sets, signal-dependent filtering and statistical methods were used to obtain subsets of data.

2.5.2. Principal Component Analysis

The main problem encountered by statistical methods in microarray data analysis is the multi-dimensionality of the data. Microarray experiments measure expression of thousands of genes across different conditions or individuals. Every transcript is potentially related to every other transcript indicating a large number of possible associations. Moreover expression levels of each transcript determined by different arrays under different conditions or in different individuals increase the complexity exponentially. Therefore it is impossible to derive a conclusion on relationship between genes or conditions. The consensus is to reduce dimensions into 2 or 3 to visualize trends or relations. Principal Component Analysis (PCA) integrated into GeneSpring GX was used in this work, to reduce data dimensionality. Mean-centered PCA on conditions was performed for detection of similarity or correlation between arrays, discriminated by major trends in the data.

2.5.3. Filtering and Statistical Data Analysis

After RMA preprocessing and summarization, initial filtering according to signal intensities was performed to reduce the number of probe sets. All probe sets were filtered by percentile, based on raw signal intensities. Probe sets with intensity values higher than the 20^{th} percentile in at least one out of all hybridizations were retained. Statistical analyses were performed with the normalized signal intensities of the remaining probe sets.

Analysis of variance (ANOVA) at $P < 0.05$ – with asymptotic P-value computation – was used for statistical analysis of data. Benjamini Hochberg false discovery rate (FDR) multiple testing corrections were performed to correct P-values (Benjamini and Hochberg, 1995). Fold change of at least 2 was considered as an indication of differential expression. Comparisons were performed between data of control groups and each of treated groups. Regulation was determined according to expression under control conditions.

2.5.4. Clustering

Hierarchical clustering on conditions and transcripts were performed to build clusters or groups. Clustering based on signal intensities of transcripts were used to discover patterns or

reveal similarities or differences in expression. Euclidean distances were calculated using centroid linkage rule.

2.5.5. Probe Set Annotation

Target sequences of probe sets were obtained from NetAffx™ Analysis Center (Affymetrix) and the Plant Expression Database (PLEXdb; http://plexdb.org). The probe sets that showed differential expression under treatments were annotated using HarvEST:Barley (version 1.83, assembly 35; http://harvest.ucr.edu). PLEXdb was used to annotate probe sets, find out orthologs in model genomes such as rice (*Oryza sativa*), and predict gene functions (Shen *et al.*, 2005). Moreover, array data was uploaded to PLEXdb and the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) for open access for research community.

2.5.6. Functional Analysis Using MapMan

Differentially regulated genes were displayed visually in the context of existing diagrams of metabolic pathways and processes using MapMan 3.5.1 software (Thimm *et al.*, 2004). The mapping file was Hvu_Affy 1.1. Probe sets, identifiers used in GeneChip platform, were mapped to hierarchical categories, BINs and subBINs. Experimental data was organized and displayed onto diagrams of processes.

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

Expression profiles obtained using microarray analyses were validated by two-step real-time reverse transcriptase PCR (RT-PCR). The entire process is known as two-step, since reverse transcription and real-time PCR are carried out in separate tubes. Real-time RT-PCR allows accurate quantitation of starting amounts of an amplicon. Usually, the amount of product is directly related to the fluorescence of a reporter dye such as SYBR Green. SYBR Green binds to double-stranded DNA without any sequence specificity and fluoresces only when bound. Total RNA samples used for array hybridizations were used as starting material for real-time RT-PCR analyses and relative quantitation was performed to validate microarray data.

2.6.1. Primer Design and Validation

Probe sets used for real-time RT-PCR validation included those representing genes which showed significant regulation or no response upon B treatment. Primer pairs were designed using Vector NTI to amplify a region of target sequence. Forward primers were positioned in the coding regions and reverse primers in the 3' untranslated regions (UTRs). Barley genes encoding for 18S rRNA (*Hv18SrRNA*) and GAPDH (*HvGAPDH*) were used as house-keeping controls and were used in quantitation of relative amounts of genes investigated with real-time RT-PCR. Sequences of primer pairs along with Tm values and sizes of amplicons are given in Table 2.6.

Co-amplification of non-specific secondary products such as primer-dimers hugely affects the accurate quantitation in real-time PCR, especially when SYBR Green is employed for quantitation. Therefore, conventional PCR and subsequent agarose gel electrophoresis were employed to validate primer pairs and to confirm amplification of a single gene-specific amplicon without any non-specific secondary products.

Table 2.6. Primer sequences for the probe sets subjected to validation by real-time RT-PCR.

Probe set ID / Gene name	Direction	Sequence (5' – 3')	Tm (°C)	Amplicon size (bp)
Contig3239_at	Forward	CGCTCTTCGCCTCTGACTTTGTGAC	62.2	335
	Reverse	TAGAGGATTGCATGCACACGAGCTG	62.2	
Contig3112_at	Forward	CTTCAGGGGCTCGTGGCTCATCATC	65.6	326
	Reverse	GGAAACATCGCCGAGACAGTTCATC	61.7	
Contig3097_at	Forward	GGTTGAGTTCACCGGCGTCACC	62.2	220
	Reverse	GCTGCGAAGCAACCGAACAAGA	61.2	
Contig2209_at	Forward	CCAGAGCTACGCCAACCAGAGGATC	63.0	382
	Reverse	CGTGAGGAACGAGGGACTACTGGAC	61.0	
Contig13632_at	Forward	CTTGGGCTGCTCCTGGGTCTTG	61.7	231
	Reverse	GAACAATCTGGCTTGCCCCACA	60.6	
Contig2113_at	Forward	CAATCTGGACGTGTCGACCCCTTAC	61.5	374
	Reverse	GGCCTTTATGGCTTTGCACATTGAC	61.5	
Hv18SrRNA	Forward	CTGCCAGTAGTCATATGCTTGTCT	52.3	450
	Reverse	CCCCGTGTCAGGATTGG	51.3	
HvGAPDH	Forward	GTGAGGCTGGTGCTGATTACG	54.7	198
	Reverse	TGGTGCAGCTAGCATTTGAGAC	54.6	

Total RNA samples were pooled by mixing 1 µL of each in a clean new tube. The concentration of pooled RNA was determined as described elsewhere (Section 2.3.2.4). Pooled RNA was used to synthesize pooled cDNA (Section 2.6.2) which was used as template in conventional PCR (Section 2.3.10) to validate primer pairs. For specific amplification of fragments of target sequences represented by probe sets, 2 µL of pooled cDNA containing 10 ng/µL of initial RNA was used as template in a 20 µL-reaction. Concentration of $MgCl_2$ was increased to 2.5 mM since it was the concentration used in real-time PCR. In the 35 cycles-amplification step, annealing temperature was set to 60°C and duration of extension step was set to 30 sec. Other reagents and details of cycling conditions employed have been presented elsewhere (Section 2.3.10).

Amplified PCR products were separated on 2% (w/v) agarose gel (Section 2.3.5) to visually check presence of a single amplicon. The amplicons were extracted from gel (Section 2.3.6) and sequenced (Section 2.3.11) for verification. The primer pairs which produced single gene-specific amplicons were selected to be used in real-time RT-PCR.

2.6.2. Reverse Transcription for Real-Time PCR

Total RNA preparations used for array hybridizations were used as starting material for real-time RT-PCR analyses. Possible contaminations of genomic DNA and impurities such as salts were removed by DNase I treatment followed by ethanol precipitation (Section 2.3.2.3). Single-stranded cDNA synthesis was performed using QuantiTect® Reverse Transcription Kit (Qiagen) according to protocols provided by manufacturer.

At the initial step of cDNA synthesis, 1 µg of total RNA was mixed with 2 µL of 7X gDNA Wipeout Buffer (provided by manufacturer) in a total volume of 14 µL. The mixture was incubated at 42°C for 2 min and chilled on ice. Subsequently, 4 µL of 5X Quantiscript RT Buffer, 1 µL of RT Primer Mix and 1 µL of Quantiscript RT was added to samples. All components were provided with the kit by the manufacturer. After incubation in a thermal cycler at 42°C for 15 min, the reaction was stopped by inactivation of Quantiscript RT at 95°C for 3 min. Reverse transcription reaction mixtures were finally diluted 10 times with RNase-free ultrapure water and aliquots of 200 µL-cDNA preparations containing 5 ng/µL of initial RNA were used in real-time PCR.

2.6.3. Real-Time PCR

Real-time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) and fluorescence measurements were done with Rotor-Gene Q (Qiagen) real-time cycler according to instructions of the manufacturer. The reagents and thermal cycling conditions used in real-time RT-PCR are provided in Table 2.7 and Table 2.8, respectively. Relative quantitation of target sequences was performed by normalization with quantity of an endogenous reference gene, *HvGAPDH*. The fragments of target and reference genes were amplified from the same sample and normalized values of initial quantities of target were determined.

Table 2.7. Reagents used in real-time RT-PCR.

Reagent	Stock concentration	Final concentration	Volume (µL)
GS primer I *	5 µM	0.3 µM	0.6
GS primer II *	5 µM	0.3 µM	0.6
QuantiTect SYBR Green PCR Master Mix **	2 X	1 X	5
Template cDNA ***	5 ng/µL	10 ng‡	2
RNase-free water			1.8
Total			**10**

* Gene-specific (GS) primers I and II were sense and antisense (forward and reverse) primers for the fragment to be amplified.
** QuantiTect SYBR Green PCR Master Mix contains HotStarTaq DNA polymerase, QuantiTect SYBR Green PCR buffer, fluorescent dye SYBR Green I, KCl, $(NH_4)_2SO_4$ and $MgCl_2$ providing a final concentration of 2.5 mM $MgCl_2$ in the reaction mixture.
*** Concentrations of template cDNA are indicated as concentrations of initial total RNA used for reverse transcription prior to real-time PCR.
‡ Final concentration of template cDNA is indicated as total amount per reaction.

Table 2.8. Thermal cycling conditions used in real-time RT-PCR.

		Temperature (°C)	Duration	Number of cycles	Fluorescence measurement
Initial enzyme activation		95	10 min	1	
Quantitation	Denaturation	95	30 sec	35	
	Annealing	60	30 sec		
	Extension	72	30 sec		Green channel
Melting curve (1°C/step)		50 – 99	5 sec/°C	1	Green channel

2.6.4. Generation of Standard Curves

The relative quantitation procedure differed depending on whether the target and the endogenous reference gene were amplified with comparable or different efficiencies. Amplification efficiencies were determined by generating standard curves with a dilution series of pooled cDNA. Real-time PCR was performed with dilutions of pooled cDNA containing 10, 1, 0.1, 0.01 and 0.001 ng of initial RNA using primer pairs for selected target sequences and reference house-keeping gene. Threshold cycle (C_T) values obtained were used to construct standard curves for each target sequence and house-keeping gene. Standard curves were generated by plotting C_T values against the logarithm ($\log_{10}$) of input amount of dilutions of template. The slopes of standard curves were used to calculate amplification efficiencies in real-time PCR. A slope of -3.322 indicated that the PCR has an efficiency of 1, or 100%, and the amount of PCR product doubled during each cycle. Efficiencies of 80 – 105%, corresponding to slopes of -3.917 – -3.208, were accepted as an indication of efficient amplification in real-time PCR.

For comparison of amplification efficiencies of target sequences and house-keeping gene, differences between C_T values of target and that of reference (ΔC_T) were plotted against the logarithm ($\log_{10}$) of input amount of template dilutions. Amplification efficiencies were comparable if the slope of the resulting straight line was < 0.1.

2.6.5. Relative Quantitation

Real-time PCR (Section 2.6.3) was used to determine C_T values of amplification of fragments from target sequences and house-keeping reference gene in cDNA samples which were synthesized (Section 2.6.2) from total RNA samples used for microarray analyses. The reactions were performed with 3 biological and 3 technical replicates. At least 2 no-template control (NTC) reactions were included in every run. The amounts of target and reference in the samples were calculated using their C_T values and the corresponding standard curves. The amount of target was divided by the amount of reference to calculate the normalized amount of target sequence. The average of replicates was calculated and log-transformed ($\log_2$). The log-transformed relative expression values were compared with those obtained by microarray analysis.

2.7. Cloning of 3H B Tolerance Gene in Barley

In barley and wheat, QTL for tolerance to B toxicity and genes coding for B transporters or channel proteins have been reported (Schnurbusch *et al.*, 2010a). Major QTL on 2H, 3H, 4H and 6H were identified in a Clipper X Sahara F1-derived doubled haploid (DH) mapping population of barley (Jefferies *et al.*, 1999). Moreover, *HvBot1* and *HvNIP2;1*, which were genetically mapped to QTL on 4H and 6H, respectively, have been characterized in barley (Sutton *et al.*, 2007; Schnurbusch *et al.*, 2010b). However nothing is known about genes mapping to QTL on 2H and 3H. A candidate gene approach was employed in this study to clone 3H B tolerance gene in barley.

2.7.1. Available EST Sequences and Primer Design

Three probe sets of Barley Genome Array represent transcripts which show high sequence similarity to putative B transporters in *O. sativa* and *A. thaliana* genomes. Details of these three probe sets and annotations based on BLAST are listed in Table 2.9. Additionally, results of BLAST on other organisms in public databases and target sequences of probe sets are provided in Appendix E. Other available barley ESTs showing similarity to B transporters have not been printed on Barley Genome Array. Moreover, numbers of B transporters or channel proteins in barley genome are not known exactly.

Target sequences of Contig14139_at and Contig21126_at showed high similarity to B transporter gene located on chromosome 1 of *O. sativa*, whereas Contig19634_at showed similarity to B transporter gene on chromosome 12. Therefore, barley genes represented by Contig14139_at and Contig21126_at were transitorily named *HvBor1a* and *HvBor1b*, respectively, whereas Contig19634_at was named *HvBor12*. Among the three genes, *HvBor1b* had been characterized, mapped to 4H and was named as *HvBot1* (Sutton *et al.*, 2007). Target sequence of Contig14139_at was used to design primers for the method of Rapid Amplification of cDNA Ends (RACE). For a second round of RACE reactions, primers were designed according to sequence of amplified product in the first round. Vector NTI software was used for primer design. Sequences of primers together with Tm values are presented in Table 2.10.

Table 2.9. Probe sets of Barley Genome Array representing putative B transporters. Target sequences were blasted against *O. sativa* and *A. thaliana* genomes. Top hits, descriptions of subjects, e-values and identity percentages of results are listed.

Probe set ID	E-value	Identity/ Match	Identity (%)
Contig14139_at			
Target sequence: 970 bp			
BLASTx: MSU *O. sativa* Genome: LOC_Os01g08020.1: B transporter protein, putative, expressed	2e-86	151/188	80.3
BLASTx: *A. thaliana* Genome: AT1G15460.1: Symbols: BOR4: HCO3-transporter family	2e-59	111/183	60.7
Contig21126_at			
Target sequence: 789 bp			
BLASTx: MSU *O. sativa* Genome: LOC_Os01g08020.1: B transporter protein, putative, expressed	1e-32	69/102	67.6
BLASTx: *A. thaliana* Genome: AT1G74810.1: Symbols: BOR5: HCO3- transporter family	6e-15	47/102	46.1
Contig19634_at			
Target sequence: 815 bp			
BLASTx: MSU *O. sativa* Genome: LOC_Os12g37840.1: B transporter protein, putative, expressed	1e-92	170/186	91.4
BLASTx: *A. thaliana* Genome: AT2G47160.2: Symbols: BOR1: HCO3- transporter family	1e-54	106/186	57.0

Table 2.10. Gene-specific primer sequences for RACE. Reverse outer (OUT) and inner (INN) primers were designed for PCR with RACE-ready cDNA and subsequent nested PCR, respectively. Forward gene-specific primers (GSP) were used in validation. Number 2 appended to the names of the primers indicate utilization in the second round of RACE reactions (RACE2).

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
RACE_OUT	Reverse	ATATTGTTCTTGAAGGCACCGCCTC	60.5
RACE_INN	Reverse	AGAAGCTGTAACCTTTCCCAAAACTG	57.4
RACE_GSP	Forward	CTATGCCGGCTATCAAGATGATACC	57.1
RACE2_OUT2	Reverse	GTTCTGCGACTCCAACGATCAACAGTGG	66.3
RACE2_INN2	Reverse	CGCCAAAGTTTCAACAGTGCTTACGATACC	65.1
RACE2_GSP2	Forward	TGTATATTTTCTTTGCCTCTGCACTCCCTG	63.3

2.7.2. Rapid Amplification of cDNA Ends (RACE)

The method of RACE comprised two steps, RACE-ready cDNA synthesis and subsequent runs of PCR (RACE-PCR). Depending on the region of transcript amplified, the method was named either 5'RACE or 3'RACE. SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc; Mountain View, CA, US) was utilized with minor modifications for RACE reactions. The 5'RACE method made use of template switching by RT during first strand cDNA synthesis performed with a modified oligo(dT) primer (5'RACE CDS primer A) and SMART II™ A oligonucleotide. The sequences of oligonucleotides and universal primers (UP) are provided in Appendix F. First strand synthesis coupled with (dC) tailing by RT provided a binding site at the 5'end of cDNA for SMART II A oligonucleotide which in turn served as an extended template for RT (Figure 2.4). Thus template switching and cDNA extension added a binding site for long UP during first cycles of 5'RACE-PCR.

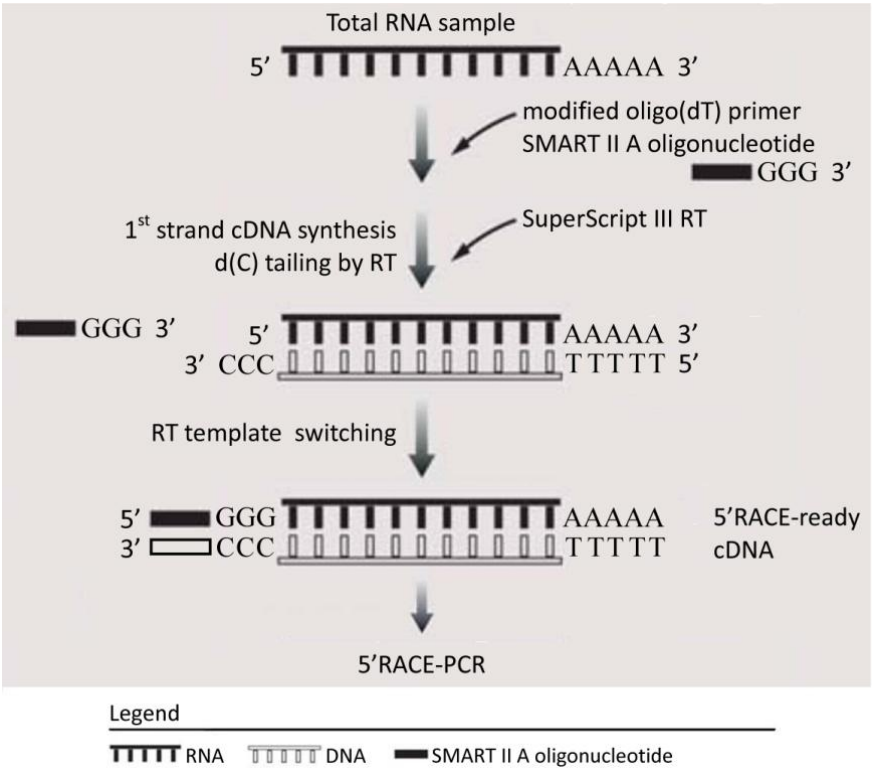

Figure 2.4. 5'RACE-ready cDNA synthesis. (Adapted from SMART RACE cDNA Amplification Kit User Manual)

Total RNA extracted (Section 2.3.2.2) from leaf tissues of barley cultivars Clipper, Sahara, Tarm-92 and Hamidiye were used to synthesize 5'RACE-ready cDNA. In 0.2 mL-tubes, 3 µL of total RNA samples were mixed with 1 µL of 12 µM 5'RACE CDS primer A and 1 µL of 12 µM SMART II A. After gentle mixing, the tubes were incubated at 70°C for 4 min and subsequently cooled on ice for 2 min. The tubes were spun briefly to collect contents at the bottom and then 1 µL of 20 mM DTT, 1 µL of 10 mM dNTP mix, 2 µL of 5X First-Strand buffer (Invitrogen) and 1 µL of SuperScript III RT (Invitrogen) were added to tubes. After gentle mixing the reaction mixtures were incubated in a thermal cycler at 42°C for 1.5 h. The reaction mixtures were then diluted with 100 µL of 0.5X TE buffer and subsequently incubated at 72°C for 7 min. The first strand reaction product, 5'RACE-ready cDNA, was utilized in 5'RACE-PCR to amplify regions of *HvBor1α*. The regions amplified and primers employed in two rounds of 5'RACE were schematically presented in Figure 2.5.

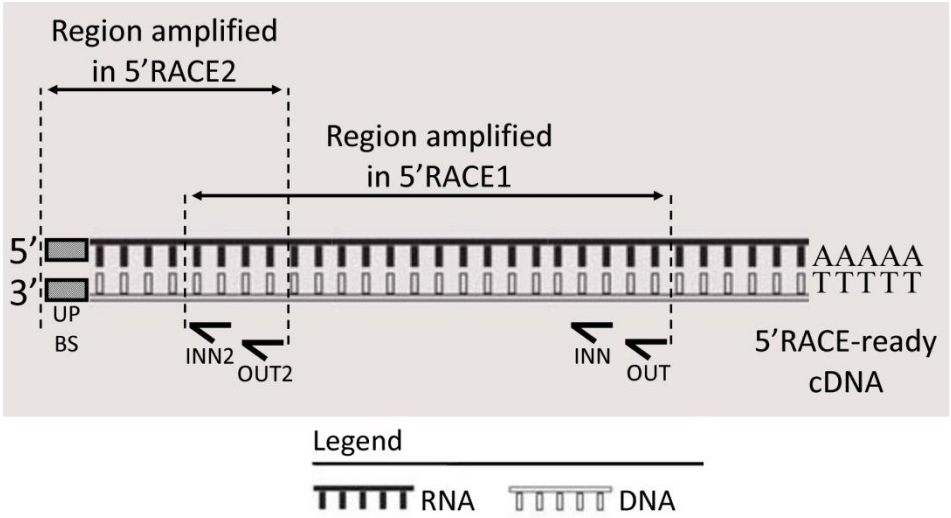

Figure 2.5. Schematic representation of regions amplified in two rounds of 5'RACE. The primers used for RACE reactions and universal primer (UP) binding sites (BS) are displayed. The details of primers are provided in Table 2.10. In the scheme the regions and primers are not in scale.

For amplification of 5’ end of transcript expressed by *HvBor1a*, 2 µL of 5’RACE-ready cDNA was used as template in 5’RACE-PCR in a total volume of 50 µL. The primers used were 0.25 µM RACE_OUT (Table 2.10) and 5 µL of 10X Universal Primer A Mix (UPM; provided by the supplier) containing 0.4 µM long UP and 2 µM short UP. During amplification in a thermal cycler, annealing temperature was set to 64°C. Duration of extension step was adjusted to 2 min since expected amplicon size was more than 1.5 kb. The other reagents used and cycling conditions employed have been presented elsewhere (Section 2.3.10).

During first few cycles of 5’RACE-PCR, long UP incorporated a binding site for short UP. In the remaining cycles, RACE_OUT and short UP provided amplification of 5’ end of the transcript. Subsequently nested PCR was carried out using a fraction of product from 5’RACE-PCR. Nested PCR was performed with 0.25 µM RACE_INN (Table 2.10) and 0.25 µM Nested Universal Primer A (NUP; provided by the supplier). Cycling conditions used for nested PCR were same as 5’RACE-PCR. Validation of fragments from RACE reactions were performed with amplification by PCR using RACE_GSP (Table 2.10).

A second round of RACE reactions (RACE2) was performed to amplify the very end of *HvBor1a* mRNA. The primers were designed according to the sequence of amplified product from first round of RACE. In a total volume of 50 µL, 2 µL of 5’RACE-ready cDNA was used as template in 5’RACE2-PCR. The primers utilized were 0.25 µM RACE2_OUT2 (Table 2.10) and 5 µL of 10X UPM. Subsequently nested PCR was carried out using a fraction of product from 5’RACE2-PCR. Nested PCR was performed with 0.25 µM RACE2_INN2 (Table 2.10) and 0.25 µM NUP. The reagents used and cycling conditions employed were same as first round of 5’RACE-PCR. Validation of fragments from second round of RACE reactions were performed with amplification by PCR using RACE2_GSP2 (Table 2.10).

Amplified products from nested PCR were separated on 1% (w/v) agarose gel (Section 2.3.5) and bands of fragments were extracted from gel (Section 2.3.6). The purified fragments were either sequenced (Section 2.3.11) or introduced into pCR8/GW/TOPO (Section 2.3.8), multiplied in *E. coli* TOP10 (Section 2.3.7) and then sequenced (Section 2.3.11). Sequence reads obtained by two rounds of RACE reactions were aligned using ContigExpress module of Vector NTI. Binding sites for UP were excluded and sequences of coding region and open reading frame (ORF) of *HvBor1a* was determined.

2.7.3. Cloning of Full Length CDS of *HvBor1a*

The sequence obtained by two rounds of RACE reactions were utilized to design primers for cloning of full length CDS of *HvBor1a*. A pair of primers with binding sites located in 5' and 3' UTR and a second pair of primers to amplify the CDS from translation initiation site (AUG) to translation stop site (UGA) were designed using Vector NTI. The sequences of the primers and Tm values calculated using Vector NTI are provided in Table 2.11.

Table 2.11. Primer sequences used for cloning of full length CDS of *HvBor1a*.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
3HBor1a_5UTR_F	Forward	GACCCGCGCGCCGCGTCCTTAGCCG	78.2
3HBor1a_3UTR_R	Reverse	GAGCTTTACTTCCTAGCGTACACGATCACG	63.2
3HBor1a_C_ATG	Forward	ATGGATCTACTAGGGAACCCTTTCAAGG	60.2
3HBor1a_C_TGA	Reverse	TCACACGCTCGGCTGAACTGCATT	64.6

Total RNA extracted (Section 2.3.2.2) from leaf tissues of barley were used to synthesize cDNA (Section 2.3.3). In an initial PCR using cDNA as template, primers with binding sites in UTRs (3HBor1a_5UTR_F and 3HBor1a_3UTR_R) were employed to amplify transcript of *HvBor1a*. Nested PCR, using a fraction of the 10X diluted product from the initial PCR as template, was performed with gene-specific primers, 3HBor1a_C_ATG and 3HBor1a_C_TGA. In certain cases, instead of nested PCR, full length CDS was amplified with 3HBor1a_C_ATG and 3HBor1a_C_TGA from cDNA used as template. A proofreading enzyme, Phusion® High-Fidelity DNA polymerase (NEB), was employed for multiplication of *HvBor1a* CDS. The components of PCR and cycling conditions are provided in Table 2.12 and Table 2.13, respectively.

Tm values of primers intended for use with Phusion DNA polymerase were calculated using Tm calculator at NEB website (http://www.neb.com/nebecomm/tech_reference/tmcalc) according to thermodynamic data described previously (Breslauer *et al.*, 1986). Annealing temperature recommended was 72°C; therefore, a two-step-PCR without a separate annealing step was employed for amplification of *HvBor1a* CDS (Table 2.13).

Table 2.12. Components of PCR used for cloning of *HvBor1a*.

Reagent	Stock concentration	Final concentration	Volume (µL)
GS primer I *	5 µM	0.5 µM	5
GS primer II *	5 µM	0.5 µM	5
dNTP mix	2.5 mM	0.2 mM	4
Phusion HF Buffer **	5 X	1 X	10
Phusion DNA polymerase	2 U/µL	0.02 U/µL	0.5
DMSO	100 %	3 %	1.5
Template	~25 ng/µL	variable	2.5
Ultrapure water			21.5
Total			**50**

* Gene-specific (GS) primers I and II were sense and antisense (forward and reverse) primers for the fragment to be amplified.

** Phusion HF Buffer provides a final concentration of 1.5 mM $MgCl_2$ in the reaction mixture.

Table 2.13. Thermal cycling conditions used for cloning of *HvBor1a*.

		Temperature (°C)	Duration	Number of cycles
Initial denaturation		98	1 min	1
Amplification	Denaturation	98	10 sec	30
	Extension	72	1 min 10 sec	
Final extension		72	10 min	1

Products from PCR were checked using electrophoretic separation on 1% (w/v) agarose gel (Section 2.3.5). Amplicons were isolated either by recovery from agarose gel (Section 2.3.6) or by direct PCR clean-up (Section 2.3.10). Since fragments produced by Phusion DNA polymerase were blunt-ended, addition of A overhangs was performed using *Taq* polymerase as described previously (Section 2.3.8). The fragment was introduced into pCR8/GW/TOPO using TA cloning (Section 2.3.8). For directional cloning, fragment which was amplified with a modified forward primer containing a 4 bp-sequence (CACC) at the 5' end was introduced into pENTR/D-TOPO (Section 2.3.8). The plasmids were multiplied in TOP10 (Section 2.3.7) and then sequenced (Section 2.3.11).

2.7.4. Analyses of Sequence at Nucleotide and Amino Acid Levels

The nucleotide and amino acid sequences of *HvBor1a* were handled using VectorNTI. The nucleotide sequence was blasted against non-redundant database at NCBI using BLASTN and BLASTX (Zhang *et al.*, 2000; Altschul *et al.*, 1997), whereas the amino acid sequence was blasted using BLASTP (Altschul et al., 1997; Altschul *et al.*, 2005). Multiple sequence alignments were performed with ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2). The conserved domains were predicted with InterProScan (http://www.ebi.ac.uk/tools/pfa/iprscan).

2.8. Characterization of 3H B Tolerance Gene

Gene and protein product of the putative B transporter, *HvBor1a*, was characterized using heterologous expression in yeast, subcellular localization and determination of endogenous expression levels in barley tissues.

2.8.1. Heterologous Expression in Yeast

2.8.1.1. Cloning of *HvBor1a* with a Yeast Consensus Sequence

The transcript of *HvBor1a* was amplified with a yeast consensus sequence at the 5'end. The consensus sequence for proper initiation of translation in yeast was inserted via PCR with a modified sense primer. The bases added upstream of initiation codon AUG was AAA. Moreover, a three-nucleotide codon UCU for serine was inserted downstream of AUG (Romanos *et al.*, 1992). The sequences of the sense and antisense primers used for amplification of *HvBor1a* are given in Table 2.14. The inserted nucleotides are shown underlined (Table 2.14).

Table 2.14. Primer sequences used for amplification of *HvBor1a* with a yeast consensus sequence. The nucleotides underlined were inserted for initiation of translation in yeast.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
3HBor1a_C_ATG_Ycon	Forward	AAAATGTCTGATCTACTAGGGAACCCTTTCAAGG	64.3
3HBor1a_C_TGA	Reverse	TCACACGCTCGGCTGAACTGCATT	64.6

The CDS of *HvBor1a* with a yeast consensus sequence was amplified in PCR using Phusion High-Fidelity DNA polymerase (Section 2.7.3). The purified fragments were inserted into pCR8/GW/TOPO after addition of A overhangs. The plasmids were multiplied in TOP10 which was selected on solid LB containing 100 µg/mL spectinomycin (Section 2.3.7). Then the plasmids were sequenced (Section 2.3.11) for verification of sequence and direction of insertion. The entry plasmid carrying full CDS of *HvBor1a* was used in recombination to transfer the GOI into yeast expression vector.

2.8.1.2. Recombination Using Gateway Cloning System

Entry vector pCR8/GW/TOPO carrying CDS of *HvBor1a* and destination vector pYES-DEST52 were recombined *in vitro* for cloning of *HvBor1a* into yeast expression vector. *In vitro* recombination was performed as described previously (Section 2.3.9). The recombination product was transformed into TOP10 cells (Section 2.3.7.2) and selection was done on solid LB media containing 100 µg/mL ampicillin. Selected colonies were streaked onto solid LB containing 12.5 µg/mL chloramphenicol for negative selection. Recombinant pYES-DEST52 expression clones were isolated using QIAprep Spin Miniprep Kit where elution was done in 50 µL of ultrapure water. Concentration and purity of expression vectors were determined (Section 2.3.4.4). And recombinant pYES-DEST52 vectors were used in transformation of yeast.

2.8.1.3. Transformation of Yeast

A single colony of yeast strain INVSc2 was inoculated into 10 mL of SD medium containing glucose, histidine and uracil. Culture was incubated in a 30°C-incubator for 24 h with gentle shaking. Titer of the initial culture was determined using a double-beam spectrophotometer. A 100-fold dilution of culture was prepared and absorption was recorded where water was the blank. Conventionally, for most yeast strains an OD of 1.0 at 600 nm corresponds to a concentration of $1x10^7$ cells/mL. Pre-warmed 50 mL of double-strength YPD medium in a pre-warmed 250 mL-flask was inoculated with $2.5x10^8$ cells to give a final concentration of $5x10^6$ cells/mL. The culture was incubated at 30°C with 200 rpm shaking for 4 h. Spectrophotometry was used to determine the titer of the culture. Absorption of a 20-fold diluted culture was recorded at 600 nm against double-strength YPD which was diluted at

the same proportions. The recommended concentration was $2x10^7$ yeast cells/mL (10^9 cells in 50 mL culture) for successful transformation.

Yeast cells were harvested in 50 mL-tubes by centrifugation at 3,000*g* for 5 min at 4°C in a refrigerated centrifuge. The supernatant was discarded and cells were washed in 25 mL of sterile water. After complete suspension of cells, a second centrifugation at 3,000*g* for 5 min at 4°C was performed to collect cells. The supernatant was discarded and pellet of cells was re-suspended in 1 mL of sterile water. The mixture was vortexed thoroughly and transferred into pre-cooled 2 mL-tubes. Cells were precipitated by centrifugation at 18,000*g* for 30 sec in a bench-top centrifuge. After removal of supernatant, cells were re-suspended in sterile water and final volume of mixture was adjusted to 1 mL. In case cell concentration was different from $2x10^7$ cells/mL at the time of harvest, the final volume might be adjusted accordingly. The titer of final suspension was maintained at 10^9 cells.

Yeast cell suspension was dispensed as 100 µL-aliquots (approximately 10^8 cells) into sterile 1.5 mL-tubes on ice. Each aliquot was used for one transformation. Cells were harvested by centrifugation at 18,000*g* for 30 sec at 4°C. After removal of supernatant, yeast cells were kept on ice during preparation of transformation mixture (Table 2.15). After addition of transformation mixture onto pellet of cells, the mixture was vortexed briefly to re-suspend cells. Subsequently, the mixture was incubated at 42°C in a water bath for 40 min.

Table 2.15. Components of mixture used for yeast transformation.

Reagent	**Stock concentration**		**Volume (µL)**
PEG-3500 *	50	% (w/v)	240
Lithium acetate, pH 7.5 **	1	M	36
Denatured fragmented salmon sperm DNA ***	~10	mg/mL	50
Sterile ultrapure water			33
pYES-DEST52 carrying *HvBor1a*	~0.5	µg/µL	1
Total			**360**

* Stock solution of PEG-3500 was autoclaved for sterilization.

** Lithium acetate solution was filter-sterilized after adjustment of pH.

*** Previously sonicated and ethanol precipitated salmon sperm DNA was boiled for 10 min for denaturation and subsequently chilled on ice, prior to preparation of transformation mixture.

After transformation, cells were collected by centrifugation at 18,000*g* for 30 sec in a bench-top centrifuge and transformation mixture was removed carefully with a pipette. Transformed cells were gently re-suspended in 1 mL of sterile water. After complete mixing, 10- and 100-fold dilutions of suspension were prepared with water. Selection of transformants was performed on uracil-lacking medium. A 100 µL-aliquot of each dilution was gently spread onto solid SD media containing glucose and histidine. The surface of the medium was dried completely in a laminar flow and plates were incubated at 30°C for 2 – 3 days, until separate colonies appeared. Transformations of yeast with empty pYES-DEST52 and water were performed as negative controls. Transformation with water did not produce colonies on selective medium.

Three colonies per transformation were selected as three independent replicates. Selected colonies of transformed yeast cells were inoculated into 2 mL of liquid SD medium containing galactose and histidine for expression of *HvBor1a*. Cultures were incubated at 30°C for 2 days with 200 rpm shaking. Yeast cells transformed with pYES-DEST52 carrying *HvBor1a* or with empty plasmid DNA were used in a bioassay for tolerance to B toxicity.

2.8.1.4. B Tolerance Bioassay with Transformed Yeast

The concentrations of cultures of transformed yeast cells were determined at 600 nm using a double-beam spectrophotometer. The cell density of each culture was adjusted with proper dilutions with sterile water to obtain cultures at approximately the same density. Culture with the lowest OD recording was selected as a baseline and others were adjusted accordingly. Subsequently, 4 different 10-fold serial dilutions of each culture were prepared.

Bioassay for tolerance to B toxicity was performed on solid SD medium containing galactose, histidine and H_3BO_3. Yeast transformants expressing *HvBor1a* were tested for tolerance on media containing 15 and 20 mM of H_3BO_3 whereas control media did not contain H_3BO_3. Three independent replicates of transformants together with three independent replicates of control yeast carrying empty vector were spotted onto all 3 media. During spotting 10 µL of each of 4 different dilutions were placed onto the surface of solid media and spots were allowed to dry in a laminar flow. The cultures were incubated at 30°C for 2 days and photos of plates were taken.

2.8.2. Determination of Introns and Untranslated Regions

The nucleotide sequences of introns and UTRs of *HvBor1a* gene were determined in Clipper and Sahara to locate single nucleotide polymorphisms (SNPs), deletions or insertions. The intention was to develop genetic markers for tolerance to B toxicity using variations in sequence.

2.8.2.1. Prediction of Introns and Primer Design

The exons and exon-intron junctions of *HvBor1a* were predicted according to the sequences of exons and introns of putative rice gene, LOC_Os01g08020.1 which was the best blast hit of *HvBor1a* in rice genome. The gene encoding for a boron transporter was annotated by Rice Genome Annotation Project (MSU; http://rice.plantbiology.msu.edu). The sequences of exons and introns were obtained from Gramene (http://www.gramene.org) Ensembl34 build of *O. sativa* Japonica Group.

After prediction of exon-intron boundaries, sets of primer pairs were designed using Vector NTI. The primer pairs were designed such that the amplicon spanned at least two introns. The sequences of the primers are provided in Table 2.16. Moreover, binding sites of primers and locations of predicted exon-intron boundaries are presented in Figure 2.6. The primers together with the ones designed for cloning (Table 2.11) and RACE reactions (Table 2.10) were used in various combinations in PCR on genomic DNA.

Table 2.16. Primer sequences used for amplification of introns of *HvBor1a*.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
3H_SPM1_F	Forward	GGTGATTGTGTGGACAGCATTGTCATTTAC	63.2
3H_SPM1_R	Reverse	ATGGGGGAATGCCAAGCAAACCACATAG	67.5
3H_SPM2_F	Forward	AGGCTAAGCAACCTGCTGCAATCCTTAC	63.6
3H_SPM2_R	Reverse	TGAGTTCATCCAACATTTCAGCGTCGTC	64.8
3H_SPM3_F	Forward	TGTATATTTTCTTTGCCTCTGCACTCCCTG	63.3
3H_SPM3_R	Reverse	GGTGAGCTGGGGTCAGAAATCTCATCATC	65.5

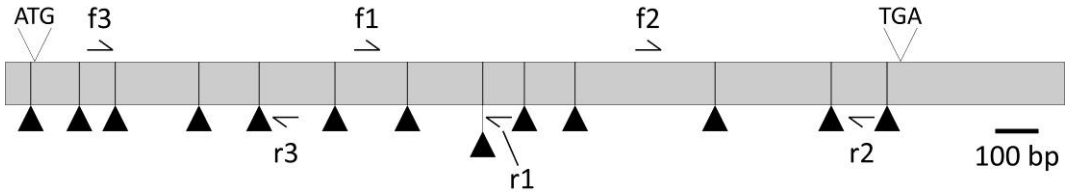

Figure 2.6. Binding sites of primers and predicted exon-intron boundaries of *HvBor1a*. Exon-intron junctions are shown with filled arrowheads whereas primers are presented with half arrows. The lengths of 14 predicted exons of *HvBor1a* are in scale however primers are not. For detailed information on primer pairs, see Table 2.16. (f1: 3H_SPM1_F; r1: 3H_SPM1_R; f2: 3H_SPM2_F; r2: 3H_SPM2_R; f3: 3H_SPM3_F; r3: 3H_SPM3_R)

Moreover, a set of primers were designed for amplification of last intron of *HvBor1a* from barley cultivars Clipper and Sahara. The primer pairs were designed according to the sequence information from Clipper such that the amplicon spanned the last intron. The sequences and binding sites of the primers are provided in Table 2.17 and Figure 2.7, respectively. The primers together with the ones designed for amplification of introns (Table 2.16) were used in various combinations in PCR on genomic DNA and cDNA.

Table 2.17. Primer sequences used for amplification of last intron of *HvBor1a*.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
3HBor1a_e14_LO2	Reverse	GAGCTTTACTTCCTAGCGTACACGATCACG	63.2
3HBor1a_e14_LO1	Reverse	GGAAAAGCGCTAGCTACAGTACAATGC	59.8
3HBor1a_e14_LO5	Reverse	GATAATCTACACTTCTGACAGAATCG	50.5
3HBor1a_e14_LO3	Reverse	CAGAATCGTCATCAAAGCCTCGGACATC	65.4
3HBor1a_e14_LO4	Reverse	CAAAGCCTCGGACATCGGGACG	64.3

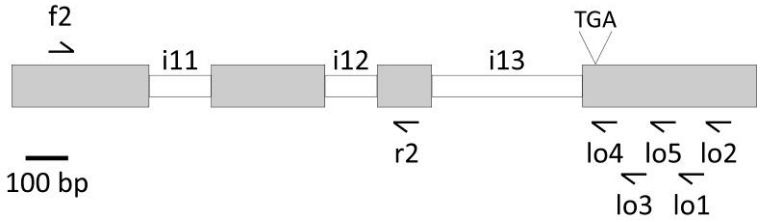

Figure 2.7. Binding sites of primers designed for amplification of last intron of *HvBor1a*. Primers are presented with half arrows. The lengths of predicted exons and introns (i) of *HvBor1a* are in scale however primers are not. For detailed information on primers, see Table 2.16 and 2.17. (f2: 3H_SPM2_F; r2: 3H_SPM2_R; lo2: 3HBor1a_e14_LO2; lo1: 3HBor1a_e14_LO1; lo5: 3HBor1a_e14_LO5; lo3: 3HBor1a_e14_LO3; lo4: 3HBor1a_e14_LO4)

2.8.2.2. PCR on Genomic DNA

Genomic DNA samples isolated (Section 2.3.4) from barley cultivars Clipper and Sahara were used as templates in PCR to amplify intron regions of *HvBor1a*. In 25 µL-reaction mixtures, 2.5 µL of 25-fold diluted genomic DNA was used as template and amplification with various combinations of primer pairs was performed with Immolase. Primers designed for cloning such as ones with binding sites in UTRs (Table 2.11) and primers designed for RACE reactions such as outer and inner primers for amplification of cDNA ends (Table 2.10) was employed in PCR for amplification of introns from genomic DNA. In certain cases nested PCR was employed to increase specificity and reduce number of unexpected products. Annealing temperature was set to 66°C and duration of extension step was adjusted to 2 min. Initial denaturation was performed for 8 min since activation of Immolase required heat treatment. Other reagents and details of cycling conditions used have been presented previously (Section 2.3.10).

Amplified products were separated on 1.5% (w/v) agarose gel (Section 2.3.5) and bands of fragments were extracted from gel (Section 2.3.6). The purified fragments were either sequenced (Section 2.3.11) or introduced into pCR8/GW/TOPO (Section 2.3.8), multiplied in *E. coli* TOP10 (Section 2.3.7) and then sequenced (Section 2.3.11). Chromatograms and sequence reads obtained from Clipper and Sahara were aligned using ContigExpress. Sequence of each intron was determined and reads were manually inspected for variations such as SNPs between two cultivars.

2.8.2.3. Genome Walking Using Restriction Enzyme Digestion

Genome walking was performed for determination of 5' and 3' UTR of *HvBor1a* in Clipper and Sahara genomes. For DNA walking, GenomeWalker™ Universal Kit (Clontech) was adapted with minor modifications. Genome walking procedure employed restriction enzyme digestion of genomic DNA, ligation of adaptors to digested DNA fragments and PCR amplification (Figure 2.8). Genomic DNA isolated (Section 2.3.4) from Clipper and Sahara were digested with 6 different restriction enzymes which had 6 bp-recognition strings and produced blunt-ended fragments in 6 individual tubes. Enzymes used in digestion were *Dra*I, *Eco*RV, *Ssp*I, *Stu*I, *Sca*I and *Pml*I (NEB). Digestion of 2.5 µg of genomic DNA was performed with 80 U of restriction enzyme in a total of 100 µL-reaction buffered with optimal buffer of the enzyme supplied by the manufacturer. The reaction mixtures were supplemented with BSA at a final concentration of 100 ng/µL. Digestion mixtures, 6 for Clipper and 6 for Sahara, were gently flicked and incubated at 37°C in a thermal cycler for 2 h. The tubes were vortexed gently at a slow speed and returned to 37°C overnight. Subsequently, the digests were purified using QIAquick® PCR Purification Kit (Qiagen). Elution of DNA fragments was performed in 30 µL of 10 mM Tris-HCl (pH 8.5).

The adaptor was prepared by sequence-dependent hybridization of 2 single-stranded oligonucleotides, which were designed such that after hybridization the resulting adaptor molecule had 5' extension on one end and blunt end on the other. Moreover the 3' end of shorter oligonucleotide was blocked by an amine group to prevent extension. The sequences of oligonucleotides are provided in Appendix F. The adaptor had no binding site for adaptor primer (AP) used in subsequent PCR. On the other hand, binding sites could only be generated by extension of gene-specific primer in first few cycles of touchdown PCR (Figure 2.8b). Mixture of 12.5 µL of 200 µM Adaptor_Oligo1 and 12.5 µL of 200 µM Adaptor_Oligo2 was prepared in a total volume of 50 µL buffered with 5 mM final concentration of Tris-HCl (pH 8.5). The mixture was incubated at 95°C for 10 min and afterwards allowed to stand at room temperature overnight for hybridization of oligonucleotides. Then the adaptor molecules were ligated to the fragments of genomic DNA to generate genome walking libraries.

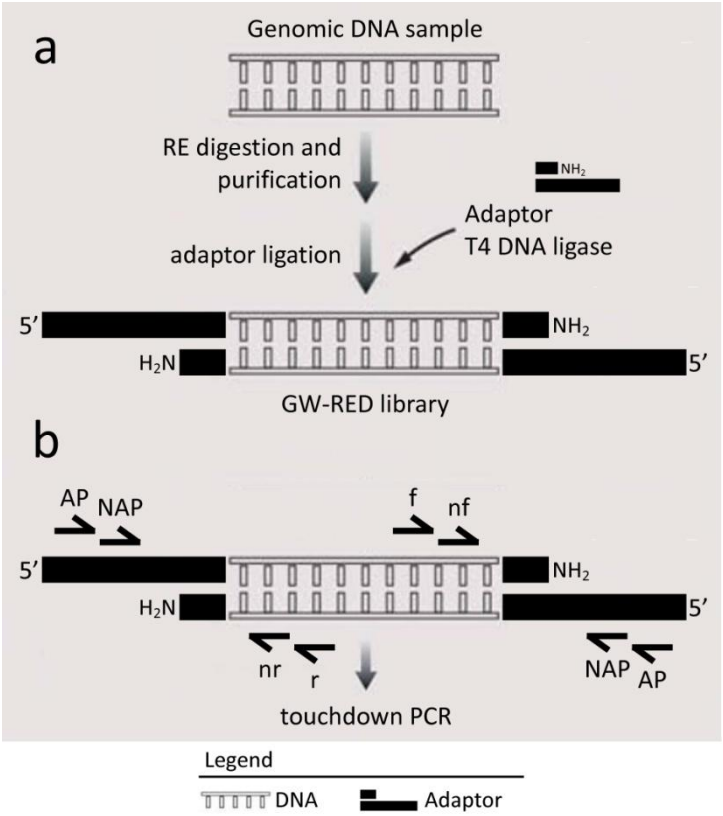

Figure 2.8. Schematic representation of method employed for genome walking using restriction enzyme digestion (GW-RED). Generation of GW-RED library (a) and relative locations of binding sites of primers (b) used in touchdown and nested PCR are displayed. For detailed information on gene-specific primers (GSP) see Table 2.18. (AP: Adaptor primer; NAP: Nested AP; f: forward GSP; nf: nested f; r: reverse GSP; nr: nested r) (Adapted from GenomeWalker Universal Kit User Manual)

Ligation of adaptor to DNA fragments was achieved with the aid of T4 DNA ligase (Figure 2.8a). In a total volume of 20 µL, 10 µL of purified and digested genomic DNA was mixed with 4 µL of 50 µM adaptor, 4 µL of 5X T4 DNA ligase buffer (Invitrogen) and 2 µL of 1 U/µL T4 DNA ligase (Invitrogen). The reaction mixture was vortexed gently, spun and then incubated at 16°C overnight in a thermal cycler. The reaction was stopped by incubation at 70°C for 5 min. Subsequently 180 µL of 10 mM Tris-Hcl (pH 8.5) was added to dilute the DNA library 10-fold. Generated genome walking libraries, 6 for Clipper and 6 for Sahara, were used as templates in touchdown PCR.

PCR based genome walking was performed with touchdown PCR where 2.5 µL of library was used as template in 50 µL-reactions. Amplification of fragments was achieved with 0.25 µM AP and 0.25 µM gene-specific primers. The sequences of gene-specific primers designed for genome walking are presented in Table 2.18. Moreover binding sites of the primers were schematically presented in Figure 2.9.

Table 2.18. Sequences of primers used in genome walking. Forward and reverse primers were employed for genome walking downstream and upstream of *HvBor1a*, respectively.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
3HBor1a_e11_F	Forward	AACCAGTTTTGGGAAAGGTTACAGCTTCTG	63.5
3HBor1a_e13_F1	Forward	AAATTCAGGAAGCCATGACAGCATAGAC	60.8
3HBor1a_e13_F2	Forward	CCGTGGAGAGCTGAAGCACAGATC	61.0
3HBor1a_i13_F	Forward	ACGGAAGAACAAGTACCGCGCATACCAG	66.4
3HBor1a_i1_R1	Reverse	CAGCTCATCGCTCACCTTACATGTCAG	62.0
3HBor1a_i1_R2	Reverse	ACTCGTCGAGAATCAGACACACGCCG	66.1

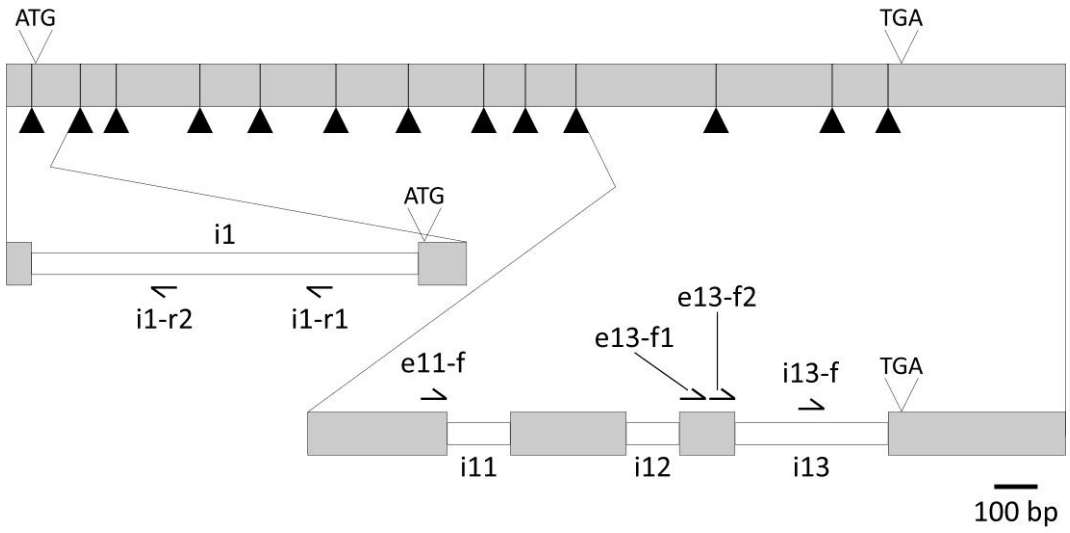

Figure 2.9. Binding sites of primers used for touchdown PCR during genome walking using restriction enzyme digestion. Exon-intron boundaries are indicated with filled arrowheads whereas primers are presented with half arrows. Introns (i) are shown as numbered empty boxes. The sizes of displayed exons and introns of *HvBor1a* are in scale however primers are not. For detailed information on primer pairs, see Table 2.18. (i1-r1: 3HBor1a_i1_R1; i1-r2: 3HBor1a_i1_R2; e11-f: 3HBor1a_e11_F; e13-f1: 3HBor1a_e13_F1; e13-f2: 3HBor1a_e13_F2; i13-f: 3HBor1a_i13_F)

Components of touchdown PCR were same as the components of conventional PCR (Section 2.3.10) whereas thermal cycling conditions employed differed notably (Table 2.19). During first cycles of PCR, extension by gene-specific primers generated binding site for AP and in the remaining cycles amplification with gene-specific primers and AP was achieved. Subsequently nested PCR was performed using a diluted fraction of product from touchdown PCR. In nested PCR amplification was done with 0.25 µM NAP and 0.25 µM nested gene-specific primers. Annealing temperature was set to 62°C and duration of extension step was adjusted to 3 min since expected amplicon sizes were more than 2 kb. Other cycling conditions and reagents used in nested PCR have been summarized elsewhere (Section 2.3.10).

Amplified products from touchdown and nested PCR were separated on 1% (w/v) agarose gel (Section 2.3.5) and bands of fragments were extracted from gel (Section 2.3.6). The purified fragments were sequenced (Section 2.3.11) and sequences obtained by genome walking were aligned using ContigExpress.

Table 2.19. Thermal cycling conditions used in touchdown PCR.

		Temperature (°C)	Duration	Number of cycles
Initial denaturation		95	9 min	1
Amplification	Denaturation	94	15 sec	7
	Extension	72	3 min	
Amplification	Denaturation	94	15 sec	33
	Extension	67	3 min	
Final extension		72	10 min	1

2.8.2.4. Genome Walking Using Multiple Displacement Amplification

A second method of genome walking based on multiple displacement amplification (MDA) of whole genomic DNA with Phi29 DNA polymerase was employed for verification of sequences of UTRs determined (Reddy *et al.*, 2008). PCR based genome walking using MDA involved

random introduction of walker primer (WP) binding sites by walker adaptors (WA), rolling circle mode of DNA synthesis by Phi29 DNA polymerase and subsequent PCR amplifications. The sequences of WAs and WPs are provided in Appendix F. Approximately 150 ng of genomic DNA preparations from Clipper and Sahara were mixed with 1.5 µL of 10 µM WA and 2 µL of 10 mM dNTP mix in a 10 µL-reaction. DNA was heat-denatured at 95°C for 2 min and hybridized to each of 6 different WAs in 6 individual tubes by incubation of mixtures at 20°C for 2 min. MDA using Phi29 DNA polymerase was performed with components of REPLI-g® Kit (Qiagen) with minor modifications to the procedure provided by the supplier. After annealing of WAs to genomic DNA, 40 µL-mixture containing 10 µL of ultrapure water, 29 µL of REPLI-g reaction buffer and 1 µL of REPLI-g DNA polymerase was added to each tube making the total volume 50 µL. The mixtures were incubated at 30°C for 17 h in a thermal cycler. The reaction was terminated by inactivation of REPLI-g DNA polymerase by incubating the mixtures for 10 min at 65°C. Generated genomic DNA libraries were used as templates in PCR.

Amplification by conventional PCR was performed with 0.25 µM WP (Appendix F) and 0.25 µM gene-specific primers (Table 2.18) which were designed for genome walking based on restriction enzyme digestion. In 25 µL-reactions 2 µL of each of 6 libraries were used as template. Subsequently nested PCR was carried out with 2 µL of 5 fold diluted products of primary PCR as templates along with 0.25 µM NWP (Appendix F) and 0.25 µM nested gene-specific primers (Table 2.18) in 50 µL-reactions. Annealing temperatures were set to 62°C and extension steps were performed for 1 min and 30 sec for both primary and nested PCR. Other reagents and cycling conditions have been described previously (Section 2.3.10). Amplified products from primary and nested PCR were separated on 1% (w/v) agarose gel (Section 2.3.5) and bands of major fragments were extracted from gel (Section 2.3.6). The purified fragments were sequenced (Section 2.3.11) and chromatograms obtained by genome walking were aligned using ContigExpress.

2.8.3. Southern Blotting

Localization of particular sequences within genomic DNA can be accomplished by Southern blotting. Fragments generated by restriction enzyme digestion of genomic DNA were separated according to size by electrophoresis. The DNA was then denatured *in situ* and

transferred to a solid support. The relative positions of the DNA fragments were preserved during their transfer to the support. A radiolabelled fragment of DNA or RNA (probe) was then hybridized to the DNA attached to the filter support, and autoradiography was used to locate the positions of fragments containing sequences which are complementary to the probe. Southern analysis in this study was performed to confirm the variation in sequence at the 3' end of *HvBor1a* in the Sahara genome.

2.8.3.1. Preparation of Membrane and Probe

Genomic DNA isolated (Section 2.3.4) from leaf tissue of Sahara and Clipper were used for Southern hybridization which was performed by Margaret Pallotta at ACPFG. DNA quality was checked by electrophoresis of 1 – 2 µL of genomic DNA on a 1.5% (w/v) agarose gel (Section 2.3.5) and relative concentrations of DNA were estimated. Approximately 4 µg of each DNA sample was digested with 20 U *Hind*III (NEB) in the presence of 1X NEBuffer 2 (NEB) in a final volume of 14 µL. The mixtures were incubated at 37°C for 4 – 5 h. After digestion samples were size separated on a 1% (w/v) agarose gel in 1X Tris-Acetic acid-EDTA (TAE) buffer at 34 V for 16 – 18 h (Section 2.3.5). The 1X TAE buffer contained 40 mM Tris base (pH 8.0), 20 mM Acetic acid, and 1 mM EDTA. Following electrophoresis gels were stained for 10 min in approximately 100 mL of de-ionized water containing 1 µg/mL ethidium bromide. The ethidium bromide solution was decanted and the gel was rinsed briefly with de-ionized water. Digested DNA was visualized on a UV illuminator.

The DNA fragments were transferred from the gel to nylon N+ membrane (Biodyne B®, Pall Corporation) by a capillary transfer method (Southern, 1975), using a solution of 0.4 M NaOH. Transfer was performed for a minimum of 5 h. Subsequently membranes were rinsed for 1 min in 100 mL of 2X SSC (pH 7.0) containing 0.3 M NaCl and 30 mM trisodium citrate. Membranes were then blotted dry on Whatman 3MM filter paper, wrapped in plastic film and stored at 4°C or -20°C until required.

The probe was amplified in a conventional PCR using forward and reverse primers, 3HBor1a_i13_F (Table 2.18) and 3HBor1a_3UTR_R (Table 2.11), which amplified a 403 bp fragment from Clipper genomic DNA. The fragment covered a region from the final intron, through the final exon and into the 3' UTR. The first 113 bp region within the final intron was

common both in Clipper and Sahara whereas the remaining 290 bp was unique to Clipper. Amplification was performed in a thermal cycler using an annealing temperature of 60°C and extension duration of 1.5 min. Other reagents and cycling conditions used in PCR have been summarized elsewhere (Section 2.3.10).

Probes were labeled with α-dCT^{32}P (Perkin Elmer) by incubation of the denatured probe for 1.5 h at 37°C in the presence of Klenow fragment (NEB) and unlabeled dATP, dGTP and dTTP. Labeled probe was separated from un-incorporated radioisotope using a mini Sephadex G-100 column. The labeled probe was denatured by boiling for 5 min and then added to the hybridization bottles (tubes) containing the hybridization solution and membrane.

2.8.3.2. Southern Hybridization

Southern membranes were pre-hybridized for approximately 24 h at 65°C in Southern pre-hybridization solution containing 5X SSC solution, 5X Denhardt's III solution, and 250 µg/mL salmon sperm DNA. Pre-hybridization solution was replaced with hybridization solution prior to addition of the probe. Hybridization solution for Southern membranes contained 1.5X HSB buffer, 1.5X Denhardt's III solution, 7.5% (w/v) dextran sulphate, and 125 µg/mL salmon sperm DNA. Hybridization was allowed to proceed at 65°C for 16 – 24 h. After hybridization membranes were washed for 20 min at 65°C successively in (1) 2X SSC, 0.1% (w/v) SDS, (2) 1X SSC, 0.1% (w/v) SDS and then (3) 0.5X SSC, 0.1% (w/v) SDS. Subsequently the membranes were blotted on paper toweling, encased in plastic bags and then exposed to film in X-ray cassettes containing intensifying screens at -80°C for up to 10 days. Films were developed using standard methods.

2.8.4. Expression Analyses of 3H B Tolerance Gene

Transcript abundance of *HvBor1a* in leaf and root tissues of barley cultivars was determined using real-time PCR under various B treatments. One- or two-step real-time RT-PCR was employed where appropriate. Moreover, Northern blotting as an independent expression analysis was carried out for confirmation of results obtained.

Expression levels of *HvBor1a* were determined in two B-stress series. Seedlings of Sahara and Clipper were treated with varying final concentrations of H_3BO_3, at 0, 20, 50, 500 and 1,000 µM for B toxicity in hydroponics using basal growth solution. Leaf and root tissues were harvested for RNA isolation, subsequent expression analysis with real-time PCR and Northern blotting. Second B-stress series included Tarm-92 and Hamidiye, seedlings of which were grown in hydroponics using basal growth solution and were treated with final concentrations of 15 µM H_3BO_3, and 0.5, 1, 5 and 10 mM H_3BO_3 for B toxicity. Total RNA was isolated from leaf and root tissues and one-step real-time RT-PCR was employed for determination of mRNA levels.

2.8.4.1. Primer Design for Real-Time PCR

Gene-specific primers were designed using Vector NTI for amplification of a 117 bp-region of mRNA of *HvBor1a* (Table 2.20). The primer pair was positioned such that a region within ORF was amplified (Figure 2.10). Conventional PCR (Section 2.3.10) and gel electrophoresis of PCR products (Section 2.3.5) were employed to validate primers and to confirm amplification of a single gene-specific amplicon without any non-specific secondary products. Amplification by gene-specific primers in conventional PCR was carried out with 2.5 µL of cDNA containing approximately 10 ng/µL of initial RNA from Sahara and Clipper in a 25 µL-reaction. Annealing temperature was adjusted to 58°C and duration of extension step was set to 50 sec. Other components and conditions used in PCR have been described previously (Section 2.3.10). Amplified products were purified and cycle-sequenced (Section 2.3.11) for verification of sequence. Moreover, the primer pair was checked in an initial run of real-time PCR and amplified product in real-time PCR was sequenced as well (Section 2.3.11).

Table 2.20. Primer sequences used in real-time PCR for amplification of a fragment of *HvBor1a* mRNA.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)	Amplicon size (bp)
Bor1a_GSP4_F	Forward	ATAACATGGATACCAGTAGCAGGGATCCTC	61.7	117
Bor1a_GSP4_R	Reverse	ATCCAGTTCTCGCAAGTCATTGGG	60.2	

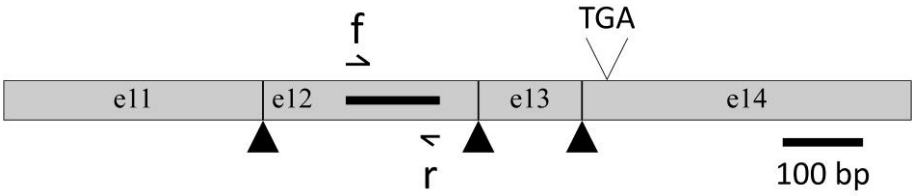

Figure 2.10. Schematic representation of the region amplified in real-time PCR. Exon-intron boundaries are indicated with filled arrowheads whereas primers are presented with half arrows. The sizes of displayed exons (e) of *HvBor1a* and primers are in scale. The fragment amplified (117 bp) in real-time PCR is shown with a filled line within exon 12 (e12). For detailed information on primers, see Table 2.20. (f: Bor1a_GSP4_F and r: Bor1a_GSP4_R)

2.8.4.2. Two-Step Real-Time RT-PCR

Materials obtained from B-stress series of Sahara and Clipper were used in expression analysis of *HvBor1a* using two-step real-time RT-PCR. Total RNA extraction using TRIzol and cDNA synthesis with Superscript III RT were performed by Dr. Tim Sutton at ACPFG. Generated cDNA samples were used in real-time RT-PCR which was performed by Dr. Neil Shirley at School of Agriculture, Food and Wine, University of Adelaide. The methods for reverse transcription and real-time PCR were done according to Section 2.6.2 and Section 2.6.3. Relative quantitation (Burton *et al.*, 2004) was employed for determination of expression levels of *HvBor1a* in leaf and root tissues of Sahara and Clipper. Moreover, *HvBor1a* transcript levels were determined in 1 cm-segments of roots of un-stressed plants. Transcript levels in segments of roots were investigated for determination of possible localized or tissue-dependent expression of putative B transporter gene *HvBor1a*.

2.8.4.3. One-Step Real-Time RT-PCR

Plant material obtained from B-stress series of Tarm-92 and Hamidiye were used for determination of endogenous transcript levels of *HvBor1a* using one-step real-time RT-PCR. Total RNA was extracted from leaf and root tissues according to procedures explained in Section 2.3.2. Contaminating genomic DNA and salts were removed by DNase I treatment followed by ethanol precipitation (Section 2.3.2.3). Quant-iT RiboGreen RNA Assay Kit

(Invitrogen) was employed for quantitation of RNA in solution and the fluorescence was recorded with NanoDrop 3300 Fluorospectrometer (Section 2.3.2.4). Final concentrations of samples were adjusted to 125 ng/µL and 2 µL of RNA preparations were used in one-step real-time RT-PCR.

Real-time RT-PCR where reverse transcription and PCR amplification were performed in a single tube was carried out using QuantiTect SYBR Green RT-PCR Kit (Qiagen). Real-time measurements of fluorescence were done with Rotor-Gene Q (Qiagen) real-time cycler according to instructions of the manufacturer. Approximately 250 ng of RNA was used as template in a 20 µL-reaction. The components and cycling conditions of real-time RT-PCR are provided in Table 2.21 and Table 2.22, respectively.

Table 2.21. Components of one-step real-time RT-PCR.

Reagent	Stock concentration		Final concentration		Volume (µL)
GS primer I *	5	µM	0.5	µM	2
GS primer II *	5	µM	0.5	µM	2
QuantiTect SYBR Green RT-PCR Master Mix **	2	X	1	X	10
QuantiTect RT Mix ***					0.2
Template RNA	125	ng/µL	250	ng‡	2
RNase-free water					3.8
Total					**20**

* Gene-specific (GS) primers I and II were Bor1a_GSP4_F and Bor1a_GSP4_R. See Table 2.20 for details.

** QuantiTect SYBR Green RT-PCR Master Mix contains HotStarTaq DNA polymerase, QuantiTect SYBR Green PCR buffer, fluorescent dye SYBR Green I, KCl, $(NH_4)_2SO_4$ and $MgCl_2$ providing a final concentration of 2.5 mM $MgCl_2$ in the reaction mixture.

*** QuantiTect RT mix contains a blend of Omniscript RT and Sensiscript RT.

‡ Final concentration of template RNA is indicated as total amount per reaction.

Table 2.22. Thermal cycling conditions used in one-step real-time RT-PCR.

		Temperature (°C)	Duration	Number of cycles	Fluorescence measurement
Reverse transcription		50	30 min	1	
Initial enzyme activation		95	15 min	1	
Quantitation	Denaturation	94	15 sec		
	Annealing	54	30 sec	45	
	Extension	72	30 sec		Green channel
Melting curve (1°C/step)		50 – 99	5 sec/°C	1	Green channel

Quantitation after one-step real-time RT-PCR was performed via absolute quantitation according to an external standard of plasmid DNA, pENTR/D-TOPO, carrying *HvBor1a* ORF. After spectrophotometric determination of plasmid concentration, the copy number of standard DNA was calculated using Equation 2.1, where C is the concentration and N is the number of molecules.

$$(\text{C g/µL DNA} / [\text{plasmid length in base pairs} \times 660]) \times 6.022 \times 10^{23} = \text{N molecules/µL} \quad (2.1)$$

Standard curve was generated using triplicates of 5 different 10-fold dilutions of plasmid DNA providing initial numbers of $10^7 – 10^3$ molecules per reaction. Values of C_T obtained for each dilution were plotted against $\log_{10}$ of initial amounts of standards. The slope of the curve was -3.844 indicating an efficiency of 82% for amplification with gene-specific primers (Table 2.20).

Quantitative real-time RT-PCR was performed in triple technical replicates of each RNA sample from three biological replicates. At least 2 reactions of NTC which contained all PCR components except the RNA template was included in each run of real-time PCR. Specificity of primer pair was confirmed with melting curve analysis performed after every run. The C_T values of reactions including RNA samples obtained from control and stressed barley seedlings were compared with the standard curve to determine the copy number of transcript of *HvBor1a* in the samples.

2.8.4.4. Northern Blotting

Expression levels of *HvBor1a*, determined using real-time RT-PCR, were confirmed with Northern blotting which provided higher specificity. Though sensitivity was low compared to real-time RT-PCR, Northern blotting was used to estimate expression levels of the gene. The method employed adsorption of total RNA to a solid support after size separation and hybridization with a radiolabelled probe. Densitometry-based comparison provided estimates of expression levels.

Total RNA extracted (Section 2.3.2) from leaf and root tissues of Sahara and Clipper, treated with varying concentrations of H_3BO_3, were used for Northern hybridization which was performed by Margaret Pallotta at ACPFG. Approximately 10 µg of RNA was size separated via electrophoresis using a 1.5% (w/v) agarose gel and 1X MOPS-EDTA buffer (50 mM MOPS and 1 mM EDTA) containing 1.1% (v/v) formaldehyde. RNA was suspended in sample loading buffer containing 1X MOPS-EDTA buffer, 6.5% (v/v) formaldehyde, 50% (v/v) formamide, 40% (w/v) sucrose, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol. Subsequently samples were heated at 65°C for 10 min and then chilled on ice prior to electrophoresis. Electrophoresis was performed at 60 V for 30 min and then at 100 V for a further 1 – 3 h (Section 2.3.5). Following electrophoresis gels were stained in a 1 µg/mL ethidium bromide solution for 10 min and RNA was visualized using UV illumination. Gels were photographed to record relative RNA loading per lane.

RNA was then transferred to positively charged nylon membrane (Biodyne B®, Pall Corporation) by capillary action using 20X SSC buffer. Transfer was performed for a minimum of 5 h. Membranes were then blotted dry on Whatman 3MM filter paper, wrapped in plastic film and stored at 4°C or -20°C until required.

The probe used for Southern hybridization was also used for Northern hybridization and it was prepared as described previously (Section 2.8.3.1). Northern membranes were pre-hybridized for approximately 24 h at 42°C in Northern pre-hybridization solution containing 5X SSPE solution, 7.5X Denhardt's III solution, 0.5% (w/v) SDS, 1.0 mg/mL salmon sperm DNA, and 45% (v/v) formamide. Pre-hybridization solution was replaced with hybridization solution prior to addition of the probe. Hybridization solution for Northern membranes

contained 5X SSPE solution, 5X Denhardt's III solution, 0.5% (w/v) SDS, 0.5 mg/mL salmon sperm DNA, 45% (v/v) formamide, and 2.5% (w/v) dextran sulphate. Hybridization was allowed to proceed at 42°C for 16 – 24 h.

After hybridization Northern membranes were washed for 20 min at 42°C in 2X SSC, 0.1% (w/v) SDS and then washed for 20 min at 42°C successively in 1X SSC, 0.1% (w/v) SDS and then 0.5X SSC, 0.1% (w/v) SDS. Subsequently the membranes were blotted on paper toweling, encased in plastic bags and then exposed to film in X-ray cassettes containing intensifying screens at -80°C for up to 10 days. Films were developed using standard methods.

2.8.5. Subcellular Localization of HvBor1a

Subcellular location of putative B transporter was determined using transient expression of HvBor1a-mGFP5 fusion protein in epidermal cells of onion.

2.8.5.1. Construct Preparation for Expression of Fluorescent Fusion Protein

The coding sequence (CDS) of *mGFP5* was fused to downstream of *HvBor1a* ORF that was lacking a stop codon to provide expression of a C-terminal fluorescent fusion protein. A PCR based method was carried out for construction of translational fusion (Hobert, 2002). The method employed a reaction in which two PCR products of overlapping DNA fragments were fused by PCR with a set of primers. The CDS of *mGFP5* was amplified from pCAMBIA1304 vector (http://www.cambia.org) without a start codon in an initial reaction. In a parallel second PCR, *HvBor1a* ORF was amplified with a modified reverse primer which introduced a 41 bp-extension to the 3' end of the fragment. The 3' extension had 26 bp-overhang complementary to the CDS of *mGFP5*. In the final PCR (fusion PCR) overlapping nucleotide sequences provided fusion of *mGFP5* in frame to the downstream of *HvBor1a* ORF. The sequences of primers used for construct preparation are provided in Table 2.23.

Table 2.23. Primer sequences used for PCR based fusion of ORFs of *HvBor1a* and *mGFP5*.

Primer ID	Direction	Sequence (5' – 3')	Tm (°C)
GFP_NOATG_F	Forward	AGTAAAGGAGAAGAACTTTTCACTGG	53.0
GFP_CA3UTR_R	Reverse	GGGGTTTCTACAGGACGTAAACTAGC	56.9
3HBor1a_5UTR_F	Forward	GACCCGCGCGCCGCGTCCTTAGCCG	78.2
3HBor1a_GFP_fs	Reverse	CCAGTGAAAAGTTCTTCTCCTTTACTCATTGTTA--TATCTCCCACGCTCGGCTGAACTGCATTTG	81.9
3HBor1a_C_ATG	Forward	ATGGATCTACTAGGGAACCCTTTCAAGG	60.2
GFP_TAA_R	Reverse	TTATTTGTATAGTTCATCCATGCCATGTGTAATCCC	65.4

Amplification of *mGFP5* CDS without a start codon was achieved in a PCR where approximately 10 ng of pCAMBIA1304 vector was used as template together with 0.25 µM of each forward and reverse primers, GFP_NOATG_F and GFP_CA3UTR_R. Annealing temperature was set to 58°C. In a parallel PCR run, ORF of *HvBor1a* was amplified with 0.25 µM 3HBor1a_5UTR_F and 0.25 µM 3HBor1a_GFP_fs. A two-step cycling without an annealing step was performed for the amplification of *HvBor1a* since the Tm values of oligonucleotides were high. The concentration of product obtained in two initial PCR runs was estimated by agarose gel electrophoresis (Section 2.3.5) with comparison to size markers. Approximately 10 – 50 ng/µL of products were used in fusion PCR with no purification or extraction.

Fusion PCR was performed with products of initial reactions as templates together with 0.25 µM of each nested primers, 3HBor1a_C_ATG and GFP_TAA_R. In a 35 cycles-amplification step annealing temperature was adjusted to 60°C and duration of extension was set to 2 min. The cycling was performed without the primer pair for the first 5 cycles, allowing hybridization between 3' extension introduced to ORF of *HvBor1a* and CDS of *mGFP5*. The nested primer pair added for the rest of cycles of amplification step provided amplification of fused ORFs of *HvBor1a* and *mGFP5*. Other components and cycling conditions used in all three PCR have been summarized elsewhere (Section 2.3.10).

Amplified product from fusion PCR was visualized on 1% (w/v) agarose gel (Section 2.3.5) and band of fragment was extracted from gel (Section 2.3.6). The purified fragment was introduced into pCR8/GW/TOPO (Section 2.3.8) and multiplied in *E. coli* TOP10 which was selected on solid LB containing 100 µg/mL spectinomycin (Section 2.3.7). Colony PCR with

gene- and vector-specific primers was employed for selection of true transformants carrying true recombinant molecules (Section 2.3.10). After plasmid isolation, the construct was sequenced (Section 2.3.11) for verification of sequence and direction of insertion. Then the entry vector carrying *HvBor1a-mGFP5* fusion was used in recombination to transfer the GOI into plant transformation vector, pEarleyGate100.

2.8.5.2. Preparation of Plant Expression Vector

Entry vector pCR8/GW/TOPO carrying *HvBor1a-mGFP5* and destination vector pEarleyGate100 were recombined *in vitro* for cloning into plant expression vector (Section 2.3.9). The recombination product was transformed into TOP10 cells (Section 2.3.7.2) and selection was done on solid LB media containing 100 µg/mL kanamycin. Selected colonies were streaked onto solid LB containing 12.5 µg/mL chloramphenicol for negative selection. Colony PCR with gene- and vector-specific primers was employed for selection of true transformants (Section 2.3.10). Recombinant pEarleyGate100 expression clones were isolated using QIAprep Spin Miniprep Kit where elution was done in 50 µL of ultrapure water. Concentration and purity of expression vectors were determined as described previously (Section 2.3.4.4).

Electroporation-competent cells of *A. tumefaciens* C58C1 were transformed with recombinant pEarleyGate100 vector carrying *HvBor1a-mGFP5* fusion (Section 2.3.7.2). Selection of transformed C58C1 was done on solid YEB media containing 100 µg/mL of each rifampicin, ampicillin and kanamycin. Colony PCR with gene- and vector-specific primers (Section 2.3.10) was employed for selection of true transformants which were used for plant transformation and expression of fluorescent fusion protein in plant cells.

2.8.5.3. Transient Expression in Onion Epidermal Cells

Transformation of plant epidermal cells and transient expression of fluorescent fusion protein were performed according to an infiltration-based method (Sparkes *et al.*, 2006) with minor modifications. A single colony of C58C1 transformed with binary vector pEarleyGate100 carrying ORF for *HvBor1a-mGFP5* fusion was used for inoculation of 5 mL of liquid YEB containing 100 µg/mL of each rifampicin, ampicillin and kanamycin. The culture

was incubated overnight at 28°C and 200 rpm in an orbital shaker. The cells were collected by centrifugation at 1,000*g* for 10 min at room temperature. After removal of supernatant, cells were re-suspended in 1 mL of freshly-prepared infiltration medium (Table 2.24). Centrifugation, removal of supernatant, and re-suspension in filtration medium were repeated once more to wash the cells and remove traces of antibiotics. The absorbance of the mixture was determined at 600 nm using a 10-fold dilution of cells. The final titer of the culture was adjusted to 0.5 OD units in a final volume of 1 mL.

Table 2.24. Infiltration medium used for transformation of plant epidermal cells.

Reagent	Stock concentration		Volume (mL)
Glucose	2	% (w/v)	12.5
MES *	500	mM	5
$Na_3PO_4.12H_2O$ *	20	mM	5
Acetosyringone **	1	M	5×10^{-3}
Ultrapure water			variable
Total			**50**

* Stock solutions of MES and $Na_3PO_4.12H_2O$ were stored at 4°C.

** Acetosyringone was prepared with DMSO and stored at -20°C.

The leaves of an onion bulb were cut into squares measuring 2 cm on each side. The samples without peeling the epidermis were placed on solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), turning onion core side up. The samples were placed under a white fluorescent lamb for 1 h before infiltration. The re-suspended C58C1 culture in infiltration medium was taken up in a 1 mL-syringe. The tip of the syringe without a needle was placed tightly on epidermis and the plunger was gently and slowly pressed down without damaging the tissue. The visual diffusion of liquid through epidermal layer was watched carefully until a region of 1 – 2 cm^2 was infiltrated. After infiltration the segments of onion leaves on solid MS medium were placed in dark in a growth chamber under normal physical conditions. For monitoring the expression under confocal microscope, the epidermal layer was peeled and infiltrated zone was mounted in water on glass microscope

slides under coverslips. Expression was monitored every 24 h for up to 4 – 5 days. Confocal microscopy was performed at the Middle East Technical University Central Laboratory.

2.9. Mapping of B Tolerance Gene on Barley 3H

The QTL on 3H for B tolerance was identified in a Clipper X Sahara F1-derived DH population (Jefferies *et al.*, 1999). The locus was reported to be associated with root length response under B toxicity. On the other hand, root length response was also determined by locus on 4H which was linked to *HvBot1*. The genetic marker *xAWBMA15* on 3H locus showed most significant association with relative root length (RRL), where a LOD score of 9.8 was determined for the region on 3H. In the current study, a candidate gene approach was employed to clone B transporter gene, *HvBor1a*. Moreover, positional cloning was considered to genetically map *HvBor1a* to QTL on 3H. Screening of DH population and progenies with previously designed markers was carried out. Furthermore development of new genetic markers and screening of aforementioned populations was performed *ad hoc* to narrow down the genetic distance between markers located on 3H QTL.

2.9.1. EST Sequence Mining and Primer Design

Gene colinearity with the syntenic region on rice chromosome 1 was used to generate markers. Predicted rice gene, LOC_Os01g08020.1 which was the best blast hit of *HvBor1a* in rice genome was used as a starting point. Genome sequences 150 kb upstream and 100 kb downstream of LOC_Os01g08020.1 was obtained from Gramene (http://www.gramene.org) Ensembl34 build of *O. sativa* Japonica Group. The DNA sequence was blasted against barley ESTs using BLASTN delimiting the database to non-human, non-mouse ESTs (est_others) and the organism to Hordeum (taxid:4512). Barley ESTs showing highest similarity and alignment score and lowest e-value were employed for primer design. Sequences of ESTs were handled using Vector NTI. After prediction of exon-intron boundaries according to sequences from rice genome, primer pairs were designed using Vector NTI. The primer pairs were designed such that the amplicon spanned at least one of the predicted introns where convenient. The sequences of the primers together with GenBank accession numbers of ESTs are provided in Table 2.25. The primer pairs were used to amplify DNA sequences from barley cultivars Sahara and Clipper to locate SNPs.

Table 2.25. Primer sequences for the barley ESTs employed for marker development.

	GenBank ID	Direction	Sequence (5' – 3')	Tm (°C)
EST1	BQ469119	Forward	ACAACTACCATTTGTTCTGAAGGGC	56.7
		Reverse	AGAACAAGTATCTTGGTCCGTTTCAG	56.1
EST2	EX584292	Forward	ATGGAGAGAGCATGCAGATTTTGAAG	58.9
		Reverse	TTCATCGGTGCAACCATCATCC	59.1
EST3	FD526638	Forward	AAGGTTCAGCTGCGGTTTATTTTAC	56.3
		Reverse	CGCCAATGTGTCACCAGATGATATC	59.2
EST4	EX599622	Forward	GCCAGCTGGTCTCAACTGTTGGAAAC	62.9
		Reverse	CCTCCTGTGAGCTCTCAATTTTGTTC	58.5
EST5	BG365872	Forward	TTTTAAGTCGGTGCACGGGAACATG	62.6
		Reverse	TGCGGAAAGGTAGGCCAATTACTG	60.2
EST6	EX594048	Forward	GGTTGAAATGTTGTTTCCTTTGACCAC	59.8
		Reverse	GCCTTCCGTAGTTCACGTTCAATC	57.9
EST7	CA031717	Forward	CTCCAGCCGCGAGTACGAACGAAAC	65.5
		Reverse	GTGCGTCCCTTGTCATCTGCTTTC	60.8
EST8	EX594732 *			
EST9	AU252391	Forward	GCAAAGAAGCTTGTCTGCCCTCTAG	58.9
		Reverse	CGCTCGAAGGTCAAATTCCTCTTG	60.1
EST10	CA006353	Forward	GCTCACCTGTGAGCCTGGTACAAG	58.9
		Reverse	GTGCTGAAAATCGGGGTGGAGGTC	63.4
EST11	GH209979	Forward	TCTAAGTGCCACAGCCGATCAGCC	63.2
		Reverse	TGTCACCGGTCCACCAGTGCATCAC	66.5
EST12	GH216431	Forward	GCGAAGACAGGGACGACGGCATTG	67.2
		Reverse	TGCGCCTTAAGGTAGGTGTCCACGG	65.6
EST13	EX585096	Forward	CGTTGATAAGTGGCTCCATATGCTC	57.9
		Reverse	ATAGCCGTATCTGCGATCGTCGTC	60.3
EST14	BF262108	Forward	CCACAACGCAGAAGCTGCAGGAGTTC	65.7
		Reverse	CCTTGCTGAACGGGAGGAAAGC	60.8
EST15	BF256699	Forward	GGGGGTCGATGTGAGCCCTAACGTAAAC	66.6
		Reverse	GAGGATGCGGCATGGCAGTTTATC	62.4
EST16	CB879639	Forward	GTTGAAGCCGCACCAGACGTTG	61.3
		Reverse	CGGTGCAGATCAAGACGAGCTG	59.5
EST17	BI952473	Forward	AGTTCTTCCAGCCCTCCGACACCAG	64.4
		Reverse	TCTGACCGGCCGAAAACCACGTACC	67.2
EST18	BY850842	Forward	ATGGCGGTGGAGGAGATAACGGAG	63.1
		Reverse	CTTCAGAATCTCCGCCACAGTCAC	58.8
EST19	AU252391	Forward	GCAAAGAAGCTTGTCTGCCCTCTAG	58.9
		Reverse	CGCTCGAAGGTCAAATTCCTCTTG	60.1
EST20	BQ765196	Forward	TGGTGAACACGGCGTCGGCGGGCG	77.3
		Reverse	TCCGCCTGCAGCAGATGCACGCCG	74.6

* EX594732 showed significant similarity to *HvBot1* and *HvBor1a*, therefore the sequence of EST was not employed for primer design.

2.9.2. CAPS Marker Development for 3H QTL

The cleaved amplified polymorphic sequence (CAPS) markers employ PCR amplification and restriction enzyme digestion for determination of genetic differences resulting from SNPs. Sequence differences between cultivars might create or abolish restriction sites and the differences might be detected in length of DNA fragment after digestion. For development of novel CAPS markers, the primer pairs (Table 2.25) were used to amplify DNA sequences from barley cultivars Sahara and Clipper. Subsequently the resulting amplicons were sequenced to locate SNPs which might have potential to be used as CAPS markers.

Approximately 20 ng of genomic DNA samples extracted from Sahara and Clipper were used in conventional PCR with 1 µM of each forward and reverse primer. The reaction mixture at a final volume of 50 µL was incubated with thermal cycling conditions where annealing temperature was adjusted to 62°C and duration of extension was set to 1.5 min. Other components and cycling conditions of PCR have been described elsewhere (Section 2.3.10). Amplified products were fractionated on 1.5% (w/v) agarose gel (Section 2.3.5) and bands of fragments were extracted from gel (Section 2.3.6). The purified fragments were sequenced as stated previously (Section 2.3.11). Sequence reads obtained from two different cultivars were aligned and investigated using ContigExpress. In case an SNP was located, the amplicon was digested with a proper restriction enzyme. Approximately 10 – 12 µL of PCR product without purification was used in digestion reaction in a final volume of 15 µL in the presence of 20 U of restriction enzyme and optimal buffer of the enzyme supplied by the manufacturer. The reaction mixtures were supplemented with BSA at a final concentration of 300 ng/µL. Digestion mixtures were gently flicked and incubated at 37°C in a thermal cycler for 2 h. Digestion products were fractionated on 2% (w/v) agarose gel (Section 2.3.5) and genetic differences were scored.

2.9.3. Screening of Doubled Haploid Population and F2 Progenies

The Clipper X Sahara DH population consisting of 150 individuals was screened using two simple sequence repeat (SSR) markers, EBmac0761 and EBmac0848 (Ramsay *et al.*, 2000). The sequences of primers and some properties of SSR markers are provided in Table 2.26. Diluted genomic DNA samples extracted from individuals of DH population were used as

templates in touchdown PCR. The components and cycling conditions are provided in Table 2.27 and Table 2.28, respectively. The products of PCR without purification were fractionated on either 3% (w/v) agarose gel (Section 2.3.5) or 12% (w/v) polyacrylamide gel according to standard procedures using Dual Adjustable Mega-Gel Kit vertical electrophoresis system (C.B.S. Scientific Company Inc). The fragments were visualized after staining with ethidium bromide under UV illumination using a gel documentation system. The DHs were genetically scored according to parental cultivars, Sahara and Clipper.

Table 2.26. Sequences of primers, the repeat motif and the expected amplicon size of SSR markers.

SSR	Direction	Sequence (5' – 3')	Tm (°C)	Amplicon size (bp)	Repeat motif
EBmac0761	Forward	TTCCTTCGTTCTCTTGGA	44.8	~176	$(AC)_9(CA)_7$
	Reverse	GAGCAACATAAAGCTAGCG	45.6		
EBmac0848	Forward	CTTGCAAAGTGTGAAGTAGC	45.1	~160	$(AC)_6(CA)_{10}$
	Reverse	TCTACCGCATACTCAAAGTG	45.4		

Table 2.27. Components used in PCR for genetic screening with SSR markers.

Reagent	Stock concentration	Final concentration	Volume (µL)
Primer mix *	10 µM	2.5 µM	2.5
dNTP mix	2 mM	0.2 mM	1
PCR buffer **, ‡	10 X	1 X	1
Q-solution **	5 X	1 X	2
Taq DNA polymerase **	5 U/µL	0.025 U/µL	0.05
Template	~5 ng/µL	variable	2
Ultrapure water			1.45
Total			**10**

* Primer mix contained 10 µM of each forward and reverse primer for SSR markers.
** Taq DNA polymerase, PCR buffer and Q-solution were purchased from Qiagen.
‡ PCR buffer provides a final concentration of 1.5 mM $MgCl_2$ in the reaction mixture.

Table 2.28. Thermal cycling conditions used in touchdown PCR for SSR markers.

		Temperature (°C)	Duration	Number of cycles
Amplification	Denaturation	94	30 sec	20
	Annealing	60 *	30 sec	
	Extension	72	30 sec	
Amplification	Denaturation	94	30 sec	30
	Annealing	50	30 sec	
	Extension	72	30 sec	
Final extension		72	5 min	1

* Annealing temperature was decreased by 0.5°C every cycle during first amplification step.

Moreover, Clipper X Sahara DH population and F2 progenies derived from crosses between four DHs were scored genetically using a CAPS marker developed in the context of this study. The F2 mapping population used for recombinant screening was derived from the crosses DH105 X DH11 and DH113 X DH120. The parent DH plants differed for alleles at B tolerance locus on 3H but not for alleles at other known B tolerance loci. The DH plants DH11 and DH113 displayed Clipper allele for the 3H locus whereas DH105 and DH120 displayed Sahara allele. Both DH and F2 populations were screened with the CAPS marker developed using the sequence of barley EST, BF262108 (EST14 in Table 2.25). The primer pair amplified 532 bp fragments, which differed for purine at position 266, from genomic DNA of Clipper and Sahara. The identified SNP introduced a restriction site for *Pst*I on the sequence amplified from Sahara but not Clipper.

Approximately 10 ng of DNA extracted from DH and F2 populations were used as templates in conventional PCR with 0.5 µM of each forward and reverse primer. The reaction mixture at a final volume of 10 µL was incubated in a thermal cycler with cycling conditions where annealing and extension were performed at 62°C for 20 sec and at 72°C for 50 sec, respectively, during 35 cycles-amplification step. Other components and cycling conditions of conventional PCR have been described elsewhere (Section 2.3.10). Subsequently total PCR product without purification was used in digestion reaction in a final volume of 15 µL in the presence of 20 U *Pst*I and 1X NEBuffer 3 (both provided by NEB). The reaction mixtures were supplemented with BSA at a final concentration of 300 ng/µL. Digestion mixtures were gently flicked and incubated at 37°C in a thermal cycler for 3 h. Digestion products were

fractionated on 2% (w/v) agarose gel (Section 2.3.5) and visualized after staining with ethidium bromide under UV illumination using a gel documentation system. The Clipper allele produced a single band of 532 bp fragment whereas Sahara allele produced a single band of two digested 266 bp fragments. Heterozygous individuals from F2 mapping population produced both bands of 532 bp and 266 bp fragments.

2.9.4. Relative Root Length Assay

Individual F2 plants derived from the crosses DH105 X DH11 and DH113 X DH120 were grown in hydroponics and RRL was measured after 14 days to determine the tolerance phenotype. Approximately 50 seeds from each cross were surface sterilized (Section 2.3.1.1) and germinated on filter papers for 2 days in dark at 4°C. Imbibition and seedling growth were performed in basal growth solution (Section 2.1.1 and Appendix A). Germinated seeds were placed on porous mashes and transferred to hydroponics with aeration for 14 days (Section 2.3.1.2). Seedlings were grown in plastic boxes containing basal growth solution supplemented with 10 mM H_3BO_3 for B toxicity treatment. Control groups were grown in basal growth solution containing 15 µM H_3BO_3. After 14 days of growth root length of seedlings were recorded and RRL was calculated. The individuals were also scored genetically using the CAPS marker developed (Section 2.9.3) to follow the recombination on 3H and to observe the variation in RRL controlled by segregation at 3H locus.

CHAPTER 3

RESULTS AND DISCUSSION

Boron (B) deficiency or toxicity impairs plant growth and reduces crop yield in agriculture. On the other hand, our understanding of the molecular responses of plants to B stress and the sources of impairments in physiology and metabolism to tackle this problem is limited. Though function of several B transporters in uptake and translocation, structural role of B in cell wall, and functional importance of B complexes have been demonstrated, various aspects of response under B deficient and toxic conditions such as signal perception and transduction, adjustment of gene expression, modulation of metabolism and physiology, and key cellular components underlying these mechanisms are unknown.

Expression profiling together with biochemical and physiological studies should shed light on key components of plant responses to B toxicity or deficiency. Moreover, determination of responsive genes should enlighten successive studies aiming to elucidate molecular mechanism of tolerance to B stress. Therefore, transcriptional responses of barley to B toxicity and deficiency were investigated using microarrays in this study (Section 3.3). Additionally, a putative B transporter gene from barley was cloned and examined for its possible roles in tolerance to B toxicity (Section 3.4).

3.1. Quality and Quantity of Nucleic Acids Isolated

Integrity and purity of RNA are the main factors affecting efficiency of target preparation for hybridizations and quality of the microarray data. Purity of RNA was assessed by recording ratio of absorbance readings at 260 and 280 nm (Table 3.1).

Table 3.1. Concentration and purity of RNA samples used for microarray analysis. Total RNA was isolated from at least 3 biological replicates of leaf and root tissues of Hamidiye and Tarm-92. For abbreviations and experimental design in microarray analysis, see Section 2.4.1 and Table 2.4. (Ha: Hamidiye; Ta: Tarm-92)

Cultivar	Treatment ($[H_3BO_3]$)	Abbreviation	Tissue	OD_{260}/OD_{280}	Concentration (µg/µL)
Hamidiye	Control (10 µM)	Ha-C	Leaf	2.02	2.84
				2.05	3.31
				1.98	3.20
			Root	2.01	2.75
				2.11	2.93
				1.97	2.81
				2.10	3.11
	B toxicity (5 mM)	Ha-T	Leaf	1.94	2.99
				1.99	3.47
				2.01	3.11
			Root	1.97	2.59
				2.09	2.83
				2.04	3.13
	B toxicity (10 mM)	Ha-TT	Leaf	1.94	3.26
				2.03	3.05
				1.95	3.67
			Root	N/A	N/A
Tarm-92	Control (10 µM)	Ta-C	Leaf	2.10	2.90
				2.04	3.39
				1.94	2.98
				2.05	3.05
			Root	1.96	3.22
				1.96	3.56
				2.08	3.49
	B deficiency (0.02 µM)	Ta-D	Leaf	2.03	3.89
				2.09	3.43
				2.11	3.50
				2.12	3.66
			Root	2.00	3.02
				1.89	2.64
				1.98	2.92
	B toxicity (5 mM)	Ta-T	Leaf	2.00	2.97
				2.03	3.22
				2.03	3.15
				2.12	3.01
			Root	1.99	2.71
				2.05	2.44
				2.08	2.63

N/A: Not available

Concentrations of RNA preparations determined on a spectrophotometer and NanoDrop 3300 Fluorospectrometer are presented in Table 3.1. The integrity of RNA was checked by separation of rRNA species using agarose gel electrophoresis and microfluidic analysis with Agilent 2100 Bioanalyzer. Discrete bands of rRNA species, with no smearing between bands in electrophoresis and discrete peaks, with no degradation products between peaks in microfluidic analysis demonstrated integrity of RNA samples (Figure 3.1).

Throughout this study genomic DNA of barley was used for certain analyses such as southern blotting, genome walking, and mapping of QTL for tolerance to B toxicity. Quantity and purity of DNA preparations are presented in Table 3.2. Contaminating biological or cellular material as well as phenol, ethanol, and salts used during isolation procedures, and any other impurities in DNA or RNA preparations either inhibit reactions or lower the efficiency of the reactions. Therefore, critical measures were taken and decontamination of surfaces, solutions, and equipment, which come in contact with nucleic acids during isolation or storage, was performed cautiously.

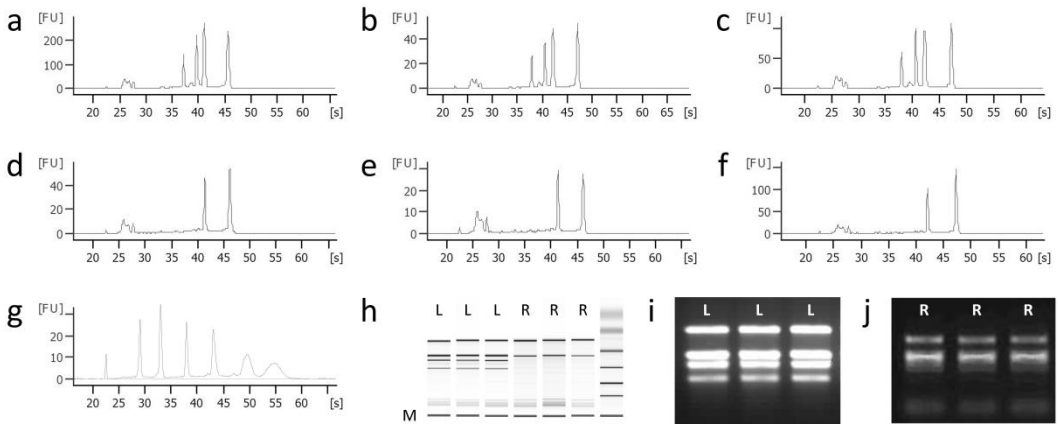

Figure 3.1. Electrophoretic separation of representative total RNA samples used for microarray analysis. Electropherograms of RNA extracted from leaf (a – c) and root (d – f) tissues, electropherogram of the ladder (g), and pseudo-gel image of RNA samples (h) obtained by microfluidic separation using Agilent 2100 Bioanalyzer are displayed. The last lane in pseudo-gel image displays ladder. A spiked-in marker (M) is used for alignment of pseudo-bands of rRNA species. Electrophoresis gel images (i, j) of representative total RNA, isolated from leaf (L) and root (R) tissues and subsequently separated on 1.5% (w/v) agarose gel, are also presented.

Table 3.2. Concentration and purity of DNA samples. Genomic DNA was isolated from leaf tissues of Hamidiye, Tarm-92, Sahara and Clipper.

Cultivar	OD_{260}/OD_{280}	Concentration (µg/µL)
Hamidiye	1.92	3.18
Tarm-92	1.98	2.92
Sahara	1.90	3.56
Clipper	1.88	3.09

3.2. Quality Control and MIAME Compliance of Microarray Hybridizations

Target preparation and sample handling during experimental part of microarray analysis include series of reactions such as reverse transcription, *in vitro* transcription, biotin labeling, and hybridization. The efficiency of these reactions is another important factor – other than quality of RNA samples – affecting the quality of microarray data. Certain metrics such as yield and size distribution of products are assessed prior to hybridization of target to array. Size distribution of labeled un-fragmented or fragmented aRNA target was estimated using electrophoresis or microfluidic analysis (Figure 3.2). Labeled un-fragmented aRNA samples showed a wide distribution of sizes from 250 – 5500 nucleotides (nt) with most of the population having sizes between 600 – 1200 nt. On the other hand, fragmented aRNA

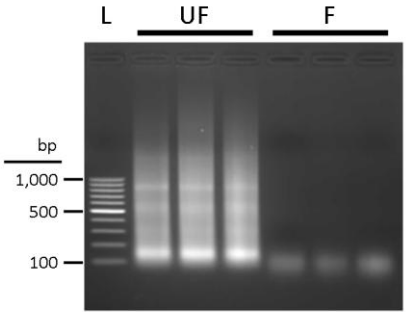

Figure 3.2. Electrophoretic separation of representative un-fragmented and fragmented aRNA samples used for microarray hybridizations. Electrophoresis gel image of 3 purified un-fragmented (UF) and 3 fragmented (F) aRNA samples, synthesized from total RNA and subsequently separated on 1.5% (w/v) agarose gel, is displayed. (L: Ladder)

samples showed a distribution of fragments that are sized between 35 – 200 nt with an accumulation around 100 – 120 nt. Thus size distribution of target at critical experimental steps provided evaluation of efficiency of the reactions and initial level of quality control for microarray analysis. Moreover, yield and concentration of labeled aRNA also provided an assessment of efficiency of the reactions.

Quality control of array data after scan was primarily evaluated using report files (.RPT files) generated by GCOS. Reports summarized critical metrics such as scaling factor and percentage of probe sets with P – present – calls, which were considered important quality control criteria (Hoffman *et al.*, 2004). According to the report files, it was observed that percentages of probe sets with P calls were 40 – 70% which indicated a good quality target, highly efficient target preparation and high quality array scan. Moreover, scaling factor values were less than 3 and similar among hybridizations within the project. Averages of background signal values were less than 100 and were lower than the average signals for probe sets flagged P. Average values of noise were from 1 – 5 and similar for different array scans. All spike-in controls *bioB*, *bioC*, *bioD* and *cre* had P calls, which indicated successful hybridizations. Similarly, poly-A controls *dap*, *lys*, *phe*, *thr*, and *trp* had P calls, which indicated efficient target preparation. In certain cases where some of the poly-A controls were called A – absent – other quality metrics were evaluated once more thoroughly and expected values for these metrics indicated the overall merit of the array data. A representative report file is provided in Appendix G.

Furthermore, visual inspection of border around the array, checkerboard corners, central plus sign, the GeneChip array name, and control regions in the center indicated the high caliber of hybridizations. All array images displayed expected patterns. Additionally, hybridization uniformity was checked visually by inspecting areas of high background or areas of low signal intensity. All array images showed uniform hybridization over the entire area of the array. Small defects, caused by foreign insoluble material or air bubbles, were masked since they were less than 1% of the total probes for the array. Masking was done using GCOS. Signal intensities from defected area were excluded as outliers without affecting the quality of the data generated.

MIAME (Minimum Information About a Microarray Experiment) describes the minimum information that is needed to enable the interpretation of the results of a microarray based gene expression monitoring experiment unambiguously and to reproduce and verify the experiment independently (Brazma *et al.*, 2001). Although details for particular experiments are different, MIAME aims to define the core that is common to most experiments. MIAME is not a formal specification, but a set of guidelines on information to be provided for description of a microarray experiment.

The six components of a microarray experiment include array, sample, experimental design, hybridization procedures and conditions, normalization, and data. MIAME defines information required in each of these components in six parts. Additionally, MIAME/Plant extends the guidelines set by MIAME by adding parameters and ontologies for description of the sample and the experimental design (Zimmermann *et al.*, 2006). In this study all the guidelines by MIAME and MIAME/Plant have been considered and information provided in relevant sections (Chapter 2) describes all the details. Moreover, microarray data has been submitted to the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) and the Plant Expression Database (PLEXdb; http://plexdb.org) which are MIAME- and MIAME/Plant-compliant, respectively. The expression data generated for intra-varietal comparison of responses under B toxicity are deposited at GEO under series GSE14521 and at PLEXdb under experiment BB63 (Öz *et al.*, 2009).

3.3. Expression Profiling of Barley under B Stress

Plant responses to B stress appears to be multigenic and controlled temporally and spatially. Global monitoring of gene expression at specific developmental stage or in specific tissues is therefore crucial to gain insight into such multigenic responses. In this study, global expression profiling of barley under B toxicity or deficiency at seedling stage was performed using total RNA isolated from leaf and root tissues of Turkish cultivars, Tarm-92 (tolerant to B toxicity) and Hamidiye (sensitive to B toxicity). GeneChip Barley Genome Array was utilized for microarray based determination of changes in expression levels associated with B stress.

Hydroponically grown barley seedlings were subjected to shock treatments of B toxicity or deficiency under aseptic and controlled environmental conditions. Field conditions differ

extremely from laboratory conditions and B stress that plants are exposed to in the field is mild. In this study, we have profiled the transcriptomes under controlled physical conditions and extreme B treatments in order to reveal responses associated solely with B toxicity or deficiency. Microarray analysis was designed to investigate both inter- and intra-varietal differences in gene expression. Intra-varietal comparisons included investigations of transcriptional responses in sensitive cultivar, Hamidiye, under B toxicity at two different toxic levels of B, applied as 5 and 10 mM H_3BO_3. Moreover, gene expression levels were examined in Tarm-92 under B deficiency or toxicity to make a second intra-varietal comparison. Inter-varietal comparison was performed to reveal differences of expression between Tarm-92 and Hamidiye under B toxicity applied as 5 mM H_3BO_3.

3.3.1. Intra-varietal Comparison I: Response of Sensitive Cultivar to B Toxicity

Effects of B toxicity on transcriptome of a sensitive barley cultivar, Hamidiye, were investigated at two toxic concentrations (5 mM and 10 mM) of H_3BO_3 (Öz *et al.*, 2009) which were previously reported to induce cellular membrane damage in leaf tissues of barley (Karabal *et al.*, 2003). At the end of treatment period, necrosis and leaf symptoms that are characteristic to B toxicity were observed at tips and margins of mature leaves of Hamidiye treated with high concentrations of H_3BO_3 (Figure 3.3). These symptoms indicated accumulation of B at the end of transpiration stream. For microarray analysis, a total of 9 hybridizations were performed with total RNA extracted from leaf tissues of 3 biological replicates of seedlings grown under control conditions (C), 5 mM H_3BO_3 treatment (T), and 10 mM H_3BO_3 treatment (TT).

Figure 3.3. Leaf symptoms characteristic to B toxicity. Necrotic zones observed at tips and margins of first emerging leaves of Hamidiye (Ha) under B toxicity are presented. (TT: Toxicity applied as 10 mM H_3BO_3 treatment)

3.3.1.1. Filtering and Principal Component Analysis

Filtering performed after RMA preprocessing and normalization, resulted in 19,424 probe sets with intensity values higher than the 20th percentile in at least 1 out of 9 hybridizations. Data analysis was carried out with the resulting 19,424 probe sets. Subsequently one-way ANOVA ($P < 0.05$) was performed to obtain a subset of data that contains 999 probe sets displaying significant expression levels with P values lower than the cut-off (Appendix H). In order to reduce the dimensions of microarray data, principle component analysis (PCA) on conditions was performed both with 19,424 probe sets and with 999 significantly regulated probe sets, limiting the number of uncorrelated variables also called principal components to three.

The PCA of all hybridizations performed with 19,424 probe sets indicated that hybridizations with control samples were distinct from hybridizations with treated samples according to second component which accounted for 21.07% of variation. The variance represented by the first component (61.04%) in this analysis can be explained by the possible biological variation among barley seedlings used as replicates of a condition (Öz *et al.*, 2009) or the presence of outliers in the data since PCA is sensitive to outliers. On the other hand, PCA performed with 999 significantly regulated probe sets provided a more informative depiction of the trends in the data. Plot visualization of the PCA performed using significantly regulated probe sets with hybridizations as separate data points is presented in Figure 3.4. The uncorrelated variables were reduced to three components and first two were plotted. The three principal components accounted for 66.8%, 24.63% and 8.57% of the variation in the data.

When plotted as data points, hybridizations with biological replicates of a condition were located in close proximity and distinct from other samples according to both components 1 and 2 which accounted for a total of more than 91% of variation (Figure 3.4). Moreover, data points representing hybridizations with untreated control samples (Ha-C) were separate from other two groups of data points (Ha-T and Ha-TT) according to component 1 which represented 66.8% of variation. These observations indicated that treatments of toxic levels of B resulted in global expression differences in leaf tissues of Hamidiye and the main source of variation among conditions was the B toxicity applied.

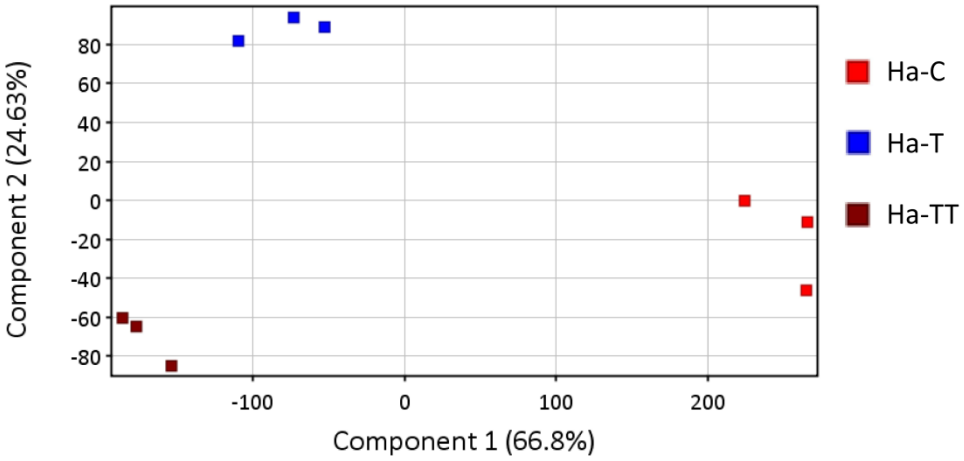

Figure 3.4. Principle component analysis of all hybridizations performed for intra-varietal comparison of responses of Hamidiye (Ha) under B toxicity. Percentages indicate variance. Biological replicates of a condition are indicated with the same color. Analysis was performed with significantly regulated probe sets. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment)

3.3.1.2. Selection of Genes Responsive to B Toxicity

Expression value-dependent filtering and statistical methods were used to obtain responsive genes in Hamidiye under B toxicity. Fold change was calculated as the ratio of expression values under treatment to that under control conditions using GeneSpring GX. Fold change of at least 2 was considered as an indication of differential expression, whereas P value of at most 0.05 was considered as an indication of significant alteration in expression.

Number of probe sets – representing genes – differentially regulated in leaf tissues of Hamidiye (Ha) under B toxicity are presented in Table 3.3. Filtering on expression values revealed that 168 and 312 genes were differentially expressed at least two-fold compared to control conditions under 5 mM and 10 mM H_3BO_3 treatments, respectively. Scatter plots shown in Figure 3.5 display these differentially expressed genes and their expression values under two levels of B toxicity. Among these, 35 were down-regulated and 133 were up-regulated under 5 mM H_3BO_3 (T) treatment, whereas 70 were down-regulated and 242 were up-regulated under 10 mM H_3BO_3 (TT) treatment (Table 3.3). Among differentially expressed genes, 132 were common to both treatments (Figure 3.6a).

Table 3.3. Number of probe sets differentially regulated in leaf tissues of Hamidiye (Ha) under B toxicity. Up- and down-regulation is assigned relative to control conditions. (FC: Fold change; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment)

Abbreviation (Cultivar-Treatment)	Differential Regulation (FC ≥ 2)			Significant Regulation (P < 0.05)
	Up	Down	Total	
Ha-T	133	35	168	37
Ha-TT	242	70	312	61

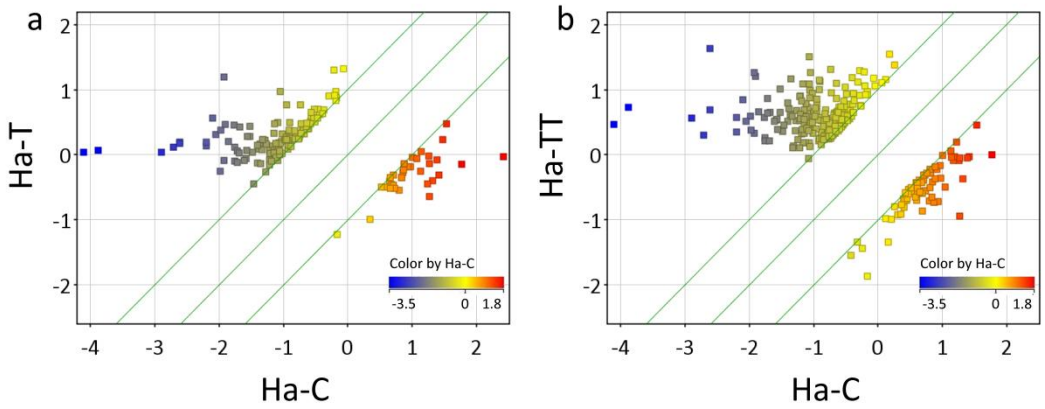

Figure 3.5. Scatter plots displaying genes and their expression values in leaf tissues of Hamidiye (Ha) under B toxicity. Expression values of differentially regulated genes under 5 mM H_3BO_3 (a) and 10 mM H_3BO_3 (b) treatments are displayed. Both axis in both graphs show normalized expression values. Diagonal lines indicate two-fold (2X) difference lines. Points above and below the 2X diagonal lines indicate up- and down-regulated genes, respectively. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment)

Furthermore, significant (P < 0.05) differences were observed in expression levels of 37 and 61 genes under 5 mM (T) and 10 mM (TT) H_3BO_3 treatments, respectively (Table 3.3). Among significantly altered genes, 31 were common to both treatments (Figure 3.6a). Expression patterns of genes which showed distinctive regulation and common to both treatments are shown in Figure 3.6b and 3.6c. All the 31 genes that were significantly altered showed up-regulation in leaf tissues of Hamidiye under both treatments (Figure 3.6c).

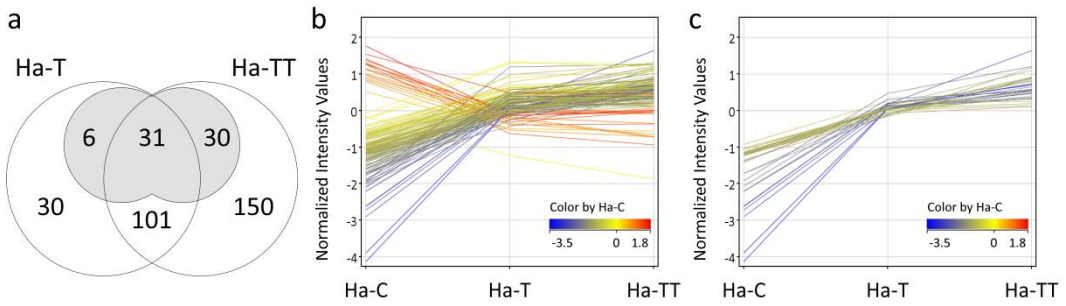

Figure 3.6. Number and expression patterns of differentially and significantly regulated genes in Hamidiye (Ha) under B toxicity. Venn diagram (a) shows number of genes differentially (outer light circles) and significantly (inner dark circles) regulated. Expression patterns of 132 differentially (b) and 31 significantly (c) regulated genes which are common to both treatments are displayed. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment)

Number of differentially expressed genes almost doubled when the B toxicity was increased from 5 mM (168 genes) to 10 mM (312 genes). Similarly, number of up- and down-regulated genes under TT treatment was approximately two times the number under T (Figure 3a). Thus it might be concluded that differential regulation in expression is dose-dependent. Furthermore, number of up-regulated genes (133 in T and 242 in TT) was more than three times the number of down-regulated genes (35 in T and 70 in TT). Additionally, all significantly regulated genes common to both T and TT showed up-regulation. In a global view it might be stated that B toxicity regulates gene expression in leaf tissues of Hamidiye by induction rather than repression and response to B involves induction of genes.

3.3.1.3. Clustering of Responsive Genes

Hierarchical clustering on responsive genes and conditions was done for 348 differentially (Figure 3.7a) and for 67 significantly (Figure 3.7b) expressed genes which constitute the entire subset of genes showing distinct expression values under T and TT treatments. Hierarchical clustering on conditions placed Ha-T and Ha-TT on the same branch separating Ha-C from both treatments (Figure 3.7). This result, consistent with the variation represented by PCA component 1 (Figure 3.4), further indicated that the main source of transcriptional differences among conditions was the B toxicity applied.

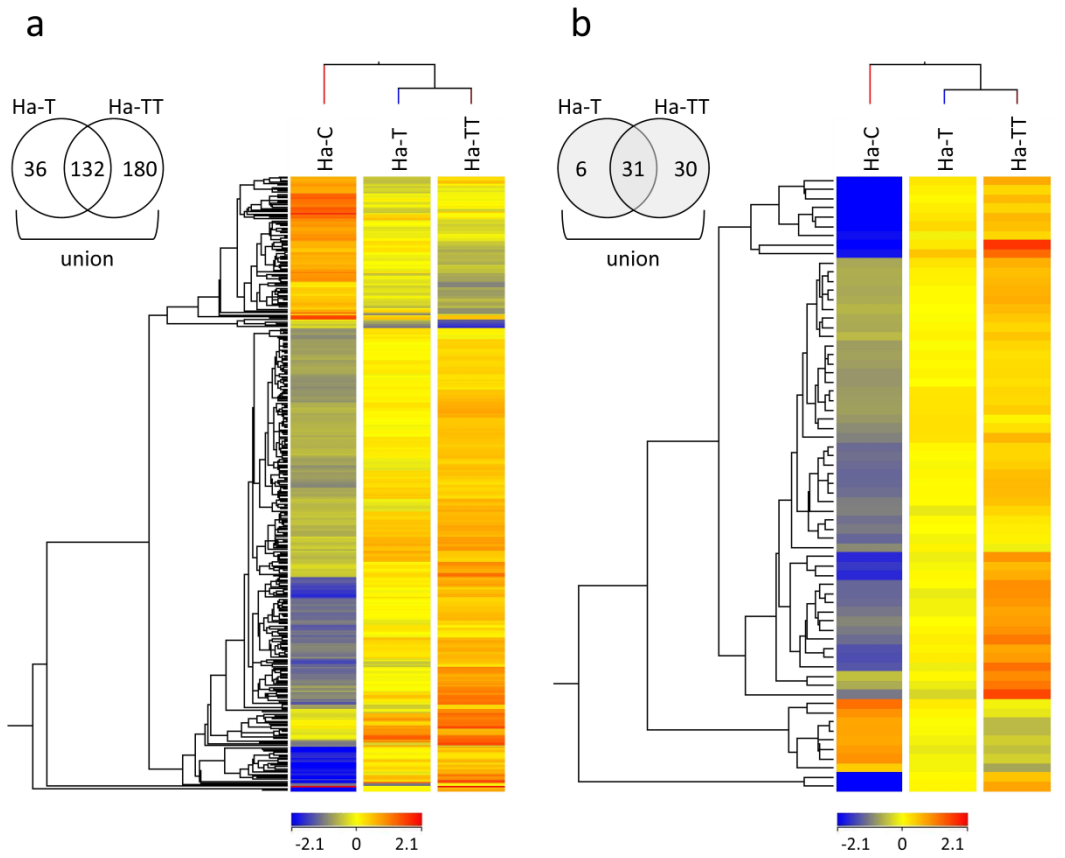

Figure 3.7. Hierarchical clustering of genes and treatments for intra-varietal analysis of transcriptional responses of Hamidiye (Ha) under B toxicity. Clustering of 348 differentially (a) and 67 significantly (b) regulated genes, which constitute the entire genes (union) showing distinct expression values under treatments, are displayed. Rows and columns represent genes and treatments, respectively. The color bars represent the corresponding expression values. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment)

3.3.1.4. Functional Categories of Differentially Regulated Genes in Hamidiye

Genes that showed differential regulation under treatments of B toxicity were annotated using HarvEST:Barley (version 1.83, assembly 35). Additionally, PLEXdb was used to annotate probe sets, find out orthologs in model genomes such as rice, and predict gene functions (Shen *et al.*, 2005). Annotations of probe sets, representing uncharacterized genes or unigenes, provide useful and suggestive information since they are frequently based on sequence similarity to a known protein or EST in another organism (Clarke and Zhu, 2006).

On the other hand, information in model organisms evolves in a rapid pace. Thus, recent version of HarvEST:Barley and updated annotations for probe sets were utilized in analysis. The output from HarvEST:Barley included the information on best BLASTX hits from UniProt database (http://www.uniprot.org; February 2010) and gene models of rice MSU version 6 (http://rice.plantbiology.msu.edu; January 2009), *Arabidopsis thaliana* TAIR version 10 (http://www.arabidopsis.org; November 2010), and *Brachypodium distachyon* BRADI version 1 (http://www.brachypodium.org; May 2009). Besides descriptions of the best hits, the output also included UniProt, rice, *Arabidopsis*, and *Brachypodium* accession numbers of the best hits. The output also provided information on unigenes that were represented by the probe sets.

Putative functions based on annotations were used to classify genes involved in or related to various biological processes. A selected list of probe sets, putative functions, and regulation of the genes under B toxicity are provided in Table 3.4. All 348 genes that showed differential regulation in leaf tissues of Hamidiye at least by two-fold under B treatments and their annotations were listed in Supplementary Table S.1 which included information obtained from HarvEST:Barley, regulation of genes under 5 mM (T) and 10 mM (TT) H_3BO_3 treatments, and fold changes in expression values compared to control conditions.

Categories of biological processes included genes encoding proteins induced by jasmonic acid (JA) or related to JA synthesis, associated with pathogenesis or senescence, having glutathione *S*-transferase (GST) activity, functioning in regulation of transcription and transport, showing kinase, transferase, and monooxygenase activities, and binding unfolded proteins (Table 3.4). Two concentrations of H_3BO_3 treatment in our experimental conditions resulted in up-regulation of JA related genes and genes encoding GST, cytochrome P450 (CYP), and pathogenesis related (PR) and senescence associated (SA) proteins in leaf tissues of Hamidiye. Moreover, alterations were observed in expressions of genes having transcription factor (TF), chaperone, and transporter activities. Microarray expression profiling revealed up-regulation of ATP-binding cassette (ABC) transporters and down-regulation of NOD26-like membrane integral proteins (NIP) in Hamidiye under B toxicity applied in this study (Table 3.4). Genes coding TFs including Spl7 protein, MYB-type TFs, C2H2 zinc finger protein, NAC domain containing TFs, and CBF1-like protein BCBF1 were up-regulated. On the other hand, WRKY family and DRE binding TFs were down-regulated.

Table 3.4. Selected differentially regulated genes which are categorized according to putative molecular function. (JA: Jasmonic Acid; GST: Glutathione *S*-Transferase; PR: Pathogenesis Related; SA: Senescence Associated; TF: Transcription factor; HSP: Heat Shock Protein; CYP: Cytochrome P450; FC: Fold Change; Ha: Hamidiye; C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; TT: Toxicity applied as 10 mM H_3BO_3 treatment)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C*	FC and regulation Ha-TT/Ha-C
JA related genes			
Contig7886_at	JA-induced protein	2.63 ↑	2.37 ↑
Contig1675_s_at	23 kDa JA-induced protein	-	2.99 ↑
rbags15p13_s_at	23 kDa JA-induced protein	-	2.19 ↑
Contig1678_s_at	23 kDa JA-induced protein	-	2.14 ↓
Contig1684_x_at	JA-induced protein	-	2.38 ↓
HV11O04r_s_at	Glutamine-dependent asparagine synthetase	-	2.06 ↑
Contig3097_at	Allene oxide synthase	2.37 ↑	4.38 ↑
HV_CEb0020D05r2_s_at	Allene oxide cyclase precursor	-	2.76 ↑
Contig4986_at	Allene oxide cyclase precursor	-	2.51 ↑
Contig26053_at	S-adenosyl-L-methionine:JA carboxyl methyltransferase	2.47 ↑	3.28 ↑
HVSMEf0011J01r2_s_at	Lectin protein kinase family protein	2.10 ↑	2.29 ↑
Contig13905_at	Lectin protein kinase family protein	-	2.19 ↑
Contig21059_at	Lectin protein kinase	-	2.14 ↑
Contig3548_at	O-methyltransferase	2.18 ↓	-
Contig393_at	Alcohol dehydrogenase 3	2.22 ↓	2.50 ↓
GST genes			
Contig5838_at	GST	5.20 ↑	8.67 ↑
Contig2248_at	GST	2.86 ↑	4.17 ↑
Contig13901_at	GST	2.71 ↑	3.88 ↑
Contig9764_at	GST	2.78 ↑	4.30 ↑
Contig12776_at	GST	2.36 ↑	3.09 ↑
Contig6008_s_at	GST 31	2.03 ↑	2.26 ↑
HV_CEb0004O15r2_s_at	GST 42	2.14 ↑	4.08 ↑
Contig18367_at	GST 42	-	2.09 ↑
HVSMEa0014H14r2_s_at	GST 22	-	2.04 ↑
Contig9632_at	GST 22	-	2.05 ↑
HVSMEa0011L14r2_s_at	GST	-	2.39 ↑
Contig12776_s_at	GST	-	2.59 ↑
PR genes			
Contig2550_x_at	Wheatwin-2 precursor	2.04 ↑	3.53 ↑
Contig1637_s_at	Glucan endo-1,3-beta-glucosidase GII precursor	3.17 ↑	4.09 ↑

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Expression differences less than two-fold are indicated with -.

Table 3.4. (continued)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C	FC and regulation Ha-TT/Ha-C
PR genes (continued)			
Contig1637_at	Glucan endo-1,3-beta-glucosidase GII precursor	2.03 ↑	-
Contig2210_at	PR protein PRB1-2 precursor	-	2.03 ↑
Contig2212_s_at	PR protein PRB1-2 precursor	-	2.81 ↑
Contig15882_s_at	Fatty acid alpha-oxidase	-	2.17 ↑
SA genes			
Contig2787_s_at	Thaumatin-like protein TLP5	3.70 ↑	3.95 ↑
Contig11118_at	B12D protein	2.29 ↑	3.25 ↑
Contig8605_at	B12D protein	-	2.28 ↑
Contig8605_s_at	B12D protein	-	2.03 ↑
Contig14377_at	Glycerophosphoryl diester phosphodisterase	-	2.21 ↑
TF genes			
Contig18961_at	Spl7 protein	2.35 ↑	2.93 ↑
Contig23823_at	C2H2 zinc finger protein	2.32 ↑	2.12 ↑
Contig3667_s_at	GAMyb	2.02 ↑	3.56 ↑
EBem10_SQ002_I10_s_at	GAMyb	2.89 ↑	4.39 ↑
Contig8369_at	AP2D23-like TF	2.10 ↑	-
Contig4395_at	Ethylene-insensitive-3-like protein	-	2.01 ↑
Contig13201_at	CCT motif family protein	-	2.19 ↑
HM07L17r_at	NAC domain TF	-	2.02 ↑
Contig15617_at	CBF1-like protein BCBF1	-	2.20 ↑
Contig18390_at	DRE binding TF	2.16 ↓	3.14 ↓
Contig12005_at	WRKY family TF	2.13 ↓	2.25 ↓
Contig21110_at	TF WRKY69	-	2.18 ↓
Contig2479_at	CBF3A-6.1	-	2.32 ↓
rbaal35o24_at	Heat shock TF	-	2.34 ↓
HSP genes			
EBem05_SQ003_L06_at	Small HSP, chloroplast precursor	3.33 ↑	-
Contig998_s_at	Heat shock cognate 70 kDa protein 2	-	2.02 ↓
Contig2008_s_at	16.9 kDa class I HSP	-	2.02 ↓
Contig10029_at	17.8 kDa class II HSP	-	2.92 ↓
Transporter genes			
Contig20774_at	MDR-like ABC transporter	5.30 ↑	6.90 ↑
Contig20553_at	PDR-like ABC transporter	-	2.09 ↑
HO15C14S_s_at	ABC transporter-like protein	-	2.14 ↑
Contig25386_at	peptide transporter protein	2.29 ↑	3.51 ↑

Table 3.4. (continued)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C	FC and regulation Ha-TT/Ha-C
Transporter genes (continued)			
HV_CEb0022J21r2_at	peptide transporter PTR2	-	2.50 ↑
Contig8001_at	amino acid transporter A1	2.03 ↑	-
Contig21251_at	proton-dependent oligopeptide transporter	-	2.06 ↓
Contig11285_at	mitochondrial phosphate transporter	2.27 ↑	2.80 ↑
Contig20673_at	phosphate translocator	-	2.05 ↑
Contig24175_at	anion/sugar transporter	-	2.48 ↑
Contig14075_at	P-type ATPase	-	2.12 ↑
Contig5632_at	NOD26-like membrane integral protein	2.52 ↓	2.83 ↓
Contig5632_s_at	NOD26-like membrane integral protein	3.74 ↓	4.62 ↓
Contig15329_at	Probable auxin efflux carrier component 6	-	2.04 ↓
Contig25699_at	Integral membrane-like protein	2.51 ↓	-
CYP genes			
Contig3045_at	CYP709C1	3.01 ↑	3.13 ↑
Contig3047_s_at	CYP709C1	7.13 ↑	8.15 ↑
Contig15560_at	CYP71C4	2.09 ↑	2.38 ↑
Contig15561_s_at	CYP	3.62 ↑	3.90 ↑
EBro08_SQ004_B22_at	CYP72A26	2.13 ↑	3.78 ↑
Contig4271_at	CYP	2.03 ↑	-
Contig17080_at	CYP family protein	-	2.17 ↑

High degree of regulation, more than five-fold increase, was observed among up-regulated genes in leaf tissues of Hamidiye. Approximately 13 and 22 probe sets displayed more than five-fold up-regulation under 5 mM (T) and 10 mM (TT) H_3BO_3 treatments, respectively (Table S.1). Most pronounced inductions were observed in expression levels of genes coding for esterases, oxalate oxidases, and enolases. Genes of esterases were up-regulated approximately 16- and 24-fold under 5 mM and 10 mM H_3BO_3 treatments, respectively, in leaves of Hamidiye (Table S.1). On the other hand, most marked reduction in expression was observed for Contig5632_s_at representing a putative *NIP* with nearly a 4-fold decrease in expression. Higher degrees of up-regulation indicated induction rather than repression of expression as a global response to B toxicity in leaves of sensitive barley cultivar Hamidiye.

3.3.1.5. Regulation of Jasmonic Acid Related Genes under B Toxicity

Phytohormones are a collection of trace amount growth regulators such as auxin, cytokinin, abscisic acid (ABA), jasmonic acid (JA), ethylene, salicylic acid (SA), and few others (Tuteja and Sopory, 2008). Lipid-derived JA and its metabolites, collectively known as jasmonates, are important plant signaling molecules that mediate plant responses to environmental stress and function in various aspects of growth and development (Wasternack, 2007; Balbi and Devoto, 2008). JA is a stress hormone produced when the plant is exposed to pathogens or other environmental stress conditions (Truman *et al.*, 2007).

Boric acid treatment in our experimental conditions resulted in up-regulation of genes involved in JA biosynthesis or genes responding to elevated levels of JA (Table 3.4). Among JA-induced genes both up-regulation and down-regulation were observed, whereas most of the JA biosynthetic genes were up-regulated. JA is one of the signal molecules produced in an integrated signaling network (Devoto and Turner, 2005) and B toxicity might be inducing a response which is connected to the JA regulated responses.

It might be suggested that JA is involved in signaling of B toxicity after perception of signal or it is used for amplification of signal and modulation of gene expression in barley leaves under B toxicity. On the other hand, it might also be suggested that signal transduction pathways involved in JA signaling and signaling under B toxicity are highly connected. This proposed connection or crosstalk might be achieved by various kinases, phosphatases, or transcription factors and regulators. Walia *et al.* (2006) also reported induction of JA related or responsive genes as a key feature of response to salinity in barley. Similarly, Ozturk *et al.* (2002) reported up-regulation of genes encoding JA-responsive proteins under drought stress in barley.

The proposed involvement of JA in cellular signaling of or molecular responses to B toxicity should be investigated in succeeding studies at the protein and metabolite level. Regulation of protein abundances or enzyme activities and relationship between JA and B contents of cells or tissues should be investigated in barley under B toxicity.

3.3.2. Intra-varietal Comparison II: Differences between Responses under B Toxicity and Deficiency

The window of B sufficiency for plants is narrow and both deficient and toxic concentrations of B result in growth retardation and large losses in yield. Moreover, physiological, biochemical, and transcriptional responses of plants to deficiency and toxicity might vary significantly. Therefore it is crucial to investigate global expression profiles under B deficiency and toxicity to unravel the responses of plants and gain insight into functional roles of B in plant biology. Effects of B deficiency and toxicity on transcriptome of leaf and root tissues of a barley cultivar, Tarm-92, were investigated in this study under treatments of deficient and toxic concentrations of B. Deficiency and toxicity was applied in nutrient solutions containing 0.02 µM and 5 mM H_3BO_3, respectively. Hybridizations were performed with total RNA extracted from leaf and root tissues of at least 3 biological replicates of seedlings grown under control conditions (C), B deficiency (D), and B toxicity (T).

3.3.2.1. Tissue Differences Revealed by Principal Component Analysis

As a result of initial filtering performed after RMA normalization 20,139 probe sets, with intensity values greater than the 20^{th} percentile in at least one hybridization, were retained for further data analysis. The remaining probe sets were considered either not expressed or expressed extremely low under all experimental conditions that analysis would lead to misjudgments. Subsequently two-way ANOVA ($P < 0.05$) was performed to obtain a subset of data that contains 16,567 probe sets displaying significant expression levels with P values lower than the cut-off (Appendix H). PCA on conditions was performed with significantly regulated probe sets to discover trends and reduce the dimensions of the data. The components were limited to three in the analysis and first two were plotted (Figure 3.8).

The PCA of all hybridizations revealed that tissue difference comprised the main source of variation according to the first component which represented 76.99% of variation. When plotted as data points, hybridizations with samples from leaf and root tissues were located at distinct areas of the plot, away from each other (Figure 3.8). This apparent result indicated existence of distinct transcriptomes expressed in leaf and root tissues.

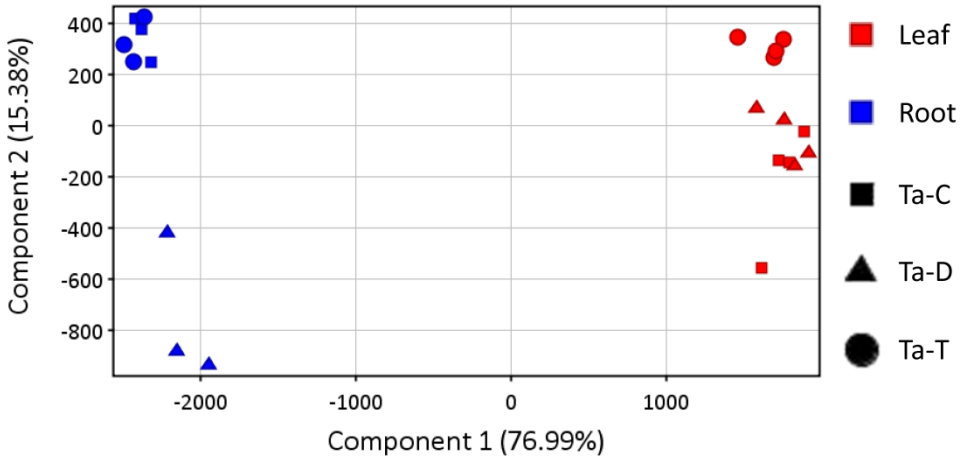

Figure 3.8. Principle component analysis of all hybridizations performed for intra-varietal comparison of transcriptional responses of Tarm-92 (Ta) under B toxicity and deficiency. Percentages indicate variance. Tissues are indicated with different colors whereas biological replicates of a condition are indicated with the same shape. Analysis was performed with significantly regulated probe sets. (C: Control; D: Deficiency applied as 0.02 µM H_3BO_3 treatment; T: Toxicity applied as 5 mM H_3BO_3 treatment)

For better depiction of trends in the data, hybridizations with samples from leaf and root tissues were evaluated in separate analysis. In the group comprising only the arrays hybridized with RNA from leaf tissues, 19,980 probe sets out of 22,840 displayed signal intensities higher than the 20th percentile in at least one array. Data analysis was carried out with this subset of data containing 19,980 probe sets. One-way ANOVA ($P < 0.05$) was performed to obtain significantly regulated subset of probe sets. Statistical analysis retained 3,412 probe sets which were used in PCA on conditions.

Plot visualization of PCA components and hybridizations as data points is presented in Figure 3.9a. The three principal components accounted for 76.15%, 15.21% and 8.64% of the variation in the data. According to component 1, data points representing hybridizations with RNA from leaf tissues treated with B toxicity (Ta-T) were isolated from other two groups of data points (Ta-C and Ta-D). The data points Ta-C and Ta-D were located at distinct areas of plot according to component 2 which represented only 15.21% of variation. These results indicated that treatment with B toxicity led to more pronounced differences in transcriptome of leaf tissues of Tarm-92 compared to B deficiency.

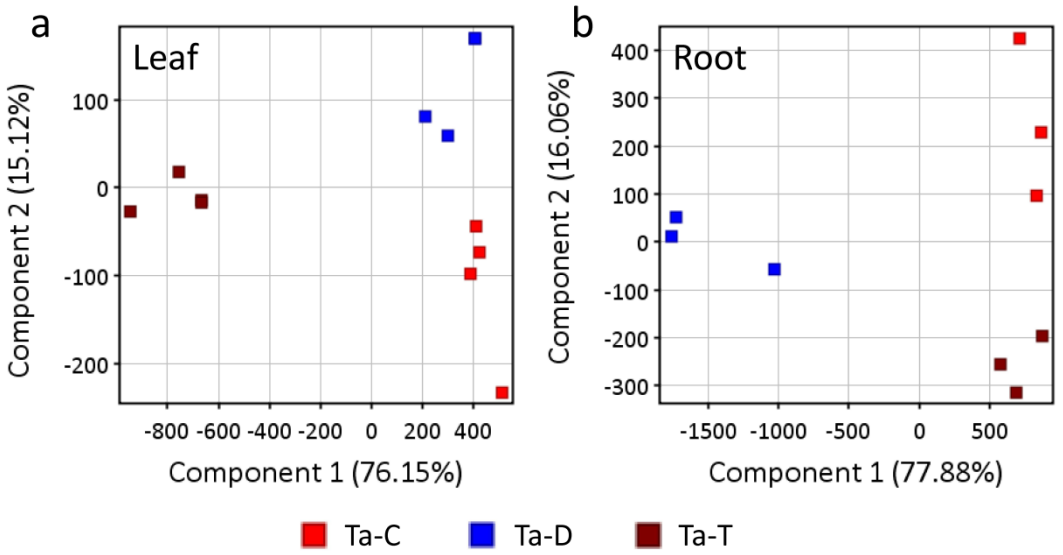

Figure 3.9. Principle component analysis of hybridizations performed for intra-varietal comparison of transcriptional responses in leaf and root tissues of Tarm-92 (Ta) under B toxicity and deficiency. Percentages indicate variance. Biological replicates of a condition are indicated with the same color. Analysis was performed with significantly regulated probe sets. (C: Control; D: Deficiency applied as 0.02 µM H_3BO_3 treatment; T: Toxicity applied as 5 mM H_3BO_3 treatment)

On the other hand, in the group comprising only the arrays hybridized with RNA from root tissues, 19,183 probe sets displayed signal intensities higher than the 20th percentile in at least one array. One-way ANOVA ($P < 0.05$) retained 4,927 significantly regulated probe sets which were used in PCA on conditions. Plot of hybridizations as data points according to resultant PCA components is presented in Figure 3.9b.

The three principal components represented 77.88%, 16.06% and 6.06% of the variation in the data. Interestingly, data points representing hybridizations with samples from root tissues treated with B deficiency (Ta-D) were isolated from other two groups of data points (Ta-C and Ta-T) according to component 1. Whereas, the data points Ta-C and Ta-T were located at distinct areas of plot according to component 2 which represented only 16.06% of variation. These results indicated that treatment with B deficiency led to more pronounced differences in transcriptome of root tissues of Tarm-92 compared to B toxicity.

3.3.2.2. Determination of Regulated Genes in Leaf and Root Tissues

Differentially or significantly regulated genes were determined using signal intensity-based filtering and statistical analysis where hybridizations with samples from leaf and root tissues were evaluated separately. Number of probe sets differentially or significantly regulated in Tarm-92 (Ta) under B deficiency (D) and toxicity (T) are presented in Table 3.5.

Table 3.5. Number of probe sets differentially regulated in leaf and root tissues of Tarm-92 (Ta) under B deficiency and toxicity. Up- and down-regulation is assigned relative to control conditions. (FC: Fold change; D: Deficiency applied as 0.02 µM H_3BO_3 treatment; T: Toxicity applied as 5 mM H_3BO_3 treatment)

Abbreviation (Cultivar-Treatment)	**Differential Regulation (FC ≥ 2)**			**Significant Regulation (P < 0.05)**
	Up	**Down**	**Total**	
Leaf				
Ta-D	24	3	27	3
Ta-T	286	139	425	303
Root				
Ta-D	340	663	1003	366
Ta-T	118	320	438	7

In the analysis of hybridizations with RNA from leaf tissues, it was observed that 425 and 27 genes were differentially expressed under toxic and deficient concentrations of B, respectively. Among these, 286 genes were up-regulated and 139 were down-regulated under B toxicity (T), whereas 24 genes were up-regulated and 3 were down-regulated under B deficiency (D).

In the analysis of hybridizations with RNA from root tissues, it was observed that 438 and 1003 genes were differentially regulated under B toxicity (T) and B deficiency (D), respectively. Among these, 118 genes were up-regulated and 320 were down-regulated under B toxicity, whereas 340 genes were up-regulated and 663 were down-regulated under B deficiency. Scatter plots provided in Figure 3.10 display these differentially regulated genes and their expression values in leaf and root tissues of Tarm-92.

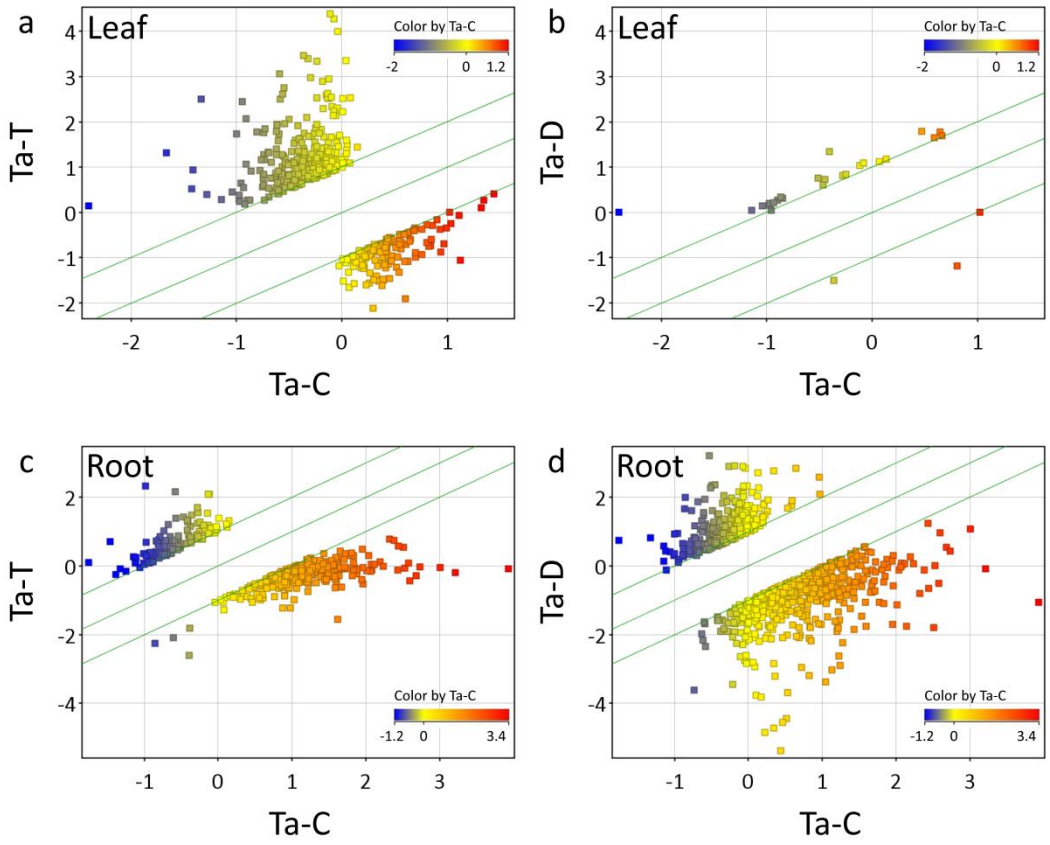

Figure 3.10. Scatter plots displaying genes and their expression values in leaf and root tissues of Tarm-92 (Ta) under B toxicity and deficiency. Expression values of differentially regulated genes under toxicity (a, c) and deficiency (b, d) are displayed. Both axis in all graphs show normalized expression values. Diagonal lines indicate two-fold (2X) difference lines. Points above and below the 2X diagonal lines indicate up- and down-regulated genes, respectively. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; D: Deficiency applied as 0.02 µM H_3BO_3 treatment)

Moreover, number of significantly regulated genes in leaf tissues was determined as 303 and 3 under B toxicity (T) and deficiency (D), respectively. On the other hand, 7 and 366 genes were significantly regulated in root tissues of Tarm-92 under B toxicity (T) and deficiency (D), respectively (Table 3.5). Venn diagrams and plots of expression profiles presented in Figure 3.11 display differentially or significantly regulated genes that are common to both treatments (Ta-D and Ta-T). Among significantly regulated genes, only 3 were common to both treatments in leaf tissues (Figure 3.11a).

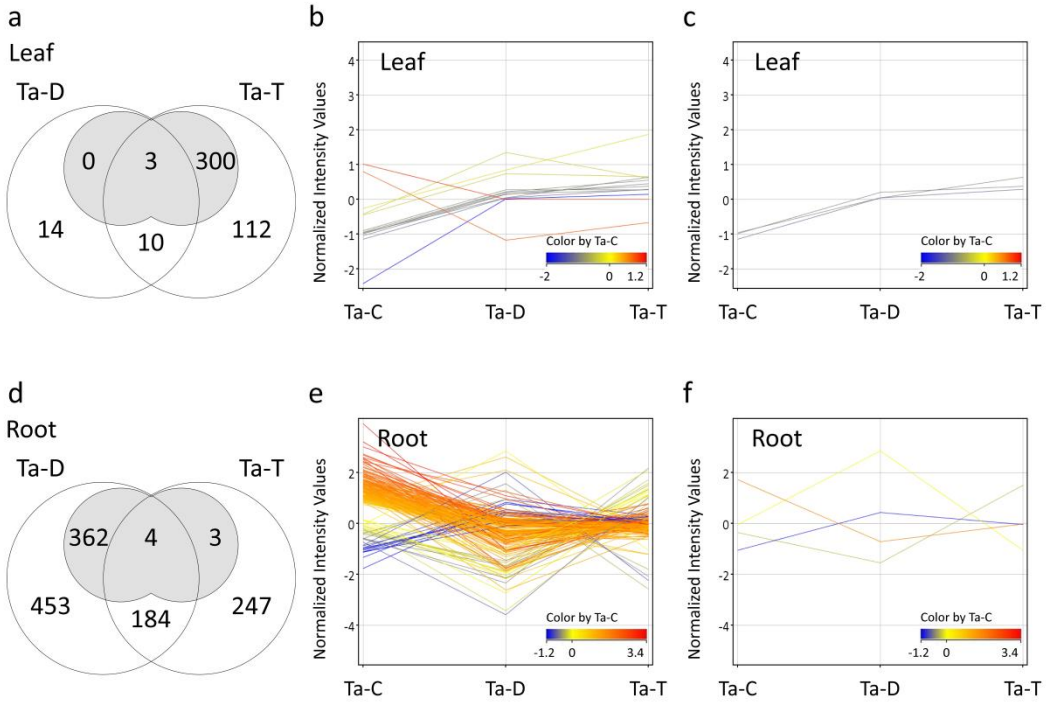

Figure 3.11. Number and expression patterns of differentially and significantly regulated genes in leaf and root tissues of Tarm-92 (Ta) under B deficiency and toxicity. Venn diagrams (a, d) show numbers of genes differentially (outer light circles) and significantly (inner dark circles) regulated. Profile plots display expression patterns of differentially (b, e) and significantly (c, f) regulated genes which are common to both treatments. Expression profiles of 13 differentially (b) and 3 significantly (c) regulated genes in leaf tissues and expression patterns of 188 differentially (e) and 4 significantly (f) regulated genes in root tissues are displayed. (C: Control; D: Deficiency applied as 0.02 µM H_3BO_3 treatment; T: Toxicity applied as 5 mM H_3BO_3 treatment)

The 3 genes coding for allene oxide synthase (AOS) and two predicted proteins displayed significant up-regulation under both toxicity and deficiency (Figure 3.11c). *AOS* (Contig3097_at) and two uncharacterized genes (Contig11993_at and Contig4797_at) showed 2.02-, 2.29-, and 2.29-fold up-regulation under B deficiency and 3.04-, 2.71-, and 2.57-fold up-regulation under B toxicity, respectively. Similarly in root tissues, only 4 among significantly regulated genes were common to both treatments (Figure 3.11d). Interestingly, expression of the gene (Contig8872_at) coding for CONSTANS-like protein was up-regulated 7.39-fold under B deficiency and down-regulated 2.02-fold under B toxicity. The expression of the gene (Contig1466_s_at) coding an avenin-like protein was down-regulated 2.30-fold under deficiency and up-regulated 3.61-fold under toxicity. Other two uncharacterized genes

(HW08C04u_at and Contig2098_at) showed 5.38-fold down-regulation and 2.81-fold up-regulation under B deficiency, and 3.34-fold down-regulation and 2.02-fold up-regulation under B toxicity (Figure 3.11f).

Interestingly, high number of genes was significantly regulated under B toxicity in leaf tissues, whereas similar significant regulation of a high number of genes was observed in root tissues under B deficiency (Table 3.5 and inner dark circles of Venn diagrams in Figure 3.11a and 3.11d). These observations and plots of PCA components provided in Figure 3.9 indicated that toxicity and deficiency resulted in substantial changes in gene expressions in leaf and root tissues, respectively. Major shift in transcriptome observed in leaf tissues under B toxicity might be explained by nature of stress and translocation of B in plant tissues. It is well known that B readily moves into plant cells through apoplastic stream, is transported in xylem, and accumulates in leaf tissues (Raven, 1980; Brown and Hu, 1994; Miwa and Fujiwara, 2010). Therefore, a greater shift in gene expression in leaf tissues – rather than roots – under B toxicity is expected and well correlates with the findings of this study. Although B toxicity applied as high concentration of H_3BO_3 solution was in contact with root tissues but not leaves, transportation of the micronutrient B within plant tissues ascertained transcriptional responses in leaf tissues under B toxicity. Additionally, B toxicity resulted in significant regulation of only 7 genes in root tissues further indicating a small effect on gene expression. This observation might be the result of the fact that it was the tolerant cultivar, expression profiles were investigated in or transportation of B rescued root tissues from toxic effects of high B.

On the other hand, ample shift in transcriptome observed in root tissues under B deficiency might be explained by the requirement of B or functional roles of B in plant tissues. Since the site of absorption of B is root tissue and B is applied in solution as H_3BO_3, plant cells may be modulating the transcriptome to take up more B from the environment to fulfill its requirements or may not be performing some metabolic processes where role of B is critical and shutting down expression. Additionally, B deficiency resulted in significant regulation of only 3 genes in leaf tissues further indicating a small effect on gene expression in leaves. This observation might be the result of the fact that expression profiles were investigated at seedling stage. Effects of B deficiency are mainly observed at reproductive tissues at later stages of plant development rather than seedling stage (Dell and Huang, 1997).

3.3.2.3. Expression Profiles of Genes Responsive to B Deficiency and Toxicity

Hierarchical clustering on genes and conditions was performed for 303 (Figure 3.12a) and 369 (Figure 3.12b) significantly regulated genes which constitute the entire subset of genes showing significant regulation in leaf and root tissues, respectively, under B toxicity or deficiency. For the leaf tissues, control (Ta-C) and deficiency (Ta-D) were clustered together whereas for the root tissues, control (Ta-C) and toxicity (Ta-T) were clustered together. This observation was consistent with the percentages of variation determined by PCA (Figure 3.9) and number of genes significantly regulated in leaf or root tissues (Table 3.5).

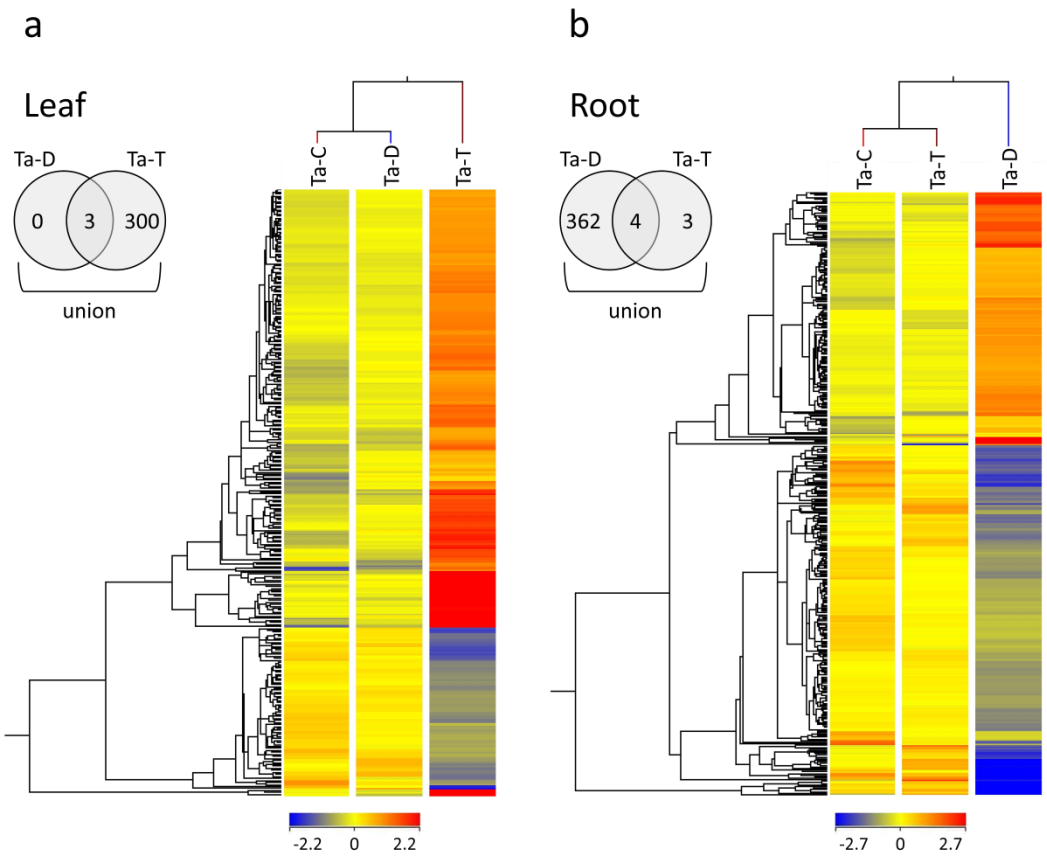

Figure 3.12. Hierarchical clustering of genes and treatments for intra-varietal analysis of transcriptional responses of leaf and root tissues of Tarm-92 (Ta) under B deficiency and toxicity. Clustering of 303 (a) and 369 (b) significantly regulated genes, which constitute the entire (union) significantly regulated genes under both treatments in leaf and root tissues, respectively, are displayed. Rows and columns represent genes and treatments, respectively. The color bars represent the corresponding expression values. (C: Control; D: Deficiency applied as 0.02 µM H_3BO_3 treatment; T: Toxicity applied as 5 mM H_3BO_3 treatment)

3.3.2.4. Significantly Regulated Genes upon B Toxicity

Since major changes in transcriptome of leaf tissues were observed under B toxicity, only the list of genes that showed significant regulation in leaves under B toxicity was further investigated. Expression profiles of these genes are provided in Figure 3.13a. On the other hand, list of genes that showed significant regulation under B deficiency in root tissues was further investigated since major shifts in transcriptome of root tissues were observed under B deficiency. Expression profiles of these genes are provided in Figure 3.13b. Putative functions which were assigned to probe sets or genes using HarvEST:Barley and PLEXdb were used to categorize genes involved in or related to various biological processes. The entire lists of genes, fold changes in expression values, putative functions, and regulation under B toxicity (T) and deficiency (D) are provided in Supplementary Table S.2 and S.3.

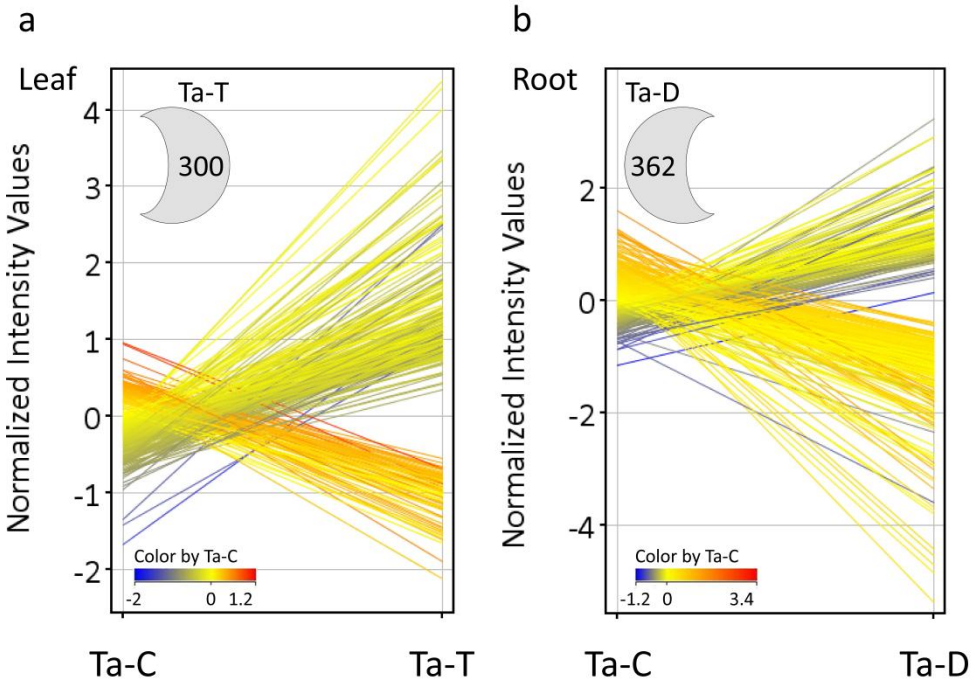

Figure 3.13. Expression patterns of significantly regulated genes in leaf and root tissues of Tarm-92 (Ta). Profile plots of genes significantly regulated under B toxicity (T) in leaf tissues (a) and B deficiency (D) in root tissues (b) are displayed. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; D: Deficiency applied as 0.02 µM H_3BO_3 treatment)

Supplementary Table S.2 lists all the genes significantly regulated in leaf tissues under B toxicity whereas Table S.3 lists all the genes significantly regulated in root tissues under B deficiency. Additionally, a list of probe sets significantly regulated at least five-fold in leaf tissues under B toxicity is provided in Table 3.6. Among these, only two genes that were represented by Contig2672_at and HV_CEb0009D09r2_at were down-regulated upon B toxicity and all the others were up-regulated. High degree of up-regulation was determined in expression levels of esterases, oxalate oxidases, thionin family proteins, and JA-induced proteins. Most of the up-regulated genes were involved in or related to responses to environmental stresses especially biotic stress. Thus it might be concluded that response to B toxicity might involve production of defensive compounds and it might be highly interconnected with responses to other biotic and abiotic stresses.

Table 3.6. Genes that are significantly regulated at least five-fold in leaf tissues of Tarm-92 (Ta) under B toxicity. Presented P values ($P < 0.05$) were corrected with multiple testing corrections in statistical analyses. (JA: Jasmonic Acid; CYP: Cytochrome P450; ABA: Abscisic acid; FC: Fold Change; Ta: Tarm-92; C: Control; T: 5 mM H_3BO_3 treatment)

Probe Set ID	Putative function	P value	FC and regulation Ta-T/Ta-C*
Contig10057_at	Esterase PIR7B	<0.0001	22.63 ↑
Contig3017_at	Oxalate oxidase GF-2.8	<0.0001	20.59 ↑
Contig10057_s_at	Esterase PIR7B	<0.0001	16.51 ↑
Contig1579_s_at	THION9 - Plant thionin family protein	0.0100	14.35 ↑
Contig11149_at	Metallo-beta-lactamase family protein	<0.0001	14.34 ↑
Contig4111_at	Nuclease I	0.0002	13.25 ↑
Contig1675_s_at	23 kDa JA-induced protein	0.0021	12.71 ↑
Contig13248_at	-	<0.0001	12.00 ↑
Contig5887_at	-	0.0002	10.52 ↑
Contig8703_at	Isocitrate lyase	0.0340	10.02 ↑
Contig6541_at	Putative nuclear protein	0.0002	9.89 ↑
rbaal21f05_s_at	Cysteine proteinase	0.0023	9.72 ↑
rbaal17b01_s_at	23 kDa JA-induced protein	0.0024	9.13 ↑
HV_CEa0009O07r2_s_at	-	0.0003	8.56 ↑
HV12E23u_at	Cysteine synthase	<0.0001	8.50 ↑
Contig1298_at	Enolase 1	<0.0001	8.46 ↑

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

Table 3.6. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-T/Ta-C
Contig1570_s_at	THION9 - Plant thionin family protein	0.0350	8.00 ↑
Contig20774_at	MDR-like ABC transporter	<0.0001	6.88 ↑
EBro08_SQ004_B22_at	CYP72A26	<0.0001	6.84 ↑
Contig5838_at	-	0.0001	6.73 ↑
Contig7437_at	Expressed protein	0.0001	6.39 ↑
HV12F07u_at	Plant viral response family protein	<0.0001	6.30 ↑
Contig1582_x_at	Acidic protein, THION9 - Plant thionin family protein	0.0038	6.29 ↑
HD07M22r_s_at	Putative protease inhibitor, BBTI11 - Bowman-Birk type bran trypsin inhibitor precursor	0.0044	6.03 ↑
Contig3047_s_at	CYP709C1	0.0002	6.01 ↑
Contig5433_at	Oxidoreductase, aldo/keto reductase family protein	0.0009	5.93 ↑
Contig15882_s_at	alpha-DOX2	<0.0001	5.90 ↑
Contig6276_s_at	18,9 kDa ABA-induced protein	0.0153	5.90 ↑
Contig3018_at	Oxalate oxidase GF-2.8	<0.0001	5.73 ↑
Contig1326_s_at	Cold-regulated protein BLT14	0.0318	5.71 ↑
Contig10115_at	Indole-3-glycerol phosphate synthase, chloroplast precursor	0.0006	5.71 ↑
Contig2672_at	Glycosyl hydrolases family 16, Xyloglucan xyloglucosyl transferase	0.0024	5.66 ↓
Contig12219_at	BLN1-2	0.0001	5.55 ↑
Contig1568_x_at	Thionin precursor	0.0001	5.49 ↑
rbaal10h14_at	-	0.0240	5.45 ↑
Contig3901_s_at	Cysteine proteinase	0.0055	5.43 ↑
Contig10263_at	-	0.0001	5.37 ↑
HV_CEb0009D09r2_at	-	0.0021	5.30 ↓
Contig4113_at	-	0.0030	5.14 ↑
Contig4954_s_at	Phosphatase	0.0190	5.13 ↑
Contig7064_s_at	Saccharopin dehydrogenase-like protein	0.0069	5.08 ↑
Contig2990_at	Chitinase	0.0392	5.06 ↑
Contig20974_at	-	<0.0001	5.02 ↑

A list of selected genes from the ones significantly regulated in leaf tissues of Tarm-92 under B toxicity (Table S.2) is provided in Table 3.7. Putative functions were used to classify genes involved in various biological processes. Categories of processes and genes in these categories were almost the same as the ones observed for intra-varietal analysis of Hamidiye leaf tissues under B toxicity (Section 3.3.1.4). Moreover, nature of regulation – induction or repression of expression – was almost the same for selected genes. For instance genes encoding proteins induced by JA or related to JA synthesis were regulated in same manners in Tarm-92 and Hamidiye under B toxicity. Additionally, categories of biological processes included genes coding pathogenesis related (PR), GST, and CYP proteins, transcription factors (TFs), transporters, and proteins showing kinase and transferase activities (Table 3.7).

Table 3.7. Selected genes that are significantly regulated in leaf tissues of Tarm-92 (Ta) under B toxicity. Genes are categorized according to putative molecular function. Presented P values ($P < 0.05$) were corrected with multiple testing corrections in statistical analyses. (JA: Jasmonic Acid; GST: Glutathione *S*-Transferase; CYP: Cytochrome P450; PR: Pathogenesis Related; WI: Wound Induced; TF: Transcription factor; TM: Transmembrane; FC: Fold Change; Ta: Tarm-92; C: Control; T: 5 mM H_3BO_3 treatment)

Probe Set ID	Putative function	P value	FC and regulation Ta-T/Ta-C*
JA related genes			
Contig1675_s_at	23 kDa JA-induced protein	0.0021	12.71 ↑
rbaal17b01_s_at	23 kDa JA-induced protein	0.0024	9.13 ↑
rbags15p13_s_at	23 kDa JA-induced protein	<0.0001	3.93 ↑
HV11O04r_s_at	Glutamine-dependent asparagine synthetase	0.0143	3.75 ↑
Contig1684_x_at	23 kDa JA-induced protein	0.0178	3.31 ↓
Contig3097_at	Allene oxide synthase	0.0021	3.04 ↑
Contig393_at	Alcohol dehydrogenase	0.0001	2.87 ↓
Contig26053_at	S-adenosyl-L-methionine:JA carboxyl methyltransferase	0.0128	2.36 ↑
HV_CEb0020D05r2_s_at	Allene oxide cyclase	<0.0001	2.35 ↑
Contig1678_s_at	23 kDa JA-induced protein	0.0029	2.31 ↓
Contig6194_s_at	12-oxophytodienoic acid reductase	0.0047	2.24 ↑
Contig2900_at	JA-induced protein	0.0049	2.11 ↑

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively.

Table 3.7. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-T/Ta-C
JA related genes (continued)			
Contig1687_x_at	23 kDa JA-induced protein	0.0241	2.34 ↓
Contig1681_x_at	JA-induced protein	0.0212	2.62 ↓
Contig4986_at	Allene oxide cyclase	0.0044	2.17 ↑
GST genes			
Contig5838_at	GST	0.0001	6.73 ↑
Contig9764_at	GST Cla47	<0.0001	4.61 ↑
Contig15264_at	GST	0.0009	4.10 ↑
Contig13901_at	GSTU6	0.0001	3.22 ↑
Contig9632_at	GSTU6	0.0008	3.02 ↑
Contig6008_s_at	GST	<0.0001	2.95 ↑
HV_CEb0004O15r2_s_at	GST	0.0023	2.35 ↑
HVSMEa0014H14r2_s_at	GSTU6	0.0005	2.17 ↑
CYP genes			
EBro08_sq004_B22_at	CYP72A26	<0.0001	6.84 ↑
Contig3047_s_at	CYP709C1	0.0002	6.01 ↑
Contig20974_at	CYP	<0.0001	5.02 ↑
Contig15561_s_at	CYP	0.0021	4.59 ↑
Contig3869_at	CYP71E1	0.0109	4.10 ↑
Contig15560_at	CYP	0.0056	2.71 ↑
Contig17080_at	CYP-like protein	0.0004	2.39 ↑
Contig6581_at	CYP	0.0234	2.29 ↑
Biotic stress related genes			
Contig1579_s_at	Thionin	0.0099	14.35 ↑
Contig1570_s_at	Thionin	0.0350	8.00 ↑
Contig1567_x_at	Thionin precursor	0.0153	3.53 ↑
Contig1568_x_at	Thionin precursor	0.0001	5.49 ↑
Contig1582_x_at	Thionin precursor	0.0038	6.29 ↑
Contig2990_at	Chitinase	0.0392	5.06 ↑
Contig4173_at	Chitinase	0.0058	2.25 ↑
Contig538_at	Benzothiadiazole-induced protein	0.0002	3.12 ↑
Contig13517_s_at	Cf2/Cf5 disease resistance protein	0.0007	2.08 ↓
Contig9060_at	Chitinase	0.0190	2.16 ↓
PR genes / WI genes			
Contig15882_s_at	alpha-DOX2, fatty acid alpha-oxidase	<0.0001	5.89 ↑
Contig2550_x_at	Wheatwin-2 precursor	0.0305	4.12 ↑

Table 3.7. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-T/Ta-C
PR genes / WI genes (continued)			
Contig18116_at	beta-1,3-glucanase	0.0366	3.29 ↓
Contig15099_s_at	PR protein	0.0101	2.86 ↓
Contig6576_s_at	PR protein 4	0.0300	2.46 ↑
TF genes			
Contig23823_at	ZOS1-15, C2H2 zinc finger TF protein	0.0002	3.23 ↑
HM07L17r_at	NAC domain TF	0.0361	3.34 ↑
Contig13885_at	MYB family TF	0.0211	2.93 ↓
Contig6946_at	MYB family TF	0.0181	2.31 ↑
Contig5688_at	Zinc finger CCCH domain-containing TF protein 2	0.0255	2.21 ↑
Transporter genes			
Contig20774_at	MDR-like ABC transporter	<0.0001	6.88 ↑
Contig21298_at	MDR protein 1 homolog	0.0001	4.69 ↑
Contig9422_at	ABC transporter family protein	<0.0001	3.35 ↑
Contig20553_at	PDR-type ABC transporter	0.0095	3.13 ↑
Contig6707_at	Transporter family protein	0.0088	2.77 ↓
Contig4952_s_at	NAR2.1, high affinity nitrate transporter	0.0187	2.58 ↑
HV_CEb0022J21r2_at	Peptide transporter PTR2	0.0008	2.41 ↑
Contig5632_at	Silicon transporter, aquaporin	0.0007	2.34 ↓
Contig6761_at	Major facilitator superfamily antiporter, Carbohydrate transporter, sugar porter	<0.0001	2.29 ↑
Receptor kinases			
Contig10292_at	Leucine-rich repeat TM kinase	0.0172	2.12 ↓
Contig22980_at	Leucine-rich repeat TM kinase	0.0003	3.02 ↓
Contig19845_at	Protein kinase Xa21	0.0019	2.19 ↓
Contig15476_at	Serine/threonine kinase-like protein	0.0089	2.26 ↓
Contig11886_at	Wall-associated kinase 1	0.0030	2.19 ↓
Contig11886_s_at	Wall-associated kinase 1	0.0001	2.03 ↓
Contig20719_at	Receptor-like kinase	0.0031	2.32 ↓

3.3.2.5. Significantly Regulated Genes upon B Deficiency

List of probe sets significantly regulated at least five-fold in root tissues of Tarm-92 under B deficiency is provided in Table 3.8. Most of the genes among highly regulated genes were down-regulated upon B deficiency. The most pronounced levels of down-regulation were observed for genes coding lipid transfer proteins (LTPs), genes specific for iron deficiency (IDS3), and genes involved in nicotianamine biosynthesis. These results indicated that repression of expression was a global response to B deficiency in root tissues of barley cultivar Tarm-92. On the other hand, most of the down-regulated genes were involved in iron metabolism. Thus it might be concluded that plant root cells might be modulating the transcriptome under B deficiency in such a way that uptake of other micronutrients such as iron was turned off or decreased. This might be explained by a possible competition between uptake of B and other micronutrients.

Table 3.8. Genes that are significantly regulated at least five-fold in root tissues of Tarm-92 (Ta) under B deficiency. Presented P values ($P < 0.05$) were corrected with multiple testing corrections in statistical analyses. (LTP: Lipid Transfer Protein; 2OG-Fe oxygenase: 2-oxoglutarate and Fe(II)-dependent oxygenase; FC: Fold Change; Ta: Tarm-92; C: Control; D: 0.02 µM H_3BO_3 treatment)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C*
Contig3782_x_at	LTPL114 - Protease inhibitor/seed storage/LTP family protein precursor	0.0123	55.65 ↓
Contig12916_s_at	IDS3, 2'-deoxymugineic-acid 2'-dioxygenase, 2OG-Fe oxygenase	0.0457	33.53 ↓
Contig3774_s_at	LTPL114 - Protease inhibitor/seed storage/LTP family protein precursor	0.0193	33.08 ↓
D37796_at	IDS3, 2'-deoxymugineic-acid 2'-dioxygenase, 2OG-Fe oxygenase	0.0341	32.17 ↓
Contig10741_at	Nicotianamine synthase 8	0.0190	30.85 ↓
Contig5348_s_at	Retrotransposon, Ty1-copia subclass	0.0084	21.17 ↓
Contig22416_at	Mugineic-acid 3-dioxygenase, oxidoreductase, 2OG-Fe oxygenase	0.0138	18.48 ↓
HU12N23u_s_at	B12D protein	0.0138	17.62 ↓
Contig6938_at	B12D protein	0.0256	17.62 ↓

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

Table 3.8. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C
Contig7288_at	Nicotianamine aminotransferase A	0.0241	15.99 ↓
AB011264_at	-	0.0176	15.68 ↓
Contig3017_at	Oxalate oxidase GF-2.8	0.0208	14.42 ↓
Contig3810_at	Galactinol synthase	0.0144	13.54 ↑
Contig15882_s_at	alpha-DOX2	0.0070	12.70 ↓
AB011266_at	-	0.0256	11.84 ↓
Contig4330_at	Universal stress protein domain containing protein	0.0282	11.39 ↓
EBed01_SQ003_L20_s_at	Cytosolic orthophosphate dikinase	0.0427	10.09 ↓
Contig8733_at	DNA-directed RNA polymerase II subunit RPB4	0.0334	9.08 ↓
Contig3810_s_at	-	0.0155	9.04 ↑
Contig1739_s_at	Cupin, RmlC-type	0.0291	8.47 ↓
Contig13422_at	1-aminocyclopropane-1-carboxylate oxidase homolog 1	0.0291	8.14 ↓
Contig19300_at	-	0.0416	8.11 ↓
Contig10740_at	-	0.0457	8.05 ↓
AF091115_at	BCH2, nitrate transporter 2:1	0.0319	7.90 ↑
Contig1741_at	Cupin, RmlC-type	0.0332	7.74 ↓
Contig3362_at	NAC domain TF family	0.0125	7.59 ↓
Contig8872_at	CONSTANS-like protein CO6, CCT/B-box zinc finger protein	0.0022	7.39 ↑
Contig3432_s_at	High molecular mass early light-inducible protein HV58, chloroplastic	0.0107	7.25 ↑
Contig17064_s_at	THION35 - Plant thionin family protein precursor	0.0416	7.25 ↓
HW05E10u_at	O-methyltransferase	0.0284	7.20 ↓
Contig15682_at	Putative ZmEBE-1 protein	0.0243	7.12 ↑
HVSMEm0022K13r2_at	Peroxidase precursor	0.0003	7.01 ↓
Contig25762_at	PVR3-like protein, LTPL40 - Protease inhibitor/seed storage/LTP family protein precursor	0.0241	6.94 ↑
HU13D04u_x_at	-	0.0441	6.87 ↓
HW09E23u_at	-	0.0108	6.75 ↓
Contig17317_at	Wound induced protein	0.0319	6.51 ↓
HVSMEa0015G15r2_s_at	Putative NAC domain TF	0.0114	6.48 ↓
Contig3018_at	Oxalate oxidase GF-2.8	0.0179	6.41 ↓
Contig3512_s_at	Retrotransposon protein, Ty1-copia subclass	0.0319	6.35 ↓
Contig1125_x_at	-	0.0427	6.18 ↓

Table 3.8. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C
EBem04_SQ002_C07_s_at	1-aminocyclopropane-1-carboxylate oxidase homolog 1	0.0364	6.12 ↓
Contig8812_x_at	-	0.0126	5.89 ↑
EBro03_SQ006_C07_at	-	0.0067	5.87 ↓
Contig6278_at	Homeodomain protein	0.0463	5.83 ↓
AB024007_at	IDS3, 2'-deoxymugineic-acid 2'-dioxygenase, 2OG-Fe oxygenase	0.0317	5.82 ↓
Contig13024_at	-	0.0084	5.81 ↑
Contig7710_at	-	0.0479	5.79 ↑
Contig16761_at	-	0.0442	5.52 ↓
Contig3057_s_at	Heavy metal-associated domain containing protein	0.0488	5.46 ↓
HW08C04u_at	Receptor-like protein kinase	0.0086	5.38 ↓
Contig19813_at	-	0.0126	5.30 ↑
Contig2358_s_at	CBS domain containing membrane protein	0.0330	5.26 ↑
Contig6074_at	-	0.0393	5.09 ↑
Contig13088_at	Cation efflux family protein	0.0341	5.09 ↓

Among down-regulated genes in root tissues, genes (Contig3017_at and Contig 3018_at) coding for oxalate oxidase GF-2.8 proteins were highly repressed under B deficiency. On the other hand, these genes were up-regulated in leaf tissues under B toxicity. The product of oxalate oxidase GF-2.8, also known as germin GF-2.8, results in developmental and stress-related release of hydrogen peroxide in the apoplast. This protein might be playing important roles in several aspects of defense mechanisms. Moreover, it is known that oxalate oxidase GF-2.8 proteins are induced by biotic stress agents and metal ions (Berna and Bernier, 1999). Induction by B toxicity in our study suggests a possible role for oxalate oxidase GF-2.8 in either sensing or response to B toxicity in barley cells.

High degree of regulation in expression levels of biotic stress related, senescence associated, iron deficiency specific, and metal induced genes supports the previously proposed possible interaction or crosstalk between responses to B and other environmental stresses. Stress responsive genes and genes functioning in metal binding or metabolism that showed significant regulation under B deficiency are listed in Table 3.9.

Table 3.9. Stress responsive genes that are significantly regulated in root tissues of Tarm-92 (Ta) under B deficiency. Genes are categorized according to putative molecular function. Presented P values ($P < 0.05$) were corrected with multiple testing corrections in statistical analyses. (FC: Fold Change; Ta: Tarm-92; C: Control; D: 0.02 µM H_3BO_3 treatment)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C*
Abiotic / Biotic stress			
Contig5348_s_at	Wound induced protein	0.0081	21.17 ↓
Contig3017_at	Oxalate oxidase	0.0213	14.42 ↓
Contig17317_at	Wound induced protein	0.0318	6.51 ↓
Contig3018_at	Oxalate oxidase 2 precursor (Germin subunit)	0.0178	6.41 ↓
Contig3512_s_at	Wound induced protein	0.0322	6.35 ↓
Contig13968_at	Seven transmembrane protein	0.0133	2.78 ↓
Contig1518_at	Oxalate oxidase	0.0089	2.70 ↓
AF250937_s_at	Germin E	0.0171	2.68 ↓
Contig8905_at	Xylanase inhibitor protein I	0.0222	2.63 ↓
Contig2390_at	-	0.0092	2.41 ↓
Contig2390_s_at	-	0.0178	2.28 ↓
Contig4701_at	Wound responsive protein	0.0392	2.17 ↓
Contig11487_at	DnaJ protein	0.0411	2.02 ↓
Contig3810_at	WSI76 protein induced by water stress	0.0139	13.54 ↑
Contig3810_s_at	WSI76 protein induced by water stress	0.0158	9.04 ↑
Contig3812_at	WSI76 protein induced by water stress	0.0092	4.90 ↑
Contig3431_x_at	Low molecular mass early light-inducible protein HV90	0.0381	2.75 ↑
Contig9808_at	Low-temperature induced membrane protein	0.0489	2.83 ↑
Contig3081_at	-	0.0141	4.57 ↑
Contig3432_s_at	High molecular mass early light-inducible protein HV58	0.0109	7.25 ↑
Metal binding / Chelation			
Contig12916_s_at	IDS3	0.0458	33.53 ↓
D37796_at	IDS3	0.0337	32.17 ↓
Contig10741_at	Nicotianamine synthase 1	0.0187	30.85 ↓
Contig7288_at	Nicotianamine aminotransferase A	0.0241	15.99 ↓
AB011264_at	Nicotianamine synthase 3	0.0181	15.68 ↓
AB011266_at	Nicotianamine synthase 4	0.0263	11.84 ↓

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

Table 3.9. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C
Metal binding / Chelation (continued)			
Contig10740_at	Nicotianamine synthase 2	0.0457	8.05 ↓
AB024007_at	IDS3	0.0319	5.82 ↓
Contig3057_s_at	Similar to farnesylated protein 3, ATFP3	0.0488	5.46 ↓
Contig6074_at	Similar to heavy metal-associated domain containing protein	0.0389	5.09 ↑

Biotic stress related genes constitute a large portion of significantly regulated genes responsive to environmental stresses (Table 3.9). High degree of down-regulation was observed in expression levels of the biotic stress related genes. On the other hand, genes (Contig3810_at, Contig3810_s_at, Contig3812_at) coding water stress induced 76 (WSI76) proteins and genes (Contig3431_x_at, Contig3432_s_at) induced by light were up-regulated upon B deficiency in root tissues of Tarm-92 (Table 3.9). These results indicated a possible crosstalk between components of signaling or cellular responses to various environmental stresses and B stress.

Additionally genes (Contig12916_s_at, D37796_at, AB024007_at) specific for iron deficiency (IDS3) that were shown to be induced by iron deficiency in barley roots (Nakanishi *et al.*, 1993) were extremely down-regulated under B deficiency (Table 3.9). Moreover, genes (Contig10741_at, AB011264_at, AB011266_at, Contig10740_at) coding nicotianamine synthases (NAS) and a gene (Contig7288_at) coding nicotianamine aminotransferase (NAAT) that function in iron acquisition and homeostasis (Takahashi *et al.*, 1999; Herbik *et al.*, 1999) were down-regulated by B deficiency in roots of Tarm-92. Repression in expression of these genes indicated either a possible competition between uptake of B and of other micronutrients or common transcription factors or cellular signal transducers upstream of signal transduction pathways responsible for sensing micronutrient deficiencies.

A list of selected genes that are involved in transcription, translation, and posttranslational modifications and are significantly regulated under B deficiency is provided in Table 3.10. Both repression and induction in expression levels of various transcription factors were observed under B deficiency. Similarly various kinases and phosphatases which might be critical nodes in signal transduction were significantly regulated upon B deficiency in root tissues of Tarm-92 (Table 3.10).

Table 3.10. Selected genes that are involved in transcription and translation and significantly regulated in root tissues of Tarm-92 (Ta) under B deficiency. Genes are categorized according to putative molecular function. Presented P values were corrected with multiple testing corrections in statistical analyses. (TF: Transcription factor; FC: Fold Change; Ta: Tarm-92; C: Control; D: 0.02 µM H_3BO_3 treatment)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C*
Transcription / Regulation of transcription			
HT11a18u_s_at	U2 snRNP auxiliary factor	0.0347	2.59 ↓
Contig11045_at	Ribonuclease HI large subunit	0.0286	2.00 ↓
Contig22529_at	DNA-directed RNA polymerase alpha subunit	0.0072	2.26 ↓
Contig8732_at	15.9 kDa subunit of RNA polymerase II	0.0487	2.36 ↑
Contig8733_at	15.9 kDa subunit of RNA polymerase II	0.0331	9.08 ↓
HVSMEh0088A15r2_x_at	-	0.0082	2.01 ↑
AF442489_at	CRT/DRE binding factor 2	0.0428	2.34 ↑
Contig24931_at	Auxin response TF (ARF6)	0.0092	2.12 ↓
Contig8872_at	CONSTANS-like protein CO6	0.0023	7.39 ↑
HZ40B21r_s_at	C2H2 zinc finger family protein	0.0178	2.78 ↑
Contig10074_at	CCAAT box binding factor family protein, RAPB protein	0.0359	2.08 ↑
Contig11270_at	CCAAT box binding factor family protein, RAPB protein	0.0323	3.08 ↑
Contig13817_at	CCAAT box binding factor family protein, RAPB protein	0.0072	2.75 ↑
Contig13817_s_at	CCAAT box binding factor family protein, RAPB protein	0.0141	2.92 ↑
Contig8396_at	CCAAT box binding factor family protein, RAPB protein	0.0167	2.62 ↑

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

Table 3.10. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C
Transcription / Regulation of transcription (continued)			
Contig19794_at	EIN3-like(EIL) TF family, TEIL	0.0467	2.36 ↑
Contig23893_at	Heat shock TF family	0.0069	2.26 ↓
HB03A08_T3_at	Heat shock TF family	0.0172	2.17 ↑
Contig13064_at	MADS box TF family	0.0168	2.39 ↑
Contig4517_at	MADS box-like protein	0.0221	2.63 ↑
Contig3875_s_at	MYB-related TF (CCA1)	0.0242	2.02 ↑
Contig3873_at	MYB-related TF family, LHY protein	0.0322	2.01↑
Contig3362_at	NAC domain TF family	0.0129	7.59 ↓
Contig6278_at	Triple-Helix TF family	0.0458	5.83 ↓
Contig8538_at	bZIP TF family, Light-inducible protein CPRF-2	0.0456	2.39 ↑
Contig15369_at	bZIP TF family protein HY5	0.0383	2.06 ↑
Contig15982_at	ABA responsive element binding factor (ABF3)	0.0092	2.50 ↑
Contig13695_at	Putative transcription regulatory protein	0.0212	2.08 ↓
Contig5347_at	SET-domain transcriptional regulator family	0.0229	2.05 ↑
Protein synthesis / targeting			
Contig11738_at	Ribosomal protein L16	0.0081	2.89 ↓
Contig11739_at	Ribosomal protein S8	0.0073	4.17 ↓
Contig11882_at	Ribosomal protein S3	0.0071	4.45 ↓
Contig2098_at	40S ribosomal protein S23	0.0078	2.81 ↑
Contig2725_at	IDI2	0.0109	2.69 ↓
Contig10883_at	Vacuolar targeting receptor bp-80	0.0157	2.84 ↓
Contig16182_at	Spot 3 protein and vacuolar sorting receptor homolog	0.0033	4.98 ↓
Posttranslational modification			
Contig13161_at	Protein phosphatase 2C-like protein	0.0224	4.22 ↑
Contig9099_at	Protein phosphatase 2C-like protein	0.0091	2.21 ↑
Contig14415_at	Protein kinase-like protein	0.0132	2.61 ↓
Contig14572_at	Putative protein kinase	0.0201	2.54 ↑
Contig18153_at	Protein kinase-like protein	0.0072	4.50 ↓
Contig20540_at	-	0.0257	2.65 ↓
Contig20608_at	Protein kinase-like protein	0.0068	2.27 ↓
Contig8698_s_at	Nonphototrophic hypocotyl 1b	0.0178	2.35 ↑

Table 3.10. (continued)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C
Protein degradation			
Contig2281_at	-	0.0459	2.33 ↓
Contig600_at	Carboxypeptidase C	0.0108	2.35 ↓
Contig14719_s_at	Ubiquitin E2	0.0127	2.22 ↑
Contig4595_at	Ubiquitin E3 RING	0.0141	2.21 ↑
Contig10401_s_at	Ubiquitin E3 RING	0.0323	2.57 ↓
Contig20563_at	Ubiquitin E3 RING	0.0426	2.25 ↑
Contig18043_at	Ubiquitin E3 RING	0.0069	2.09 ↓
Contig12211_at	F-box protein family	0.0102	2.05 ↑
Contig17820_at	Ubiquitin E3 BTB/POZ	0.0054	2.53 ↓

Among genes involved in regulation of transcription, genes coding for CCAAT box binding transcription factor (TF) family proteins, also known as RAPB proteins, were up-regulated upon B deficiency. Additionally, TF genes such as MADS box family, MYB-related family, and bZIP family were also up-regulated after treatment with B deficiency. Moreover a high degree of induction was observed in expression level of a gene (Contig8872_at; 7.39-fold) coding CONSTANS-like protein CO6. On the other hand, a high degree of reduction was observed in expression level of a gene (Contig3362_at; 7.60-fold) coding a NAC domain TF family protein. These TFs and regulation of their expression might confer tolerance to B deficiency in barley roots by regulating other genes or TFs.

It is well known that TF families such as MYB-related, NAC domain, and bZIP families comprise large numbers of proteins, some of which function in response to biotic and abiotic stresses (Yamasaki *et al.*, 2008; Golldack *et al.*, 2011). Regulation of various abiotic and biotic stress related TF genes under B deficiency supports the idea of crosstalk between certain components of environmental stresses like B deficiency, cold, salt, or pathogen attack. A similar observation for crosstalk between salinity, heat, cold, and dehydration stresses in barley was also reported previously (Walia *et al.*, 2006).

Results of expression profiling in this study proposed a critical role for CONSTANS-like protein CO6 in signal transduction of B stress in root tissues. Expression of *CO6* was induced under B deficiency whereas it was repressed under B toxicity (Table 3.10 and Table S.3). The CO6 is a plant specific TF containing a CCT domain. Though regulation of *CO6* expression might be a result of possible interaction or crosstalk between various TFs, kinases, and phosphatases, it seems expression of *CO6* is sensitive to available concentration of B. The proposed role based on transcript abundances determined in this study should be verified with heterologous or homologous expression, loss-of-function or gain-of-function mutants, and chromosome immunoprecipitation assays in subsequent studies.

Certain TFs such as ABA responsive element binding factor (ABF), phosphatases such as protein phosphatase 2C (PP2C), and kinases are key components of ABA-dependent signal transduction (Umezawa *et al.*, 2010). The B deficiency applied in this study significantly induced expression of genes coding ABF3 and PP2C-like protein. Thus involvement of ABA in sensing or signaling of B deficiency in roots of barley might be proposed.

It is well known that transport of B through cellular membranes is mediated by passive or facilitated diffusion or by energy-dependent transport against concentration gradients (Miwa and Fujiwara, 2010). Thus, expression levels of genes coding putative transporters were examined thoroughly and a list of selected genes significantly regulated under B deficiency in root tissues is provided in Table 3.11.

Table 3.11. Selected transporter genes that are significantly regulated in root tissues of Tarm-92 (Ta) under B deficiency. Genes are categorized according to putative molecular function. Presented P values ($P < 0.05$) were corrected with multiple testing corrections in statistical analyses. (FC: Fold Change; Ta: Tarm-92; C: Control; D: 0.02 µM H_3BO_3 treatment)

Probe Set ID	Putative function	P value	FC and regulation Ta-D/Ta-C*
Transporter activity			
Contig19105_at	Mannitol transporter	0.0109	3.18 ↓
Contig16464_at	Iron-phytosiderophore transporter protein yellow stripe 1	0.0168	2.59 ↓
Contig7897_at	Similar to peptide transporter	0.0141	2.43 ↓
Contig4728_at	Plastidic ATP/ADP-transporter	0.0348	2.42 ↓
Contig17276_at	ABC transporter family protein	0.0319	2.19 ↓
Contig12553_at	ABC transporter family protein	0.0362	2.49 ↑
Contig7895_at	Similar to peptide transporter	0.0241	2.16 ↓
Contig17502_at	Urea active transport protein	0.0021	2.11 ↑
Contig7377_at	Tonoplast membrane integral protein, TIP	0.0068	2.27 ↑
Contig7293_at	Similar to sugar transporter	0.0258	2.30 ↑
rbaal41j07_at	Sodium-dicarboxylate cotransporter-like protein	0.0121	2.42 ↑
HV_CEb0001H12r2_at	Amino acid permease 6	0.0071	2.91 ↑
Contig16352_at	Zinc transporter protein	0.0169	2.95 ↑
Contig16901_at	NOD26-like intrinsic protein, NIP	0.0117	3.80 ↑
Contig19634_at	Anion exchange protein	0.0083	2.44 ↑
Contig7710_at	Nitrate transporter	0.0478	5.79 ↑
AF091115_at	High affinity nitrate transporter	0.0318	7.90 ↑

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively.

Expression levels of genes coding a NOD26-like intrinsic protein (NIP) and an anion exchange protein, which were determined as orthologs of *Arabidopsis* NIP5;1 and BOR1, respectively, were up-regulated under B deficiency in barley roots. The genes represented by Contig16901_at and Contig19634_at were induced 3.8- and 2.4-fold, respectively, in root tissues (Table 3.11). It was shown that NIP5;1 and BOR1 are crucial for efficient transport of B across the plasma membrane under B deficiency in *Arabidopsis* (Takano *et al.*, 2002; 2006).

Our results are well correlated with previous reports indicating the requirement of these proteins for uptake of B. Therefore gene expression results of this study and report of Takano *et al.* (2006) substantiate the involvement of an aquaporin and an anion exchange protein for B uptake.

Moreover, genes coding ABC transporter family proteins, tonoplast membrane integral proteins (TIP), zinc transporters, and nitrate transporters were significantly regulated upon B deficiency. Our results suggested involvement of these transporters in uptake of B in barley.

3.3.3. Inter-varietal Comparison: Transcriptional Differences Between Sensitive and Tolerant Cultivars under B Toxicity

Effects of B toxicity on transcriptome of two barley cultivars, Tarm-92 and Hamidiye, which were previously reported as tolerant and sensitive to B toxicity, respectively (Torun *et al.*, 2003; Karabal *et al.*, 2003), were investigated at toxic concentrations (5 mM) of H_3BO_3.

3.3.3.1. Accumulation of B and Development of Leaf Symptoms under B Toxicity

Leaf symptoms observed in sensitive cultivar and concentrations of accumulated B determined with inductively coupled plasma optical emission spectrometry (ICP-OES) are presented in Figure 3.14. It was shown that accumulation, predominantly in first emerging leaves, was higher in sensitive cultivar Hamidiye compared to tolerant Tarm-92. Sensitive cultivars of barley are known to accumulate more B in leaf tissues compared to tolerant ones (Sutton *et al.*, 2007). Moreover, phloem-mediated translocation of B is limited (Mengel and Kirkby, 2001) leading to accumulation of B at the end of transpiration stream in mature leaves (Marschner, 1995). Thus, our results, well correlated with previous reports, indicated the relation between development of toxicity symptoms in barley leaves and concentrations of accumulated B.

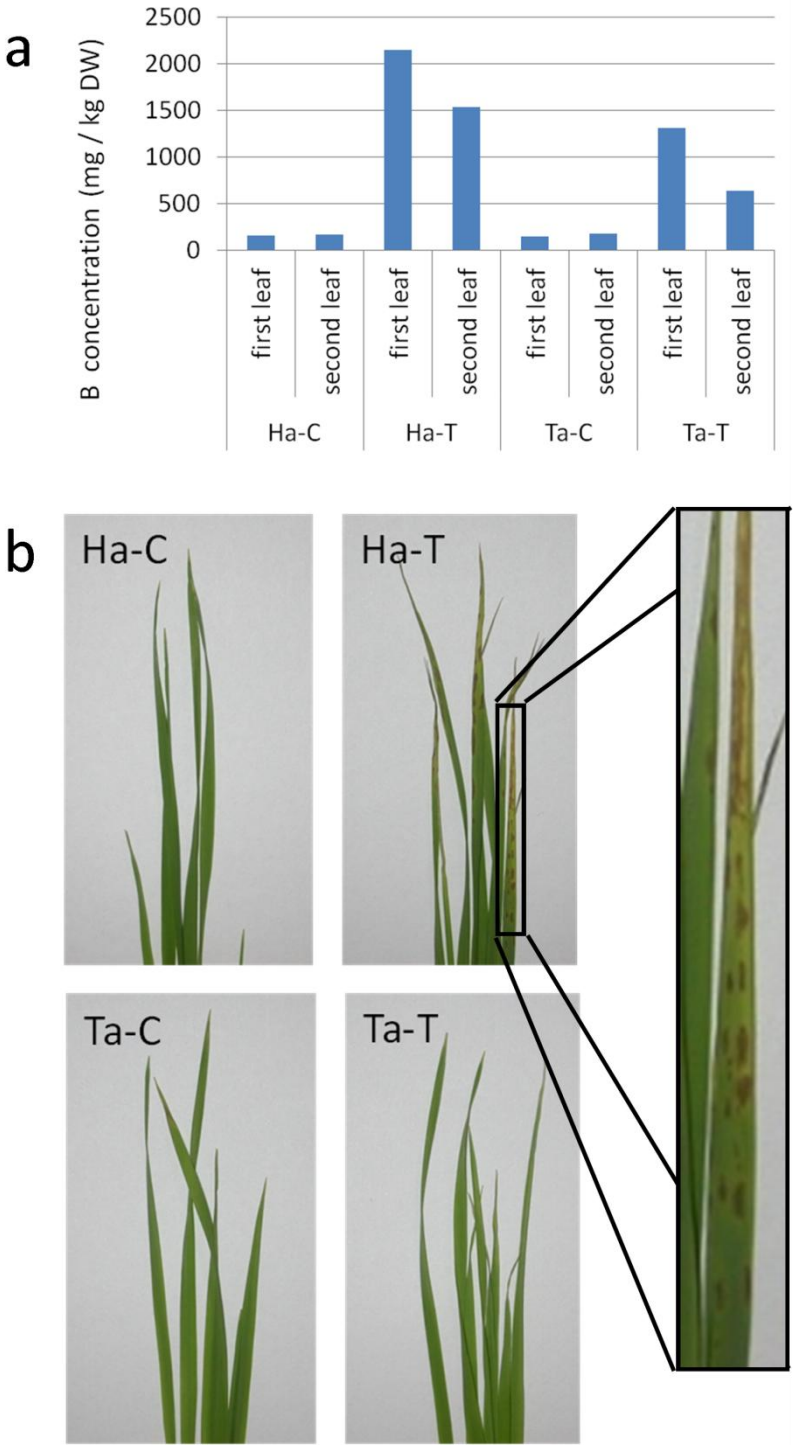

Figure 3.14. Accumulation of B and symptoms observed on leaf tissues under B toxicity. Accumulated B concentrations (a) in dried samples of first and second emerging leaves of Hamidiye (Ha) and Tarm-92 (Ta) were determined under control conditions (C) and B toxicity (T) applied as 5 mM H_3BO_3 treatment. Images of leaves (b) were recorded after 5 days of treatment with high concentrations of B.

3.3.3.2. Patterns Identified with Principal Component Analysis of Microarray Data

Microarray-based expression profiling was used to make an inter-varietal comparison between tolerant and sensitive cultivars of barley under B toxicity. Hybridizations were performed with total RNA extracted from leaf and root tissues of at least 3 biological replicates of seedlings of Tarm-92 (Ta) and Hamidiye (Ha) grown under control conditions (C) and B toxicity (T) applied as 5 mM H_3BO_3 in solution.

As a result of initial filtering 20275 probe sets, with intensity values greater than the 20th percentile in at least one of the hybridizations, were retained for further data analysis. Subsequently three-way ANOVA ($P < 0.05$) was performed to obtain a subset of data that contains 18,007 probe sets displaying significant expression levels with P values lower than the cut-off (Appendix H). The plot visualization of PCA on conditions performed with significantly regulated subset of probe sets is presented in Figure 3.15. When plotted as data points, hybridizations with samples from leaf and root tissues were located at distinct areas of the plot. Variance presented by the first component (74.14%) indicated tissue differences as the main source of variation.

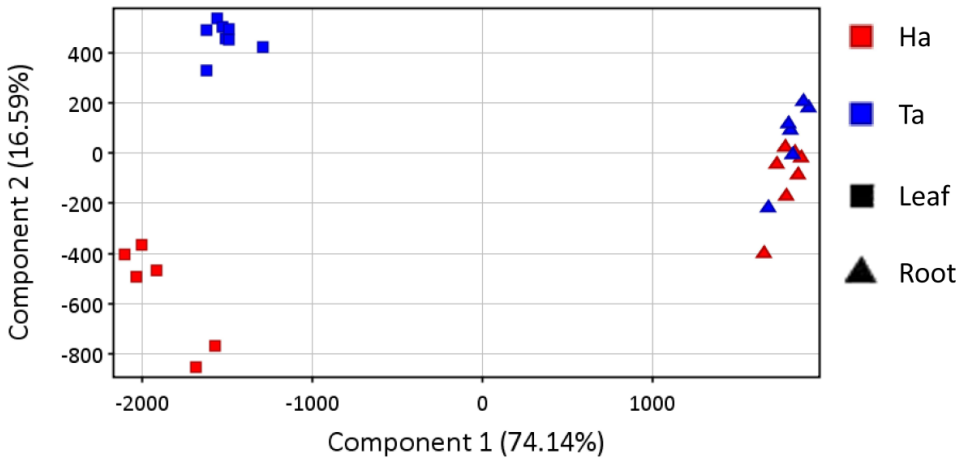

Figure 3.15. Principle component analysis of all hybridizations performed for inter-varietal comparison of transcriptional responses of Hamidiye and Tarm-92 under B toxicity. Percentages indicate variance. Cultivars are indicated with different colors whereas tissues are shown with the same shape. Analysis was performed with significantly regulated probe sets. (Ha: Hamidiye, Ta: Tarm-92)

Subsequently, hybridizations with samples from leaf and root tissues were analyzed separately. In the group comprising only the arrays hybridized with RNA from leaf tissues, 20,053 probe sets displayed signal intensities higher than the 20th percentile in at least one of the hybridizations. Statistical data analysis was carried out with the subset of data containing 20,053 probe sets. Two-way ANOVA ($P < 0.05$) was performed to obtain significantly regulated subset of probe sets. Statistical analysis retained 9,957 probe sets which were used in PCA on conditions. Plot visualization of hybridizations as data points after PCA is presented in Figure 3.16a. According to the first component, difference in genotype was the source of 64.81% of variance. Additionally, data points representing hybridizations with RNA from leaf tissues of Tarm-92 seedlings treated with B toxicity (Ta-T) were isolated from those of Tarm-92 seedlings grown under control conditions (Ta-C) according to the second component. However, a clear distinction was not observed among data points of hybridizations with RNA from leaf tissues of Hamidiye (Ha) seedlings. These results indicated a more pronounced effect of B toxicity on transcriptome of leaf tissues of Tarm-92 compared to that of Hamidiye.

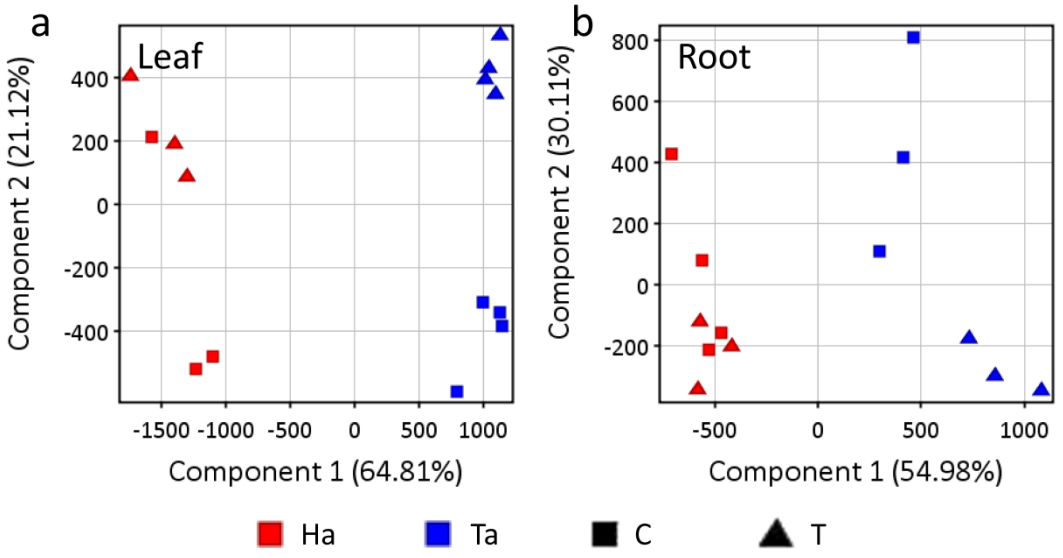

Figure 3.16. Principle component analysis of hybridizations performed for inter-varietal comparison of transcriptional responses in leaf and root tissues of Hamidiye (Ha) and Tarm-92 (Ta) under B toxicity. Percentages indicate variance. Cultivars are shown with same color whereas biological replicates of a condition are indicated with the same shape. Analysis was performed with significantly regulated probe sets. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; Ha: Hamidiye; Ta: Tarm-92)

On the other hand, in the group comprising only the arrays hybridized with RNA from root tissues, 19,169 probe sets displayed signal intensities higher than the 20th percentile in at least one of the arrays. Two-way ANOVA ($P < 0.05$) retained 5,892 significantly regulated probe sets which were used in PCA on conditions. Plot of hybridizations as data points according to the resultant PCA components is presented in Figure 3.16b. Difference in genotype was again the main source of variation in data according to the first component which represented 54.98% of variance.

3.3.3.3. Determination of B Responsive Genes in Barley Cultivars

Signal intensity-based filtering and statistical analysis, where hybridizations with samples from leaf and root tissues were evaluated separately, were used to determine B responsive genes. Number of probe sets differentially or significantly regulated in Hamidiye (Ha) and Tarm-92 (Ta) under B toxicity (T) are presented in Table 3.12. Analysis of hybridizations with RNA from leaf tissues revealed that 170 and 442 genes were differentially expressed under B toxicity in leaf tissues of Hamidiye and Tarm-92, respectively. Among these, 136 genes were up-regulated and 34 were down-regulated in Hamidiye (Ha-T), whereas 310 genes were up-regulated and 132 were down-regulated in Tarm-92 (Ta-T). Additionally, in root tissues 118 and 476 genes were differentially regulated in Hamidiye and Tarm-92, respectively. Among these, 46 genes were up-regulated and 72 were down-regulated in Hamidiye (Ha-T), whereas 126 genes were up-regulated and 350 were down-regulated in Tarm-92 (Ta-T).

Table 3.12. Number of probe sets differentially regulated in leaf and root tissues of barley cultivars under B toxicity. Up- and down-regulation is assigned relative to control conditions. (Ha: Hamidiye; Ta: Tarm-92; FC: Fold change; T: Toxicity applied as 5 mM H_3BO_3 treatment)

Abbreviation (Cultivar-Treatment)	**Differential Regulation (FC ≥ 2)**			**Significant Regulation (P < 0.05)**
	Up	**Down**	**Total**	
Leaf				
Ha-T	136	34	170	125
Ta-T	310	132	442	290
Root				
Ha-T	46	72	118	24
Ta-T	126	350	476	33

Scatter plots provided in Figure 3.17 display the differentially regulated genes and their expression values in leaf and root tissues of Hamidiye and Tarm-92. Additionally, number of significantly regulated genes in leaf tissues was determined as 125 and 290 under B toxicity in Hamidiye (Ha) and Tarm-92 (Ta), respectively. On the other hand, 24 and 33 genes were significantly regulated under B toxicity in root tissues of Hamidiye (Ha) and Tarm-92 (Ta), respectively (Table 3.12). Venn diagrams and plots of expression profiles presented in Figure 3.18 display differentially or significantly regulated genes that are common to both genotypes under B toxicity (Ha-T and Ta-T).

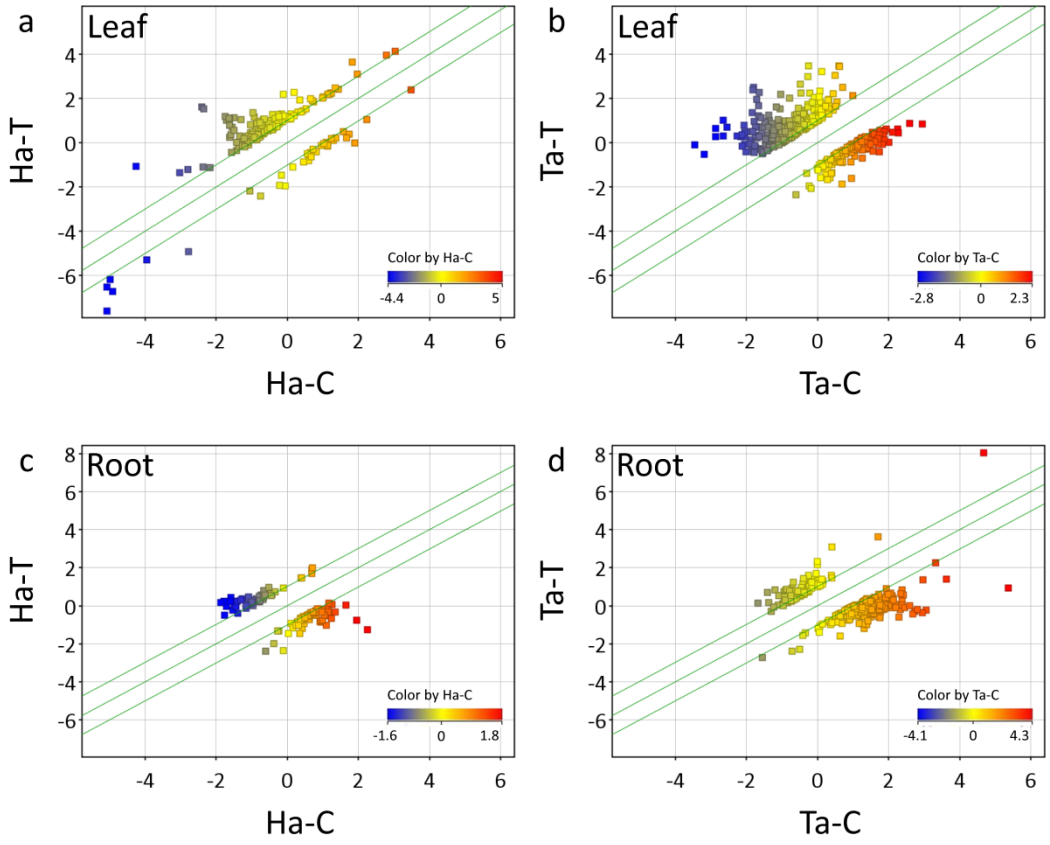

Figure 3.17. Scatter plots displaying genes and their expression values in leaf and root tissues of Hamidiye (Ha) and Tarm-92 (Ta) under B toxicity. Expression values of differentially regulated genes in leaf (a, b) and root (c, d) tissues under toxicity are displayed. Both axis in all graphs show normalized expression values. Diagonal lines indicate two-fold (2X) difference lines. Points above and below the 2X diagonal lines indicate up- and down-regulated genes, respectively. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; Ha: Hamidiye; Ta: Tarm-92)

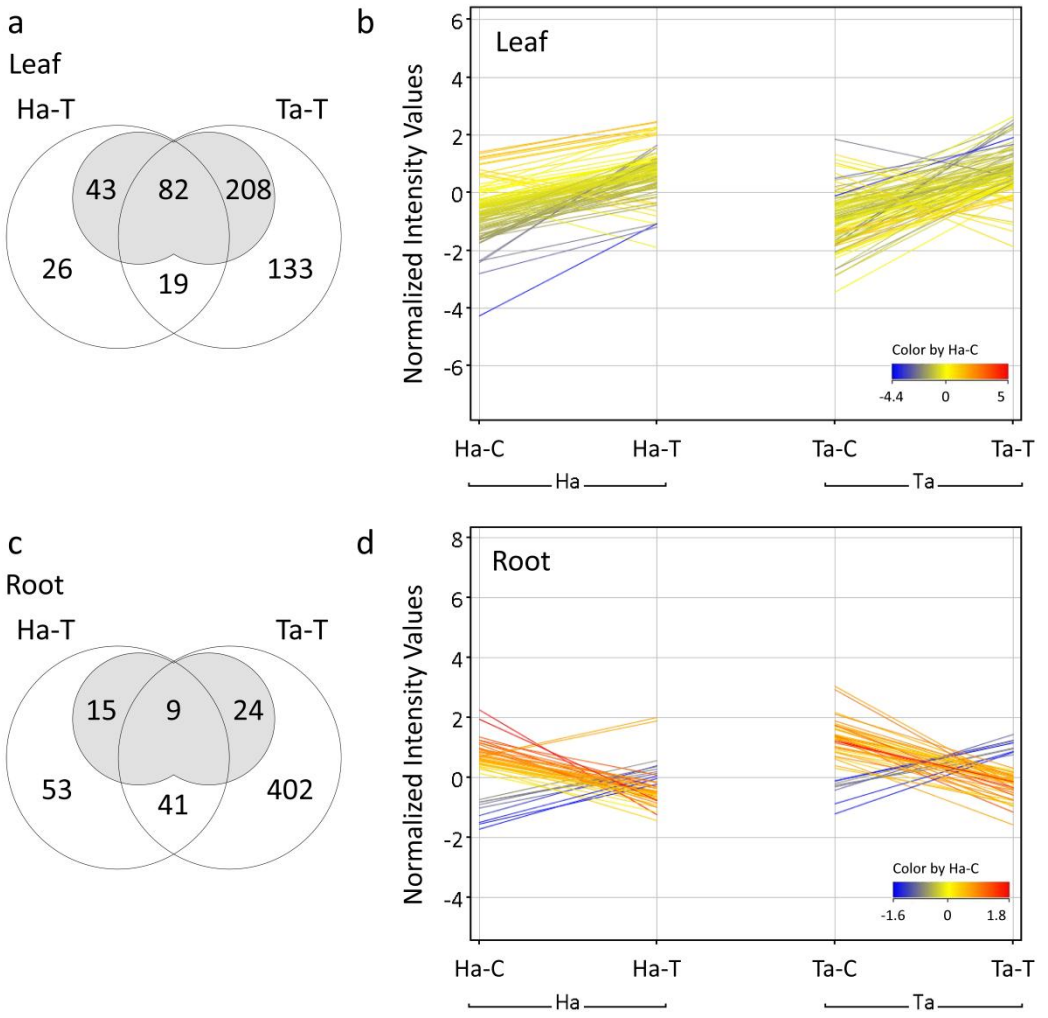

Figure 3.18. Number and expression patterns of differentially and significantly regulated genes in leaf and root tissues of Hamidiye and Tarm-92 under B toxicity. Venn diagrams (a, c) show numbers of genes differentially (outer light circles) and significantly (inner dark circles) regulated. Profile plots display expression patterns of differentially (b, d) regulated genes which are common to both genotypes. Expression profiles of 101 differentially regulated genes in leaf tissues (b) and 50 differentially regulated genes in root tissues (d) are displayed. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; Ha: Hamidiye; Ta: Tarm-92)

Among differentially regulated genes in leaf tissues, 101 genes (Figure 3.18a) and similarly in root tissues, 50 genes (Figure 3.18c) were common to both genotypes. These subsets of genes might be regarded as basal response genes regulated in barley upon B toxicity. Functional annotation of basal response genes might provide clues for signaling or molecular responses to B toxicity in barley.

3.3.3.4. Clustering of Responding Genes upon B Toxicity Treatment

Hierarchical clustering on genes and conditions was performed for 333 (Figure 3.19a) and 48 (Figure 3.19b) significantly regulated genes which constitute the entire subset of genes showing significant regulation under B toxicity in leaf and root tissues, respectively. For both leaf and root tissues Ha-C and Ha-T as well as Ta-C and Ta-T were clustered together according to expression levels of significantly regulated genes. Clustering on conditions indicated a more pronounced effect of genotype on transcriptome of barley.

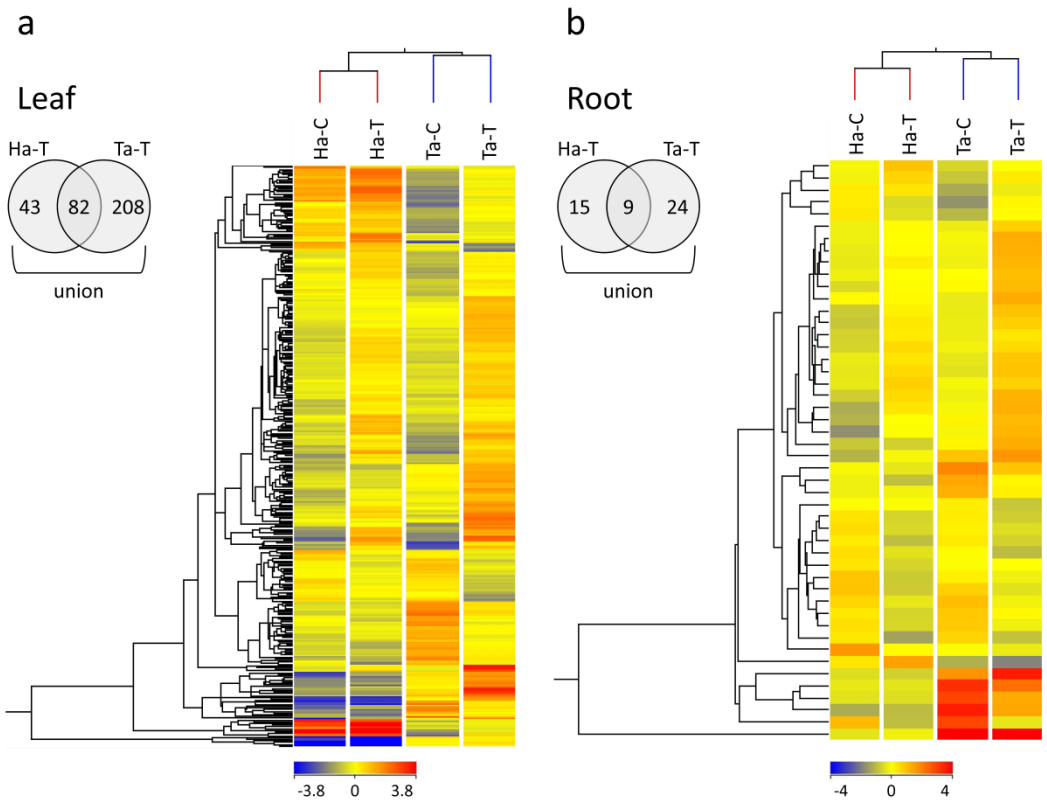

Figure 3.19. Hierarchical clustering of genes and treatments for inter-varietal comparison of transcriptional responses in leaf and root tissues of Hamidiye (Ha) and Tarm-92 (Ta) under B toxicity. Clustering of 333 (a) and 48 (b) significantly regulated genes, which constitute the entire (union) significantly regulated genes in both cultivars in leaf and root tissues, respectively, are displayed. Rows and columns represent genes and treatments, respectively. The color bars represent the corresponding expression values. (C: Control; T: Toxicity applied as 5 mM H_3BO_3 treatment; Ha: Hamidiye; Ta: Tarm-92)

3.3.3.5. Functional Analysis of Differentially Regulated Genes

Putative functions were assigned to probe sets or genes using HarvEST:Barley and PLEXdb. Differentially regulated genes grouped using Venn diagrams (Figure 3.18a and 3.18c) were discussed separately. The first group comprised basal response genes regulated upon B toxicity in both genotypes (Section 3.3.3.6). On the other hand, the second group comprised genes regulated in tolerant (Tarm-92) but not in sensitive (Hamidiye) cultivar (Section 3.3.3.7) whereas the third group comprised genes regulated in sensitive (Hamidiye) but not in tolerant (Tarm-92) cultivar (Section 3.3.3.8).

3.3.3.6. Basal Response Genes under B Toxicity

Investigation of transcriptomes of leaf tissues revealed that 101 genes were regulated differentially in both Hamidiye and Tarm-92. Among these, 82 genes were significantly regulated upon B toxicity (Figure 3.20a). The entire list of differentially regulated genes, fold changes in expression values, putative functions, and regulation under B toxicity is provided in Supplementary Table S.4.

Similarly, investigation of transcriptomes of root tissues revealed that 50 genes were regulated differentially in both Hamidiye and Tarm-92. Among these, 9 genes were significantly regulated upon B toxicity (Figure 3.20c). The entire list of differentially regulated genes, fold changes in expression values, putative functions, and regulation under B toxicity is provided in Supplementary Table S.5.

Regulations of selected differentially expressed genes which are represented by colored square boxes are presented in Figure 3.20b and 3.20d. Color-based visual display was generated according to fold changes in expression values using BINs or subBINs defined in MapMan software (Thimm *et al.*, 2004). A general induction of gene expression was observed in leaf tissues upon B toxicity. On the other hand, a general repression of expression was observed in root tissues. The nature of regulation – induction or repression – was same for differentially regulated genes in Hamidiye and Tarm-92 except for 3 genes in leaf and 2 genes in root tissues. Expressions of these 5 genes were induced in Hamidiye whereas they were repressed in Tarm-92 (Table S.4 and S.5).

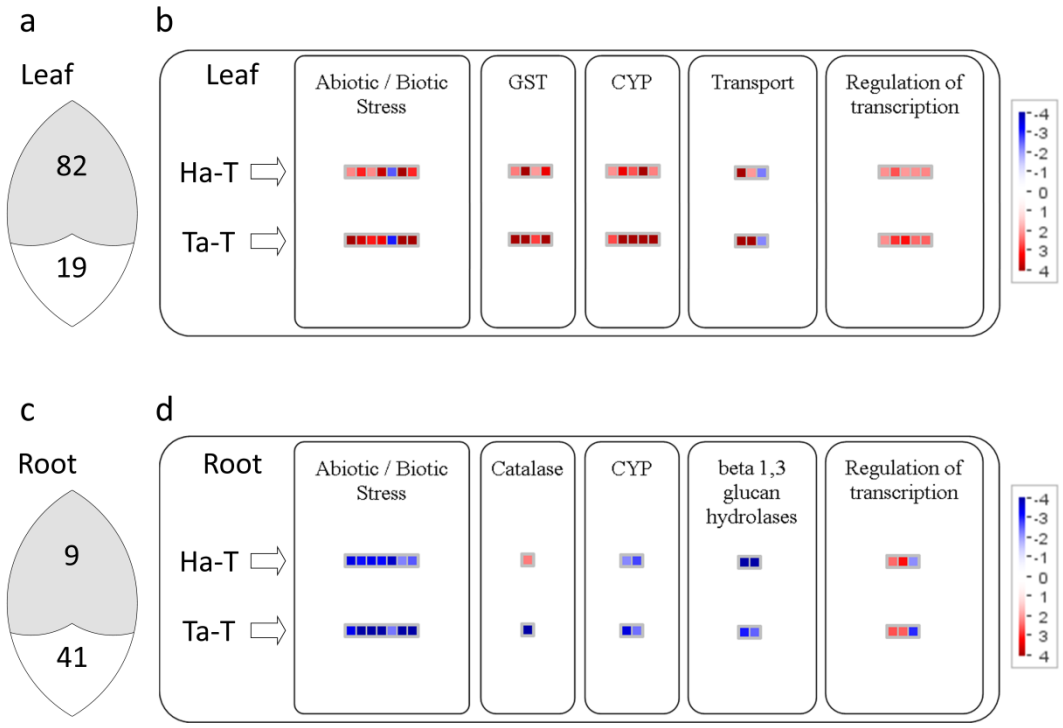

Figure 3.20. Number and regulation of differentially expressed genes in leaf and root tissues of Hamidiye and Tarm-92 under B toxicity. Diagrams (a, c) show numbers of genes that are differentially (outer light region) and significantly (inner dark region) regulated. Blocks (b, d) display regulation of selected differentially expressed genes which are represented by squares. Up-regulation compared to control conditions is presented with red color whereas down-regulation is presented with blue color. (T: Toxicity applied as 5 mM H_3BO_3 treatment; Ha: Hamidiye; Ta: Tarm-92)

A list of selected basal response genes in leaf tissues of Hamidiye and Tarm-92 is presented in Table 3.13. Genes which showed at least four-fold regulation under B toxicity in either cultivar are retained in the list. As indicated previously, the nature of regulation was almost the same in two barley genotypes. Moreover, high degrees of regulation were observed for the same set of genes such as esterases. However, ratio of regulation was different for an important set of genes. Ratio of regulation was calculated by simply dividing the fold change in expression recorded in tolerant cultivar, Tarm-92 by the fold change in sensitive cultivar, Hamidiye. Thus, it might be concluded that tolerance to B toxicity in Tarm-92 might be a result of high degree of regulation of specific genes such as the ones represented by Contig11149_at, HV12E23u_at, Contig3017_at, and EBro08_SQ004_B22_at.

Table 3.13. Common response genes that are differentially regulated in leaf tissues of Hamidiye (Ha) and Tarm-92 (Ta) under B toxicity. Genes which showed at least four-fold regulation under B toxicity in either cultivar are retained. (GST: Glutathione-S-transferase; CYP: Cytochrome P450; BBTI: Bowman-Birk type bran Trypsin Inhibitor; FC: Fold Change; RoR: Ratio of Regulation; Ha: Hamidiye; Ta: Tarm-92; C: Control; T: 5 mM H_3BO_3 treatment)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C*	FC and regulation Ta-T/Ta-C	RoR Ta/Ha
Contig11149_at	Metallo-beta-lactamase family protein	2.67 ↑	12.79 ↑	4.80
HV12E23u_at	Cysteine synthase	2.71 ↑	8.82 ↑	3.26
Contig3017_at	Oxalate oxidase GF-2.8	6.69 ↑	19.96 ↑	2.98
EBro08_SQ004_B22_at	CYP72A1	2.28 ↑	6.66 ↑	2.92
Contig7437_at	-	2.16 ↑	5.97 ↑	2.76
Contig21298_at	-	2.05 ↑	5.61 ↑	2.73
Contig4111_at	Nuclease I	4.37 ↑	11.54 ↑	2.64
Contig6541_at	Putative nuclear protein	3.44 ↑	8.95 ↑	2.60
Contig4954_s_at	Phosphatase	2.09 ↑	5.07 ↑	2.43
Contig8703_at	Isocitrate lyase	4.33 ↑	10.33 ↑	2.39
HV_CEb0009D09r2_at	-	2.44 ↓	5.80 ↓	2.38
Contig2990_at	-	2.23 ↑	4.92 ↑	2.21
Contig5433_at	Oxidoreductase, aldo/keto reductase family protein	2.52 ↑	5.55 ↑	2.20
Contig10115_at	Indole-3-glycerol phosphate synthase, chloroplast precursor	2.22 ↑	4.82 ↑	2.17
Contig3018_at	Oxalate oxidase GF-2.8	2.99 ↑	6.06 ↑	2.02
Contig12075_at	-	2.03 ↑	4.08 ↑	2.01
HT05D14u_s_at	-	2.57 ↑	5.04 ↑	1.96
Contig6682_at	Universal stress protein domain containing protein	2.15 ↑	4.06 ↑	1.89
Contig13901_at	GST U6	2.32 ↑	4.03 ↑	1.74
Contig10263_at	-	3.19 ↑	5.47 ↑	1.72
Contig20974_at	-	2.67 ↑	4.38 ↑	1.64
Contig15561_s_at	Putative ferulate 5-hydroxylase, CYP	3.39 ↑	5.54 ↑	1.64
Contig7064_s_at	Saccharopin dehydrogenase-like protein	2.77 ↑	4.48 ↑	1.62
Contig4113_at	-	3.24 ↑	5.12 ↑	1.58
Contig5888_at	Alternative oxidase	2.77 ↑	4.25 ↑	1.54

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

Table 3.13. (continued)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C	FC and regulation Ta-T/Ta-C	RoR Ta/Ha
Contig1298_at	Enolase 1	6.36 ↑	9.54 ↑	1.50
HV12F07u_at	Plant viral response family protein	5.07 ↑	7.32 ↑	1.44
HD07M22r_s_at	Putative protease inhibitor, BBTI11 precursor	4.23 ↑	5.77 ↑	1.36
Contig5838_at	-	5.14 ↑	6.93 ↑	1.35
Contig9764_at	GST Cla47	3.40 ↑	4.49 ↑	1.32
Contig20774_at	MDR-like ABC transporter	5.38 ↑	6.80 ↑	1.26
Contig5887_at	-	7.79 ↑	9.31 ↑	1.19
HV_CEa0009O07r2_s_at	-	6.77 ↑	7.89 ↑	1.17
rbaal10h14_at	-	4.95 ↑	5.59 ↑	1.13
Contig10057_at	Esterase PIR7B	16.64 ↑	18.66 ↑	1.12
Contig10057_s_at	Esterase PIR7B	15.12 ↑	15.40 ↑	1.02
Contig3047_s_at	CYP709C1	6.80 ↑	6.17 ↑	0.91
Contig15475_at	Retrotransposon protein, Ty1-copia subclass	4.85 ↑	3.11 ↑	0.64
Contig639_at	-	9.17 ↑	4.03 ↑	0.44
Contig4520_at	Late embryogenesis abundant protein, LEA	2.46 ↑	2.53 ↓	-1.03
Contig10779_at	-	2.05 ↑	2.45 ↓	-1.19
Contig22452_at	-	2.18 ↑	2.08 ↓	-0.95

Additionally, list of genes presented in Table 3.13 also includes the 3 genes which showed up-regulation in leaf tissues of Hamidiye and down-regulation in Tarm-92. The gene represented by Contig4520_at was annotated as a gene coding for a late embryogenesis abundant (LEA) protein. On the other hand, genes represented by Contig10779_at and Contig22452_at were uncharacterized.

A list of selected basal response genes in root tissues of Hamidiye and Tarm-92 is presented in Table 3.14. Genes which showed at least four-fold regulation under B toxicity in either cultivar are retained in the list. High ratios of regulation were observed in expression levels of genes represented by Contig5311_at, EBem05_SQ002_D05_s_at, and Contig10115_at. Regulation of these genes might be important for conferring tolerance to Tarm-92.

Additionally, couple of genes coding pathogenesis related (PR) proteins were down-regulated in root tissues of both cultivars. This finding indicated possible crosstalk in response or signaling pathways involved in responses to biotic stress and B toxicity. Additionally, list of genes presented in Table 3.14 also includes the 2 genes which showed up-regulation in root tissues of Hamidiye and down-regulation in Tarm-92. The genes represented by HT06F11u_s_at and Contig13248_at were annotated as genes coding for catalase 2 (CAT2) and UDP-glucosyl transferase, respectively. Regulation of these genes might be important in responses to B toxicity in root tissues. Similarly, an increase in activity of CAT was reported in root tissues of both sensitive and tolerant cultivars of barley (Karabal *et al.*, 2003). Repression of *CAT2* expression in tolerant cultivar in our study might be a genotype-dependent response in Tarm-92.

Table 3.14. Common response genes that are differentially regulated in root tissues of Hamidiye (Ha) and Tarm-92 (Ta) under B toxicity. Genes which showed at least four-fold regulation under B toxicity in either cultivar are retained. (PR: Pathogenesis Related; FC: Fold Change; RoR: Ratio of Regulation; Ha: Hamidiye; Ta: Tarm-92; C: Control; T: 5 mM H_3BO_3 treatment)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C*	FC and regulation Ta-T/Ta-C	RoR Ta/Ha
Contig5311_at	O-methyltransferase	4.24 ↓	9.75 ↓	2.30
EBem05_SQ002_D05_s_at	Endochitinase	2.26 ↓	5.07 ↓	2.24
Contig10115_at	Indole-3-glycerol phosphate synthase precursor	2.07 ↓	4.33 ↓	2.09
Contig2210_at	PR protein PRB1-2	2.61 ↓	4.67 ↓	1.79
Contig2209_at	PR protein PRB1-2	3.16 ↓	5.55 ↓	1.76
Contig14609_at	Putative peroxidase	2.49 ↑	4.16 ↑	1.67
Contig2214_s_at	PR protein PRB1-2	3.20 ↓	5.08 ↓	1.59
Contig2212_s_at	PR protein PRB1-2	3.37 ↓	4.49 ↓	1.33
HVSMEm0003C15r2_s_at	Glucan endo-1,3-beta-glucosidase GII	6.50 ↓	2.54 ↓	0.39
Contig1637_s_at	Glucan endo-1,3-beta-glucosidase GII	11.30 ↓	3.08 ↓	0.27
HT06F11u_s_at	Catalase 2	2.32 ↑	4.62 ↓	-1.99
Contig13248_at	Putative UDP-glucosyl transferase	2.49 ↑	9.62 ↓	-3.86

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

3.3.3.7. Genes Regulated in Tolerant but not in Sensitive Cultivar

Transcripts that were differentially regulated in Tarm-92 but did not respond significantly to B toxicity in Hamidiye might hold the clue to how Tarm-92 seedlings manage to overcome B stress. Number of genes differentially regulated in leaf and root tissues of Tarm-92 were 341 and 426, respectively (Figure 3.18a and 3.18c). List of genes, expression levels of which were regulated more than four-fold in leaf and root tissues of Tarm-92 is provided in Table 3.15, whereas complete lists of genes are provided in Supplementary Table S.6 and S.7.

Table 3.15. Selected genes differentially regulated under B toxicity in leaf and root tissues of Tarm-92 (Ta) but not in Hamidiye. Genes which showed at least four-fold regulation under B toxicity are retained. (JA: Jasmonic acid; FC: Fold Change; Ta: Tarm-92; C: Control; T: 5 mM H_3BO_3 treatment)

Probe Set ID	Putative function	FC and regulation Ta-T/Ta-C*
Leaf tissue		
Contig1675_s_at	23 kDa JA-induced protein	13.29 ↑
Contig1579_s_at	THION9 - Plant thionin family protein	12.87 ↑
Contig13248_at	-	10.13 ↑
rbaal17b01_s_at	23 kDa JA-induced protein	9.64 ↑
rbaal21f05_s_at	Cysteine proteinase	9.61 ↑
Contig6155_at	JA induced protein	8.71 ↑
Contig1570_s_at	THION9 - Plant thionin family protein	7.46 ↑
Contig1582_x_at	THION9 - Plant thionin family protein	7.43 ↑
Contig11773_at	-	7.20 ↑
Contig12219_at	BLN1-2	7.02 ↑
Contig15882_s_at	alpha-DOX2	6.73 ↑
Contig5780_at	Alternative oxidase	6.41 ↑
Contig6276_s_at	18,9 kDa ABA-induced protein	5.94 ↑
Contig2672_at	Xyloglucan xyloglucosyl transferase	5.78 ↓
Contig1568_x_at	-	5.59 ↑
Contig3901_s_at	Cysteine proteinase	5.36 ↑
Contig1326_s_at	Cold-regulated protein BLT14	5.34 ↑
Contig8733_at	DNA-directed RNA polymerase II subunit RPB4	4.49 ↑
Contig2638_at	-	4.36 ↓

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

Table 3.15. (continued)

Probe Set ID	Putative function	FC and regulation Ta-T/Ta-C
Leaf tissue (continued)		
Contig13523_at	CRT/DRE binding factor 1	4.25 ↓
Contig24068_at	-	4.24 ↑
Contig2783_s_at	Legumain, vacuolar-processing enzyme precursor	4.13 ↑
Contig11623_at	Tryptophan decarboxylase	4.10 ↑
HY03N19u_s_at	Lipoxygenase	4.09 ↑
Root tissue		
Contig10179_s_at	-	21.60 ↓
Contig3777_at	Lipid transfer protein	10.54 ↑
Contig20720_at	Abscisic stress-ripening protein	8.53 ↓
Contig7018_at	EF hand family protein	8.04↓
HV12E23u_at	Cysteine synthase	7.95 ↓
Contig21640_at	-	7.17 ↓
Contig2415_at	AMBP1 - Antimicrobial peptide MBP-1 family protein	6.55 ↑
HVSMEl0007C14r2_at	Tryptophan decarboxylase	5.92 ↓
Contig24993_at	-	5.69 ↓
Contig12336_at	Adhesive/proline-rich protein	5.66 ↓
EBma01_SQ002_F07_s_at	Papain-like cysteine proteinase	5.13 ↑
Contig22733_at	-	5.00 ↓
Contig24933_at	RING-H2 finger protein ATL2B	4.85 ↓
Contig13288_at	Lipoxygenase, precursor	4.79 ↓
Contig20981_at	C2H2 zinc finger protein	4.79 ↓
Contig3778_x_at	-	4.60 ↑
Contig26496_at	BLN2	4.60 ↓
HS17D15r_s_at	Early nodulin protein	4.53 ↓
Contig25242_at	Class III peroxidase	4.40 ↑
Contig14114_at	-	4.18 ↓
Contig12590_at	Calmodulin-related calcium sensor protein	4.17 ↓
Contig5469_at	Cys-rich domain containing protein	4.15 ↓
Contig2008_s_at	16.9 kDa class I heat shock protein 1	4.13 ↓
Contig20755_at	-	4.10 ↓
Contig21298_at	-	4.10 ↓
Contig8829_at	Calmodulin-related calcium sensor protein	4.05 ↓
EBro02_SQ006_C05_at	-	4.00 ↓

High degree of regulation was observed in expression levels of genes coding JA-induced proteins and thionin family proteins (Table 3.15). Thionins are cell wall-associated proteins known to have roles in plant defense (Gausing 1987; Bohlmann *et al.*, 1988). It was also demonstrated that thionin gene expression was induced by JA, wounding, and sorbitol in *Arabidopsis* (Bohlmann *et al.*, 1998; Xu *et al.*, 2001). Thionins are small, cysteine-rich peptides with antimicrobial properties. They are believed to be involved in protection against bacterial and fungal pathogens of plants presumably by attacking the cell membrane and rendering it permeable (Stec, 2006). Genes up-regulated in Tarm-92 might be broadly categorized into defense related and JA-induced. Genes coding alternative oxidases, cysteine proteinases, and lipoxygenases that were up-regulated under B toxicity have been shown to be induced by JA and involved in defense (Grudkowska and Zagdanska 2004; Gao *et al.*, 2008). Induction of genes related to JA in our study suggests a critical role for JA in leaf tissues of barley under B toxicity.

In root tissues of Tarm-92, genes involved in intracellular communication such as genes encoding calmodulin-related calcium sensor proteins from the EF hand superfamily were down-regulated. The highest degree of down-regulation was observed in expression level of a gene represented by Contig10179_s_at (Table 3.15). The gene was uncharacterized however might be a novel gene important in response to B toxicity in root tissues.

3.3.3.8. Genes Regulated in Sensitive but not in Tolerant Cultivar

Genes that were differentially regulated in Hamidiye but did not respond significantly to B toxicity in Tarm-92 might be the reason for sensitivity of Hamidiye seedlings to B toxicity. Number of genes differentially regulated in leaf and root tissues of Hamidiye were 69 and 68, respectively (Figure 3.18a and 3.18c). List of genes, expression levels of which were regulated more than four-fold in leaf and root tissues of Hamidiye is provided in Table 3.16, whereas complete lists of genes are provided in Supplementary Table S.8 and S.9.

Induction in expression of genes coding nicotianamine synthases (NAS) were observed in root tissues of Hamidiye. NAS is the key enzyme in the biosynthetic pathway for the mugineic acid family of phytosiderophores, chelating compounds that sequester iron (Higuchi *et al.*, 1999).

Table 3.16. Selected genes differentially regulated under B toxicity in leaf and root tissues of Hamidiye (Ta) but not in Tarm-92. Genes which showed at least four-fold regulation under B toxicity are retained. (FC: Fold Change; Ha: Hamidiye; C: Control; T: 5 mM H_3BO_3 treatment)

Probe Set ID	Putative function	FC and regulation Ha-T/Ha-C*
Leaf tissues		
Contig5058_x_at	RNase S-like protein	5.70 ↓
Contig6681_at	-	4.34 ↓
Root tissues		
Contig12866_at	-	4.63 ↓
Contig21662_at	Nicotianamine synthase 1	4.19 ↑
Contig10741_at	Nicotianamine synthase 8	4.09 ↑
Contig10740_at	-	4.08 ↑

* Regulation of gene expression is represented with ↑ and ↓ for up- and down-regulation, respectively. Uncharacterized proteins are indicated with -.

3.3.4. Level of Transcriptional Regulation

Investigations of intra-varietal responses of the sensitive cultivar Hamidiye (Section 3.3.1) and tolerant cultivar Tarm-92 (Section 3.3.2) under B toxicity revealed similar sets of genes differentially regulated in leaf tissues. Moreover, inter-varietal comparison of the two cultivars (Section 3.3.3) and ratios of regulation determined for basal response genes indicated differences in level of transcriptional regulation between Tarm-92 and Hamidiye. Though similar sets of genes were regulated in the same manner in tolerant and sensitive cultivars, level of regulation was much higher in the tolerant cultivar Tarm-92 compared to the sensitive Hamidiye. This result indicates that tolerance to B toxicity in Tarm-92 might be a consequence of stronger level of transcriptional regulation under B toxicity.

For instance, the genes – represented by Contig3017_at and Contig3018_at – coding for germin-like oxalate oxidases were up-regulated upon B toxicity in both Tarm-92 and Hamidiye (Table 3.13). However, the expression levels were induced by 19.92- and 6.06-fold in Tarm-92 but 6.69- and 3.00-fold in Hamidiye. Similarly, expression of an uncharacterized gene represented by Contig639_at was induced 9.17-fold in leaf tissues of Hamidiye and

4.03-fold in leaf tissues of Tarm-92 (Table 3.13). Therefore, it is concluded that higher degrees and stronger levels of regulation might be conferring tolerance to Tarm-92 under B toxic conditions.

On the other hand, gene represented by Contig5311_at and coding for O-methyltransferase was down-regulated upon B toxicity in root tissues of both cultivars (Table 3.14). However, the expression level was repressed by 9.75-fold in Tarm-92 but 4.24-fold in Hamidiye. Similarly, expression of genes coding glucan endo-1,3-beta-glucosidase GII – represented by HVSMEm0003C15r2_s_at and Contig1637_s_at – was repressed at higher levels in root tissues of Hamidiye compared to that of Tarm-92. Ratio of regulation, which was determined as the ratio of fold change in expression recorded in Tarm-92 to that in Hamidiye, was 0.39 and 0.27 for these two probe sets (Table 3.14).

Differences between strength of transcriptional regulation in tolerant and sensitive cultivars might be explained by transcription factors (TFs) or proteins involved in regulation of aforementioned genes which showed different levels of up- or down-regulation. It is well known that different number of TFs in different combinations, besides single TFs, bind to regulatory regions of genes to give rise to a wide spectrum of expression patterns (Singh, 1998). Regulatory regions of genes often contain more than one *cis*-acting element such as TATA box, upstream elements, and enhancers. Additionally, a group of proteins, TFs, activators, repressors, or associated factors bind to *cis*-acting elements and each other via DNA-protein or protein-protein interactions (Maniatis *et al.*, 1987; Buratowski, 1997; Lee and Young, 1998). Thus, protein complexes formed at the regulatory region of a gene impose a combinatorial effect and determine level of expression leading to tissue-specific, developmental, or stress-responsive expression.

Level of transcriptional regulation upon B toxicity might be explained by combinatorial effect of various TFs, activators, or repressors presumably functioning more effectively in tolerant barley cultivar under B toxicity to confer tolerance. Importance of combinatorial control provided by enhanceosomes comprising certain number and specific combination of TFs bound to a regulatory region has been shown in regulation of genes involved in anthocyanin biosynthesis in maize (Mol *et al.*, 1998), genes responsive to chilling stress in rice (Yun *et al.*, 2010), and genes induced by abscisic acid (Busk and Pages, 1998; Abe *et al.*, 2003).

3.3.5. Regulation of Transporter Genes

Categories of transporter genes included various ATP-binding cassette (ABC), peptide, nitrate, and phosphate transporter genes which were up-regulated as a result of B toxicity treatments (Table 3.4 and Table 3.7). Certain ABC transporters function in pumping cationic or neutral compounds out of the cell. Barley cells might be inducing genes of ABC transporters to remove excess B accumulated within the cytoplasm. The direction of transport might be out of the cell as well as into vacuoles. This assumption is based on the proposed mechanism of compartmentation of B under toxicity (Reid *et al.*, 2004). Importance of vacuolar transport mechanisms under B toxicity was also demonstrated in a proteomic study which described increases in abundance of the vacuolar proton-translocating ATPase (V-ATPase) subunit E protein in tolerant barley genotype (Atik *et al.*, 2011). Functional role of ABC transporters specifically MDR-like ABC transporters, transcripts of which showed 4- to 7-fold up-regulation under B toxicity in leaf tissues of both Hamidiye and Tarm-92 (Table 3.4 and Table 3.7), should be investigated in subsequent studies.

It was previously shown that *AtNIP5;1* – coding an aquaporin – is induced in *Arabidopsis* plants under conditions of limiting B (Takano *et al.*, 2006) and AtBOR1 – a B transporter – is required for efficient B uptake (Takano *et al.*, 2002). In the present study, it was demonstrated that transcripts coding NOD26-like membrane integral proteins (NIPs) which display high similarity to *AtNIP5;1* was up-regulated in root tissues of Tarm-92 under deficient concentrations of B (Table 3.11). Moreover, expression of a gene which is the ortholog of predicted B transporter gene on chromosome 12 of rice was induced in root tissues of Tarm-92 upon B deficiency (Table 3.11). Though complete CDS of this gene – represented by Contig19634_at – is not known, the consensus of EST sequences shows high similarity to *AtBOR1*-like transporter genes. Thus, gene expression results of this study and previous reports (Takano *et al.*, 2002; 2006) confirm the involvement of B transporters and aquaporins for B uptake in barley.

On the other hand, transcripts coding NIPs which are similar to *AtNIP5;1* was down-regulated in leaf tissues upon high level of B exposure (Table 3.4 and Table 3.7). Repression of the NIPs in leaf tissues of barley might help the cell to prevent B influx and keep excess B out. Based on demonstrated expression of AtNIP6;1 in shoots, involvement of NIPs in

translocation of B in shoot tissues of *A. thaliana* was proposed previously (Miwa and Fujiwara, 2010). According to overall results of the present study it might be stated that induction of ABC transporters and repression of NIPs in barley leaf tissues might be working together to lower the amount of B within the cytoplasm. Moreover, it might be hypothesized that both B exclusion and compartmentation mechanisms might be contributing to tolerance to B toxicity in barley. Contribution by these mechanisms, tissue-specific expression of genes coding B transporters and NIPs, and subcellular localization of protein products should be investigated in succeeding studies.

3.3.6. Crosstalk between Environmental Stresses and B Toxicity

A large number of probe sets representing genes which were annotated as glutathione *S*-transferases (GSTs) were found to be up-regulated at least by two-fold under B toxicity (Table 3.4 and Table 3.7). Catalytic functions of GSTs include conjugation and resulting detoxification of herbicides and reduction of organic hydroperoxides formed during oxidative stress. They bind flavonoid natural products in the cytosol prior to their deposition in the vacuole and are potential regulators of apoptosis. GST is proposed to be functioning in protection of plants from oxidative tissue damage during wounding or pathogen attack (Kim *et al.*, 1994). Our results indicated involvement of GST in protection of barley leaf tissues under prolonged B toxicity. Additionally, up-regulation in expression levels of differentially regulated genes encoding pathogenesis related (PR) proteins were observed after high level B exposure (Table 3.4). Induction of such genes involved in responses to biotic stress reveals a possible crosstalk between signaling of B toxicity and that of biotic stresses.

Patterns of up-regulation under B toxicity among differentially expressed genes of GST and PR proteins were also observed in senescence associated (SA) genes (Table 3.4). Necrotic and chlorotic patches on leaves are characteristic symptoms of B toxicity. Induction of the SA genes might lead to development of chlorotic patches under B toxicity. On the other hand, GST genes might be induced to protect plants from tissue or cell damage during B toxicity induced chlorosis or necrosis.

A category of molecular function included genes having monooxygenase activity. Expression levels of genes annotated as cytochrome P450 (CYP) were up-regulated upon high level of B

exposure (Table 3.4 and Table 3.7). CYPs are involved in various biosynthetic reactions producing fatty acid conjugates, hormones or defensive compounds. They are also known to metabolize various endogenous or exogenous compounds in detoxification reactions. Transcript of CYP709C1 which was up-regulated 7- to 8-folds under B toxic conditions in leaf tissues of Hamidiye (Table 3.4) and 6-fold in leaf tissues of Tarm-92 (Table 3.7), was proposed to be involved in plant defense by producing hydroxylated fatty acids (Kandel *et al.*, 2005). Additionally, genes represented by Contig15560_at and Contig17080_s_at were annotated as CYPs that function in phenylpropanoid biosynthesis. Phenylpropanoids have a wide variety of functions including defense against pathogens or injury (Golkari *et al.*, 2007), protection from UV, and as signaling molecules or structural components of cell walls. Although B toxicity is an abiotic stress, responses to B toxicity might involve alteration of metabolism and production of defensive compounds and it might be highly associated with responses to other biotic and abiotic stresses. Induction of abiotic and biotic stress related genes supports the idea of crosstalk between certain components of environmental stresses such as B toxicity, cold, salt or pathogen attack. A similar observation for crosstalk between salinity, heat, cold and dehydration stresses in barley was also reported previously (Walia *et al.*, 2006).

3.3.7. Real-Time RT-PCR Validation of Microarray Results

The gene expression profiles obtained using microarray analyses were validated by two-step real-time reverse transcriptase PCR (RT-PCR). Total RNA samples used for array hybridizations were used as starting material for real-time RT-PCR analyses and relative quantitation was performed to validate microarray data. Probe sets used for real-time RT-PCR validation included those representing genes which showed significant regulation or no response upon B treatment. Relative quantitation of target sequences was performed by normalization with quantity of an endogenous reference gene, *HvGAPDH*. The amount of target was divided by the amount of reference to calculate the normalized amount of target expression. The average of replicates was calculated and log-transformed (log_2). The log-transformed relative expression values were compared with those obtained by microarray analysis. Expression profiles obtained from the microarray analysis showed high correlation with the real-time RT-PCR data (Figure 3.21). This analysis confirmed validity of expression profiles obtained from microarray data.

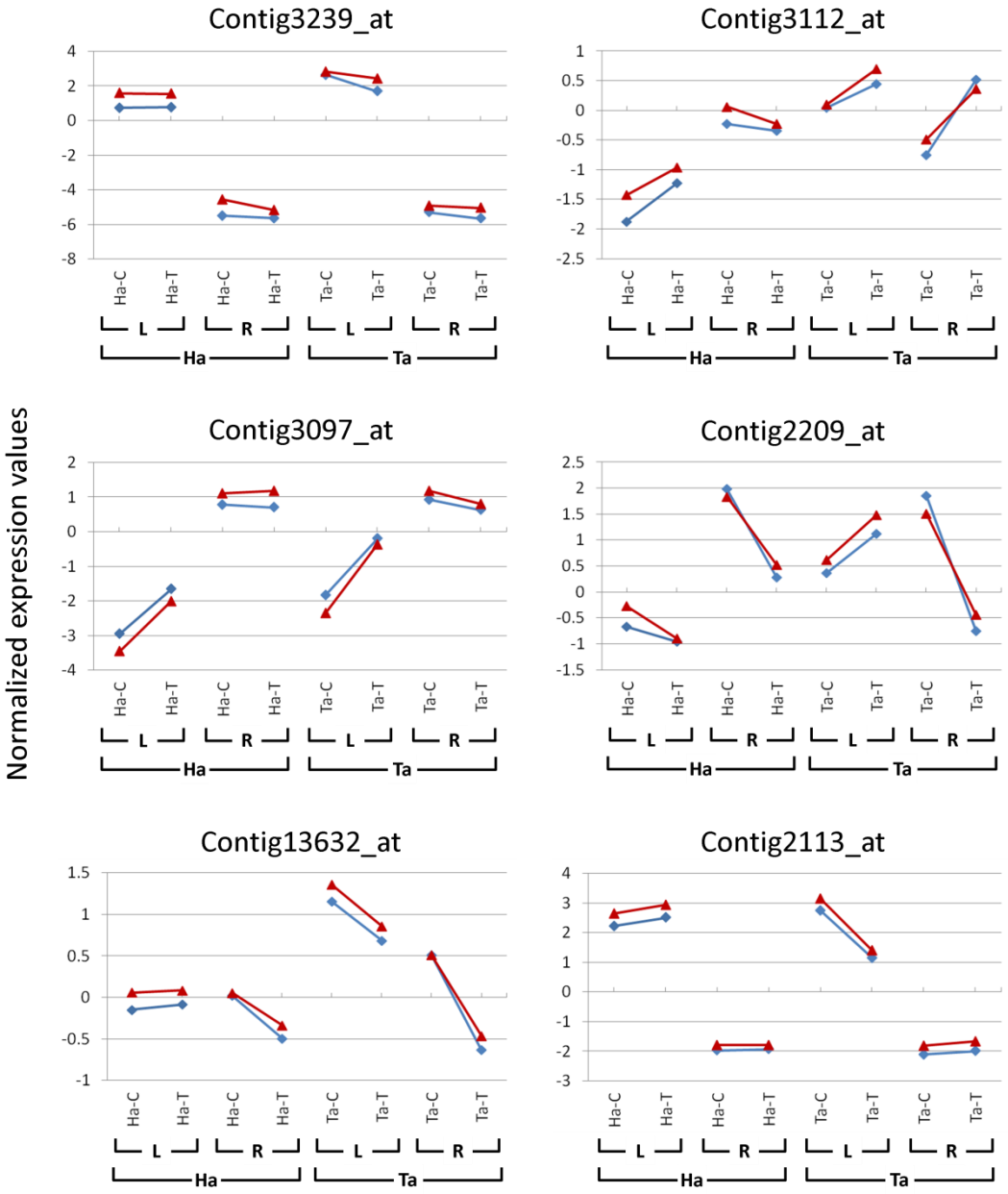

Figure 3.21. Comparison of microarray expression profiles of selected probe sets with expression values obtained from real-time RT-PCR analyses. Blue lines represent expression data from microarray and red lines represent expression values from real-time RT-PCR. The microarray data was log transformed, RMA normalized, and centered about the average over biological replicates. The real-time RT-PCR data was normalized using an endogenous reference gene *HvGAPDH*, log transformed, and centered about the average over replicates. (Ha: Hamidiye, Ta: Tarm-92, L: Leaf, R: Root, C: Control; T: 5 mM H_3BO_3 treatment)

3.4. Cloning and Functional Characterization of B Tolerance Gene on Barley 3H

In barley and wheat, QTL for tolerance to B toxicity and genes coding for B transporters or channel proteins have been identified (Schnurbusch *et al.*, 2010a). The major QTL on 2H, 3H, 4H and 6H were identified in a Clipper X Sahara F1-derived DH mapping population of barley (Jefferies *et al.*, 1999). Moreover, *HvBot1* and *HvNIP2;1*, which were genetically mapped to QTL on 4H and 6H, respectively, have been characterized (Sutton *et al.*, 2007; Schnurbusch *et al.*, 2010b). A candidate gene approach was employed in this study to clone 3H B tolerance gene in barley.

On the other hand, only 3 probe sets of Barley Genome Array represent transcripts which show high sequence similarity to putative B transporter genes in *O. sativa* and *A. thaliana*. Among these 3 probe sets, target sequence of Contig14139_at shows high similarity to B transporter gene located on chromosome 1 of *O. sativa* (Table E.2). Therefore, the barley gene represented by Contig14139_at was transitorily named *HvBor1a* and cloned in this study. The gene is predicted to be located on 3H based on homology and colinearity between rice chromosome 1 and barley 3H (Sato *et al.*, 2011).

Additionally, sequences of introns were determined with an intention to develop genetic markers. Heterologous transient expression was used to assess functional role and subcellular localization of protein product. Moreover, endogenous expression patterns of *HvBor1a* in leaf and root tissues of various cultivars of barley grown under B toxic conditions were investigated.

3.4.1. Full Length CDS of *HvBor1a* in Clipper

The 970 bp sequence information from Contig14139_at (Table E1) and method of 5'RACE was employed to obtain 2,466 bp-long full length nucleotide sequence of the *HvBor1a* mRNA (Table 3.17). Amplified products from RACE reactions were sequenced and reads obtained were aligned using ContigExpress module of Vector NTI. Consensus sequence of amplified fragments from two rounds of 5'RACE was used to determine the coding sequence (CDS) and open reading frame (ORF) of *HvBor1a*. The sequence was utilized to design primers and clone full length *HvBor1a*.

Table 3.17. Full length CDS of putative B transporter gene *HvBor1a*. The open reading frame is shown underlined whereas the translational start and stop codons are highlighted.

```
       1 TGCCGATCGG ACCCGCGCGC CGCGTCCTTA GCCGTCGCCG CCGCCGCCGC
         GACTTCACCG CTAGCTGATG GATCTACTAG GGAACCCTTT CAAGGGAGTC
     101 GTCGCGGATG TCAAAGGGAG AGCATCTTGG TACAAGGACG ATTGGGTTGC
         AGGGCTCCGA ACTGGCTTCA GGATATTGGC ACCTACCATG TATATTTTCT
     201 TTGCCTCTGC ACTCCCTGTA ATCTCCTTCG GAGAGCAGCT GAGCAACGAA
         ACAGATGGTA TCGTAAGCAC TGTTGAAACT TTGGCGTCTA CGGCGATATG
     301 TGGGATAATA CACTCGATTC TTGGAGGGCA GCCACTGTTG ATCGTTGGAG
         TCGCAGAACC TACTATTATC ATGTATACGT ATCTCTACAA GTTTGCCAAG
     401 AAGCAGCCAG ATCTGGGAGA ACGGCTATAT TTGGCTTGGG CTGGATGGGT
         CTGCATTTGG ACTGCTATCA TGCTGTTTCT TTTGGCAATG TTCAATGCTT
     501 CCAATGTTAT AAGCAGATTC ACGAGGGTTG CAGGAGAGCT TTTTGGTATG
         TTGATCACTG TCCTGTTCCT GCAGCAAGCT ATCAAGGGAA TTGTAAGTGA
     601 GTTCAGTGTG CCGAAAGATG ATGAGATTTC TGACCCCAGC TCACCTATAT
         ACCAGTTCCA GTGGATTTAT GTCAATGGCC TACTTGGTGT TATATTTTCC
     701 ATTGGCTTGC TGTACACTGC ACTGAAGACT AGGCGTGCAA GGTCATGGCT
         GTATGGCGTA GGATGGCTTA GAAGCTTCAT TGCCGATTAC GGTGTACCGC
     801 TGATGGTGAT TGTGTGGACA GCATTGTCAT TTACACTACC AAGCAAAGTC
         CCTTCAGGAG TGCCTAGGAG GCTCTTCAGT CCACTTCCCT GGGAGTCAAT
     901 CTCACTGAGA CATTGGACCG TAGCAAAGGA TTTGTTTTCT GTCCCTCCAA
         CATATATATT TGCAGCCATC GTGCCTGCTT TGATGGTCGC AGGACTTTAT
    1001 TTCTTTGACC ACAGTGTAGC TTCACAGTTG GCTCAGCAGA AGGAGTTTAA
         TTTGAAGAAG GCTTCTGCCT ACCATTATGA CATTTTGGTA CTTGGATTCA
    1101 TGGTCCTACT ATGTGGTTTG CTTGGCATTC CCCCATCAAA TGGAGTACTT
         CCTCAGTCCC CCATGCATAC AAGAAGCCTT GCTGTCCTCA AGGGGCAGCT
    1201 GCTACGCAGA AAGATGCTTC AAACTGCCAA AGAGGGCATG TCAAACCGTG
         CGAGCAGTTT GGAAATCTAT GGCAAGATGC AGGAAGTGTT CATCCAAATG
    1301 GATAGCAACC AGAATGCTAA TTCTGTTGAC AAGGACTTGA AGAGCTTGAA
         GGATGCTGTG CTGCGGGAAG GTGACGAAGA AGGGAAATTG GCTGGAGAAT
    1401 TTGATCCTAG CAAACACATT GAAGCACATT TGCCTGTTCG TGTGAACGAA
         CAGAGGCTAA GCAACCTGCT GCAATCCTTA CTTGTTGGTG GCTGTGTTGG
    1501 AGCTATGCCG GCTATCAAGA TGATACCGAC TTCGGTCCTC TGGGGTTACT
         TTGCCTACAT GGCCATTGAT AGCCTACCTG GGAACCAGTT TTGGGAAAGG
    1601 TTACAGCTTC TGTGCATTGG AGCAAGCCGA CGCTACAAGG TCTTGGAAGG
         CCCCCATGCA TCTTTCGTGG AGGCGGTGCC TTCAAGAACA ATATCTGCCT
    1701 TTACGGTCTT CCAGTTTGTG TATCTCTTGA TATGCTTCGG TATAACATGG
         ATACCAGTAG CAGGGATCCT CTTCCCGCTG CCTTTCTTCA TTATGATTCT
    1801 CATCAGGCAA CACCTACTCC CAAAGTTCTT TGAGCCCAAT GACTTGCGAG
         AACTGGATGC AGCTGAGTAT GAAGAACTTG AAGGCGTCCC ACATGAACAA
    1901 ACACTGGAGG AAGATGGCTC AAATTCAGGA AGCCATGACA GCATAGACGA
         CGCTGAAATG TTGGATGAAC TCACGACAAA CCGTGGAGAG CTGAAGCACA
    2001 GATCTGCAAG CCATCCTGAA GAAAGGCACC TTCAGGTCCA TTCAAATGCA
         GTTCAGCCGA GCGTGTGAAG ATGGAAAAAC GTCCCGATGT CCGAGGCTTT
    2101 GATGACGATT CTGTCAGAAG TGTAGATTAT CCTGAAGCCA TTGTTCATTT
         CCGAATACGC CATTGTTCAG CAATGTGCAT TGTACTGTAG CTAGCGCTTT
    2201 TCCGGTAAGG CGTGATCGTG TACGCTAGGA AGTAAAGCTC AGGTAATTAG
         CAGTGAGATC AGACTGAAGA AAGTGTATTG GATATGGCAG GGGAACTTGG
    2301 CAGAATAGTA TTAGTAATGG TTAGAAGTAG AAAGACTGTT GCTCCGTGAC
         CTGTTATTGC AAGACTGTTG CTCTGTGACC TGTTGCCTGT GTAAAGTCCA
    2401 GCTTTCTGTG TCTGAATGTA AAATGCCAGG CATAAGAATT TCTTGAAAAA
         AAAAAAAAAA AAAAAA
```

The full length CDS of *HvBor1a* – including start and stop codons, excluding UTRs – was cloned from Clipper cDNA synthesized from the total RNA isolated from leaf tissues. The fragment was amplified in PCR by a proofreading enzyme. Subsequently size of the fragment was checked using electrophoresis on agarose gel (Figure 3.22). The fragment was then recovered from gel and cloned into entry vectors for Gateway cloning. The plasmids were multiplied in TOP10 and then sequenced for verification of the direction and sequence of the insert. The entry clone was utilized in succeeding studies such as heterologous expression after *in vitro* recombination with appropriate expression vectors.

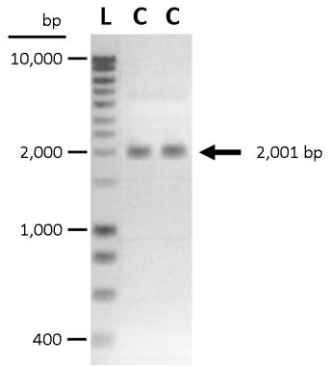

Figure 3.22. Amplified full length CDS of *HvBor1a* separated on agarose gel. Electrophoresis gel image of 2,001 bp-long CDS of *HvBor1a* cloned from Clipper (C) is displayed. The fragment was amplified by PCR from cDNA that was prepared from leaf tissues of Clipper. Amplified fragment was separated on 1% (w/v) agarose gel. (L: Ladder)

The nucleotide sequence of *HvBor1a* was handled using VectorNTI and the CDS was blasted against non-redundant (nr/nt) database at NCBI using BLASTN (Table 3.18). The full list and details of BLASTN results are provided in Appendix I. The CDS of *HvBor1a* showed the highest similarity to the sequence of mRNA coding *T. aestivum* BOR2 (TaBOR2) which is the putative B transporter identified in wheat (Reid, 2007). Additionally, at nucleotide level the CDS of *HvBor1a* showed 89% and 83% similarity to *HvBot1* and *OsBOR3*, respectively.

Table 3.18. BLASTN results displaying sequences which produce significant alignments with *HvBor1a*. The CDS of *HvBor1a* was blasted against nucleotide collection (nr/nt) database at NCBI using BLASTN delimiting the source organism to higher plants (taxid:3193). Accession numbers and descriptions of hit subjects, query coverage, e-values, and identity percentages of results are listed.

Accession	Description	Query coverage	E-value	Identity (%) Identity/Match
EU220225.1	*T. aestivum* B transporter 2 mRNA, cds	100%	0.0	96% (1912/2002)
XM_003569470.1	*B. distachyon* B transporter 4-like (LOC100824289), mRNA	100%	0.0	89% (1797/2018)
EF660437.1	*H. vulgare* ssp. *vulgare* B transporter (Bot1) mRNA, cds	100%	0.0	89% (1792/2014)
DQ421408.1	*O. sativa* (japonica) B transporter mRNA, cds	99%	0.0	83% (1695/2031)
NM_001154326.1	*Z. mays* B transporter-like protein 2 (LOC100281408), mRNA, cds	97%	0.0	83% (1658/1997)
XM_002455044.1	*S. bicolor* hypothetical protein, mRNA	97%	0.0	83% (1652/1999)
DQ421409.1	*O. sativa* (japonica) B transporter (BOR4) mRNA, cds	88%	0.0	77% (1408/1820)
XM_002440654.1	*S. bicolor* hypothetical protein, mRNA	62%	0.0	77% (990/1284)
NM_001061328.1	*O. sativa* (japonica) (Os05g0176800) mRNA, cds	47%	5e-141	77% (767/996)
NM_001195926.1	*Z. mays* uncharacterized (LOC100501111), mRNA	15%	3e-94	87% (278/321)
BT084558.1	*Z. mays* full-length cDNA clone ZM_BFb0132E07 mRNA, cds	15%	3e-94	87% (278/321)

3.4.2. Predicted Protein Structure of HvBor1a

The ORF of *HvBor1a* was predicted using VectorNTI. It was determined that the 1998 bp-long ORF encodes a putative protein of 666 amino acids with a theoretical molecular weight (MW) of 74,253.35 Da and isoelectric point (pI) of 6.13 (http://www.expasy.ch/tools/pi_tool; Bjellqvist *et al.*, 1993). The amino acid sequence is presented in Table 3.19.

Table 3.19. Amino acid sequence of HvBor1a.

```
  1 MDLLGNPFKG VVADVKGRAS WYKDDWVAGL RTGFRILAPT MYIFFASALP
 51 VISFGEQLSN ETDGIVSTVE TLASTAICGI IHSILGGQPL LIVGVAEPTI
101 IMYTYLYKFA KKQPDLGERL YLAWAGWVCI WTAIMLFLLA MFNASNVISR
151 FTRVAGELFG MLITVLFLQQ AIKGIVSEFS VPKDDEISDP SSPIYQFQWI
201 YVNGLLGVIF SIGLLYTALK TRRARSWLYG VGWLRSFIAD YGVPLMVIVW
251 TALSFTLPSK VPSGVPRRLF SPLPWESISL RHWTVAKDLF SVPPTYIFAA
301 IVPALMVAGL YFFDHSVASQ LAQQKEFNLK KASAYHYDIL VLGFMVLLCG
351 LLGIPPSNGV LPQSPMHTRS LAVLKGQLLR RKMLQTAKEG MSNRASSLEI
401 YGKMQEVFIQ MDSNQNANSV DKDLKSLKDA VLREGDEEGK LAGEFDPSKH
451 IEAHLPVRVN EQRLSNLLQS LLVGGCVGAM PAIKMIPTSV LWGYFAYMAI
501 DSLPGNQFWE RLQLLCIGAS RRYKVLEGPH ASFVEAVPSR TISAFTVFQF
551 VYLLICFGIT WIPVAGILFP LPFFIMILIR QHLLPKFFEP NDLRELDAAE
601 YEELEGVPHE QTLEEDGSNS GSHDSIDDAE MLDELTTNRG ELKHRSASHP
651 EERHLQVHSN AVQPSV
```

The secondary structure of HvBor1a was predicted using PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred; Jones, 1999; McGuffin *et al.*, 2000). Prediction was performed on amino acid sequence. The predicted secondary structure is presented in Appendix J. Additionally the amino acid sequence of HvBor1a was analyzed using InterProScan (http://www.ebi.ac.uk/tools/pfa/iprscan) to locate conserved domains. The graphical display of results of InterProScan is provided in Figure 3.23 and results as a table are summarized in Appendix K. It was revealed that the predicted protein contains a bicarbonate transporter domain and an anion exchange protein-related domain which are characteristic domains of B transporters (Frommer and von Wiren, 2002).

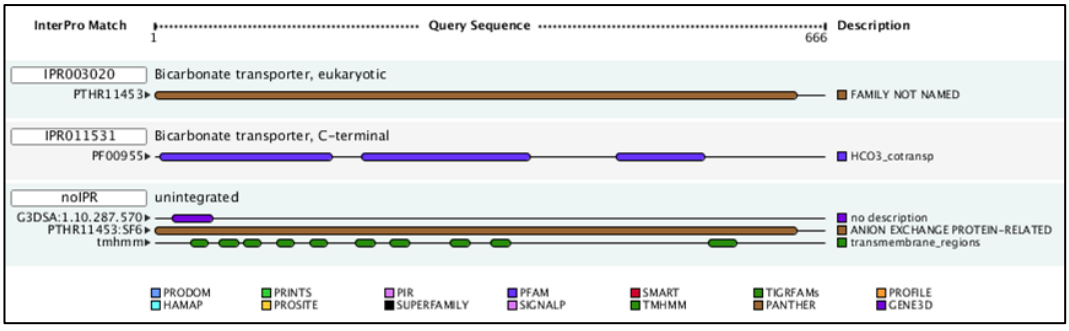

Figure 3.23. Graphical display of InterProScan results. The sequence of 666 amino acids was queried against databases of conserved domains at InterPro. Conserved domains are shown colored.

The analysis using InterProScan also revealed that the predicted protein contains transmembrane segments. According to the method of TMHMM at least 10 transmembrane domains were determined (Figure 3.23 and Table J.1). The amino acid sequence of HvBor1a was further analyzed using DAS (Dense Alignment Surface) transmembrane prediction server (http://www.sbc.su.se/~miklos/DAS; Cserzo *et al.*, 1997). The graphical representation of DAS profile scores calculated on amino acid sequence is provided in Figure 3.24 and the potential transmembrane regions are listed in Table L.1 (Appendix L). The analysis indicated presence of 10 – 12 potential transmembrane segments. Similarly, the protein product of *HvBot1*, which was the first B transporter identified in barley, was demonstrated to contain 10 – 12 putative transmembrane helices (Sutton *et al.*, 2007). Though sequence based predictions indicated that HvBor1a is a protein spanning the membrane, secondary and tertiary structure of the protein should be analyzed further and biochemical work should be carried out in succeeding studies to ascertain that HvBor1a is a transmembrane protein.

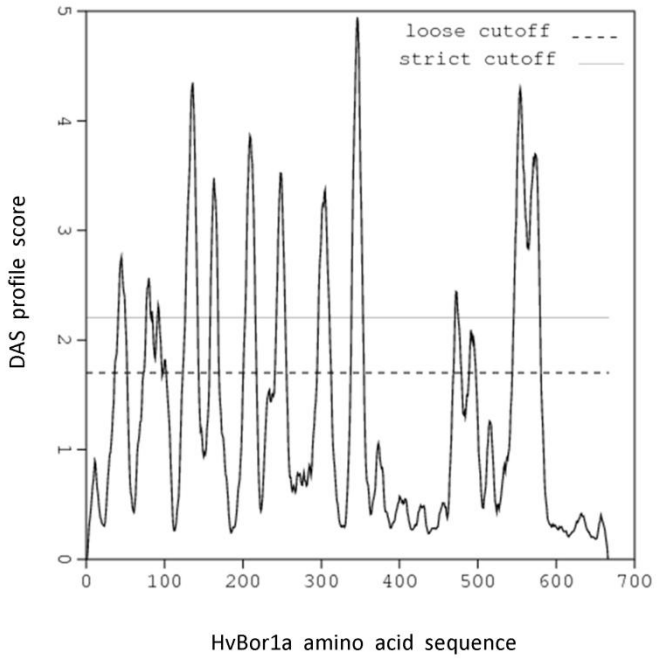

Figure 3.24. Graphical representation of DAS profile scores indicating transmembrane segments of HvBor1a. Loose cutoff of 1.7 and strict cutoff of 2.2 are shown with dashed and straight lines, respectively. The 666 amino acid long sequence of HvBor1a was analyzed using DAS (Dense Alignment Surface) transmembrane prediction server.

3.4.3. Multiple Alignments of B Transporter Genes

Multiple sequence alignment was carried out for comparison of CDS of *HvBor1a* to genes encoding AtBOR1-like B transporters in *A. thaliana*, *B. distachyon*, rice, wheat, and barley. The sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2). The alignment is provided in Appendix M and the match scores are presented in Table 3.20.

The CDS of the predicted B transporter genes from *A. thaliana*, *B. distachyon*, and rice were obtained from Gramene (http://www.gramene.org). The sequence comparison also included CDS of genes encoding TaBOR2 and HvBot1 which showed high sequence similarity to CDS of *HvBor1a* according to BLASTN (Table 3.18). The sequences of *TaBOR2* and *HvBot1* were obtained from NCBI (http://www.ncbi.nlm.nih.gov).

Table 3.20. Match scores of alignment with ClustalW2. The CDS of *HvBor1a* and *AtBOR1*-like genes from *A. thaliana*, *B. distachyon*, rice, wheat, and barley were used in alignment with ClustalW2. The length of nucleotide sequences and match scores are displayed. The locus identifiers for genes from *A. thaliana*, *B. distachyon*, and rice were obtained from Gramene.

Sequence A	Name	Length	Sequence B	Name	Length	Score (%)
1	HvBor1a	2001				
			2	TaBOR2	2001	95.0
			3	Bot1_HvBor1b	2001	88.0
			4	BRADI2G04690	2001	88.0
			5	OsBOR3_LOC_Os01g08020.1	2019	83.0
			6	OsBOR4_LOC_Os05g08430.1	2034	75.0
			7	OsBOR1_LOC_Os12g37840.1	2136	66.0
			8	AtBOR4_AT1G15460	2052	69.0
			9	AtBOR1_AT2G47160.2	2190	66.0
			10	AtBOR5_AT1G74810	2052	68.0
			11	AtBOR7_AT4G32510	2022	66.0
			12	AtBOR6_AT5G25430	2016	66.0
			13	AtBOR3_AT3G06450.1	2199	64.0
			14	AtBOR2_AT3G62270	2112	66.0

According to Gramene, genomes of *A. thaliana* and *O. sativa* accommodate 7 and 3 putative *AtBOR1*-like B transporter genes, respectively. Previously, it was predicted that the rice genome contained 4 putative B transporter genes. Two of the genes, *OsBOR3* and *OsBOR2* with locus identifiers LOC_Os01g08020.1 and LOC_Os01g08040.1 were located on chromosome 1. However, the predicted gene, *OsBOR2* with the locus identifier LOC_Os01g08040.1 was not listed as a gene in Ensembl34 build of *O. sativa* Japonica Group and in MSU6 (http://rice.plantbiology.msu.edu). Additionally, updated annotations in the final release, MSU7 (released on October 31, 2011) did not list the locus, LOC_Os01g08040.1 as a predicted gene.

Multiple sequence alignment indicated high percentages of similarity between CDS of *HvBor1a* and those of genes encoding TaBOR2, the putative B transporter from *B. distachyon*, HvBot1, and OsBOR3. The closest rice ortholog of *HvBor1a* is *OsBOR3* located on chromosome 1.

Additionally, unrooted phylogenetic tree (Figure 3.25) was constructed using the alignment generated with ClustalW2. The tree was constructed in the PHYLIP format with scaled branches using GeneBee TreeTop phylogenetic tree prediction server (http://www.genebee.msu.su/services/phtree_reduced.html). According to the phylogenetic tree *HvBor1a* and genes encoding B transporters in wheat, barley, and *B. distachyon* were clustered together. Wheat, barley, and *B. distachyon* are members of the grass subfamily Pooideae. The closest B transporter gene from rice to the Pooideae cluster is *OsBOR3*.

The closest ortholog of *HvBor1a* is *TaBOR2*, which was cloned from roots of wheat grown under B toxic conditions (Reid, 2007). The amino acid sequences of HvBor1a and TaBOR2 were aligned using BLASTP (Table 3.21) to locate variations between two polypeptides. Alignment with 97% identities and no gaps displayed 21 amino acid differences between two sequences.

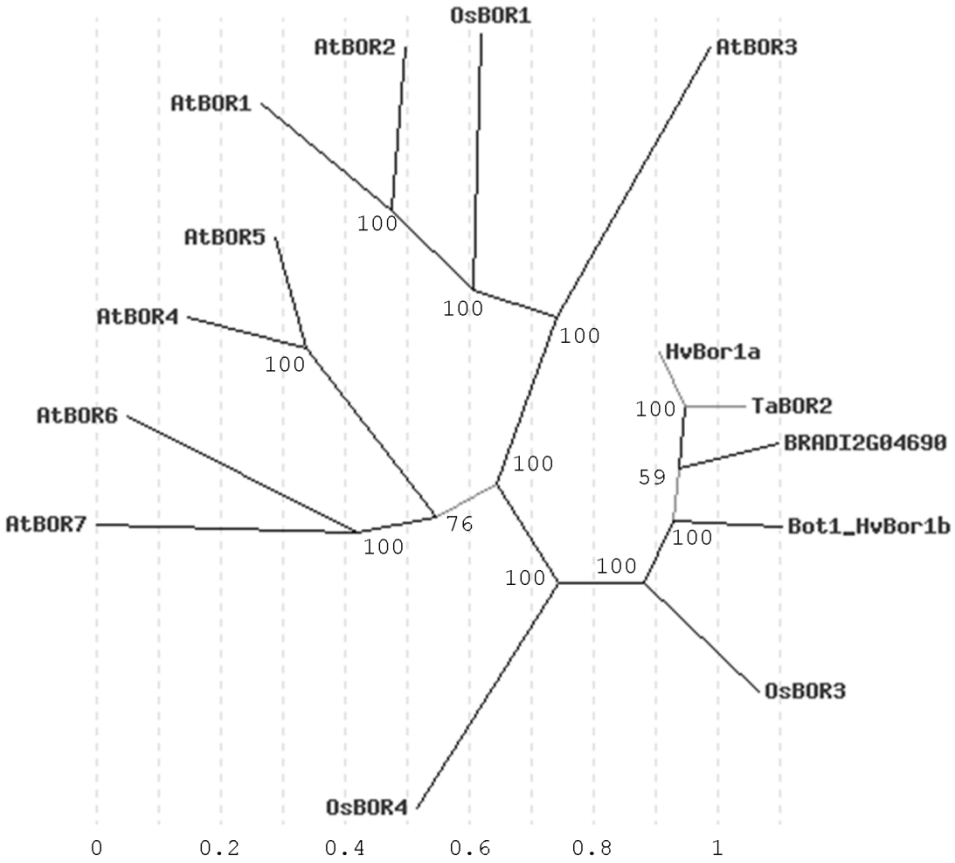

Figure 3.25. Phylogenetic tree including *HvBor1a* and *AtBOR1*-like genes. The tree was constructed with PHYLIP tree format using GeneBee TreeTop phylogenetic tree prediction server. Numbers separating junctions are bootstrap values. The branches are scaled where scaling is indicated with dashed grid lines.

Table 3.21. Alignment of amino acid sequences of HvBor1a and TaBOR2. Alignment was performed using BLASTP. Identities are indicated with dots.

```
Score = 1328 bits (3436), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 645/666 (97%), Positives = 656/666 (98%), Gaps = 0/666 (0%)

HvBor1a 1   MDLLGNPFKGVVADVKGRASWYKDDWVAGLRTGFRILAPTMYIFFASALPVISFGEQLSN  60
TaBOR2  1   ..............AP..............................................  60

HvBor1a 61  ETDGIVSTVETLASTAICGIIHSILGGQPLLIVGVAEPTIIMYTYLYKFAKKQPDLGERL  120
TaBOR2  61  .....L.................A.................................Q.  120

HvBor1a 121 YLAWAGWVCIWTAIMLFLLAMFNASNVISRFTRVAGELFGMLITVLFLQQAIKGIVSEFS  180
TaBOR2  121 ............................................................  180

HvBor1a 181 VPKDDEISDPSSPIYQFQWIYVNGLLGVIFSIGLLYTALKTRRARSWLYGVGWLRSFIAD  240
TaBOR2  181 M...........................................................I.........  240

HvBor1a 241 YGVPLMVIVWTALSFTLPSKVPSGVPRRLFSPLPWESISLRHWTVAKDLFSVPPTYIFAA  300
TaBOR2  241 ............F...............................................  300

HvBor1a 301 IVPALMVAGLYFFDHSVASQLAQQKEFNLKKASAYHYDILVLGFMVLLCGLLGIPPSNGV  360
TaBOR2  301 ............................P...........................  360

HvBor1a 361 LPQSPMHTRSLAVLKGQLLRRKMLQTAKEGMSNRASSLEIYGKMQEVFIQMDSNQNANSV  420
TaBOR2  361 ....................K.....................................  420

HvBor1a 421 DKDLKSLKDAVLREGDEEGKLAGEFDPSKHIEAHLPVRVNEQRLSNLLQSLLVGGCVGAM  480
TaBOR2  421 ............W.................Y.............................  480

HvBor1a 481 PAIKMIPTSVLWGYFAYMAIDSLPGNQFWERLQLLCIGASRRYKVLEGPHASFVEAVPSR  540
TaBOR2  481 .V..............................M.......................S....  540

HvBor1a 541 TISAFTVFQFVYLLICFGITWIPVAGILFPLPFFIMILIRQHLLPKFFEPNDLRELDAAE  600
TaBOR2  541 ............................................................  600

HvBor1a 601 YEELEGVPHEQTLEEDGSNSGSHDSIDDAEMLDELTTNRGELKHRSASHPEERHLQVHSN  660
TaBOR2  601 ......................C..R..S.I..............V........F.....  660

HvBor1a 661 AVQPSV  666
TaBOR2  661 ......  666
```

3.4.4. Intron Structure of *HvBor1a* in Clipper and Sahara

Nucleotide sequences of introns and UTRs of *HvBor1a* gene were determined with an intention to develop genetic markers for tolerance to B toxicity. Possible variations such as SNPs, deletions or insertions in the sequence of *HvBor1a* among versions of the gene in different cultivars of barley might be used for designing novel genetic markers. After prediction of exon-intron junctions according to the sequence of *OsBOR3*, primer pairs were designed and conventional PCR was employed to amplify intron sequences from genomic DNA isolated from Clipper and Sahara. Amplified fragments were sequenced and reads obtained were aligned using ContigExpress. Sequence of each intron was determined and reads were manually inspected for variations such as SNPs between genomic sequences of two cultivars of barley.

The graphical representation of intron structure of *HvBor1a* in Clipper genome is displayed in Figure 3.26. Additionally the complete sequences of introns are provided in Appendix N. The *HvBor1a* gene contains 14 exons and 13 introns. The first intron is upstream of translation initiation site, within 5' UTR, and is 892 bp-long (Table M.1). The first 8 bp region of exon2 is also within 5' UTR. The exact sequences of introns 6 and 10 have not been determined. The former was predicted to be larger than 350 bp and latter 600 bp. The counterpart of last intron – intron 13 – which is 353 bp-long in *HvBor1a* was lacking in *HvBot1* which contained 12 introns (Sutton *et al.*, 2007).

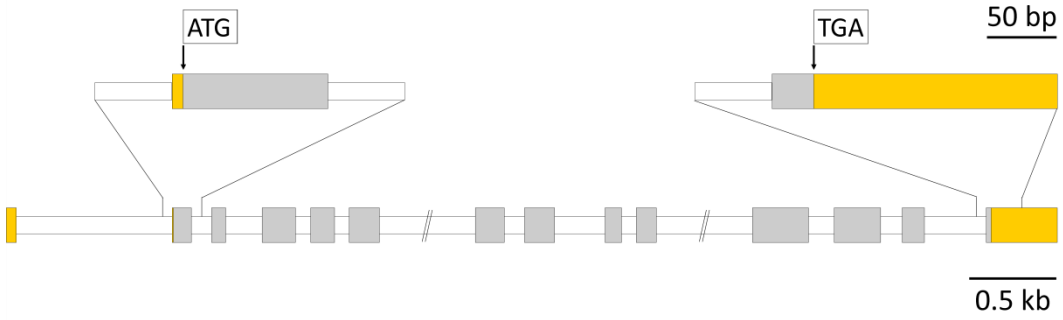

Figure 3.26. Graphical display of intron structure of *HvBor1a* in Clipper. Introns are depicted with empty boxes whereas exons and UTRs with filled boxes. Exons are shown in gray color whereas UTRs in gold. The lengths of exons, introns, and UTRs of *HvBor1a* are in scale. Translation initiation and stop sites are indicated with arrows.

The sequences of introns of *HvBor1a* in genomes of Clipper and Sahara were exactly identical except for intron 13. The first 268 bp region within last intron was also found to be identical in Clipper and Sahara genomes. However, the remaining sequence diverged in Sahara probably because of an insertion or deletion within intron 13.

The work has been concentrated on final intron since a corresponding intron was lacking in *HvBot1* and this region, showing variance in Clipper and Sahara, might be critical for regulation of the expression of *HvBor1a* in B-sensitive Clipper and B-tolerant Sahara. Besides regulation of the gene, variation in final intron might lead to a variation in polypeptide sequence since the last 10 amino acids were encoded by the exon downstream of intron 13 in Clipper. The final stretch of amino acids forming the carboxy-terminus of the protein product might be critical for regulation of the activity of protein. Carboxy-terminal domains of proteins are widely utilized for posttranslational regulations or modifications and may contain signals for protein sorting and anchoring.

3.4.5. Last Intron and 3' End of *HvBor1a* in Sahara

The sequence of final intron – intron 13 – was determined in Clipper. On the other hand, the sequence showed a significant variation in Sahara. Though the first 268 bp long region of the final intron was identical in Clipper and Sahara, the remaining 85 bp segment was unique to Clipper. The variation in genomic sequence of *HvBor1a* in Clipper and Sahara was confirmed with a series of PCR amplifications (Figure 3.27).

Primers were designed for amplification of last intron of *HvBor1a* from barley cultivars Clipper and Sahara. The primer pairs were designed according to the sequence information from Clipper such that the amplicons spanned the last intron (Figure 3.27c). The primers were used in various combinations in PCR on genomic DNA and cDNA from Clipper and Sahara. One of the primer pairs was used as a positive control. The expected amplicon from positive control did not span the last intron but instead spanned the two upstream introns (Figure 3.27c). Amplification of fragments from both genomic DNA and cDNA of Clipper and Sahara verified the variation in sequence within the final intron of *HvBor1a* in Sahara genome (Figure 3.27a and 3.27b).

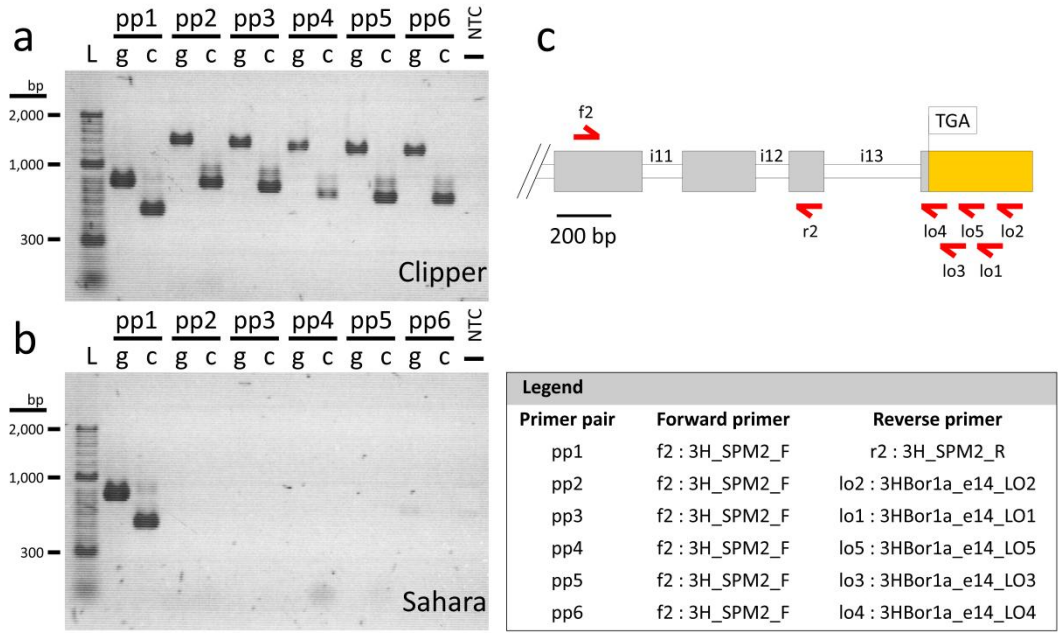

Figure 3.27. Verification of variation in last intron and 3' end of *HvBor1a* in Sahara. Electrophoresis gel images (a, b) of amplified fragments from genomic DNA (g) and cDNA (c) preparations from Clipper and Sahara are displayed. Primer pairs (pp) used in PCR amplifications are listed in legend. Primers are shown with half arrows and binding sites of primers (c) are displayed relative to exons and introns (i) of *HvBor1a*. The lengths of exons and introns are in scale however primers are not. For detailed information on primers, see Section 2.8.2.1. (L: Ladder, NTC: No template control)

The amplification with primer pair employed as positive control indicated the intact nature of the gene upto final intron in both Clipper and Sahara genomes. Additionally amplification with the same primer pair on cDNA indicated the expression of transcript in Clipper as well as Sahara. However, none of the remaining primer pairs resulted in amplification from genomic DNA or cDNA of Sahara. The absence of amplification from genomic DNA of Sahara (Figure 3.27b) might be explained by either the absence of binding sites of primers as a result of a deletion in this region or significant increases in size of expected fragments because of an insertion within final intron in Sahara. On the other hand, absence of amplification from cDNA of Sahara (Figure 3.27b) might be explained by either an early translational stop site within final intron or a new splice site and translation stop codon generated after a deletion or insertion within last intron of *HvBor1a* in Sahara.

Furthermore genome walking with two different approaches – restriction enzyme digestion (GW-RED) and multiple displacement amplification (GW-MDA) – was employed for determination of last intron as well as 5' and 3' UTR regions of *HvBor1a* in Clipper and Sahara genomes. The fragments amplified and subsequently separated on agarose gel (Figure 3.28 and 3.29) were sequenced for genome walking.

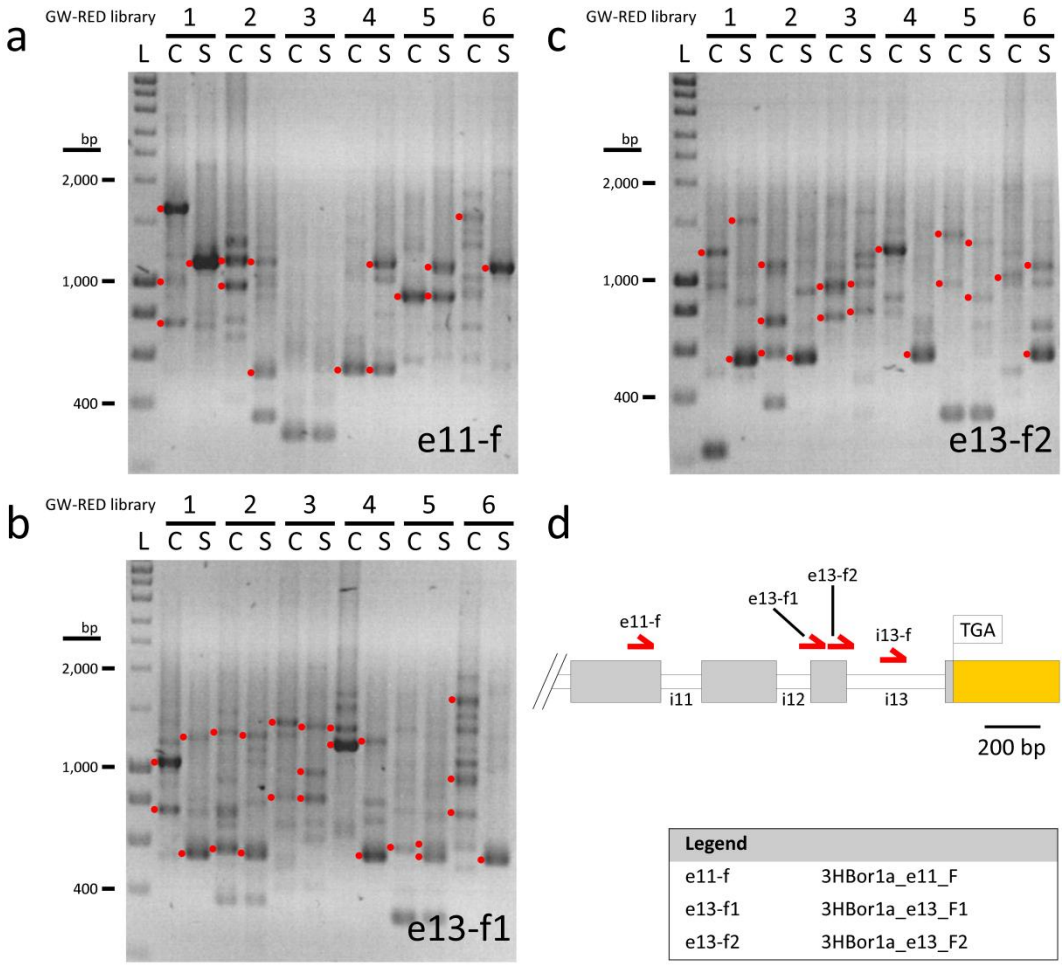

Figure 3.28. The fragments sequenced and primers employed for genome walking using restriction enzyme digestion (GW-RED). Electrophoresis gel images (a, b, c) of fragments amplified from 6 different GW-RED libraries of Clipper (C) and Sahara (S) are displayed. The fragments indicated with dots on gel images were sequenced for genome walking. Primers used in PCR amplifications are listed in legend. Primers are shown with half arrows (d) and binding sites of primers are displayed relative to exons and introns (i) of *HvBor1a*. The lengths of exons and introns are in scale however primers are not. For detailed information on the method and primers, see Section 2.8.2.3. (L: Ladder)

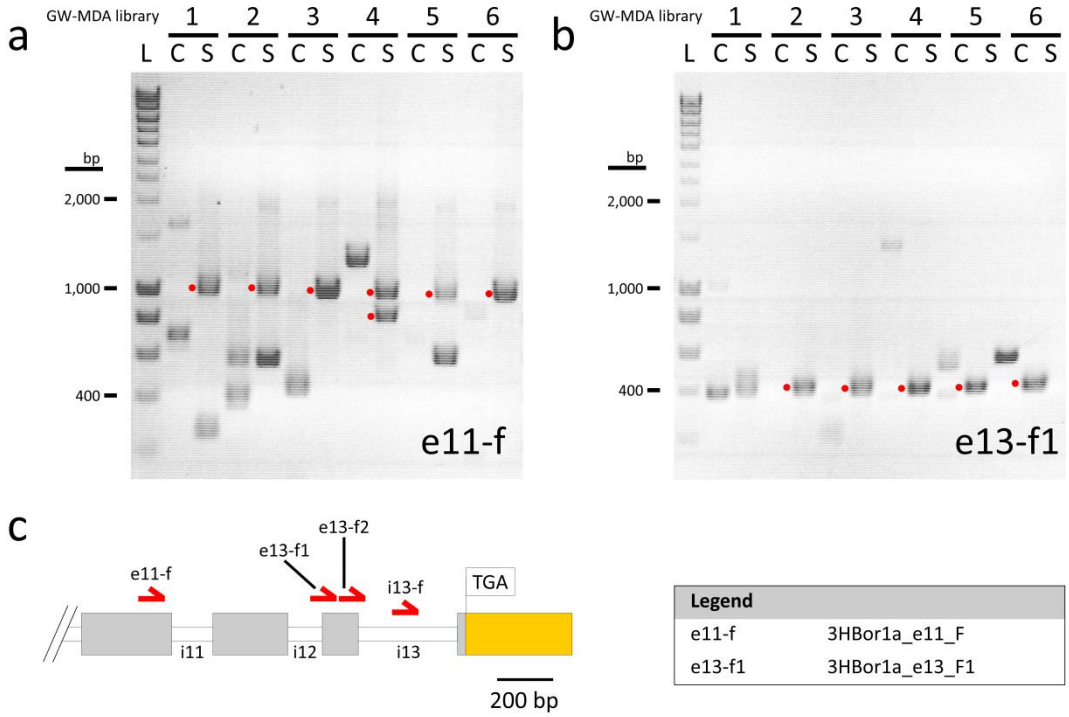

Figure 3.29. The fragments sequenced and primers employed for genome walking using multiple displacement amplification (GW-MDA). Electrophoresis gel images (a, b) of fragments amplified from 6 different GW-MDA libraries of Clipper (C) and Sahara (S) are displayed. The fragments indicated with dots on gel images were sequenced for genome walking. Primers used in PCR amplifications are listed in legend. Primers are shown with half arrows (c) and binding sites of primers are displayed relative to exons and introns (i) of *HvBor1a*. The lengths of exons and introns are in scale however primers are not. For detailed information on the method and primers, see Section 2.8.2.3 and 2.8.2.4. (L: Ladder)

The sequences obtained by genome walking were aligned using ContigExpress. Genome walking on Clipper DNA confirmed the sequence of the last intron – intron 13 (Table M.1). Furthermore, the sequence of a 636 bp region that was determined downstream of the translation stop codon (Table O.1, Appendix O) verified the sequence of 3' UTR of *HvBor1a* in Clipper. On the other hand, genome walking on Sahara DNA did not produce fragments with sequences having an in-frame final exon and translation stop codon. Additionally in some libraries of GW-RED and GW-MDA, fragments of *HvBot1* were amplified because of high sequence similarity between *HvBot1* and *HvBor1a*. The sequences of these fragments of *HvBot1* were excluded from analyses.

As judged by sizes of amplified fragments from Sahara genomic DNA (Figure 3.29) genome walking on Sahara DNA did not produce fragments with significant lengths. Consensus sequence of the fragments from genome walking resulted in identification of an approximately 390 bp long sequence downstream of exon 13. The determined sequence covered the common region within intron 13 and a 120 bp long unique sequence in Sahara (Table O.2, Appendix O). However the sequence unique to Sahara genome contained neither the final exon – exon 14 – of Clipper *HvBor1a* nor a new splice site to generate an in-frame stop codon.

Couple of other attempts to determine an in-frame final exon and translation stop codon in Sahara genome failed (data not shown). In another attempt, 3'RACE method was employed to determine 3' end of *HvBor1a* in Sahara. The 3'RACE-PCR was performed with the gene specific primer 3HBor1a_e13_F2 which had a binding site within exon 13 (Table 2.18 and Figure 2.9). The 3'RACE method generated two fragments from RACE-ready cDNA prepared from Sahara. The 362 bp long consensus sequence of the fragments is provided in Table N.3 (Appendix O). The first 53 bp region of the sequence was common to both Clipper and Sahara, whereas the remaining region was unique to Sahara. The common region of transcript was identical to exon 13 of Clipper *HvBor1a*. Additionally, the unique region contained an in-frame new translation stop site (TAG). The region unique to Sahara might be the result of the proposed insertion or deletion within intron 13 of *HvBor1a* in Sahara genome. Determination of a unique region of the transcript in Sahara cDNA supported the idea that a new translation stop site is generated by an insertion or deletion. The sequence of the fragment of transcript obtained in 3'RACE was used to design new primers. An attempt to amplify the final intron and exon from Sahara genomic DNA using these primers failed (data not shown).

Though a fragment with a potential to provide a stop codon was amplified from cDNA of Sahara, the sequence could not be verified with amplification from genomic DNA. Furthermore, the sequence identified with genome walking (Table N.2) was different from the sequence identified in 3'RACE (Table N.3). Therefore it is highly likely that the unique region in the sequence of the transcript from cDNA is further downstream of the exon 13, resulting in a large final intron.

3.4.6. Validation of Genomic Structure with Southern Blotting

The variation in sequence at the 3' end of *HvBor1a* in Sahara genome was confirmed with southern analysis. *Hind*III digested genomic DNA of Clipper and Sahara were size separated on agarose gel. Subsequently the fragments were blotted to membrane and hybridized to the radiolabeled probe. The 403 bp long probe was amplified from genomic DNA of Clipper and covered a region from final intron, through the final exon and into the 3' UTR. The first 113 bp region within the final intron was common both in Clipper and Sahara whereas the remaining 290 bp was unique to Clipper (Figure 3.30a). Southern analysis indicated that the expected fragment from 3' end of *HvBor1a* is missing in Sahara genome (Figure 3.30b).

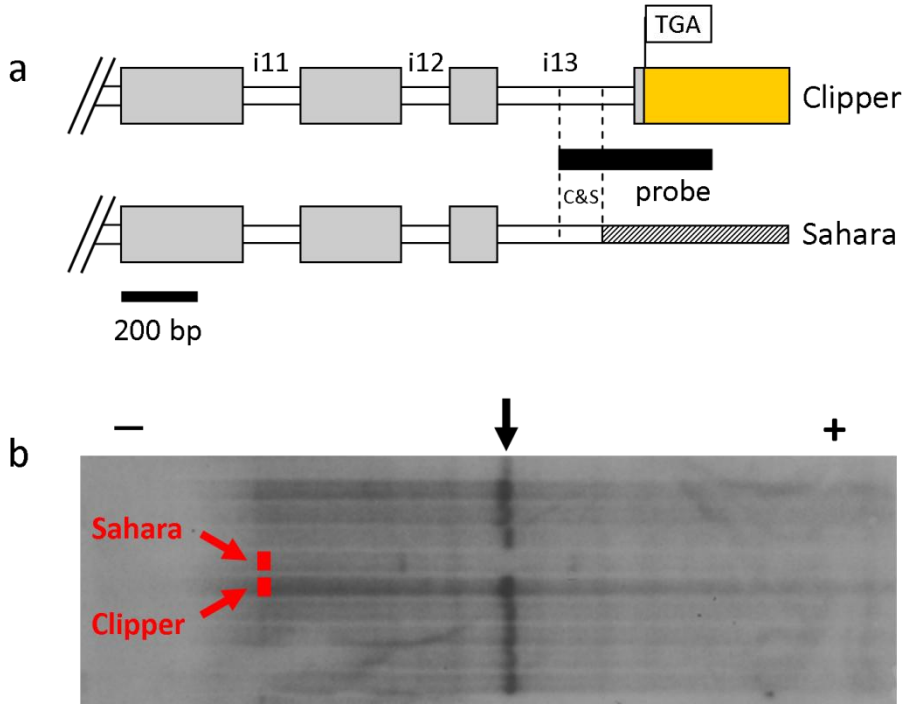

Figure 3.30. Graphical display of probe and autoradiography image of southern blotting. The probe and its hybridization region (a) are displayed relative to the sequence of *HvBor1a* in Clipper and Sahara. The region common to both Clipper and Sahara is indicated with dashed lines and C&S. Exons, introns, and the probe are in scale. Image of autoradiography after hybridization with probe (b) is displayed. Negative and positive poles of electrophoresis are represented with – and +, respectively. Red arrows indicate the lanes genomic DNA from Sahara and Clipper are loaded to. Black arrow indicates the expected fragment. (i: intron)

Results of the southern hybridization indicated the possibility of a deletion or insertion within Sahara genomic DNA. The sequence at the 3' end of *HvBor1a* might be determined by sequencing of BAC clones which would provide a more informative picture of the event of deletion or insertion.

3.4.7. Intron Structure of *HvBor1a* in Hamidiye and Tarm-92

The full CDS of *HvBor1a* was cloned from cDNA preparations of barley genotypes Hamidiye and Tarm-92 cultivated in Turkey. The full CDS was amplified in PCR by a proofreading enzyme. Subsequently, the fragments were introduced into entry vector pCR8/GW/TOPO for Gateway cloning. The plasmids were multiplied in TOP10 and then sequenced for verification of the direction and sequence of the insert.

Interestingly, in certain clones from both Hamidiye and Tarm-92 larger insert sizes were observed. Fragments of 3' end of the full CDS of *HvBor1a* were amplified from the cloned inserts in the entry vectors and size separated on agarose gel (Figure 3.31). A larger fragment size and the sequence of the clones in entry vectors verified an alternative splicing of *HvBor1a* in both Hamidiye and Tarm-92. It was demonstrated that during splicing of *HvBor1a*, a region within final intron – intron 13 – was spliced in to generate an alternatively spliced mature transcript (Figure 3.32).

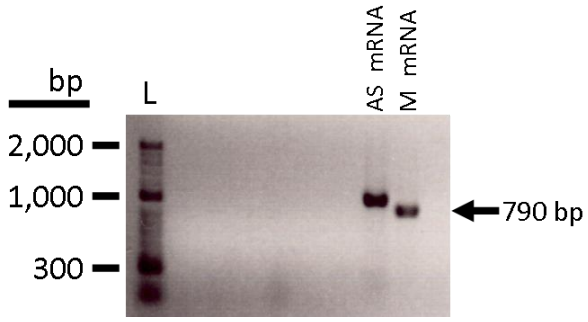

Figure 3.31. Image of electrophoresis gel displaying alternative splicing of *HvBor1a*. The expected size of fragment (790 bp) is indicated with an arrow. The fragments were amplified from alternatively spliced clone (AS mRNA) and mature clone (M mRNA) in a PCR. (L: Ladder)

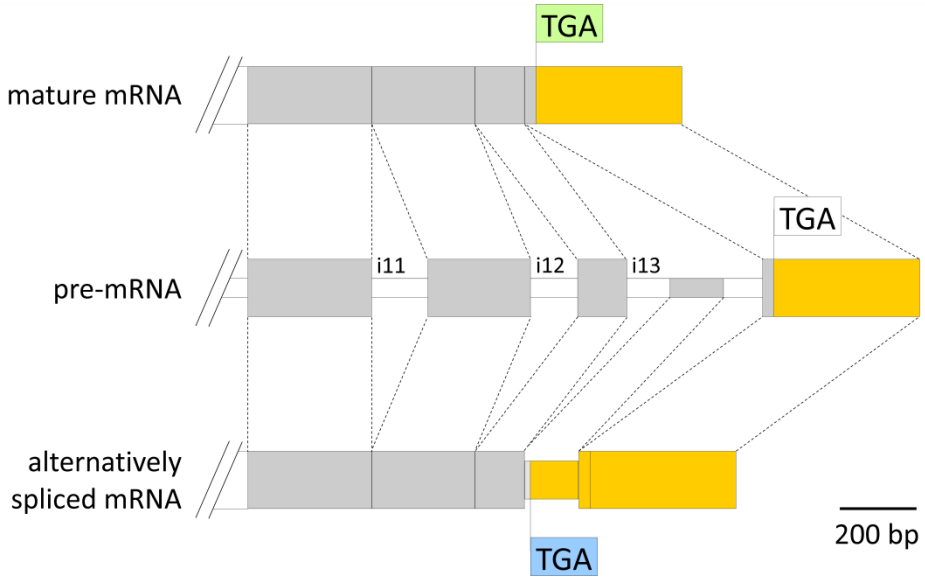

Figure 3.32. Graphical display of alternative splicing of *HvBor1a* in Hamidiye and Tarm-92. Exons and introns (i) which are shown with filled and empty boxes, respectively, are in scale. The 3' UTR downstream of translation stop codon (TGA) is indicated with gold coloring. The stop codon generated after alternative splicing is indicated in blue whereas the stop codon in mature mRNA is indicated in green.

The sequence of the region spliced in is provided in Table 3.22. Alternative splicing generated a larger transcript both in Hamidiye and Tarm-92. On the other hand, splicing generated an in-frame stop codon (TGA) resulting in a shorter polypeptide of HvBor1a (Table 3.22). It was predicted that the alternatively spliced transcript encoded for 661 amino acids where the normal transcript of *HvBor1a* encoded for 666 amino acids.

Observation of an alternative splicing in both Hamidiye and Tarm-92 further indicated the importance of final intron of *HvBor1a*. Though both normal and alternatively spliced transcripts were cloned from the same cDNA preparations of B-sensitive Hamidiye and B-tolerant Tarm-92, alternative splicing might be employed for regulation of protein activity. The protein product of alternatively spliced transcript was 5 amino acids shorter than that of normal transcript. The change in carboxy-terminus might be used in regulation of protein product under B stresses. This hypothesis should be verified in succeeding biochemical studies.

Table 3.22. The sequence of region spliced in to generate alternatively spliced *HvBor1a*. The 138 bp long region within intron 13 is indicated underlined whereas the in-frame new translation stop codon is highlighted.

Intron 13	
1	GTAAACGATG GGCATGCATA GCATTGCAGT TGCAACAAGT GCACATTGAA CCGTAGGTGC
61	TATTATACTG TGATGGCTCA CAGTCTGACC TTTCTGCTGG CTTCACATAG GTCCAGCCAA
121	CAGATTGAAC TTGTGAGCTG TTTTTTTCTT TTTTTACGGA AGAACAAGTA CCGCGCATAC
181	CAGTAGATGG TTCTTTTGTG CCTGTTATGG GCAGATTTCA CGGGCGCAAA TTCATAAATG
241	TTCCCGTCGT GAGTTGATCC TATTCCACAC CTTTTTTCTT CTCGGAAAGA GAGGGGTTTT
301	GATGCCAATA TGCCATTTTT CTGAATTTGA CAGTTTCAAA TTACACCGTG CAG

3.5. Heterologous Expression in Yeast

Functional characterization of protein product of *HvBor1a* was performed with heterologous expression in yeast. The full CDS of *HvBor1a* from Clipper was recombined into a yeast expression vector pYES-DEST52 which in turn was transformed into yeast strain INVSc2. A tolerance bioassay with transformed yeast cells was performed to assess the function of *HvBor1a* under B toxicity. Additionally the bioassay included the yeast transformed with Sahara *HvBot1* which was employed as a positive control. The yeast transformed with empty vector was used as a negative control.

The ten-fold serial dilutions of transformed yeast were plated on solid media containing high concentrations of H_3BO_3. Following two days of incubation, responses of yeast were recorded by taking the photos of plates (Figure 3.33). The results of the bioassay and comparison to negative control indicated that Clipper *HvBor1a* provided tolerance to B toxicity in yeast. The level of tolerance was as high as the tolerance provided by Sahara *HvBot1* as judged by the growth of diluted yeast cells. It might be proposed that tolerance to B toxicity in yeast might be provided by the efflux of B out of the cytoplasm. Additionally the bioassay with yeast demonstrated the B transporting activity of the protein product of *HvBor1a*. This proposal should be investigated in succeeding studies using heterologous expression in plant cells or tissues.

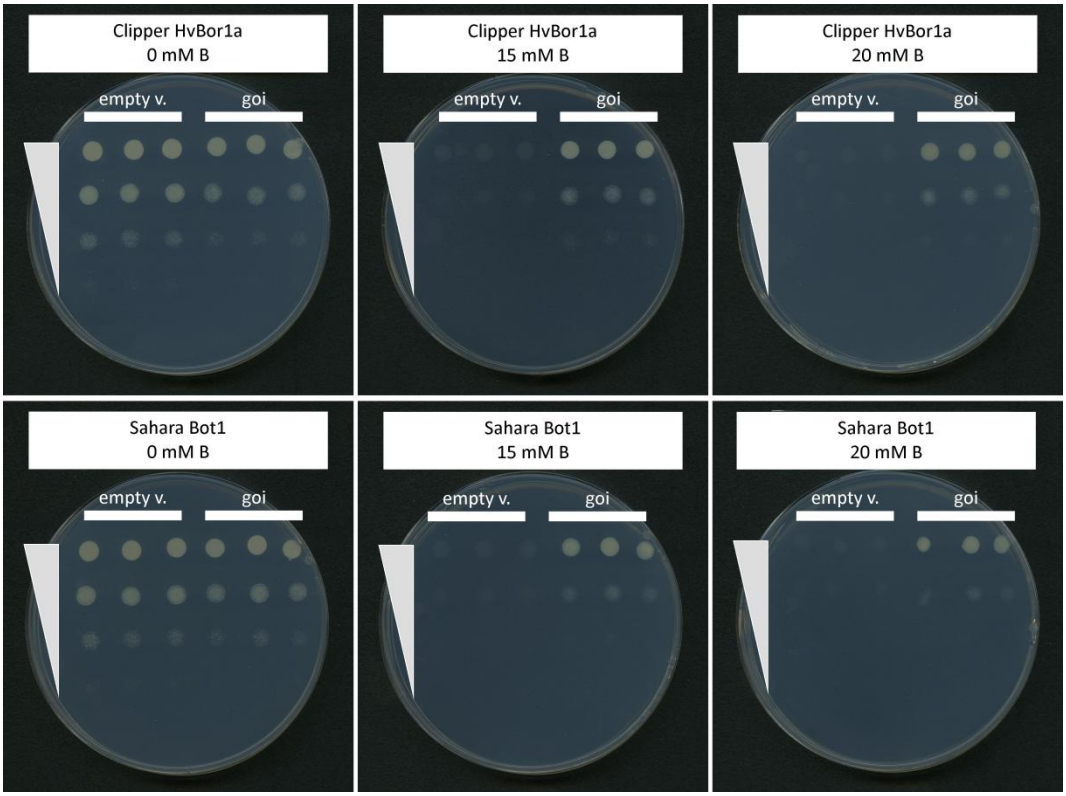

Figure 3.33. Tolerance bioassay with yeast expressing Clipper *HvBor1a*. Growth of *S. cerevisiae* strain INVSc2 on solid media containing 0, 15, and 20 mM H_3BO_3 is displayed. Each plate shows three independent yeast clones containing either empty vector or gene of interest (goi) left to right. Top panel displays bioassay with yeast expressing Clipper *HvBor1a* whereas bottom panel displays bioassay with yeast expressing Sahara *HvBot1*. A 10 µL aliquot of ten-fold serial dilutions were plated top to bottom where dilution is indicated with a triangular box on each plate.

3.6. Expression Analyses of *HvBor1a*

Endogenous expression of *HvBor1a* in leaf and root tissues of barley cultivars under various B treatments was determined using real-time PCR. Additionally, northern blotting as an independent expression analysis was carried out for confirmation of results obtained.

3.6.1. Expression of *HvBor1a* under B Toxicity

Transcript abundances of *HvBor1a* in Clipper and Sahara were investigated under B toxicity. Normalized mRNA copies of *HvBor1a* in leaf and root tissues of Clipper and Sahara are presented in Figure 3.34. Though the expression levels were low, it was determined that the expression of *HvBor1a* was significantly higher in leaf tissues compared to roots in both Clipper and Sahara. Expression of *HvBor1a* was extremely low in root tissues and non-significant over the concentration range applied. Moreover the expression in root was similar in both B-sensitive Clipper and B-tolerant Sahara. On the other hand, in leaf tissues a decrease in expression was observed in both cultivars upon application of 50 µM H_3BO_3.

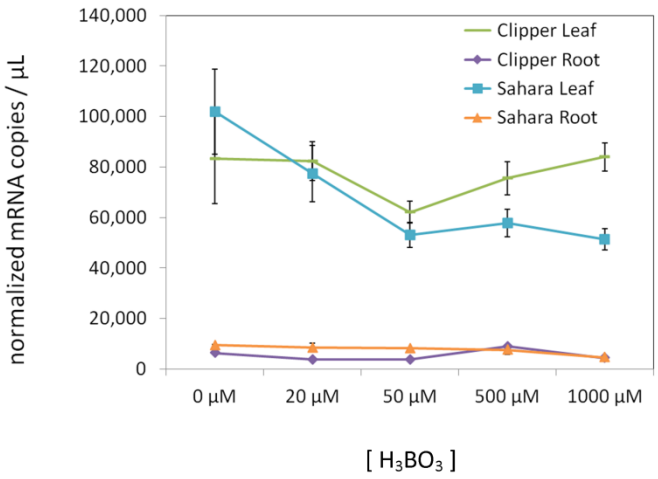

Figure 3.34. Transcript abundances of *HvBor1a* in leaf and root tissues of Sahara and Clipper. Normalized mRNA copies were determined by two-step real-time RT-PCR using *Hv18SrRNA* and *HvGAPDH* as house-keeping controls in relative quantitation. Three biological replicates of total RNA samples were used in three independent real-time RT-PCR runs.

According to similar expression patterns in Clipper and Sahara, it might be concluded that the contribution of HvBor1a to tolerance of barley to B toxicity might be small. It was shown that the major contribution to tolerance was achieved by HvBot1 in barley cultivar Sahara (Sutton *et al.*, 2007). On the other hand higher levels of expression in leaf tissues suggested a role for HvBor1a in distribution of B within above ground tissues of barley.

Furthermore expression levels of *HvBor1a* were investigated in Hamidiye and Tarm-92 under B toxicity. Normalized mRNA copies of *HvBor1a* in leaf and root tissues are presented in Figure 3.35. Though the normalized mRNA copy numbers were low, a marked decrease in expression was observed in leaf tissues of Tarm-92 upon 0.5 mM H_3BO_3 treatment. The expression levels were almost constant over the concentration range where B toxicity was applied. The expression in leaf tissues of Hamidiye also declined. However the decrease was observed at 5 mM H_3BO_3 application.

On the other hand, transcript abundances in root tissues showed a slight increase upon 0.5 mM H_3BO_3 application and decreased sharply at 1 mM H_3BO_3. Similar to leaf tissues the expression in root tissues was constant over 1, 5, and 10 mM H_3BO_3 applications.

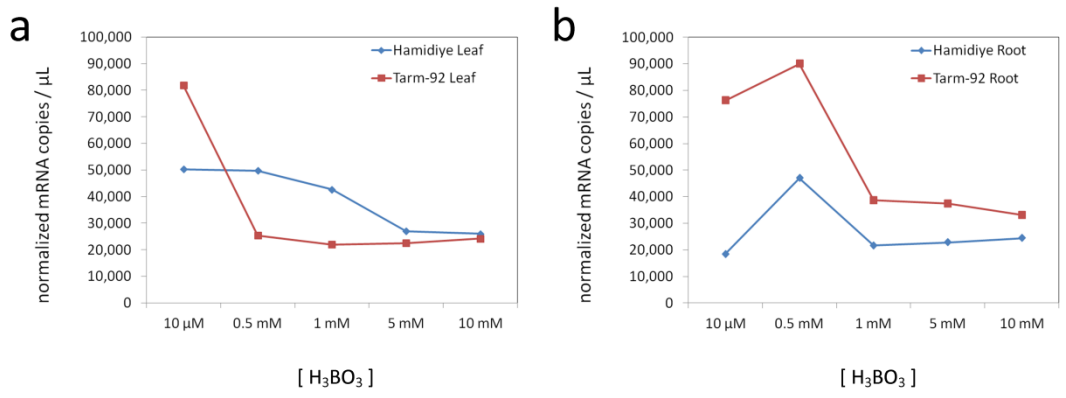

Figure 3.35. Transcript abundances of *HvBor1a* in leaf and root tissues of Hamidiye and Tarm-92. Normalized mRNA copies were determined in leaf (a) and root (b) tissues by one-step real-time RT-PCR using absolute quantitation. Three biological replicates of total RNA samples were used in two independent real-time RT-PCR runs.

Though the contribution of HvBor1a to tolerance in Sahara is low, it might be high in B-tolerant Turkish cultivar Tarm-92. Therefore the contribution of HvBor1a to tolerance should be investigated in DH populations of Hamidiye and Tarm-92. Additionally conclusive information might be achieved after investigation of expression levels of HvBor1a in specific tissues such as phloem or endodermis in leaves.

3.6.2. Expression of *HvBor1a* in Root Tips

Transcript abundances of *HvBor1a* were also investigated in 1 cm-segments of roots of un-stressed seedlings of Clipper and Sahara. Transcript levels in segments of roots were investigated for determination of possible localized or tissue-dependent expression of putative B transporter gene *HvBor1a*. Normalized mRNA copies of *HvBor1a* in root segments of Clipper and Sahara are presented in Figure 3.36. The determined expression levels were extremely low similar to those obtained in B-stress series. Though the transcript levels were low, a high level of expression was determined at root tip compared to other segments. On the other hand, transcript copy numbers in segments of root were non-significant among barley cultivars Clipper and Sahara.

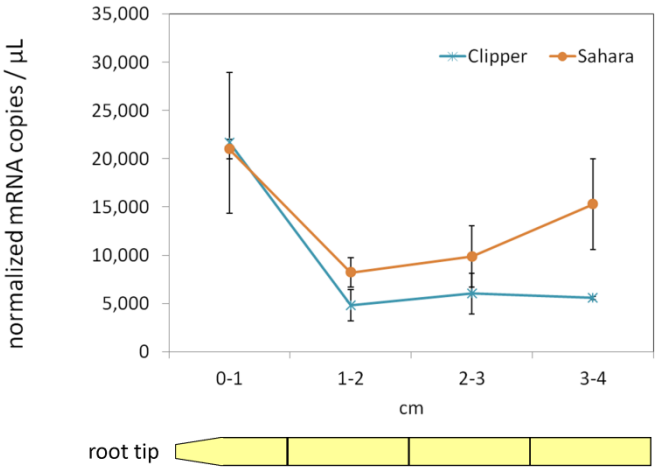

Figure 3.36. Transcript levels in root segments of Clipper and Sahara. Normalized mRNA copies were determined in 1 cm-segments taken along the root by two-step real-time RT-PCR using *Hv18SrRNA* and *HvGAPDH* as house-keeping controls in relative quantitation. Three biological replicates of total RNA samples were used in three independent real-time RT-PCR runs. Segments of root are graphically represented below the graph.

3.6.3. Validation of Expression Levels with Northern Blotting

Northern hybridization was employed to verify the expression levels obtained in real-time RT-PCR. Though sensitivity was low compared to RT-PCR northern blotting provided higher levels of specificity. The probe used for Southern hybridization was also used for Northern hybridization (Figure 3.30a). Total RNA from B-stress series of Clipper and Sahara were size separated on agarose gel (Figure 3.37a) and subsequently blotted to membrane and hybridized to the radiolabeled probe. Image of autoradiography is presented in Figure 3.37b. Northern blotting confirmed the low levels of expression of *HvBor1a* as well as the relatively high level of expression in leaf tissues compared to roots determined by real-time RT-PCR. Since the 290 bp region out of 403 bp total length of probe was unique to Clipper, hybridization with Sahara RNA produced no signals.

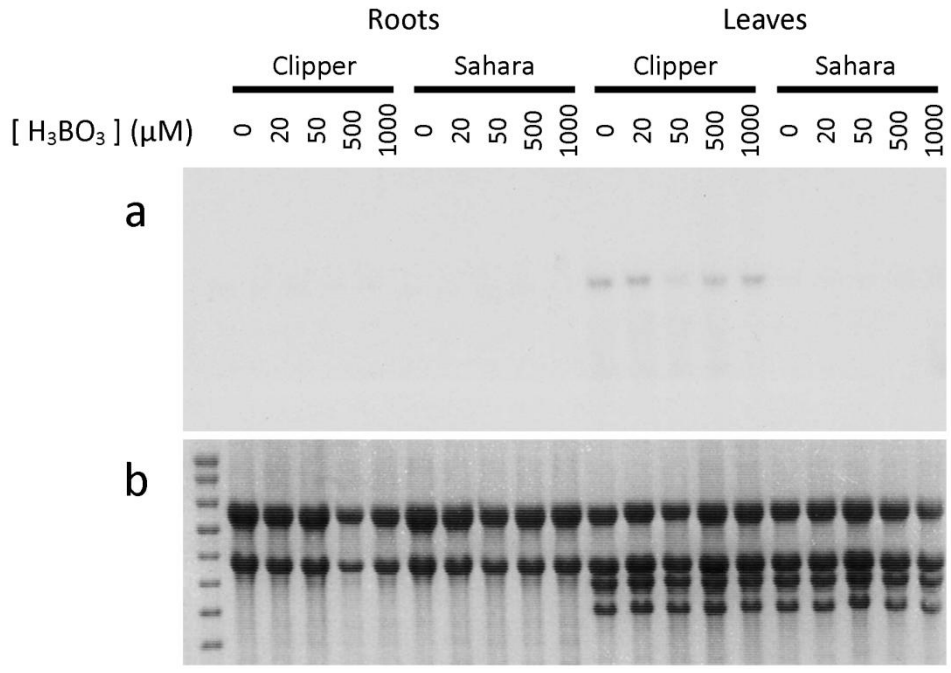

Figure 3.37. Autoradiography image of northern blotting. Image of autoradiography after hybridization with probe (a) and gel image of electrophoresis of total RNA prior to transfer to membrane (b) are displayed. For detailed information on probe, see Figure 3.30a.

3.7. Subcellular Localization of HvBor1a

Subcellular location of putative B transporter was determined using transient expression of HvBor1a-mGFP5 fusion protein in epidermal cells of onion. Onion epidermal cells were infiltrated with *A. tumefaciens* C58C1 carrying pEarleyGate100 with *HvBor1a-mGFP5* inserted downstream of CaMV35S promoter. The expression of fusion protein was monitored using confocal microscopy (Figure 3.38).

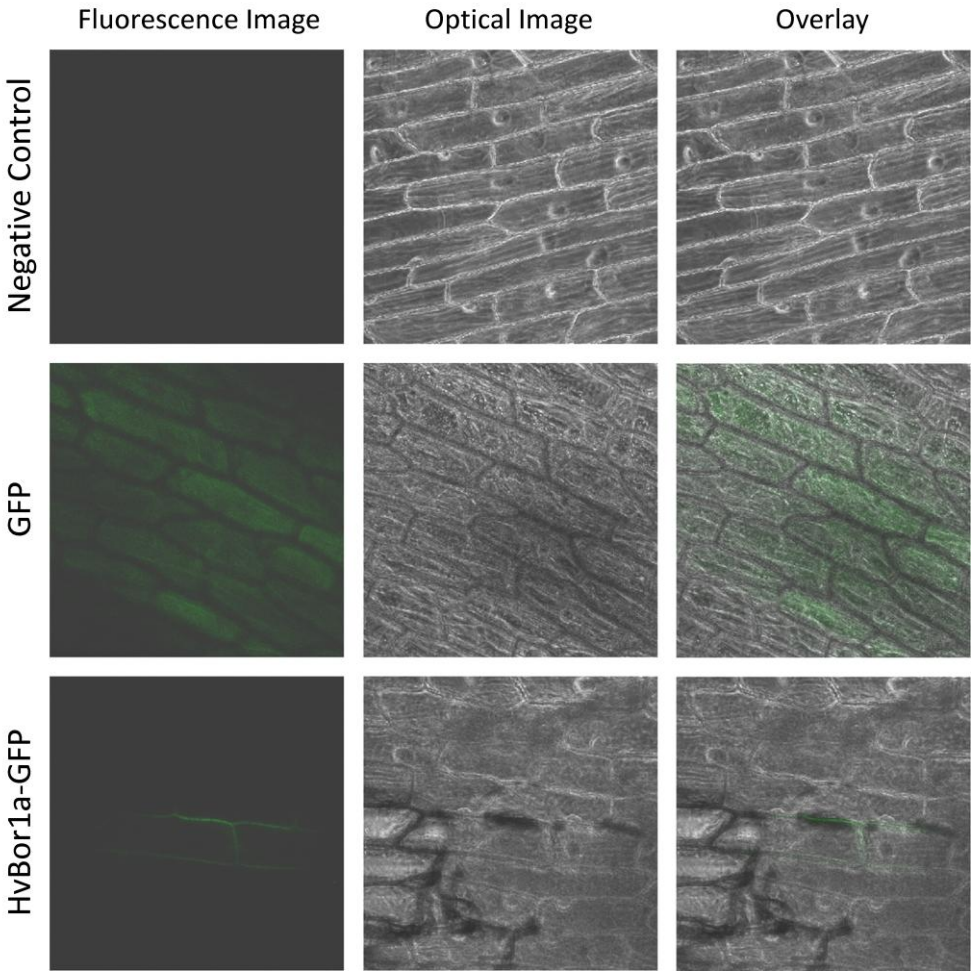

Figure 3.38. Subcellular location of HvBor1a. Fluorescence and optical images as well as overlay of the two images are displayed. Fluorescence and optical images of untransformed onion epidermal cells or cells transformed with expression vector carrying *HvBor1a-GFP* fusion are recorded with a confocal microscope.

Transient expression of HvBor1a-mGFP5 fusion protein in plant cells demonstrated that the B transporter HvBor1a was localized to plasma membrane. Similarly the B transporter AtBOR1 was reported to be localized to cell membrane in *A. thaliana* using expression of GFP fusion protein (Takano *et al.*, 2002; 2005). The transient expression system employed in the present study used constitutive expression under CaMV35S promoter. Thus the results confirmed that the putative B transporter HvBor1a is a transmembrane protein localized to plasma membrane. Succeeding studies should investigate the tissue-specific expression of *HvBor1a* in barley plants. Moreover, analysis of promoter region and fusion of the promoter to *GFP* should be performed to determine spatial and temporal expression of *HvBor1a*.

3.8. Mapping of 3H B Tolerance QTL

The QTL on 3H for B tolerance was identified in a Clipper X Sahara F1-derived DH population (Jefferies *et al.*, 1999). The locus was reported to be associated with root length response under B toxicity. However the tolerance gene located on 3H is unknown. Cloning of *HvBor1a* which was predicted to be located in the region was achieved using a candidate gene approach in the present study. Moreover, positional cloning was considered to genetically map *HvBor1a* to QTL on 3H. Thus, screening of DH population and progenies with previously designed markers was carried out. Furthermore development of new genetic markers and screening of populations was performed *ad hoc* to narrow down the genetic distance between markers located on 3H QTL.

The Clipper X Sahara DH population consisting of 150 individuals was screened using two SSR markers, EBmac0761 and EBmac0848 (Ramsay *et al.*, 2000). The images of gel electrophoresis for screening with EBmac0761 and EBmac0848 are presented in Figure 3.39 and 3.40, respectively. The DHs were genetically scored according to parental cultivars, Sahara and Clipper.

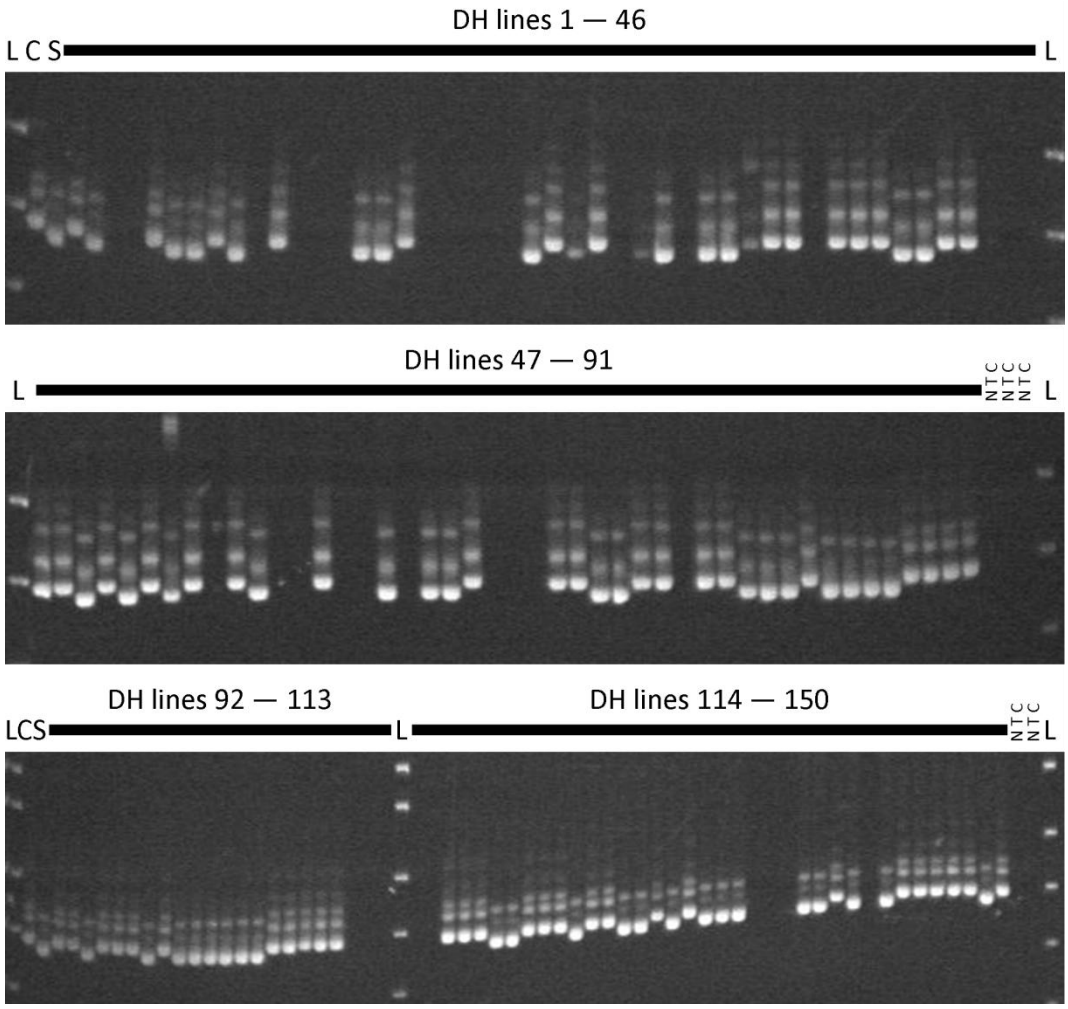

Figure 3.39. Images of gel electrophoresis for screening of DH population with SSR marker EBmac0761. The parents Clipper and Sahara are indicated with C and S, respectively. (L: Ladder, NTC: No template control)

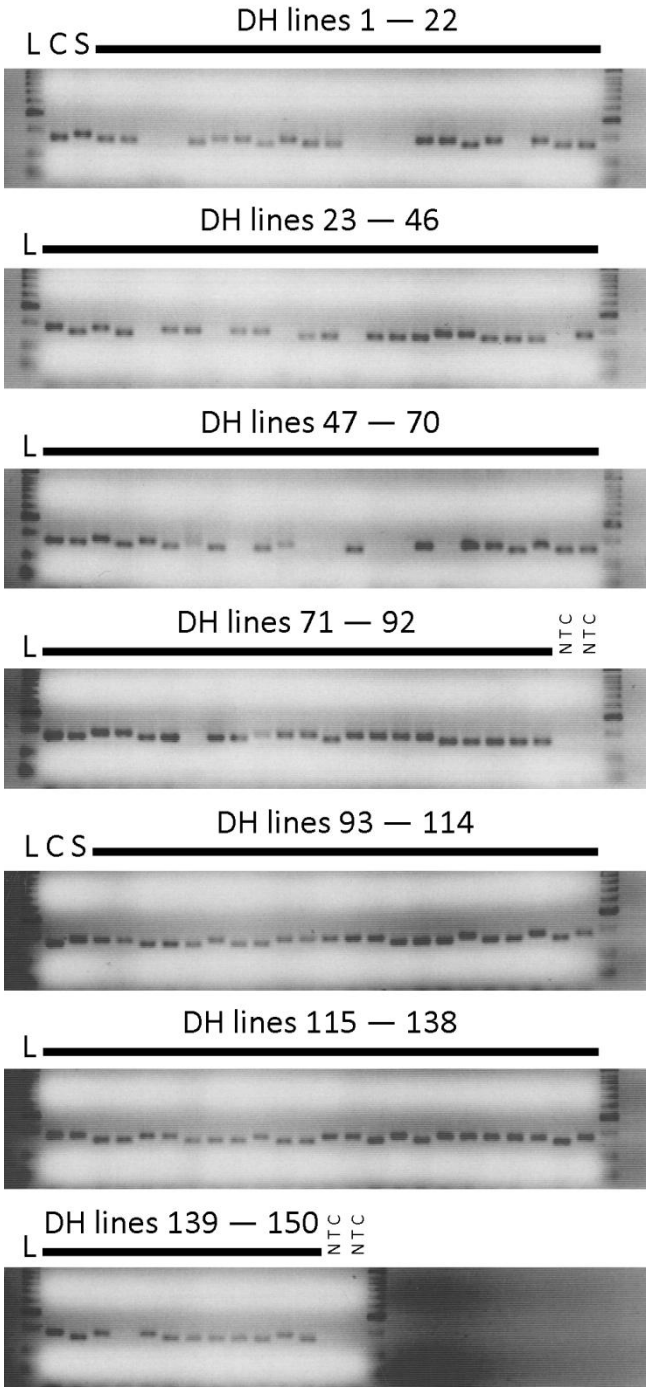

Figure 3.40. Images of gel electrophoresis for screening of DH population with SSR marker EBmac0848. The parents Clipper and Sahara are indicated with C and S, respectively. (L: Ladder, NTC: No template control)

Additionally, gene colinearity with the syntenic region on rice chromosome 1 was used for development of novel CAPS markers which employ PCR amplification and restriction enzyme digestion for determination of genetic differences resulting from SNPs. Predicted rice gene with locus identifier LOC_Os01g08020.1 which was the best blast hit of *HvBor1a* in rice genome was used as a starting point. Genome sequences 150 kb upstream and 100 kb downstream of LOC_Os01g08020.1 was employed for determination of barley ESTs and primer design. The primer pairs were used to amplify DNA sequences from barley cultivars Sahara and Clipper. A representative image of gel electrophoresis performed for separation of fragments amplified from Clipper and Sahara is displayed in Figure 3.41. Subsequently the resulting amplicons were sequenced to locate SNPs which might have potential to be used as CAPS markers.

A total of 19 primer pairs design for barley ESTs were used in attempts for development of CAPS markers (Table 3.23). Five of the primer pairs produced no amplicons (nulls) from genomic DNA of barley whereas one resulted in non-specific amplification. The fragments amplified with the remaining primer pairs were size separated, recovered from gel, and subsequently sequenced.

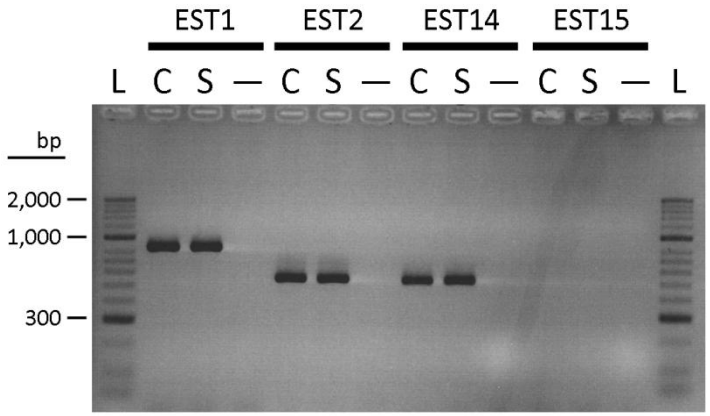

Figure 3.41. Representative image of gel electrophoresis for marker development. Primer pairs designed for barley ESTs, EST1, EST2, EST14, and EST15 were used in amplification from DNA preparations from Clipper (C) and Sahara (S). The GeneBank accession numbers for EST1, EST2, EST14, and EST15 are BQ469119, EX584292, BF262108, and BF256699, respectively. For detailed information on primer pairs, see Table 2.25. (L: Ladder, – : No template control)

Table 3.23. Results table of attempts for development of novel CAPS markers. For detailed information on primer pairs and barley ESTs, see Table 2.25. (bp: base pairs)

	Amplification	**Size on gel (bp)**	**Sequence read (bases)**	**Polymorphism**
EST1	Single	~900	827	Non-polymorphic
EST2	Single	~550	525	Non-polymorphic
EST3	Single	~900	868	Non-polymorphic
EST4	Single	~1000	903	Non-polymorphic
EST5	Single	~2200	994 + 691	Non-polymorphic
EST6	Null	N/A	N/A	N/A
EST7	Single	~1500	1373	Non-polymorphic
EST9	Single	~750	669	Non-polymorphic
EST10	Single	~400	288	Non-polymorphic
EST11	Non-specific	N/A	N/A	N/A
EST12	Null	N/A	N/A	N/A
EST13	Single	~450	319	Non-polymorphic
EST14	Single	~550	520	Polymorphic
EST15	Null	N/A	N/A	N/A
EST16	Single	~350	302	Non-polymorphic
EST17	Single	~600	519	Non-polymorphic
EST18	Null	N/A	N/A	N/A
EST19	Single	~650	644	Non-polymorphic
EST20	Null	N/A	N/A	N/A

N/A: Not applicable

Alignment of the sequence reads were achieved using ContigExpress. Additionally each chromatogram was scanned manually to locate SNPs. Interestingly, among primer pairs that amplified a single fragment from genomic DNA of both Clipper and Sahara such as primer pairs for EST1 (Figure 3.41), 12 of them amplified fragments that were non-polymorphic (Table 3.23). Since the fragments from Clipper and Sahara were identical in sequence, the corresponding DNA sequences and ESTs could not be used for CAPS marker design. Only one of the primer pairs, which was design for EST14, resulted in amplification of a fragment which carried an SNP between Clipper and Sahara.

3.8.1. CAPS Marker xHvMYB

The fragments amplified by the primer pair designed according to barley EST14 (Table 2.25) were size separated (Figure 3.41), recovered from gel, and subsequently sequenced in both directions – forward and reverse. The sequence reads obtained for both Clipper and Sahara are provided in Table P.1 (Appendix P). Additionally the sequence chromatograms of the region and the SNP determined are displayed in Figure 3.42. The fragments that were 532 bp long differed for purine at the position 266 – according to the consensus of the sequence reads. Clipper contained a guanidine (G) whereas Sahara contained an adenine (A) at position 266. It was determined that the identified SNP introduced a restriction site for *Pst*I (5'CTGCAG3') on the genomic sequence of Sahara but not Clipper. Thus it was concluded that the SNP could be employed as a CAPS marker to differentiate Sahara and Clipper backgrounds.

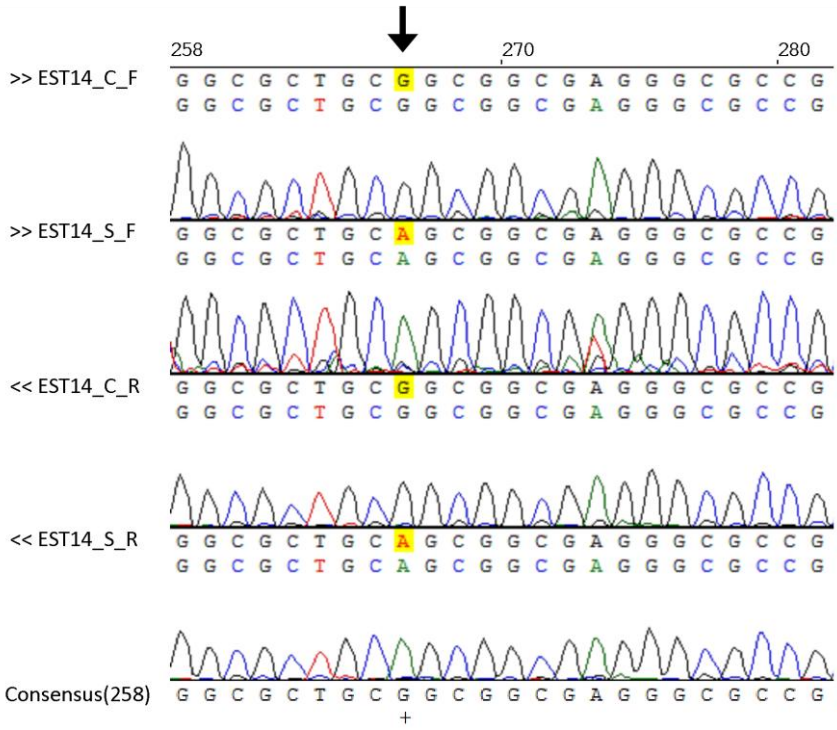

Figure 3.42. Chromatograms of the sequence reads locating the SNP in barley EST14. The fragments amplified from genomic DNA of Clipper (C) and Sahara (S) were sequenced in forward (F) and reverse (R) directions. The highlighted SNP is indicated with an arrow.

The sequence of the fragment amplified using primer pairs designed for EST14 was blasted against non-redundant database at NCBI using BLASTN delimiting the organism to higher plants (taxid:3193). It was determined that the best blast hit was a predicted gene encoding a MYB family transcription factor in rice. Therefore the novel marker developed in the context of the present study was named xHvMYB.

For confirmation of the CAPS marker xHvMYB, DNA preparations from Sahara and Clipper were subjected to PCR amplification with EST14 primer pairs and subsequently the reaction mixtures were used in *Pst*I digestion. Then the digestion products were fractionated on agarose gel (Figure 3.43). The Clipper allele produced a single band of 532 bp fragment whereas Sahara allele produced a single band of two digested 266 bp long fragments.

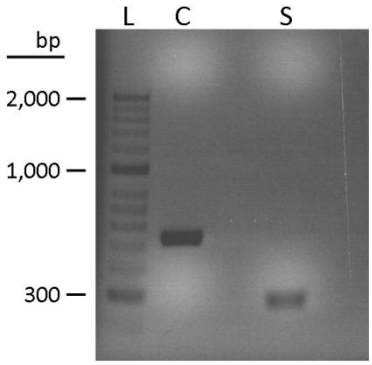

Figure 3.43. Image of electrophoresis gel for confirmation of CAPS marker xHvMYB. Fragments amplified from Clipper (C) and Sahara (S) DNA preparations were digested with *Pst*I and subsequently size separated on agarose gel. (L: Ladder)

3.8.2. Segregation of xHvMYB and *HvBor1a*

The individuals from Clipper X Sahara DH population were screened using CAPS marker xHvMYB (Figure 3.44). Genetic background of each DH was determined according to parental cultivars Clipper and Sahara.

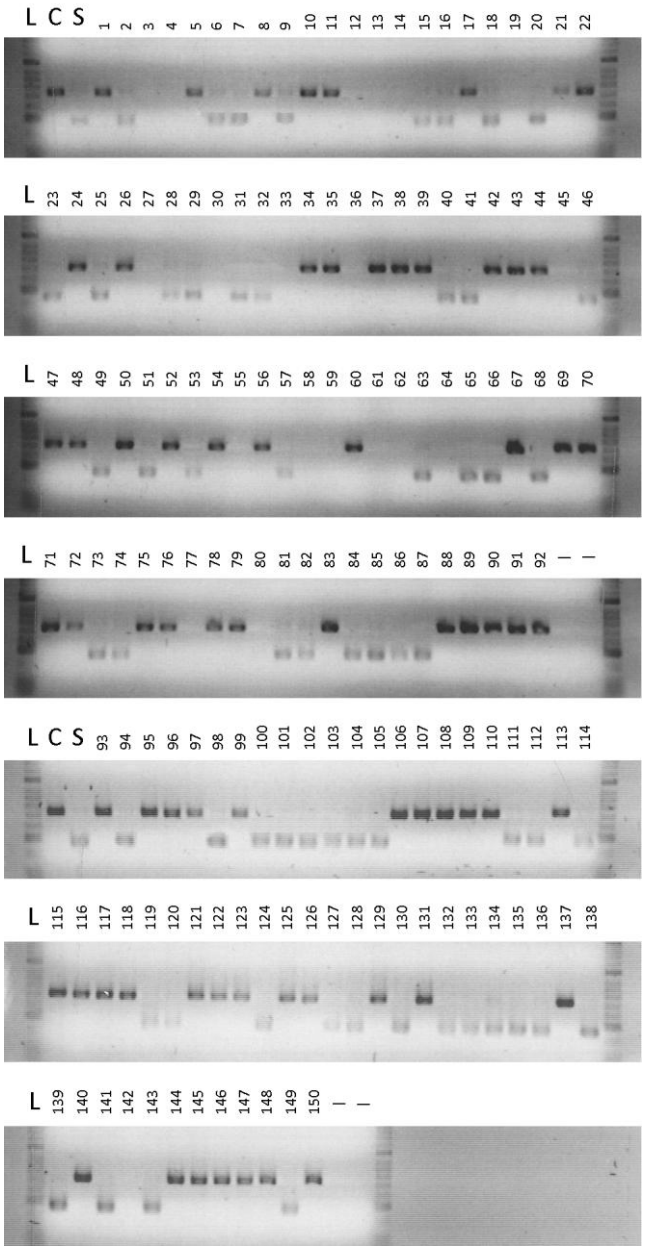

Figure 3.44. Images of gel electrophoresis for screening of DH population with CAPS marker xHvMYB. The parents Clipper and Sahara are indicated with C and S, respectively. (L: Ladder, – : No template control)

Genotyping data from the screen with CAPS marker xHvMYB were integrated into interval mapping developed previously at ACPFG. The map generated is displayed in Figure 3.45. No recombinants were determined between xHvMYB and *HvBor1a* in the DH population derived from the cross between Clipper and Sahara. Thus it was concluded that the CAPS marker xHvMYB co-segregated with *HvBor1a* in this mapping population.

3.8.3. Evaluation of F2 Mapping Populations

Furthermore to narrow down the genetic distance between markers on 3H B tolerance QTL, F2 progenies derived from crosses of DH lines were evaluated for recombinant screening. The F2 mapping populations used for screening were derived from crosses DH105 X DH11 and DH113 X DH120.

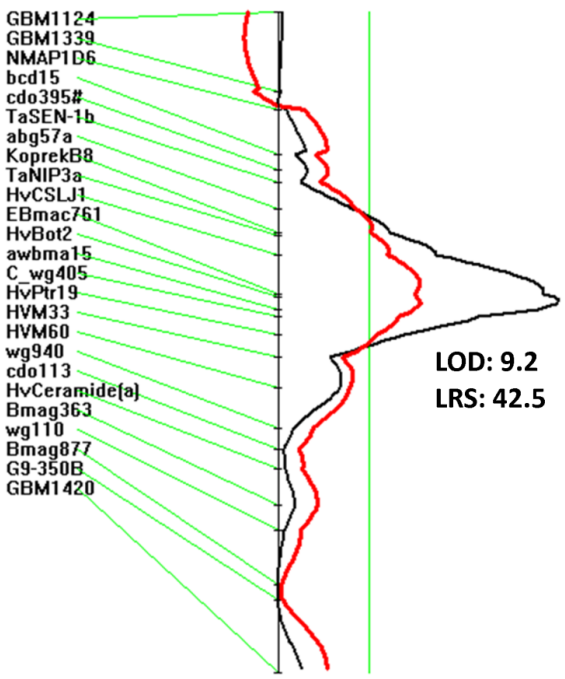

Figure 3.45. Interval mapping of 3H B tolerance locus. The map was developed based on analyses of 150 individuals of Clipper X Sahara DH population with SSR markers EBmac0761 and EBmac0848 and CAPS marker xHvMYB as well as available mapping information generated previously at ACPFG. (LOD: Log of odds; LRS: Likelihood ratio statistics)

The genetic makeup of 3H locus of four parental DH lines is presented in Table 3.24. The DH plants DH11 and DH113 displayed Clipper allele for the 3H locus whereas DH105 and DH120 displayed Sahara allele. The parental DH plants differed for alleles at B tolerance locus on 3H but not for alleles at other known B tolerance loci which were fixed in these crosses. The cross DH105 X DH11 carried the Clipper background whereas DH113 X DH120 the Sahara background.

Table 3.24. Genetic makeup of 3H B tolerance locus of four DH lines. (A: Clipper allele, B: Sahara allele, X: Not available)

	DH11	DH105	DH113	DH120
TaNIP3a#	A	B	B	B
HvCSLJ1	A	B	B	B
EBmac761	A	B	A	B
EBmac760	A	X	X	X
CAPS_EST14_xHvMYB	A	B	A	B
HvBor1a#	A	B	A	B
awbma15	A	B	A	B
Bmac67	A	B	X	B
Bmag6	A	B	A	B
EBmac848	A	B	A	B

Genotype and phenotype of a subset of F2 progenies were determined for evaluation of applicability of the population for recombinant screening. Approximately 50 individuals from each cross were grown in hydroponics and root lengths were measured after 14 days to determine the tolerance phenotype. On the other hand, the same F2 plants were scored genetically using CAPS marker xHvMYB. A representative image of gel electrophoresis displaying the banding pattern of Clipper and Sahara as well as heterozygous individuals from F2 population is presented in Figure 3.46. The results indicated availability of recombinants among individuals of F2 population. Additionally distribution of root lengths (Figure 3.47) and relative root lengths (Figure 3.48) of seedlings were determined in a bioassay.

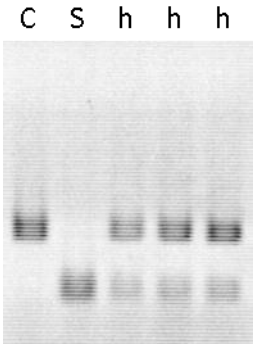

Figure 3.46. Representative image of gel electrophoresis displaying banding pattern of F2 individuals. Fragments amplified from DNA preparations of homozygous Clipper (C) and Sahara (S) and heterozygous (h) F2 progenies were digested with *Pst*I and subsequently size separated on agarose gel.

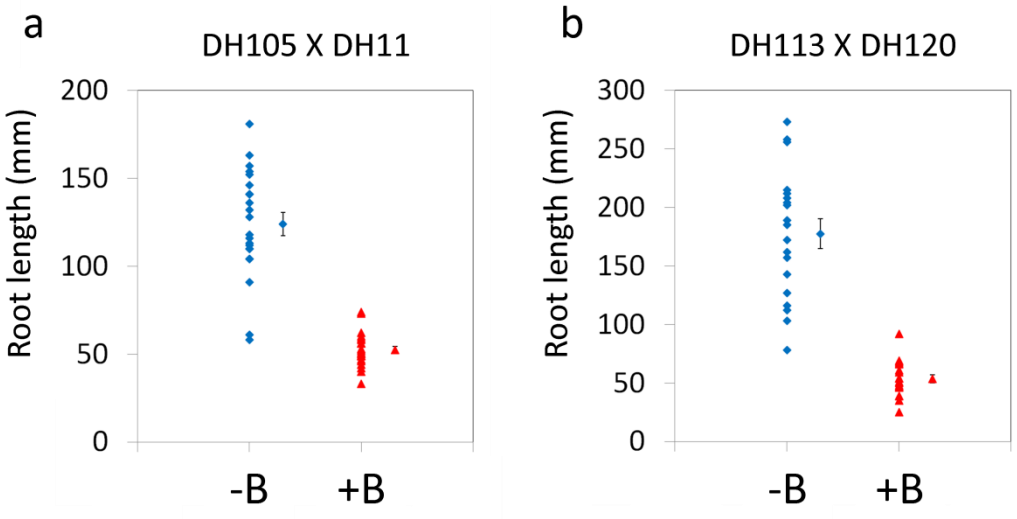

Figure 3.47. Distribution of root length of F2 plants. Seedlings were grown in basal solution supplemented with 15 µM H_3BO_3 (-B) for control conditions or with 10 mM H_3BO_3 (+B) for B toxicity treatment. The averages of root lengths are displayed next to the distributions. Bars represent standard errors of mean.

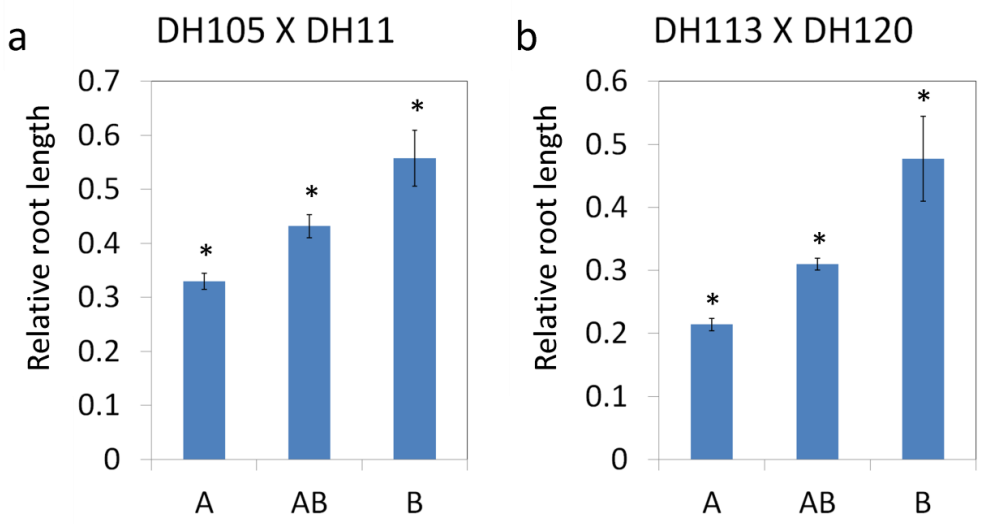

Figure 3.48. Relative root length of F2 plants. Genotyping was performed with CAPS marker xHvMYB. Bars represent standard errors of mean whereas asterisks indicate significance at the level of $P < 0.05$ (A: Clipper allele, B: Sahara allele, AB: Heterozygous)

Results of genotyping with xHvMYB and phenotyping with determination of relative root length indicated that the F2 populations derived from DH105 X DH11 as well as DH113 X DH120 might be employed for recombinant screening. The F2 populations each consisting of approximately 300 individuals should be scored genetically using xHvMYB and phenotypically using relative root length in succeeding studies to narrow down the genetic distance between xHvMYB and *HvBor1α*.

CHAPTER 4

CONCLUSION

Boron (B) deficiency and toxicity are important constraints limiting plant growth and reducing crop yield in agriculture worldwide. On the other hand, our understanding of functional roles and significance of B in plant biology, the molecular responses of plants to B stresses, and the sources of impairments in physiology and metabolism to tackle the crop loss is limited. Moreover, molecular mechanisms providing tolerance to B toxicity or deficiency are not revealed completely. Proposed mechanisms for constitutive tolerance to B toxicity include existence of compounds binding to B once it accumulates to toxic concentrations within the cell, compartmentation of B upon accumulation in cytoplasm, and active efflux of B by transporters.

The present work concentrated mainly on determination of transcriptional responses of two barley cultivars to B toxicity and deficiency at seedling stage. Analyses of expression profiles performed using microarrays were designed to investigate both inter- and intra-varietal differences in gene expression. Global transcript profiling of barley and monitoring of stress responsive gene expression under B toxicity and deficiency was achieved using a high throughput, reliable and highly standardized microarray platform. To the best of our knowledge this work is the first report on transcriptome profiling under B toxicity or deficiency in *Triticeae* including wheat and barley.

The data generated and comparisons performed should enlighten succeeding studies to unravel molecular mechanisms of tolerance to B stresses. Additionally the data should provide novel tools for conventional or biotechnological approaches for reduction of the

crop loss due to B toxicity or deficiency. The differentially regulated genes specifically coding for transcription factors (TFs) or key enzymes of certain metabolic pathways might be cloned and used in improvement of B-sensitive high yielding barley cultivars. Additionally, transcriptional differences determined between sensitive and tolerant cultivars might be used for development of genetic markers for breeding or marker assisted selection for tolerance to B toxicity or deficiency.

Induction of genes encoding enzymes involved in jasmonic acid (JA) biosynthesis or proteins responding to elevated levels of JA was determined as a key feature of response to B toxicity in barley leaves. A noticeable feature of response to B toxicity was the crosstalk between responses to various environmental stresses and B toxicity. Specifically, regulation in expression levels of genes coding for biotic stress related, pathogenesis associated, and germin-like proteins suggested a high level of interconnection between certain components of signaling or response to biotic stresses and B toxicity.

Inter-varietal comparison of B-sensitive Hamidiye and B-tolerant Tarm-92 under toxic levels of B revealed that the degree of transcriptional regulation under B toxicity was stronger in Tarm-92 compared to that in Hamidiye. Thus, it was concluded that a combinatorial effect of TFs, activators, repressors, or associated factors functioning in enhanceosomes in tolerant barley cultivar might be providing tolerance to B toxicity. However in sensitive cultivar absence, underexpression, or misregulation of any of these proteins or factors might result in weak responses and thus sensitivity to B toxicity.

It is known that B transport through cellular membranes is mediated by passive or facilitated diffusion or by active transport against concentration gradients. Besides regulation of certain genes coding B transporters, anion exchange proteins, and NOD26-like membrane integral proteins (NIPs), results of expression profiling in the present work suggested putative functions for ATP-binding cassette (ABC) transporters under B toxicity or deficiency based on differential regulation of genes coding these proteins.

Expression profiles obtained and numbers of genes differentially regulated indicated that toxicity and deficiency resulted in substantial changes in gene expressions in leaf and root tissues, respectively. It might be concluded that transportation of B readily to above ground

tissues rescued roots from toxic effects of high B. On the other hand, plant cells might be modulating the transcriptome of roots to take up more B from the environment to fulfill its requirements or might be unable to perform some metabolic processes where role of B is critical and thus shutting down expression.

Among various TF genes regulated differentially under B stresses, *CO6* coding for a putative CONSTANS-like protein CO6 was up-regulated under B deficiency and down-regulated under B toxicity in root tissues. Expression of *CO6* was determined to be regulated by available B supply. Thus it was proposed that regulation of *CO6* expression might be critical in signal transduction under B stress. The *CO6* might be a promising candidate for development of B efficient crop plants. The proposed role should be verified in systems such as overexpressor lines and loss-of-function or gain-of-function mutants.

According to the putative functions of genes differentially regulated under B toxicity or deficiency it might be concluded that response mechanisms such as compartmentation by vacuolar sorting, active efflux by transporters, re-distribution of excess B within tissues, and stimulation of biotic stress related response mechanisms might contribute to the overall constitutive tolerance to B stresses in barley. Contribution of each mechanism might vary among genotypes and should be determined in succeeding studies using physiological or biochemical approaches.

Besides expression profiling under B stresses, this study also comprised cloning and characterization of a putative B transporter gene *HvBor1a* in barley. The full length CDS of the gene as well as non-coding regions were identified. Additionally, examination of last intron of *HvBor1a* has led to identification of an alternatively spliced variant in Turkish barley cultivars.

Transporter activity and localization of the protein product which were demonstrated in heterologous expression systems indicated the potential of HvBor1a in tolerance to B stresses. Moreover, high expression levels in leaf tissues pointed out the importance of HvBor1a in re-distribution of B within plant tissues.

Therefore future studies should investigate potential of HvBor1a in tolerance to B stresses using stably transformed plants or mutants. Furthermore spatial and temporal expression of *HvBor1a* and regulatory regions possibly providing tissue-specific expression should be investigated to draw a clear picture of functional roles of the transporter.

In barley 4 QTL for tolerance to B toxicity have been identified in a Clipper X Sahara F1-derived DH population. The candidate gene *HvBor1a* located on 3H QTL has been characterized in the present study. Furthermore positional cloning was considered to locate the gene in the region and a novel CAPS marker was developed to narrow the genetic distances at the locus. It was shown that the CAPS marker xHvMYB co-segregated with *HvBor1a* in the DH population. Additionally F2 populations derived from crosses of DHs were evaluated for applicability of the populations for recombinant screening. According to genotyping and phenotyping performed it was concluded that the individuals from F2 populations might be used in succeeding studies to determine recombinants and narrow the genetic distance between CAPS marker xHvMYB and *HvBor1a*.

REFERENCES

Abbo S, Lev-Yadun S, Gopher A (2010) Agricultural origins: Centers and noncenters; a Near Eastern reappraisal. *Crit. Rev. Plant Sci.* 29: 317 – 328.

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* 15: 63 – 78.

Aftab T, Khan MMA, Idrees M, Naeem M, Ram M (2010) Boron induced oxidative stress, antioxidant defence response and changes in artemisinin content in *Artemisia annua* L. *J. Agron. Crop Sci.* 196: 423 – 430.

Agarwala S, Sharma P, Chatterjee C, Sharma C (1981) Development and enzymatic changes during pollen development in boron deficient maize plants. *J. Plant Nutr.* 3: 329 – 336.

Alpaslan M and Gunes A (2001) Interactive effects of boron and salinity stress on the growth, membrane permeability and mineral composition of tomato and cucumber plants. *Plant Soil* 236: 123 – 128.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389 – 3402.

Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, Yu Y (2005) Protein database searches using compositionally adjusted substitution matrices. *FEBS J.* 272: 5101 – 5109.

Amin SA, Kupper FC, Green DH, Harris WR, Carrano CJ (2007) Boron binding by a siderophore isolated from marine bacteria associated with the toxic dinoflagellate *Gymnodinium catenatum*. *J. Am. Chem. Soc.* 129: 478 – 479.

Ardic M, Sekmen AH, Tokur S, Ozdemir F, Turkan I (2009) Antioxidant responses of chickpea plants subjected to boron toxicity. *Plant Biol.* 11: 328 – 338.

Atik AE, Bozdag GO, Akinci E, Kaya A, Koc A, Yalcin T, Karakaya HC (2011) Proteomic changes during boron tolerance in barley (*Hordeum vulgare*) and the role of vacuolar proton-translocating ATPase subunit E. *Turk. J. Bot.* 35: 379 – 388.

Avigad G (1982) Sucrose and other disaccharides. In: Loewus FA and Tanner W (Ed.) Plant Carbohydrates I. Intercellular Carbohydrates. Encyclopedia of Plant Physiology. New Series. Vol 13A. Springer-Verlag, Berlin, pp 217 – 347.

Ayars JE, Hutmacher RB, Schoneman RA, Vail SS, Pflaum T (1993) Long term use of saline water for irrigation. *Irrigation Sci*. 14: 27 – 34.

Baik BK and Ullrich SE (2008) Barley for food: Characteristics, improvement, and renewed interest. *J. Cereal Sci.* 48: 233 – 242.

Balbi V and Devoto A (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. *New Phytol*. 177: 301 – 318.

Banuelos GS, Mackey B, Wu L, Zambrzuski S, Akohoue S (1995) Bioextraction of soil boron by tall fescue. *Ecotox. Environ. Safe*. 31: 110 – 116.

Barnes LD, Robinson AK, Mumford CH, Garrison PN (1985) Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. *Anal. Biochem*. 144: 296 – 304.

Bassil E, Hu H, Brown PH (2004) Use of phenylboronic acids to investigate boron function in plants. Possible role of boron in transvacuolar cytoplasmic strands and cell-to-wall adhesion. *Plant Physiol*. 136: 3383 – 3395.

Bellaloui N, Brown PH, Dandekar AM (1999) Manipulation of in vivo sorbitol production alters boron uptake and transport in tobacco. *Plant Physiol*. 119: 735 – 741.

Bellaloui N, Yadavc RC, Chern MS, Hu H, Gillen AM, Greve C, Dandekar AM, Ronald PC, Brown PH (2003) Transgenically enhanced sorbitol synthesis facilitates phloem-boron mobility in rice. *Physiol. Plantarum* 117: 79 – 84.

Benjamini Y and Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B Met*. 57: 289 – 300.

Bennett A, Rowe RI, Soch N, Eckhert CD (1999) Boron stimulates yeast (*Saccharomyces cerevisiae*) growth. *J. Nutr.* 129: 2236 – 2238.

Berna A and Bernier F (1999) Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H_2O_2-producing enzyme. *Plant Mol. Biol*. 39:539 – 549.

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, Frutiger S, Hochstrasser DF (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14: 1023 – 1031.

Blaser-Grill J, Knoppik D, Amberger A, Goldbach H (1989) Influence of boron on the membrane potential in *Elodea densa* and *Helianthus annuus* roots and H+ extrusion of suspension cultured *Daucus carota* cells. *Plant Physiol*. 90: 280 – 284.

Blevins DG and Lukaszewski KM (1998) Boron in plant structure and function. *Annu. Rev. Plant Physiol. Mol. Biol*. 49: 481 – 500.

Bohlmann H, Clausen S, Behnke S, Giese H, Hiller C, Reimann-Philipp U, Schrader G, Barkholt V, Apel K (1988) Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to

plant-pathogenic fungi and possibly involved in the defence mechanism of plants. *EMBO J*. 7: 1559 – 1565.

Bohlmann H, Vignutelli A, Hilpert B, Miersch O, Wasternack C, Apel K (1998) Wounding and chemicals induce expression of the *Arabidopsis thaliana* gene Thi2.1, encoding a fungal defense thionin, via the octadecanoid pathway. *FEBS Lett*. 437: 281 – 286.

Bolanos L, Lukaszewski K, Bonilla I, Blevins D (2004) Why boron? *Plant Physiol. Bioch*. 42: 907 – 912.

Bonilla I, Cadahia C, Carpena O, Hernando V (1980) Effects of boron on nitrogen metabolism and sugar levels of sugar beet. *Plant Soil* 57: 3 – 9.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FCP, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M (2001) Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. *Nat. Genet*. 29: 365 – 371.

Brennan RF and Adcock KG (2004) Incidence of boron toxicity in spring barley in Southwestern Australia*. J. Plant Nutr*. 27: 411 – 425.

Breslauer KJ, Frank R, Blocker H, Marky LA (1986) Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. USA* 83: 3746 – 3750.

Brown PH and Hu H (1994) Boron uptake by sunflower, squash and cultured tobacco cells. *Physiol. Plantarum* 91: 435 – 441.

Brown PH and Hu H (1996) Phloem mobility of boron is species dependent: evidence for phloem mobility in sorbitol-rich species. *Ann. Bot*. 77: 497 – 505.

Brown PH, Bellaloui N, Hu H, Dandekar A (1999) Transgenically enhanced sorbitol synthesis facilitates phloem boron transport and increases tolerance of tobacco to boron deficiency. *Plant Physiol*. 119: 17 – 20.

Brown PH, Bellaloui N, Wimmer MA, Bassil ES, Ruiz J, Hu H, Pfeffer H, Dannel F, Römheld V (2002) Boron in plant biology. *Plant Biol*. 4: 205 – 223.

Buratowski S (1997) Multiple TATA-binding factors come back into style. *Cell* 91: 13 – 15.

Burton RA, Shirley NJ, King BJ, Harvey AJ, Fincher GB (2004) The CesA gene family of barley. Quantitative analysis of transcripts reveals two groups of co-expressed genes. *Plant Physiol.* 134: 224 – 236.

Busk PK and Pages M (1998) Regulation of abscisic acid-induced transcription. *Plant Mol. Biol*. 37: 425 – 435.

Cakmak I and Römheld V (1997) Boron deficiency-induced impairments of cellular functions in plants. *Plant Soil* 193: 71 – 83.

Cakmak I, Kurz H, Marschner H (1995) Short-term effects of boron, germanium and high light intensity on membrane permeability in boron deficient leaves of sunflower. *Physiol*. *Plantarum* 95: 11 – 18.

Camacho-Cristobal JJ, Herrera-Rodríguez MB, Beato VM, Rexach J, Navarro-Gochicoa MT, Maldonado JM, González-Fontes A (2008) The expression of several cell wall-related genes in *Arabidopsis* roots is down-regulated under boron deficiency. *Environ*. *Exp*. *Bot*. 63: 351 – 358.

Camacho-Cristobal JJ, Rexach J, Herrera-Rodríguez MB, Navarro-Gochicoa MT, González-Fontes A (2011) Boron deficiency and transcript level changes. *Plant Sci*. 181: 85 – 89.

Cartwright B, Zarcinas BA, Mayfield AH (1984) Toxic concentrations of boron in red-brown earth at Gladstone, South Australia. *Aust. J. Soil Res*. 22: 261 – 272.

Cattivelli L, Baldi P, Crosatti C, Di Fonzo N, Faccioli P, Grossi M, Mastrangelo AM, Pecchioni N, Stanca AM (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in *Triticeae*. *Plant Mol. Biol*. 48: 649 – 665.

Cervilla LM, Blasco B, Rıos J, Romero L, Ruiz J (2007) Oxidative stress and antioxidants in tomato (*Solanum lycopericum*) plants subjected to boron toxicity. *Ann*. *Bot*. 100: 747 – 756.

Cervilla LM, Blasco B, Ríos JJ, Rosales MA, Rubio-Wilhelmi MM, Sánchez-Rodríguez E, Romero L, Ruiz JM (2009) Response of nitrogen metabolism to boron toxicity in tomato plants. *Plant Biol*. 11: 671 – 677.

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* 415: 545 – 549.

Cho S, Garvin DF, Muehlbauer GJ (2006) Transcriptome analysis and physical mapping of barley genes in wheat-barley chromosome addition lines. *Genetics* 172: 1277 – 1285.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal*. *Biochem*. 162: 156 – 159.

Clark RB (1982) Plant response to mineral element toxicity and deficiency. In: Christiansen MN and Lewis CF (Ed.) Breeding Plants for Less Favourable Environments. John Wiley & Sons, New York, NY, pp 71 – 142.

Clarke JD and Zhu T (2006) Microarray analysis of the transcriptome as a stepping stone towards understanding biological systems: practical considerations and perspectives. *Plant J*. 45: 630 – 650.

Close TJ, Wanamaker SI, Caldo RA, Turner SM, Ashlock DA, Dickerson JA, Wing RA, Muehlbauer GJ, Kleinhofs A, Wise RP (2004) A new resource for cereal genomics: 22K Barley GeneChip comes of age. *Plant Physiol*. 134: 960 – 968.

Cserzo M, Wallin F, Simon I, von Heijne G, Elofsson A (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng*. 10: 673 – 676.

Cushman JC and Bohnert HJ (2000) Genomic approaches to plant stress tolerance. *Curr*. *Opin. Plant Biol*. 3: 117 – 124.

Dell B and Huang L (1997) Physiological response of plants to low boron. *Plant Soil* 193: 103 – 120.

Devoto A and Turner JG (2005) Jasmonate-regulated Arabidopsis stress signalling network. *Physiol. Plantarum* 123: 161 – 172.

Dolferus R, Ji XM, Richards RA (2011) Abiotic stress and control of grain number in cereals *Plant Sci.* 181: 331 – 341.

Dordas C, Chrispeels MJ, Brown PH (2000) Permeability and channel-mediated transport of boric acid across membrane vesicles isolated from squash roots. *Plant Physiol*. 124: 1349 – 1362.

Dugger WM (1983) Boron in plant metabolism. In: Lauchli A and Bieleski RL (Ed.) Encyclopedia of Plant Physiology, New Series, Vol. 15, Springer-Verlag, Berlin, pp 626 – 650.

Dugger WM and Humphreys TE (1960) Influence of boron on enzymatic reactions associated with biosynthesis of sucrose. *Plant Physiol*. 35: 523 – 530.

Dugger WM and Palmer RL (1980) Effect of boron on the incorporation of glucose from UDP-glucose into cotton fibers grown in vitro. *Plant Physiol*. 65: 266 – 273.

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* 45: 616 – 629.

FAOSTAT Food and Agriculture Organization of The United Nations (FAO) Statistical Databases (2009) last access date: November 2011, http://faostat.fao.org

Ferreyra RE, Aljara AU, Ruiz RS, Rojas LP, Oster JD (1997) Behavior of 42 crop species grown in saline soils with high boron concentrations. *Agric. Water Manage*. 34: 111 – 124.

Fitzpatrick KL and Reid RJ (2009) The involvement of aquaglyceroporins in transport of boron in barley roots. *Plant Cell Environ*. 32: 1357 – 1365.

Furlani AMC, Carvalho CP, de Freitas JG, Verdial MF (2003) Wheat cultivar tolerance to boron deficiency and toxicity in nutrient solution. *Sci. Agric.* 60: 359 – 370.

Frommer WB and von Wiren N (2002) Plant biology: Ping-pong with boron. *Nature* 420: 282 – 283.

Gao X, Stumpe M, Feussner I, Kolomiets M (2008) A novel plastidial lipoxygenase of maize (*Zea mays*) ZmLOX6 encodes for a fatty acid hydroperoxide lyase and is uniquely regulated by phytohormones and pathogen infection. *Planta* 227: 491 – 503.

Gauch HG and Dugger WM (1954) The physiological action of boron in higher plants: review and interpretation. University Maryland *AES Techn. Bull.* 80: 1 – 43.

Gausing K (1987) Thionin genes specifically expressed in barley leaves. *Planta* 171: 241 – 246.

Goldbach HE and Wimmer MA (2007) Boron in plants and animals: is there a role beyond cell-wall structure? *J. Plant Nutr. Soil Sci.* 170: 39 – 48.

Goldberg S (1997) Reactions of boron with soils. *Plant Soil* 193: 35 – 48.

Golkari S, Gilbert J, Prashar S, Procunier JD (2007) Microarray analysis of *Fusarium graminearum*-induced wheat genes: identification of organ-specific and differentially expressed genes. *Plant Biotechnol. J.* 5: 38 – 49.

Golldack D, Lüking I, Yang O (2011) Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Rep*. 30: 1383 – 1391.

Grieve CM and Poss JA (2000) Wheat response to interactive effects of boron and salinity. *J. Plant Nutr.* 23: 1217 – 1226.

Grudkowska M and Zagdanska B (2004) Multifunctional role of plant cysteine proteinases. *Acta Biochimica Polonica* 51: 609 – 624.

Gunes A, Soylemezoglu G, Inal A, Bagci EG, Coban S, Sahin O (2006) Antioxidant and stomatal responses of grapevine (*Vitis vinifera* L) to boron toxicity. *Sci. Hortic*. 110: 279 – 284.

Habash DZ, Kehel Z, Nachit M (2009) Genomic approaches for designing durum wheat ready for climate change with a focus on drought. *J. Exp. Bot*. 60: 2805 – 2815.

Hanson EJ, Chaplin MH, Breen PJ (1985) Movement of foliar applied boron out of leaves and accumulation in flower buds and flower parts of “Italian” prune. *HortScience* 20: 747 – 748.

Hayes JE and Reid RJ (2004) Boron tolerance in barley is mediated by efflux of boron from the roots. *Plant Physiol*. 136: 3376 – 3382.

Hazen SP, Pathan MS, Sanchez A, Baxter I, Dunn M, Estes B, Chang HS, Zhu T, Kreps JA, Nguyen HT (2005) Expression profiling of rice segregating for drought tolerance QTLs using a rice genome array. *Funct. Integr. Genomic*. 5: 104 – 116.

Herbik A, Koch G, Mock HP, Dushkov D, Czihal A, Thielmann J, Stephan UW, Bäumlein H (1999) Isolation, characterization and cDNA cloning of nicotianamine synthase from barley. A key enzyme for iron homeostasis in plants. *Eur. J. Biochem*. 265: 231 – 239.

Higuchi K, Suzuki K, Nakanishi H, Yamaguchi H, Nishizawa NK, Mori S (1999) Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. *Plant Physiol.* 119: 471 – 480.

Himmelbach A, Zierold U, Hensel G, Riechen J, Douchkov D, Schweizer P, Kumlehn J (2007) A set of modular binary vectors for the transformation of cereals. *Plant Physiol*. 145: 1192 – 1200.

Hoagland DR and Arnon DI (1950) The water-culture method for growing plants without soil. *Cal. Agric. Exp. Sta. Cir.* 347: 1 – 32.

Hobert O (2002) PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic *C. elegans*. *Biotechniques* 32: 728 – 730.

Hoffman EP, The Tumor Analysis Best Practices Working Group (2004) Expression profiling – best practices for data generation and interpretation in clinical trials. *Nat. Rev. Genet*. 5: 229 – 237.

Holtorf H, Guitton MC, Reski R (2002) Plant functional genomics. *Naturwissenschaften* 89: 235 – 249.

Hu H, Brown PH, Labavitch JM (1996) Species variability in boron requirement is correlated with cell wall pectin. *J. Exp. Bot*. 47: 227 – 232.

Hu H, Penn SG, Lebrilla CB, Brown PH (1997) Isolation and characterization of soluble boron complexes in higher plants (The mechanism of phloem mobility of boron). *Plant Physiol*. 113: 649 – 655.

Huang L, Bell RW, Dell B (2001) Boron supply into wheat (*Triticum aestivum* L. cv. Wilgoyne) ears whilst still enclosed within leaf sheaths. *J. Exp. Bot.* 52: 1731 – 1738.

Hunt CD (2002) Boron-binding-biomolecules: a key to understanding the beneficial physiologic effects of dietary boron from prokaryotes to humans. In: Goldbach HE, Rerkasem B, Wimmer MA, Brown PH, Thellier M, Bell RW (Ed.) Boron in Plant and Animal Nutrition. Kluwer Academic Publishers, New York, NY, pp 21 – 36.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TB (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 31: e15.

Jadhav SJ, Lutz SE, Ghorpade VM, Salunkhe DK (1998) Barley: Chemistry and value-added processing. *Crit. Rev. Food Sci. Nutr*. 38: 123 – 171.

Jefferies SP, Barr AR, Karakousis A, Kretschmer JM, Manning S, Chalmers KJ, Nelson JC, Islam AKMR, Langridge P (1999) Mapping of chromosome regions conferring boron toxicity tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet*. 98: 1293 – 1303.

Jefferies SP, Pallotta MA, Paull JG, Karakousis A, Kretschmer JM, Manning S, Islam A, Langridge P, Chalmers KJ (2000) Mapping and validation of chromosome regions conferring boron toxicity tolerance in wheat (*Triticum aestivum*). *Theor. Appl. Genet*. 101: 767 – 777.

Jones DT (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol*. 292: 195 – 202.

Kalayci M, Alkan A, Cakmak I, Bayramoglu O, Yilmaz A, Aydin M, Ozbek V, Ekiz H, Ozberisoy F (1998) Studies on differential response of wheat cultivars to boron toxicity. *Euphytica* 100: 123 – 129.

Kandel S, Morant M, Benveniste I, Blee E, Werck-Reichhart D, Pinot F (2005) Cloning, functional expression, and characterization of CYP709C1, the first sub-terminal hydroxylase of long chain fatty acid in plants – Induction by chemicals and methyl jasmonate. *J. Biol. Chem*. 280: 35881 – 35889.

Kar Y, Şen N, Demirbaş A (2006) Boron minerals in Turkey, their application areas and importance for the country's economy. *Minerals & Energy - Raw Materials Report*, 20, 3 – 4: 2 – 10.

Karabal E, Yucel M, Oktem HA (2003) Antioxidant responses of tolerant and sensitive barley cultivars to boron toxicity. *Plant Sci*. 164: 925 – 933.

Kasajima I, Ide Y, Hirai MY, Fujiwara T (2010) WRKY6 is involved in the response to boron deficiency in *Arabidopsis thaliana*. *Physiol. Plant*. 139: 80 – 92.

Kastori R and Petrovic N (1989) Effect of boron on nitrate reductase activity in young sunflower plants*. J. Plant Nutr*. 12: 621 – 632.

Kim CS, Kwak JM, Nam HG, Kim KC, Cho BH (1994) Isolation and characterization of two cDNA clones that are rapidly induced during the wound response of *Arabidopsis thaliana*. *Plant Cell* 13: 340 – 343.

Kluge R and Podlesak W (1985) Plant critical levels for the evaluation of boron toxicity in spring barley (*Hordeum vulgare* L.). *Plant Soil* 83: 381 – 388.

Kobayashi M, Matoh T, Azuma J (1996) Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiol*. 110: 1017 – 1020.

Kobayashi M, Mutoh T, Matoh T (2004) Boron nutrition of cultured tobacco BY-2 cells. IV. Genes induced under low boron supply. *J. Exp. Bot*. 55: 1441 – 1443.

Kobayashi M, Ohno K, Matoh T (1997) Boron nutrition of cultured tobacco BY-2 cells. II. Characterization of the boron-polysaccharide complex. *Plant Cell Physiol*. 38: 676 – 683.

Komatsuda T and Mano Y (2002) Molecular mapping of the intermedium spike-c (int-c) and non-brittle rachis 1 (btr1) loci in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet*. 105: 85 – 90.

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. *Proc. Natl. Acad. Sci. USA* 104: 1424 – 1429.

Koornneef M, Alonso-Blanco C, Peeters AJM (1997) Genetic approaches in plant physiology. *New Phytol*. 137: 1 – 8.

Kuhn E (2001) From library screening to microarray technology: Strategies to determine gene expression profiles and to identify differentially regulated genes in plants. *Ann. Bot*. 87: 139 – 155.

Landers JP, Oda RP, Schuchard MD (1992) Separation of boron-complexed diol compounds using high performance capillary electrophoresis. *Anal. Chem*. 64: 2846 – 2851.

Lanoue L, Trollinger DR, Strong PL, Keen CL (2000) Functional impairments in preimplantation mouse embryos following boron deficiency. *FASEB J.* 14A: 539.

Lee TI and Young RA (1998) Regulation of gene expression by TBP-associated proteins. *Genes Dev.* 12: 1398 – 1408.

Li CJ, Pfeffer H, Dannel F, Romheld V, Bangerth F (2001) Effects of boron starvation on boron compartmentation, and possibly hormone-mediated elongation growth and apical dominance of pea (*Pisum sativum*) plants. *Physiol. Plant*. 111: 212 – 219.

Lockhart DJ and Winzeler EA (2000) Genomics, gene expression and DNA arrays. *Nature* 405: 827 – 836.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol*. 13: 1675 – 1680.

Loomis WD and Durst RW (1992) Chemistry and biology of boron. *BioFactors* 3: 229 – 239.

Lukaszewski KM and Blevins DG (1996) Root growth inhibition in boron-deficient or aluminum-stressed squash may be a result of impaired ascorbate metabolism. *Plant Physiol*. 112: 1135 – 1140.

MacGregor AW (1993) Barley. In: Macrae R, Robinson RK, Sadler MJ (Ed.) Encyclopaedia of Food Science, Food Technology, and Nutrition. Academic Press, New York, NY, pp 308.

Mahboobi H, Yucel M, Oktem HA (2000) Changes in total protein profiles of barley cultivars in response to toxic boron concentration. *J. Plant Nutr*. 23: 391 – 399.

Mahboobi H, Yucel M, Oktem HA (2001) Cell wall uronic acid concentrations of resistant and sensitive cultivars of wheat and barley under boron toxicity. *J. Plant Nutr*. 24: 1965 – 1973.

Mahboobi H, Yucel M, Oktem HA (2002) Nitrate reductase and glutamate dehydrogenase activities of resistant and sensitive cultivars of wheat and barley under boron toxicity. *J. Plant Nutr*. 25: 1829 – 1837.

Maniatis T, Goodbourn S, Fischer JA (1987) Regulation of inducible and tissue-specific gene expression. *Science* 236: 1237 – 1245.

Marschner H (1995) Mineral Nutrition of Higher Plants. Academic Press, London, UK, pp 461 – 465.

Matoh T and Ochiai K (2005) Distribution and partitioning of newly taken-up boron in sunflower. *Plant Soil* 278: 351 – 360.

Matoh T, Ishigaki K, Ohno K, Azuma J (1993) Isolation and characterization of a boron-polysaccharide complex from radish roots. *Plant Cell Physiol*. 34, 639 – 642.

McDonald GK, Eglinton JK, Barr AR (2009) Assessment of the agronomic value of QTL on chromosomes 2H and 4H linked to tolerance to boron toxicity in barley (*Hordeum vulgare* L.). *Plant Soil* 326: 275 – 290.

McGuffin LJ, Bryson K, Jones DT (2000) The PSIPRED protein structure prediction server. *Bioinformatics* 16: 404 – 405.

Mengel K and Kirkby EA (2001) Principles of Plant Nutrition. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 621 – 638.

Miwa K and Fujiwara (2010) Boron transport in plants: co-ordinated regulation of transporters. *Ann. Bot*. 105: 1103 – 1108.

Miwa K, Takano J, Omori H, Seki M, Shinozaki K, Fujiwara T (2007) Plants tolerant of high boron levels. *Science* 318: 1417.

Mol J, Grotewold E, Koes R (1998) How genes paint flowers and seeds. *Trends Plant Sci.* 3: 212 – 217.

Munns R and Tester M (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol*. 59: 651 – 681.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473 – 479.

Murray MG and Thompson WF (1980) Rapid isolation of high molecular-weight plant DNA. *Nucleic Acids Res*. 8: 4321 – 4325.

Nable RO (1988) Resistance to boron toxicity amongst several barley and wheat cultivars: a preliminary examination of the resistance mechanism. *Plant Soil* 112: 45 – 52.

Nable RO, Banuelos GS, Paull JG (1997) Boron toxicity. *Plant Soil* 193: 181 – 198.

Nakagawa Y, Hanaoka H, Kobayashi M, Miyoshi K, Miwa K, Fujiwara T (2007) Cell-type specificity of the expression of OsBOR1, a rice efflux boron transporter gene, is regulated in response to boron availability for efficient boron uptake and xylem loading. *Plant Cell* 19: 2624 – 2635.

Nakanishi H, Okumura N, Umehara Y, Nishizawa NK, Chino M, Mori S (1993) Expression of a gene specific for iron deficiency (Ids3) in the roots of Hordeum vulgare. *Plant Cell Physiol*. 34: 401 – 410.

Nasuda S, Kikkawa Y, Ashida T, Islam AKMR, Sato K, Endo TR (2005) Chromosomal assignment and deletion mapping of barley EST markers. *Genes Genet Syst*. 80: 357 – 366.

Nevo E and Chen GX (2010) Drought and salt tolerances in wild relatives for wheat and barley improvement. *Plant Cell Environ*. 33: 670 – 685.

Nielsen FH (1997) Boron in human and animal nutrition. *Plant Soil* 193: 199 – 208.

O'Neill MA, Eberhard S, Albersheim P, Darvill AG (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* 249: 846 – 849.

O'Neill MA, Ishii T, Albersheim P, Darvill AG (2004) Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol*. 55: 109 – 139.

O'Neill MA, Warrenfeltz D, Kates K, Pellerin P, Doco T, Darvill A, Albersheim P (1996) Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. *J. Biol. Chem*. 271: 22923 – 22930.

Ochiai K, Shimizu A, Okumoto Y, Fujiwara T, Matoh T (2011) Suppression of a NAC-like transcription factor gene improves boron-toxicity tolerance in rice (*Oryza sativa* L.). *Plant Physiol*. 156: 1457 – 1463.

Oertli J (1962) Loss of boron from plants through guttation. *Soil Sci*. 94: 214 – 219.

Öktem HA, Eyidoğan F, Selçuk F, Öz MT, Teixeira da Silva JA, Yücel M (2008) Revealing response of plants to biotic and abiotic stresses with microarray technology. *Genes Genomes Genomics* 2: 14 – 48.

Öz MT, Yılmaz R, Eyidoğan F, de Graaff L, Yücel M, Öktem HA (2009) Microarray analysis of late response to boron toxicity in barley (*Hordeum vulgare* L.) leaves. *Turk. J. Agric. For*. 33: 191 – 202.

Ozturk ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Mol. Biol.* 48: 551 – 573.

Parr AJ and Loughman BC (1983) Boron and membrane function in plants. In: Robb DA and Pierpoint WS (Ed.) Metals and Micronutrients: Uptake and Utilization by Plants. Academic Press, London, UK, pp. 87 – 107.

Patterson J, Ford K, Cassin A, Natera S, Bacic A (2007) Increased abundance of proteins involved in phytosiderophore production in boron-tolerant barley. *Plant Physiol*. 144: 1612 – 1631.

Paull JG, Nable RO, Rathjen AJ (1992) Physiological and genetic control of the tolerance of wheat to high concentrations of boron and implications for plant breeding. *Plant Soil* 146: 251 – 260.

Paull JG, Rathjen AJ, Cartwright B (1991) Major gene control of tolerance of bread wheat (Triticum aestivum L.) to high concentrations of soil boron. *Euphytica* 55: 217 – 228.

Pollard AS, Parr AJ, Loughman BC (1977) Boron in relation to membrane function in higher plants. *J. Exp. Bot*. 28: 831 – 841.

Power PP and Woods WG (1997) The chemistry of boron and its speciation in plants. *Plant Soil* 193: 1 – 13.

Ralston NVC and Hunt CD (2001) Diadenosine phosphates and S-adenosylmethionine: novel boron binding biomolecules detected by capillary electrophoresis. *Biochim. Biophys. Acta* 1527: 20 – 30.

Ramsay L, Macaulay M, degli Ivanissevich S, MacLean K, Cardle L, Fuller J, Edwards KJ, Tuvesson S, Morgante M, Massari A, Maestri E, Marmiroli N, Sjakste T, Ganal M, Powell W, Waugh R (2000) A simple sequence repeat-based linkage map of barley. *Genetics* 156: 1997 – 2005.

Raven JA (1980) Short and long distance transport of boric acid in plants. *New Phytol*. 84: 231 – 249.

Rawson HM (1996) The developmental stage during which boron limitation causes sterility in wheat genotypes and the recovery of fertility. *Aust. J. Plant Physiol*. 23: 709 – 717.

Reddy PS, Mahanty S, Kaul T, Nair S, Sopory SK, Reddy MK (2008) A high-throughput genome-walking method and its use for cloning unknown flanking sequences. *Anal. Biochem.* 381: 248 – 253.

Reid R (2007) Identification of boron transporter genes likely to be responsible for tolerance to boron toxicity in wheat and barley. *Plant Cell Physiol*. 48: 1673 – 1678.

Reid R (2010) Can we really increase yields by making crop plants tolerant to boron toxicity? *Plant Sci*. 178: 9 – 11.

Reid R and Fitzpatrick K (2009) Influence of leaf tolerance mechanisms and rain on boron toxicity in barley and wheat. *Plant Physiol*. 151: 413 – 420.

Reid RJ, Hayes JE, Post A, Stangoulis JCR, Graham RD (2004) A critical analysis of the causes of boron toxicity in plants. *Plant Cell Environ*. 25: 1405 – 1414.

Rerkasem B and Loneragan JF (1994) Boron deficiency in two wheat genotypes in a warm, subtropical region. *Agron. J.* 86: 887 – 890.

Ricardo A, Carrigan MA, Olcott AN, Benner SA (2004) Borate minerals stabilize ribose. *Science* 303: 196 – 196.

Riley MM, Robson AD, Deller GA, Gartell JW (1994) Critical toxic concentrations are variable in barley. *J. Plant Nutr*. 17: 1701 – 1719.

Roessner U, Patterson JH, Forbes MG, Fincher GB, Langridge P, Bacic A (2006) An investigation of boron toxicity in barley using metabolomics. *Plant Physiol*. 142: 1087 – 1101.

Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: A review. *Yeast* 8: 423 – 488.

Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M, Prado FE (2009) Soluble sugars—Metabolism, sensing and abiotic stress. A complex network in the life of plants. *Plant Signal Behav*. 4: 388 – 393.

Rowe RI and Eckhert CD (1999) Boron is required for zebrafish embryogenesis. *J. Exp. Biol*. 202: 1649 – 54.

Sakuma S, Salomon B, Komatsuda T (2011) The domestication syndrome genes responsible for the major changes in plant form in the *Triticeae* crops. *Plant Cell Physiol*. 52: 738 – 749.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463 – 5467.

Sato K, Motoi Y, Yamaji N, Yoshida H (2011) 454 sequencing of pooled BAC clones on chromosome 3H of barley. *BMC Genomics* 12: 246.

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467 – 470.

Schnurbusch T, Hayes J, Hrmova M, Baumann U, Ramesh SA, Tyerman SD, Langridge P, Sutton T (2010b) Boron toxicity tolerance in barley through reduced expression of the multifunctional aquaporin HvNIP2;1. *Plant Physiol*. 153: 1706 – 1715.

Schnurbusch T, Hayes J, Sutton T (2010a) Boron toxicity tolerance in wheat and barley: Australian perspectives. *Breeding Sci.* 60: 297 – 304.

Schon MK, Novacky A, Blevins DG (1990) Boron induces hyperpolarisation of sunflower root cell membranes and increases membrane permeability to K+. *Plant Physiol*. 93: 566 – 571.

Shen L, Gong J, Caldo RA, Nettleton D, Cook D, Wise RP, Dickerson JA (2005) BarleyBase - an expression profiling database for plant genomics. *Nucl. Acids Res*. 33: D614 – D618.

Shorrocks VM (1997) The occurrence and correction of boron deficiency. *Plant Soil* 193: 121 – 148.

Singh KB (1998) Transcriptional regulation in plants: The importance of combinatorial control. *Plant Physiol*. 118: 1111 – 1120.

Smith TE, Grattan SR, Grieve CM, Poss JA, Suarez DL (2010a) Salinity's influence on boron toxicity in broccoli: I. Impacts on yield, biomass distribution, and water use. *Agric. Water Manage*. 97: 777 – 782.

Smith TE, Grattan SR, Grieve CM, Poss JA, Suarez DL (2010b) Salinity's influence on boron toxicity in broccoli: II. Impacts on boron uptake, uptake mechanisms and tissue ion relations. *Agric. Water Manage*. 97: 783 – 791.

Sommer AL and Lipman CD (1926) Evidence on the indispensable nature of zinc and boron for higher green plants. *Plant Physiol.* 1: 231 – 249.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel-electrophoresis. *J. Mol. Biol*. 98: 503 – 508.

Sparkes IA, Runions J, Kearns A, Hawes C (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc*. 1: 2019 – 2025.

Stangoulis JC, Reid RJ, Brown PH, Graham RD (2001) Kinetic analysis of boron transport in Chara. *Planta* 213: 142 – 146.

Stec B (2006) Plant thionins – the structural perspective. *Cell. Mol. Life Sci.* 63: 1370 – 1385.

Stein N (2007) *Triticeae* genomics: advances in sequence analysis of large genome cereal crops. *Chromosome Res.* 15: 21 – 31.

Sutton T, Baumann U, Hayes J, Collins NC, Shi BJ, Schnurbusch T, Hay A, Mayo G, Pallotta M, Tester M, Langridge P (2007) Boron-toxicity tolerance in barley arising from efflux transporter amplification. *Science* 318: 1446 – 1449.

Suzuki M, Takahashi M, Tsukamoto T, Watanabe S, Matsuhashi S, Yazaki J, Kishimoto N, Kikuchi S, Nakanishi H, Mori S, Nishizawa NK (2006) Biosynthesis and secretion of mugineic acid family phytosiderophores in zinc-deficient barley. *Plant J.* 48: 85 – 97.

Takahashi M, Yamaguchi H, Nakanishi H, Shioiri T, Nishizawa NK, Mori S (1999) Cloning two genes for nicotianamine aminotransferase, a critical enzyme in iron acquisition (Strategy II) in graminaceous plants. *Plant Physiol*. 121: 947 – 956.

Takano J, Miwa K, Fujiwara T (2008) Boron transport mechanisms: collaboration of channels and transporters. *Trends Plant Sci.* 13: 451 – 457.

Takano J, Miwa K, Yuan L, von Wirén N, Fujiwara T (2005) Endocytosis and degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. *Proc. Natl. Acad. Sci. USA* 102: 12276 – 12281.

Takano J, Noguchi K, Yasumori M, Kobayashi M, Gajdos Z, Miwa K, Hayashi H, Yoneyama T, Fujiwara T (2002) *Arabidopsis* boron transporter for xylem loading. *Nature* 420: 337 – 340.

Takano J, Wada M, Ludewig U, Schaaf G, Von Wirén N, Fujiwara T (2006) The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell* 18: 1498 – 1509.

Takano J, Yamagami M, Noguchi K, Hayashi H, Fujiwara T (2001) Preferential translocation of boron to young leaves in *Arabidopsis thaliana* regulated by the BOR1 gene. *Soil Sci. Plant Nutr*. 47: 345 – 357.

Talame V, Ozturk NZ, Bohnert HJ, Tuberosa R (2007) Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. *J. Exp. Bot.* 58: 229 – 240.

Tanaka M, Takano J, Chiba Y, Lombardo F, Ogasawara Y, Onouchi H, Naito S, Fujiwara T (2011) Boron-dependent degradation of NIP5;1 mRNA for acclimation to excess boron conditions in *Arabidopsis. Plant Cell* 23: 3547 – 3559.

Tanaka M, Wallace IS, Takano J, Roberts DM, Fujiwara T (2008) NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in *Arabidopsis. Plant Cell* 20: 2860 – 2875.

Thimm O, Blaesing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J.* 37: 914 – 939.

Torun A, Gultekin I, Kalayci M, Yilmaz A, Eker S, Cakmak I (2001) Effects of zinc fertilization on grain yield and shoot concentrations of zinc, boron, and phosphorus of 25 wheat cultivars grown on a zinc-deficient and boron-toxic soil. *J. Plant Nutr*. 24: 1817 – 1829.

Torun AA, Yazici A, Erdem H, Çakmak I (2006) Genotypic variation in tolerance to boron toxicity in 70 durum wheat genotypes. *Turk. J. Agric. For*. 30: 49 – 58.

Torun B, Kalayci M, Ozturk L, Torun A, Aydin M, Cakmak I (2003) Differences in shoot boron concentrations, leaf symptoms, and yield of Turkish barley cultivars grown on boron-toxic soil in field. *J. Plant Nutr*. 26: 1735 – 1747.

Truman W, Bennettt MH, Kubigsteltig I, Turnbull C, Grant M (2007) *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc. Natl. Acad. Sci. USA* 104: 1075 – 1080.

Tuteja N and Sopory SK (2008) Chemical signaling under abiotic stress environment in plants. *Plant Signal. Beh.* 3: 525 – 536.

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant Cell Physiol*. 51: 1821 – 1839.

Walia H, Wilson C, Wahid A, Condamine P, Cui X, Close TJ (2006) Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress. *Funct. Integr. Genomics* 6: 143 – 156.

Wang G, Romheld V, Li C, Bangerth F (2006) Involvement of auxin and CKs in boron deficiency induced changes in apical dominance of pea plants (*Pisum sativum* L.). *J. Plant Physiol*. 163:591 – 600.

Warington K (1923) The effect of boric acid and borax on the broad bean and certain other plants. *Ann. Bot*. 37: 629 – 672.

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* 100: 681 – 697.

Watson L and Henry RJ (2005) Microarray analysis of gene expression in germinating barley embryos (*Hordeum vulgare* L.). *Funct. Integr. Genomics* 5: 155 – 162.

Wimmer MA, Bassil ES, Brown PH, Lauchli A (2005) Boron response in wheat is genotype-dependent and related to boron uptake, translocation, allocation, plant phenological development and growth rate. *Funct. Plant Biol.* 32: 507 – 515.

Wimmer MA, Lochnit G, Bassil E, Mühling KH, Goldbach HE (2009) Membrane-associated, boron-interacting proteins isolated by boronate affinity chromatography. *Plant Cell Physiol*. 50: 1292 - 1304.

Wimmer MA, Muhling KH, Lauchli A, Brown PH, Goldbach HE (2003) The interaction between salinity and boron toxicity affects the subcellular distribution of ions and proteins in wheat leaves. *Plant Cell Environ*. 26: 1267 – 1274.

Woods WG (1996) Review of possible boron speciation relating to its essentiality. *J. Trace Elem. Exp. Med.* 9: 153 – 163.

Xu L, Liu F, Wang Z, Peng W, Huang R, Huang D, Xie D (2001) An *Arabidopsis* mutant cex1 exhibits constant accumulation of jasmonate-regulated AtVSP, Thi2.1 and PDF1.2. *FEBS Lett.* 494: 161 – 164.

Yadav HD, Yadav OP, Dhankar OP, Oswal MC (1989) Effect of chloride salinity and boron on germination, growth, and mineral composition of chickpea (*Cicer arietinum* L.). *Ann. Arid Zone* 28: 63 – 67.

Yamaguchi T and Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci*. 10: 615 – 620.

Yamasaki K, Kigawa T, Inoue M, Watanabe S, Tateno M, Seki M, Shinozaki K, Yokoyama S (2008) Structures and evolutionary origins of plant-specific transcription factor DNA-binding domains. *Plant Physiol. Biochem*. 46: 394 – 401.

Yau SK, Nachit M, Ryan J (1997) Variation in growth, development, and yield of durum wheat in response to high soil boron II. Differences between genotypes. *Aust. J. Agr. Res*. 48: 951 – 957.

Yermiyahu U, Ben-Gal A, Keren R, Reid RJ (2008) Combined effect of salinity and excess boron on plant growth and yield. *Plant Soil* 304: 73 – 87.

Yun KY, Park MR, Mohanty B, Herath V, Xu F, Mauleon R, Wijaya E, Bruskiewich R, de Los Reyes BG (2010) Transcriptional regulatory network triggered by oxidative signals configures the early response mechanisms of japonica rice to chilling stress. *BMC Plant Biol*. 10: 16.

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 7: 203 – 214.

Zimmermann P, Schildknecht B, Craigon D, Garcia-Hernandez M, Gruissem W, May S, Mukherjee G, Parkinson H, Rhee S, Wagner U, Hennig L (2006) MIAME/Plant – adding value to plant microarray experiments. *Plant Methods* 2: 1.

APPENDIX A

COMPOSITIONS OF NUTRIENT SOLUTIONS FOR PLANT GROWTH

Table A.1. Composition of Hoagland's nutrient solution.

Component	Stock concentration	Stock solution (g/L)	Final concentration	Volume of stock for half-strength solution (mL)
$MgSO_4.7H_2O$	1 M	246.5	0.5 mM	0.5
$Ca(NO_3)_2.4H_2O$	1 M	236.2	1.15 mM	1.15
KH_2PO_4	1 M	136.1	0.25 mM	0.25
KNO_3	1 M	101.1	1.25 mM	1.25
$MnCl_2.4H_2O$	9.2 mM	1.82	2.3 µM	0.25
$ZnSO_4.7H_2O$	0.8 mM	0.22	0.2 µM	0.25
$Na_2MoO_4.2H_2O$	0.4 mM	0.09	0.1 µM	0.25
$CuSO_4.5H_2O$	0.4 mM	0.09	0.1 µM	0.25
$FeCl_3.6H_2O$	1.8 mM	0.484	18 µM	10
EDTA	5 mM	1.500	50 µM	10
H_3BO_3 *	5 mM	0.309	10 µM	2
Total				**1,000**
* For application of B deficiency				
H_3BO_3	0.05 mM	0.0031	0.02 µM	0.4
* For application of B toxicity				
H_3BO_3	0.5 M	30.92	5 mM	10
	0.5 M	30.92	10 mM	20

Table A.2. Composition of basal growth solution.

Component	Stock concentration	Stock solution (g/L)	Final concentration	Volume of stock for full-strength solution (mL)
$Ca(NO_3)_2$	1 M	164.2	0.5 mM	0.5
$ZnSO_4$	1 mM	0.162	2.5 µM	2.5
H_3BO_3 *	5 mM	0.309	15 µM	3
Total				**1,000**
* For application of B toxicity				
H_3BO_3	0.5 M	30.92	10 mM	20

APPENDIX B

COMPOSITIONS OF BACTERIAL GROWTH MEDIA

Table B.1. Composition of Luria Bertani (LB) medium.

Component	Concentration	Amount (g/L)
Yeast extract	0.5% (w/v)	5
Tryptone	1% (w/v)	10
NaCl	1% (w/v)	10
Microbiological agar *	1.5% (w/v)	15

* Agar is added in case solid medium is required.

Table B.2. Composition of S.O.C. medium.

Component	Concentration	Amount (g/L)
Yeast extract	0.5% (w/v)	5
Tryptone	2% (w/v)	20
NaCl	10 mM	0.5844
KCl	2.5 mM	0.1864
$MgCl_2$	10 mM	0.9521
$MgSO_4$	10 mM	1.2037
Glucose *	20 mM	3.6032

* Glucose is filter-sterilized and added immediately before use.

Table B.3. Composition of Yeast Extract Broth (YEB) medium.

Component	Concentration	Amount (g/L)
Yeast extract	0.1% (w/v)	1
Nutrient broth	1.35% (w/v)	13.5
$MgSO_4.7H_2O$	2 mM	0.493
Sucrose	0.5% (w/v)	5
Microbiological agar *	1.5% (w/v)	15

* Agar is added in case solid medium is required.

APPENDIX C

COMPOSITIONS OF MEDIA USED FOR GROWTH AND TRANSFORMATION OF YEAST

Table C.1. Composition of Yeast extract Peptone Dextrose (YPD) medium.

Component	Concentration	Amount (g/L) **
Yeast extract	1% (w/v)	10
Peptone	2% (w/v)	20
Glucose	2% (w/v)	20
Microbiological agar *	2% (w/v)	20

* Agar is added in case solid medium is required.

** Double-strength liquid YPD, which is used in transformation of yeast, is prepared by doubling the amounts of components.

Table C.2. Composition of Synthetic Dextrose (SD) minimal medium.

Component	Stock solution	Final concentration	Amount
Yeast nitrogen base without amino acids	-	0.67% (w/v)	6.7 g/L
Glucose solution *	20% (w/v)	2% (w/v)	100 mL/L
Histidine solution **	1% (w/v)	0.002% (w/v)	2 mL/L
Uracil solution ***	0.2% (w/v)	0.002% (w/v)	10 mL/L
Microbiological agar ‡	-	2% (w/v)	20 g/L

* Glucose solution is replaced with 20% (w/v) galactose stock solution in case induction medium is required. Glucose and galactose solutions are filter-sterilized and added to autoclaved and cooled medium.

** Histidine solution is filter-sterilized and added to autoclaved medium.

*** Uracil is included in case medium is intended for growth of untransformed yeast strain INVSc2. Selective and induction media should not contain uracil. Solution of uracil is filter-sterilized and added to autoclaved medium.

‡ Agar is added in case solid medium is required.

APPENDIX D

MAPS OF VECTORS

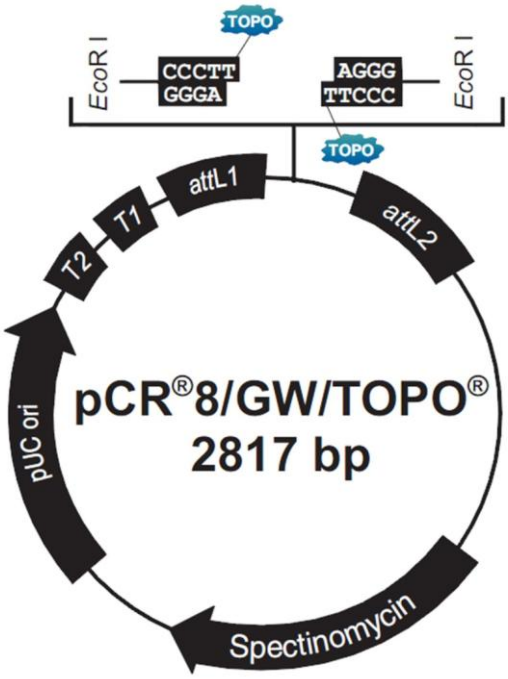

Figure D.1. Map and key features of pCR8/GW/TOPO vector. (*att*L1 and *att*L2: recombination sites; TOPO: Topoisomerase I; T1 and T2: *rrn*B transcription termination sequences; Spectinomycin: bacterial spectinomycin resistance gene; pUC ori: pUC origin; Property of Invitrogen Corporation)

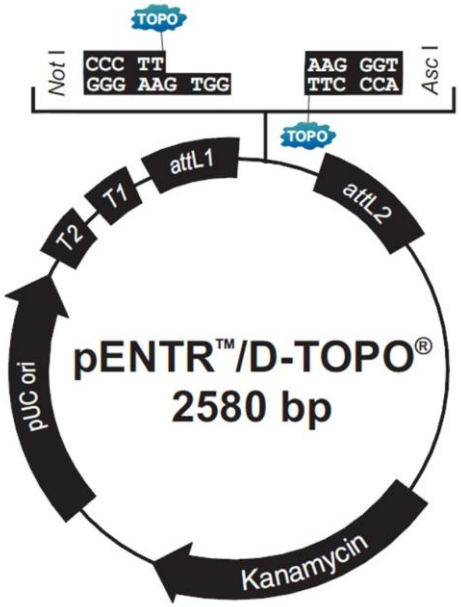

Figure D.2. Map and key features of pENTR/D-TOPO vector. (Kanamycin: bacterial kanamycin resistance gene; for other features see legend of Figure D.1; Property of Invitrogen Corporation)

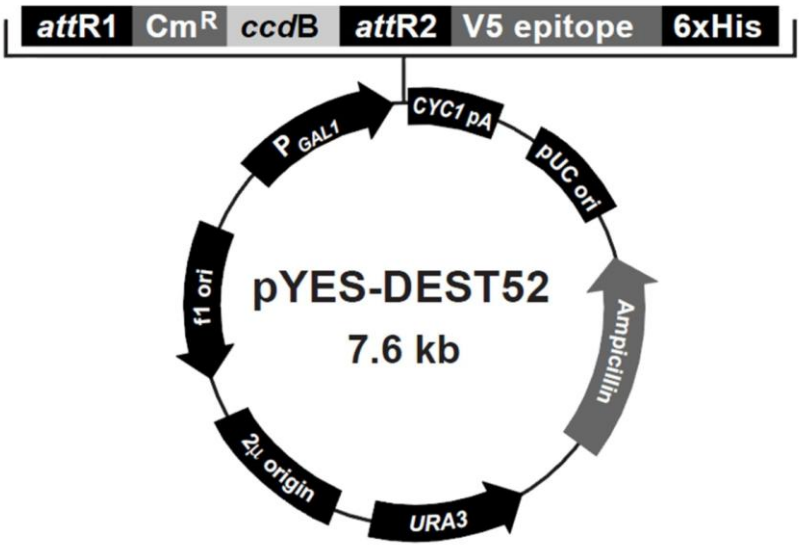

Figure D.3. Map and key features of pYES-DEST52 vector. (*att*R1 and *att*R2: recombination sites; Cm^R: Chloramphenicol resistance gene; *ccd*B: lethal gene; 6xHis: polyhistidine tagging region; *CYC1* pA: *CYC1* polyadenylation region; pUC ori: pUC origin; Ampicillin: bacterial ampicillin (*bla*) resistance gene; *URA*3: *URA*3 gene; f1 ori: f1 origin; P_{GAL1}: *GAL1* promoter; Property of Invitrogen Corporation)

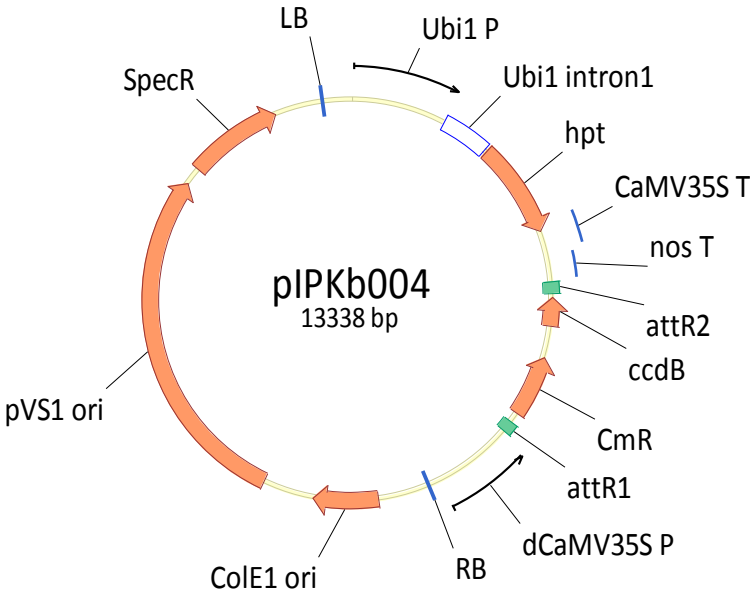

Figure D.4. Map and key features of pIPKb004 vector. (LB: left border of T-DNA; RB: right border of T-DNA; Ubi1 P: ubiquitin 1 gene promoter; hpt: hygromycin phosphotransferase; CaMV35S T: CaMV35S terminator; nos T: nopaline synthase terminator; *att*R1 and *att*R2: recombination sites; CmR: Chloramphenicol resistance gene; *ccd*B: lethal gene; dCaMV35S P: doubled enhanced CaMV35S promoter; ColE1 ori: ColE1 origin; pVS1 ori: pVS1 origin; SpecR: bacterial spectinomycin resistance gene; vector was kindly provided by IPK)

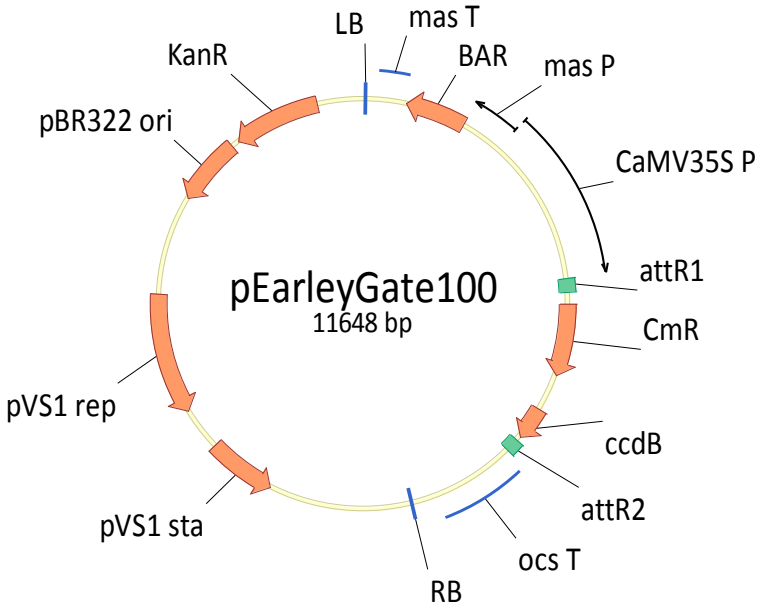

Figure D.5. Map and key features of pEarleyGate100 vector. (mas T: mannopine synthase terminator; BAR: Basta resistance gene; mas P: mannopine synthase promoter; CaMV35S P: CaMV35S promoter; ocs T: octopine synthase terminator; pBR322 ori: pBR322 origin; pVS1 rep: pVS1 replication; pVS1 sta: pVS1 stability; KanR: bacterial kanamycin resistance gene; for other features see legend of Figure D.4; vector was provided by TAIR)

APPENDIX E

PROBE SETS REPRESENTING GENES HAVING SEQUENCE SIMILARITY TO B TRANSPORTERS

Table E.1. Target sequences of probe sets representing putative B transporter genes.

Probe set ID	
Contig14139_at	
1	GGTTGGAGCT ATGCCGGCTA TCAAGATGAT ACCGACTTCG GTCCTCTGGG GTTACTTTGC
61	CTACATGGCC ATTGATAGCC TACCTGGGAA CCAGTTTTGG GAAAGGTTAC AGCTTCTGTG
121	CATTGGAGCA AGCCGACGCT ACAAGGTCTT GGAAGGCCCC CATGCATCTT TCGTGGAGGC
181	GGTGCCTTCA AGAACAATAT CTGCCTTTAC GGTCTTCCAG TTTGTGTATC TCTTGATATG
241	CTTCGGTATA ACATGGATAC CAGTAGCAGG GATCCTCTTC CCGCTGCCTT TCTTCATTAT
301	GATTCTCATC AGGCAACACC TACTCCCAAA GTTCTTTGAG CCCAATGACT TGCGAGAACT
361	GGATGCAGCT GAGTATGAAG AACTTGAAGG CGTCCCACAT GAACAAACAC TGGAGGAAGA
421	TGGCTCAAAT TCAGGAAGCC ATGACAGCAT AGACGACGCT GAAATGTTGG ATGAACTCAC
481	GACAAACCGT GGAGAGCTGA AGCACAGATC TGCAAGCCAT CCTGAAGAAA GGCACCTTCA
541	GGTCCATTCA AATGCAGTTC AGCCGAGCGT GTGAAGATGG AAAAACGTCC CGATGTCCGA
601	GGCTTTGATG ACGATTCTGT CAGAAGTGTA GATTATCCTG AAGCCATTGT TCATTTCCGA
661	ATACGCCATT GTTCAGCAAT GTGCATTGTA CTGTAGCTAG CGCTTTTCCG GTAAGGCGTG
721	ATCGTGTACG CTAGGAAGTA AAGCTCAGGT AATTAGCAGT GAGATCAGAC TGAAGAAAGT
781	GTATTGGATA TGGCAGGGGA ACTTGGCAGA ATAGTATTAG TAATGGTTAG AAGTAGAAAG
841	ACTGTTGCTC CGTGACCTGT TATTGCAAGA CTGTTGCTCT GTGACCTGTT GCCTGTGTAA
901	AGTCCAGCTT TCTGTGTCTG AATGTAAAAT GCCAGGCATA AGAATTTCTT GAGAAAAAAA
961	AAAAAAAAAA
Contig21126_at	
1	TACCGATTGC CGGGATCCTT TTCCCGCTAC CTTTCTTCCT CATGATTCTC ATCAGGCAGT
61	ACCTGCTCCC CAAGTTTTTT GAGCCCAATG ACTTGCGGGA ACTGGACGCG GCTGAGTACG
121	ATGAACTTGA AGGGGTCCAA CATGAACACA CATTGGAGGA AGATGGCTCC ATTTCAGGAA
181	GCTGCGACGG CAGGATTGAC GCTGAGATAT TGGATGAACT CACAACACAC CGTGGGGAGT
241	TGAAACACAG GGTTGTGAGC CATCGTGAAG AAAGACACCT TCAGGTCCAT TCAAATGCCG
301	TTCAGCCAAG CGTGTGAAGA TAGAACAATG GCCCAGGACC GACCGAGGAT TTCATCAATG
361	ATTCTATCGG AAATGAGGAG GGAAATCCTC AGGCAGTCGT TCTTTGCCGA CTGCACTATT
421	ATCTTTCCGT GATAATATGC ACGGTACTGG TAGTATAGCA CTTTCCATAA GACAGGTAGC
481	AGTGAGAGCA GCAGACTGAA GGAAGTATTG CCGGAATAGC AACGGTGAGG AGTAGAAAGA
541	TTAGCTGATT TGGTATTGGA GGAAAACTGT CCCTCTGTGA TCTGTTGTTG CTTGTGTGCC
601	TGGCTTTGTT CTGTTTGAGT GTACCATGTT AAAAGTATTG CTTGATAAGC AACGGCACCG
661	GGATTTCTCT CCCCGCCACC AACTACCGGA GTGGCATGCA CCGAAGCATC CACGGGTTCG
721	CCGGTGAAGC TTGATAGATG AGACTAGCCT TAGCCGCCGT ACGAAATAAG TCGATAATGC
781	AATAACACC
Contig19634_at	
1	ACAAGGTGCT AGAAGAGTAC CACACAACAT TTGTCGAGAC CGTGCCATTC AAGACGATAG
61	CCATGTTCAC ACTTTTCCAG ACAACATATC TGCTTGTTTG CTTTGGGATC ACATGGATCC
121	CGATAGCTGG GGTTCTTTTC CCCCTCATGA TCATGCTCCT GGTTCCAGTC AAGCAGTATA
181	TCCTCCCAAA GCTCTTCAAA GGTGCACATC TCAATGACCT GGACGCAGCG GAGTATGAGG
241	AATCACCGGC TATACCATTC AACCTTGCTA CGCAAGATAT TGATGTTGCA TTGGGACACA
301	CCCAGAGCGC AGAGATCCTT GATGACATGG TCACCAGAAG TCGCGGTGAG ATCAAGCGCC
361	TGAATAGTCC GAAGATCACC AGCTCAGGTG GCACTCCTGT CACAGAACTG AAAGGCATCC
421	GCAGTCCTTG TATTTCTGAG AAGGCCTACA GCCCTCGCAT CACCGAGCTG AGGCATGAAC
481	GCAGCCCTCT AGGGGGGCGA GACAGTCCAC GGACGGGAGA GGCCCGACCA TCCAAGCTCG
541	GTGAAGGCTC AACACCAAAG TGAAGCAGTG ATGCTGCATT GCACATATAA GCTATGGCGC
601	TGCTGGAAAT AAATTATATC GATAACTCGA GCATCAGTCT AATATAAGAT GTCTACTGAT
661	GCGGTATGAT GCCACTATAT GATGGCCAGT GATGAAGTAT GAAAATAGGC TGATGTGTAT
721	CTATCTGTAG CCTCCTGATC CAAGGGAGTA CTGTATGTGT AATCTCTGAA TGAGTAGAAT
781	ATTATAGCGT CTACTTTGAA AAAAAAAAAA AAAAA

Table E.2. BLAST results of probe sets representing putative B transporters. Target sequences were blasted against *O. sativa*, *B. distachyon* and *A. thaliana* genomes and databases of UniProt, RefSeq and *H. vulgare* Gene Index. Top hits, descriptions of subjects, e-values and identity percentages of results are listed.

Probe set ID	E-value	Identity/ Match	Identity (%)
Contig14139_at			
Target sequence: 970 bp			
BLASTx: MSU *O. sativa* Genome: LOC_Os01g08020.1: B transporter protein, putative, expressed	2e-86	151/188	80.3
BLASTx: *B. distachyon* Genome: Bradi2g04690.1	7e-93	163/190	85.8
BLASTx: *A. thaliana* Genome: AT1G15460.1: Symbols: BOR4: HCO3-transporter family	2e-59	111/183	60.7
BLASTx: UniProt: UniRef90_A8WCD9: B transporter 2 n=2 Tax=Triticeae RepID=A8WCD9_WHEAT	1e-101	181/190	95.3
BLASTx: NCBI RefSeq: NP_001042174: Os01g0175600 [*O. sativa* Japonica Group]	9e-86	151/188	80.3
BLASTn: DFCI *H. vulgare* Gene Index: TC199419	0	953/953	100
Contig21126_at			
Target sequence: 789 bp			
BLASTx: MSU *O. sativa* Genome: LOC_Os01g08020.1: B transporter protein, putative, expressed	1e-32	69/102	67.6
BLASTx: *B. distachyon* Genome: Bradi2g04690.1	1e-40	83/104	79.8
BLASTx: *A. thaliana* Genome: AT1G74810.1: Symbols: BOR5: HCO3- transporter family	6e-15	47/102	46.1
BLASTx: UniProt: UniRef90_A9XTK3: B transporter n=1 Tax=*H. vulgare* subsp. vulgare RepID=A9XTK3_HORVD	1e-43	91/104	87.5
BLASTx: NCBI RefSeq: NP_001042174: Os01g0175600 [*O. sativa* Japonica Group]	4e-32	69/102	67.6
BLASTn: DFCI *H. vulgare* Gene Index: TC195114	0	2591/787	99.7
Contig19634_at			
Target sequence: 815 bp			
BLASTx: MSU *O. sativa* Genome: LOC_Os12g37840.1: B transporter protein, putative, expressed	1e-92	170/186	91.4
BLASTx: *B. distachyon* Genome: Bradi4g04420.1	4e-99	178/186	95.7
BLASTx: *A. thaliana* Genome: AT2G47160.2: Symbols: BOR1: HCO3- transporter family	1e-54	106/186	57.0
BLASTx: UniProt: UniRef90_Q2QNH0: Os12g0566000 protein n=5 Tax=Poaceae RepID=Q2QNH0_ORYSJ	7e-91	170/186	91.4
BLASTx: NCBI RefSeq: NP_001067049: Os12g0566000 [*O. sativa* Japonica Group]	6e-92	170/186	91.4
BLASTn: DFCI *H. vulgare* Gene Index: TC220748	0	1033/789	99.9

APPENDIX F

SEQUENCES OF OLIGONUCLEOTIDES EMPLOYED FOR RACE AND GENOME WALKING

Table F.1. Sequences of oligonucleotides and universal primers (UP) used for 5'RACE.

Oligonucleotide ID	Sequence (5' – 3')
SMART II A	AAGCAGTGGTATCAACGCAGAGTACGCGGG
5'RACE CDS primer A	$(T)_{25}$VN *
UP A Mix (UPM)	
Long UP	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Short UP	CTAATACGACTCACTATAGGGC
Nested UP A (NUP)	AAGCAGTGGTATCAACGCAGAGT
	* V = A, G, or C; N = A, G, C, or T

Table F.2. Sequences of oligonucleotides and adaptor primers (AP) used for genome walking.

Oligonucleotide ID	Sequence (5' – 3')
Adaptor_Oligo1	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT
Adaptor_Oligo2	ACCAGCCC/3Amine *
AP	GTAATACGACTCACTATAGGGC
Nested AP (NAP)	ACTATAGGGCACGCGTGGT
	* 3' end is blocked with an amine group

Table F.3. Sequences of walker adaptors (WAs) and walker primers (WP) used for genome walking based on multiple displacement amplification.

Oligonucleotide ID	Sequence (5' – 3')
WA1	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNATGC *
WA2	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNGATC
WA3	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNTAGC
WA4	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNCTAG
WA5	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNTAC
WA6	GTGAGCGCGCGTAATACGACTCACTATAGGGNNNNTAG
WP	GTGAGCGCGCGTAATACGA
Nested WP (NWP)	GTAATACGACTCACTATAGGG
	* N = A, G, C, or T

APPENDIX G

REPRESENTATIVE REPORT FILE USED FOR EVALUATION OF QUALITY OF ARRAY DATA

Table G.1. Report file (.RPT file) generated by GCOS.

Report Type:	Expression Report			
Date:	11:49PM 01/08/2011			
Filename:	TaC1R.CHP			
Probe Array Type:	Barley1			
Algorithm:	Statistical			
Probe Pair Thr:	8			
Controls:	Antisense			
Alpha1:	0.05			
Alpha2:	0.065			
Tau:	0.015			
Noise (RawQ):	2.220			
Scale Factor (SF):	1.000			
Norm Factor (NF):	1.000			
Background:	Avg: 53.22	Std: 0.84	Min: 51.70	Max: 55.50
Noise:	Avg: 2.90	Std: 0.12	Min: 2.60	Max: 3.20
Corner+	Avg: 205	Count: 32		
Corner-	Avg: 12651	Count: 32		
Central-	Avg: 10416	Count: 9		
The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".				
Total Probe Sets:	22840			
Number Present:	15859	69.4%		
Number Absent:	6733	29.5%		
Number Marginal:	248	1.1%		
Average Signal (P):	592.0			
Average Signal (A):	14.8			
Average Signal (M):	49.9			
Average Signal (All):	416.0			

Table G.1. (continued)

Spike Controls:								
Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
AFFX-BIOB	93.8	P	116.4	P	81.6	P	97.24	0.87
AFFX-BIOC	266.2	P			288.5	P	277.34	1.08
AFFX-BIOD	537.8	P			1486.3	P	1012.07	2.76
AFFX-CRE	3307.6	P			6317.9	P	4812.75	1.91
AFFX-DAP	4.0	A	11.9	P	16.6	A	10.82	4.12
AFFX-LYS	6.0	A	12.9	A	18.2	P	12.38	3.06
AFFX-PHE	0.9	A	1.3	A	3.7	A	1.97	3.96
AFFX-THR	1.6	A	1.7	A	4.3	A	2.51	2.71
AFFX-TRP	16.8	A	0.7	A	0.8	A	6.11	0.05
AFFX-R2-EC-BIOB	111.6	P	166.3	P	134.2	P	137.37	1.20
AFFX-R2-EC-BIOC	292.9	P			557.2	P	425.04	1.90
AFFX-R2-EC-BIOD	1507.9	P			1915.6	P	1711.75	1.27
AFFX-R2-P1-CRE	7141.2	P			8565.9	P	7853.56	1.20
AFFX-R2-BS-DAP	2.7	A	6.6	A	5.1	A	4.83	1.86
AFFX-R2-BS-LYS	5.3	A	4.8	A	5.3	A	5.16	1.00
AFFX-R2-BS-PHE	4.9	A	0.5	A	16.3	A	7.24	3.33
AFFX-R2-BS-THR	8.5	A	12.7	A	1.8	A	7.65	0.21

APPENDIX H

STATISTICAL ANALYSES RESULTS

Table H.1. One-way ANOVA results of intra-varietal comparison of B Toxicity in sensitive cultivar. The single independent variable (treatment) has 3 levels, control, toxicity applied as 5 mM H_3BO_3, and toxicity applied as 10 mM H_3BO_3.

	P < 0.05
Number of probe sets (treatment)	999

Table H.2. Two-way ANOVA results of intra-varietal comparison of differences between responses under B toxicity and deficiency. Among the 2 independent variables, the first independent variable (tissue) has 2 levels, leaf and root. The second independent variable (treatment) has 3 levels, control, deficiency applied as 0.02 µM H_3BO_3, and toxicity applied as 5 mM H_3BO_3.

	P < 0.05
Number of probe sets (tissue)	14,717
Number of probe sets (tissue-treatment)	5,260
Number of probe sets (treatment)	5,461
Total	16,567

Table H.3. Three-way ANOVA results of inter-varietal comparison between sensitive and tolerant cultivars under B toxicity. Among the 3 independent variables, the first independent variable (cultivar) has 2 levels, sensitive (Hamidiye) and tolerant (Tarm-92). The second independent variable (tissue) has 2 levels, leaf and root. The third independent variable (treatment) has 2 levels control and toxicity applied as 5 mM H_3BO_3.

	P < 0.05
Number of probe sets (tissue)	15,691
Number of probe sets (tissue-cultivar)	6,604
Number of probe sets (tissue-cultivar-treatment)	1,386
Number of probe sets (tissue-treatment)	2,272
Number of probe sets (cultivar)	7,413
Number of probe sets (cultivar-treatment)	907
Number of probe sets (treatment)	2,798
Total	18,007

APPENDIX I

SEQUENCES PRODUCING SIGNIFICANT ALIGNMENTS WITH *HvBor1a*

Table I.1. BLASTN results displaying sequences which produce significant alignments with *HvBor1a*. The CDS of *HvBor1a* was blasted against nucleotide collection (nr/nt) database at NCBI using BLASTN delimiting the source organism to higher plants (taxid:3193). Accession numbers, descriptions, and total length of hit subjects, query coverage, e-values, and identity and gap percentages of results are listed.

Accession Description	**Total length**	**Total score**	**Query coverage**	**E-value**	**Identity (%) Identity/Match**	**Gap (%) Gaps/Match**
EU220225.1 T. aestivum B transporter 2 mRNA, cds						
	2338	3197	100%	0.0	96% (1912/2002)	0% (2/2002)
XM_003569470.1 B. distachyon B transporter 4-like (LOC100824289), mRNA						
	2423	2471	100%	0.0	89% (1797/2018)	2% (34/2018)
EF660437.1 H. vulgare ssp. vulgare B transporter (Bot1) mRNA, cds						
	2001	2466	100%	0.0	89% (1792/2014)	1% (26/2014)
DQ421408.1 O. sativa (japonica) B transporter mRNA, cds						
	2019	1823	99%	0.0	83% (1695/2031)	4% (72/2031)
NM_001154326.1 Z. mays B transporter-like protein 2 (LOC100281408), mRNA, cds						
	2630	1729	97%	0.0	83% (1658/1997)	4% (87/1997)
XM_002455044.1 S. bicolor hypothetical protein, mRNA						
	2181	1685	97%	0.0	83% (1652/1999)	5% (91/1999)
DQ421409.1 O. sativa (japonica) B transporter (BOR4) mRNA, cds						
	2034	987	88%	0.0	77% (1408/1820)	5% (99/1820)
XM_002440654.1 S. bicolor hypothetical protein, mRNA						
	1983	665	62%	0.0	77% (990/1284)	7% (84/1284)

Table I.1. (continued)

Accession Description	**Total length**	**Total score**	**Query coverage**	**E-value**	**Identity (%) Identity/Match**	**Gap (%) Gaps/Match**
NM_001061328.1 O. sativa (japonica) (Os05g0176800) mRNA, cds						
	2238	508	47%	5e-141	77% (767/996)	7% (68/996)
NM_001195926.1 Z. mays uncharacterized (LOC100501111), mRNA						
	1399	353	15%	3e-94	87% (278/321)	1% (2/321)
BT084558.1 Z. mays full-length cDNA clone ZM_BFb0132E07 mRNA, cds						
	1399	353	15%	3e-94	87% (278/321)	1% (2/321)

APPENDIX J

PREDICTED SECONDARY STRUCTURE OF HvBor1a

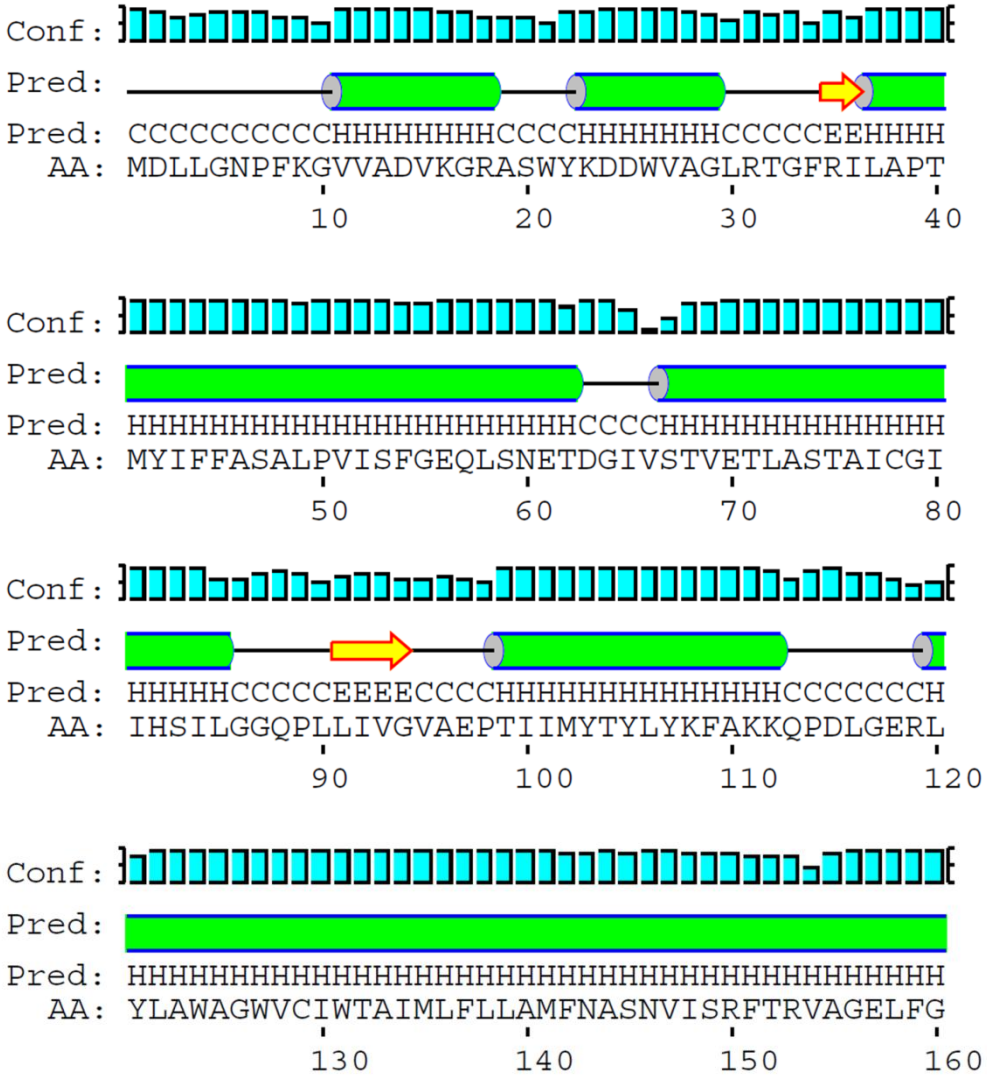

Figure J.1. Predicted secondary structure of HvBor1a. Prediction was performed on amino acid sequence using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) Protein Structure Prediction Server.

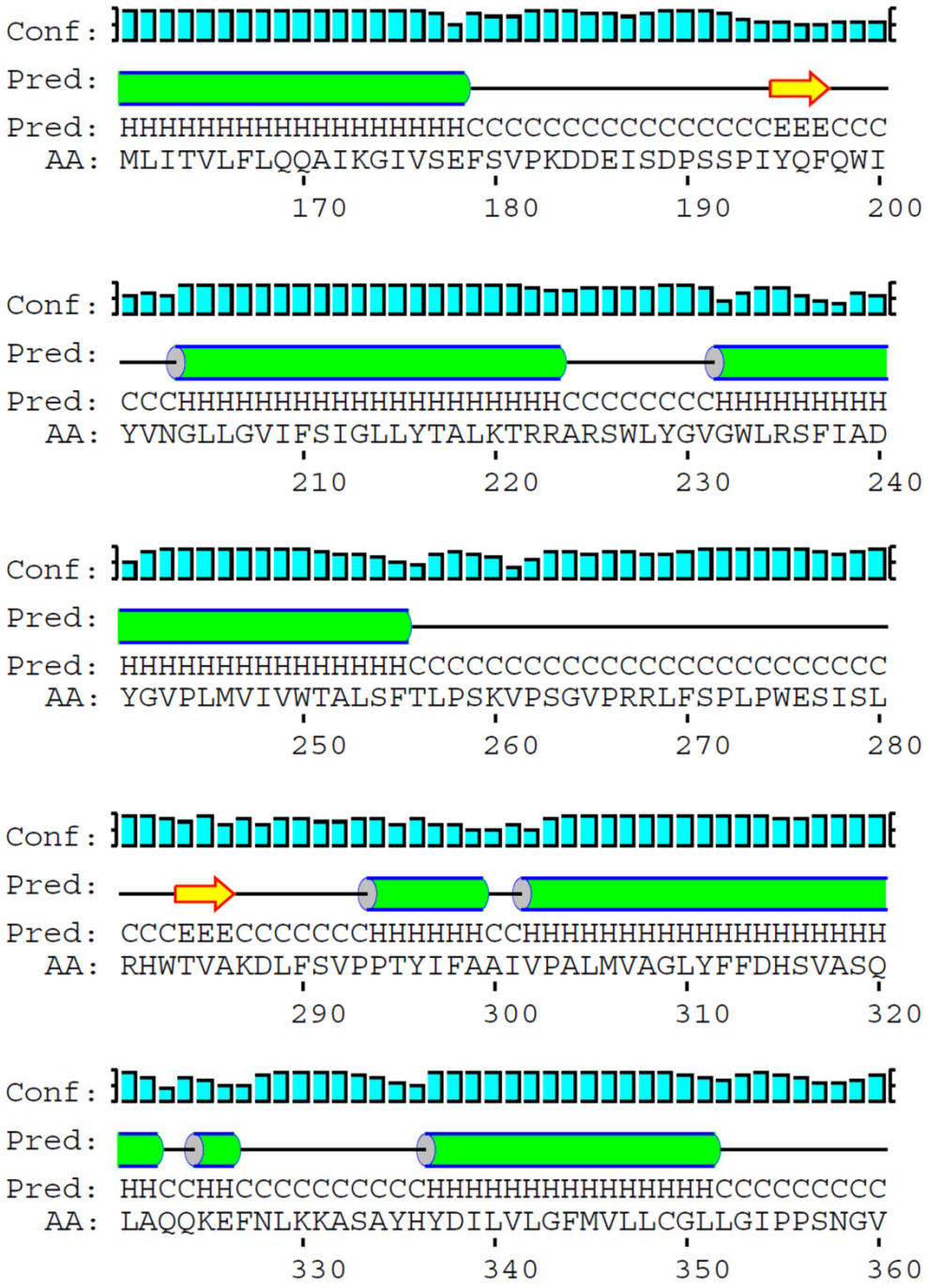

Figure J.1. (continued)

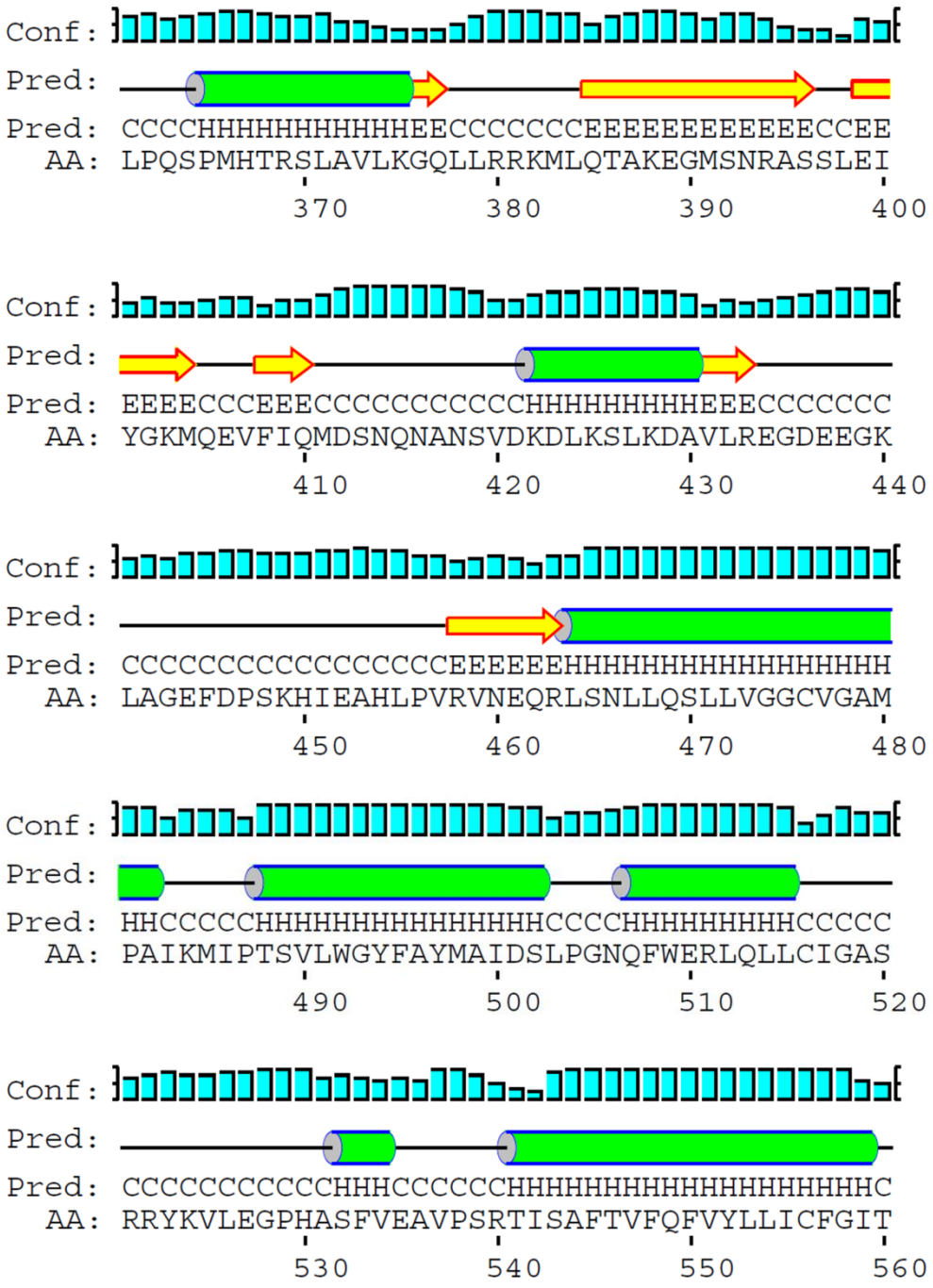

Figure J.1. (continued)

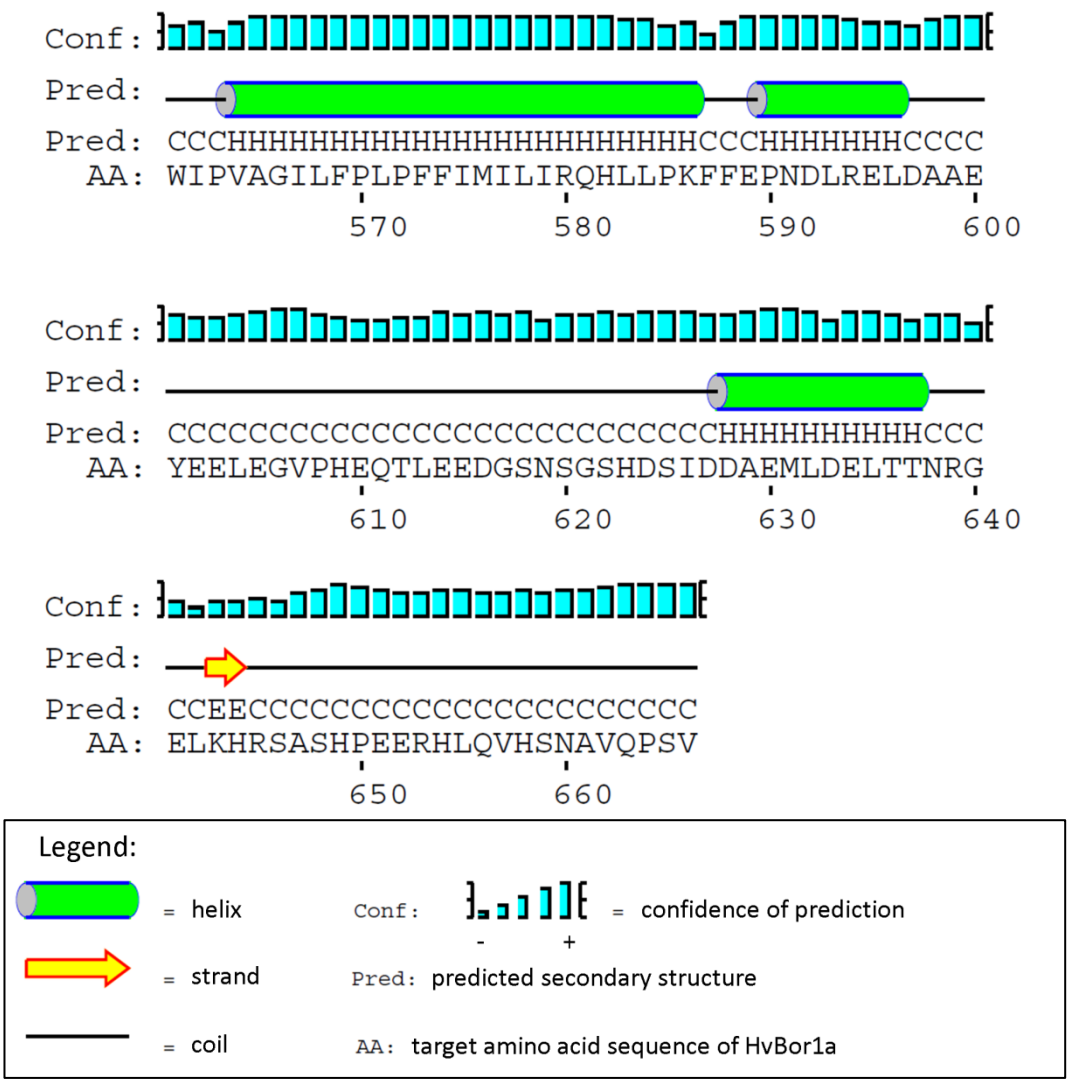

Figure J.1. (continued)

APPENDIX K

INTERPROSCAN RESULT SUMMARY

Table K.1. InterProScan result summary. Domain prediction was performed on amino acid sequence of HvBor1a using InterProScan (http://www.ebi.ac.uk/tools/pfa/iprscan). Numbers in brackets provide amino acid number in the sequence matching the predicted domain.

IPR003020 Bicarbonate transporter, eukaryotic			
Method	**Identifier**	**Description**	**Matches**
PANTHER	PTHR11453	FAMILY NOT NAMED	• 0.0 [1-638]
Parent	No parent		
Children	• IPR001717 • IPR003024		
Found in	No entries		
Contains	• IPR011531 • IPR013769 • IPR018241		
GO terms	• GO:0005452 inorganic anion exchanger activity • GO:0006820 anion transport • GO:0016020 membrane		
IPR011531 Bicarbonate transporter, C-terminal			
Method	**Identifier**	**Description**	**Matches**
PFAM	PF00955	HCO3_cotransp	• 3.0E-34 [5-177] • 5.2E-19 [205-373] • 1.6E-13 [458-547]
Parent	No parent		
Children	No children		
Found in	• IPR001717 • IPR003020 • IPR003024		
Contains	• IPR018241		
GO terms	• GO:0006820 anion transport • GO:0016021 integral to membrane		

Table K.1. (continued)

noIPR unintegrated			
Method	**Identifier**	**Description**	**Matches**
GENE3D	G3DSA:1.10.287.570	no description	• 1.7E-5 [18-59]
PANTHER	PTHR11453:SF6	ANION EXCHANGE PROTEIN-RELATED	• 0.0 [1-638]
TMHMM	tmhmm	transmembrane-regions	• -1.0 [36-54] • -1.0 [64-84] • -1.0 [89-107] • -1.0 [121-139] • -1.0 [154-172] • -1.0 [199-219] • -1.0 [234-254] • -1.0 [293-313] • -1.0 [334-354] • -1.0 [550-579]
Parent	No parent		
Children	No children		
Found in	No entries		
Contains	No entries		
GO terms	None		

APPENDIX L

POTENTIAL TRANSMEMBRANE SEGMENTS OF HvBor1a

Table L.1. Potential transmembrane segments of HvBor1a. The prediction was done with DAS (Dense Alignment Surface) transmembrane prediction server.

Start	Stop	Length (aa)	DAS score cutoff
37	52	16	1.7
41	49	9	2.2
74	96	23	1.7
77	82	6	2.2
84	84	1	2.2
91	93	3	2.2
98	102	5	1.7
124	143	20	1.7
127	142	16	2.2
158	169	12	1.7
159	168	10	2.2
201	217	17	1.7
203	216	14	2.2
241	255	15	1.7
243	254	12	2.2
295	312	18	1.7
297	310	14	2.2
338	354	17	1.7
339	353	15	2.2
469	479	11	1.7
471	474	4	2.2
489	497	9	1.7
544	580	37	1.7
546	579	34	2.2

APPENDIX M

MULTIPLE NUCLEIC ACID SEQUENCE ALIGNMENT

Table M.1. Multiple sequence alignment of *HvBor1a* and B transporter genes. Alignment was performed with ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2).

```
HvBor1a           ATG------GATCTACTAGGGAAC---------CCTTTCAAGGGAGTCGTCGCGGATGTC 45
TaBOR2            ATG------GATCTACTAGGGAAC---------CCTTTCAAGGGGGTCGTCGCCGATGCC 45
Bot1_HvBor1b      ATG------GATCTACTGAGGAAC---------CCCTTCAAGGGAGTGGTCGCAGATGTC 45
BRADI2G04690      ATG------GATTTACTAAGGAAC---------CCCTTCAAGGGAGTCGTCGCCGATGTC 45
OsBOR3            ATG------GATCTACTAAGGACT---------CCCTTTAAGGGAGTCGTCGCTGATATC 45
OsBOR4            ATGACGG--GAACTG-TGAAAGCC---------CCATTTGAGGGAGTGGTTAATGATTTC 48
AtBOR7            ATGGAGGG----AGTTAAATT--C---------CCATTTGGTGGGATTATTAACGATTTC 45
AtBOR6            ATGAAGAGTGAAGGAGAAAGTGGT---------CCATTTCAGGGAATTCTTCGAGATATC 51
AtBOR4            ATGGAGGAAGAAAGAGTGGATAGCTCGAAGAGGCTATTCAGAGGTATAGTAGCAGATCTT 60
AtBOR5            ATGGAGGAAGAAAGAGTGGAAGGCTCGAAAAGGCCATTTCAAGGTATCATAAGAGATGTC 60
AtBOR1            ATG------GA------AGAGACTTTTGT---GCCGTTTGAAGGAATCAAGAATGATCTT 45
AtBOR2            ATG------GA------AGAGACTTTTGT---TCCGTTTGAAGGTATCAAGAATGATCTT 45
OsBOR1            ATG------GA------GGAGAGCTTCGT---GCCCCTCCGTGGCATCAAGAACGACCTC 45
AtBOR3            ATG------GACGAAGCAGAGAGCTTTGT---CCCGTTTCAGGGTATAAAGAAAGATGTC 51
                  ***                               *   *    **  *       **

HvBor1a           AAAGGGAGAGCATCTTGGTACAAGGACGATTGGGTTGCAGGGCTCCGAACTGGCTTCAGG 105
TaBOR2            CCAGGGAGAGCGTCTTGGTACAAGGACGACTGGGTTGCAGGGCTCCGAACTGGCTTCAGG 105
Bot1_HvBor1b      AAAGGGAGAGCGCCTTGGTACAAGGACGACTGGCTTGCAGGGCTCCGAGCTGGCTTCGGG 105
BRADI2G04690      AAAGGGAGAGCATCTTGGTACAAGGACGACTGGGTTGCTGGGCTCCGTGCTGGTTTCAGG 105
OsBOR3            GAAGGCCGAGTAGCTTGGTACAAGCATGACTGGGTTGCTGGATTCCGCTCTGGCTTCAGG 105
OsBOR4            AAAGGAAGATTATCTTGCTACAAACAAGATTGGATAGATGGGTTCCGTACTGGATTCAGG 108
AtBOR7            AATGGAAGAAGAAAGTGTTACAAACAAGACTGGCTTGCTGCCTTCAATTCTGGTGTTAGG 105
AtBOR6            GAAGGTAGACGAAAATGTTACAAACAAGATTGGATTCGTGGCATAAAAACCGGTATTAGA 111
AtBOR4            AGAGGAAGAGCCTTGTGTTACAAGGAAGACTGGGTCGCTGGTCTCCGTTCTGGTTTCGGG 120
AtBOR5            AAAGGAAGAGCCTTGTGTTACAAGCAAGACTGGATCGCTGGTCTACGTTCTGGTTTCGGG 120
AtBOR1            AAAGGAAGATTGATGTGCTATAAGCAAGATTGGACTGGTGGATTCAAAGCTGGATTTAGG 105
AtBOR2            AAAGGAAGGTTAATGTGCTACAAACAAGATTGGACCGGAGGAATCAAAGCTGGGTTTAGG 105
OsBOR1            CATGGGAGGCTCCAGTGCTACAAGCAGGATTGGACCGGAGGATTCCGCGCCGGTATCAGG 105
AtBOR3            AAAGGGAGACTCAACTGCTACAAGCAAGATTGGATCAGTGGTCTCAGAGCTGGATTTAGG 111
                     **  *       ** ** **  * ** ***      *   *     * **  *  *

HvBor1a           ATATTGGCACCTACCATGTATATTTTCTTTGCCTCTGCACTCCCTGTAATCTCCTTCGGA 165
TaBOR2            ATATTGGCACCTACCATGTATATATTCTTTGCCTCTGCACTCCCTGTCATCTCCTTCGGA 165
Bot1_HvBor1b      ATATTGGCACCTACCATGTACATATTCTTTGCCTCCGCGCTCCCTGTCATCGCCTTCGGA 165
BRADI2G04690      ATATTGGCCCCTACCATGTATATCTTTTTTGCCTCTGCACTCCCCGTCATCGCCTTTGGA 165
OsBOR3            ATATTGGCACCTACCATGTATATCTTCTTTGCCTCTGCCCTCCCAGTCATCGCCTTCGGA 165
OsBOR4            ATATTGGCACCTACATTGTATATCTTCTTTGCATCTGCACTACCTGTTGTTGCCTTTGGG 168
AtBOR7            ATACTGGCTCCAACTCTCTATATTTTCATTGCCTCTGCACTACCTGTCATTGCATTCGGC 165
AtBOR6            ATTTTGGCTCCGACTTGCTATATTTTCTTTGCGTCGTCTCTTCCTGTAGTTGCCTTTGGT 171
AtBOR4            ATTTTAGCACCCACAACATATATTTTTTTCGCTTCCGCTCTTCCGGTTATCGCCTTTGGG 180
AtBOR5            ATTTTAGCACCGACAACATATGTTTTCTTCGCCTCTGCGCTTCCTGTTATTGCCTTTGGC 180
AtBOR1            ATTCTGGCTCCCACCACTTACATATTTTTCGCTTCTGCGATTCCTGTCATCTCATTTGGT 165
AtBOR2            ATTCTGGCTCCTACCACATACATATTCTTCGCGTCTGCGATTCCTGTTATTTCATTCGGC 165
OsBOR1            ATCCTTGCGCCGACCACCTACATATTCTTCGCTTCGGCCATACCGGTGATATCGTTCGGA 165
AtBOR3            ATTCTGGCACCAACAACTTACATATTCTTTGCATCCGCTATTCCGGTGATTACATTTGGA 171
                  **  * ** ** **    **  * **  * ** **  *  * ** **  *  * ** **

HvBor1a           GAGCAGCTGAGCAACGAAACAG---------------------------------------- 187
TaBOR2            GAGCAGCTGAGCAACGAAACAG---------------------------------------- 187
Bot1_HvBor1b      GAGCAGCTCAGCAACGAAACAA---------------------------------------- 187
BRADI2G04690      GAGCAGCTGAGCAGAGAAACAA---------------------------------------- 187
OsBOR3            GCGCAACTGAGCAGAGAAACAA---------------------------------------- 187
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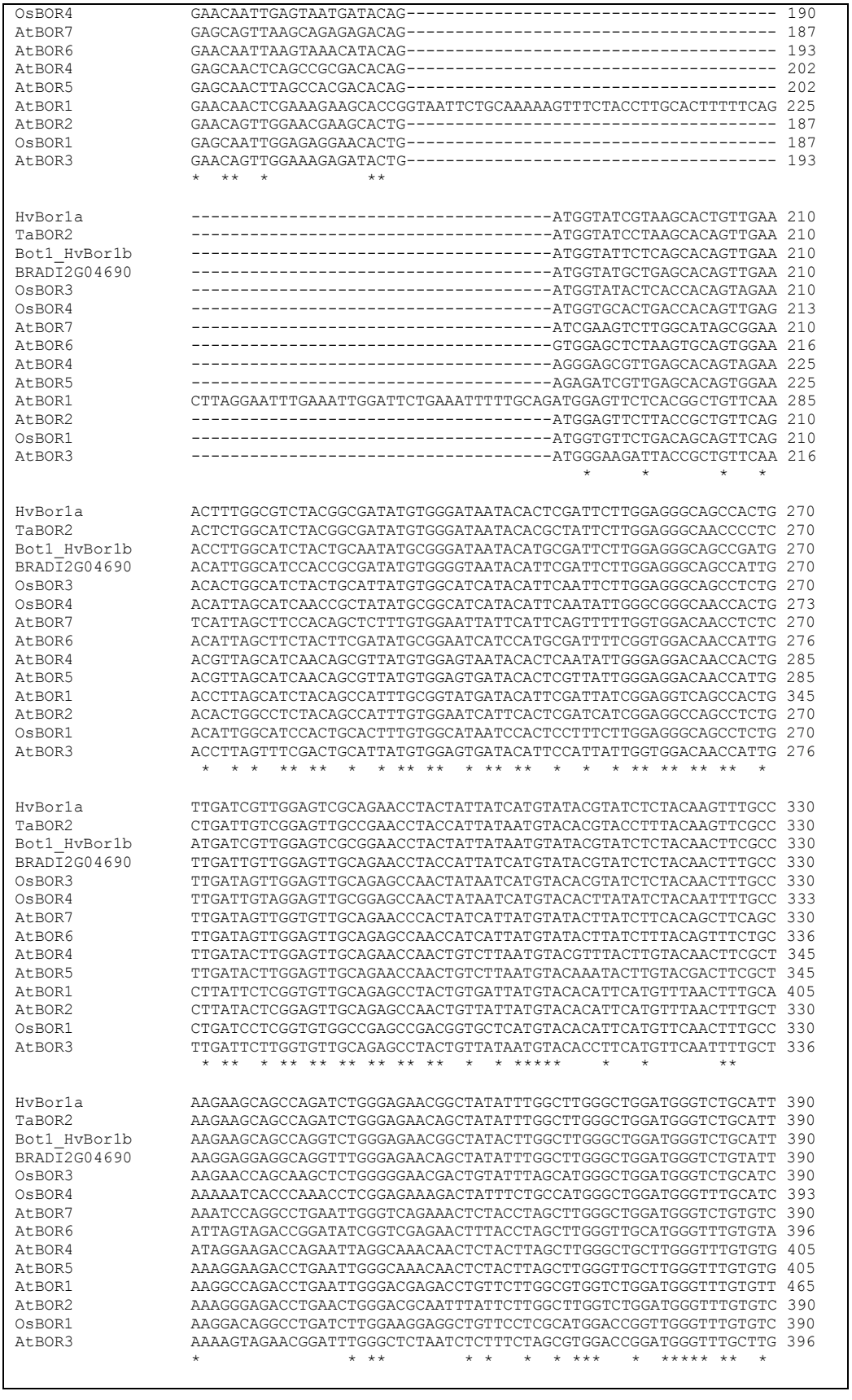
OsBOR4 GAACAATTGAGTAATGATACAG---------- 190
AtBOR7 GAGCAGTTAAGCAGAGAGACAG---------- 187
AtBOR6 GAACAATTAAGTAAACATACAG---------- 193
AtBOR4 GAGCAACTCAGCCGCGACACAG---------- 202
AtBOR5 GAGCAACTTAGCCACGACACAG---------- 202
AtBOR1 GAACAACTCGAAAGAAGCACCGGTAATTCTGCAAAAAGTTTCTACCTTGCACTTTTTCAG 225
AtBOR2 GAACAGTTGGAACGAAGCACTG---------- 187
OsBOR1 GAGCAATTGGAGAGGAACACTG---------- 187
AtBOR3 GAACAGTTGGAAAGAGATACTG---------- 193
HvBor1a ----------ATGGTATCGTAAGCACTGTTGAA 210
TaBOR2 ----------ATGGTATCCTAAGCACAGTTGAA 210
Bot1_HvBor1b ----------ATGGTATTCTCAGCACAGTTGAA 210
BRADI2G04690 ----------ATGGTATGCTGAGCACAGTTGAA 210
OsBOR3 ----------ATGGTATACTCACCACAGTAGAA 210
OsBOR4 ----------ATGGTGCACTGACCACAGTTGAG 213
AtBOR7 ----------ATCGAAGTCTTGGCATAGCGGAA 210
AtBOR6 ----------GTGGAGCTCTAAGTGCAGTGGAA 216
AtBOR4 ----------AGGGAGCGTTGAGCACAGTAGAA 225
AtBOR5 ----------AGAGATCGTTGAGCACAGTGGAA 225
AtBOR1 CTTAGGAATTTGAAATTGGATTCTGAAATTTTTGCAGATGGAGTTCTCACGGCTGTTCAA 285
AtBOR2 ----------ATGGAGTTCTTACCGCTGTTCAG 210
OsBOR1 ----------ATGGTGTTCTGACAGCAGTTCAG 210
AtBOR3 ----------ATGGGAAGATTACCGCTGTTCAA 216
HvBor1a ACTTTGGCGTCTACGGCGATATGTGGGATAATACACTCGATTCTTGGAGGGCAGCCACTG 270
TaBOR2 ACTCTGGCATCTACGGCGATATGTGGGATAATACACGCTATTCTTGGAGGGCAACCCCTC 270
Bot1_HvBor1b ACCTTGGCATCTACTGCAATATGCGGGATAATACATGCGATTCTTGGAGGGCAGCCGATG 270
BRADI2G04690 ACATTGGCATCCACCGCGATATGTGGGGTAATACATTCGATTCTTGGAGGGCAGCCATTG 270
OsBOR3 ACACTGGCATCTACTGCATTATGTGGCATCATACATTCAATTCTTGGAGGGCAGCCTCTG 270
OsBOR4 ACATTAGCATCAACCGCTATATGCGGCATCATACATTCAATATTGGGCGGGCAACCACTG 273
AtBOR7 TCATTAGCTTCCACAGCTCTTTGTGGAATTATTCATTCAGTTTTTGGTGGACAACCTCTC 270
AtBOR6 ACATTAGCTTCTACTTCGATATGCGGAATCATCCATGCGATTTTCGGTGGACAACCATTG 276
AtBOR4 ACGTTAGCATCAACAGCGTTATGTGGAGTAATACACTCAATATTGGGAGGACAACCACTG 285
AtBOR5 ACGTTAGCATCAACAGCGTTATGTGGAGTGATACACTCGTTATTGGGAGGACAACCATTG 285
AtBOR1 ACCTTAGCATCTACAGCCATTTGCGGTATGATACATTCGATTATCGGAGGTCAGCCACTG 345
AtBOR2 ACACTGGCCTCTACAGCCATTTGTGGAATCATTCACTCGATCATCGGAGGCCAGCCTCTG 270
OsBOR1 ACATTGGCATCCACTGCACTTTGTGGCATAATCCACTCCTTTCTTGGAGGGCAGCCTCTG 270
AtBOR3 ACCTTAGTTTCGACTGCATTATGTGGAGTGATACATTCCATTATTGGTGGACAACCATTG 276
HvBor1a TTGATCGTTGGAGTCGCAGAACCTACTATTATCATGTATACGTATCTCTACAAGTTTGCC 330
TaBOR2 CTGATTGTCGGAGTTGCCGAACCTACCATTATAATGTACACGTACCTTTACAAGTTCGCC 330
Bot1_HvBor1b ATGATCGTTGGAGTCGCGGAACCTACTATTATAATGTATACGTATCTCTACAACTTCGCC 330
BRADI2G04690 TTGATTGTTGGAGTTGCAGAACCTACCATTATCATGTATACGTATCTCTACAACTTTGCC 330
OsBOR3 TTGATAGTTGGAGTTGCAGAGCCAACTATAATCATGTACACGTATCTCTACAACTTTGCC 330
OsBOR4 TTGATTGTAGGAGTTGCGGAGCCAACTATAATCATGTACACTTATATCTACAATTTTGCC 333
AtBOR7 TTGATAGTTGGTGTTGCAGAACCCACTATCATTATGTATACTTATCTTCACAGCTTCAGC 330
AtBOR6 TTGATAGTTGGAGTTGCAGAGCCAACCATCATTATGTATACTTATCTTTACAGTTTCTGC 336
AtBOR4 TTGATACTTGGAGTTGCAGAACCAACTGTCTTAATGTACGTTTACTTGTACAACTTCGCT 345
AtBOR5 TTGATACTTGGAGTTGCAGAACCAACTGTCTTAATGTACAAATACTTGTACGACTTCGCT 345
AtBOR1 CTTATTCTCGGTGTTGCAGAGCCTACTGTGATTATGTACACATTCATGTTTAACTTTGCA 405
AtBOR2 CTTATACTCGGAGTTGCAGAGCCAACTGTTATTATGTACACATTCATGTTTAACTTTGCT 330
OsBOR1 CTGATCCTCGGTGTGGCCGAGCCGACGGTGCTCATGTACACATTCATGTTCAACTTTGCC 330
AtBOR3 TTGATTCTTGGTGTTGCAGAGCCTACTGTTATAATGTACACCTTCATGTTCAATTTTGCT 336
HvBor1a AAGAAGCAGCCAGATCTGGGAGAACGGCTATATTTGGCTTGGGCTGGATGGGTCTGCATT 390
TaBOR2 AAGAAGCAGCCAGATCTGGGAGAACAGCTATATTTGGCTTGGGCTGGATGGGTCTGCATT 390
Bot1_HvBor1b AAGAAGCAGCCAGGTCTGGGAGAACGGCTATACTTGGCTTGGGCTGGATGGGTCTGCATT 390
BRADI2G04690 AAGGAGGAGGCAGGTTTGGGAGAACAGCTATATTTGGCTTGGGCTGGATGGGTCTGTATT 390
OsBOR3 AAGAACCAGCAAGCTCTGGGGGAACGACTGTATTTAGCATGGGCTGGATGGGTCTGCATC 390
OsBOR4 AAAAATCACCCAAACCTCGGAGAAAGACTATTTCTGCCATGGGCTGGATGGGTTTGCATC 393
AtBOR7 AAATCCAGGCCTGAATTGGGTCAGAAACTCTACCTAGCTTGGGCTGGATGGGTCTGTGTC 390
AtBOR6 ATTAGTAGACCGGATATCGGTCGAGAACTTTACCTAGCTTGGGTTGCATGGGTTTGTGTA 396
AtBOR4 ATAGGAAGACCAGAATTAGGCAAACAACTCTACTTAGCTTGGGCTGCTTGGGTTTGTGTG 405
AtBOR5 AAAGGAAGACCTGAATTGGGCAAACAACTCTACTTAGCTTGGGTTGCTTGGGTTTGTGTG 405
AtBOR1 AAGGCCAGACCTGAATTGGGACGAGACCTGTTCTTGGCGTGGTCTGGATGGGTTTGTGTT 465
AtBOR2 AAAGGGAGACCTGAACTGGGACGCAATTTATTCTTGGCTTGGTCTGGATGGGTTTGTGTC 390
OsBOR1 AAGGACAGGCCTGATCTTGGAAGGAGGCTGTTCCTCGCATGGACCGGTTGGGTTTGTGTC 390
AtBOR3 AAAAGTAGAACGGATTTGGGCTCTAATCTCTTTCTAGCGTGGACCGGATGGGTTTGCTTG 396

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HvBor1a             TGGACTGCTATCATGCTGTTTCTTTTGGCAATGTTCAATGCTTCCAATGTTATAAGCAGA 450
TaBOR2              TGGACTGCTATCATGCTCTTTCTGTTGGCAATGTTCAATGCTTCCAATGTTATAAGCAGA 450
Bot1_HvBor1b        TGGACTGCTATCATGTTGTTTCTCTTGGCAATGTTCAATGCTTCCAATGTTATAAGCAGA 450
BRADI2G04690        TGGACTGCTATCATGTTGTTTTTTCTGGCAATGTTCAATGCTTCCAATGTGATTAGCAGA 450
OsBOR3              TGGACTGCGCTCATGTTGTTTCTTCTGGCAATGTTCAATGCTTCTAATGTTATTAGCAGG 450
OsBOR4              TGGACTGCCTTCATGCTATTCCTCATGGCAATGTTTAATGCTGCAGTCGTTATAAACAGA 453
AtBOR7              TGGACAGCAGTTTTGCTTATGCTTCTTGCTATGTTAAACGCATGCAACATCATTTCTAGG 450
AtBOR6              TGGACTTCAGTTTTGCTTATCCTTCTTTCGATATTTAACGCGGGCACAATCATCACGAGG 456
AtBOR4              TGGACGGCTCTGTTACTGTTCGTAATGGCAATCCTCAATACGGCTGATATCATTAACCGG 465
AtBOR5              TGGACGGCTTTGTTACTATTCCTAATGGCGATATTCAACATGGCTTATATCATCAACCGG 465
AtBOR1              TGGACTGCTTTGATGCTGTTTGTGTTGGCAATATGTGGAGCTTGTTCTATCATCAATAGG 525
AtBOR2              TGGACTTCGTTGATACTGTTTGTGTTGGCTATATGTGGAGCTTGTTCATTTATCAACAGA 450
OsBOR1              TGGACAGCAATTTGCTGTTCTTGCTGGCGATACTAGGCGCGTGCTCGATCATCAACCGG 450
AtBOR3              TGGACGGGGCTGTTGTTGTTCTTGTTAGCTGTATTAGGCGCTTGCACCTTCATCAATCGA 456
                    *****     *  *  *  *  *  *  *  *                  * **     *

HvBor1a             TTCACGAGGGTTGCAGGAGAGCTTTTTGGTATGTTGATCACTGTCCTGTTCCTGCAGCAA 510
TaBOR2              TTCACGAGGGTTGCAGGAGAGCTTTTTGGTATGCTTATCACCGTCCTCTTCCTGCAACAA 510
Bot1_HvBor1b        TTCACGAGGGTTGCAGGAGAACTTTTTGGGATGTTGATCACCGTTCTCTTCCTGCAAGAA 510
BRADI2G04690        TTTACAAGGGTTGCAGGAGAACTATTTGGGATGTTGATCACTGTTCTGTTCCTGCAACAA 510
OsBOR3              TTTACAAGGGTTGCAGGAGAACTTTTTGGGATGTTGATTACTGTTCTGTTCCTGCAACAA 510
OsBOR4              TTTACAAGGTTTGCTGGAGAACTATTTGGAATGTTGATTACGATTTTGTTCATGCAGGAA 513
AtBOR7              TTTACAAGAATTGCAGGAGAGCTCTTTGGAATGCTCATAACTGTTCTTTTCATCCAAGAG 510
AtBOR6              TTTACGAGAATCGCTGGGGAACTTTTTGGCATGTTGATTGCTGTTCTGTTTTTACAAGAA 516
AtBOR4              TTTACGAGGGTTGCTGGTGAGCTGTTTGGTATGTTGATCTCCGTTCTGTTCATTCAACAA 525
AtBOR5              TTCACGAGGATCGCTGGTGAGCTGTTTGGTATGTTGATCGCTGTTCTATTTCTCCAACAA 525
AtBOR1              TTTACTCGAGTAGCTGGAGAATTGTTTGGACTGCTTATTGCTATGCTTTTCATGCAGCAA 585
AtBOR2              TTCACCAGAGTAGCTGGAGAATTGTTTGGCCTTCTTATAGCTATGCTCTTCATGCAACAA 510
OsBOR1              TTCACCCGCATCGCGGGTGAGCTGTTTGGGCTCCTGATTGCAATGCTCTTCATGCAGCAG 510
AtBOR3              TTCACTCGACTTGCTGGGGAACTGTTTGGTATTCTAATAGCCATGCTTTTCATGCAAGAA 516
                    ** **  *  * ** ** **  * *****  *  * **  *  *  * **  * **  *

HvBor1a             GCTATCAAGGGAATTGTAAGTGAGTTCAGTGTGCCGAAAGATGATGAGATTTCTGACCCC 570
TaBOR2              GCTATCAAGGGAATTGTAAGCGAGTTCAGTATGCCGAAAGATGATGAGATTTCTGACCCC 570
Bot1_HvBor1b        GCTATCAAGGGAATCGTGGGCGAATTCAGTATGCCGAAAGATGCTGAGATATTTGACCGC 570
BRADI2G04690        GCAATCAAGGGAATTGTAAGTGAGTTCAGTTTGCCTAAAGCGGCTGAAATTGTTGACCGC 570
OsBOR3              GCCATTAAAGGAATTATAGAGGAATTCAAGGTGCCTAGAGATG---------CAGACCAT 561
OsBOR4              GCAGTCAAGGGGATGTTGGGTGAATTCAGTGTGCCCGAGGGTAA---------AGACCAC 564
AtBOR7              GCTGTTAAGGGACTTATCGGCGAGTTTCTTGTCCCAAAATCTGA---------CGATCCA 561
AtBOR6              GCTATAAAGGGATTGATCAGTGAGTTTCACGCCCCCGAGATTAA---------AAATCAA 567
AtBOR4              GCCATTAAGGGTATGGTGAGTGAGTTTGGGATGCCAAAAGATGA---------GGACTCA 576
AtBOR5              ACCATAAAGGGAATGGTGAGTGAATTTAGGATTCCAAAAGGTGA---------AGACTCA 576
AtBOR1              GCCATCAAAGGGCTAGTTGATGAATTCCGCATTCCTGAACGAGA---------AAATCAG 636
AtBOR2              GCCATCAAAGGATTAGTCGATGAATTTCGCGCTCCTGCGAGAGA---------GGATCTG 561
OsBOR1              GCTATCAAGGGGCTTGTTGACGAGTTCCGCATCCCTGAGAGGGA---------GAACAGA 561
AtBOR3              GCCATTAGAGGCATTGTGGATGAGTTTGGTGTCCCCGGAAGAAC---------AAATCCA 567
                     *  * *  **  *  *    ** **       **                   *

HvBor1a             AGCTCACCTATATACCAGTTCCAGTGGATTTATGTCAATGGCCTACTTGGTGTTATATTT 630
TaBOR2              AGCTCACCCATATACCAGTTCCAGTGGATTTATGTCAATGGCCTACTTGGTGTTATATTT 630
Bot1_HvBor1b        AGTTCGCCGATATACCAGTTCCAATGGATATATGTCAATGGCCTACTTGGGGTTATCTTC 630
BRADI2G04690        AGTTCACTGATATATGATTTCCAGTGGATATACGTCAATGGCCTGCTTGGTGTCATTTTT 630
OsBOR3              AGTTCACCTATATATCAATTCCAGTGGCTGTATGTCAACGGCCTGCTTGGTGTTATCTTT 621
OsBOR4              AGTCTACCGATATACCAATTCCAATGGGCTTATGTTAATGGTCTGCTTGGAATTATCTTT 624
AtBOR7              AGTTTGGAAGTGTATCAGTTCCAGTGGCGGTATACCAATGGTCTGCTTGCAGTCATTTTC 621
AtBOR6              GAAACAGGGAAATCTCATTTCCTCTTGATTTATGCAAATGGTTTGCTTGCGGTAATTTTC 627
AtBOR4              AAACTAGAAAAGTATAAGTTTGAGTGGCTCTATACAAACGGACTTCTCGGCCTCATTTTC 636
AtBOR5              AAACTTGAAAAGTATCAGTTTGAGTGGCTCTACACAAACGGACTTCTTGGCCTTATTTTC 636
AtBOR1              AAGCTGAAGGAGTTCTTACCTTCCTGGAGGTTTGCTAATGGGATGTTTGCTCTGGTTCTC 696
AtBOR2              AAACTTGTGGAGTTTTTACCTTCCTGGAGATTTGCAAATGGGATGTTTGCTTTGGTTCTT 621
OsBOR1              AAGGCATTAGAGTTTGTTTCATCATGGAGGTTCGCCAACGGAATGTTTGCTATCGTCTTG 621
AtBOR3              AGATCAGCTGAGTTTCAACCTGCTTGGGTGTTTGCAAATGGAATGTTCGGTTTGGTTTTG 627
                               *           * *   *     ** **  *  * *   *  *  *

HvBor1a             TCCATTGGCTTGCTGTACACTGCACTGAAGACTAGGCGTGCAAGGTCATGGCTGTATGGC 690
TaBOR2              TCCATTGGCTTGCTGTACACTGCACTGAAGACTAGGCGTGCAAGGTCATGGCTGTATGGC 690
Bot1_HvBor1b        TCAATTGGCCTGCTATACAGTGCACTCAAGACTAGGCGGGCAAGGTCATGGCTGTATGGT 690
BRADI2G04690        TCAATTGGCTTGCTATACACTGCATTGAAGAGTAGGCGGGCAAGGTCATGGCTGTATGGC 690
OsBOR3              TCAATTGGGCTGTTATACACTGCATTAAGGTCAAGAAGGGCAAGGTCATGGGTGTATGGT 681
OsBOR4              TCAATGGGCCTGCTATACACAGCAATACGTAGCAGGAGTGCAAGATCATCGCTATACGGC 684
AtBOR7              TCATTCGGTCTTCTTTACACTGCTCTGAAAAGCAGGAGGGCAAGATCATGGAAATATGGC 681
AtBOR6              TCGCTAGGCCTTCTAATCACCGCGCTAAAGAGTAGGAGAGCAAAATCTTGGAAATATGGT 687
AtBOR4              ACCTTTGGCCTTCTCTACACCGCTTTGAAGAGTCGAAAAGCAAGGTCTTGGCGATACGGA 696
AtBOR5              ACAGTCGGTCTTGTCTACACCGCTTTGAAGAGCAGAAAAGCAAGGTCTTGGCCATACGGA 696
AtBOR1              TCCTTTGGCCTTCTTCTGACTGGACTTAGAAGCAGAAAAGCCAGATCATGGCGGTACGGA 756
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AtBOR2              TCCTTTGGTCTTCTGATAACTGCACTTAGAAGCAGAAAAGCAAGATCATGGAGATATGGA 681
OsBOR1              TCGTTTGGCCTTTTGCTCACTGCACTGCGGAGCAGGAAGGCTCGATCATGGCGCTATGGA 681
AtBOR3              TCTTCTGGACTTCTCTATACTGGACTAAAAAGCCGAAAAGCAAGGTCTTGGAGATTTGGT 687
                     *    **  *  *    *  *   *        *    **    ** * *   *  **

HvBor1a             GTAGGATGGCTTAGAAGCTTCATTGCCGATTACGGTGTACCGCTGATGGTGATTGTGTGG 750
TaBOR2              ATAGGATGGCTTAGAAGCTTCATTGCCGATTATGGCGTACCGCTGATGGTGATTGTGTGG 750
Bot1_HvBor1b        ATAGGATGGCTTAGGAGCTTCATTGCCGATTATGGTGTCCCGCTTATGGTGATCGTGTGG 750
BRADI2G04690        ATAGGATGCCTTAGAAGCTTCATTGCCGATTATGGTGTCCCGCTAATGGTGATTGTGTGG 750
OsBOR3              CAAGGGTGGCTCAGAGGCTTCATTGCTGATTATGGTGTCCCACTTATGGTGATTGTTTGG 741
OsBOR4              ACTGGGTGGCAAAGAAGCTTCATTGCTGACTATGGCGTTCCACTCATGGTTGTAGTCTGG 744
AtBOR7              TTTAGGTGGATGCGAGGTTTTATCGGGGATTATGGAACTCTCCTCATGCTTGTGTTGTGG 741
AtBOR6              TTTGGGTGGCTTCGAAGTTTCATTGGAGATTACGGTGTTCCTTTAATGGTCTTGTTATGG 747
AtBOR4              ACAGGATGGTACAGAAGCTTCATCGCAGACTATGGAGTTCCTTTGATGGTTGTGGTTTGG 756
AtBOR5              ACAGGATGTTGCCGAAGCTTCGTTGCAGACTACGGAGTTCCGTTGATGGTTGTGGTTTGG 756
AtBOR1              ACTGGCTGGCTCAGAAGCTTAATAGCTGACTATGGTGTACCACTCATGGTGCTCGTGTGG 816
AtBOR2              ACAGGCTGGCTTAGAAGTTTAGTAGCTGACTATGGTGTGCCACTCATGGTTCTGGTGTGG 741
OsBOR1              ACAGGTTGGCTCCGTGGCTTCATCGCCGACTATGGTGTCCCACTGATGGTGCTAGTATGG 741
AtBOR3              GCTGAATGGTTACGTGGGTTTATAGCAGATTATGGTGTTCCAGTAATGGTGGTAGTGTGG 747
                           **     *  * **  * *  ** ** **    *   * *** *  *  * ***

HvBor1a             ACAGCATTGTCATTTACACTACCAAGCAAAGTCCCTTCAGGAGTGCCTAGGAGGCTCTTC 810
TaBOR2              ACAGCATTTTCATTTACACTACCAAGCAAAGTCCCTTCAGGAGTGCCTAGGAGGCTCTTC 810
Bot1_HvBor1b        ACGGCATTTTCGTACGCGCTACCGAGCGGGGTCCCTTCAGGAGTGCCTAGGAGACTCTTC 810
BRADI2G04690        ACAGCATTATCGTACACACTACCAAGCAAAGTCCCTTCAGGAGTGCCTCGGAGGCTCTTC 810
OsBOR3              ACAGCATTCTCGTACACGTTACCAAAGGATGTTCCTTCAGGAGTTCCTAGGAGGCTGTTC 801
OsBOR4              ACAGCTTTGTCGTATTCATTGCCAAGCAAGATCCCTTCAGGGGTTCCTAGGAGGCTCTTC 804
AtBOR7              AGCGCATTTTCATACACAGTCCCTAGAAACCTTCCTGAAGGAGTTCCAAGGAGGCTGGAA 801
AtBOR6              ACGGCATTGTCTTACACAGTTCCTAGTGAAGTCCTTCCAAGTGTTCCCCGGAGACTGTTT 807
AtBOR4              ACAGCATTGTCTTTTAGTACACCATCAAAACTCCCCTCTGGTGTCCCGAGAAGACTCTTT 816
AtBOR5              ACAGCATTGTCTTTCAGTACGCCATCAAAACTACCCTCTGGTGTCCCGAGAAGACTCGTT 816
AtBOR1              ACCGGTGTCTCCTACATTCCAGCAGGAGATGTTCCAAAAGGAATTCCTCGGCGACTTTTT 876
AtBOR2              ACCGGTGTCTCCTACATCCCAACAGGAGATGTTCCAAAAGGAATTCCTCGGCGACTTTTT 801
OsBOR1              ACAGGAGTTCCTACATTCCATATGGTAGTGTTCCAAAAGGAATTCCACGGCGCCTTTTC 801
AtBOR3              ACTTGTATATCATACATACCTTGGAAAAGTGTTCCTCAAGGGATACCAAGACGTCTTGTT 807
                    *      * ** *                  * *      *  * **  *  * **

HvBor1a             AGTCCACTTCCCTGGGAGTCAATCTCACTGAGACATTGGACCGTAGCAAAGGATTTGTTT 870
TaBOR2              AGTCCACTTCCCTGGGAGTCAATCTCACTGCGACATTGGACCGTAGCAAAGGATTTGTTT 870
Bot1_HvBor1b        AGTCCACTTCCTTGGGAGTCAAGTTCATTGGGTCATTGGACCGTAGCAAAGGATTTGTTT 870
BRADI2G04690        AGTCCACTTCCCTGGGAGTCAAGTTCACTGGGACATTGGACCGTAGCAAAGGATCTGTTT 870
OsBOR3              AGTCCACTTCCCTGGGAGTCGAGTTCACTGCAACATTGGACGGTAGCAAAAGATCTGTTT 861
OsBOR4              ACCCCACTTCCTTGGGAACCAAAGTCATTGCAGCATTGGACAGTAGCAAAGGATTTGTTT 864
AtBOR7              TTGCCACTTCCTTGGGCATCCGAGTCCTTGTATCACTGGACAGTCGTCAAGGATATGGCG 861
AtBOR6              TGTCCTCTTCCATGGGAGCCAGCTTCATTGTATCATTGGACTGTAGTCAAGGACATGGGG 867
AtBOR4              AGTCCTTTACCATGGGACTCTCCTTCTTTATCACATTGGACTGTCATCAAGGACATGGGA 876
AtBOR5              AGTCCTCTTCCATGGGACTCTGTTTCTTTAACACATTGGACTGTCATCAAGGACATGGGT 876
AtBOR1              AGCCCAAATCCTTGGTCTCCTGGTGCTTATGGGAACTGGACCGTAGTAAAGGAGATGCTT 936
AtBOR2              AGCCCAAATCCTTGGTCCCCTGGTGCTTATGAGAATTGGACTGTTGTAAAGGAGATGCTT 861
OsBOR1              AGCCCCAACCCATGGTCCCCTGGTGCATATGATAATTGGACAGTCATCAGGGACATGCCA 861
AtBOR3              AGTCCTAATCCATGGTCTCCTGGTGCATATCAGAATTGGACTGTCATTAAGGAGATGGTG 867
                       **    ** ***    *     *        * ***** **    *  **  **

HvBor1a             TCTGTCCCTCCAACATATATATTTGCAGCCATCGTGCCTGCTTTGATGGTCGCAGGACTT 930
TaBOR2              TCTGTTCCTCCAACATATATATTTGCAGCCATCGTGCCTGCTTTGATGGTCGCAGGACTT 930
Bot1_HvBor1b        TCTGTCCCTCCGGCATATATATTTGCAGCCATCGTGCCAGCTTTGATGGTTGCGGGACTC 930
BRADI2G04690        TCTGTCCCTCCGGCATTTATATTTGCAGCCATCGTGCCTGCTTTGATGGTTGCAGGGCTT 930
OsBOR3              TCAGTTCCTCCAGCATACATATTTGCTGCCATTCTGCCGGCTTTGATGGTTGCAGGACTT 921
OsBOR4              TCTGTCCCTCCACCATACATATTTTTGGCTATTGTGCCTGCTGTAATGGTTGCCGGGCTC 924
AtBOR7              AAGGTCCCGCCTCTTTACATCCTTGCTGCATTTATACCAGCAATCATGATCGCTGGCCTA 921
AtBOR6              AAGGTACCGATAATGTATATCTTAGCTGCGTTTATACCTGGTGTGATGATAGCAGGACTT 927
AtBOR4              AAAGTCTCTCCGGGTTACATATTTGCGGCATTTATACCCGCATTGATGATCGCAGGGCTT 936
AtBOR5              AAAGTCTCTCCCGGTTACATATTTGCAGCGTTTATACCCGCATTGATGATCGCAGGCCTC 936
AtBOR1              GATGTTCCAATCGTCTACATAATTGGAGCTTTCATTCCAGCATCAATGATTGCTGTGCTT 996
AtBOR2              CAAGTTCCAATTGTCTACATAATTGGAGCATTCATTCCAGCAACAATGATTGCAGTTCTT 921
OsBOR1              AATGTGCCACTCCTCTACATTATTGGTGCCTTCATACCAGCAACGATGATAGCCGTCCTG 921
AtBOR3              GATGTACCTGTGCTTTACATTCTTTTAGCGGTTGTTCCAGCGTCAATGATTGCGGTTCTT 927
                       **  *       *  **  *    **  *  * ** *     *** * ** *  **

HvBor1a             TATTTCTTTGACCACAGTGTAGCTTCACAGTTGGCTCAGCAGAAGGAGTTTAATTTGAAG 990
TaBOR2              TATTTCTTTGATCACAGTGTAGCTTCACAGTTGGCTCAGCAAAAGGAGTTCAATTTGAAG 990
Bot1_HvBor1b        TACTTCTTTGATCATAGTGTAGCTTCACAGTTGGCTCAGCAACAGGAGTTCAATCTGAAG 990
BRADI2G04690        TATTTCTTTGACCACAGTGTAGCTTCACAGTTGGCGCAGCAAAAGGAGTTCAATTTGAAG 990
OsBOR3              TACTTCTTTGATCATAGCGTGGCTTCACAATTGGCGCAACAAAAAGAGTTTAACTTGAAG 981
OsBOR4              TATTTCTTTGATCATAGTGTAGCTTCACAACTGGCTCAGCAGAAGGAGTTTAATTTGAAA 984
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AtBOR7              TACTTCTTTGATCACTGTGTATCTGCACAAATGGCTCAGCAAAAGGAGTTCAATCTCAAA 981
AtBOR6              TACTTCTTCGACCATAGTGTAGCTTCACAAATGGCACAACAGAAAGAGTTTAATCTAAAG 987
AtBOR4              TACTTCTTTGACCACAGCGTTGCCTCGCAGCTCGCTCAGCAGAAGGAGTTCAACCTCAAG 996
AtBOR5              TACTTCTTTGACCACAGCGTTGTCTCGCAGCTCGCGCAGCAGAAGGAGTTTAACCTCAAG 996
AtBOR1              TACTACTTCGACCATAGTGTAGCTTCACAGCTCGCGCAGCAGAAAGAATTCAATTTGAGA 1056
AtBOR2              TACTACTTCGATCATAGTGTAGCATCACAGCTTGCGCAGCAGAAAGAATTCAATTTGAGA 981
OsBOR1              TACTACTTCGATCACAGTGTTGCTTCTCAGCTTGCTCAGCAGAAGGAGTTCAATTTGAGG 981
AtBOR3              TACTACTTTGACCATAGTGTAGCCTCGCAGCTCGCACAGCAGGAAGATTTCAATCTGAGA 987
                    ** * *** ** **  * **     * **  * ** ** **  * ** ** **  * *

HvBor1a             AAGGCTTCTGCCTACCATTATGACATTTTGGTACTTGGATTCATGGTCCTACTATGTGGT 1050
TaBOR2              AAGCCTTCTGCCTACCATTATGACATTTTGGTACTTGGATTCATGGTCCTACTATGTGGT 1050
Bot1_HvBor1b        AAACCTTCCGCCTACCATTACGACATTTTGGTACTTGGATTCATGGTCCTACTGTGTGGT 1050
BRADI2G04690        AAGCCTTCTGCCTACCATTACGACATTTTGGTACTTGGGTTCATGGTACTACTATGTGGT 1050
OsBOR3              AAACCCTCTGCCTACCATTATGACATTTTGGTCCTCGGATTCATGGTCCTGCTTTGTGGT 1041
OsBOR4              AACCCATCGGCTTACCATTATGACATTTTGGTCCTCAGCTTTATGGTCTTGATTTGTGGT 1044
AtBOR7              AATCCGACGGCTTATCACTATGATATCTTTATTTTAGGAATCATGACACTAATCTGTGGC 1041
AtBOR6              AATCCTTCTGCTTATCACTATGACATCTTCTTGCTTGGAATTATTACTTTGATATGTGGA 1047
AtBOR4              AAGCCTTCAGCGTATCACTATGACATTCTCTTGTTGGGTTTCATGACGTTGATATGTGGA 1056
AtBOR5              AACCCTTCTGCATATCACTACGACATTCTCTTGTTAGGTTTCATGGTATTGATCTGTGGA 1056
AtBOR1              AAACCGTCTTCTTACCACTATGACTTGCTTCTTCTTGGGTTTCTGACATTAATGTGTGGT 1116
AtBOR2              AAACCATCTTCTTACCACTATGATCTGCTTCTTCTTGGATTTCTGACCTTAATGTGTGGT 1041
OsBOR1              AAGCCCCCATCTTTCCATTATGATTTGCTTCTCCTGGGTTTCCTGACATTATTGTGTGGC 1041
AtBOR3              AAGCCTCCTGCTTACCATTATGATCTGTTTCTTCTAGGTTTCTTGACAATCCTTTGCGGT 1047
                    **  *  *  * *  ** ** **  *  *  *  *  *  *  *     *  * ** **

HvBor1a             TTGCTTGGCATTCCCCCATCAAATGGAGTACTTCCTCAGTCCCCCATGCATACAAGAAGC 1110
TaBOR2              TTGCTTGGCATTCCTCCATCAAATGGAGTACTTCCTCAGTCCCCAATGCATACAAGAAGC 1110
Bot1_HvBor1b        TTAATTGGCATTCCTCCAGCAAATGGAGTACTTCCTCAGTCCCCCATGCATACAAGAAGC 1110
BRADI2G04690        TTGATTGGCATCCCTCCATCAAATGGAGTACTTCCTCAGTCCCCAATGCATACAAGAAGC 1110
OsBOR3              TTGATCGGCATCCCTCCATCTAATGGAGTACTTCCTCAGTCTCCGATGCATACAAGAAGC 1101
OsBOR4              CTTATTGGCATCCCTCCATCTAATGGAGTACTTCCCCAGTCCCCCATGCACACTAGAAGC 1104
AtBOR7              CTACTTGGACTTCCTCCTTCGAATGGGGTCATTCCACAATCCCCTATGCACACAAAGAGT 1101
AtBOR6              CTACTTGGACTTCCACCTTCAAATGGTGTTCTTCCTCAGGCTCCAATGCACACAAAGAGT 1107
AtBOR4              TTGCTAGGTCTGCCTCCTTCAAATGGAGTCCTCCCTCAGTCTCCTATGCATACCAAAAGC 1116
AtBOR5              ATGCTCGGTCTACCGCCTTCCAACGGAGTCCTCCCGCAGTCTCCTATGCATACCAAAAGC 1116
AtBOR1              CTACTTGGAGTCCCTCCATCAAACGGTGTCATTCCTCAATCTCCAATGCATACCAAGAGC 1176
AtBOR2              CTACTTGGAATTCCTCCATCAAATGGAGTCATCCCTCAATCACCAATGCATACTAAGAGC 1101
OsBOR1              CTTATTGGTATCCCTCCGGCGAATGGTGTCATTCCACAGTCTCCAATGCATACGAAGAGT 1101
AtBOR3              CTCATCGGAATTCCTCCATCCAATGGTGTCATCCCTCAGTCTCCAATGCACACAAAAAGC 1107
                     *  * **  * ** **  * ** ** **  * ** **  * ** ***** ** *  **

HvBor1a             CTTGCTGTCCTCAAGGGGCAGCTGCTACGCAGAAAGATGCTTCAAACTGCCAAAGAGGGC 1170
TaBOR2              CTTGCTGTCCTCAAGGGGCAGTTGCTACGCAAAAAGATGCTTCAAACTGCCAAAGAGGGC 1170
Bot1_HvBor1b        CTTGCTGTCCTCAAGGGGCAGCTAATGCGCAAAAGGATGCTTCGAACTGCCAAAGAAGGC 1170
BRADI2G04690        CTTGCTGTCCTCAAGGGTCAGTTGATGCGCAACAAGATGCTCCAAACTGCCAAAGAGGGC 1170
OsBOR3              CTTGCAGTCCTTAAGGGGCAGCTGCTACGCAAAAAGATGGTTCAAACTGCCAACGAAGGC 1161
OsBOR4              CTTGCCGTCCTCAAGGGGCAGTTGCTTCGCAAAAAGATGGTCCAAACTGCCAAGGAGGGT 1164
AtBOR7              CTTGCAGTTCTTAAGAAGCAGCAAATGAGAAAGAAAATGGTCCAGAAAGCAAAAGAATGC 1161
AtBOR6              CTTGCGGTTCTCAATCGTCAACTTATACGCAAGAAAATGGTGAAGAAAGCAAAGGAGTGT 1167
AtBOR4              CTTGCTGTTCTTAAACGACAGTTGATCCGGAGGAAGATGGTAAAGACAGCCAAAGAAAGC 1176
AtBOR5              CTAGCTGTTTTCAAACGACAGTTAATGCGGAGGAAGATGGTGATGACAGCCAAAGAAAGC 1176
AtBOR1              TTAGCAACTCTTAAATACCAGTTGCTTCGTAACAGACTGGTCGCAACAGCACGAAGAAGT 1236
AtBOR2              TTAGCAACACTAAAATATCAGCTGCTTCGGAACAGACTAGTTGCAACTGCTCGCAAAAGC 1161
OsBOR1              TTGGCTACTCTCAAACATCAACTACTCCGTAACCGACTAGTAGCCACAGCCCGACAAAGC 1161
AtBOR3              TTGGCAACACTGAATCACCAGTTACTTCGAAACAAACTCGTGGCAGCTGCGCGTAAATGC 1167
                     * **     * **    **     *  * *      *  *       **        *

HvBor1a             ATGTCAAACCGTGCGAGCAGTTTGGAAATCTATGGCAAGATGCAGGAAGTGTTCATCCAA 1230
TaBOR2              ATGTCAAACCGTGCGAGCAGTTTGGAAATCTATGGCAAGATGCAGGAAGTGTTCATCCAA 1230
Bot1_HvBor1b        ATGTCGAACCGTGCAAGCAGTTTGGAAATCTATGGAAAGATGCATGAAGTGTTCATCGAA 1230
BRADI2G04690        ATGACGAACCGTGCTAGCAGTTTGGAAATATATGGCAAGATGCAGGAAGTTTTCATCCAA 1230
OsBOR3              CTGATGAATCGTGCTAGCAGTTTGGAAATCTATGGCAAGATTCAAGGGGTTTTCATCGAG 1221
OsBOR4              ATGATGAACAATGCTAGCAGTTCAGAAGTTTATGGAAAGATGCAAGAAGTTTTTATCAAA 1224
AtBOR7              ATGAGGGAAAAAGCAAGTAACTCAGAGATCTATGGGAGGATGCAAGATGTGTTTATAGAA 1221
AtBOR6              ATGAAAATGAAAGCAAGCAAATCAGAAATATATGGGAGAATGCAATCAGTGTTTATAGAG 1227
AtBOR4              ATCAGGAAGAGAGAAACATCCTCACAAGTGTACGAGAATATGCAAGAAGTCTTCATAGAA 1236
AtBOR5              ATCAGACAGAAAGCAACGTCCTCTCAAGTGTACGAGGATATGGAACAAGTCTTCATAGAA 1236
AtBOR1              ATCAAAACAAATGCGAGTTTGGGTCAACTCTATGACAATATGCAAGAAGCTTACCATCAC 1296
AtBOR2              ATCAAGCAGAATGCGAGTCTAGGACAGTTGTATGGAAACATGCAAGATGTTTATAATCAA 1221
OsBOR1              ATGAGCCAGAATGCGAGCTTGAGCCAGCTGTATGGCAGCATGCAGGAAGCTTACCAGCAG 1221
AtBOR3              ATCAGAAACAATGCAACAATTGGAGAAGTCTATGGAAGCATGGAAGAAGCTTACCAACAA 1227
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HvBor1a           ATGGATAGCAACCAGAA-TGCTAA---------TTCTGTTGACAAGGACTTGAAGAGCTT 1280
TaBOR2            ATGGATAGCAACCAGAA-TGCTAA---------TTCTGTTGACAAGGACTTGAAGAGCTT 1280
Bot1_HvBor1b      ATGGATAATAAACAGGA-TGCTGA---------TTCTGTTGACAAGGACTTGAAGAGTTT 1280
BRADI2G04690      ATGGATAGAGAAAAGAA-TACTGA---------TTCTGTTGACAAGGAGTTGAAGAGTTT 1280
OsBOR3            ATGGACTGCGAAAAAAA-TACTGA---------TTCTGTTGACAAGGAGTTAAAGAGCTT 1271
OsBOR4            ATGGATGATAAATCCAA-TGCCAA---------ATCTGTACGCAAAGAGCTGAAGGAATT 1274
AtBOR7            ATGGAAACATCCCC-------TAAGG---CTACTTCAGTGGTGAAAGAGTTAGAAAACTT 1271
AtBOR6            ATGGAGACATCCCCAC----CTCAGG---ATAATTCTGTAGCAACAGATTTAAAAGAGTT 1280
AtBOR4            ATGGATAAAAGTCCACT-TGCTCAGACAGATCCATCAGTGATAATTGAGCTTCAAGATCT 1295
AtBOR5            ATGGACAAAAGCCCACT-TGCTGAGACACACACAACACTGATAAATGAGCTGCAAGATCT 1295
AtBOR1            ATGCAGACACCATTAGTATACCAGCA-------ACC------CCAAGGTCTAAAAGAGCT 1343
AtBOR2            ATGCAGACTCCATTAGTCTACCAACA-------GCC------TCAGGGTCTGAAAGAGCT 1268
OsBOR1            ATGCAGACACCACTGATTTACCAGCA-------ACCGTCAGTCAAGGGATTGAATGAGCT 1274
AtBOR3            ATGCAGAGCCCTCTGATACACCAAGA-------GCC-----TTCTCGGATTCAAGGA-CT 1274
                  *** *                              *          *   *  *     *

HvBor1a           GAAGGATGCTGTG-CTGCGGGAAGGT---------GACGAAGAAGGGAAATTGGCTGGAG 1330
TaBOR2            GAAGGATGCTGTG-CTGTGGGAAGGT---------GACGAAGAAGGGAAGTTGGCTGGAG 1330
Bot1_HvBor1b      GAAGGATGCTGTG-CTGCGTGAAGGC---------GACGAGGATGGAAAATTGGCTGGAG 1330
BRADI2G04690      GAAGGATGCTGTG-CTGAGAGAAGGC---------GACGAAGAAGGAAAATTGGCTGGAG 1330
OsBOR3            GAAGGATGCTATG-CTGCAAGAAGGT---------GATAAAGAAGGGACATTGGCTGAAG 1321
OsBOR4            GAAGGATGCAGTT-ATTCCAGAGGGA---------AATGGAGCAGGGAGGGTGTCTGAGG 1324
AtBOR7            GAAAGAAGCAGTG-ATGAAAGCAGAC---------GATGGAGGTGGAGAAACAAAGGGGA 1321
AtBOR6            GAAAGAAGTTGTG-ATGAGACCAGAC---------GAAGGAGGCG---ATACAAAAGGAA 1327
AtBOR4            GAAAGAAGCAGTA-ATGAAGAGCAAT---------GATGAAGAAAGAGAGGGAGACGAGG 1345
AtBOR5            GAAAGAGGCAGTG-ATGAAGAAGAGT---------GACGACGACGGGGATACCGGCGAAG 1345
AtBOR1            CAAGGAATCGACA-ATCCAAGCTACTACATTCACCGGAAACCTCAATGCTCCAGTTGATG 1402
AtBOR2            AAGAGAATCAACA-ATCCAAGCAACAACATTCACAGGAAATCTCGATGCTCCAGTTGATG 1327
OsBOR1            CAAGGACTCAACA-GTCCAAATGGCTTCAAGCATGGGCAACATCGATGCGCCAGTTGATG 1333
AtBOR3            CAAGCAGTC-ACATATTCAAAAGGCTTCA---------AACGCCGATGCACTGGTCGATG 1324
                   *   *         *                                    *

HvBor1a           AA------TTTGATCCTAGCAAACACATTGAAGCACATTTGCCTGTTCGTGTGAACGAAC 1384
TaBOR2            AA------TTTGATCCTAGCAAATATATCGAAGCACATTTGCCTGTTCGCGTGAACGAAC 1384
Bot1_HvBor1b      AA------TTTGATCCAAGAAAACATATTGAAGCACATTTGCCTGTTCGTGTCAACGAAC 1384
BRADI2G04690      AA------TTTGATCCAAGAAAACATATCGAAGCCCATTTGCCCGTTCGGGTAAATGAAC 1384
OsBOR3            AA------TTTGACCCTATAAAACATATTGAAGCACATTTGCCTGTTCGAGTGAATGAGC 1375
OsBOR4            TA------TTTGATCCTGAAAAGCATATAGAAGCTTACTTGCCTGTCCGGGTGAATGAGC 1378
AtBOR7            AGAAG---TTTGATCCAGAGGTACATATCGAAGACCATTTGCCGGTTAGAGTTAACGAGC 1378
AtBOR6            AA------TTCGATCCCGATGTTCATATAGAGGCTAATTTACCGGTTAGAGTAAACGAGC 1381
AtBOR4            AGAGTGGTTTTGATCCAGAGAAGCACCTTGACGCTTACTTGCCTGTTCGAGTCAACGAGC 1405
AtBOR5            AGAGTGGTTTCGATCCAGAGAAGCACGTTGACGCTTACTTGCCTGTTCGAGTCAACGAGC 1405
AtBOR1            AAACTCTGTTCGACATAGAGAAAGAAATAGATGATTTACTACCAGTTGAAGTCAAAGAAC 1462
AtBOR2            AGACTCTGTTTGATATCGAGAAAGAGATTGATGATTTATTGCCAATTGAAGTCAAAGAAC 1387
OsBOR1            AGACAGTCTTTGACATCGAGAAAGAAATCGATGACCTGCTGCCTATCGAGGTCAAGGAGC 1393
AtBOR3            AAACAGTGTTCGATATAGAGACGGAAGTGGAGAATATTCTGCCAGTAGAAGTAAAAGAAC 1384
                         ** **           *  * **        * **  *    ** ** ** *

HvBor1a           AGAGGCTAAGCAACCTGCTGCAATCCTTACTTGTTGGTGGCT----GTGTTGGAGCTATG 1440
TaBOR2            AGAGACTAAGCAACCTGCTGCAATCCTTACTTGTTGGTGGTT----GTGTGGGAGCTATG 1440
Bot1_HvBor1b      AGAGACTAAGCAACCTGCTACAATCCTTGCTGGTTGGTGGCT----GTGTTGGAGCTATG 1440
BRADI2G04690      AGAGACTGAGTAACTTGCTGCAATCCTTACTGGTTGGTGGCT----GTGTTGGGGCTATG 1440
OsBOR3            AGAGACTAAGTAACTTGCTGCAATCCTTATTAGTTGGTGCTT----GTGTTGGAGCTATG 1431
OsBOR4            AGAGAGTGAGCAATCTGTTGCAGTCCCTGTTGATCGCTGGTT----GTGTTGGAGTCATG 1434
AtBOR7            AGAGAGTGAGCAATCTATTGCAATCAGTCCTTGT--TGGATTGTTGATACTAG--CGGTA 1434
AtBOR6            AAAGAGTGAGCAATCTTTTGCAATCAGTTCTTGT--TGGTTTAACACTTCTTG--CAGTG 1437
AtBOR4            AGAGAGTTAGCAACTTGTTGCAGTCACTGCTTGTGGCAGGT--GCAGTGTTGG--CTATG 1461
AtBOR5            AGAGAGTGAGCAACCTGTTGCAATCATTGCTAGTGATAGGT--GCAGTGTTTG--CTCTA 1461
AtBOR1            AACGGGTAAGCAACTTGCTTCAGTCTACAATGGTAGGAGGAT----GCGTTGCAGCTATG 1518
AtBOR2            AGAGAGTAAGCAACTTGCTTCAAGCAGTAATGGTTGGAGGGT----GTGTTGCAGCTATG 1443
OsBOR1            AGAGGTTGAGCAACTTGCTCCAGGCCACAATGGTTGGGGGTT----GTGTTGCTGCTATG 1449
AtBOR3            AAAGAGTAAGCAACTTTCTTCAAGCTATGATGGTGGCTGGAT----GTGTTGCAGCAATG 1440
                  *  *  * ** **  *  * **  *     *  *    *                   *

HvBor1a           CCGGCTATCAAGATGATACCGACTTCGGTCCTCTGGGGTTACTTTGCCTACATGGCCATT 1500
TaBOR2            CCGGTTATCAAGATGATACCAACTTCGGTCCTCTGGGGTTACTTTGCCTACATGGCCATT 1500
Bot1_HvBor1b      CCGGTTATCAAGATGATACCGACTTCAGTCCTCTGGGGTTACTTTGCCTACATGGCCATT 1500
BRADI2G04690      CCAGTTATCAAGATGATACCGACTTCTGTCCTCTGGGGTTACTTTGCTTACATGGCAATT 1500
OsBOR3            CCAGTCATCAAGATGATACCGACATCGGTCCTCTGGGGTTACTTTGCTTATATGGCCATT 1491
OsBOR4            CCAATCATTCAGAAGATACCAACATCAGTCCTTTGGGGTTACTTTGCTTATATGTCCATT 1494
AtBOR7            CCAGTTCTCAGAATGATACCAACTTCAGTTCTATGGGGTTACTTCACTTACATGGCTGTT 1494
AtBOR6            ACAGTCATTAAAATGATCCCAAGTTCAGTACTTTGGGGTTACTTTGCTTATATGGCGATA 1497
AtBOR4            CCGGCCATTAAGCTCATACCGACTTCCATTCTATGGGGATACTTTGCTTACATGGCCATC 1521
AtBOR5            CCGGTCATTAAGCTCATACCGACTTCACTTCTATGGGGATATTTTGCTTACATGGCCATT 1521
AtBOR1            CCTATCCTTAAAATGATCCCAACATCAGTCCTTTGGGGCTATTTTGCCTTCATGGCCATC 1578
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AtBOR2              CCTCTCCTTAAAATGATCCCTACATCAGTCCTTTGGGGCTACTTTGCCTTCATGGCAATC 1503
OsBOR1              CCATTGCTCAAGAAGATCCCGACTTCTGTCCTCTGGGGCTACTTCGCCTTCATGGCCATT 1509
AtBOR3              CCTTTGATCAAAAGAATCCCAAGCTCGGTTCTTTGGGGTTACTTCGCTTACATGGCAATC 1500
                     *     *       ** ** *  **  * ** ***** ** **  * *  *** *  *

HvBor1a             GATAGCCTACCTGGGAACCAGTTTTGGGAAAGGTTACAGCTTCTGTGCATTGGAGCAAGC 1560
TaBOR2              GATAGCCTACCCGGGAACCAGTTTTGGGAAAGGATGCAGCTTCTGTGCATTGGAGCAAGC 1560
Bot1_HvBor1b        GATAGCCTACCCGGGAACCAGTTTTGGGAAAGGATACAACTTTTATTCGTTGGAGCAAGC 1560
BRADI2G04690        GATAGCCTACCTGGTAACCAATTTTGGGAAAGGATAAAGCTTCTGTTCATCGGATCAACC 1560
OsBOR3              GACAGTCTACCTGGCAACCAGTTTTGGGAAAGAATAAGACTTATATTCATCCCATCAAGC 1551
OsBOR4              GACAGCGTTCCGGGAAACCAGTTCTGGGAGAGGACACAACTTCTGTTCATTTCACCTCAA 1554
AtBOR7              GATAGTCTCCCTGGGAATCAATTCTGGGAAAGACTTCAGTTACTATTCATCACTCCTGGA 1554
AtBOR6              GACAGTCTCCCGGGAAACCAATTTTGGGAGAGATTGTTGCTTCTCTTTATTCCTCCTAGC 1557
AtBOR4              GACAGCCTCCCGGGAAATCAATTCTTCGAACGCTTAACGCTTCTCTTCGTTCCAACAAGC 1581
AtBOR5              GATAGCCTCCCAGACAATCAATTCTTCGAACGAACAGTACTTCTCTTCGTCCCACCAACC 1581
AtBOR1              GAAAGCTTACCCGGAAACCAATTCTGGGAAAGAATCTTACTTCTGTTCACCGCCCCAAGT 1638
AtBOR2              GAAAGCTTACCCGGAAACCAATTCTGGGAAAGAATCTTACTTCTCTTCACAGCTCCTAGT 1563
OsBOR1              GAGAGCTTGCCTGGTAACCAGTTCTGGGAGAGGATCTTGCTGCTCTTCACTGCTCCCAGC 1569
AtBOR3              GAAAGCCTCCCAGGGAATCAATTCTGGGAAAGGATCGTGCTTCTCTTCACTGCCCCAAGT 1560
                    ** **  * ** *  ** ** ** *  **  *        *  * *        *

HvBor1a             CGACGCTACAAGGTCTTGGAAGGCCCCCATGCATCTTTCGTGGAGGCGGTGCCT-TCAAG 1619
TaBOR2              CGACGCTACAAGGTCTTGGAAGGCCCACATGCATCTTTCGTGGAGTCAGTGCCT-TCAAG 1619
Bot1_HvBor1b        CGACGCTACAAGGTTTTGGAAGGTCCCCATGCATCTTTTGTGGAGTCGGTGTCT-TCGAG 1619
BRADI2G04690        CGACGCTACAAGGTCCTGGAAGGTCCACATGCATCTTTCGTGGAGTCAGTGCCT-TCAAA 1619
OsBOR3              CGACGCTACAAGGTTTTGGAGGGTCCCCATGCGTCTTTCATGGAGTCAGTGCCT-TCAAA 1610
OsBOR4              CGGCGCTACAAGCTTCTGGAAGGCGCTCATGCATCCTTCATGGAGTCAGTACCTATCAAG 1614
AtBOR7              CGTCGATTCAAGGTTCTTGAAGGTTTACATGCATCATTTGTGGAGATAGTACCA-TACAA 1613
AtBOR6              CGGCTTTTCAAAGTCTTGGAAGGAGTGCATGCTTCATTTGTGGAGTTGGTACCA-TACAG 1616
AtBOR4              CGGAGATTCAAGGTCTTGGAGGGAGCACACGCGTCGTTCGTGGAGAAAGTTCCT-TACAA 1640
AtBOR5              CGGAGATTCAAGGTCTTGGAAGGAGCGCATGCATCGTTCGTGGAGAAAGTTCCG-CATAA 1640
AtBOR1              CGCCGCTTCAAGGTTCTTGAAGATTACCACGCGACATTCGTGGAAACCGTTCCA-TTCAA 1697
AtBOR2              CGTCGATTCAAGGTCCTTGAAGATAACCACGCGACATTCGTGGAAACCGTTCCA-TTCAA 1622
OsBOR1              AGAAGATACAAGGTGTTAGAAGAGTACCACACCACGTTTGTCGAGACCGTGCCA-TTCAA 1628
AtBOR3              AGAAGATTCAAAGTTCTTGAGGATAATCATGCTGTGTTTATTGAAACGGTTCCG-TTTAA 1619
                     *    * ***  *  * ** *     **  *    **  * **    **  *     *

HvBor1a             AACAATATCTGCCTTTACGGTCTTCCAGTTTGTGTATCTCTTGATATGCTTCGGTATAAC 1679
TaBOR2              AACAATATCTGCCTTTACGGTCTTCCAGTTTGTGTATCTCTTGATATGCTTCGGCATAAC 1679
Bot1_HvBor1b        AACGATATATGTCTTTACGATCTTTCAGATTGTGTACTTCTTGATATGTTTCGGCACAAC 1679
BRADI2G04690        AACAATATCTGTCTTCACGATCTTCCAGCTGGTCTATCTCTTGATATGCTTCGGCACAAC 1679
OsBOR3              AACAATTACTGTCTTCACGATCTTCCAGTTGGTTTACCTCTTGATATGCTTCGGCATAAC 1670
OsBOR4              AA-AATATCTGCCTTCACTATTTTCCAGCTGGTTTATCTCTTGATCGTCTGGGGGATGAC 1673
AtBOR7              GTCGATTGTTATGTTCACACTCTTCCAGCTTCTATATTTTCTGATATGCTATGGAGTGAC 1673
AtBOR6              AGTGATTGTAACATTCACACTTTTCCAGTTGGTTTATTTTCTCTTGTGCTATGGTATGAC 1676
AtBOR4              GTCAATGGCTGCGTTCACACTGTTGCAGATATTCTACTTTGGGCTGTGCTATGGGGTGAC 1700
AtBOR5              GTCAATCGCTGCATTCACGCTATTTCAGATACTCTACTTTGGGCTTTGCTACGGAGTGAC 1700
AtBOR1              GACGATTGCAATGTTTACTCTTTTCCAAACGACTTATCTCTTGATCTGCTTTGGTCTCAC 1757
AtBOR2              AACGATCGCAATGTTCACAATTTTCCAAACAACTTATCTCTTAACTTGCTTTGGTCTCAC 1682
OsBOR1              GACGATAGCCATGTTCACACTCTTCCAGACAATGTATCTACTCGTCTGCTTCGGGATCAC 1688
AtBOR3              GACAATGGCGATGTTTACTCTGTTTCAAACCGCTTACTTACTGGTCTGCTTTGGCATCAC 1679
                        **       ** **  * ** **       **  *          *  **    **

HvBor1a             ATGGATACCAGTAGCAGGGATCCTCTTCCCGCTGCCTTTCTTCATTATGATTCTCATCAG 1739
TaBOR2              ATGGATACCAGTAGCAGGGATCCTCTTCCCGCTGCCTTTCTTCATTATGATTCTCATCAG 1739
Bot1_HvBor1b        ATGGATACCGATTGCCGGGATCCTTTTCCCGCTACCTTTCTTCCTCATGATTCTCATCAG 1739
BRADI2G04690        ATGGATACCAATAGCAGGGATCCTCTTCCCAGTGCCTTTCTTTCTCATGATTCTCATCAG 1739
OsBOR3              ATGGATACCAATAGCAGGGATCCTGTTCCCGTTGCCATTCTTCCTTATGATTCTTATCAG 1730
OsBOR4              ATGGATACCAGTAGCTGGAATCCTCTTCCCACTGCTGTTCTTCTTTCTCATTGTCATTAG 1733
AtBOR7              ATGGATACCTGTAGGAGGAATATTGTTCCCTTTGCCATTCTTCATCCTCATTGCGTTACG 1733
AtBOR6              ATGGATTCCTATGGCCGGGATATTTTTCCCCGCACTCTTCTTTCTACTTATAAGTATAAG 1736
AtBOR4              GTGGATTCCGGTGGCTGGAATCATGTTTCCGGTTCCTTTCTTCCTCTTAATAGCTATCAG 1760
AtBOR5              GTGGATTCCAGTGGCCGGAATCATGTTTCCGGTTCTTTTCTTCCTTTTAGTAGCCATCAG 1760
AtBOR1              ATGGATACCAATCGCAGGAGTCATGTTCCCTTTAATGATCATGTTCTTAATCCCCGTACG 1817
AtBOR2              GTGGATACCAATCGCTGGAGTTATGTTCCCTCTACTGATCATGTTTCTGATACCCGTAAG 1742
OsBOR1              ATGGATCCCGATTGCTGGGGTTCTTTTCCCCCTCATGATCATGCTGCTGGTTCCAGTCAG 1748
AtBOR3              GTGGGTTCCGGTGGCCGGAGTTTTGTTCCCGTTGATGATAATGTTTCTTGTTCCAGTTAG 1739
                     *** * **  * *  **  *  * ** **        *  *  *  *  *     *  *

HvBor1a             GCAACACCTACTCCCAAAGTTCTTTGAGCCCAATGACTTGCGAGAACTGGATGCAGCTGA 1799
TaBOR2              GCAACACCTACTCCCAAAGTTCTTTGAGCCCAATGACTTGCGGGAACTGGACGCAGCCGA 1799
Bot1_HvBor1b        GCAGTACCTGCTCCCCAAGTTTTTTGAGCCCAATGACTTGCGGGAACTGGACGCGGCTGA 1799
BRADI2G04690        GCAGCACCTACTCCCAAAGTTTTTCGATCCCAATCACTTGAGGGAACTAGACGCTGCAGA 1799
OsBOR3              GCAGCATGTCCTCCCAAAGTTTTTTGAGCCAAATGACCTTCGGGAATTGGATGCAGCTGA 1790
OsBOR4              ACAGTATATCCTCCCGAAATTCTTCGATCCACGCCACCTGTGGGAATTGGATGCAGCTGA 1793
```

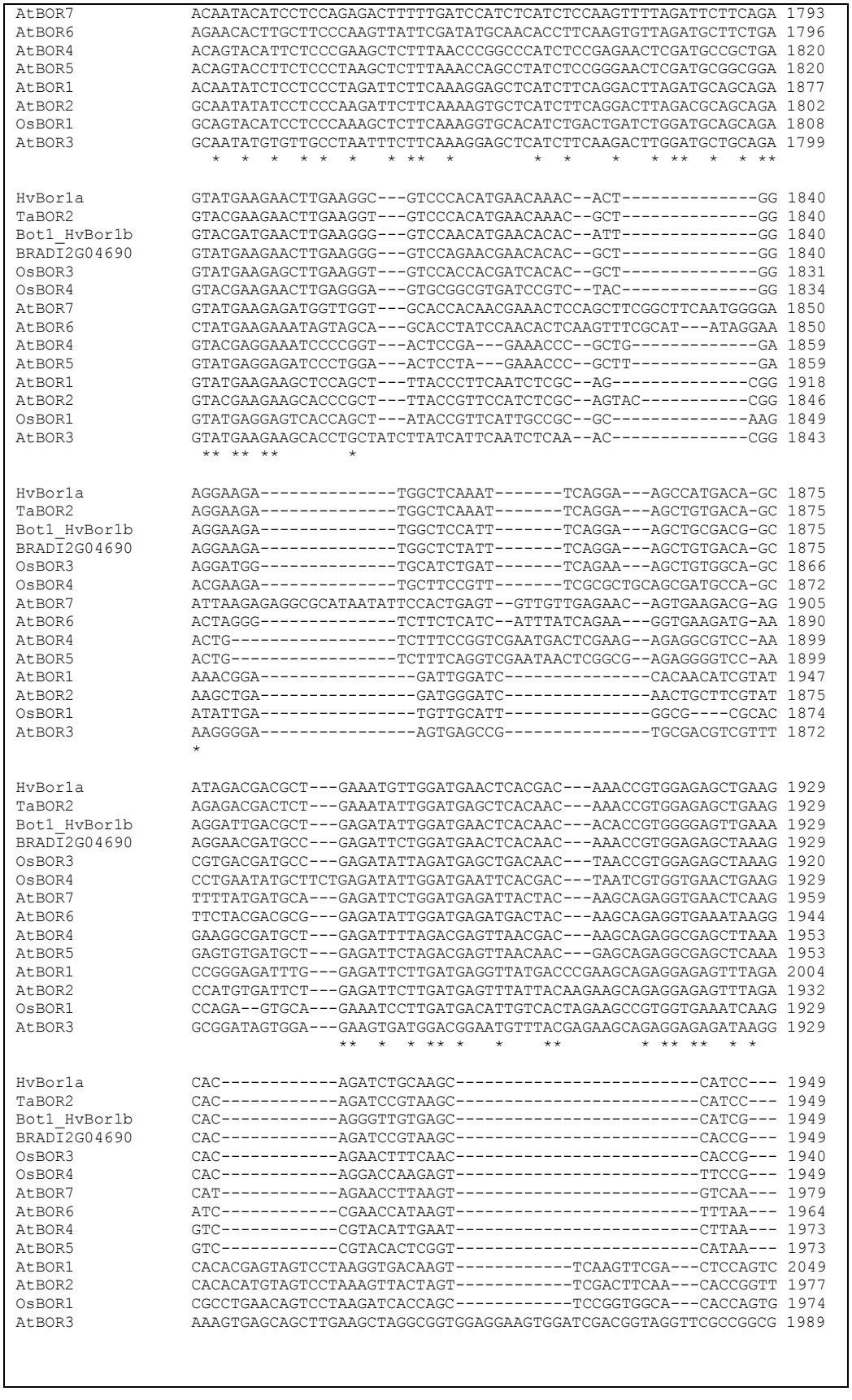
AtBOR7 ACAATACATCCTCCAGAGACTTTTTGATCCATCTCATCTCCAAGTTTTAGATTCTTCAGA 1793
AtBOR6 AGAACACTTGCTTCCCAAGTTATTCGATATGCAACACCTTCAAGTGTTAGATGCTTCTGA 1796
AtBOR4 ACAGTACATTCTCCCGAAGCTCTTTAACCCGGCCCATCTCCGAGAACTCGATGCCGCTGA 1820
AtBOR5 ACAGTACCTTCTCCCTAAGCTCTTTAAACCAGCCTATCTCCGGGAACTCGATGCGGCGGA 1820
AtBOR1 ACAATATCTCCTCCCTAGATTCTTCAAAGGAGCTCATCTTCAGGACTTAGATGCAGCAGA 1877
AtBOR2 GCAATATATCCTCCCAAGATTCTTCAAAAGTGCTCATCTTCAGGACTTAGACGCAGCAGA 1802
OsBOR1 GCAGTACATCCTCCCAAAGCTCTTCAAAGGTGCACATCTGACTGATCTGGATGCAGCAGA 1808
AtBOR3 GCAATATGTGTTGCCTAATTTCTTCAAAGGAGCTCATCTTCAAGACTTGGATGCTGCAGA 1799
HvBor1a GTATGAAGAACTTGAAGGC---GTCCCACATGAACAAAC--ACT--------------GG 1840
TaBOR2 GTACGAAGAACTTGAAGGT---GTCCCACATGAACAAAC--GCT--------------GG 1840
Bot1_HvBor1b GTACGATGAACTTGAAGGG---GTCCAACATGAACACAC--ATT--------------GG 1840
BRADI2G04690 GTATGAAGAACTTGAAGGG---GTCCAGAACGAACACAC--GCT--------------GG 1840
OsBOR3 GTATGAAGAGCTTGAAGGT---GTCCACCACGATCACAC--GCT--------------GG 1831
OsBOR4 GTACGAAGAACTTGAGGGA---GTGCGGCGTGATCCGTC--TAC--------------GG 1834
AtBOR7 GTATGAAGAGATGGTTGGT---GCACCACAACGAAACTCCAGCTTCGGCTTCAATGGGGA 1850
AtBOR6 CTATGAAGAAATAGTAGCA---GCACCTATCCAACACTCAAGTTTCGCAT---ATAGGAA 1850
AtBOR4 GTACGAGGAAATCCCCGGT---ACTCCGA---GAAACCC--GCTG-------------GA 1859
AtBOR5 GTATGAGGAGATCCCTGGA---ACTCCTA---GAAACCC--GCTT-------------GA 1859
AtBOR1 GTATGAAGAAGCTCCAGCT---TTACCCTTCAATCTCGC--AG--------------CGG 1918
AtBOR2 GTACGAAGAAGCACCCGCT---TTACCGTTCCATCTCGC--AGTAC-----------CGG 1846
OsBOR1 GTATGAGGAGTCACCAGCT---ATACCGTTCATTGCCGC--GC--------------AAG 1849
AtBOR3 GTATGAAGAAGCACCTGCTATCTTATCATTCAATCTCAA--AC--------------CGG 1843
HvBor1a AGGAAGA--------------TGGCTCAAAT-------TCAGGA---AGCCATGACA-GC 1875
TaBOR2 AGGAAGA--------------TGGCTCAAAT-------TCAGGA---AGCTGTGACA-GC 1875
Bot1_HvBor1b AGGAAGA--------------TGGCTCCATT-------TCAGGA---AGCTGCGACG-GC 1875
BRADI2G04690 AGGAAGA--------------TGGCTCTATT-------TCAGGA---AGCTGTGACA-GC 1875
OsBOR3 AGGATGG--------------TGCATCTGAT-------TCAGAA---AGCTGTGGCA-GC 1866
OsBOR4 ACGAAGA--------------TGCTTCCGTT-------TCGCGCTGCAGCGATGCCA-GC 1872
AtBOR7 ATTAAGAGAGGCGCATAATATTCCACTGAGT--GTTGTTGAGAAC--AGTGAAGACG-AG 1905
AtBOR6 ACTAGGG--------------TCTTCTCATC--ATTTATCAGAA---GGTGAAGATG-AA 1890
AtBOR4 ACTG-----------------TCTTTCCGGTCGAATGACTCGAAG--AGAGGCGTCC-AA 1899
AtBOR5 ACTG-----------------TCTTTCAGGTCGAATAACTCGGCG--AGAGGGGTCC-AA 1899
AtBOR1 AAACGGA----------------GATTGGATC---------------CACAACATCGTAT 1947
AtBOR2 AAGCTGA----------------GATGGGATC---------------AACTGCTTCGTAT 1875
OsBOR1 ATATTGA----------------TGTTGCATT---------------GGCG----CGCAC 1874
AtBOR3 AAGGGGA----------------AGTGAGCCG---------------TGCGACGTCGTTT 1872
HvBor1a ATAGACGACGCT---GAAATGTTGGATGAACTCACGAC---AAACCGTGGAGAGCTGAAG 1929
TaBOR2 AGAGACGACTCT---GAAATATTGGATGAGCTCACAAC---AAACCGTGGAGAGCTGAAG 1929
Bot1_HvBor1b AGGATTGACGCT---GAGATATTGGATGAACTCACAAC---ACACCGTGGGGAGTTGAAA 1929
BRADI2G04690 AGGAACGATGCC---GAGATTCTGGATGAACTCACAAC---AAACCGTGGAGAGCTAAAG 1929
OsBOR3 CGTGACGATGCC---GAGATATTAGATGAGCTGACAAC---TAACCGTGGAGAGCTAAAG 1920
OsBOR4 CCTGAATATGCTTCTGAGATATTGGATGAATTCACGAC---TAATCGTGGTGAACTGAAG 1929
AtBOR7 TTTTATGATGCA---GAGATTCTGGATGAGATTACTAC---AAGCAGAGGTGAACTCAAG 1959
AtBOR6 TTCTACGACGCG---GAGATATTGGATGAGATGACTAC---AAGCAGAGGTGAAATAAGG 1944
AtBOR4 GAAGGCGATGCT---GAGATTTTAGACGAGTTAACGAC---AAGCAGAGGCGAGCTTAAA 1953
AtBOR5 GAGTGTGATGCT---GAGATTCTAGACGAGTTAACAAC---GAGCAGAGGCGAGCTCAAA 1953
AtBOR1 CCGGGAGATTTG---GAGATTCTTGATGAGGTTATGACCCGAAGCAGAGGAGAGTTTAGA 2004
AtBOR2 CCATGTGATTCT---GAGATTCTTGATGAGTTTATTACAAGAAGCAGAGGAGAGTTTAGA 1932
OsBOR1 CCAGA--GTGCA---GAAATCCTTGATGACATTGTCACTAGAAGCCGTGGTGAAATCAAG 1929
AtBOR3 GCGGATAGTGGA---GAAGTGATGGACGGAATGTTTACGAGAAGCAGAGGAGAGATAAGG 1929
HvBor1a CAC------------AGATCTGCAAGC-------------------------CATCC--- 1949
TaBOR2 CAC------------AGATCCGTAAGC-------------------------CATCC--- 1949
Bot1_HvBor1b CAC------------AGGGTTGTGAGC-------------------------CATCG--- 1949
BRADI2G04690 CAC------------AGATCCGTAAGC-------------------------CACCG--- 1949
OsBOR3 CAC------------AGAACTTTCAAC-------------------------CACCG--- 1940
OsBOR4 CAC------------AGGACCAAGAGT-------------------------TTCCG--- 1949
AtBOR7 CAT------------AGAACCTTAAGT-------------------------GTCAA--- 1979
AtBOR6 ATC------------CGAACCATAAGT-------------------------TTTAA--- 1964
AtBOR4 GTC------------CGTACATTGAAT-------------------------CTTAA--- 1973
AtBOR5 GTC------------CGTACACTCGGT-------------------------CATAA--- 1973
AtBOR1 CACACGAGTAGTCCTAAGGTGACAAGT------------TCAAGTTCGA---CTCCAGTC 2049
AtBOR2 CACACATGTAGTCCTAAAGTTACTAGT------------TCGACTTCAA---CACCGGTT 1977
OsBOR1 CGCCTGAACAGTCCTAAGATCACCAGC------------TCCGGTGGCA---CACCAGTG 1974
AtBOR3 AAAGTGAGCAGCTTGAAGCTAGGCGGTGGAGGAAGTGGATCGACGGTAGGTTCGCCGGCG 1989

```
HvBor1a           -----------TGAAG-----AAAGG--------------CACCTTCAGGTCCATTCAA- 1978
TaBOR2            -----------TGAAG-----AAAGG--------------CACTTTCAGGTCCACTCAA- 1978
Bot1_HvBor1b      -----------TGAAG-----AAAGA--------------CACCTTCAGGTCCATTCAA- 1978
BRADI2G04690      -----------TGAAG-----AAAGG--------------CACCTTCAGGTGCATTCAA- 1978
OsBOR3            -----------TGAAG-----AAAGG--------------CACCCGCAGGCGCATACAA- 1969
OsBOR4            -----------TGACG-----AGAGG--------------CTGATACAGCTTAATTCGGT 1979
AtBOR7            -----------AGAAG-----ACAGA-----------------------------TCCCA- 1994
AtBOR6            -----------AGAGGT----GCA-------------------------------CCCG- 1977
AtBOR4            -----------CGAAG-----ACAAA--------------GGCAACCAGATTTATCCCA- 2002
AtBOR5            -----------CGAAG-----ACAAA--------------GGCCACCAGATATATCCCA- 2002
AtBOR1            ----------AATAATC----GGAG------------TTTGT--CACAAGTGTTT----- 2076
AtBOR2            ----------TATAATC----GGAA------------TCTCT--CTCAAGTGTTT----- 2004
OsBOR1            G---------CAGAACTTAAAGGAA------------TCCGCAGCCCTTGTATCTCTGA- 2012
AtBOR3            GGAGGAGGAGTGGAGTTGATGAGAAGAGTGGTGAGTTTTCAGAATCCAAGGGTGTCGGA- 2048
                             *         *

HvBor1a           -ATGCAGTTCAGCC----------------------GAGCGTGT--GA------------ 2001
TaBOR2            -ATGCAGTTCAGCC----------------------AAGCGTGT--GA------------ 2001
Bot1_HvBor1b      -ATGCCGTTCAGCC----------------------AAGCGTGT--GA------------ 2001
BRADI2G04690      -ACGCCGTTCAGCC----------------------AAGCGTGT--GA------------ 2001
OsBOR3            -AAGCCGTTCAGCC----------------------CAG-GTGT--GGAGATACAGAAAA 2003
OsBOR4            GAAGATGACCAGAG----------------------AACTTTCTCGGATTCCGACTTTTA 2017
AtBOR7            GA---TGGTAAA------------------------AA-TA--------------TATAA 2012
AtBOR6            GAGCCTGAAGAG------------------------AAGCA--------------TGTAA 1999
AtBOR4            AGGAGAAAGTAA------------------------AAGCAGGA---GACGGGGACATGA 2035
AtBOR5            AGGAGATAGTAG------------------------AAGTAGGG---GATGGGGACATGA 2035
AtBOR1            --AGTCCAAGAGTG--------AGTGGAATCAGGTTGGGTC-----AGATGAGTCCTCGA 2121
AtBOR2            --AGTCCTAGAGTG--------ATTG-ATTTAAG--AGGTG-----AGATGAGTCCAAGG 2046
OsBOR1            GAGGGCATACAGCC--------CTTGTATCACCG--AGTTG-----AGGCATGACCGCAG 2057
AtBOR3            GAAAGTGTACATTCGGAGCTTAAGTGATTTCAGAGGAGGTGGAG--AGATTAGTCCACGA 2106
                           *

HvBor1a           ------------------------------------------------------------
TaBOR2            ------------------------------------------------------------
Bot1_HvBor1b      ------------------------------------------------------------
BRADI2G04690      ------------------------------------------------------------
OsBOR3            CTGGTCC--------------GAGTGCTAA------------------------------ 2019
OsBOR4            CTCCTCC--------------ACGATCCTAA------------------------------ 2034
AtBOR7            CCATTC----------------------TTGA------------------------------ 2022
AtBOR6            CCTTTGA--------------ACCACATTAA------------------------------ 2016
AtBOR4            GCACGAC--------------AAGGGAGTGA------------------------------ 2052
AtBOR5            GTTCTTC--------------GAGAGAGTGA------------------------------ 2052
AtBOR1            GTCGTCG--GGAAT----------AGTCCAAAGCCGGC-----GAGTTGTGGGAGGA--- 2161
AtBOR2            CTCTCCG--GGAA-----------AGGCCAAAAT----------AGTCCAAAGCCGA--- 2080
OsBOR1            CCCTCTA--GGAGGAAG---AGGCAGCCCAAGGAC---------CGGTGAGACCCGATC- 2102
AtBOR3            TCGTCCGCTGGAAGAGCTCCGTTCAGTCCACGATCCGCCACGGGTGGTGGAGGAGGAGAA 2166


HvBor1a           ---------------------------------------
TaBOR2            ---------------------------------------
Bot1_HvBor1b      ---------------------------------------
BRADI2G04690      ---------------------------------------
OsBOR3            ---------------------------------------
OsBOR4            ---------------------------------------
AtBOR7            ---------------------------------------
AtBOR6            ---------------------------------------
AtBOR4            ---------------------------------------
AtBOR5            ---------------------------------------
AtBOR1            ----GTCCC--TTGAACCAGTCGTCATCG---AACTGA 2190
AtBOR2            ----GTCCT--TTAAACCCATCATCATCGTCGAAATGA 2112
OsBOR1            ----GTCCAAGTTGGGCGAAGGATCAACACCAAAGTGA 2136
AtBOR3            CAGAGACTT--TCTAATTTAGGAAAGTCTGTTTAG--- 2199
```

APPENDIX N

SEQUENCES OF INTRONS OF *HvBor1a*

Table N.1. Sequences of introns of *HvBor1a* in Clipper. The sequences of introns 6 and 10 have been determined partially. All introns are identical in genomes of Clipper and Sahara except for intron 13. The variation within intron 13 is presented underlined which is unique in genome of Clipper but different in Sahara.

Introns	
Intron 1 (892 bp)	
1	GGTACGGCCC CCCGACTCCC CGTCTCCGAT TCCTCCCCTC TACCCGCGTC TCGGTTTGTT
61	CCTACTAGAT CCGTGAGCGC GCGAGGAGCA TGCCCCTCCC GTGCCGTCCG GTCGCCGTCG
121	GCCGCTCGAT GTAGAGTTCA TGCGACGGGT TTTTCAGTGG GTTTTGGGGT GGTTCCCCGT
181	TCCATTTTAC CTTTTTTTCC CCGCGAGTCC GTTCGTATCG TGGACAACCA ATTTCAGTAA
241	ATACAAACCG ATCGCGTCAG CCGTCGCGCG CCTGCGGCGT GTGTCTGATT CTCGACGAGT
301	GGATTTTTCC GGGGGTTTTA ATTAGTCGCC GATTTTGATC CGTTTACTGC TGGAATCTTC
361	GACTTGTTCG TAGCTATATG CGGAGGACAA ACGAGCGTTT CAGACTCCGC TCAATGTGCA
421	TGTGGACGCG CAGAGGGTTC CATTTGGATC TGCATGTCGA TTTTCTCCAA CCGTGCAGAG
481	ATTTTCTACT AACATATTCT ATTGTCCGCG TCAGTTGTCA TGTAGCACAG TGCTCCTGTT
541	TCCATATTCT TGCCGCAGCC GATCTGCGGT GCGTGTGTTT TTACGGCAAA GTTTGATCGC
601	CTGCGAACGT AGCAGCTTGA GTTGACGTGT TTTCTGACAT GTAAGGTGAG CGATGAGCTG
661	CTTGTTCTTC GTCAAGTCGT GTATACAATA ATTCACCCCG TCCCGTCCTT TAAGTTGGTG
721	TAGCATCCGT GCAATCACTA GTATACTAGC AGTAGTACCA ACTCCGGGTC AAAGTAAATG
781	CAATCTTTTG GACAGATTTT CCCTGCGACT GTTCACAGTT TGATTCATCT CATCTCCATT
841	TTCCATGGAA ATTCTCCGCA CGGTTACTGA TTCTCTCAAG GTTTTGTGAC CA
Intron 2 (111 bp)	
1	GTGAGTTTTG TTTCGACAGA CCGAACAAGT TCATCTGCAG CCCAGTCGAT TTTTACATGT
61	ACAAATATTG CATCCATCGA TTTGCTTATG CTTCTTGCCA ATGTTTGCCA G
Intron 3 (209 bp)	
1	GTGAATGCTT GCCCTCTTTG GTTTGCTACC AACATTTGGC AAGCCAACAT TTGGCAACCT
61	TTTGTGGCAT TGGCAACAGA AATTGCATGC AACCAATCTC TAGCCAACAT TTGGCGGTTG
121	CCAACATTTG GCAGTTGCCA AAATTTGGCA TGCCAACTTT TGGCATCAAA CCAATTAGGC
181	TCTAACAAGA ACGAAGTGAT CTGTTTCAG
Intron 4 (84 bp)	
1	GTAGGTACTA ATATAGAGTA CACCCAATCC TGTGATAGCT TCGGTATAAC ATAAATGCTC
61	TCTGTGTTTG TTTGCTGTTG ACAG
Intron 5 (79 bp)	
1	GTATGCCATG CGTAGCAAAT CGTAGCTATT TATTCCCACC CTGCTGAAAA TACCTAACAC
61	TGGATCCTAT CTGTTGCAG

Table N.1. (continued)

Introns
Intron 6 (> 350 bp) GTTGGGTCAC ACGTTTTCTT TGCGCTCCCA TCATCTTGTT TTCTCTATAG TTTAGTGGCA GACAGAACTC CCAGGTAACA CCACACATTT TCAA // TTCTTTGGAA CACTTGTAGT AGTCCCAAAT AGGGGCAGTT TTAAAATTTG GTTCCTTCAG GCGGTTGAAT ATCTTTCTTC TGTAGTTCTG AATCAGCTTA TAGCTGTTTC TAAAGTATAA TTTATCACCA GGGAAAATCA TATATAAAGT TTCTAGTTGT GTGTAAAATA TACATGCACT CATCCACTGA TCACCGTGCC TTTGAGTTCC CATTTGCAG
Intron 7 (112 bp) 1 GTATTAAGTC AAATCACTTG TGATTCTAGA TGTGTAAATA TTATTTATGT CTCTGAGCTT 61 GGTAAAGATG TATTGTATGC CATAATTCTG ATTTTTTGGT TTTCACTTTC AG
Intron 8 (287 bp) 1 GTATGTCGTA TCTAAATCAT TCTGTAGATA ATGTGTTCAT TTACAGAGTC TCTTCTTCTG 61 TAAACAAAAT CATGATTGTC TTAAGAACCT AACTATGTAT GCCAGTGCAA ATTAGGACAA 121 TCCATTTTTC ATATTCCTTG TATATAATGT GGATTAGTGA GGAGGGGAGA TAGGAGGCCT 181 GTATAGACGA CCCCTCCCAA TCTATCCTGA GACCTGACTG CATTTTACCT CAACATTCTG 241 ATTTACCATG TCTCGTTTCC TCTTCTCATA GTATGTTAAT GATGTAG
Intron 9 (83 bp) 1 GTTAGTGAGA TTCAGACTGA TCCGAGTCAT ATGCTAAGTT AGTCAGCTTT GCTCATCTGT 61 CTTCTCTTTT TCTGCCAATT TAG
Intron 10 (> 600 bp) GTGAGTCTTT CAGTCTGTTA TCTTGCTATC TATATCTGGG GTGAGTG // GTTAAAGAAG TAGGGTCCCT ACTATGTGGT TTGCCTGGCA ATCCCCCCCT TAAATTGAAG ACTTTCTCCA TTCCCCCATA GAAACAAGAA AGCCTGCTGT CCTCAAGGGG GCAGGTTGTG AGATTTGGAT GATTCGAGGC ATATGGTAAA GTAGTTAGCT TTGGTTATCT GTTTTTTCTT TTTTTGCCCA ATTAGGGGTA AGCAGAAAAG AAGGTTCAAA ATGCCCAAGA AGGCAAGTCC AACCCGGGGG GCCGGTTGGG AATTTAAGGC AAAAAGCCGG AAAGGTTTAT CCCAAAGGAA AGCCACCCGA AAGGGGGTTT TTCCATTTGT TTTTTTGCCA TTTAAATTTC CCGGGGGGGA AAGGTTTTTT TTTTTGAGAG AGCATAAATT AGAGGAAAAA CCAAAATGAC TTCTAGGCTA AAGTAATGTA GTTGCTTCTG CATCCTTGAT GTTTCCATAC ATTAGTTTCT TTTCAGTAAA TGTTCGAATA CTAATATTTT TTATTAGTTT TGTAAGGAAA TGCATAAAGT ACATACTCGT ATCGCTAAAA AAAATATGGC CTGTAG
Intron 11 (145 bp) 1 GTAATGTCTA TTCTGCATAT CTGTTCATCT CATCTGAACA CATGAACTTG CTGTGTGCTT 61 CCTTTTGGAC CACACCATTT TTGATAAGAT ACAGGGACTA TATAATAGCA GATTGCACTA 121 ACACGTAGCG TTGTAACCTT TGCAG
Intron 12 (122 bp) 1 GTATGAATTC AGCTTGGGTT CTTGGATAAA TATTTTGTCT GTCCACATTG TTTCATTATC 61 TGAGAATCCC CTAGGTGTAT TGTCATCTTT TGTTAATCTG AAACTCTCGG ATCGTCATGC 121 AG

Table N.1. (continued)

Introns
Intron 13 (353 bp)

```
      1  GTAAACGATG GGCATGCATA GCATTGCAGT TGCAACAAGT GCACATTGAA CCGTAGGTGC
     61  TATTATACTG TGATGGCTCA CAGTCTGACC TTTCTGCTGG CTTCACATAG GTCCAGCCAA
    121  CAGATTGAAC TTGTGAGCTG TTTTTTTCTT TTTTTACGGA AGAACAAGTA CCGCGCATAC
    181  CAGTAGATGG TTCTTTTGTG CCTGTTATGG GCAGATTTCA CGGGCGCAAA TTCATAAATG
    241  TTCCCGTCGT GAGTTGATCC TATTCCACAC CTTTTTTCTT CTCGGAAAGA GAGGGGTTTT
    301  GATGCCAATA TGCCATTTTT CTGAATTTGA CAGTTTCAAA TTACACCGTG CAG
```

APPENDIX O

SEQUENCES OBTAINED BY GENOME WALKING AND 3'RACE

Table O.1. Sequence of 3' UTR of *HvBor1a* in Clipper. Translation stop codon is highlighted.

(639 bp)

```
  1  TGAAGATGGA AAAACGTCCC GATGTCCGAG GCTTTGATGA CGATTCTGTC AGAAGTGTAG
 61  ATTATCCTGA AGCCATTGTT CATTTCCGAA TACGCCATTG TTCAGCAATG TGCATTGTAC
121  TGTAGCTAGC GCTTTTCCGG TAAGGCGTGA TCGTGTACGC TAGGAAGTAA AGCTCAGGTA
181  ATTAGCAGTG AGATCAGACT GAAGAAAGTG TATTGGATAT GGCAGGGGAA CTTGGCAGAA
241  TAGTATTAGT AATGGTTAGA AGTAGAAAGA CTGTTGCTCC GTGACCTGTT ATTGCAAGAC
301  TGTTGCTCTG TGACCTGTTG CCTGTGTAAA GTCCAGCTTT CTGTGTCTGA ATGTAAAATG
361  CCAGGCATAA GAATTTCTTG AGAACCAGTG AATCTATTCT GCCGCGTGTT CTGGCTGCAT
421  TTTGATGCCT CTGTTCTTCA AGCTTTTTTT GGCAGCCACA TTTTGTACCA CTGCCTTAGA
481  GCATCTCCCA CAGGTGTGCC GTGCAAAAAA TTACTTTACA ACGTCGAGAT AGTCAGTTTT
541  TGCGCGCTCC AGCAGGTGAT GTAATTTTTT TTTAAGCCGG GCAAACACAC CTTTCCATTA
601  CAATGAACAA AAATACAACA GTTCCAGAGA GTATCAGGA
```

Table O.2. Sequence of last intron of *HvBor1a* in Sahara. The region common to both Clipper and Sahara is underlined.

(388 bp)

```
  1  GTAAACGATG GGCATGCATA GCATTGCAGT TGCAACAAGT GCACATTGAA CCGTAGGTGC
 61  TATTATACTG TGATGGCTCA CAGTCTGACC TTTCTGCTGG CTTCACATAG GTCCAGCCAA
121  CAGATTGAAC TTGTGAGCTG TTTTTTTTCT TTTTTTACGG AAGAACAAGT ACCGCGCATA
181  CCAGTAGATG GTTCTTTTGT GCCTGTTATG GGCAGATTTC ACGGGCGCAA ATTCATAAAT
241  GTTCCCGTCG TGAGTTGATC CTATTCCACC GTACTTCGGC TTTTTATTGA TATGCATAGC
301  TAGGATCATT GCAAGGTCCT CCTCCTCTTC AATATCAAAT TCTTCTTCGG AAGAATCATA
361  TGATGAACTC ATCTACAATA TTGAATTT
```

Table O.3. Sequence of 3' end of *HvBor1a* determined in 3'RACE on RACE-ready cDNA from Sahara. The region common to both Clipper and Sahara is underlined. The putative stop codon is highlighted.

(362 bp)

```
  1  GTGGAGAGCT GAAGCACAGA TCTGCAAGCC ATCCTGAAGA AAGGCACCTT CAGACCCCGA
 61  GATAGACACT ACTACCGGGT ACGACTACCC TCCTGACGGC CAACCCTTTG CCGCAGAGCA
121  ACAAGACGAT GCAACCCAGG AGGTGGCATA TCCCGTCGTC GACGACTTCT ACCCCGACGG
181  TGCCTACTAC TATGTGGAGG CCGCTGAAGA CCAGGAGTAG TTTAGGAGGT TCCCATGCTG
241  GAGTCCTCGC CTTGTTCGAT CCCTGTATAT TTTGTGCCAA CCTTCTCTAA GGCATTATCA
301  TGTTTCATGT GTTTACTTTA TATTTGTTGC TCCCGCGTAC TCTGCGTTGA TACCACTGCT
361  TA
```

APPENDIX P

SEQUENCE OF THE FRAGMENT AMPLIFIED BY PRIMERS OF CAPS MARKER xHvMYB

Table P.1. Aligned sequences of fragments amplified from Clipper (C) and Sahara (S) genomic DNA. Fragments amplified by primer pair designed for EST14 (xHvMYB) were sequenced in forward (F) and reverse (R) direction. Single nucleotide polymorphism (SNP) in the sequence reads used for development of CAPS marker xHvMYB is displayed shaded.

```
                    1                                                  50
» EST14C_F      (1)                                              GACGAGCGG
» EST14S_F      (1)
« EST14C_R      (1)            AAGCTGCAGGAGTTCCTGGCCCGCCTCGACGACGAGCGG
« EST14S_R      (2) CCACAACGCAGAAGCTGCAGGAGTTCCTGGCCCGCCTCGACGACGAGCGG
  Consensus     (2) CCACAACGCAGAAGCTGCAGGAGTTCCTGGCCCGCCTCGACGACGAGCGG
                (1)
                    51                                                 100
» EST14C_F     (11) CTCAAGATCGACGCCTTCAAGCGCGAGCTCCCGCTCTGCATGCAGCTGCT
» EST14S_F      (1)                                 CCGCTCTGCATGCAGCTGCT
« EST14C_R     (41) CTCAAGATCGACGCCTTCAAGCGCGAGCTCCCGCTCTGCATGCAGCTGCT
« EST14S_R     (52) CTCAAGATCGACGCCTTCAAGCGCGAGCTCCCGCTCTGCATGCAGCTGCT
  Consensus    (52) CTCAAGATCGACGCCTTCAAGCGCGAGCTCCCGCTCTGCATGCAGCTGCT
               (51)
                    101                                                150
» EST14C_F     (61) CAACCAAGGTAATGAACGTGCATGCCGAACTGCCTATGCATGCGATGCGA
» EST14S_F     (22) CAACCAAGGTAATGAACGTGCATGCCGAACTGCCTATGCATGCGATGCGA
« EST14C_R     (91) CAACCAAGGTAATGAACGTGCATGCCGAACTGCCTATGCATGCGATGCGA
« EST14S_R    (102) CAACCAAGGTAATGAACGTGCATGCCGAACTGCCTATGCATGCGATGCGA
  Consensus   (102) CAACCAAGGTAATGAACGTGCATGCCGAACTGCCTATGCATGCGATGCGA
              (101)
                    151                                                200
» EST14C_F    (111) GCTTACGTAGAAGGCGATGGAGCTCTGCCTGACTGTATAATGTATGTGTG
» EST14S_F     (72) GCTTACGTAGAAGGCGATGGAGCTCTGCCTGACTGTATAATGTATGTGTG
« EST14C_R    (141) GCTTACGTAGAAGGCGATGGAGCTCTGCCTGACTGTATAATGTATGTGTG
« EST14S_R    (152) GCTTACGTAGAAGGCGATGGAGCTCTGCCTGACTGTATAATGTATGTGTG
  Consensus   (152) GCTTACGTAGAAGGCGATGGAGCTCTGCCTGACTGTATAATGTATGTGTG
              (151)
                    201                                                250
» EST14C_F    (161) GGTGCAGCTATGGAGGCGTACAGGCAGCAGCTGGAAGCGTGCCAGATGGG
» EST14S_F    (122) GGTGCAGCTATGGAGGCGTACAGGCAGCAGCTGGAAGCGTGCCAGATGGG
« EST14C_R    (191) GGTGCAGCTATGGAGGCGTACAGGCAGCAGCTGGAAGCGTGCCAGATGGG
« EST14S_R    (202) GGTGCAGCTATGGAGGCGTACAGGCAGCAGCTGGAAGCGTGCCAGATGGG
  Consensus   (202) GGTGCAGCTATGGAGGCGTACAGGCAGCAGCTGGAAGCGTGCCAGATGGG
              (201)
                    251                                                300
» EST14C_F    (211) GAGCCATGGCGCTGCGGCGGCGAGGGCGCCGCTGGTGCTCGAGGAATTCA
» EST14S_F    (172) GAGCCATGGCGCTGCAGCGGCGAGGGCGCCGCTGGTGCTCGAGGAATTCA
« EST14C_R    (241) GAGCCATGGCGCTGCGGCGGCGAGGGCGCCGCTGGTGCTCGAGGAATTCA
« EST14S_R    (252) GAGCCATGGCGCTGCAGCGGCGAGGGCGCCGCTGGTGCTCGAGGAATTCA
  Consensus   (252) GAGCCATGGCGCTGC GCGGCGAGGGCGCCGCTGGTGCTCGAGGAATTCA
              (251)               +
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                    301                                                 350
» EST14C_F   (261) TACCGCTGAAGAACATCGGGATCGACGAGGCGGAGAAGGCGGCCGGGAAC
» EST14S_F   (222) TACCGCTGAAGAACATCGGGATCGACGAGGCGGAGAAGGCGGCCGGGAAC
« EST14C_R   (291) TACCGCTGAAGAACATCGGGATCGACGAGGCGGAGAAGGCGGCCGGGAAC
« EST14S_R   (302) TACCGCTGAAGAACATCGGGATCGACGAGGCGGAGAAGGCGGCCGGGAAC
  Consensus  (302) TACCGCTGAAGAACATCGGGATCGACGAGGCGGAGAAGGCGGCCGGGAAC
             (301)
                    351                                                 400
» EST14C_F   (311) GCGCCGTCAGAGAAGGCGAGCTGGATGGTGTCGGCGCAGCTGTGGAACGG
» EST14S_F   (272) GCGCCGTCAGAGAAGGCGAGCTGGATGGTGTCGGCGCAGCTGTGGAACGG
« EST14C_R   (341) GCGCCGTCAGAGAAGGCGAGCTGGATGGTGTCGGCGCAGCTGTGGAACGG
« EST14S_R   (352) GCGCCGTCAGAGAAGGCGAGCTGGATGGTGTCGGCGCAGCTGTGGAACGG
  Consensus  (352) GCGCCGTCAGAGAAGGCGAGCTGGATGGTGTCGGCGCAGCTGTGGAACGG
             (351)
                    401                                                 450
» EST14C_F   (361) GCCGGCTACGGGGGACGCGGCGGCCAAGGGCCCGCAGACTCCCAAGGAGC
» EST14S_F   (322) GCCGGCTACGGGGGACGCGGCGGCCAAGGGCCCGCAGACTCCCAAGGAGC
« EST14C_R   (391) GCCGGCTACGGGGGACGCGGCGGCCAAGGGCCCGCAGACTCCCAAGGAGC
« EST14S_R   (402) GCCGGCTACGGGGGACGCGGCGGCCAAGGGCCCGCAGACTCCCAAGGAGC
  Consensus  (402) GCCGGCTACGGGGGACGCGGCGGCCAAGGGCCCGCAGACTCCCAAGGAGC
             (401)
                    451                                                 500
» EST14C_F   (411) GCTCGGAGCACCCGCTGGACACGAGCCCCATGCTCGGCGCCCTCGACGGC
» EST14S_F   (372) GCTCGGAGCACCCGCTGGACACGAGCCCCATGCTCGGCGCCCTCGACGGC
« EST14C_R   (441) GCTCGGAGCACCCGCTGGACACGAGCCCCATGCTC
« EST14S_R   (452) GCTCGGAGCACCCGCTGGACACGAGCCCCATGCTC
  Consensus  (452) GCTCGGAGCACCCGCTGGACACGAGCCCCATGCTCGGCGCCCTCGACGGC
             (451)
                    501             520
» EST14C_F   (461) GGCGGCGGTGCTTTCCTCCC
» EST14S_F   (422) GGCGGCGGTGCTTTCCTCCC
« EST14C_R   (474)
« EST14S_R   (485)
  Consensus  (502) GGCGGCGGTGCTTTCCTCCC
             (501)
```

SUPPLEMENTARY DATA

CURRICULUM VITAE

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Education:

2012	PhD	METU, Biotechnology Dept.
2005	MSc	METU, Biotechnology Dept.
2002	BS	METU, Dept. of Biological Sciences

Experiences:

2008 – 2009	Occupational Trainee, Australian Centre for Plant Functional Genomics Pty Ltd (ACPFG) Adelaide SA, Australia
2002 – 2011	Research Assistant, METU, Faculty Development Program (ÖYP)
2002 – 2011	Teaching Assistant, METU, Dept. of Biological Sciences
2001 – 2002	Intern, METU, Dept. of Biological Sciences, Plant Biochemistry and Biotechnology Research Laboratory
2001	Intern, Refik Saydam Hygiene Center, Contagious Diseases Research Dept.

Peer-reviewed Publications in SCI, SSCI and AHCI:

1. Yılmaz R, Akça O, Baloğlu MC, **Öz MT**, Öktem HA, Yücel M (2012) Optimization of yeast (*Saccharomyces cerevisiae*) RNA isolation method for real-time quantitative PCR and microarray analysis. Afr. J. Biotechnol. 11(5): 1046-1053.
2. **Öz MT**, Eyidoğan F, Yücel M, Öktem HA (2009) Optimized selection and regeneration conditions for *Agrobacterium*-mediated transformation of chickpea cotyledonary nodes. Pak. J. Bot. 41(4): 2043-2054.
3. **Öz MT**, Yılmaz R, Eyidoğan F, de Graaff L, Yücel M, Öktem HA (2009) Microarray analysis of late response to boron toxicity in barley (*Hordeum vulgare* L.) leaves. Turk. J. Agric. For. 33: 191-202.
4. Öktem HA, Eyidoğan F, Demirbaş D, Bayraç AT, **Öz MT**, Özgür E, Selçuk F, Yücel M (2008) Antioxidant responses of lentil to cold and drought stress. J. Plant Biochem. Biot. 17(1): 15-21.
5. Eyidoğan F and **Öz MT** (2007) Effect of salinity on antioxidant responses of chickpea seedlings. Acta Physiol. Plant. 29: 485-493.

Peer-reviewed International Publications – Book, Chapter, Review:

1. Eyidoğan F, **Öz MT**, Yücel M, Öktem HA (2012) Signal transduction of pytohormones under abiotic stresses. In: Khan NA, Nazar R, Iqbal N, and Anjum NA (Ed.) Phytohormones and Abiotic Stress Tolerance in Plants. Springer, Germany.
2. Yılmaz R, Eyidoğan F, **Öz MT**, Yücel M, Öktem HA (2010) Gıda Örneklerinde Genetiği Değiştirilmiş Organizma Analizleri. Translated book: Querci M, Jermini M, Van den Eede G, and Aygün SB (Ed.) The Analysis of Food Samples for the Presence of Genetically Modified Organisms. European Commission Joint Research Center, Italy.
3. Öktem HA, Eyidoğan F, Selçuk F, **Öz MT**, Teixeira da Silva JA, Yücel M (2008) Revealing response of plants to biotic and abiotic stresses with microarray technology. Invited Review. Genes, Genomes and Genomics, 2(1): 14-48.

Presentations in an International Conference:

1. **Öz MT**, Gumusel D, Eyidogan F, Sutton T, Hay A, Pallotta M, Langridge P, Yucel M, Oktem HA "Determination of expression profiles of barley (*Hordeum vulgare* L) under boron toxicity and characterization of 3H boron transporter gene" 9th PlantGEM, Abstract Book P110, Istanbul, Turkey, May 4-7, 2011.

2. Inan Genc A, Jahya E, Atilgan S, Patir G, Unal Y, **Öz MT**, Baloglu MC, Ercan Akca O, Battal A, Oktem HA, Yucel M "Physiological and biochemical effects of boron toxicity on local cultivars of lentil (*Lens culinaris*)" 9th PlantGEM, Abstract Book P66, Istanbul, Turkey, May 4-7, 2011.
3. **Öz MT**, Eyidoğan F, Yücel M, Öktem HA "Expression profiles of barley (*Hordeum vulgare* L) reveal differences between responses under boron toxicity and deficiency" Plant Gene Discovery Technologies, International Conference, Book of Abstracts pp 41, Vienna, Austria, February 23-26, 2011.
4. Baloğlu MC, Kavas M, **Öz MT**, Battal A, Eroğlu A, Kayıhan C, Öktem HA, Yücel M "Cloning of wheat NAC-type transcription factors and *Agrobacterium* mediated transformation of wheat mature and immature embryos" Plant Transformation Technologies II, International Conference, Book of Abstracts pp 71, Vienna, Austria, February 19-22, 2011.
5. Eroğlu A, Baloğlu MC, **Öz MT**, Öktem HA, Yücel M "*Agrobacterium* mediated transformation of tobacco with a NAC-type transcription factor, *TaNAC69-1*" Plant Transformation Technologies II, International Conference, Book of Abstracts pp 76, Vienna, Austria, February 19-22, 2011.
6. Ünsal BG, **Öz MT**, Yılmaz R, Hamamcı H, Öktem HA, Yücel M "Transcription Profiling of Wheat Seedlings (*Tricitum aestivum* L.) under Salt and Drought Stress Conditions" Nanomat 2007 International Workshop on Nanobiotechnology and Genome Technologies, Book of Abstracts pp 39, Antalya, Turkey, 2007.
7. **Öz MT**, Yılmaz R, Eyidoğan F, de Graaff L, Yücel M, Öktem HA "Expression Profiling of Barley (*Hordeum vulgare* L.) under Boron Toxicity via DNA Microarrays" 5th PlantGEM, Abstract Book P1.26, Venice, Italy, October 2006.
8. Eyidoğan F and **Öz MT** "Antioxidant responses of shoots and roots of chickpea to NaCl stress" 2nd EPSO Conference (Interactions in Plant Biology: cells, plants and communities), Abstract Book, P111, Ischia, Italy, October 2004.

Presentations in a National Conference:

1. Kayıhan C, **Öz MT**, Eyidoğan F, Ekmekçi Y, Yücel M, Öktem HA "Bor Uygulamaları ve Tarımborun Buğday Bitkisinin Fotosistem II Aktivitesi Üzerine Etkisi", 20th National Biology Congress, Abstract published in Proceedings and Poster Book, Denizli, Turkey, 2010.

2. **Öz MT**, Yılmaz R, Eyidoğan F, de Graaff L, Yücel M, Öktem HA "Bor Toksisitesine Karşı Arpanın (*Hordeum vulgare* L.) Gen İfade Seviyelerinin Mikroarray Yöntemi ile Belirlenmesi" XVth National Biotechnology Congress, Full paper published in Proceedings and Poster Book pp 70-73, S-BHB2, Antalya, Turkey, 2007.
3. **Öz MT**, Eyidoğan F, Yücel M, Öktem HA "Nohutta Transformasyon Sonrası Seçme Koşullarının Optimizasyonu", XIVth National Biotechnology Congress, Abstract published in Proceedings and Poster Book, Eskişehir, Turkey, 2005.
4. **Öz MT** and Eyidoğan F "Tuz Stresinin Nohut Antioksidan Sistemleri Üzerine Etkisi" XIVth National Biotechnology Congress, Full paper published in Proceedings and Poster Book, Eskişehir, Turkey, 2005.
5. Eyidoğan F, **Öz MT**, Yücel M, Öktem HA, Szabados L "Geri Dönüşüm İnhibisyonu Engellenmiş Mutant *AtP5CS* Geninin Tütüne Transferi ve Transgenik Bitkilerin Analizi" XIIIth National Biotechnology Congress, Full paper published in Proceedings Book, Çanakkale, Turkey, 2003.
6. Öktem HA, Cicerali IN, Demirbaş D, Bayraç AT, **Öz MT**, Yol BD, Özgür E, Eyidoğan F, Yücel M "Sıcak, Soğuk ve Kuraklık Stresinin Mercimek (*Lens culinaris* M.) Antioksidan Sistemi Üzerine Etkileri" XIIIth National Biotechnology Congress, Full paper published in Proceedings Book, Çanakkale, Turkey, 2003.

Seminars, Congress and Courses Participated as an Organizer or Instructor:

- 1st National Biotechnology Student Summit, Organizers: Öktem HA, Çökmüş C, Eyidoğan F, Tuncer AS, Ercan O, Baloğlu C, **Öz MT**, METU, Culture and Convention Center, Ankara, Turkey, September 23 – 24, 2011.
- 3rd Microarray Training and Data Analysis Course, Organizer: Yücel M, Instructors: Yılmaz R, **Öz MT**, Ercan O, Baloğlu MC, METU, Central Laboratory, Molecular Biology and Biotechnology R&D Center, Ankara, Turkey, December 20 – 25, 2010.
- Microarray Training and Data Analysis Course, Organizer: Yücel M, Instructors: Yılmaz R, **Öz MT,** METU, Central Laboratory, Molecular Biology and Biotechnology R&D Center, Ankara, Turkey, November 24 – 25, 2008.

Memberships:

- Member of the Federation of European Societies of Plant Biology (FESPB).
- Member of Executive Board of Turkish Biotechnology Association.
- Member of the Young European Biotech Network (YEBN).

Scholarships, Certificates and Awards:

- Certificate for "Quarantine Awareness" endorsed by Australian Quarantine and Inspection Service (AQIS), Australian Government Department of Agriculture, Fisheries and Forestry, attainment number and date: 16611, 11.03.2009.
- Certificate for "Quarantine Approved Premises for Accredited Persons (Classes 2 to 9)" endorsed by Australian Quarantine and Inspection Service (AQIS), Australian Government Department of Agriculture, Fisheries and Forestry, attainment number and date: 16611, 17.03.2009.
- Occupational Trainee, November 2008 – December 2009, Australian Centre for Plant Functional Genomics Pty Ltd (ACPFG), Adelaide SA, Australia, scholarship provided by Faculty Development Program (ÖYP) of METU, Ankara Turkey.
- Bronze Award, iGEM 2007 (International Genetically Engineered Machines) Competition, MIT, Massachusetts, USA.
- Young Scientist Award, 5th PlantGEM (Plant Genomics European Meetings), Venice, Italy.
- MSc Program Honor Student of the Department of Biotechnology in the Academic Year 2003-2004, METU, Graduate School of Natural and Applied Sciences, Ankara, Turkey.

Computer Skills:

Microsoft Office Applications (Word, Excel, PowerPoint, Publisher), MINITAB, Bioconductor, R, Vector NTI, GCOS, GeneSpring GX, HarvEST, MeV, SPSS, SAS and Internet Applications

Projects:

1. **TUBITAK** 108O786. NAC tipi transkripsiyon faktörleri kullanılarak abiyotik stres dirençli transgenik buğday çeşitlerinin geliştirilmesi ve elde edilen bitkilerde abiyotik stres koşullarında gen ifade profillerinin mikroarray yöntemiyle incelenmesi. Yücel M, Gürel E, Çelikkol Akçay U, Kavas M, Öz MT, Kalemtaş G, Kayıhan C, Ercan O, Aysin F, Aksoy E, Baloğlu MC.
2. **BOREN** BOREN-2009-Ç0217. Bor stresi altında buğday bitkisinin gen ifade profillerinin incelenmesi ve Tarımborun bu strese tepki üzerine etkilerinin moleküler seviyelerde belirlenmesi. Öktem HA, Yücel M, Eyidoğan F, Öz MT, Ercan O, Kayhan C.
3. **BOREN** BOREN-2006-25-Ç24-25. Bor stresinin bitkiler üzerinde oluşturduğu etkilerin DNA çip teknolojileri ile araştırılması. Öktem HA, Yücel M, de Graaff L, Eyidoğan F, Öz MT.
4. **ODTÜ-BAP-ÖYP** BAP-08-11-DPT 2002 K120510. Arpada abiyotik stres koşullarının genel gen ifadesi üzerine etkileri. Öktem HA, Yücel M, Eyidoğan F, Öz MT.
5. **ODTÜ-BAP-YUUP** BAP-08-11-DPT 2002 K120510. Yemeklik baklagillerin biyoteknolojik yöntemlerle iyileştirilmesi. Öktem HA, Yücel M, Önde S, Öz MT, Çelikkol Akçay U, Bayraç T, Kamçı H.
6. **ODTÜ-BAP-ÖYP** BAP-08-11-DPT 2002 K120510. Nohutun genetik transformasyonu ve herbisit dirençli transgenik bitkilerin geliştirilmesi. Öktem HA, Eyidoğan F, Öz MT.

Languages: Turkish: native speaker
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Interests: Reading, Music, Sailing.