IDENTIFICATION AND CHARACTERIZATION OF BORON TOLERANT GENES IN *HORDEUM VULGARE* (BARLEY) BY USING mRNA DIFFERENTIAL DISPLAY AND RT-PCR TECHNIQUES

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF BORON TOLERANT GENES IN *HORDEUM VULGARE* (BARLEY) BY USING mRNA DIFFERENTIAL DISPLAY AND RT-PCR TECHNIQUES

Boron, is a microelement that plays a role in plant development. In contrast, excess amount of boron is toxic for plants. Turkey is the second country that has the largest boron reserve in the world, thus boron is one of the major problems in agriculture in Turkey. Barley (*Hordeum vulgare*) is the second widely produced cereal after wheat. Because barley is used in human diet, animal feeding and beer industry it is an economically valuable crop. There are ten different barley varieties in Turkey and these varieties show different genetic variations against boron toxicity. In this study, Hamidiye (boron sensitive) and Anadolu (boron tolerant) varieties were used to identify genes responsible for boron tolerance. RT-PCR and mRNA Differential Display techniques were used from root and leaf samples of Hamidiye and Anadolu varieties grown in laboratory with or without boron conditions. Eight differentially expressed genes identified by using mRNA Differential Display technique. Sequence of these genes gave homology to an eukaryotic translation initiation factor in *Arabidopsis thaliana*, a chlorophyll a/b binding protein precursor in *Triticum aestivum*, an elongation factor in Oryza sativa, short-chain dehydrogenase/reductase family protein in *Arabidopsis thaliana*, a thioredoxin h isoform in *Triticum aestivum*, a shaggy-like kinase protein in *Triticum aestivum*, chloroplast genome in *Hordeum vulgare*, a hypothetical protein in *Arabidopsis thaliana*. Expression level of six of forty three antiporter genes showed differences between Anadolu and Hamidiye cultivars in Real Time PCR.

ÖZET

BOR TOKSİSİTESİNE TOLERANS GÖSTEREN ARPA ÇEŞİDİNDEKİ GENLERİN mRNA FARKLILIĞIN GÖSTERİMİ TEKNİĞİ VE RT-PCR KULLANARAK İZOLASYONU VE KARAKTERİZASYONU

Bor, bitkilerin gelişmesinde rol oynayan bir mikroelementtir. Ancak fazla miktardaki bor bitkiler için toksiktir. Türkiye, dünyada en fazla bor rezervine sahip ikinci ülkedir ve bu yüzden bor tarımda Türkiye için ciddi bir problemdir. Arpa (*Hordeum vulgare*), Türkiye'de buğdaydan sonra en çok üretilen tahıldır. Arpa insan beslenmesinde, hayvan yemciliğinde ve bira sanayinde de kullanıldığı için ekonomik değeri yüksek bir tarım ürünüdür. Türkiye'de 10 farklı arpa çeşidi vardır ve bunlar bor toksisitesine karşı farklı genetik varyasyonlar gösterir. Bu çalışmada Hamidiye (bora karşı dirençsiz) ve Anadolu (bora karşı dirençli) çeşitleri kullanılmıştır. İki çeşit arasındaki genetik farklılığı ortaya çıkarmak için mRNA Farklılık Gösterim tekniği kullanılmıştır. Hem mRNA Farklılık Gösterim tekniği ile bulunan sekanslar hem de bilinen bazı antiporter sekanslar RT-PZR ile de çalışılmıştır. Bu deneylerde kullanılan kök ve yaprak örnekleri, laboratuar koşullarında borlu ve borsuz ortamda yetiştirilen Hamidiye ve Anadolu çeşitlerinden farklı zamanlarda toplanmıştır. mRNA Farklılık Gösterim tekniği ile farklı eksprese olan 8 gen tespit edilmiştir. Sekans analizi sonucunda bu genlerin *Arabidopsis thaliana*'nadaki bir ökaryotik translasyon başlatıcı proteinine, *Triticum aestivum*'daki bir klorofil a/b bağlayıcı protein öncüsüne, *Oryza sativa*'daki bir elongasyon faktörüne, *Arabidopsis thaliana*'daki bir kısa-zincir dehidrogenaz/redüktaz ailesi proteinine, *Triticum aestivum*'daki bir tioredoksin h izoformuna, *Triticum aestivum*'daki bir shaggy benzeri kinaz proteinine, *Hordeum vulgare*'deki kloroplast genomuna ve *Arabidopsis thaliana*'daki bir hipotetik proteine homoloji gösterdiği bulunmuştur. Ayrıca, RT-PZR ile 43 antiporter geninden 6'sının Anadolu ve Hamidiye çeşitleri arasında farklı düzeylerde eksprese oldukları gösterilmiştir.

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ABBREVIATIONS

B	Boron
mg	Milligram
kg	Kilogram
RG-II-B	Rhamnogalacturonan-II-boron
ATPase	Adenosine triphosphatase
DNA	Deoxiribonucleic acid
RNA	Ribonucleic acid
mM	Millimolar
µM	Micromolar
ATP	Adenosine triphosphate
ROS	Reactive oxygen species
kDa	Kilodalton
RFLP	Restriction fragment length polymorphism
QTL	Quantitative trait loci
mRNA	Messenger ribonucleic acid
hrs	Hour
ml	Milliliter
µl	Microliter
HCL	Hamidiye control leave sample
ACL	Anadolu control leave sample
HBL2	Hamidiye boron treated leave sample picked up at 2 hour
HBL6	Hamidiye boron treated leave sample picked up at 6 hour
HBL24	Hamidiye boron treated leave sample picked up at 24 hour
HBL48	Hamidiye boron treated leave sample picked up at 48 hour
ABL2	Anadolu boron treated leave sample picked up at 2 hour
ABL6	Anadolu boron treated leave sample picked up at 6 hour
ABL24	Anadolu boron treated leave sample picked up at 24 hour
ABL48	Anadolu boron treated leave sample picked up at 48 hour
HCR	Hamidiye control root sample
ACR	Anadolu control root sample
HBR2	Hamidiye boron treated root sample picked up at 2 hour

HBR6	Hamidiye boron treated root sample picked up at 6 hour
HBR24	Hamidiye boron treated root sample picked up at 24 hour
HBR48	Hamidiye boron treated root sample picked up at 48 hour
ABR2	Anadolu boron treated root sample picked up at 2 hour
ABR6	Anadolu boron treated root sample picked up at 6 hour
ABR24	Anadolu boron treated root sample picked up at 24 hour
ABR48	Anadolu boron treated root sample picked up at 48 hour
DNase	Deoxiribonuclease
RNase	Ribonuclease
M	Molar
rpm	Revolution per minute
µg	Microgram
dNTP	Deoxiribonucleotide triphosphate
cDNA	Complementary DNA
PCR	Polymerase chain reaction
min	Minute
sec	Second
EtBr	Ethidium Bromide
TAE	Tris-Acetate electrophoresis buffer
V	Voltage
dH_2O	Distilled water
E. coli	*Escherichia coli*
LB	Luria-Bertani
IPTG	Isopropyl-thio-β-D-galactopyranoside
X-Gal	5-bromo-4-chloro-3 indolyl-B-D-galactoside

CHAPTER 1

INTRODUCTION

1.1. Boron

Boron is a nonmetallic element that belongs to Group IIIA of the periodic table and has an oxidation state of +3 (EPA 2004). The boron atom is small with only three valance electrons (Bolanos et al. 2004). It has an atomic number of 5 and atomic weight of 10.81 (EPA 2004). Boron is actually a mixture of two stable isotopes, ^{10}B (19.8%) and ^{11}B (80.2%) (World Health Organization 1998). As a member of the semiconductor group of elements, boron has properties intermediate between metals and non-metals (Bolanos et al. 2004). At the near neutral pH as being in most biological fluids, boron exists primarily as undissociated boric acid which is soluble in water, $B(OH)_3$ and exists a small amount of borate anion, $B(OH)_4^-$ (Bolanos et al. 2004). Boric acid is a very weak acid in aqueous solution; its activity as an acid appears to be related to OH^- acceptance by $B(OH)_3$ rather than to H^+ donation. The primary sources of boron in soils are tourmaline and the volatile emanations of volcanoes (Nable et al, 1997, Chesworth 1991).

Boric acid and sodium salts of boron (primarily borax, or disodium tetraborate decahydrate) are widely used for a variety of industrial purposes including manufacture of glass, fiberglass insulation, porcelain enamel, ceramic glazes, and metal alloys (EPA 2004). These compounds are also used as fire retardants in cellulose insulation, laundry additives, fertilizers, herbicides and insecticides (Woods 1994).

Most soils have low concentrations of B (under 10 mg B kg-1) and many areas of land are deficient in B. The concentration in sea water (1 – 10 mg B kg-1) is about 350 times that found in river water (Nuttall 2000, Power & Woods 1997).

Boron is an essential micronutrient for growth and development of higher plants (Takano et al. 2005, Hu & Brown 1997). It is widely distributed through the earth's land and water (Takano et al. 2005, Hu & Brown 1997). In intact tissues of plants, boron occurs as water-soluble forms, the majority of which seems to be localized in the apoplastic region as boric acid, and water insoluble forms (Mahboobi et al. 2001). Soils with high concentrated boron are found in parts of the western United States and Turkey

(Nuttall 2000, Power & Woods 1997), South Australia (Nable et al. 1997, Cartwright et al. 1984), the west coast of Malaysia (Nable et al. 1997, Shorrocks 1964), southern coast of Peru (Nable et al. 1997, Masson 1967), the Andes foothills in northern Chile (Nable et al. 1997, Caceres et al. 1992), ferralsols of India (Nable et al. 1997, Takkar 1982), rendzinas in Israel (Nable et al. 1997, Ravikovitch et al. 1961).

Table 1.1 Chemical and physical properties of boron and selected boron compounds (Source: EPA 2004)

	Boron	Boric Acid	Borax	Borax Pentahydrate	Anhydrous Borax	Boron Oxide
Molecular Formula	B	H_3BO_3	$Na_2B_4O_7.10H2O$	$Na_2B_4O_7.5H2O$	$Na_2B_4O_7$	B_2O_3
Molecular Weight	10.81	61.83	381.43	291.35	201.27	69.62
Boron Content (%)	100	17.48	11.34	14.85	21.49	31.06
Physical Form	black crystal or yellow-brown amorphous powder	white or colorless crystalline granules or powder	white or colorless crystalline granules or powder	white or colorless crystalline granules or powder	white or colorless vitreous granules	white or colorless vitreous granules
Specific Gravity (@ 20°C)	2.34	1.51	1.73	1.81	2.37	2.46
Melting Point (°C) closed space	2300	171	>62	<200	No data	No data
Melting Point (°C) anhydrous form	2300	450	742	742	742	450
Water Solubility (% w/w)	insoluble	4.7@ 20°C 27.5@100°C	4.71@ 20°C 65.63@ 100°C	3.6@ 20°C 50.13@ 100°C	2.4@ 20°C 34.5@100°C	rapidly hydrates to boric acid

1.2. Necessity, Function and Deficiency of Boron

Existence of boron in plants was firstly reported by Agulhon in 1910 but its importance for plants was demonstrated later (Blewins & Lukaszewski 1998). After the discovery of necessity of boron for plants, using of boron fertilizer for crops increased which led to an observation that different plant species and even different varieties of the same species differ according to their boron requirement (Blewins & Lukaszewski

1998). This observation forced researchers to classify the plants according to their boron requirement. Plants can be classified into three groups according to their boron requirement (Mengel and Kirkby 1987): graminaceous monocots have the lowest demand for boron; non-graminaceous monocots and many of the dicots have an intermediate requirement and finally latex-forming plants have the highest boron requirement among plant species (Blewins & Lukaszewski 1998, Mengel & Kirkby 1987). In addition, Shkolnik classified the plants based on stage of growth and localization of boron deficiency symptoms: in some dicots such as sunflower, tomato, squash and alfalfa inhibition of root growth and degeneration of meristematic regions under boron free conditions appear rapidly and simultaneously; in other dicots such as pea, soybean and lupine degeneration of growing points is delayed; some monocots such as maize, sorghum, millet and onion are capable to display normal root growth and vegetative growth much longer than dicots under boron free conditions (Blewins & Lukaszewski 1998, Shkolnik 1984). Different boron requirement of dicots and monocots is due to the differences in their cell wall composition; the primary cell wall of graminaceous plants contains very little pectic material which affects the boron binding capacity of the cell wall (Nuttall 2000, Darvill et al. 1980). However, it should take into consideration that boron binding capacity of the cell wall may not be the only factor which designates the boron requirement of a plant.

Boron has many functions in plant cells. In many studies it is clearly demonstrated that boron is functional in the structure of cell wall, in membrane functions and in the some metabolic activities of plants (Blewins & Lukaszewski 1998). Although it is known that boron is necessary for many functions of plants there is no evidence that boron forms the part of any enzyme or it plays a direct role in enzyme activity (Nuttall 2000, Çakmak & Romheld 1997). It is also widely accepted that these vital roles of boron are related to its capacity to form diester bridges between cis-hydroxyl-containing molecules (Bolanos et al. 2004, Nuttall 2000).

It was reported that approximately 90% of cellular boron has been localized in the cell wall fraction (Blewins & Lukaszewski 1998, Loomis & Durst 1992). In the boron deprivation, abnormalities in cell wall and middle lamella organization have been reported (Blewins & Lukaszewski 1998, Loomis & Durst 1992, Hu & Brown 1994, Matoh et al. 1992). The key observation in the relationship between cell wall and boron is the isolation and characterization of rhamnogalacturonan-II-boron (RG-II-B) from the cell walls of root cells of radish by Kobayashi et al. (Blewins & Lukaszewski 1998,

Bolanos et al. 2004, Nuttall 2000). Subsequently, Matoh et al. (1993) demonstrated the presence of RG-II-B in cell walls of 22 other plant species belonging Leguminosae, Apiaceae, Chenopodiaceae, Solanaceae, Asteraceae, Liliaceae, Araceae, Amaryllidaceae and Gramineae (Blewins & Lukaszewski 1998, Matoh et al. 1996). RG-II is a pectic polymer (Thomas et al. 1989) that forms a dimmer with the help of boron cross-link (Nuttall, 2000, Bolanos et al. 2004, O`Neill et al. 1996). Not only in cell wall but also in the cytoplasm there are a large number of biological compounds that can form complexes with boron (Hu & Brown 1994) such as sugars, sugar alcohols, phenols, organic acids and some polymers (Hu & Brown 1994, Boesken 1949, Raeven 1980).

The role of boron in membranes is not well defined as done in cell wall. On the other hand, many of performed experiments by different researchers related to boron treatment of low-boron plants resulted in the hyperpolarization of root membranes and stimulation of ferricyanide-dependent H^+ release, ATPase activity, NADH oxidase activity, and ion transport all of which these changes can be associated with membrane function (Blewins & Lukaszewski 1998, Ferrol et al. 1993, Loughman & White 1984, Roldan et al. 1992, Schon et al. 1990). Marschner et al. proposed that boron stabilized the structure of the plasma membrane by complexing membrane constituents (Blewins & Lukaszewski 1998). Either H-bonding or ester formation with glycolipids and/or glycoproteins could easily keep enzymes or channels in an optimum conformation and anchored in the membrane (Blewins & Lukaszewski 1998). The presence of less phospholipids and galactoplipid in membranes of boron deficient plants also supports the relationship between boron and membranes (Blewins & Lukaszewski 1998 Shkolnik 1984). Moreover, Shkolnik demonstrated the reason of retardation in membrane function of dicots under boron deprivation. According to Shkolnik, under boron deficient conditions some enzymes including ribonuclease, glucose-6-phosphate dehydrogenase, phenylalanine ammonia lyase, β-glucosidase and polyphenoloxidase activation of which alters plant metabolism, depletes RNA and increases phenolic synthesis become active. However, these enzymes normally bind to membranes or walls in a latent form (Blewins & Lukaszewski 1998, Shkolnik 1984). Increased phenolics, potential growth inhibitors, inhibit ion uptake thereby retarding membrane function (Blewins & Lukaszewski 1998, Glass & Dunlop 1974).

The other physiologic actions in which boron take parts can be summarized as follows: reproduction (Loomis & Durst 1992), pollen tube growth, pollen germination,

flowering, fruiting (Blewins & Lukaszewski 1998), nitrogen fixation (Matco et al. 1986), auxin metabolism (MacVicar & Tottingham 1947, Moniat 1943), sugar transport, carbohydrate metabolism, respiration, lignification, phenol metabolism and RNA metabolism (Nuttall 2000). Although physiological basis for the high boron demand for reproduction of a plant is not fully understand, it is observed that boron requirement of many plant species in reproductive growth is much higher than in vegetative growth (Blewins & Lukaszewski 1998, Gauch & Dugger 1954). Because of the reasons previously listed, boron deficiency causes defects in assembly and mechanical properties of cell walls, in structural and functional integrity of plasma membrane, and in several metabolic and physiological processes (Yu et al. 2002, Loomis & Durst 1991, Goldbach 1997, Blevins & Lukaszewski 1998, Goldbach et al. 2001, Brown et al. 2002).

1.3. Boron Absorption by Plant Roots

Absorption of boron from soil solution is performed by roots mainly as the undissociated boric acid (Hu & Brown 1994). However the key question `Is boron absorption is active or passive?` is still unclear. For years many researchers have been focusing on this question and performing many studies with varied and conflicting results.

By computing the permeability of the plant cells to boric acid on the basis of the lipid solution mechanism, Raven (1980) predicted that boron absorption by roots would be a passive process (Hu & Brown 1994, Bingham et al. 1970). He claimed that the use of active transport of boric acid to maintain boron distribution across a membrane away from thermodynamic equilibrium is likely to be energetically expensive.

Wilders and Neales (1971) reported that disks of carrot and red beet absorbs boron to equilibrium internal concentration which is greater than that of the external solution and that this accumulation of boron in tissues is inhibited by anoxia, by DNP and also by low temperature, suggesting active uptake (Hu & Brown 1994, Wilders & Neales 1971). However, in the same study boron content of storage tissues, after a period of absorption, was rapidly and completely desorbed into a boron free solution, suggesting a passive diffusion type process (Wilders & Neales 1971). Because of these results, Wilders and Neales explained the boron uptake with two components, one of

which is passive diffusion of boric acid and the other of which is active transport for borate anion (Wilders & Neales 1971). On the other hand, Hu and Brown (1997) reported that conclusion of active uptake may be the result of insufficient tissue rinsing and may also have been influenced by the relatively high concentrations of boron-binding ligands in carrot such as mannitol and fructose in sugar beet (Hu & Brown 1994).

Oertli and Grgurevic (1975) examined the effects of pH on boron absorption in barley root. They observed changes in boron concentration of tissues from 0.093mM to 0.93mM when pH changes from 6 to10 suggesting that boron in root tissues and external solutions tends to approach a diffusion equilibrium which is governed by the proportion of boric acid and borate anion in the system (Hu & Brown 1994, Oertli & Grgurevic 1975).

Bowen and Nissen (1977) demonstrated the inhibitor effects of DNP, NaN_3, NaCN, arsenate and dicoumarol on boron uptake in barley suggesting active uptake of boron (Hu & Brown 1994, Bowen & Nissen 1976). Hu and Brown (1997) also oppose the accuracy of these results. According to them, Bowen and Nissen used much more boron concentration which is physiologically relevant for barley and the transitions observed by Bowen and Nissen may be better explained by the saturation of the boron-binding capacity of various cellular compartments (Hu & Brown 1994).

It can be inferred from the experiments and their results mentioned above, boron uptake by plants is still a paradox for scientists. The general opinion is the absorption of boron in a passive process the rate of which influenced by passive diffusion of boron through the membrane, the formation of boron complexes within the cell wall and plant water fluxes (Hu & Brown 1994). However, there is doubtful evidence that boron uptake differs between species and even within varieties of a same species, for instance barley and wheat (Hu & Brown 1994, Nable 1988). To explain this paradox, several mechanisms have been postulated (Hu & Brown 1994, Nable 1988, Nable et al. 1990) some of which as follows: 1) Boron uptake may be partially under metabolic control and an active boron exclusion mechanism may explain the different extent of active uptake from genotype to genotype (Hu & Brown 1994). 2) Organic compounds containing cis-diols can complex with boron the resultant of which is an anion and it would have greatly reduced membrane permeability in comparison to uncharged boric acid, and hence absorption would be reduced (Hu & Brown 1994). 3) Root boron absorption capacity may differ significantly and therefore may affect the boron uptake

(Hu & Brown 1994). 4) Physical barriers related to root cell wall structure may determine boron uptake rates (Hu & Brown 1994).

1.4. Boron Distribution

The distribution of boron in plants is much more obvious than the absorption of boron by roots. To start with, in plants xylem and phloem are two members of the vascular system by which the long distance translocation of nutrient elements take place and also in which water is the translocating agent. In this vascular system, nonliving cells of xylem are responsible for upward movement from the roots to the shoots and this translocation is driven predominantly by the gradient in water potential resulting from surface water loss (transpiration) during the day (Brown & Shelp 1997). On the other hand, living cells of phloem are responsible for both upward and downward movement of the nutrients and also this movement does not depend on transpiration. In addition, nutrients could be transferred between xylem and phloem by extensive exchange process during translocation (Brown & Shelp 1997). For instance, DaSilva and Shelp (1990) demonstrated that in the steam and leaf veins xylem and phloem are separated by only a few cell layers and direct exchange of unmetabolised nutrients between these two pathways may occur (Brown & Shelp 1997). In terms of boron, there is no doubt that boron is translocated to sites of greatest water lost by xylem (Brown & Shelp 1997), but it is also assumed that phloem also plays a role in providing boron to sites that do not lose water readily (Brown & Shelp 1997). For example, the studies of both Shelp & Kitheka (1987), and Tammes & Van Die (1966) suggest that phloem, rather than xylem, is the predominant source of boron for developing sinks (Brown & Shelp 1997). In addition, Brown and Hu (1996) attributed the phloem mobility of boron to the presence of sorbitol as a primary photosynthetic product in plants. Their hypothesis is based on the effectively complexing of boron with polyols such as sorbitol, mannitol, or dulcitol which are the primary photosynthesis products. Brown and Hu (1996) also proposed that the occurrence of significant phloem mobility of boron is species dependent (Brown & Shelp 1997). Via their studies based upon movement of isotopic boron and a review of Makkee et al. (1985) with existing chemical data they hypothesized that the apparent mobility of boron in Prunus, Malus and Pyrus is a consequence of the use of sorbitol as a primary translocated

photosynthate in these species (Brown & Shelp 1997). Based upon these explanations, it can be inferred that influence of environmental and phenological factors on production and distribution of polyols can affect boron mobility in plants.

In addition to these physiological studies, Fujivara et al. (1997) used molecular approach to understand the boron metabolism in plants. They described and identified a novel *Arabidopsis thaliana* mutant, bor1-1 that requires high amounts of boron to complete its life cycle (Noguchi et al. 1997). Under boron sufficient medium (30μM boron) bor1-1 mutant was able to develop normal flowers but failed to set seeds the result of which indicates that bor1-1 mutants are female-sterile grown with 30μM boron (Noguchi et al. 1997). The other defects they detected in bor1-1 mutant were reduced expansion of rosette leaves and loss of apical dominance all of which can be recovered by application of excess boron (Noguchi et al. 1997). Also the boron concentration in bor1-1 mutants was lower than those that in wild type *Arabidopsis thaliana*. Interestingly, they demonstrated that when both mutants and wild types were grown under high boron concentration (at least 12μM boron), there were no differences in growth of both lines (Noguchi et al. 1997) suggesting that bor1-1 mutant plants are sensitive to boron deficiency.

1.5. Boron Toxicity

Although boron is an essential micronutrient for plant growth and development, excessive boron in the soil or in irrigation water causes the toxicity in plants and also reduces the crop yield (Takana et al. 2005). This toxicity is a significant problem in agricultural regions across the world (Hayes & Reid 2004, Cartwright et al. 1986, Nable et al. 1997). Normally different plant species can express different symptoms against boron toxicity. The main visible symptom of boron toxicity in many of plant species is necrotic and/or chlorotic spots at the margins and tips of older leaves (Nable et al. 1997, Bennett 1999, Bergmann 1992, Eaton 1944). Fruit disorders such as gummy nuts and internal necrosis, bark necrosis caused by death of cambial tissues and stem die back are the other symptoms resulted from boron toxicity (Nable et al. 1997, Brown & Hu 1996). In addition there is no reported symptom of boron toxicity in roots which may due to the fact that boron concentration in roots is much more lower than those in leaves and they may not reach to toxic levels (Nuttall 2000, Nable et al. 1997).

Today, the reason of boron toxicity is still unclear. Complex-forming ability of boron may be the cause of this toxicity (Nuttall 2000). Complexation with boron is limited to those compounds with two hydroxyl groups in the cis-conformation, classified as cis-diols (Reid et al. 2004), for instance RG-II-B complex. The other biological compound with cis-diols on a furanoid ring is ribose which is the main component of ATP, NADH or NADPH and RNA (Reid et al. 2004). Such being the case excessive amount of boron may bind to the ribose of these compounds and may cause the metabolic disruption. Reid et al. (2004) demonstrated that boron could interfere with metabolism by complexation of NAD^+ and to a lesser extent NADH and $NADP^+$. They also showed that membrane transport of amino acids is affected by boron but protein synthesis itself is not (Reid et al. 2004).

1.6. Boron Tolerance and Barley (*Hordeum vulgare*)

There is a large genetic variation in response to boron toxicity at both inter- and intra-specific levels in plants (Nable et al. 1997). Some of reported boron tolerant plant species are as follows: saltbush (Nable et al. 1997, Watson et al. 1994), milkvetch (Nable et al. 1997, Parker et al. 1991), barley (Nable et al. 1997, Nable 1990), wheat (Nable et al. 1997, Paull et al. 1988), Indian mustard (Nable et al. 1997, Banuelos et al. 1993), tall fescue (Nable et al. 1997, Banuelos et al. 1995), and some tree species (Nable et al. 1997, El-Motaium et al. 1994, Francois & Clark 1979). In addition, recent studies have found out the fact that a wide range of intra-specific variation in response to boron occurs in a number of crops (Nable et al. 1997), including bread wheat (Nable et al. 1997, Chatterjee et al. 1980, Mehrotra et al. 1980, Paull et al. 1988), durum wheat, (Nable et al. 1997, Brooks 1991, Jamjod 1996, Yau et al. 1995), barley (Nable et al. 1997, Nable 1988), rice (Cayton 1985, Paliwal & Mehta 1973), peas (Bagheri et al. 1992, Materne 1989), annual medics (Paull et al. 1992), citrus (Chapman & Vanselow 1955, Haas 1945), pecan (Picchioni & Miyamoto 1991), and strawberry (Blatt 1976). Among these plants, barley (*Hordeum vulgare*) is an important and widely grown cereal crop for Turkey (Avci & Akar 2004). Ten different varieties of boron are seen in Turkey: Tokak, Cumhuriyet, Hamidiye, Erginel, Obruk, Anadolu, Bülbül, Yesevi, Yea-1868 and Tarm-92, all of which show different genotypic variations in tolerance to boron toxicity (Torun et al. 2003).

Belonging to family of Graminea, barley is the most important cereal after the wheat. It is produced to feed animals, produce malt and be used in human foods (Katerji et al. 2006). Moreover, its importance rises from the ability to grow and produce in extreme conditions such as drought, low temperature and salinity (Katerji et al. 2006, Van Oosterom et al. 1993, Baum et al. 2004, Maas & Hoffman 1997).

Among the cereal species barley is the most sensitive to boron toxicity (Torun et al. 2003). However, there is a wide genotypic variation in response to boron toxicity in barley cultivars (Torun et al. 2003). Öktem et al. (2003) and Çakmak et al. (2003) revealed that Hamidiye and Bülbül are the most sensitive varieties, Anadolu and Tarm-92 are the most tolerant varieties in response to boron toxicity among barley cultivars in Turkey (Takana et al. 2005, Torun et al. 2003). Although many studies have been done regarding boron tolerance in barley, the exact mechanism of boron resistance is still unclear.

Reid and Hayes (2004) explained a possibility that tolerance against boron in some barley cultivars might be due to a mechanism involves efflux of the borate anion from root (Hayes & Reid 2004). They used two Australian barley cultivars both of which show very different tolerance to high boron, Sahara as the tolerant cultivar and Schooner as the sensitive cultivar. After boron treatment to these two cultivars they detected that boron concentration in the root of Schooner had equal to boron in the growth solution, while boron concentration in root of Sahara were 53% less than outside and they observed a similar result that xylem and shoot boron concentrations were lower in Sahara compared to Schooner. In addition, in microscopy studies of root they could not find any anatomical differences between Sahara and Schooner such as a compartment within the roots of Sahara with a capacity to store boron, results of which indicate that the roots of Sahara are able to efflux boron resulting in lower boron concentration in the xylem and less accumulation of boron in the shoot (Hayes & Reid 2004).

On the other hand, unlike Reid and Hayes, in their field study with Turkish barley cultivars Çakmak et al. (2003) explained that Hamidiye as the most sensitive cultivar against boron toxicity contained lower boron concentration in shoots at the tillering stage than many boron tolerant cultivars and had the lowest boron concentration in flag leaves (Torun et al. 2003). However, in a different greenhouse study researchers could not detect the considerable differences in shoot boron levels among the Turkish barley cultivars, even they found out that sensitive cultivars tended

to contain more boron in shoot than the tolerant cultivars (Torun et al. 2003). Boron tolerance of Turkish varieties might depend on the internal tolerance mechanism such as complexation of boron in cell walls or within the cells and differential distribution or compartmentation of boron at the cellular or organ levels (Torun et al. 2003). According to Çakmak et al., other possible tolerance mechanisms against boron toxicity for barley cultivars are altered distribution of boron between cytosol and vacuole, and expression of different antioxidant defense mechanisms against ROS produced under boron toxicity (Torun et al. 2003).

However, in their study Öktem et al. (2003) proved that membrane damage caused by boron toxicity is not related to oxygen free radicals and also antioxidant enzymes do not have a role in boron toxicity tolerance mechanism in barley (Takana et al. 2005). Öktem et al. (2001, 2002) also investigated the relationships between cell wall uronic acid content, a significant structural component of cell wall pectins, and boron toxicity tolerance (Mahboobi et al. 2001), and nitrate reductase and glutamate dehydrogenase activities, both of which are functional enzymes in inorganic nitrogen assimilation, and boron toxicity tolerance (Mahboobi et al. 2002) but they could not find any correlation between them.

Mahboobi et al. (2000) compared the protein contents of Anadolu and Hamidiye cultivars by using two-dimensional gel electrophoresis (Mahboobi et al. 2000). Results of this study demonstrated that boron treated seedlings of each cultivar showed an increase and decrease in a number of proteins in root and leaf tissues. They detected a newly synthesized protein, which was absent in Anadolu control cultivar and Hamidiye boron treated cultivar, with relative molecular weight of 35.0 kDa and pI value of 7.8 in the root of boron treated Anadolu cultivar. They also demonstrated the increase of three proteins with relative molecular weights of 23.0, 45.0, and 45.0 kDa and pI values of 6.1, 6.0, and 6.1 in root profile of Anadolu cultivar, but there was no increase of these proteins in Hamidiye cultivar.

Jenkin (1993) studied inheritance and location of genes conferring boron tolerance in barley by using leaf symptom data (Jefferies et al. 1999). By using RFLP linkage map and based on reduced leaf symptoms data, Jenkin proposed that the control of boron tolerance is mediated by three major gene loci (Jefferies et al. 1999). In addition, by using RFLP linkage map Jefferies et al. (1998) detected four chromosomal regions associated with boron tolerance in two of Australian barley cultivars, Clipper (intolerant to boron toxicity) and Sahara (tolerant to boron toxicity) (Jefferies et al.

1999). These chromosomal regions are on 2H associated with leaf symptom expression, on 3H associated with a reduction of the effect of boron toxicity on root growth suppression, on 6H associated with reduced boron uptake, and on 4H associated with the control of boron uptake, root length response, dry matter production and symptom expression (Jefferies et al. 1999). On the other hand, because some barley genotypes produce very few leaf symptoms yet accumulate high concentrations of boron in plant tissues (Mahalakshmi et al. 1995), mapping of major genes or quantitative trait loci (QTL) associated with boron tolerance based on data from leaf symptoms alone is insufficient to identify all important loci involved in the boron tolerance mechanism, since it is likely that some or all of the mechanisms involved in boron tolerance are under separate genetic control (Jefferies et al. 1999). In 2006, Fujiwara et al. performed a complex study by using *Arabidopsis thaliana* cDNAs and yeast mutants to isolate genes functional in boron tolerance (Nozawa et al. 2006). They introduced the cDNA library of *Arabidopsis thaliana* into a *Saccharomyces cerevisiae* mutant that lacks ScBOR1 (YNL275W), a boron efflux transporter. Screening of these mutant yeasts under 80 mM boron conditions, identified five *Arabidopsis thaliana* genes, AtPAB2, AtRPS20B, AtRBP47c, AtMYB13 and AtMYB68, that conferred elevated tolerance to boric acid in both Scbor1Δ and wild type yeast (Nozawa et al. 2006). Also two yeast genes, ScRPS20 and ScHRB1 were shown to be important in boron tolerance (Nozawa et al. 2006). However, yeasts over-expressing AtPAB2, AtRPS20B, AtMYB13 and AtMYB68 showed a little reduction in cell boron concentration and also yeast over-expressing AtRBP47c showed no reduction in cell boron concentration (Nozawa et al. 2006). Moreover, sequence analysis of these genes demonstrated that AtPAB2, AtRBP47c, ScRPS20 and ScHRB1 are responsible for several RNA-dependent processes such as transcription, RNA splicing, nuclear export of RNA, translation and mRNA degradation (Nozawa et al. 2006). It is a known fact that boron can complex with a variety of molecules containing cis-diol thereby inhibiting their activity and RNA is one of such cis-diol-containing molecules (Nozawa et al. 2006). Taken together, Fujiwara et al. proposed that those genes identified in their study may promote some RNA-dependent processes described thereby conferring boron tolerance (Nozawa et al. 2006). Moreover, AtMYB13 and AtMYB68 do not have an RNA-binding domain the mean of which may be the fact that these genes are involved in boron tolerance by different mechanisms (Nozawa et al. 2006).

Genetic mechanism of boron tolerance in plants is not completely identified. The techniques using in molecular genetics can be powerful tool for understanding the roles of essential elements in plants. The differences in the mRNA level of an organism demonstrate the response of organism against stress factors. Comparison of mRNAs between stress tolerant and intolerant organisms enables us to identify differentially expressed genes. For this purpose, we used mRNA differential display techniques to identify and characterize the genes conferring boron tolerance between two Turkish barley cultivars Hamidiye, as the intolerant genotype and Anadolu, as the tolerant genotype against boron toxicity. To detect the expression level of differentially expressed genes between cultivars in question, we also used Real Time PCR with proper primer pairs. Some of anti-porters were also screened in Real Time PCR to find out whether anti-porter genes are functional in boron tolerance of plants or not.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

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

2.2. Methods

2.2.1. Growth of Plants

Boron resistant (Anadolu) and sensitive (Hamidiye) cultivars of barley, *Hordeum vulgare*, were used in this study. Hundred healthy seeds for each cultivar were chosen. The seeds were subjected to 10% bleach for 20 min in petri dishes followed by distillated water for 1 hour for sterilization. The seeds were subjected to 5% HCL for 20 min followed by distillated water for 1 day. After surface sterilization they were placed on Whatmen papers in petri dishes for germination (Figure 2.1.). After two days, germinated seeds were transferred to beakers filled with Hoagland solution and plants were grown in these beakers under the controlled conditions at 28^0C (Figure 2.2.). Every two days Hoagland solution was refreshed.

2.2.2. Boron Treatment

After 10 days, 10mM boric acid solution was applied to plants. One beaker for Hamidiye and one beaker for Anadolu were selected as control plants and no boric acid was supplied to these beakers. Other two beakers one of which containing Hamidiye and the other one containing Anadolu were supplied to 10mM boric acid in Hoagland solution.

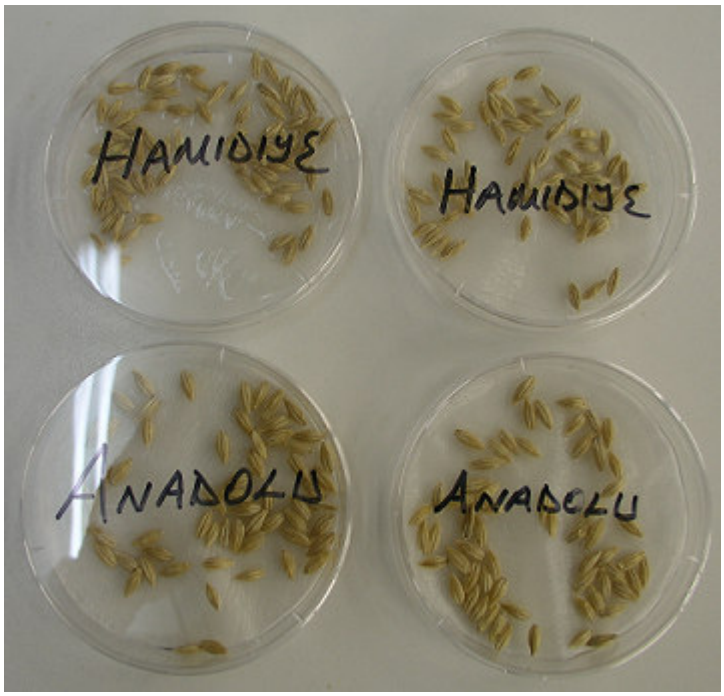

Figure 2.1. Germination of surface sterilized seeds in Petri dishes

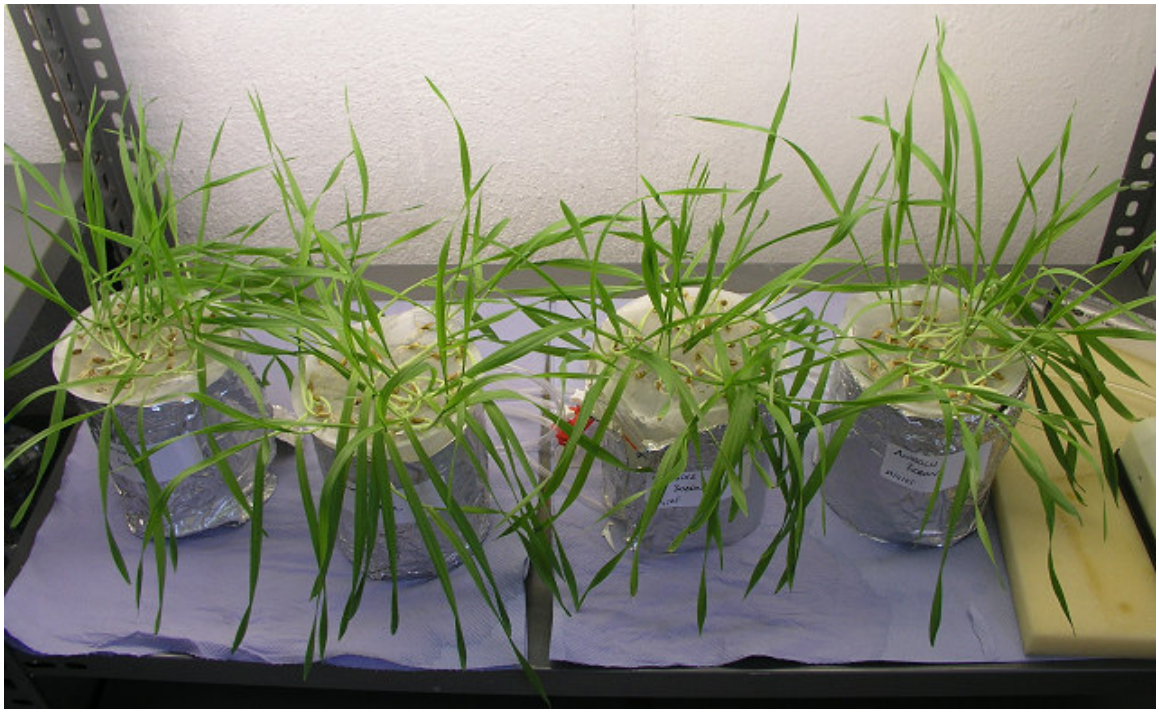

Figure 2.2. Growing of plants in beakers filled with Hoagland solution

2.2.3. Harvesting of Samples

After boron treatment, both root and leaf samples were collected from each plant at 2hrs, 6hrs, 24hrs and 48hrs and stored in -80^{0}C for further experiments.

2.2.4. RNA Isolation

Total RNA isolation was performed with TRIzol from frozen root and leaf samples. Tissues were homogenized by using mortal and pestle in liquid nitrogen. One ml TRIzol was used per 50-100 mg of tissue. Homogenates were centrifuged in falcon tubes at 12,000x g for 10 min at 4^{0}C to remove the insoluble materials. Clear supernatants containing RNA were transferred to fresh falcon tubes and 0.2 ml of chloroform per ml of TRIzol was added to falcon tubes. Falcon tubes were shaken vigorously for 15 seconds and allowed to stand for 15 min at room temperature. Resulting mixtures were centrifuged at 12,000x g for 15 min at 4^{0}C. After centrifugation, colorless upper aqueous phases containing RNA were transferred to fresh falcon tubes and 0.5 ml of isopropanol per ml of TRIzol was added. Resulting mixtures were allowed to stand for 10 min at room temperature and centrifuged at 12,000x g for 10 min at 4^{0}C. After this centrifugation, RNA precipitates formed pellets on the side and bottom of the tubes. Supernatants were removed and RNA pellets were washed by adding 1 ml of 75% ethanol per 1 ml of TRIzol. Falcon tubes were vortexed and centrifuged at 7,500x g for 5 min at 4^{0}C. Ethanol was removed and pellets were dried under vacuum. RNA pellets were dissolved in 100 µl of nuclease free water. The amount of isolated total RNAs was measured by using spectrophotometer. Isolated RNAs are also loaded to agarose gel to check the quality (Figure 2.3.).

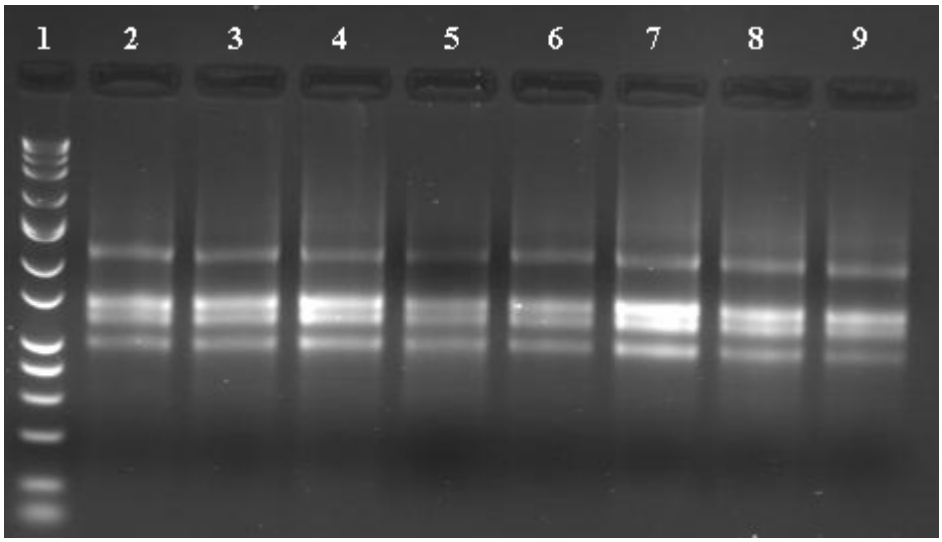

Figure 2.3. Total RNA samples. (1: marker DNA, 2: HCL, 3: ACL, 4: HBL6, 5: ABL6, 6: HBL24, 7: ABL24, 8: HBL48, 9: ABL48)

2.2.5. DNase I Treatment of Total RNAs

An equal volume of DNase mixture for each total RNA sample was prepared to prevent DNA contamination. One µl of 0.5M Tris-HCl (pH 7.5), 1 µl of 0.5M $MgCl_2$, 22 µl of sterile H_2O and 1 µl of RNase-free DNase I (1 unit/µl) were added to tube and this mixture was combined with 25 µl of RNA sample in a 0.5-ml microcentrifuge tube. Tubes were incubated at 37^0C for 30 min 2.5 µl of 0.2M EDTA and 2 µl of 3M NaOAc were added to tubes. Fifty µl of phenol: chloroform: isoamyl alcohol (25:24:1) equal was added to reactions. Tubes were vortexed and centrifuged at 14,000 rpm for 10 min. Aqueous layers were transferred to clean 0.5-ml tubes and a volume of chloroform: isoamyl alcohol (24:1) equal was added to the volume of aqueous layers. Tubes were vortexed and centrifuged at 14,000 rpm for 10 min. The top aqueous layers were transferred to clean 0.5-ml tubes. To each tube, 1/10 volume of 3M NaOAc and 2.5 volumes of 95% ethanol were added. Tubes were vortexed and centrifuged at 14,000 rpm for 20 min. Supernatants were removed and pellets were overlaid with 200 µl of %80 ethanol. After centrifugation at 14,000 rpm for 5 min, supernatants were removed and pellets were air dried for 10 min. Each pellet was dissolve in 1.5 µl of H_2O for each 2 µg of starting RNA.

2.2.6. First-Strand cDNA Synthesis

For each RNA sample; 2 µg total RNA, 1 µl cDNA synthesis primer and dH_2O up to 5 µl were combined in 0.5 ml tubes. Contents were mixed and spined briefly in a microcentrifuge. Tubes were incubated at 70^0C for 3 min and cooled on ice for 2 min. A master mix was prepared as follow: 2 µl of 5X First-strand buffer, 2 µl of dNTP mix (5mM) and 1 µl of MMLV reverse transcriptase (200 units/ µl) were mixed and added to each first-strand cDNA synthesis reaction. Contents were mixed and centrifuged briefly. The tubes were incubated at 42^0C for 1 hour in the air incubator. Reactions were terminated by incubating at 75^0C for 10 min. The tubes were immediately placed on ice and centrifuged in a microcentrifuge. Two different dilutions were prepared from newly synthesized cDNAs. First dilutions were prepared by transferring 2 µl of reaction to another tube and by adding 78 µl of sterile H_2O into this tube. Second dilutions were prepared by adding 72 µl of sterile H_2O to the tubes containing the remaining 8 µl of each single strand cDNA.

2.2.7. Differential Display Technique

2.2.7.1. PCR

Master mix for all of the PCRs was prepared as follows: 2 µl of 10X Klen Taq PCR reaction buffer (Sigma), 14.4 µl of sterile H_2O, 0.2 µl of dNTP mix (5mM) and 0.4 µl of Advantage Klen Taq polymerase (Sigma) were mixed in a proper tube. For each sample; 1 µl of cDNAs as the template, 1 µl T primer and 1 µl P primer were added to master mix thereby resulting total 20 µl final volume. Different primer pairs were used for each cDNAs. All types of P and T primers and sequences of these primers are presented in Appendix C. Differential display PCR consisted of four main parts. The first part of reaction contained 1 cycle which was made up of a denaturation step at 94^0C for 5 min, an annealing step at 40^0C for 5 min, an extension step at 68^0C for 5 min. The second part of reaction contained 2 cycles which was made up of a denaturation step at 94^0C for 30 sec, an annealin step at 40^0C for 30 sec, an extension step at 68^0C for 5 min. The third and main part of reaction contained 23 cycles which was made up of a denaturation step at 94^0C for 20 sec, an annealin step at 60^0C for 30

sec, an extension step 68^0C for 2 min. The reaction was terminated with an extension step at 68^0C for 7 min.

2.2.7.2. Agarose Gel Electrophoresis

Agarose gels (0.8-1.5%) were prepared in 1X TAE electrophoresis buffer. EtBr was added at the concentration of 0.5 µg/ ml from a 10 mg/ml stock and it was poured into a horizontal gel apparatus and gel combs were placed and the gel was allowed to harden. DNA samples prepared with 10X gel loading buffer were loaded into the wells of the gel and they were exposed to an electric constant at 80V. The movement of the DNA molecules could be observed with bromophenol blue present in the gel loading dye and thus the power supply was turned of when bromophenol blue was exceeding half of the gel. DNA fragments were visualized in gel documentation system.

2.2.8. cDNA Purification From Agarose Gel and Reamplification of Purified Bands

QIAquick Gel Extraction Kit (Qiagen) was used in order to isolate the bands from agarose gel. Differentially expressed cDNA bands were excised from agarose gel with a clean scalpel and minimized by removing extra agarose. Gel slices were put in microcentrifuge tubes and 3 volumes of Buffer QG to 1 volume of gel in the tubes were added. Tubes were incubated at 50^0C until the gel slices were completely dissolved. One volume of isopropanol was added to each sample. Samples then were applied to QIAquick columns placed in collection tubes and centrifuged at maximum speed for 1 min. Flow-through was discarded and QIAquick columns were placed in the same collection tubes. Five hundred µl of Buffer QG was added to each column and centrifuged for 1 min. Seven hundred fifty µl of Buffer PE was added to each QIAquick column and centrifuged for one minute. Flow-through was discarded and columns were centrifuged for an additional 1 min at 13,000 rpm. QIAquick columns were placed into clean 1.5-ml microcentrifuge tubes and 50 µl of dH_2O was added to the center of each QIAquick membrane. By centrifugation cDNAs were eluted.

Reamplification of cDNAs, purified from agarose gel, was performed by using P and T primers in PCR. Proper P and T primer pairs were chosen for each band and

reamplified in PCR. Reaction mixture was set up as follows: 0.5 µl of Taq polymerase (Sigma) , 5 µl of 10X buffer (Sigma), 0.5 µl of dNTP (10mM) mix, 2.5 µl of P primer, 2.5 µl of T primer, 7 µl of cDNA and 32 µl of dH_2O were mixed in 0.2-ml microcentrifuge tube. The first part of reaction contained 1 cycle which was made up of a denaturation step at 94^0C for 5 min, an annealing step at 40^0C for 5 min, an extension step at 72^0C for 5 min. The second part of reaction contained 2 cycles which was made up of a denaturation step at 94^0C for 30 sec, an annealin step at 40^0C for 30 sec, an extension step at 72^0C for 5 min. The third and main part of reaction contained 23 cycles which was made up of a denaturation step at 94^0C for 20 sec, an annealin step at 60^0C for 30 sec, an extension step 72^0C for 2 min. The reaction was terminated with an extension step at 72^0C for 7 min. These PCR products would be used as template in ligation reactions.

2.2.9. Cloning of cDNAs into *E. coli* Lines

2.2.9.1. Preparation of Competent *E. coli* Cells

Dh5α strain of *E. coli* bacteria was used to prepare competent cells with $CaCl_2$ treatment. A single colony was picked from *E. coli* stock and inoculated into 100 ml LB broth and grown overnight at 180 rpm at 37^oC. The next day, in order to allow the culture reach the mid-log phase, 1 ml overnight culture was transferred into 100 ml fresh LB broth (1: 100 dilution) and incubation was continued for approximately 3 hours at 37°C with continuous agitation. Following this incubation step, 100 ml culture was transferred into 2 sterile, ice-cold 50 ml falcon tubes each with the volume of 40 ml culture. Falcon tubes were incubated on ice for 20 min to cool the culture. Cells were pelleted by centrifugation at 5000 rpm for 10 min at 4°C. After discarding supernatant completely, the cell pellet was resuspended in 8 ml of ice-cold 100mM $CaCl_2$ and centrifuged at 5000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet was treated with 8 ml of ice-cold 100mM $CaCl_2$ again, and centrifuged at the same conditions told above. Supernatant was removed again and finally the cells were resuspended in 1600 µl ice-cold 100mM $CaCl_2$ (1600 µl for the cells in one falcon tube) very gently. Final suspension volume was divided into aliquots. Sterile microcentrifuge tubes were already kept on ice. One hundred sixty µl of this $CaCl_2$ treated cell

suspension was transferred into one microcentrifuge tube, then 40 µl sterile glycerol was added and mixed gently.

The prepared competent cells were either used immediately for transformation or stored at -80°C for later use.

2.2.9.2. Ligation of cDNAs into Plasmid

pGEM-T Easy Vector System (Promega) was used for ligation reactions. A ligation reaction was set up as follows: 5 µl of 2X Rapid Ligation Buffer, 1 µl of pGEM-T Easy Vector (50 ng), 4 µl of PCR products, 1 µl of T4 DNA Ligase and 4 µl of dH_2O were mixed in a 0.5-ml microcentrifuge tube by pipetting and incubated overnight at 4^0C.

2.2.9.3. Transformation of pGEM-T Easy Vector into Competent *E. coli* Cells

LB plates with ampicillin/ IPTG/ X-Gal were prepared by adding 15 g agar, 100µg/ ml ampicillin, 100µl of 100mM IPTG and 20 µl of 50µg/ ml X-Gal.

Prepared ligation reaction and competent cells were placed on the ice. Four µl of each ligation reaction was added to sterile 1.5-ml microcentrifuge tubes and 100 µl of competent cells was transferred into each tube. Tubes were gently mixed and placed on ice for 20 min. Cells were heat-shocked for 90 sec in a water bath at exactly 42^0C and immediately returned to ice for 2 min. Nine hundred µl of SOC medium was added to tubes containing cells transformed with ligation reactions. Reaction was incubated for 90 min at 37^0C with shaking (~150 rpm). After incubation, 200 µl of each transformation culture was plated onto LB/ ampicilin/ IPTG/ X-Gal plates and incubated overnight at 37^0C in order to see the blue-white colonies. White colonies, containing inserts, were picked up after overnight incubation for plasmid isolation.

2.2.10. Colony PCR

cDNAs of interest in plasmids were amplified by PCR using SP6 and T7 primers. White colonies were picked up from plates via toothpicks and suspended in 30

µl of H_2O. These dilutions were heated for 5 min at 95^0C in water bath. Mixtures were centrifuged in a microcentrifuge for 2 min at 14,000 rpm. Five µl from supernatant was taken for each colony and transferred into a 0.2-ml microcentrifuge tube. PCR components were added as follows: 0.5 µl of Taq polymerase, 1 µl of SP6 Primer (25µM), 1 µl of T7 Primer (25µM), 0.5 µl of dNTP and 37 µl of dH_2O. Reaction contained 30 cycles which started with an initial denaturation step at 94^0C for 3 min, followed by consisting a 30 sec denaturation step at 94 ºC, a 30 sec annealing at 50 ºC, and a 1 min extension at 72 ºC. A 7 min primer extension at 72 ºC completed the sequence.

Sequence of SP6 and T7 primer pairs are given in Appendix C.

2.2.11. Plasmid DNA Isolation

Plasmid DNAs from selected white colonies were isolated by using Wizard Plus SV Minipreps DNA Purification System (Promega). White colonies were transferred to 7 ml LB broth with ampicillin in the 15 ml falcon tubes and incubated overnight at 37^0C in a shaking incubator. Bacterial cultures were harvested by centrifugation for 15 min at 4000x g. Supernatant was poured and pellets were dried by inverting the tubes on paper towels. Two hundred fifty µl of cell resuspension solution was added to each pellet and resuspended completely by vortexing. Resuspended cells were transferred to sterile microcentrifuge tubes. Two hundred fifty µl of cell lysis solution was added to tubes and mixed by inverting the tubes four times. Suspension was incubated until it became clear. Ten µl of alkaline protease solution was added to each tube and mixed by inverting the tubes four times. Suspension was incubated for 5 min at room temperature. Three hundred fifty µl of Wizard Plus SV neutralization solution was added to each tube and mixed immediately by inverting the tubes four times. Bacterial lysates were centrifuged at 14,000x g in a microcentrifuge at room temperature. Plasmid DNA purification units were prepared by inserting spin columns into 2 ml collection tubes for each sample. Cleared lysates were transferred to prepared spin column. Supernatant was centrifuged at maximum speed in a microcentirfuge for 1 min at room temperature. Flow-through was discarded after centrifugation. Seven hundred fifty µl of column wash solution diluted with 95% ethanol was added to each spin column and centrifuged at maximum speed in a microcentrifuge for 1 min at room temperature. Flow-through

was discarded after centrifugation. Wash procedure was repeated using 250 µl of column wash solution and centrifuged at maximum speed in a microcentrifuge for 2 min at room temperature. Spin columns were transferred to new sterile 1.5-ml microcentrifuge tubes. Hundred µl of nuclease-free water was added to each spin columns. By centrifugation of spin columns at maximum speed for 1 min at room temperature in a microcentrifuge, plasmid DNAs were eluted. The amount of each plasmid was measured by spectrophotometry.

2.2.12. Digestion of Plasmid DNAs:

Plasmid DNAs were digested by EcoRI enzyme (Fermantas). Digestion reaction was set up for each plasmid DNA as follows: 24 µl of dH_2O, 5 µl of 1X buffer, 20 µl of plasmid DNA and 1 µl of EcoRI enzyme were mixed in a microcentrifuge tube and incubated at 37^0C for 3 hours. After incubation, samples were observed at 1% agarose gel.

2.2.13. Sequence Analysis of cDNAs of Interest:

2.2.13.1. Purification of Colony PCR Products

Colony PCR products were purified by using Montage PCR Centrifugal Devices Kit (Millipore). According to instructions of manufacturer, Montage PCR sample reservoirs were inserted into 1.5-ml microcentrifuge tubes. Three hundred fifty µl of dH_2O was added to each sample reservoirs. Fifty µl of colony PCR product was also added to each reservoir and centrifuged at 1000x g for 15 min. After centrifugation, samples were inverted the reservoir into a clean 1.5-ml microcentrifuge tube and added 20 µl dH_2O. Purified cDNAs were obtained by centrifugation at 1000x g for 2 min.

2.2.13.2. Sequence Reaction

2.2.13.2.1. Preparation of DNA Sequencing Reaction

For sequence analysis of samples, GenomeLab Methods Development Kit Dye Terminator Cycle Sequencing (Beckman Coulter) was used. According to instructions of manufacturer, a premix was prepared as follows: 200 µl of 10X Sequencing Reaction Buffer, 100 µl of dNTP mix, 200 µl of ddUTP dye terminator, 100 µl of ddGTP dye terminator, 200 µl of ddCTP dye terminator, 200 µl of ddATP dye terminator and 100 µl of polymerase enzyme were mixed in a 1.5-ml microcentrifuge tube and this aliquot was divided into the sterile 0.5 ml microcentrifuge tubes.

Then, 20 µl sequencing reaction was prepared in 0.2-ml thin-wall tubes for each sample as follows: 6 µl of dH_2O, 1 µl of DNA (60 ng), 2 µl of T7 primer (1.6µM) and 11 µl of premix were mixed. All reagents were kept on ice while preparing sequencing reactions. Samples mixed with premix and other necessary components were placed in a thermal cycler. Reaction contained 30 cycles which was made up of a denaturation step at 96^0C for 20 sec, an annealing step at 50^0C for 20 sec and an extension step at 60^0C for 4 min.

2.2.13.2.2. Ethanol Precipitation

Fresh stop solution/ glycogen mixture was prepared for each sequencing reaction as follows: 2 µl of 3M sodium acetate, 2 µl of 100mM Na_2-EDTA and 1 µl of 20 mg/ ml of glycogen. Five µl of the stop solution/ glycogen mixture was transferred to labeled new 0.5-ml microcentrifuges tubes. Sequencing reactions were also transferred to same tubes and mixed. Sixty µl of cold 95% ethanol was added to each reaction and all of the tubes were centrifuged at 14,000 rpm at 4^0C for 15 min. Supernatants were removed carefully by micropipette without touching the pellets. Pellets were rinsed 2 times with 200 µl of cold 70% ethanol. For each rinse, samples were centrifuged at 14,000 rpm at 4^0C for minimum 2 min. After centrifugation supernatants were removed and pellets were vacuum dried for until 10 min. Each pellet was resuspended in 40 µl of sample loading solution.

2.2.13.2.3. Sample Preparation for Loading into the Instrument

Sequencing of samples was performed in Beckman Coulter CEQ 8800 Genetic Analysis System. Resuspended samples were transferred to the appropriate 96-well plates recommended for the instrument. Each of the resuspended samples was overlaid with one drop of light mineral oil. Sample plate was loaded into the instrument and sequencing of samples was performed.

2.2.14. Real Time PCR Analysis

2.2.14.1. Primer Designing and PCR

Sequences encode anti-porter proteins in plants were obtained from http://plantst.genomics.purdue.edu/plantst/html/cation.shtml, `Functional Genomics of Plant Transporters`. Of these anti-porter sequences, forty three primer pairs were designed. The list of ordered anti-porter primer pairs and sequences were listed in Appendix C.

Primer pairs were amplified by PCR using Hamidiye and Anadolu cDNA. Six primer pairs showed differential expression level. These primer pairs were anti-porter 6 (PDR-like ABC transporter), 7 (MRP-like ABC transporter), 8 (ABC transporter family protein), 19 (Multi antimicrobial extrusion protein MatE family protein), 24 (MATE efflux family protein) and 25 (MATE efflux family protein).

Primers of differentially expressed genes, P7T2 ABL24 3kb, P3T3 HBL2 408bp, P7T5 HBL24 574bp, P3T8 HBL24 440bp were listed in Appendix C.

2.2.14.2. Real Time PCR

Hamidiye and Anadolu leaf and root samples were collected at 0, 6, 24, 48 and 144 hours. cDNAs were synthesized from RNAs of collected samples by using Fermentas cDNA Synthesis Kit. Real Time PCR was set up as follows: 12.5 µl of IQ SYBR green supermix (BioRAD), 1 µl of forward and reverse primers, 1 µl of cDNA template and 9.5 µl of dH_2O. Reaction was set up for each anti-porter primer pairs and cDNA template. GAPDH and 18S templates were also used for each primer pairs to

normalize the results. Real Time PCR consisted of five main parts. The first part of reaction contained 1 cycle which was made up of a denaturation step at 95^0C for 90 sec. The second part of reaction contained 40 cycles which was made up of a denaturation step at 94^0C for 50 sec, an annealing step at 58^0C for 45 sec, an extension step at 72^0C for 45 sec. The third part of reaction contained 1 cycles which was made up of a denaturation step at 95^0C for 60sec. The forth part of reaction contained 1 cycles which was made up of an annealing step at 55^0C for 60 sec. The reaction was terminated with 81 cycles step at 55^0C for 30 sec.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Differential Display

In differential display, primer pairs used for 2 hours boron treated samples were as follows: P1T1, P1T9, P2T2, P2T6, P3T2, P3T3, P3T7, P4T3, P4T4, P5T1, P5T5, P5T6, P6T3, P6T5, P6T6, P7T6, P7T7, P8T2, P8T4, P8T7, P8T8, P9T5, P9T9, P10T1, P10T9. Primer pairs used for 24 hours boron treated samples were as follows: P1T3, P1T5, P1T7, P2T1, P2T9, P3T4, P3T5, P3T8, P4T2, P4T6, P4T9, P5T3, P6T7, P7T1, P7T2, P7T5, P7T8, P9T5, P10T4, P10T9. Of these primer pairs P4T4, P3T3, P2T2, P6T6, P8T8, P3T7, P6T5, P8T7 and P4T3 for 2 hours boron treated samples and P2T9, P3T8, P7T2 and P7T5 for 24 hours boron treated samples showed differentially expressed bands. Agarose gel pictures of differentially expressed bands also indicated the length of bands (Figure 3.1). As a result, 4 down-regulated, 2 up-regulated bands from 24 hours samples and 3 down-regulated, 2 up-regulated bands from 2 hours samples were obtained.

3.2. Sequence Analysis of Differentially Expressed cDNAs

Sequences of these differentially expressed cDNAs are given in Appendix A.

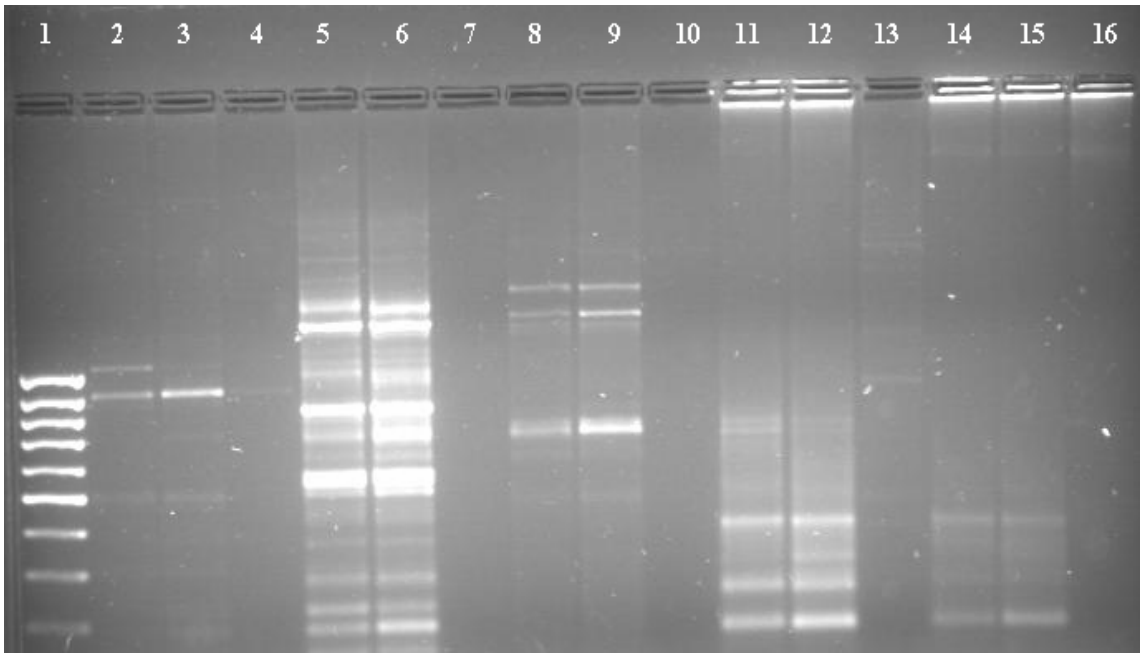

Figure 3.1. Agarose gel picture of differentially display PCR; 1: Marker 1000bp, 2: ABL2 P2T2, 3: HBL2 P2T2, 4: RNA P2T2, 5: ABL2 P6T6, 6: HBL2 P6T6, 7: RNA P6T6, 8: ABL2 P7T7, 9: HBL2 P7T7, 10: RNA P7T7, 11: ABL2 P8T8, 12: HBL2 P8T8, 13: RNA P8T8, 14: ABL2 P8T4, 15: HBL2 P8T4, 16: RNA P8T4

3.3. Blast Analysis

Blast analysis of sequences indicated that P7T5 HBL24 730 bp cDNA and P7T2 HBL24 724 bp cDNA gave homology to an eukaryotic translation initiation factor TIF3B1 in *Arabidopsis thaliana* (ref NM_001036877.2). P7T5 HBL24 574 bp cDNA gave homology to a chlorophll a/b binding protein WCAB precursor in *Triticum aestivum* (gb U73218.1 TAU73218). P7T2 ABL24 3 kb cDNA gave homology to an elongation factor in *Oryza sativa* (gb EF122482). P2T9 ABL24 747 bp cDNA gave homology to a short-chain dehydrogenase/reductase (SDR) family protein in *Arabidopsis thaliana* (ref NM_129309.3) and an oxidoreductase protein in *Arabidopsis thaliana* (gb AY099558.1). P3T8 HBL24 440 bp cDNA gave homology to thioredoxin H isoform in *Triticum aestivum* (gb AF286593.2). P3T3 HBL2 408 bp cDNA gave homology to a shaggy-like kinase protein in *Triticum aestivum* (dbj AB281487.1). P3T3 HBL2 466 bp cDNA, P4T4 HBL2 385 bp cDNA and P4T4 ABL2 649 bp cDNA gave homology to chloroplast genome in *Hordeum vulgare* (gb EF115541.1). P3T3 ABL2

524 bp cDNA gave homology to a hypothetical protein in *Arabidopsis thaliana* (dbj AK226302.1) and hydroxyproline-rich glycoprotein family in *Arabidopsis thaliana* (ref NM116335.2).

It can be inferred from the results of differential display indicated above; boron is directly related to photosynthesis because it either causes activation or over-expression of some genes in chloroplast genome. It is also known that excessive amount of boron gives rise to some defects in photosynthesis. One of the possible boron tolerance mechanisms in Anadolu cultivar of barley may be the alteration of gene expression in chloroplast genome. However, the observation of change in chloroplast genome of Hamidiye cultivar is weakened the theory about direct relation of chloroplast genome in boron tolerance. In HBL24 samples, differentially expression of cDNA similar to eukaryotic translation initiation factor TIF3B1 demonstrates that Hamidiye cultivar is trying to manage the translation of mRNAs because TIF3B1 is functional in the initiation of ribosome-mediated translation of mRNAs into polypeptides The effect of boron toxicity on RNA metabolism also supports that. Over-expression of cDNAs like a short-chain dehydrogenase/reductase (SDR) family protein and oxidoreductase protein in ABL24 samples proves the relation between membrane activity and boron in plants. Dehydrogenase/reductase (SDR) family proteins are proteins catalyse an oxidation-reduction reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One of the possible boron sensitivity in Hamidiye cultivar may be the inhibition of some redox reactions. Differentially expression of thioredoxin h isoform like cDNA in HBL24 samples also demonstrates the cellular protection role of thioredoxin h against oxidative stress increased by boron (Gelhaye et al., 2004). Expression of shaggy-like kinase protein like cDNA in HBL2 may be assumed that Hamidiye cultivar is trying to manage the cell elongation which is retarded by boron. It is known that shaggy-like kinase protein plays an important role in cell elongation especially in flower development (Claisse et al., 2007).

3.4. Real Time PCR of Differentially Expressed Genes

Quantitative results of Real Time PCR and expression level graphics of the differentially expressed genes were given below. Results were normalized using GAPDH, as an internal control.

The Real Time PCR result of P7T2 ABL24 3kb, shows similarity with the result of differential display technique (Table 3.2). In differentially display PCR this gene also performed much more expression in Anadolu cultivar than it did in Hamidiye cultivar. In control plants, expression of P3T3 HBL2 408bp, coding kinase like protein, is higher than Anadolu cultivar than it is in Hamidiye cultivar. However, after boron treatment it shows nearly equal expression in both cultivars thereby proving the idea that both cultivar are trying to fix the cell elongation retarded by boron by expressing a kinase like protein (Table 3.3). P7T5 HBL24 574bp, which encodes chlorophyll a/b binding protein, also shows similar patterns like P3T3 HBL2 408bp (Table 3.4). Its expression is higher in Anadolu control plants than it is in Hamidiye control plants. However, in boron treated samples it is much more expressed in Hamidiye cultivars than it is done in Anadolu cultivars.

Table 3.1. Quantitative results of Real Time PCR of Differentially Expressed Genes

	HCL	ACL	HBL24	ABL24	HBL48	ABL48
P7T2 ABL24 3kb	24.5	21.79	23.47	22.44	25.39	24.2
P7T2 ABL24 3kb N	27.53	29.13	26.55	26.48	25.39	26.21
P3T3 HBL2 408bp	23.82	22.13	23.63	21.96	24.3	22.35
P3T3 HBL2 408bp N	26.77	29.59	26.73	25.91	24.43	24.20
P7T5 HBL24 574bp	23.98	21.89	24.36	22.54	25.8	21.31
P7T5 HBL24 574bp N	26.95	29.27	27.56	26.6	25.8	23.08
P3T8 HBL24 440bp	22.98	19.79	22.14	20.9	24.43	22.45
P3T8 HBL24 440bp N	25.83	26.46	25.05	24.67	24.43	24.31
GAPDH	21.2	17.82	21.06	20.19	23.83	22
GAPDHN	1.124	1.33	1.13	1.18	1	1.08

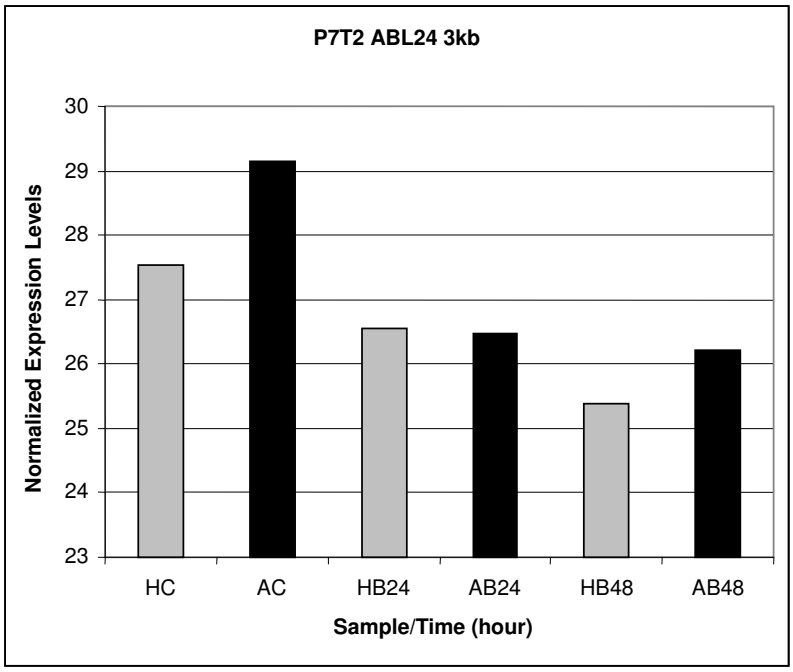

Figure 3.2. Expression level of P7T2 ABL24 3kb (elongation factor) in barley cultivars

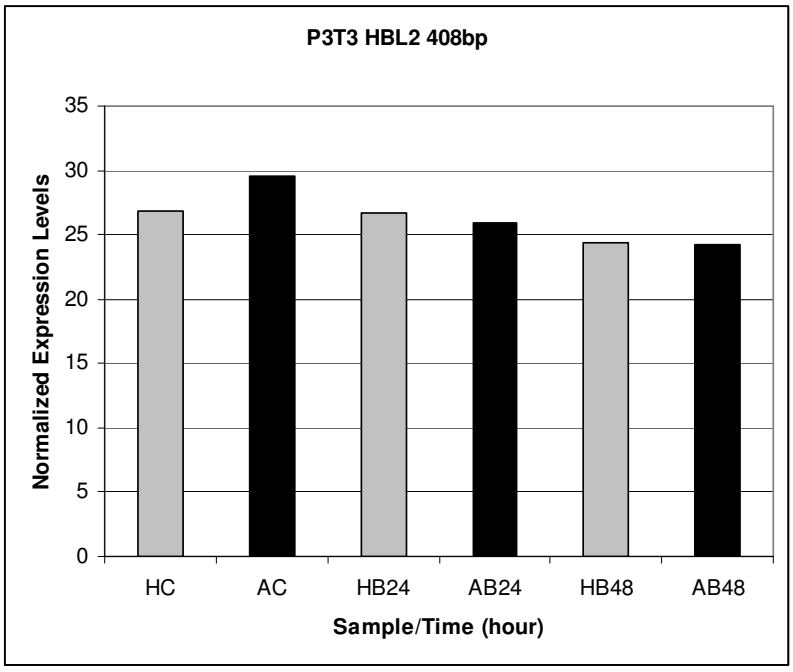

Figure 3.3. Expression level of P3T3 HBL2 408bp (kinase like protein) in barley cultivars

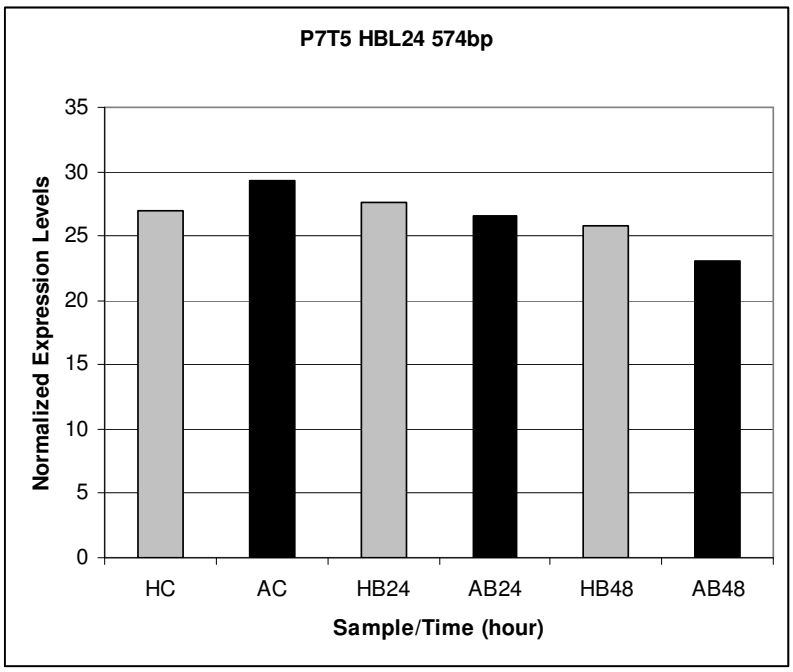

Figure 3.4. Expression level of P7T5 HBL24 574bp (chlorophyll a/b binding protein) in barley cultivars.

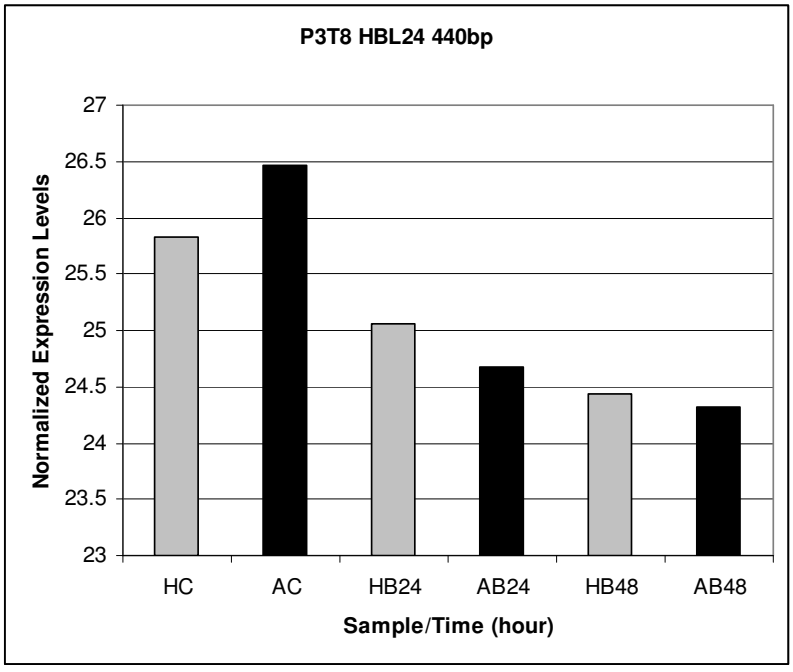

Figure 3.5. Expression level of P3T8 HBL24 440bp (thioredoxin h isoform) in barley cultivars

3.5. Real Time PCR of Anti-Porter Genes

Boron tolerance can be related with transporting of excessive boron from the cell. Efflux protein and anti-porter proteins have ability to transfer boron. Therefore, activity of known anti-porter genes from barley genome was tested in this research.

Quantitative results of Real Time PCR and expression level graphics of the differentially expressed genes were given below. Results were normalized using GAPDH, as an internal control.

Expression level of anti-porter 6 (PDR-like ABC transporter) in Anadolu samples is higher than Hamidiye samples except 144 hours samples (Table 3.7). Expression level of anti-porter 7 (MRP-like ABC transporter) in Anadolu samples is higher than Hamidiye samples except 24 hours samples (Table 3.8). Expression level of anti-porter 8 (ABC transporter family protein) in all Anadolu samples is higher than Hamidiye samples (Table 3.9). Expression level of anti-porter 19 (Multi antimicrobial extrusion protein MatE family protein) in Anadolu samples is higher than Hamidiye samples except 48 hours samples (Table 3.10). Expression level of anti-porter 24 in Anadolu samples is higher than Hamidiye samples except 48 hours samples (Table 3.11). Expression level of anti-porter 25 in all Anadolu samples is higher than Hamidiye samples (Table 3.12). Higher expression of anti-porter genes in Anadolu cultivar is not related to presence of boron, because it is also high in Anadolu control samples when compared with Hamidiye control samples. On the other hand, tolerance of Anadolu cultivar against boron may be due to higher expression of anti-porter genes thereby emitting excess amount of boron from cell.

Table 3.2. Quantitative results of Real Time PCR of anti-porter genes.

	HCR 0	ACR 0	HBR 6	ABR 6	HBR 24	ABR 24	HBR 48	ABR 48	HBR 144	ABR 144
GN	1.08	1.21	1.06	1.11	1.04	1.23	1.13	1.16	1	1.24
G	31.17	27.77	31.72	30.17	32.18	27.33	29.59	28.86	33.72	27.07
18SN	1.02	1.05	1	1.08	1.07	1.20	1.14	1.17	1.03	1.24
18S	35	33.75	35.72	32.81	33.11	29.72	31.29	30.32	34.41	28.7
7	35.22	33.27	33.47	35.09	35.94	29.95	31.73	31.69	35.4	30.56
7NG	38.10	40.39	35.58	39.21	37.65	36.95	36.15	37.02	35.4	38.06
6	N/A	35.19	34.87	38.06	36.09	32.84	31.97	35.76	35.73	28.35
6NG	37	42.72	37.06	42.53	37.81	40.51	36.43	41.78	35.73	35.31
8	37.36	33.69	33.46	35.29	33.67	30.31	31.13	33.63	30	29.04
8NG	40.41	40.90	35.56	39.44	35.28	37.39	35.47	39.29	30	36.17
19	33.7	33.39	33.38	34.46	33.47	33.63	33.42	31.15	33.1	31.66
19NG	36.45	40.54	35.48	38.51	35.07	41.49	38.08	36.39	33.1	39.43
24	33.44	33.91	33.14	33.14	35.45	32.9	33.69	32.65	33.87	29.73
24NG	36.17	41.17	35.22	37.03	37.14	40.59	38.39	38.14	33.87	37.03
25	29.06	27.92	28.32	28.33	28.72	28.09	28.35	28.6	28.94	26.97
25NG	31.43	33.90	30.10	31.66	30.09	34.65	32.30	33.41	33	33.59

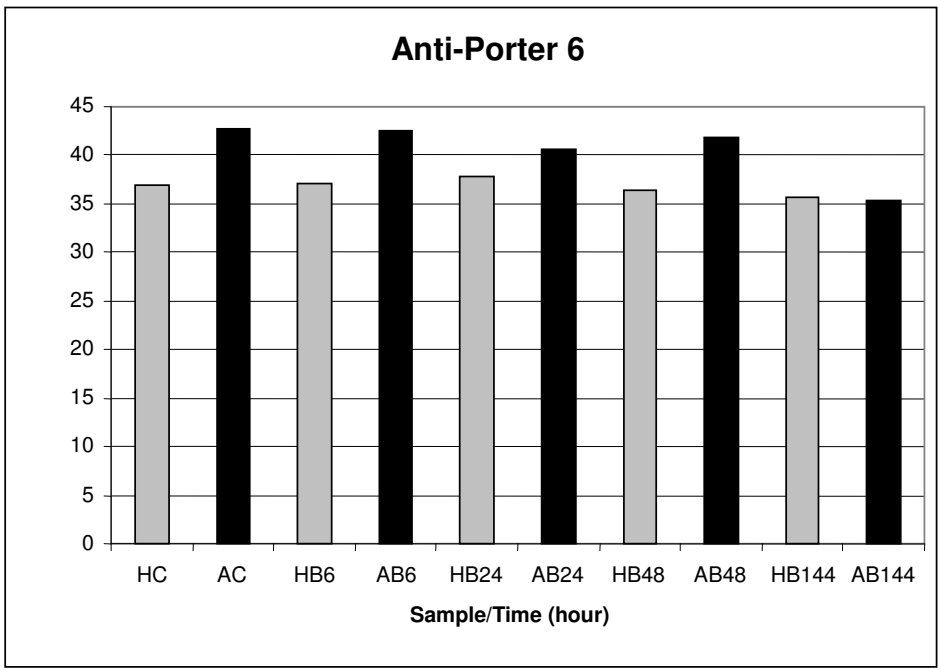

Figure 3.6. Expression levels of anti-porter 6 (PDR-like ABC transporter) in barley cultivars.

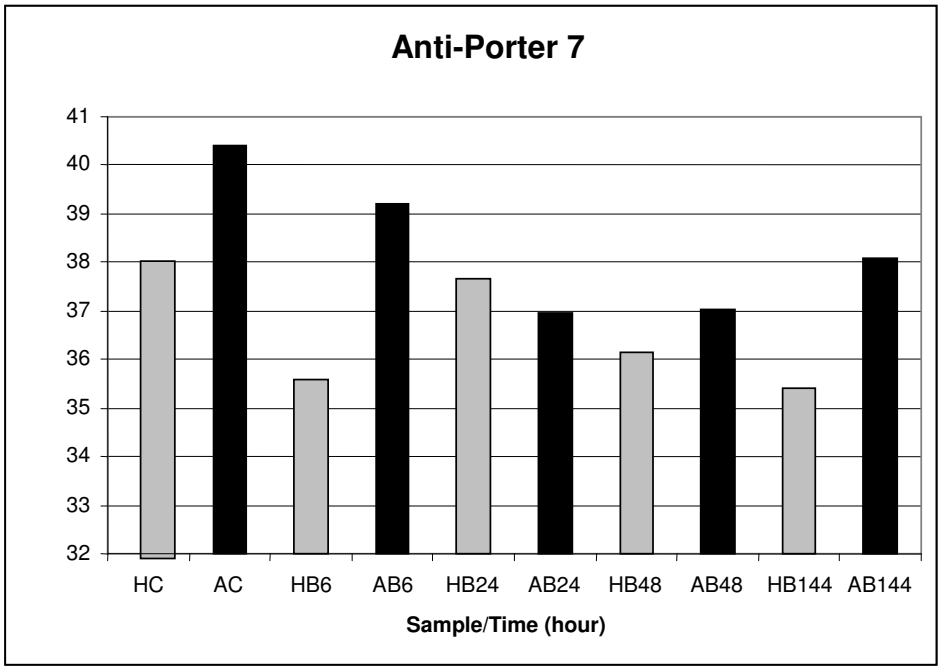

Figure 3.7. Expression levels of anti-porter 7 (MRP-like ABC transporter) in barley cultivars

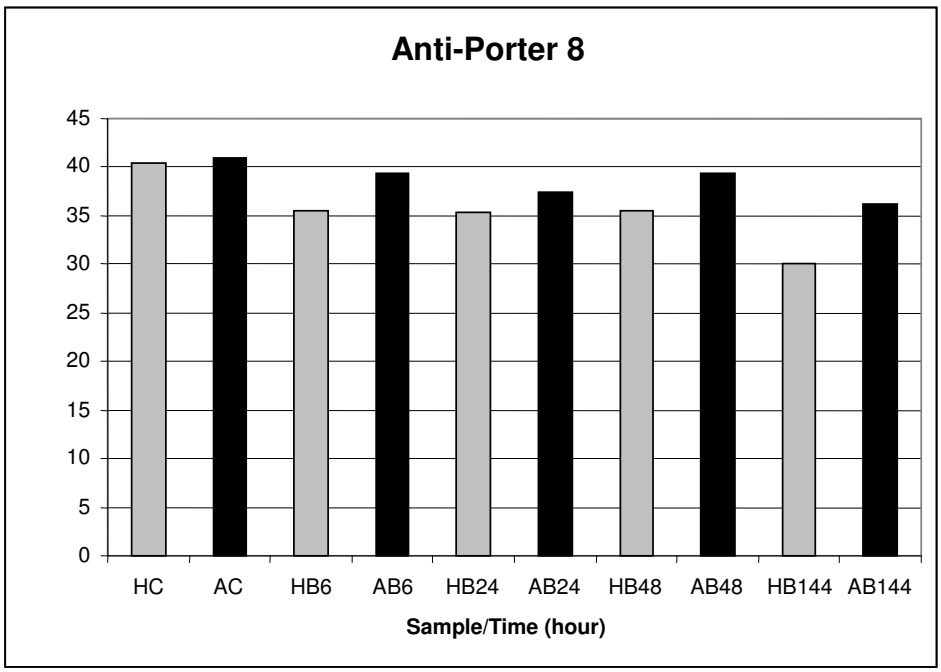

Figure 3.8. Expression levels of anti-porter 8 (ABC transporter family protein) in barley cultivars

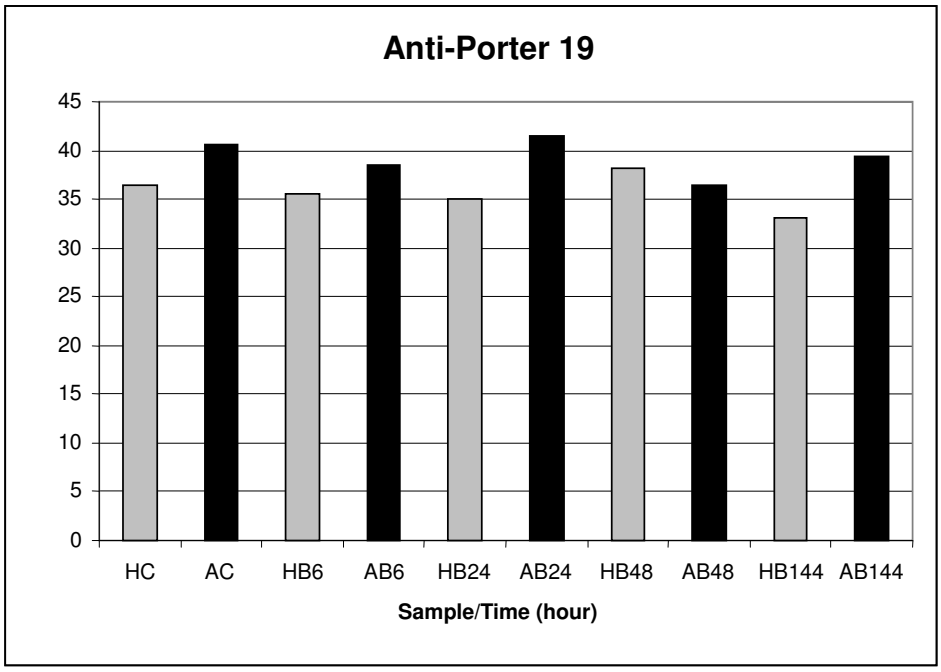

Figure 3.9. Expression levels of anti-porter 19 (Multi antimicrobial extrusion protein MatE family protein) in barley cultivars

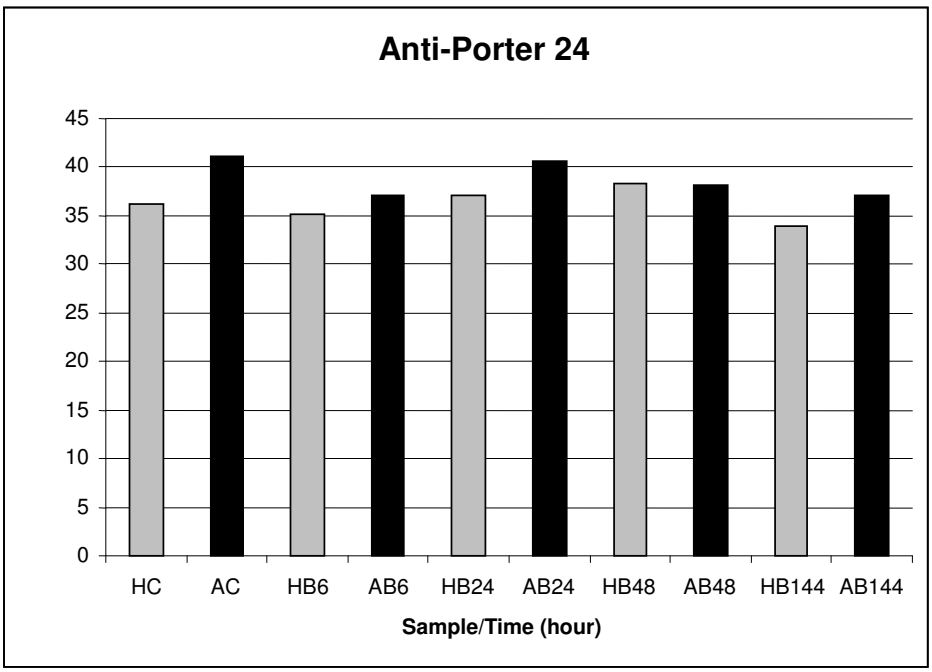

Figure 3.10. Expression levels of anti-porter 24 (MATE efflux family protein) in barley cultivars

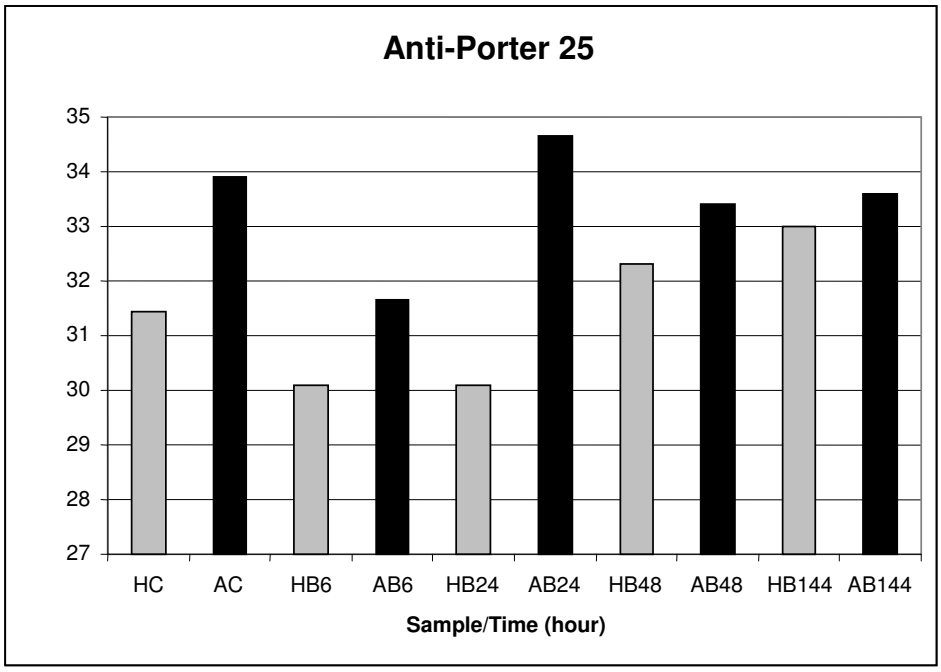

Figure 3.11. Expression levels of anti-porter 25 (MATE efflux family protein) in barley cultivars

CHAPTER 4

CONCLUSION

The aim of this research was to find out the genes responsible in boron tolerance between Turkish barley cultivars. For this purpose, three different methods were carried out: mRNA Differential Display, RT-PCR with known boron tolerant genes and RT-PCR with known anti-porter genes. Anadolu (resistant against boron stress) and Hamidiye (sensitive against boron stress) Turkish barley (*Hordeum vulgare*) cultivars were grown under laboratory conditions. Leaf and root samples were harvested following boron treatment to plants. Eight differentially expressed genes identified by using mRNA Differential Display technique. Analysis of BOR1 gene, responsible for boron efflux in *Arabidopsis*, did not show any gene expression difference between Anadolu and Hamidiye varieties indicating BOR1 gene is not responsible boron tolerance in Turkish barley cultivars. In addition, Real Time PCR analysis of 6 anti-porter genes showed different expression level in boron tolerant Anadolu. Transcriptional regulation of boron tolerance was conducted in this study. Posttranslational modification of boron tolerance might be related with this mechanism. For this reason, proteomic approach should be conducted to identify proteins responsible for boron tolerance in barley.

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APPENDIX A

SEQUENCES OF DIFFERENTIALLY EXPRESSED GENES IN BARLEY CULTIVARS

1. P7T5 HBL24 730bp:

5`ATTAACCCTCACTAAATGCTGTATGTTTGATAGGTGCAGCGCGGAAGAAC
AAAGAGGAATACTTCGATTTTGTAAATTCTTATAAGTTTTTCTGTCGCCGTC
GTAATTCTCGTGGCATAATTGTAAAATCTGGCTTCGGGAGGTTACGCGCTTC
CGATCTAAAAACTAATGTAATCCGCCTTTGTCGCGGTATTACCCCTGGAGAA
AATGAATGGCGATTGCAACCGCAGAATGAAACTGTCCTTAATGCTCACAAT
AAAAATCTGATGCGGACTTTGTAGCGTCAGATTGCAAACCAGCTTGATATTG
ATGACCAGCTTGATATTGATGATGGCATGGAATTTGAGGACTTTGACAACC
ACCTGAATGTATCTCATGGTTGGAAGCCTGCTGAAATCAAGCCACACATGC
CAGGAAAAAGGCTATCTGTTGAGAAGGTCAGATGTCAGTTTCTGGTAAATG
CTGGTCCTTGTGGGGAAGTTTATACGAATGATGCCGAGAAGACAACATCTG
ACCCTGTGTACCAAATGCAGTACCGCACATGTGATTTAATTCAATGGTCACC
TCTTGGAACACACTTGGTGACGGTGGATAGGCAGGGCGCACTTATGTGGGG
CGGTGATGATAAGTTTGCGTGTCTCATGAGCTTCGGAGATCAGCAGGTGAA
ATTGGTCGATTTCTCTCCTGGCGAGAGATACTTGGTCACATACAGCATTTAG
TGAGGGTTAAT3`

2. P7T5 HBL24 574 bp (chlorophyll a/b binding protein):

5'ATTAACCCTCACTAAATGCTGTATGGTTCAAGGCAGGCTCCCAAATCTTCA
GCGACGGTGGCCTCGACTACCTCGGCAACCCTAGCCTCGTGCATGCACAAA
GCATCCTTGCTATCTGGGCATGTCAAGTCGTGCTCATGGGCGCCGTTGAAGG
GTACCGCATTGCTGGTGGCCCACTCGGTGAGGTGGTTGATCCACTCTACCCT
GGTGGCAGCTTCGACCCGCTCGGCCTAGCCGATGACCCAGAGGCATTCGCG
GAGCTCAAGGTAAAAGAGGTCAAGAATGGGCGCCTTGCCATGTTCTCCATG
TTTGGCTTCTTCGTGCAGGCTATTGTCACCGGCAAGGGCCCCCTCGAGAACC
TTGCTGACCATCTCGCCGATCCTGTCAACAACAACGCATGGGCGTTTGCCAC
TAACTTTGTTCCCGGCAAGTGAGGCGTTGGCGCTAGTTAGCTGCGAGTTGTA
GCCATTAAGGCGACTACAGTGAACTGCACACGTATTGTGTGGTTTTGTAACA

TCATGATGTAAATTACAAGGATCTTTTGGGAAAAAAAAAGATATCACTCAG
CATAATG3’

3. P7T2 HBL24 724 bp:
5’ATTAACCCTCACTAAATGCTGTATGTTTGATAGGTGCAGCGCGGAAGAAC
AAAGAGGAATACTTCGATTTTGTAAATTCTTATAAGTTTTTCTGTCGCCGTC
GTAATTCTCGTGGCATAATTGTAAAATCTGGCTTCGGGAGGTTACGCGCTTC
CGATCTAAAAACTAATGTAATCCGCCTTTGTCGCGGTATTACCCCTGGAGAA
AATGAATGGCGATTGCAACCGCAGAATGAAACTGTCCTTAATGCTCACAAT
AAAAATCTGATGCGGACTTTGAAGCGTCAGATTGCAAACCAGCTTGATATT
GATGACCAGCTTGATATTGATGATGGCATGGAATTTGAGGACTTTGACAAC
CACCTGAATGTATCTCATGGTTGGAAGCCTGCTGAAATCAAGCCACACATG
CCAGGAAAAAGGCTATCTGTTGAGAAGGTCAGATGTCAGTTTCTGGTAAAT
GCTGGTCCTTGTGGGGAAGTTTATACGAATGATGCCGAGAAGACAACATCT
GACCCTGTGTACCAAATGCAGTACCGCACATGTGATTTAATTCAATGGTCAC
CTCTTGGAACACACTTGGTGACGGTGGATAGGCAGGGCGCACTTATGTGGG
GCGGTGATGATAAGTTTGCGTGTCTCATGAGCTTCGGAGATCAGCAGGTGA
AATTGGTCGATTTCTCTCCTGGCGAGAGATACTTGGTCACATACAGCATTTA
GTGAGG3`

4. P7T2 ABL24 3 kb (elongation factor):
5’CATTATGCTTCAGTGATATCTTTTTTTTTACCAGTGGACAACAGATACATA
ATCTAAGTTCAACATCCGTTACATCACCGGACATCAAAACATAGTTCTCTTC
CCAGTATCACCACAAACAGGTCATTTAACAGCAAGATAGTAAGATTGACTC
ATCGATACAAAATAGTTCAACTCAACAAGAGCAGGTAAGGGATAACAGCAC
CAAAAATTTAGAGCTTGTCCTCGAAATCAGATAGAGGGGTCATCTGCTCCTT
GAGACCCTTCCTCTTGCGGATCTCCGTGACAAGCGTCGCCGACTGGGTGCCC
GGGTCCAAAGGATCAGAAGCCATGATGTCCCAGTGGTCAAACACACACTGG
GGGAAGGCCTGGCCGGAGGTCGCGGCCCTCAGGGTGCTTGAGAATCCAAAG
GACTCAATGACGGGGAGGTAGGCCTTGATGTTGTAGAGCGGGGTACCTTGC
CTCTGCATCTCCTCGAACACGTGCCCTCTCTTCTGGTTCAGAACACCATAGA
TGCCACCAAGTGCATTCTCCGGGGCCTGGATCTCAACCAGGTAGACGGGCT
CCAGCAGCCTTGGCTTGGCAGTGAGCTGNAGAAGCATAAATGACCCTCCTG
GCCGTTGGGATGACCTGACCACCACCCCTGTGNATGGCATCAGTGTGGNAG

AACAACATCACAGACCTCAAAGCAAATGCCACGCATGTTTTCCTCAGCCAA
TGCACCTTCCTTTGATGCCCACTGGAACCCCAC3

5. P2T9 ABL24 747 bp:
3'ATTAACCCTCACTAAATCGGTCATAGGAGGATCTAGTGGCATTGGTTTTGA
GACCTCGAGAGTCTTTGCCCTGAGAGGAGCCCATGTCGTCATCGCTGCGAG
AAACACAGAGGCTGCATCAGAGGCAAAGAAACGCATCATGAAGATACACC
CAGTAGCCCGCATCGATGTTCTGAAGCTTGACCTTAGCTCCCTCAAGTCTGT
CAGGGCCTTCGCTGACCAGTTCAACTCCATGAATCTTCCTCTAAACATCTTG
ATAAATAATGCAGGTGTTATGTTCTGTCCTTTCCAACTGTCTGAAGATGAAG
TTGAGATGCAGTTTGCCACAAATCATCTTGGTCACTTCCTACTGACCAACCT
TCTCCTTGAGAACATGAAAACTACTGCTAAGTCTACCGGTATTGAGGGTCGC
ATTGTGAACTTGTCATCAGTTGCCCACCTCCATACATATCCAAAGGGGATTC
AGTTTGATCAGCTCAATGACAAGAAAACATACAATGATAAAATGGCCTATG
GACAATCTAAGCTTGCAAACATACTGCACGCAAAAGAGCTCTCTAGGCGGC
TCAAGGAGGAAGGAGCTAACATCACAGTTAATTGCGTTCATCCTGGATTGA
TCATGACCAATTTGATGAGGCATTCCTTTGCTCTCATGACGTTATGATTTATC
TTTTGCTTTCATTTGCAGAGGTAATTCAAGTCGTCACTTACGTGTTCTGGAA
GAATGTACCCCAGGGAGCAGCCACT5`

6. P3T8 HBL24 440 bp (thioredoxin h isoform):
5'ATTAACCCTCACTAAATGCTGGTGGTGATTGACTTCACTGCATCATGGTGC
GGACCATGCCGCATCATGGCTCCAGTTTTCGCTGATCTCGCCAAGAAGTTCC
TAAATGCTGTTTTCCTCAAGGTCGACGTGGATGAACTGAAGCCCATTGCTGA
GCAATTCAGTGTCGAGGCCATGCCAACGTTCCTGTTCATGAAGGAAGGAGA
CGTCAAGGACAGGGTTGTCGGAGCTATCAAGGAGGAACTGACCGCCAAGGT
TGGGCTTCACGCGGCGGCCCAGTAATTACCTATTGGTGTAGTATTCGCCTAA
ATAAAATTGCCGCTCAAGAAGACTATGAATGCTGTGTACTGCTTGCTACTTG
TTGTTGGTTTATGGATACTGCGATGCTTGATCCAAGCTAGTGTGCTTTGGCA
AAAAAAAAGATATCACTCAGCATAATG3'

7. P3T3 HBL2 408 bp (kinase like protein):

5`ATTAACCCTCACTAAATGCTGGTGGGTATGTGGGTTTACCAGAAGATTTTG
TGGCTTGATATCCCTGTGGCAAACTCCAATGCTGCCATGAATGTAAGCCAGA
GCCCTACATATCTGGTATGTATACAGCTTCACATAGATTAGTGGCATGCGCT
GGGTTCATCTTGTTGTAATGCTTGACAACACGATGGACAGTCTCAGGCACAT
ACTCGAGAACCAAGTTTAGATACAGTTCATCCTTCTCAGTTGTAGAAAAGA
AGCAATGCTTCAGAGCTACGACATTTGGGTGGTCAAGCAGGCGCATGGTCT
GTAGCTCACGGTTCTTGTAGCGCTTGTCCTGAAAGACCTTCTTAATGGCAAC
AGTTTCCCCTGTCCCCAAACATTAAGCCTGGAATACAATACCAAATG3`

8. P3T3 HBL2 466 bp:

5`ATTAACCCTCACTAAATGCTGGTGGGTGACCGATAGCGAAGTAGTACCGT
GAGGGAAAGGTGAAAAGAACCCCCAGTGGGTAGTGAAATAGAACGTGAAA
CCGTGCTGAGCTCCCAAGCAGTGGGAGGGGAAAGTGATCTCTGACCGCGTG
CCTGTTGAAGAATGGGCCGGCGACTCATAGGCAGTGGCTTGGTTAAGGGAA
CGGAACCCACCGGAGCCGTAGCGAAAGCGAGTCTTAATAGGGCGATTGTCA
CTGCTTATGGACCCGAACCTGGGTGATCTATCCATGACCAGGATGAAGCTTG
GATGAAACTAAGCAGAGGTCCGAACCGACTGATGTTGAAGAATCAGCGGAT
GAGTTGTGGTTAGGGGTGAAATGCCACTCGAACCCAGAGCTAGCTGGTTCT
CCCCGAAATGCGTGGAGGCGCAGCAGTTGACTGGACATCTAGGGGTAAGCA
CTGTTCGG3`

9. P3T3 ABL2 524 bp:

5`ATTAACCCTCACTAAATGCTGGTGGGGGATGGCAGAGCAGTGATCTTCCC
TGGATTGCTCCCAAGAAGGGATTCAGTTTGGACTTCGGTGAGCTATTTGGGG
ATGGTTCTCAAGGTTTGCCTGTCACAATTGGTCTTGCTGCAGCTACTGGTTT
GGGAATACCTGCCTACGCAGAGATAGAAACCGTGCTGCAGTTTCTGGGTTC
AGCTGCCATTGTCCAGGTTGTGGCAAGCAAGCTCGTCTATGCCGAGGACCG
AAAGAAGACCCTGCAACAGGTTGATGACTTCTTTAACAAGAAGATTGCACC
AAAGGAGCTTGTTGATGAAATTAAGGAAATCGGGCAGGCTCTCCTGCCTTC
ATCTGGTGAAGCTAAGAGCCAACCAGCAACAGCGGCAGCGCCAGCTGCTGC
CACGGCAACTGCTGCACCAGCAGCAGCACCAGCAGCCCCAGCTGCTGCCAC
GGGGGCTGCTGCACCAGTAGCAGAACCAGCAGCCCCAGCTGCAGAAGCCA
GCACAGAATTGCCT3`

10. P4T4 HBL2 385 bp:

5`ATTAATCCCTCACTAAATGCTGGTAGCCAAGTGCGGAGAGGATAACTGCT
GAAAGCATATAGGTAGTAAGCCCACCCCAAGATGAGTGCTCTCTCCTCCGA
CTTCCCTAGAGCCTCCGGTATCACAGCCGAGACAGCGACGGGTTCTCCACCC
ATACGGGGATGGAGCGACAGAAGTATGGAAATAGGATAAGGTAGCGGCGA
GACGAGCCGTTTAAATAGGTGTCAAGTGGAAAGTGCAGTGATGTATGCAGC
TGAGGCATCCTAACGAACGAACGATTTGAACCTTGTTCCTACACGGTCCTGA
TCAAATCGATCAGGCACTTGCCATCTATCTTCATTGTTCAACTCTTTGATGA
AAAAAAAAGATATCACTCAGCATAATG3`

11. P4T4 ABL2 649 bp:

5`ATTAACCCTCACTAAATGCTGGTAGGCGAAGCGGTTGAGTGCCGCACCCT
AGATGGCTAAAGTCCAGTAGCCGAAAGCATCACTAGCTTATGCTCTGACCC
GAGTAGCATGGGGCACGTGGAATCCCGTGTGAATCAGCAAGGACCACCTTG
CAAGGCTAAATACTCCTGGGTGACCGATAGCGAAGTAGTACCGTGAGGGAA
AGGTGAAAAGAACCCCCAGTGGGTAGTGAAATAGAACGTGAAACCGTGCT
GAGCTCCCAAGCAGTGGGAGGGGAAAGTGATCTCTGACCGCGTGCCTGTTG
AAGAATGAGCCGGCGACTCATAGGCAGTGGCTTGGTTAAGGGAACGGAACC
CACCGGAGCCGTAGCGAAAGCGAGTCTTAATAGGGCGATTGTCACTGCTTA
TGGACCCGAACCTGGGTGATCTATCCATGACCAGGATGAAGCTTGGATGAA
ACTAAGCAGAGGTCCGAACCGACTGATGTTGAAGAATCAGCGGATGAGTTG
TGGTTAGGGGTGAAATGCCACTCGAACCCAGAGCTAGCTGGTTCTCCCCGA
AACGCGTTGAGGCGCAGCAGTTGACTGGACATCTAGGGGTAAAGCACTGTT
TCGGTGCGGGGCTGCGCGAGCGGTACCAAATCGAGGCAA3`

APPENDIX B

SEQUENCES AND PRIMER PAIRS OF ANTI-PORTER GENES IN PLANTS

1- DN157074

GCTCGAGCGGCCGCTCACATGCCCTGCGCTCATTCAAATATCAGAATAAGA
TTTCTAGCTAGCTATGAGCCTGTGACTGTGACAACAAATGGGTCAGCATTCC
AGCTGCACACAAGAATTCCGTTTTGAGTTTTTTGTTTGGCTAACTTTTTGCTG
GATTCTGCATATGGATGTAGGAGGGGAAAAAGTTTCTATTCTATACAGGTA
AAGATTCCTCCTTCAAAACTCCCCCCATCTCCATTCTCCACGAGCGGTCTAC
CAGCGGGTATAGTTCTCTTAGGTCGTGGCTTACAACCAGTATCGTGTGGTCT
TTCTTTAGGTCCTTCAGGAGATTCACAACATCAGCCCGAGCTTTCCAATCAA
GACCAGCAAGGGGCTCATCAAGCAACAATAAATCGGGAGTTTGAACCAGTT
GAATTGCCAGCGGCCGCTAGTAAATCATTTCTGATGAAGGAGGCAGAAATT
TATGTTATGAGCATGATTCATAGGTGGTGCAGAAGAAGTGAACAAGGATAA
TATTTTACCACAAGAGTTGACAGGTATAAGGACACTTTAATCTGCGTGTTAC
GCAGGCAGTGAGCCACGCTTCATGCTGTCCAATCAGCGGGGAACTCTTTTGT
GCGAGTGTTTCCAGTCACAGAAGCACTATTGTAGCACTTGGTGGGGATAGG
ATTGATTGATGTGAAACCCCCACTTCTTT

AP1F TCAGCATTCCAGCTGCACACAAGA
AP1R CTGATTGGACAGCATGAAGCGTGG

2- CB883458

GCGTCCGCCCACGCGTCCGGCCCAGGCCCATCATCTCCTATCGCCCTCGCCG
ACGATGGCGCACAGCCTTACCGGCGGCGCCACCCCCTTCTGCTGCTACCGTC
ACCCGCCCTGCTGCGCCGCCGGGCGAGCTACCCTGACCGCTCCCCCTTCCAC
CCGCTGCCGTGTGGCCGCCTCGGCCTCCCCTCCGCCGCCCCCGCCGGCCATC
GAGGGCCGCGGGGTGGGATTTTCGGTGACGACGAGGCGGGGCCTGGTGCTG
CCGGTGCTCAAGGACTGCTCGCTGTGCGTTCCGTCGGGGCAGCTCTGGATGC
TCCTCGGCCCCAACGGCTGCGGCAAGTCCACCCTCCTCAAGGTTTTGGCAGG
ATTTCAAAATCCCTCTGCTGGTACAGTGCATATTAATAGGCCATTCAGCTAT

GTCTTCCAAAATCCTGATCACCAGGTTGTGATGCCCACGGTGGAATCCGATG
TTGCATTTGGACTTGGTAAGCTCAATCTTTCATTGGATGAGGTTAGATCAAG
AGTGTCACAATCTCTGGATGCAGTTGGAATGTTGAGCTACTCTCAAGTAGGC
AGCTCACATGCACATTTTGGATTATAATCTACTATTGTCTGCACAAGGTTCA
CAAACATCCTTCATCCAAAACAGAGGCCAATCCAAACTCTGAGTGGTGGGC

AP2F ATCATCTCCTATCGCCCTCGCC
AP2R TGCATGTGAGCTGCCTACTTGAGA

3-CD054547
CCTGTTCTAGAGCGTTCGGCACGAGGGGCTTACCGCCTCCGTCAAGGAGAC
TGGGCAGCAGATCCTAAAAGGCGTCGACCTCACCGTTCGCGAGGGCGAGGT
TCATGCGATCATGGGGAAGAACGGCTCCGGCAAGAGCACCCTCACAAAGGT
TCTTGTTGGCCATCCACATTACGAGGTAACTGGTGGTACCATTCTCTTCAAG
GGTGAGAACTTGATTGACATGGAGCCAGAGGAAAGATCTCTTGCAGGCCTC
TTTATGAGCTTCCAAGCGCCTATTGAGATTCCTGGAGTGAGCAATTATGATT
TTCTGCTCATGGCAATAAATGCTCGCAGAGAGAAGGACGGTCTCCCAGCTTT
GGGACCTCTTGAGTTCTATTCAGTTGTATCACAAAAAGTCGAGGCCTTAAAG
ATGGAACCAAAGATCCTTGATCGTAATGTAAACGAAGGATTTAGTGGTGGA
GAAAGAAAGCGCAATGAGATACTGCAACTTTCTGTCCTTGGGGCTGATTTA
GCCCTCCTCGACGAAATCGATTCAGGATTAGATGTTGATGCACTTGAAGATG
TAGCTCATGCAGTGAATGGGCTTTTGACTCCCCAAAACTCTGTTTTGATGAT
TACACATTACCAACGTCTTTTGGATCTTATTAAGCCAAGCTATGTTCACATC
ATGGAAAGCG GCAAGATAGT GAAAG

AP3F GCACCCTCACAAAGGTTCTTGTTGG
AP3R TGAATCGATTTCGTCGAGGAGGGCT

4-BQ467726
CGGCACGAGGCTTCAACCTCCGCGTCGCCCCCGGCTCCCGCTGCCTCCTCGT
CGGCGCCAACGGATCCGGCAAGACCACTCTCTTGAAGATTCTTGCAGGAAA
ACATATGGTTGGAGGGAAGGATGTTGTTCGTGTTCTTAATGGTTCTGCTTTT
CATGACACACAGTTCGTGTGCAGTGGTGACCTTTCCTACTTGGGTGGTTCGT
GGAGTCGGACTGTCAGTTCAGTTGGTGATGTTCCACTCCAAGGCGATTTCTC

TGCTGAGCACATGATATTTGGAGTTGATGGGGTTGATCCTGTTAGGCGAGAC
AAGCTGATTGATCTACTAGATATTGATCTGCAATGGCGCATGCATAAAGTTT
CTGACGGACAACGCCGCAGGGTACAAATTTGCATGGGTCTTCTTCATCCGTA
CAAGGTCCTCTTACTCGATGAGATCACGGTTGATCTCGATGTCGTGACCAGG
ATGGATCTCCTCGACTTCTTCAAGGAAGAGTGCGAGCAGAGAGAAGCCACG
ATCGTCTACGCCACCCACATATTCGACGGGCTGGAGACATGGGCGACCGAC
ATCGCGTACATCCAAGAGGGTGAGCTGAAAAGGTCAGCGAAATACT

AP4F TGGAGGGAAGGATGTTGTTCGTGT
AP4R TTCAGCTCACCCTCTTGGATGTACG

5- BE231217
GAGAGAGAACCCTTTCCAAAGAAAGCAGTTAAAGATCAGCTTTCCTGAACG
TGGGAGAAGCGGTAGAACTGTGTTAGCAATAAATAATCTCAAGTTTGGTTTT
GGGGATAAGATATTGTTCAACAATGCTAATCTAATAGTCGAGAGAGGCGAA
AAGATAGCAATTATTGGCCCCAATGGATGTGGTAAGAGCACATTACTGAAG
CTTGCTTTGGGAACAGAGAAGCCACAAGAAGGTGAAGTCATTCTTGGGGAG
CATAATGTCCTGCCTAACTATTTCGAGCAGAATCAGGCAGAAGCTCTTGATT
TAGAGAAGACTGTACTGGACACTGTAGCTGAAGCTGCAGAGGATTGGAAAA
TTGATGATATCAAAGGTCTCCTTGGTCGTTGTAACTTTCGGGATGACATGCT
GAATAGAAAGGTTCGGTTTCTAAGTGGTGGAGAGAAGGCGAGGCTTTCCTT
TTGCAAGTTCATGGTGACTCCATCGACTTTACTAATCTTGGATGAACCAACA
AATCACCTCGATATCCCATCAAAGGAAATGCTTGAGGAGGCAATATCAGAA
TACACGGGCACTGTAATAACAGNTTCTCATGATCGGTATTTTGTTAAGCAAA
TAGTTAACAGAGGTCATTGGAGTGGAAGATCAAACTATCCAGGACTTTCAA
GGAGATTCAATTATTACCTTCTAAGGAACCTGGAAGCCCGAGAAGGGACTT
GCCCGTGCCGCAAACCTTGAGAAAAGCCCCCAAGTAAGCCAATCAAATGGC
AAGGCGGAAACTGGAGGAGAAACAAAGGCGGCCCTCCACAAGCGGCGAAT
CAATCTTGAAACCTAAAGGGGACTGGATCTCCCCGTATGGGGGCTATCGGC
TTAGAATTATGGGGCGGCGGCCTCTGTGTA

AP5F AGCTGAAGCTGCAGAGGATTGGAA
AP5R CCAGTTTCCGCCTTGCCATTTGAT

6-BF253854 (PDR-like ABC transporter)

AGTGGATAGATTCGAAGAAGTATCACCCTCTCCGGCTACCCTAAAAAACAA
GAAACTTTTGCCCGCATCAGTGGCTATTGTGAACAGACTGATATCCATTCAC
CAAATGTCACTGTATATGAATCCATTCTCTACTCTGCCTGGCTTCGCCTTTCC
TCAGATGTTGACGAAAAAACAAGAAAGTTGTTTGTGGAGGAAGTCATGACT
CTTGTAGAGCTTGATGTGTTGCGTAATGCTATGGTTGGTCTCCCTGGAGTGG
ACGGATTATCGACTGAACAGAGAAAGAGACTGACAATTGCCGTGGAGCTGG
TAGCAAATCCTTCAATCATATTCATGGATGAGCCAACTTCTGGTCTTGATGC
TAGAGCCGCAGCGATTGTAATGCGGGCGGTGAGAAATACAGTCAACACTGG
GCGAACTGTGGTTTGCACTATCCATCAGCCCAGCATCGATATATTCGAGTCT
TTTGATGAGCTTCTGCTTATGAAAAGAGGAGGACAGGTTATTTATGCTGGTG
AACTTGGTCACCACTCTTATAAACTAGTTGAATATTTTGAGGCAATTCCAGG
TGTTGAAAAGATCACAGAAGGATATAATCCCGCAACATGGATGCTGGAAAG
TAGCTCCCCTTTAGCCGAAGCTCGCCTGAACGTCAACTTTGCTGAAATTATG
CTAATTCTGAACTTTTAGGAAAAACCACAACTTATTAAGGAATTAAGCGTCC
CCGCCAGGCTATGAGATCTCTCGTTTCCTACAAAGACTCTCAGAACTTCTAC
ACCAATGCATTGCAAAACTCTGGAAGCATACAAACTTATTTGAAGATTCGC
CCACCAGGCATGCCCTTTTGAGACCA

AP6F TGGCTTCGCCTTTCCTCAGATGTT

AP6R TCCAGCATCCATGTTGCGGGATTA

7-BQ471758 (MRP-like ABC transporter)

CGGCACGAGGATCATCAACAGATTTTCGAAGGATTTGGGTGACATTGACAG
GAATCTTGCTGTCTTCGTCAACATGTTTATGGCACAAATATCTCAGTTGCTCT
CAACATTTATTCTCATCGGTGTTGTCAGCACTATGTCTCTTTGGGCTATCATG
CCGCTTCTGATTTTATTTTATGCAGCCTACCTTTATTACCAGACCACATCACG
CGAGGTAAAGCGCATGGATTCTATTACCAGGTCTCCTGTGTATGCTCAATTT
TCAGAGGCATTAAATGGTCTGTCCACAATCCGTGCCTACAAAGCCTATGATA
GAATGTCAAACATCAATGGGAAATCAATGGACAACAACATCAGATTCACAC
TCGTGAACATGAGTTCAAATAGGTGGCTAGCCATCCGGCTGGAAACATTGG
GTGGCATCATGATATGGTTCACGGCAACATTTGCAGTCATGCAAAACCAAC
GAGCAGAGAATCAGAAGGCCTTTGCTTCCACGATGGGTCTTCTTCTTACTTA

CACCCTCAATATCACCAATCTGCTCACAGCTGTTCTTCGTCTTGCTAGTCTTG
CTGAAAATAGTATGAATGCTGTTGAACGCGTGGGGACATACATTG

AP7F TCTTTGGGCTATCATGCCGCTTCT
AP7R CCCACGCGTTCAACAGCATTCATAC

8-BE602103 (ABC transporter family protein)
GATAGTTTTGGCAATTAGACTGCAGAGGTACTATCTAGCCTCGGCAAAGGA
ACTGATGCGGATCAATGGTACCACCAAGTCTGCTCTTGCGAATCACTTAGGT
GAATCGATTGCAGGGGCTATAACCATAAGGGCCTTTGAGGAGGAAGATCGT
TTCTTCACTAAAAATTTGGACCTTGTTGACAAGAATGCCAGTCCATATTTCT
ATAATTTTGCATCAACTGAATGGTTGATTCAACGTCTGGAGATAATGAGCGC
CGCAGTTCTTTCTTTTTCTGCCTTTGTCATGGCCCTTCTTCCTCAAGGAACTT
TTAGCCCTGGTTTTGTGGGAATGGCATTGTCCTATGGTCTTTCCCTAAATATG
TCATTTGTATTCTCTATTCAAAACCAATGCAACCTGGCGAATCAAATAATCT
CGGTGGAACGGGTGAACCAGTACATGGACATACAAAGTGAAGCAGCAGAA
GTTGTTGAGGAAAATCGACCATCACCAGATTGGCCCCAAGATGGTAATGTG
GAGCTTAAAGATTTGAAGATCAGGTATAGAAAAGATGCTCCCCTTGTACTA
CATGGAATCACTTGCAGGTTTGAAGGTGGAAATAAGATTGGTATAGTTGGT
CGAACGGGAAGTGGCAAAACAACGTTAATTGG

AP8F TACCACCAAGTCTGCTCTTGCGAA
AP8R GGGCCAATCTGGTGATGGTCGATTT

9- BI953506
CAGAAAGGAAAATTCTTAATGGGGCCACTTTCACTGTACCAGCAGGGAAAG
AGTGTGGCAATTGTTGGAACTAGTGGCAGCGGAAAATCAACCATACTTAGA
CTTCTCTTTAGATTTTTTGATACAACTTCAGGATCTATACGAATAGACGGCC
AAGATATTCGGGGAGTCACACTGGAGAGTCTTCGGAAGTCTCTTGGTGTTGT
GCCACAGGATACGGTACTTTTCAATGACACGATTAAGCATAATATACAATA
TGGGCTGTTATCAGCAAATGATGAAGAGGTTTACGATGCTGCTCGACGTGC
CTCTATCCATGATACAATTATGAACTTTCCTGAAAAGTATGACACGGTTGTG
GGAGAGCGCGGATTGAAGTTAAGTGGTGGTGAGAAACAACCACTCTCAATT

GCTCGTGTATTCTTGAAGGAGCCTCACATTCTGTTATGTGATGAAGCTACAA
GCGCACTGGATAGCACCACTGAATCATCGATTTTGAATTCTTTAATGTCTNT
ATCCATCGATCGAACCTCAATTTTATTGCTCATCGACTTCAAACTGCCATGC
TATGCGACGAGATCATTGTCTTCGAAAAATGAACACTATTGGAAACAGGGC
CACATGATCTTCCTCCTGTCCAAACACGGCCGGTTGCCTAACTTTGGTTCTA
CCAGAAACCCCCTTGAGCCAGTTGTTCCTCTCGCTTTCGGCTCATAAGTCGG
ATANACCCAATCCGTTTTCCCTTTGCGGAGGGCAAACACAGCCGTCAGCCTG
TTCTATGGCTGGCCGGGTCCGCTAATTCGCCATGGAATGTACCGTCTGAGAG
CTGCTCCCGGGGAAGTGGGAATATGCTGGGCCGGG

AP9F TCTCTTGGTGTTGTGCCACAGGAT
AP9R TCAGACGGTACATTCCATGGCGAA

10- BQ660845
ACAGTATAACGGATTCATGTTGGATGTCATGGATGAATGTACACGCAAAAT
GTAATTTGTCCCTTTACTCATCATAGCTGTCCCATAATTTACAACCAAAATC
ACATCAAGATTTATGGAACATGTGCAAGAAAGGGATTCAGGCTTTGGTACT
ACTCGTAAGTAGAAGATGCAGAGAAGCAAGCAGAGTGGTGTGAGGAGGAT
GTGAGCATCAATGCGGATCGTGCGGCGGCCCTGAGCGTGAGGCTGCAGCGG
ACCCGGAGCACGGCGTAGCCGAGCACCCGGTACAAGGCGACGAACCCCAC
CATGACGGCCACGTTCCTCCACCGGCACTCCTCCCCGATCCCCTCCCGCCGG
AGCACCTCGTCGCCGGTCGCCACGCACACCCCCATCGCCCTCGCCGCGCACC
GGCCCCCGAACTCGTTCAGCAGCAGCGCCTCGAACGGCCACTTGAAGAGCG
ACAGGTAGTGCATCGGCACCCAGTACGCCGGCATCGCCGACCGCCTGAT

AP10F GCAAGAAAGGGATTCAGGCTTTGG
AP10R TACTGGGTGCCGATGCACTACCT

11-AU090180
AATTCGGCACGAGGATTTGGTATCATTCAGTAATATCCTTTATGGAATCTAT
CCACCATTATTCGTTGTTCTTATCGTTTATTCTCTTGGAGGGACCGCTATTAG
TGTCTTCCTTGGTAAGAGTTTGGTCAACTTGAACTTCATGCAAGAAAAGAAA
GAAGCTGATTTCCGTTACGGGCTTGTCCGTGTTAGAGAAAATGCTGAATCAA
TTGCCTTTTATGGTGGTGAGGAAAATGAATTGCAACTTCTGTTGGATCGGTT

CAGGAGGGCTTACCAAAACCTAACTGAATTACTGATAGCATCCCGGAATCT
GGAGTTCTTCACCAATGGTTACCGATATTTAATTCAAATCCTGCCAGCTGCA
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AP11F TCGGCACGAGGATTTGGTATCATTC
AP11R CAACTGCAGCTGGCAGGATTTGAA

12- CD056233
AATTGATCCAACTAGTGATGAAGGCATAAAGCTAGAGAAAATTGATGGCAA
CATAGATTTCAACCATGTGAGCTTAAGTACCCGTCCCGCCCAGATGTCCAAG
TATTCAATGACTTTACTTTGGGTATTCCCTCGGGAAAGACTACTGCACTTGT
TGGAGAGAGTGGCAGTGGCAAGTCCACAGTAATTGCTTTGCTAGAGCGATT
CTATGATCCAGACTCTGGCACAATCTCACTAGATGGAATAGAAATCAAAAA
CTTAACACTGAGTTGGTTAAGAGACCAGATGGGGCTGGTAAGCCAAGAACC
AGTACTTTTCAATGACACGATTCGTGCCAACATAGCATATGGAAAGCGCGG
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GAGGAACACAACTCTCTGGTGGGCAAAAACAACGGGTAGCTATTGCCAGGG
CGATCTTGAAGGACCCAAGAGTACTTCTGCTAGACGAGGCAACAAGTGCCC
TCGACGCTGAATCGGAGCGTATTGTTCAAG

AP12 FTGGCAGTGGCAAGTCCACAGTAAT
AP12R AACAATACGCTCCGATTCAGCGTCG

13-AV833152
TCGGCACGAGGGTGATCTGAAGCTGTTCAATGTGAGATGGCTGCGGAGCCA
CATGGGACTGGTTCCTCAGGACCCTGTCATATTCTCCACAACCATAAGAGAG
AACATCATATACGCGAGGCACAATGCGACGGAGTCCGAGATGAAGGAGGC
TGCCAGGATCGCGAATGCCCACCATTTCATCAGCAGCCTGCCCCACGGCTAC
GACACGCACGTGGGGATGCGCGGAGTTGACTTGACGCCCGGGCAGAAGCA
GCGGATCGCCATTGCCCGTGTCGTGCTGAAGAATGCGCCCATTGTGTTGCTT
GATGAAGCCAGCTCTGCTATCGAGTCTGAGTCAAGCAGGGTGGTGCAGGAG
GCCCTTGATACGTTGATCATGGGGAACAAGACGACGATCCTCATCGCGCAC
AGGGCAGCCATGATGAAGCATGTGGACAACATCGTGGTGCTCAATGGTGGC

AAGATCGTGGAGCAAGGGACGCACGACTCGCTGGTGCAGATGAATGGATTG
TACATCAAGCTGATGCAGCCCTC

AP13F TCATATACGCGAGGCACAATGCGA
AP13R ATCCATTCATCTGCACCAGCGAGT

14-AU252340
ATCGCGGCTTATATGGGAAGCCAGGTGCTTACGGTGTAGTTACATTACCATT
TTCGGTCCGAGAAGGTATCAATATTTTCCTGGCTGGATTTGTTCCAACAGAA
AACCTTCGGTTTCGAGATGAATCTCTTACATTTAAGATTGCGGAGACCCAGG
AAAACGCAGAGGAAGTTGCTACGTACCAGCGGTACAAGTACCCTAACATGA
GCAAAACACAGGGAAATTTCAAGCTTTCTGTTGTTGAGGGTGAATTCACTG
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TCATCAGAATGCTGGCCGGGTTGTTGAAGCCAGATACCATGGAAGGAACCG
AGGTTGAAATTCCTGAATTCAATGTGTCCTACAAGCCTCAAAAGATCAGTCC
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TATACTCATCCTCAGTTTGTATCTGATGTTATGAAACCACTACAAATTGAGC
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TAGCATTATGTTTGTGCCTTGGAAAGCCTGCAGATATTTATTTAATTGATGA
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TCATCATGGCAACCTACTTAGCGGACAAGGTTATTGTTTACGAAGGACTTGC
TTCTATTGACTGTACTGCCAATGCACCACAGTCTTTGGTATCTGGGATGAAT
AAATTCTTATCGCATCTTGATATTACATTTCGAAGAGACCCGACCAATTATA
AGCCGCGAT

AP14F ACCATGGAAGGAACCGAGGTTGAA
AP14R CAAAGACTGTGGTGCATTGGCAGT

15- BU971343
CGGCACGAGGCTCCTCGTCTACGTGCCCCTGCAGTGCCCATACGGTTCTTGC
AGTCGCAGACCATCGTCCTGCCCGTGACGGCCAGCTCCGGCGCCACCGCGC
TCTGCCACCCGCTCGTGTGCTGACTGCTGGTGTTCAAGGCCGGCCTGGGGAG
CAAGGGCGCCGCGCTCAGTAACGCCGTCTCCTACGGCATCAATCTGGTCAT

ACTGGCTCTGTACGTCAGGCTGTCCGCCACCTGCAAGAACACCTGGAGCGG
CTTCTCCCGGGAGGCCTTCAAGGAGCTGCGCCAGTTCACCGCGCTCGCCATG
CCGTCCGCCATGATGATCTGCTTGGAGTGGTGGCCATTTGAGGTCCTTGTGC
TTCTCTCTGGGCTTCTGCCCAATCCTCAGCTTGAGACATCAGTGCTGTCAAT
ATGCCTTAACACAGGTGCTCTGTTGTACATGGTACCACTTGGCCTTTCTTCTT
CTATCAGCACGCGCGTCTCAAACGAACTTGGGGCAGGCCACCCAGAAGCAG
CAAAGCTCGCGATGCGAGTGGTCATGTACATGGCCTTGTCTGTAGGATTTGT
GCTGGCCTTGACCATGATCTTGCTACGGAATGTTTGGGGGTACCTGTACAGC
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AP15F GGCATCAATCTGGTCATACTGGCTCT
AP15R TGACGATTTCCTGCTCGTTGCTGT-451

16- DN177172
GCACGAGGCTGCGATTGGGTTGAAGTGATTGTCCTTGGTCTTTACATTAAGT
TCTCGCCTTCTTGTGAGAAAACACGTGCTCCACTCACGTGGGAAGCTTTTAA
AGGAATTGGCAGTTTCATGCGTTTGGCTGTACCGTCGGCTCTTATGATTTGC
CTTGAGTGGTGGTCTTACGAGCTGCTTGTTCTGCTTTCTGGGATCTTACCAA
ATCCAGCACTTGAAACTTCTGTGCTTTCTATATGCATATCTACAGTGGTACT
GTTGTACAATCTTCCTTACGGTATTGGAACAGCTGCAAGTGTTCGTGTCTCG
AATGAACTAGGTGCTGGCAACCCAGAAGGTGCCCGCTTGGTGGTAGGTGTT
GCTTTGTCTATTGTAGTTTGTTCAGCAGCACTGGTGAGCACAACTCTTCTCG
CATCGCGTCACTTCATTGGAATTGCATTCAGCAATGAGGAGGAGGTTATAG
ATTATGTCACCAGAATGGTGCCCGTACTTTCCATTTCAGTTATTACAGACAG
CCTCCAAGGAGTCCTTTCAGGTGTTTCTCGGGGCTGTGGATGGCAGCATTTA
NGCGCGTACGTTAACCTGGGTGCATTCTATCTTGTTGGGATTCCAGTTGCAC
TCTTTTTTGGTTT

AP16F GGCTGCGATTGGGTTGAAGTGATT
AP16R ACGGGCACCATTCTGGTGACATAA

17- CA015342
CGGCACGAGGGTACTACAAGTGCTTGTGTTGCTTACGGGGTACTTGCCGAA
CGCTGAGATCGCCGTGGACGCCCTCTCATATGCTTGACGATCAACGGATGG

GAGATGATGATTCCCATAGGGTTCCTGGCGGCCACTGGCGTGCGGGTGGCA
AACGAGCTCGGCGCGGGCAGCGGCAAGGGGGCGCGCTTCTCCATTGTCGTC
TCCATCACCACCTCCG
TGGTGATCGGGCTCGTCTTCTGGTGCCTCATCCTCACCTACAACGACCAGAT
CGCGCTCCTCTTCTCGTCGGGGAAGGCCGTGCTCGACGCCGTGCACAATCTC
TCCATGCTGCTAGCATTCACCATCCTCCTCAACAGCGTGCAGCCCGTACTCT
CTGGGGTGGCTATTGGTTCTGGATGGCAAGCATTGGTCGCCTATGTCAACAT
CGGATCCTACTACCTGGTCGGGGTGCCGATCGGGGTCATACTGGGCTGGCC
ACTCGGATTCGGAGTTCGGGGAATTTGGTCTGGGTTGATTGGCGGGACCGCT
GTTCAGACGCTCGTGTTGGTCTATCTCACCATGAGATGTGATTGGGACGATG
AGGCCAAGACCACCAGTGCACGGATGAGAAAATGGGCCAGCACAAAATGA
GAGCTACTCG

AP17F ATGCTTGACGATCAACGGATGGGA
AP17R GATAGACCAACACGAGCGTCTGAACA

18- Barley chip
TCCTGACAATCTCCATCGTGCTTGACTCCGCGCAGGGCGTGCTGTCAGGGGT
GGCAAGGGGCTGTGGATGGCAGCACCTGGCGGCCATGACGAACCTGGTGGC
GTTCTACTTGGCCGGCATGCCCGTGGCCATCTTCCTGGCCTTCAAGCTCAAC
TTGTACACCCACGGTTTATGGTTGGGTCTAATCACCGGGCTGGCATGCCAGA
CCAGCGTGATGGTGCTCATCACCCTCCGCACGAAATGGTCCAACCTCGTGG
ACGCCATGGAGAAAAACCGGGATGGCTATGTCGCTTGATCGATCCATGCTT
AACTTGGGTGCTAGTTCAAGATGCACCAATTGTTGTATTTGTCTACTAGTTC
AAGATGCACCAATTGTTGTATTTGTCGACGTAACTGTTGTATTTGTAAGTTTT
GACCGTATCTCTGAGTTGATTTTACCCTGTAAAAATGGAGGCTGCCTAACAG
ATGGCATTTGCCCAACGTTAATTTTTTTTTGACTGAAATGTACACCAATGAG
GAACA

AP18F TGACAATCTCCATCGTGCTTGACTCC
AP18R TTGGGCAAATGCCATCTGTTAGGC

19- BI960363 (Multi antimicrobial extrusion protein MatE family protein)

AGGTTCAACTCAAATGGCCGCATTCCAGATTTGCTTGCAGATCTGGTTGGCA
TCTTCCCTTCTTGCTGATGGTTTGGCTTTTGCTGGACAGGCTATACTTGCAAG
TGCATTTGCTCGTAAGGACCATTCAAAGGCGAAGGCCACAGCTTCCCGCAT
ACTGCAGCTCGGATTGATCTTGGGGCTTCTCCTGAGCCTACTTCTTGGAGTT
GGCCTCCATACAGGTTCAAGATTATTTACTGAAGACAAGGGTGTACTGCATC
ATATTTACGTGGCAACACCGTTTGTTGCTCTAACTCAACCGATCAACGCTTT
AGCCTTTGTTTTCGACGGCGTCAATTACGGTGCATCTGATTTTGCGTATGCT
GCTTATTCATTGATACTTGTGGCTATTGTCAGCATTGCTTGCATTGTCACCCT
CGCCAGTTACAGTGGTTTTGTTGGGATCTGGATAGCCTTGTCGATCTACATG
TGCCTCCGCATGTTTGCCGGATTATGGAGGATTGGGACTGCACGAGGGCCA
TGGGTTTTCCTTCGCAGCTGAACATGTAACATTTAATCTGGTAGAATATTTA
CACTTGTAAACAACTTTGGAGTAACCCTGAAACATGTAAGCCTCAAACTGT
GCCACCATGATTTTTTTTTTTAACCGGGCAAAA

AP19F TTGGAGTTGGCCTCCATACAGGTT

AP19R TGGTGGCACAGTTTGAGGCTTACA

20- CB860075

GGTGTCTCAAAGCTTGCTTTCAACAAGATATTTTTTCCCCATCTCTAATAATA
AGTAACAATCAGCAAAATCTTTACATGGCTACATTAGCAAATGCGAACCAA
AATTTGTCACCCTGTCAGCGCCCTAAGCTCCTCCCCCCCGCCCCGGCCAAAA
TATGGCCAGCAGGGGAAGGAAATAAAAATGGTAAAAATAAGAAGCAGGGG
GAGTAGATTACAATCACACGCCCCCCCGGCTCCCTCGCGCTGCGTAGCGTA
GCTATCAACACCGATCATTCGGCTGCTCGATGTCTGCTGCGTCGATAAGCAG
GCGCGACTTCTCGTCTCCGTCGGCCGCGCCAACTTTGTCCTGGCCGGTGAGC
TGCTTCGAGCGCTTGGCCTCGGCCGCCCAGTCCGTCCGCCCGATGACCAGCA
GCATGCGCACCATGCAGGTCGCCTGCGCCGCCAGCAGCCCGAACCACAGGC
CCTTGAAGTCGTAGTGGAACCAGAAGGCCAGCACCAGCGCCACCGGCGTCC
CGACG

AP20F AAATTTGTCACCCTGTCAGCGCC

AP20R GCTGGTGCTGGCCTTCTGGTT

21- BF620839

CCCATCCTCATCCTCCTCGGCGAGTCCCCCGAGATAGCCCGCGCCGCGGCCA
TCTTCGTCTACGGCCTCATCCCGCAGATCTTTGCCTACGCTGCCAACTTCCCC
ATTCAAAAGTTCATGCAGGCGCAGAGCATCATGGCGCCCAGCGCCTACATC
TCCGCAGGCACACTCGCCATCCACCTCGTCCTCAGCTACCTCGTCGTGTACA
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AGATACTCGTGCTCATTGCTGGCCTCCTCAAAGACCCCGAAATGGCTCTTGC
ATCGCTCTCCGTCTGCATGACCATTTCAGGATGGGTGTTCATGATTTCTGTTG
GATTCAATGCAGCGGCCAGCGTGAGGGTGAGCAATGAGCTTGGCGCAGGCA
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TACATTTACACGGGGGGCGACGATGTGGCGGCGGCAGTGTCCAAGCTGACG
CCGNNTCTAGCGCTCACCCTCATCCTCAACGGCATCCAGCCAGTATTGTCAG
GTGTGGCCCGTGGGGGTGTGGGTGGCAAGCGTTCGTCGCCTACGTCAATGT
CGGCTGCTAT

AP21F AGATCTTTGCCTACGCTGCCAACT

AP21R TCCTGAAATGGTCATGCAGACGGA

22- BE558861

AATCGGCCGAGAAAATCACCCGTCTCGAGCGCGCGCGCGATGGCGGAGGCG
CCCCTGCTGCCGCGGAAGGACCAGGAGGACGCCGGCGAGGATGGTGCGGG
CCGCTGGCGGGGCGAGGCCGGGAAGCTGGCGTACCTGGCGCTGCCGATGGT
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CGGCCACCTCCCCGGCGTCCTCCCGCTCTCCTCCGCCGCCATCGCCACCTCC
CTCGCCTCCGTCTCCGGCTTCAGCCTCCTCATCGGCATGGCAAGCGCACTGG
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GCTCCTGTGGGCGTTCATGGGCAAGATCCTGGTGCTGATCGGGCAAGACCC
CCTGATCGCGCGCGGCGCCGGGAGGTACATCGTTTGGCTCATCCCGGGGCT
CTTCGGCAACGCGGTCATTCAGCCCATCACCAAGTTCCTGCAGTCACAGAGC

CTCACCATGCCCGTGGTGCTGTCGTCCGTGGCCACCCTTGGGCTTCACGTCC
CCCTTGTGGCTGGGCAATGGGGTCAAGGACGGGGATGGGGGTCACCGGGGC
CAACCCGGGCAATAAGCGGGGGTAAAGGGGCTTAACTGGGGCAAGGCCTG
GGGCCTAAATTTGGGATAGTAAGCCACTCTTAAAGGGAAAACCCCCACCCC
CCCCCAATAAAGGGCCTTAGGGGGGGGGGGTGTGGGTTCC

AP22F TGGCGGTGAGCCTGTCGCAGTA
AP22R CTCTGTGACTGCAGGAACTTGGTGAT

23- CA018179
CGGCACGAGGCGCGTCAGGGCCTCGCCGCGGCGGAGGTGAAGCGGCTGGT
GCGGCTCGACGGGCCCCTCGTCGCCAGCTGCATCCTCCAGAACGTCGTCAA
CATGGTGTCTGTCATGGTCGTCGGCCACCTCGGCGAGCTGCCCCTCGCCGGC
GCCTCCCTCGCCACCTCCCTCGCCAACGTCACCGGCTACAGCCTCCTCGCGG
GCATGGCGACGGCGATGGACACGCTCTGCGGCCAGGCATACGGCGCGCGGA
TGTACCACCGGCTCGGCGTCTACAAGCAGTGCGCCATGGTGGTGCTCTCGCT
CGCCTGCGTCCCCATCGTCCTCATCTGGGTCACACCACCAGGATCCTCGTCT
TCCTCGGCCAGGACCCGGCCATATCGGCCGTGGCCGGCGAGTACGCGCGGT
GGACGATCCCGTCGCTCCTCGTGTACGTGCCCCTGCAGTGCCACATACGGTT
CCTGCAGTCGCAGACCACCGTCCTGCCCGTGACGGCCAGCTCCGGC
GCCACGGCAC TCTGCCACCC GCTCGTGTGCTGGCTG

AP23F TGCATCCTCCAGAACGTCGTCAA
AP23R TCTGCGACTGCAGGAACCGTATGT

24- AU252296 (MATE efflux family protein)
TACCCAGGAGCGTCAAAGGAGAGCTTGGTGGTGATTGAGGTCAAGAAGCAG
CTGTACCTGGCCGGGCCTCTCATCGCCGGATGCCTGCTGCAGAACGTCGTGC
AGATGATCTCGGTCATGTTTGTCGGCCATCTCGGTGAGCTCGCTCTCTCGAG
TGCCTCCATCGCCACCTCCTTTGCCGGTGTCACCGGCTTCAGCTTGTTGGCC
GGCATGGCGAGCAGCTTGGACACGCTGTGTGGGCAAGCCTTCGGGGCAAAG
CAGTACTACCTGCTCGGCATCTACAAGCAGAGGGCGATCCTTGTGCTCACGC
TGGTGAGCGTTGTGGTTGCGGTGGTCTGGGCCTACACCGGGCAGCTCCTCCT
GCTCTTCGGCCAAGACCCAGAGATCGCCATGGGGGCGGGGAGCTACATCCG

GTGGATGATTCCGGCATTGTTCGTGTACGGCCCGCTGCAGTGTCACGTCCGG
TTCCTCCAGACTCAGAACATCGTCCTTCCGGTGATGGCGAGCTCGGGCGTNA
CNACGCTGAACCACGTGNTCGTGTGCTGGTTGCTAGTGTACAAGCTTGGGCT
GGGTAACAAGGGGGCTGCCCTGGCCAACGCCATCTCGTACNTGGCCAACGT
GTCAATCTTGGCTCTCTACATCAGGTTCTCTCCATCCTGCAAGAGCACCTGG
ACAGGGGTCTCAAAGGAGGCGTTCCGCGGCATCCTTAGCTTCATGAAGCTT
GCCGNACCATCTGCGCTCCTGGGT

AP24F ATCTCGGTCATGTTTGTCGGCCAT
AP24R TGTACACTAGCAACCAGCACACGA

25- BU989688 (MATE efflux family protein)
CTCATCCTGGCTCTCTACATCAGGCTCTCTCCATCCTGTAAGAGGACTTGGA
CAGGGTTGTCAATGGAGGCATTTCGCGACATCCTTAGCTTTTTGAGGCTTGC
GGTTCCATCTGCTCTGATGGTTTGTTTGGAGTGGTGGTCATTTGAGCTGCTTG
TACTTTTTTCTGGATTTCTTCCAAATCCTAAGCTCGAGGCGTCCGTGTTGTCC
ATCAGTTTAAATACTCTTTCATTAGTATTCAGAATCCCATCTGGACTTGGTG
CAGCTATAAGCACCCGTGTATCAAATGAGCTCGGTGCTGGGCGACCTGATG
CTGCCCGTTTAGCGACCCATGTGATCATGGTTCTGGGTCTTGTGTCGAGCGT
ATCGGTCGGTCTTGCCATCATTTTGGTGCGCAATTTATGGGGGTACGCATAC
AGCAACGAGAAGGAAGTGGTGGAATACATTGCCAGAATGATGCCAATTCTC
GCCGTCACATTCTTGTTTGATGACCTGCAGTGTGTCCTTTCAGGTATTGTTAG
GGGCTGTGGCTTGCAGAAGATTGGCGCGTATGTCAATCTTAGCGCATACTAC
CTTGTCGGCATCCCGGCGGCTTTATATTTTGCGTTTGTGTCCCATCTTGG

AP25F TCTACATCAGGCTCTCTCCATCCTGT
AP25R TTCCACCACTTCCTTCTCGTTGCT

26- DN179985
GCACGAGGCATAGGTCAGCAACCAGAAATTGCAAATGAGGCTGGGAAATA
TGCATTATGGCTTATCCCTGGTTTATTTGCCTTCAGTGTTGCTCAATGCTTTT
CAAAGTTTCTGCAGTGTCAGAGCCTCATCTTTCCTATGGTTCTTAGCTCCATG
ATCACACTCGCTGTATTTATTCCTCTGTGTTGGTTCATGGTTTATAAAGTTGG
TATGGGTAATGCTGGAGCTGCTTTATCCGTCAGCATCTGCGATTGGGTTGAA

GTGATTGTCCTTGGTCTTTACATTAAGTTCTCGCCTTCTTGTGAGAAAACAC
GTGCTCCACTCACGTGGGAAGCTTTTAAAGGAATTGGCAGTTTCATGCGTTT
GGCTGTACCGTCGGCTCTTATGATTTGCCTTGAGTGGTGGTCTTACGAGCTG
CTTGTTCTGCTTTCTGGGATCTTACCAAATCCAGCACTTGAAACTTCTGTGCT
TTCTATATGCATATCTACAGTGGTACTGTTGTACAATCTTCCTTACGGTATTG
GAACAGC

AP26F CGAGGCATAGGTCAGCAACCAGAAAT
AP26R AACAAGCAGCTCGTAAGACCACCA

27- BJ479276
GGAGGAGCCCCTTGTTCACAAGACAGGAGAGCAGAGCCTGGTAGTGATTGA
GGTGAAGAAGCAGTTGTACCTTGCCGGTCCTCTAATCGTCGGAAGCCTCTTG
CAAGATGTGGTCCAAATGATATCCGTCATGTTTGTGGGTCATCTCGGCGAAC
TCGCTCTCTCGAGTGCCTCCATCGCAACCTCCTTCGCCGGTGTAACTGGCTT
CAGCTTGTTGTCGGGCATGTCGAGCAGCTTGGACACGCTGTGTGGGCAAGC
CTTTGGAGCAAAGCAGCACCATCTTCTTGGCATATACATGCAGAGGGCAAT
CCTTGTGCTCACTCCGGTGAGCGCCGTGGTTGCCATAATCTGGGGATACACC
GGTCAGATCCTTTTGTTGTTTGGGCAAGACCCTGAAATTGCCATGGAAGCAG
GGAGCTACATCCGATGGATGATTCCGTCGCTTTTTGTCTACGGCCCGCTGCA
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AGCTCGGGCGTCACAGCGCTGAACCACATCTTG
GTGTGCTGGCTGCTCGTGTACAAGCTTGGCCTGGGCAACAAGGG
AP27F AAGAAGCAGTTGTACCTTGCCGGT
AP27R TCTGCGTCTGCAGAAATCGGACAT

28- AV941525
GGAGATTGCAAGTTTCAATCNTGTTGCCGGCCATGGTGGAGCCCTTTGTTGG
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TGACCGAGGTGAAGAAGCAGCTGTACCTCGCCGGGCCTCTCATCGCCGGAT
GCCTGCTGCAGAACGTCGTGCAGATGATCTCGGTCATGTACGTCGGCCATCT
CGGCGAGCTCGCCCTCTCCAGCGCCTCCATCGCCACATCCTTCGCGAATGTC
ACCGGCTTCAGCTTGATGTCTGGCATGGCGAGCAGCTTGGACACGCTGTGTG
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GGGCGATCCTTGTGCTCACTCTGGTGAGCGTCGTGGTTGCGGTGCTCTGGGC
GTTCACCGGGCAGATCCTCCTGCTCTTGGGCCAGGACCCGGAGATCGCCAT
GGGGGCAGGGAGCTACATCCGGTGGATGATCCCGTCGCTCTTCGCTTACGC
GCTGCTGCAGTGCC

AP28F TGACCGAGGTGAAGAAGCAGCTGTA
AP28R GTAAGCGAAGAGCGACGGGATCAT

29- BU978824
CGGCACGAGGGGGCTGGGGAGGTTCAGGCTTGATGAGGTTGGGATGGACAT
CCTGGGCATCGCCGTGCCCGCGGTGCTTGCACTCGCCGCTGACCCCATCACG
GCGCTCGTTGACACCGCCTTCGTCGGACATATCGGCTCGGTTGAACTTGCTG
CTGTTGGTGTATCCATATCTGTCTTCAACCTGGTGTCCAAGCTGTTTAATGTG
CCACTGCTTAATGTCACCACATCCTTTGTTGCTGAGCAGCAAGCAGTGGATG
CCAAGTATAGCGGTGTAGGAGAAATTCTCACCTAGGAGATGAAGTGTCGAG
CACCCGAGAGCAGGCGAGT
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TGGACATCGTTGGTATACCTGTCGATTCACCGATGCGAGCACCGGCAGAAC
AATTTCTTACTTTAAGGGCATATGGTGCTCCGCCAGTCGTAGTAGCACTTGC
AGCACAAGGTGCAT
TTCGTGGTTTCATGGATACAAAGACACCTTTGTATGCTGTGGTTGCTGGCAA
CCTAGTAAATGCAATACTGGATGCCATATTTATC

AP29F TCTTCAACCTGGTGTCCAAGCTGT
AP29R TGCCAGCAACCACAGCATACAAAG

30- BF260781
CTGCAGAGCCAGCTCAAGAACTGGGTCACCGCCGCCACCGCGGGGGTCACG
CTCGCGCTCCACGTCCTCGTCACCTACCTCCTCGTCACCCGATTCCAGCTCG
GATACGCCGGCGTGGTCGTCGCCGCCGACGTCGCGTGGTGGGTCGCCGAGC
TGGGGCAGTTCTTGTACGTCGTCTGCGGCGGCTGCCCGCTCTCGTGGAGGGG
CTTCTCCGTGGAGGCCTTCGCCGACTTCTGGGACTTGATCAAGCTCTCCACC
GAATCCGGCGTCATGCTCTGCCTGGAGAACTGGTACTACAAAGTGCTTGTGT

TGCTTACGGTGTACTTGCCAAACGCTGAGATCGCCGTGGACGCCCTCTCCAT
ATGCTTGACGATCAACGGATGGGAGATGATGATTCCCATAGGGTTCCTGGC
GGCCACTGGCGTGCGGGTGGCAAACGAGCTCGGCGCGGGCAGCGGCAAGG
GGGCGCGCTTCTNCATTGTCGTCTACATTACCACCTACGTGGTGATCGGGCT
CGTCTTTTGGTGCCTCATCCTCACCTG

AP30F AGCCAGCTCAAGAACTGGGTCAC
AP30R GCCAGGAACCCTATGGGAATCATCA

31- CB652291

CGGCACGAGGGCGCTAACGAACCTTGTGGCGTTCTACTTCATCGGCATGCC
GTTGGCCATCCTCTTTGCCTTCAAGCTCAACTTCTACACCAAGGGTTTATGG
TCGGGTCTGATCTGCGGGCTGACTTGCCAGACCAGCACGCTGGTGGTGATC
ACCGCCCGCACGAAATGGTCCAAGATCGTGGATGCGATGCGGCAAGAGAA
GGACAACTATGTCGCTTGATCGTTAAAGATCGTCCGTGGCTTGCACTTGTAT
GTACGGAGCGTGCGATGCTTTAACTGTTGTTTAGCTTTATGAGGTAAGCTAT
AGCTCGTTTTAACCGTGTACTTCGAGGGAATTCATGTCTCAGGAAGATCGAA
CCTGTAATGGCAGAGAGTAACCACGTGACATATGTGGTAAGCAATACAAAC
AATTCAAAAAAATATATACAGGAGAAACGTACATAACGGGACATGGATGTT
CCGTCCCCGCGGACCCATGCATGGATGTTCATATTTTCAAGGACGCAAATGT
ATCGTGGCTGTTGCTAACCTCGTCAACACAACA

AP31F TGTGGCGTTCTACTTCATCGGCAT
AP31R AGCAACAGCCACGATACATTTGCG

32- BF631546

CAGCCCACCAACGTCTCGACTAATATGTACCAACAGCGCCCAGCTCTCATCT
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ACGTCACCGGCTTCAGCTTGCTGGTGGGCATGTCGAGCGCCCTGGACACGCT
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GCCCGTCATGGTCAGCTTGGGCGTACGTTCGCTGAACACATACTCGTGTGCT
GGGTGCTGTGGCCCAGGGGGGCCTGGGGAACAAAGGGCCCGGGCCTGGCA
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GAACTGCCCATTGCGGGGACTCCCGTCCC

AP32F AGCTCGTGTCCGTGATGTTCGTT
AP32R ACCCAGCACACGAGTATGTGTTCA

33- BJ465445
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AGTGGTTGTGTTGTTCTTCCGCGATCGCATCTCCTACCTGTTCACAGGTGGC
GAAGCCGTTGCCAACGCAGTGTCCGACCTCTGCCCGCTACTCGCCATCACGC
TCATACTCAATGGCGTTCAACCCGTGCTTTCTGGTGTGGCTGTGGGGTGCGG
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AP33F TCATGCTCTGCCTAGAGACGTGGTA
AP33R ACGTATGCCACAAACACCTGCCAA

34- BI958584
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CCTACTCGGCATGGGGAGCGCGCTGGAGACGCTGTGCGGGCAGGCGTTCGG
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CTCATAGCCGCCGCCATCCTCATGGTGCCCTTCTACGTCTTCGCCGAGCCGC
TGCTGCTCGCCCTCGGCCAGGACGCCACCGTCGCGCGCGAGGCCGCCCGCT
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GCCAAGTTCCTCCAGGCGCAGAGCAGGGTCCTCGTGCTCGCCCTGGATCGG
CGT CGGCGG

AP34F GGATGGTGTGGGAGGAGTCCAA
AP34R AAGTTGACGCGAAAGGAGAAGGCG

35- CB882565
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GCCTCTTGGTCATAGATGCTTCATCCCAGATTATTAGCGATGCTGCTTTGAG
TAGCTTGGCTGTCCCACTCTGTTTTGTGAATCCACAAATATCCCCATCGTCTA
TTTTGAGTGGAATCTTGAACCTTGAGTGTGCGGTCCTCCCTCCGGGCATTAT
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GCGTATTGTGGCCAAGAGCGCCCTATAAAGAAATGTTTTTCCCGTGCCTCCA
GGTCCATCAACAAAGAATATTCCTC
CTCTTTGGTGGTCAATTGCTGCTAGGATTTCGTCGTAGGCCTCCCTCTGCTCG
TTGTTTAGGTTCTTATGCAAAGATGTGTCCTCATAGTCCACCTCGATGGAGG
TCTCCTCCATGATCTCCCTTGGTATGTCATCTGTCGTCTCGTGTTGTTGGTCT
ATCTCCGGAAGAGGATACGATTCTATGTC

AP35F TCCACCGTTTGCCTCTTGGTCATA
AP35R CCAAGGGAGATCATGGAGGAGACC
39- BI946613
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AAAGATGAGTCGGGGGGAAATTAATAAAAATAGATCGTGGGTATTCGCTCA
CAAGTATAGAAAGAACGCCGCATGTCCGGAGTGAGTATATATCTTTCACTC
GTGTGAACTTAAGTGAACACTGCGTGTCAGTAGCG

AP36F AGCTGTTCTGCTTCCTCCAAGGAT
AP36R ATATACTCACTCCGGACATGCGGC

37- BU988568
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GGTCGCAACCTCCAACATGGTGGCCACCTCCTTAGCTAACAAGGATGAAGA
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TAGTCTAGGCATGAAAGATTCTTGGGGTCCTCTGAAGGCATTGGCAGCAGC
TAGTGTTATAAATGCTGTTGGCGATATATTTCTTTGCTCTGTATGTGGCTATG
GAATTGCTGGT

AP37F AGGCCGTGTTCTGCGACTATCT
AP37R TAACACTAGCTGCTGCCAATGCCT

38- BQ460607
CGGCACGAGGATCCTTAAAAGACCAAAGATTTCCGGTGCACAGCTTCTTGC
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AGCCTTGGGAAGGTTGCAGTGTCATTCACACATACAATCAAGGCCATGGAG
CCTTTCTTCTCAGTTCTACTTTCTGCAATGTTCCTTGGCGAGCTGCCTACTCC
TTGGGTTGTTTTGTCTCTTCTTCCTATTGTTGGTGGTGTGGCATTGGCATCTA
TTTCTGAAGCTTCTTTTAAC

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TACCAACAGGTGTCATACATGATCCTCGCAAGGGTGTCACCAGTTACCCACT
CGGTAGGTAACTGTGTCAAG

AP38F CCAAAGATTTCCGGTGCACAGCTT
AP38R TGACACCCTTGCGAGGATCATGT

39- BQ464301
AATCACCACCGGCTTCTTCTTCTTCATGTGGTACTTCCTGAACGTGATATTCA
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CGGTGGCACTGTGCCATGCTCTTGGTCATGTCACAAGCAACGTGTCCTTTGC
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GCACTTATTATTGAAGGACCACAACTAATGCAGTATGGATTAAACGATGCA
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TGTTCTACCATCTCTATAATCAG

AP39F TCACCACCGGCTTCTTCTTCTTCA
AP39R AGGGATGCCATTGAAACACCAAGC

40- BI777343
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GGGCATCCTGATCCAGACCACGTCGCTCATCTACATCTTCCCTTCGTCGCTC
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GGCGCCTTGGCCTCCGCGTTCGCGTTCCTGGTGCGGGACGTGTGGGCCAGCA
TGTTCACGGCCGACCCGGCGATCATCGCGC
TCACCGCGTCGGTGCTGCCGATCCTGGGCCTG

AP40F CAGCTCATCACCCTGGCCCT
AP40R GACGCGGTGAGCGCGATGAT

41- DN186269
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CATGTTGCTGCCACAGTGAGCATGTCAAAGGTGGCAGTGTCATTCACACAC
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GGTTGTGGTCTAGCTGCTGCGACAGAGCTGAACTTTAATATGATCGGATTTA
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TTCAATTATGTCACTGGTCATACTCGCACCATTTGCTATTGCTATGGAAGGC
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AGGTGTCCTACATGTCTTTGGACCAGATTTCTCCATTGACGTTTAGCATTGG
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ACACCTGTCCGTCCTGTAAATGCACT

AP41F TCCGGTTGCTGTGGCTCATACAAT
AP41R AGTGCATTTACAGGACGGACAGGT

42- AI2
ACTCGTTCACCCATTTGTGAACAAAGCGGTGCTTACACCACACGATCGCTCT
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GTCTCTATGGCACATGCCGATGGAACCGTCCGAATATGGGACTGCGGACAC
GACGATGAAATCGAAAATGGCGACGTAGTGCAAGTTGATCTTGCCCGCGCC
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TCATGCGAGATGTCTATTGGACTGAAGACCGGAGAGCTTGCCGTCTTCCGCT
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GTCGGATGTCAATAAGGTTCCTAAGCCGTGCAATTTCCTCAAAATTGGGGGC
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AI2F AACAAAGCGGTGCTTACACCACAC
AI2R TCCGACATGTGGGCAGTGTGTATT

43- AI1

ACTCGTTCACCCATTTGTGAACAAAGCGGTGCTTACACCAGTCGATCGCTCT
GCCTGGCTCGGTCTCAAAGAGCGCCGATTGCAAGGTCCCAAATTTGTGGTG
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GCTTAAAAAATGCTCACCCAGAATGGGCTACTCAGATTGAATT TGGG
AI1F TGCAAGGTCCCAAATTTGTGGTGG
AI1R AGTAGCCCATTCTGGGTGAGCATT

APPENDIX C

PRIMER PAIRS USED IN DIFFERENTIAL DISPLAY PCR

P1: 5`-ATTAACCCTCACTAAATGCTGGGGA-3`

P2: 5`-ATTAACCCTCACTAAATCGGTCATAG-3`

P3: 5`-ATTAACCCTCACTAAATGCTGGTGG-3`

P4: 5`-ATTAACCCTCACTAAATGCTGGTAG-3`

P5: 5`-ATTAACCCTCACTAAAGATCTGACTG-3`

P6: 5`-ATTAACCCTCACTAAATGCTGGGTG-3`

P7: 5`-ATTAACCCTCACTAAATGCTGTATG-3`

P8: 5`-ATTAACCCTCACTAAATGGAGCTGG-3`

P9: 5`-ATTAACCCTCACTAAATGTGGCAGG-3`

P10: 5`-ATTAACCCTCACTAAAGCACCGTCC-3`

T1: 5`-CATTATGCTGAGTGATATCTTTTTTTTTAA-3`

T2: 5`-CATTATGCTGAGTGATATCTTTTTTTTTAC-3`

T3: 5`-CATTATGCTGAGTGATATCTTTTTTTTTAG-3`

T4: 5`-CATTATGCTGAGTGATATCTTTTTTTTTCA-3`

T5: 5`-CATTATGCTGAGTGATATCTTTTTTTTTCC-3`

T6: 5`-CATTATGCTGAGTGATATCTTTTTTTTTCG-3`

T7: 5`-CATTATGCTGAGTGATATCTTTTTTTTTGA-3`

T8: 5`-CATTATGCTGAGTGATATCTTTTTTTTTGC-3`

T9: 5`-CATTATGCTGAGTGATATCTTTTTTTTTGG-3`

SP6: 5`-CATTTAGGTGACACTATAG-3`

T7: 5`-TAATACGACTCACTATAGGG-3`

APPENDIX D

COMPOSITION OF BUFFERS AND STOCK SOLUTIONS

1. Hoagland Solution, per liter
    - $Ca(NO_3)_2$ 7 ml
    - KNO_3 5 ml
    - KH_2PO_4 2 ml
    - $MgSO_4$ 2 ml
    - FeEDTA 1 ml
    - Trace elements 1 ml
    - dH_2O to 1 L

2. Boric Acid Solution, 10mM
    - 0.6 g Boric acid in 1L dH_2O

3. TRIzol, per liter
    - Phenol in saturated buffer 380 ml/liter
    - Guanidine thiocyanate 118.16 g
    - Ammonium thiocyanate 73.12 g
    - Sodium acetate, pH5 33.4 ml of 3M stock
    - Glycerol 50 ml
    - dH_2O to 1 L

4. 5x First-Strand Buffer (Clontech)
    - 250 mM Tris (pH 8.3)
    - 30mM $MgCl_2$
    - 375mM KCL

5. 10X Klen Taq PCR Reaction Buffer (Sigma)
    - 400mM Tricine-KOH
    - 150mM KOAc
    - 35mM $Mg(OAc)_2$

- 750 µg/ml Bovine serum albumin

6. 50x TAE Electrophoresis Buffer, per 1 L
   - 242 g Tris base
   - 37.2 g Na_2EDTA (H_20)
   - 57.1 ml Glacial acetic acid
   - dH_2O to 1 L

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

8. 10x PCR Buffer (Sigma)
   - 100mM Tris-HCL
   - 500mM KCl
   - 15mM $MgCl_2$
   - 0.01% Gelatin

9. 10mM dNTP
   - 10mM each dATP, dTTP, dCTP, dGTP

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

11. 100mM $CaCl_2$ Solution

    1M $CaCl_2$ solution was prepared by dissolving 11.1 g $CaCl_2$ in 100 ml ultra pure H_2O. Filter sterilized solution was divided to 10 ml aliquots and stores at -20^0C. Before use, one aliquot was taken and let to be thawn. By adding 90 ml of ultra pure H_2O freshly prepared 100mM 100ml $CaCl_2$ was obtained.

12. 2x Rapid Ligation Buffer (Promega)
    - 60mM Tris-HCL (pH 7.8)

- 20mM $MgCl_2$
- 20mM DTT
- 2mM ATP
- 10% polyethylene glycol

13. SOC Medium, per 100 ml
    - 2 g Tryptone
    - 0.5 g Yeast Extract
    - 1 ml 1M NaCL
    - 0.25 ml 1M KCL
    - 1 ml 2M Mg^{2+} Stock
    - 1 ml 2m Glucose
    - dH_2O up to 100 ml