The Transcriptional Response of Barley (*Hordeum vulgare* L.) to Boron Toxicity

By

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ABSTRACT

The occurrence of Boron (B) toxicity in Australian soils is recognised as a limiting factor for cereal productivity. A number of loci conferring tolerance to B toxicity have been identified in barley and chromosomally mapped. However, a lack of knowledge relating to the physiological and molecular events that occur under B toxicity and the molecular basis for B stress tolerance has been a bottleneck in harnessing available genetic diversity in barley and wheat. The recent advances in functional genomics provided an opportunity to examine B stress in barley in more detail. The aim of this project was to analyse genes differentially expressed under B stress in tolerant and intolerant barley to identify candidate genes involved in B toxicity tolerance. Two experimental approaches, Suppression Subtractive Hybridization (SSH) and microarray were adopted.

Firstly, SSH was performed to examine gene expression in roots of selected tolerant and intolerant doubled haploid lines from a Clipper (B intolerant) X Sahara 3771 (B tolerant) mapping population, grown under moderate B stress. The SSH experiment aimed to investigate the early transcriptional response of B tolerant barley lines to B stress in order to identify the basis for B toxicity tolerance in roots.

Differential screening of the subtracted library generated from B treated plants identified a total of 111 non-redundant clones up-regulated in bulked tolerant lines. On the other hand 94 clones were differentially expressed under non-treated conditions. Among the clones identified from subtracted library generated from B treated plants, metabolism was the largest functional category, representing 21% of the clones. The largest functional category in the subtracted library generated from non treated plants was cellular transport, representing 19% of the clones. Based on sequence similarity, about 170 transcripts identified in this experiment were assigned to chromosomal segments (bins) on the three homoeologous genomes of bread wheat. In total, 36 clones from the subtracted library generated from B treated plants were analysed as candidates. Nine were genetically mapped within the region of B tolerance QTL on three chromosomes (2H, 4H and 6H). The genes mapped to 4H and 6H QTL have the highest association with these loci in the Clipper X Sahara 3771 doubled haploid mapping population. A 4H B tolerance QTL candidate gene was identified as a B transporter gene with similarity to the *Arabidopsis BOR1* gene. Genes identified to be differentially expressed in the tolerant lines from SSH suggest activation of a diverse defence response in the roots of barley plants under B stress. Data from SSH experiment indicate that cell wall-plasma membrane-

cytoskeleton continuum constitute the first action site against B toxicity and the influence of toxic B on K^+ uptake could be the key initiating factor.

In the second approach, the Affymetrix 22K Barley1 GeneChip™ was used to investigate B stress adaptation processes in barley. Gene expression was profiled in leaves of Sahara 3771 and Clipper plants grown under various B concentrations. The results show that the two genotypes respond differently to B toxicity. The B intolerance of Clipper is expressed through the induction of a high number of probe sets (2310) even at a low B concentration of 100 µM. In contrast, Sahara 3771 responded to a high B concentration (2000 µM) through the induction of only a few hundred (266) probe sets. In Sahara 3771 no change in the expression level of any probe sets was observed at 100 µM B. Altogether 286 probe sets showed differential expression in Sahara 3771 under three levels of B treatment (500, 1000 and 2000 µM). About 30% of these were down-regulated and about 70% were up-regulated in Sahara 3771 in response to B treatment. Most of the probe sets (59%) up-regulated in Sahara 3771 did not respond to B treatment in Clipper. These genes are either salt stress responsive or related to plant defense and thus could play a key role in protecting barley plants from the toxic effects of B.

Two differentially expressed probe sets annotated as B transporters were identified between Sahara 3771 and Clipper under control condition. These two B transporter probe sets did not respond to B treatment but showed opposing expression patterns in the two varieties. One of these probe sets (Contig21126_at) is similar to the B transporter gene isolated from the SSH experiment that maps to the 4H tolerance locus. The map location and expression of this B transporter gene suggest that it could be the borate anion efflux transporter predicted by the proposed efflux model of B tolerance in Sahara 3771 barley. The other B transporter gene (Contig14139_at) showed over expression in Clipper under control condition and could be contributing to high B accumulation in Clipper which needs further investigation.

Data from both experiments have indicated that B toxicity triggers oxidative stress and that jasmonate-based signaling plays a key role in B toxicity tolerance. SSH data indicate that Sahara 3771 which evolved in the harsh environment of Africa is more efficient in osmoregulation and ROS scavenging than Clipper. This trait is likely to give Sahara 3771 an edge over Clipper in tolerating toxic the effect of B. In addition to the efflux mechanism, which becomes less efficient with increasing B supply, Sahara 3771 appears to apply a number of other mechanisms for alleviating or withstanding toxic B induced stress to sustain growth. Some of these mechanisms are already known to be used by plants to cope with a number of stresses.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in University Library, being available for loan and photocopying.

Mahmood Hassan

December 2007

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List of Abbreviations

$(B[OH]_3)$ or H_3BO_3 = boric acid

(O_2^-) =superoxide

μF =microfarrad

μg = microgram

μM = micromole

12-OPDA = 12-oxo-phytodienoic acid

13S-HPOT = 13(S)-hydroperoxy-9(Z),ll(E),15(Z)- octadecatrienoic acid; a-ketol, 12-oxo-13-hydroxy-9(Z),15(Z)-octadecadienoic acid

4CL = 4-coumarate:CoA ligase

A9C = anthracene-9-carboxylic acid

ABC = ATP binding cassette

ACPFG = Australian Centre for Plant Functional Genomics

ADP = adenosine diphosphate

AE = anion exchanger

AGRF = Australian Genome Research Facility

AOC = allene oxide cyclase

AOS = allene oxide synthase

AQP9 = animal aquaporin9

ATP = adenosine 5'-triphosphate

B = boron

$B[OH]^-_4$ = borate

BAC = bacterial artificial chromosome

BADH = betaine aldehyde dehydrogenase

BLAST = Basic Local Alignment Search Tool

BOR1= boron transporter 1

Br^- = bromine ion

BSA = Bovine Serum Albumin

bZIP = Basic Leucine Zipper

C X S = Clipper X Sahara

C4H = cinnamate 4-hydroxylase

CA II = carbonic anhydrase II

Ca^{2+} = calcium ion

CaMV = Cauliflower Mosaic Virus

cDNA = complementary DNA

CDS = coding sequence

CeSA 1= cellulose synthase A catalytic subunit 1

CeSA 3= cellulose synthase A catalytic subunit 3

CEV1= constitutive expression of VSP1 protein 1

CHCA = α-cyano-4-hydroxycinnamic acid

CHS = chalcone synthase

CIMMIT = International Maize and Wheat Improvement Center (Centro Internacional de Mejoramiento del Maíz y del Trigo)

Cl^- = chlorine ion

cM = centimorgan

cm = centimeter

CPRF2 = light-inducible protein CPRF-2

cRNA = complementary RNA

CS = Chinese Spring

Cyt = cytochrome

dATP = 2-deoxyadenosine 5’-triphophate

dCTP =2-deoxycytidine 5’-triphophate

DEPC =diethylpolycarbonate

dGTP = 2-deoxyguanosine5’-triphosphate

DH = doubled haploid

DHA = dehydroascorbate

DI = deionized

DIDS = 4,4'-di-isothiocyanostilbene-2,2'-disulfonic acid

DPC = diphenylamine-2carboxilic acid

dTTP = 2-deoxythymidine 5'-triphophate

dw= dry weight

ECM = extra-cellular matrix

EDTA = ethylendiaminetetracacetic acid

EST = expressed sequence tag

FDR = false discovery rate

fw = fresh weight

GB = glycine betaine

GDH = glutamate dehydrogenase

GLP = germin-like protein

GONST = golgi nucleotide sugar transporter

GPI= glucosylphospatidyl-inositol

GPX = glutathione peroxidise

GRP94 =94-kDa glucose related protein

GSH = reduced glutathione

GST = glutathione S transferase

H^+ = hydrogen ion/ proton

H_2O_2 = hydrogen peroxide

HAC1 = histone acetyltransferase HAC1

HAK = high-affinity K^+ uptake systems

HATS = high-affinity nitrate transport system

HCO_3^- = bicarbonate ion

$HgCl_2$ = mercuric chloride

HIF = heterogeneous inbred families

HsBTR1= *Homo sapiens* bicarbonate transporter-related protein 1

HSP = heat shock protein

HVGI = TIGR Barley gene index

I^- = iodine ion

ICARDA = International Center for Agricultural Research in the Dry Areas

ICP-MS = Inductively Coupled Plasma Mass Spectrometry

ITB = intolerant boron

ITC = intolerant control

JA = jasmonic acid

JAFA = Joined Assembly of Function Annotations

kDa = kiloDalton

kg = kilogram

K_m = Michaelis-Menten kinetics coefficient

LB = Luria broth

LIMMA = Linear Models for Microarray Data Package

LOD = Log of the Odds

LOX = lipoxygenase

LRS = Likelihood Ratio Statistics

M = mole

MAPK = mitogen-activated protein kinase

MAPKK = mitogen-activated protein kinase kinase

MAPKKK = mitogen-activated protein kinase kinase kinase

MDHA = monodehydroascorbate

mg = milligram

$MgCl_2$ = magnesium chloride

MIPS = Munich Information Center for Protein Sequences

MIPs = major intrinsic proteins

mM = millimole

MOPS = 3-(N-morpholino)propanesulfonic acid

mRNA= messenger ribonucleic acid

MRP = multidrug resistance-associated protein

N = nitrogen

Na^+ = sodium ion

Na_2CO_3 = sodium carbonate

NaBC1 = sodium borate cotransporter 1

NaCl = sodium chloride

NaCl = sodium chloride

NAD = nicotinamide adenine dinucleotide

NADH = reduced form of nicotinamide adenine dinucleotide

NADP = nicotinamide adenine dinucleotide phosphate

NADPH = reduced form of nicotinamide adenine dinucleotide phosphate

NADP-ME = NADP-malic enzyme

$NaHCO_3$ = sodium bicarbonate

NCBI = National Center for Biotechnology Information

NDH = NADH-quinone oxidoreductase

NdhK = NADH-plastoquinone oxidoreductase subunit K

ng = nanogram

NHX = sodium/hydrogen exchanger

NIL = near isogenic lines

NIP = nodulin 26 like intrinsic protein

NO_3 = nitrate

nr = non-redundant

NRT = nitrate transporter

O_2 = Oxygen molecule

OH^- = hydroxyl ion

P = probability

P^{32} = phosphorus-32

PAL = phenylalanine ammonia-lyase

PCR = polymerase chain reaction

PEG = poly ethylene glycol

Pf_b = lipid permeability of boric acid

PIP = plasma membrane intrinsic protein

PIPES = piperazine-1-4-bis[2-ethanesulfonic acid]

PO_4^{3-} = phosphate ion

ppm = parts per million

PRR73 = pseudo-response regulator 73

PS = photosystem

PS1-A = photosystem I P700 apoprotein A1

qPCR = quantitative polymerase chain reaction

QTL = quantitative trait loci

RAB1C= Ras-related protein Rab-35

RAV2 = regulator of V-ATPase in vacuolar membrane protein 2

RFLP = restriction fragment length polymorphism

RG-II = rhamnogalacturonan- II

RNA = ribonucleic acid

ROS = reactive oxygen species

rpm = revolutions per minute

rRNA = ribosomal RNA

RT = room temperature

SAMDC = *S*- adenosylmethionine decarboxylase

SARDI = South Australian Research and Development Institute

SDS = sodium dodecyl sulfate

SFP = single feature polymorphism

SIP = small basic intrinsic protein

SLC4 = solute carrier family 4

SLC4A11= sodium bicarbonate transporter-like protein 11

SNP = single nucleotide polymorphism

SO_4^{2-} = sulfate ion

SOB = super-optimal broth

SOD = superoxide dismutase

SOS = salt overly sensitive

SSC = sodium chloride/ sodium citrate

SSH = Suppression Subtractive Hybridization

t = metric ton

TAE = Tris/acetate/EDTA

TB = tolerant boron

TC = tolerant control

T-DNA = transferred DNA

TIGR = The Institute for Genomic Research

TIP = tonoplast intrinsic protein

TM = trans-membrane

TPX= thiol peroxidase

Ub = ubiquitin

UDP = uridine diphosphoglucose

USPA =universal stress protein A

UTR = untranslated region

UV = ultra violet

V = voltage

V-ATPase = vacuolar type H^+-ATPase

VDAC = voltage-dependent anion-selective channel protein

VSP = vegetative storage protein

W/V = weight/volume

YNL275w = nonglycosylated anion transport protein from yeast

CHAPTER 1

LITERATURE REVIEW

1.1. Barley and mineral stress

1.1.1 The barley crop

Domesticated about 10,000 years ago in the Fertile Crescent (Zohary and Hopf, 1993) barley (*Hordeum vulgare* L.) is one of the eight so-called founder crops of agriculture. With a world production of 138 million t in 2005, barley is the fourth most important cereal crop after maize, wheat and rice Woldeab *et al.* (2007). Genetic variation in barley has made it possible to be cultivated in diverse environments ranging from the sub-Arctic to the sub-tropic. Barley is mostly produced in sub-humid to semi-arid regions which are not considered suitable for growing other major cereals. Because of its high protein content barley is mainly used as animal feed. It is also used for malt production which is used for brewing alcoholic beverages. Barley is also a popular staple being used in soups as a source of plant protein. Other uses include in the bakery and confectionary industries.

In Australia, barley is the second largest grain crop after wheat. Average Australian barley production for the last five years was 7.14 million t (worth 1.4 billion AUD), accounting for 3% of world output. Australia's average export was nearly 4.9 million t in the last five years, comprising 18% of the world barley trade and worth about 1.2 billion AUD. Averaged over the last five years South Australian production was 2.2 million t.

Other than its agronomic and economic importance, barley, having wide diversity in physiology, morphology and genetics (Forster *et al.*, 2000) is considered as a classic model species for genetic and physiological studies (Koornneef *et al.*, 1997). Barley is a diploid organism with only seven pairs of chromosomes representing the least complex *Triticeae* genome. Over the last decade a wealth of genetic and genomics resources for barley has been developed. These include 400,000 ESTs, a barley Affymetrix chip, a transcript map representing more than 1000 genes and BAC clones containing 30,000 genes. The barley genome shares synteny with other *Triticeae* species such as wheat, rye and ryegrass (Linde-Laursen *et al.*, 1997; Ellis *et al.*, 1997). Wheat-barley substitution lines present strong evidence of the syntenic relationship between wheat and barley (Islam and Shepherd, 1990). The wheat barley addition lines display the genetic equivalence of wheat and barley chromosomes, where a pair of barley chromosome is substituted for a particular pair of wheat chromosome. Barley also shares a large number of agronomic traits with *Triticeae* species such as plant height, resistance

to shattering, grain yield, winter hardiness, drought tolerance, disease and insect resistance, straw strength, etc.

1.1.2 *Hordeum* species and their relationship with wheat

According to modern taxonomy, barley (*Hordeum vulgare*) includes three subspecies: *H. vulgare* L., *H. spontaneum* C. Koch, and *H. agriocrithon* Åberg (Badr *et al*., 2000). Subspecies *H. spontaneum* is morphologically similar to cultivated *H. vulgare* and considered to be its progenitor. Compared with cultivated *H. vulgare*, *H. spontaneum* has narrower leaves, slightly smaller seeds, longer stems and longer and thinner rachis (Badr *et al*., 2000). A number of other *Hordeum* species are still found in their primary habitats of the Fertile Crescent.

Barley and wheat originated from a common ancestor of the *Triticeae* species diverging from each other 12 million years ago (Li and Gill, 2002). The modern cultivated barley is a selfing diploid with seven chromosome pairs and is very closely related to wheat. The close relationship between barley and wheat is demonstrated by the fact that barley could be successfully hybridized with wheat at all three ploidy levels. Islam *et al*. (1981) developed six euplasmic wheat-barley addition lines from barley (*Hordeum vulgare, cv* Betzes) and wheat (*cv* Chinese Spring). These disomic addition lines contain barley chromosomes 1, 2, 3, 4, 6, or 7 in addition to the full complement of wheat chromosomes in a wheat cytoplasmic background. The disomic form of addition line carrying chromosome 5 of barley is self-sterile (Islam *et al*., 1981). The predicted homoeologous relationship of wheat and barley chromosomes was proposed by Islam and Shepherd (1981) on the basis of the morphological similarities of the wheat–barley addition lines and wheat tetrasomics and the presence of similar isoenzymes.

1.1.3. Mineral stress

Abiotic stresses are now known to cause more productivity losses than any other factor. It is estimated that about a quarter of the world's soils are currently suffering from some form of mineral stress (Clark, 1982), the majority of these soil ailments are not easily correctable by conventional soil amelioration practices such as liming. Crop yields suffer heavily under these conditions. Toxic soil with excess aluminium, manganese, boron (B) and copper imposes the most agriculturally important mineral stresses for crops.

The possibility of breeding varieties adapted to mineral stressed soil was first suggested by Gregory and Growther (1928) following Moers's (1922) observation of genotypic difference of crop plant's response to soil fertility status. Recently, advances in genetics and an understanding of the molecular basis of stress responses have led to the identification of a large number of

single loci, quantitative trait loci (QTL) and genes related to stress tolerance. These recent developments suggest the existence of common tolerance mechanisms for different stresses, or that the different stress tolerance processes are controlled by clusters of genes (Cattivelli *et al.*, 2002). This review will concentrate only on B toxicity stress particularly in barley and wheat.

1.2. B toxicity and tolerance

1.2.1. Natural toxic occurrence of B

Although B has a low natural abundance (Reeves, 1974), it is reported to be widely distributed in both the lithosphere and hydrosphere (Morgan, 1980). Most soils, except those associated with recent volcanism usually have a low B content (Power and Woods, 1997). Occurrence of high concentrations of B in soil has been reported in the arid and semi arid areas (Leyhon and Wu, 1993) and is frequently associated with saline soils and high B ground water for irrigation (Gupta, 1979). In saline soils, excessive concentrations of B in the soil solution are often the result of lack of sufficient drainage (Goldberg, 1997). Boron is very mobile and is readily leached. As B is prone to adsorption to clay particles (Goldberg, 1997) accumulation of B in the subsoil takes place following leaching (Walsh and Golden, 1953; Kubota *et al.*, 1948). Boron toxicity has been recognised as an important problem that limits crop productivity in the low rainfall areas of southern Australia (Cartwright *et al.*, 1984), West Asia and North Africa including Iraq, Jordan, Syria, Turkey, Egypt, Morocco and Libya (Sillanpaa, 1982; ICARDA Annual Report 1993), Indian sub-continent, China, the Philippines, Japan and parts of South America (Campbell *et al.*, 1998). Paull *et al.* (1991) reported that imbalances in B nutrition are widespread as the range between B deficiency and toxicity is rather narrow (Eaton, 1944; Goldberg, 1997).

In Australia, toxic levels of B in soil have been reported for cereal crops grown under semi-arid (250-450mm p.a.) rain-fed conditions (Cartwright *et al.*, 1984, 1986, 1987) and have most frequently been associated with soils derived from marine parent materials (Norrish, 1975).

In the subsoils of extensive areas of southern Australia (Cartwright, *et al.*, 1986) B occurs in concentrations sufficient to cause plant toxicity. The soil B concentration has been demonstrated to increase with depth (Cartwright, *et al.*, 1984, 1987), thus, amelioration through soil modification has been precluded (Jefferies *et al.*, 2000). Keren and Bingham (1985) observed that reclamation of high B soils would require about three times more water as reclamation of saline soils.

1.2.2. B toxicity

B toxicity to crop plants was recognized as early as the 1930s by Christensen (1934). Cartwright *et al*. (1984) reported significant yield reduction in barley due to high B concentration in soil. B tolerant wheat lines have been reported to demonstrate an average of 3% yield advantage over B sensitive lines across South Australia and up to 11% yield advantage at high B sites (Moody *et al*., 1993). Similarly a 17% yield penalty has been attributed to B toxicity in barley in South Australia (Cartwright *et al*., 1984). Tolerance to B is therefore of major economic importance in South Australia (Paull *et al*., 1995) and selection for tolerance to B toxicity has become a priority of the South Australian breeding programmes (Paull *et al*., 1992). A clear explanation of the primary causes of B toxicity is not available yet (Nuttal, 2000, Ruiz *et al*., 2003) except the demonstrated effect of high foliar application of B in increasing reactive oxygen species (ROS) in tobacco plants by Garcia *et al*. (2001). It is now known that ROS are the key molecules that elicit hypersensitive cell (death) response (Hirasawa *et al*., 2005).

Crop plants are reported to suffer toxic stress when the level of hot water soluble B in the soil exceeds 5.0 mg kg^{-1} (Mengel and Kirkby, 1987). However, a wide range of values for the critical tissue B concentration have been reported, above which toxicity symptoms appear (Furlani *et al*., 2003). Leaf B concentrations of susceptible and tolerant species have been found to vary as much as ten times (Gupta, 1993). Crop species and cultivars have varying ranges at which B is considered adequate. These ranges lie in the narrow differences between the critical values for B deficiency and B toxicity. B concentrations also vary widely between different plant parts (leaf, root, shoot or whole plant) and at different growth stages. In barley and wheat, critical values have been reported to range between 10 and 130 mg B kg^{-1} dry weight (Gupta, 1977; Kluge and Podlesak, 1985; Paull *et al*., 1988a; Riley *et al*., 1994). Ayars *et al*. (1990, 1994) reported that the critical mean leaf B concentration for wheat were 701 mg B kg^{-1} dry weight. Table 1 shows critical tissue B concentrations for B toxicity according to Marschner, 1995.

Table 1.1. Critical tissue B concentrations for B toxicity (Marschner, 1995)**.**

Plant / crop	Critical toxicity (mg B kg^{-1}dw)
Wheat	100-270
Maize	100
Snap-bean	100
Cowpea	>330
Cucumber	400
Squash	1000

1.2.3. Symptoms of B toxicity

The typical symptoms of B toxicity in a wide range of plant species including barley and wheat is leaf burn, characterized by chlorotic and/or necrotic patches of the margins and tips of older leaves (Bennett, 1993; Bergmann, 1992; Eaton, 1944). Nable *et al.* (1997) suggested that these symptoms represent the distribution of B in most species, which indicates that B accumulates at the end of the transpiration stream. Oertli and Roth (1969) reported that the chlorotic / necrotic patches have been found to have much higher B concentrations compared to the surrounding leaf tissues. Loomis and Durst (1992) observed that soluble B concentration in plants may possibly play a major role in the occurrence of B toxicity symptoms. Only a poor correlation is reported to exist in general between the expressions of B toxicity symptoms in leaves and overall shoot B concentrations (Jefferies *et al.*, 2000). For example, there are reports that more often increased B toxicity symptoms occurred while total shoot B concentrations are not affected or even reduced such as in wheat (Bingham *et al.*, 1987); chick pea (Yadav *et al.*, 1989) and also in some trees. Nable *et al.* (1997) observed that in species lacking phloem mobility of B, leaf burn is predominantly the symptom of B toxicity. For example, wheat, in which B is not known to be phloem mobile. On the other hand, symptoms of B toxicity in plant species in which B is phloem mobile include fruit disorder, bark necrosis and stem die back (Brown and Hu, 1996). In contrast to this generally held view, recent research (Bellaloui, 2003) has shown B to be phloem mobile in rice, which also manifests similar foliar symptoms under B toxicity as barley.

Both salinity (Wimmer *et al.*, 2003) and drought have been reported to worsen B toxicity symptoms in wheat. Salinity is also associated with a further increase in shoot B concentration in wheat and a variety of other species (Wimmer *et al.*, 2003; Grieve and Poss, 2000).

1.2.4. Mechanism of tolerance to B toxicity

The mechanisms associated with tolerance of high B concentrations are not well understood (McDonald *et al.*, 2003, Ruiz *et al.*, 2003). Jenkin (1993) and Nable *et al.* (1997) pointed out that B toxicity tolerance mechanisms operate at the organ and cellular level. Huang and Graham (1990) showed that in excised wheat root culture, genotypes classified as susceptible from field and glasshouse experiments produced shorter root axes and less lateral roots than tolerant genotypes in high B containing culture media. They also reported that root explants of the B toxicity susceptible varieties produced less callus compared to that of the tolerant genotypes.

There are indications from recent research that B toxicity tolerance in barley is associated with increased root elongation along with a proportional increase in cell density within the zone of

cell division at the root tip (Choi, 2004). The concentration of reducing sugars has also been found to increase in the root tips of tolerant genotypes (Choi, 2004). This may indicate that roots are able to utilize more sucrose and therefore continue to grow even under high soil B. Another B tolerance mechanism is the redistribution of root biomass to the top-soil enabling the plants to avoid coming into contact with B toxicity in the subsoil (McDonald, 2003).

A survey of literature on B toxicity tolerance reveals that over the last several decades a large body of investigation has been carried out on different aspects of B in relation to plants. These include:

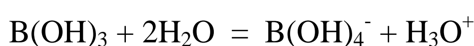
i. B chemistry and B complexes
ii. Functional role of B in plants
iii. B uptake and translocation within the plants
iv. B re-translocation within the plants
v. Carbohydrate metabolism under B toxicity
vi. Protein profile changes under B toxicity.
vii. Symptoms of B toxicity
viii. Accumulation and partitioning of B in different plant parts at stages of growth under toxic conditions
ix. B efficiency of crop plants/cultivars under both deficient and toxic conditions
x. Genetics of tolerance to B toxicity

In the following sections these area are reviewed.

1.3. B chemistry and B complexes

B is the first member of the metalloid or semiconductor family of elements, which includes silicon and geranium, and is the only non-metal of group III elements and thus has properties between metals and electronegative non-metals. Like carbon, B has a tendency to form double bonds and macromolecules. It also tends to form anionic rather than cationic complexes. Since B has high ionisation potentials, B chemistry is of covalent B compounds and not of B^{3+} ions (Keren, 2003).

B is exclusively found as borates in nature (Power and Woods, 1997), which are formed spontaneously (Keren, 2003). Whereas in plants at apoplastic pH of 5.5, more than 99.95% B exists as boric acid ($B[OH]_3$) and the rest in the form of ($B[OH]_4^-$) (Power and Woods, 1997; Brown *et al.*, 2002). In accordance with the electron configuration of B, boric acid acts as a weak Lewis acid that accepts a hydroxyl (OH^-) ion leaving an excess of protons:



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

Since B forms complexes with organic compounds containing hydroxyl groups, it is capable of interacting with a wide range of biological molecules including sugars and polysaccharides, adenosine-5-phosphate, pyridoxine, riboflavin, dehydro-ascorbic acid and pyridine nucleotide (Zittle, 1951). B forms esters and complexes with different hydroxyl groups of cell wall carbohydrates (Loomis and Durst, 1992, Woods 1996) and also with glycoproteins (Loomis and Durst, 1992). The stability of these complexes depends on several factors, especially on pH (Friedman *et al*., 1974) and they are found to be more stable in *cis*-diols than in *trans*-diols (Boesken, 1949). Mazurek and Perlin (1963) demonstrated that borate esters formed with *cis*–diols on a furanoid ring generally are the most stable ones. These structures are rare in nature (Power and Woods, 1997). Only ribose and apiose have this configuration among the biological compounds found in plants (Loomis and Durst, 1992). Apiose is found in the rhamnogalacturonan-II (RG-II) - a complex polysaccharide of the pectic fraction of primary cell walls. Apiose is found in a variety of monocotyledonous and dicotyledonous plant species including the grass family (Darvill *et al*., 1978; Carpita and Gibeut, 1993). About 30 glycosyl residues are also found in RG-II (Power and Woods, 1997). In fact, borate complexes with polyols and RG-II represent the vast majority of the biological compounds of B isolated from micro-organisms and plants. The functional significance of rhamnogalacturonan-II boron complex in plants has been described by Hu *et al*. (1997), Kobayashi *et al*. (1996) and O'Neill *et al*. (1996). The presence of Ca^{2+} greatly enhances the stability of rhamnogalacturonan-II boron complex (Kobayashi, *et al*., 1997). However, in graminaceous plants polysaccharides contain very little pectin (Darvill *et al*., 1980; Thomas *et al*., 1989), for instance, the complexable polysaccharides in rice cell walls contain only 0.1% RG-II (Thomas *et al*., 1989). Ribose present abundantly in ribonucleotides (such as co-enzyme NAD) is supposed to have a role in the chemistry of B toxicity (Loomis and Durst, 1992). Goldbach *et al*. (1991) hypothesised that excess borate may interfere with protein synthesis by esterifying the *cis*-diol of the ribose group of RNA, while Krueger *et al*. (1987) viewed this as the proposed essential role of B in protein synthesis. A strong binding affinity of B has also been reported for pyridine nucleotide coenzyme (NAD^+), ATP, and several sugars (Ralston and Hunt, 2000).

Power and Woods (1997) predicted that because of the ubiquitous presence of –OH groups in biological molecules, more B complexes could be found. It is also probable that other types of B complexation may be observed, particularly with nitrogen e.g. as with serine (Woods, 1996). Even small structural changes upon binding of B to these molecules could result in alteration of enzyme activities causing metabolic disruption (Wimmer *et al*., 2003). Insights into B complexation in cereals, especially wheat and barley, might provide fundamental understanding of B toxicity in these plants/crops.

1.4. Functional roles of B in plants

The presence of B in diverse plant species and its importance was reported in the first decade of the last century (Agluhon, 1910; Mazé, 1915, 1919). However, B was first definitively proved to be essential for higher plants by Warington (1923). A few years later Sommer and Lipman (1926) established the B requirement of six non-leguminous dicots and one graminaceous plant, barley.

The various postulated functions of B in higher plants are based on the observation and identification of a number of B deficiency symptoms. B is involved in:

i. Sugar transport and carbohydrate metabolism
ii. Cell wall synthesis and lignification
iii. Maintenance of membrane integrity and function
iv. Stimulation of nucleic acid metabolism
v. Indole acetic acid metabolism
vi. Role in the ascorbate/glutathione cycle
vii. Phenol metabolism
viii. Activation of enzymes
ix. In pollen tube formation
x. Nitrogen metabolism and N fixation
xi. In photosynthesis
xii. Amelioration of aluminium–induced root growth inhibition.

However, Cakmak and Römheld (1997) reported that there is no evidence that B forms a part of an enzyme or that it plays a direct role in enzyme activities. Brown *et al*. (2002) has discounted the likelihood that B regulates metabolism through the formation of an exchangeable complex. In a recent experiment Reid *et al*. (2004) conducted an *in vitro* analysis of enzyme reactions with B binding substrates. They concluded that, B at a concentration between 5-20 mM has the ability to disrupt metabolism by forming complexes with NAD^+.

The primary function of B has not yet been conclusively defined. Brown *et al*. (2002) hypothesized that the role of B involves the formation of B complexes of high stability either as structural components of cellular organs or critical molecules. Primary cell wall structure and membrane function are now closely linked to B nutrition (Blevins and Lukaszweski, 1998) and are discussed below.

1.4.1. Role in the cell wall

Under B deficiency, apical meristem growth in shoots and roots ceases (Brown *et al.*, 2002). B is also critical for the growth of pollen tubes (Schmucker, 1933). Leaves and petioles develop brittleness due to impaired cell wall development under B deficient conditions (Shorrocks, 1997; Loomis and Durst, 1992; Goldbach, 1997). Under B deficiency, loosely packed (Matoh, 1997), abnormally thick cell walls form (Kouchi and Kumazawa, 1976; Matoh *et al.*, 2000) with courser texture and sometimes deformed structure (Lee and Aronoff, 1966; Hu and Brown, 1994). Experimental evidence suggests that B influences the incorporation of proteins, pectins and/or precursors into the existing and growing cell wall (Torosell, 1956; Spurr, 1957). It has been experimentally shown that B predominantly complexes with the pectic fraction of the cell wall (Loomis and Durst, 1992; Yamauchi and Yamamoto, 1986; Hu and Brown, 1994). B cross-links two chains of monomeric RG-II through B containing complexes. The chains of monomeric RG-II actually form part of a long chain of pectic polymers (Thomas *et al.*, 1989). RG-II contains two apiosyl residues, and at this point B cross-links the two pectic chains (O'Neill *et al.*, 1996). It has been demonstrated that under B deficiency the formation of the cross-link is inhibited, which results in pore size larger than normal (Fleischer *et al.*, 1999). Dannel *et al.* (2002) also concluded that B complexes with RG-II to form a dimer and regulates the pore size of the cell wall, identifying this as the primary function of B. Yang and Li (1999) reported that cross sections of walls from B deficient plants show much less microfibrillar structure than those from plants grown under normal B supply. However, no compelling evidence has been reported to suggest that B is directly involved in the synthesis of cell wall material and no decrease in the production of pectic substances, or cell wall precursors is observed when plants are B deficient (Goldbach, 1997; Fleischer *et al.*, 1999; Hu *et al.*, 1996; Kouchi and Kumazawa, 1976).

1.4.2. Role in membrane structure and functions

Recent research has revealed that B also plays essential roles in organisms lacking cellulose or cell walls. This strongly suggests a biological role of B beyond its known function in cell wall structure (Läuchli, 2002; Blevins and Lukaszweski, 1998). Addition of B to B deficient tissue membranes has been reported to induce rapid changes in membrane function (Blevin and Lukaszweski, 1998). It has been found that B deficiency disrupts membrane transport processes, the activity of membrane localized proteins and the composition of the cell (Brown *et al.*, 2002). Adequate B supply to B deficient plants triggers a number of events including root cell membrane hyper-polarization (Schon *et al.*, 1990), stimulation of ATPase and NADH oxidase activity (Schon *et al.*, 1990; Barr and Crane, 1991; Barr *et al.*, 1993) and enhanced ion transport (Loughman and White, 1984) (reviewed by Blevins and Lukaszweski, 1998). Cakmak *et al.*

(1995) proposed that B stabilizes the structure of the plasma membrane by forming complexes with membrane constituents. They demonstrated leakage of potassium, sucrose, phenolics and amino acids in B deficient sunflower leaves. Polard *et al*. (1977) and Parr and Loughman (1983) suggested that B is likely to form complexes with glycoprotein or glycolipid components in the cell membrane or with sugars and may cause alterations in membrane transport activity. Thus, several researchers have proposed that B plays a structural role in the membrane, which may explain the large number of reported effects on membrane processes (Brown *et al*., 2002). Brown *et al*. (2002) hypothesized that B has a specific function in cellular membranes through its role in the formation and function of 'membrane rafts', which are physiologically active membrane domains with discrete function. 'Membrane rafts' are particularly prevalent in highly active and rapidly developing membrane system (Simons and Ikonen, 1997). 'Membrane rafts' are characterized by concentrations of glycolipids and glycoproteins and thus provide a significant number of B complexing sites. They also contain various B binding molecules (such as sugars like galactose, mannose and amino acids like serine, tyrosine). It is now thought that 'Membrane rafts' have a specific role in membrane signal transduction and serve as the sites for glucosylphospatidyl-inositol (GPI) protein association (Brown *et al*., 2002).

1.5. B uptake and translocation

About 10% of the total soil B content is usually present in soluble form and only this soluble B is available to plants (Power and Woods, 1997). Occurrence and toxicity of B in plants is profoundly influenced by the uptake of B from soil and the movement/translocation of B within the plants.

The subject of B uptake has been controversial for over 30 years. Scientists have put forward significant evidence to support both the active and passive uptake of B in higher plants (Brown and Shelp, 1997; Hu and Brown, 1997).

1.5.1. Passive B uptake

Until recently B uptake has been known to be a non-metabolic process, and the rate of uptake is governed by B concentration of the external solution, internal B complexation and plant water use.

Raven (1980) first postulated that B uptake would occur via passive diffusion based on a theoretically calculated value for lipid permeability of boric acid (Pf_b) at physiological pH ($8x10^{-6}cm\ s^{-1}$), which he believed adequate to satisfy the B requirement of plants. Recent direct measurement of membrane permeability of B (Pf_b) by Dordas and Brown (2000) and Dordas *et*

al. (2000) and Stangoulis *et al*. (2001) has supported Raven's assertion with some degree of variability. The variation observed in the Pf_b values from direct measurement was attributed to variation in membrane composition between species and crop varieties and was supported by experimentation conducted with mutant lines of *Arabidopsis thaliana* (Dordas and Brown, 2000). Brown *et al*. (2002) presented a theoretical estimation of the permeability coefficient of B in canola and tobacco based on the measured Pf_b for membrane of squash and made a similar conclusion to Raven (1980). Brown *et al*. (2002) also pointed out that passive permeation of B would be adequate to provide the observed B requirement for both canola and tobacco under normal conditions of B supply (10 µM B) but not at reduced B supply (1 µM B). Thus they did not preclude the role of membrane proteins in the facilitation of transmembrane movement of B. Recent experimentation by Dordas *et al*. (2000) and Nuttall (2000) provided evidence that channel proteins are involved in B uptake. This was further supported by the finding that the expression of one of the MIPs (major intrinsic proteins), namely PIP1 (plasma intrinsic protein I) with homology to non-electrolyte transporting channels from other species, in *Xenopus laevis* oocytes resulted in 30% increase in Pf_b. Nuttall (2000) verified the effectiveness of additional MIP's at increasing B transport. However, B movement through diffusion channel mediated transport could account for B uptake only when B supply is adequate (Brown *et al*., 2002).

One major shortcoming of the concept of passive uptake of B is that it cannot explain the observed differences in B uptake among plant species or cultivars. It has been found that intolerant varieties acquire seven times as much B as tolerant varieties even if they were grown under the same conditions (Nable, 1988). Such differences in B uptake cannot easily be explained through difference in water use efficiency alone. The water use efficiency in barley and wheat cultivars has not been found to vary as greatly as required to explain the above situation (Passioura, 1997).

1.5.2. Active carrier mediated B uptake

Dannel *et al*. (1997) and Pfeffer *et al*. (1999) after conducting a series of experiments suggested that when B supply is inadequate, B uptake is carried out through a metabolically active, carrier mediated transport process. After detailed characterisation of B uptake in sunflower, Dannel *et al*. (2000) suggested that B uptake occurs by a saturable (saturation with substrate) carrier mediated transport at low B concentration and non-saturable diffusion driven process at higher concentrations. Pfeffer *et al*. (1999) and Stangoulis *et al*. (2001) suggested that B uptake at low B supply follows Michaelis-Menten kinetics, where the initial reaction rate is dependent upon the concentration of the substrate. Brown *et al*. (2002) observed that the idea that an active B uptake process exists in plants should be considered cautiously. This is because the cytoplasmic

B concentration cannot yet be directly determined (Brown *et al*., 2002). The Michaelis-Menten kinetics coefficient (K_m) for the putative high affinity B transporter determined by Dannel *et al*., (2002) and Stangoulis *et al*., (2001), is also much higher than the minimal concentration of B at which plants can maintain their normal growth (Asad *et al*., 1997) and is significantly higher than the coefficient of other micronutrients required by plants in equivalent concentrations (Veltrup, 1978, Mullins and Sommers, 1986).

In the recent years experimental evidence has emerged in support of carrier mediated transport of B. An efflux type B transporter much similar to the animal bicarbonate transporters (SLC4) and aquaporins has been shown to be involved in xylem loading of B in *Arabidopsis* plants under deficient B supply (Takano *et al*., 2002; Takano *et al*., 2006).

1.5.3. The role of B transporters

The evidence that membrane bound transporters could be involved in B transport first emerged from an investigation into the sensitivity of the *Arabidopsis thaliana* mutant *bor1-1* to B deficiency by Noguchi *et al*. (1997). Later Takano *et al*. (2002) identified the *BOR1* gene using map-based cloning and showed that it shares similarity with solute carrier transporters (also known as bicarbonate transporters) in animals. They demonstrated that AtBOR1 is an efflux type B transporter by expressing it in yeast. They reported the existence of six more predicted proteins in *Arabidopsis* similar to BOR1. Recently, Fujiwara *et al*. (2006) reported that all of these genes code for proteins with B transport activities and at least some of them are required for plant growth under B deficient conditions. They also reported tissue specific expression of these genes (Fujiwara *et al*., 2006). The latest findings about *BOR1* gene products under elevated B conditions are discussed in the last part of this section.

Frommer and Wirén (2002) showed by phylogenetic analysis of the SLC4 (Solute carrier family 4) anion exchanger superfamily that BOR1 and yeast protein YNL275w are in the same clade with a human bicarbonate transporter related protein (HsBTR1), along with the other six predicted proteins of *A. thaliana*. The ten members of *SLC4* gene family (Romero *et al*., 2004) are classified into three main classes. They are (i) Na^+ independent Cl^-- HCO_3^- exchangers, (ii) electrogenic and electro-neutral Na^+ - HCO_3^- co-transporters, and (iii) Na^+ dependent Cl^--HCO_3^- exchangers.

Na^+ independent Cl^-- HCO_3^- exchangers include the electroneutral transporters AE1, AE2 and AE3. AE4 is also included in this family although such inclusion is not universally accepted, pending confirmation of its functionality (Romero *et al*., 2004). These membrane proteins

transport HCO_3^- across the plasma membrane. Human AE1 or Band 3 is a 911-amino-acid glycoprotein abundantly present in the plasma membrane of red blood cells (erythrocytes). Its function is to carry out the electro-neutral exchange of chloride and bicarbonate across the plasma membrane and to lower the cytosolic pH by effectively removing "excess" OH^- ions (Romero *et al.*, 2004). Exchange of cytosolic HCO_3^- for extracellular Cl^- is propelled by the import of Cl^- down its concentration gradient. These exchanges also regulate cell volume and membrane potential by controlling the transmembrane Cl^- gradient (Romero *et al.*, 2004; Alper, 2006). The 404 amino acid long cytosolic N-terminal domain binds to the red blood cell cytoskeleton and cytosolic proteins and acts as an anchor. The active function of transport is carried out by its C-terminal membrane domain, which is about 475 amino acids long (Zhu *et al.*, 2003). The membrane domain has been reported to consist of 12–14 trans-membrane (TM) segments (Reithmeier *et al.*, 1996) consistent with the other members of the SLC4 family (Romero *et al.*, 2004). Part of the remaining 40 amino acids of the cytoplasmic C terminal section is anchored in the soluble carbonic anhydrase II (CA II) enzyme (Vince and Reithmeier, 1998). Although the preferred substrates are HCO_3^- and Cl^-, the Band 3 protein has been reported to be able to bind and transport several other anions such as SO_4^{2-}, PO_4^{3-}, Br^- and I^- (Passow,1986). It has been claimed that Band 3 can also co-transport SO_4^{2-} and H^+ in exchange for Cl^- at a very low rate (Romero *et al.*, 2004). There are suggestions that Band 3 proteins do not function only as conventional anion transporters, but they can also act as flippase. In this case they translocate membrane-intercalated anionic amphiphiles that come towards the transporters from the lipid domain (Ortwein *et al.*, 1994). Ortwein *et al.* (1994) observed that the flippase mode of operation of Band 3 is quite a different mechanism from the conventional anion exchange.

SLC4A11 or HsBTR1 (also known as NaBC1) is a unique member of the SLC4 transporter family (Romero *et al.*, 2004) having only distant similarity to the other members (Pushkin and Kurtz, 2005). HsBTR1 is an electrogenic Na^+ -borate co-transporter (Park *et al.*, 2004) and has the highest sequence similarity with the *S. cerevisiae* borate transporter YNL275w (Alper, 2006). Park *et al.* (2004, 2005) have shown that HsBTR1 functions as a Na^+ - dependent borate transporter and determines the steady state concentration of cytoplasmic borate. They showed that this membrane transporter functions differently in the presence or absence of borate. In the absence of borate HsBTR1 acts as a channel that permeates uncoupled Na^+ - and OH^- /(H^+) in both directions (influx and efflux). However, as borate is the physiological substrate of this protein it does not transport OH^- or H^+ in the presence of B. Rather it acts as a voltage regulated, electrogenic Na^+- $B(OH)_4^-$ co-transporter. Park *et al.* (2004, 2005) added that HsBTR1 is especially useful for $B(OH)_4^-$ uptake from a low $B(OH)_4^-$ concentration due to tight coupling

and selectivity for Na^+- and $B(OH)_4^-$. They also showed that HsBTR1 does not transport similar metalloid arsenate.

Phylogenetically the oldest SLC4 transporter, YNL275w (Alper, 2006) is reported to have 27% sequence identity with BOR1 (Takano *et al*., 2002) and 26% with Band 3 protein (Zhao and Reithmeier, 2001). Zhao and Reithmeier (2001) reported that a six-histidine (His6)-tagged Ynl275wp protein shows similar anion binding properties as the Band 3 protein. They added that this non-glycosylated membrane protein can bind to a number of substrates that include HCO_3^-, I^-, NO_3, Br^- and Cl^- (Zhao and Reithmeier, 2001). Using a *Saccharomyces cerevisiae* mutant containing an insertion mutation in YNL275w, Takano *et al*. (2002) showed that YNL275w can efflux B, keeping the soluble B concentration in the wild type yeast cells 13 times less than their mutant counterparts.

In a recent paper Takano *et al*. (2005) looked at *BOR1* mRNA accumulation as affected by the amount of B supply. Previously Takano *et al*. (2002) showed that BOR1 is localized in the plasma membrane and is expressed in the pericycle cells of the root stele where it facilitates xylem loading of B under deficient B supply. In 2005 Takano *et al*. showed that the xylem loading of B carried out by BOR1 is regulated by B supply. With increasing B supply BOR1 mediated xylem loading is drastically reduced. Takano *et al*. (2005) reported that mRNA accumulation was not affected by the B availability although BOR1 protein diminished with the increase in B supply. Takano *et al*. (2005) presented circumstantial evidence that post-translational down regulation of BOR1 protein took place in pericycle cells. They hypothesized that in order to fine tune B homeostasis plants employ a sophisticated down regulation mechanism of BOR1 proteins. This eventually helps the plants to avoid accumulation of excessive B in the tissue, averting the toxic effect of B when supply of B is in excess. Finally they concluded that the supply of B determines the fate of BOR1 proteins, which are continuously fed into the endocytic pathway. Under limited B supply they are sorted by the early endosome for recycling into the plasma membrane, while under high B supply they are sent to the late endosome for eventual degradation in the vacuole.

1.5.4. The role of aquaporins

A decade after the first discovery of aquaporins, it is now known that plants use an array of aquaporins as selective pathways for water movement across cell membranes. Plant aquaporins are hydrophobic transmembrane proteins with six membrane domains (Tyerman *et al*., 2002). These proteins make up a superfamily also known as major intrinsic proteins or MIPS. MIPS are clustered into four subfamilies having euphonic acronyms: PIPs, TIPs, NIPs and SIPs.

PIPs are intrinsic proteins mostly localized to the plasma membrane, while TIPs are localized to tonoplast or vacuolar membranes. NIPs (nodulin 26 like intrinsic proteins) were first localized in the peribacteroid membrane of soybean nodule cells. Their subcellular location in non-leguminous plants is not known (Tyerman *et al*., 2002, Chaumont *et al*., 2005). SIPs are small basic intrinsic proteins whose subcellular localisation is yet to be established (Chaumont *et al*., 2005; Johanson and Gustavsson, 2002; Johanson *et al*., 2001). SIPs are the most distant members of the MIPs super family (Dordas *et al*., 2002). Aquaporins are now known to function as water selective channels or non-selective channels for water and non-electrolytes such as urea and glycerol (Tsukaguchi *et al*., 1998; Borgnia and Agre, 2001). They are also known to allow ion permeation (Yasui *et al*., 1999). Animal aquaporin AQP9 has been reported to be able to transport a range of other solutes that include sorbitol, carbamides, polyols and pyrimidines (Tsukaguchi *et al*., 1998). It has been reported that nodulin 26 has a high permeability to water, glycerol and formamide. Kaldenhoff and Fischer (2006) observed that TIPs are important in plants for urea homeostasis among cellular compartments.

Based on the observation of the transport of non-electrolytes like urea through channels, Dordas *et al*. (2000) first suggested that aquaporins may transport boric acid. They demonstrated that boric acid permeation of the plasma membrane could be partially inhibited by $HgCl_2$ and phloretin. This evidence points to the channel- mediated transport of B. Dordas *et al*. (2000) also showed that the inhibition by $HgCl_2$ can be reversed by 2-mecaptoethanol. By expressing *Zea mays* PIP1 in *Xenopus laveis* oocytes, they further showed that B permeability of the oocyte could be increased by 30%. In another experiment Dordas and Brown (2001) showed that the presence of channel inhibitors such as $HgCl_2$, phloretin and DIDS (4,4'-di-isothiocyanostilbene-2,2'-disulfonic acid) reduced B uptake by squash roots by 40-90%. They also showed that the presence of some small solutes like urea and glycerol could suppress B uptake by squash roots by up to 54%.

The next advance came in 2006 when Takano *et al*. (2006) identified NIP5;1, an aquaporin of the NIP subclass, to be up-regulated in B deficient *Arabidopsis thaliana* roots. NIPs are reported to be multifunctional transporters that are likely to transport water, glycerol, NH_3 and other small solutes (Kaldenhoff and Fischer 2006). NIPs are classified on the basis of the similarity and dissimilarity of their aromatic/argininine (ar/R) region with that of the archetypal Nodulin 26. NIP Group I possesses the conserved ar/R region of Nodulin 26 whereas NIP Group II possesses divergent ar/R tetrad. The latter group includes NIP5;1, NIP6;1 and NIP7;1. Analysis by homology modelling suggested that NIP6;1 might accommodate larger solutes (Wallace and Roberts, 2004).

Takano *et al*. (2006) showed that NIP5;1 is strongly up-regulated in the plasma membranes of the root elongation zone. By heterologous expression of NIP5;1 in *Xenopus* oocytes they demonstrated that NIP5;1 protein transports boric acid. They demonstrated that NIP5;1 mutation by T-DNA insertion reduced B uptake in mutant lines under limited B supply, indicating the requirement of NIP5;1 for efficient B uptake under these conditions. Growth retardation of NIP5;1 mutant lines under B deficient conditions was also presented as evidence of its involvement in B transport. Recently Takano *et al*. (2006) reported a role of another member of the Group II NIPs, NIP6;1 in B transport. Their interpretation followed the observation that the growth of NIP6;1 T-DNA insertion line is impaired under B deficient conditions. They concluded that NIP6;1 might play a different role from NIP5;1 in B transport as its tissue specificity was different from that of NIP5;1.

1.5.5. B re-translocation

Brown and Shelp (1997) observed that B re-translocation has a profound effect on the expression of B deficiency and toxicity symptoms. B is unique among the essential elements because its phloem mobility varies dramatically among species. Generally, B is immobile in phloem of most plant species because it accumulates in leaves and is not re-translocated to other parts of the plant (Brown *et al*., 2002; Blevins and Lukaszewski, 1998). However, in polyol-producing species such as celery and apple B is readily transported in the phloem (Brown and Hu, 1996). In these species B is translocated as a consequence of the formation of B-polyol complexes towards growing meristem tissues (Brown and Shelp, 1997).

Nable *et al*. (1997) observed that most species in which B is phloem mobile are susceptible to B toxicity. They viewed phloem immobility of B as an internal tolerance mechanism. Their explanation being that, in a phloem immobile species the retention of B in the leaf margins keeps the B away from important metabolic sites. Thus, although these plants suffer leaf burn, they are still able to maintain considerable photosynthetic area. However, there is no indication in the available literature that B is phloem mobile in wheat or barley. Wheat and barley are not known to produce polyols.

Of special interest are the findings by Brown *et al*. (1999) that the elevated production of sorbitol in transgenic tobacco significantly increases B uptake, tissue B concentrations and B transport to meristematic tissue, compared with plants not containing sorbitol. Bellaloui *et al*. (2003) also obtained a similar result in transgenic rice, which was engineered to produce extra sorbitol that enhanced remobilization of B within the plant by forming B-sugar alcohol complexes. They also detected the presence of sorbitol (0.89%) in mature leaves of the rice

cultivar Tai-pei 309, a fact previously unreported. Kawamata (1977) noted that within a single species, different varieties can have very different sorbitol concentrations. Substantial difference in sorbitol concentrations are also seen between different tissues and organs of the same plant (Bieleski, 1982). It is important to note in this context that polyols are easily masked by sugars in a variety of separation techniques, and thus can easily be missed even when present in large amounts (Bieleski, 1982). Unfortunately, many techniques used by physiologists for carbohydrate analyses do not reveal the presence of polyols. Gas and high-pressure liquid chromatography can, however, be used to separate and identify polyols and enzymatic analysis is also available for sorbitol (Loescher, 1987).

1.6. Carbohydrate metabolism under B toxicity

Alterations in sucrose levels are among the plant responses reported due to various environmental adversities, including extreme temperature, salinity, prolonged or shortened illumination, drought, infection, and other extreme conditions (Levitt, 1980; Good and Bell, 1980). B has been shown in many studies to have a variable effect on the biosynthesis of plant glycosides including sucrose (Dugger and Humphreys, 1960). The rate of replenishing the UDP–glucose (uridine diphosphoglucose) pool and particularly its differential utilization by different biosynthetic reactions is affected by B (Avigad, 1982). Delmer *et al*. (1977) and Dugger and Palmer (1980) observed that, since sucrose synthesis and degradation are closely associated with this UDP-glucose pool, it is not surprising that its levels and utilization are significantly influenced by B deficiency. Probably this is also true under B toxicity. For example, B has been reported to inhibit the formation of starch from sugar (http://www.knowledgebank.irri.or/). Reducing sugars have been found to increase in the root tip when soil B concentrations are high (McDonald *et al*., 2003). In intolerant barley varieties under B toxicity, invertase activity was seen to increase with a concomitant decrease in sucrose and an increase in glucose and fructose content (Stangoulis, J., personal communication). Long *et al*. (1975) suggested that sucrose is hydrolysed by an acid invertase to two hexoses (glucose, fructose).

1.7. Protein profile changes under B toxicity

The stress-induced alteration in protein synthesis in plants, animals and microbes is well known. Higher plants have been found to synthesize proteins that are involved in stress tolerance mechanisms under various stress conditions such as under B toxicity (Wimmer *et al*., 2003; Mahboobi *et al*., 2000 and references therein), heat (Waters *et al.,* 1996), cold (Sing and Laroche, 1990), salt (Serrano and Gaxiola, 1994), drought (Bray, 1993) and heavy metals (Rauser, 1990). Wimmer *et al*. (2003) recently showed that in wheat high B concentration

increased both inter- and intra-cellular soluble protein concentrations. From SDS page gel electrophoresis they found that B stress treatment induced a change in inter-cellular fluid protein profile in comparison with the control treatment. This included an increase of a 19 kDa protein and decrease of proteins of apparent molecular mass of 58 and 51 kDa. However, they did not characterise these proteins and hence their functions are unknown. Mahboobi *et al.* (2000) also reported that B toxicity caused an increase or decrease in a number of uncharacterised proteins in root and leaf tissue of barley seedlings. They found that a new protein with relative molecular weight of 35 kDa was synthesized in the root tissue of the tolerant barley variety Anadolu but not in the susceptible variety Hamidiye. They also observed a quantitative increase of three proteins in the root tissue of the tolerant varieties but not in the susceptible variety. They found that in leaf tissue, the abundance of seven proteins quantitatively increased in the tolerant varieties but were unchanged in the susceptible variety. In the susceptible variety some protein were seen to decrease in amount while others were completely missing under high B treatment in their experiment. In another solution culture experiment Mahboobi *et al.* (2002) found that under B toxicity in wheat and barley, nitrate reductase activity reduced by an average of 15% in root and leaf tissue of both tolerant and sensitive varieties. Similar reduction in the nitrate reductase activity has also been reported in sunflower seedlings under both B deficiency and toxicity (Kastori and Petrovic, 1989). Mahboobi *et al.* (2002) observed that the activity of glutamate dehydrogenase (GDH) increased by 30% in leaves and 81% in roots in both sensitive and tolerant varieties under B toxicity. GDH activity was significantly higher in tolerant varieties. Boussama *et al.* (1999) and Lutts *et al.* (1999) also reported enhancement in GDH activity in barley seedlings under cadmium toxicity and in rice seedling roots under salt stress. In the presence of substantial level of ammonia in tissues GDH is directly involved in the formation of glutamate, which is considered the principal precursor of proline biosynthesis (Mahboobi *et al.*, 2002). Proline is classified as a glucogenic amino acid, which is degraded into the precursors of glucose if present in excess in tissue (Hames and Hooper, 2000). Probably this is why McDonald *et al.* (2003) reported an increase of reducing sugars in the roots of barley under B stress. Mahboobi *et al.* (2002) hypothetised that production of increased amounts of glutamate dehydrogenase could be an adaptive mechanism in these species and possibly GDH has a protective role under B stress.

1.8. Accumulation and partitioning of B under toxic conditions

The major mechanism of tolerance for all crop species is the ability to maintain low concentration of B in plant tissue (B exclusion) (Mcdonald *et al*., 2003, Nable *et al*., 1997; Paull *et al*., 1992, 1995). Reduced uptake of B forms the basis of tolerance rather than differences in a critical level of B within tissues. Tolerant genotypes accumulate less B in both root and shoots

(Nable, 1988). In solution culture experiments, the examination of B concentration in both root and shoot indicated that exclusion mechanisms operate in a wide range of species (Nable, *et al*., 1997). B-tolerant genotypes of wheat and barley (Nable, 1988) as well as peas and medics (Paull *et al*., 1992) maintain lower B concentrations both in roots and shoots than the susceptible genotypes. Nable *et al*. (1997) reported that the ranking of genotypes for B tolerance and leaf B concentration have been found to be the same in solution culture, in pot culture or even under field conditions. From this observation they concluded that the exclusion mechanism operates under all conditions. However, the nature of the exclusion mechanism remains elusive.

In sharp contrast to Nable's assertion that an exclusion mechanism is operating in tolerant wheat varieties, Furlani *et al*. (2003) presented data showing that the tolerant wheat variety IAC 287 used in their solution culture experiment accumulated higher amounts of B at harvest (112 days of age) compared to the intolerant varieties. No reduction in dry matter and grain yield was observed in this wheat cultivar even with high B concentrations (up to 2 mM) in the solution.

Ralph (1991/1992) suggested that genotypes tolerant to B toxicity should be susceptible to B deficiency and vice-versa as strong selection pressure operates in low- and high-B soils. He concluded that a reversal of this fact could add worrying complications to the widely accepted concept of 'exclusion mechanism'.

1.9. B efficiency of crop cultivars under B deficiency and toxicity

Variation in the degree of tolerance to B deficiency and toxicity in wheat and barley has been reported. It is generally thought that wheat species are more efficient in the uptake and use of B in comparison to other species and more tolerant to low soil B concentration (Furlani *et al*., 2003). However, Furlani *et al*. (2003) observed that B efficiency and tolerance of wheat depend strongly on the genotypes considered and pointed out the need for re-evaluating this statement. Varieties with a large B requirement are said to be less susceptible to B toxicity, and vice versa. In fact, Furlani and co-workers showed that 'IAC 24', a B toxicity tolerant variety, actually required more B for growth and grain yield, as inferred from the gradually higher response obtained for the total-plant-dry matter and spike-dry matter in relation to spike–straw B concentration. They also observed a gradual increase in the number of grains per spike with increasing B concentration for this variety. Rerkasem *et al*. (1993) and Rerkasem and Loneragan (1994) put forward the opinion that it might be necessary to consider vegetative and reproductive efficiency separately. It has been reported that small grains and grasses have normal vegetative growth under B- free conditions and show B deficiency symptoms only

during the formation of reproductive organs (Blevins and Lukaszweski, 1998). High demand for B during reproductive growth is a common feature among plant species (Gauch and Dugger, 1954; Loomis and Durst, 1992; Blevins and Lukaszweski, 1998).

Furlani *et al*. (2003) reported that 'IAC 287' and 'IAC 60' showed considerable B efficiency and were able to produce the highest shoot, spike and grain dry matter under B deficient conditions among the tested varieties. In their experiment 'IAC 287' was also found to be B toxicity tolerant. They reported that 'IAC 287' produced the highest grain yield in solution culture with a B concentration of 12.9 mM and no toxicity symptom was expressed at a B concentration as high as 32.4 mM in the growing media. Punchana *et al*. (2004) cautioned against generalising B efficient wheat varieties as prone to B toxicity and/or vice- versa. They added that it is not uncommon to find varieties showing sensitivity to both B deficiency and toxicity. In an experiment they evaluated the response of three B efficient and inefficient wheat varieties under potentially toxic B conditions. They found that Bonza, a B inefficient variety was most B tolerant while Fang 60 a B efficient variety was the least B toxicity tolerant. In another experiment they evaluated 180 entries from the 18th Semi-arid Wheat Screening nursery of CIMMIT. They classified the responses in three distinctive groups. These are a) B toxicity intolerant and B efficient. b) B toxicity tolerant and B inefficient and c) B toxicity intolerant and B inefficient. They concluded that the ideal combination of B efficient and B tolerant was not found in this international collection of germplasm.

1.10. Exclusion or efflux as a tolerance mechanism? The tolerance mechanism revisited

In the recent years the observation that an Algerian barley landrace, Sahara 3771, has the ability to maintain a low internal B content over a range of potentially toxic B concentration has strengthened the argument that this barley landrace is able to exclude B. However, the nature of the exclusion mechanism still remains elusive. One would wonder is it simply a passive exclusion mechanism influenced by membrane lipid composition or an active process whereby B is actively effluxed.

In 2000, Dordas and Brown using *Arabidopsis thaliana* mutants differing in plasma membrane lipid composition showed clearly that lipid composition could affect B uptake. They demonstrated that a chilling sensitive *Arabidopsis* mutant *chs1-1* having 20% less sterols permits 30% higher B uptake compared with the wild type. In contrast, *act1-1* mutant having higher percentage of longer fatty acids, restricted B uptake by 35% in comparison to the wild type. They also reported that lipid composition changes in several other mutants influenced B uptake to various extents.

Recently Hayes and Reid (2004) proposed an efflux mechanism involving an anion channel or anion exchanger in Sahara 3771 to prevent B build up within plant tissue. They demonstrated that after growing the plants at 5mM B for 16 days, B concentration in root, shoot and xylem exudates was 2.7, 4.5 and 2.7 fold lower respectively, in Sahara 3771 compared to the B sensitive variety Schooner. The ability of Sahara 3771 to exclude B held until the B concentration in the growing medium was increased to 10 mM. They also showed that Sahara 3771 was able to establish a minimal tissue B concentration within 3 hours of exposure to high B concentration (Figure A). They observed that these facts are indicative of a constitutively expressed mechanism operating in Sahara 3771 which enables this barley variety to maintain low B in tissues. Garnett *et al*. (1993) examined the B influx in two wheat varieties differing in their ability to exclude B (Figure B and Figure C). They reported that within four hours no apparent difference in B influx was observed between Halberd an efficient B excluding (B tolerant) wheat variety and (WI*MMC)/W1/10, an intolerant wheat variety unable to exclude B. This indicates that the B excluding mechanism is not the same in Sahara 3771 barley and the B tolerant wheat variety Halberd. It is worth noting that the pattern of B efflux in wheat and barley after removal of B shown by Garnett *et al*. (1993) and Hayes and Reid (2004) was very similar.

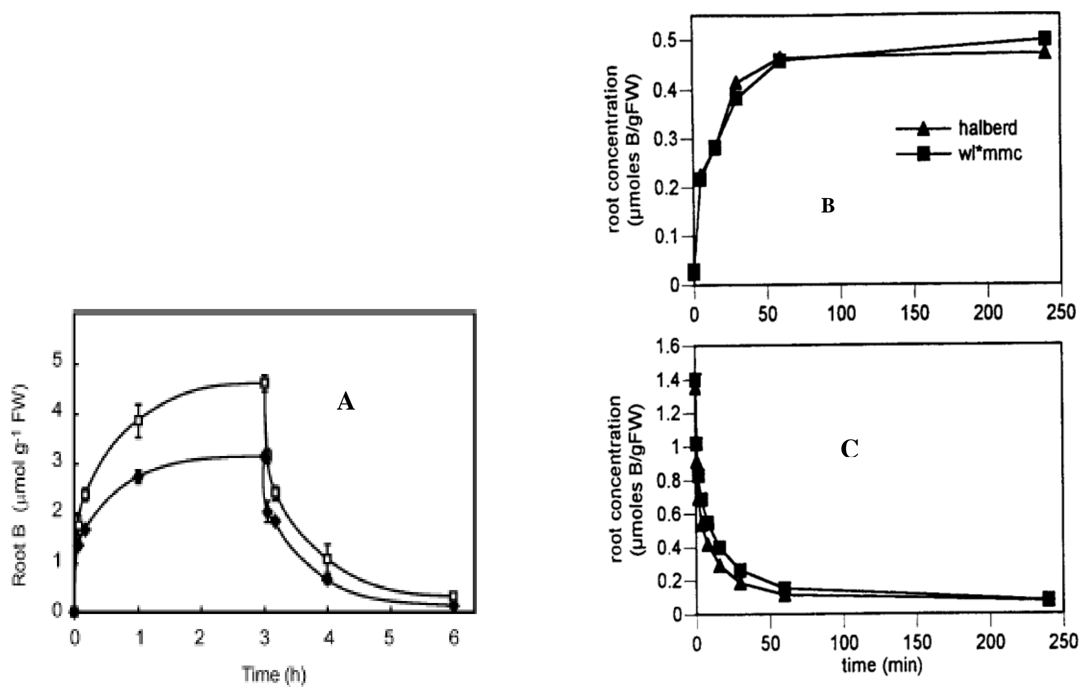


Figure A. Influx of B in Sahara 3771 and Schooner barley roots during short –term exposure to nutrient solution containing 5 mM B and efflux after removal of B. (Closed box – Sahara 3771, open box–Schooner) Adopted from Hayes and Reid (2004).

Figure B[§]. Short-term influx of B to the roots of Halberd and WI*MMC wheat exposed to 1 mM B (Triangle- Halberd, Square- WI*MMC)

Figure C[§]. Efflux of B from wheat roots after removal of B. (Triangle- Halberd, Square- WI*MMC)

[§] Figure B and Figure C were adopted from Garnett *et al*. (1993).

Most interestingly Hayes and Reid (2004) showed that the shoot B concentration of Sahara 3771 was not linearly related to root B concentration unlike in Schooner where this was the case. The ability of Sahara 3771 shoot to further restrict B influx from root broke down when the external B concentration reached to 5 mM.

Hayes and Reid (2004) also looked into the effect of metabolic inhibitors and channel inhibitors on the influx /efflux of B in Sahara 3771 and the intolerant variety Schooner. They found that 0.5 mM sodium azide – a metabolic inhibitor, resulted in 66% increase in root B concentration in Sahara 3771 while the root B concentration of Schooner was not altered. This indicates that the efflux mechanism of Sahara 3771 is an energy demanding process. However, low temperature treatment had no effect on root B concentration of either of the barley varieties contradicting the result observed with sodium azide. This could also be interpreted as utilization of remaining ATP reserves counteracting the chilling effect. Hayes and Reid (2004) reported

that application of ethacrynic acid, A9C (anthracene-9-carboxilic acid), DIDS (4,4'-di-isothiocyanostilbene-2,2'-disulfonic acid), CHCA (α--cyano-4-hydroxycinnamic acid) at a concentration of 100 μM for 2 hours did not alter influx/efflux property of Sahara 3771 roots. But, DPC (diphenylamine-2carboxilic acid) and niflumate increased the B concentration by 50 and 80%, respectively, in Sahara 3771. Root B concentration of Schooner was unaffected by the application of any of the above mentioned channel inhibitors. These results are in sharp contrast to the results obtained by Dordas and Brown (2001), who reported that the presence of channel inhibitors like $HgCl_2$, phloretin and DIDS reduced B uptake by squash roots by 40-90%. However, B influx/efflux mechanisms of grasses and dicots are likely to be different.

DIDS has been long known for inhibiting anion transport activities of band 3 proteins (Cabanthick and Greger, 1992). Not all members of the SLC4 anion exchanger family are inhibited by DIDS (Romero *et al*., 2004). For example electroneutral sodium bicarbonate co-transporter (NBC3) (Pushkin *et al*., 2000) and rabbit AE4a (Usui *et al*., 2001) are not sensitive to DIDS. The inhibitory effect of DIDS and the stilbene inhibitors has been reported to transcend the SLC4 family members and DIDS inhibits other anion exchangers that do not have any sequence similarity with SLC4 transporters (Pushkin and Kurtz, 2005).

The pH sensitivity of the anion exchanger family members also varies considerably (Alper *et al*., 2002). For example mouse AE1 when expressed in *Xenopus* oocytes does not show pH sensitivity between pH 5 to 9 (Alper *et al*., 2001). On the other hand AE2 is highly sensitive to pH changes (Jiang *et al*., 1994). It has been reported previously that boric acid (H_3BO_3) permeability across lipid bi-layers is also affected by pH. Dordas and Brown (2001) reported that H_3BO_3 and $B(OH)^-_4$ permeability apparently declined sharply with an increase in pH. They observed that this decline of B permeability was not related linearly with the increase in pH**.** Charged B species are known to have very low permeability across membranes (Gutknecht and Walter, 1981; Chakrabarti *et al*., 1992; Xiang and Anderson, 1994).

Hayes and Reid (2004) investigated the effect of pH of the external solution on the B uptake of Sahara 3771 and Schooner at an external B concentration of 5 mM. They reported that an increase in pH in the external medium altered the proportion of H_3BO_3 and $B(OH)^-_4$ inversely. The falling H_3BO_3 concentration reduced B uptake by Schooner root whereas the B concentration in Sahara 3771 root remained unaffected. They suggested that in an efflux mechanism would most likely extrude B in the form of $B(OH)^-_4$ ions. They explained that the higher concentration of $B(OH)^-_4$ in the external medium would naturally discourage efflux because of the diminishing outward electrochemical gradient. An alternative explanation of this

$B(OH)^{-}_{4}$ effluxes would be that an increased energy input will be required in such a situation for the efflux mechanism to operate and hence render the efflux mechanism less efficient. Finally Hayes and Reid (2004) proposed two alternative models for this efflux mechanism involving either an anion channel or an anion exchanger. The second model was originally proposed by Frommer and von Wirén (2002) to explain the possible mechanism of action of *Arabidopsis* BOR1 in xylem loading.

1.11. Genetics of tolerance to B toxicity

Genetic variation in tolerance to B toxicity exists in wheat and barley and also in several other crops (Cartwright *et al*., 1987; Paull, *et al*., 1986, 1988a, 1988b, 1992; Nable, 1988; Moody *et al*., 1988, 1993; Bagheri *et al*., 1992; Jamjod, 1996). A wide range of intra-specific variation in response to B has been reported in bread wheat (Chatterjee *et al*., 1980; Mehrotra *et al*., 1980; Paull *et al*., 1988a) and durum wheat (Brooks, 1991; Jamjod, 1996; Yau *et al*., 1995). However, the degree of dominance expressed by F1 hybrids varies with the level of B treatment. At low B treatment (i.e., near the threshold level of the tolerant parent) the responses of the F1 population resembled that of the tolerant parent, while at high B treatment their responses shifted towards the sensitive parent (Jamjod, 1996; Jenkin, 1993; Paull *et al*., 1991). Yau *et al*. (1997) and Kalayci *et al*. (1998) reported that durum wheat is much less tolerant to high soil B concentration than bread wheat based on a leaf symptom score, shoot B concentrations and grain yield. A high degree of genotype × environment interaction for B tolerance has been reported for wheat (Kalayci *et al*., 1998) and barley (Jenkin, 1993). B tolerance in bread and durum wheat (as in barley and field pea) is controlled by partially dominant nuclear genes. Genes responsible for tolerance to B have been located on chromosomes of wheat by several methods including inter-varietal substitution lines (Paull et al., 1988b, 1995), inter-specific substitution and addition lines and monosomic analyses (Chantachume, 1995; Chantachume et al., 1994; Jamjod, 1996).

In the case of barley, leaf symptom data was used by Jenkin (1993) to study the chromosomal location and inheritance of genes providing B tolerance in barley. Jenkin (1993) conducted a genetic study of B tolerance which involved all possible cross combination using B toxicity tolerant Algerian landrace Sahara 3771, a moderately tolerant variety CM 72 and an intolerant variety Stirling. The inheritance patterns of B toxicity tolerance were followed in F1, F2 and F2 derived F3 populations from these crosses. Jenkin proposed that at least three major genes confer B tolerance in Sahara 3771 while two genes are involved in CM 72. The genetic effect of B tolerance appeared to be acting in an additive manner. An RFLP linkage map of 43 doubled haploid lines derived from a cross between the B susceptible Australian variety Clipper and the

tolerant Sahara 3771 was used to determine the location of these three genes on barley chromosomes (Jenkin, 1993). A region on chromosome 2HL and another on 7HS was identified as having significant association with leaf symptom expression caused by B toxicity. However, the reported association was meagre having a LOD score less than 3.0. The small size of the doubled haploid population was cited to be the probable cause for such weak association.

1.11.1. B tolerance genes and QTL in wheat

Three partially dominant genes *Bo1*, *Bo2* and *Bo3* located on homoeologous chromosomal groups 4 and 7 have been reported to control B toxicity tolerance in tolerant wheat varieties by acting additively (Paull 1990, 1991).

A review of the literature on the genetics of B toxicity reveals some controversy in assigning the role of *Bo* genes and their locations. Chromosome 7B has been implicated in B tolerance in durum wheat (Jamjod, 1996) and also in an analysis by Paull *et al*. (1992) of lines derived from Halberd X monosomic Condor selection lines. Chantachume (1995) also located the tolerance gene *Bo1* of Halberd on chromosome 7B from a genetic analysis using a Condor monosomic series. From linkage analysis using RFLP, Jamjod (1996) predicted that in durum wheat two genes (*BoT1* and *BoT2*) are located on chromosome 7B and segregate independently. Analysing 161 doubled haploid Halberd lines Jefferies *et al*. (2000) reported leaf symptom expression to be controlled by at least two genes located on chromosome 7B and 7D. Paull *et al*. (1991) has associated the *Bo3* gene with the mid-leaf necrosis symptom [a distinctive symptom expressed only in S(KF4A), CF and (W1*MMC) and CS (Sapporo 4A) in response to excess B in addition to the usual leaf symptom which progresses from the leaf tips (Paull *et al*., 1992)]. From an analysis of single recombinant lines developed from Chinese Spring X Chinese Spring (Kenya farmer 4A) Paull *et al*. (1991) identified *Bo2* to be linked to Sr7a and hence indicate that it is located on chromosome 4A and more precisely on the proximal segment of 4AL. Chantachume (1995) associated the 4A locus (*Bo4*) with B exclusion. Jefferies, *et al*. (2000) suggested that it is possible that wheat and barley may possess a common B tolerance gene on 4A and 4H. They suggested that the B tolerance gene on chromosome 4H of barley is associated with B uptake as well as being associated with root length response, dry matter and symptom expression.

Jefferies, *et al*. (2000) found regions on chromosome 7B and 7D are associated with leaf symptom expression behaving in a largely additive manner. They suggested that the 7D locus might be involved in the translocation of B in leaf tissue, which may contribute to differences in leaf symptom expression. They found that the region on 7B was also associated with the control of B uptake (control of a B exclusion mechanism) and with a reduction in the effect of B

toxicity on root growth suppression (i.e. relative root length). They reported association of Halberd alleles at the chromosome 7B locus with the concentration of B in whole shoot and grain. Correlative evidence was presented by Jefferies *et al*. (2000) in associating the 7B locus (*Xpsr680*-7B) with improved grain yield on B toxic soils.

Paull (1990) proposed that the genes for B tolerance, derived from an exotic Greek line G61450 are located on chromosome 4A and 7D. Jefferies, *et al*. (2000) suggested that it is possible that the Halberd 7D locus they identified and 7D locus derived from Paull (1990) are common loci. From results of backcross reciprocal monosomic analyses, Chantachume (1995) indicated that 4A was the location of genes controlling B tolerance also in exotic lines India 126 and Benventuto Inca. Jefferies *et al*. (2000) suggested that additional minor genes conferring B tolerance might be present in Halberd.

1.11.2. B tolerance QTL in barley

In 1999 Jefferies *et al*. studied the genetic basis of B toxicity tolerance in a mapping population of 150 doubled haploid lines derived from a cross between Clipper and Sahara 3771. They screened these lines in hydroponic and soil-based assays over two consecutive years and identified four significant quantitative trait loci (QTL) controlling various aspects of B toxicity tolerance. By internal regression mapping they detected a major QTL on the long arm of 4H to be significantly controlling all four parameters of B toxicity tolerance, namely: shoot B concentration, leaf symptom expression, relative root length and dry matter production. A region on chromosome 2H was associated with leaf symptom expression having a much stronger effect on this parameter than the 4H QTL. Another QTL on chromosome 3H was identified to control relative root length at toxic B concentrations having a lesser effect than that of 4H QTL and acting in additive manner with the 4H QTL. A region on chromosome 6H was identified as contributing to whole shoot B concentration again behaving largely in additive manner with the 4H QTL. They reported that the 4H QTL had a substantially larger effect than chromosome 6H QTL on this most important B toxicity tolerance trait. They proposed that these two QTL are most likely to be involved in the B exclusion mechanism in Sahara 3771. They concluded that since 4H QTL is effective in keeping B from entering into the plant and preventing it from the deleterious effects of toxic B, it thus is associated with all B toxicity tolerance traits.

1.12. Breeding for B toxicity

The distribution of B tolerant wheat varieties in Australia is concentrated in those regions where a predominantly high concentration of soil B exists. Thus, the breeding and selection of wheat

varieties in South Australia provides a good example of both passive and active selection for tolerance to high B concentrations. Halberd has been identified as a moderately B tolerant variety and it is recognised as the most tolerant among Australian varieties. The moderately tolerant varieties of Australia are related to Federation and Currawa (Nable *et al*., 1997). Varieties in this family include Ghurka, Quadrat, Insignia, Heron, Olympic and Spear. These varieties were the dominant and most widely cultivated varieties of South Australia and Victoria during most of the 20th century. By transferring the Bo1 allele from Halberd to moderately sensitive varieties through back crossing several sets of near isogenic lines (NIL) of wheat have been developed (Nable *et al*., 1997). When grown at high B sites, these NILs have been found to consistently demonstrate a yield advantage of 5-10% (Campbell *et al*., 1994; Moody *et al*., 1993). Active selection for B tolerance has led to the release of a number of high yielding, widely adapted varieties including Spear, Dagger, Trident, and more recently Frame and Krichauff. About 50% of Victorian varieties now carry B tolerance traits.

As for wheat, introducing B tolerance in barley has been a major objective of barley breeding in South Australia, especially at the Waite Institute. The Algerian landrace Sahara 3771 was identified as a source of B tolerance in early work. This 6 – row tall variety stands out for its B tolerance. However, subsequent breeding efforts for improving B tolerance in barley have not been very successful (McDonald *et al*., 2003). It was reported that progeny derived from Sahara 3771 as one of the parents, demonstrated reduced leaf symptom and shoot B concentration without significant yield advantage in high B soils. In 2002 lines were selected for the presence and absence of the Sahara 3771 B tolerance loci at 2H and 4H in experiments using backcross lines of B intolerant Sloop and the Victorian breeders line VB9104. The presence of either of the allele or both in combination reduced visual symptom expression without any yield gain over the parents. Further analysis subsequently revealed that a large section of the 2H chromosome unrelated to B tolerance (as much as 50cM) from the agronomically unsuitable Sahara 3771 was transferred due to backcross lag (linkage drag) (McDonald *et al*., 2003). Therefore new breeding strategies have been adopted to overcome the linkage drag associated with the effort of introducing B intolerance trait sourced from Sahara 3771. Recently some of the B tolerance characteristics from Sahara 3771 have been successfully incorporated in a Victorian breeding line called Sloop Vic (I).

1.13. Genomic approaches

Abiotic environmental stresses including toxic concentration of mineral nutrients in soils contribute most significantly to the reduced yields (Flowers and Yeo, 1995). Plants have the ability to dramatically alter their gene expression patterns to cope with a variety of

environmental stresses. These transcriptional changes are sometimes successful adaptations leading to tolerance while in other instances the plant ultimately fails to adapt to the new environment showing susceptibility or intolerance (Hazen *et al.*, 2005). Plants streamline defense resources at the cellular level upon the imposition of stress. However, our ability to improve plant tolerance to environmental stresses has remained limited due to our lack of insight and understanding of the inherent complexity of stress signaling and adaptation processes (Cushman and Bohnert, 2000). Breeding for increased abiotic stress tolerance has been found to be difficult partly due to the multigenicity of abiotic stress tolerance (Bohnert *et al.*, 2001). Recent insights into the molecular basis of stress tolerance have begun to suggest new strategies for crop improvement. The first step of this approach is to learn more about gene regulation and signal transduction pathways involved in stress tolerance.

The gene complement expressed by a cell is dynamic and responds rapidly and dramatically to external stimuli or even to normal cellular events such as DNA replication and cell division (Spellman *et al.*, 1998; Cho *et al.*, 1998). Therefore, measurement of gene expression can provide clues about regulatory mechanisms, biochemical pathways and broader cellular function (Clarke *et al.*, 2001).

Advances and technical developments in genomics, bioinformatics and 'functional genomics' made in recent years have offered the opportunity to gain a more complete understanding of how many genes become integrated to effect abiotic stress tolerance. Thus it is now possible to address the complexity of a stress response on a large scale through genome wide 'expression profiling' (Reymond *et al.*, 2000; Richmond and Somerville, 2000). Scientists are now equipped to perform gene expression analysis to characterize and define the functional roles of all genes essential, important, and ancillary to the stress response of tolerance phenotypes. Novel genes and regulators identified by gene expression profiling can be explored further for their specific role(s) in the tolerance or susceptibility to the stress in question. The new and novel genes can also serve as genetic markers for diversity in commercial and exotic germplasm. The results obtained through genomic approaches can also be helpful in the development of gene constructs, which could be used to genetically modify crop plants for elevated stress tolerance.

1.14. Conclusion

After wheat, barley is the second largest grain crop in Australia. On average Australian barley production is worth 1.4 billion AUD. Excessive B content in soil, a major toxic component of the semiarid regions of Australia and many other parts of the world have been identified as a serious problem that causes significant yield loses in susceptible barley varieties. But the

primary causes of B toxicity are yet to be fully understood. An Algerian barley landrace, Sahara 3771 has been identified as exceptionally B tolerant. Several quantitative trait loci have previously been identified that confer B toxicity tolerance in this barley variety. But the molecular basis of this resistance mechanism remained elusive. This has been a major obstacle in harnessing the full potential of the tolerant traits in breeding varieties that can stand tall in terms of yield under potentially toxic concentrations of soil B. The advent of modern genomic approaches provides new hope and opportunity for unlocking the elusive mechanisms of B toxicity and tolerance in barley. Structural and functional characterization of B-stress induced genes using genomic approaches may contribute to a better understanding of how barley plants respond and adapt to this specific stress. The identification of B stress responsive genes will be useful in germplasm screening and genetic improvement of barley for B toxicity tolerance.

1.15. Aims of the project

- Comprehensive genome wide survey and monitoring of stress responsive gene expression under B toxicity.
- Compare and study overall differences in gene expression between B stressed and non-stressed barley plants of tolerant and intolerant varieties.
- Isolation and characterization of genes up or down regulated in the event of B toxicity.
- Functional identification and analysis of genes important or involved in stress signaling and tolerance using array based expression screening.
- To gain novel insight into the molecular mechanism that co-coordinates metabolic pathways, regulatory and signaling networks under B toxicity of barley.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Seed Germination

Seeds were surface sterilized by immersing them in 70% ethanol for five minutes with continuous shaking. After discarding the ethanol solution seeds were washed three times with autoclaved milliQ water five minutes each. The seeds were then treated with 0.5% sodium hypochlorite solution for 15 minutes. 0.1% Triton X was added to the hypochlorite solution as a wetting agent. After removing the seeds from sodium hypochlorite solution they were again washed with autoclaved milliQ water for five times for five minutes each. All sterilisation and washing steps were performed in falcon tubes mounted on a flask shaker (Gallenkamp, Loughborough, UK). Seeds were then placed on Whatman filter papers in sterile Petri dishes and soaked with autoclaved water. Surface sterilized seeds were handled in a clean-bench to avoid contaminations. Germination was carried out for four days in the dark at room temperature.

2.2. Hydroponic system

For growing plants aerated plastic tanks filled with hydroponic solution formulated in our lab for growing cereals (ACPFG cereal growth nutrient solution) were used. The tanks' lids were fitted with 24 plastic tubes which were suspended through holes cut in the tank lid. The tubes had narrow openings at the bottom to allow the roots to grow in the aerated solution. Plastic tanks used to grow plants were either made of black plastic or covered with black PVC sheet to provide a dark environment in the root zone. Seedlings were transferred to the hydroponic tanks on the 5th day after sowing. They were placed near the openings of the tube with roots hanging outside. After placing the seedlings in the hydroponic tanks they were allowed to grow in the same solution for one week. From the second week onward fresh solution was provided every three days. The solution was aerated with an air pump intermittently. The roots of the tolerant and intolerant lines were kept separate using cheesecloth screen. Figure 2.1 and 2.2 shows photographs of hydroponic system used to grow plant materials.

Figure 2.1. View of the hydroponic tanks from above showing the arrangement of plants.

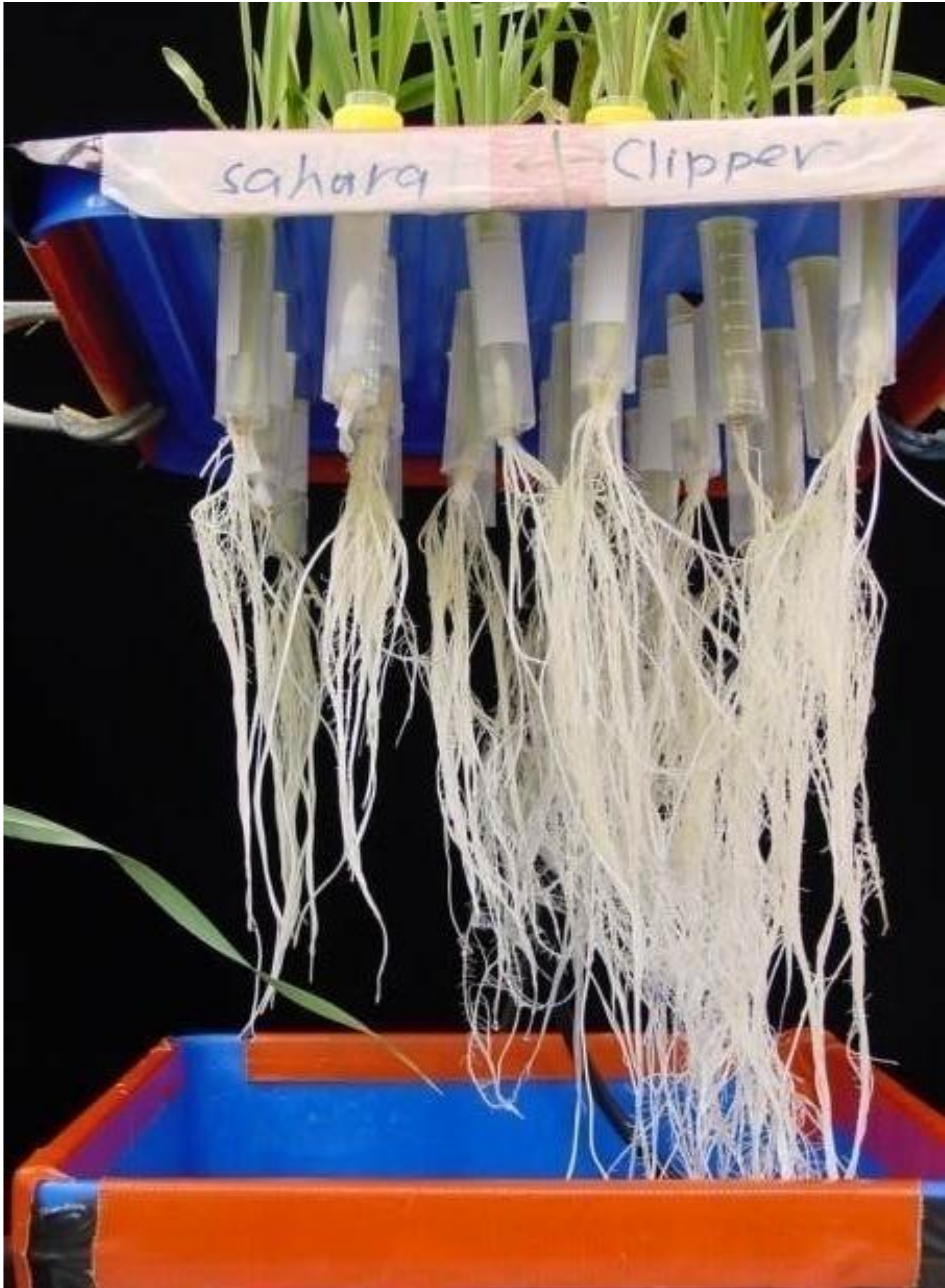


Figure 2.2. Photo of the plants growing in a hydroponic tank with the lid uncovered to show how the plants were grown.

2.3. Nutrient solution

Plants were grown using ACPFG cereal growth nutrient solution. The composition of the solution is shown in Table 2.1. Each of the (macro and micro nutrient) stock solutions was made separately to avoid precipitation. For each tank 10 litres of solution was used.

Table 2.1. Composition of the ACPFG cereal growth nutrient solution used to grow plants hydroponically.

	Salts	Stock solution concentration	Final concentration
1	Macronutrients	(M)	(mM)
	NH_4NO_3	1.0	5.0
	KNO_3	1.0	5.0
2	$Ca(NO_3)_2 \cdot 4H_2O$	0.4	2.0
3	$MgSO_4 \cdot 7H_2O$	0.4	2.0
	KH_2PO_4	0.02	0.1
4*	Na_2SiO_3	0.5	0.5
5	NaFe(III)EDTA	0.05	0.05
6[a]	Micronutrients	(mM)	(μM)
	H_3BO_3	10.0	10.0
	$MnCl_2 \cdot 4H_2O$	5.0	5.0
	$ZnSO_4 \cdot 7H_2O$	5.0	5.0
	$CuSO_4 \cdot 5H_2O$	0.5	0.5
	Na_2MoO_3	0.1	0.1

Na_2SiO_3 was not included in the nutrient solution

[a] for microarray experiment 20.0 (μM) H_3BO_3 was used instead of 10.0 (μM) as final concentration in the nutrient solution.

2.4. Growth chamber conditions

The growth chamber was provided with a 16 hour photoperiod. The growth chamber temperature was set at 22 ℃ throughout the plant growth.

2.5. Leaf symptom score

For assessing the degree of leaf damage, leaf symptoms were scored using a scale of 1 to 9 adopted from Kluge and Podlesak (1985). The scale is presented in Table 2.2.

Table 2.2 Scale used to score leaf symptoms in barley due to B toxicity.

Score	Visual observation of symptom	Extent of damage
9	No visual symptom	No damage
8	Initiation of necrotic damage at the leaf tip	Pre-initial
7	Brown point necroses affecting 1-2 cm from the tip of the leaves	Initial
6	Brown point necrosis coalescing with leaf tip start to turn dirty- white	Post-initial
5	Brown point and area necroses, leaf margin in the upper third of the leaf, about 1cm of leaf-tip necrotic	Moderate
4	One third leaf area affected and necrotic	More than moderate
3	Brown point and area necroses of about two third of the leaf area, only leaf base is free from damage. About one third of leaf area from the top to downwards dirty white necrotic.	Heavy
2	Brown necrosis spreading towards the leaf bases.	Very heavy
1	Strongly developed brown point area necroses up to the leaf bases. About half of the leaf area from top become dirty white necrotic in the downward direction	Severe

2.6. ICP-AES analysis

For determining B concentration plant samples were analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The plant samples (shoots in this case) were placed in paper bags and oven dried at 70 ºC for 72 hours. Subsequently the samples were ground to powder using ball bearings and shaker in falcon tubes. The ground samples were sent to the Waite Analytical Facilities for the analysis using SPECTRO-CIROS^{CCD} ICP-spetrometer (SPECTRO Analytical Instruments GMBH, Kleve, Germany).

2.7. Collection and preservation of plant materials for RNA isolation

Roots and shoots or leaves were excised using RNase free scissors. The scissors were frequently treated with RNase Zap (Ambion, Austin, TX, USA) and subsequently washed with double autoclaved water. Hand gloves were used during the handling of the plant materials and changed frequently between harvesting individual plants. The scissors were also washed with ample double autoclaved water in between individual plant harvests. Roots were pat dried with autoclaved Whatman filter paper immediately before excision. Excised plant materials were placed in falcon tubes and frozen immediately in liquid Nitrogen. Plant materials were preserved at -80 ºC until used for RNA extraction.

2.8. RNA preparation and handling

2.8.1. Total RNA extraction

Total RNA was extracted using Trizol (Ambion, Austin, TX, USA) reagent according to the manufacturer's instructions. In short, the plant material was ground using a pre-chilled (using liquid Nitrogen) mortar and pestle. All mortars and pestles used for RNA extractions were rinsed with double autoclaved milliQ water and baked at 180 ºC overnight prior to use.

About 200 mg ground tissue were taken 10 ml RNase free plastic tubes and RNA extraction was carried out according to the protocol supplied by the manufacturer. After phase separation the supernatant was transferred to 3x 2 ml Eppendorf tubes for RNA precipitation. RNA pellets were resuspended in DEPC treated water (Ambion, Austin, TX, USA) stored at -20Cº.

2.8.2. Total RNA cleanup

Total RNA was cleaned up using Qiagen RNeasy minikit (Qiagen, Australia) according to the manufacturer's instructions. Cleaned up RNA was eluted with RNase-free water supplied with the kit.

2.8.3. Poly(A)$^+$ RNA isolation from total RNA

For poly(A)$^+$ RNA isolation Poly(A)Purist^{TM} kits (Ambion, Austin, TX, USA) were used following the manufacturer's instructions. Poly(A)$^+$ RNA was eluted with RNA storage solution supplied with the kit and stored at -80 ºC after spectrometric quantification.

2.8.4. Spectrometric quantification of RNA

All the RNA quantification was carried out using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). This machine allows quantification of one µl of nucleic acid sample. For poly(A)$^+$ RNA, 1 µl of the RNA was diluted to 9 µl of double autoclaved milliQ water and quantified using three replicates.

2.8.5. Electrophoresis of RNA on denaturing gel

Size separation of RNA was carried out on a 1.25% agarose gel in presence of a denaturing agent such as 0.05% formaldehyde and 1% 10x MOPS buffer (0.5 M MOPS, 0.01 M EDTA, pH adjusted 7.0) in order to prevent formation of secondary structures. 5-10 µg RNA was run on each lane which was dried under vacuum and resuspended in 15 µl formaldehyde, deionized (DI) formamide (Ambion, Austin, TX, USA) and 10x MOPS buffer solution (2.75 µl formaldehyde, 7.5 µl DI formamide, 0.75 µl 10x MOPS and 4 µl water). For re-suspension the RNA was vortexed briefly. Re-suspended RNA was spun at 8000 rpm for three minutes and

heated at 55 ºC in a dry heat block for 15 minutes and chilled on ice. Prior to loading on the gel 1.5 µl RNase free RNA loading buffer (322 µl 10x MOPS buffer, 5 mg xylene cyanol, 5mg bromocresol green, 178 µl 37% formaldehyde, 500 µl DI formamide and 400 mg sucrose) was added to the re-suspended RNA. Electrophoresis was carried out in RNase free gel tank containing 1x MOPS buffer at low voltage (40V). Following electrophoresis the gel was stained in RNase free ethidium bromide solution for 15 minutes and de-stained in double autoclaved milliQ water for about an hour. The gel was visualized under UV illumination using a GeneFlash gel doc system (Syngene Bio Imaging, Cambridge, UK) and documented.

2.8.6. Transferring RNA to nylon membrane by capillary blotting

Following documentation of the RNA gel it was incubated in RNase free milliQ water for 15 minutes with slight agitation. The gel was then transferred in sterile 10x SSC (0.15 M NaCl, 0.015 M Tri-sodium citrate, pH 7-8) and soaked for 15 minutes with gentle agitation and this step was repeated. Overnight transfer of RNA onto nylon membrane (Hybond_N^{+}, Amersham UK) by capillary blotting was carried out using 20x SSC as transfer buffer. Following transfer the membrane was rinsed in 5x SSC for a minute and then air dried. The RNA was cross-linked to the membrane by exposing the membrane to UV for 150 seconds and preserved inside a plastic bag at -20 ºC until use.

2.9. cDNA synthesis

First strand and second strand cDNA was synthesized from poly(A)$^{+}$ RNA according to Clontech PCR-select^{TM} cDNA Subtraction Kit User Manual using components supplied with the kit. In this case 2-3 µg poly(A)$^{+}$ RNA was used.

2.10. Agarose gel electrophoresis of DNA

PCR amplified inserts were cleaned using the QIAquick PCR Purification Kit (Qiagen, Australia) according to manufacturer's instructions. Usually 5–10 µl of cleaned up DNA product was electrophoresed on each lane of (1-3% depending on resolution required) Agarose-1x TAE (0.4 M Tris-acetate, 1.0 mM Na_2EDTA, pH 8.0) gel. 0.2 volumes of 6x Ficoll dye (15% (w/v) Ficoll 400, 0.25 % (w/v) bromophenol blue 0.25% (w/v) xylene cyanol) was added to the DNA sample before loading. Gel electrophoresis was carried out in 1x TAE and the gel was stained in 10 µg/ml ethidium bromide solution for about half an hour. The gel was then visualized under UV and documented by a Gene Flash gel doc system (Syngene Bio Imaging, Cambridge, UK).

2.11. Isolation and purification of DNA fragments from agarose gel

Following size separation and ethidium bromide staining gels were illuminated using UV lamps and the desired fragment band(s) was excised with a sharp scalpel. DNA was purified using QIAquick Gel Extraction Kit (Qiagen, Australia) according to the manufacturer's protocol. DNA was eluted with an appropriate volume of autoclaved milliQ water.

2.12. Ligation of DNA to cloning vectors

30 ng of cleaned up subtracted cDNA clones were ligated into pGM®-T easy vector (Promega, Madison, WI, USA) in 10 µl reactions using 1µl of T4 DNA ligase (Promega, Madison, WI, USA) and 5 µl 2x Rapid ligation buffer (Promega, Madison, WI, USA). The reaction was incubated at room temperature for an hour and subsequently at 4 ºC overnight. The ligation reaction was then used for transforming electro-competent *E. coli.*

2.13. Introduction of ligated cDNA into *E. Coli* by electroporation

XL1 – Blue Electroporation – Competent Cells (Stratagene, Santa Clara, CA, USA) were used. Transformation was carried out by Gene-Pulse (Bio-Rad, Hercules, CA, USA) according to the method supplied by Bio-Rad (Hercules, CA, USA). Briefly, competent cells were gently thawed at room temperature and then were immediately placed on ice. 2 µl of plasmid ligated DNA was mixed well with 30 µl of cell suspension in 1.5 ml Eppendorf tubes and was allowed to chill on ice. Then the cell mixture was transferred in pre-chilled sterile cuvette. Gene-Pulser apparatus was set at 25 µF and the pulse controller at 200 Ω. The cuvette was pulsed once at 1.80 kV. After pulsing the cuvette was removed and 1 ml SOB (20 g tryptone, 5 g yeast extract, 0.5 g NaCl/l) was added and the cells were gently resuspended with a pipette. $MgCl_2$ (5 ml of 2 M $MgCl_2$/l SOB) was added just before the use of SOB. The cell suspension was quickly transferred to 1.5 ml Eppendorf tubes and incubated at 37 ºC with vigorous shaking (225 rpm). The transformed cell suspension was plated on LB (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl, pH 7.5) agar (LB media plus 15g/l bacto-agar) containing 50 µg/ml ampicillin plates in a clean-bench. The plates were allowed to dry and were then sealed with parafilm. Plates were incubated at 37 ºC overnight in an inverted position.

2.14. Colony picking and sub-culturing colonies

The colonies were viewed against fluorescent light and only larger white colonies were picked (leaving the blue colonies) with sterile tooth picks. Picked colonies were transferred into 96 well plates containing 150 µl LB-ampicillin media/well (100 µl of ampicillin solution (50 mg/ml) added to 100 ml LB). After transfer the plates were sealed with adhesive 3M plastic

seals (Qiagen, Australia) and incubated at 37 ºC overnight with vigorous shaking (250 rpm). The plates were stored at 4 ºC.

2.15. Restriction digestions of DNA

Restriction digestion of PCR amplified inserts was carried out in 15 µl reaction volume at 37 ºC for three hours. The reaction contained 10 µl PCR product, 0.5 µl (4-6 units) restriction enzyme and 1.5 µl appropriate 10x restriction buffer (supplied by the manufacturer). Commonly used restriction enzyme included *Hae* III, and *Sml* I.

2.16. Labeling of DNA probes with ^{32}P

For DNA probe preparation approximately 50 ng of purified DNA insert was used. Labeling was carried out using the random oligo-priming method described by Feinberg and Vogelstein (1983). 3 µl of 9mer random primer was added to the DNA sample and the volume was brought up to 7.5 µl (using autoclaved milliQ water if required). In order to denature DNA the mixture was boiled for 8 minutes in a water bath and immediately chilled in ice for 5 minutes to allow primer annealing. 12.5 µl oligo labeling mix [40 µM d(ATP, GTP, TTP), 100 mM Tris pH 7.6, NaCl, 20mM $MgCl_2$, 200 µg/ml acetylated DNase free BSA (Fraction V, Sigma)] and 1 µl DNA polymerase (Klenow fraction) and 4 µl [α-^{32}P]-dCTP (Amersham Biosciences, Australia) was added to the mix and briefly spun. The mixture was incubated at 37 ºC in a water bath for one hour. Subsequently the mixture was cleaned using the Qiaquick PCR Purification Kit (Qiagen) according to the manufacturer's supplied protocol. Probes were boiled in a water bath for 8 minutes to denature and chilled on ice for 5 minutes before membrane hybridization.

2.17. Southern hybridization

Membranes were pre-hybridized with 10 ml pre-hybridization solution made from 500 µl sterile milliQ water, 3 ml 5x HSB (3 M NaCl, 100 mM PIPES, 25 mM Na_2 EDTA, pH 6.8), 3 ml Denhart's III solution (2 % (w/v) ficoll, 2 % (w/v) PVP, 10 % (w/v) SDS, 5 % (w/v) tetra sodium phosphate, filtered through 1MM Whatman paper), 3 ml 25 % (w/v) dextran sulphate, and 500 µl denatured salmon sperm DNA (5µg/ml). The membranes were soaked in 5x SSC and placed in hybridization bottles (Hybaid, Hampshire, UK). Pre-hybridization was carried out at 65 ºC for 4 hours to overnight. The pre-hybridization solution was replaced with freshly made warm (60 ºC) hybridization solution (same as pre-hybridization solution) and the chilled probe was added directly into the hybridization bottle. Hybridization was carried out at 65 ºC overnight. Following hybridization, membranes were washed under increasingly more stringent conditions at 65 ºC for half an hour at each wash step. All wash solutions contained 0.1% SDS, where as the SSC concentration was step wise reduced from 2x SSC to 1x SSC, 0.5 x SSC and 0.1 x SSC. Membranes were blotted dry, sealed in clear plastic bags and exposed to Fuji

Medical X-ray films (Super Hr-G30, Fuji Photofilm, Tokyo, Japan) at –80 ºC for 6 h to 14 days. X-ray films were developed in an Agfa Curix 60 automatic X-ray film processor (Agfa-Gavaert, Burwood, Victoria, Australia). In case of low signal intensity, membranes were exposed to Kodak BioMax MS film (Kodak, USA) inserted in a Kodak BioMax MS Intensifying Screen Cassette (Amersham Bioscience, UK) for several hours to 5 days.

2.18. Stripping radio labelled probes from membranes

Removal of bound radio labelled DNA probe was achieved by incubating membranes in 500 ml of boiling stripping solution (0.1% SDS, 2mM Na_2EDTA, pH 8.0) for 30 minutes or until the solution temperature equilibrated with RT. Membranes were blotted dry and sealed in plastic bags and exposed to X-ray film for 48 hours at –80 ºC to ensure that the radio-labelled probe was removed completely. Membranes were stored at -20 ºC.

CHAPTER 3

DIFFERENCE IN GENE EXPRESSION BETWEEN ROOTS OF B TOLERANT AND B INTOLERANT BARLEY GROWN AT HIGH AND LOW B CONCENTRATIONS ASSAYED USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION (SSH)

3.1. Introduction

Boron (B) toxicity in the low rainfall areas of southern Australia is recognised as a limiting factor for the productivity of cereals including barley and wheat (Cartwright *et al*., 1984, 1986, 1987). Cartwright *et al*. (1984) reported significant yield reduction in barley due to high B concentration in soil. However, primary causes of B toxicity remain unexplained.

An African barley landrace Sahara 3771 (abbreviated to Sahara henceforth in this thesis) was found to be able to tolerate a very high concentration of B in the soil (Cartwright *et al*., 1987) by restricting B accumulation. Sahara is also able to accumulate more dry matter, maintain greater relative root length and manifest lesser leaf symptom expression compared to intolerant genotypes in high B environments. However, a lack of understanding of the molecular basis of tolerance of high B concentration in Sahara has been a bottleneck to the exploitation of its genetic advantage related to B toxicity tolerance fully. Recent advances in functional genomics provide an opportunity to examine B toxicity tolerance using a holistic approach. The identification and characterization of genes differentially expressed under B toxicity in the tolerant genotype through technologies such as gene expression profiling could lead to a greater understanding of B stress recognition, signal transduction and biochemical pathways of B tolerance in plants.

Different technologies are available for gene expression profiling that have been described as 'open' or 'closed' systems (Stratowa and Wilgenbus, 1999). Open systems are not limited to available genomic resources (Velculescu *et al*., 1995, Brenner *et al*., 2000, Sutcliffe *et al*., 2000). They are generally better suited to identify uncharacterized genes within biological systems (Clarke *et al*., 2001). Open systems include technologies such as differential display (Liang and Pardee, 1992), subtractive hybridization approaches (Sargent and Dawid, 1983; Davis *et al*., 1984) serial analysis of gene expression (Velculescu *et al*., 1995) or massive parallel signature sequencing (Brenner *et al*., 2000). Closed systems such as gene arrays are well suited to high throughput screening of multiple samples (Clarke *et al*., 2001) and its use is rapidly growing (Hazen *et al*., 2003). However, although comprehensive microarrays for

Arabidopsis and rice are available, barley microarrays provide only a partial representation of the barley genome, and hence are a limitation for a comprehensive expression analysis.
In this study Suppression Subtractive Hybridization (SSH) was chosen because of its ability to efficiently detect differentially expressed rare transcripts (Diatchenko *et al.,* 1996). Other advantages of this technique include the ability to isolate genes with no prior knowledge of their sequence or identity and the use of common molecular biology methods that do not require specialized equipment or analyses (Moody, 2001). SSH has been successfully used to study differential gene expression in plants, for example in detecting the cold induced expression of a plant defensin and lipid transfer protein transcript in wheat (Gaudet *et al*, 2003), to study early transcriptional changes due to powdery mildew infection in barley (Hein *et al.*, 2004), to study genes differentially expressed during desiccation in fucoid algae (Pearson *et al.,* 2001), and to identify several genes associated with submergence in adventitious root primordia of *Sesbania* (Caturla *et al.,* 2002).

B tolerance is a quantitative trait controlled by multiple genes and their alleles**.** Regions of the chromosomes that control quantitative traits are called quantitative trait loci (QTL). Quantitative trait locus (QTL) mapping has been in wide use for about two decades. QTL mapping aims to determine the genetic loci that are responsible for variation in complex, quantitative traits. It provides the opportunity to identify the genes controlling the phenotype and to analyze their functions. Using a mapping population of 150 DH lines from a cross between B tolerant Sahara and B sensitive Clipper, Jefferies *et al.* (1999) mapped two QTL on barley chromosomes 4H and 6H associated with B exclusion. The 4H locus was also associated with the other three tolerance traits mentioned above (page 40, paragraph 2). These QTL mapping results presented an opportunity to target gene expression analysis specifically to those QTL regions in order to identify genes (candidate genes) controlling B tolerance.

However, QTL mapping has limited resolution (Paterson *et al*., 1990) and to obtain precise map information of the loci associated with the expression further experiments needs to be conducted (Tuinstra *et al.,* 1997). The map position of QTL can be confirmed in near isogenic lines (NILs) or heterogeneous inbred families (HIFs) (Loudet *et al*., 2002; Alonso-Blanco and Koornneef, 2000). NILs contain a small introgressed fragment(s), for example one or a few loci in an isogenic or identical genetic background, whereas HIFs are derived from a single recombinant inbred line that segregates a single QTL region in an inbred genetic background containing a mixture of the two parents. B exclusion QTL identified by Jefferies *et al.* (1999) could not be subsequently confirmed using NILs or HIFs (differing for B tolerance QTL) due to their unavailability.

3.1.1. Experimental strategy

Gene expression studies involving comparisons between NILs that differ for short chromosome segments offer an attractive means of identifying candidate genes for QTL located within such segments (Borevitz and Chory, 2004). As NILS have very similar genetic make-up, the background noise due to variable genome regions is expected to be minimal (Shi *et al*., 2005). NILs have been used in a number of gene expression studies, for example for pathogen related peroxidase gene expression in tomato (Mohan and Kolattukudy 1990), for low temperature tolerance related gene expression in wheat (Limin and Fowler, 2002), for mosaic virus resistance related gene expression in sugarcane (Shi *et al*., 2005), and for seed callus proliferation associated genes expression in rice (Taguchi-Shiobara *et al*., 2006). Although NILs could be useful for expression studies the resources required to develop appropriate NILs has often been a constraint.

In this expression study using SSH a novel approach was adopted to overcome the unavailability of NILs that differ for B tolerance QTL. As an alternative to NILs bulked DH lines (from a population that were used for the identification of B tolerance QTL by Jefferies *et al*., 1999) were used. It is now established that the B tolerant barley genotype Sahara accumulates less B, when excess amount of B is present in the soil or growth media, either by effectively restricting B influx or by adopting an active B efflux mechanism. Jefferies *et al.* (1999) identified a major QTL on barley chromosome 4H, which they showed to be associated with this trait. Moreover they also reported that the presence of the 4H marker allele of Sahara accounts for 64% reduction in whole shoot B concentration. The 4H locus was also found to control relative root length and dry matter accumulation in Sahara. This has been viewed as the resulting effect of a B exclusion mechanism whereby the toxic effects of high B on relative root growth and dry matter production is avoided. In addition, Jefferies *et al.* (1999) identified another QTL on 6H and also associated it with the B exclusion trait that accounted for 31% reduction in shoot B concentration. Therefore DH lines were selected on the basis of shoot B concentration of individual lines of the Clipper X Sahara (C X S) mapping population to make up B tolerant and intolerant bulks. Care was taken so that the bulks also differ by the coupled presence of Sahara allele for tolerant lines and Clipper allele for intolerant lines at 4H and 6H loci. Thus it was ensured that the individual DH lines of the tolerant and intolerant pool were identical for the exclusion trait, but had random genotypes at loci unlinked to the target region. It was expected that by bulking DH lines in this manner it would be possible to smooth out the background differences. In order to maximize the contrast in tolerance traits and minimize the background differences sufficiently, 20 DH lines were chosen from among the lowest and highest B accumulating lines to make up each of the tolerant and intolerant bulks, respectively.

The aim was to identify genes differentially expressed between the two bulks of plants that differed only in relation to their 4H and 6H B tolerance locus phenotype.

3.2. Materials and methods

3.2.1. Suppression subtractive hybridization

First described in early 1980s (Sargent and Dawid, 1983; Davis *et al*., 1984), subtractive hybridization techniques have been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Hendrick *et al*. 1984; Duguid and Dinauer, 1990; Hara *et al.,* 1991). They involve hybridization of cDNA from one population (tester) to excess mRNA from a second population (driver). Transcripts expressed in both the tester and driver would form an mRNA/cDNA hybrid whereas cDNA sequences unique to the tester would remain single stranded. Unhybridized single stranded sequences are separated using hydroxylapatite chromatography (Hendrick *et al*., 1984), avidin- biotin binding (Sargent and Dawid, 1983), or oligo $(dT)_{30}$-latex beads (Hara *et al.,* 1991) from double stranded sequences. Significant limitations of the original protocol include the requirement of large quantities of mRNA (Diatchenko *et al.,* 1996; Moody, 2001), involvement of multiple and repeated subtraction steps, being labour intensive (Diatchenko *et al*, 1996) and a bias against the identification of rare transcripts (Moody, 2001). In 1996, Diatchenko and colleagues described a PCR-based protocol termed as 'Suppression subtractive hybridization' (SSH) designed to selectively amplify differentially expressed transcripts while suppressing the amplification of abundant transcript. This method eliminated the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard subtraction kinetics (Diatchenko *et al.,* 1996). This method is currently available as a commercial kit, namely: PCR-Select cDNA Subtraction Kit (Catalog No. 637401; Clontech, Palo Alto, CA).

3.2.2. An over-view of the experimental procedures

Clontech PCR –Select™ cDNA Subtraction Kit is designed for selective amplification of differentially expressed transcripts. This kit offers to combine normalization and subtraction of transcripts in a single procedure and uses suppression PCR to efficiently select and amplify the differentially up-regulated transcripts from the test sample. A brief overview of the experimental procedure is described here according to the Clontech PCR Select™ cDNA Subtraction Kit User Manual. Figure 3.1 outlines the steps involved in SSH. In Figure 3.2 the molecular events of the SSH is presented schematically.

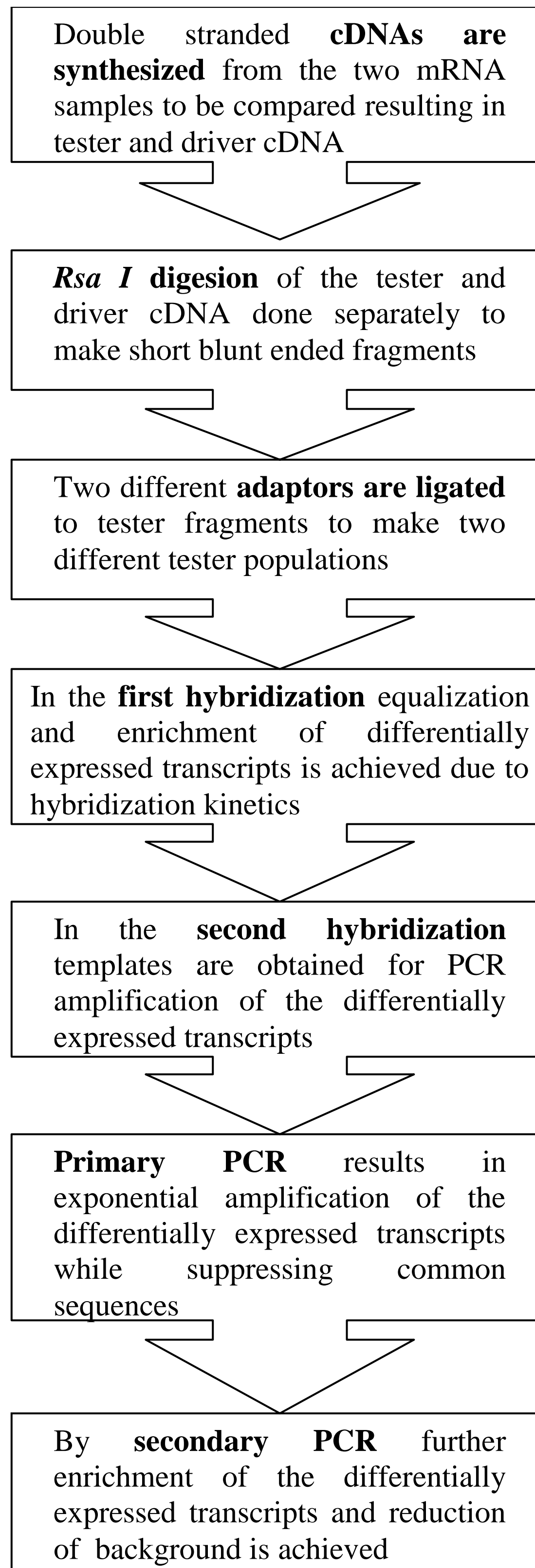


Figure 3.1. Flowchart of the steps involved in the suppression subtractive hybridization (adopted from the Clontech PCR –Select^{TM} cDNA Subtraction Kit User Manual). (Tester: cDNA population screened for differentially expressed transcripts; Driver: the reference cDNA population)

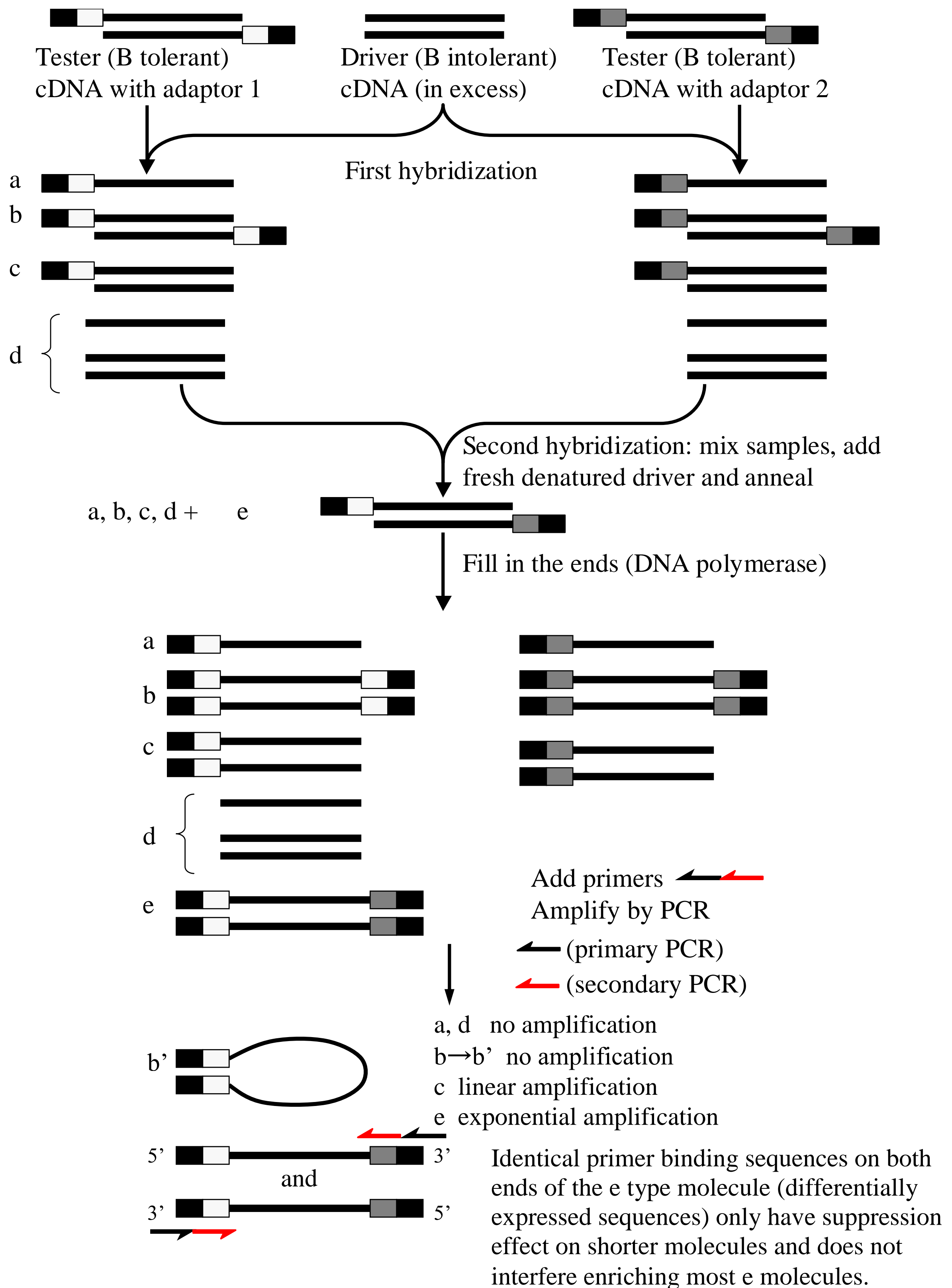


Figure 3.2. Schematic presentation of the molecular events of the SSH (adopted from the Clontech PCR –Select™ cDNA Subtraction Kit User Manual). Transcripts up-regulated in tester give rise to 'e' type molecules. Here, the solid lines represent *Rsa* I digested tester or driver cDNAs. The solid boxes represent outer sections of the adaptors and their corresponding primary PCR primer sequence. Clear boxes represent the inner section of the one type of the adaptors and corresponding secondary PCR primer sequence. Shaded box represent the inner part of other type of adaptor and the corresponding secondary PCR primer sequence.

cDNA is synthesized from two mRNA samples under comparison. The cDNA population containing differentially expressed transcripts is termed 'tester', while the reference cDNA population is known as 'driver'. Both the tester and the driver double stranded cDNAs are digested with a frequent DNA cutter, such as *Rsa* I (a four-base-cutting restriction enzyme), in order to produce blunt ended fragments. The two halves of the tester population are then ligated with two different adaptor sequences. As the adaptors lack a phosphate group at the ends, only one strand of the adaptor ligates to the 5' ends of the tester cDNAs. Both types of adaptors have a section of identical sequences that allows PCR primers to anneal to them after the ends have been filled in.

The procedure consists of two subsequent hybridisations. In the first round of hybridisation an excess of driver is added to the two different tester populations. The mixtures are then heat denatured and allowed to anneal. This leads to the formation of four different types of molecules, which include (a) single and (b) double stranded tester molecules, (c) hybrids of single-stranded tester and driver molecules and (d) single and double stranded driver molecules (Figure 3.2). Equalization of the high and low abundance sequences among the single stranded tester molecules (type a) is achieved due to the second-order of kinetics of hybridization leading to faster re-annealing of abundant molecules. At the same time differentially expressed sequences among the single stranded tester molecules are significantly enriched. cDNAs that are not differentially expressed hybridize to form hybrid of single stranded tester and driver molecules (type c).

In the second round of hybridisation the two resulting samples arising from the first hybridisation are mixed together without denaturing. In this hybridisation step remaining equalized and subtracted single stranded cDNAs (from type a) reassociate resulting in the formation of new hybrid double stranded tester molecules with different adaptor ends (type e molecule in Figure 3.2). These double stranded tester molecules represent truly differentially expressed transcripts. These molecules have different annealing sites for the secondary (nested) PCR primers on their 5' and 3' ends, which correspond to the two different adaptors. Freshly denatured driver is added again in order to further enrich the e molecules. Ends of these e molecules are filled with DNA polymerase so that they have different annealing sites for the nested primers on both ends.

The resulting mixture from the second round of hybridisation is subjected to PCR amplification, which will exponentially amplify only 'e' type molecules. Type a and d molecules are not amplified because they lack primer annealing sites. Type b molecules form a panhandle

structure as a result of the suppression PCR effect. Type c molecules having only one primer annealing site, can only amplify in a linear fashion.

In the secondary PCR further amplification of the e type molecules is achieved using nested primers. In this PCR step the background PCR products from the first round of PCR are also reduced in proportion. These enriched differentially expressed PCR products are then directly inserted into a T/A vector and subsequently subcloned.

3.2.3. Plant materials

Plant materials used for the SSH experiment were selected from a DH population of 150 lines originating from a cross between an Australian barley variety Clipper and an Algerian landrace Sahara (Jefferies *et al,.* 1999; Karakousis *et al.*, 2003). Clipper was bred at the Waite Campus and was a commercially important barley variety in Australia until the mid 80's. This variety is very sensitive to B (Jefferies *et al,.* 1999). The other parent Sahara on the other hand is outstanding in its ability to tolerate high B concentrations. These C X S DH lines were produced by the *Hordeum bulbosum* method (Islam and Shephard, 1981). The seeds of the DH lines and Clipper and Sahara parents were obtained from the Waite germplasm collection. Initially 20 plants were selected based on the shoot B concentration scores representing either tolerant or sensitive phenotype. The shoot B concentrations of the selected individual line are listed in Table 3.1.

Table 3.1. Shoot B concentrations (ppm) of the C X S DH lines used for SSH. These values were used as the basis for line selection for making up the tolerant and intolerant bulks (data obtained from Ms Margaret Pallotta, ACPFG)

Tolerant lines		Intolerant lines	
DNA #.	Shoot B Concentration (ppm)	DNA #	Shoot B Concentration (ppm)
4	93	6	466
5	94	11	471
12	120	15	510
19	97	18	404
26	87	27	477
30	96	29	456
32	102	53	496
48	91	57	440
50	103	63	450
58	120	68	442
69	92	70	499
74	88	72	513
76	93	81	412
77	85	82	578
83	91	88	427
112	95	91	419
122	94	101	407
134	94	105	558
136	93	138	461
140	92	141	440

Table 3.2 shows the genetic makeup at different B tolerance loci of the bulks. The numbers represent the percentage of lines that contained Sahara or Clipper allele at 2H, 3H, 4H and 6H loci. A full graphical representation of the genetic make-up of all loci of chromosome 2H, 3H, 4H and 6H of individual lines of the bulks is given in appendix A (Figures 1-4).

Table 3.2. Genetic make-up at different B tolerance loci of the bulks. Figures represent percentage of lines containing a Sahara or Clipper allele (data obtained from Ms Margaret Pallotta, ACPFG).

	Tolerant bulk			Intolerant bulk		
B tolerance locus	Sahara allele	Clipper allele	Unknown*	Sahara allele	Clipper allele	Unknown**
2H	55	40	5	30	70	0
3H	35	60	5	65	30	5
4H	95	0	5	10	90	0
6H	95	0	5	10	85	5

*The genetic makeup of one of the lines from among the tolerant bulk was not available. This line was later discarded from the tolerant bulk.
** Not determined

Seeds of the above lines were germinated at room temperature (Chapter 2, section 2.1). Four day old seedlings were transferred to hydroponic system housed in a growth chamber (see Chapter 2, section 2.4 for growth conditions). The seedlings were grown for ten days in hydroponic solution as described in Chapter 2, section 2.3. One half of the plants were then treated with 200 µM B. Roots were collected after 24 hours of B treatment. Scissors were treated with RNase Zap (Ambion, Austin, TX, USA) and frequently washed with RNase free water in between excisions. Hand gloves were frequently changed in order to avoid cross contaminations. Excised roots were quickly frozen in liquid nitrogen and stored at –80 ºC until RNA was extracted.

3.2.4. RNA Extraction

Total RNA was isolated using the Trizol method as described in Chapter 2, section 2.8.1. After extraction RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and randomly selected RNA samples were run on a denaturing gel as described in Chapter 2 (section 2.8.4), in order to check RNA quality. Bulking of the RNA was done by pooling equal amounts of total RNA from individual lines. In total four bulks of total RNA were made. These are from:

a) B treated tolerant lines
b) Non- treated tolerant lines
c) B treated intolerant lines

d) Non- treated intolerant lines

3.2.5. Poly(A)$^+$ RNA isolation

Two mg total RNA from each bulk was used for poly(A)$^+$ RNA isolation. Poly(A)$^+$ RNA was isolated using Poly(A) Purist™ mRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. One µl purified poly(A)$^+$ RNA was run on a denaturing gel to check its integrity. The concentration of poly(A)$^+$ RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific,Wilmington, DE, USA).

3.2.6. Subtractive hybridisation and library construction

A PCR Select cDNA Subtraction Kit (BD Biosciences, Palo Alto, CA, USA) was used to carry out cDNA synthesis and SSH according to the protocol outlined in the user manual. In short, 2 µg of poly(A)$^+$ RNA from each of the four bulks (B treated and untreated tolerant and intolerant bulks) were used for cDNA synthesis. Double stranded tester cDNAs were digested with *Rsa* I following procedure described in the Clontech PCR Select cDNA Subtraction Kit Manual. *Rsa* I digested tester cDNA was divided into two equal portions, which was ligated to adaptor 1 and adaptor 2R separately. In the first hybridisation an excess of driver cDNA was added to each adaptor ligated tester cDNA samples and the mixtures were heat denatured and allowed to anneal for an incubation period of 8 h at 68 ºC. In the second hybridization, the two samples from first hybridisation were mixed and freshly denatured driver cDNA was added. The resulting mixture was incubated overnight at 68 ºC. Following the second hybridisation step, two rounds of PCR were performed to selectively amplify and enrich the differentially expressed sequences. Subtractions were conducted between tolerant and intolerant bulks in both directions. The final PCR products were cleaned up using QIAquick PCR purification kit (Qiagen, Australia) according to manufacturer's instructions. The cleaned up DNA was cloned into pGEM T-Easy vector (Promega, Madison, WI, USA) by T/A cloning as described in Chapter 2 (section 2.12) and transformed into XL 1 –BLUE electroporation competent *Escherichia coli* cells (Stratagene, Santa Clara, CA, USA) according to manufacturer's instructions (see Chapter 2, section 2.13). Most transformant colonies were picked following a white blue selection (only white colonies were picked) and subcultured in 96-well microtiter plates as described in Chapter 2, section 2.14. In total four subtracted libraries were constructed. These were:

Forward subtractions:

I. B treated tolerant bulk (TB) – B treated intolerant bulk (ITB)
II. Untreated tolerant bulk (TC) – Untreated intolerant bulk (ITC)

Reverse subtractions:

III. B treated intolerant bulk (ITB) – B treated tolerant bulk (TB)

IV. Untreated intolerant bulk (ITC) – Untreated tolerant bulk (TC)

3.2.7. Construction of macroarrays (dot-blots)

All colonies from each subtracted cDNA library (forward and reverse subtraction) were amplified by PCR in 96-well microtiter plates. For the PCR amplifications 2 µl template DNA (culture) was used in 50 µl PCR reactions. Nested primer 1 (5' – TCGAGCGCCGCCCGGGCAGGT- 3') and 2R (5'- AGCGGTCGCGGCCGAGGT- 3') were used for amplification. PCR products were selected randomly and amplification of the inserts was checked by gel electrophoresis as described in Chapter 2, section 2.10.

The PCR products were denatured by adding NaOH to a final concentration of 0.3 M before printing onto Amersham Hybond™-N+ positively charged nylon membranes (GE Healthcare). The DNA was printed with the help of a Bio-Rad VersArray CPAS Robot. A 384 pin gridding (1 mm diameter) was used. Dots were printed by a single transfer involving 0.2 µl of liquid. After printing the DNA spots were left to dry on the membrane at room temperature. Three types of membranes were made. Type F1 and F2 were printed with I and II subtracted libraries (forward subtracted), respectively. Type R1 was printed with III and IV subtracted libraries (reverse subtracted). Type F1 and F2 membranes jointly contained 1152 spots and the R1 type membranes contained 864 spots. Twenty four of the forward subtracted clones could not be fitted on the dot-blot. The PCR products of these remaining clones were sequenced directly.

3.2.8. Southern blot hybridisation for differential screening

For differential screening forward and reverse subtracted libraries (printed on the nylon membranes) were hybridised with forward and reverse subtracted ^{32}P-labeled probes. Subtracted cDNAs were digested with *Rsa I* in order to remove the adaptor sequences before preparation of probes. After *Rsa I* digestion the subtracted cDNAs were cleaned up using QIAquick PCR purification kit (Qiagen) and DNA concentrations were quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

About 50 ng DNA was used to make each probe. The Southern hybridisations and subsequent washings were performed as outlined in Chapter 2 (section 2.17). The hybridised membranes were exposed to Fuji Medical X-ray films (Super Hr-G30, Fuji Photofilm, Tokyo, Japan). A series of exposure time was used to get uniform exposures.

3.2.9. Quantification of spot intensities

The resulting hybridisation signals were quantified by GeneTools (Syngene, USA) software. Spot detection was done manually and background corrections were done using the inbuilt auto-background subtraction method of the software. The spot signal intensities (incident values) were transferred to Microsoft EXCEL and comparisons were made between forward and reverse subtracted cDNA probe hybridisations.

3.2.10. Sequence Analysis

Differentially expressed clones were sequenced using ABI Prism BigDye Terminator version 3.1 (Perkin – Elmer, Applied Biosystems). Following $MgSO_4$/ethanol precipitation, samples were sent to the Australian Genome Research Facility, Brisbane (AGRF) for automated sequencing using an ABI 3730xl 96-capillary automatic sequencer (Perkin – Elmer, Applied Biosystems). All the sequences were subjected to nucleotide similarity searches at the National Centre for Biotechnology Information (NCBI) using BLASTn and BLASTx (Altschul, 1997). A threshold E value of e^{-30} for BLASTn and e^{-10} for BLASTx results was used. Sequences were also searched against the TIGR barley gene index (http://www.tigr.org/tdb/tgi/plant.shtml). Sequences that had over 90% similarity with barley ESTs were chosen for *in silico* comparative mapping to wheat deletion bins using http://wheat.pw.usda.gov/wEST/blast/ (Mapped ESTs in wheat).

3.2.11. Southern blot hybridisation for mapping candidates

Southern blot hybridizations and subsequent washings were carried out by methods described in Chapter 2, section 2.17. The hybridized filters were exposed to Fuji Medical X-ray films (Super Hr-G30, Fuji Photofilm, Tokyo, Japan) or Kodak Biomax X- ray films depending on the signal intensity monitored by a Mini-Monitor G-M Tube Geiger Counter (Mini-Instruments Ltd., Essex, UK).

3.2.12. cDNA preparation for qPCR analysis

To generate cDNA for qPCR analysis Sahara and Clipper plants were grown hydroponically (as described in Chapter 2, section 2.2) in a glasshouse under natural sunlight. B treatments used for Clipper were 0, 200 and 500 µM B. The Sahara plants were treated with 0, 200 and 2000 µM B. B treatment started when the plants were 2 weeks old. Roots and whole shoots were harvested 1, 7, and 14 days after the B treatment started. Total RNA was isolated from these roots and shoots using the Trizol method as described in Chapter 2, section 2.8.1. Total RNA was cleaned up using Qiagen RNeasy Kit following manufacturer's instructions. Cleaned up RNA was quantified by a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific,

Wilmington, DE, USA). First strand cDNA was prepared from total RNA using Superscript III (Invitrogen) following protocol supplied by the manufacturer. For each reaction about 2 µg total RNA was used. In addition 0.2 µl of 9mer primer was added to each 20 µl reaction.

3.3. Library construction and differential screening

3.3.1. Library construction

Two different libraries were generated from subtractive hybridization. The 'B treated library' was constructed from the subtraction of tester and driver cDNAs of B treated tolerant and intolerant bulks, respectively. A 'non-treated library' was constructed from the subtraction of tester and driver cDNAs of non- treated tolerant and intolerant bulks, respectively. In either case subtractions were carried out in both forward (tolerant – intolerant bulk) and reverse (intolerant – tolerant bulk) direction. Forward and reverse subtracted cDNAs from B treated bulks were ligated to pGEM T-Easy and cloned by *Escherichia coli* transformation. A total of 384 clones from the forward subtraction of the B treated bulks were picked for the B treated library construction. Another 780 clones were picked from the forward subtraction of the non-treated bulks for the non-treated library construction. These cloned inserts were printed on 'F1' and 'F2' membrane respectively. From the reverse subtraction 864 clones were also picked to construct a reverse subtracted library and the inserts were printed on the 'R1' membrane.

3.3.2. Differential screening for genes up-regulated by B treatment in the tolerant bulk

In order to identify genes up-regulated by B treatment in the tolerant bulk two differential screening approaches were used. In the first approach the forward subtracted (TB-ITB) library were printed on the nylon membrane, which is termed as 'F1 membrane'. For differential analysis this 'F1 membrane' was hybridised with ^{32}P labelled probes generated from the forward (TB-ITB) and reverse (ITB-TB) subtracted cDNAs obtained from the secondary PCR and the signal intensities were compared (Figure 3.3 i and ii. respectively). This approach has previously been used by Lukyanov *et al*. (1996) and Wang and Brown (1991). The rationale here is that the truly differentially expressed genes will hybridise only with the forward subtracted cDNA probes, while clones that hybridise with the reverse subtracted probes may represent background (Clontech Manual). However, it is likely that some low abundant differentially expressed genes could be missed out as background in this differential screening procedure.

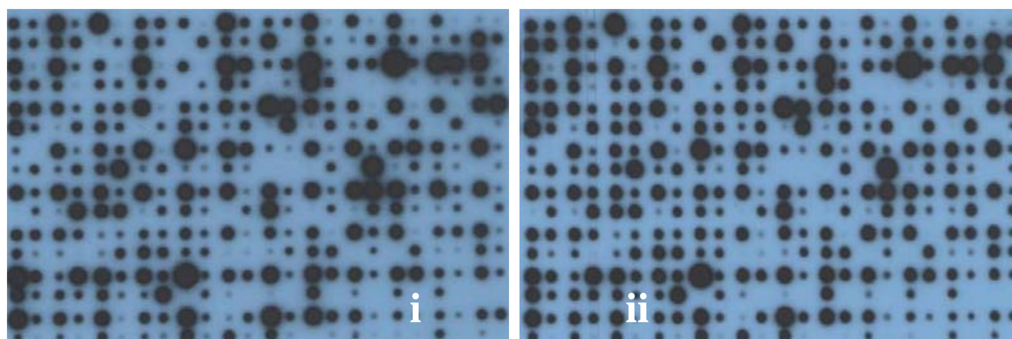


Figure 3.3. Autoradiograph of type F1 membrane hybridized with forward (i) and reverse (ii) subtracted ^{32}P labelled cDNA probes.

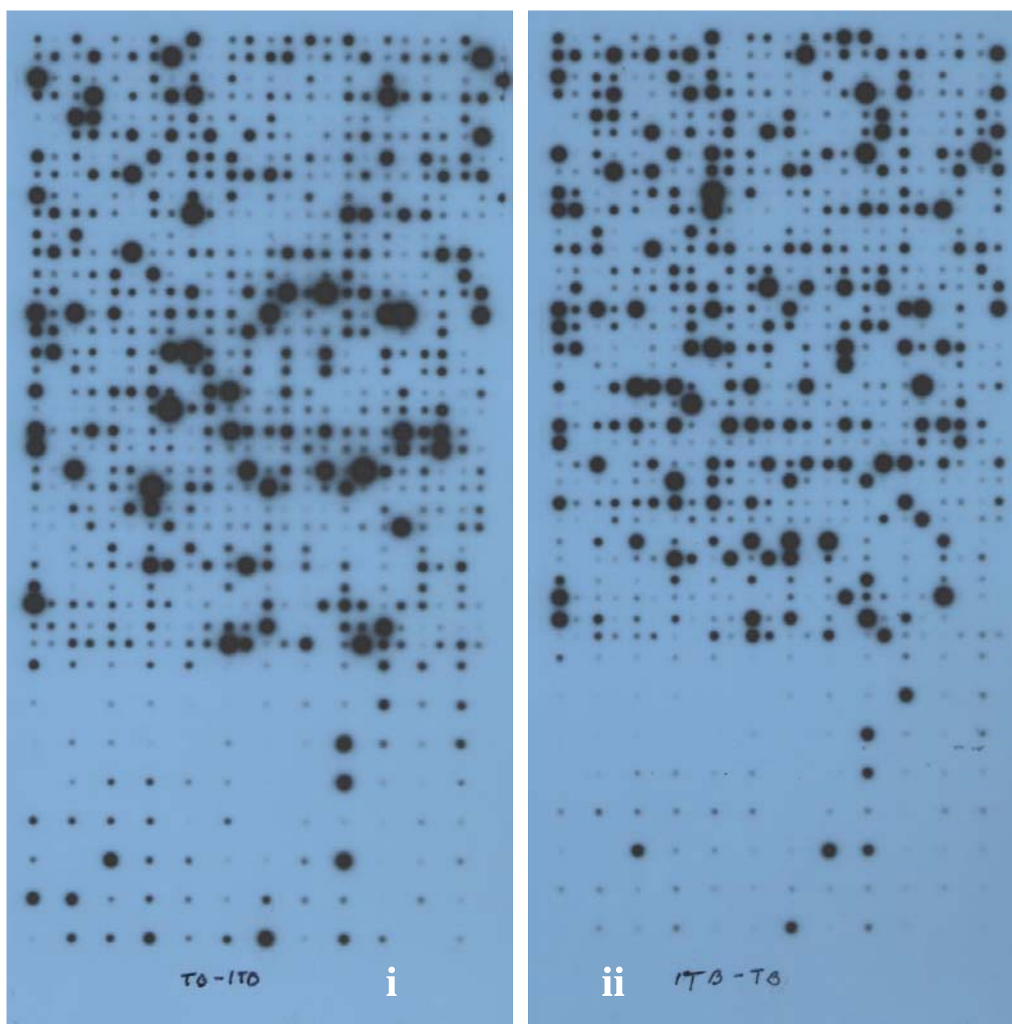


Figure 3.4. Autoradiograph of type R1 membrane hybridized with forward (i) and reverse (ii) subtracted ^{32}P labelled cDNA probes.

In the second approach the reverse subtracted library was printed onto the nylon membrane, which is termed as ‘R1 membrane’. The R1 membrane was hybridised again both with the forward (TB-ITB) and reverse (ITB-TB) subtracted cDNA probes as before (Figure 3.4 i and ii, respectively). This was done in order to capture less abundant but differentially expressed transcripts that were not captured or missed out as background in the first screening approach. The working hypothesis was that the differentially expressed genes due to B treatment would

also hybridise more strongly with the forward subtracted (TB-ITB) probes than with the reverse subtracted (ITB-TB) probes in this case as it did in the first approach. Thus by applying both of the screening approaches in conjunction it would be possible to isolate a more complete set of B inducible differentially expressed genes.

3.3.3. Differential screening for genes over-expressed in the tolerant bulk under control conditions

In order to identify genes more abundantly expressed in the tolerant bulk compared to the intolerant bulk under normal conditions the amplified inserts of the forward subtracted non-treated library (TC-ITC) were printed on the nylon membrane which is called the F2 membrane. For differential screening F2 membranes were hybridised with ^{32}P labelled probes generated from forward (TC-ITC) and reverse subtracted (ITC-TC) cDNA of the non-treated bulks (Figure 3.5 i and ii, respectively). Clones that hybridised strongly with the forward subtracted probes were selected for sequencing.

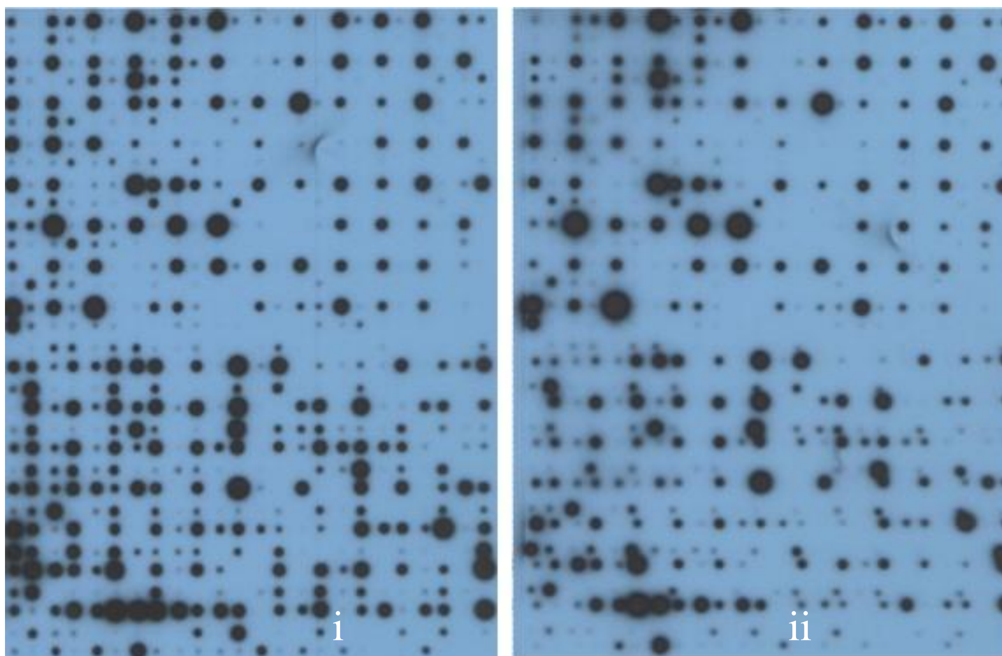


Figure 3.5. Autoradiograph of type F2 membrane hybridized with forward (i) and reverse (ii) subtracted ^{32}P labelled cDNA probes

3.4. Results

3.4.1. Verification of the selected C x S DH Lines

Plant materials used in the experiment were verified for the B tolerance phenotype. Seeds of selected DH lines were germinated at room temperature grown in a growth chamber in hydroponic solutions as described in Chapter 2, section 2.2. B treatment (1 mM H_3BO_3) started when the seedlings were two weeks old and the treatment continued for two weeks. Leaf symptoms were recorded for each line using a scale of 1 to 9 adopted from Kluge and Podlesak (1985) as described in Chapter 2, section 2.5. Shoots of these plants were analysed for B concentration by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry) analysis. The shoot B concentrations of the selected lines and their leaf symptom scores are presented in Figure 3.6. Figure 3.6 shows that the tolerant lines and intolerant lines were distinctly different in their shoot B concentrations. However there was an overlap in leaf symptom scores between the tolerant and intolerant lines. Moreover, 40% tolerant lines contained Clipper allele and 70% intolerant lines contained Sahara allele at the 2H locus, which is associated with leaf symptom expression. This is explainable as the lines were selected for the shoot B concentration and not for the leaf symptom scores. Lines represented by circled dots had inconsistent shoot B concentrations between replicates and thus were removed from the initial selection. The lines that were removed include DNA # 12, 58, 76 (from tolerant bulk) and DNA # 105 (from intolerant bulk).

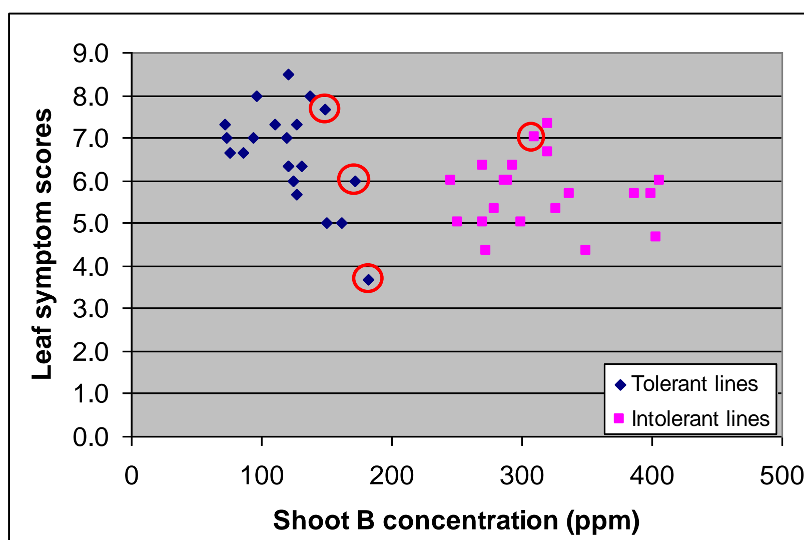


Figure 3.6. Results of the verification of the individual lines of the bulks showing shoot B concentrations and leaf symptom scores (results are average of three replicates).

Figure 3.7 shows the average shoot B concentration of the bulks as found by this verification experiment excluding the discarded lines. Figure 3.7 shows that the lines in the tolerant bulk accumulated about 3 times as much B in their shoots compared to the tolerant lines over two

weeks when grown with 1 mM B. This signifies the contrast between the two bulks in B exclusion trait.

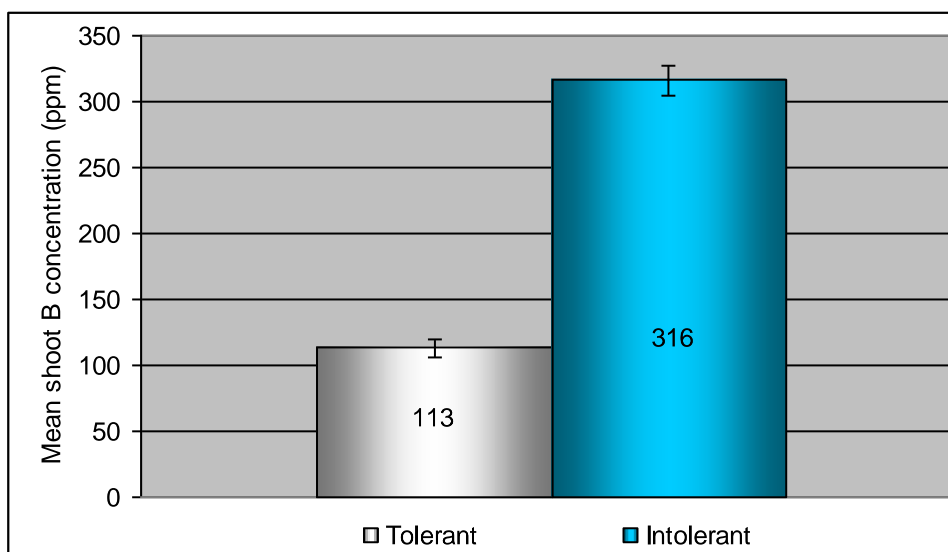


Figure 3.7. Mean shoot B concentration of the tolerant and intolerant bulks after growing the plants for two weeks at 1 mM B (the error bars represent standard errors, for tolerant bulk n=17, for intolerant bulk n=19).

3.4.2. Expression profiles from the differential screening for genes up-regulated by B treatment in the tolerant bulk

For differential screening a threshold expression ratio of 1.5 was used (conventionally a threshold expression ratio between 1.5 and 2.0 is commonly used for gene expression studies). The signal intensity data from the differential screening of the membrane F1 (Figure 3.5) and R1 (Figure 3.7) were plotted in the scatter plots shown in Figure 3.8. As expected the signal intensities were much lower in the R1 membrane compared to that in the F1 membrane. In the F1 membrane most of the clones hybridised with similar signal intensities with the forward and reverse subtracted probes falling within the 1.5 cut-off lines. The opposite was true for the R1 membrane reassuring that the second approach for differential screening of the B treated library complemented the first approach. From F1 membrane 20% of clones (with signal intensities > 1000 incidence value) showed greater than 1.5 fold up-regulation in the tolerant bulk. Only four out of 380 clones spotted on F1 membrane showed up regulation beyond 1.5 as background (hybridising strongly with the reverse subtracted cDNA probe).

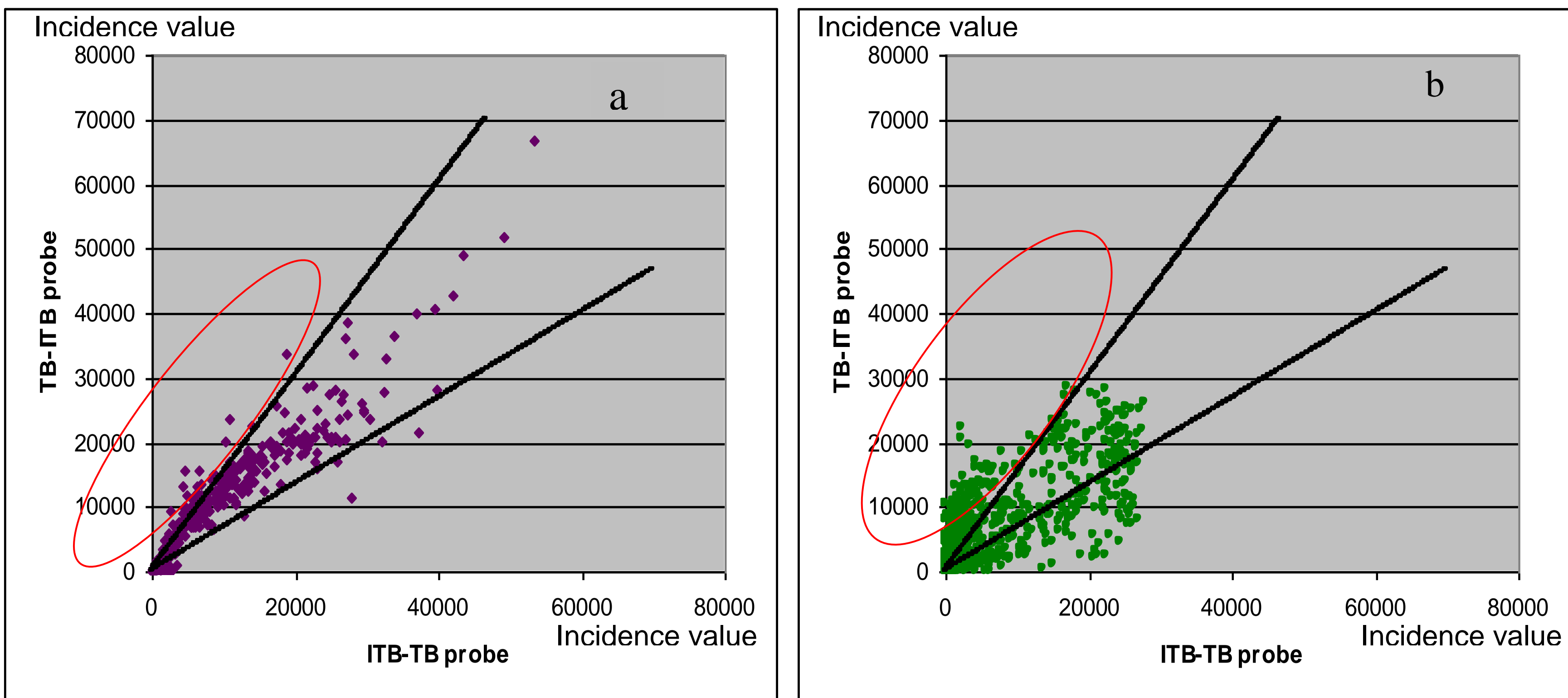


Figure 3.8. Scatter plot of signal intensities of each spot on membrane F1 (a) and R1 (b). Signal intensities measured as incidence values (using GeneTools software, Syngene, USA) were derived from the hybridisation of the reverse subtracted (ITB-TB) ^{32}P-labeled probe and forward subtracted (TB-ITB) ^{32}P-labeled probe and were plotted along the *x* and *y* axes, respectively. The diagonal lines represent 1.5 fold ratio cut-offs. The spots inside the red ellipse represent truly differentially expressed genes as they hybridize strongly with forward subtracted cDNA probe and were sequenced for further investigations. The spots below the lower diagonal line represent the background since they hybridized more strongly with reverse subtracted cDNA probes.

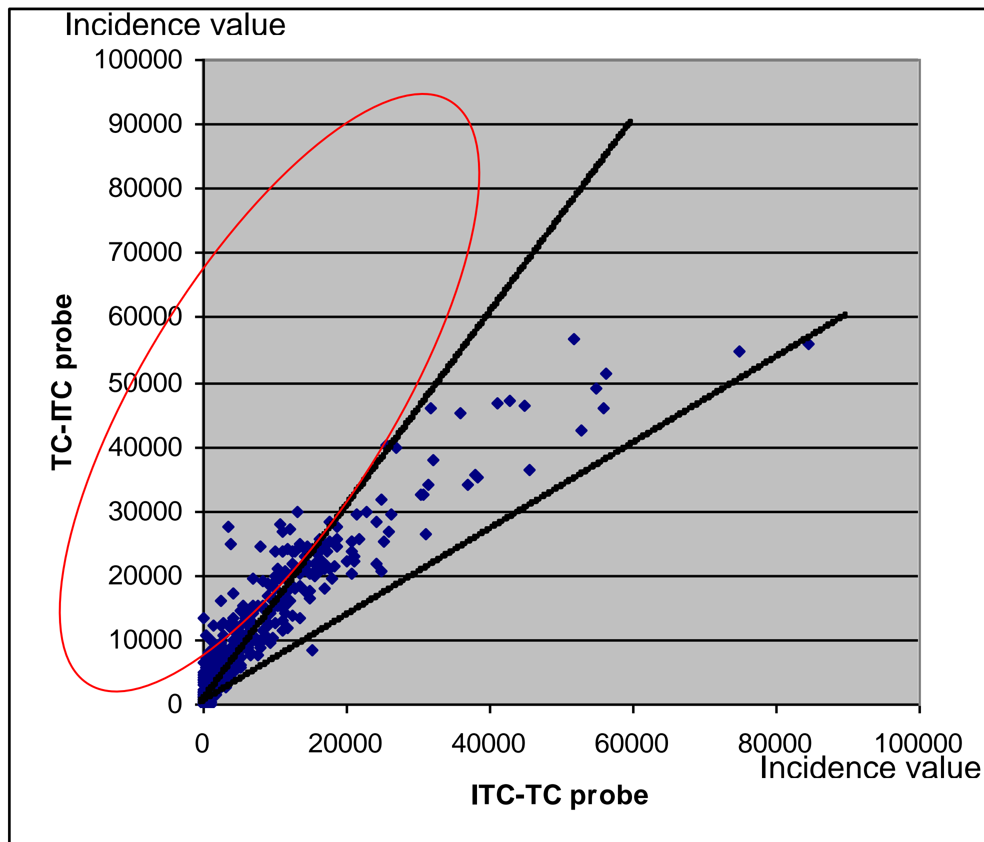


Figure 3.9. Scatter plot of signal intensities of each spot on membrane F2. Signal intensities measured as incidence values (using GeneTools software, Syngene, USA) were derived from the hybridisation of the reverse subtracted (ITC-TC) ^{32}P-labeled probe and forward subtracted (TC-ITC) ^{32}P-labeled probe and were plotted along the *x* and *y* axes, respectively. The diagonal lines represent 1.5 fold ratio cut-offs. The spots inside the red ellipse represent truly differentially expressed genes as they hybridize strongly with forward subtracted cDNA probe and were sequenced for further investigations. The spots below the lower diagonal line represent background since they hybridized more strongly with reverse subtracted cDNA probes.

This indicates the efficient enrichment of differentially expressed genes in the tolerant bulk due to subtraction. In the R1 membrane the spread of the signal intensities was much greater as the spots represented the reverse subtracted library and the reverse subtracted probes hybridised strongly with large number of spots making the background more apparent. When the hybridisation result of membrane F1 and R1 are viewed in conjunction the efficiency of the subtraction and subsequent enrichment of differentially expressed genes from the tolerant bulk becomes apparent. From R1 membrane 17% of clones (spots above upper diagonal line) hybridized strongly with B treated forward subtracted probes to give greater than 1.5 fold ratios over B treated reverse subtracted probes. On the other hand 20% clones (spots below lower diagonal line) from R1 membrane hybridized strongly with B treated reverse subtracted probes to give greater than 1.5 fold ratios over B treated forward subtracted probes. These spots represent background as they hybridise strongly with the reverse subtracted cDNA probe.

3.4.3. Expression profiles from the differential screening for genes over-expressed in the tolerant bulk under control condition

Spot signal intensities from the 'F2' membrane resulting from the hybridisation by forward and reverse subtracted probes obtained from non-treated (control) bulks were plotted in Figure 3.9. The majority of differentially expressed genes having a fold change greater than 1.5 hybridise strongly to forward subtracted probe while a few hybridise strongly to reverse subtracted probe representing background. This demonstrates the efficiency of the subtraction and subsequent enrichment of the differentially expressed genes.

In all differential screening 1.5 fold ratio cut-offs were used. Spot intensities of each corresponding spots were examined visually on both autoradiographs (i and ii of Figures 3.3, 3.4 and 3.5) while comparing fold changes to get rid of low abundance (defined by low spot intensity/ incidence value) clones.

3.4.4 BLAST search

All differentially expressed genes were sequenced and subjected to sequence similarity searches using BLASTx against the NCBI non-redundant (nr) database. The lengths of sequenced fragments ranged between 98 bp and 845 bp. Table 3.3 shows a summary of the results of the sequence similarity searches.

Table 3.3. Summary of the sequence similarity searches using BLASTx against the NCBI non-redundant (nr) database.

Search results	Clones from subtracted libraries generated from B treated plants	Clones from subtracted library generated from non-treated plats
E value < e^{-10}	70	39
No hits	80	70
Unknown proteins	12	13
Hypothetical proteins	7	3
Known proteins	136	112
Total number of clone sequenced	305	237

A full listing of NCBI BLASTx results of all sequenced clones from B treated (305) and non-treated library (237) is given in Table 1 and Table 2 in Appendix B, respectively. In both groups about eight percent of the genes were found to be redundant (22 in the B treated and 18 in the non-treated group). The most redundant sequences in both groups were those encoding for a senescence-associated protein. In the B treated group there were 13 representatives of this sequence while in the non-treated group this gene was represented 7 times. The second most redundant sequence in the B treated group was cytochrome P450 monooxygenase being represented four times. In the non-treated group the second largest representation was by sequences that code for 'band 3 anion transport protein –like protein'. It is worth mentioning that some of the sequences that failed to produce any meaningful annotation by NCBI BLASTx search were found to code for this protein when they were subjected to BLASTx search at TIGR barley gene index (HVGI). According to the BLASTx result from the TIGR (HVGI) database band 3 anion transport protein–like protein (F9L1.41 protein) is the highest redundant group among the clones from non-treated library and was represented nine times. In the B treated group this protein was represented six times as per TIGR annotation, making it the second largest redundant group.

In total 43 sequences were common to both groups. Figure 3.10 shows a venn diagram to represent the distribution of sequenced clones from the B treated and non-treated libraries.

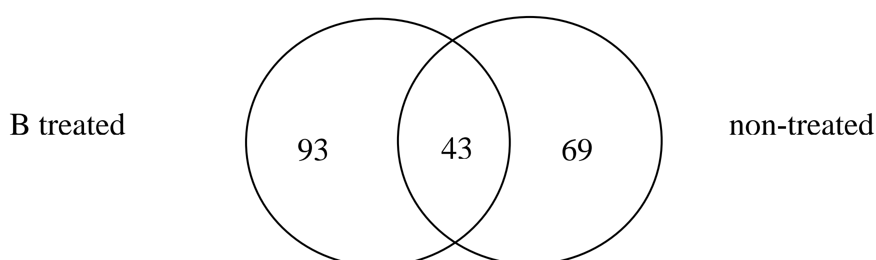


Figure 3.10. Distribution and overlap of the candidates producing known protein hits from the B treated and non-treated library

Table 3.4 shows the genes represented in both groups with their relative abundance. These gene products also constituted the list of common genes in both groups.

Table 3.4. Redundant clones from the B treated and non-treated library with their relative abundance.

NCBI BLASTx (nr) annotations of the clones	From B treated library	From non-treated library
putative senescence-associated protein [*Pisum sativum*]	13	7
putative glutathione S-transferase [*Oryza sativa*]	1	1
band 3 anion transport protein –like protein [*Oryza sativa*]	1	3
putative F-box protein [*Triticum aestivum*]	1	1
putative fructose 1-,6-biphosphate aldolase [*Triticum aestivum*]	1	1
putative AdoMet synthase 3 [*Hordeum vulgare* subsp. *vulgare*]	1	1
S-adenosyl-L-homocysteine hydrolase [*Anaeromyxobacter dehalogenans* 2CP-C]	1	1
putative dihydrolipoamide dehydrogenase [*Oryza sativa*]	2	1
CI2D [*Hordeum vulgare*]	1	1
probable cytochrome P450 monooxygenase - maize (fragment)	3	1

3.4.5. Functional categories of the clones

The sequences encoding known proteins from both groups were subjected to BLAST search at Munich Information Center for Protein Sequences (MIPS) *A. thaliana* proteome database in order to classify them according to their functions (http://mips.gsf.de/proj/thal/db/search/search_frame.html). Clones from both B treated and non-treated library fell in 11 functional classes. Only 5% (total 7) of the clones from B treated library and 6% (total 7) of clones from the non-treated could not be functionally classified. The proteins with unknown functions of the non-treated library included an ankyrin-like protein, pollen specific protein C13 precursor, ethylene responsive factor, 200 kDa antigen p200 -like protein, COP1 (Constitutive Photomorphogenic 1), cytoplasmic protein of eukaryotic origin (38.3 kD)-like and C2 domain-containing protein-like protein. The functionally unclassified genes from the B treated group included auxin-regulated protein-like protein, photosystem I P700 apoprotein A2, putative DNAJ heat shock N-terminal domain-containing protein, CREG2 (cellular repressor of E1A-stimulated genes 2) protein-like protein, TMV-MP30 binding protein 2C-like protein, APC5 (Anaphase promoting complex subunit 5) and DHHC-type zinc finger domain-containing protein–like protein. The largest functional category in the non-treated group was 'Cellular transport, transport facilitation and transport routes' comprising 19% of the differentially expressed genes. This functional category represented 14% of the genes subtracted under B treatment. Among the clones isolated from B treated library 'Metabolism' was the largest functional category accounting for 21% of the differentially expressed genes.

Figure 3.11. Distribution of the clones (producing known protein hits) from the B treated library into functional categories. Each gene was assigned to a functional category using Munich Information Center for Protein Sequences (MIPS) classification system by using BLASTn analysis with a threshold E value of -30. 'Unclassified' means that no function of the protein was assigned by MIPS. Figures indicate number of clones belonging to each functional category. The percentages of clones are shown in parenthesis.

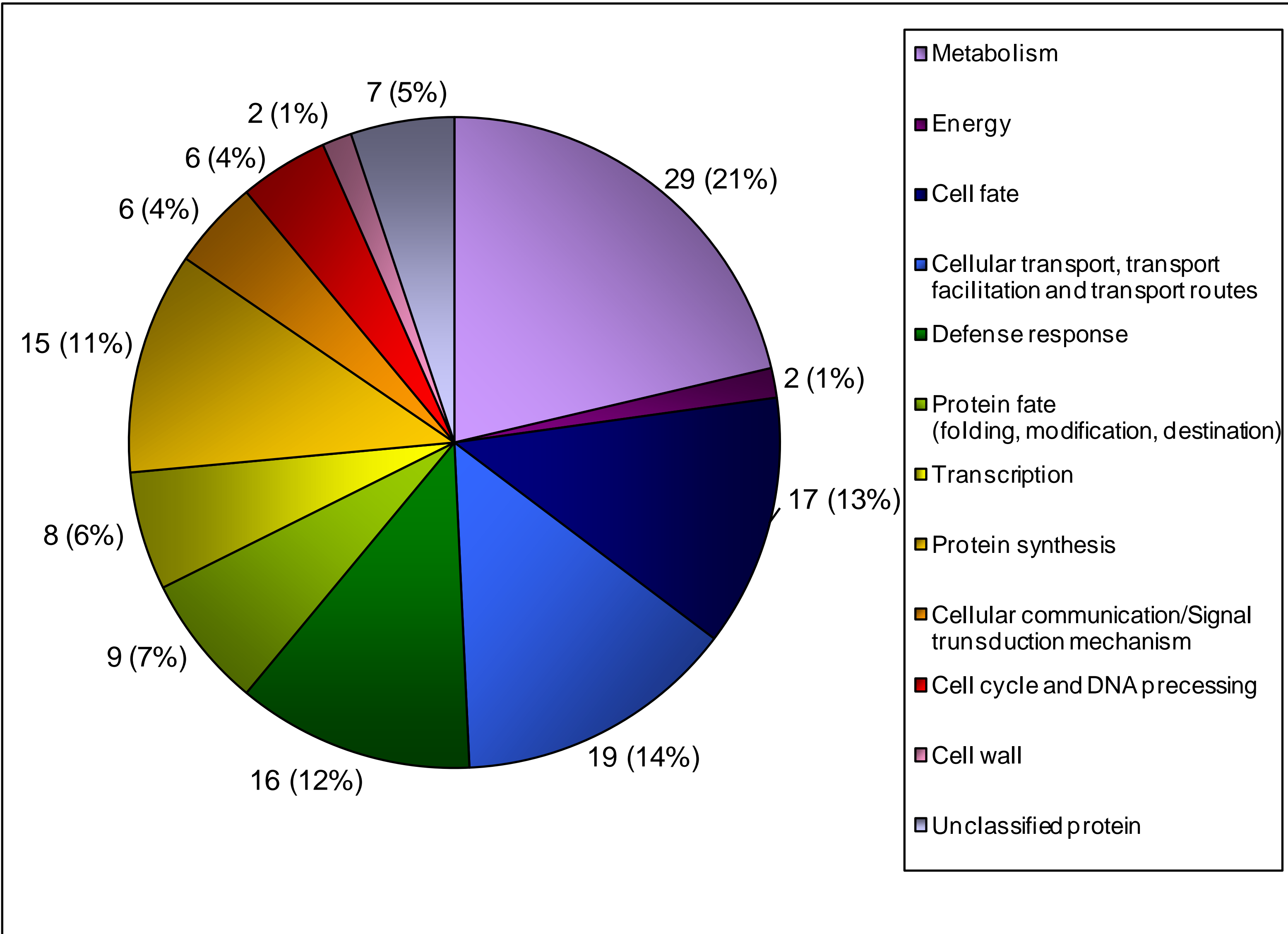
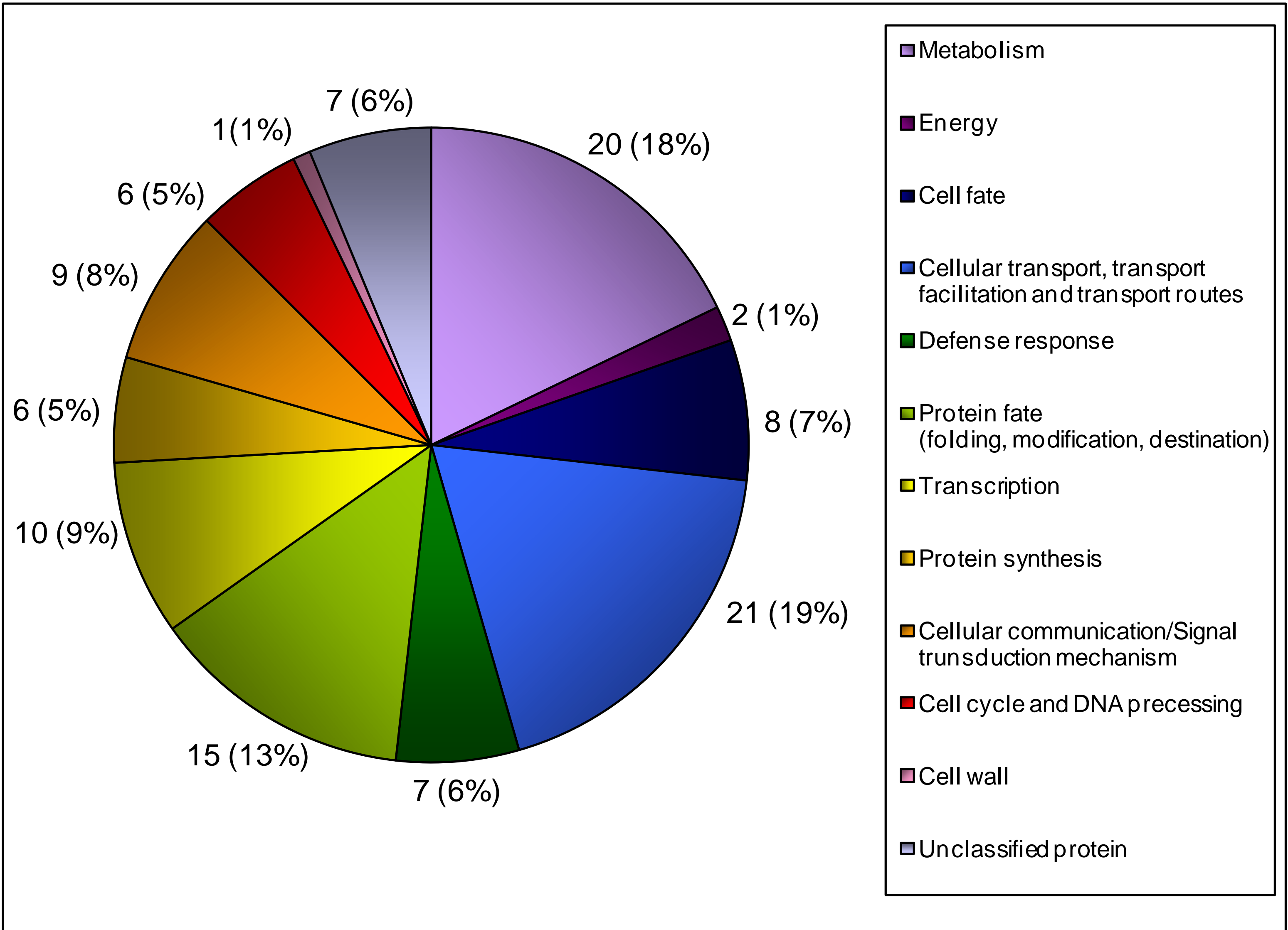

29 (21%)
2 (1%)
17 (13%)
19 (14%)
16 (12%)
9 (7%)
8 (6%)
15 (11%)
6 (4%)
6 (4%)
2 (1%)
7 (5%)
Metabolism
Energy
Cell fate
Cellular transport, transport facilitation and transport routes
Defense response
Protein fate (folding, modification, destination)
Transcription
Protein synthesis
Cellular communication/Signal trunsduction mechanism
Cell cycle and DNA precessing
Cell wall
Unclassified protein

Figure 3.12. Distribution of the clones (producing known protein hits) from the non-treated library into functional categories. Each gene was assigned to a functional category using Munich Information Center for Protein Sequences (MIPS) classification system by using BLASTn analysis with a threshold E value of -30. 'Unclassified' means that no function of the protein was assigned by MIPS. Figures indicate number of clones belonging to each functional category. The percentages of clones are shown in parenthesis.


20 (18%)
2 (1%)
8 (7%)
21 (19%)
7 (6%)
15 (13%)
10 (9%)
6 (5%)
9 (8%)
6 (5%)
1(1%)
7 (6%)
Metabolism
Energy
Cell fate
Cellular transport, transport facilitation and transport routes
Defense response
Protein fate (folding, modification, destination)
Transcription
Protein synthesis
Cellular communication/Signal trunsduction mechanism
Cell cycle and DNA precessing
Cell wall
Unclassified protein

3.4.6. *In silico* comparative mapping of the clones to wheat deletion bins

In order to further characterize the isolated clones from the libraries it is important to know the map locations of the clones. It was not possible to physically map all clones on barley chromosomes within the timeframe of this project. *In silico* mapping was adopted as a quick way of assigning genes to chromosomal segments by exploiting the synteny among the grass genomes.

Genomic synteny between barley, wheat and rice is an established fact (Gale and Devos 1998; Sorrels *et al*., 2003). The wheat barley addition lines show the genetic equivalence of wheat and barley chromosomes, where a pair of wheat chromosomes is substituted for a particular pair of barley chromosomes. Gale and Devos, (1998) observed that comparisons of the D genome of bread wheat and the barley genome demonstrates almost complete colinearity. In this context all the clones were assigned map locations on wheat deletion bin maps* by inferring the map-locations of homologous mapped wheat ESTs through BLASTn searches at http://wheat.pw.usda.gov/GG2/blast.shtml. In addition this can also provide additional support for chromosomal mapping of selected clones by Southern blot hybridisation carried out as a part of this experiment (section 3.4.8).

The sequences were first subjected to homology search at the TIGR barley gene index (HVGI) database. Sequences having more than 90% sequence identity and E value smaller than e^{-30} were then used to search against the wheat mapped EST database (GrainGenes). About 41% of the sequences could thus be assigned (with significant hit, E value smaller than e^{-30}) to bins of all seven wheat homoeologous chromosomal groups. Bin assignment of the clones on different wheat homoeologous chromosomal group is diagrammatically presented in Figures 3.13 through to 3.19. Full listing of TIGR- HVGI annotations along with NCBI BLASTx results of all sequenced clones from B treated (305) and non-treated libraries (237) is given in Table 1 and Table 2 in appendix B, respectively. Table 3.5 shows the percentages of sequences that could be assigned to different chromosomal groups of wheat from both the treated and non-treated library.

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* Mapping was done on a set of aneuploid lines having a different terminal deletion of one chromosome arm in each line. Deletion bins are defined as chromosomal segments lying between the breakpoints of two deletion lines (GrainGenes).

Table 3.5. Percentage of clones from both the B treated and non-treated libraries that could be assigned to different wheat homoeologous chromosomal groups. The total number of clones for each chromosomal group is shown in parenthesis.

Wheat chromosomal group	B treated % (Total)	Non-treated% (Total)
1	4.9 (15)	4.6 (11)
2	6.6 (20)	7.6 (18)
3	6.6 (20)	5.9 (14)
4	5.9 (18)	6.8 (16)
5	8.2 (25)	6.8 (16)
6	4.9 (15)	5.5 (13)
7	16.1 (49)	13.5 (32)

3.4.6.1. Clones bin mapped to wheat homoeologous chromosomal group 1

The lowest percentage of clones could be assigned to chromosome group 1 (4.9% and 4.6% for treated and non-treated library, respectively). The only redundant clone was Dihydrolipoamide dehydrogenase being represented three times and present in both libraries.

3.4.6.2. Clones bin mapped to wheat homoeologous chromosomal group 2

Chromosomal group 2 represented 6.6 % of the clones from B treated library and 7.6% of the clones from non-treated library. Heat shock protein cognate 70 arising from the B treated library was the one of the few redundant clones and was being represented twice. The other redundant clones were Phenylalanine ammonia-lyase and Transmembrane protein like protein (TC140847) arising from the non-treated library and each represented two times.

3.4.6.3. Clones bin mapped to wheat homoeologous chromosomal group 3

6.6% and 5.9% clones from the B treated and non-treated library, respectively, could be assigned to group 3 chromosomes. Among the redundant clones Casein kinase I, delta isoform and Heat shock protein cognate 70 was represented twice in the B treated library. Ribosomal protein L28-like protein and an F-box protein were represented twice in the non-treated library.

3.4.6.4. Clones bin mapped to wheat homoeologous chromosomal group 4

5.9% and 6.8% clones of the B treated and non-treated library, respectively, could be assigned to group 4 chromosomes. Vacuolar H^+-ATPase, Actin, Apyrase-like protein and Heat shock protein cognate 70 were among the redundant genes. Vacuolar H^+-ATPase, and Actin were present in both the library while Heat shock protein cognate 70 was present twice in the B treated library. Apyrase-like protein was present twice in the non-treated library.

3.4.6.5. Clones bin mapped to wheat homoeologous chromosomal group 5

After chromosome group 7 chromosome group 5 could be assigned with the largest numbers of genes. 8.2% from the B treated library and 6.8% from the non-treated library could be assigned to the group 5 chromosomes. There were only three genes that were represented more than once and hence redundant. One of these is Vacuolar H^+-ATPase represented twice in B treated library and once in the non-treated library. Among the others were S- adenosyl methionine decarboxylase and Heat shock protein cognate 70 both being represented twice in the B treated library.

3.4.6.6. Clones bin mapped to wheat homoeologous chromosomal group 6

After chromosome group 1, chromosome group 6 was assigned with the least number of clones from both B treated (4.9%) and non-treated (5.5%) library. Among the few redundant genes ethylene responsive protein was represented once in both libraries and heat shock protein cognate 70 was represented in the B treated library twice.

3.4.6.7. Clones bin mapped to wheat homoeologous chromosomal group 7

The highest percentage of clones could be assigned to chromosome group 7 from both (16.1%) treated and (13.5%) non-treated libraries. A majority of these clones (57%) code for ribosomal RNA (rRNA). There was also a very high degree of redundancy (75%) among these clones. Clones having similarity to *T. aestivum* mitochondrion rrna26 gene for rRNA large subunit (26S) accounted for 24.7% of the clones of which 70% came from the B treated library. Large subunit 26S ribosomal RNA gene (*Acorus gramineus*) accounted for 18.5% of the clones of which 64 % came from the B treated library. Copia-like retro-element pol polyprotein accounted for 13.6% of the clones of which 73% came from the non-treated library. Putative senescence-associated protein (*Pisum sativum*) accounted for 8.6% of the clones of which 57% came from the B treated library.

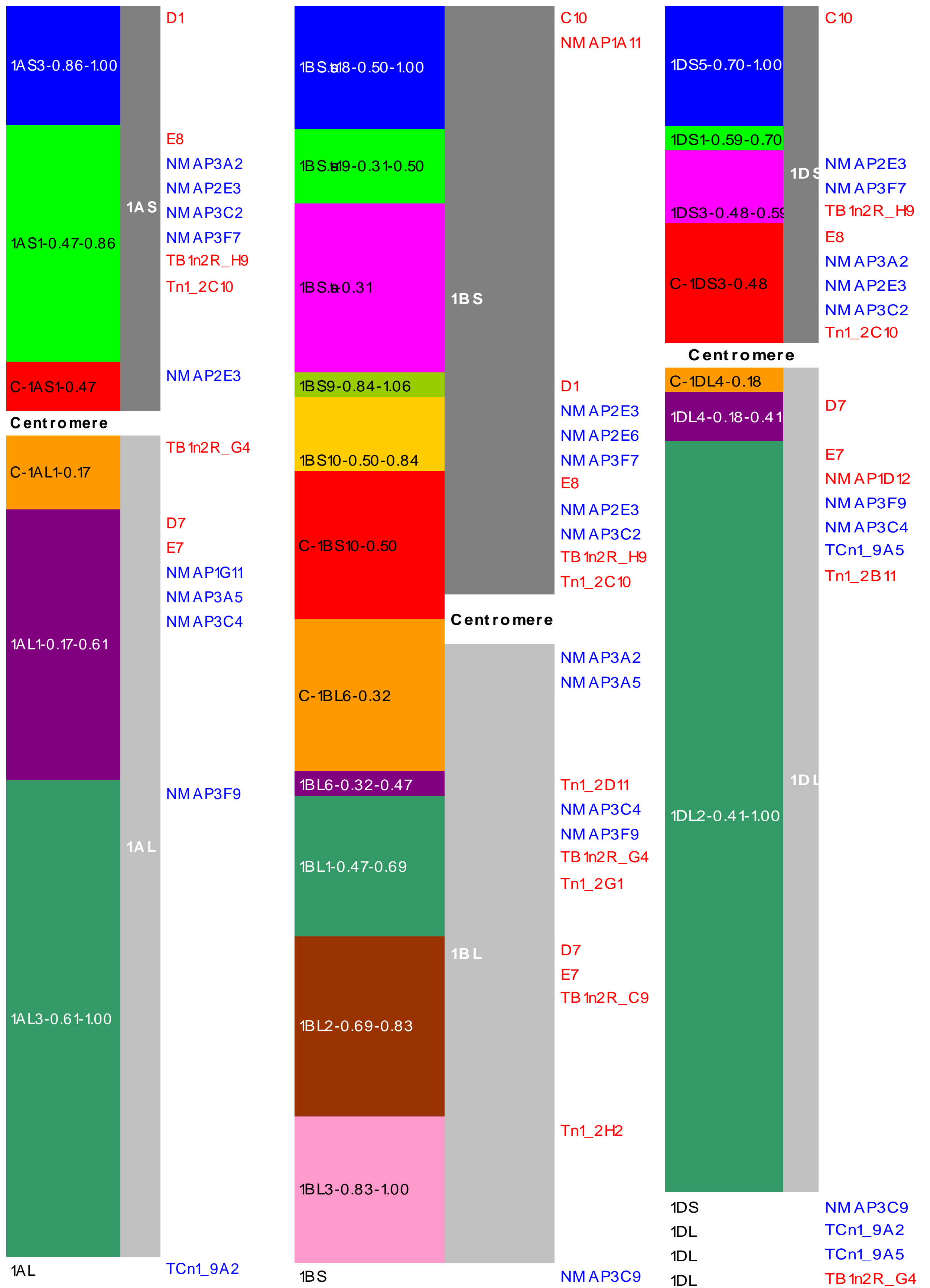


Figure 3.13. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 1.

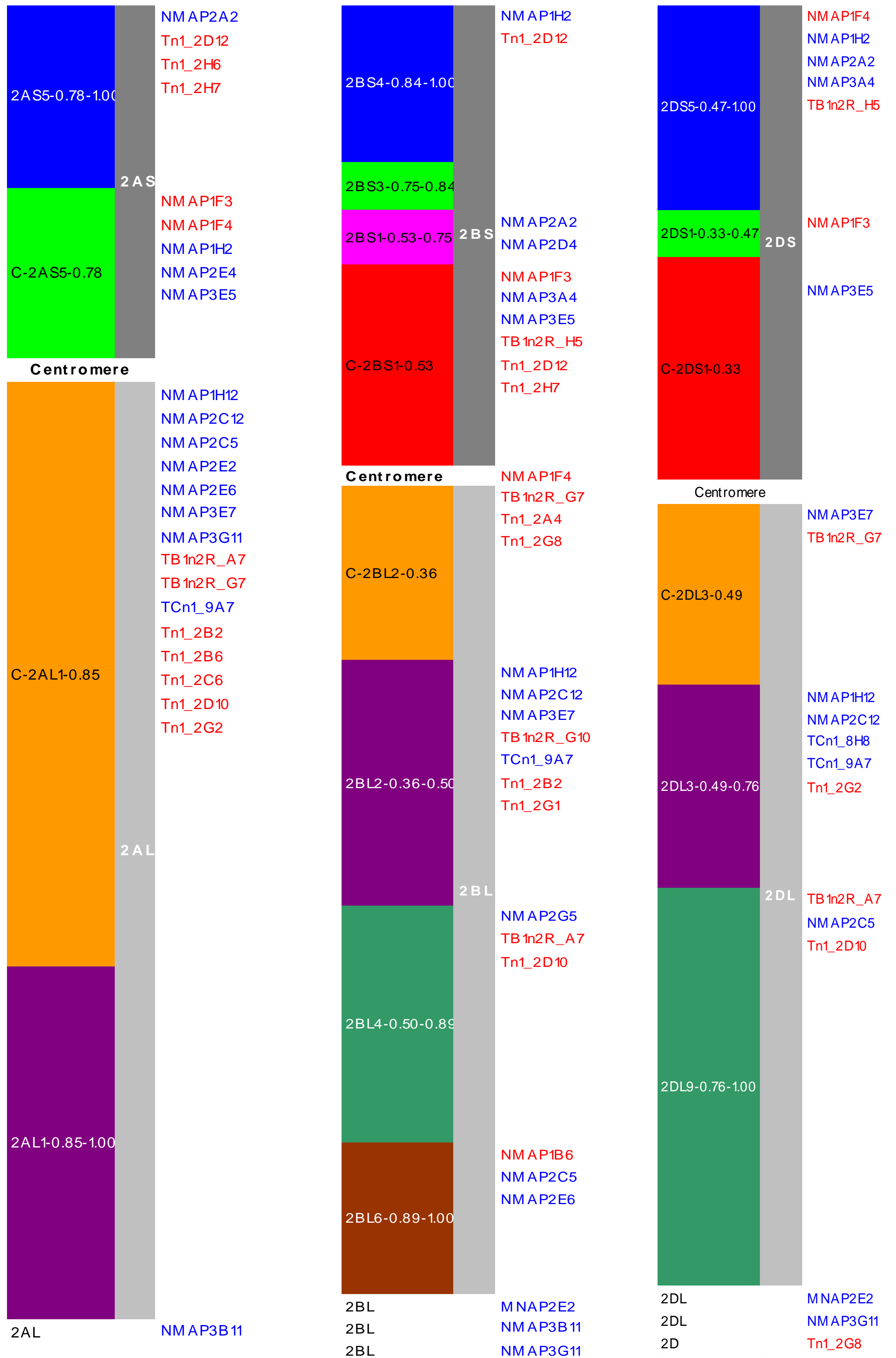


Figure 3.14. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 2.

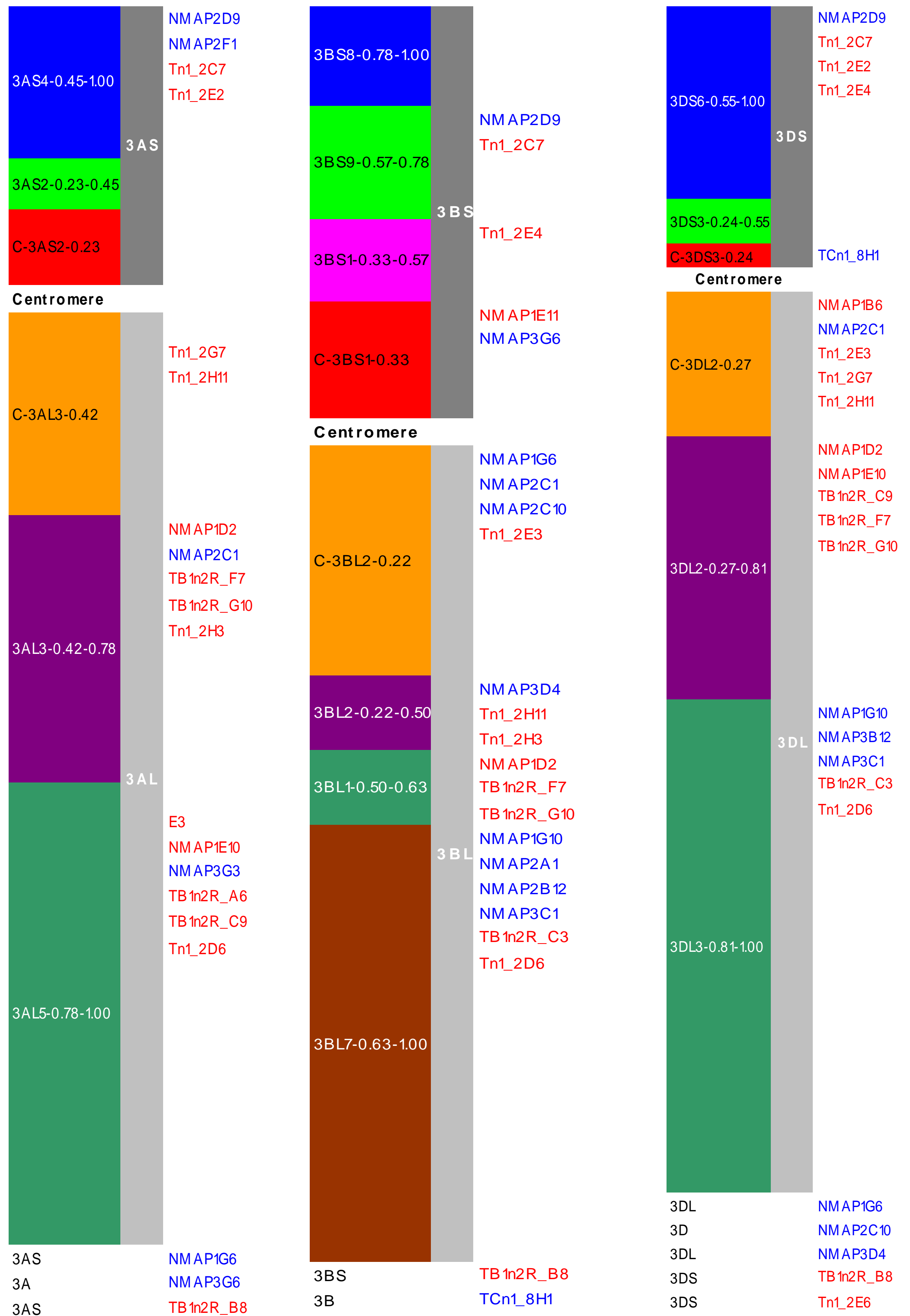


Figure 3.15. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 3.

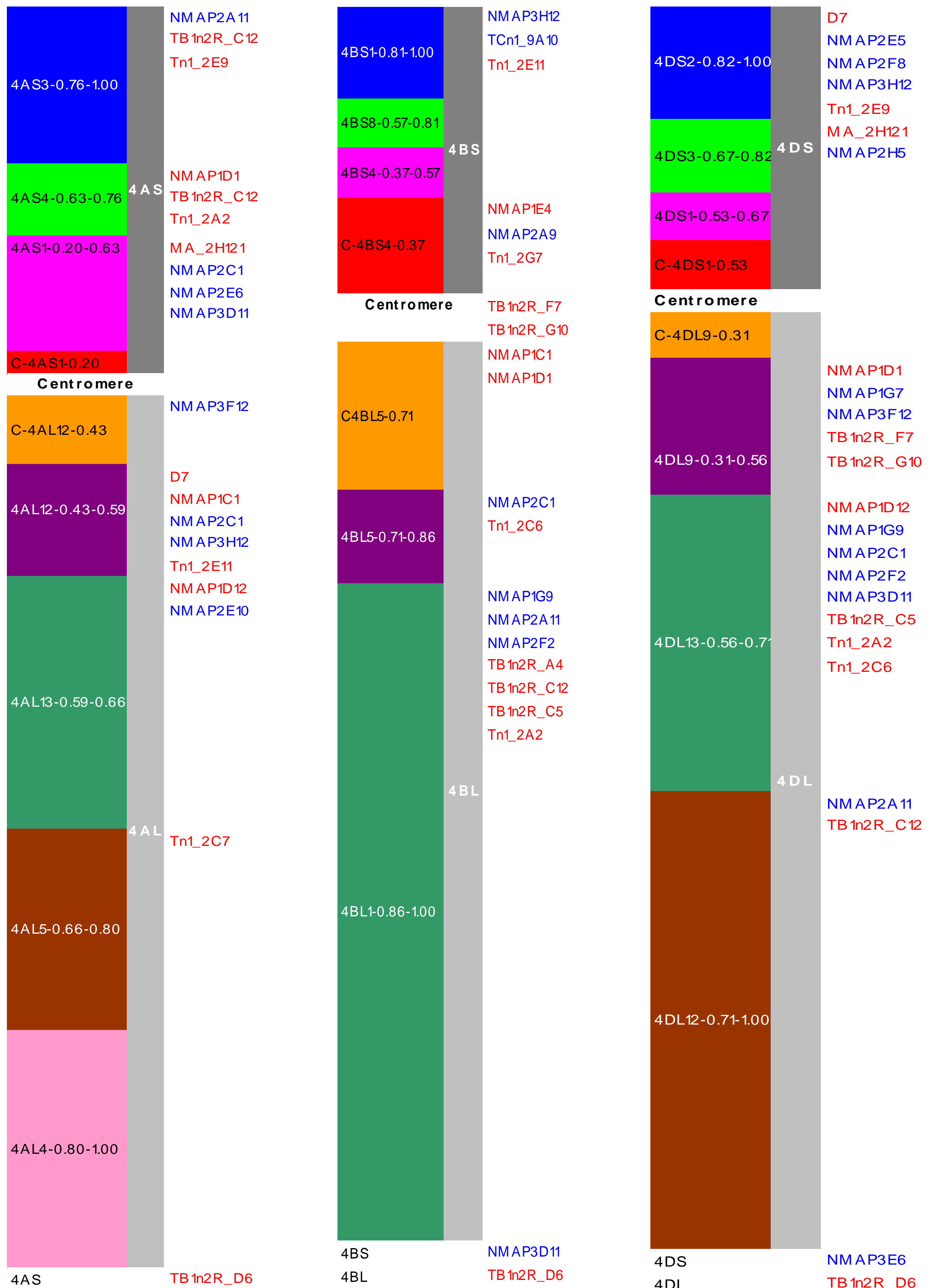


Figure 3.16. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 4.

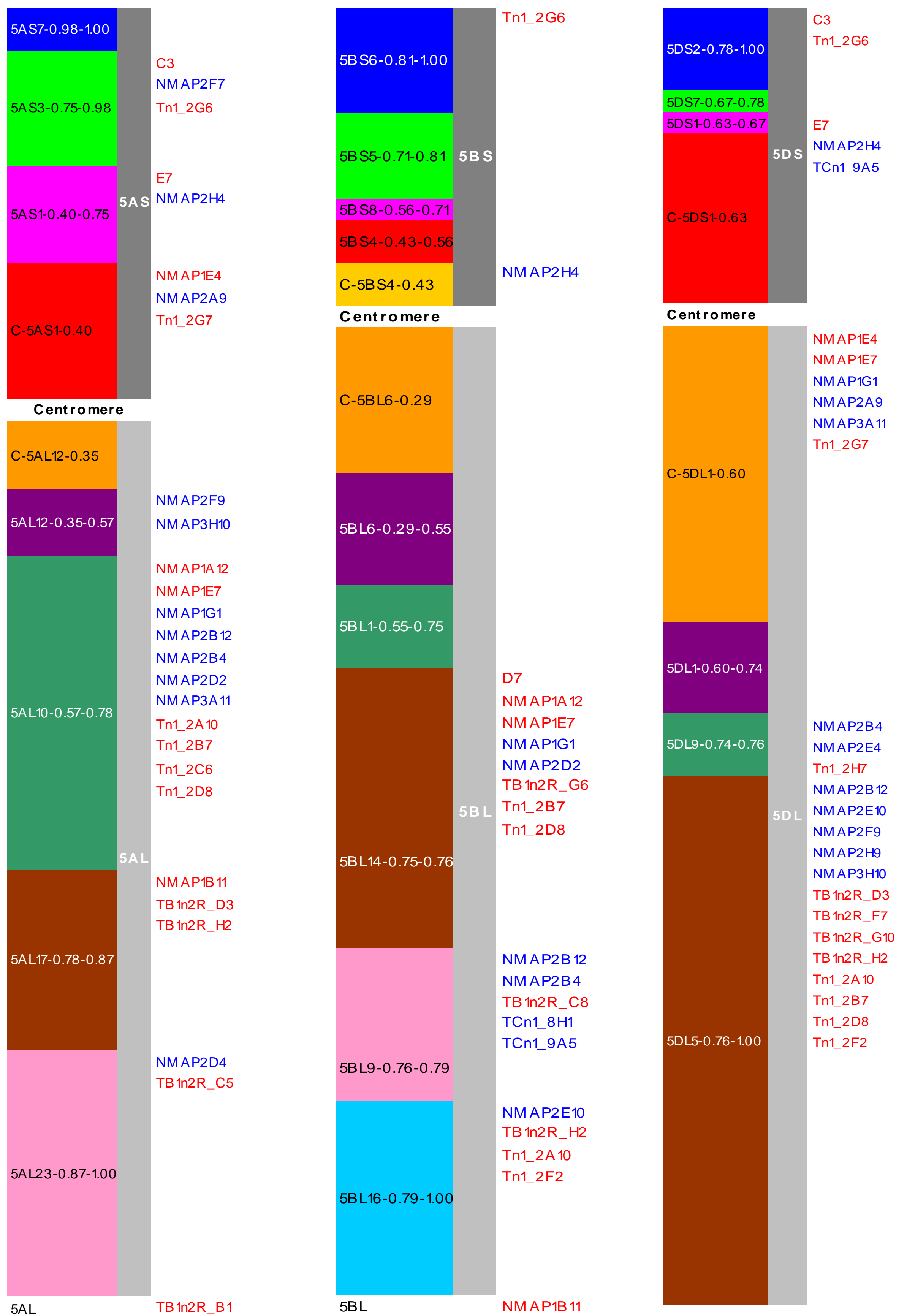


Figure 3.17. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 5.

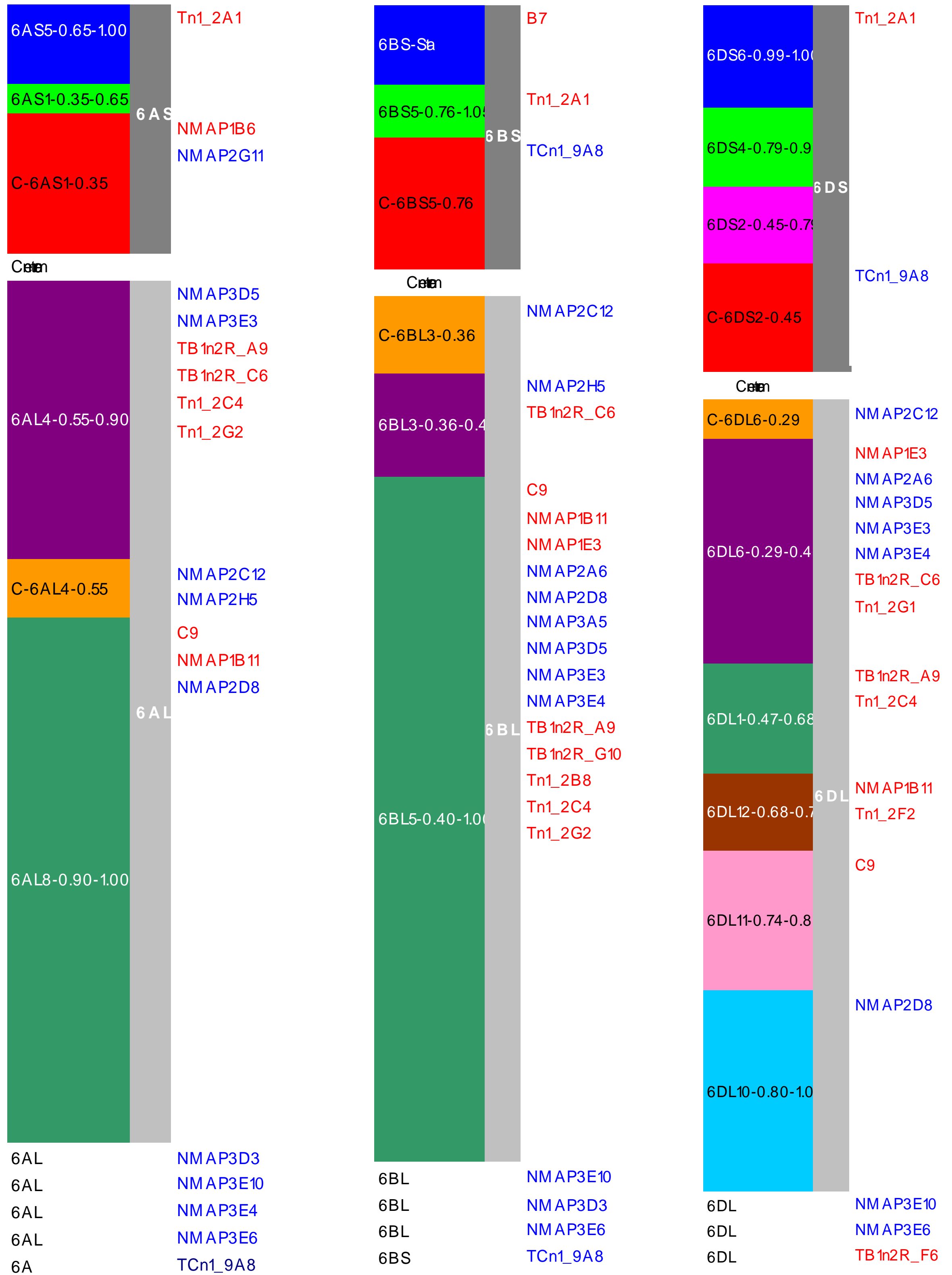
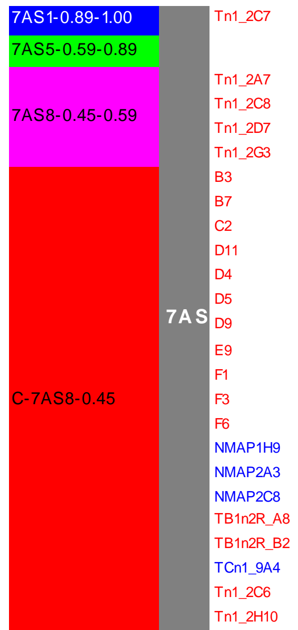


Figure 3.18. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 6.

Figure 3.19. Candidates from non-treated (blue font) and B treated (red font) library mapped on deletion bins of wheat homoeologous chromosomal group 7.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B7
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D4
D5
D9
E9
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F3
F6
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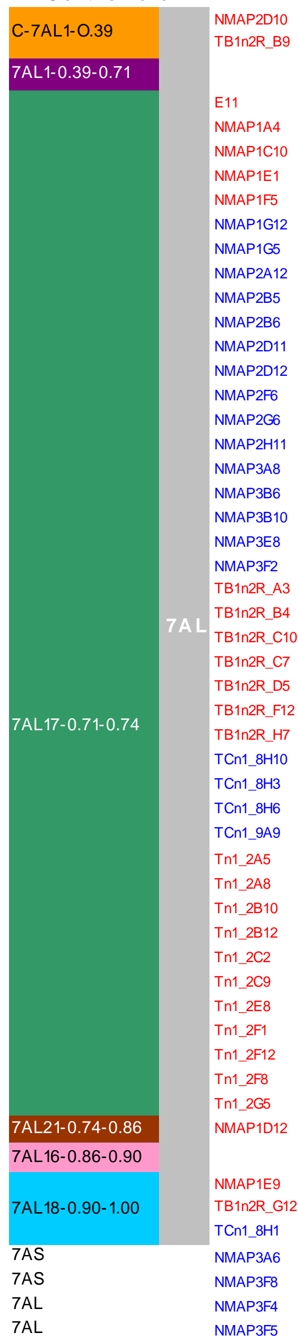
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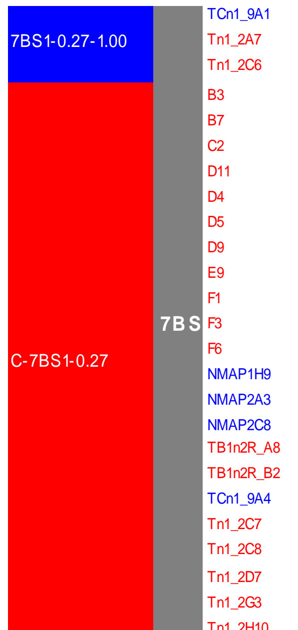
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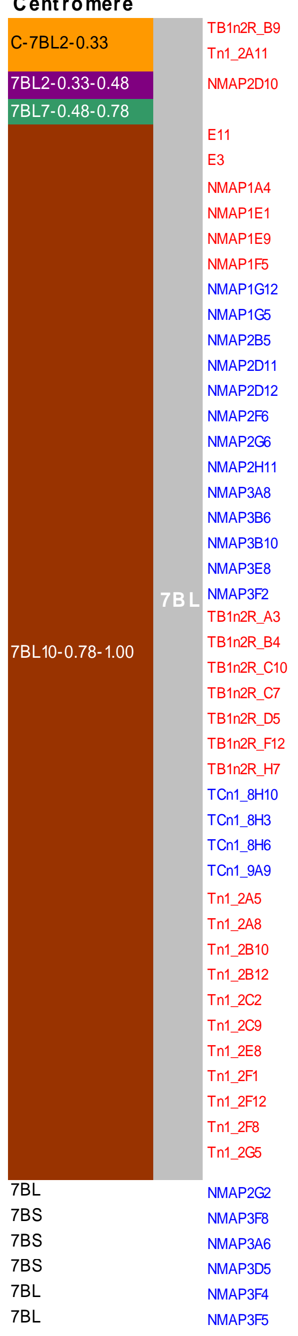
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NMAP1G5
NMAP2B5
NMAP2D11
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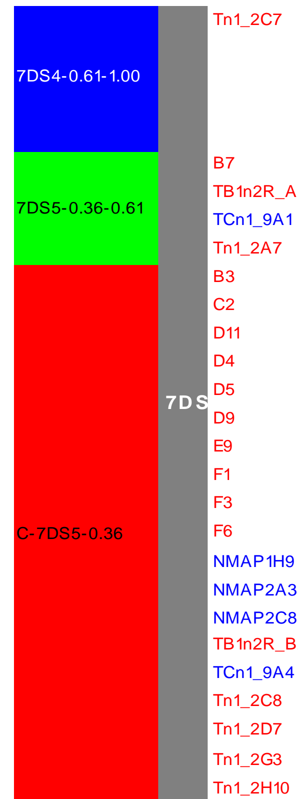
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D4
D5
D9
E9
F1
F3
F6
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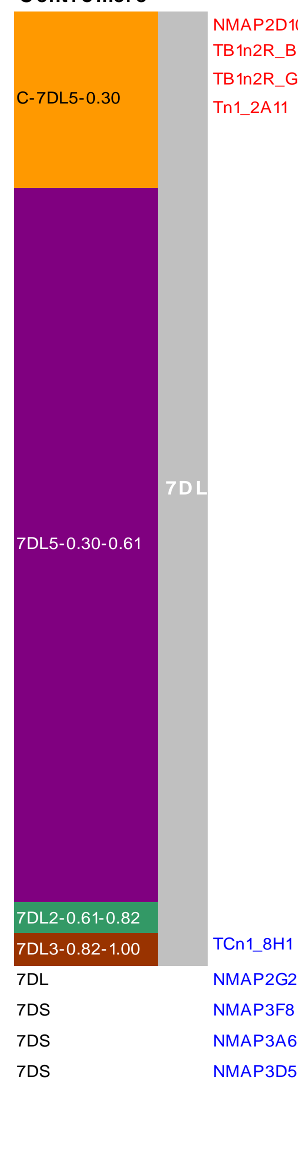
Tn1_2H10
Centromere

C-7DL5-0.30
7DL5-0.30-0.61
7DL2-0.61-0.82
7DL3-0.82-1.00
7DL
NMAP2D10
TB1n2R_B9
TB1n2R_G11
Tn1_2A11
TCn1_8H1
7DL NMAP2G2
7DS NMAP3F8
7DS NMAP3A6
7DS NMAP3D5

3.4.7. Short listing clones for further analysis

Two moderately large libraries were generated as a result of the subtractive hybridizations. The sizes of the libraries could have been minimized by picking colonies at random as opposed to picking as many colonies as possible. This was done deliberately so that the chances of capturing candidates responsible for B tolerance in the tolerant genotypes could be maximized.

Hayes and Reid (2004) showed that Sahara was able to establish a minimal tissue B concentration within 3 hours of exposure to high B concentration. They asserted that these facts indicate that gene(s) responsible for the exclusion mechanism of Sahara in order to maintain low B in tissues is constitutively expressed. However, previous experimental results also indicated that B tolerance is multi-genic in nature and several mechanisms may be involved. It is logical to assume that not all genes responsible for these mechanisms would be constitutively expressed (since all B responsive genes were not represented in the non-treated library). Genes responsible for B tolerance in barley can either be constitutively expressed or B induced (these scenarios are depicted in Figure 3.20). If the genes responsible for tolerance were constitutively expressed, in that case they could be found both in non-treated and B treated library. On the other hand if the expressions of genes responsible for the tolerance mechanism are B inducible then they can be only found in the B treated library. Therefore, the working hypothesis was that whether genes responsible for B stress tolerance are constitutively expressed or their expression is B inducible the best chance of identifying the genes responsible for B stress tolerance would be to analyse genes differentially expressed in the B treated library.

NCBI BLASTx (nr) database annotations and molecular functions (MIPS *Arabidopsis* proteome database) of the 111 non-redundant clones from the B treated library were carefully considered. Further analysis on clones related to transport, metabolism, energy, cell rescue and defence response (responses to environmental stimuli), transcription, protein fate, protein synthesis and signal transduction was carried out on the assumption that these functional categories may be more relevant to B exclusion or other tolerance mechanism than clones related to cell fate and cell cycle. Literature pertaining to various stress physiology was consulted to further narrow down analysis to 36 clones as initial candidates. Table 3.6 shows the list of these candidates with their NCBI BLASTx (nr) annotations and E values, the size of the fragments, expression ratio in differential screening Southern blot hybridization, and fold changes. Initially these clones were mapped onto barley chromosomes by Southern blot analysis using wheat-barley chromosome addition lines. It was decided that clones mapping

on chromosome 1H and 7H not to be carried forward for any further analysis as because previous QTL mapping studies (Jefferies *et al*, 2000) did not identify any QTL for B tolerance on these two chromosomes. Candidates mapping on other chromosomes were subjected to genetic mapping using C X S or other mapping populations. A tentative decision was made to carry out expression analysis using real time qPCR on candidates that locate within the QTL windows previously mapped by Jefferies *et al*. (1999). The whole process of deciding on the initial candidates is shown in the decision tree of Figure 3.20.

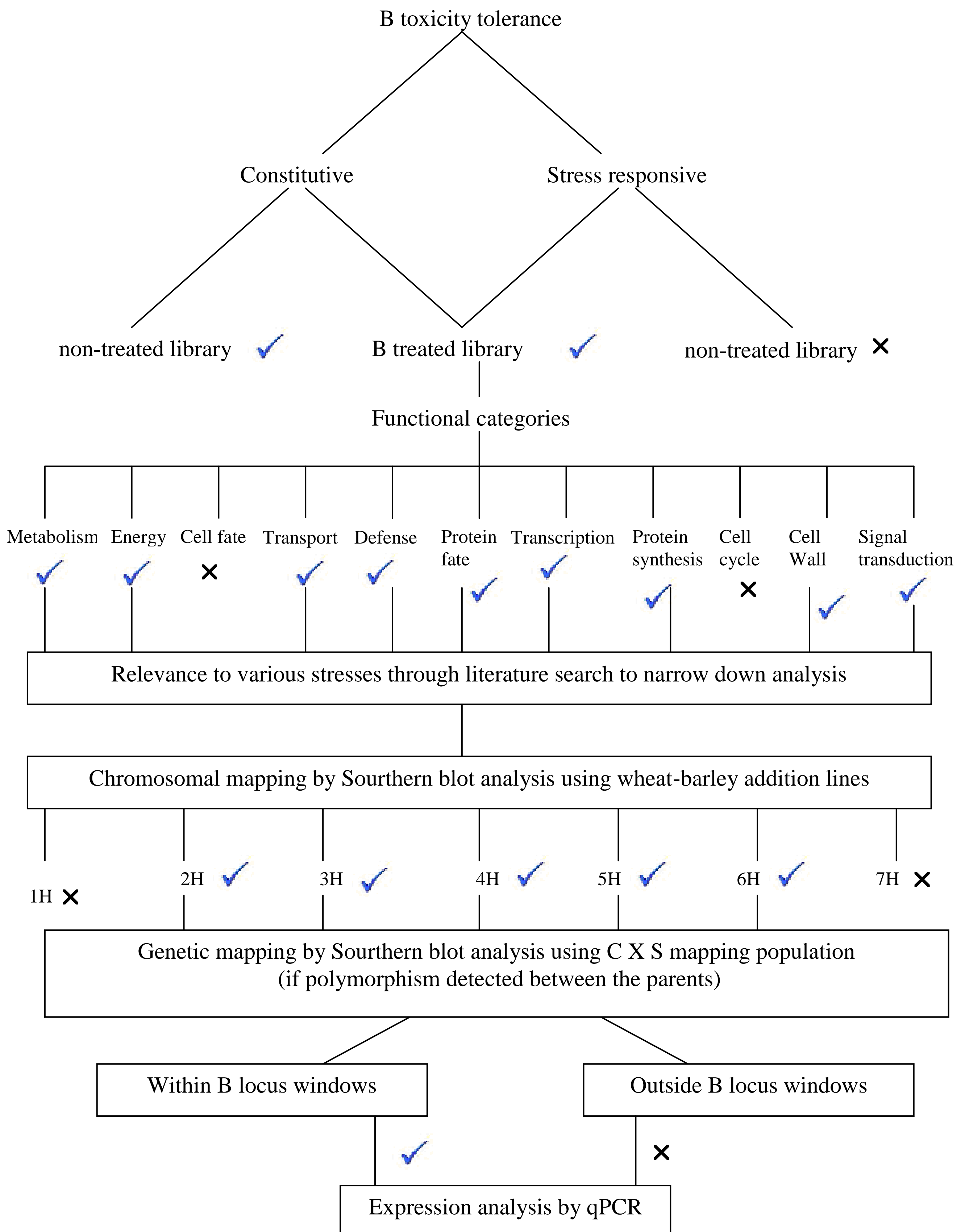


Figure 3.20. Decision tree used to shortlist initial candidates for further analysis.

Table 3.6. List of the short-listed initial candidates for further analysis.

MIPS Functional categories	Clone Name	Blast X results	E value	DNA size (bp)	Expression ratio	Fold change
Transport	B4.1	putative Potassium-efflux system protein [O. sativa]	2.00E-31	463	10136/7274	1.5
	B9	NAR2.1 [H. vulgare]	2.00E-03	267	12805/2172	5.9
	NMAP1D5	band 3 anion transport protein -like [O. sativa]	1.00E-36	282	20304/10235	2
	NMAP1D6	putative Na+/H+ antiporter [O. sativa]	2.00E-15	191	7443/4545	1.6
	NMAP1D8	outer mitochondrial membrane protein porin (VDAC)	2.00E-22	218	9515/5768	1.6
	Tn1_2A12	GONST3 Golgi Nucleotide sugar transporter [A. thaliana]	2.00E-24	358	5281/1070	4.9
	Tn1_2A4	OSJNBa0027G07.7 [O. sativa] (sugar porter activity)	9.00E-18	172	8303/5297	1.6
	Tn1_2C4	ATP/ADP carrier protein [T. turgidum]	2.00E-57	349	13598/4031	3.4
	Tn1_2D10	Oryza sativa HAK11 gene for putative K+ transporter	3.00E-30	259	15347/6153	2.5
	Tn1_2G10	O. sativa, mrp9 gene for MRP-like ABC transporter	1.00E-17	217	15852/4178	3.8
	Tn1_2G2	tonoplast intrinsic protein [O. sativa]	3.00E-12	132	7236/1398	5.2
	Tn1_2G7	putative Vacuolar ATP synthase subunit d [O. sativa]	8.00E-19	161	11360/2942	3.9
Cell wall	NMAP1D4	putative cellulose synthase catalytic subunit [H.vulgare](CesA1)	9.00E-20	182	11721/4722	2.5
Protein fate	NMAP1E9	putative beta 3 proteasome subunit [N.tabacum]	2.00E-15	163	9601/5522	1.7
Metabolism	D10	lipase-like protein [A. thaliana]	1.00E-31	273	6734/2637	2.6
	MA1C81	methionine synthase 1 enzyme [H. vulgare]	7.00E-33	281	16125/6797	2.4
	NMAP1E12	enolase [O. sativa]	2.00E-50	350	15100/9568	3.3
	Tn1_2D8	putative AdoMet synthase 2 [H. vulgare]	2.00E-18	349	100070/1555	6.5
	Tn1_2G6	sucrose-phosphatase [H. vulgare]	6.00E-38	276	8623/808	10.7
Energy	NMAP1C11	NADP malic enzyme [O. sativa]	5.00E-16	174	9652/5444	1.8
Protein synthesis	Tn1_2B7	S-adenosylmethionine decarboxylase precursor [T. aestivum]	3.00E-10	321	15738/6177	2.5
	Tn1_2E5	S-adenosylmethionine decarboxylase [O. sativa)]	1.00E-25	410	12206/2403	5.1
Cell rescue, defence(Environmental stimuli responses)	B7	dnaK-type molecular chaperone HSP70 [H. vulgare]	1.00E-60	380	10401/2849	3.7
	C2	putative monodehydroascorbate reductase [O. sativa)]	1.00E-18	255	10607/1011	10.5
	C5	putative glutathione S-transferase [O. sativa]	9.00E-26	295	10074/2213	4.6
	C9	allene oxide cyclase [H. vulgare]	2.00E-12	363	8076/3228	2.5
	NMAP1A11	RGH1A [H. vulgare]	4.00E-15	210	6842/4511	1.5
	NMAP1B5	betaine aldehyde dehydrogenase [H vulgare]	1.00E-18	176	8812/5513	1.6
	NMAP1E3	ethylene-responsive protein-like [O. sativa]	5.00E-29	234	4704/2694	1.7
	NMAP1F11	glutathione-S-transferase, I subunit [H. vulgare]	2.00E-35	251	7798/4091	1.9
	Tn1_2B4	GPX12Hv, glutathione peroxidase-like protein [H. vulgare]	1.00E-44	334	8805/2946	3
	Tn1_2C5	GRP94 homologue [H. vulgare]	2.00E-24	304	10399/5252	2.5
	Tn1_2H8	putative glycosyltransferase [O. sativa]	2.00E-37	304	11425/4849	2.4
Signal transduction	NMAP1D1	pseudo response regulator	5.00E-41	240	13339/6150	2.2
	NMAP1D3	putative MAP kinase kinase kinase [O. sativa]	4.00E-24	251	9838/4535	2.2
Transcription	B4.2	bromo-adjacent homology (BAH) domain-containing protein-like	2.00E-26	582	10136/7275	1.5
	NMAP1A8	putative CREB-binding protein [O. sativa]	1.00E-14	176	6805/3378	2
	Tn1_2H7	SUSIBA2 (susiba2) mRNA [H. vulgare]	1.00E-62	398	9996/2443	4.1

3.4.8. Chromosomal mapping of the candidates using wheat-barley chromosome addition lines by Southern blot analysis

Chromosomal mapping was achieved by using wheat–barley chromosome addition lines (Islam *et al*., 1975). The wheat-barley chromosome addition lines contain the full complement of wheat chromosomes from Chinese Spring (CS) and a pair of one of the homoeologous chromosomes of barley *cv*. Betzes. Southern hybridizations were carried out using wheat-barley chromosome addition line filters, kindly provided by Ms Margaret Pallotta. A wheat-barley chromosome addition line filter contains the genomic DNA from each of the seven wheat-barley chromosome addition lines (CS Add-1H to CS Add-7H) in each lane. In addition the filters also contain the genomic DNA of Chinese spring wheat and barley *cv*. Betzes in two additional lanes. The analysis is reliant on the identification of barley-specific RFLP bands. The barley specific band(s) will be absent in all other lanes except that containing the chromosome on which the fragment is residing. CS Add–1H wheat-barley addition line contains only the short arm of 1H (the long arm being totally absent). When a clear barley band is evident but none of the addition line lanes show the barley band, the location of fragments is considered to be on the long arm of 1H.

The results of chromosomal mapping using wheat–barley chromosome addition lines by Southern blot analysis are shown in Table 3.7. In some cases more than one band was apparent making it difficult to assign the candidates to a single chromosome with confidence. However it is not unlikely that a gene can be present on more than one chromosome. Chromosomal mapping results were compared with *in silico* mapping results described in section 3.4.6. In few cases barley chromosomal mapping did not correspond to wheat *in silico* mapping, for example clone C2 and NMAP1A11. This could be explained by the presence of cross hybridizing gene family members in barley or by genome rearrangement resulting from speciation. The chromosomal locations of the candidate genes in the rice genome was also reviewed and compared with syntenic location in the wheat genome. Recently Cho *et al*. (2006) physically mapped 1787 barley specific genes (expressed only in Betzes barley but not in Chinese Spring wheat) to barley chromosomes using wheat barley addition lines. The results of chromosomal mapping were also compared with that of Cho *et al*. (2006). These results are included in Table 3.7.

Table 3.7. Results of chromosomal mapping of the short listed candidates using wheat-barley addition lines.

Accesssion No.	Chromosomal mapping result using wheat barley addition lines	In silico mapping to wheat chromosomal groups	Rice chromosome location	Rice synteny to wheat group	Cho et al. (2006)	Genetic mapping using mapping population	Nearest RFLP /SSR markers
B4.1	7H		-				
B4.2	6H		2	6		6H	AMY(1A)
B7	6H	6BS/6AS	2	6		6H	Between bcd342 and psr627
B9	5H,6H		2	6		5H	centromeric, next to HvUXS147
C2	5H,7H	7AS/7DS/7BS	8	7			
C5	4H		3	4			
C9	5H,6H	6AL/6DL/6BL	3	4		6H	bcd269
D10	2H		4	2			
MA1C81	N/A		12	5			
NMAP1A11	5H (weak 6H band)	1BS	-				
NMAP1A8	6H		1	3		5H	between cdo749 and HvUXS147
NMAP1B5	2H/5H/6H		4	2	2H		
NMAP1C11	5H/6H		5	1			
NMAP1D1	5H						
NMAP1D3	5H		6	7			
NMAP1D4	2H,5H		7	2			
NMAP1D5	4H		-			4H	WG114
NMAP1D6	3H		8	7		3H	G9-415Db*
NMAP1D8	5H,6H		9	5	5H		
NMAP1E12	5H/6H		10	1	5H		
NMAP1E3	6H	6BL/6DL	2	6			
NMAP1E9	5H,6H		2	6	6HS		
NMAP1F11	4H/5H		1	3		5H	between cdo749 and HvUXS147
Tn1_2A12	4H,5H		3&2	4&6		4H	abc152b
Tn1_2A4	5H,6H		1	3			
Tn1_2B4	2H,6H		2	6			
Tn1_2B7	2H		4	2		2H	CD0366
Tn1_2C4	5H,6H	6AL/6BL/6DL	2	6		6H	abc163
Tn1_2C5	6H, 7H		6	7			
Tn1_2D10	2H,5H	2BL/2AL/2DL	4	2	2H		
Tn1_2D8	5H	5BL/5DL/5AL	4	2			
Tn1_2E5	5H		9	5		5H	PSR128
Tn1_2G10	2H,5H,4H,7H		-			2H	WG516
Tn1_2G2	5H,6H	6Bl/6AL/2DL/2AL	2	6	6HL	6H	Bmag210 Bmag9, EBmac787 also abc163
Tn1_2G6	5H	5DS/5BS/5AS	1&2	3&6			
Tn1_2G7	3H	3DL/3AL	1	3	3H		
Tn1_2H7	N/A	C-2AS/C-2BS	7	2			
Tn1_2H8	7H (weak 6H band)		-				

3.4.9. Genetic mapping of candidates using mapping populations by Southern blot analysis

Fragments were mapped genetically to assign a relative location on a particular chromosome. This can be achieved when a suitable polymorphism is detected in the parental lines of a pre-existing mapping population. Polymorphisms can be detected using either RFLP-based Southern blot hybridization or PCR-based methods. Once a suitable polymorphism is detected members of the mapping population are scored for the polymorphic bands. Subsequently comparison against scores for all other mapped polymorphisms is used to determine the most likely relative location for the new polymorphism using computer software (MapManager). The results of genetic mapping of candidates are shown in Table 3.7.

3.4.9.1. Candidates mapped onto 4H QTL

Two candidates were mapped within the QTL region of 4H. These are NMAP1D5 (band 3 anion transport protein –like protein) and Tn1_2A12 (GONST3 Golgi Nucleotide Sugar transporter [*A. thaliana*]). The map locations of these two candidates are shown in Figure 3.21. NMAP1D5 was mapped close to the RFLP marker WG114. This marker was known to have the highest association (Stat[§] 47.8, P=0[¤]) with the B toxicity tolerance trait including B exclusion trait controlled by the 4H QTL. NMAP1D5 has been found to have even higher association with the B exclusion trait (Stat 47.9, P=0) controlled by 4H QTL and it co-segregates with the trait among 150 DH lines of the CXS mapping population. Tn1_2A12 was mapped close to the RFLP marker ABC152(B). This locus is at the border of the QTL and is outside the significant region of the QTL.

[§] Stat denotes the likelihood ratio statistics (LRS) for the association of the phenotype with this marker locus.

[¤] P denotes the probability of such an a strong association happening by chance

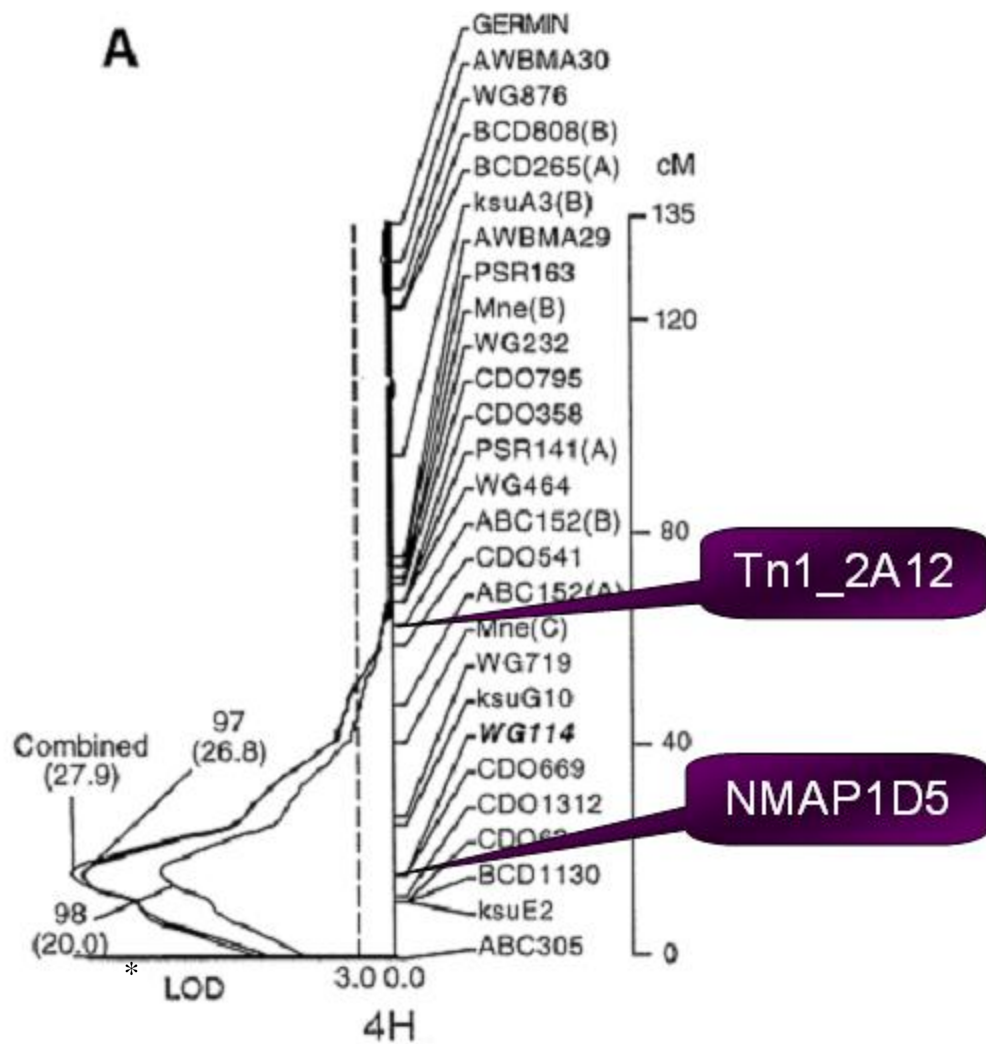


Figure 3.21. Candidates genetically mapped on the 4H B tolerance QTL (Jefferies *et al.*, 1999).

LOD score is the Log of the odds supporting linkage of two loci. A LOD score of 3.0 or greater is accepted as significant evidence of linkage between a marker and a gene controlling a phenotype.

3.4.9.2. Candidates mapped onto 2H QTL

Two candidates were mapped to the QTL region of chromosome 2H. These are Tn1_2B7 (S-adenosylmethionine decarboxylase precursor [*Triticum aestivum*]) and Tn1_2G10 (mrp9 gene for MRP-like ABC transporter [*Oryza sativa*]). The map locations of these two candidates are shown in Figure 3.22. The nearest RFLP marker for Tn1_2B7 is CDO366 which lies in the significant region of the previously mapped 2H QTL. RFLP marker CDO370 has previously been identified to have the highest association with the B leaf symptom score. Tn1_2G10 was mapped at the border of the QTL in a non-significant region. The nearest RFLP marker was identified to be WG516.

3.4.9.3. Candidates mapped onto 6H QTL

Five candidates were successfully mapped to the QTL region of chromosome 6H. These are B4.2 (bromo-adjacent homology (BAH) domain-containing protein-like protein), Tn1_2C4 (ATP/ADP carrier protein [*Triticum turgidum]),* Tn1_2G2 (tonoplast intrinsic protein [*Oryza sativa*]), C9 (allene oxide cyclase [*Hordeum vulgare*]) and B7 (dnaK-type molecular chaperone HSP70 [*Hordeum vulgare*]). B4.2, Tn1_2C4 and Tn1_2G2 were mapped within the significant region of the 6H QTL. The map locations of this group of candidates are shown in Figure 3.23.

B4.2 was mapped close to the RFLP marker AMY (1A). The precise map location is shown in Figure 3.24. AMY (1A) marker was known to have the highest association (Stat 20.1, P=0.00001) with the B exclusion trait controlled by the 6H QTL. B4.2 has been found to have even higher association (Stat 21.2, P=0) with the B exclusion trait controlled by this QTL and it co-segregates with the trait among 150 DH lines of the CXS mapping population.

Tn1_2C4 and Tn1_2G2 were mapped between AMY (1A) and PSR 167. C9 was mapped in the proximity of the RFLP marker BCD269. Although this locus is outside the significant region of the QTL, but it is characterised by an abrupt increase in the LOD score* which is slightly smaller than 3.0. B7 was mapped near the border of the QTL and located between the RFLP markers pSR627 and BCD342.

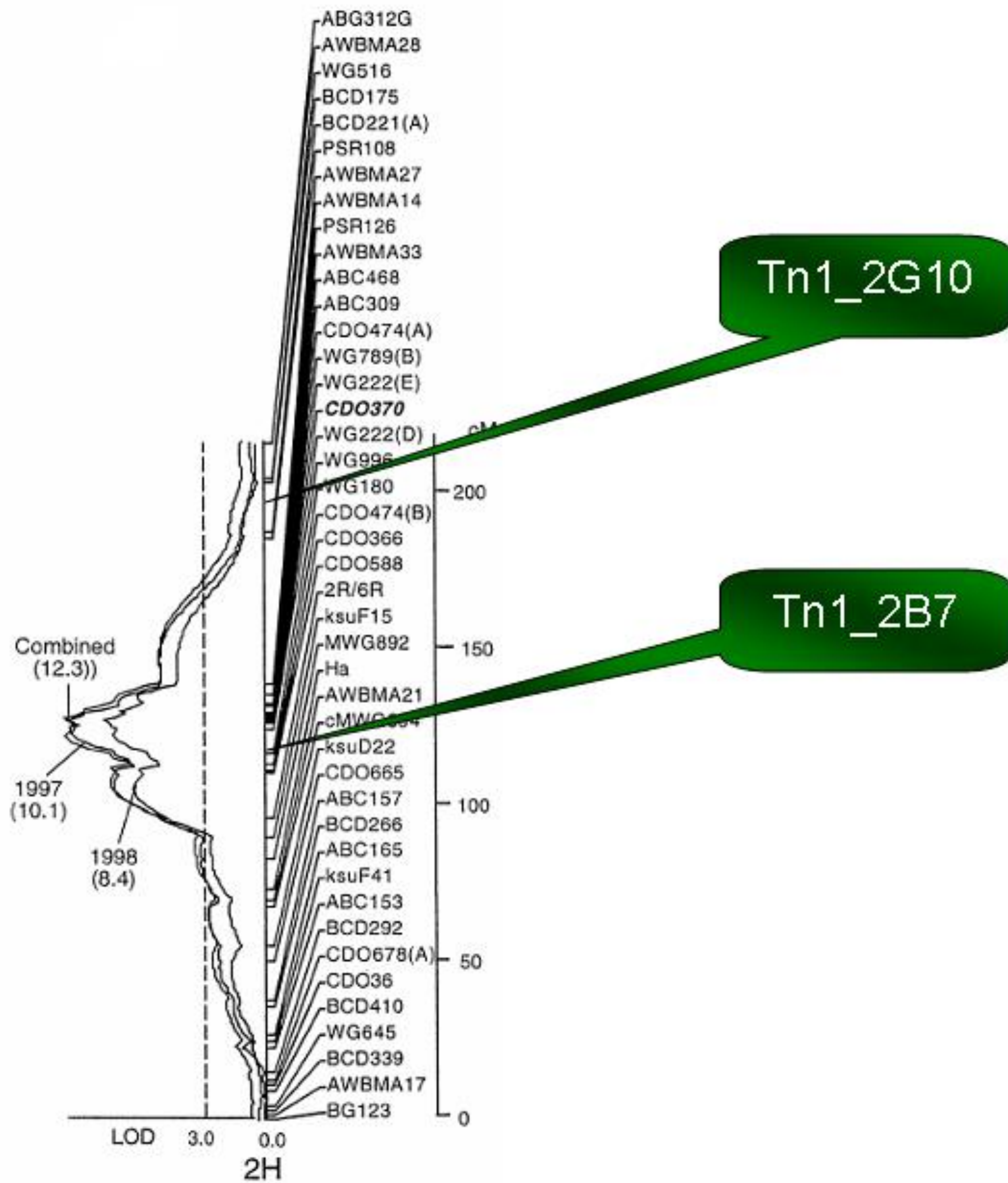


Figure 3.22. Candidates genetically mapped on the 2H B tolerance QTL (Jefferies *et al*., 1999).

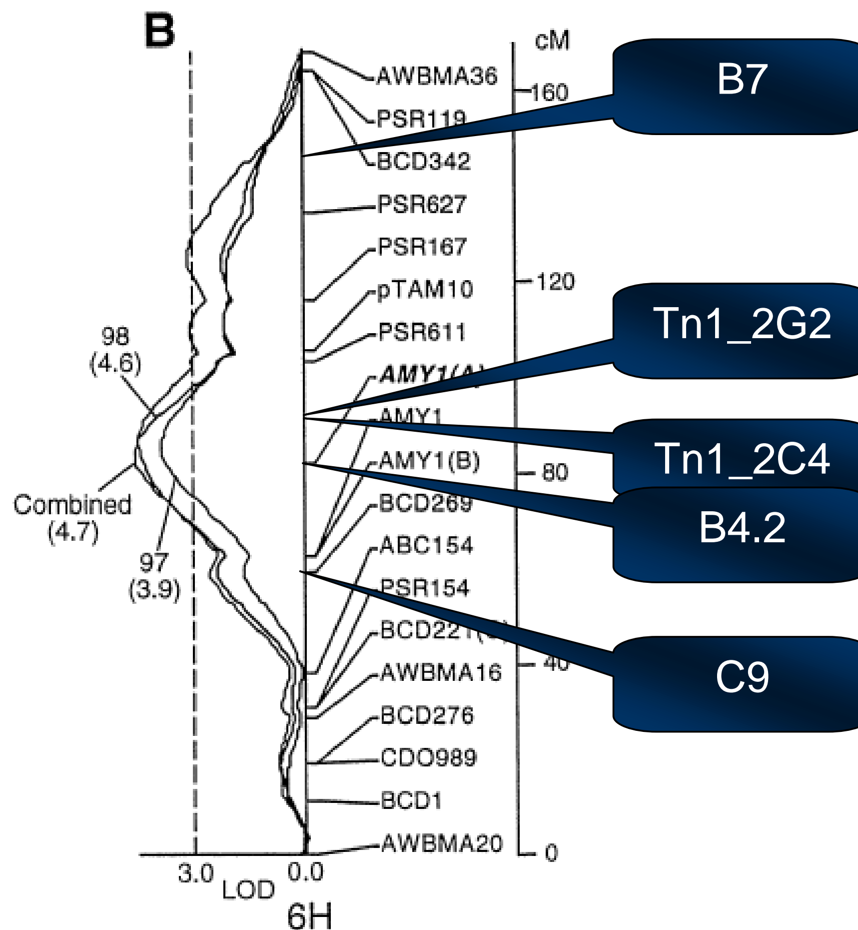


Figure 3.23. Candidates genetically mapped on the 6H B tolerance QTL (Jefferies *et al.*, 1999).

Figure 3.24. The precise map location of B4.2 on the 6H B tolerance QTL (map diagram obtained from Ms Margaret Pallotta, ACPFG).

3.4.10. Expression analysis by qPCR.

Quantitative real time PCR (qPCR) was performed in order to determine the transcript levels of some of the candidate genes in bulked total RNA (extracted from roots) of the tolerant and intolerant lines under non-treated and B treatment condition. Gene specific PCR analyses

were carried out using Corbett qPCR instruments (Corbett Life Science, Sydney, Australia). The raw data was normalized using methods described by Vandesoemple *et al*. (2002).

3.4.10. 1. Expression of a candidate mapped onto 2H QTL

The expression was examined for Tn1_ 2B7 (S-adenosylmethionine decarboxylase precursor) which had been mapped in the vicinity of the B tolerance locus on chromosome 2H. The expression was checked in bulked total RNA extracted from roots of the tolerant and intolerant lines under non-treated and B treatment conditions. The expression of Tn1_ 2B7 by qPCR analysis is shown in Figure 3.25.

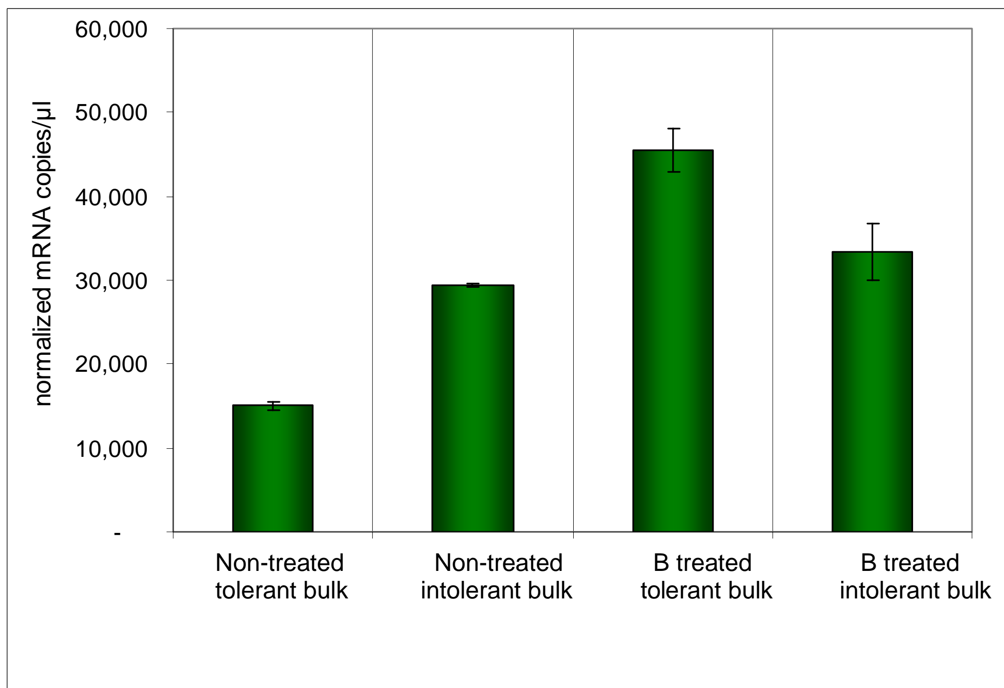


Figure 3.25. Accumulation of Tn1_2B7 (S-adenosylmethionine decarboxylase precursor) transcripts (normalized mRNA copy/µl) in bulked root RNA of the tolerant and intolerant lines without B and 200 µM B treatment for 24 hours. Data represent means of three technical replications. Error bars represent standard errors.

Figure 3.25 shows that the expression of this gene was up-regulated only in the tolerant bulk root RNA due to B treatment. Figure 3.25 also shows that under non-treated condition the mRNA of Tn1_ 2B7 was nearly 2-fold more abundant in the intolerant bulk root RNA (29,400 normalized mRNA copies/µl) than in the tolerant bulk (15,000 normalized mRNA copies/µl). However, B treatment resulted in a 3-fold up regulation of this gene in the tolerant bulk root RNA raising the transcript level to 45,500 normalized mRNA copies/µl. While in the intolerant bulk up-regulation due to B treatment of was insignificant (33,400 normalized mRNA copies/µl).

3.4.10.2. Expression of candidates mapped onto 6H QTL

The expression of two genes namely B4.2 (bromo-adjacent homology (BAH) domain-containing protein-like protein) and Tn1_2C4 (ATP/ADP carrier protein) that were mapped to the 6H QTL were tested. B4.2 was mapped on the 6H locus and Tn1_2C4 was mapped in the vicinity of the B tolerance locus on chromosome 6H. The transcript levels of B4.2 and Tn1_ 2C4 in the tolerant and intolerant bulk root RNA both under non-treated condition and B treatment is presented in Figure 3.26 and Figure 3.27, respectively.

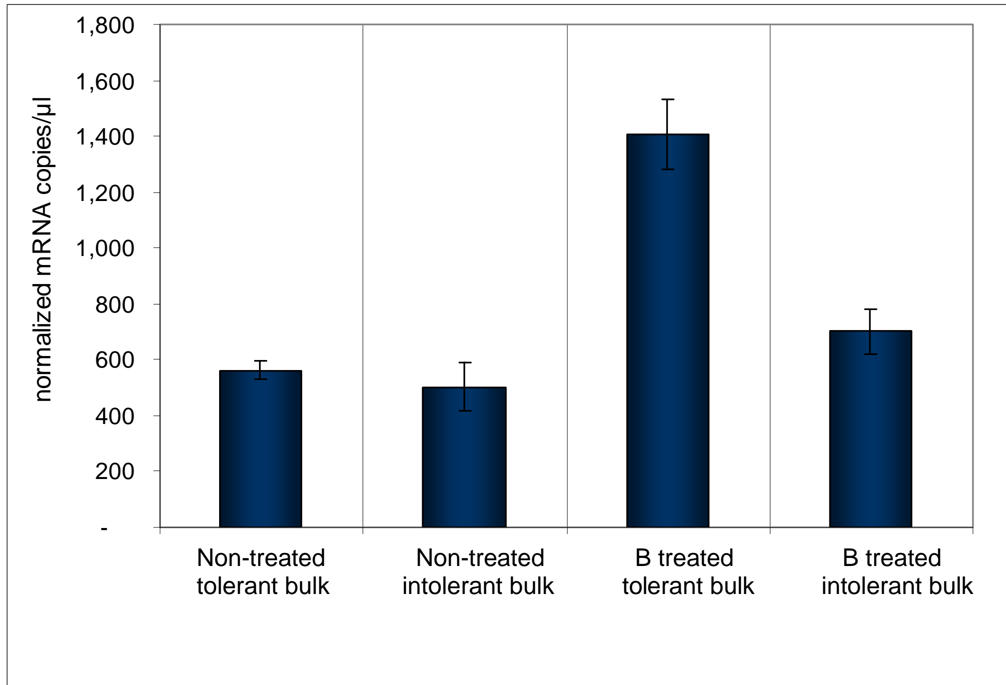


Figure 3.26. Accumulation of B4.2 (bromo-adjacent homology (BAH) domain-containing protein-like protein) transcripts (normalized mRNA copy/µl) in bulked root RNA of the tolerant and intolerant lines without B and 200 µM B treatment for 24 hours. Data represent means of three technical replications. Error bars represent standard errors.

B4.2 generally showed a low expression level under all conditions ranging between 500 copies in intolerant bulk under non-treated condition to 1,400 copies in the tolerant bulk root RNA under B treatment. Under non-treated condition B4.2 mRNA expression was increased slightly in the tolerant bulk root RNA compared to the intolerant bulk root RNA. But the abundance of this mRNA almost tripled in the tolerant bulk due to B treatment. In the intolerant bulk however, the abundance increased by only about 40%.

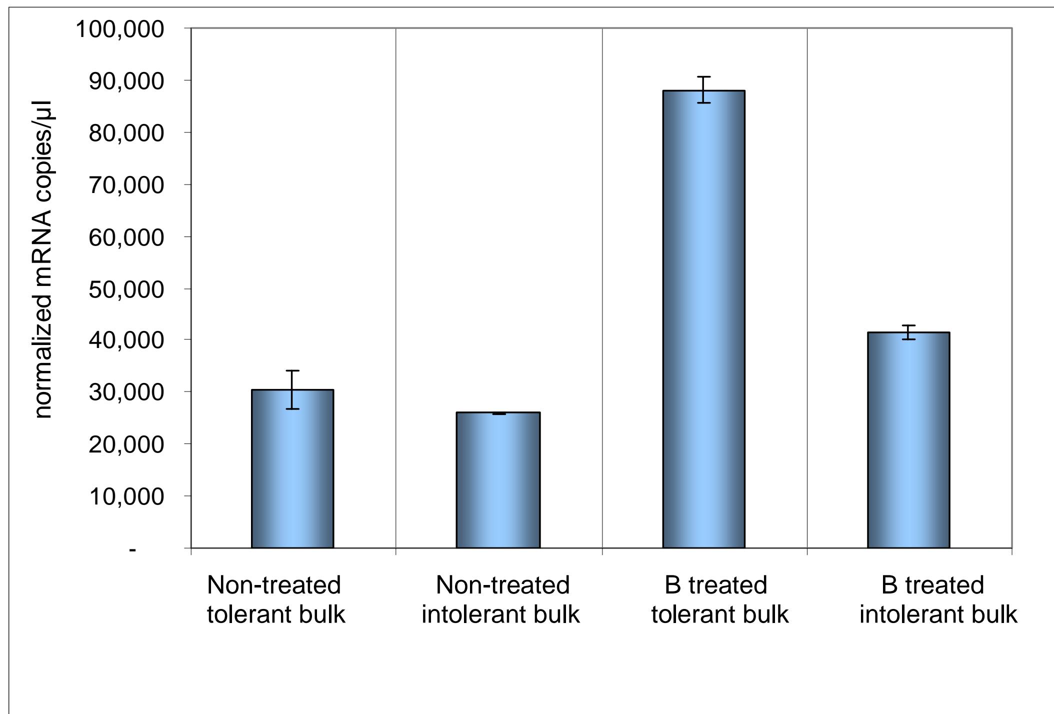


Figure 3.27. Accumulation of Tn1_2C4 (ATP/ADP carrier protein) transcripts (normalized mRNA copy/µl) in bulked root RNA of the tolerant and intolerant lines without B and 200 µM B treatment for 24 hours. Data represent means of three technical replications. Error bars represent standard errors.

The expression of Tn1_2C4 was the highest among all the genes tested for expression analysis by qPCR. Transcript abundance varied from 26,000 copies in intolerant bulk under non-treated condition to 88,100 copies in the tolerant bulk under B treatment. Under non-treated condition the tolerant bulk had similar expression to that in the intolerant bulk. B treatment resulted in 2.9 fold increase in expression of this mRNA in the tolerant bulk. However, in the intolerant bulk there was a 1.6-fold increase in the abundance of this transport related gene mRNA.

3.4.10.3. Expression of candidates mapped onto 4H QTL

The expressions of both genes mapped on the 4H QTL were tested. These are Tn1_2A12 (GONST3 Golgi Nucleotide sugar transporter) and NMAP1D5 (band 3 anion transport protein –like protein). Figure 3.28 and Figure 3.29 show the expression pattern of Tn1_2A12 and NMAP1D5, respectively. Tn1_2A12 showed rather low abundance both in the tolerant (800 copies) and intolerant bulk (700 copies). The gene showed B responsiveness in both bulks. B treatment caused a 4-fold increase in the expression of this gene in the tolerant bulk and reached the level of 3250 normalized mRNA copies/µl. While in the intolerant bulk the increase was modest and only 1.5-fold.

NMAP1D5 was found to be constitutively over-expressed in the tolerant bulk. The expression of this gene was not detectable in the intolerant bulk both under non-treated and B treatment conditions. In the tolerant bulk although this gene was not expressed in large quantities under non-treated condition (650 copies) but B treatment caused a clear 5.2 fold increase in expression during the short treatment period of 24 hours.

To further investigate the expression pattern of NMAP1D5 Clipper and Sahara root and shoot RNA was extracted from plants grown at different B concentration and harvested at different time points. Randomly selected shoot samples of these plant materials were subjected to ICP-AES analysis. Figure 3.30 shows the results of this ICP-AES analysis.

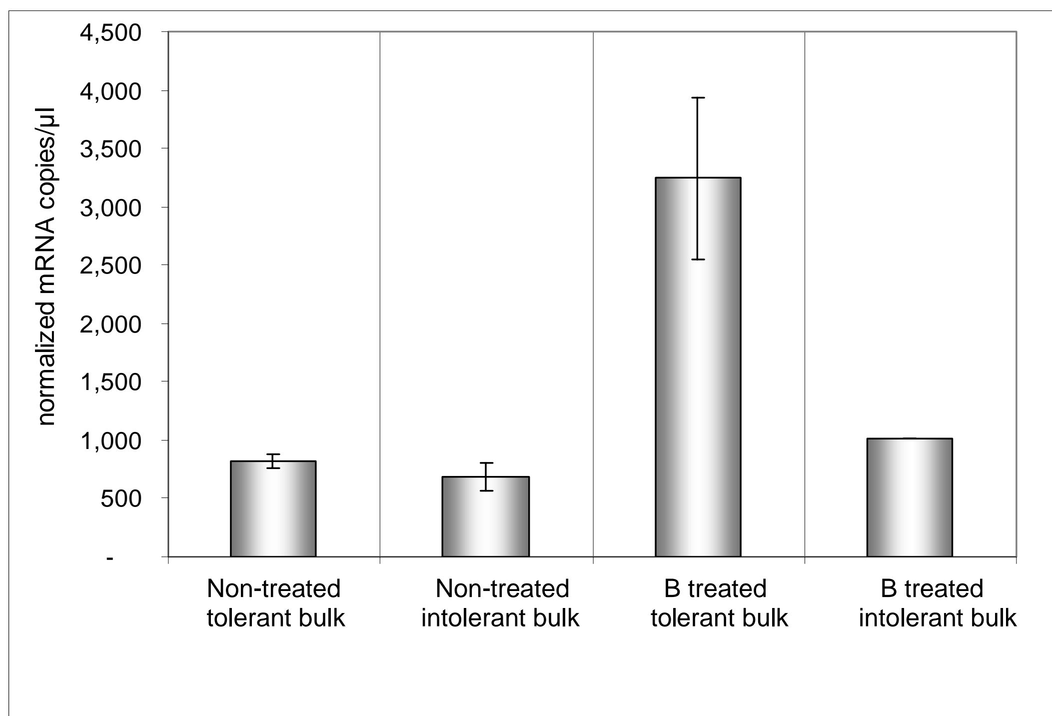


Figure 3.28. Accumulation of Tn1_2A12 transcripts (normalized mRNA copy/µl) in bulked root RNA of the tolerant and intolerant lines without B and 200 µM B treatment for 24 hours. Data represent means of three technical replications. Error bars represent standard deviations.

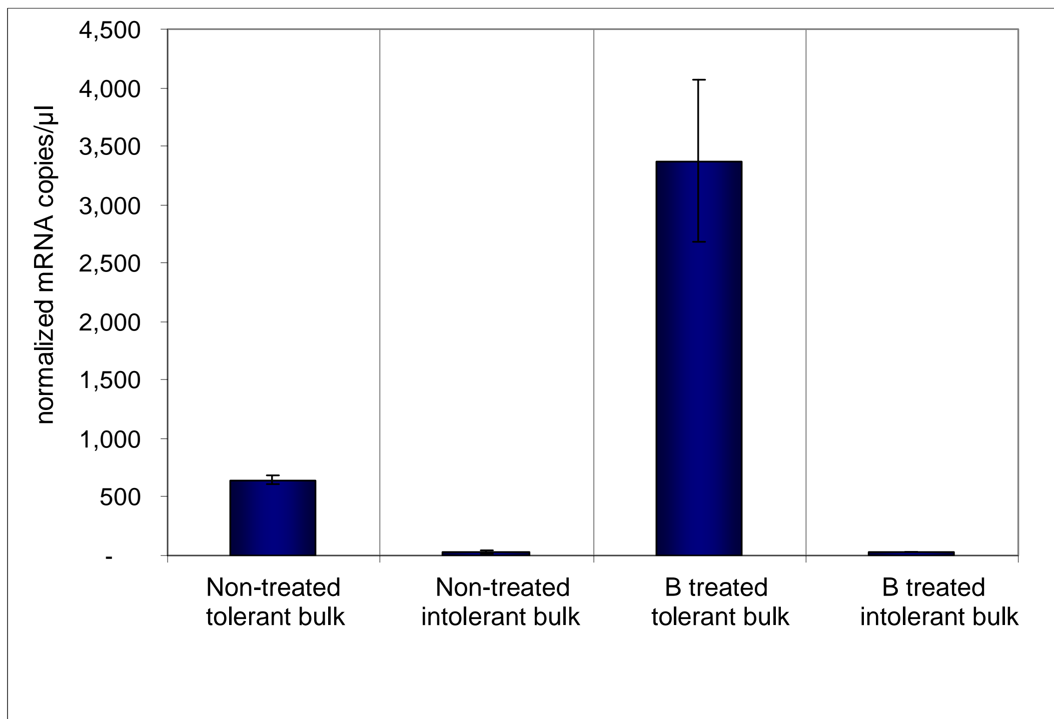


Figure 3.29. Accumulation of NMAP1D5 transcripts (normalized mRNA copy/µl) in bulked root RNA of the tolerant and intolerant lines without B and 200 µM B treatment for 24 hours. Data represent means of three technical replications. Error bars represent standard deviations.

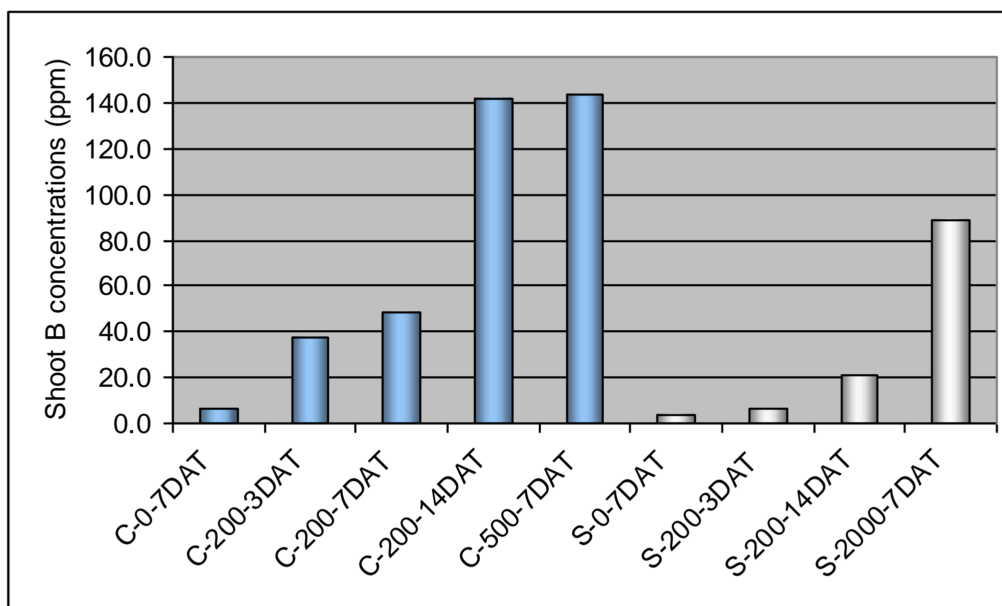


Figure 3.30. B concentrations in the shoots of Clipper and Sahara plants used to isolate RNA for qPCR analysis. Samples were randomly selected for analysis. Legend for each bar represents the Clipper (C) / Sahara (S) - B treatment in µM - harvest time of plants after treatment in days.

Figure 3.31 shows the expression of NMAP1D5 in Clipper and Sahara roots. The graph shows that in Clipper NMAP1D5 expression was hardly detectable by qPCR analysis, ranging

between 600 copies to 2750 copies normalized mRNA/µl. There was no increase in the transcript level of this gene due to B treatment even after two weeks. The expression in Sahara however was clearly higher, even without any B treatment. Figure 3.31 shows that after 24 hours the expression was decreased from 88,900 copies to 70,400 (21%) due to 200 µM B and to 56,300 (36%) due to 2000 µM B treatment. After 7 days there was an increase of 24% transcript level upon 2000 µM B treatment. After 14 days the transcript level went further down by 58%. However, the data shows a decrease in the expression of this gene in Sahara root as the plant matured.

Figure 3.32 shows the expression of NMAP1D5 in Clipper and Sahara shoots at different time points under different B treatments. The expression of this gene was also much lower in Clipper shoots compared to Sahara and was not B responsive as was seen in the case of Clipper roots. This gene did not show B responsiveness in Sahara shoots either.

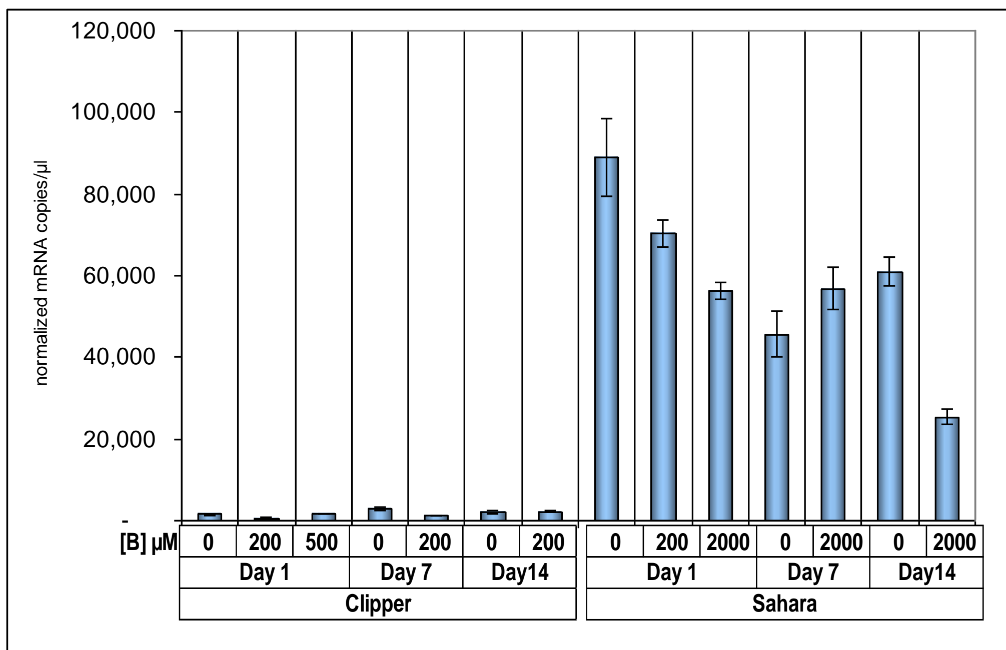


Figure 3.31. Accumulation of NMAP1D5 transcripts (normalized transcript copy/µl cDNA) in the Clipper and Sahara roots under non-treatment condition and different B treatments at different time-points. RNA was extracted from plants harvested after 1, 7 and 14 days after B treatment. Clipper plants were grown at 200 and 500 µM B. Sahara plants were grown at 200 and 2000 µM B. Data represent means of three technical replications. Error bars represent standard errors.

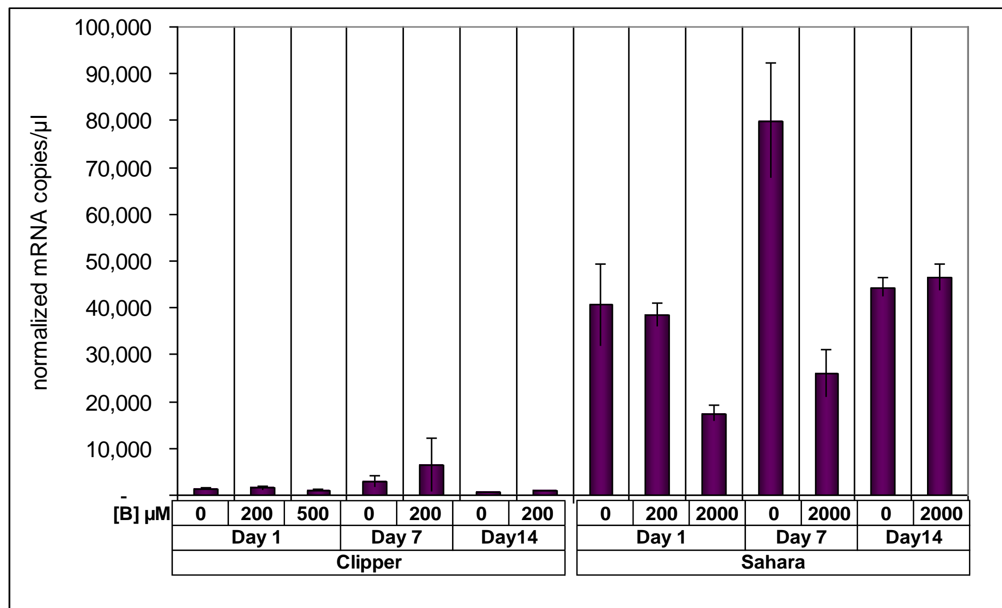


Figure 3.32. Accumulation of NMAP1D5 transcripts (normalized transcript copy/µl cDNA) in the Clipper and Sahara shoots under non-treatment condition and different B treatments at different time-points. RNA was extracted from plants harvested after 1, 7 and 14 days after B treatment. Clipper plants were grown at 0, 200 and 500 µM B. Sahara plants were grown at 0, 200 and 2000 µM B. Data represent means of three technical replications. Error bars represent standard errors.

CHAPTER 4

DISCUSSION (SSH)

4.1. Introduction

Plant responses to abiotic stresses are multigenic and genetically complex (Bohnert *et al*., 2001). Detrimental abiotic factors such as drought, salinity, cold and chemical toxicity cause cellular damage and osmotic and oxidative stress (Vinocur and Altman, 2005). These may result in metabolic changes, generation of reactive oxygen species, membrane disorganization, deterioration in photosynthetic capability and alterations in nutrient uptake and metabolism as reported for drought (Bray, 1993; Ingram and Bartels, 1996) and salinity (Hasegawa *et al*., 2000).

Plants are reported to have both stress specific adaptive responses and common adaptive response that protect them from a variety of environmental stresses (Chinnusamy *et al*, 2004). It is possible that the same is true for the observed tolerance to high B concentrations in the barley genotype Sahara 3771. Avoidance of the toxic B by removing excess B (efflux/ sequestration) and stress responses such as ROS scavenging and activation of defence mechanisms could reflect specific and general responses to high B concentrations. In addition, a third mechanism namely tissue tolerance is also possible. For example, recently Roessner *et al*. (2006) reported that Sahara 3771 (grown at 5 mM B) developed leaf symptoms five days later than the intolerant Clipper (grown at 1 mM B) even though both varieties had similar leaf B concentration. Choi (2004) reported that the B tolerant barley line VB9953 showed tissue B tolerance by accumulating high B without compromising growth.

To our knowledge, this research is the first approach to understand B tolerance mechanisms at a molecular level. The objective of this approach was to analyse genes differentially expressed between tolerant and intolerant lines of barley under B stress and thus to identify genes potentially involved in B tolerance. Using a Suppression Subtractive Hybridization (SSH) technique 111 non-redundant known genes were found to be up-regulated in bulked tolerant lines after stressing the plants for only 24 hours. It can be imagined that during this short and early stage of stress the plants would most probably be responding by gene expression in order to activate tolerance mechanisms. This would probably include avoidance

of B (exclusion or efflux) and readying other tolerance mechanisms that help the plant to withstand stress. The mechanisms related to tissue tolerance would probably come into play further down the track. This is because at 200 μM B, tolerant lines are expected to accumulate little B in the root tissue due to the exclusion/efflux mechanism shown by Hayes and Reid (2004) to be operating within 3 hours of exposure to high B concentrations.

Jefferies *et al*. (1999) identified a major QTL on chromosome 4H, and an associated minor QTL on 6H acting additively for B exclusion. Moreover, they also reported that the presence of the 4H Sahara allele accounts for 64% reduction in whole shoot B concentration while the presence of the 6H Sahara allele accounted for about 31% reduction. The experimental strategy specifically targeted the B exclusion genes by bulking tolerant and intolerant DH lines (from a cross between Clipper and Sahara 3771) distinct for this trait. The tolerant lines only contained the Sahara allele at the 4H and 6H loci, the intolerant lines contained only the Clipper allele at the 4H locus and most of them (90%) also at the 6H locus.

In the following the possible role of the isolated SSH clones will be discussed on the basis of their homology to known proteins and in the context of two possible B tolerance mechanisms, firstly B exclusion/efflux and secondly mechanisms that would allow plants to withstand stress imposed by elevated B in the root zone. The first mechanism can be viewed as B specific where the latter could either be B specific or a shared stress response.

4.2. The 4H and 6H B tolerance locus candidates for B exclusion

Subsequent genetic mapping of the SSH clones presented strong indication that the experimental strategy was very useful in isolating strong candidates for the 4H and 6H loci. Among the SSH clones a B transporter gene represented by NMAP1D5 was found to have the highest association with B exclusion trait (associated with 4H QTL) and to co-segregate with the trait. The top NCBI BLASTx search hit of NMAP1D5 is band 3 anion transport protein–like protein. In the subtracted libraries another 14 clones similar to NMAP1D5 were identified. The TIGR hits of these sequences are annotated as F9L1.41 protein, which is synonymous to B transporter like protein. In fact, Band 3 is the prototype anion exchanger found in red blood cells and has been shown to have homology with B transporter genes identified in *Arabidopsis* (Takano *et al*., 2002; Frommer and von Wirén, 2002). It appears that there is a major weakness in the annotations of B transporter genes in the databases because little is known about B transporters in plants other than *Arabidopsis* and rice. Recently, it was shown in our laboratory that barley has four B transporter genes (Ute Baumann, personal

communications). One of them is located at the 4H locus previously identified by Jefferies *et al*. (1999) by QTL mapping. Our lab has successfully isolated the full-length cDNA sequence of this B transporter gene. Sequence alignment confirmed that all of the 15 gene fragments identified in the subtracted libraries (B treated and control) belong to the 4H locus B transporter gene. Out of these 15 clones, nine came from the non-treated library and six from the B treated library. The transcript abundance in the non-treated library indicates that this gene is constitutively expressed in the tolerant genotype Sahara. Subsequent qPCR analysis of NMAP1D5 in the tolerant and intolerant bulk roots used for the subtraction (Figure 3.29) and also in Clipper and Sahara root and shoot (Figure 3.31 and 3.32) confirmed that this B transporter gene is constitutively over expressed in Sahara 3771. Hayes and Reid (2004) showed that Sahara plants are able to establish a minimal tissue B concentration within three hours of exposure to high B, which they considered a strong indication for constitutive expression of the trait in Sahara. Expression data presented here about this B transporter (NMAP1D5) are in agreement with the observation of Hayes and Reid (2004). NMAP1D5 therefore appears to be a very good candidate for the efflux mechanism predicted by Hayes and Reid (2004). The authors proposed two models involving anion transporters to depict a probable efflux mechanism for B tolerant Sahara. There is a strong possibility that the B transporter represented by NMAP1D5 (and other 14 related clones) is indeed the anion transporter proposed in Hayes and Reid's (2004) B efflux model. Choi (2004) observed that reduction in B is apparently initiated at the point of xylem loading. Further work needs to be done to determine tissue localization and to characterize the mode of action of the barley B transporter gene identified by SSH in this work.

Another SSH clone, namely B4.2, was genetically mapped onto the 6H B tolerance locus identified by Jefferies *et al*. (1999). Annotation of TIGR hits for this clone suggests homology to a bromo-adjacent homology (BAH) domain-containing protein-like protein. qPCR analysis of its expression in the tolerant and intolerant bulk roots (Figure 3.26) confirmed that B4.2 is B responsive and expressed more abundantly in the roots of the tolerant bulk. qPCR analysis on Clipper and Sahara root cDNAs grown with and without B also shows B responsiveness of this gene after 24 hours (Table 1 in Appendix C). However, there was no significant difference in the expression of this gene in Clipper and Sahara leaf after 24 hours of B treatment (data not shown).

A number of proteins have been identified to contain a BAH domain. The functions of most of these proteins are unknown (Goodwin and Nicolas, 2001). By performing a database

analysis Callebaut *et al*. (1999) postulated that the BAH domain plays an important role in coupling DNA methylation, replication and chromatin mediated gene inactivation. The authors added that proteins containing a BAH domain are predominantly involved in gene transcription and repression through a likely involvement in protein-protein interaction. A well characterized BAH domain containing barley ES43 protein also contain a PHD finger immediately downstream of the BAH domain. This PHD finger is responsible for protein-protein interaction and transcriptional regulation (Callebaut *et al.*, 1999). However, sequence alignment indicates that B4.2 is not related to ES43 protein (TC14765). Müssig and Altman (2003) observed that BAH domains may change their partner specific interactions with changes in their structure for example through variable insertion lengths. Further research is required to determine the role of B4.2 (bromo-adjacent homology (BAH) domain-containing protein-like) in B tolerance.

4.3. Further SSH clones with potential involvement in B tolerance

A tolerance mechanism that allows plants to withstand environmental stress includes a number of physiological adjustments. Some of them are common to various abiotic and biotic stresses. These involve stress perception and signal transduction, transcriptional adjustment, ionic homeostasis, altered metabolism, osmotic adjustment detoxification of cellular environment and degradation of damaged and misfolded proteins. For the purpose of narrowing down the search for potential candidates for B toxicity tolerance, SSH clones related to energy, cellular transport, metabolism, cell rescue and defense, cell wall, transcription, protein synthesis and protein fate and signal transduction were considered. It was not possible to establish the role of these clones in B tolerance experimentally within the time frame of this project. However, an analysis of stress related literature indicates their potential involvement in alleviation of various stresses. In the following the potential roles of selected clones from the above-mentioned functional categories in B toxicity tolerance is discussed based on their homology to functionally characterized genes.

4.3.1. Enhanced respiration and meeting energy demand for B efflux

According to the first efflux model proposed by Hayes and Reid (2004) energy input to a H^+-ATPase is needed. Two clones were isolated that are directly related to mitochondrial respiration - the source of ATP. These are an ADP/ATP carrier protein (Tn1_2C4) and a voltage-dependent anion-selective channel protein (VDAC) (NMAP1D8). There are also reports that environmental stresses strongly affect mitochondrial respiration (Hanson, 1982). For coupled cellular respiration it is essential that ATP and ADP are efficiently exchanged

between the cytosol and the mitochondrial matrix (Heiden *et al*., 2000). The principal mediators of the ATP/ADP exchange between the mitochondrial matrix and the cytosol are the ADP/ATP carrier on the inner membrane and the voltage-dependent anion channel (VDAC) on the outer membrane of mitochondria (Rostovtseva and Colombini, 1997; Wallace, 1999; Ledesma *et al*., 2002). Baker and Leaver (1985) observed that ADP/ATP carriers are likely to play a pivotal role in plants in maintaining cellular ATP levels. Bitar *et al*., (2003) reported that environmental stresses affecting respiration have an effect on expressions of genes encoding VDACs.

From the observed inhibitory effect of the metabolic inhibitor sodium azide (0.5mM) on the efflux of B in Sahara that resulted in a 66% increase in root B concentration in Sahara Hayes and Reid (2004) postulated that the efflux mechanism of Sahara is an energy demanding active process meaning an increased supply of ATP will be required. This can be achieved by enhanced ATP/ADP exchange between the mitochondrial matrix and the cytosol requiring elevated transcription of proteins like the ADP/ATP carrier protein and the VDAC protein. The ADP/ATP carrier protein (Tn1_2C4) and VDAC (NMAP1D8) were found to be 3.4 fold and 1.6 fold up-regulated in the tolerant B treated library, respectively. The fold change of ADP/ATP carrier protein was confirmed by qPCR (Figure 3.27) and was found to be highly B responsive in the tolerant bulk. Hayes and Reid (2004) also found that low temperature treatment had no effect on the root B concentration of Sahara indicating that the efflux mechanism was provided with a continued supply of ATP even at a low temperature, again indicating the possible contribution of the over expressed mediators of ATP/ADP exchange that was found in the B treated library. No gene coding for either VDAC or the ADP/ATP carrier protein was present in the non-treated library.

4.3. 2. K^+ ion homeostasis

Disturbance in the homeostasis of one particular ion onsets rapid modification in the transport of other nutrients in order to bring about an overall nutrient balance within the plant (Maathius *et al*. 2003). Membrane transporters are responsible for nutrient and water uptake and for maintaining intra or extracellular ionic homeostasis. High concentration of a particular ion in the growth media may induce up-regulation of certain transporters to facilitate efflux of the toxic ion from the cytosol (Maathius *et al*., 2003). A high concentration of one ion may also inhibit the uptake of other ions (Marschner, 1995; Santa-Maria *et al*., 1997).

Analysing *Arabidopsis* K^+-dependent transcripts Armengaud *et al*. (2004) concluded that jasmonic acid plays a prominent role in nutrient signaling. These authors reported that

transcript levels for the JA biosynthetic enzymes allene oxide synthase (AOS), and allene oxide cyclase (AOC) were strongly increased during K^+ starvation and quickly decreased after K^+ re-supply. In the B treated subtracted library AOC was found to be 2.5 fold up-regulated indicating that plants might have suffered from K deficiency under B toxicity, at least mildly. Nable (1989) presented data showing increasing B supply (from 15 µM - 2000 µM) caused significant reduction in shoot K content in the B intolerant cultivars Schooner and Galleon but no significant change was apparent in the B tolerant Sahara 3763 and Sahara 3768. K^+ is an essential nutrient for living cells. Cells rely on K^+ uptake, and eventually on K^+ efflux for growth and function (Rodríguez-Navarro, 2000). K^+ carries out vital functions in metabolism, growth, and stress adaptation (Armengaud *et al*, 2004) and thus plants' survival is very much dependent upon high-affinity K^+ (HAK) uptake systems. Studies on the regulation of HAK system has shown that it is rapidly up-regulated when the supply of exogenous K^+ is arrested (Glass, 1978; Glass and Dunlop, 1978). In agreement with a possible interaction between B and K^+ uptake a rice homologue of a 'probable potassium transporter 11' (OsHAK11) was identified in the B treated library which was up-regulated 2.5 fold.

K^+ deficiency initially results in proportional replacement of K^+ content by H^+. This decreases the internal pH (Ramos *et al*., 1990; Walker *et al*., 1996; Walker *et al*., 1998), which exerts a detrimental effect by itself (low pH) by inhibiting protein synthesis (Walker *et al*., 1998). However, if the K^+ deficiency occurs in the presence of Na^+, Na^+ is rather taken up as a substitute for K^+. The final ratio between the K^+ and Na^+ contents in the cell is not determined only by the influx ratio between the two cations, but also by the effluxes of individuals of the two (Rodríguez-Navarro, 2000). In agreement with this situation a clone homologous to potassium-efflux system protein (*Oryza sativa*) was identified with a 1.5 fold-change in the B treated library. Another clone homologous to a putative Na^+/H^+ antiporter (*Oryza sativa*) or *SOS1* gene was also identified in the B treated library 1.6 fold up-regulated. *SOS1* is the seventh (NHX7) member of the *NHX* gene family. *NHX* genes or the $Na^+:H^+$ antiporters play an important role in salt tolerance by sequestrating Na^+ into the vacuole and also by effluxing Na^+ through plasma membranes (Apse *et al*., 2003). Yamaguchi *et al*. (2001) postulated that the primary role of NHX exchangers is to regulate pH under non-saline conditions. From an investigation of *sos1* mutants in *A. thaliana* Wu *et al*. (1991) concluded that SOS1 is also involved in K^+ uptake.

A number of ramifications have been reported in the literature resulting from K^+ starvation. The decrease of the K^+ content may cause osmotic stress (Leigh and Wyn Jones, 1984; Walker *et al*., 1996). In other cases K^+ movement provides a charge-balancing counter-flux,

which is essential for maintaining the movement of other ions. Thus, energy production through H^+-ATPases is reliant on the overall H^+/K^+ exchange (Tester and Blatt, 1989; Wu *et al*., 1991). K^+ deficiency also actuates reactive oxygen species generation as an early response (Rodríguez-Navarro and Rubio, 2006).

It is well established that K^+ starvation leads to impaired nitrogen and sugar balance due to inhibition of protein synthesis, photosynthesis, and long-distance transport. Repression of protein synthesis reported in germinating soybean seed under high B (Haba *et al*., 1985) and a low protein content reported in sugarcane root tip under high B supply (Bowen, 1972) may potentially be an indicative of K^+ starvation-induced N imbalance. Armengaud *et al*. (2004) reported that a nitrate transporter NRT2.1 was one of the highly significant genes among their library of K^+-responsive genes. NRT2 and NAR2.1 constitute a two-component high-affinity nitrate transport system (HATS) (Orsel *et al*., 2006). In rice NAR2.1, is thought to be an activator gene for the function of NRT2 (Araki and Hasegawa, 2006). It appears that the tolerant barley lines are able to boost N uptake to rectify N imbalance caused by apparent K^+ starvation resulting from higher B concentration (as discussed above). In the present experiment a barley NAR2.1 gene was found 5.9 fold up-regulated in the B treated library. No nitrogen transporter was found in the non-treated library indicating that it was B responsive. In agreement to this finding Bonilla *et al*. (1980) reported increased accumulation of N in sugar beet leaf and root under excess B supply (measured as NO_3^-). Orsel *et al*. (2006) demonstrated by characterising two *Arabidopsis* gene knockout mutants, atnar2.1-1 and atnar2.1-1, that both of these proteins are necessary for N uptake at 0.2 mM nitrate, but atnar2.1-1 plants suffered more severely.

4.3.3. Carbohydrate metabolism

Alterations in sucrose levels have been reported due to various adverse environmental factors (Levitt, 1980; Good and Bell, 1980). B has been shown in many studies to affect glycosides biosynthesis including sucrose in plant (Dugger and Humphreys, 1960). Choi (2004) presented data showing that root tips of a B tolerant barley line VB9953 has less than one third the sucrose content of that in Clipper under control condition (15 µM B). Under toxic B conditions sucrose concentration increased by 50% in the B tolerant VB9953 but decreased significantly in Clipper root tips. In agreement with Choi's (2004) findings a clone coding for sucrose phosphatase (Tn1_2G6) was found to be 10.5 fold up-regulated in the B treated library. Sucrose phosphatase is the last enzyme in the pathway of sucrose synthesis (Lunn, 2002). Sucrose phosphatase was not found in the non-treated library indicating that it was

induced by B. It is likely that higher sucrose content in the tolerant VB9953 under toxic B observed by Choi (2004) resulted from the up-regulation of a sucrose phosphatase gene. Tn1_2G6 was mapped to 5H chromosome. Jefferies *et al* (1999) found association of the 5H QTL to relative root length. It is apprehensible that sucrose, which plays a pivotal role in carbohydrate metabolism, (Lunn, 2002) is also required for root elongation.

Root growth is also dependent upon a continuous supply of carbohydrate for respiration and building new tissues as roots have rather a low buffering capacity against fluctuation in carbon supply. Recently metabolite profiling by Roessner *et al.* (2006) showed the concentrations of most sugars including glucose and fructose increased significantly in Sahara roots due to high B treatment (1 mM) compared to those in treated Clipper roots. Similarly, Choi (2004) also showed that glucose and fructose concentrations were increased significantly in the root tip of B tolerant VB9953 when grown at high B concentrations. Choi (2004) also presented data showing the opposite was true for the root tip of Clipper zone. These observations did not correlate with invertase activity measured in the roots of either VB9953 or Clipper. In both cases Choi (2004) found a major reduction in invertase activity (74% in Clipper and 54% in Sahara). Carbohydrate transport is essential for cell division (Muller *et al*., 1998) and thereby for root elongation (Jones and Kiniry, 1986). It is obvious that the significant increase of glucose and fructose in the root tip of a tolerant barley variety would certainly require elevated activity of sugar transporters since carbohydrate is only synthesized in the above ground part of the plant. A gene similar to a membrane bound sugar/carbohydrate transporter namely- OSJNBa0027G07.7, was found to be 1.6 fold up-regulated in the tolerant bulk under B treatment. The fact that tolerant barley varieties display an increase in glucose and fructose content in the root tip zones can be attributed to the higher expression of sugar transporters like OSJNBa0027G07.7, which was not found in the non-treated library meaning that this gene was potentially induced by B treatment.

4.3.4. Cell wall synthesis

Recent investigations have shown that B tolerance in barley is associated with increased root elongation along with a proportional increase in cell density within the zone of cell division at the root tip (McDonald *et al*. 2003; Choi, 2004). Huang and Graham (1990) showed that excised roots of susceptible wheat cultured in high B containing media produced fewer lateral roots than tolerant genotypes. They also reported less callus production on the root of the varieties susceptible to toxic B concentration than tolerant genotypes. These results indicate that tolerant varieties are more efficient in synthesizing cell wall under toxic B conditions.

Cellulose, hemicellulose and pectin are the major carbohydrates that make up the primary cell wall. Several genes related to cell wall formation were identified in the B treated library which is in line with the above results. For example barley *CesA1* gene was found in the B treated library to be up-regulated 2.5 times but not in the non-treated library. In *Arabidopsis CesA1* synthesizes cellulose for primary cell walls, and this was directly demonstrated by expanding root cells (Sugimoto *et al.*, 2001), while the cotton *CesA1* gene expression was enhanced in cotton fibres at the onset of secondary wall synthesis (Richmond and Somerville, 2000).

In addition Pear *et al*. (1996) reported that a glycosyltransferase gene was strongly expressed during cellulose deposition in the developing cotton fibre. In fact, Golgi localized glycosyltransferases catalyse pectin synthesis in the Golgi apparatus (Sterling *et al*., 2001). Nucleotide sugars are the substrates for the glycosyltransferases and are made in the cytosol (Gerardy-Schahn *et al*., 2001). Transport of these nucleotide sugars across the Golgi membrane is mediated by Golgi nucleotide sugar transporters (GONSTs) (Handford *et al*., 2004). In keeping with these facts both putative glycosyltransferase (*Oryza sativa*) (Tn1_2H8) and GONST3 Golgi nucleotide sugar transporter (*Arabidopsis thaliana*) (Tn1_2A12) were found up-regulated 2.4 and 4.9 fold, respectively, in the B treated library. None of these were found in the non-treated library hinting that these genes were B induced. Thus the differential expression of cell wall related genes in the present experiment provides an explanation as to how the B tolerant barley varieties are able to maintain higher relative root length and a proportional increase in cell density within the zone of cell division at the root tip.

4.3.5. Osmoregulation

Osmoregulation is prompted by modified ionic fluxes to cope with resulting osmotic stress (Munnik and Meijer, 2001; Csonka, 1989). Osmotic stress and the associated oxidative stress have been reported in the literature as inevitable consequences when plants are exposed to drought, salinity and low temperature (Wang *et al*., 2003). Several clones isolated from the B treated library provide some indications that the tolerant barley lines were in the process of embarking on a osmoprotection scheme.

For example one of the key enzymes involved in polyamine biosynthesis in plants *S*-adenosylmethionine decarboxylase (SAMDC) was found to be up-regulated 6.5 fold in the B treated library. This clone was mapped close to the B tolerance locus (Figure 3.22) of chromosome 2H. qPCR data shows that this gene was up-regulated about three fold in the

tolerant bulk roots due to B treatment, whereas in the intolerant bulk only 10% up-regulation was observed. qPCR analysis also showed a higher level of SAMDC in Sahara shoot than in Clipper shoot under all conditions (Table 2 in Appendix C). Recently a complementation test using yeast in our laboratory has shown that SAMDC confers B tolerance to yeast (Julie Hayes, personal communication). Besford *et al*. (1993) reported that exogenous application of polyamines provided protection to oat leaves against osmotic stress. Polyamines play important roles in plant defense to a variety of environmental stresses (Kasukabe *et al*., 2004) and have been found to accumulate under several abiotic stress conditions, including salt and drought (Galston *et al*, 1997). Various authors have shown that a high cellular level of polyamines correlates with plant tolerance to a wide range of environmental stresses such as salinity (Krishnamurthy and Bhagwat, 1989; Aziz *et al*., 1998), low and high temperatures (Roy and Ghosh, 1996; Shen *et al*., 2000; He *et al*., 2002), hyper-osmosis (Besford *et al*., 1993) and oxidative stress (Langebartels *et al*., 1991; Kurepa *et al*., 1998). In the case of salt stress, tolerant plants are capable of producing 2- to 3 - fold more polyamines than in unstressed plants in response to stress compared to intolerant lines (Kasukabe *et al*., 2004).

Another example is the key enzyme betaine aldehyde dehydrogenase (BADH) which is responsible for catalizing glycine betaine (GB); it was found to be up-regulated 1.6 fold in the B treated library. Stress inducible GB is a compatible solute, which plays important role in plants under abiotic stresses. Plants produce GB as a mechanism for increasing solute concentrations (Bray *et al.*, 2000). It increases cellular osmolarity under stress related hyperosmotic conditions and has been shown that it helps in stabilizing enzymes and maintaining the cellular membrane integrity in a variety of abiotic stresses including salinity, heat, cold and freezing (Gorham, 1995). Neither SAMDC nor BADH was found in the non-treated library indicating that they were induced by B toxicity.

4.3.6. Detoxification of the cellular environment (ROS scavenging, detoxification and repairing stress damage)

A number of glutathione dependent enzymes such as Glutathione S transferase or GST (C5, fold change 4.6), Glutathione S transferase I subunit (NMAP1F11, fold change 1.9) and glutathione peroxidise or GPX (Tn1_2B4, fold change 3.0) were identified in the B treated library indicating that toxic B may induce the production of reactive oxygen species (ROS). It has been shown that plants when exposed to arsenic, which is a metalloid similar to B, triggers an increase in reactive oxygen species (ROS) (Hartley-Whitaker *et al*. 2001a). Ruiz *et al*. (1999) also reported that altered B nutritional status leads to formation of ROS.

All biotic and a number of abiotic stresses (including, heat, cold, UV, drought, salinity and heavy metals) impose oxidative stress in plants, promoting the formation of reactive oxygen species (ROS) such as superoxide radicals, ($\cdot O_2^-$), hydroxyl ($\cdot OH$), hydrogen peroxide (H_2O_2) and perhydroxyl ($\cdot O_2H$) (Bray *et al*., 2000; Apel and Hirt, 2004). ROS causes damage to lipid, nucleic acids and protein through oxidation (Hernandez *et al*., 2001) and also causes cell death (Noctor and Foyer, 1998). In plants, the ascorbate–glutathione pathway is considered as a major antioxidative defence mechanism (Noctor and Foyer, 1998; Hou *et al*., 2000, Ann *et al*., 2000), which is responsible for scavenging peroxide radicals resulting from oxidative stress. GPX is one of the major antioxidative enzymes that catalyse the reduction of hydrogen peroxide, organic hydroperoxides and lipid hydroperoxides where glutathione (GSH) acts as a substrate for the enzyme (Navari-Izzo and Izzo, 1994, Churin *et al.,* 1999). GPX may metabolize lipid hydroperoxides and generate glutathione disulphide, which in turn is re-reduced to GSH by NADPH (Navari-Izzo and Izzo, 1994). Thus GPX repairs membrane damage from lipid peroxidation and acts as the main line of enzymatic defense against oxidative membrane damage (Kühn and Borchert, 2002; Ursini *et al*., 1995). Both GST and GPX activities have been reported to be activated by a large number of stresses causing oxidative stress to plants (Marrs, 1996, Gueta-Dahan *et al*., 1997, Churin *et al*., 1999). In the present experiment HVGPX (Tn1_2B4) was found to be up-regulated three fold by B treatment in the tolerant bulk. None of the glutathione dependent enzymes were found in the non-treated library. In agreement with the identification with glutathione dependent enzymes in the B treated library in the present experiment Ruiz *et al*. (2003) demonstrated that external application of glutathione significantly reduced B toxicity in sunflower.

The most important reducing substrate for H_2O_2 detoxification in plant cells is ascorbate (Mehlhorn, 1996; Nakano and Asada, 1987). Ascorbate is produced from monodehydroascorbate (MDHA) either directly or via dehydroascorbate (DHA). Conversion of MDHA to ascorbate is catalysed by monodehydroascorbate reductase (Asada, 1997) and from DHA to ascorbate by dehydroascorbate reductase using glutathione as the reducing substrate (Foyer and Halliwell, 1976). Heber *et al*. (1996) observed that MDHA is a sensitive endogenous index of oxidative stress in plant leaves. In the present experiment a clone coding for MDHA was found to be very strongly up-regulated in the tolerant bulk under B treatment (fold change 10.5) and was not found in the non-treated library indicating *MDHA* was B induced. This provides further evidence that barley plants may indeed suffer from oxidative

stress and tolerant barley plants are more efficient in the detoxifying cellular environment from such consequences.

4.3.7. Sequestration as a possible B tolerance mechanism

In addition to the possible role of GSTs in B tolerance discussed under detoxification of cellular environment, they are also known to conjugate toxic electrophiles generated by xenobiotic treatment to glutathione in order to neutralise their harmful effects (Dixon *et al*., 1997). A complex family of ATP binding cassette (ABC) transporter (including MRP) proteins that resides in the tonoplast membrane are capable of sequestrating the glutathione conjugates (which is inhibitive to GSTs) into the vacuoles (Coleman *et al*, 1997; Rea *et al*., 1998; Lu *et al*, 1997). A clone homologue to the mrp 9 gene for MRP-like ABC transporter was found to be 3.8 fold up-regulated in the B treated library raising the possibility of such sequestration in case of B toxicity in barley as well. Dixon *et al* (1997) reported that the *Zm* GST I subunit demonstrated wide conjugating ability toward diverse substrates. Further study may be necessary to determine whether in case of B toxicity in barley glutathione acts as a precursor of phytochelatins as it does to bind toxic concentrations of heavy metals (Grill *et al*., 1987; 1989) and also arsenate- a similar metalloid to B (Schmöger *et al.,* 2001; Hartley-Whitaker *et al.,* 2001b) where GSTs may catalyse the conjugation.

In addition two more genes bound to vacuolar membrane were found in the B treated library significantly up-regulated, which could also be involved in sequestration mechanism. In plants under environmental and biotic stress vacuoles play a crucial role for general cell homeostasis and in processes of detoxification (Marty, 1999). Vacuolar type H^+-ATPases, which energize secondary transport processes across the tonoplast (Lüttge and Ratajczak, 1997), are involved in defense against environmental stress in plants (Sze *et al*., 2002; Magnotta and Gogarten, 2002). In agreement with these facts a clone coding for a V-ATPase d subunit was identified in the B treated library being up-regulated 3.9 fold. A tonoplast intrinsic protein (*Oryza sativa*) (Tn1_2G2) was isolated and was mapped within the significant region of the B tolerance locus of the 6H chromosome. It was found to be 5.2 fold up-regulated in the B treated library. This clone corresponds to δ-TIP of *Arabidopsis* (Daniels *et al*., 1996). Vacuoles containing δ-TIP proteins may act as storage compartments for vegetative storage proteins (VSPs). VSPs are synthesized in response to developmental and environmental cues including oxidative stress (Jauh *et al*., 1998). VSPs have also been reported to be induced by jasmonic acid (Xu et al., 2001) and by the action of agents causing oxidative stress (Mira *et al*., 2002). It has been shown that VSPs bind to copper in order to

maintain copper ion homeostasis and detoxification under toxic copper supply (Kung *et al*., 2006; Mira *et al*., 2002). Further investigation would be needed to determine whether sequestration is a part of the overall B tolerance mechanism in barley and if so how the vacuolar membrane bound proteins mentioned above may contribute to such a mechanism.

4.3.8. A hint of lignification

Recently Ghanati *et al*. (2002) reported under that B toxicity in tobacco both suberin and lignin increase in cell wall indicating that also in case of barley lignification of the cells is a possibility. It needs to be explored whether this lignification acts to form a physical barrier in order to lower boric acid permeability and thereby reduce B permeability. Choi (2004) concluded that an exclusion mechanism involving a physical barrier between the root cortical cells and the xylem is likely.

Reduced glutathione (GSH), which is known to increase under various oxidative stress conditions, was reported to induce increase in the activity and amount of NADP-malic enzyme (NADP-ME) (Barber and Ride, 1988). In the present experiment a clone coding for NADP-ME was found to be1.8 fold up-regulated in the tolerant bulk only under B treatment. NADP-ME up-regulation has been implicated in defense related lignification in the literature (Walther *et al*., 1993; Whetten and Sederoff, 1995; Schaaf *et al*., 1995; Casati *et al*., 1997). In vascular plants lignin is mainly synthesized in cells that become a part of the transport system (Quiroga *et al*., 2000). Suberin is synthesized in root cells from the endodermis and exodermis in order to strengthen the cell wall and to control water movement (Quiroga *et al*., 2000). It is difficult to predict from the results of this experiment whether NADP-MEs could have any role in lignin biosynthesis in the case of B toxicity which has been previously linked to plant defense responses through lignification by providing NADPH (Whetten and Sederoff, 1995; Schaaf *et al*., 1995). Efforts might be directed to explore such a possibility.

4.3.9. Stress perception and cellular signaling under B stress

A number of clones were identified in the B treated library related to intercellular stress signaling pathways. A review of their role in stress signaling provides an idea of how tolerant barley lines may perceive B toxicity and associated stresses. For example a two-component response regulator-like PRR73 was identified and was up regulated 2.2 fold in the B treated library. The two-component system is a simple and elaborate signaling module that transduces extracellular signals to the cytoplasm through phosphotransfer between two components (Swanson *et al*., 1994; Appleby, 1996). Ideally the two-component system is

composed of a sensory histidine kinase and a response regulator (Urao *et al*., 2001). In the present experiment a rice homologue of a putative histidine kinase was found to be 4.6 fold up-regulated in the B treated library. Two-component regulatory systems also act as osmosensors (Bray, 1997) and have been found to regulate mitogen-activated protein kinase (MAPK) cascades (Wurgler-Murphy and Saito, 1997). Reports also indicate that redox sensitive receptor like kinases and two component histidine kinases are potential ROS sensors that supposedly activate a MAPK module (Chinnusamy *et al*., 2005). A MAPK cascade consists of MAPKKK-MAPKK-MAPK module at the least (Nakagami *et al*., 2005). Components of the MAPK cascade are activated by more than one stress and may act as converging points of multiple abiotic as well as biotic stress signaling (Chinnusamy *et al*., 2004). Kovtun *et al*. (2000) and recently Moon *et al*. (2003) demonstrated that reactive oxygen species activate a MAPKKK (ANP1) in *Arabidopsis*. Recently it has been shown that by constitutively expressing tobacco MAPKKK (NPK1) in maize an oxidative signal cascade could be activated under heat, salinity and cold stress in order to enhance tolerance (Shou *et al.,* 2004). Teige *et al*. (2004) reported that in *Arabidopsis* MAPKKK activates MAPKK in abiotic stress. MAPKKK can also be activated by osmotic stress in *Arabidopsis* (Droillard *et al.*, 2002). In the present experiment a rice homologue of a putative MAPKKK was found to be up-regulated 2.2 fold in the B treated library. Identification of the above mentioned signaling related clones in the B treated library bolsters the argument that B toxicity may impose osmotic and oxidative stress in barley plants.

Further to this, two clones identified in the B treated library indicate that jasmonate dependent signaling pathways were activated under B stress in tolerant barley lines as are also known to be activated under drought (Reymond and Farmer, 1999; Wierstra and Kloppstech, 2000) and saline conditions (Jiang and Deyholos, 2006) in plants including in barley (Ozturk *et al*., 2002). These clones code for lipase like protein and allene oxide cyclase (AOC). The role of lipases in JA-dependent defense signaling has been described in the literature (Jakab *et al*., 2003, Jiang and Deyholos, 2006). Allene oxide synthases (AOS) are involved in the biosynthesis of jasmonate and Ozturk *et al*., (2002) found allene oxide synthase most pronouncedly up-regulated in barley in response to drought and salt stress. AOS and AOC-catalyses a step in jasmonate (JA) biosynthesis where AOC catalyzes the stereospecific cyclization of an unstable allene oxide formed by an AOS (Ziegler *et al*., 2000). Yamada *et al*. (2002) reported that expression of mangrove AOC enhances tolerance to salt in bacteria (*E. coli*), yeasts and tobacco cells. Lipase and allene oxide cyclase were not found to be up-

regulated in the non-treated library. Thus it appears that jasmonate based signaling may also play an important role in B toxicity tolerance in barley.

4.3.10. Involvement of the Ubiquitin (Ub)/proteasome pathway

Extreme environments often adversely affect proteins by increasing free radicals that cause denaturation and damage (Smalle and Vierstra, 2004; Santos *et al*, 2006). For cell survival it is critical to degrade these proteins by various quality control pathways within the Ub/26S proteasome system (Kopito, 2000; Kostova and Wolf, 2003; Varshavsky, 2003), which continuously monitor mature proteins for post-synthetic denaturation or chemical damage (Goldberg 2003).

Identification of beta-3 proteasome subunit, Cullin and F-Box protein (Deshaies, 1999) up-regulated in the B treated library may indicate tolerant barley varieties are able to efficiently degrade damaged protein that may result from B toxicity. Hartley-Whitaker *et al*. (2001b) suggested that a conjugation of salvage and proteolytic machineries including Heat shock protein (HSP) synthesis and the Ub/proteasome pathway is induced under low arsenic toxicity in plants. Apparently a similar mechanism may also be operational in case of low B toxicity. HSP70 are also involved in the ubiquitination and subsequent removal of misfolded proteins (Smalle and Vierstra, 2004). Evidence has been presented that in plants oxidative stress results in the accumulation of some HSPs (Dat *et al*. 1998). The accumulation of HSPs may thus indicate that plants are under oxidative stress (Liu and Thiele, 1996; Ahn and Thiele, 2003; Smalle and Vierstra, 2004). A barley dnaK-type molecular chaperone HSP70 was found to be up-regulated in the B treated library 3.7 fold. This again adds weight to the argument that efficient degradation of damaged and misfolded protein may constitute a part of the overall B tolerance mechanism in B tolerant barley.

4.3.11. Involvement of transcription factors

Among the transcription factors (besides the BAH domain protein, section 4.2) an Ethylene responsive protein, a putative CREB binding protein and a GRP94 (94-kDa glucose related protein) were identified in the B treated library. The ethylene responsive protein is similar to the USPA (universal stress protein A of *Escherichia coli*) domain of bacteria and a large variety of stimuli were found to be responsible for the production of the protein, such as stationary phase, carbon, nitrogen, phosphate, sulphate and amino acids starvation, and exposure to heat, oxidants, metals, uncouplers, polymyxin, cycloserine, ethanol, antibiotics and other stimulants (Van Bogelen *et al*., 1990). HAC1/CREB binding protein form tri-element complexes boosting promoter recognition efficiency markedly and activating

transcription in plant cells (Bharti *et al*., 2004). The GRP94 belongs to a class of HSP90 proteins. Gjetting *et al*., (2004) postulated that it might be involved in preventing apoptosis as it has been shown to prevent cell death from oxidative stress (Punyiczki and Fésüs, 1998). Thus GRP94 may also play a role in reduced or delayed leaf symptom expression in Sahara. These clones were not found in the non-treated library indicating that they are likely to be B responsive. It would require further investigation to determine roles of these clones to B toxicity tolerance in barley.

4.4. Summary

This experiment aimed to characterize the B toxicity tolerance mechanism at the molecular level. By bulking DH lines from a cross between Clipper and Sahara on the basis of the B exclusion trait it was possible to isolate a B transporter gene at the previously identified major locus on chromosome 4H. The results indicate that this B transporter is constitutively expressed in Sahara, which is in agreement with Hayes and Reid's (2004) observation that the efflux mechanism is constitutive in nature. These findings warrant further investigation in order to determine the nature and the mode of action of this B transporter of barley. In addition a clone mapping on the 6H locus was also isolated whose function is not described in currently available literature. The association of this candidate to the B exclusion trait also provides adequate ground for further investigation to determine how this candidate could be related to facilitating B exclusion. A large number of other clones were also identified from the subtractive hybridization. Analysis of B tolerance and several other stress tolerance related investigations indicate that the adaptive mechanism of B tolerant Sahara may involve (i) elevated energy production through mitochondrial respiration to ensure sustained availability of ATP probably to keep energy demanding efflux mechanism operating, (ii) nutrient homeostasis specially K^+ ion in order to maintain osmotic balance and to protect plants from K deficiency related adversities including rectification of impaired N balance, (iii) efficient cell wall synthesis and mobilization of C to maintain root growth, (iv) efficient osmosensing and osmotic adjustment through polyamine production, (v) ROS scavenging and detoxification of the cellular environment through the ascorbate–glutathione pathway and (vi) elevating activity of proteolytic machinery to remove damaged proteins caused by ROS quickly from the system. Although the data indicated that B stress may induce lignifications, no strong evidence was observed to suggest that Sahara barley attempts to exclude B by reinforcing the physical barrier between the root cortical cells and the xylem in order to reduce B permeability. However, since GB and GPX prevent oxidative membrane damage they may also help in reducing passive influx of B. Another possible tolerance mechanism

through chelation involving GST seems plausible as it takes place in the case of the similar metalloid arsenic. Finally, the data indicate that B toxicity causes secondary stresses like ROS generation and osmotic imbalances as seen for a number of abiotic stresses especially nutrient toxicity. Since Sahara barley evolved in a rather harsh environment it is possible that this barley genotype is more efficient at osmoregulation and ROS scavenging than Clipper, which was bred to produce high yield in an agronomically managed environment. Other than the efflux mechanism, which is not known to be able to completely prevent B uptake, the tolerant barley genotype Sahara may apply a number of other mechanisms for alleviating/ withstanding high B induced stress and to sustain growth. Some of these mechanisms are already known to be used by plants to cope with a number of other stresses.

CHAPTER 5

EXPRESSION PROFILING OF B TOLERANT AND INTOLERANT BARLEY USING GENECHIP^{TM} BARLEY GENOME ARRAY

5.1. Introduction

The basic difference between B tolerant and susceptible barley varieties lies in their ability to prevent B accumulation in their tissue and most notably in leaf blades. B accumulation is most pronounced at the end of the transpiration stream, that is, in leaf tips and leaf margins (Brown *et al*., 2002; Blevins and Lukaszewski, 1998; Nable *et al*., 1997). B is generally phloem immobile in barley and is not re-translocated to other parts of the plant. Thus toxic concentrations of B build up in the leaf over time causing stress in the leaf tissues and subsequently affecting the whole plant. The necrotic B toxicity symptoms first appear at the sites of highest B accumulation in leaf blades. However, Sahara takes longer to manifest leaf symptoms compared to the B toxicity susceptible variety Clipper at similar tissue B concentrations (Choi, 2004; Roessner *et al*., 2006). This suggests that Sahara may have a better ability to tolerate B internally - a trait known as 'internal B tolerance' (Stangoulis and Reid, 2002). B toxicity susceptible barley varieties also demonstrate reduced above ground vegetative growth compared to Sahara, when grown under high B conditions. This suggests that Sahara has a superior metabolic system that allows it to sustain growth bypassing the toxic effect of high tissue B content. In order to provide an insight into the molecular basis of such superiority of Sahara in restricting leaf B accumulation as well as in tolerating higher tissue B concentrations over B intolerant Clipper, it is important to gather comprehensive information about gene expression in the leaf tissues of these barley genotypes through technologies such as transcript profiling under B toxicity. Recent developments in microarray technologies provide opportunities for such an examination. Transcript profiling using microarrays has already established insights into the adaptive mechanism of tolerant rice cultivars against salinity stress (Kawasaki *et al*., 2001). Microarrays have been employed to investigate a range of abiotic stresses at the transcriptome level, for example drought and salt stress responses in barley (Ozturk *et al*., 2002), low-oxygen response in *Arabidopsis* root culture (Klok *et al*., 2002), oxidative stress response in *Arabidopsis* (Desikan *et al*., 2001), salinity, drought and cold stress responses in *Arabidopsis* (Seki *et al*., 2001) and drought stress and heat shock responses in tobacco (Rizhsky *et al*., 2002).

Recently the first commercially available barley microarray was released by Affymetrix called Barley 1 GeneChip^{TM} (Affymetrix, Santa Clara, CA). This microarray contains a comprehensive collection of barley transcript sequences, including all known barley genes. The availability of such a comprehensive array offered an opportunity to examine and compare B stress responses in Sahara and Clipper.

5.2. Experimental strategy

The plant response to B, like to other abiotic stress appears to be multigenic. For example Jefferies *et al*. (1999) identified QTL on four chromosomes of barley that have significant effects on B tolerance. In order to cast insight into such multigenic responses the global monitoring of gene expression changes is important. The comparison of gene expression in Sahara and Clipper grown under different B concentrations could reveal the adaptive and non-adaptive responses of barley to high B environments. Such comparison can also provide information about the response of barley to high B environments that confers tolerance.

However, a direct comparison between the responses to B of two diverse barley varieties like Clipper and Sahara using Affymetrix Barley 1 GeneChip^{TM} could present serious challenges. Because of the high sensitivity to small regions of sequence mismatch offered by short oligonucleotide arrays one needs to be cautious when comparing gene expression between Sahara and Clipper directly using Affymetrix microarrays. Such comparison could be jeopardised by excessive single feature polymorphisms (SFPs). SFPs can be the result of single nucleotide polymorphisms (SNP) within exons and also polymorphisms generated during posttranscriptional processing such as alternative splicing and polyadenylation (Rostoks *et al*., 2005). Such polymorphisms, if present in a region complementary to the probe can affect the hybridization of the target RNA to the GeneChip^{TM} probes (Rostoks *et al*., 2005).

Thus, a microarray experiment was designed to facilitate intra-varietal comparison of gene expression within Sahara and Clipper under different B concentrations. Preliminary tests showed that Sahara plants are able to withstand up to 2000 µM B for about two weeks without showing severe leaf symptoms. On the other hand Clipper plants manifest moderate leaf symptoms at a B concentration of 150 µM by two weeks. Thus in order to produce a comparable stress response in Sahara it was necessary to treat them with B concentrations an order of magnitude higher than that required for Clipper.

5.3. Materials and methods

5.3.1. GeneChip^{TM} Barley Genome Array (Affymetrix 22K Barley1 GeneChip^{TM})

GeneChip^{TM} Genome Arrays (Affymetrix, Santa Clara, CA) are photolithographically synthesized oligonucleotide arrays. They consist of nucleic acids (16-25bp) synthesized onto silica slides by a photolithographic process at a very high density (250,000 oligonucleotide/cm^2) (Kehoe, 1999). High-density oligonucleotide arrays provide direct information about the expression levels of thousands of mRNA simultaneously.

The Affymetrix 22K Barley1 GeneChip^{TM} features 22,792 probe sets including more than 1,000 barley genes from the NCBI nr database along with more than 20,000 barley genes identified from EST sequences. On the Barley1 GeneChip^{TM} each gene is represented by a probe set composed of 11 pairs of 25mer oligonucleotides with a bias toward the 3' end of the gene. Each probe pair consists of a perfect match and a mismatch oligonucleotide. The mismatch oligonucleotide contains a single base substitution at the 13th position. The sequence of the probe is chosen to represent a particular gene specifically. In general the perfect match probes hybridise more strongly with a given RNA sample than their mismatch counterparts. This provides the basis to determine the presence or absence of a particular RNA. In general for each probe set, the value representing the expression level of the corresponding gene (the quantitative RNA abundance) is calculated by subtracting the average difference between the set of perfect match probes and the set of mismatch probes. The mismatched oligonucleotides are also used as a measure of non-specific hybridization and local background signals. However, not all methods for analysis of Affymetrix arrays use the mismatch probes (Ute Baumann, personal communications).

The processing of the mRNA used to query the GeneChip^{TM} arrays is straight forward. The mRNA is reverse transcribed into cDNA, which is subjected to an *in vitro* transcription reaction that yields biotin-labeled cRNA. The biotin-labeled cRNA is then hybridized to the GeneChip^{TM} and the hybridized arrays are washed to remove unbound probe. Hybridization signals are developed using immunochemistry and detected using a high-resolution scanner.

Oligo nucleotide arrays are inherently more consistent from array to array (Lemieux *et al.*, 1998; Eisen and Brown, 1999) than cDNA arrays. Furthermore, an overall false change rate of only 0.76% to 0.81% was reported when Barley1 GeneChip^{TM} was verified by hybridizing with independent biological replicates from the same experiment (Close *et al.*, 2004). It has

been suggested that technical variability between GeneChip^{TM} is smaller than variability between independent biological replicates (Close *et al*., 2004).

5.3.2. Plant materials

Clipper and Sahara seeds were obtained from the Waite germplasm collection (Waite Campus, Adelaide University). Surface sterilized seeds (Chapter 2, Section 2.1) were germinated at room temperature. Four day old seedlings were transferred to a hydroponic system housed in a growth chamber (see Chapter 2, section 2.3 for the composition of the hydroponic solution and 2.4 for growth conditions). The seedlings were then subjected to various B treatments for the next two weeks as shown in Table 5.1. Plants were photographed to document stress conditions one day before harvesting.

Table 5.1. Various B treatments for Sahara and Clipper plants. B was supplied in the form of H_3BO_3.

Sahara	0 µM	100 µM	500 µM	1000 µM	2000 µM
Clipper	0 µM	50 µM	100 µM	150 µM	-

For each treatment, the four oldest leaf blades (excluding the first leaf) of three plants (representing three replicates) were harvested after two weeks of growth with B. The first leaf was excluded because in Clipper high B treatment damages the first leaf severely. Harvesting was carried out under RNase-free conditions and harvesting of leaf blades was finished within an hour to avoid circadian effects. Excised leaf blades were placed in 50 ml falcon tubes and immediately frozen in liquid nitrogen. Samples were stored at –80 ºC until RNA was extracted.

5.3.3. RNA extraction and processing

Total RNA was isolated using the Trizol method as described in Chapter 2 (section 2.8.1). Following RNA extraction total RNA was cleaned up using Qiagen RNeasy minikit (Qiagen, Australia) according to the manufacturer’s instructions. RNA was eluted with RNase-free water supplied with the kit and quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All RNA samples were electrophoresed on a 1.5% denaturing gel as described in Chapter 2 (section 2.8.4), in order to check RNA quality. RNA was stored at -20Cº.

5.3.4. Microarray hybridisation

RNA samples were sent to the Australian Genome Research Facility (AGRF) for cDNA synthesis, biotin labelled cRNA generation and Affymetrix 22K Barley1 GeneChip array hybridization.

5.3.5. Statistical analysis of microarray data

The microarray data was normalized using the Robust Multiarray Average (RMA) method (Irizarry *et al*., 2003) implemented in the statistical software package R. Differentially expressed genes were identified using the Linear Models for Microarray Data Package (LIMMA) (Smyth 2004). With LIMMA a linear model is fitted to the expression data of each gene. Comparisons were performed between data of control experiments and each of the boron-treated experiments employing an empirical Bayes' t-test. P-values were corrected according to the false discovery rate (FDR) method by Benjamini and Hochberg (1995). A cut-off value was set at $p < 0.05$, meaning the expected proportion of false positives should be less than 0.05% of identified genes.

5.3.6. Probe set annotations

Probe set sequences showing significant differential expression due to B treatment were blasted against NCBI non-redundant (nr) database for annotation (BLASTx; *E*-value < e^{-10}). The probe set sequences showing differences in expression between Clipper and Sahara in control treatment were also searched against NCBI rice database using BLASTn (BLASTn; *E*-value < e^{-20}) for annotation. The PLEXdb web site (http://www.plexdb.org/modules//PD_probeset/contig_barley1.php) was used to obtain predicted protein sequences and protein functions were assigned to probe sets using GO (Gene Ontology) terms through blast search of the probe sets' predicted protein sequences using JAFA (http://jafa.burnham.org/).

5.3.7. Microarray expression validation by Real-Time qPCR

The gene expression profiles obtained from the microarray analysis were validated by qPCR using single-stranded cDNA synthesized from the same RNA samples used for microarray hybridization. DNAse I treatment to remove possible genomic DNA contamination in total RNA samples was carried out according to the manufacturer's protocol (Ambion, Austin, TX, USA). Subsequently, first-strand cDNA synthesis was performed using Superscript III (Invitrogen, Australia) with only minor changes to the manufacturer's protocol: for each 20 µl reaction 2 µl total RNA was used together with 0.2 µl of 9mer random primers (10 µM). Barley 18S ribosomal RNA gene primers (forward- CTGCCAGTAGTCATATGCTTGTCT,

reverse- CCCCGTGTCAGGATTGG) were used to quantify relative amounts of cDNA used in each cDNA sample (normalisation). Each cDNA was diluted 20-times and 2 µl of single-stranded cDNA was used per 10 µl real-time PCR reaction volume containing 5 µl of 1x Quanti-Tect PCR master mix (Qiagen, Valencia, CA), 0.3 µM each gene-specific primer, and 0.6 µl of a 100-fold dilution of SYBR Green I dye (Applied Biosystems, Foster City, CA). PCR cycling and fluorescence measurements were performed with a Rotorgene 2000 Real-Time Cycler RG2072 (Corbett, Sydney, Australia). A three step thermal cycling program was used, beginning with an initial activation step at 95 ºC for 10 minutes followed by 35 cycles of denaturation at 95 ºC for 30s, annealing at 60 ºC for 30s and extension at 72 ºC for 30s. The probe sets used for qPCR validation included those showing up- or down-regulation, or no response due to B treatment. Forward primers for each probe set were positioned in the coding sequence and reverse primers in 3' UTR. A list of these probe sets along with their forward and reverse primer sequences is given in Table 5.2.

Table 5.2. Primer sequences of the probe sets subjected to validation test by real-time qPCR

Probe set names	Direction	Primer sequence	Tm (ºC)
Contig11160_at	Forward	ACGGCGCACAACGTCGTCTC	60.0
	Reverse	AACCCGTTCCGCCCATGTCT	60.0
Contig13632_at	Forward	CTTGGGCTGCTCCTGGGTCTTG	60.0
	Reverse	GAACAATCTGGCTTGCCCCACA	60.0
Contig2113_at	Forward	CAATCTGGACGTGTCGACCCCTTAC	61.5
	Reverse	GGCCTTTATGGCTTTGCACATTGAC	61.5
Contig21141_at	Forward	CAACATATATGCCTCGGAGAGGATG	57.8
	Reverse	GCACTCTTGTCTCAGCTTCTTTTCC	56.8
Contig2209_at	Forward	CCAGAGCTACGCCAACCAGAGGATC	63.0
	Reverse	CGTGAGGAACGAGGGACTACTGGAC	61.0
Contig22666_at	Forward	CCTCAACTACATCACGGGCAACGAG	62.5
	Reverse	CCGATCCACGAACACCAGTATAACC	59.4
Contig24328_at	Forward	CCGGCATATGGCTGAGAATGTACC	60.0
	Reverse	GCACGTTTGCCATCCTTACACTTCTAGT	60.0
Contig3097_at	Forward	GGTTGAGTTCACCGGCGTCACC	61.0
	Reverse	GCTGCGAAGCAACCGAACAAGA	61.0
Contig3112_at	Forward	CTTCAGGGGCTCGTGGCTCATCATC	65.6
	Reverse	GGAAACATCGCCGAGACAGTTCATC	61.7
Contig3239_at	Forward	CGCTCTTCGCCTCTGACTTTGTGAC	62.2
	Reverse	TAGAGGATTGCATGCACACGAGCTG	62.2
Contig8538_at	Forward	AAACATGACCTCTCAGAGGTAGCAACCC	61.0
	Reverse	CGACAAAACTTAGCCCACTGGAGCATTA	61.0
Contig8635_at	Forward	TGGAGGCTTGCTGTACATGAAGGAG	60.5
	Reverse	GCTGTGTCTCCGTAATTTTGCCTAC	57.3

5.4. Results

For microarray hybridizations RNA form three biological replicates were used. Differentially expressed genes were identified using LIMMA (Smyth 2004) and by comparing data of control experiments to each of the boron-treated experiments using Bayes' t-test. A cut-off P-value of 0.05 was employed.

The expression of approximately 22400 transcripts was analysed in tolerant (Sahara) and intolerant (Clipper) barley varieties at different B treatments using the Affymetrix Barley1 GeneChip^{TM}. The target tissue was leaf blades as they are not directly exposed to B, rather B stress in leaf is the result of gradual accumulation of B over time. B accumulation in the leaf blades of susceptible and tolerant varieties varies distinctively over time and the level of tissue tolerance also varies between the susceptible and tolerant varieties which becomes evident by delayed leaf symptom expression in the tolerant barley variety Sahara.

5.4.1. Development of leaf symptoms due to B stress in Clipper and Sahara

Plants were photographed on the 13th day of B treatment (one day before harvest) to keep a photographic record of the extent of B stress in Clipper and Sahara plants at different B concentrations. These photographs are shown in Figure 5.4.1 and Figure 5.4.2, respectively. Clipper plants were quite healthy at the control B treatment but showed moderate necrotic symptoms at 50 µM B after about two weeks (Figure 5.4.1). However these symptoms were mostly limited to the first leaf. At 100 and 150 µM B (Figure 5.4.1) the severity increased with B concentration and leaves were seen to develop chlorosis (pale green to yellow). At 150 µM B smaller necrotic patches started to coalesce and leaf damage became more apparent. On the other hand Sahara plants were found to be healthy even at 500 µM B (Figure 5.4.2) after two weeks. The symptoms expressed in Sahara at 1000 and 2000 µM B were much less severe than that seen in Clipper plants even at 50 µM B.

0 µM

50 µM

100 µM

150 µM

Figure 5.4.1. Leaf symptom expression in Clipper barley grown at a range of B concentrations. Photographs were taken on the 13th day of B treatment, one day before harvest.

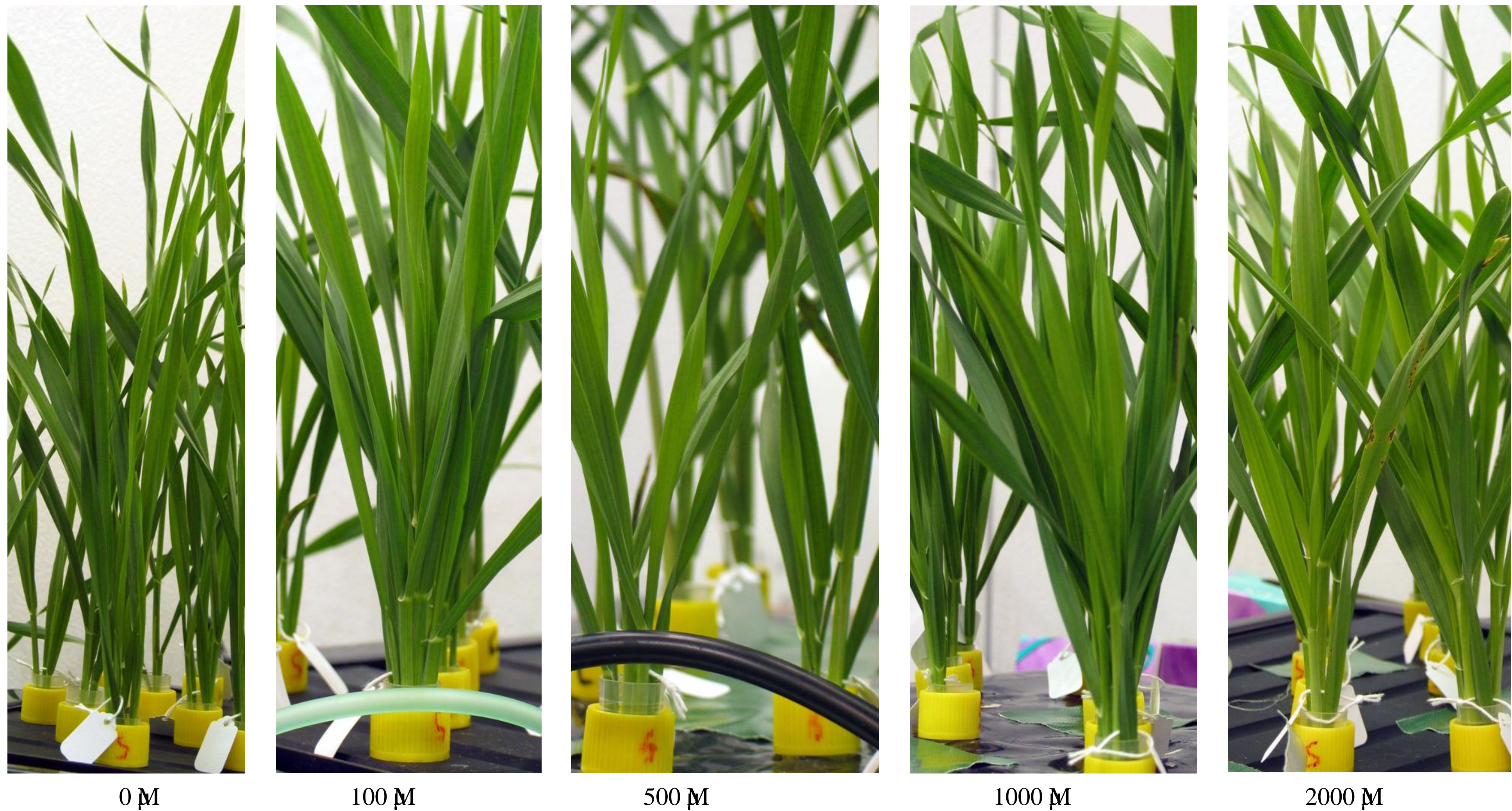


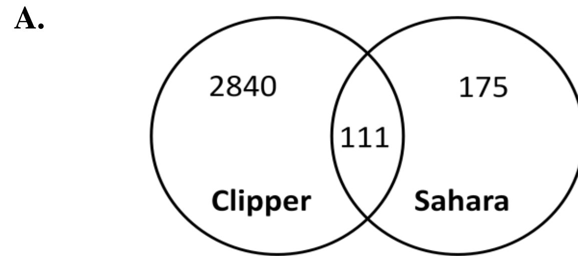
Figure 5.4.2. Leaf symptom expression in Sahara barley grown at a range of B concentrations. Photographs were taken on the 13th day of B treatment, one day before harvest.

5.4.2. The transcriptional responses of Clipper and Sahara barley to B treatment

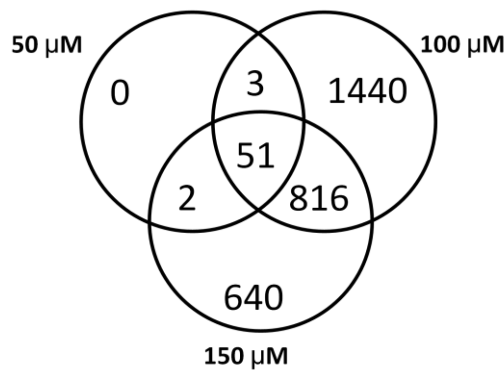
The susceptible nature of Clipper was reflected at the expression level, as a large number of probe sets (2851) were differentially regulated. In contrast, in Sahara the number of probe sets differentially regulated was about one tenth (286) of that seen in Clipper. Similar results were reported when comparative transcriptional profiling was carried out between salinity sensitive rice variety IR29 and tolerant FL478 under salt stress (Walia *et al*., 2006). These authors also found that the response of the sensitive variety was characterized by induction of a larger number of probe sets than the tolerant variety. Figure 5.4.3A illustrates the number of probe sets induced by B treatments in Clipper and Sahara and the overlap between these two genotypes. Figure 5.4.3B and Figure 5.4.3C illustrate the number of probe sets induced by different B treatments in Clipper and Sahara, respectively and the overlap between B treatments. A total of 111 probe sets were differentially regulated commonly in Clipper and Sahara by B treatment. This represents less than 4% of the total probe sets in Clipper and about 40% of probe sets in Sahara that responded to B stress. In Clipper the highest number of probe sets found to be differentially regulated (2310) was when plants were treated with 100 μM B. Interestingly, when Clipper plants were treated with a harsher B regime of 150 μM, the number of probe sets differentially regulated was about two thirds (1509) of that seen with 100 μM B. Although at 50 μM B necrotic symptoms were visible in Clipper leaf blades only 56 probe sets were differentially regulated. A total of 51 probe sets were differentially regulated under all treatment levels in Clipper. Only five of these were also differentially regulated in Sahara. Two of the probe sets code for photosystem I P700 apoprotein A1 (*Triticum aestivum*) and were highly up-regulated in Clipper (up to 32 fold) but significantly down-regulated in Sahara. Two probe sets coding for pathogenesis-related protein 1a (barley) that has previously been reported to be up-regulated in the common ice plant following salt stress (Kore-eda *et al.*, 2004), showed up to 64 fold up-regulation in Clipper and up to 16 fold up-regulation in Sahara. No sequence similarity was found for the fifth probe set sequence by NCBI (nr) BLASTx search. A full listing of NCBI (nr) BLASTx best sequence similarities of these 51 probe sets is given in Table 1 in Appendix D.

In Sahara, most of the differentially regulated probe sets (approximately 90%) were detected only in response to the high B concentration of 2000 μM. At 100 μM B no change in the expression level of any probe set was observed. Although at 500 μM B the number of probe sets differentially regulated was 30, at 1000 μM B only seven probe sets were seen to be differentially regulated. There were only four probe sets that were commonly induced by all three B treatment levels in Sahara.

A.



B. **Clipper**



C. **Sahara**

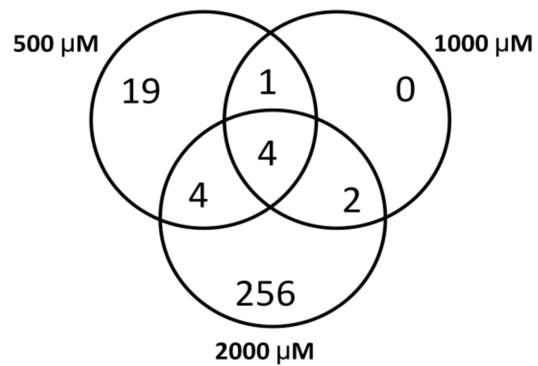


Figure 5.4.3. Venn diagrams illustrating the number of probe sets differentially regulated by B: **A.** Clipper and Sahara over all B treatments, **B.** by different B treatments in Clipper and **C.** by different B treatments in Sahara.

The sequences of the 286 probe sets differentially regulated by B in Sahara were subjected to nucleotide similarity searches at the NCBI using BLASTx (Altschul, 1997). A threshold E value of e^{-10} was used. The results of the sequence similarity searches are shown in Figure 5.4.4. A full listing of BLASTx (NCBI nr) results of all probe sets that responded to B treatment in Sahara is given in Table 2 in Appendix D.

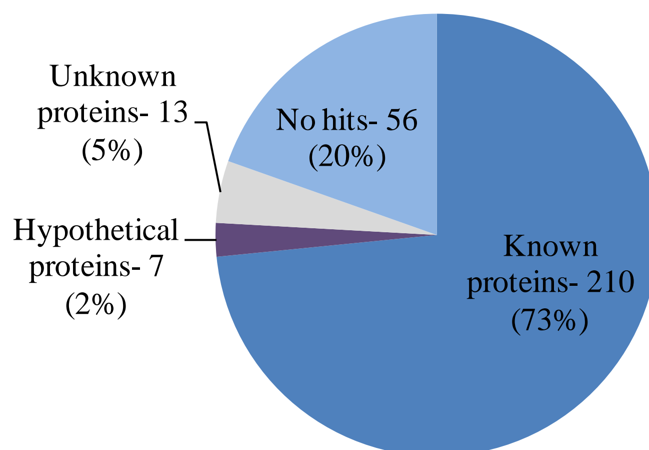


Figure 5.4.4. Summary of sequence similarity searches of 286 probe sets differentially regulated by B in Sahara using BLASTx against the NCBI non-redundant (nr) database.

Figure 5.4.5 shows the response of these 286 probe sets in Sahara and Clipper. About 30% of these were down-regulated and about 70% were up-regulated in Sahara in response to B treatments. Approximately 66% of probe sets down-regulated in Sahara did not respond to B treatment in Clipper. About 26% of probe sets down-regulated in Sahara were also found to be down-regulated in Clipper in response to B treatment. Only about 8% of these probe sets (down-regulated in Sahara) were up-regulated in Clipper. Similarly most of the probe sets (59%) up-regulated in Sahara did not respond to B treatments in Clipper. Only 3.5% of probe sets up-regulated in Sahara were also seen to be up-regulated in Clipper. About 37% of these probe sets (up-regulated in Sahara) were down-regulated in Clipper.

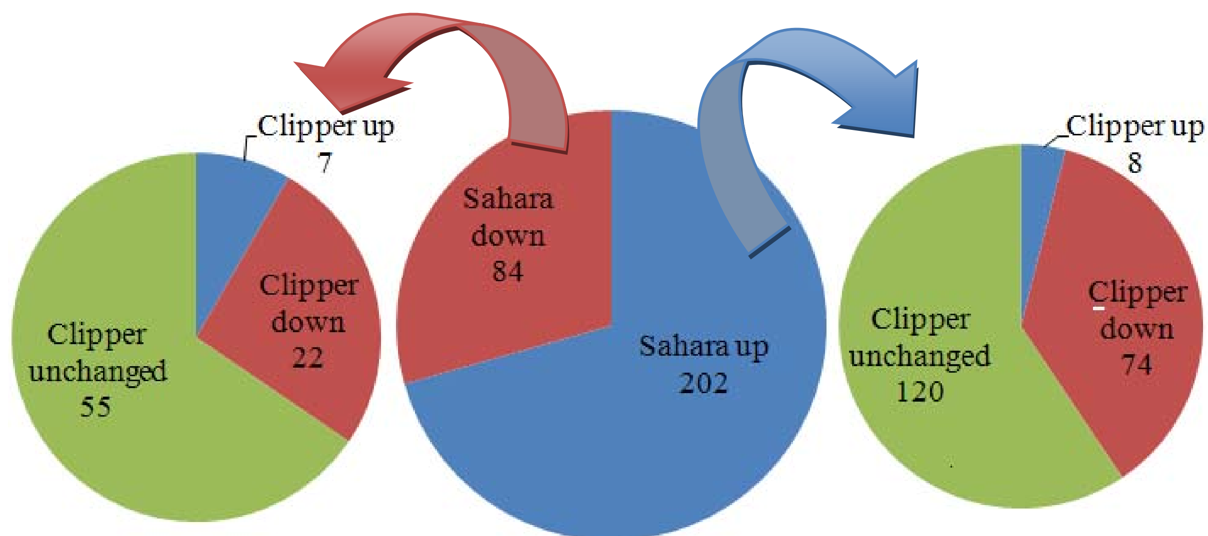


Figure 5.4.5. Responses of probe sets in Clipper that were seen to be up- or down- regulated in Sahara

5.4.3. Real-Time qPCR validation of microarray expression results

The gene expression profiles obtained from microarray analysis were validated by qPCR using cDNA synthesized from the RNA samples used for microarray hybridizations. A total of 12 primers pairs were designed in order to generate amplicons representative of all Affymetrix GeneChip probe sets. Transcripts were selected to represent up- or down-regulation or no response due to B treatment. qPCR data showed high correlation with the expression profiles obtained from microarray analysis (correlation coefficients ranged between 0.829 and 0.999 where every two out of three microarray and qPCR datasets had a correlation coefficient of 0.99) (Figure 5.4.6). This analysis suggested that the expression profiles obtained from the microarray data were valid.

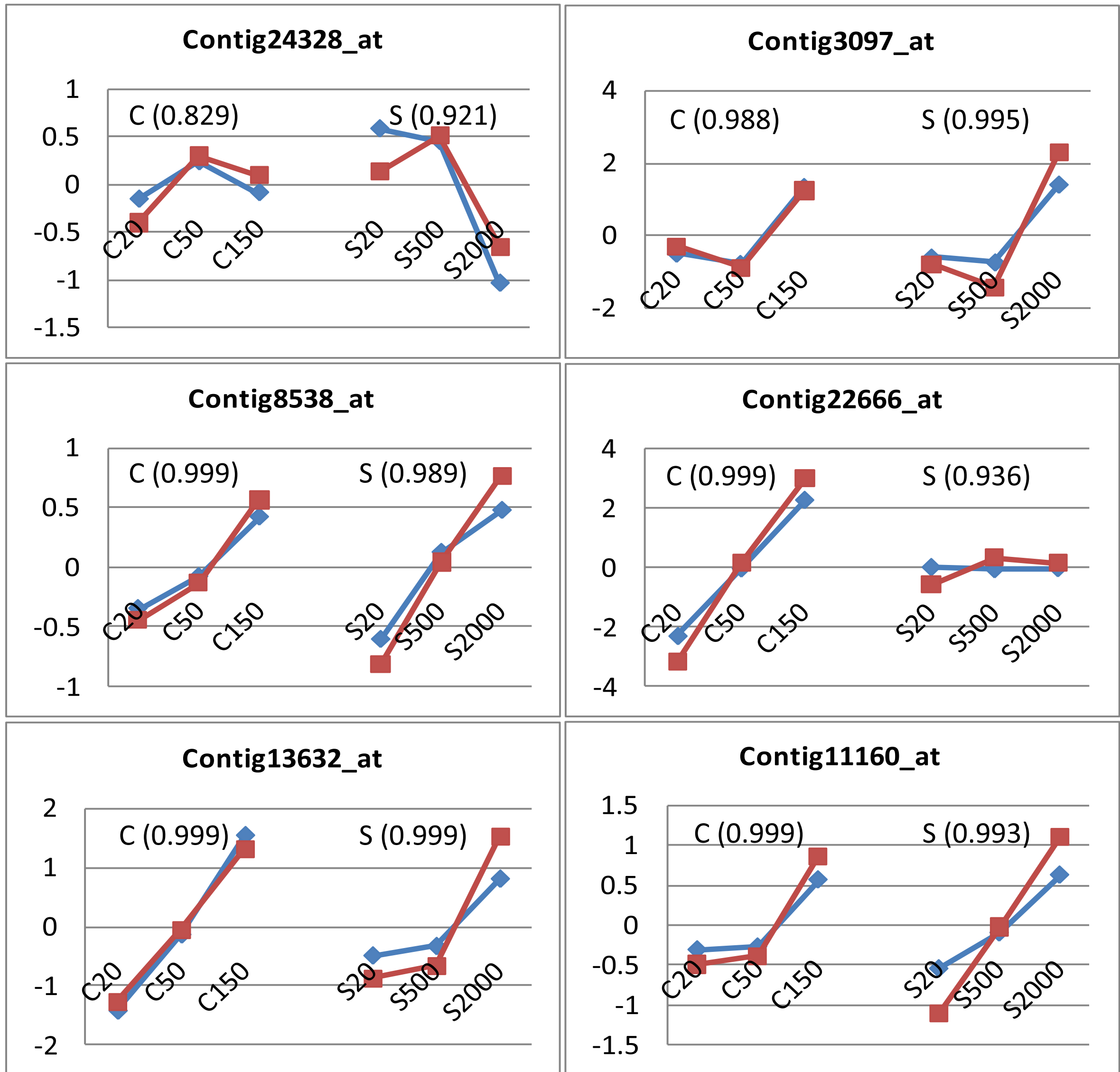


Figure 5.4.6. Comparison of microarray expression profile of selected probe sets with expression data obtained from real-time qPCR analysis. Blue lines represent expression data from microarray and the orange lines represent real-time qPCR data. The microarray data is log base 2, RMA normalized and centered about the average over three treatment levels for Clipper and Sahara. The qPCR data is log base 2, normalized mRNA copies/µL and centered. In each chart the correlation coefficient between the microarray and qPCR data sets are shown in parenthesis indicated by C and S for Clipper and Sahara, respectively.

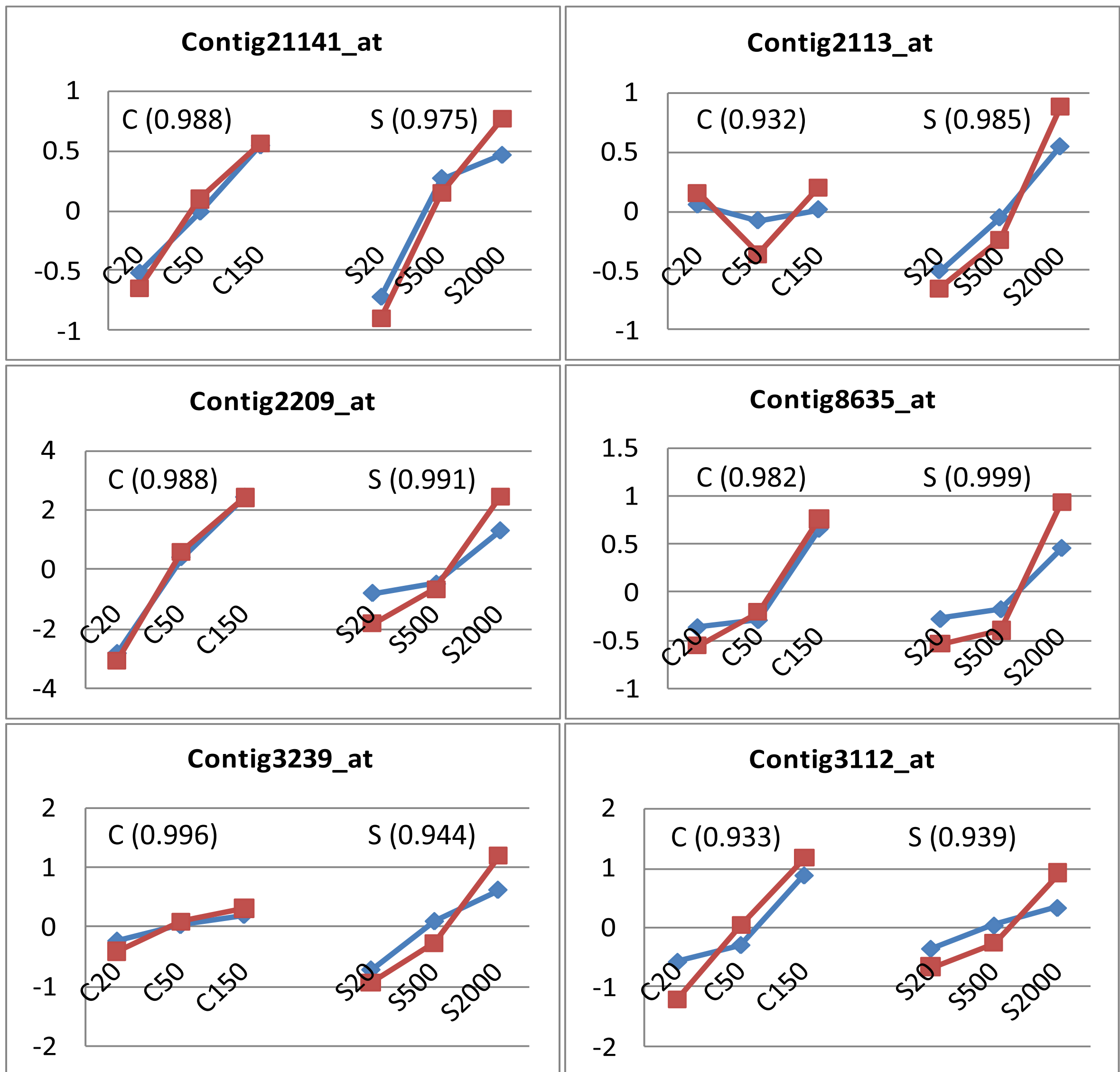


Figure 5.4.6 (contd).

5.4.4. Down-regulated transcripts in Sahara

Out of the 286 probe sets differentially regulated in Sahara, 59 were seen to be up- or down-regulated more than two- fold. Twenty of these probe sets did not respond significantly to B treatment in Clipper. Only three of these 20 probe sets were down- regulated (in Sahara). The maximum three- fold down-regulation was seen for 'Contig24328_at' which codes for an unknown protein [*Oryza sativa* (japonica cultivar-group)] (NCBI nr). The other two down-regulated probe sets are 'X74365_at'- an ATP synthase subunit 9 (*Hordeum vulgare)* and 'HVSMEc0015H24f_at'- a NADH-plastoquinone oxidoreductase subunit K (*Hordeum vulgare*). Both were down-regulated about two- fold. Given these sequences are very short (CDS < 280 bp, Ute Baumann, personal communications) the observed regulation needs to be considered with caution.

Recently, subunit 9 of *Arabidopsis* mitochondrial ATP synthase was identified, however its function is not clear (Heazlewood *et al*., 2003). Zhang *et al*. (2005) reported that the expression of rice mitochondrial ATP synthase subunit 9 (*RMtATP9)* was slightly affected in leaf blades under various stresses (80 mM NaCl, 30 mM $NaHCO_3$, 15 mM Na_2CO_3 and 10% PEG 6000), but the expression of *RMtATP9* in roots was clearly down-regulated by Na_2CO_3 and PEG treatment. However, the stress treatments adopted in this experiment lasted for only 24 hours which would probably only stress the roots and not the leaves. Compared to this the two week long B treatment used in the current experiment is likely to stress plant leaves to a much greater extent. Not much information is available about the expression of F_1F_0-ATPase subunit genes in response to environmental stresses. Sweetlove *et al*. (2002) found that the ATP synthase α-subunit and ß-subunit expression were significantly down-regulated by oxidative stress treatments in *Arabidopsis* cells which led the authors to speculate that oxidative stress largely affects the ATP synthase subunit**s** negatively (suppressing expression).

Increased expression and activity of NADH-plastoquinone-oxidoreductase (NDH) under abiotic stress conditions has previously been reported (Casano *et al*., 2000, 2001; Yao *et al*., 2001). Yao *et al*. (2001) reported increased NDH activity and NdhK (NADH-plastoquinone oxidoreductase subunit K) in chloroplasts of heat stressed (at 50 ºC) tobacco (*Nicotiana tabacum*) plants. Wang *et al*. (2006) postulated that NDH-mediated cyclic electron transport may alleviate the stress. However, Guera *et al.* (2004) showed that the expression of NDH in the thylakoid membranes of barley to alleviate ozone induced oxidative stress is leaf-age-dependent. They found significant amount of the NDH complex in mature barley leaves, but

not in young barley leaves. Guera *et al.* (2004) observed that young barley leaves behaved like *ndh*-deficient leaves. The decreased expression of NdhK in the present experiment is thus probably not surprising as only young immature leaf blades were used.

The other 39 probe sets that were differentially regulated in Sahara were also seen to be differentially regulated in Clipper. Again three probe sets from this group of 39 were down regulated beyond two- fold (in Sahara). These probe sets are 'HV_CEa0013J19f_at', a photosystem I P700 apoprotein A1 (*Triticum aestivum*), 'HVSMEc0016D02f_at', a photosystem I P700 chlorophyll A apoprotein A1 (*Oryza sativa*) and 'HVSMEa0022N20f_at', a Photosystem I subunit IX (*Nicotiana tabacum*). Again all of these three probe sets represent short sequences and their regulation needs to be carefully considered. The first two probe sets were strongly up-regulated in Clipper (beyond 16 fold) even at 50 µM B concentration. Jantaro *et al.* (2005) investigated the effect of ionic stress on photosystem I P700 chlorophyll A apoprotein A1 (psaA) in a moderately halotolerant cyanobacterium (*Synechocystis* spp.) by treating them with 22, 170, 370 and 570 mM NaCl. They showed that moderate ionic stress (up to 370 mM) decreased the *psaA* transcript level whereas high salt treatment of 570 mM had no significant effect. Marin *et al*. (2004) also reported a decrease in the amount of *psaA* transcript in *Synechocystis* soon after the onset of salinity stress. In the current experiment *psaA* expression in B tolerant Sahara and intolerant Clipper under B stress apparently is in agreement with the results of Jantaro *et al.* (2005). It appears that *psaA* may initially be affected by ionic stress but its expression is bolstered in the later stages of ionic stress in order to increase photosynthetic activity in the undamaged chlorophyll to compensate for the lost photosynthetic area due to necrosis/apoptosis seen in Clipper leaf blades.

Photosystem I subunit IX was up-regulated a little over two- fold in Clipper at all B concentrations and was down-regulated in Sahara. Kreps *et al*. (2002) reported that Photosystem I subunit III and IV mRNA was up-regulated 2-3 fold in *Arabidopsis* after three hours of salt treatment. It is apparent that Photosystem I subunit IX shows a similar response to B stress in the B intolerant barley.

5.4.5. Up-regulated transcripts in Sahara

5.4.5.1. Up-regulated in Sahara, no change in Clipper

Among the probe sets that were differentially regulated only in Sahara 17 showed up-regulation beyond two- fold. Database searches revealed no information about putative function for three probe set sequences and one sequence showed significant similarity to a

database sequence of an unknown protein. Table 5.3 lists the putative ID and fold changes and functional annotations of these probe sets due to various B treatments in Sahara. These transcripts could be crucial in protecting the barley plants against B stress as they are B responsive in Sahara but not in Clipper. Several of these genes have been described in the literature to have defense response activity. Others have also been reported to be induced by salinity stress.

Two of these probe sets 'Contig3239_at' (bacterial-induced peroxidase precursor) and 'Contig2113_at' (peroxidase) are involved in oxidative stress response. Particularly 'Contig3239_at' was seen to be up-regulated at 500 µM B. This provides an indication that B toxicity in barley may cause oxidative stress. This is not unlikely as salt and other ionic stress (for example aluminium) causes accelerated production of reactive oxygen species in plants such as superoxide, hydrogen peroxide, and the hydroxyl radical resulting in oxidative stress (Bellaire *et al.,* 2000, Hernández *et al*., 2001; Pastori and Foyer, 2002; Hamel *et al*., 1998). Up-regulation of peroxidases to alleviate B induced oxidative stress in barley is further supported by the reported up-regulation of peroxidases in tobacco cells upon exposure to aluminium (Ezaki *et al*., 1996).

One of the most strongly up-regulated transcripts in Sahara encoded a barley germin-like protein (GLP) (Contig3155_s_at). GLPs are associated with the cell wall (Lane *et al*., 1992; Heintzen *et al*., 1994; Lane, 1994; Berna and Bernier, 1997). It has been reported that transcription of some *GLP* genes is influenced by stress conditions (Bernier and Berna, 2001). Tabuchi *et al.* (2003) showed that GLP isolated from the halophyte *Atriplex lentiformis* has superoxide dismutase (SOD) activity. Since SOD rapidly catalyzes the dismutation of superoxide to H_2O_2, the enzyme is thought to inhibit hydroxyl radical production, and protect living cells from damage. Among various defense responses upon powdery mildew fungus infection in wheat and barley, the localized production of reactive oxygen species is well known (Huckelhoven and Kogel, 1998; Thordal-Christensen *et al.,* 1997). Accumulation of wheat *TaGLP2a* transcripts in epidermal cells of leaf blades after inoculation with powdery mildew fungus has previously been reported by Schweizer *et al*. (1999). Ozturk *et al.* (2002) also reported 1.7 fold up-regulation of *GLP* in barley within 24 hours of salt stress. Thus the up-regulation of a germin-like protein in Sahara leaf blades in the present experiment is a likely response towards B induced oxidative stress.

'Contig1580_x_at' coding for thionin was found up-regulated in Sahara almost to the similar extent as *GLP*. Thionins are also wall proteins known to have a role in plant defense (Gausing 1987; Bohlmann *et al*., 1988). Bohlmann *et al.* (1998) demonstrated that in *Arabidopsis* seedlings the thionin gene *Thi2.1* is inducible by methyl jasmonate, wounding, silver nitrate, coronatine, and sorbitol.

Allene oxide synthases (AOS) are involved in the biosynthesis of jasmonate. 'Contig3097_at' coding for AOS was two fold up-regulated in Sahara under 2000 µM B and could contribute towards the up-regulation of thionin precursors in Sahara. The reason for a down-regulation of thionin precursors in response to 500 µM B is not clear. However, AOS was also seen to be slightly down-regulated at this B concentration in Sahara. Another gene involved in the jasmonic acid biosynthesis lipoxygenase 2.2 also showed similar pattern of induction.

The other defense related gene up-regulated in Sahara codes for a beta glucan binding protein (Contig9476_at) and is part of the plasma membrane-localized pathogen receptor complex and is able to bind to microbial cell wall elicitors (Fliegmann *et al.,* 2005). It has been reported to activate defence responses in soybean (Sharp *et al.,* 1984).

A number of salt responsive genes and associated transcription factors were induced by B in Sahara. For example a chalcone synthase-1 gene (Contig11944_at) was up-regulated over two fold, and is involved in flavonoid biosynthesis. Flavonoids are known to have roles in stress protection (Winkel-Shirley, 2002). Kreps *et al*. (2002) reported 1.6-2.4 fold changes of expression of chalcone synthase genes in *Arabidopsis* in response to salt stress. Another example is a barley homologue of wheat ribulose-1·5-bisphosphate carboxylase/oxygenase small subunit (Contig346_at) that was seen to be 1.2 fold up-regulated in Sahara. Recently, ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) has also been linked to salinity stress (Walia *et al.,* 2007). Walia *et al.* (2007) reported 2.2 fold up-regulation of expression of gene encoding rubisco subunit 1A in barley seedling. A bZIP transcription factor CPRF2 can act as a transcriptional activator or repressor for both ribulose-1·5-bisphosphate carboxylase/oxygenase small subunit and chalcone synthase, by binding to the hexameric G-box located in their promoter regions (Kircher *et al*., 1999). Thus the up-regulation of the barley homologue of parsley CPRF-2 (Contig8538_at) appears to be in line with up-regulation of the above mentioned two probe sets namely 'Contig11944_at' and 'Contig346_at'.

A gene coding for a cold-regulated protein up-regulated by B treatment in Sahara in this experiment has previously been reported to be up-regulated by salt treatment. The gene encodes a cold-regulated protein *COR15B* was reported to be 78-fold induced in *Arabidopsis* upon salt stress (Kreps *et al*., 2002). A gene coding for RAV2-like protein (Contig7481_at) was also seen to be up-regulated in Sahara. A similar degree of up-regulation of a gene coding for RAV2-like protein was also reported in *Arabidopsis* by salinity treatment (Kreps *et al*., 2002). RAV2 are a cold inducible transcription factor (Sung *et al.,* 2003). However, the types of genes regulated by RAV2 is not identified yet (Guilfoyle and Hagen, 2001). Several genes encoding isoforms of lipoxygenase were reported to be up regulated in barley including LOX2:Hv:2 by salt stress (Walia *et al.,* 2007). Similar observation was also made in *Arabidopsis* due to salt stress (Kreps *et al.*, 2002). In agreement with this, *LOX2:Hv:2* was up-regulated in Sahara by B in this experiment.

Two dormancy related proteins, 'Contig1762_s_at' and 'Contig7516_at', were up-regulated in Sahara in response to 500 µM B. However, an exact role for their function in the plant's response to abiotic stress has not been reported in the literature and thus further study is required to explain the response.

5.4.5.2. Up-regulated both in Sahara and Clipper

Another 35 probe sets that were up-regulated in Sahara beyond 2 fold (up to 8 fold) were also seen to be up-regulated in Clipper. Only a few of these probe sets (7) were seen more strongly up-regulated in Sahara plants than in Clipper. However, the degree of up-regulation of these genes over Clipper was small. Twenty out of 35 probe sets up-regulated in Sahara were even more strongly up-regulated (up to 42 fold) in Clipper at 150 µM B than in Sahara at 2000 µM B (Appendix C, Table 1 and Table 2.). Some of these probe sets showed even stronger up-regulation in Clipper at 100 µM B than in Sahara at 2000 µM B. The up-regulation of these probe sets in Clipper may be considered as non-adaptive response of Clipper to toxic B. Here the term non-adaptive response means transcriptional responses in Clipper under B stress that does not provide any protection against the toxic effect of B in these plants.

5.4.5.3. Up-regulated in Sahara and down-regulated in Clipper

There was only one probe set in this category, namely 'Contig12563_s_at' which has sequence similarity (8E-32) with a putative chalcone synthase (*Oryza sativa*). This gene was two fold up-regulated at 2000 µM B in Sahara but down-regulated (1.5 fold) at all B concentrations in Clipper.

Table 5.3. Probe sets up-regulated (≥2 fold) in Sahara alone but not differentially regulated in Clipper by B treatments.

Probe set name	Putative ID (BLASTx) (nr)	E- value	Expression fold change (log2)			GO biological process
			500 µM	1000 µM	2000 µM	
Contig8658_at	unknown protein (*Oryza sativa*)	6E-91	0.82	0.47	1.00	no hits found
Contig4281_s_at	cold-regulated protein *(Hordeum vulgare)*	3E-77	0.66	0.38	1.01	no hits found
Contig2113_at	peroxidase [Oryza sativa]	1E-113	-0.21	0.51	1.06	response to oxidative stress
Contig9476_at	beta-glucan binding protein *(Phaseolus vulgaris)*	0	0.40	0.37	1.06	no hits found
Contig14625_at	no hit		0.14	0.11	1.09	-
Contig8538_at	light-induced protein CPRF-2 - parsley	9E-46	0.66	0.75	1.09	regulation of transcription
Contig2305_at	Lipoxygenase 2.2, chloroplast precursor (LOX2:Hv:2)	0	-0.19	0.09	1.12	electron transport
Contig2632_s_at	no hit		0.79	0.56	1.14	-
Contig7481_at	DNA-binding protein RAV2-like (*Oryza sativa*)	1E-23	0.87	0.77	1.16	no hits found
Contig11944_at	putative chalcone synthase 1 (*Oryza sativa*)	1E-111	-0.11	0.26	1.19	flavanoid biosynthsis
Contig346_at	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*Triticum aestivum*)	1E-09	-0.13	0.05	1.20	no hits found
Contig7516_at	Drm3 *(Pisum sativum)*	1.00E-06	0.78	0.67	1.28	no hits found
Contig3239_at	bacterial-induced peroxidase precursor (*Gossypium hirsutum*)	1E-106	0.94	0.80	1.35	response to oxidative stress
HV_CEb0009D09r2_at	no hit		0.23	0.20	1.49	-
Contig1762_s_at	dormancy-associated protein - apple tree	4E-25	0.91	0.82	1.52	no hits found
Contig1580_x_at	thionin precursor, leaf *(Hordeum vulgare)*	5E-70	-1.15	-0.03	1.80	defense response
Contig3155_s_at	germin-like protein *(Hordeum vulgare)*	1E-113	0.33	0.38	1.84	no hits found

5.4.6. Expression difference between Clipper and Sahara control plants

Current evidence suggests that in Sahara the expression of the genes responsible for B tolerance through exclusion is constitutive (Hayes and Reid, 2004). A comparison of differences in gene expression between Clipper and Sahara under control condition was done to identify any transcript that may account for such possibility.

Only 1.6 % (368) probe sets showed more than two fold expression in Sahara over Clipper. On the other hand 2.3% (515) probe sets showed more than two fold expression in Clipper over Sahara. Maximum over-expression in Sahara over Clipper was found to be up to 212 fold. The maximum over-expression in Clipper over Sahara was 153 fold. Pie charts in Figures 5.4.7 and 5.4.8 show the number of probe sets showing different degrees of over-expression (fold change categories) over the other variety in Sahara and Clipper, respectively. Table 5.4 and Table 5.5 lists the putative ID of the probe sets that were over expressed 16 fold and above in Sahara and Clipper relative to the other variety, respectively. The results from BLASTn search against the rice database are also shown in Table 5.4 and Table 5.5. However, more work is needed to be certain whether these expression differences are indeed real or due to SFP.

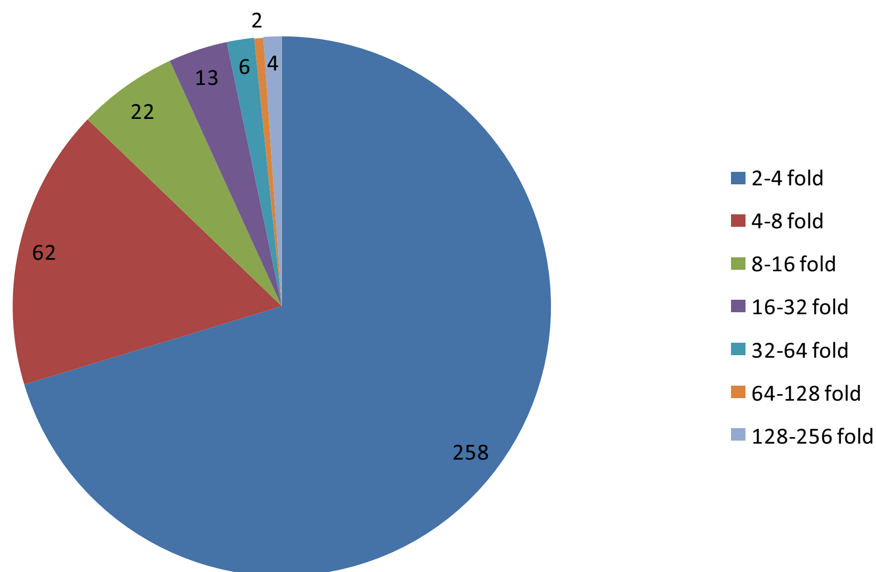


Figure 5.4.7. Fold change categories and the category wise numbers of probe sets over-expressed in Sahara relative to their expression in Clipper.

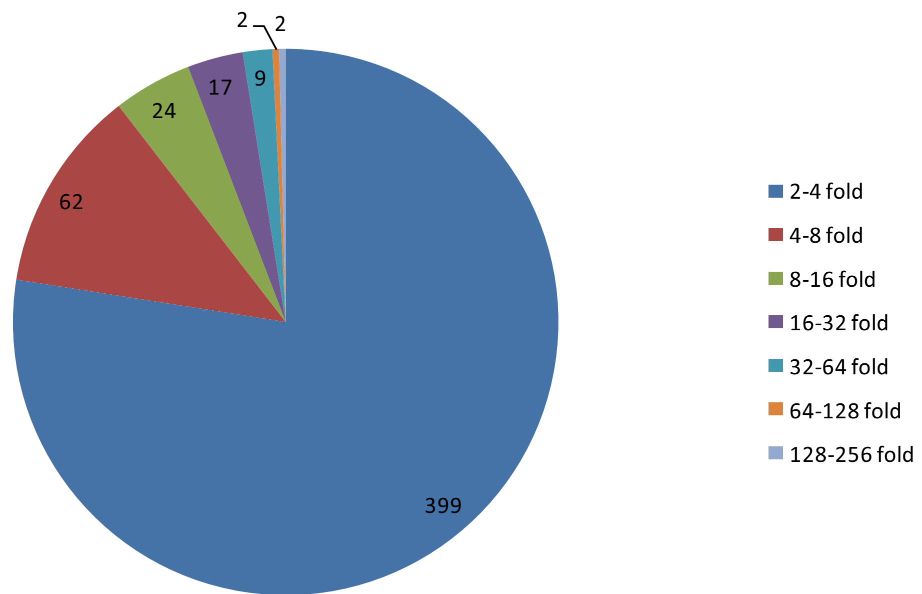


Figure 5.4.8. Fold change categories and the category wise numbers of probe sets over-expressed in Clipper relative to their expression in Sahara.

Table 5.4. Annotations of probe sets over-expressed in Sahara with a fold change ratio of 16 and above their expression in Clipper.

Fold change (Log2)	Probe set Names	BLASTx nr	E -value	BLASTn against rice db (cut-off e-20)	E- value
7.76	baak46e09_s_at	no hit		no hits found	
7.54	Contig5586_at	putative ribosomal protein L18 *(Oryza sativa)*	7E-62	putative ribosomal protein L18	e-126
7.39	Contig9843_s_at	hypothetical protein *(Oryza sativa)*	2E-34	hypothetical protein	3E-46
7.25	Contig11524_at	no hit		no hits found	
6.71	baak16l04_x_at	chlorophyll a/b-binding protein precursor - swollen duckweed	4E-37	chlorophyll a/b binding protein 1	5E-62
6.57	Contig3339_at	OSJNBa0020P07.12 *(Oryza sativa)*	1E -114	expressed protein	e-119
5.66	HVSMEm0006I15r2_at	unknown protein *(Oryza sativa)*	1E-41	no hits found	
5.65	rbah11m03_at	expressed protein *(Arabidopsis thaliana)*	3E-14	PQ loop repeat, putative	4E-49
5.20	Contig3552_at	O-methyltransferase *(Secale cereal)*	1E -168	O-methyltransferase	1E-32
5.19	Contig21126_at	P0013F10.1 *(Oryza sativa)*	9E-30	is a member of the PF|00955 Anion exchanger family.	1E-21
5.18	Contig6615_at	CAA30376.1 protein *(Oryza sativa)*	4E-33	no hits found	
5.03	Contig9927_s_at	heat shock factor protein hsf8 -related *(Arabidopsis thaliana)*	1E -114	S1 RNA binding domain, putative	e-176
5.00	Contig9177_at	OSJNBb0079B02.2 [*(Oryza sativa)*	3E-83	shikimate kinase, putative	0
4.79	Contig5370_at	OSJNBb0067G11.7 *(Oryza sativa)*	1E -124	copper amine oxidase-like protein, A. thaliana	e-135
4.75	Contig4910_at	O-methyltransferase *(Secale cereal)*	1E -117	no hits found	
4.43	Contig4621_at	proline rich protein homolog WCOR518 *(Triticum aestivum)*	8E-41	proline rich protein - apple tree	1E-29
4.41	Contig422_at	chlorophyll a/b-binding protein WCAB precursor *(Triticum aestivum)*	1E -146	chlorophyll A-B binding protein, putative	e-158
4.35	Contig2279_at	no hit		no hits found	
4.32	Contig5058_x_at	RNase S-like protein *(Hordeum vulgare)*	2E-53	RNase S-like protein	2E-43
4.27	Contig6075_at	unnamed protein product *(Oryza sativa)*	1E-114	phosphorylase family	5E-90
4.27	Contig3216_at	defensin *(Triticum aestivum)*	5E-20	no hits found	
4.21	Contig143_at	putative microtubial binding protein *(Oryza sativa)*	6E-58	microtubule associated protein 1A/1B, light chain 3	e-110
4.17	Contig2279_s_at	no hit		no hits found	
4.09	Contig2769_s_at	adenosine diphosphate glucose pyrophosphatase *(Triticum aestivum)*	2E-79	cupin, putative	e-159
4.00	HVSMEc0017M18f_x_at	hypothetical protein *(Oenothera elata)*	9E-12	new cDNA-based Gene	e-113

Table 5.5. Annotations of probe sets over-expressed in Clipper with a fold change ratio of 16 and above their expression in Sahara.

Fold change (Log2)	**Probe set Names**	**BLASTx nr**	**E - value**	**BLASTn against rice db (cut-off e-20)**	**E - value**
7.26	rbaal9i05_at	putative protein kinase Xa21, receptor type precursor *(Oryza sativa)*	6E-55	-no hits found	
7.05	Contig3241_at	probable peroxidase (EC 1.11.1.7) (clone PC44) - spinach	7E-47	bacterial-induced peroxidase precursor	2E-97
6.70	Contig993_at	no hit		expressed protein	1E-22
6.52	Contig3842_at	putative embryogenesis-abundant protein *(Oryza sativa)*	1E-57	late embryogenesis abundant protein	5E-76
5.89	Contig3140_at	probable acyl-CoA oxidase (EC 1.3.3.6), peroxisomal *(Hordeum vulgare)*	5E-41	acyl-CoA oxidase, putative	4E-86
5.88	Contig3660_at	putative ABC transporter *(Oryza sativa)*	0	FeS assembly protein SufB	0
5.83	Contig6215_at	no hit		no hits found	
5.82	rbaal14f06_s_at	no hit		no hits found	
5.78	HV09F14u_at	no hit		no hits found	
5.40	Contig4873_s_at	putative ribosomal protein *(Oryza sativa)*	1E-30	putative ribosomal protein	1E-48
5.39	Contig2710_s_at	B1146B04.15 *(Oryza sativa)*	1E-12	no hits found	
5.35	Contig16182_at	OSJNBa0085I10.18 *(Oryza sativa)*	8E-35	PA domain, putative	5E-42
5.28	Contig25218_at	hypothetical protein *(Oryza sativa)*	5E-43	putative cyclase	1E-79
4.98	Contig16307_at	putative cytochrome P450, 5'-partial *(Oryza sativa)*	2E-77	no hits found	
4.97	Contig3618_at	putative LIM-domain protein *(Oryza sativa)*	3E-90	LIM domain, putative	e-166
4.95	Contig11886_s_at	OSJNBa0023J03.15 *(Oryza sativa)*	3E-66	protein kinase domain, putative	1E-39
4.92	Contig11295_at	putative cytochrome P450 (Populus x canescens)	8E-58	no hits found	
4.90	Contig2787_s_at	thaumatin-like protein TLP6 *(Hordeum vulgare)*	1E-126	thaumatin-like protein TLP7	5E-46
4.79	Contig1078_x_at	polyubiquitin *(Sporobolus stapfianus)*	0	polyubiquitin 6 - rice	0
4.60	Contig14139_at	P0013F10.1 *(Oryza sativa)*	2E-84	is a member of the PF|00955 Anion exchanger family.	1E-96
4.51	Contig2704_s_at	no hit		no hits found	
4.50	HT12D12u_s_at	glutaredoxin *(Triticum aestivum)*	3E-48	glutaredoxin - rice	1E-60
4.28	Contig6206_s_at	putative protein kinase Xa21, receptor type precursor *(Oryza sativa)*	9E-25	no hits found	
4.27	Contig4782_at	putative cell death suppressor protein *(Oryza sativa)*	2E-90	putative iron-sulfur cluster-binding protein	1E-68
4.26	Contig852_at	unknown protein *(Oryza sativa)*	2E-60	expressed protein	1E-25
4.21	Contig568_s_at	no hit		no hits found	
4.15	HV05A09u_s_at	signal recognition particle 54 kd protein 3 (SRP54)	1E-22	no hits found	
4.12	Contig5830_s_at	unknown protein *(Oryza sativa)*	2E-72	expressed protein	3E-44
4.06	EBed02_SQ002_H13_x_at	no hit		no hits found	
4.03	Contig5371_at	OSJNBb0067G11.7 *(Oryza sativa)*	1E-137	copper amine oxidase-like protein, A. thaliana	e-137

Sahara and Clipper barley have quite different phenology and genomic background. To examine all genes that show constitutive differential expression between these two genotypes could be misleading as the majority of the genes are unlikely to be involved in B tolerance. The SSH experiment (Chapter 3) identified a B transporter that maps onto the 4H locus which is known to have major contribution towards B tolerance trait in Sahara. Thus, a search was focussed on genes potentially encoding B transporters. A comparison between Table 5.4 and Table 5.5 reveals that two probe sets 'Contig21126_at' and 'Contig14139_at' from among the probe sets over-expressing in Sahara and Clipper respectively, generate a common hit. The top BLASTx hit for these two probe sets is P0013F10.1 (*Oryza sativa*) and BLASTn (rice) hit is a member of the PF|00955 anion exchanger family. The TIGR hits of these sequences are annotated as F9L1.41 protein, which is synonymous to B transporter like protein. Several clones were identified from the SSH experiment (Chapter 3) having the same TIGR hit. This protein shares similarity with the gene product of B transporter identified in *Arabidopsis* (Takano *et al*., 2002; Frommer and von Wirén, 2002). There is one more probe set (Contig19634_at) featured in the Affymetrix 22K Barley1 GeneChip^{TM} which is annotated (BLASTx nr) as a putative B transporter [*Oryza sativa* (japonica cultivar-group)]. The expression of these probe sets in response to B and under control condition in both the varieties is shown in Figure 5.4.9. 'Contig19634_at' was not differentially expressed between Clipper and Sahara under control condition. Whereas the expression of 'Contig21126_at' was about 40 fold more abundant in Sahara compared to that in Clipper. On the other hand 'Contig14139_at' showed a 15 fold over expression in Clipper relative to its expression in Sahara. None of these probe sets were induced by B treatment in either of the varieties.

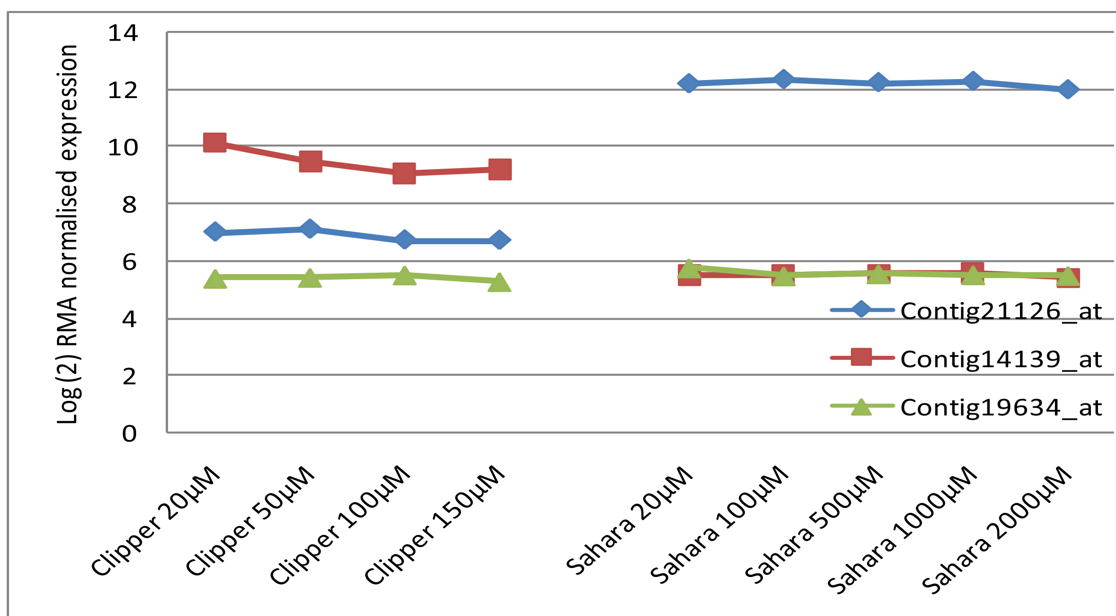


Figure 5.4.9. RMA normalised expression (expressed as log base 2) in Clipper and Sahara plants under control condition and various B treatments of all three probe sets having sequence similarity with B transporters featured in the Affymetrix 22K Barley1 GeneChip^{TM} (Contig21126_at, Contig14139_at and Contig19634_at).

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CHAPTER 6

DISCUSSION (MICROARRAY)

6.1. Introduction

A genome wide gene expression analysis of a highly B tolerant barley landrace Sahara and a B toxicity susceptible barley variety Clipper grown under various B concentrations was carried out using leaf RNA in this study. The results show that the two genotypes respond differently to B toxicity at the transcriptome level. The intolerance of Clipper barley to B is expressed through the induction of a very high number of transcripts (2310) even at a B concentration of 100 μM. On the other hand, B tolerant Sahara responded to a very high B concentration (2000 μM) through induction of only a few hundred (266) transcripts. This difference in the number of transcripts induced is commensurate to the extent of necrotic damage seen on the leaf tissue of the two genotypes in response to B toxicity.

6.2. Is the differential expression of transcripts enabling the Sahara plant to overcome B stress?

Transcripts significantly up or down regulated in Sahara that either did not respond significantly to B treatments in Clipper or responded in the opposite manner may hold the clue as to how Sahara plants manage to overcome B stress. Genes constitutively differentially expressed in Sahara or Clipper could also contribute to B tolerance or susceptibility in these barley genotypes. Transcripts significantly up or down regulated in both the varieties may be discounted as they supposedly would only divulge common stress responses to B toxicity in both the barley genotypes.

6.2.1. Down-regulated transcripts in Sahara

There were only six transcripts that were significantly (beyond 2 fold) suppressed in Sahara in response to B toxicity. Half of them were non-responsive in Clipper, while the other half were up-regulated in Clipper. These are ATP synthase subunit 9 (*Hordeum vulgare),* NADH-plastoquinone oxidoreductase subunit K and, photosystem I P700 apoprotein A1 (*Triticum aestivum)*, photosystem I P700 chlorophyll A apoprotein A1 (*Oryza sativa*) and photosystem I subunit IX (*Nicotiana tabacum*), respectively. Interestingly, all of these transcripts are localized to the chloroplast thylakoid membrane and are related to photosynthesis.

Photosynthesis is a physico-chemical process which is greatly regulated by the ionic environment (Jajoo *et al*., 2001). However, Reid *et al.* (2004) found no inhibition of photosynthesis in barley leaf slices (cv. Schooner, a moderately B sensitive barley variety) by treatment with 50 mM B for 3 hours (intracellular B is expected to reach a similar concentration to that in the medium within 3 hours). Only at a B concentration of 100 mM was photosynthesis reduced by 23%. Contrary to the findings of Reid *et al*. (2004), Kaur *et al.* (2006) reported that in the B sensitive *Brassica rapa* variety 'Shillong' photosynthesis was greatly reduced at an average B concentration above 50 mM in the leaf cell sap. Similarly, reduction of the rate of photosynthesis has also been reported in orange (Sotiropoulos *et al.,* 2002), and kiwi fruit (Papadakis *et al*., 2004) due to high B concentrations. Sotiropoulos *et al*., (2002) found that orange plants grown under excess B had fewer and smaller mesophyll cell chloroplasts with a darker appearance of the stroma. These authors also found that the total volume of thylakoid membranes was severely reduced and the thylakoids dilated, particularly those interconnecting grana. The thylakoid membrane is the site of photosynthetic electron transport. There are four multi-protein complexes embedded in the thylakoid membranes and involved in the electron transport process, namely: photosystem 2 (PS2), cytochrome (Cyt) *b6*/*f*, photosystem 1 (PS1), and the ATP synthase complex. It has been reported that many anions stimulate PS1- mediated electron transport. Itoh (1979a, 1979b) has reported that the rates of P700 (primary electron donor of PS1) reduction by anionic donors are determined by their concentration at the surface of the thylakoid membrane. Later Jajoo and Bharti (1995) observed that as anions promote PS1 rates it is likely that anions also affect the photosystem I P700 apoprotein A1(PS1-A fraction). Thus it appears that presence of excess B in leaf tissue is likely to affect the thylakoids in barley leaves and eventually photosynthesis depending on its concentration in the mesophyll cell sap.

Direct transfer of electron from PS1 to oxygen produces superoxides in the chloroplast (Bray *et al*, 2000). Good (1962) observed that the reduction of oxidants by chloroplast under irradiation is significantly enhanced by high concentrations of many anions including arsenate. Arsenic is a similar metalloid as boron. Thus enhanced electrons transfer by PS1 may lead to enhanced production of superoxide causing oxidative stress. Sahara may have evolved a mechanism to suppress PS1 in order to suppress superoxide production under ionic stress and thereby preventing leaf tissue from oxidative stress. On the other hand up-regulation of PS1 in Clipper may augment superoxide production to the detriment of leaf tissue through superoxide related cell death. However, as it has been mentioned earlier, the probe sets representing the above mentioned photosynthesis related transcripts in this experiment have short CDSs, therefore the results need further verification. Finally, to clarify

the effect of high B concentrations on photosynthetic machinery and photosynthesis itself, systematic studies need to be carried out. These may include measurement of chlorophyll content and fluorescence gas exchange characteristics, anatomical and morphological studies of mesophyll tissue under toxic B in susceptible and tolerant barley genotypes.

6.2.2. Up-regulated transcripts in Sahara

Genes up-regulated in Sahara due to B treatment which were not differentially regulated in Clipper could broadly be categorized into defense related and/or genes similar to those responding to salt stress. The defense related genes are germin-like protein (GLP), thionin, peroxidase and bacterial-induced peroxidase precursor. All of these genes are also associated with the cell wall and have been shown to be involved in defence related cell wall modification (Membŕe *et al.*, 2000; Amaya *et al.* 1999; Kang and Buchenauer, 2003). The other defense related gene codes for a beta glucan binding protein which is a part of the plasma membrane-localized pathogen receptor complex. Based on its expression pattern GLP can also act as a cell surface receptor (Bernier and Berna, 2001). Besides this, some GLPs have been shown to have superoxide dismutase (SOD) activity (Tabuchi *et al.*, 2003; Carter and Thornburg, 2000; Yamahara *et al.*, 1999).

Recently ROS accumulation under toxic B conditions was reported in apple rootstock (Molassiotis *et al.,* 2006) and citrus leaves (Keles *et al.*, 2004). It is likely that B toxicity may result in ROS accumulation in barley leaves as well. Antioxidant enzymes such as superoxide dismutase (SOD) catalyze the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2). However, H_2O_2 is also toxic to cells and has to be further detoxified by catalase and/or peroxidase to water and oxygen (Zhu *et al.*, 2004).

Garcia *et al.* (2001) reported increased SOD activity in tobacco leaves under B toxicity. While Karabal *et al.* (2003) have shown increased SOD activity under B toxicity in barley roots but not in the leaves. However, it is possible that B tolerant barley such as Sahara utilize GLP to dismutate superoxides instead of SOD as an alternative mechanism. Khuri *et al.* (2001) proposed that GLPs function primarily as SODs in order to protect plants from the effects of oxidative stress, Berna and Bernier (1999) have shown that GLP expression is induced by some heavy metals including cadmium which is known to cause oxidative stress in barley (Hegedüs *et al.*, 2001).

Recently Gunes *et al.* (2006) showed that in grape vine leaves the concentration of H_2O_2 was increased significantly to around 30 mmol g^-1(fw) level by B treatments compared to what

was found in control plants (22.74 mmol g^{-1}, fw). In contrast Karabal *et al.* (2003) showed that high B treatment (5 mM and 10 mM for five days) caused a decline in H_2O_2 concentration in the B tolerant barley variety Anadolu. Such decline in H_2O_2 concentration in B tolerant barley may be attributed to the enhanced expression of peroxidases under B toxicity as found in the current experiment. This may explain why Sahara plants show tissue tolerance to toxic B.

Since GLP, peroxidases and thionin are also capable of modifying cell wall structure, they may also play an alternative role in protecting cells from B toxicity by means of reducing passive inflow of B. GLPs are localized in the extra-cellular matrix (ECM) of cells where they may act as structural proteins under stressed conditions (Schweizer *et al.,* 1999; Bernier and Berna, 2001). Schweizer *et al.* (1999) reported that in wheat epidermal cells transiently expressing the gf-2.8 germin gene, the gene product became insoluble at the site of attempted fungal penetration where localized production of H_2O_2 was observed, reducing the penetration of fungus. They concluded that germins and GLPs may play a role in cell wall reinforcement during pathogen attack. Bernier and Berna (2001) also reported such insolubility of GLPs upon heat treatment to barley plants which provides resistance to pathogens through cell wall modification and without the need of papilla formation at the site of infection. Peroxidases in addition to detoxifying H_2O_2 have also been reported to play a role in the structural modification of cell walls. For example the *TPX2* gene encoding a cell-wall-associated peroxidase was reported to be involved in modification of cell wall architecture in tomato (Amaya *et al*., 1999). Amaya *et al.* (1999) investigated ten independent transgenic tobacco plants transformed with tomato *TPX2* under the control of the CaMV 35S promoter. They found that the germination rate of transgenic plants increased greatly under conditions of high salt (250 mM NaCl) or osmotic stress (470 mM mannitol). Amaya *et al.* (1999) reported that thermoporometry calculations indicated a lower mean pore size in the cell walls of transgenic seeds. Modifications in plant cell walls as a part of defence responses also include deposition of lignin-like material, callose, phenolic compounds and proteins such as thionins and hydroxyproline-rich glycoproteins (Kang and Buchenauer, 2003). The production and accumulation of thionins as part of a defence response against attacks by a variety of pathogens has been reported in many plants (Bohlmann *et al.,* 1988; Ebrahim-Nesbat *et al.*, 1993; Epple *et al.*, 1997; Kang and Buchenauer, 2003).

Thus it is tempting to speculate that structural modification of the cell wall by the combined effect of germin-like protein (GLP), thionin and peroxidases which were seen to be up-regulated in Sahara in response to high B treatment, may prevent B from entering the leaf

cells and thereby avoiding subsequent cell damage. Further research would be required to understand the actual role of these genes in B toxicity tolerance in barley.

It was mentioned earlier that thionin is jasmonate (jasmonic acid and its derivatives) inducible (Bohlmann *et al*., 1988). Significant up-regulation of two genes involved in the jasmonic acid biosynthesis namely allene oxide synthases (AOS) and lipoxygenase 2.2 (LOX2:Hv:2) were seen in Sahara grown under high B concentrations. This is in agreement with the findings of Molassiotis *et al*. (2006) who recently reported increased LOX activity in apple rootstock grown under B toxicity. Jasmonates are formed from 12-OPDA (12-oxo-phytodienoic acid) (Walia *et al*., 2006). Lipoxygenase oxidation of linolenic acid followed by a sequential action of AOS and allene oxide cyclase (AOC) on 13S-HPOT [13(S)-hydroperoxy-9(Z), ll(E),15(Z)-octadecatrienoic acid; a-ketol, 12-oxo-13-hydroxy-9(Z),15(Z)-octadecadienoic acid] - a lipoxygenase product, results in the formation of 12-OPDA (Hamberg and Gardner, 1992). Jasmonic acid (JA) may play an important role in adaptation of barley to toxic B conditions as it does for salinity conditions (Tsonev *et al*., 1998, Walia *et al*., 2006). JA has been described to play a key role in stress induced signaling pathways and modulating changes in gene expression (Wasternack *et al*., 1998).

A number of salt responsive genes and associated transcription factors are also induced by B toxicity in Sahara. These include chalcone synthase-1, ribulose-1·5-bisphosphate carboxylase/oxygenase small subunit and a bZIP transcription factor CPRF2 which can act as a transcriptional activator or repressor for both ribulose-1·5-bisphosphate carboxylase/oxygenase small subunit and chalcone synthase. Chalcone synthase is a key enzyme in the synthesis of flavonoids (Mackerness *et al.*, 1997). Flavonoids are polyphenolic compounds produced in plants in response to diverse environmental cues (Chalker-Scott, 1999) with an apparent role in stress protection (Delalonde *et al.* 1996). B toxicity has been related to alterations in the content of phenolic compounds and their metabolism (Ruiz *et al*., 1998). They showed that high B treatment (leaf sprayed with 20 μM boric acid) significantly increased the activity of phenylalanine ammonia lyase (PAL) compared to normal level of B treatment. PAL catalyzes the conversion of phenylalanine to cinnamate and tyrosine to *p*-coumarate. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate and 4-coumarate:CoA ligase (4CL) converts *p*-coumarate to its coenzyme-A ester and activates it for reaction with malonyl CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Ruiz *et al.* (1998) speculated that PAL activity at the

high B level induces the accumulation of phenolics because they are complexed with B and thus become unavailable for oxidation.

The increase in concentration of phenolic compounds in several plant species, e.g., winter oilseed rape (Solecka and Kacperska 2003; Stefanowska *et al.,* 2002), soybean (Janas *et al*., 2000) and petunia (Pennycooke *et al.,* 2005) due to cold acclimation (2-10 ºC) has been shown. The *COR15B* gene coding for a cold-regulated protein was up-regulated by B treatment in Sahara which has previously been reported to be up-regulated by salt treatment. Up-regulation of *COR15B* could be linked to an accumulation of flavonoid/phenolics under conditions of B toxicity (Ruiz *et al*., 1998) or salinity (Kreps *et al*., 2002).

Flavonoids, with other phenolics have a role in UV-attenuation (Raven 1991*a*,*b*). In flowering plants, flavonoids, including anthocyanins, accumulate in the vacuoles of epidermal cells where they attenuate the UV component of sunlight with minimal absorption of photosynthetically active radiation (Stapleton and Walbot, 1994; Landry et al., 1995). It has been reported that rubisco small and large subunits are up-regulated when UV-A and UV-B were both depleted (excluded by filtering) (Casati and Walbot, 2003). Again the bZIP transcription factor CPRF2 up-regulated in Sahara due to high B treatment can act as a transcriptional activator or repressor for both ribulose-1·5-bisphosphate carboxylase/oxygenase small subunit and chalcone synthase (Kircher *et al*., 1999). Thus the up-regulation of ribulose-1·5-bisphosphate carboxylase/oxygenase (rubisco) small subunit could be an in-built mechanism of photosynthetic plants to compensate for UV attenuation caused by flavonoid accumulation and may not play any active role in B tolerance mechanism.

6.3. Constitutively differentially expressed transcripts in relation to B toxicity tolerance

One of the most notable differences in gene expression between Sahara and Clipper under control condition was that of the two probe sets annotated as B transporters. As mentioned earlier 'Contig21126_at' showed about 40 fold higher expression in Sahara compared to its expression in Clipper. Recently, it has been shown in our laboratory that this B transporter gene maps to the 4H B tolerance QTL region previously identified by Jefferies *et al*. (1999). The gene co-segregates with B tolerance in the Clipper X Sahara doubled haploid mapping population (Tim Sutton, personal communication). It was mentioned in chapter 4 that the full-length cDNA sequence of this gene has recently been isolated in our laboratory. Several (15) gene fragments representing this gene were also isolated from both the control and B treated library arising from the SSH experiment. Taken together this information identifies this gene

as a prime candidate for B toxicity tolerance in barley. Further research is needed to explore the mode of action of this B transporter gene and to gain conclusive evidence that the gene is an efflux type B transporter actually providing the B toxicity tolerance in Sahara through B efflux as predicted by earlier workers.

The other B transporter gene represented by 'Contig14139_at' showed about 15 fold higher expression in Clipper compared to Sahara. Since this gene also shows similarity to B transporters it is tempting to speculate that this gene may be associated with B uptake in Clipper and the reason why Clipper plants accumulate so much B in its tissue within a short time. If this speculation is true then Clipper would probably be a more B efficient cultivar than Sahara when grown under B deficient conditions. Further research is required to understand the significance of the over-expression of this gene in Clipper.

Another noteworthy difference in gene expression between Sahara and Clipper was that of probe set 'Contig2769_s_at'. This probe set showed 16 fold higher expression in Sahara compared to that in Clipper and is annotated as adenosine diphosphate glucose pyrophosphatase (BLASTx nr) or putative cupin (BLASTn against rice db). Cupins represent a superfamily of proteins that includes germin like proteins (Membré *et al.*, 2000). The N-terminal amino acid sequence of barley germin like proteins (HvGLP 1) and adenosine diphosphate glucose pyrophosphatase is 100% identical (Rodríguez-López *et al.*, 2001; Segarra *et al.*, 2003). Since GLPs may function primarily as SODs in order to protect plants from the effects of oxidative stress (Khuri *et al.,* 2001) the 16 fold higher expression of this gene in Sahara may provide certain advantage in tolerating ROS accumulation under toxic B conditions as reported in leaves of apple rootstock (Molassiotis *et al.,* 2006). This may explain why leaf symptom expression in Sahara plants is delayed when Clipper plants show leaf damage at similar leaf B concentrations.

6.4. Summary

Transcript profiling was conducted on B tolerant Sahara and sensitive Clipper grown under control condition and those covering a range of B toxicity. The aim of these experiments was to examine the molecular basis for the ability of Sahara to both restrict leaf B accumulation and tolerate high tissue B. Perturbations in a relatively smaller number of genes in Sahara even at a very high B concentration seen in this experiment signify its ability to cope with toxic B. On the other hand induction of a large number of genes in Clipper under elevated B regimes indicates its intolerance to B.

Two B transporter genes were identified that did not respond to B treatments but showed opposing expression patterns in the two varieties under test. One of these B transporters (represented by Contig21126_at) was mapped onto the 4H tolerance locus and is now known to be co-segregating with the B exclusion trait (Tim Sutton, personal communication). Thus it indicates that this gene may be an efflux type B transporter. Further research is needed to confirm this possibility. The finding that this gene is over expressed in Sahara under control condition also agrees with the predictions by earlier workers (Hayes and Reid, 2004) that the efflux mechanism is constitutive in nature. The over expression of the other B transporter gene (represented by Contig14139_at) in Clipper is interesting and suggests a possible role in the influx of B. In this case the constitutive expression of this gene would render B efficiency to Clipper under deficient B conditions. It is not known whether Clipper is a B efficient barley cultivar or not. Future research in exploring such possibility is needed.

A cupin family gene homologous to barley GLP 1 with suggested SOD activity was also found to be constitutively over-expressing in Sahara (and was not B responsive in either of the genotypes) may help these plants to withstand B associated oxidative stress, if any. The constitutive over expression of this gene in Sahara may be an important adaptive mechanism of this African landrace as it evolved in the harsh environment of Africa. GLPs are glycoproteins and may also play roles in defense related cell wall modification and can act as a cell surface receptor. Most interestingly Durka *et al.* (2002) reported that a nine-GLP gene cluster resides on the sub telomeric region of chromosome 4H (bin13, Steptoe X Morex). Taking together these observations suggest that GLP may play a crucial role in B toxicity tolerance in Sahara.

The combined action of GLP (as SOD) and peroxidases up-regulated in Sahara by B treatment may provide a healthier environment within the cell under B toxicity. Again the combined action of GLP peroxidases and thionin in defense related cell wall modification/ reinforcement could be helpful to prevent B from entering inside the cell. These possibilities attract further scrutiny.

Up-regulation of two genes (AOS and LOX) in the jasmonate synthesis pathway in Sahara due to B treatment reinforces the argument that JA may play an important role (Chapter 4) in adaptation of barley to toxic B conditions as it does under salinity. The up-regulation of several salt inducible genes indicates that under B stress Sahara barley may at least partially utilize some of the mechanism adopted by plants to overcome salt stress.

Lastly down-regulation of a number of photosynthesis related genes in Sahara under toxic B may indicate that Sahara has evolved a mechanism to suppress superoxide production under ionic stress and thereby saving leaf tissue from oxidative stress. On the other hand, up-regulation of these genes in Clipper may indicate that these plants were trying to compensate for photosynthetic loss incurred by reduced photosynthetic area resulting from B toxicity related cell death.

CHAPTER 7

GENERAL DISCUSSION

In this work the boron tolerance mechanism in barley was investigated at the transcriptome level. Two genomic approaches were adopted, namely: suppression subtractive hybridization (SSH) and transcriptional profiling using microarray. The SSH experiment aimed to analyse the early response of B tolerant barley lines and to identify the basis for B toxicity tolerance in the roots of these plants. The experiment was designed to target the B exclusion mechanism controlled by the 4H B tolerance QTL previously identified by QTL mapping (Jefferies *et al.*, 1999). On the other hand the microarray experiment was designed to examine the basis of how Sahara plants cope with B toxicity in the longer term (adaptive response). The data presented here showed that when the tolerant plants are challenged with B, in the initial phase a diverse defence response is activated particularly in the roots.

Arguably it would simply be too naïve to try to compare inducible B tolerance mechanisms gleaned from the data analysis of these two approaches. This is because of the difference in spatial and temporal expression patterns of genes that was investigated in the SSH and microarray experiment. However, the data obtained from the two experiments could be directly comparable if the basis of the B toxicity tolerance is constitutively expressed as previously predicted by Hayes and Reid (2004). A B transporter gene having similarity with the *Arabidopsis BOR 1* gene (Takano *et al.*, 2002) was identified from both SSH and microarray experiment and shown to map to the 4H B tolerance locus. It was found to be constitutively more abundantly expressed in Sahara and B tolerant lines descending from a cross between Clipper and Sahara. This strongly indicates that the basic B toxicity tolerance mechanism (through B exclusion) could indeed be constitutive in nature. As the map location of the identified B transporter gene from both experiments coincides with the B tolerance locus identified by Jefferies *et al.* (1999) on 4H one can conclude that this finding is a big step forward in the right direction in understanding B toxicity tolerance mechanism involving B efflux.

Hayes and Reid (2004) proposed an efflux model to explain the B tolerance mechanism in Sahara barley where they predicted the presence of a borate anion efflux transporter in Sahara similar to efflux type borate transporter BOR1. In the SSH experiment subtractive hybridization was carried out between the root transcriptomes of the tolerant and intolerant lines that were selected on the basis of the presence and absence of 4H Sahara locus coupled

with B exclusion and non-exclusion criteria, respectively. Fragments of the above mentioned B transporter gene were found to be abundantly represented in both B treated and control subtracted libraries. In addition, qPCR expression analysis has indicated that this gene is up-regulated in the root in response to B treatment within 24 hours in the tolerant lines and it co-segregates with the B exclusion trait in the Clipper X Sahara population. Taking all these facts together, one could speculate that this gene might have a possible role in B toxicity tolerance, possibly by actively effluxing B from the roots. The constitutive over expression of this gene in the leaves of Sahara may indicate that it might efflux B from leaves as well. Future experiments including gene knockout, overexpressing transgenic and complementation tests need to be designed to further characterize this gene.

Data from both experiments have indicated that B toxicity most likely triggers oxidative stress. This is in agreement with the recent findings that ROS accumulates in apple root stock (Molassiotis *et al.,* 2006) and citrus leaves (Keles *et al.*, 2004) due to B toxicity. Data from SSH indicated that in the earlier stage of exposure to high B, a major antioxidative defence mechanism, namely the ascorbate –glutathione pathway was induced in the roots of tolerant barley lines. On the other hand microararry data showed that in the later stage GLPs and peroxidases which are known to protect plants from the effects of oxidative stress, were up-regulated in the leaves of Sahara. Microarray data also indicated that constitutive GLP1 expression is much higher in Sahara leaves compared to that in Clipper implying that Sahara may be naturally better prepared for managing oxidative stress. In either case (at the early stage of B toxicity in the roots and at the later stages in the leaves) Sahara demonstrated a better oxidative stress management ability, which is crucial for maintaining a healthy cellular environment and overall wellbeing of the plant.

It appears from the results of both experiments that jasmonate-based signaling plays a key role in B toxicity tolerance. JA has been described to play a key role in nutrient signaling, stress induced signaling pathways and modulating changes in gene expression (Wasternack *et al.,* 1998; Armengaud *et al*., 2004). Evidence has been presented in the literature to support the idea that JA originates from the plasma membrane. Ryan (1992) has proposed that JA biosynthesis is stimulated by pathogens or insect pests through the production of elicitors and systemic signaling molecules that interact with specific receptors on the plasma membrane. JA arises from the release of cell membrane fatty acids through the action of lipase in response to wounding or autolytic events (Farmer and Ryan, 1992). Creelman and Mullet (1995) suggested that changes in ion transport may stimulate lipase and lipoxygenase activity which eventually induce JA. In agreement with these findings a lipase like protein was found

to be 2.6 fold up-regulated in the B treated library arising from the SSH experiment and LOX2 was seen to be up-regulated in Sahara leaves about 2.3 fold (microarray). Besides this several other observations made in this work can be attributed to enhanced JA activity in tolerant barley under B stress. These observations are discussed in the following paragraph.

JA has been reported to influence vegetative sink and storage regulation (Becker and Spoel, 2006) which was indicated by the up-regulation (over five fold) of a tonoplast intrinsic protein (corresponding to δ-TIP of *Arabidopsis*, see Chaper 4) in the B treated library from the SSH experiment. It has been reported that low concentrations of JA induce genes coding for proteinase inhibitor (Framer and Ryan, 1990), chalcone synthase (Dittrich *et al*., 1992; Creelman *et al*., 1992) and thionin (Anderson *et al.,* 1992; Epple *et al*., 1995). All of these genes were found to be up-regulated in Sahara upon high B treatment in the microarray experiment. JA also influences photosynthesis (Becker and Spoel, 2006). Modulation of photosynthesis related gene expression by B treatment has indicated (see Chapter 5 and 6) that high B concentrations influence photosynthesis in barley as has been reported in a number of other plant species (Kaur *et al.,* 2006; Sotiropoulos *et al.,* 2002; Papadakis *et al*., 2004). Study of the *coi1-1* mutant of *Arabidopsis* showed that JA influences *COI1*gene expression which codes for a protein with an F-box protein motif (Gagne *et al*., 2002), that may function in the modification and ubiquination of proteins (Xie *et al*., 1998) and subsequent degradation through the proteasome. Induction of beta-3 proteasome subunit, cullin and F-Box protein in the B treated library from SSH experiment has indicated that an Ub/proteasome pathway was induced by B toxicity as was shown under low arsenic (a similar metalloid to B) toxicity in plants (Hartley-Whitaker *et al*., 2001). Thus it is apparent that a number of JA-mediated protective mechanisms were activated in tolerant barley in response to toxic B levels.

B has been shown to affect K uptake by plants (Robertson and Loughman, 1997; Pollard *et al*., 1977), which in turn induces JA activity. Armengaud *et al*. (2004) reported that transcript levels for the JA biosynthetic enzymes allene oxide synthase (AOS), and allene oxide cyclase (AOC) were strongly increased during K^+ starvation. A possible K^+ starvation was discussed earlier (Chapter 4) as a consequence of a likely interaction between B and K^+ uptake (indicated by the HAK11 gene identified in the B treated library which was found up-regulated 2.5 fold). It was shown by Pollard *et al*. (1977) that B toxicity like B deficiency affects K^+ uptake. Pollard *et al*. (1977) using rubidium as an analogue for K^+ uptake (Lauchli and Epstein, 1970), showed that rubidium accumulation by maize roots excised from seedlings grown with 100 µM boric acid, gradually decreased with 10 and 100 fold increase

in boric acid concentration above 10 µM in the incubation media. They showed that rubidium uptake was highest when the boric acid concentration of the incubation media was 10 µM. Like JA, K^+ ion homeostasis apparently also plays a central role in B toxicity tolerance mechanism. Schon *et al*. (1990) reported that the effect of B on K^+ conductance through the plasma membrane is instantaneous. B has been reported to play important roles in the plasma membrane structure and function. A large number of experimental results have shown that B is important for membrane transport processes, ATP-dependent proton pumping and changes in membrane polarization (Brown *et al*., 2002). Some of the effects of B on the membrane could actually be mediated by or as a result of K^+ depletion. It has been reported that K^+ depletion or starvation can also affect the function of anion exchangers located in the plasma membrane and inhibit Cl^- uptake (Madshus *et al.,* 1987a). In agreement with this Pollard *et al*. (1977) also showed that B toxicity reduced Cl^- uptake by excised maize roots. Thus it is important to examine more carefully the relation between toxic B concentration and its effect on K^+ conductance and absorption by the plasma membrane.

Cell membranes and cell walls have been the focus of many studies involving B. In fact B plays an essential role in cell wall structure and function as it does in cell membranes (Brown *et al.,* 2002). From a study of the *cev1* mutant of *Arabidopsis* (*CEV1* encodes the cellulose synthase CeSA3) Ellis *et al.* (2002) reported that cell walls are involved in JA mediated stress and defence responses (Devoto and Turner, 2005). Up-regulation of *CeSA1* gene, (2.5 fold) and a number of tubulin genes, for example, alpha-tubulin 1 (4.6 fold), alpha-tubulin (4.1 fold), beta-tubulin (1.7 fold), and an actin gene (1.8 fold) in the B treated library from the SSH experiment was observed. The induction of tubulin and actin genes reflects changes in the structure of the cell wall (Yu *et al*., 2002b). Interestingly, induction of tubulin and actin genes has also been reported due to short-term B deprivation in several plant species. Using anti-actin and anti-tubulin monoclonal antibodies, Yu *et al.* (2001) showed that levels of actin and tubulin proteins were increased upon short-term B deprivation in roots of hydroponically grown *Arabidopsis*. Similar responses were also observed in the cytoskeleton of cells of maize (*Zea mays*) root apices (Yu *et al.,* 2002a) upon short term B deprivation. Yu *et al.* (2002b) showed that short term B deprivation caused rapid increase of pectin in the cell wall in meristematic cells of maize and wheat root apices. These authors concluded that rapid accumulation of tubulin and actin in root cytoskeleton are related to alterations of cell wall pectins due to the inhibition of receptor-mediated endocytosis (internalization) of cell wall pectin. This rapid alteration in the distribution patterns of cell wall pectins resulted in changes in cell wall structure and porosity (Yu *et al*., 2002b). Heuser and Anderson (1989) reported that formation of endocytotic vesicle through invagination of the plasma membrane is

controlled by clathrin lattice, which is the major coat forming cytoplasmic protein. Clathrin is a trimer of heavy-chain subunits having a triskelion-shape which polymerize into a polyhedral lattice (Ybe *et al.,* 1999). A gene coding for clathrin heavy chain was identified (up-regulated 1.6 fold) in the B treated library (SSH experiment) which could indicate that B toxicity may also interfere with internalization of cell wall pectin in Sahara. In this context it can be mentioned that another protein involved in the organization of cytoskeleton and vesicular transport, namely RAB1C (belonging to the Ras superfamily of proteins) was also seen to be 1.5 fold up-regulated in the B treated library. Depletion of intracellular K^+ is also known to inhibit receptor-mediated endocytosis by clathrin coated vesicles (Larkin *et al*., 1986; Madshus *et al*., 1987b). Thus up-regulation of *HAK11*, tubulin and actin genes, *RAB1C* and clathrin heavy chain in the tolerant lines within 24 hours of B treatment suggests that B toxicity initially may stimulate similar responses due to short-term B depletion mediated by K^+ starvation. Again, induction of tubulin and actin genes, *RAB1C* and clathrin heavy chain raises the question whether B toxicity also inhibits receptor-mediated endocytosis of pectin and thereby causes an increase in pectin content in the cell wall of the tolerant lines. Such structural change in the cell wall caused by increased pectin level may reduce the passive inflow of B through modification of cell wall structures. In addition NADP-ME up-regulation in the B treated library also hints that defence-related lignification may be initiated in the root cell wall of the tolerant lines upon B treatment (within 24 hours). No transcriptional changes were found for actin, tubulin or cellulose synthase genes in the leaf tissue of Sahara grown at high B concentration through microarray profiling. However, several other genes, such as thionin, GLPs and peroxidase were up-regulated in the microarray experiment which could also contribute to the structural reinforcement of the cell wall. Thus both experiments point to the possibility of B exclusion by means of structural reinforcement of the cell wall both in the root and the leaf and needs to be examined through further investigations. The above observations lead to the conclusion that the cell wall- plasma membrane-cytoskeleton continuum may constitute the first action site against B toxicity and the influence of toxic B on K^+ uptake may be the key initiating factor.

B is an essential plant nutrient. An interesting outcome of this work is that genes differentially regulated in the tolerant barley are in line with the various postulated functions of B reported in the literature. These functions are largely based on the observation and identification of a number of B deficiency symptoms and include:

i) Sugar transport and carbohydrate metabolism
ii) Cell wall synthesis and lignification
iii) Maintenance of membrane integrity and function

iv) Role in the ascorbate/glutathione cycle

v) Phenol metabolism

vi) Nitrogen Metabolism

vii) Photosynthesis

Thus, it appears that B toxicity and B deficiency affect similar metabolic processes in plants eliciting similar responses at least at the initial phase. This may be attributed to K^+ ion imbalances caused by both B toxic and deficient conditions, which may play a pivotal role in both stresses.

Roots and leaves play distinctly different roles in plant biology and are exposed to very different environments. Thus their responses to environmental challenges should be related to their distinct biological roles. Plant roots are involved in the absorption of minerals and water and their supply to the above ground part. When plant roots are challenged with toxic nutrient concentration/composition they may primarily try to exclude the toxic nutrient from entering into the root system. If the element inevitably enters into the root system at a toxic level the next option would be effluxing or sequestrating the toxic element. In order to maintain a healthier cellular environment and to reduce the impact of the toxic effect of the rogue element the plant may activate cell rescue and defence mechanisms. Plants could also try to redistribute or reorganize root arrangement in order to avoid coming into contact with toxic element(s) as has been reported by McDonald (2003). This would require the root to undertake quick elongation driven by enhanced cell division in the meristematic zone of root. The data from the SSH experiment is essentially in line with the above mentioned strategies of plants that involve (i) up-regulation of a transporter mediated B efflux and maintenance of the energy demanding efflux mechanism through elevated energy (ATP) supply, (ii) ROS scavenging and detoxification of cellular environment and quick removal of damaged proteins caused by ROS from the system, (iii) enhanced osmolite synthesis and nutrient (Na^+ and K^+) homeostasis in order to maintain osmotic balance which would facilitate root growth through maintaining a high turgor pressure (Pritchard *et al.,* 2000) and (iv) efficient cell wall synthesis and mobilization of carbon to maintain root growth. As has been discussed earlier, the SSH results support the idea that exclusion of B may also be achieved by reducing passive uptake of B through structural reinforcement of the cell wall and minimizing ROS related membrane damage.

On the other hand plant leaves are responsible for carbohydrate synthesis to sustain plant growth and development. In the event of over-supply of toxic elements through the

transpiration stream leaf cells could try to protect their photosynthetic machinery in order to minimize interruptions in carbon assimilation. This could be achieved through efflux of or preventing influx of the toxic element and by maintaining a healthy cellular environment consequently minimizing interference with photosynthetic activities. The data from of the microarray support these strategies through (i) B efflux through constitutive over-expression of an efflux type B transporter or alternatively by reducing passive inflow of B in the cytoplasm through the structural reinforcement of the cell wall, (ii) maintaining a healthier cellular environment through effective ROS scavenging and (iii) adjusting photosynthetic activity.

The results obtained from this work are also signified by genetic mapping of a number of B responsive genes within the B tolerance QTL on three chromosomes (2H, 4H and 6H). The genes that were mapped to 4H and 6H B tolerance loci have the highest association with these loci in our doubled haploid mapping population. These can serve as valuable markers in marker-assisted selection work in Australian barley breeding programmes. Since selecting for B tolerance has been one of the priorities of barley breeding programs in Southern Australia these mapping results could be of great help to increase the efficiency of breeding for B tolerance in barley. *In silico* bin mapping carried out in this work can also serve as reference for future mapping works. About 170 non-redundant transcripts were assigned to various chromosomal segments (bins) on all three genomes of bread wheat. Since the D genome of bread wheat is co-linear to the barley genome (Gale and Devos, 1998) the *in silico* bin mapping could be useful for both wheat and barley mapping studies.

The work presented here has examined the responses in B tolerant barley at the level of gene expression against B toxicity and identified some of the physiological responses to B adapted by barley plants. This can be considered as a positive step forward in B tolerance research. However, the outcomes of the current work leave many questions to be investigated in further detail to complete our understanding of B toxicity tolerance mechanisms in barley. These include:

1) Determine tissue and cellular localization of the barley B transporter gene (represented by Contig21126_at) identified at the 4H B tolerance locus in this work. Analysis of the protein encoded by this gene to confirm B transporter activity and studying the mode of action of the protein through producing gene knockouts, over-expressing transgenics and complementation tests. In addition, characterizing the other B transporter gene (represented by Contig14139_at) that was more abundantly expressed

in Clipper would complement our understanding of the role of B transporters in barley.

2) Interaction between B transporter genes located at the 4H B tolerance locus and other nutrient transporters also warrants further investigations.
3) Examination of the effect of toxic B on the cell wall-plasma membrane-cytoskeleton continuum to reveal whether tolerant barley plants adopt any mechanism to minimize passive inflow of B under toxic concentrations through cell wall modification as indicated in this study.
4) Investigation of K^+ conductance and absorption by the plasma membrane under B toxicity and the downstream consequences thereof is important as it can affect B absorption and has diverse effects on overall plant health.
5) Investigate JA signaling in relation to B toxicity and genes induced by JA as it appears that JA signaling plays a central role to help tolerant barley in streamlining several defence mechanisms in order to cope with toxic B.
6) Research is needed to examine the extent of oxidative stress induced by toxic B and mechanisms adopted by barley plants to alleviate such stress.
7) Further investigation would be needed to determine whether sequestration involving GST is a part of the overall B tolerance mechanism in barley and if so, how the vacuolar membrane bound genes found up-regulated in the B treated library from SSH experiment (ABC transporter, V-ATPase d subunit and tonoplast intrinsic protein) may contribute to such a mechanism.
8) Further work is required to determine the role of bromo-adjacent homology (BAH) domain-containing protein-like protein identified from the SSH experiment in B toxicity tolerance as this gene was found to co-segregate with the 6H B exclusion trait.
9) Genomic studies through transcript profiling represent a mammoth task. Here transcript profiling was carried out by means of SSH and 22K Barley 1 GeneChip™ microarray. 111 non- redundant clones were isolated from the B treated library arising from the SSH experiment. It was not possible to characterize all isolated clones and discount false positives because of the limited time frame of this project. Further analysis of many of these clones could provide more information about B tolerance mechanism not outlined in this thesis. Mapping of the B responsive genes from Sahara identified by the microarray experiment also could generate valuable information for future work on B toxicity tolerance.

Lastly, breeding efforts aiming to incorporate B tolerance trait from Sahara 3771 in elite barley varieties have been limited by a lack of understanding of B tolerance mechanism

and also due to linkage drag (McDonald *et al*., 2003). Further evaluation and identification of the most effective B tolerance mechanisms from among those outlined in this work, followed by isolation of genes involved could quicken the development of high yielding barley varieties with enhanced B toxicity tolerance.

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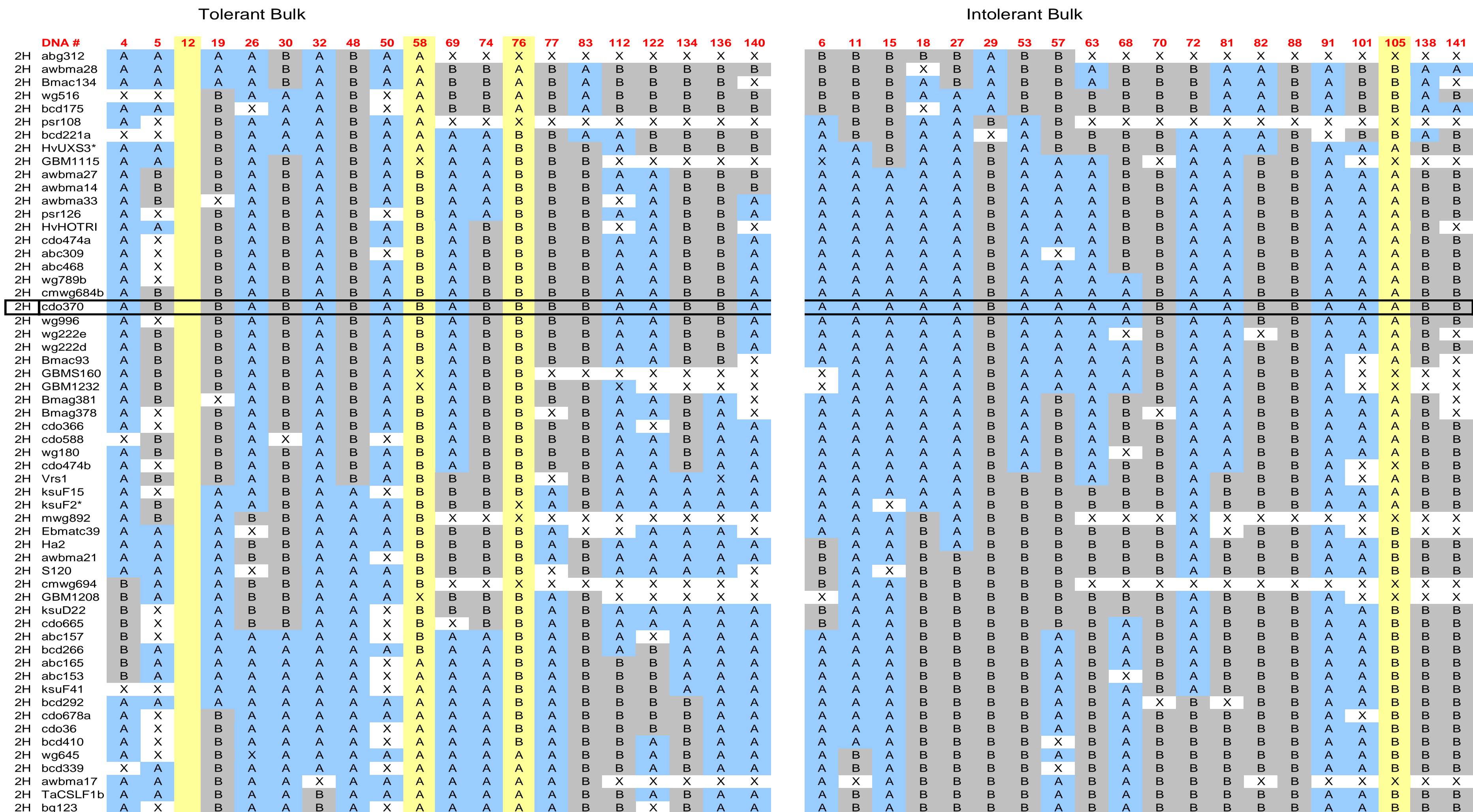
APPENDICES

APPENDIX A



Tolerant Bulk

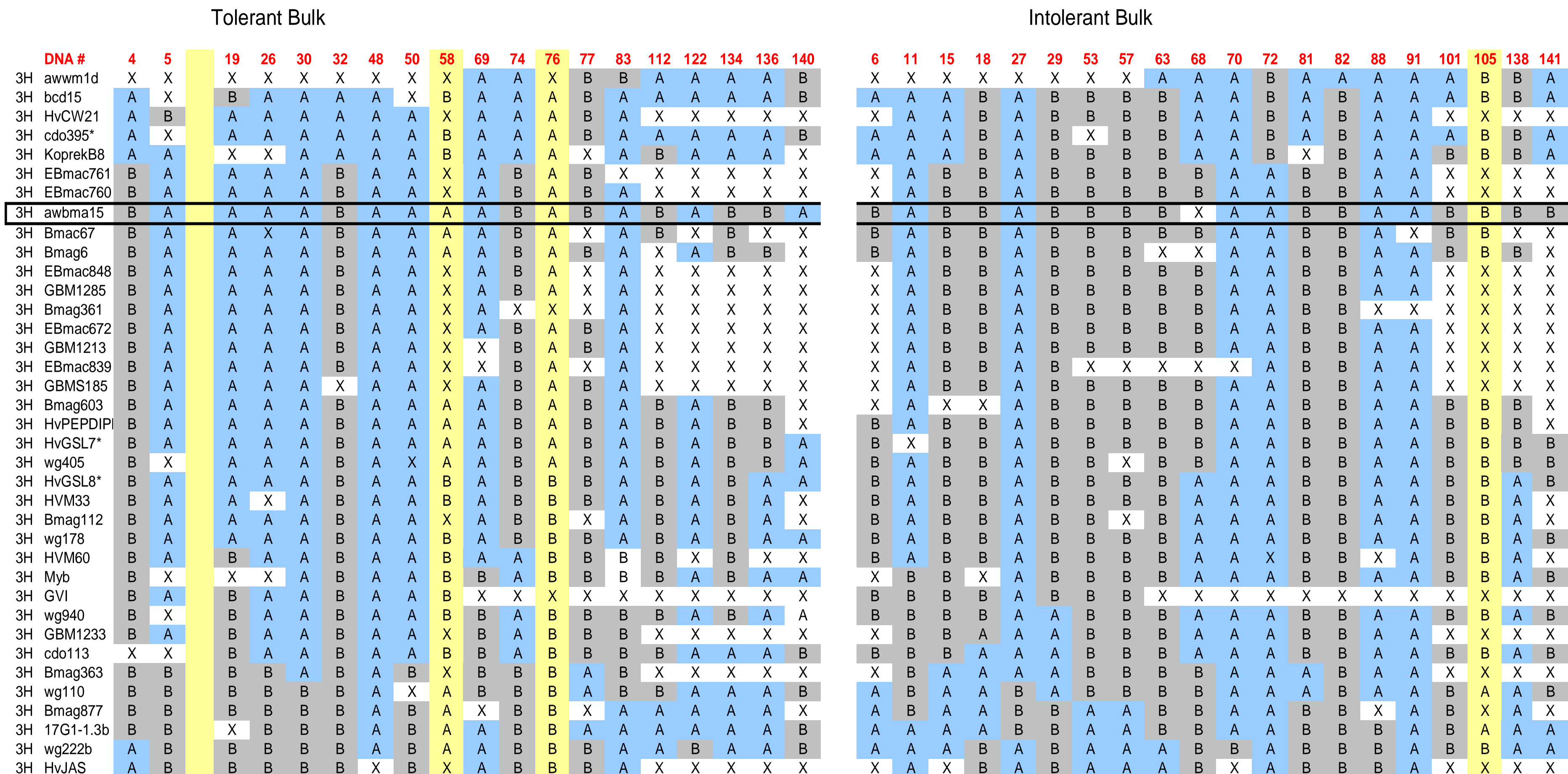
	DNA #	4	5	12	19	26	30	32	48	50	58	69	74	76	77	83	112	122	134	136	140
2H	abg312	A	A		A	A	B	A	B	A	A	X	X	X	X	X	X	X	X	X	X
2H	awbma28	A	A		A	A	B	A	B	A	A	B	B	A	B	A	B	B	B	B	B
2H	Bmac134	A	A		A	A	B	A	B	A	A	B	B	A	B	A	B	B	B	B	X
2H	wg516	X	X		B	A	A	A	B	X	A	B	B	A	B	A	B	B	B	B	B
2H	bcd175	A	A		B	X	A	A	B	X	A	B	B	A	B	A	B	B	B	B	B
2H	psr108	A	X		B	A	A	A	B	A	A	X	X	X	X	X	X	X	X	X	X
2H	bcd221a	X	X		B	A	A	A	B	A	A	A	A	B	B	A	A	B	B	B	B
2H	HvUXS3*	A	A		B	A	A	A	B	A	A	A	A	B	B	B	A	B	B	B	B
2H	GBM1115	A	A		B	A	B	A	B	A	X	A	A	B	B	B	X	X	X	X	X
2H	awbma27	A	B		B	A	B	A	B	A	B	A	A	B	B	B	A	A	B	B	B
2H	awbma14	A	B		B	A	B	A	B	A	B	A	A	B	B	B	A	A	B	B	B
2H	awbma33	A	B		X	A	B	A	B	A	B	A	A	B	B	B	X	A	B	B	A
2H	psr126	A	X		B	A	B	A	B	X	B	A	A	B	B	B	A	A	B	B	A
2H	HvHOTRI	A	A		B	A	B	A	B	A	B	A	B	B	B	B	X	A	B	B	X
2H	cdo474a	A	X		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	abc309	A	X		B	A	B	A	B	X	B	A	B	B	B	B	A	A	B	B	A
2H	abc468	A	X		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	wg789b	A	X		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	cmwg684b	A	B		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	cdo370	A	B		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	wg996	A	X		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	wg222e	A	B		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	wg222d	A	B		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	A
2H	Bmac93	A	B		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	B	X
2H	GBMS160	A	B		B	A	B	A	B	A	X	A	B	B	X	X	X	X	X	X	X
2H	GBM1232	A	B		B	A	B	A	B	A	X	A	B	B	B	B	X	X	X	X	X
2H	Bmag381	A	B		X	A	B	A	B	A	B	A	B	B	B	B	A	A	B	A	X
2H	Bmag378	A	X		B	A	B	A	B	A	B	A	B	B	X	B	A	A	B	A	X
2H	cdo366	A	X		B	A	B	A	B	A	B	A	B	B	B	B	A	X	B	A	A
2H	cdo588	X	B		B	A	X	A	B	X	B	A	B	B	B	B	A	A	B	A	A
2H	wg180	A	B		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	A	A
2H	cdo474b	A	X		B	A	B	A	B	A	B	A	B	B	B	B	A	A	B	A	A
2H	Vrs1	A	B		B	A	B	A	B	A	B	B	B	B	X	B	A	A	A	X	A
2H	ksuF15	A	X		A	A	B	A	A	X	B	B	B	B	A	B	A	A	A	A	A
2H	ksuF2*	A	B		A	A	B	A	A	A	B	B	B	X	A	B	A	A	A	A	A
2H	mwg892	A	B		A	B	B	A	A	A	B	X	X	X	X	X	X	X	X	X	X
2H	Ebmatc39	A	A		A	X	B	A	A	A	B	B	B	B	A	X	X	A	A	A	X
2H	Ha2	A	A		A	B	B	A	A	A	B	B	B	B	A	B	A	A	A	A	A
2H	awbma21	A	A		A	B	B	A	A	X	B	B	B	B	A	B	A	A	A	A	A
2H	S120	A	A		A	X	B	A	A	A	B	B	B	B	X	B	A	A	A	A	X
2H	cmwg694	B	A		A	B	B	A	A	A	B	X	X	X	X	X	X	X	X	X	X
2H	GBM1208	B	A		A	B	B	A	A	A	X	B	B	B	A	B	X	X	X	X	X
2H	ksuD22	B	X		A	B	B	A	A	X	B	B	B	B	A	B	A	A	A	A	A
2H	cdo665	B	X		A	B	B	A	A	X	B	X	B	B	A	B	A	A	A	A	A
2H	abc157	B	X		A	A	A	A	A	X	B	A	A	B	A	B	A	X	A	A	A
2H	bcd266	B	A		A	A	A	A	A	A	B	A	A	B	A	B	A	B	A	A	A
2H	abc165	B	A		A	A	A	A	A	X	A	A	A	B	A	B	B	B	A	A	A
2H	abc153	B	A		A	A	A	A	A	X	A	A	A	B	A	B	B	B	A	A	A
2H	ksuF41	X	X		A	A	A	A	A	X	A	A	A	B	A	B	B	B	A	A	A
2H	bcd292	A	A		A	A	A	A	A	A	A	A	A	B	A	B	B	B	B	A	A
2H	cdo678a	A	X		B	A	A	A	A	A	A	A	A	B	A	B	B	B	B	A	A
2H	cdo36	A	X		B	A	A	A	A	X	A	A	A	B	A	B	B	B	B	A	A
2H	bcd410	A	X		B	A	A	A	A	X	A	A	A	B	A	B	B	A	B	A	A
2H	wg645	A	X		B	X	A	A	A	A	A	A	A	A	A	B	B	A	B	A	A
2H	bcd339	X	A		B	A	A	A	A	X	A	A	A	A	A	B	B	A	B	A	A
2H	awbma17	A	A		B	A	A	X	A	A	A	A	A	A	A	B	X	X	X	X	X
2H	TaCSLF1b	A	A		B	A	A	B	A	A	A	A	A	A	A	B	B	A	B	A	A
2H	bg123	A	X		B	A	A	B	A	X	A	A	A	A	A	B	B	X	B	A	A

Intolerant Bulk

	DNA #	6	11	15	18	27	29	53	57	63	68	70	72	81	82	88	91	101	105	138	141
2H	abg312	B	B	B	B	B	A	B	B	X	X	X	X	X	X	X	X	X	X	X	X
2H	awbma28	B	B	B	X	B	A	B	B	A	B	B	B	A	A	B	A	B	B	A	A
2H	Bmac134	B	B	B	A	B	A	B	B	A	B	B	B	A	A	B	A	B	B	A	X
2H	wg516	B	B	B	A	A	A	B	B	B	B	B	B	A	A	B	A	B	B	A	B
2H	bcd175	B	B	B	X	A	A	B	B	B	B	B	B	A	A	B	A	B	B	A	A
2H	psr108	A	B	B	A	A	B	A	B	X	X	X	X	X	X	X	X	X	X	X	X
2H	bcd221a	A	B	B	A	A	X	A	B	B	B	B	A	A	A	B	X	B	B	A	B
2H	HvUXS3*	A	A	B	A	A	B	A	B	B	B	B	A	A	A	B	A	A	A	B	B
2H	GBM1115	X	A	B	A	A	B	A	A	A	B	X	A	A	B	B	A	X	X	X	X
2H	awbma27	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	B
2H	awbma14	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	B
2H	awbma33	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	B
2H	psr126	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	B
2H	HvHOTRI	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	X
2H	cdo474a	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	B
2H	abc309	A	A	A	A	A	B	A	X	A	B	B	A	A	B	B	A	A	A	B	B
2H	abc468	A	A	A	A	A	B	A	A	A	B	B	A	A	B	B	A	A	A	B	B
2H	wg789b	A	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	A	A	B	B
2H	cmwg684b	A	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	A	A	B	B
2H	cdo370	A	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	A	A	B	B
2H	wg996	A	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	A	A	B	B
2H	wg222e	A	A	A	A	A	B	A	A	A	X	B	A	A	X	B	A	A	A	B	X
2H	wg222d	A	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	A	A	B	B
2H	Bmac93	A	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	X	A	B	X
2H	GBMS160	X	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	X	X	X	X
2H	GBM1232	X	A	A	A	A	B	A	A	A	A	B	A	A	B	B	A	X	X	X	X
2H	Bmag381	A	A	A	A	A	B	A	B	A	B	B	A	A	B	B	A	A	A	B	X
2H	Bmag378	A	A	A	A	A	B	A	B	A	B	X	A	A	B	B	A	A	A	B	X
2H	cdo366	A	A	A	A	A	B	A	B	A	B	B	A	A	B	B	A	A	A	B	B
2H	cdo588	A	A	A	A	A	B	A	B	A	B	B	A	A	B	B	A	A	A	B	B
2H	wg180	A	A	A	A	A	B	A	B	A	X	B	A	A	B	B	A	A	A	B	B
2H	cdo474b	A	A	A	A	A	B	A	B	A	B	B	A	A	B	B	A	X	X	B	B
2H	Vrs1	A	A	A	A	A	B	B	B	A	B	B	A	B	B	B	A	X	A	B	B
2H	ksuF15	A	A	A	A	A	B	B	B	B	B	B	A	B	B	B	A	A	A	B	B
2H	ksuF2*	A	A	X	A	A	B	B	B	B	B	B	A	B	B	B	A	A	A	B	B
2H	mwg892	A	A	A	B	A	B	B	B	X	X	X	X	X	X	X	X	X	X	X	X
2H	Ebmatc39	A	A	A	B	A	B	B	B	B	B	B	A	X	B	B	A	X	B	X	X
2H	Ha2	B	A	A	B	A	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B
2H	awbma21	B	A	A	B	B	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B
2H	S120	B	A	X	B	B	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B
2H	cmwg694	B	A	A	B	B	B	B	B	X	X	X	X	X	X	X	X	X	X	X	X
2H	GBM1208	X	A	A	B	B	B	B	B	B	B	B	A	B	B	B	A	X	X	X	X
2H	ksuD22	B	A	A	B	B	B	B	B	B	B	B	A	B	B	B	A	A	B	B	B
2H	cdo665	B	A	A	B	B	B	B	B	B	A	B	A	B	B	B	A	A	B	B	B
2H	abc157	A	A	A	B	B	B	B	A	B	A	B	A	B	B	B	A	A	B	B	B
2H	bcd266	A	A	A	B	B	B	B	A	B	A	B	A	B	B	B	A	A	B	B	B
2H	abc165	A	A	A	B	B	B	B	A	B	A	B	A	B	B	B	A	A	B	B	B
2H	abc153	A	A	A	B	B	B	B	A	B	X	B	A	B	B	B	A	A	B	B	B
2H	ksuF41	A	A	A	B	B	B	B	A	B	A	B	A	B	B	B	A	A	B	B	B
2H	bcd292	A	A	A	B	B	B	B	A	B	A	X	B	X	B	B	A	A	B	B	B
2H	cdo678a	A	A	A	B	B	B	B	A	B	A	B	B	B	B	B	A	X	B	B	B
2H	cdo36	A	A	A	B	B	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B
2H	bcd410	A	A	A	B	B	B	B	X	B	A	B	B	B	B	B	A	A	B	B	B
2H	wg645	A	B	A	B	B	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B
2H	bcd339	A	B	A	B	B	B	B	X	B	A	B	B	B	B	B	A	A	B	B	B
2H	awbma17	A	X	A	B	B	B	B	A	B	A	B	B	B	X	B	X	X	X	X	X
2H	TaCSLF1b	A	B	A	B	B	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B
2H	bg123	A	B	A	B	B	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B

Figure 1. Genetic makeup of all loci (graphical genotype) of individual C X S DH lines of each bulk for chromosome 2H.

A- Clipper allele, B Sahara 3771 Allele, X- not available. DNA #epresented by yellow columns was removed from the bulks (data obtained from Ms Margaret Pallotta, ACPFG).



Tolerant Bulk

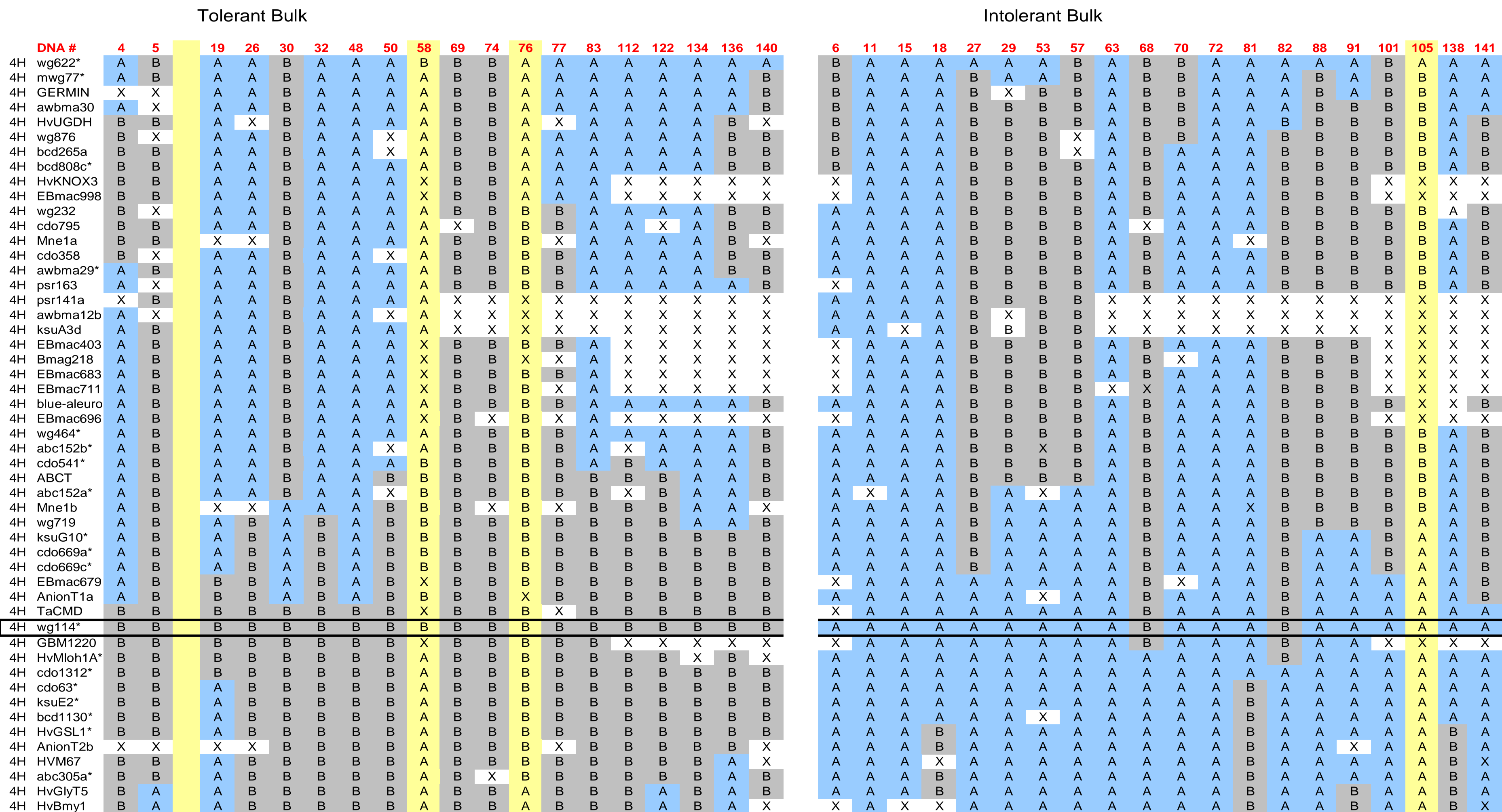
Chr	DNA #	4	5		19	26	30	32	48	50	58	69	74	76	77	83	112	122	134	136	140
3H	awwm1d	X	X		X	X	X	X	X	X	X	A	A	X	B	B	A	A	A	A	B
3H	bcd15	A	X		B	A	A	A	A	X	B	A	A	A	B	A	A	A	A	A	B
3H	HvCW21	A	B		A	A	A	A	A	A	X	A	A	A	B	A	X	X	X	X	X
3H	cdo395*	A	X		A	A	A	A	A	A	B	A	A	A	B	A	A	A	A	A	B
3H	KoprekB8	A	A		X	X	A	A	A	A	B	A	A	A	X	A	B	A	A	A	X
3H	EBmac761	B	A		A	A	A	B	A	A	X	A	B	A	B	X	X	X	X	X	X
3H	EBmac760	B	A		A	A	A	B	A	A	X	A	B	A	B	A	X	X	X	X	X
3H	awbma15	B	A		A	A	A	B	A	A	A	A	B	A	B	A	B	A	B	B	A
3H	Bmac67	B	A		A	X	A	B	A	A	A	A	B	A	X	A	B	X	B	X	X
3H	Bmag6	B	A		A	A	A	B	A	A	A	A	B	A	B	A	X	A	B	B	X
3H	EBmac848	B	A		A	A	A	B	A	A	X	A	B	A	X	A	X	X	X	X	X
3H	GBM1285	B	A		A	A	A	B	A	A	X	A	B	A	X	A	X	X	X	X	X
3H	Bmag361	B	A		A	A	A	B	A	A	X	A	X	X	X	A	X	X	X	X	X
3H	EBmac672	B	A		A	A	A	B	A	A	X	A	B	A	B	A	X	X	X	X	X
3H	GBM1213	B	A		A	A	A	B	A	A	X	X	B	A	B	A	X	X	X	X	X
3H	EBmac839	B	A		A	A	A	B	A	A	X	X	B	A	X	A	X	X	X	X	X
3H	GBMS185	B	A		A	A	A	X	A	A	X	A	B	A	B	A	X	X	X	X	X
3H	Bmag603	B	A		A	A	A	B	A	A	A	A	B	A	B	A	B	A	B	B	X
3H	HvPEPDIPI	B	A		A	A	A	B	A	A	A	A	B	A	B	A	B	A	B	B	X
3H	HvGSL7*	B	A		A	A	A	B	A	A	A	A	B	A	B	A	B	A	B	B	A
3H	wg405	B	X		A	A	A	B	A	X	A	A	B	A	B	A	B	A	B	B	A
3H	HvGSL8*	B	A		A	A	A	B	A	A	B	A	B	B	B	A	B	A	B	A	A
3H	HVM33	B	A		A	X	A	B	A	A	B	A	B	B	B	A	B	A	B	A	X
3H	Bmag112	B	A		A	A	A	B	A	A	X	A	B	B	X	A	B	A	B	A	X
3H	wg178	B	A		A	A	A	B	A	A	B	A	B	B	B	A	B	A	B	A	A
3H	HVM60	B	A		B	A	A	B	A	A	B	A	A	B	B	B	B	X	B	X	X
3H	Myb	B	X		X	X	A	B	A	A	B	B	A	B	B	B	B	A	B	A	A
3H	GVI	B	A		B	A	A	B	A	A	B	X	X	X	X	X	X	X	X	X	X
3H	wg940	B	X		B	A	A	B	A	A	B	B	A	B	B	B	B	A	B	A	A
3H	GBM1233	B	A		B	A	A	B	A	A	X	B	A	B	B	B	X	X	X	X	X
3H	cdo113	X	X		B	A	A	B	A	A	B	B	A	B	B	B	B	A	A	A	B
3H	Bmag363	B	B		B	B	A	B	A	B	X	B	B	B	A	B	X	X	X	X	X
3H	wg110	B	B		B	B	B	B	A	X	A	B	B	B	A	B	B	A	A	A	B
3H	Bmag877	B	B		B	B	B	B	A	B	A	X	B	B	X	A	A	A	A	A	X
3H	17G1-1.3b	B	B		X	B	B	B	A	B	A	A	B	B	A	A	A	A	A	A	B
3H	wg222b	A	B		B	B	B	B	A	B	A	A	B	B	B	A	A	B	A	A	B
3H	HvJAS	A	B		B	B	B	B	X	B	X	A	B	B	B	A	X	X	X	X	X

Intolerant Bulk

Chr	DNA #	6	11	15	18	27	29	53	57	63	68	70	72	81	82	88	91	101	105	138	141
3H	awwm1d	X	X	X	X	X	X	X	X	A	A	A	B	A	A	A	A	A	B	B	A
3H	bcd15	A	A	A	B	A	B	B	B	B	A	A	B	A	B	A	A	A	B	B	A
3H	HvCW21	X	A	A	B	A	B	B	B	B	A	A	B	A	B	A	A	X	X	X	X
3H	cdo395*	A	A	A	B	A	B	X	B	B	A	A	B	A	B	A	A	A	B	B	A
3H	KoprekB8	A	A	A	B	A	B	B	B	B	A	A	B	X	B	A	A	B	B	B	A
3H	EBmac761	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	EBmac760	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	awbma15	B	A	B	B	A	B	B	B	B	X	A	A	B	B	A	A	B	B	B	B
3H	Bmac67	B	A	B	B	A	B	B	B	B	B	A	A	B	B	A	X	B	B	X	X
3H	Bmag6	B	A	B	B	A	B	B	B	X	X	A	A	B	B	A	A	B	B	B	X
3H	EBmac848	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	GBM1285	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	Bmag361	X	A	B	B	A	B	B	B	B	B	A	A	B	B	X	X	X	X	X	X
3H	EBmac672	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	GBM1213	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	EBmac839	X	A	B	B	A	B	X	X	X	X	X	A	B	B	A	A	X	X	X	X
3H	GBMS185	X	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	X	X	X	X
3H	Bmag603	X	A	X	X	A	B	B	B	B	B	A	A	B	B	A	A	B	B	B	X
3H	HvPEPDIPI	B	A	B	B	A	B	B	B	B	B	A	A	B	B	A	A	B	B	B	X
3H	HvGSL7*	B	X	B	B	A	B	B	B	B	B	A	A	B	B	A	A	B	B	B	B
3H	wg405	B	A	B	B	A	B	B	X	B	B	A	A	B	B	A	A	B	B	B	B
3H	HvGSL8*	B	A	B	B	A	B	B	B	B	A	A	A	B	B	A	A	B	B	A	B
3H	HVM33	B	A	B	B	A	B	B	B	B	A	A	A	B	B	A	A	B	B	A	X
3H	Bmag112	B	A	B	B	A	B	B	X	B	A	A	A	B	B	A	A	B	B	A	X
3H	wg178	B	A	B	B	A	B	B	B	B	A	A	A	B	B	A	A	B	B	A	B
3H	HVM60	B	A	B	B	A	B	B	B	B	A	A	X	B	B	X	A	B	B	A	X
3H	Myb	X	B	B	X	A	B	B	B	B	A	A	A	B	B	A	A	B	B	A	B
3H	GVI	B	B	B	B	A	B	B	B	X	X	X	X	X	X	X	X	X	X	X	X
3H	wg940	B	B	B	B	A	A	B	B	B	A	A	A	B	B	A	A	B	B	A	B
3H	GBM1233	X	B	B	A	A	A	B	B	B	A	A	A	B	B	A	A	X	X	X	X
3H	cdo113	B	B	B	A	A	A	B	B	B	A	A	A	B	B	A	A	B	B	A	B
3H	Bmag363	X	B	A	A	A	A	B	B	B	B	A	A	A	B	A	A	X	X	X	X
3H	wg110	A	B	A	A	B	A	B	B	B	B	A	A	A	B	A	A	B	A	A	B
3H	Bmag877	A	B	A	A	B	B	A	A	B	B	A	A	B	B	X	A	B	X	A	X
3H	17G1-1.3b	A	A	A	A	B	B	A	A	B	B	A	A	B	B	B	A	B	A	A	A
3H	wg222b	A	A	A	B	A	B	A	A	A	B	B	A	B	B	B	A	B	B	A	A
3H	HvJAS	X	A	X	B	A	B	A	A	A	B	X	A	B	B	B	A	X	X	X	X

Figure 2. Genetic makeup of all loci (graphical genotype) of individual C X S DH lines of each bulk for chromosome 3H.

A- Clipper allele, B Sahara 3771 Allele, X- not available. DNA #epresented by yellow columns were removed from the bulks (data obtained from Ms Margaret Pallotta, ACPFG).



Tolerant Bulk

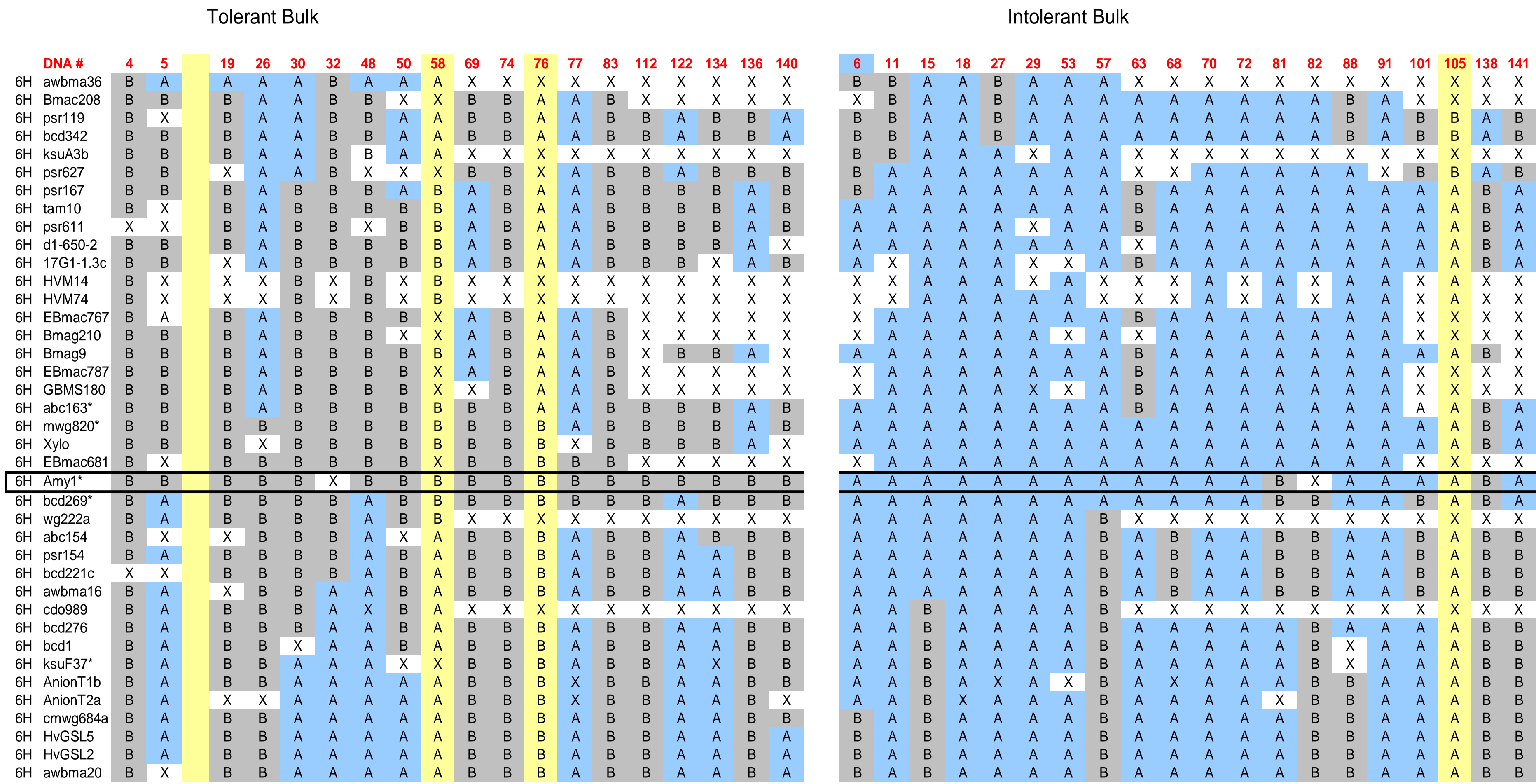
	DNA #	4	5	19	26	30	32	48	50	58	69	74	76	77	83	112	122	134	136	140
4H	wg622*	A	B	A	A	B	A	A	A	B	B	B	A	A	A	A	A	A	A	A
4H	mwg77*	A	B	A	A	B	A	A	A	A	B	B	A	A	A	A	A	A	A	B
4H	GERMIN	X	X	A	A	B	A	A	A	A	B	B	A	A	A	A	A	A	A	B
4H	awbma30	A	X	A	A	B	A	A	A	A	B	B	A	A	A	A	A	A	A	B
4H	HvUGDH	B	B	A	X	B	A	A	A	A	B	B	A	X	A	A	A	A	B	X
4H	wg876	B	X	A	A	B	A	A	X	A	B	B	A	A	A	A	A	A	B	B
4H	bcd265a	B	B	A	A	B	A	A	X	A	B	B	A	A	A	A	A	A	B	B
4H	bcd808c*	B	B	A	A	B	A	A	A	A	B	B	A	A	A	A	A	A	B	B
4H	HvKNOX3	B	B	A	A	B	A	A	A	X	B	B	A	A	A	X	X	X	X	X
4H	EBmac998	B	B	A	A	B	A	A	A	X	B	B	A	A	A	X	X	X	X	X
4H	wg232	B	X	A	A	B	A	A	A	A	B	B	B	B	A	A	A	A	B	B
4H	cdo795	B	B	A	A	B	A	A	A	A	X	B	B	B	A	A	X	A	B	B
4H	Mne1a	B	B	X	X	B	A	A	A	A	B	B	B	X	A	A	A	A	B	X
4H	cdo358	B	X	A	A	B	A	A	X	A	B	B	B	B	A	A	A	A	B	B
4H	awbma29*	A	B	A	A	B	A	A	A	A	B	B	B	B	A	A	A	A	B	B
4H	psr163	A	X	A	A	B	A	A	A	A	B	B	B	B	A	A	A	A	A	B
4H	psr141a	X	B	A	A	B	A	A	A	A	X	X	X	X	X	X	X	X	X	X
4H	awbma12b	A	X	A	A	B	A	A	X	A	X	X	X	X	X	X	X	X	X	X
4H	ksuA3d	A	B	A	A	B	A	A	A	A	X	X	X	X	X	X	X	X	X	X
4H	EBmac403	A	B	A	A	B	A	A	A	X	B	B	B	B	A	X	X	X	X	X
4H	Bmag218	A	B	A	A	B	A	A	A	X	B	B	X	X	A	X	X	X	X	X
4H	EBmac683	A	B	A	A	B	A	A	A	X	B	B	B	B	A	X	X	X	X	X
4H	EBmac711	A	B	A	A	B	A	A	A	X	B	B	B	X	A	X	X	X	X	X
4H	blue-aleuro	A	B	A	A	B	A	A	A	A	B	B	B	B	A	A	A	A	A	B
4H	EBmac696	A	B	A	A	B	A	A	A	X	B	X	B	X	A	X	X	X	X	X
4H	wg464*	A	B	A	A	B	A	A	A	A	B	B	B	B	A	A	A	A	A	B
4H	abc152b*	A	B	A	A	B	A	A	X	A	B	B	B	B	A	X	A	A	A	B
4H	cdo541*	A	B	A	A	B	A	A	A	B	B	B	B	B	A	B	A	A	A	B
4H	ABCT	A	B	A	A	B	A	A	B	B	B	B	B	B	B	B	B	A	A	B
4H	abc152a*	A	B	A	A	B	A	A	X	B	B	B	B	B	B	X	B	A	A	B
4H	Mne1b	A	B	X	X	A	A	A	B	B	B	X	B	X	B	B	B	A	A	X
4H	wg719	A	B	A	B	A	B	A	B	B	B	B	B	B	B	B	B	A	A	B
4H	ksuG10*	A	B	A	B	A	B	A	B	B	B	B	B	B	B	B	B	B	B	B
4H	cdo669a*	A	B	A	B	A	B	A	B	B	B	B	B	B	B	B	B	B	B	B
4H	cdo669c*	A	B	A	B	A	B	A	B	B	B	B	B	B	B	B	B	B	B	B
4H	EBmac679	A	B	B	B	A	B	A	B	X	B	B	B	B	B	B	B	B	B	B
4H	AnionT1a	A	B	B	B	A	B	A	B	B	B	B	X	B	B	B	B	B	B	B
4H	TaCMD	B	B	B	B	B	B	B	B	X	B	B	B	X	B	B	B	B	B	B
4H	wg114*	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
4H	GBM1220	B	B	B	B	B	B	B	B	X	B	B	B	B	B	X	X	X	X	X
4H	HvMloh1A*	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	B	X	B	X
4H	cdo1312*	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B
4H	cdo63*	B	B	A	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B
4H	ksuE2*	B	B	A	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B
4H	bcd1130*	B	B	A	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B
4H	HvGSL1*	B	B	A	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B
4H	AnionT2b	X	X	X	X	B	B	B	B	A	B	B	B	X	B	B	B	B	B	X
4H	HVM67	B	B	A	B	B	B	B	B	A	B	B	B	B	B	B	B	B	A	X
4H	abc305a*	B	B	A	B	B	B	B	B	A	B	X	B	B	B	B	B	B	A	B
4H	HvGlyT5	B	A	A	B	B	B	B	B	A	B	B	A	B	B	B	A	B	A	B
4H	HvBmy1	B	A	A	B	B	B	B	B	A	B	B	A	B	B	B	A	B	A	X

Intolerant Bulk

	DNA #	6	11	15	18	27	29	53	57	63	68	70	72	81	82	88	91	101	105	138	141
4H	wg622*	B	A	A	A	A	A	A	B	A	B	B	A	A	A	A	A	B	A	A	A
4H	mwg77*	B	A	A	A	B	A	A	B	A	B	B	A	A	A	B	A	B	B	A	A
4H	GERMIN	B	A	A	A	B	X	B	B	A	B	B	A	A	A	B	A	B	B	A	A
4H	awbma30	B	A	A	A	B	B	B	B	A	B	B	A	A	A	B	B	B	B	A	A
4H	HvUGDH	B	A	A	A	B	B	B	B	A	B	B	A	A	B	B	B	B	B	A	B
4H	wg876	B	A	A	A	B	B	B	X	A	B	B	A	A	B	B	B	B	B	A	B
4H	bcd265a	B	A	A	A	B	B	B	X	A	B	A	A	A	B	B	B	B	B	A	B
4H	bcd808c*	B	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	HvKNOX3	X	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	X	X	X	X
4H	EBmac998	X	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	X	X	X	X
4H	wg232	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	cdo795	A	A	A	A	B	B	B	B	A	X	A	A	A	B	B	B	B	B	A	B
4H	Mne1a	A	A	A	A	B	B	B	B	A	B	A	A	X	B	B	B	B	B	A	B
4H	cdo358	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	awbma29*	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	psr163	X	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	psr141a	A	A	A	A	B	B	B	B	X	X	X	X	X	X	X	X	X	X	X	X
4H	awbma12b	A	A	A	A	B	X	B	B	X	X	X	X	X	X	X	X	X	X	X	X
4H	ksuA3d	A	A	X	A	B	B	B	B	X	X	X	X	X	X	X	X	X	X	X	X
4H	EBmac403	X	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	X	X	X	X
4H	Bmag218	X	A	A	A	B	B	B	B	A	B	X	A	A	B	B	B	X	X	X	X
4H	EBmac683	X	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	X	X	X	X
4H	EBmac711	X	A	A	A	B	B	B	B	X	X	A	A	A	B	B	B	X	X	X	X
4H	blue-aleuro	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	X	X	B
4H	EBmac696	X	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	X	X	X	X
4H	wg464*	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	abc152b*	A	A	A	A	B	B	X	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	cdo541*	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	ABCT	A	A	A	A	B	B	B	B	A	B	A	A	A	B	B	B	B	B	A	B
4H	abc152a*	A	X	A	A	B	A	X	A	A	B	A	A	A	B	B	B	B	B	A	B
4H	Mne1b	A	A	A	A	B	A	A	A	A	B	A	A	X	B	B	B	B	B	A	B
4H	wg719	A	A	A	A	B	A	A	A	A	B	A	A	A	B	B	B	B	A	A	B
4H	ksuG10*	A	A	A	A	B	A	A	A	A	B	A	A	A	B	A	A	B	A	A	B
4H	cdo669a*	A	A	A	A	B	A	A	A	A	B	A	A	A	B	A	A	B	A	A	B
4H	cdo669c*	A	A	A	A	B	A	A	A	A	B	A	A	A	B	A	A	B	A	A	B
4H	EBmac679	X	A	A	A	A	A	A	A	A	B	X	A	A	B	A	A	A	A	A	B
4H	AnionT1a	A	A	A	A	A	A	X	A	A	B	A	A	A	B	A	A	A	A	A	B
4H	TaCMD	X	A	A	A	A	A	A	A	A	B	A	A	A	B	A	A	A	A	A	A
4H	wg114*	A	A	A	A	A	A	A	A	A	B	A	A	A	B	A	A	A	A	A	A
4H	GBM1220	X	A	A	A	A	A	A	A	A	B	A	A	A	B	A	A	X	X	X	X
4H	HvMloh1A*	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A
4H	cdo1312*	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
4H	cdo63*	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A
4H	ksuE2*	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A
4H	bcd1130*	A	A	A	A	A	A	X	A	A	A	A	A	B	A	A	A	A	A	A	A
4H	HvGSL1*	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	A	A	A	B	A
4H	AnionT2b	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	X	A	A	B	A
4H	HVM67	A	A	A	X	A	A	A	A	A	A	A	A	B	A	A	A	A	A	B	X
4H	abc305a*	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	A	A	A	B	A
4H	HvGlyT5	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	A	B	A
4H	HvBmy1	X	A	X	X	A	A	A	A	A	A	A	A	B	A	A	B	A	A	B	X

Figure 3. Genetic makeup of all loci (graphical genotype) of individual C X S DH lines of each bulk for chromosome 4H.

A- Clipper allele, B Sahara 3771 Allele, X- not available. DNA #epresented by yellow colu mns were removed from the bulks (data obtained from Ms Margaret Pallotta, ACPFG).



Tolerant Bulk

Chr	DNA #	4	5	19	26	30	32	48	50	58	69	74	76	77	83	112	122	134	136	140
6H	awbma36	B	A	A	A	A	B	A	A	A	X	X	X	X	X	X	X	X	X	X
6H	Bmac208	B	B	B	A	A	B	B	X	X	B	B	A	A	B	X	X	X	X	X
6H	psr119	B	X	B	A	A	B	B	A	A	B	B	A	A	B	B	A	B	B	A
6H	bcd342	B	B	B	A	A	B	B	A	A	B	B	A	A	B	B	A	B	B	A
6H	ksuA3b	B	B	B	A	A	B	B	A	A	X	X	X	X	X	X	X	X	X	X
6H	psr627	B	B	X	A	A	B	X	X	X	B	B	X	A	B	B	A	B	B	B
6H	psr167	B	B	B	A	B	B	B	A	B	A	B	A	A	B	B	B	B	A	B
6H	tam10	B	X	B	A	B	B	B	B	B	A	B	A	A	B	B	B	B	A	B
6H	psr611	X	X	B	A	B	B	X	B	B	A	B	A	A	B	B	B	B	A	B
6H	d1-650-2	B	B	B	A	B	B	B	B	B	A	B	A	A	B	B	B	B	A	X
6H	17G1-1.3c	B	B	X	A	B	B	B	B	B	A	B	A	A	B	B	B	X	A	B
6H	HVM14	B	X	X	X	B	X	B	X	B	X	X	X	X	X	X	X	X	X	X
6H	HVM74	B	X	X	X	B	X	B	X	B	X	X	X	X	X	X	X	X	X	X
6H	EBmac767	B	A	B	A	B	B	B	B	X	A	B	A	A	B	X	X	X	X	X
6H	Bmag210	B	B	B	A	B	B	B	X	X	A	B	A	A	B	X	X	X	X	X
6H	Bmag9	B	B	B	A	B	B	B	B	B	A	B	A	A	B	X	B	B	A	X
6H	EBmac787	B	B	B	A	B	B	B	B	X	A	B	A	A	B	X	X	X	X	X
6H	GBMS180	B	B	B	A	B	B	B	B	X	X	B	A	A	B	X	X	X	X	X
6H	abc163*	B	B	B	A	B	B	B	B	B	B	B	A	A	B	B	B	B	A	B
6H	mwg820*	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B	B	B	A	B
6H	Xylo	B	B	B	X	B	B	B	B	B	B	B	B	X	B	B	B	B	A	X
6H	EBmac681	B	X	B	B	B	B	B	B	X	B	B	B	B	B	X	X	X	X	X
6H	Amy1*	B	B	B	B	B	X	B	B	B	B	B	B	B	B	B	B	B	B	B
6H	bcd269*	B	A	B	B	B	B	A	B	B	B	B	B	B	B	B	A	B	B	B
6H	wg222a	B	A	B	B	B	B	A	B	B	X	X	X	X	X	X	X	X	X	X
6H	abc154	B	X	X	B	B	B	A	X	A	B	B	B	A	B	B	A	B	B	B
6H	psr154	B	A	B	B	B	B	A	B	A	B	B	B	A	B	B	A	A	B	B
6H	bcd221c	X	X	B	B	B	B	A	B	A	B	B	B	A	B	B	A	A	B	B
6H	awbma16	B	A	X	B	B	A	A	B	A	B	B	B	A	B	B	A	A	B	B
6H	cdo989	B	A	B	B	B	A	X	B	A	X	X	X	X	X	X	X	X	X	X
6H	bcd276	B	A	B	B	B	A	A	B	A	B	B	B	A	B	B	A	A	B	B
6H	bcd1	B	A	B	B	X	A	A	B	A	B	B	B	A	B	B	A	A	B	B
6H	ksuF37*	B	A	B	B	A	A	A	X	X	B	B	B	A	B	B	A	X	B	B
6H	AnionT1b	B	A	B	B	A	A	A	A	A	B	B	B	X	B	B	A	A	B	B
6H	AnionT2a	B	A	X	X	A	A	A	A	A	B	B	B	X	B	B	A	A	B	X
6H	cmwg684a	B	A	B	B	A	A	A	A	A	B	B	B	A	B	B	A	A	B	B
6H	HvGSL5	B	A	B	B	A	A	A	A	A	B	B	B	A	B	B	A	A	B	A
6H	HvGSL2	B	A	B	B	A	A	A	A	A	B	B	B	A	B	B	A	A	B	A
6H	awbma20	B	X	B	B	A	A	A	A	A	B	B	B	A	B	B	A	A	B	A

Intolerant Bulk

Chr	DNA #	6	11	15	18	27	29	53	57	63	68	70	72	81	82	88	91	101	105	138	141
6H	awbma36	B	B	A	A	B	A	A	A	X	X	X	X	X	X	X	X	X	X	X	X
6H	Bmac208	X	B	A	A	B	A	A	A	A	A	A	A	A	A	B	A	X	X	X	X
6H	psr119	B	B	A	A	B	A	A	A	A	A	A	A	A	A	B	A	B	B	A	B
6H	bcd342	B	B	A	A	B	A	A	A	A	A	A	A	A	A	B	A	B	B	A	B
6H	ksuA3b	B	B	A	A	A	X	A	A	X	X	X	X	X	X	X	X	X	X	X	X
6H	psr627	B	A	A	A	A	A	A	A	X	X	A	A	A	A	A	X	B	B	A	B
6H	psr167	B	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	B	A
6H	tam10	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	B	A
6H	psr611	A	A	A	A	A	X	A	A	B	A	A	A	A	A	A	A	A	A	B	A
6H	d1-650-2	A	A	A	A	A	A	A	A	X	A	A	A	A	A	A	A	A	A	B	A
6H	17G1-1.3c	A	X	A	A	A	X	X	A	B	A	A	A	A	A	A	A	A	A	B	A
6H	HVM14	X	X	A	A	A	X	A	X	X	X	A	X	A	X	A	A	X	A	X	X
6H	HVM74	X	X	A	A	A	A	A	X	X	X	A	X	A	X	A	A	X	A	X	X
6H	EBmac767	X	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	X	X	X	X
6H	Bmag210	X	A	A	A	A	A	X	A	X	A	A	A	A	A	A	A	X	X	X	X
6H	Bmag9	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	B	X
6H	EBmac787	X	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	X	X	X	X
6H	GBMS180	X	A	A	A	A	X	X	A	B	A	A	A	A	A	A	A	X	X	X	X
6H	abc163*	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	B	A
6H	mwg820*	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A
6H	Xylo	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A
6H	EBmac681	X	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	X	X	X	X
6H	Amy1*	A	A	A	A	A	A	A	A	A	A	A	A	B	X	A	A	A	A	B	A
6H	bcd269*	A	A	A	A	A	A	A	A	A	A	A	A	B	B	A	A	B	A	B	A
6H	wg222a	A	A	A	A	A	A	A	B	X	X	X	X	X	X	X	X	X	X	X	X
6H	abc154	A	A	A	A	A	A	A	B	A	B	A	A	B	B	A	A	B	A	B	B
6H	psr154	A	A	A	A	A	A	A	B	A	B	A	A	B	B	A	A	B	A	B	B
6H	bcd221c	A	A	A	A	A	A	A	B	A	B	A	A	B	B	A	A	B	A	B	B
6H	awbma16	A	A	A	A	A	A	A	B	A	B	A	A	B	B	A	A	B	A	B	B
6H	cdo989	A	A	B	A	A	A	A	B	X	X	X	X	X	X	X	X	X	X	X	X
6H	bcd276	A	A	B	A	A	A	A	B	A	A	A	A	A	B	A	A	A	A	B	B
6H	bcd1	A	A	B	A	A	A	A	B	A	A	A	A	A	B	X	A	A	A	B	B
6H	ksuF37*	A	A	B	A	A	A	A	B	A	A	A	A	A	B	X	A	A	A	B	B
6H	AnionT1b	A	A	B	A	X	A	X	B	A	X	A	A	A	B	B	A	A	A	B	B
6H	AnionT2a	A	A	B	X	A	A	A	B	A	A	A	A	X	B	B	A	A	A	B	B
6H	cmwg684a	B	A	B	A	A	A	A	B	A	A	A	A	A	B	B	A	A	A	B	B
6H	HvGSL5	B	A	B	A	A	A	A	B	A	A	A	A	A	B	B	A	A	A	B	B
6H	HvGSL2	B	A	B	A	A	A	A	B	A	A	A	A	A	B	B	A	A	A	B	B
6H	awbma20	B	A	B	A	A	A	A	B	A	A	A	A	A	B	B	A	A	A	B	B

Figure 4. Genetic makeup of all loci (graphical genotype) of individual C X S DH lines of each bulk for chromosome 6H.

A- Clipper allele, B Sahara 3771 Allele, X- not available. DNA #epresented by yellow columns was removed from the bulks (data obtained from Ms Margaret Pallotta, ACPFG).

APPENDIX B

Appendix B. Table 1. NCBI BLASTx and TIGR BLASTn annotations of clones sequenced from B treated library

Clone name	Length (bp)	NCBI BLASTx (nr)	E- value	TIGR BLASTn (HVGI)	E- value
A1	235	putative histone H2B [Oryza sativa (japonica cultivar-group)]	4.00E-06	TC138806 homologue to UP|H2B1_WHEAT (P27807) Histone H2B, partial (91%)	5.00E-56
A10	327	no hits found	-	TC140111 weakly similar to UP|CRK7_HUMAN (Q9NYV4) Cell division cycle 2-related protein kinase 7 (CDC2-related protein	7.00E-90
A11	316	putative senescence-associated protein [Pisum sativum]	2.00E-41	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-155
A3	278	unknown protein [Oryza sativa (japonica cultivar-group)]	4.00E-04	TC132055 weakly similar to UP|Q6Z707 (Q6Z707) Non-ribosomal peptide synthetase modules and related proteins-like, partial	5.00E-35
A4	312	unknown [Saccharomyces cerevisiae]	1.00E-07	TC130828 homologue to emb|X00755.1|OSRRN17S Rice gene for 17S ribosomal RNA, complete	4.00E-60
A6	278	no hits found	-	TC135279 weakly similar to UP|Q43528 (Q43528) Xyloglycan endo-transglycosylase precursor , partial (62%)	e-121
A7	365	no hits found	-	TC131598 GB|AAP47482.1|32331909|AY164908 RTNLB31 {Hordeum vulgare;} , complete	3.00E-86
A8	294	putative dihydrolipoamide dehydrogenase [Oryza sativa (japonica cultivar-group)]	6.00E-06	TC147317 similar to UP|Q8GT30 (Q8GT30) Dihydrolipoamide dehydrogenase , partial (95%)	2.00E-68
A9	364	hypothetical protein CE2794 [Corynebacterium efficiens YS-314]	1.00E-05	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	2.00E-93
B1	236	conserved hypothetical protein [Frankia sp. EAN1pec]	0.01	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-104
B10	312	hypothetical protein [Oryza sativa (japonica cultivar-group)]	9.00E-23	TC146455 homologue to gb|AF036494.1|AF036494 Eucryphia lucida large subunit 26S ribosomal RNA gene, partial sequence,	e-154
B11	314	no hits found	-	no hits found	-
B12	306	no hits found	-	BF625049	0.008
B2	316	no hits found	-	TC138151 similar to UP|GRP1_PETHY (P09789) Glycine-rich cell wall structural protein 1 precursor, partial (7%)	3.00E-95
B3	225	hypothetical protein CE2804 [Corynebacterium efficiens YS-314]	0.002	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-102
B4	494	putative Potassium-efflux system protein [Oryza sativa (japonica cultivar-group)]	2.00E-31	CD055614	e-123
B7	404	dnaK-type molecular chaperone HSP70 - barley	1.00E-60	TC147130 UP|Q40058 (Q40058) HSP70 precursor, complete	0
B8	291	no hits found	-	no hits found	-
B9	285	NAR2.1 [Hordeum vulgare subsp. vulgare]	2.00E-43	TC141528 UP|Q6X679 (Q6X679) NAR2.1, complete	e-143
C1	259	no hits found	-	TC140109 similar to UP|Q8W417 (Q8W417) Cig3, partial (4%)	e-115
C10	341	CI2D [Hordeum vulgare]	2.00E-20	BM816648	e-117
C12	334	putative HD-zip transcription factor [Oryza sativa (japonica cultivar-group)]	4.00E-05	BQ767072 weakly similar to PIR|T01364|T013 homeodomain transcription factor (ATHB-14) [imported] - Arabidopsis	1.00E-91
C2	272	putative monodehydroascorbate reductase [Oryza sativa (japonica cultivar-group)]	1.00E-18	TC139665 similar to UP|Q6ZJ08 (Q6ZJ08) Monodehydroascorbate reductase, complete	1.00E-81
C3	452	OSJNBa0086B14.2 [Oryza sativa (japonica cultivar-group)]-sugar transport	3.00E-27	TC138807 similar to UP|WSC4_YEAST (P38739) Cell wall integrity and stress response component 4 precursor, partial (3%)	e-107
C4	236	probable cytochrome P450 monooxygenase - maize (fragment)	2.00E-05	TC146396 homologue to gb|AF223066.1|AF223066 Humulus lupulus 26S ribosomal RNA gene, partial sequence; 18S ribosomal RNA	9.00E-33
C5	318	Putative glutathione S-transferase [Oryza sativa (japonica cultivar-group)]	9.00E-26	TC140104 similar to UP|Q9FQC9 (Q9FQC9) Glutathione S-transferase GST 10 , partial (84%)	e-112
C6	282	putative senescence-associated protein [Pisum sativum]	2.00E-05	AJ434911	5.00E-44
C7	323	hypothetical protein CE1541 [Corynebacterium efficiens YS-314]	0.003	BU983255	7.00E-19
C8	521	putative histidine kinase [Oryza sativa (japonica cultivar-group)]	4.00E-10	TC141011 similar to UP|Q84TZ8 (Q84TZ8) Histidine kinase (Fragment), partial (27%)	2.00E-88
C9	386	allene oxide cyclase [Hordeum vulgare]	2.00E-12	TC147438 homologue to UP|Q711R0 (Q711R0) Allene oxide cyclase precursor , complete	7.00E-81

D1	260	unknown protein [Oryza sativa (japonica cultivar-group)]	0.003	TC143032	1.00E-63
D10	298	lipase-like protein [Arabidopsis thaliana]	1.00E-31	TC148384 similar to UP|Q94F26 (Q94F26) Lipase-like protein, partial (78%)	e-147
D11	334	hypothetical protein CE2794 [Corynebacterium efficiens YS-314]	1.00E-05	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	1.00E-88
D3	480	hypothetical protein 3 [Microplitis demolitor bracovirus]	2.00E-13	CA002129 similar to GP|4097684|gb|A ubiquitin conjugating enzyme {Metarhizium anisopliae}, partial (12%)	2.00E-84
D4	224	conserved hypothetical protein [Frankia sp. EAN1pec]	0.01	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-102
D5	316	hypothetical protein CE2794 [Corynebacterium efficiens YS-314]	8.00E-06	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	2.00E-99
D6	512	putative reverse transcriptase [Cicer arietinum]	6.00E-08	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	1.00E-05
D7	333	alpha-tubulin 2 [Hordeum vulgare subsp. vulgare]	1.00E-47	TC131375 UP|TBA2_HORVU (Q96460) Tubulin alpha-2 chain, complete	e-162
D8	301	no hits found	-	TC146303 emb|Z14059.1|MISCREPU3 S.cereale mitochondrion fMet, 18S, 5S repeat unit DNA, partial (60%)	3.00E-33
D9	479	catalase [Campylobacter jejuni]	3.00E-09	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	1.00E-95
MA_1C81	421	methionine synthase 1 enzyme [Hordeum vulgare subsp. vulgare]	3.00E-32	AL505571 Methionine synthase [Hordeum vulgare (Barley)]	e-155
MA_2D111	458	methionine synthase 1 enzyme [Hordeum vulgare subsp. vulgare]	2.00E-65	AL505571 Methionine synthase [Hordeum vulgare (Barley)]	0
MA_2H121	418	14-3-3 protein [Triticum aestivum]	7.00E-51	BG342895 14-3-3-like protein A [Hordeum vulgare (Barley)]	0
NMAP1A1	262	hypothetical protein UM04102.1 [Ustilago may	3.2	TA45791_4513	e-123
NMAP1A10	251	no hits found	-	TC133752 weakly similar to UP|O65450 (O65450) Glycine-rich protein, partial (19%)	5.00E-93
NMAP1A11	210	RGH1A [Hordeum vulgare]	4.00E-15	NP461006 RGH1A [Hordeum vulgare]	4.00E-44
NMAP1A12	244	arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II [Hordeum vulgare]	4.00E-33	TC132139 UP|Q9ATV7 (Q9ATV7) Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II, complete	e-111
NMAP1A2	169	unnamed protein product [Hordeum vulgare]	1.00E-16	TC138639 similar to UP|JI23_HORVU (P32024) 23 kDa jasmonate-induced protein, complete	1.00E-68
NMAP1A3	185	OSJNBa0072K14.18 [Oryza sativa (japonica cultivar-group)]	1.00E-10	TC143213 weakly similar to UP|Q9LW29 (Q9LW29) Transport inhibitor response-like protein (At3g26830), partial (35%)	1.00E-62
NMAP1A4	313	putative senescence-associated protein [Pisum sativum]	6.00E-41	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-152
NMAP1A5	219	putative floral homeotic protein HUA1 [Oryza sativa (japonica cultivar-group)]	4.00E-27	TC135310	8.00E-98
NMAP1A6	187	cytoplasmatic ribosomal protein S13 [Triticum aestivum]	2.00E-21	BE422386 homologue to SP|Q05761|RS13 40S ribosomal protein S13. [Maize] {Zea mays}, partial (84%)	3.00E-81
NMAP1A7	192	no hits found	-	AJ465155	2.00E-05
NMAP1A8	176	putative CREB-binding protein [Oryza sativa (japonica cultivar-group)]	1.00E-14	TC151571 weakly similar to UP|P300_HUMAN (Q09472) E1A-associated protein p300 , partial (3%)	4.00E-59
NMAP1A9	160	putative Potential phospholipid-transporting ATPase 8 [Oryza sativa (japonica cultivar-group)]	0.16	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	7.00E-05
NMAP1B1	201	Moco containing protein(OsMCP) [Oryza sativa (japonica cultivar-group)]	6.00E-22	TC132212 homologue to UP|Q8LP96 (Q8LP96) Moco containing protein (Moco containing protein(OsMCP)), complete	4.00E-96
NMAP1B10	186	no hits found	-	TC134029 weakly similar to UP|Q9FR48 (Q9FR48) Acyl-CoA binding protein 2 (Fragment), partial (12%)	4.00E-65
NMAP1B11	210	no hits found	-	TC131571 homologue to UP|Q9AYS8 (Q9AYS8) 1-aminocyclopropane-1-carboxylate oxidase, partial (95%)	0.001
NMAP1B12	208	hypothetical protein [Oryza sativa (japonica cultivar-group)]	1.00E-16	TC150893 weakly similar to UP|Q9Y6D4 (Q9Y6D4) Microrchidia, partial (5%)	1.00E-80
NMAP1B2	209	PREDICTED: similar to hairless protein isoform a; hairless (mouse) homolog [Pan troglodytes]	0.76	TC133657 weakly similar to UP|Q07524 (Q07524) Cellulase , partial (89%)	4.00E-50
NMAP1B3	159	no hits found	-	TC133450 similar to UP|Q761Z4 (Q761Z4) BRI1-KD interacting protein 120 (Fragment), partial (36%)	9.00E-66
NMAP1B4	187	band 3 anion transport protein -like [Oryza sativa (japonica cultivar-group)]	0.005	BJ468992 similar to GP|11034532|db P0013F10.1 {Oryza sativa (japonica cultivar-group)}, partial (18%)	7.00E-33
NMAP1B5	176	betaine aldehyde dehydrogenase [Hordeum vulgare subsp. vulgare]	1.00E-18	TC140076 UP|Q94IC0 (Q94IC0) Betaine aldehyde dehydrogenase, complete	2.00E-67
NMAP1B6	263	putative cyclin Ia [Oryza sativa (japonica cultivar-group)]	5.00E-19	TC139865 similar to UP|Q41734 (Q41734) Cyclin IaZm (Fragment), partial (58%)	1.00E-56
NMAP1B7	210	putative histidine kinase [Oryza sativa (japonica cultivar-group)]	2.00E-18	TC141011 similar to UP|Q84TZ8 (Q84TZ8) Histidine kinase (Fragment), partial (27%)	8.00E-70

NMAP1B8	278	P0497A05.15 [Oryza sativa (japonica cultivar-group)]	0.58	TC143981 similar to GB|AAR24650.1|38603810|BT010872 At5g63160 {Arabidopsis thaliana;} , partial (12%)	6.00E-25
NMAP1B9	332	S-adenosyl-L-homocysteine hydrolase [Anaeromyxobacter dehalogenans 2CP-C]	8.00E-35	no hits found	-
NMAP1C1	529	At2g43970/F6E13.10 [Oryza sativa (japonica cultivar-group)]	7.00E-74	TC140426 similar to GB|AAM91060.1|22136548|AY129474 At2g43970/F6E13.10 {Arabidopsis thaliana;} , partial	0
NMAP1C10	119	no hits found	-	AJ435560	9.00E-22
NMAP1C11	174	NADP malic enzyme [Oryza sativa (japonica cultivar-group)]	5.00E-16	TC139308 homologue to UP|Q6T5D1 (Q6T5D1) NADP malic enzyme , complete	2.00E-64
NMAP1C12	290	clathrin heavy chain-Cellular component: clathrin coat of coated pit	5.00E-32	no hits found	-
NMAP1C2	227	SMC3 protein [Oryza sativa]	9.00E-26	TC132351 partial sequence, homology to chromosome scaffold proteins [Hordeum vulgare]	8.00E-95
NMAP1C3	241	unknown protein [Oryza sativa (japonica cultivar-group)]	6.00E-17	TC133635 weakly similar to UP|Q6IDJ6 (Q6IDJ6) At1g35180, partial (51%)	e-109
NMAP1C4	185	no hits found	-	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	3.00E-04
NMAP1C5	233	putative methylcrotonyl-CoA carboxylase beta chain, mitochondrial precursor [Oryza sativa (japonica cultivar-group)]	1.00E-24	TC148913 similar to UP|MCCB_ARATH (Q9LDD8) Methylcrotonyl-CoA carboxylase beta chain, mitochondrial precursor	1.00E-87
NMAP1C6	292	p41-Arc [Dictyostelium discoideum]	4.00E-04	no hits found	-
NMAP1C7	264	no hits found	-	TC131477 homologue to UP|Q9FSE2 (Q9FSE2) D-TDP-glucose dehydratase, partial (97%)	8.00E-43
NMAP1C8	157	no hits found	-	BM371382 weakly similar to GP|20269071|emb pectin methylesterase {Sesbania rostrata}, partial (9%)	4.00E-06
NMAP1C9	197	putative dihydrolipoamide dehydrogenase [Oryza sativa (japonica cultivar-group)]	8.00E-14	TC147317 similar to UP|Q8GT30 (Q8GT30) Dihydrolipoamide dehydrogenase , partial (95%)	3.00E-63
NMAP1D1	240	putative pseudo-response regulator [Oryza sativa (japonica cultivar-group)]	1.00E-06	TC149085 similar to UP|Q6UV11 (Q6UV11) Pseudo-response regulator protein (Fragment), partial (36%)	e-109
NMAP1D10	259	no hits found	-	no hits found	-
NMAP1D11	231	auxin-regulated protein-like [Oryza sativa (japonica cultivar-group)]	2.00E-21	TC147891 homologue to UP|Q84ZE8 (Q84ZE8) Auxin-regulated protein-like, complete	e-104
NMAP1D12	212	tubulin beta chain [Oryza sativa (japonica cultivar-group)]	9.00E-26	TC130782 UP|TBB5_WHEAT (Q9ZRA8) Tubulin beta-5 chain (Beta-5 tubulin), complete	1.00E-90
NMAP1D2	339	P0506B12.23 [Oryza sativa (japonica cultivar-group)]	1.00E-44	TC140125 weakly similar to UP|SFR4_HUMAN (Q08170) Splicing factor, arginine/serine-rich 4 (Pre-mRNA splicing factor SRP75)	e-158
NMAP1D3	251	putative MAP kinase kinase kinase [Oryza sativa (japonica cultivar-group)]	4.00E-24	TC143170	6.00E-28
NMAP1D4	182	putative cellulose synthase catalytic subunit [Hordeum vulgare]	1.00E-14	TC139373 homologue to UP|Q84ZN6 (Q84ZN6) Cellulose synthase-4, complete	4.00E-59
NMAP1D5	282	band 3 anion transport protein -like [Oryza sativa (japonica cultivar-group)]	1.00E-36	BJ452857 similar to GP|5103843|gb| Is a member of the PF|00955 Anion exchanger family. {Arabidopsis thaliana}, partial	8.00E-40
NMAP1D6	191	putative Na+/H+ antiporter [Oryza sativa (japonica cultivar-group)]	2.00E-15	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	0.001
NMAP1D7	229	acetyl CoA synthetase [Deschampsia antarctica]	7.00E-10	TC133889 homologue to GB|CAD40664.1|21741488|OSJN00124 OSJNBb0118P14.2 {Oryza sativa (japonica	6.00E-37
NMAP1D8	218	Outer mitochondrial membrane protein porin (Voltage-dependent anion-selective channel protein) (VDAC)	2.00E-22	TC146872 homologue to UP|POR1_WHEAT (P46274) Outer mitochondrial membrane protein porin (Voltage-dependent	3.00E-94
NMAP1D9	292	no hits found	-	TC142893 similar to UP|Q08529 (Q08529) Glycine-rich protein, partial (11%)	9.00E-49
NMAP1E1	116	putative senescence-associated protein [Pisum sativum]	4.00E-06	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	2.00E-41
NMAP1E10	206	no hits found	-	TC140237 similar to UP|Q96332 (Q96332) Glyoxalase II, partial (94%)	2.00E-86
NMAP1E11	227	RAB1C [Lotus corniculatus var. japonicus]	5.00E-27	TC139501 homologue to UP|RIC1_ORYSA (P40392) Ras-related protein RIC1, complete	5.00E-99
NMAP1E12	350	enolase [Oryza sativa (japonica cultivar-group)]	2.00E-50	TC138622 homologue to UP|ENO_ORYSA (Q42971) Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate	e-163
NMAP1E2	213	no hits found	-	TC141303 UP|Q9BX68 (Q9BX68) HIT-17kDa (Histidine triad nucleotide binding protein 2) (HINT2) (PKCI-1-related HIT protein)	1.00E-93
NMAP1E3	234	ethylene-responsive protein-like [Oryza sativa (japonica cultivar-group)]	5.00E-29	TC147921 similar to UP|Q8S3R7 (Q8S3R7) Ethylene-responsive protein-like, complete	e-107
NMAP1E4	316	vacuolar ATPase subunit c isoform [Pennisetum glaucum]	6.00E-04	TC131509 UP|Q945E8 (Q945E8) Vacuolar H+-ATPase 16 kDa proteolipid subunit c, complete	e-134
NMAP1E5	181	putative cellulose synthase catalytic subunit [Hordeum vulgare]	9.00E-20	TC139386 homologue to UP|Q851L8 (Q851L8) Cellulose synthase, complete	1.00E-74
NMAP1E6	193	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	1.00E-86

NMAP1E7	177	unknown [Arabidopsis thaliana]	0.2	TC130856	2.00E-67
NMAP1E8	196	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	2.00E-79
NMAP1E9	163	putative beta 3 proteasome subunit [Nicotiana tabacum]	2.00E-15	TC131966 homologue to UP|Q6H800 (Q6H800) Proteasome subunit beta type 3, complete	1.00E-61
NMAP1F1	545	photosystem I P700 apoprotein A2 [Typha latifolia]	2.00E-93	TC134796 homologue to UP|PSAB_ANTMA (Q33332) Photosystem I P700 chlorophyll A apoprotein A2 (PsaB) (PSI-B), partial	0
NMAP1F10	106	sucrose synthase [Bambusa oldhamii]	2.00E-04	TC131392 homologue to UP|SUS2_ORYSA (P31924) Sucrose synthase 2 (Sucrose-UDP glucosyltransferase 2) , partial (63%)	3.00E-18
NMAP1F11	251	glutathione-S-transferase, I subunit [Hordeum vulgare subsp. vulgare]	2.00E-35	TC151803 UP|Q8LPD5 (Q8LPD5) Glutathione-S-transferase, I subunit, partial (81%)	e-116
NMAP1F12	247	At1g16570 [Arabidopsis thaliana]	8.00E-17	TC150852 similar to GB|AAP49524.1|31376395|BT008762 At1g16570 {Arabidopsis thaliana;} , partial (41%)	9.00E-30
NMAP1F2	179	no hits found	-	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	2.00E-05
NMAP1F3	343	translation initiation factor 5A [Oryza sativa (japonica cultivar-group)]	1.00E-39	TC146867 similar to UP|Q9AUW3 (Q9AUW3) Translation initiation factor 5A, complete	e-167
NMAP1F4	142	no hits found	-	TC130754 similar to UP|RSP4_DAUCA (O80377) 40S ribosomal protein SA (p40), partial (79%)	1.00E-58
NMAP1F5	167	putative HAC5 [Oryza sativa (japonica cultivar-group)]	2.00E-11	BM376094	0.004
NMAP1F6	207	At1g22140/F2E2_13 [Arabidopsis thaliana]	3.00E-04	TC149011 similar to GB|AAM16206.1|20334900|AY094050 At1g22140/F2E2_13 {Arabidopsis thaliana;} , partial	3.00E-94
NMAP1F7	186	no hits found	-	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	2.00E-05
NMAP1F8	148	putative NIC2 [Oryza sativa (japonica cultivar-group)]	3.00E-08	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	0.001
NMAP1F9	197	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	8.00E-70
TB1n2R_A1	301	no hits found	-	TC149984 similar to GB|CAB10420.1|2245000|ATFCA6 LET1 like protein {Arabidopsis thaliana;} , partial (7%)	6.00E-25
TB1n2R_A10	310	WD-40 repeat protein-like [Oryza sativa (japonica cultivar-group)]	4.00E-33	TC139941 homologue to UP|Q7EYE2 (Q7EYE2) WD-40 repeat protein-like, partial (67%)	4.00E-59
TB1n2R_A11	235	putative methylcrotonyl-CoA carboxylase beta chain, mitochondrial precursor [Oryza sativa (japonica cultivar-group)]	1.00E-24	TC148913 similar to UP|MCCB_ARATH (Q9LDD8) Methylcrotonyl-CoA carboxylase beta chain, mitochondrial precursor	1.00E-37
TB1n2R_A12	226	COG1216: Predicted glycosyltransferases [Desulfovibrio desulfuricans G20]	0.57	no hits found	-
TB1n2R_A2	290	probable cytochrome P450 monooxygenase - maize (fragment)	5.00E-28	TC146396 homologue to gb|AF223066.1|AF223066 Humulus lupulus 26S ribosomal RNA gene, partial sequence; 18S ribosomal RNA	4.00E-50
TB1n2R_A3	310	putative senescence-associated protein [Pisum sativum]	2.00E-26	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	4E-54
TB1n2R_A4	198	Hypothetical protein [Oryza sativa (japonica cultivar-group)]	2.00E-08	TC140381	1.00E-28
TB1n2R_A5	221	no hits found	-	TC142864	7E-29
TB1n2R_A6	540	putative fructose 1-,6-biphosphate aldolase [Triticum aestivum]	4.00E-56	TC146554 homologue to UP|Q6QWQ3 (Q6QWQ3) Fructose 1,6-bisphosphate aldolase , complete	e-108
TB1n2R_A7	249	no hits found	-	TC148705	9E-20
TB1n2R_A8	200	no hits found	-	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	2E-16
TB1n2R_A9	320	putative dihydroorotate dehydrogenase [Oryza sativa (japonica cultivar-group)]	6.00E-12	TC132419 similar to UP|Q9ST36 (Q9ST36) JPR ORF1 protein, partial (88%)	1.00E-30
TB1n2R_B1	381	unknown protein [Arabidopsis thaliana]	3.00E-20	TC150066 similar to UP|OLF4_CANFA (Q95157) Olfactory receptor-like protein OLF4, partial (7%)	9E-67
TB1n2R_B10	641	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	4.00E-32	TC146396 homologue to gb|AF223066.1|AF223066 Humulus lupulus 26S ribosomal RNA gene, partial sequence; 18S ribosomal RNA	e-130
TB1n2R_B11	420	unknown protein [Oryza sativa (japonica cultivar-group)]	1.00E-09	BQ660616	5.00E-79
TB1n2R_B12	461	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-51	TC135928 similar to UP|Q9LQY8 (Q9LQY8) T24P13.2, partial (26%)	2.00E-36
TB1n2R_B2	225	conserved hypothetical protein [Paracoccus denitrificans PD1222]	2.00E-04	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	6E-36
TB1n2R_B3	191	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	2E-25
TB1n2R_B4	302	putative senescence-associated protein [Pyrus communis]	2.00E-20	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	7E-30

TB1n2R_B5	194	no hits found	-	BE421735 homologue to GP|169061|gb|AA chlorophyll a/b-binding protein {Pisum sativum}, partial (30%)	0.01
TB1n2R_B6	615	putative AdoMet synthase 3 [Hordeum vulgare subsp. vulgare]	6.00E-41	TC131483 UP|METK_HORVU (P50299) S-adenosylmethionine synthetase 1 (Methionine adenosyltransferase 1) (AdoMet synthetase 1)	e-119
TB1n2R_B7	126	no hits found	-	no hits found	-
TB1n2R_B8	474	pyruvate decarboxylase [Dianthus caryophyllus]	0.052	TC139454 homologue to UP|Q8RUU6 (Q8RUU6) Pyruvate decarboxylase, partial (72%)	5.00E-22
TB1n2R_B9	251	no hits found	-	TC147371 similar to UP|Q41901 (Q41901) NADH ubiquinone oxidoreductase subunit, partial (67%)	1E-17
TB1n2R_C1	468	aconitase [Arabidopsis thaliana]	3.00E-30	TC139409 similar to UP|ACOC_CUCMA (P49608) Aconitate hydratase, cytoplasmic (Citrate hydro-lyase) (Aconitase) , partial	6E-49
TB1n2R_C10	192	no hits found	-	BE421808	2.00E-30
TB1n2R_C11	365	PREDICTED: hypothetical protein XP_580193 [Rattus norvegicus]	9.00E-07	TC146455 homologue to gb|AF036494.1|AF036494 Eucryphia lucida large subunit 26S ribosomal RNA gene, partial sequence, partial	6E-15
TB1n2R_C12	460	hypothetical protein AdehDRAFT_1322 [Anaeromyxobacter dehalogenans 2CP-C]	0.74	TC138657 similar to UP|Q9AUH6 (Q9AUH6) F-box containing protein TIR1, partial (34%)	4.00E-19
TB1n2R_C2	198	ribosomal protein S8e [Georissus sp. APV-2005]	1.00E-07	TC131434 homologue to UP|RS8_ORYSA (P49199) 40S ribosomal protein S8, partial (95%)	6.00E-09
TB1n2R_C3	208	putative F-box protein [Triticum aestivum]	2.00E-10	TC141398 weakly similar to UP|Q9FZK1 (Q9FZK1) F17L21.13, partial (50%)	4E-25
TB1n2R_C4	427	At3g62310 [Arabidopsis thaliana]	3.00E-52	TC149790 homologue to UP|Q9LZQ9 (Q9LZQ9) ATP-dependent RNA helicase-like protein (At3g62310), partial (60%)	3E-80
TB1n2R_C5	190	putative transcription regulatory protein [Oryza sativa (japonica cultivar-group)]	1.00E-20	TC133231 similar to UP|O81909 (O81909) T7I23.15 protein, partial (14%)	3.00E-30
TB1n2R_C6	283	OSJNBa0084A10.17 [Oryza sativa (japonica cultivar-group)]	2.00E-24	TC133043 weakly similar to GB|AAP49513.1|31376373|BT008751 At1g35620 {Arabidopsis thaliana;} , partial (70%)	9E-36
TB1n2R_C7	308	putative senescence-associated protein [Pisum sativum]	4.00E-32	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	1E-50
TB1n2R_C8	238	IAA1 protein [Triticum aestivum]	2.00E-14	TC131541 UP|Q7XTK5 (Q7XTK5) IAA1 protein, complete	1E-30
TB1n2R_C9	240	ATPase beta	0.031	TC130729 homologue to UP|Q41534 (Q41534) ATP synthase beta subunit , complete	8E-20
TB1n2R_D1	267	no hits found	-	CA011340	2.00E-10
TB1n2R_D10	332	putative uridine kinase/uracil phosphoribosyltransferase [Oryza sativa (japonica cultivar-group)]	3.00E-25	TC146038 similar to GB|AAM10488.1|29465725|AY089970 uracil phosphoribosyltransferase {Arabidopsis thaliana;}	2.00E-27
TB1n2R_D11	225	no hits found	-	no hits found	-
TB1n2R_D12	689	putative AFG1-like ATPase [Oryza sativa (japonica cultivar-group)]	7.00E-95	TC140885	e-141
TB1n2R_D2	310	putative DNAJ heat shock N-terminal domain-containing protein [Oryza sativa (japonica cultivar-group)]	7.00E-10	no hits found	-
TB1n2R_D3	207	ribosomal protein S7 [Secale cereale]	2.00E-05	BE603137 homologue to GP|4588906|gb| ribosomal protein S7 {Secale cereale}, partial (76%)	3E-21
TB1n2R_D4	220	no hits found	-	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	3E-11
TB1n2R_D5	307	putative senescence-associated protein [Pyrus communis]	6.00E-09	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	6E-38
TB1n2R_D6	542	Putative Nonclathrin coat protein gamma - like protein [Oryza sativa (japonica cultivar-group)]	2.00E-30	TC147625 similar to UP|O65673 (O65673) Nonclathrin coat protein gamma-like protein, partial (94%)	1E-67
TB1n2R_D7	122	no hits found	-	no hits found	-
TB1n2R_D8	184	no hits found	-	TC139548 homologue to emb|Z00028.1|CHZMRRNA Zea mays chloroplast rRNA-operon, partial (9%)	0.00007
TB1n2R_D9	284	putative multicatalytic endopeptidase [Arabidopsis thaliana]	4.00E-23	TC147116 UP|Q6H852 (Q6H852) Alpha 2 subunit of 20S proteasome, complete	2E-41
TB1n2R_E1	231	no hits found	-	BM815945	2E-18
TB1n2R_E10	235	SD07261p [Drosophila melanogaster]	0.12	TC146472 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (41%)	0.006
TB1n2R_E11	208	unknown protein [Oryza sativa (japonica cultivar-group)]	7.00E-13	no hits found	-
TB1n2R_E12	208	no hits found	-	TC131158 homologue to gb|K02202.1|MZERG17S Maize 17S ribosomal RNA gene and flanks, partial (17%)	1E-12
TB1n2R_E2	262	putative HECT ubiquitin-protein ligase 3 [Oryza sativa (japonica cultivar-group)]	2.00E-14	no hits found	-
TB1n2R_E3	100	no hits found	-	no hits found	-
TB1n2R_E4	106	no hits found	-	no hits found	-

TB1n2R_E5	274	putative leucine zipper-EF-hand containing transmembrane protein 1 [Oryza sativa (japonica cultivar-group)]	2.00E-06	TC149177 weakly similar to UP|O95202 (O95202) Leucine zipper-EF-hand containing transmembrane protein 1,	9E-12
TB1n2R_E6	265	no hits found	-	no hits found	-
TB1n2R_E7	252	putative ubiquitin conjugating enzyme [Oryza sativa (japonica cultivar-group)]	1.00E-10	TC147574 similar to UP|Q43780 (Q43780) Ubiquitin conjugating enzyme, partial (92%)	5E-19
TB1n2R_E8	207	no hits found	-	TC141947 homologue to UP|Q86EE5 (Q86EE5) Clone ZZD480 mRNA sequence, partial (5%)	2E-14
TB1n2R_E9	463	CREG2-protein-like [Oryza sativa (japonica cultivar-group)]	9.00E-58	TC138822 weakly similar to UP|Q8RY62 (Q8RY62) At2g04690/F28I8.27, partial (71%)	4.00E-91
TB1n2R_F1	246	unnamed protein product [Hordeum vulgare]	5.00E-05	TC138639 similar to UP|JI23_HORVU (P32024) 23 kDa jasmonate-induced protein, complete	7E-18
TB1n2R_F10	363	putative senescence-associated protein [Pisum sativum]	1.00E-04	TC146472 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (41%)	8.00E-09
TB1n2R_F11	309	no hits found	-	TC132502 homologue to UP|Q8LPA2 (Q8LPA2) Chloride channel, partial (50%)	1E-17
TB1n2R_F12	310	putative senescence-associated protein [Pisum sativum]	2.00E-28	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	9E-54
TB1n2R_F2	259	PROBABLE 30S RIBOSOMAL SUBUNIT PROTEIN S4 [Sinorhizobium meliloti]	2.00E-19	no hits found	-
TB1n2R_F3	117	no hits found	-	no hits found	-
TB1n2R_F4	308	no hits found	-	TC145943 similar to UP|Q6PJU0 (Q6PJU0) WBSCR21 protein (Fragment), partial (5%)	7E-23
TB1n2R_F5	327	putative glutamate dehydrogenase [Arabidopsis thaliana]	7.00E-12	TC131994 homologue to UP|Q852M0 (Q852M0) Glutamate dehydrogenase, complete	5E-25
TB1n2R_F6	371	no hits found	-	TC141890 weakly similar to UP|Q7S2M4 (Q7S2M4) Predicted protein, partial (6%)	1E-57
TB1n2R_F7	249	heat shock cognate 70-1 [Arabidopsis thaliana]	2.00E-04	TC138926 homologue to UP|Q84TA1 (Q84TA1) Heat shock protein cognate 70, complete	2E-22
TB1n2R_F8	253	no hits found	-	TC145611 UP|Q9Z5X4 (Q9Z5X4) Ferredoxin oxidoreductase a-subunit, partial (3%)	2E-33
TB1n2R_F9	319	no hits found	-	TC138527	5.00E-05
TB1n2R_G1	450	hypothetical protein [Plasmodium yoelii yoelii]	0.04	TC131173 gb|AF168852.1|AF168852 Hordeum jubatum 18S small subunit ribosomal RNA gene, complete sequence, partial (30%)	3E-79
TB1n2R_G10	252	heat shock cognate 70-1 [Arabidopsis thaliana]	4.00E-12	TC138926 homologue to UP|Q84TA1 (Q84TA1) Heat shock protein cognate 70, complete	8E-43
TB1n2R_G11	333	unknown protein [Arabidopsis thaliana]	0.039	TC148723 similar to UP|AF9_HUMAN (P42568) AF-9 protein, partial (3%)	1E-19
TB1n2R_G12	338	unknown protein [Arabidopsis thaliana]	2.00E-08	BU984252	3E-51
TB1n2R_G2	207	no hits found	-	AJ432072 similar to GP|14488078|gb At1g05170/YUP8H12_22 {Arabidopsis thaliana}, partial (44%)	0.0000001
TB1n2R_G3	323	no hits found	-	TC130737 similar to UP|Q6Z4N6 (Q6Z4N6) R40g2 protein, partial (95%)	1E-42
TB1n2R_G4	304	Unknown protein [Arabidopsis thaliana]	3.00E-27	TC140166 weakly similar to UP|Q9SEZ9 (Q9SEZ9) At2g40160/T7M7.25 (At2g40160 protein), partial (50%)	1.00E-58
TB1n2R_G5	444	probable cytochrome P450 monooxygenase - maize (fragment)	9.00E-23	TC146396 homologue to gb|AF223066.1|AF223066 Humulus lupulus 26S ribosomal RNA gene, partial sequence; 18S ribosomal RNA	8E-77
TB1n2R_G6	248	putative endomembrane protein 70 [Arabidopsis thaliana]	2.00E-09	TC133738 homologue to UP|O04091 (O04091) Endomembrane protein EMP70 precusor isolog; 68664-64364, partial (63%)	3E-35
TB1n2R_G7	429	calcineurin-like phosphoesterase family-like protein [Oryza sativa (japonica cultivar-group)]	3.00E-57	TC133143 similar to UP|Q8H5T7 (Q8H5T7) Calcineurin-like phosphoesterase family-like protein, partial (95%)	5E-86
TB1n2R_G8	336	B1045D11.16 [Oryza sativa (japonica cultivar-group)]	0.089	TC150993	2E-58
TB1n2R_G9	298	putative cationic amino acid transporter [Oryza sativa (japonica cultivar-group)]	2.00E-27	TC130848 similar to UP|Q9ASS7 (Q9ASS7) AT5g36940/MLF18_60, partial (80%)	8.00E-53
TB1n2R_H10	197	no hits found	-	no hits found	-
TB1n2R_H11	319	no hits found	-	TC148179 similar to UP|PM14_ARATH (Q9FMP4) Pre-mRNA branch site p14-like protein, partial (92%)	9.00E-21
TB1n2R_H12	308	At3g09630 [Arabidopsis thaliana]	9.00E-05	AV919133 similar to SP|Q9XF97|RL4_ 60S ribosomal protein L4 (L1). [Apricot] {Prunus armeniaca}, partial (30%)	3.00E-17
TB1n2R_H2	293	putative leukotriene A-4 hydrolase [Oryza sativa (japonica cultivar-group)]	4.00E-17	TC141288 similar to GB|CAC05429.1|9955544|ATT6I14 leukotriene-A4 hydrolase-like protein {Arabidopsis thaliana;} , partial	2.00E-34
TB1n2R_H3	318	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-27	TC145175 weakly similar to UP|Q9FGT4 (Q9FGT4) Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K21L19, partial	1.00E-61
TB1n2R_H4	231	nuclear protein-like [Arabidopsis thaliana]	4.00E-07	TC151191 similar to UP|Q9FMF9 (Q9FMF9) Nuclear protein-like, partial (9%)	1.00E-16

TB1n2R_H5	389	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-46	TC140069	2.00E-67
TB1n2R_H6	141	no hits found	-	no hits found	-
TB1n2R_H7	310	putative senescence-associated protein [Pisum sativum]	2.00E-33	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	3.00E-60
TB1n2R_H8	446	methionine synthase 2 enzyme [Hordeum vulgare subsp. vulgare]	3.00E-20	TC131380 homologue to UP|Q8W0Q7 (Q8W0Q7) Methionine synthase protein, complete	1E-49
TB1n2R_H9	287	putative dihydrolipoamide dehydrogenase [Oryza sativa (japonica cultivar-group)]	1.00E-10	TC147317 similar to UP|Q8GT30 (Q8GT30) Dihydrolipoamide dehydrogenase , partial (95%)	9E-27
Tn1_2A1	235	sucrose:sucrose 1-fructosyltransferase [Triticum aestivum]	1.00E-08	TC147336 homologue to UP|Q8W430 (Q8W430) Sucrose:sucrose 1-fructosytransferase , complete	2.00E-80
Tn1_2A10	317	r-protein BnS15a [Brassica napus]	9.00E-36	TC131038 homologue to UP|RS1A_ARATH (P42798) 40S ribosomal protein S15a, complete	e-135
Tn1_2A11	170	putative 60S ribosomal protein L31 [Oryza sativa (japonica cultivar-group)]	5.00E-10	TC130806 similar to UP|RL31_NICGU (P46290) 60S ribosomal protein L31, partial (94%)	4.00E-62
Tn1_2A12	437	GONST3 Golgi Nucleotide sugar transporter [Arabidopsis thaliana]	2.00E-24	TC133055 weakly similar to UP|Q84L08 (Q84L08) GONST4 Golgi Nucleotide sugar transporter (Fragment), partial (65%)	0
Tn1_2A2	251	Unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-23	TC147919 similar to UP|Q9I6T2 (Q9I6T2) Probable ATP-binding component of ABC transporter, partial (5%)	e-112
Tn1_2A3	240	no hits found	-	BU983597 SP|Q05761|RS13 40S ribosomal protein S13. [Maize] {Zea mays}, partial (41%)	e-109
Tn1_2A4	183	OSJNBa0027G07.7 [Oryza sativa (japonica cultivar-group)] sugar transporter	9.00E-18	TC153042 weakly similar to UP|Q9FRT5 (Q9FRT5) Monosaccharide transporter 3, partial (38%)	8.00E-08
Tn1_2A5	311	putative senescence-associated protein [Pisum sativum]	8.00E-42	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-157
Tn1_2A6	144	no hits found	-	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	4.00E-43
Tn1_2A7	230	conserved hypothetical protein [Frankia sp. EAN1pec]	0.01	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	2.00E-95
Tn1_2A8	317	putative senescence-associated protein [Pisum sativum]	0.008	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	2.00E-56
Tn1_2A9	288	unnamed protein product [Oryza sativa (japonica cultivar-group)]	5.00E-28	TC138293 homologue to UP|Q9LI45 (Q9LI45) ESTs D39300(R3292), partial (56%)	e-137
Tn1_2B1	407	APC5 [Arabidopsis thaliana]-function unknown	2.00E-29	TC150057 weakly similar to UP|Q9SHK3 (Q9SHK3) F12K11.7, partial (44%)	0
Tn1_2B10	179	no hits found	-	TC138990 homologue to PRF|1604470A.0|226763|1604470A poly-ubiquitin. {Zea mays;} , partial (78%)	3.00E-75
Tn1_2B11	261	no hits found	-	TC139135 similar to UP|ASSY_ARATH (Q9SZX3) Argininosuccinate synthase, chloroplast precursor (Citrulline--aspartate	e-104
Tn1_2B12	130	no hits found	-	TC146445 homologue to dbj|AB026819.1| Magnaporthe grisea genes for 18S rRNA, 5.8S rRNA, 26S rRNA, complete sequence, rDNA	2.00E-17
Tn1_2B2	413	ribosomal protein S8 [Hordeum vulgare subsp. vulgare]	3.00E-52	TC131434 homologue to UP|RS8_ORYSA (P49199) 40S ribosomal protein S8, partial (95%)	0
Tn1_2B3	321	TMV-MP30 binding protein 2C-like [Oryza sativa (japonica cultivar-group)]	4.00E-33	AJ461195	3.00E-27
Tn1_2B4	381	GPX12Hv, glutathione peroxidase-like protein [Hordeum vulgare subsp. vulgare]	1.00E-41	TC131780 homologue to UP|Q9SME6 (Q9SME6) GPX12Hv, glutathione peroxidase-like protein, complete	e-179
Tn1_2B5	346	no hits found	-	AL503159	e-145
Tn1_2B6	396	putative 60S ribosomal protein L5 [Oryza sativa (japonica cultivar-group)]	1.00E-37	TC131090 similar to UP|RL5_ORYSA (P49625) 60S ribosomal protein L5, partial (97%)	e-174
Tn1_2B7	362	S-adenosylmethionine decarboxylase precursor [Triticum aestivum]	3.00E-10	TC130707 homologue to UP|Q9ZPJ1 (Q9ZPJ1) S-adenosylmethionine decarboxylase, complete	e-150
Tn1_2B8	299	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-08	TC132508 similar to UP|Q9LMB4 (Q9LMB4) F14D16.29, partial (69%)	e-127
Tn1_2B9	281	hypothetical protein [Plasmodium yoelii yoelii]	2.00E-09	TC146455 homologue to gb|AF036494.1|AF036494 Eucryphia lucida large subunit 26S ribosomal RNA gene, partial sequence, partial	7.00E-37
Tn1_2C1	450	unnamed protein product [Kluyveromyces lactis NRRL Y-1140]	3.00E-22	TC130828 homologue to emb|X00755.1|OSRRN17S Rice gene for 17S ribosomal RNA, complete	0
Tn1_2C10	321	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-34	TC132867	e-159
Tn1_2C11	231	putative bromelain-like thiol protaease [Oryza sativa (japonica cultivar-group)]	4.00E-04	BQ763183	6.00E-06
Tn1_2C12	196	P0492F05.25 [Oryza sativa (japonica cultivar-group)]-Hypothetical protein	1.00E-05	TC131020 similar to UP|Q7QHQ0 (Q7QHQ0) AgCP7099 (Fragment), partial (5%)	5.00E-65

Tn1_2C2	280	putative senescence-associated protein [Pisum sativum]	3.00E-27	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	e-134
Tn1_2C3	300	P0491F11.10(hypothetical protein) [Oryza sativa (japonica cultivar-group)]Hypothetical protein	2.00E-21	CB869717 similar to GP|15408716|db P0039A07.31 {Oryza sativa (japonica cultivar-group)}, partial (33%)	2.00E-09
Tn1_2C4	379	ATP/ADP carrier protein [Triticum turgidum]	2.00E-57	TC146246 homologue to UP|ADT_ORYSA (P31691) ADP,ATP carrier protein, mitochondrial precursor (ADP/ATP translocase) (Adenine	0
Tn1_2C5	416	GRP94 homologue [Hordeum vulgare]	2.00E-24	TC146764 UP|ENPL_HORVU (P36183) Endoplasmin homolog precursor (GRP94 homolog), complete	e-105
Tn1_2C6	204	p34cdc2 (ATP binding-prot. kinase)[Triticum aestivum]	8.00E-13	BM377914 homologue to GP|4096105|gb| p34cdc2 {Triticum aestivum}, partial (36%)	2.00E-82
Tn1_2C7	163	no hits found	-	TC141442 similar to UP|Q8LJT8 (Q8LJT8) Myb/SANT domain protein, partial (4%)	1.00E-55
Tn1_2C8	207	hypothetical protein CE2794 [Corynebacterium efficiens YS-314]	8.00E-05	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	6.00E-77
Tn1_2C9	207	putative senescence-associated protein [Pisum sativum]	2.00E-16	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	9.00E-82
Tn1_2D1	316	predicted mRNA(O. sativa)	1.00E-07	TC130889 GB|AAP47481.1|32331907|AY164907 RTNLB32 {Hordeum vulgare;} , complete	e-151
Tn1_2D10	288	OSJNBa0070C17.23 [Oryza sativa (japonica cultivar-group)];putative potasium transporter	3.00E-30	TC149191 similar to UP|PT11_ARATH (O64769) Potassium transporter 11 (AtPOT11), partial (56%)	e-130
Tn1_2D11	255	putative cytochrome [Oryza sativa (japonica cultivar-group)]	4.00E-25	TC132033 similar to UP|Q8H293 (Q8H293) Cytochrome b5, partial (96%)	e-102
Tn1_2D12	262	ribosomal protein s6 RPS6-2 [Zea mays]	2.00E-10	TC131464 homologue to UP|Q9FYS0 (Q9FYS0) Ribosomal protein s6 RPS6-2, partial (95%)	e-108
Tn1_2D2	400	putative AdoMet synthase 2 [Hordeum vulgare subsp. vulgare]	2.00E-18	TC131481 homologue to UP|METK_HORVU (P50299) S-adenosylmethionine synthetase 1 (Methionine adenosyltransferase 1) (AdoMet	1.00E-85
Tn1_2D3	174	putative TGF(transfoming growth factor) beta inducible nuclear protein TINP1 [Oryza sativa (japonica cultivar-group)]	8.00E-13	TC131771 weakly similar to GB|AAC03224.1|603365|SCE9781 Yer126cp {Saccharomyces cerevisiae;} , partial (93%)	1.00E-68
Tn1_2D4	419	OSJNBa0042N22.7 [Oryza sativa (japonica cultivar-group)] ATP binding	1.00E-21	no hits found	-
Tn1_2D5	181	no hits found	-	CB863220	2.00E-17
Tn1_2D6	473	cullin 1B-like [Oryza sativa (japonica cultivar-group)]	3.00E-25	TC142370	0
Tn1_2D7	227	conserved hypothetical protein [Frankia sp. EAN1pec]	0.01	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	4.00E-87
Tn1_2D8	517	S-adenosylmethionine decarboxylase precursor [Triticum aestivum]	5.00E-08	TC130707 homologue to UP|Q9ZPJ1 (Q9ZPJ1) S-adenosylmethionine decarboxylase, complete	3.00E-96
Tn1_2D9	184	no hits found	-	TC138340 homologue to UP|Q96Q97 (Q96Q97) Bradeion alpha, partial (7%)	3.00E-72
Tn1_2E1	217	hypothetical protein YfreA_01004244 [Yersinia	2.00E-05	TC146297 homologue to emb|Z00028.1|CHZMRRNA Zea mays chloroplast rRNA-operon, partial (5%)	2.00E-12
Tn1_2E10	348	OSJNBa0045O17.11 [Oryza sativa (japonica cultivar-group)]/actin	3.00E-16	BM443521	e-104
Tn1_2E11	359	actin [Elaeis oleifera]	2.00E-47	TC131420 homologue to UP|Q75LK6 (Q75LK6) Actin, complete	e-164
Tn1_2E12	108	putative senescence-associated protein [Pisum sativum]	2.00E-25	AJ434911	1.00E-26
Tn1_2E2	221	asparagine synthetase [Securigera parviflora]	6.00E-09	TC147318 similar to UP|ASNS_MAIZE (P49094) Asparagine synthetase [glutamine-hydrolyzing] (Glutamine-dependent asparagine	3.00E-91
Tn1_2E3	167	At1g72710 [Arabidopsis thaliana]	2.00E-07	TC139857 similar to UP|KC1D_HUMAN (P48730) Casein kinase I, delta isoform (CKI-delta) (CKId) , partial (67%)	1.00E-58
Tn1_2E4	222	putative tetrafunctional protein of glyoxysomal fatty acid beta-oxidation [Oryza sativa (japonica cultivar-group)]	4.00E-16	TC139491 similar to UP|MFPA_BRANA (O49809) Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a [Includes:	5.00E-68
Tn1_2E5	553	S-adenosylmethionine decarboxylase [Oryza sativa (japonica cultivar-group)]	1.00E-25	TC142944 similar to UP|Q9FSM2 (Q9FSM2) S-adenosylmethionine decarboxylase, partial (57%)	0
Tn1_2E6	179	no hits found	-	TC138861 homologue to UP|RL30_MAIZE (O48558) 60S ribosomal protein L30, partial (95%)	3.00E-69
Tn1_2E7	267	no hits found	-	TC148346 similar to UP|Q6YWR7 (Q6YWR7) ACT domain-containing protein-like, partial (32%)	e-120
Tn1_2E8	264	putative senescence-associated protein [Pisum sativum]	4.00E-28	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-114
Tn1_2E9	466	40S ribosomal protein S23 [Oryza sativa (japonica cultivar-group)]	1.00E-46	TC131490 UP|Q8L4F2 (Q8L4F2) 40S ribosomal protein S23, complete	0

Tn1_2F1	378	putative senescence-associated protein [Pisum s	1.00E-26	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-102
Tn1_2F10	160	no hits found	-	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	3.00E-04
Tn1_2F11	179	no hits found	-	TC131228 homologue to gb|AF168852.1|AF168852 Hordeum jubatum 18S small subunit ribosomal RNA gene, complete sequence	1.00E-62
Tn1_2F12	131	OSJNBa0073L04.5 [Oryza sativa (japonica cultivar-group)] AB_hydrolase_1.	1.00E-13	TC146472 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (41%)	1.00E-24
Tn1_2F2	354	elongation factor 1 alpha; EF-1a [Naegleria andersoni]	6.00E-37	TC149773 homologue to UP|EF1A_AURPU (Q00251) Elongation factor 1-alpha (EF-1-alpha), partial (83%)	0.01
Tn1_2F3	183	no hits found	-	TC145217 similar to UP|Q84VB4 (Q84VB4) WD repeat protein (Fragment), partial (50%)	1.00E-59
Tn1_2F4	244	beta tubulin 2 [Anemia phyllitidis]	4.00E-13	NP315693 beta tubulin 3 [Hordeum vulgare]	6.00E-62
Tn1_2F5	279	no hits found	-	TC150832	1.00E-63
Tn1_2F6	486	no hits found	-	no hits found	-
Tn1_2F7	192	40S ribosomal protein S30-like [Oryza sativa (japonica cultivar-group)]	6.00E-05	TC131072 homologue to UP|Q6K853 (Q6K853) 40S ribosomal protein S30-like, partial (98%)	3.00E-60
Tn1_2F8	314	putative senescence-associated protein [Pisum sativum]	4.00E-40	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-144
Tn1_2F9	362	hypothetical protein [Oryza sativa (japonica cultivar-group)]	4.00E-24	TC146455 homologue to gb|AF036494.1|AF036494 Eucryphia lucida large subunit 26S ribosomal RNA gene, partial sequence,	0
Tn1_2G1	166	OSJNBa0073L04.5 [Oryza sativa hydrolase act	3.00E-15	TC133605	2.00E-70
Tn1_2G10	237	OSJNBa0035O13.14 [Oryza sativa (japonica cultivar-group)];MRP-like ABC transporter	1.00E-17	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	4.00E-04
Tn1_2G11	192	DHHC-type zinc finger domain-containing protein -like [Oryza sativa (japonica cultivar-group)]	3.00E-15	TC151063 similar to UP|Q6ZJ21 (Q6ZJ21) DHHC-type zinc finger domain-containing protein-like, partial (18%)	2.00E-82
Tn1_2G12	220	no hits found	-	BQ757111	4.00E-35
Tn1_2G2	152	tonoplast intrinsic protein [Oryza sativa (japonica cultivar-group)]	3.00E-12	TC130898 homologue to UP|Q7XA61 (Q7XA61) Tonoplast intrinsic protein, complete	1.00E-52
Tn1_2G3	127	hypothetical protein Bd1752.1 [Bdellovibrio bacteriovorus HD100]	0.11	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	1.00E-27
Tn1_2G4	225	hypothetical protein [Oryza sativa (japonica cultivar-group)]	0.96	TC132430 similar to UP|Q99MK4 (Q99MK4) AF9 (Fragment), partial (4%)	2.00E-95
Tn1_2G5	142	no hits found	-	BM376094	2.00E-05
Tn1_2G6	299	sucrose-phosphatase [Hordeum vulgare subsp. vulgare]	6.00E-38	TC139444 UP|Q84ZX7 (Q84ZX7) Sucrose-phosphatase , complete	e-136
Tn1_2G7	176	putative Vacuolar ATP synthase subunit d [Oryza sativa (japonica cultivar-group)]	8.00E-19	TC131672 similar to UP|V0D2_ARATH (Q9LHA4) Probable vacuolar ATP synthase subunit d 2 (V-ATPase d subunit 2) (Vacuolar	2.00E-73
Tn1_2G8	226	conserved hypothetical protein [Frankia sp. EAN1pec]	0.051	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	1.00E-87
Tn1_2G9	123	no hits found	-	no hits found	-
Tn1_2H10	217	conserved hypothetical protein [Frankia sp. EAN1pec]	0.067	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	3.00E-85
Tn1_2H11	172	At1g72710(Putative casein kinase I) [Arabidopsis thaliana]	6.00E-06	TC139857 similar to UP|KC1D_HUMAN (P48730) Casein kinase I, delta isoform (CKI-delta) (CKId) , partial (67%)	5.00E-46
Tn1_2H12	338	ribosomal protein L2 [Lycopersicon esculentum]	3.00E-13	BU978953 homologue to SP|P29766|RL2_ 60S ribosomal protein L2 (L8) (Ribosomal protein TL2). [Tomato] {Lycopersicon	1.00E-54
Tn1_2H2	277	no hits found	-	TC148812 similar to UP|Q93ZD7 (Q93ZD7) AT3g58970/F17J16_20 (MRS2-4), partial (68%)	e-115
Tn1_2H3	180	CCAAT-binding transcription factor subunit A (CBF-A) (NF-Y protein chain B) (NF-YB) (CAAT-box DNA binding protein subunit	0.001	TC134420 similar to UP|Q84NF0 (Q84NF0) HAP3 (Fragment), partial (92%)	6.00E-55
Tn1_2H4	569	major outer membrane lipoprotein, putative [Shewanella amazonensis SB2B]	0.53	no hits found	-
Tn1_2H5	546	putative leucine rich repeat protein [Oryza sativa (japonica cultivar-group)]	1.00E-55	TC144277 similar to PIR|T51336|T51336 auxin-induced protein AIR9 [imported] - Arabidopsis thaliana (fragment)	0
Tn1_2H6	195	no hits found	-	TC130940 similar to UP|Q9LRH5 (Q9LRH5) Mitochondrial ribosomal protein S10, partial (84%)	9.00E-85
Tn1_2H7	398	SUSIBA2 [Hordeum vulgare]	3.00E-63	TC132606 UP|Q6VWJ6 (Q6VWJ6) SUSIBA2, complete	0
Tn1_2H8	324	putative glycosyltransferase [Oryza sativa (japonica cultivar-group)]	2.00E-37	CA017915	1.00E-08
Tn1_2H9	153	no hits found	-	TC147813	1.00E-49

Appendix B. Table 2. NCBI BLASTx and TIGR BLASTn annotations of clones sequenced from non-treated library

Clone name	Length (bp)	NCBI BLASTx (nr)	E- value	TIGR BLASTn (HVGI)	E- value
NMAP1G1	206	beta-D-galactosidase [Pyrus pyrifolia]	6.00E-20	TC148880 weakly similar to GB|AAF70821.1|7939617|AF154420 beta-galactosidase {Lycopersicon esculentum;} , partial	3.00E-72
NMAP1G2	355	unknown protein [Oryza sativa (japonica cultivar-group)]	4.00E-21	TC150488 similar to GB|AAP37779.1|30725514|BT008420 At2g40980 {Arabidopsis thaliana;} , partial (24%)	2.00E-87
NMAP1G3	245	ESTs AU031435(E61570),AU078245(E61570) correspond to a region of the predicted gene.~hypothetical protein [Oryza sativa	2.00E-07	TC133189 similar to UP|Q9SNJ3 (Q9SNJ3) ESTs AU031435(E61570), partial (42%)	e-101
NMAP1G4	720	putative PIR7A protein [Oryza sativa (japonica cultivar-group)]	1.00E-99	TC148724 similar to GB|AAL11601.1|15983466|AF424607 At1g33990/F12G12_220 {Arabidopsis thaliana;} , partial	0
NMAP1G5	313	putative senescence-associated protein [Pisum sativum]	1.00E-42	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-153
NMAP1G6	371	beta-tubulin 1 [Hordeum vulgare subsp. vulgare]	2.00E-58	TC131561 UP|TBB_HORVU (P93176) Tubulin beta chain (Beta tubulin), complete	0
NMAP1G7	425	Putative endosomal protein [Oryza sativa (japonica cultivar-group)]	4.00E-65	TC131860 weakly similar to UP|T9S1_HUMAN (O15321) Transmembrane 9 superfamily protein member 1 precursor (hMP70), partial	0
NMAP1G8	347	band 3 anion transport protein -like [Oryza sativa (japonica cultivar-group)]	2.00E-14	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	e-162
NMAP1G9	244	S-type apyrase [Oryza sativa (japonica cultivar-group)]	3.00E-20	TC146987 weakly similar to UP|Q84UD8 (Q84UD8) Apyrase-like protein, partial (51%)	e-111
NMAP1G10	291	putative F-box protein [Triticum aestivum]	2.00E-43	TC141398 weakly similar to UP|Q9FZK1 (Q9FZK1) F17L21.13, partial (50%)	e-136
NMAP1G11	224	no hits found	-	BQ468413	5.00E-90
NMAP1G12	316	putative senescence-associated protein [Pisum sativum]	2.00E-40	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-146
NMAP1H2	351	putative proteinase inhibitor [Hordeum vulgare subsp. vulgare]	1.00E-32	BM816647 similar to GP|4127862|emb| glyoxalase I {Glycine max}, partial (52%)	e-134
NMAP1H3	343	At1g05520/T25N20_16 [Oryza sativa (japonica cultivar-group)]	3.00E-38	TC147293 similar to UP|Q9ZVY6 (Q9ZVY6) T25N20.17, partial (26%)	e-173
NMAP1H4	599	band 3 anion transport protein -like [Oryza sativa (japonica cultivar-group)]	3.00E-66	BJ452857 similar to GP|5103843|gb| Is a member of the PF|00955 Anion exchanger family. {Arabidopsis thaliana}, partial	6.00E-98
NMAP1H5	470	no hits found	-	TC152303 weakly similar to UP|Q6PVH9 (Q6PVH9) P54, partial (3%)	0
NMAP1H6	450	no hits found	-	TC133752 weakly similar to UP|O65450 (O65450) Glycine-rich protein, partial (19%)	0
NMAP1H7	297	putative AKT1-like potassium channel [Hordeum vulgare]	3.00E-36	TC151596 homologue to UP|Q9M671 (Q9M671) AKT1-like potassium channel, partial (52%)	e-143
NMAP1H8	350	band 3 anion transport protein -like [Oryza sativa (japonica cultivar-group)]	5.00E-18	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	e-174
NMAP1H9	292	conserved hypothetical protein [Frankia sp. EAN1pec]	0.011	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-100
NMAP1H10	296	PREDICTED P0516G10.26 gene product [Oryza sativa (japonica cultivar-group)]	6.00E-44	TC135973 weakly similar to UP|Q8BYV2 (Q8BYV2) Mus musculus 16 days neonate thymus cDNA, RIKEN full-length enriched library,	e-132
NMAP1H11	654	no hits found	-	AJ466164	e-170
NMAP1H12	379	phenylalanine ammonia-lyase [Hordeum vulgare subsp. vulgare]	7.00E-20	TC130847 homologue to UP|PALY_WHEAT (Q43210) Phenylalanine ammonia-lyase , partial (82%)	0
NMAP2A1	452	putative ribosomal protein L10a [Oryza sativa (japonica cultivar-group)]	5.00E-39	TC131049 homologue to UP|R10A_ARATH (Q8VZB9) 60S ribosomal protein L10a-1, partial (97%)	e-178
NMAP2A2	285	OSJNBa0027H09.17 [Oryza sativa (japonica cultivar-group)]	5.00E-34	TC146779 weakly similar to UP|S24B_HUMAN (O95487) Protein transport protein Sec24B (SEC24-related protein B), partial (15%)	e-131
NMAP2A3	226	conserved hypothetical protein [Frankia sp. EAN1pec]	0.011	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-102
NMAP2A4	197	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	9.00E-82
NMAP2A5	324	putative adaptor-related protein complex AP-3, beta 2 subunit [Oryza sativa (japonica cultivar-group)]	1.00E-18	CD058393	e-148
NMAP2A6	175	no hits found	-	TC147922 similar to UP|Q8S3R7 (Q8S3R7) Ethylene-responsive protein-like, partial (55%)	1.00E-31
NMAP2A7	195	oligopeptidase A-like [Oryza sativa (japonica cultivar-group)]	0.053	TC139653 similar to UP|Q6K9T1 (Q6K9T1) Oligopeptidase A-like, partial (90%)	1.00E-22

NMAP2A8	370	unknown protein [Oryza sativa (japonica cultivar-group)]	3.00E-45	TC142546	7.00E-81
NMAP2A9	386	OSJNBa0063C18.17 [Oryza sativa (japonica cultivar-group)]	5.00E-22	TC147882 similar to UP|VATD_ARATH (Q9XGM1) Vacuolar ATP synthase subunit D (V-ATPase D subunit) (Vacuolar proton pump D	0
NMAP2A10	333	glutathione S-transferase [Triticum aestivum]	4.00E-20	TC146838 UP|Q8LPD5 (Q8LPD5) Glutathione-S-transferase, I subunit, complete	e-158
NMAP2A11	156	no hits found	-	TC147515 homologue to UP|Q8S7U0 (Q8S7U0) Serine/threonine protein phosphatase PP2A-4 catalytic subunit, partial (91%)	3.00E-56
NMAP2A12	118	no hits found	-	AJ435560	1.00E-20
NMAP2B1	157	putative transmembrane protein [Oryza sativa (japonica cultivar-group)]	1.00E-14	TC135126 similar to UP|Q9FYQ8 (Q9FYQ8) Endosomal protein-like, partial (15%)	1.00E-58
NMAP2B2	625	transcription factor MADS23 [Oryza sativa (japonica cultivar-group)]	5.00E-19	AJ461395	5.00E-83
NMAP2B3	528	unknown protein [Oryza sativa (japonica cultivar-group)]	3.00E-11	TC141653 weakly similar to PIR|E86186|E86186 YUP8H12.18 [imported] - Arabidopsis thaliana {Arabidopsis thaliana;} , partial	e-139
NMAP2B4	644	proline-rich protein [Triticum aestivum]	0.36	TC142529 similar to UP|Q01979 (Q01979) Proline-rich protein, partial (33%)	1.00E-49
NMAP2B5	117	putative senescence-associated protein [Pisum sativum]	0.001	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	3.00E-34
NMAP2B6	110	no hits found	-	AJ435560	8.00E-16
NMAP2B7	622	ankyrin-like protein [Oryza sativa (japonica cultivar-group)]	8.00E-37	TC131507 homologue to UP|RK16_WHEAT (Q95H50) Chloroplast 50S ribosomal protein L16, partial (98%)	e-137
NMAP2B8	313	putative ribosomal protein [Oryza sativa (japonica cultivar-group)]	2.00E-45	TC146673 similar to UP|Q9FJA6 (Q9FJA6) 40S ribosomal protein S3 (AT5g35530/MOK9_14), partial (62%)	e-155
NMAP2B9	102	no hits found	-	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	6.00E-04
NMAP2B10	99	no hits found	-	TC133185 similar to UP|Q8VZJ3 (Q8VZJ3) AT3g02910/F13E7_14, partial (39%)	5.00E-20
NMAP2B11	323	putative 26S proteasome regulatory particle triple-A ATPase subunit4 [Oryza sativa (japonica cultivar-group)]	4.00E-35	TC147414 homologue to UP|Q9FXT8 (Q9FXT8) 26S proteasome regulatory particle triple-A ATPase subunit4, complete	e-152
NMAP2B12	273	putative succinate dehydrogenase flavoprotein alpha subunit [Oryza sativa (japonica cultivar-group)]	7.00E-23	TC131740 homologue to UP|DHSA_ARATH (O82663) Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (FP)	e-130
NMAP2C1	604	alpha-tubulin [Miscanthus floridulus]	1.00E-65	TC139660 UP|TBA1_HORVU (Q43473) Tubulin alpha-1 chain, complete	0
NMAP2C2	196	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	2.00E-76
NMAP2C3	160	no hits found	-	TC148416 homologue to GB|AAM70547.1|21700847|AY124838 AT3g07590/MLP3_4 {Arabidopsis thaliana;} , complete	4.00E-65
NMAP2C4	248	no hits found	-	TC140470 similar to UP|Q84UY9 (Q84UY9) BHLH transcription factor PTF1, partial (75%)	e-103
NMAP2C5	295	OSJNBa0032F06.20 [Oryza sativa (japonica cultivar-group)]	3.00E-35	TC140411 similar to UP|Q9LQ76 (Q9LQ76) T1N6.21 protein, partial (65%)	e-132
NMAP2C6	771	unknown protein [Arabidopsis thaliana]	4.00E-34	TC140411 similar to UP|Q9LQ76 (Q9LQ76) T1N6.21 protein, partial (65%)	4.00E-04
NMAP2C7	421	unnamed protein product [Oryza sativa (japonica cultivar-group)]	2.00E-13	TC133965	0
NMAP2C8	218	no hits found	-	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	7.00E-21
NMAP2C9	354	putative callose synthase [Hordeum vulgare subsp. vulgare]	4.00E-54	TC148129 homologue to UP|Q6WGM8 (Q6WGM8) Beta 1,3 glucan synthase, partial (55%)	e-180
NMAP2C10	233	ribosomal protein L28-like [Oryza sativa (japonica cultivar-group)]	0.004	TC130723 similar to UP|Q8S0P2 (Q8S0P2) Ribosomal protein L28-like, complete	e-102
NMAP2C11	471	AT5g52200/F17P19_10 [Arabidopsis thaliana]	8.00E-04	TC140755 similar to UP|Q9LTK0 (Q9LTK0) Arabidopsis thaliana genomic DNA, chromosome 5, BAC clone:F17P19	0
NMAP2C12	472	OSJNBa0073E02.14 [Oryza sativa (japonica cultivar-group)]	1.00E-32	TC138810 phenylalanine ammonia-lyase [Hordeum vulgare]	e-112
NMAP2D1	246	putative floral activator, relative of early flowering 6 [Oryza sativa (japonica cultivar-group)]	3.00E-13	TC146657 weakly similar to UP|Q9Y2P0 (Q9Y2P0) BC37295_3, partial (3%)	8.00E-86
NMAP2D2	163	no hits found	-	TC132079 similar to GB|AAG53613.1|12407658|AF285832 eukaryotic initiation factor 3E subunit {Arabidopsis thaliana;}	9.00E-60
NMAP2D3	163	Porin [Pisum sativum]	4.00E-09	TC136480 weakly similar to UP|Q6W2J1 (Q6W2J1) VDAC3.1, partial (61%)	1.00E-71

NMAP2D4	276	no hits found	-	TC150765 homologue to UP|Q9M7I2 (Q9M7I2) WD-repeat cell cycle regulatory protein, partial (65%)	e-103
NMAP2D5	303	no hits found	-	no hits found	-
NMAP2D6	187	no hits found	-	no hits found	-
NMAP2D7	254	putative Xaa-Pro aminopeptidase 2 [Oryza sativa (japonica cultivar-group)]	8.00E-27	TC146990 similar to UP|Q93X45 (Q93X45) Xaa-Pro aminopeptidase 2 , partial (91%)	e-103
NMAP2D8	223	putative plicing factor 3B subunit 2 [Oryza sativa (japonica cultivar-group)]	5.00E-08	TC141102 similar to UP|Q8H0V8 (Q8H0V8) Spliceosome associated protein-like (At4g21660), partial (48%)	4.00E-81
NMAP2D9	848	TPA: reverse transcriptase/ribonuclease H [Coprinopsis cinerea]	0.16	TC147059 UP|O24001 (O24001) 14-3-3 protein, complete	e-126
NMAP2D10	303	hypothetical protein [Sporobolus stapfianus]	1.00E-04	TC131211 homologue to UP|Q9ZWJ2 (Q9ZWJ2) Glyoxalase I, complete	e-121
NMAP2D11	113	putative senescence-associated protein [Pisum sativum]	3.00E-07	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	2.00E-41
NMAP2D12	115	putative senescence-associated protein [Pisum sativum]	2.00E-06	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	3.00E-40
NMAP2E1	400	P0692C11.17 [Oryza sativa (japonica cultivar-group)]	2.00E-22	TC150837 similar to UP|Q9SP08 (Q9SP08) Exonuclease RRP41, partial (10%)	3.00E-18
NMAP2E2	369	putative transmembrane protein [Oryza sativa (japonica cultivar-group)]	2.00E-48	TC140847 similar to UP|Q6K5X9 (Q6K5X9) Transmembrane protein-like, partial (94%)	0
NMAP2E3	510	unnamed protein product [Oryza sativa (japonica cultivar-group)]	5.00E-21	TC139339 homologue to UP|Q9LWN0 (Q9LWN0) ESTs AU032448(S10057), partial (95%)	5.00E-73
NMAP2E4	212	mitochondrial ribosomal protein S10 [Oryza sativa (japonica cultivar-group)]	2.00E-11	TC130940 similar to UP|Q9LRH5 (Q9LRH5) Mitochondrial ribosomal protein S10, partial (84%)	1.00E-62
NMAP2E5	275	predicted protein [Aspergillus nidulans FGSC A4]	0.99	TC147031 weakly similar to UP|O82767 (O82767) PRT1 protein, partial (43%)	e-131
NMAP2E6	305	JIOsPR10 [Oryza sativa]	3.00E-12	TC139972 similar to UP|Q945E9 (Q945E9) Pathogen-related protein (JIOsPR10), complete	e-144
NMAP2E7	258	putative DNA-binding protein GBP16 [Oryza sativa (japonica cultivar-group)]	3.00E-28	TC146911 similar to UP|O22523 (O22523) DNA-binding protein GBP16, complete	e-110
NMAP2E8	243	band 3 anion transport protein -like [Oryza sativa (japonica cultivar-group)]	1.00E-16	TC149698 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (22%)	1.00E-41
NMAP2E9	216	hypothetical protein [Plasmodium yoelii yoelii]	0.34	AV837046	2.00E-73
NMAP2E10	209	no hits found	-	TC131993 similar to GB|AAD03446.1|4115936|T4B21 coded for by A. thaliana cDNA R89964 {Arabidopsis thaliana;} , partial	3.00E-69
NMAP2E11	532	katanin p80 (WD40-containing) subunit B 1-like protein [Oryza sativa (japonica cultivar-group)]	5.00E-43	BJ464057 homologue to GP|15289878|dbj P0010B10.21 {Oryza sativa (japonica cultivar-group)}, partial (2%)	e-128
NMAP2E12	477	OSJNBb0026L04.10 [Oryza sativa (japonica cultivar-group)]	5.00E-62	TC135190	6.00E-42
NMAP2F1	317	no hits found	-	TC140351 homologue to UP|Q6K3S8 (Q6K3S8) Prefoldin-like, partial (82%)	6.00E-47
NMAP2F2	249	putative Xaa-Pro aminopeptidase 2 [Oryza sativa (japonica cultivar-group)]	1.00E-20	TC146990 similar to UP|Q93X45 (Q93X45) Xaa-Pro aminopeptidase 2 , partial (91%)	2.00E-77
NMAP2F3	205	no hits found	-	CB880538	1.00E-56
NMAP2F4	144	putative peroxidase [Oryza sativa (japonica cultivar-group)]	7.00E-10	BI779578	2.00E-42
NMAP2F5	151	putative heat shock protein Hsp70 [Oryza sativa (japonica cultivar-group)]	2.00E-13	TC146888 similar to GB|AAO11541.1|27363244|BT002625 At1g79930/F19K16_11 {Arabidopsis thaliana;} , partial	5.00E-58
NMAP2F6	117	putative senescence-associated protein [Pisum sativum]	0.001	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	3.00E-34
NMAP2F7	155	no hits found	-	TC149247 homologue to GB|BAC65973.1|29027857|AP005448 ubiquitin-related modifier-1 {Oryza sativa (japonica	2.00E-51
NMAP2F8	161	no hits found	-	TC148011 similar to UP|Q9FLQ4 (Q9FLQ4) 2-oxoglutarate dehydrogenase E2 subunit, partial (73%)	3.00E-16
NMAP2F9	289	Lipoxygenase 1	1.00E-31	TC146955 UP|LOX1_HORVU (P29114) Lipoxygenase 1 , complete	e-121
NMAP2F10	163	unknown protein,3'-partial [Oryza sativa (japonica cultivar-group)]	4.00E-10	BU991227	3.00E-44
NMAP2F11	149	hypothetical protein Tfu_1628 [Thermobifida fusca YX]	0.054	TC143886	8.00E-57
NMAP2F12	536	unknown protein [Oryza sativa (japonica cultivar-group)]	9.00E-28	TC132794 similar to GB|AAQ56809.1|34098853|BT010366 At4g12700 {Arabidopsis thaliana;} , partial (58%)	0
NMAP2G1	187	putative AdoMet synthase 3 [Hordeum vulgare subsp. vulgare]	1.00E-21	CB863508	1.00E-77
NMAP2G2	161	putative aldose reductase [Oryza sativa (japonica cultivar-group)]	4.00E-11	TC133329 similar to UP|Q84TF0 (Q84TF0) At2g37790, partial (95%)	5.00E-49
NMAP2G3	167	no hits found	-	TC140203 similar to UP|Q940S3 (Q940S3) At1g31070/F17F8_1, partial (91%)	8.00E-51

NMAP2G4	288	putative high mobility group protein [Oryza sativa (japonica cultivar-group)]	2.00E-07	BU991530 similar to GP|18700443|dbj high mobility group box protein 2 {Oryza sativa (japonica cultivar-group)}, partial	e-127
NMAP2G5	244	putative Pollen specific protein C13 precursor [Oryza sativa (japonica cultivar-group)]	6.00E-19	TC139730 similar to GB|AAN31783.1|23396189|AC134516 Putataive pollen specific protein C13 precursor {Oryza sativa}	6.00E-99
NMAP2G6	312	putative senescence-associated protein [Pisum sativum]	2.00E-42	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-138
NMAP2G7	340	putative thionin Osthi1 [Oryza sativa (japonica cultivar-group)]	0.58	TC131935	e-119
NMAP2G8	198	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	2.00E-70
NMAP2G9	213	no hits found	-	no hits found	-
NMAP2G10	343	putative protein kinase [Oryza sativa (japonica cultivar-group)]	2.00E-48	TC142932 similar to UP|Q9FDV7 (Q9FDV7) Protein kinase (PK) (Fragment), partial (45%)	e-165
NMAP2G11	342	Is a member of the PF|00171 aldehyde dehydrogenase family. ESTs gb|T21534, gb|N65241 and gb|AA395614 come from this	6.00E-35	TC132154 similar to GB|AAL16297.1|16226915|AF428367 At1g79440/T8K14_14 {Arabidopsis thaliana;} , partial	e-167
NMAP2G12	350	no hits found	-	TC136394 weakly similar to UP|Q9NJB6 (Q9NJB6) Fibrillarin, partial (12%)	6.00E-75
NMAP2H2	846	putative ethylene responsive factor [Oryza sativa]	1.00E-85	CD055231	0
NMAP2H3	375	putative oligosaccharyl transferase STT3 [Oryza sativa (japonica cultivar-group)]	1.00E-19	TC140322 similar to UP|Q94A42 (Q94A42) AT5g19690/T29J13_110, partial (58%)	0
NMAP2H4	196	200 kDa antigen p200 -like protein [Oryza sativa (japonica cultivar-group)]	2.00E-18	TC139404 similar to UP|Q8RXD0 (Q8RXD0) Auxilin-like protein (At4g12780), partial (56%)	3.00E-69
NMAP2H5	679	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	e-105	TC146536 homologue to UP|Q7FAH2 (Q7FAH2) OJ000223_09.15 protein, complete	0
NMAP2H6	193	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	5.00E-77
NMAP2H7	189	3-phosphoinositide-dependent protein kinase-1-like [Oryza sativa (japonica cultivar-group)]	2.00E-10	TC145943 similar to UP|Q6PJU0 (Q6PJU0) WBSCR21 protein (Fragment), partial (5%)	2.00E-85
NMAP2H8	200	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	9.00E-79
NMAP2H9	256	putative eukaryotic initiation factor subunit [Oryza sativa (japonica cultivar-group)]	8.00E-30	TC146441 similar to UP|IF39_ARATH (Q9C5Z1) Eukaryotic translation initiation factor 3 subunit 9 (eIF-3 eta) (eIF3 p110)	5.00E-84
NMAP2H10	385	BEL1-like homeodomain protein HB2 [Oryza sativa (japonica cultivar-group)]	6.00E-35	TC133619 similar to UP|Q6QUW0 (Q6QUW0) Benzothiadiazole-induced homeodomain protein 1, partial (44%)	0
NMAP2H11	310	putative senescence-associated protein [Pyrus communis]	1.00E-25	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-110
NMAP2H12	282	ly200 protein [Capsicum annuum]	1.00E-08	TC131170 similar to UP|Q6RW44 (Q6RW44) Ly200 protein, complete	e-131
NMAP3A1	161	Porin [Pisum sativum]	9.00E-10	TC136480 weakly similar to UP|Q6W2J1 (Q6W2J1) VDAC3.1, partial (61%)	3.00E-72
NMAP3A10	317	unknown protein [Oryza sativa (japonica cultivar-group)]	5.00E-19	TC136169 similar to UP|Q6NKU3 (Q6NKU3) At1g55340, partial (21%)	7.00E-93
NMAP3A11	444	phosphoenolpyruvate carboxylase [Triticum aestivum]	2.00E-74	TC146895 UP|O82072 (O82072) Phospoenolpyruvate carboxylase, complete	0
NMAP3A12	300	hypothetical protein [Oryza sativa (japonica cultivar-group)]	0.35	TC150450 similar to UP|VNUA_PRVKA (P33485) Probable nuclear antigen, partial (3%)	e-111
NMAP3A2	184	putative leucine aminopeptidase [Oryza sativa (japonica cultivar-group)]	6.00E-12	TC147191 similar to UP|Q8GZD8 (Q8GZD8) Neutral leucine aminopeptidase preprotein precursor , partial (80%)	1.00E-59
NMAP3A3	342	carotenoid cleavage dioxygenase [Zea mays]	3.00E-47	TC140416 similar to UP|Q84KG5 (Q84KG5) Crocetin dialdehyde, partial (36%)	e-160
NMAP3A4	324	unknown protein [Oryza sativa (japonica cultivar-group)]	3.00E-15	BM816970	e-139
NMAP3A5	278	no hits found	-	TC148313	9.00E-12
NMAP3A6	191	putative histone H2B [Oryza sativa (japonica cultivar-group)]	4.00E-16	TC138783 homologue to UP|Q43215 (Q43215) Protein H2B153, complete	6.00E-83
NMAP3A7	170	no hits found	-	TC132364 UP|Q7PSX2 (Q7PSX2) ENSANGP00000018625 (Fragment), partial (6%)	2.00E-64
NMAP3A8	241	putative HIRA [Oryza sativa (japonica cultivar-group)]	3.00E-31	BM376094	3.00E-05
NMAP3A9	321	putative DNA-binding protein GBP16 [Oryza sativa (japonica cultivar-group)]	2.00E-20	TC146911 similar to UP|O22523 (O22523) DNA-binding protein GBP16, complete	e-139
NMAP3B1	216	serine/threonine kinase receptor precursor-like protein [Oryza sativa (japonica cultivar-group)]	3.00E-10	TC152731 similar to UP|Q84SG8 (Q84SG8) Serine/threonine kinase receptor-like protein, partial (25%)	7.00E-89

NMAP3B10	149	no hits found	-	TC140277 similar to UP|Q6R0G3 (Q6R0G3) MYB transcription factor, partial (32%)	3.00E-50
NMAP3B11	325	no hits found	-	TC131449 similar to UP|Q8T032 (Q8T032) LD35343p, partial (3%)	e-170
NMAP3B12	153	putative phosphate translocator [Oryza sativa (japonica cultivar-group)]	6.00E-10	TC139942 similar to GB|AAP42755.1|30984584|BT008742 At2g30460 {Arabidopsis thaliana;} , partial (90%)	7.00E-51
NMAP3B2	546	putative glycine-rich protein [Oryza sativa (japonica cultivar-group)]	6.00E-40	TC140019	0
NMAP3B3	282	putative RAD23 protein [Oryza sativa (japonica cultivar-group)]	3.00E-12	TC139414 similar to UP|Q40742 (Q40742) OsRAD23, partial (62%)	e-135
NMAP3B4	158	no hits found	-	TC148416 homologue to GB|AAM70547.1|21700847|AY124838 AT3g07590/MLP3_4 {Arabidopsis thaliana;} , complete	9.00E-69
NMAP3B5	483	putative oligouridylate binding protein [Oryza sativa (japonica cultivar-group)]	2.00E-73	TC140356 similar to UP|Q9LJH8 (Q9LJH8) RNA binding protein nucleolysin; oligouridylate binding protein	0
NMAP3B6	125	putative senescence-associated protein [Pisum sativum]	6.00E-07	TC146426 similar to UP|Q8L9C3 (Q8L9C3) Copia-like retroelement pol polyprotein, partial (32%)	8.00E-38
NMAP3B7	240	putative PDR-type ABC transporter 2 [Oryza sativa (japonica cultivar-group)]	1.00E-27	TC140581 homologue to UP|Q8GU89 (Q8GU89) PDR-like ABC transporter (PDR4 ABC transporter), partial (29%)	e-105
NMAP3B8	317	putative protein [Arabidopsis thaliana]	3.00E-20	AL510043	4.00E-79
NMAP3B9	336	COP1 [Oryza sativa subsp. indica]	4.00E-35	TC141586 homologue to UP|Q6ZHH4 (Q6ZHH4) COP1, constitutive photomorphogenesis 1, partial (59%)	e-119
NMAP3C1	259	putative F-box protein [Triticum aestivum]	5.00E-37	TC141398 weakly similar to UP|Q9FZK1 (Q9FZK1) F17L21.13, partial (50%)	e-118
NMAP3C10	228	no hits found	-	TC148295	1.00E-99
NMAP3C11	5	no hits found	-	no hits found	-
NMAP3C12	318	putative VHS domain-containing protein [Oryza sativa (japonica cultivar-group)]	7.00E-08	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	6.00E-04
NMAP3C2	199	putative dihydrolipoamide dehydrogenase [Oryza sativa (japonica cultivar-group)]	2.00E-18	TC147317 similar to UP|Q8GT30 (Q8GT30) Dihydrolipoamide dehydrogenase , partial (95%)	6.00E-86
NMAP3C3	273	putative somatic embryogenesis protein kinase 1 [Oryza sativa (japonica cultivar-group)]	2.00E-20	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	8.00E-06
NMAP3C4	228	serine/threonine protein kinase [Oryza sativa (japonica cultivar-group)]	1.00E-27	BU974931	2.00E-92
NMAP3C5	306	Dreg-2 like protein [Oryza sativa (japonica cultivar-group)]	1.00E-42	TC147564 similar to UP|O48587 (O48587) Dreg-2 like protein, partial (93%)	e-141
NMAP3C6	321	putative acetyl-CoA C-acyltransferase [Oryza sativa (japonica cultivar-group)]	4.00E-26	TC139402 similar to UP|Q8S4Y1 (Q8S4Y1) Acetoacetyl-CoA thiolase , partial (98%)	e-156
NMAP3C7	242	putative glucosyltransferase-10 [Oryza sativa (japonica cultivar-group)]	3.00E-12	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	4.00E-04
NMAP3C8	365	MSI type nucleosome/chromatin assembly factor C, putative [Oryza sativa (japonica cultivar-group)]	3.00E-55	TC140471 homologue to UP|Q8W514 (Q8W514) MSI type nucleosome/chromatin assembly factor C, complete	e-168
NMAP3C9	236	CI2D [Hordeum vulgare]	1.00E-17	BM816648	4.00E-66
NMAP3D1	290	Profilin-1	9.00E-19	BE412652	e-119
NMAP3D10	198	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	4.00E-81
NMAP3D11	203	OSJNBa0074L08.18 [Oryza sativa (japonica cultivar-group)]	0.27	TC131071 homologue to UP|Q7Y1Z2 (Q7Y1Z2) 27K protein (Fragment), partial (90%)	1.00E-46
NMAP3D12	191	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	5.00E-77
NMAP3D2	601	putative protein phosphatase 2C [Oryza sativa (japonica cultivar-group)]	1.00E-45	TC139287 similar to UP|Q6IV73 (Q6IV73) Protein phosphatase 2C, partial (89%)	0
NMAP3D3	294	fatty aldehyde dehydrogenase 1 [Zea mays]	7.00E-21	BG344593	e-123
NMAP3D4	289	putative 60S ribosomal protein L28 [Oryza sativa (japonica cultivar-group)]	2.00E-37	TC130727 similar to UP|Q8S0P2 (Q8S0P2) Ribosomal protein L28-like, complete	e-148
NMAP3D5	447	no hits found	-	BU971431	1.00E-82
NMAP3D6	283	probable cytochrome P450 monooxygenase - maize (fragment)	4.00E-20	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-143
NMAP3D7	339	no hits found	-	TC141029	e-115
NMAP3D8	230	conserved hypothetical protein [Paracoccus denitrificans PD1222]	0.011	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	5.00E-99
NMAP3D9	241	profilin [Triticum aestivum]	1.00E-14	BE412652	2.00E-40
NMAP3E1	228	conserved hypothetical protein [Frankia sp. EAN1pec]	0.011	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-105
NMAP3E10	273	no hits found	-	TC147031 weakly similar to UP|O82767 (O82767) PRT1 protein, partial (43%)	e-130

NMAP3E11	196	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	2.00E-76
NMAP3E12	5	no hits found	-	no hits found	-
NMAP3E2	403	ACT domain, putative [Oryza sativa (japonica cultivar-group)]	1.00E-26	TC139752 weakly similar to GB|AAP13425.1|30023784|BT006317 At1g16880 {Arabidopsis thaliana;} , partial (36%)	1.00E-94
NMAP3E3	234	no hits found	-	TC139318 similar to GB|AAP80663.1|32400863|AF479044 F1-ATPase {Triticum aestivum;} , partial (96%)	3.00E-14
NMAP3E4	246	HvPIP1;3 [Hordeum vulgare subsp. vulgare]	0.53	TC146836 UP|O48518 (O48518) HvPIP1;3 protein, complete	5.00E-59
NMAP3E5	360	Os07g0498300 [Oryza sativa (japonica cultivar-group)]	2E-13	TC147226	e-172
NMAP3E6	179	no hits found	-	BE422249 homologue to SP|P26517|G3PX Glyceraldehyde 3-phosphate dehydrogenase cytosolic (EC 1.2.1.12). [Barley]	2.00E-64
NMAP3E7	316	polyubiquitin [Sporobolus stapfianus]	4.00E-29	TC138984 homologue to PRF|1604470A.0|226763|1604470A poly-ubiquitin. {Zea mays;} , partial (93%)	e-151
NMAP3E8	316	putative senescence-associated protein [Pisum sativum]	6.00E-12	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-119
NMAP3E9	206	glyceraldehyde 3-phosphate dehydrogenase [Hordeum vulgare]	3.00E-04	BE422249 homologue to SP|P26517|G3PX Glyceraldehyde 3-phosphate dehydrogenase cytosolic (EC 1.2.1.12). [Barley]	2.00E-98
NMAP3F1	230	no hits found	-	TC153017 homologue to UP|CDK8_HUMAN (P49336) Cell division protein kinase 8 (Protein kinase K35) , partial (5%)	7.00E-86
NMAP3F10	263	mitochondrial import receptor subunit tom40 -like [Oryza sativa (japonica cultivar-group)]	5.00E-12	BI776543	1.00E-44
NMAP3F11	402	ubiquitin-conjugating enzyme [Hordeum vulgare]	1.00E-62	TC139319 homologue to UP|Q8L458 (Q8L458) Ubiquitin conjugating enzyme, complete	0
NMAP3F12	558	HBP-1b [Triticum aestivum]	1.00E-43	TC132749 homologue to UP|HBPB_WHEAT (P23923) Transcription factor HBP-1b(c38), complete	0
NMAP3F2	310	putative senescence-associated protein [Pisum sativum]	1.00E-41	TC146451 homologue to gb|AF036490.1| Acorus gramineus large subunit 26S ribosomal RNA gene, partial sequence, partial (31%)	e-154
NMAP3F3	196	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	3.00E-72
NMAP3F4	229	conserved hypothetical protein [Corynebacterium efficiens YS-314]	9.00E-05	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	6.00E-71
NMAP3F5	233	conserved hypothetical protein [Corynebacterium efficiens YS-314]	6.00E-06	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	6.00E-71
NMAP3F6	221	hypothetical protein CE2804 [Corynebacterium efficiens YS-314]	2.00E-07	TC146297 homologue to emb|Z00028.1|CHZMRRNA Zea mays chloroplast rRNA-operon, partial (5%)	4.00E-10
NMAP3F7	271	unknown protein [Oryza sativa (japonica cultivar-group)]	3.00E-23	TC148497 similar to GB|AAQ89615.1|37202000|BT010593 At3g17205 {Arabidopsis thaliana;} , partial (46%)	5.00E-87
NMAP3F8	232	conserved hypothetical protein [Frankia sp. EAN1pec]	0.011	TC146264 emb|Z11889.1|MITTARRNG T.aestivum mitochondrion rrn26 gene for rRNA large subunit (26S), partial (48%)	e-104
NMAP3F9	212	no hits found	-	TC147092 similar to UP|NDK4_SPIOL (Q8RXA8) Nucleoside diphosphate kinase IV, chloroplast precursor (NDK IV) (NDP kinase)	6.00E-80
NMAP3G1	229	no hits found	-	TC144007 similar to UP|HSP1_OCTVU (P83214) Sperm protamine P1 (Po1) [Contains: Sperm protamine P2 (Po2) (Main protamine)]	5.00E-22
NMAP3G10	201	no hits found	-	TC137720 similar to UP|Q9XI23 (Q9XI23) F9L1.41 protein (At1g15460/T16N11_24), partial (9%)	4.00E-56
NMAP3G11	366	putative transmembrane protein [Oryza sativa (japonica cultivar-group)]	4.00E-44	TC140847 similar to UP|Q6K5X9 (Q6K5X9) Transmembrane protein-like, partial (94%)	e-167
NMAP3G12	196	no hits found	-	AJ477095	8.00E-05
NMAP3G2	5	no hits found	-	no hits found	-
NMAP3G3	354	putative fructose 1-,6-biphosphate aldolase [Triticum aestivum]	6.00E-52	TC146558 homologue to UP|Q6QWQ3 (Q6QWQ3) Fructose 1,6-bisphosphate aldolase , partial (61%)	e-153
NMAP3G4	282	putative peroxidase [Oryza sativa (japonica cultivar-group)]	1.00E-29	BQ762523	e-101
NMAP3G5	351	F-box protein family-like [Oryza sativa (japonica cultivar-group)]	5.00E-11	BU988862 weakly similar to PIR|D86241|D86 protein T16B5.8 [imported] - Arabidopsis thaliana, partial (23%)	2.00E-47
NMAP3G6	308	endonuclease [Hordeum vulgare subsp. vulgare]	8.00E-06	TC133379 homologue to UP|O81958 (O81958) Endonuclease , complete	e-112
NMAP3G7	195	no hits found	-	TC141282	2.00E-70
NMAP3G8	343	putative protein kinase [Oryza sativa (japonica cultivar-group)]	1.00E-44	TC142932 similar to UP|Q9FDV7 (Q9FDV7) Protein kinase (PK) (Fragment), partial (45%)	e-154

NMAP3G9	201	no hits found	-	no hits found	-
NMAP3H10	288	Lipoxygenase 1	6.00E-33	TC146955 UP|LOX1_HORVU (P29114) Lipoxygenase 1 , complete	e-124
NMAP3H11	342	putative protein kinase [Oryza sativa (japonica cultivar-group)]	3.00E-48	TC142932 similar to UP|Q9FDV7 (Q9FDV7) Protein kinase (PK) (Fragment), partial (45%)	e-161
NMAP3H12	237	no hits found	-	TC140223 homologue to UP|Q9SAR7 (Q9SAR7) Actin, complete	2.00E-89
NMAP3H2	170	putative Potential phospholipid-transporting ATPase 8 [Oryza sativa (japonica cultivar-group)]	4.00E-13	BM371381 similar to GP|9758823|dbj| cytochrome P450-like protein {Arabidopsis thaliana}, partial (18%)	7.00E-05
NMAP3H3	287	P0497A05.15 [Oryza sativa (japonica cultivar-group)]	0.21	TC143981 similar to GB|AAR24650.1|38603810|BT010872 At5g63160 {Arabidopsis thaliana;} , partial (12%)	3.00E-27
NMAP3H4	245	S-adenosyl-L-homocysteine hydrolase [Anaeromyxobacter dehalogenans 2CP-C]	3.00E-28	TC139067 homologue to UP|Q84VE1 (Q84VE1) Adenosylhomocysteinase-like protein, partial (54%)	2.00E-06
NMAP3H5	209	Hypothetical protein [Oryza sativa (japonica cultivar-group)]	2.00E-21	TC150893 weakly similar to UP|Q9Y6D4 (Q9Y6D4) Microrchidia, partial (5%)	1.00E-87
NMAP3H6	169	no hits found	-	BM371382 weakly similar to GP|20269071|emb pectin methylesterase {Sesbania rostrata}, partial (9%)	7.00E-05
NMAP3H7	278	cytoplasmatic ribosomal protein S13 [Triticum aestivum]	2.00E-11	BE422386 homologue to SP|Q05761|RS13 40S ribosomal protein S13. [Maize] {Zea mays}, partial (84%)	3.00E-48
NMAP3H8	5	no hits found	-	no hits found	-
NMAP3H9	247	aminopeptidase P [Oryza sativa (japonica cultivar-group)]	2.00E-28	TC146990 similar to UP|Q93X45 (Q93X45) Xaa-Pro aminopeptidase 2 , partial (91%)	e-109
TCn1_9A7	271	no hits found	-	DN189634	5.00E-49
TCn1_9A8	663	putative coatomer protein complex, subunit beta 2 (beta prime) [Oryza sativa (japonica cultivar-group)]	2.00E-38	TA34593_4513 Putative coatomer protein complex, subunit beta 2 [Oryza sativa (japonica cultivar-group)]	0
TCn1_9A9	138	no hits found	-	BE194592 Putative senescence-associated protein [Pisum sativum (Garden pea)]	2.00E-16
TCn1_9A10	140	no hits found	-	BJ456649 Phosphoglucomutase, cytoplasmic [Bromus inermis (Smooth brome grass)]	4.00E-23
TCn1_9A11	189	no hits found	-	TA50020_4513 Nodulin-like [Oryza sativa (japonica cultivar-group)]	6.00E-66
TCn1_9A12	567	unknown protein [Oryza sativa (japonica cultivar-group)]	1.00E-23	TA39183_4513 Hypothetical protein OSJNBa0047P18.32-2 [Oryza sativa (japonica cultivar-group)]	0
TCn1_8H1	194	no hits found	-	CD053865 Small GTP-binding protein Sar1BNt-like protein [Solanum tuberosum (Potato)]	7.00E-29
TCn1_8H2	338	putative phospholipase D [Oryza sativa (japonica cultivar-group)]	6.00E-29	TA49455_4513 Hypothetical protein OSJNBb0106M04.16 [Oryza sativa (japonica cultivar-group)]	4.00E-19
TCn1_8H3	308	putative senescence-associated protein [Pisum sativum]	4.00E-40	CB883519 Putative senescence-associated protein [Pisum sativum (Garden pea)]	e-148
TCn1_8H4	191	no hits found	-	TA53517_4513 Band 3 anion transport protein-like [Oryza sativa (japonica cultivar-group)]	5.00E-79
TCn1_8H5	155	putative beta-1,3 glucanase [Oryza sativa (japonica cultivar-group)]	2.00E-11	TA50275_4513 Putative beta-1,3 glucanase [Oryza sativa (japonica cultivar-group)]	1.00E-26
TCn1_8H6	125	putative senescence-associated protein [Pisum sativum]	1.00E-05	TA28901_4513 Putative senescence-associated protein [Pisum sativum (Garden pea)]	5.00E-38
TCn1_8H7	531	cytoplasmic protein of eukaryotic origin (38.3 kD)-like [Oryza sativa (japonica cultivar-group)]	2.00E-85	TA41544_4513 Cytoplasmic protein of eukaryotic origin (38.3 kD)-like [Oryza sativa (japonica cultivar-group)]	0
TCn1_8H8	228	no hits found	-	DN157867	e-101
TCn1_8H9	143	no hits found	-	CX629884 BGAL3; beta-galactosidase/ sugar binding [Arabidopsis thaliana]	2.00E-19
TCn1_8H10	127	no hits found	-	TA28901_4513 Putative senescence-associated protein [Pisum sativum (Garden pea)]	6.00E-28
TCn1_8H11	207	putative potasium transporter [Oryza sativa (japonica cultivar-group)]	1.00E-22	BF626788 Putative potassium transporter [Oryza sativa (japonica cultivar-group)]	2.00E-69
TCn1_8H12	124	no hits found	-	TA29694_4513 26S protease regulatory subunit 4 homolog [Oryza sativa (Rice)]	1.00E-26
TCn1_9A1	215	C2 domain-containing protein-like [Oryza sativa (japonica cultivar-group)]	6.00E-21	TA42863_4513 C2 domain-containing protein-like [Oryza sativa (japonica cultivar-group)]	3.00E-90
TCn1_9A2	220	no hits found	-	CA011268 Putative STF-1 [Oryza sativa (japonica cultivar-group)]	5.00E-70
TCn1_9A3	245	no hits found	-	BM443521	e-111
TCn1_9A4	228	conserved hypothetical protein [Frankia sp. EAN1pec]	0.018	TA30977_4513	1.00E-89
TCn1_9A5	306	no hits found	-	BI959065	e-144
TCn1_9A6	150	no hits found	-	BF623005	5.00E-29

APPENDIX C

Appendix C

Table 1. Accumulation of B4.2 (bromo adjacent homology domain) transcripts (normalized mRNA copies/µl ± SE) in roots of Clipper and Sahara plants grown without B and at different B treatments after 1 day of treatment.

Cultivars	B concentrations (µM)	Day after treatment	Normalized mRNA copies/µl
Clipper	0	0	2,556 ± 234
	200	1	3,765 ± 164
	500	1	3,764 ± 26
Sahara	0	0	5,038 ± 146
	200	1	5,810 ± 469
	2000	1	5,685 ± 320

Table 2. Accumulation of SAMDC (Tn1_2B7) transcripts (normalized mRNA copies/µl ± SE) in Clipper and Sahara root grown without B and at different B treatments after 1 day of treatment.

Cultivars	B concentrations (µM)	Day after treatment	Normalized mRNA copies/µl
Clipper	0	0	253,815 ± 17,694
	200	1	184,922 ± 10,778
	500	1	216,129 ± 20,267
	0	7	220,120 ± 15,310
	200	7	238,692 ± 7,035
	0	14	220,207 ± 15,194
	200	14	234,957 ± 4,184
Sahara	0	0	269,132 ± 11,230
	200	1	444,194 ± 21,751
	2000	1	223,788 ± 7,001
	0	7	667,473 ± 33,022
	2000	7	344,278 ± 3,914
	0	14	547,983 ± 38,043
	2000	14	631,918 ± 47,273

APPENDIX D

Appendix D. Table 1. NCBI BLASTx annotations of Affymetrix 22K Barley1 GeneChip™ probsets differentially regulated in Clipper by B treatments and their RMA normalized hybridization intensity expressed as log base 2

Probe set name	NCBI BLASTx (nr)(<e-10)	Expression data in Clipper at different B treatment levels				
		E- value	20(µM)	50(µM)	100(µM)	150(µM)
Contig10651_at	hypothetical protein [Oryza sativa]	1.00E-109	5.84	5.14	5.04	5.12
Contig10915_s_at	Putative anion transporter [Oryza sativa]	8.00E-31	10.62	10.13	9.79	10.00
Contig10987_s_at	no hits found		7.60	6.84	6.94	7.23
Contig12255_at	putative Trp-Asp repeat protein [Oryza sativa]	0	7.40	7.95	7.68	8.10
Contig12378_at	patatin family [Arabidopsis thaliana]	1.00E-101	6.01	5.58	5.67	5.69
Contig12925_at	cDNA GRAS family transcription factor, putative	0	6.09	5.04	4.86	5.30
Contig13063_at	Unknown protein [Oryza sativa]	5.00E-80	5.73	6.60	6.56	6.75
Contig13670_at	no hit		7.30	8.55	8.29	7.92
Contig14067_at	unknown [Arabidopsis thaliana]	3.00E-46	9.77	9.33	9.01	9.07
Contig15311_at	expressed protein [Arabidopsis thaliana]	1.00E-83	6.08	6.75	6.78	6.85
Contig15545_at	no hit		7.64	7.10	6.70	7.01
Contig15797_at	putative aldehyde oxidase [Oryza sativa]	0	5.88	7.31	6.88	7.18
Contig17181_at	OSJNBa0084K01.19 [Oryza sativa]	1.00E-120	9.26	8.37	8.05	8.25
Contig17422_at	expressed protein [Arabidopsis thaliana]	1.00E-49	6.70	6.04	6.02	6.28
Contig17673_at	no hit		4.98	6.18	5.77	6.23
Contig18053_at	hv711N16.16 [Hordeum vulgare]	1.00E-132	4.37	5.48	5.13	5.45
Contig20263_at	putative adenosine kinase [Oryza sativa]	4.00E-47	5.60	6.13	6.01	6.14
Contig20479_at	contains EST AU065194(E60541)~similar to oligopeptide transporter [Oryza sativa]	2.00E-57	5.60	6.13	6.01	6.14
Contig2209_at	pathogenesis-related protein 1a - barley	8.00E-81	7.49	10.68	11.94	12.87
Contig2210_at	SCP-like extracellular protein, putative	3.00E-71	7.82	10.18	11.21	12.03
Contig2211_at	SCP-like extracellular protein, putative	1.00E-82	7.83	10.64	11.94	12.90
Contig2212_s_at	Pathogenesis-related protein PRB1-3 precursor (PR-1B) (HV-8)	6.00E-88	6.15	8.95	10.34	11.48
Contig2214_s_at	pathogenesis-related protein 1a - barley	6.00E-81	8.75	11.30	12.31	12.89
Contig22627_at	iron-phytosiderophore transporter protein yellow stripe 1 [Zea mays]	1.00E-106	6.12	7.28	6.84	7.63
Contig23086_at	putative heat shock protein [Oryza sativa]	1.00E-107	7.64	7.08	6.74	6.97
Contig2318_s_at	protein T27G7.16 [imported] - Arabidopsis thaliana	1.00E-14	4.58	5.75	5.31	6.38
Contig25398_at	putative mitogen-activated protein kinase 1 [Medicago sativa]	4.00E-13	6.99	6.40	6.44	6.45
Contig2550_x_at	pathogenesis-related protein 4 - barley	4.00E-73	6.16	8.29	9.44	10.51
Contig333_5_at	alpha-tubulin 2 [Hordeum vulgare subsp. vulgare]	0	7.75	8.89	8.48	8.70
Contig333_5_x_at	alpha-tubulin 2 [Hordeum vulgare subsp. vulgare]	0	7.69	8.74	8.31	8.75
Contig361_s_at	sucrose synthase 1 (sucrose-UDP glucosyltransferase 1)	1.00E-103	5.90	7.66	7.35	7.72
Contig501_s_at	S-adenosylmethionine decarboxylase proenzyme (AdoMetDC) (SamDC)	3.00E-49	10.20	11.84	11.40	11.60
Contig5213_at	putative RNA-binding protein [Oryza sativa]	0	9.85	9.36	9.12	9.12
Contig5356_s_at	NTF2-containing RNA-binding protein, putative [Arabidopsis thaliana]	4.00E-15	5.79	6.78	6.70	6.46
Contig626_x_at	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Triticum aestivum]	7.00E-47	5.67	7.61	6.99	7.42
Contig6486_at	expressed protein [Arabidopsis thaliana]	5.00E-97	7.54	6.89	6.86	6.80
Contig6739_at	unknown protein [Oryza sativa]	0	7.42	6.82	6.54	6.95
Contig6759_at	CCCH-type zinc finger protein -related [Arabidopsis thaliana]	4.00E-60	9.13	8.27	8.55	8.46
Contig7038_at	OSJNBb0056F09.13 [Oryza sativa]	1.00E-153	9.23	8.19	8.03	8.26
Contig7080_at	putative peroxidase [Oryza sativa]	5.00E-27	5.43	7.13	7.61	8.94
Contig7408_s_at	similar to Arabidopsis thaliana transport inhibitor response 1 (TIR1) (T48087) [Oryza sativa]	2.00E-13	7.50	6.84	6.43	6.64
Contig9631_at	putative homeodomain protein [Oryza sativa]	2.00E-83	6.76	7.55	7.25	7.77
Contig9858_at	no hit		10.56	9.97	9.77	9.86
EBma01_SQ005_G16_at	hypothetical protein At2g38330 [imported] - Arabidopsis thaliana	3.00E-19	4.85	5.36	5.21	5.30
HU05B08u_s_at	no hit		4.51	4.02	4.07	4.23
HU09E15u_x_at	no hit		6.05	5.35	5.25	5.22
HU12E14u_at	glycosyltransferase - related [Arabidopsis thaliana]	1.00E-24	5.40	6.58	6.12	6.49
HV_CEa0013J19f_at	photosystem I P700 apoprotein A1 [Triticum aestivum]	1.00E-74	5.30	9.27	9.05	9.45
HVSMEc0016D02f_at	photosystem I P700 chlorophyll A apoprotein A1 [Oryza sativa]	9.00E-52	5.79	9.87	9.67	10.14
HVSMEf0022D18r2_s_at	no hit		5.39	8.41	9.39	10.94
HX10L20r_s_at	Eukaryotic initiation factor 4A (eIF4A) (eIF-4A)	8.00E-13	6.12	7.54	7.13	7.52

Appendix D. Table 2. NCBI BLASTx annotations of Affymetrix 22K Barley1 GeneChip^{TM} probsets differentially regulated in Sahara by B treatments and their RMA normalized hybridization intensity expressed as log base 2

Probe set name	NCBI BLASTx (nr)(<e-10)	Expression data in Sahara at different B treatment levels					
		E- value	20(μM)	100 (μM)	500 (μM)	1000 (μM)	2000 (μM)
Category -1. Down regulated in Sahara							
Category -1.1. Down regulated in Clipper							
Contig2257_s_at	putative EREBP-type transcription factor [Oryza sativa]	2.00E-78	6.34	6.55	6.10	6.24	5.82
Contig9315_at	CONSTANS-like protein CO7 [Hordeum vulgare subsp. vulgare]	e-100	7.44	7.43	7.25	7.02	6.80
Contig8697_at	nonphototrophic hypocotyl 1b [Oryza sativa (japonica cultivar-group)]	1.00E-54	5.40	5.38	5.25	5.09	4.99
Contig6150_at	P0031D02.12 [Oryza sativa (japonica cultivar-group)]	9.00E-58	10.14	9.92	10.09	10.05	9.74
Contig9591_at	AT4g17280/dl4675c [Arabidopsis thaliana]	2.00E-70	8.07	7.88	7.90	8.01	7.66
Contig11522_at	putative chloroplast-targeted beta-amylase [Oryza sativa (japonica cultivar-group)]	e-128	9.29	9.32	9.08	9.01	8.61
Contig24757_at	cryptochrome dash [Arabidopsis thaliana]	e-119	8.01	7.88	7.60	7.82	7.45
Contig1968_s_at	early nodulin binding protein 1 - spring vetch	5.00E-08	7.73	7.87	7.38	7.56	7.32
Contig19246_at	putative glucosyltransferase [Oryza sativa (japonica cultivar-group)]	1.00E-23	8.19	7.90	8.03	7.96	7.69
Contig11854_at	putative myosin [Oryza sativa (japonica cultivar-group)]	e-160	9.11	9.15	8.92	8.98	8.72
Contig10520_s_at	hexose transporter, putative [Arabidopsis thaliana]	3.00E-21	7.44	7.32	7.24	7.38	7.05
Contig4783_at	pyruvate kinase isozyme A, chloroplast precursor	0	8.63	8.68	8.37	8.38	8.23
Contig7617_at	unkown protein [Arabidopsis thaliana]	2.00E-67	6.77	7.00	7.81	7.00	6.76
Contig23549_at	P0446B05.22 [Oryza sativa (japonica cultivar-group)]	2.00E-61	8.47	8.48	8.14	8.16	7.90
Contig3432_s_at	high molecular mass early light-inducible protein HV58, chloroplast precursor (ELIP)	3.00E-96	11.36	11.16	10.86	11.01	10.53
Contig3431_x_at	low molecular mass early light-inducible protein HV90, chloroplast precursor (ELIP)	6.00E-61	7.88	8.03	7.42	7.54	7.02
Contig16141_at	no hit		7.79	7.88	7.64	7.77	7.48
Contig24050_at	no hit		6.85	6.56	6.42	6.41	6.22
HVSMEf0015I21r2_s_at	no hit		10.04	10.04	9.88	9.85	9.54
baak3f15_at	no hit		8.07	7.98	7.74	7.82	7.49
HVSMEh0102G02f_x_at	no hit		6.49	6.51	6.61	6.31	5.89
HU09E15u_x_at	no hit		6.27	6.25	5.82	5.91	5.59
Category -1.2. Upregulated in Clipper							
Contig9189_at	isoleucine-tRNA ligase - like protein [Arabidopsis thaliana]	e-116	3.90	3.96	3.72	3.61	3.57
Contig2905_s_at	jasmonate induced protein - barley	7.00E-86	6.92	6.52	6.81	6.35	6.33
HV_CEa0013J19f_at	photosystem I P700 apoprotein A1 [Triticum aestivum]	1.00E-74	6.89	6.43	5.64	6.14	5.50
HVSMEc0016D02f_at	photosystem I P700 chlorophyll A apoprotein A1 [Oryza sativa (japonica cultivar-group)]	9.00E-52	6.97	6.69	6.08	6.50	5.93
Contig13712_at	ESTs AU057825(S21823),AU057072(S21123) correspond to a region of the predicted gene.~Similar to Nicotiana glauca X	1.00E-18	6.50	6.32	6.44	6.28	6.07
HVSMEf0021H03r2_s_at	no hit		9.09	9.29	8.94	8.82	8.61
HVSMEa0022N20f_at	no hit		10.00	9.34	9.03	9.46	8.96
Category -1.3. No significant response in Clipper							
Contig1684_x_at	23 kd jasmonate-induced protein	e-103	9.17	9.25	9.21	9.06	8.62
Contig18173_at	no hit	5.00E-09	5.81	5.67	5.45	5.50	5.20
X74365_at	ATP synthase subunit 9; H(+)-transporting ATP synthase [Hordeum vulgare subsp. vulgare]		8.18	7.72	7.29	7.53	7.06
Contig13674_at	putative beta-xylosidase [Triticum aestivum]	e-101	8.63	8.18	8.08	8.18	7.88
Contig4621_at	proline rich protein homolog WCOR518 - wheat (fragment)	8.00E-41	10.97	11.02	10.79	10.90	10.29
Contig17619_at	FYVE finger-containing phosphoinositide kinase-like [Oryza sativa (japonica cultivar-group)]	5.00E-94	7.44	7.45	7.32	7.28	7.10
Contig8416_s_at	unknown protein [Oryza sativa (japonica cultivar-group)]	4.00E-23	6.44	6.39	6.33	6.13	5.97
Contig1230_at	HvPIP1;5 [Hordeum vulgare subsp. vulgare]	e-160	7.63	7.55	7.47	7.37	7.10
HV_CEb0016L15r2_at	inward rectifying shaker K+ channel; ZmK2.1 [Zea mays]	6.00E-30	5.64	5.64	5.31	5.40	5.04
Contig7416_at	MADS-box protein 3 [Hordeum vulgare subsp. vulgare]	e-127	6.00	5.58	5.87	5.59	5.55
Contig14747_at	mRNA capping enzyme - like protein [Arabidopsis thaliana]	e-106	6.40	6.17	6.46	6.14	6.03
HVSMEc0015H24f_at	NdhK [Hordeum vulgare]	4.00E-94	11.13	11.01	10.19	10.78	10.14
Contig5630_at	OSJNBa0053B21.1 [Oryza sativa (japonica cultivar-group)]	4.00E-18	5.61	5.34	5.15	5.09	4.77

Contig6115_at	P0431G06.23 [Oryza sativa (japonica cultivar-group)]	7.00E-43	8.87	9.08	9.44	8.89	8.85
HVSMEm0001B03r2_at	P0503E05.4 [Oryza sativa (japonica cultivar-group)]	2.00E-27	8.18	8.02	7.96	7.89	7.70
Contig12968_at	P0690B02.4 [Oryza sativa (japonica cultivar-group)]	3.00E-24	6.66	6.72	7.96	6.79	6.66
rbags23g17_s_at	OSJNBa0016N04.14 [Oryza sativa (japonica cultivar-group)]	1.00E-27	5.91	5.83	5.54	5.66	5.47
Contig20012_at	putative ABA-responsive protein [Oryza sativa (japonica cultivar-group)]	5.00E-94	8.33	8.34	8.14	8.14	7.90
Contig15176_at	probable ABC-type transport protein T23J7.90 - Arabidopsis thaliana	3.00E-37	5.98	6.02	5.74	5.57	5.50
Contig7423_at	putative Chain A, Crystal Structure Of Phosphopantothenoylcysteine Synthetase [Oryza sativa (japonica cultivar-group)]	e-128	4.56	4.59	4.42	4.32	4.01
Contig24754_at	putative inositol 1,3,4-trisphosphate 5/6-kinase [Oryza sativa (japonica cultivar-group)]	2.00E-16	7.71	7.39	7.58	7.64	7.11
Contig17768_at	L-aspartate oxidase-like protein - Arabidopsis thaliana	6.00E-81	7.04	7.02	6.89	6.86	6.55
Contig17756_at	OJ000126_13.1 [Oryza sativa (japonica cultivar-group)]	4.00E-92	10.72	10.59	10.49	10.62	10.27
Contig7711_s_at	putative nitrate transporter [Oryza sativa (japonica cultivar-group)]	e-125	10.32	10.21	10.17	10.13	9.79
Contig20429_at	putative Nucleoporin Nup43 [Oryza sativa (japonica cultivar-group)]	1.00E-96	5.07	4.99	5.04	4.82	4.57
Contig6182_at	oxidoreductase, 2OG-Fe(II) oxygenase family [Arabidopsis thaliana]	e-136	8.59	8.58	8.63	8.62	8.20
Contig6848_at	putative phytase [Oryza sativa (japonica cultivar-group)]	0	6.21	6.24	7.77	6.23	5.73
Contig21251_at	nitrite transport protein, chloroplast - cucumber	2.00E-29	5.13	4.89	4.56	4.46	4.38
Contig14921_s_at	putative purple acid phosphatase [Oryza sativa (japonica cultivar-group)]	2.00E-19	6.36	6.22	7.33	6.39	6.24
Contig21080_at	unknown protein [Arabidopsis thaliana]	1.00E-38	6.90	6.94	6.75	6.82	6.36
Contig20537_at	putative triacylglycerol lipase [Oryza sativa (japonica cultivar-group)]	3.00E-69	4.66	4.53	5.75	4.51	4.65
Contig9958_at	expressed protein [Arabidopsis thaliana]	1.00E-93	8.93	8.74	8.58	8.73	8.31
HD13B05r_s_at	ribonuclease (EC 3.1.-.-) - barley	1.00E-21	9.58	9.83	10.83	9.66	9.51
Contig18065_at	OSJNBa0019K04.6 [Oryza sativa (japonica cultivar-group)]	1.00E-21	7.67	7.91	8.81	7.70	7.46
Contig4612_at	sucrose transporter 1 [Hordeum vulgare subsp. vulgare]	0	9.25	9.27	9.23	9.11	8.75
HV_CEb0001H12r2_at	putative amino acid permease 6 [Oryza sativa (japonica cultivar-group)]	5.00E-37	7.16	7.14	7.17	7.06	6.55
Contig1035_at	fiber polyubiquitin [Gossypium barbadense]	2.00E-69	9.68	9.80	9.72	9.68	9.10
Contig12177_at	At2g13820 [Arabidopsis thaliana]	2.00E-19	7.09	6.76	6.80	6.80	6.52
Contig24061_at	hydrophobic protein OSR8	1E-14	7.75	7.84	8.74	7.67	7.66
Contig24328_at	unknown protein [Oryza sativa (japonica cultivar-group)]	3.00E-23	8.01	7.87	7.89	7.67	6.40
rbah11m03_at	expressed protein [Arabidopsis thaliana]	3E-14	9.57	9.35	9.26	9.33	9.00
HVSMEf0020J20f2_s_at	no hit		10.26	10.00	9.90	10.02	9.73
Contig10522_at	no hit		10.35	10.41	10.22	10.30	9.54
rbaal1f12_s_at	no hit		10.22	10.18	10.02	9.99	9.81
HVSMEf0015C12f_at	no hit		9.36	8.98	8.90	8.83	8.73
HV12J02u_s_at	no hit		7.29	7.19	7.37	7.20	6.73
Contig8526_s_at	no hit		10.23	10.41	10.15	10.02	9.66
Contig25806_at	no hit		8.84	8.61	8.68	8.58	8.32
Contig3834_at	no hit		8.98	8.72	8.44	8.56	8.17
HVSMEf0017C20f2_at	no hit		8.12	7.93	7.70	7.75	7.55
Contig17730_at	no hit		5.59	5.70	5.44	5.55	5.18
Contig12969_s_at	no hit		8.00	8.15	9.81	7.84	7.65
Contig16582_at	no hit		9.47	9.38	9.08	9.02	8.64
EBro02_SQ006_D14_s_at	no hit		8.49	8.00	7.89	8.25	7.74
Contig9996_at	no hit		5.17	4.76	4.97	4.84	4.71

Category -2. Up-regulated in Sahara

Category -2.1. Down regulated in Clipper

Contig6198_at	OSJNBa0005N02.2 [Oryza sativa (japonica cultivar-group)]	8.00E-28	7.51	7.72	7.83	7.93	8.00
Contig6177_at	putative casein kinase I [Oryza sativa (japonica cultivar-group)]	0	7.36	7.61	7.62	7.88	8.01
Contig12563_s_at	putative chalcone synthase [Oryza sativa (japonica cultivar-group)]	8.00E-32	6.95	7.44	7.34	7.55	7.95
Contig13148_at	homeodomain leucine zipper protein [Oryza sativa]	6E-11	7.05	7.24	7.21	7.34	7.57
rbaal9k07_at	putative myosin heavy chain [Oryza sativa]	2.00E-58	6.96	7.41	7.13	7.36	7.48
Contig935_at	putative potasium transporter [Oryza sativa (japonica cultivar-group)]	0	7.94	8.09	8.18	8.30	8.30
HVSMEf0011J01r2_s_at	no hit		6.82	6.93	7.06	7.22	7.31

Contig19410_at	no hit		7.15	7.40	7.53	7.58	7.77

Category -2.2 Up-regulated in Clipper

rbags15p13_s_at	23 kd jasmonate-induced protein	1.00E-29	5.03	4.73	4.81	5.28	6.69
Contig6708_at	expressed protein [Arabidopsis thaliana]	e-147	7.43	7.89	7.75	7.91	8.23
Contig3097_at	allene oxide synthase [Hordeum vulgare subsp. vulgare]	0	5.92	5.65	5.76	6.47	7.92
Contig3096_s_at	allene oxide synthase [Hordeum vulgare subsp. vulgare]	0	9.11	9.14	9.12	9.51	10.00
Contig15617_at	CBF1-like protein BCBF1 [Hordeum vulgare subsp. vulgare]	9.00E-96	6.43	7.19	7.21	7.45	7.35
Contig2992_s_at	chitinase (EC 3.2.1.14) cht2b precursor - barley	e-133	6.19	7.42	6.70	7.44	8.19
Contig4326_s_at	chitinase IV precursor [Triticum aestivum]	e-127	4.69	4.62	4.59	5.32	6.65
Contig3112_at	cold acclimation protein WCOR413 - wheat	4.00E-87	8.18	8.42	8.58	8.65	9.00
Contig2716_s_at	ferritin [Oryza sativa (japonica cultivar-group)]	e-102	6.64	6.16	7.34	6.70	8.09
HV12A05u_s_at	ferritin [Oryza sativa (japonica cultivar-group)]	5.00E-34	6.58	6.22	7.14	6.70	7.90
Contig21141_at	OSJNBb0072N21.3 [Oryza sativa (japonica cultivar-group)]	9.00E-43	8.21	9.04	9.19	9.06	9.39
Contig3667_s_at	myb-related protein - barley	0	5.82	5.90	5.62	6.17	6.89
Contig5247_at	gigantea-like protein [Hordeum vulgare]	0	6.98	7.20	7.24	7.41	7.73
Contig2975_s_at	glutathione S-transferase 1 (GST class-phi)	e-105	8.85	9.54	9.68	9.59	9.29
Contig5838_at	glutathione-S-transferase 2 [Aegilops tauschii]	8.00E-88	4.87	4.90	5.06	4.93	5.33
baak20j05_s_at	integral membrane protein [Arabidopsis thaliana]	2.00E-22	4.56	4.62	4.80	4.76	5.06
Contig3626_s_at	hypersensitive-induced reaction protein 3 [Hordeum vulgare subsp. vulgare]	e-159	7.77	8.39	8.07	8.14	8.71
Contig5469_at	hypothetical protein [Oryza sativa (japonica cultivar-group)]	1.00E-40	3.97	4.05	4.02	4.43	5.47
Contig3636_at	leucine-rich repeat protein LRP - sorghum	2.00E-63	4.49	5.01	4.94	5.34	5.63
Contig3635_s_at	leucine-rich repeat protein LRP - sorghum	1.00E-60	5.78	6.70	6.16	6.76	7.64
Contig11212_at	OSJNBa0064M23.14 [Oryza sativa (japonica cultivar-group)]	2.00E-85	5.48	5.99	5.91	6.56	7.63
Contig23889_at	OSJNBa0069D17.7 [Oryza sativa (japonica cultivar-group)]	1.00E-44	6.27	6.47	6.52	6.48	6.85
Contig11160_at	OSJNBa0079A21.19 [Oryza sativa (japonica cultivar-group)]	1.00E-81	6.47	6.74	6.88	6.94	7.58
Contig3746_at	OSJNBa0088H09.15 [Oryza sativa (japonica cultivar-group)]	9.00E-80	4.47	4.69	4.68	4.69	5.38
Contig3744_s_at	OSJNBa0088H09.15 [Oryza sativa (japonica cultivar-group)]	3.00E-70	6.57	6.76	6.66	6.76	7.17
Contig8635_at	vacuolar targeting receptor bp-80 [Triticum aestivum]	e-119	5.97	6.04	6.05	6.14	6.72
Contig11615_s_at	P0423B08.25 [Oryza sativa (japonica cultivar-group)]	1.00E-37	5.02	5.12	5.33	5.82	6.73
Contig2550_x_at	pathogenesis-related protein 4 - barley	4.00E-73	4.55	5.22	4.57	5.57	6.58
HVSMEm0005P05r2_at	peroxidase (EC 1.11.1.7) precursor, pathogen-induced - barley	2.00E-23	3.96	4.69	3.92	4.73	5.25
Contig2123_s_at	peroxidase [Hordeum vulgare subsp. vulgare]	2.00E-64	4.56	4.45	4.66	4.64	5.07
Contig2209_at	pathogenesis-related protein 1a - barley	8.00E-81	5.96	6.71	6.30	6.90	8.06
Contig12753_at	putative ABC transporter [Oryza sativa (japonica cultivar-group)]	2.00E-81	4.92	5.34	5.27	5.26	5.75
Contig2427_at	putative acid phosphatase [Hordeum vulgare subsp. vulgare]	e-101	5.29	5.55	5.41	6.02	6.95
Contig11494_at	unknown protein [Oryza sativa (japonica cultivar-group)]	8.00E-56	5.41	5.58	5.61	5.64	5.87
Contig19393_at	putative water stress induced tonoplast intrinsic protein [Oryza sativa (japonica cultivar-group)]	5.00E-61	9.59	9.77	9.98	10.07	10.52
Contig12286_s_at	FAD-linked oxidoreductase family [Arabidopsis thaliana]	e-116	5.14	5.03	5.24	5.20	5.49
Contig18990_at	putative cytochrome P450 [Oryza sativa (japonica cultivar-group)]	7.00E-68	4.89	4.77	4.93	4.98	5.44
Contig26313_at	putative cytochrome P450 [Populus x canescens]	6.00E-45	5.62	5.45	5.66	5.94	6.14
Contig10152_at	Putative membrane protein [Oryza sativa (japonica cultivar-group)]	e-150	6.58	6.47	6.53	6.55	7.08
Contig6541_at	putative nuclear protein [Hordeum vulgare subsp. vulgare]	7.00E-65	4.45	4.51	4.60	4.53	4.94
Contig11361_at	putative peroxidase [Oryza sativa (japonica cultivar-group)]	e-129	5.83	5.75	5.96	6.26	6.63
Contig34_s_at	putative proteinase inhibitor [Hordeum vulgare subsp. vulgare]	4.00E-33	5.99	5.70	6.48	6.92	8.98
Contig50_x_at	putative proteinase inhibitor [Hordeum vulgare subsp. vulgare]	8.00E-27	4.75	4.67	4.76	5.11	6.16
Contig88_x_at	putative proteinase inhibitor [Hordeum vulgare subsp. vulgare]	7.00E-26	6.72	6.56	7.07	7.22	8.16
Contig17478_at	tryptophan synthase-related [Arabidopsis thaliana]	e-128	6.90	6.75	7.12	7.24	7.33
Contig3054_s_at	senescence-associated protein-like protein [Oryza sativa (japonica cultivar-group)]	8.00E-85	6.49	6.44	6.84	6.94	7.42
Contig13201_at	pseudo-response regulator, APRR3 (APRR1/TOC1 family) [Arabidopsis thaliana]	1E-15	6.14	6.70	6.93	7.11	7.58
Contig2214_s_at	pathogenesis-related protein 1a - barley	6.00E-81	6.13	7.47	6.83	8.07	9.20

Contig11737_at	P0678F11.22 [Oryza sativa (japonica cultivar-group)]	1.00E-61	6.84	7.01	7.14	7.16	7.37
Contig6170_s_at	P0482C06.16 [Oryza sativa (japonica cultivar-group)]	9.00E-35	7.70	8.12	7.66	8.27	8.58
Contig6169_at	P0482C06.16 [Oryza sativa (japonica cultivar-group)]	5.00E-35	8.14	8.44	8.20	8.50	8.80
Contig10932_at	expressed protein [Arabidopsis thaliana]	6.00E-46	7.83	7.98	8.06	8.05	8.27
Contig17964_at	hypothetical protein [Arabidopsis thaliana]	4.00E-63	5.03	5.03	5.32	5.24	5.57
Contig10441_at	unknown protein [Oryza sativa (japonica cultivar-group)]	4.00E-23	6.23	5.82	6.30	6.32	7.10
Contig13073_at	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-39	6.91	7.51	7.84	7.97	8.25
Contig2163_at	no hit		7.24	7.97	7.69	8.03	8.93
Contig12237_at	no hit		7.54	8.04	7.89	8.00	8.62
Contig9923_at	no hit		5.09	5.67	5.73	5.96	6.69
Contig9922_s_at	no hit		5.55	6.20	6.15	6.35	7.08
Contig13632_at	no hit		6.19	6.14	6.34	6.50	7.52
EBem10_SQ002_I10_s_at	no hit		3.86	4.42	4.06	4.67	5.64
HD04G07u_s_at	no hit		7.94	8.16	8.09	9.02	9.87
Contig9917_at	no hit		6.84	7.70	7.13	7.90	8.65
HP01B09w_at	no hit		5.48	5.80	5.42	6.47	7.20
Contig7209_at	no hit		6.20	6.60	8.06	7.46	8.20
Contig16826_at	no hit		5.33	5.50	5.56	5.89	6.10
HVSMEb0002K02r2_s_at	no hit		8.05	8.52	8.11	8.58	8.69
Contig7210_at	no hit		5.02	5.06	5.36	5.30	5.66
HVSMEf0013K24r2_s_at	no hit		4.65	4.62	4.82	5.02	5.49
HA11K18u_s_at	no hit		4.62	4.87	4.91	4.97	5.27
HO03L07S_at	no hit		6.76	6.73	6.90	7.00	7.17
Contig4954_s_at	no hit		6.17	6.39	6.50	6.53	6.97
HZ45N21r_at	no hit		6.43	7.20	6.85	7.05	7.35
Contig11161_s_at	no hit		6.61	7.15	7.58	7.45	7.94

Category -2.3. No significant response in Clipper

Contig86_at	Thiol protease aleurain precursor	0	10.76	11.28	11.28	11.27	11.43
Contig6974_at	Nt-iaa2.3 deduced protein [Nicotiana tabacum]	2.00E-40	9.33	9.62	9.82	9.63	9.77
Contig13355_at	B1112D09.4 [Oryza sativa (japonica cultivar-group)]	2.00E-77	5.07	5.47	5.66	5.54	5.43
Contig4545_s_at	P0005H10.22 [Oryza sativa (japonica cultivar-group)]	7.00E-20	9.32	9.90	9.83	9.70	10.20
Contig6570_at	oxidase lip19 - rice	4.00E-43	8.41	8.80	8.90	8.72	8.90
Contig15334_at	bZIP transcription factor ABI5 [Hordeum vulgare subsp. vulgare]	4.00E-30	6.58	6.89	6.95	6.99	6.96
Contig4281_at	cold-regulated protein [Hordeum vulgare subsp. vulgare]	3.00E-77	6.83	6.92	6.87	7.00	7.40
Contig4281_s_at	cold-regulated protein [Hordeum vulgare subsp. vulgare]	3.00E-77	7.35	8.01	7.72	7.80	8.35
Contig8538_at	light-induced protein CPRF-2 - parsley	9.00E-46	6.89	7.55	7.64	7.73	7.98
Contig7481_at	DNA-binding protein RAV2-like [Oryza sativa (japonica cultivar-group)]	1.00E-23	6.51	7.38	7.28	7.30	7.67
Contig11487_at	DnaJ-like protein [Glycine max]	7.00E-31	7.51	7.59	7.86	8.24	8.10
Contig1765_at	dormancy-associated protein [similarity] - apple tree	8.00E-21	5.08	5.67	5.43	5.33	6.05
Contig1762_s_at	dormancy-associated protein [similarity] - apple tree	4.00E-25	8.18	9.09	9.00	8.76	9.71
HVSMEa0017I09r2_s_at	ethylene-insensitive-3-like protein [Oryza sativa (japonica cultivar-group)]	4E-12	9.31	9.51	9.58	9.68	9.95
Contig4395_at	ethylene-insensitive-3-like protein [Oryza sativa (japonica cultivar-group)]	0	9.18	9.42	9.53	9.68	9.90
Contig16651_at	putative sulfolipid synthase [Oryza sativa (japonica cultivar-group)]	0	6.42	6.83	8.44	6.83	6.67
Contig3155_s_at	germin-like protein - barley	e-113	4.47	4.80	4.85	5.49	6.31
Contig4772_s_at	light regulated protein precursor	2.00E-23	11.47	11.98	11.91	11.72	12.18
Contig2305_at	Lipoxygenase 2.2, chloroplast precursor (LOX2:Hv:2)	0	6.17	5.98	6.26	6.35	7.29
Contig6351_at	MLO protein	0	7.30	7.90	7.52	7.77	8.05
Contig10719_at	38.7K hypothetical protein F2J7.21 - Arabidopsis thaliana	4.00E-30	4.77	4.75	5.72	4.69	4.83
Contig7388_at	putative nitrate transporter NRT1-5 [Oryza sativa (japonica cultivar-group)]	e-153	7.03	7.40	7.27	7.44	7.90
Contig7305_at	O-methyltransferase ZRP4 (OMT)	1.00E-78	9.21	9.33	9.51	9.54	9.91
Contig7304_s_at	O-methyltransferase ZRP4 (OMT)	3.00E-56	9.45	9.66	9.85	9.90	10.21
Contig10193_at	FIERG2 protein - rice	7.00E-61	4.93	4.90	5.06	5.14	5.48
Contig9941_at	OSJNBa0013K16.15 [Oryza sativa (japonica cultivar-group)]	2.00E-46	7.16	7.08	7.27	7.45	7.56
Contig8667_at	OSJNBa0032F06.25 [Oryza sativa (japonica cultivar-group)]	7.00E-34	4.68	4.93	4.89	4.88	5.26
Contig18207_at	OSJNBa0036B21.18 [Oryza sativa (japonica cultivar-group)]	2.00E-30	6.77	6.89	6.88	7.14	7.17

Contig5648_s_at	OSJNBa0036B21.19 [Oryza sativa (japonica cultivar-group)]	e-164	5.81	5.90	6.07	6.19	6.39
Contig10887_at	ABC transporter family protein [Arabidopsis thaliana]	0	4.91	5.19	5.21	5.38	5.55
Contig721_s_at	OSJNBa0094P09.18 [Oryza sativa (japonica cultivar-group)]	5.00E-88	8.74	8.82	8.96	9.03	9.08
Contig4250_at	OSJNBa0086O06.23 [Oryza sativa (japonica cultivar-group)]	e-101	8.14	8.22	8.27	8.27	8.57
Contig8578_s_at	OSJNBb0013O03.10 [Oryza sativa (japonica cultivar-group)]	5.00E-38	9.56	10.02	9.87	10.07	10.09
HI02L18u_at	OSJNBb0089K06.1 [Oryza sativa (japonica cultivar-group)]	6.00E-48	4.72	4.83	4.84	5.05	5.24
Contig14754_at	P0497A05.15 [Oryza sativa (japonica cultivar-group)]	E-42	8.89	9.25	9.30	9.31	9.68
HV_CEa0006L03r2_at	P0497A05.15 [Oryza sativa (japonica cultivar-group)]	3.00E-38	8.06	8.70	8.59	8.46	8.80
Contig2113_at	peroxidase [Oryza sativa (japonica cultivar-group)]	e-113	6.53	6.33	7.05	7.11	7.59
Contig3243_x_at	bacterial-induced peroxidase precursor [Gossypium hirsutum]	e-108	8.78	8.71	8.89	8.90	9.52
Contig3239_at	bacterial-induced peroxidase precursor [Gossypium hirsutum]	e-106	9.45	10.40	10.25	10.11	10.80
rbah19o01_s_at	pseudo-response regulator, APRR3 (APRR1/TOC1 family) [Arabidopsis thaliana]	2E-15	8.29	8.74	8.77	8.80	8.94
Contig6533_at	contains similarity to two-component response regulator protein~gene_id:T31K7.5 [Arabidopsis thaliana]	6.00E-75	8.49	8.76	8.92	8.95	8.97
Contig9890_at	expressed protein [Arabidopsis thaliana]	e-122	8.31	8.61	8.64	8.80	8.88
Contig2642_at	1-aminocyclopropane-1-carboxylate oxidase (EC 1.4.3.-) ACO1 [similarity] - sorghum	e-153	8.51	8.80	8.72	8.76	9.10
Contig6174_at	putative 6-phosphogluconolactonase [Oryza sativa (japonica cultivar-group)]	e-133	9.31	9.69	9.76	9.66	9.72
Contig15250_at	auxin induced protein [Zea mays]	7.00E-29	7.07	7.29	7.05	7.22	7.69
Contig9476_at	beta-glucan binding protein [Phaseolus vulgaris]	0	6.38	6.78	6.75	6.87	7.44
Contig6664_at	cadmium-induced protein-like [Oryza sativa (japonica cultivar-group)]	2.00E-62	8.01	8.33	8.24	8.47	8.52
AJ250283_at	putative calcium binding EF-hand protein [Hordeum vulgare subsp. vulgare]	e-117	4.65	4.58	4.83	4.84	5.32
Contig15493_at	calmodulin-binding heat-shock protein - common tobacco	7.00E-16	5.38	5.36	5.45	5.53	5.90
Contig8468_at	B1114B07.18 [Oryza sativa (japonica cultivar-group)]	e-117	7.47	7.57	7.63	7.66	7.90
Contig8854_at	serine carboxypeptidase II-like protein [Arabidopsis thaliana]	e-170	7.07	7.60	7.32	7.58	7.57
bags23d05_s_at	putative casein kinase I [Oryza sativa (japonica cultivar-group)]	2.00E-18	8.13	8.40	8.43	8.57	8.77
Contig11944_at	putative chalcone synthase 1 [Oryza sativa (japonica cultivar-group)]	e-111	5.53	5.42	5.80	5.81	6.72
Contig5551_at	probable choline-phosphate cytidylyltransferase (EC 2.7.7.15) (clone CCT2) - rape	e-114	9.59	9.73	10.03	9.76	9.64
Contig9547_at	choline kinase -related [Arabidopsis thaliana]	e-136	8.57	9.07	8.91	8.87	9.03
Contig20663_at	putative lipase homolog [Oryza sativa (japonica cultivar-group)]	7.00E-64	4.47	4.53	4.68	4.90	5.16
Contig12418_at	putative glucosyltransferase [Oryza sativa (japonica cultivar-group)]	e-145	7.09	7.36	7.44	7.48	7.66
Contig1385_at	putative glutamate carboxylase [Oryza sativa (japonica cultivar-group)]	0	6.71	6.67	6.74	7.05	7.52
Contig19684_at	putative hypersensitivity-related (hsr)protein [Oryza sativa (japonica cultivar-group)]	6.00E-38	6.48	6.48	6.53	6.63	6.88
Contig4711_s_at	putative MAP kinase [Hordeum vulgare subsp. vulgare]	2.00E-65	7.70	7.98	7.98	8.06	8.28
Contig8122_at	PDR-like ABC transporter [Oryza sativa (japonica cultivar-group)]	e-128	5.68	5.74	6.00	5.97	6.16
Contig23705_at	peptide transporter [Oryza sativa (japonica cultivar-group)]	6.00E-58	4.75	5.30	5.16	5.07	5.59
Contig1867_at	putative peroxidase [Oryza sativa (japonica cultivar-group)]	e-120	9.52	9.43	9.88	9.61	10.16
Contig7525_at	putative phospholipase [Oryza sativa]	1.00E-49	5.10	5.19	5.29	5.18	5.55
Contig7362_at	putative protein kinase SPK-3 [Oryza sativa (japonica cultivar-group)]	e-174	7.23	7.33	7.55	7.78	8.10
Contig9074_at	OSJNBb0062H02.4 [Oryza sativa (japonica cultivar-group)]	2.00E-74	7.41	7.67	7.77	7.77	7.75
Contig17515_at	putative serine/threonine kinase [Hordeum vulgare]	4.00E-88	5.77	5.85	6.08	5.97	6.29
Contig14822_at	CBL-interacting protein kinase 4 [Arabidopsis thaliana]	7.00E-21	8.12	8.72	8.84	8.71	8.56
Contig2021_at	OSJNBa0070M12.12 [Oryza sativa (japonica cultivar-group)]	e-100	7.49	7.85	7.72	7.76	8.10
Contig16201_at	putative sphingosine kinase [Oryza sativa (japonica cultivar-group)]	2.00E-85	7.13	7.43	7.51	7.63	7.73
Contig2825_at	putative trehalose-phosphatase [Oryza sativa (japonica cultivar-group)]	e-141	9.22	9.45	9.35	9.45	9.73
Contig1262_at	putative ubiquitin-specific protease 3 [Oryza sativa (japonica cultivar-group)]	0	7.50	7.85	7.85	7.83	7.85
Contig12159_at	putative UVB-resistance protein [Oryza sativa (japonica cultivar-group)]	6.00E-86	7.15	7.26	8.03	7.20	7.20
Contig6354_s_at	NADPH oxidase [Nicotiana tabacum]	2.00E-57	4.84	5.11	5.20	5.29	5.57

Contig3054_at	senescence-associated protein-like protein [Oryza sativa (japonica cultivar-group)]	8.00E-85	7.50	7.70	7.69	7.98	8.29
Contig10672_at	ribonuclease (EC 3.1.-.-) - barley	e-115	7.00	7.30	8.26	7.14	7.10
Contig6902_at	wound-induced protein 1	4E-12	8.54	8.68	8.82	8.87	8.93
Contig1580_x_at	thionin precursor, leaf - barley	5.00E-70	6.73	5.58	6.70	7.59	8.53
Contig10709_at	ESTs C26000(C11448),AU082130(C11448) correspond to a region of the predicted gene.~Similar to mRNA for zinc-finger protein	3.00E-54	7.55	7.81	7.95	8.01	8.14
Contig11597_at	hypothetical protein [Oryza sativa (japonica cultivar-group)]	4.00E-90	7.95	8.13	8.30	8.18	8.45
Contig10615_at	hypothetical protein [Oryza sativa (japonica cultivar-group)]	1.00E-35	5.97	6.02	6.25	6.19	6.42
Contig8683_at	hypothetical protein [Oryza sativa (japonica cultivar-group)]	7E-12	6.13	6.57	6.55	6.90	6.86
Contig17563_at	unnamed protein product [Oryza sativa (japonica cultivar-group)]	1.00E-30	5.16	4.89	5.36	5.39	5.86
Contig10116_at	unknown protein [Oryza sativa (japonica cultivar-group)]	e-124	7.90	8.18	8.27	8.28	8.51
Contig3579_at	unknown protein [Oryza sativa (japonica cultivar-group)]	1.00E-25	7.75	8.21	8.02	8.16	8.50
Contig9110_at	expressed protein [Arabidopsis thaliana]	9E-10	5.34	5.35	5.45	5.64	6.02
Contig10015_at	expressed protein [Arabidopsis thaliana]	e-157	8.00	8.25	8.62	8.09	8.23
Contig10221_at	expressed protein [Arabidopsis thaliana]	3.00E-36	5.53	5.94	6.16	5.99	6.09
Contig4282_at	expressed protein [Arabidopsis thaliana]	1.00E-79	10.69	10.93	10.91	10.98	11.19
Contig15750_at	expressed protein [Arabidopsis thaliana]	3.00E-33	7.35	7.64	7.76	7.81	7.78
Contig5410_at	unknown protein [Oryza sativa (japonica cultivar-group)]	6.00E-61	10.26	10.47	10.54	10.51	10.75
HK03F11r_at	unknown protein [Oryza sativa (japonica cultivar-group)]	9E-12	6.86	7.33	7.37	7.37	7.39
Contig8658_at	unknown protein [Oryza sativa (japonica cultivar-group)]	6.00E-91	7.20	8.02	7.67	7.70	8.21
Contig9589_at	unknown protein [Oryza sativa (japonica cultivar-group)]	2.00E-78	8.21	8.29	8.38	8.56	8.59
Contig7516_at	no hit		7.88	8.66	8.55	8.78	9.16
Contig11383_at	no hit		10.69	10.83	10.90	11.03	11.10
Contig7450_at	no hit		8.24	8.10	9.08	8.36	8.24
Contig13690_at	no hit		7.60	7.82	8.24	8.05	8.26
Contig14625_at	no hit		4.29	4.43	4.40	4.34	5.39
baak1o23_s_at	no hit		4.25	4.47	4.46	4.57	4.72
HD07L17r_at	no hit		6.32	6.90	7.14	6.91	6.84
HV_CEb0009D09r2_at	no hit		5.24	5.47	5.44	5.82	6.74
Contig2632_s_at	no hit		8.29	9.08	8.85	8.76	9.43
Contig2630_at	no hit		7.54	8.21	8.12	7.99	8.51
Contig955_s_at	no hit		5.83	5.82	6.21	6.37	6.62
Contig12648_at	no hit		10.34	10.78	10.69	10.87	11.10
HW02O09u_s_at	no hit		3.94	3.91	4.26	4.45	4.69
Contig9774_s_at	no hit		10.41	11.04	11.09	11.06	11.17
Contig17657_at	no hit		5.67	6.08	6.22	6.26	6.40
HO15C14S_s_at	no hit		5.41	5.69	5.88	5.98	6.10
Contig10275_s_at	no hit		5.61	5.56	5.91	5.97	6.38
Contig1021_at	no hit		4.62	4.84	4.78	4.68	5.28
Contig21971_at	no hit		9.83	10.19	10.04	9.99	10.37
HB30J05r_at	no hit		6.53	6.84	7.19	7.21	7.05
HVSMEl0002L04r2_s_at	no hit		7.07	7.19	7.29	7.32	7.50
Contig15186_at	no hit		5.85	5.95	6.16	6.14	6.36
Contig346_at	no hit		3.91	3.78	3.96	4.05	5.11
Contig5929_at	no hit		7.20	7.77	7.64	7.59	8.02
Contig9101_s_at	no hit		5.36	5.57	5.60	5.65	5.77
Contig12460_at	no hit		5.75	5.72	6.09	6.11	6.53

APPENDIX E

Appendix E

Nucleotide sequences of SSH clones considered candidates in Chapter 3

>B4.1

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>B4.2

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>B7

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>B9

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>MA_1C81

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>NMAP1A8

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>NMAP1D1

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>NMAP1D3

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>NMAP1D4

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