TÜBİTAK

Turk J Agric For
33 (2009) 191-202
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doi:10.3906/tar-0806-22

Microarray Analysis of Late Response to Boron Toxicity in Barley (*Hordeum vulgare* L.) Leaves

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Received: 17.06.2008

Abstract: DNA microarrays, being high-density and high-throughput, allow quantitative analyses of thousands of genes and their expression patterns in parallel. In this study, Barley1 GeneChip was used to investigate transcriptome changes associated with boron (B) toxicity in a sensitive barley cultivar (*Hordeum vulgare* L. cv. Hamidiye). Eight-day-old aseptically grown seedlings were subjected to 5 or 10 mM boric acid ($B(OH)_3$) treatments for 5 days and expression profiles were determined with DNA microarrays using total RNA from leaf tissues. Among the 22,840 transcripts – each represented with a probe set on the GeneChip – 19,424 probe sets showed intensity values greater than 20th percentile in at least one of the hybridizations. Compared to control (10 µM $B(OH)_3$), 5 mM $B(OH)_3$ treatment resulted in differential expression of 168 genes at least by twofold. Moreover, 10 mM $B(OH)_3$ treatment resulted in at least twofold induction or reduction in expression of 312 transcripts. Among these genes, 37 and 61 exhibited significantly ($P < 0.05$) altered levels of expression under 5 and 10 mM $B(OH)_3$ treatments, respectively. Differentially expressed genes were characterized using expression-based clustering and HarvEST:Barley. Investigations of expression profiles revealed that B toxicity results in global changes in the barley transcriptome and networks of signaling or molecular responses. A noticeable feature of response to B was that it is highly interconnected with responses to various environmental stresses. Additionally, induction of jasmonic acid related genes was found to be an important late response to B toxicity. Determination of responsive genes will shed light on successive studies aiming to elucidate molecular mechanism of B toxicity or tolerance. To the best of our knowledge, this is the first report on global expression analysis of barley seedlings under B toxicity.

Key Words: Barley, Barley1 GeneChip, Boron toxicity, Gene expression, Microarray, Transcriptome analysis

Arpa (*Hordeum vulgare* L.) Yapraklarında Bor Toksisitesine Geç Tepkinin Mikroarray Analizleri

Özet: DNA mikroarrayleri, aynı anda binlerce gen ve bunlara ait ifade seviyelerinin kantitatif analizlerine izin vermektedir. Bu çalışmada, hassas arpa (*Hordeum vulgare* L.) çeşidi Hamidiye yaprak dokularında bor (B) toksisitesi ile ilgili transkriptom değişiklikleri Barley1 GeneChip kullanılarak araştırılmıştır. Aseptik koşullarda büyütülmüş 8 günlük bitkilere 5 gün boyunca 5 ya da 10 mM borik asit ($B(OH)_3$) uygulanmış ve DNA mikroarrayleri ile yaprak dokusunda gen ifade profilleri belirlenmiştir. GeneChip üzerinde herbiri bir prob seti ile temsil edilen 22.840 transkriptten 19.424'ü, en az bir hibridizasyonda, tüm sinyal değerlerinin % 20'lik en düşük diliminden daha yüksek sinyal vermiştir. Kontrol (10 µM $B(OH)_3$) ile karşılaştırıldığında, 5 mM $B(OH)_3$ uygulaması 168 genin ifade seviyelerinde en az 2 kat farklılığa neden olmuştur. Ayrıca 10 mM $B(OH)_3$ uygulaması 312 genin ifade seviyelerinde en az 2 kat artış ya da azalış ile sonuçlanmıştır. 5 ve 10 mM $B(OH)_3$ uygulaması altında bu genler arasından sırası ile 37 ve 61 gene ait ifade seviyeleri anlamlı ($P < 0.05$) farklılıklar göstermiştir. İfadesi farklılık göstermiş genler gen ifadelerine dayalı kümeleme ve HarvEST:Barley ile tanımlanmıştır. İfade profillerinin incelenmesi ile, B toksisitesinin arpa transkriptomunda ve sinyal ya da moleküler tepki ağlarında geniş çaplı değişikliklere neden olduğu ortaya konmuştur. Toksisiteye tepkinin önemli bir özelliği çeşitli çevresel streslere tepki ile

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bağlantılı olmasıdır. Buna ek olarak jasmonik asit ile ilgili genlerin ifadelerindeki artışın B toksisitesine önemli bir geç tepki olduğu bulunmuştur. Bor stresi altında arpanın genel gen ifade analizi üzerine ilk araştırma olan bu çalışma, bor toksisitesinin moleküler mekanizmalarını açıklamayı amaçlayan sonraki çalışmalara ışık tutacaktır.

Anahtar Sözcükler: Arpa, Barley1 GeneChip, Bor toksisitesi, Gen ifadesi, Mikroarray, Transkriptom analizi

Introduction

Boron (B), an essential micronutrient for plant growth and development, is toxic at concentrations slightly above the required amount. Moreover, the range between levels of toxicity and deficiency is narrow for almost all plant species. Graminaceous monocots, such as barley and wheat, require 4 – 10 µg B g^{-1} dry weight (Hu et al., 1996). Sources of high B are soil and prolonged irrigation with B-rich water. Toxic levels of soil B is a serious problem of dry and semi-arid lands (Nable et al., 1997). Soil B generally exists as borax ($Na_2[B_4O_5(OH)_4]$ $8H_2O$), colemanite ($Ca[B_3O_4(OH)_3]$ $2H_2O$), and boric acid ($B(OH)_3$).

Under physiological pH, B exists mainly as $B(OH)_3$,which behaves as a weak acid (pKa 9.24) (Power and Woods, 1997). Boric acid is permeable through the plasma membrane, allowing passive diffusion, which is also facilitated by plasma membrane-located channel proteins (Takano et al., 2006). There is an extensive body of literature concerning B uptake, transport, and functions in plants (Brown et al., 2002; Takeda and Matsuoka, 2008). The most widely accepted role for B in plant nutrition is its role in primary cell wall structure (Matoh, 1997) and maintenance of structural integrity of plasma membranes (Cakmak and Römheld, 1997).

The proposed mechanisms for B toxicity include disruption of cell wall development, cellular division, and development by binding to ribose, either as free sugar or within RNA, NADH, or NADPH (Reid et al., 2004). Several studies have questioned whether the mechanism of B tolerance is originating from uronic acid – a significant component of cell wall pectins – (Mahboobi et al., 2001) or from antioxidant enzyme systems (Karabal et al., 2003). Besides these investigations and evidence of irrelevance of uronic acid content or antioxidant enzymes to B tolerance, it was also shown that B toxicity leads to changes in protein profiles of barley (Mahboobi et al., 2000). Recently it was proposed that tolerance to B in barley is mediated by efflux of B from the roots by a plasma membrane borate anion transporter (Hayes and Reid, 2004; Sutton et al., 2007). Moreover, some B transporter genes have been identified in wheat and barley (Reid, 2007).

Former studies using physiological, biochemical, and genetic approaches concentrated on a single gene or a protein functioning in tolerance to B toxicity. However, global expression analyses of barley under B stress have not been previously investigated. Microarray analyses – one of the most widely employed tools of functional genomics – allow estimations of global gene expression under various cellular and environmental conditions such as B toxicity. The 2 major types of microarrays are cDNA- and oligonucleotide-based chips, one of the latter uses 25 base pair long oligonucleotide probes. These probes are complementary to the 3' end of expressed sequences from a genome, and a set of probes represents a single transcript. As a result of hybridization between these probes and biotin labeled RNAs, a fluorescence signal, which provides quantitative values for gene expression, is produced (Lipshutz et al., 1999; Aharoni and Vorst, 2001).

Recently both cDNA and oligonucleotide based microarrays for barley have been developed (Close et al., 2004) and used for global expression analyses under various abiotic stresses. A recent study monitored expression changes and reported differential regulation of approximately 10% of profiled transcripts in barley leaves under dehydration shock and drought stress (Talame et al., 2007). Another study reported inductions in expression of genes involved in the methionine cycle in both Zn-deficient and Fe-deficient barley roots (Suzuki et al., 2006). Walia et al. (2006) investigated early responses of barley genes to salinity stress at seedling stage using the Barley1 GeneChip. In one of the first studies involving microarrays for barley, a cDNA array was used to monitor large-scale changes in transcript abundance in drought and salt stress (Ozturk et al., 2002).

Exact mechanisms of B signaling or tolerance to toxicity have still not been elucidated. Moreover, transcriptomes of barley have not yet been investigated under B toxicity. Sensitive cultivars of barley are known

to accumulate more B in leaf tissues compared to tolerant ones. Therefore, this study aims to investigate the gene expression profiles of a sensitive barley cultivar under prolonged B toxicity to elucidate the mechanisms behind signaling and tolerance.

Materials and Methods

Plant material

Seeds of a local, B-sensitive cultivar of barley (*Hordeum vulgare* L. cv. Hamidiye) (Torun et al., 2003) were obtained from Turkish Ministry of Agriculture and Rural Affairs, Central Field Crop Research Institute (Ankara, Turkey). Surface sterilized seeds were aseptically germinated and grown on half strength Hoagland's solution (Hoagland and Arnon, 1950) (pH 5.8) solidified with Phytagel® for 8 days at 23 ± 2 °C with 16 h light (400 µmol m^{-2} s^{-1}) and 8 h dark photocycle with 70% relative humidity. Seedlings were transferred to sterile hydroponic cultures for B treatment, which was applied immediately after transfer as half strength Hoagland's solution containing either 5 mM $B(OH)_3$ (5B) or 10 mM $B(OH)_3$ (10B) for another 5 days under the same physical conditions. Control (C) groups were transferred to half strength Hoagland's solution without extra $B(OH)_3$. Each set of experiments, with a completely randomized design, was repeated 3 times and used as independent biological replicates.

RNA isolation, labeling, and array hybridization

Total RNA was isolated from leaf tissues of 13-day-old barley seedlings using TRIzol reagent (Chomczynski and Sacchi, 1987). Precipitation with ethanol and sodium acetate was performed to remove impurities. RNA integrity and yield were assessed by agarose gel electrophoresis and absorbance at 260 and 280 nm. All samples had 260 to 280 ratios of ~2.0 and clear 18S and 28S ribosomal RNA bands on the agarose gel. Complementary RNA (cRNA) synthesis, biotin labeling, hybridization, and scanning were performed according to the protocols described in the Affymetrix GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/technical/manuals.affx).

Fifteen micrograms of total RNA were used to generate double stranded cDNA by reverse transcription, using the One-Cycle cDNA Synthesis Kit (Affymetrix), SuperScript II and *E. coli* DNA polymerase I. After second-strand synthesis, cDNA was cleaned with GeneChip Sample Cleanup Module (Affymetrix). Synthesis of biotin-labeled cRNA was performed by in vitro transcription, using the GeneChip IVT Labeling Kit (Affymetrix). After another round of cleanup, quantification of labeled cRNA was performed spectrophotometrically at 260 and 280 nm. Then, 20 µg of cRNA product was fragmented by metal-induced hydrolysis at 94 °C for 35 min. The efficiency of the fragmentation procedure was checked by analyzing the size of the fragments on an agarose gel. Each fragmented cRNA sample was then used to prepare 200 µl of hybridization cocktail containing 100 mM MES, 1 M NaCl, 20 mM ethylenediamine tetraacetic acid, 0.01% Tween-20, 0.1 mg ml^{-1} herring sperm DNA (Promega), 0.5 mg ml^{-1} bovine serum albumin (Invitrogen), 0.1% DMSO, hybridization controls, and 10 µg of fragmented sample. Samples were then hybridized for 16 h to Barley1 GeneChip (Affymetrix), which contains 22,840 probe sets (Close et al., 2004) in Hybridization Oven 640 (Affymetrix) at 45 °C and 60 rpm.

After hybridization, arrays were washed in Fluidics Station 450 (Affymetrix) and stained with streptavidin-phycoerythrin (Invitrogen) and biotinylated anti-streptavidin antibody (Sigma), according to the appropriate standard protocol for each array type. Arrays were then scanned with a GeneChip Scanner 3000 (Affymetrix). Hybridization, scanning, and preliminary analyses with GeneChip Operating Software 1.4 were performed at the Middle East Technical University Central Laboratory.

Microarray data processing and analysis

Data from all hybridizations were further analyzed using a GeneSpringGX 9.0 (Agilent) and the probe annotations for the Barley1 GeneChip were updated prior to analysis. Expression values, computed from .CEL files, were processed first by Robust Multiarray Analysis (RMA), which is a model of normalization over multiple arrays. RMA uses only perfect match (PM) probes and includes probe-specific background correction, normalization across all arrays, and median polishing (Irizarry et al., 2003). Filtering on expression levels and fold changes (≥2) were performed for determination of differentially expressed genes. Statistical analyses were performed using one-way ANOVA at $P < 0.05$ – with asymptotic P-value computation – followed by Tukey HSD post hoc test and Benjamini Hochberg FDR multiple testing corrections. Fold change of at least 2 was

considered as an indication of differential expression, where P value of at most 0.05 was considered as an indication of significant alteration in expression. Hierarchical clustering on genes and treatments were performed with euclidean similarity measure and centroid linkage rule. The probe sets that showed differential expression under B treatment were annotated using HarvEST:Barley (version 1.65, assembly 35) (http://harvest.ucr.edu).

Results

Global expression differences in barley leaves under B toxicity were investigated using Barley1 GeneChip. A total of 9 hybridizations with RNA from 3 biological replicates of control (C), 5 mM $B(OH)_3$ (5B), and 10 mM $B(OH)_3$ (10B) treatments were performed. RMA-normalized intensity values from each hybridization and principal component analysis (PCA) of all hybridizations are shown in Figure 1. According to PCA component 2, C hybridizations were separated from 5B and 10B hybridizations. After RMA preprocessing and normalization, initial filtering resulted in 19,424 probe sets with normalized intensity values higher than the 20th percentile in at least 1 out of 9 hybridizations. Data analyses were carried out with resulting 19,424 genes.

Filtering on expression values revealed that 168 and 312 genes were differentially expressed at least twofold compared to C under 5B and 10B treatments, respectively. Scatter plots shown in Figure 2 display these differentially expressed genes and their expression values under 5B or 10B. Among these, 35 were down-regulated and 133 were up-regulated under 5B treatment, and 70 were down-regulated and 242 were up-regulated under 10B treatment. Among differentially expressed genes, 132 were common to both treatments.

Significant ($P < 0.05$) differences were observed in expression levels of 37 and 61 genes under 5B and 10B treatments, respectively. Among significantly altered genes, 31 were common to both 5B and 10B (Figure 3a). Expression levels of genes that showed differential regulation and were common to both treatments are shown in Figure 3b and 3c. All the 31 genes that were significantly altered showed up-regulation under both treatments (Figure 3c). Hierarchical clustering on genes and treatments were performed for 132 differentially (Figure 4a) and for 31 significantly (Figure 4b) expressed genes, which were common to both 5B and 10B treatments. The 132 differentially regulated genes were clustered into 7 subgroups, where the 31 significantly altered genes were clustered into 3 subgroups according to expression based hierarchical clustering (Figure 4).

Annotations of probe sets, representing uncharacterized genes or unigenes, provide useful and suggestive information since they are frequently based on sequence similarity to a known protein or EST in another organism (Clarke and Zhu, 2006). Therefore, recent versions of HarvEST:Barley and GeneSpringGX were utilized and updated annotations for probes were used in analyses. HarvEST:Barley was used to annotate

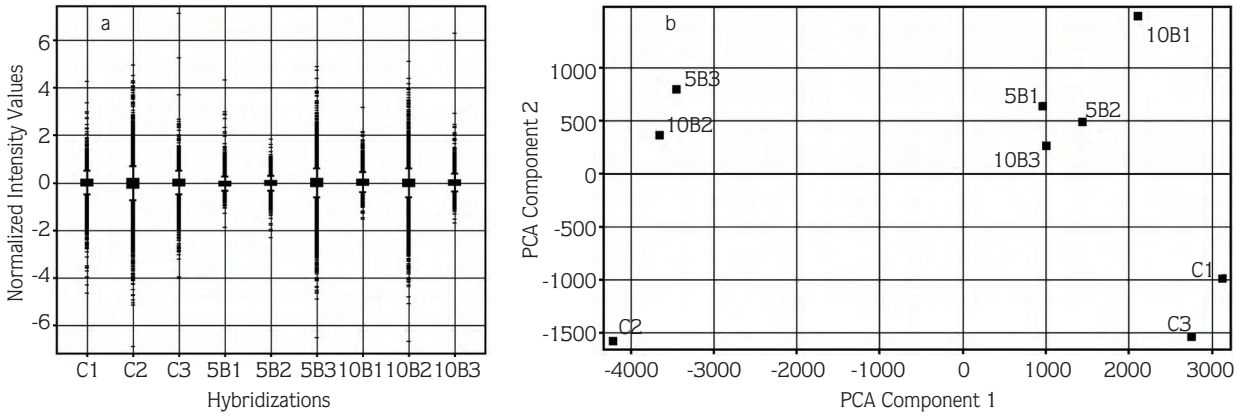

Figure 1. Normalization and principal component analysis. RMA-normalized intensity values from each hybridization (a) and principal component analysis of all hybridizations (b) are displayed. Numbers next to the names of hybridizations indicate different biological replicates (C: Control; 5B: 5 mM $B(OH)_3$ treatment; 10B: 10 mM $B(OH)_3$ treatment; PCA: Principal Component Analysis).

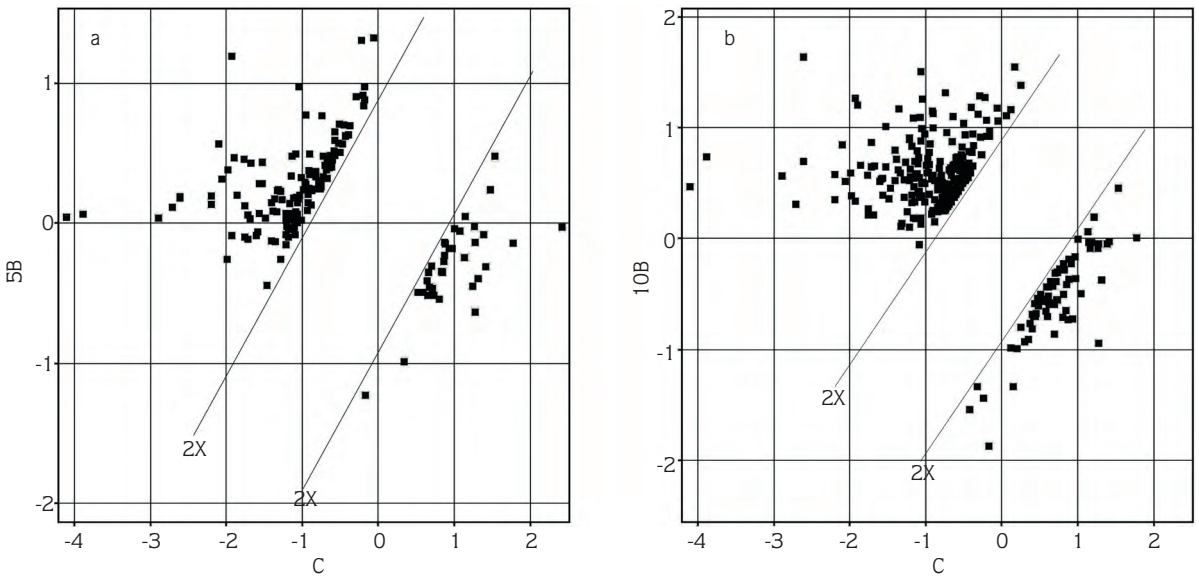

Figure 2. Scatter plots of differentially regulated genes under B treatments and their expression values. Normalized expression values of differentially regulated genes under 5 mM $B(OH)_3$ (a) and 10 mM $B(OH)_3$ (b) treatments are displayed. Both axes in both graphs show normalized expression values. Diagonal lines indicate twofold difference lines. Points above and below the 2X diagonal lines indicate up- and down-regulated genes, respectively (C: Control; 5B: 5 mM $B(OH)_3$ treatment; 10B: 10 mM $B(OH)_3$ treatment).

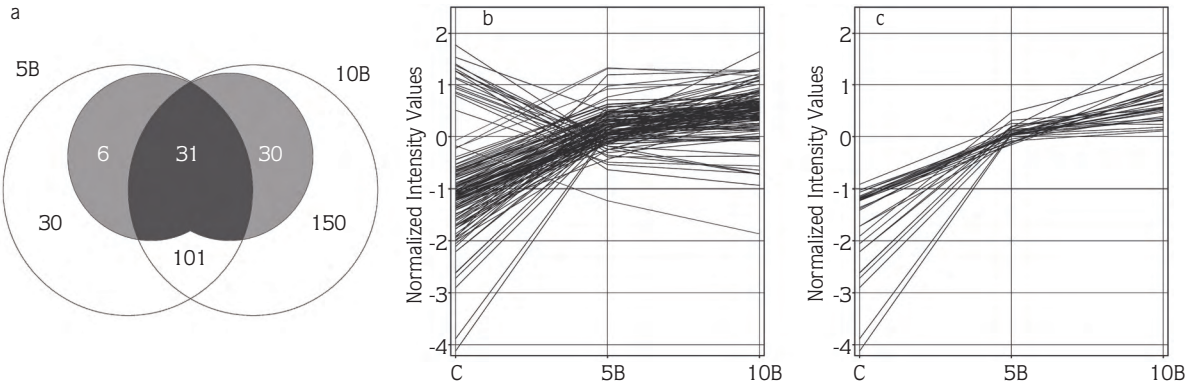

Figure 3. Number and expression patterns of differentially and significantly regulated genes. Venn diagram (a) shows numbers of genes differentially (outer light circles) or significantly (inner dark circles) regulated. Expression patterns of 132 differentially (b) and 31 significantly (c) regulated genes that are common to both treatments are displayed (C: Control; 5B: 5 mM $B(OH)_3$ treatment; 10B: 10 mM $B(OH)_3$ treatment).

differentially and significantly expressed genes. These annotations were used to classify genes involved in or related to transcription regulation, transport, kinase activity, transferase activity, ion binding, oxygen binding, or unfolded protein binding. All genes showing differential expression at least by twofold under B treatments and their annotations are listed in Supplementary Tables 1 and 2. Furthermore, all genes showing significant alterations in expression at $P < 0.05$ level and their annotations are listed in Supplementary Tables 3 and 4.

The output from HarvEST:Barley includes the best BLASTX hit from UniProt database (http://www.expasy.uniprot.org) and best BLASTX hits from *Arabidopsis* and rice TIGR databases (http://www.

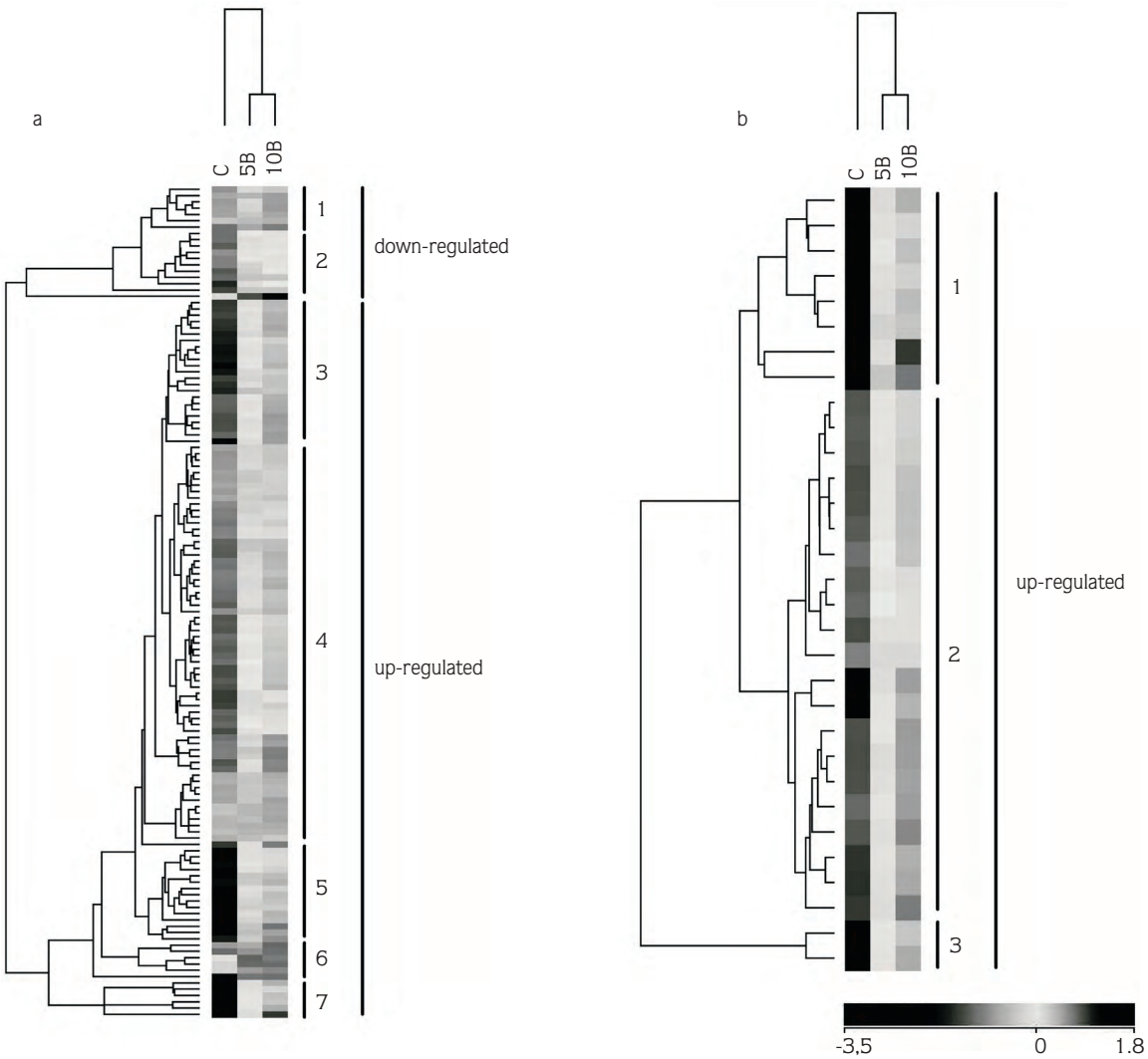

Figure 4. Hierarchical clustering of genes and treatments. Clustering of 132 differentially (a) and 31 significantly (b) regulated genes that are common to both treatments are displayed. Rows and columns represent genes and treatments, respectively. Numbers represent subgroups. Bar represents the colors corresponding to expression values (C: Control; 5B: 5 mM $B(OH)_3$ treatment; 10B: 10 mM $B(OH)_3$ treatment).

tigr.org). Besides descriptions of the best hits, the output also includes UniProt accession numbers and *Arabidopsis* and rice accession numbers of the best BLASTX hits. The output also provided information on which unigenes were represented by a particular probe set. Supplementary Tables 1, 2, 3, and 4 include selected information obtained from HarvEST:Barley output, regulation of genes in 5B and 10B, and P-values for significantly altered genes.

Differentially regulated genes under B toxicity were grouped and discussed for their possible functions in mechanisms of B signaling or tolerance. Boric acid treatment in our experimental conditions resulted in up-regulation of genes involved in jasmonic acid biosynthesis and genes encoding glutathione *S*-transferase (GST), pathogenesis related (PR), or senescence associated (SA) proteins (Table 1). Moreover, alterations were observed in expressions of genes having transcription factor (TF), chaperone, transport, and monooxygenase activities (Table 2). Microarray expression analyses of barley leaves revealed up-regulation of ATP-binding cassette (ABC) transporters and down-regulation of NOD26-like membrane integral proteins under B treatments applied in this study (Table 2).

Table 1. Selected differentially regulated genes that are involved in various plant-specific reactions (JA: Jasmonic Acid; GST: Glutathione S-Transferase; PR: Pathogenesis Related; SA: Senescence Associated; FC: Fold Change; C: Control; 5B: 5 mM $B(OH)_3$ treatment; 10B: 10 mM $B(OH)_3$ treatment).

Category	Probe Set ID	Putative function	FC and regulation C vs. 5B*	FC and regulation C vs. 10B
JA related genes	Contig7886_at	JA-induced protein	2.630 ↑	2.370 ↑
	Contig1675_s_at	23 kDa JA-induced protein	-	2.999 ↑
	rbags15p13_s_at	23 kDa JA-induced protein	-	2.188 ↑
	Contig1678_s_at	23 kDa JA-induced protein	-	2.138 ↓
	Contig1684_x_at	JA-induced protein	-	2.376 ↓
	HV11004r_s_at	Glutamine-dependent asparagine synthetase	-	2.057 ↑
	Contig3097_at	Allene oxide synthase	2.366 ↑	4.376 ↑
	HV_CEb0020D05r2_s_at	Allene oxide cyclase precursor	-	2.760 ↑
	Contig4986_at	Allene oxide cyclase precursor	-	2.512 ↑
	Contig26053_at	S-adenosyl-L-methionine:JA carboxyl methyltransferase	2.474 ↑	3.276 ↑
	HVSMEf0011J01r2_s_at	Lectin protein kinase family protein	2.100 ↑	2.296 ↑
	Contig13905_at	Lectin protein kinase family protein	-	2.193 ↑
	Contig21059_at	Lectin protein kinase	-	2.136 ↑
	Contig3548_at	O-methyltransferase	2.181 ↓	-
	Contig393_at	Alcohol dehydrogenase 3	2.218 ↓	2.501 ↓
GST genes	Contig5838_at	GST	5.200 ↑	8.670 ↑
	Contig2248_at	GST	2.857 ↑	4.173 ↑
	Contig13901_at	GST	2.709 ↑	3.877 ↑
	Contig9764_at	GST	2.781 ↑	4.301 ↑
	Contig12776_at	GST	2.357 ↑	3.096 ↑
	Contig6008_s_at	GST 31	2.033 ↑	2.259 ↑
	HV_CEb0004015r2_s_at	GST 42	2.138 ↑	4.078 ↑
	Contig18367_at	GST 42	-	2.094 ↑
	HVSMEa0014H14r2_s_at	GST 22	-	2.040 ↑
	Contig9632_at	GST 22	-	2.045 ↑
	HVSMEa0011L14r2_s_at	GST	-	2.397 ↑
	Contig12776_s_at	GST	-	2.588 ↑
PR genes	Contig2550_x_at	Wheatwin-2 precursor	2.041 ↑	3.530 ↑
	Contig1637_s_at	Glucan endo-1,3-beta-glucosidase GII precursor	3.174 ↑	4.087 ↑
	Contig1637_at	Glucan endo-1,3-beta-glucosidase GII precursor	2.028 ↑	-
	Contig2210_at	PR protein PRB1-2 precursor	-	2.030 ↑
	Contig2212_s_at	PR protein PRB1-2 precursor	-	2.813 ↑
	Contig15882_s_at	Fatty acid alpha-oxidase	-	2.165 ↑
SA genes	Contig2787_s_at	Thaumatin-like protein TLP5	3.702 ↑	3.947 ↑
	Contig11118_at	B12D protein	2.285 ↑	3.254 ↑
	Contig8605_at	B12D protein	-	2.282 ↑
	Contig8605_s_at	B12D protein	-	2.030 ↑
	Contig14377_at	Glycerophosphoryl diester phosphodisterase	-	2.208 ↑

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

Table 2. Selected differentially regulated genes categorized according to molecular function (TF: Transcription factor; HSP: Heat Shock Protein; CYP: Cytochrome P450; FC: Fold Change; C: Control; 5B: 5 mM $B(OH)_3$ treatment; 10B: 10 mM $B(OH)_3$ treatment).

Category	Probe Set ID	Putative function	FC and regulation C vs. 5B*	FC and regulation C vs. 10B
TF genes	Contig18961_at	Spl7 protein	2.352 ↑	2.928 ↑
	Contig23823_at	C2H2 zinc finger protein	2.319 ↑	2.117 ↑
	Contig3667_s_at	GAMyb	2.020 ↑	3.559 ↑
	EBem10_SQ002_I10_s_at	GAMyb	2.885 ↑	4.386 ↑
	Contig8369_at	AP2D23-like TF	2.102 ↑	-
	Contig4395_at	Ethylene-insensitive-3-like protein	-	2.005 ↑
	Contig13201_at	CCT motif family protein	-	2.198 ↑
	HM07L17r_at	NAC domain TF	-	2.020 ↑
	Contig15617_at	CBF1-like protein BCBF1	-	2.200 ↑
	Contig18390_at	DRE binding TF	2.157 ↓	3.142 ↓
	Contig12005_at	WRKY family TF	2.125 ↓	2.250 ↓
	Contig21110_at	TF WRKY69	-	2.184 ↓
	Contig2479_at	CBF3A-6.1	-	2.317 ↓
	rbaal35o24_at	Heat shock TF	-	2.338 ↓
HSP genes	EBem05_SQ003_L06_at	Small HSP, chloroplast precursor	3.327 ↑	-
	Contig998_s_at	Heat shock cognate 70 kDa protein 2	-	2.020 ↓
	Contig2008_s_at	16.9 kDa class I HSP	-	2.018 ↓
	Contig10029_at	17.8 kDa class II HSP	-	2.918 ↓
Transporter genes	Contig20774_at	MDR-like ABC transporter	5.303 ↑	6.899 ↑
	Contig20553_at	PDR-like ABC transporter	-	2.092 ↑
	H015C14S_s_at	ABC transporter-like protein	-	2.138 ↑
	Contig25386_at	peptide transporter protein	2.291 ↑	3.506 ↑
	HV_CEb0022J21r2_at	peptide transporter PTR2	-	2.501 ↑
	Contig8001_at	amino acid transporter A1	2.027 ↑	-
	Contig21251_at	proton-dependent oligopeptide transporter	-	2.058 ↓
	Contig11285_at	mitochondrial phosphate transporter	2.269 ↑	2.799 ↑
	Contig20673_at	phosphate translocator	-	2.046 ↑
	Contig24175_at	anion/sugar transporter	-	2.484 ↑
	Contig14075_at	P-type ATPase	-	2.121 ↑
	Contig5632_at	NOD26-like membrane integral protein	2.515 ↓	2.834 ↓
	Contig5632_s_at	NOD26-like membrane integral protein	3.739 ↓	4.616 ↓
	Contig15329_at	Probable auxin efflux carrier component 6	-	2.042 ↓
	Contig25699_at	Integral membrane-like protein	2.506 ↓	-
CYP genes	Contig3045_at	CYP709C1	3.011 ↑	3.130 ↑
	Contig3047_s_at	CYP709C1	7.131 ↑	8.154 ↑
	Contig15560_at	CYP71C4	2.090 ↑	2.375 ↑
	Contig15561_s_at	CYP	3.617 ↑	3.901 ↑
	EBro08_SQ004_B22_at	CYP	2.130 ↑	3.777 ↑
	Contig4271_at	CYP	2.026 ↑	-
	Contig17080_at	CYP family protein	-	2.171 ↑

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

Discussion

Effects of B toxicity on transcriptome of a sensitive barley cultivar were investigated with DNA microarrays at 2 concentrations (5 mM and 10 mM) of $B(OH)_3$ (Karabal et al., 2003). Quality of the microarray data obtained by hybridizations of RNA from C, 5B, and 10B to the Barley1 GeneChip was assessed prior to analysis. Box-whisker plot representation of expression intensities, distribution of quartiles, and medians from all hybridizations (Figure 1a) revealed that the samples and expression values obtained by microarrays are comparable. When PCA of all hybridizations (Figure 1b) were examined, it was observed that C samples comprise a separate group according to PCA component 2. This observation indicated that treatments of 5B and 10B give rise to global expression differences compared to C. On the other hand, variation presented by PCA component 1 might be a result of the large biological variation among barley seedlings, although pooling of 7 to 9 seedlings were performed for each replicate.

The number of differentially expressed genes almost doubled when the $B(OH)_3$ treatment was increased from 5 mM (168 genes) to 10 mM (312 genes). Similarly, the number of up- and down-regulated genes under 10B treatment was approximately twice the number under 5B (Figure 3a). Therefore, it might be concluded that differential regulation in expression is dose-dependent. Furthermore, the number of up-regulated genes (133 in 5B and 242 in 10B) was more than 3 times the number of down-regulated genes (35 in 5B and 70 in 10B) for each treatment. Additionally, all significantly regulated genes common to both 5B and 10B showed up-regulation. This suggests that B toxicity regulates global gene expression in barley by induction rather than repression and response to B involves induction of genes.

Hierarchical clustering of treatments placed 5B and 10B on the same branch separating C from both treatments (Figure 4). This result was consistent with the variation presented by PCA component 2 (Figure 1b). Results of hierarchical clustering were used to divide differentially and significantly regulated genes into 7 and 3 subgroups, respectively (Figure 4). Among these, differentially regulated gene subgroup 4 and significantly regulated gene subgroup 2 contained the highest number of genes. Both subgroups comprised genes whose regulation changed less drastically compared to other subgroups (data not shown).

Differentially regulated genes under B toxicity were grouped according to molecular function and discussed for their possible roles in mechanisms of B signaling or tolerance. Abscisic acid, ethylene, and jasmonic acid (JA) are important signaling molecules in plants. JA is a stress hormone produced when the plant is exposed to pathogens or other environmental stress conditions (Truman et al., 2007). Boric acid treatment in our experimental conditions resulted in up-regulation of genes involved in JA biosynthesis and genes responding to elevated levels of JA (Table 1). Among JA-induced genes both up-regulation and down-regulation were observed, whereas most of the JA biosynthesis genes were up-regulated. JA is one of the signaling molecules produced in an integrated signaling network (Devoto and Turner, 2005) and B toxicity might be inducing a response that is connected to the JA regulated response. Walia et al. (2006) also reported the induction of JA related or responsive genes as a key feature of response to salinity in barley. Similarly, Ozturk et al. (2002) reported up-regulation of genes encoding JA-responsive proteins under drought stress in barley.

A large number of probe sets representing the genes that were annotated to be GST were found to be up-regulated at least by twofold (Table 1). GST is proposed to function in protection of plants from oxidative tissue damage during wounding or pathogen attack (Kim et al., 1994). Our results indicated the involvement of GST in protection of barley leaf tissues under prolonged B toxicity. Additionally, up-regulation in expression levels of all differentially regulated genes encoding PR proteins were observed after high level B exposure (Table 1). Induction of such genes involved in responses to biotic stress reveals a possible cross talk between signaling of or response to B toxicity and biotic stresses.

Patterns of up-regulation among all differentially expressed genes of GST and PR proteins were also observed in SA genes (Table 1). Necrotic and chlorotic patches on leaves are characteristic symptoms of B toxicity. Induction of these SA genes might lead to development of chlorotic patches under B toxicity. On the other hand, GST genes might be induced to protect plants from tissue or cell damage during B toxicity induced chlorosis or necrosis.

Genes that were identified as differentially regulated were categorized according to molecular function. Major categories included genes having TF, transport,

chaperone, and monooxygenase activities (Table 2). Category of TF activity included Spl7 protein, myb-type, AP2D23-like, NAC domain containing TFs, and CBF1-like protein BCBF1, which were found to be up-regulated. On the other hand, WRKY family and DRE binding TFs were down-regulated under B toxicity. Previously, 2 *Arabidopsis* myb TFs were shown to provide boric acid tolerance to yeast (Nozawa et al., 2006). These TFs and up-regulation of their expression might confer tolerance in barley by regulating other genes or TFs. Although Spl7 and CBF1 were reported to be responsive to heat and cold stresses, respectively; they might be involved in various types of abiotic stresses. WRKY family and NAC domain TFs comprise large families and function in various plant-specific reactions like development, senescence, and response to biotic or abiotic stresses (Yamasaki et al., 2008). Moreover, WRKY and myb TFs were reported to be regulating senescence, defense against pathogens, and response to drought, cold, or salt (Sperotto et al., 2008; Walia et al., 2006; Seki et al., 2002). Induction of such abiotic (heat, cold, etc.) and biotic stress related TFs and genes, supports the idea of cross talk between certain components of environmental stresses like B toxicity, cold, salt, or pathogen attack. A similar observation for cross talk between salinity, heat, cold, and dehydration stresses in barley was also reported by Walia et al. (2006).

Most of the genes encoding heat shock proteins (HSP) and grouped under category of chaperone activity were down-regulated upon 10B treatment (Table 2). HSPs are known to respond to protein degradation upon various stresses like heat or water deprivation. They play roles in protein folding, establishment of proper protein conformation, and prevention of protein aggregations. Repression of HSP genes pointed out a lesser degree of protein abnormalities under B toxicity in our experimental conditions. Besides HSP down-regulation, a heat shock TF gene represented by rbaal35o24_at was also down-regulated after 10B exposure (Table 2). This TF might be regulating the expression of down-regulated HSPs.

The category of transporter genes included various ABC, peptide, and phosphate transporters, which were up-regulated as a result of either one of the B treatments or both 5B and 10B treatments (Table 2). ABC transporters function in pumping cationic or neutral compounds out of the cell. Barley cells might be inducing genes of ABC transporters to remove excess B accumulated within the cytoplasm. On the other hand, NOD26-like membrane integral protein (NIP), which was annotated to be AtNIP5;1 – B transporter / aquaporin-was down-regulated upon high level B exposure (Table 2). Takano et al. (2006) showed that AtNIP5;1 is induced in *Arabidopsis* plants under conditions of limiting B. Repression of this NIP in barley might help the cell to prevent B influx and keep excess B out. Therefore gene expression results of this study and report of Takano et al. (2006) substantiate the involvement of an aquaporin for B uptake. Overall, induction of ABC transporters and repression of NIPs might work together to lower the amount of B within the cell.

Another functional category included genes having monooxygenase activity. Expressions of genes annotated to be cytochrome P450 (CYP) were up-regulated upon high level of B exposure (Table 2). CYPs are involved in various biosynthetic reactions producing fatty acid conjugates, hormones, or defensive compounds. They are also known to metabolize various endogenous or exogenous compounds in detoxification reactions. CYP709C1, which was up-regulated 7- to 8-fold under B toxic conditions (Table 2), was proposed to be involved in plant defense by producing hydroxylated fatty acids (Kandel et al., 2005). Additionally, genes represented by Contig15660_at and Contig17080_s_at were annotated to be CYPs that function in phenylpropanoid biosynthesis. Phenylpropanoids have a wide variety of functions including defense against pathogens or injury (Golkari et al., 2007), protection from UV, and as signaling molecules or structural components of cell walls. Although B toxicity is an abiotic stress, responses to B toxicity might involve alteration of metabolism and production of defensive compounds and it might be highly associated with responses to other biotic and abiotic stresses.

This study aimed to determine differentially expressed genes of barley under B toxicity. Lists of genes that were significantly altered will enlighten succeeding studies aiming to elucidate molecular mechanism of B toxicity or tolerance. Quantitative PCR analyses to validate the results of this study and further microarray analyses of leaf and root tissues of barley seedlings under B toxicity or deficiency should be performed to unravel B stress.

Electronic Supplementary Material

Supplementary material is available for this article at http://plantbiotech.metu.edu.tr/plantbiotech/rint/marray.htm. All expression data are available through the Plant

Expression Database (PLEXdb) at http://www.plexdb.org/ under experiment BB63 and through the Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/ under series GSE14521.

Acknowledgements

This work was supported by BOREN-2006-25-Ç24-25, BAP-08-11-DPT2002 K120510 and FP6-NMP 17125 (METU-CENTER) grants.

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