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Transcriptome Analysis Reveals Key Genes Involved in the Response of *Triticum urartu* to Boron Toxicity Stress

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Abstract: The domestication and breeding of wheat genotypes through the years has led to the loss in their genetic variation, making them more prone to different abiotic stresses. Boron (B) toxicity is one of the stresses decreasing the wheat cultivars' yield in arid and semi-arid regions around the world. Wild wheat progenitors, such as *Triticum urartu* Thumanian ex Gandilyan, possess a broader gene pool that harbors several genes conferring tolerance to various biotic and abiotic stresses. Unfortunately, *T. urartu* is not well-explored at the molecular level for its tolerance towards B toxicity in soil. In this study, for the first time, we compared the transcriptomic changes in the leaves of a high B-tolerant *T. urartu* genotype, PI662222, grown in highly toxic B (10 mM B in the form of boric acid) with the ones grown in the control (3.1 µM B) treatment in hydroponic conditions. The obtained results suggest that several mechanisms are involved in regulating the response of the studied *T. urartu* genotype toward B toxicity. All the growth parameters of *T. urartu* genotype, including root–shoot length, root fresh weight, and root–shoot dry weight, were less affected by high boron (10 mM) as compared to the boron-tolerant bread wheat cultivar. With a significant differential expression of 654 genes, 441 and 213 genes of *T. urartu* genotype were down- and upregulated, respectively, in the PI662222 leaves in high B in comparison to the control treatment. While key upregulated genes included those encoding RNA polymerase beta subunit (chloroplast), ATP synthase subunit gamma, chloroplastic, 60S ribosomal protein, and RNA-binding protein 12-like, the main downregulated genes included those encoding photosystem II protein D, ribulose bisphosphate carboxylase small subunit, and peroxidase 2-like. Interestingly, both Gene Ontology enrichment and KEGG pathways emphasized the possible involvement of the genes related to the photosynthetic process and apparatus in the high B tolerance of the *T. urartu* genotype. The further functional characterization of the identified potential *T. urartu* genes will facilitate their utilization in crop improvement programs for B toxicity stress.

Keywords: abiotic stress; high boron; gene expression; pathways; red wild einkorn; RNA sequencing; wheat progenitor

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1. Introduction

To feed the world population in 2050, world food production must increase by approximately 70%. As one of the three predominant cereal crops, wheat represents the most extensively cultivated cereal grain on a global scale, constituting approximately one-third of the worldwide cereal output. It occupies a critical position in the maintenance of global food and nutritional security, contributing approximately one-fifth of the worldwide caloric intake and protein sources. [1]. Global wheat production was recorded to be over 781 million metric tons in the marketing year 2022–2023. However, based on the current population growth, an added 132 million metric tons per year is required by 2050 to fulfill the wheat consumption needs. Unfortunately, agriculture suffers from various climate change-related environmental concerns. Wheat productivity is threatened by the unpredictable frequency of acute climatic events, such as drought, heat, salinity, high CO_2, and nutritional deficiencies or toxicities [2]. The adverse effects of these factors on the global wheat economy and wheat quality and yield are a major concern for several wheat breeders [3].

In soil, mobile boron (B) exists in the form of boric acid and borates. Boron enters into plants and then passes through the phospholipid membrane to become distributed [4,5]. While passive diffusion is the method of B entry into plants at normal to high B concentrations, boric acid channels and borate exporters are required to facilitate its movement under low B supply [5–7]. Boron transporters participate significantly in the exclusion of boron from various tissues under conditions characterized by boron toxicity during growth [8]. The uptake of boron and its subsequent loading into the xylem are governed by the channels and transporters located in the root system, whereas the regulation of intracellular boron levels within these tissues is modulated by transporters found in seeds and anthers [9,10]. Aquaporins, especially nodulin-26-like intrinsic proteins (NIPs), actively participate in regulating B transport in plants. These maintain B homeostasis by facilitating B permeation across cell membranes in its molecular form as boric acid [11]. A decrease in the expression levels of specific aquaporins under B toxicity increases the tolerance of plants towards a high B supply [12,13]. As B mostly moves across the xylem due to transpirational force, it becomes accumulated at the tips of the leaves and leads to the chlorosis and necrosis of the edges of the mature leaves when present in excess [14]. In roots, B toxicity has a diminishing effect on length [15]. B toxicity disturbs the physiological and molecular mechanisms in plants by impairing antioxidant defense mechanisms, carbohydrate metabolism, cell division, cell wall stability, and photosynthesis [6,16]. Stunted root–shoot development, nutrient imbalance, and the necrosis of leaf tips are some of the observable symptoms of excessive B supply [17,18]. Though B deficiency in the soil can be handled up to a certain extent by the application of fertilizers, the handling of B toxicity is difficult as the artificial leaching of B toxic soils at a large scale is not realistic [19]. Hence, it is crucial to develop B toxicity-tolerant genotypes that can be grown in these high B-affected regions.

Boron (B) toxicity in the soil represents a significant abiotic stressor that adversely impacts wheat production globally, particularly within arid and semi-arid agricultural zones [20]. Nations including Turkey, Syria, Serbia, Russia, Peru, Pakistan, Morocco, Mexico, Malaysia, Libya, Jordan, Italy, Israel, Iraq, India, Hungary, Egypt, Chile, Australia, and the United States are documented as experiencing detrimental effects from soil B toxicity [21,22]. In Turkey, 25% of the agricultural soils in the Salihli, Menemen, Bursa Kemalpaşa plains, as well as in the Central Anatolia region and Iğdır plains, which are irrigated by rivers, especially the Gediz and are fed by the Simav Mountains where boron deposits are located, have boron toxicity, causing a loss of around 50% in wheat yield. In dry regions, the B present near the roots cannot be leached due to water deficiency and, thus, becomes accumulated on the top soil, leading to B toxicity symptoms in plants [5].

Due to homogeneity in modern cultivars, wheat wild relatives (WWRs) can serve as an important resource for the introgression of boron toxicity tolerance traits in wheat breeding programs [18]. In order to fully exploit their potential, it is crucial to identify the genes that could be involved in conferring tolerance to increased levels of boron. Unfortunately, there exists a paucity of research focused on the assessment of WWRs under B-toxic growing conditions [18,20,23,24]. *Triticum urartu* Thumanian ex Gandilyan, also known as wild einkorn or red wild einkorn, is a diploid wild wheat species and the A genome donor of durum and bread wheat. It is native to the Fertile Crescent region, which includes modern-day Turkey, Iran, Iraq, and Syria. Several successful attempts of the interspecific crossing of *T. urartu* with tetraploid and hexaploid wheat genotypes have been previously reported [25], which makes it an even more beneficial candidate for the breeding programs. Interestingly, despite being a potential genetic resource, *T. urartu* has not been thoroughly studied for boron toxicity tolerance to date. Thus, aiming to fill this gap in the literature, we successfully identified a *T. urartu* genotype, PI662222, that exhibits a high tolerance to boron following an extensive screening of over 150 accessions involving several wild and neglected wheat species.

Furthermore, we developed the hypothesis that the genes expressing differentially under boron toxicity can possibly be involved in providing tolerance to the identified boron-tolerant *T. urartu* genotype. Consequently, in this study, for the first time, we conducted the transcriptome analysis of the leaves of the boron-tolerant *T. urartu* genotype (PI662222) to identify the genes that are differentially expressed under high boron (10 mM boron) as compared to the control (3.1 µM B) treatment.

2. Materials and Methods

2.1. Plant Material, B Toxicity Treatment, and Measurement of Growth Parameters

Initially, in a thorough screening, the boron toxicity tolerance of 158 accessions comprising wild and neglected wheat species was compared with that of the well-known B-tolerant bread wheat (*Triticum aestivum* L.) cultivar Bolal-2973. Bolal-2973 is a Turkish bread wheat genotype whose B toxicity tolerance has been identified in a number of previous studies [26]. As a result, a high B-tolerant *T. urartu* genotype (PI662222) that was received from NSGC, USDA (National Small Grains Collection, U.S. Department of Agriculture), was identified. Furthermore, in the year 2021, the experiments were carried out in Selcuk University, Turkey, and seeds of both the accessions (Bolal and PI662222) were kept in dark for 3 days to germinate. After 3 days of growth, when they turned into seedlings, three biological replicates each of the B-tolerant *T. urartu* and *T. aestivum* genotypes were transferred to hydroponic pots containing 1/5th Hoagland's solution. Moreover, after a four-day growth period, during which the plants attained the 3–4-leaf stage, the plants were subjected to a control treatment [1/5th Hoagland with 3.1 µM B] as well as a toxic boron treatment [10 mM B], which persisted for a duration of seven days [17,20,21]. Boron was incorporated into the Hoagland solution in the form of boric acid (H3BO3). The growing conditions within the hydroponic chamber were maintained at a light intensity of 16,000 Lx/day, a temperature of 22 ± 10 °C, a light/dark photoperiod of 16/8 h, and a humidity level ranging from 45% to 55%. The growth parameters of the harvested genotypes, which included shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW), were assessed after the completion of seven days of treatment. Moreover, liquid nitrogen and Rnasezap (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithunia) were used to collect three shoot replicates of the *T. urartu* genotype from both treatments for molecular analysis, and the samples were stored at −80 °C.

2.2. RNA Extraction, Construction of cDNA Library, and RNA-Sequencing of the Collected Leaf Samples of T. urartu Genotype

A manual RNA extraction protocol utilizing 1000 µL of QIAzol-Lysis Reagent (Qiagen, Hilden, Germany) was implemented to isolate RNAs from the triplicate leaf specimens of the *T. urartu* genotype PI662222 [22]. The resultant RNA pellet was dissolved in 100 µL of double-distilled nuclease-free water. The RNA integrity score (RIS) values, quantity, and quality of the extracted RNA samples were estimated using the QIAxcel advanced fragment analyzer (Qiagen, Hilden, Germany), nanodrop spectrophotometer (Thermoscientific, Waltham, MA, USA), and 1% agarose gel, respectively. For RNA sequencing, two samples were prepared by mixing equal amounts of RNA from all three biological replicates from the control and high B treatments of the *T. urartu* PI662222 genotype. The two cDNA libraries were prepared using the RNA samples with RIN values greater than 7 and A260/A280 between 2.0–2.10. The DNBseq platform (MGI Tech, Shenzhen, China) was used to conduct sequencing. Through the utilization of the oligoDT selection methodology, approximately 96 million high-fidelity paired-end reads were generated for each sample, exhibiting over 97% of Q20 bases and exceeding 90% of Q30 bases. For the reference genome mapping and quantification of transcripts, the good quality reads with a phred score >30 from both treatment samples were deployed as clean reads.

2.3. Determining Differentially Expressed Genes

The HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts) v2.0.4 and Bowtie2 v2.2.5 software applications were employed to facilitate the alignment of high-quality reads with the *T. aestivum* reference genome (IWGSC_CS_RefSeq_v2.1) and corresponding reference genes, respectively. The RSEM and Cufflinks v2.2.2 software tools were utilized for the quantification of gene expression for each sample, as well as for the normalization of gene/transcript expression utilizing the FPKM (fragments per kilobase of transcript per million fragments mapped) methodology, respectively. FPKM values provide an idea of the differential fold change in the gene under stressed conditions and FDR (false discovery rate) values, where a higher fold change and significant expression difference are denoted by smaller FDR values. Genes exhibiting an adjusted *p*-value of $\leq$0.01, a false discovery rate (FDR) of $\leq$0.001, and a log2FoldChange of $\geq$1 were classified as significantly differentially expressed genes (DEGs).

2.4. Functional Annotation, Pathway Enrichment Analysis of DEGs, and DEG-Encoding Transcription Factors

The enrichment analysis identifies the genes that are substantially enriched in a particular pathway, molecular function, or biological process. Through Gene Ontology (GO) enrichment analysis, DEGs with particular biological functions are filtered out and GO functional terms that are significantly enriched in DEGs relative to their genomic background are identified. The enriched GO terms were deemed significantly enriched in DEGs if their Q value (corrected *p*-value) was less than 0.05. Using KEGG pathway enrichment analysis, the metabolic, signal transduction, or biochemical pathways with a Qvalue $\leq$ 0.05 were identified as being significantly enriched in DEGs. Additionally, the transcription factors (TFs) that encode DEGS and their family were identified.

2.5. Reverse Transcription and RT-qPCR of RNA Samples Isolated from the Leaves of T. urartu Genotype Grown Under Control and B Toxic Conditions

From the RNA sequencing results, three genes exhibiting significant expression under B toxicity in comparison to the control treatment were chosen at random, and their differential expression was confirmed using RT-qPCR. Target genes were normalized using the glyceraldehyde-3-phosphate dehydrogenase gene (*TaGAP*) as the internal control [27].

Primers for the chosen genes were created using the NCBI Primer-BLAST program for RT-qPCR. The purified total RNA samples from various biological replicates were reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and oligo-dT primers. Once the cDNA samples were diluted to a 1:10 ratio, 100 ng of cDNA, 2 µL (10 µM) of the forward and reverse primers, 8 µL of Maxima Sybr Green qRT-PCR Master mix (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithunia)), and 6 µL of RNase free DEPC-treated water were combined to create a total volume of 20 µL for the qPCR reactions. All qPCR reactions were pre-denatured at 95 °C for 6 min, followed by 35–45 cycles of 95 °C denaturation for 10 s, 45–60 °C annealing for 30 s, and 72 °C extension for 30 s. All of this was conducted with three biological and three technical replicates. Melting curves were then obtained after a gradual heating from 50 °C at 4 °C/s to 95 °C at 0.1 °C/s. Melting curves were used to determine the standard error of mean in the expression of target and reference genes for two treatments, as well as the specificity of the individuals. The Livak and Schmittgen [28] method was used to perform the relative quantification.

3. Results

3.1. Effect of B Toxicity Stress on Physiological Growth of T. urartu PI662222 and T. aestivum Bolal 2973

Root–shoot growth parameters have been largely used to obtain the tolerance level of wheat genotypes under different stresses [26,29]. In this paper, the level of tolerance of the *T. urartu* genotype PI662222 towards B toxicity stress was compared with that of the high B-tolerant bread wheat genotype Bolal-2973 based on the growth parameters (Tables 1 and S1). The *T. urartu* genotype showed less yellowing of leaves than the *T. aestivum* genotype Bolal 2973 under a high B treatment (Figure S1). Under high B, *T. urartu* demonstrated an increase of 8% in SL and a decrease of only 28% in RL, whereas Bolal demonstrated a decrease of 28% and 33% in SL and RL, respectively, in comparison to the control treatment (Table 1). In a similar way, under B toxicity stress, Bolal's shoot fresh weight (SFW) and root fresh weight (RFW) dropped by 23% and 52%, respectively, while *T. urartu*'s decreased by 33% and 14%. The shoot dry weight (SDW) of *T. urartu* remained unchanged under high B, but the SDW of Bolal decreased by 1%. Comparing the B-toxic growing conditions to the control treatment, the root dry weight (RDW) of *T. urartu* and Bolal dropped by 7% and 36%, respectively (Table 1).

Table 1. Percent changes in growth parameters of the check cultivar *T. aestivum* genotype, Bolal 2973, and *Triticum urartu* genotype PI662222 grown in boron (B) toxic (10 mM B) conditions as compared to the control treatment. The minus sign (−) represents the reduction in percentage.

Traits/Genotypes	Bolal 2973 *Triticum aestivum* L.	PI662222 *Triticum urartu* L.
Shoot Length	−28	8
Root Length	−33	−28
Shoot Fresh Weight	−23	−33
Root Fresh Weight	−52	−14
Shoot Dry Weight	−1	0
Root Dry Weight	−36	−7

3.2. RNA Sequencing and Genome Mapping of Sequenced Reads

In RNA sequencing, the leaf samples of the *T. urartu* genotype grown in boron-toxic conditions were compared to the leaf samples of the same genotype grown in the normal boron (control) growing conditions. Thus, two RNA samples were prepared by pooling leaves of 15 plants from three biological replicates of the B-tolerant *T. urartu* genotype from

control and B toxic treatments. The DNBseq platform produced two cDNA libraries from the RNA samples of the control and high B treatments, yielding a total of 135.26 million clean reads with an average of 67 million reads each (Table S2). A total of 12.60 and 12.15 million high-quality clean reads from the two *T. urartu* libraries were mapped to the *T. aestivum* reference genome (IWGSC_CS_RefSeq_v2.1), yielding distinct mapping ratios of 4.30 and 4.02%, respectively. The control and high B treatment libraries provided a total of 26,602 and 27,981 genes, as well as 29,378 and 30,744 transcripts, respectively. RNA sequencing provides information on novel genes or transcripts that can be involved in particular stress [30]. A total of 7402 novel transcripts and 2254 novel genes were identified in this study (Table S2).

3.3. Differentially Expressed Genes of T. urartu Leaves Under B-Toxic Growing Conditions

While a total of 32,777 genes were differentially expressed in the two libraries, high B led to significant regulation changes in 654 genes of *T. urartu* leaves, where 213 genes were upregulated and 441 genes were found to be downregulated (Figure 1).

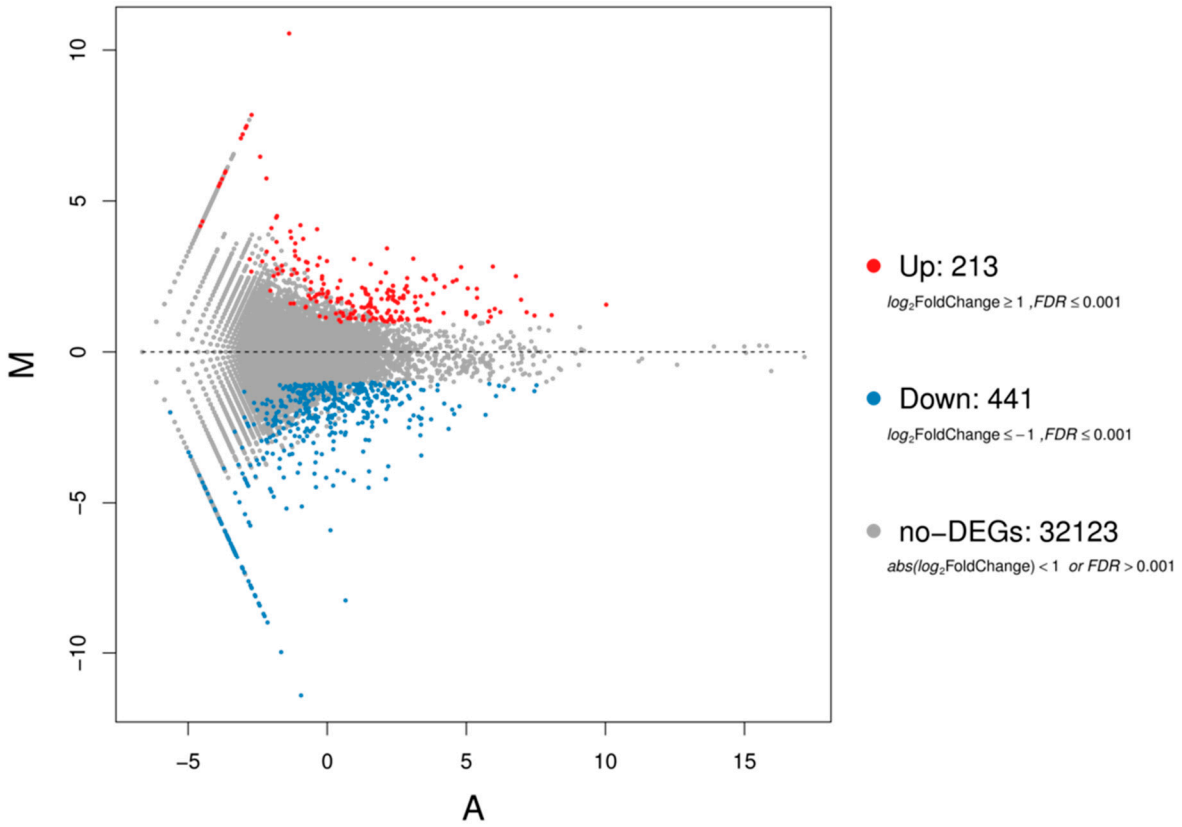

Figure 1. The MA plot displays genes that are differentially expressed (blue and red dots) in the leaves of the *T. urartu* (Tu) genotype PI662222 treated with high boron (B) (TB 10 mM) as opposed to 3.1 µM B (control) treatment. The log2-transformed mean expression level is represented by the X-axis (A) and the log2-transformed fold change by the Y-axis (M).

3.4. Functional Classification of Differentially Regulated Genes via Gene Ontology (GO) Enrichment

To determine the most highly regulated pathways under B toxicity stress, the Gene Ontology Enrichment Analysis (molecular function, biological process, and cellular compartment) was conducted on differentially regulated genes (Figures 2 and S2). The DEGs were classified into 20, 3, and 10 categories in biological process, cellular component, and molecular function, respectively. The most prominent GO terms in the biological process were cellular and metabolic processes with 327 and 315 DEGs, respectively (Figure 2).

While cellular anatomical entity and intracellular were the most dominant GO terms in cellular components with 392 and 260 DEGs, catalytic activity and binding were dominant in molecular function with 331 and 317 DEGs, respectively (Figure 2).

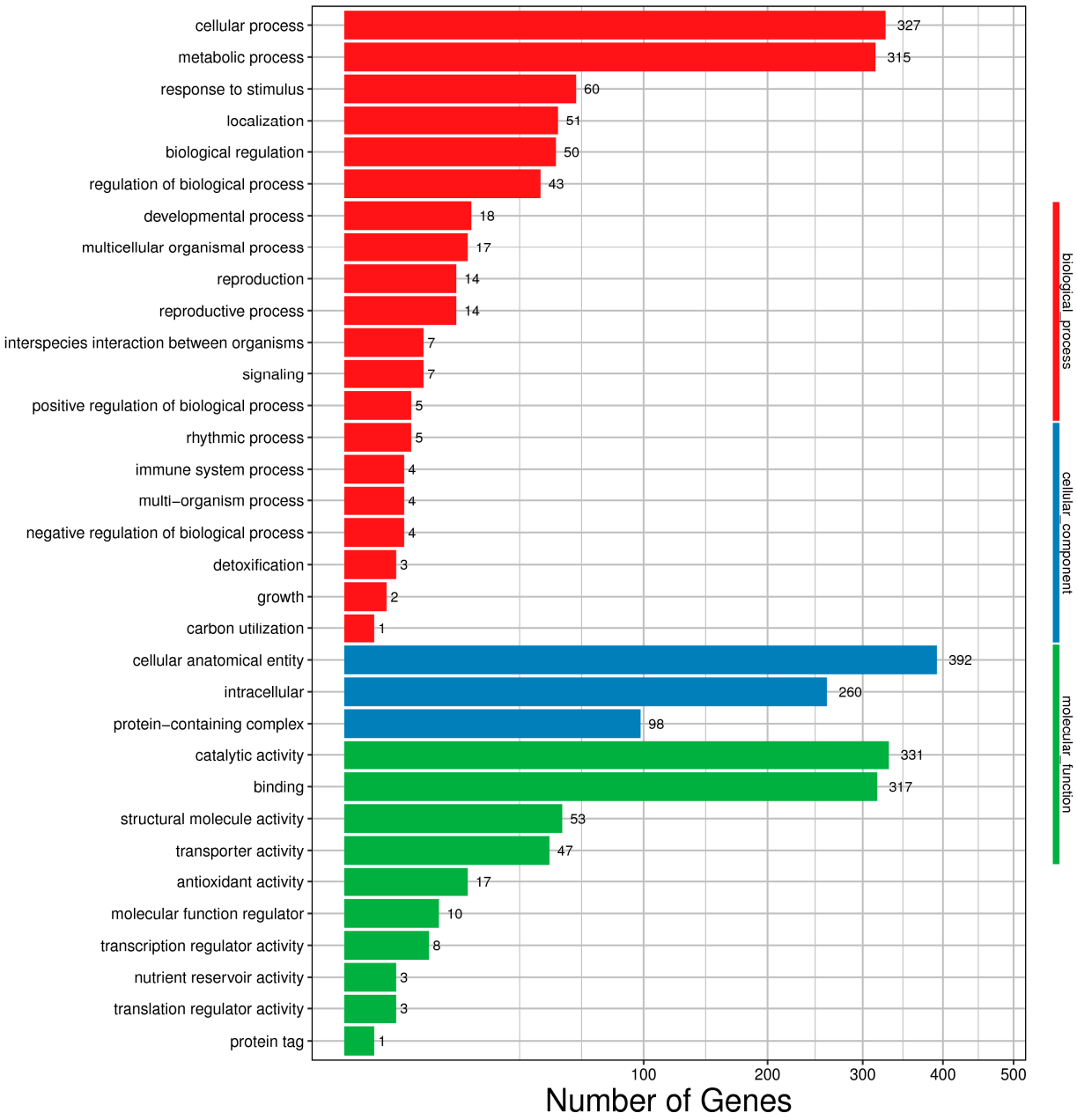

Figure 2. Gene Ontology grouping of differentially expressed genes in *T. urartu* leaves grown in a B toxic environment (10 Mm B) as compared to the control (3.1 µM B) treatment. While the X-axis shows the number of DEGs in different groups, the Y-axis denotes the different Gene Ontology groups.

3.5. KEGG Pathway Enrichment Analysis of Differentially Expressed Genes (DEGs)

Certain biological functions that are facilitated by the typical interaction of genes can be explored by using pathway-based analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was employed to perform the pathway enrichment analysis of DEGs. Consequently, pathway annotation was observed in 865 DEGs that were grouped into five key classes, including metabolism, genetic information processing, environmental information processing, organismal systems, and cellular processes, with 97 KEGG pathways (Figure S3). The pathways with the highest number of DEGs (Table 2) included

metabolic pathways, biosynthesis of secondary metabolites, oxidative phosphorylation, ribosome, and photosynthesis. In Level 2, most of the DEGs were involved in global and overview maps, energy metabolism, translation, and carbohydrate metabolism. The KEGG pathway enrichment showed a maximum enrichment ratio in photosynthesis, followed by oxidative phosphorylation (Figure 3).

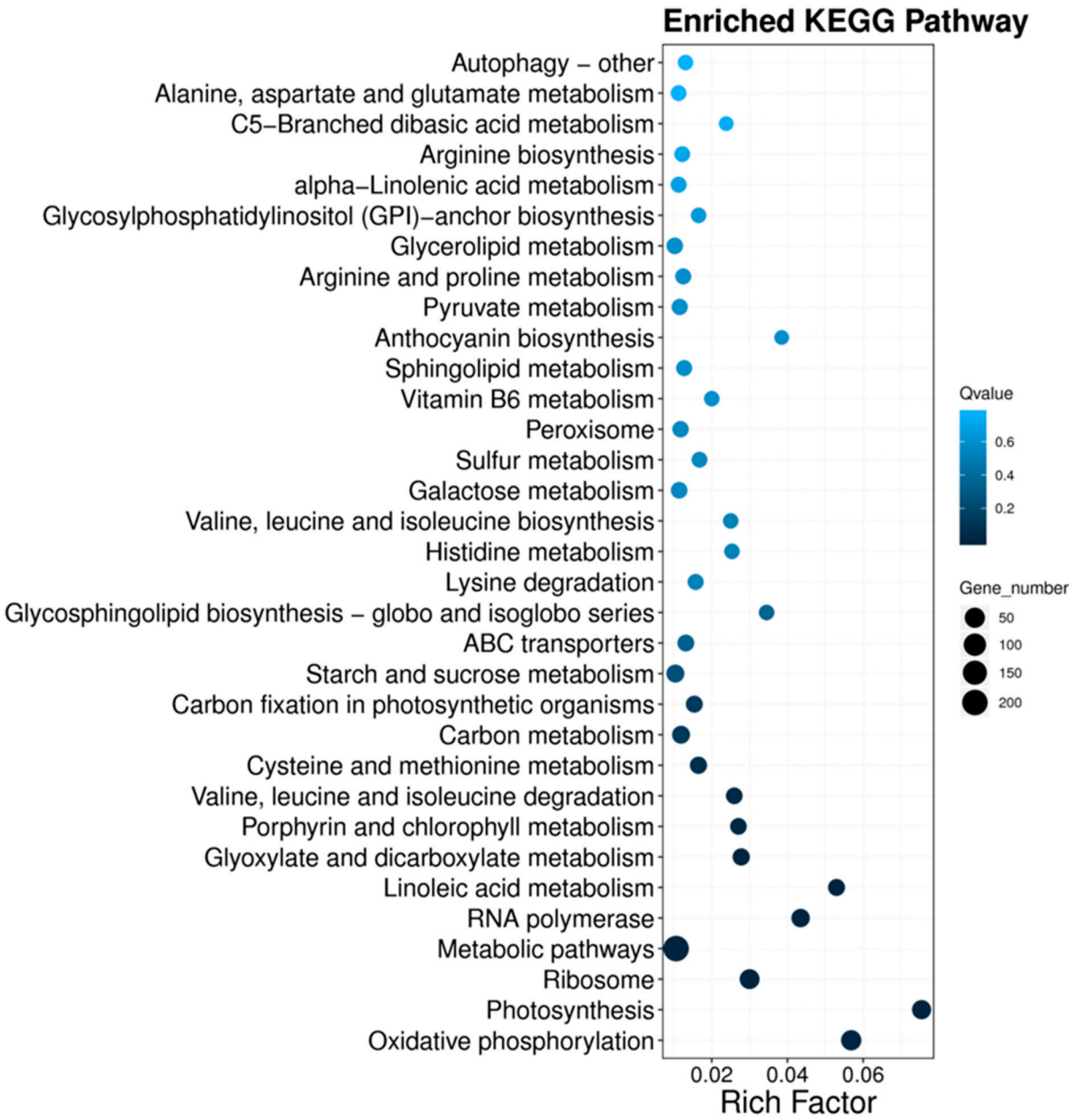

Figure 3. KEGG pathway functional enrichment of differentially expressed genes of *T. urartu* leaves under a B-toxic growing environment (10 Mm B) as compared to the control (3.1 μM B) treatment. The enrichment factor is denoted on the X-axis and the name of the pathway is shown on the Y-axis. The significant enrichment is understood by a larger enrichment factor. The point size belongs to the DEG number.

Table 2. The 10 maximum enriched KEGG pathways in the leaves of the *T. urartu* (Tu) genotype PI662222 grown under B toxicity. Pathway ID is the ID of the pathway. Number of genes is the number of DEGs annotated to a particular pathway.

Pathway ID	#Pathway	Number of Annotated Genes	Level 1	Level 2
ko01100	Metabolic pathways	207	Metabolism	Global and overview maps
ko01110	Biosynthesis of secondary metabolites	72	Metabolism	Global and overview maps
ko00190	Oxidative phosphorylation	54	Metabolism	Energy metabolism
ko03010	Ribosome	47	Genetic Information Processing	Translation
ko00195	Photosynthesis	35	Metabolism	Energy metabolism
ko03020	RNA polymerase	24	Genetic Information Processing	Transcription
ko04016	MAPK signaling pathway–plant	22	Environmental Information Processing	Signal transduction
ko04626	Plant–pathogen interaction	21	Organismal Systems	Environmental adaptation
ko00940	Phenylpropanoid biosynthesis	20	Metabolism	Biosynthesis of other secondary metabolites
ko03013	RNA transport	20	Genetic Information Processing	Translation

3.6. Transcription Factors Regulating the DEGs

Transcription factors (TFs) bind to particular cis-acting elements of genes and control the expression of those genes. Under B toxicity stress, a number of TFs belonging to 58 TF families showed differential expression in *T. urartu* leaves when compared to the control treatment. The families MYB, MYB-related, AP2-EREBP, WRKY, bHLH, NAC, FAR1, and C3H had the greatest number of differentially expressed TFs. These gene families are already known for being vast and participating in the regulation of plant responses to different abiotic stresses (Figure 4).

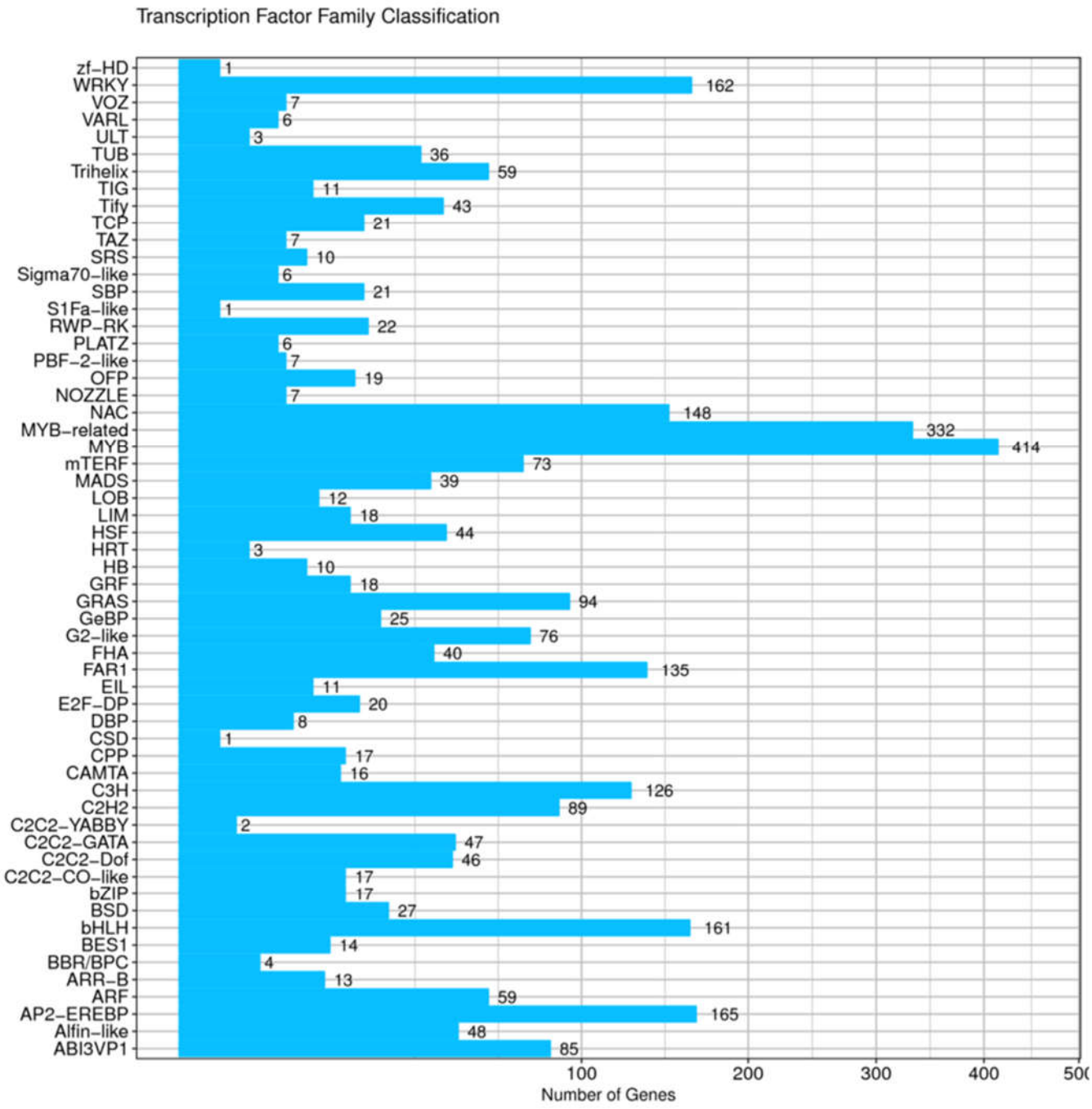

Figure 4. Transcription factor families of differentially expressed genes of *T. urartu* leaves under a B-toxic growing environment (10 mM B) as compared to the control treatment (3.1 µM B).

3.7. Validation of RNA-seq Data by RT-qPCR Analysis

The gene expression data obtained from RNA sequencing were validated via the random selection of three genes [*TuαDOX-like* (TraesCS3B02G549000; α-DOX), *TuGDSL-like* (TraesCS3B02G219300; GELP), and *TuFTSH9-like* (TraesCS3B02G252900; FTSH)) and confirming their level of expression via RT-qPCR (Table 3). The relative gene expression of all the genes under high B stress in RT-qPCR analysis was in agreement with the RNA sequencing results (Figure 5).

Table 3. List of DEGs selected across the *T. urartu* genotype based on the transcriptome sequencing results for RT-qPCR analysis and sequences of the designed primers.

Gene Code	Targeted Gene	Annotation	log2 Fold Change RNA-seq	Primer Type	Sequence (5′->3′)
TuαDOX-like (α-DOX)	TraesCS3B02G549000	PREDICTED: *Triticum dicoccoides* alpha-dioxygenase 1-like (LOC119326475), transcript variant X1, mRNA	−14.98	Forward primer	GGCATGGGTTGAGCCTCTT
				Reverse primer	AACCCATGGCTCTGGTAGAA
TuGDSL-like (GELP)	TraesCS3B02G219300	PREDICTED: *Triticum dicoccoides* GDSL esterase/lipase At4g10955-like (LOC119275874), mRNA	−14.48	Forward primer	AACAGGGAAGTTGAAGGGGA
				Reverse primer	CAATTACCAGCCCTCCCCAG
TuFTSH9-like (FTSH9)	TraesCS3B02G252900	PREDICTED: *Triticum dicoccoides* ATP-dependent zinc metalloprotease FTSH 9, chloroplastic/mitochondrial (LOC119276292), mRNA	−13.66	Forward primer	GCTGAGAAGTGCATCACGCT
				Reverse primer	GTTTCCTTTAAACAATGGCGAGGCA
Reference gene	*TaGAP*	Glyceraldehyde-3-phosphate dehydrogenase	-	Forward primer	TTCAACATCATTCCAAGCAGCA
				Reverse primer	CGTAACCCAAAATGCCCTTG

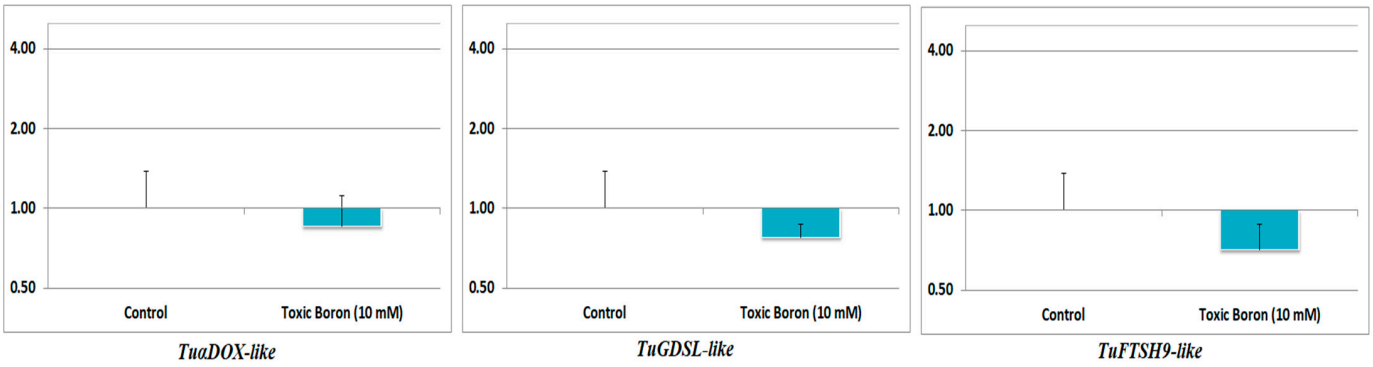

Figure 5. Relative differential expression of *TuαDOX-like*, *TuGDSL-like*, and *TuFTSH9-like* genes with respect to the reference gene, *TaGAP*. The values are provided as average expression values of three individual replicates with the standard error mean normalized to the control.

4. Discussion

4.1. Greater B Toxicity Tolerance in T. urartu Genotype PI662222 than T. aestivum Genotype Bolal-2973

B tolerance in plants is widely predicted via changes in their growth parameters under a high B growin environment [5,18,20,26,29]. The *T. urartu* genotype was less affected by B toxicity in comparison to the *T. aestivum* genotype Bolal-2973, as it showed less symptoms of chlorosis (Figure S1) and less effect on all the growth parameters other than shoot fresh weight (Table 1). The obtained results are similar to the results of previous studies, where wild or neglected wheat species were found to be less impacted by B toxicity as compared to the check bread wheat genotype [20,22–24]. Our results showing a less affected RL of the tolerant *T. urartu* genotype under B toxicity were similar to those of Schnurbusch et al. [31] and Pallotta et al. [26]. In both of these studies, the root length of the tolerant wheat genotypes was greater than that of the susceptible genotypes and, thus, root length was considered as the potential criteria for boron toxicity tolerance.

B toxicity inhibits the cell division or cell expansion, thus restricting plant growth. It can be proposed that cell division or expansion in *T. urartu* is less inhibited by high B and, thus, its shoot and root length are also less affected under high B as compared to the bread wheat genotype. Root–shoot dry mass is an important indicator for the selection of tolerant genotypes towards different stresses [20,32]. Similar to our study, the shoot and root dry weights of B-tolerant Aegilops genotypes and neglected wheat species, *Triticum zhukovskyi* Menabde & Ericzjan were also less affected due to B toxicity as compared to the check wheat cultivar Bolal 2973 [20,22]. The SDW values of both *T. urartu* and Bolal-2973 were less impacted by B toxicity, even though their RDW dropped at high B. Therefore, regardless of the genotypes' level of B tolerance, the roots appear to be more adversely impacted by high B than the shoots [22]. This is justified, as roots are the main site of B uptake. Excessive B hinders root cell division and decreases the size of root meristems, thus reducing root growth. Moreover, B toxicity decreases water uptake and its transport from roots to shoots. This declined water transport disturbs the overall plant water balance, leading to reduced transpiration rates and impaired photosynthesis in the shoots. Thus, B toxicity has a systemic nature affecting both the roots and shoots of wheat plants.

4.2. T. urartu Genes Enriched Under B Toxicity Stress via Gene Ontology Enrichment

The functioning of various upregulated and downregulated genes under B toxicity stress in *T. urartu* via GO enrichment revealed that genes involved in chloroplast and plastid, their thylakoids and thylakoid membranes (GO:0009507; GO:0009536; GO:0055035; GO:0009535; GO:0031976; GO:0009534), cytoplasm (GO:0005737), and ribo-

some (GO:0005840) were notably enriched in high B stress in cellular components. Genes linked to oxidoreductase activity, operating on NAD(P)H, quinone or a similar chemical as an acceptor (GO:0016655), qui-none binding (GO:0048038), and NADH dehydrogenase (quinone) activity (GO:0050136) were enriched in molecular function. Genes linked to the oxidation–reduction process (GO:0055114), the production of precursor metabolites and energy (GO:0006091), the electron transport chain (GO:0022900), and the reaction to photosynthesis (GO:0015979) were among the enriched genes in the biological process grouping. The most enriched GO terms in downregulated genes were cellular anatomical entity, catalytic activity, binding, cellular process, and metabolic process; in upregulated genes, the most enriched GO terms were cellular anatomical entity, cellular process, metabolic process, and intracellular processes. Similar to our results, catalytic activity was one of the well-represented molecular functions in leaf tissues of bread wheat genotypes under B toxicity in an experiment conducted by Kayihan et al. [27]; however, other molecular functions were different.

4.3. KEGG Pathways Enriched in T. urartu Under B Toxicity

The enriched metabolic pathways in KEGG pathways comprised 62 and 145 significantly up- and downregulated genes in *T. urartu* leaves under B-toxic growing conditions, respectively (Table 2, Figure 3). The network of indirect protein–protein interactions constitutes the metabolic pathways. These metabolic pathways are closely associated with photosynthesis, oxidative phosphorylation, and linoleic acid metabolism. The oxidative phosphorylation KEGG pathway comprised 30 and 24 significantly up- and downregulated genes in *T. urartu* leaves in the high B environment. In oxidative phosphorylation, nutrients are oxidized by mitochondrial cells using enzymes, and ATP is produced in the form of chemical energy. Though oxidative phosphorylation is a constructive process producing energy, it also produces reactive oxygen species (ROS) that damage different cellular components. The significant up- and downregulation of several genes of this pathway means that it may have a beneficial and a detrimental role in regulating the tolerance level of *T. urartu* under B stress, respectively. Several studies have reported a lower oxidative stress in tolerant plant genotypes, suggesting that this KEGG pathway needs to be the focus for developing B toxicity tolerance in different plant species [20,33]. Under high B, B toxicity-tolerant *T. urartu* comprised 41 and 6 significantly up- and downregulated genes in the enriched ribosome KEGG pathway, respectively. This was in agreement with the studies that highlighted the inhibitory effect of high B on translation and increased frequency of ribosomes on the stop codons under high B [34,35]. B may have a role in global translational processes as translation takes place on ribosomes. The photosynthesis KEGG pathway comprised 8 and 27 significantly up- and downregulated genes in *T. urartu* leaves in the high B environment. B stress is known to affect the photosynthetic activity of plants. The significant differential regulation of several genes of the photosynthesis KEGG pathway has been reported in tolerant genotypes in different studies [23,36]. As Gene Ontology enrichment also showed the enrichment of genes related to photosynthetic apparatus (chloroplast and thylakoid), genes participating in the photosynthetic pathway must be focused on to develop B toxicity tolerance in susceptible genotypes.

4.4. Enriched Transcription Factors in T. urartu Leaves Under B Toxicity Stress

Transcription factors are the proteins that bind to particular DNA sequences and regulate the transcription rate and expression of genes via either promoting or inhibiting their expression. These factors are considered an important source for the production of stress-tolerant crops via genetic engineering as they actively participate in regulating the expression of several stress-responsive genes as well. In this study, *T. urartu* leaves showed

the maximum number of enriched TFs in MYB and MYB-related families under high B stress (Figure 4). Different physiological and biological processes, including responses to biotic and abiotic stresses, regulation of primary and secondary metabolic reactions, cell morphogenesis, plant growth and development, signal transduction, and hormone synthesis, are regulated by MYB TFs. Parallel to our outcomes, different experiments have suggested the role of MYB and related TFs in regulating the response of tolerant genotypes toward B toxicity stress [27].

A high number of enriched TFs in *T. urartu* leaves under B toxicity stress belonged to the APETALA2/ethylene-responsive element-binding protein (AP2/EREBP) family. The TFs of the AP2/EREBP family are known to be involved in regulating plant responses to biotic stresses and abiotic stresses, such as salinity, drought, heat, and cold [37]. Though the TFs of the AP2/EREBP family are not thoroughly studied for B toxicity stress, their role in the regulation of high B responses has been reported in some plants, including *T. zhukovskyi* [22], sweet cherry [38], and *Triticum dicoccum* (Schrank) Schübl., orth. Var. [23]. WRKY are the plant-specific TFs that regulate transcription in plants' responses towards biotic and abiotic stresses, epigenetic modification, phytohormone production, mitogen-activated protein kinase activation, Ca^{2+} influx, and reactive oxygen species burst. Similar to our study, TFs in the WRKY family were enriched in the roots of *T. zhukovskyi* under B toxicity stress [22]. In addition, the downregulation of WRKY TFs in *T. urartu* leaves in this study was in line with their reduced expression in bread wheat genotypes [27] and *Arabidopsis thaliana (L.) Heynh* [39].

Flavonoid biosynthesis, which is controlled by the TFs of the basic helix–loop–helix (bHLH) family, maintains ROS equilibrium. Similar to the downregulation of bHLH family proteins in bread wheat cultivars [27] and *T. zhukovskyi* roots [22], the four enriched TFs from the bHLH family were markedly downregulated in *T. urartu* leaves under B toxicity. Different abiotic stresses, including drought, salinity, and cold, are regulated by plant-specific transcriptional regulators, NAC TFs [40]. The four enriched TFs from the NAC gene family were significantly downregulated in *T. urartu* leaves under B toxicity similar to the downregulation of the NAC-like TF gene named B EXCESS TOLERANT1 (BET1) in rice [41] and *T. zhukovskyi* roots [22], and contrary to barley leaves, where NAC-domain TFs were increasingly expressed [42].

4.5. Relative Expression of Genes Focused in RT-qPCR

The protein coding gene *TuαDOX-like* belongs to Haem peroxidase (family) is found on chromosome 3B (reverse strand). In the Gene Ontology analysis, while the molecular function of this gene is related to peroxidase activity, oxidoreductase activity, and heme binding, the biological process of this gene is related to fatty acid biosynthetic processes, response to oxidative stress, oxylipin biosynthetic processes, and cellular oxidant detoxification. The reduced expression of Tuα-DOX1 genes in *T. urartu* leaves in both the sequencing and RT-qPCR results suggests its possible role in regulating B toxicity tolerance. The significant differential expression of α-DOX genes in plants has been reported in different stress conditions, such as heavy metal stress, high salinity stress, herbivore attack, cellular signals mediating cell death, and bacterial infection [43]. While some of these experiments revealed upregulated expression, few reported downregulated expression. The differences in the type of expression within the same genotype were dependent on the studied α-DOX genes, target tissues (leaves or roots), and the duration of stress. For example, Tirajoh et al. [44] observed the expression of different α-DOX genes, including Leα-DOX1, Leα-DOX2, and Leα-DOX3, in tomato roots under salinity. Salinity stress decreased the expression of all the Leα-DOX isoforms after 0.5 h. However, after 2 h, it only decreased the transcript levels in Leα-DOX2 and Leα-DOX3. Contrary to our study, Leα-DOX1 showed an upregulation in

roots after 8 h and 24 h of salt stress. This difference can be either due to the utilization of a completely different plant (tomato) or tissue (root) in their study. In *A. thaliana*, similar to our study, the expression of the Atα-DOX1 gene was downregulated under 50 mM of NaCl supply. However, it was upregulated under 100, 150, and 200 mM of NaCl supply. Moreover, the expression of the Atα-DOX1 gene was different in both root and shoot tissues. While the Atα-DOX1 gene was not expressed in shoots in any of the treatments except 250 mM salt stress, in roots, it was expressed in all the treatments. Similarly, in a study conducted by Koeduka et al. [43], the expression of α-DOX genes in rice leaves was dependent on the type and duration of stress treatment. In their experiment, while the expression of the α-DOX gene was increased under bacterial stress, methyl viologen (MV, a generator of intracellular superoxide), sodium nitroprusside (SNP, a generator of NO), $CuSO_4$, or $NiSO_4$, it decreased under hydrogen peroxide, $ZnSO_4$, or $CoSO_4$ supply. In line with our study, Abdeen et al. [45] found that the DOX1 gene of *Arabidopsis* plants showed 1.1-fold decreased expression under drought stress.

TuGDSL-like gene that is the GDSL esterase/lipase At4g10955-like gene encodes the fungal lipase-like domain-containing protein. The gene is located on chromosome 3B (forward strand) and belongs to the GDSL esterase/lipase protein (GELP) family. In Gene Ontology analysis, while the molecular function of this gene is related to hydrolase activity, the biological process of this gene is related to the lipid metabolic process. GDSL esterase/lipases (GELPs) are largely distributed in plants, playing vital roles in growth, development, and stress responses. In line with our study, the gene showed 1.9- and 1.6-fold downregulation in wheat leaves infected by *Xanthomonas translucens* and *F. graminearum*, respectively, as compared to the no infection condition. Likewise, Ren et al. [46] examined 12 GDSL-type lipases related to gummy stem blight resistance in gummy stem blight-resistant wild watermelon accession PI 189225 (*C. amarus*) and found that 9 of them were significantly downregulated after 24 h post-inoculation as compared to 0 h after inoculation.

The ATP-dependent zinc metalloprotease FTSH 9 chloroplastic/mitochondrial (LOC119276292) gene of *Triticum dicoccoides* is homologous to the *TuFTSH9-like* gene that showed reduced expression under high B in our investigation (Figure 5). The gene is located on chromosome 3B (reverse strand) and belongs to the peptidase M41 family in the C-terminal section and the AAA ATPase family in the N-terminal section. In Gene Ontology analysis, the molecular function of this gene is related to nucleotide binding, ATP-dependent peptidase activity, metalloendopeptidase activity, ATP binding, peptidase activity, metallopeptidase activity, hydrolase activity, and ATP hydrolysis activity. Similarly, its biological process is related to proteolysis and response to heat, while its cellular function is related to the cytoplasm, mitochondrion, chloroplast thylakoid, chloroplast envelope, membrane, and intracellular membrane-bounded organelle. Several previous studies showed the downregulated expression of the *FTSH* gene, in line with our results. In agreement with our study, the gene showed 1.5-fold downregulation in wheat leaves infected by *X. translucens* as compared to the no infection condition. In line with our results, ATP-dependent zinc metalloprotease *FTSH* chloroplastic gene showed 0.44- and 0.27-fold decreases in expression after 1 and 3 days of cold stress treatment in potato leaves, respectively [47]. However, the expression was increased by 0.77- and 0.58-fold after 5 and 7 days of cold treatment in the same genotype, respectively. Plant resilience to heat and cold stress is shown to be influenced by the ATP-dependent zinc metalloprotease FtsH family [48].

5. Conclusions

In this study, with a greater tolerance than the well-known B-toxicity-tolerant bread wheat cultivar Bolal 2973, the *T. urartu* genotype showed less devastating effects of B

toxicity on all growth parameters, except the shoot fresh weight. Through the differential regulation of many genes, transcriptional profiling in this study was able to successfully identify putative functional candidate genes implicated in *T. urartu*'s tolerance to B toxicity. As Gene Ontology enrichment and KEGG pathway analyses showed the enrichment of genes related to photosynthesis and its apparatus (chloroplast and thylakoid); genes participating in the photosynthetic pathway must be focused to develop B toxicity tolerance in genotypes. As it is the first transcriptome profiling study on the B toxicity-tolerant *T. urartu* genotype, it is necessary to focus on the obtained outcomes and to conduct the functional characterization of important candidate genes to interpret the role of the gene in providing tolerance to B toxicity. The obtained data not only contribute to fulfilling the deficiency in the literature but also provide relevant information regarding the regulatory and molecular mechanisms underlying the genotype growth under B toxicity. The identified genes (e.g., RNA polymerase beta subunit, ATP synthase subunit gamma, photosystem II protein D) associated with B toxicity tolerance can be introgressed into modern wheat cultivars via marker-assisted selection (MAS) or gene editing (CRISPR-Cas9). Additionally, molecular markers associated to B toxicity tolerance traits can be established employing the transcriptomic data. These markers can facilitate the genomic selection of tolerant lines in breeding programs without demanding extensive field screening.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15010191/s1, Figure S1: Picture showing the comparison of the effect of highly toxic B (10 mM B) on the growth of the *T. urartu* genotype PI662222 and the check cultivar (*T. aestivum* genotype) Bolal 2973 grown in hydroponic conditions for 7 days. Figure S2: GO classification of up- and downregulated differentially expressed genes of *T. urartu* leaves under a B-toxic growing environment (10 Mm B) as compared to the control treatment (3.1 µM B). Figure S3: KEGG pathway classification of differentially expressed genes of *T. urartu* leaves under a B-toxic growing environment (10 Mm B) as compared to the control treatment (3.1 µM B). While the number of genes is denoted by the X-axis, functional classification of KEGG is denoted by the Y-axis. Supplementary Tables: Table S1: Individual values and means with corresponding standard errors (SEMs) of six growth parameters of *Triticum aestivum* genotype Bolal 2973 and *Triticum urartu* genotype PI662222 grown in high B (10 mM boron) growing conditions and the control (3.1 µM boron) (n = 3). Significance of the effects of genotype, treatment, and genotype-treatment interaction (G×T) obtained from two-way ANOVA are indicated as **** for $p < 0.0001$; *** for $p < 0.001$; ** for $p < 0.01$; * for $p < 0.05$; and ns for $p \geq 0.05$ (not significant). Table S2: Results of the quality estimate of reference genome alignment statistics and sequencing data of *T. urartu* (Tu) leaves grown under high B (10 mM B) growing conditions in comparison to the control treatment (3.1 µM B).

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