Insight into the Root Transcriptome of a Boron-Tolerant Triticum zhukovskyi Genotype Grown under Boron Toxicity

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Abstract: Boron (B) toxicity is an important abiotic stress that drastically damages agricultural production worldwide, mostly in arid regions. Several studies have reported large losses in wheat due to high B in arable land. The identification of different B-toxicity-tolerant wheat germplasm and using them in breeding programs to develop tolerant cultivars is a potential solution to B toxicity. However, tolerance to B toxicity in such germplasm largely relies on the molecular changes in plants under B toxicity at the transcriptome level. Thus, the aim of this study is to determine the transcriptomic response of the roots of a B-toxicity-tolerant Triticum zhukovskyi genotype, PI296968, grown in a highly toxic B environment (10 mM B) in comparison with a control (3.1 μM B) treatment. T. zhukovskyi is a hexaploid wheat species forming a separate lineage from the main wheat lineage and can be a good source of genes for various stresses. However, it has been hardly explored for tolerance to any abiotic stress condition. This study is the first in the literature reporting the B toxicity tolerance of a T. zhukovskyi genotype along with the molecular changes occurring in it under B toxicity as compared to the control treatment. In the present study, 5992 genes were found to be significantly differentially expressed, with 1679 and 4313 up- and down-regulated genes, respectively. A number of transcription factors and pathways were identified to be significantly involved in the B toxicity response of the T. zhukovskyi genotype. A total of 12582 novel transcripts were determined in the study, with 9238 and 3344 coding and noncoding transcripts, respectively. The results not only suggest several candidate genes that can be further studied to improve wheat tolerance to B toxicity in upcoming breeding programs, but also enhance the understanding of the regulatory and molecular processes behind the wheat response to B toxicity. Further experiments are suggested to functionally characterize the identified high-B-responsive genes to confirm their role in providing B toxicity tolerance to the plants.

Keywords: abiotic stress; boron toxicity; gene expression; genetic resources; pathways; RNA sequencing; transcriptome; Triticum species; wheat

1. Introduction

Despite the importance of boron (B) as a crucial micronutrient for plant growth [1,2], its excess in soil is a major abiotic constraint for crop yield around the world, mainly in semi-arid and arid agricultural regions [3]. B toxic soil has been previously reported in different countries, including Australia, Chile, Egypt, Hungary, India, Iraq, Israel, Italy, Jordan, Libya, Malaysia, Mexico, Morocco, Pakistan, Peru, Russia, Serbia, Syria, Turkey, and the US [4]. Boron is mobile in the soil either in the form of boric acid or borates. However, water is required for its leaching from the root zone. As arid and semi-arid regions have less water, the amount of water required to leach B from soil remains in-
sufficient, and thus, B accumulates in topsoil, leading to B toxicity in plants and affecting crop production [5–7]. While the application of B fertilizers can handle B deficiency in crops, large-scale washing of B toxic soils is impractical [8]. Under low or normal B conditions, B passes into plants via passive diffusion, whereas boric acid channels and borate exporters help its movement in plants under B-deficient and excess B growth conditions [9]. High B impairs the physiological and molecular processes in plants, affecting photosynthesis, cell division, cell wall stability, antioxidant defense mechanisms, and carbohydrate metabolism [1,9–11]. The visual symptoms of excess B include stunted root growth and chlorosis and necrosis of leaf tips of stressed plants [12–14].

Different processes involved in alleviating B toxicity (BT) include decreasing the cellular active B, decreasing the tissue B concentrations, and improving the physiological tolerance of plants [15]. This occurs via the prevention of B through shallow root systems, its decreased translocation to shoots, and its exclusion from roots [16]. A crucial strategy for dealing with B toxicity is the identification of B-tolerant genotypes and the use of these genotypes in breeding programs to develop tolerant cultivars [15,17]. The mechanism of tolerance towards B stress involves the activation of several molecular, biochemical, and physiological mechanisms in plants and provides the foundation for screening tolerant accessions [15]. The importance of B in the formation of complex networks of pectic polysaccharides and strengthening membrane structure is well established at the molecular level [18]. Several gene families, especially borate exporters, are involved in B efflux, thus maintaining a stable B concentration in cells. The mRNA degradation of the major intrinsic protein NIP5;1 and the decrease in the level of this protein play a role in plant adaptation under B toxic conditions [19]. BOR1 homologs regulate B transfer to the shoot by controlling B efflux in root cells. B transporters push out excess B from symplast to apoplast where greater B concentrations can have comparatively less disastrous effects [20]. The translational inhibition of the BOR1 protein prevents excess transport of B to shoots, allowing plants to survive under B toxicity [21]. The up-regulation of TaBOR1.2 under B toxicity seems to pump out excess B from the tissues, providing more tolerance towards high B [9]. The expression of another wheat ortholog, Ta-BOR2, was reported to reduce B concentration in roots, mostly in tolerant cultivars [22]. Similarly, the expression of BOR4 in root membrane tissues diminishes the entry of B into the xylem [23] and protects plants against B toxicity.

RNA sequencing (RNA-seq) can be employed to understand the presence and quantity of RNA and, consequently, the gene expression in a biological tissue at a particular time [24]. The RNA-seq-based genome-wide transcript profiling allows the detection of differential expression of genes, single nucleotide variants, and transcript isoforms without the prior genome sequence information requirement. The technique has been widely employed to understand the transcriptomic response of different crops to B toxicity stress [9]. These studies facilitate the elucidation of boron toxicity stress responsive pathways and provide information on the genetic variation in stress tolerance traits. Though a number of studies addressed the issue of B toxicity tolerance in wheat and identified differentially expressed genes associated with tolerance to boron toxicity in modern tetraploid and hexaploid wheat [9,25], no information is presented on the level of tolerance of T. zhukovskyi to boron toxicity and the genes associated with the trait.

Wild wheat relatives and their existing domesticated or cultivated forms have enormous potential to develop tolerance against different biotic and abiotic stress constraints [2]. T. zhukovskyi Menabde and Ericz., a hexaploid wheat species, exists only in cultivated forms, similar to T. aestivum. The species with AAvAvAvAvAvAvGG genome have been developed through the hybridization of cultivated einkorn T. monococcum with domesticated T. timopheevii that originated in Turkey [26]. However, Pont et al. [27] proposed hybridization between T. boeoticum and T. araraticum for the origin of T. zhukovskyi. T. timopheevii and T. zhukovskyi form a separate lineage (AAGG) apart from the main wheat lineage (AABB), which is composed of T. turgidum and T. aestivum. Despite the interesting nature of the T. timopheevii lineage, limited studies have been performed
on the group, perhaps because of its limited distribution in the Transcaucasus region [28–
30]. However, the less-explored *T. zhukovskyi* (AAGG) lineage can be a valuable source of
genes for different contemporary biotic and abiotic stress conditions. Considering the
importance of *T. zhukovskyi*, we identified a B-toxicity-tolerant *T. zhukovskyi* genotype in
one of our previous experiments (unpublished data).

The root is the primary tissue that takes part in B uptake and movement in plants. In
this study, we explored the transcriptome of the roots of a B-toxicity-tolerant *T. zhu-
kovskyi* genotype, PI296968, grown under normal and toxic B conditions to gain insight
into the molecular mechanism involved in its B toxicity tolerance. The purpose of this
experiment is to determine the transcriptional regulation, metabolic pathways, and dif-
ferentially expressed genes (DEGs) of *T. zhukovskyi* grown in B toxicity stress. The results
would not only suggest candidate genes that can be further explored to improve wheat
tolerance to B toxicity in upcoming breeding programs, but can also enhance the under-
standing of the regulatory and molecular processes behind the wheat response to B tox-
icity.

2. Materials and Methods

2.1. Plant Growth, B Toxicity Stress Treatment, and Measurement of Root and Shoot Growth
Parameters

We identified a B-toxicity-tolerant *T. zhukovskyi* genotype, PI296968, from a thor-
ough screening of 158 wheat genotypes including wild and primitive species (un-
published data) under hydroponic growth conditions. *Triticum zhukovskyi* genotype
PI296968 is a National Small Grains Collection (NSGC) material collected from Georgia.
Bolal 2973, which is a well-established B-toxicity-tolerant bread wheat genotype [31–33],
was included as a reference genotype in the study to determine the B toxicity tolerance of
PI296968. Again, both the *T. zhukovskyi* genotype and the reference genotype Bolal were
grown in a hydroponic chamber with 16,000 Lx/day light intensity, 22 ± 10 °C tempera-
ture, 16/8 h light/dark photoperiod, and 45–55% humidity. Following a randomized de-
sign, the genotypes were grown in triplicates in two treatments (1/5th Hoagland solution
containing 3.1 μM B considered as control, and 10 mM B considered as highly toxic B).
After surface sterilization, wheat seeds were retained in darkness for 3 days at 22 °C for
germination. Five seedlings of all the genotypes were passed on to sterile hydroponic
pots (containing one-fifth Hoagland’s solution) in three replicates in two different sets
(one set for each B treatment) following germination. Consequently, five plants consti-
tuted one biological replicate, and thus, all 15 plants per genotype were treated with each
of the two B treatments. Hoagland’s solution (control) and required B concentration, 10
mM B, were applied to the plants after 3 days of growth. B treatment continued for 7
days, and the nutrient solution was swapped after every 3 days. On the 7th day of B
treatment, at the tillering stage (Feekes scale 4–5), the roots and shoots were harvested for
measurement of growth parameters of both genotypes, including shoot length (SL), root
length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW),
and root dry weight (RDW). Moreover, root samples of the *T. zhukovskyi* genotype were
collected in triplicates for molecular analysis and directly frozen in liquid nitrogen and
kept at -80°C for RNA isolation.

2.2. RNA Isolation, cDNA Library Construction, and RNA Sequencing of the Collected Root
Samples of *T. zhukovskyi* Genotype

RNA was extracted from three replicates of roots of *T. zhukovskyi* PI296968 grown
under control and B toxic conditions employing the manual total RNA extraction meth-
od. About 0.1–0.2 g of root samples was crushed employing liquid nitrogen followed by
the collection of samples in 2 mL tubes and the addition of 1000 μL QIAzol-Lysis Reagent
(Qiagen, Hilden, Germany). Further, after adding 200 μL of chloroform, samples were
centrifuged at 12000 rpm for 15 min at 4 °C. Later, 500 μL isopropanol was added to the
supernatant, and the mix was again centrifuged at 12,000 rpm for 10 min at 4 °C. The pellet was washed using 1000 μL of 75% ethanol, and after centrifugation at 7500 rpm for 5 min at 4 °C, the obtained pellet was dissolved in 100 μL nuclease-free ultra-pure water. Quality, concentration, and RNA Integrity Score (RIS) values of different RNA samples were checked using 1% agarose gel in 1x TAE buffer (Sigma Aldrich, Saint Louis, MO, USA), a Nanodrop Spectrophotometer, and a QIAxcel Advanced Fragment Analyzer (Qiagen, Hilden, Germany), respectively. Equal quantities of the total RNA of every three biological replicates of T. zhukowskyi PI296968 were pooled together to prepare one sample for RNA sequencing for each of the two treatments, control and B toxic. The RIN value of the samples used for sequencing was more than 7. The cDNA library preparation and MGI sequencing were performed using a DNBseq platform (Illumina, San Diego, CA, USA).

Paired-end reads were generated for each sample with ~34.9 million reads per sample with >96% of Q20 bases and >87% of Q30 bases using the oligoDT selection method. The raw reads were quality filtered to trim adaptors and low-quality bases from 5’ and 3’ ends, and to discard poor quality reads and reads with Ns to ensure the reads selected for transcriptome analysis have a Phred score >30. Approximately 91% of the raw reads were identified to be of good quality for both samples (control and BT-treated) and thus were used as clean reads for mapping with the reference genome and the transcript quantification procedure.

2.3. Identification of Differentially Expressed Genes

The obtained clean reads were aligned to the Triticum aestivum reference genome (IWGSC RefSeq v2.1, https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_018294505.1/) using HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts) software, while the clean reads were aligned to reference genes using Bowtie2 (Johns Hopkins University, Baltimore, MD, USA) to obtain the alignment result. Further, RSEM was used to estimate the gene expression of each sample. The gene/transcript expression levels were normalized using the fragments per kilobase of transcript per million fragments mapped (FPKM) method employing Cufflinksv2.2.2 software (Seattle, WA, USA). The differential fold change of the genes and false discovery rate (FDR) values were estimated based on FPKM values. Smaller FDR values coincide with larger fold changes indicating more significant gene expression differences. Genes with log2 fold change ≥ 1, false discovery rate (FDR) ≤ 0.001, and adjusted p value ≤ 0.01 were considered as significantly differentially expressed genes (DEGs). The statistical significance for the differential expression was assessed using the iDEG R package that identifies differentially expressed genes in two conditions without requiring replicates [34,35].

2.4. Functional Annotation and Pathway Enrichment Analysis of DEGs

Enrichment analysis is conducted to determine whether a particular gene is significantly enriched in a particular biological process, molecular function, or pathway. Gene Ontology (GO) enrichment analysis gives information on the GO functional terms that are significantly enriched in DEGs in comparison with their genomic background and filters out the DEGs that are associated with specific biological functions. All the DEGs were first aligned to each entry in the Gene Ontology database to determine the significantly enriched GO functions in DEGs. The statistical significance of enriched GO terms was determined by calculating the P value and finally the Q value; the GO terms with Q value (corrected P value) ≤ 0.05 were considered to be significantly enriched in DEGs. KEGG pathway enrichment analysis was performed to determine signal transduction pathways or significantly enriched metabolic pathways in DEGs. The pathways with Q value ≤ 0.05 were accepted to be significantly enriched in DEGs, and the most significant signaling, metabolic, and biochemical pathways were determined.
2.5. DEGs Encoding Transcription Factors

The differentially expressed genes with the ability to encode transcription factors (TFs) were determined, and the families of transcription factors to which the differentially expressed genes belong were classified.

2.6. Reverse Transcription and Quantitative PCR (RT-qPCR)

The validation of gene expression patterns obtained from RNA sequencing was conducted employing RT-qPCR for three randomly selected DEGs (Table 1) using the RNA samples of three biological replicates whose samples were pooled and sent for RNA sequencing. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Taq-GAP) was used as an internal control for the normalization of target genes [25,33]. Specific primer pairs for the three selected genes were prepared by employing NCBI Primer-BLAST and are provided in Table 1. The purified total RNA samples were reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo-dT primers employing the manufacturer’s protocol. The obtained cDNA samples were further diluted to a 1:10 ratio to be used for quantitative PCR (qPCR). qPCR reactions were conducted in a LightCycler 96 (Roche, Mannheim, Germany) in a final volume of 20 μL comprising 10 μL Maxima Sybr Green qRT-PCR Master mix (Thermo Scientific, Waltham, MA, USA), 2 μL (100 ng) of 1:10 dilution of cDNA, 1 μL (10 μM) of the forward and reverse primers, and 6 μL of RNase-free DEPC-treated water. All qPCR reactions were conducted in 3 biological replicates and 3 technical replicates. PCR conditions were adjusted to 95°C for 6 min for pre-denaturation accompanied 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. Slow heating from 50 °C at 4 °C/s to 95 °C at 0.1 °C/s and continuous monitoring of the fluorescence signal provided the melting curves. For qPCR reactions of each gene, 3 biological and 3 technical replicates from each treatment and for each of the biological replicates, respectively, were used for a qPCR experiment. The average expression levels of target and reference genes and the standard error of mean in their expression levels for two treatments were determined. The specificity of the individuals was checked using melting curves. Relative quantification was performed using the ΔCt method as described by Livak and Schmittgen [36].

Table 1. List of differentially expressed genes (DEGs) selected from transcriptome sequencing results of T. zhukovskyi roots for RT-qPCR validation, the obtained log2 fold change in RNA sequencing, and the primers used in the study.

<table>
<thead>
<tr>
<th>Gene Code</th>
<th>Selected Target Gene</th>
<th>Targeted Species</th>
<th>log2 Fold Change RNA-seq</th>
<th>Primer Type</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TzG1</td>
<td>TraesCS5A02G234200</td>
<td>Predicted: Triticum dicocoides 1-aminocyclopropane-1-carboxylate oxidase 1-like, mRNA</td>
<td>8.89</td>
<td>Forward primer</td>
<td>GGGGTCCGTCATGTGT1TGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>TCCCTC1GTACCCCCA1ACTACA</td>
</tr>
<tr>
<td>TzG2</td>
<td>TraesCS3D02G325400</td>
<td>Triticum aestivum WAT1-related protein A5f07050-like, mRNA</td>
<td>6.06</td>
<td>Forward primer</td>
<td>TAAATTTGCGGGCCTGGCTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>CTCCCCCGATATGAAAACAAGAA</td>
</tr>
<tr>
<td>TzG3</td>
<td>TraesCS7A02G555600</td>
<td>Predicted: Triticum aestivum mixed-linked glucan synthase 2-like, mRNA</td>
<td>-8.28</td>
<td>Forward primer</td>
<td>AAGTTCCCGGACCTGTACACTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>CTCGGATGCCAGCAATTCACG</td>
</tr>
</tbody>
</table>
3. Results

3.1. Physiological Response of T. zhukovskyi PI296968 to High B Stress

Root–shoot growth parameters are widely used as criteria to recognize the tolerance towards B toxicity in wheat accessions [33,37–39]. Bolal 2973, a well-recognized B-toxicity-tolerant bread wheat cultivar, was used as a reference to evaluate the B toxicity tolerance of the T. zhukovskyi PI296968 genotype. T. zhukovskyi PI296968 showed fewer morphological symptoms of B toxicity such as chlorosis as compared to Bolal (Supplementary Figure S1). Among all the measured parameters, the only parameter in which PI296968 was shown to be mainly influenced by high B stress as compared to Bolal 2973 was RL; RL of PI296968 and Bolal 2973 showed a decrement of 125% and 50%, respectively. For all the other measured physiological growth parameters, the effect of B toxicity was less prominent in PI296968 as compared to Bolal 2973 (Table 2, Supplementary Table S1). The highly toxic B (10 mM) led to only a 1% reduction in the SL of PI296968, while it caused a decrease of 38% in the SL of Bolal. Similarly, SFW and RFW of PI296968 decreased by only 12% and 27%, respectively, under B toxic conditions when compared to the control treatment; while there were decrements of 30% and 108% in Bolal 2973 in these two traits. The SDW of PI296968 showed an increase of 20% under highly toxic B as compared to the control treatment, while Bolal 2973 did not show any increase under the stress treatment. The 10 mM B caused a reduction of up to 15% in RDW in PI296968 and around 53% in Bolal 2973 in comparison with the control treatment.

Table 2. Changes in percentage in growth parameters of the two genotypes, PI296968 (Triticum zhukovskyi) and Bolal 2973 (Triticum aestivum), in 10 mM B (highly toxic B) treatment in comparison with the control treatment. The genotypes were grown in triplicates in two treatments, and each replicate constituted five plants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Parameters/Species</th>
<th>PI296968</th>
<th>Bolal 2973</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot Length</td>
<td>Triticum zhukovskyi</td>
<td>Triticum aestivum</td>
</tr>
<tr>
<td></td>
<td>Root Length</td>
<td>−1</td>
<td>−125</td>
</tr>
<tr>
<td></td>
<td>Shoot Fresh Weight</td>
<td>−12</td>
<td>−30</td>
</tr>
<tr>
<td></td>
<td>Root Fresh Weight</td>
<td>−27</td>
<td>−108</td>
</tr>
<tr>
<td></td>
<td>Shoot Dry Weight</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Root Dry Weight</td>
<td>−15</td>
<td>−53</td>
</tr>
</tbody>
</table>

3.2. RNA Sequencing Data and Reference-Based Annotation

RNA-seq analyses of roots of the B-tolerant PI296968 genotype harvested from B toxicity stress and control conditions were conducted on RNA samples pooled from three independent biological replicates comprising a total of 15 plants. Two cDNA libraries were assembled from pooled RNA samples of control and B toxicity treatments and sequenced on a DNBseq platform, and 69.95 million raw reads were obtained (Table 3). A total of 64.17 million clean reads (91.73% of the raw reads) were obtained following the removal of low-quality reads and adaptors. From each sample, an average of 32.08 million clean reads (3.21 Gb) was found (Table 3). The mean GC content was determined as 55.03%, and the percentages of Phred-like quality scores at the Q30 level ranged from 88.9 to 89.25% (Table 3). From two libraries, 43.34 and 49.68 high-quality clean reads were mapped to the Triticum aestivum reference genome (IWGSC CS RefSeq v2.1, https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_018294505.1/), and the obtained uniquely mapping ratios were 2.29 and 2.89% (Table 3). From the two libraries, control and treatment, 67,704 and 69,713 genes were identified and the total numbers of transcripts were 76,288 and 79,518, respectively (Table 3).
Table 3. Statistics of quality evaluation of RNA-seq data of *T. zhukovskyi* (Tz) roots grown in control (3.1 μM B) and 10 mM B (highly toxic B, TB), information of filtered reads and statistics of alignment with reference genome.

<table>
<thead>
<tr>
<th>Parameters/Sample</th>
<th>Tz_Control</th>
<th>Tz_TB</th>
<th>Parameters/Sample</th>
<th>Tz_Control</th>
<th>Tz_TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Raw Reads (M)</td>
<td>31.44</td>
<td>38.51</td>
<td>Total Gene Mapping Ratio (%)</td>
<td>50.39</td>
<td>60.7</td>
</tr>
<tr>
<td>Total Clean Reads (M)</td>
<td>28.72</td>
<td>35.45</td>
<td>Uniquely Gene Mapping Ratio (%)</td>
<td>16.69</td>
<td>20.76</td>
</tr>
<tr>
<td>Total Clean Bases (Gb)</td>
<td>2.87</td>
<td>3.54</td>
<td>Total Gene Number</td>
<td>67704</td>
<td>69713</td>
</tr>
<tr>
<td>Clean Reads Q20 (%)</td>
<td>97.17</td>
<td>97.17</td>
<td>Known Gene Number</td>
<td>65670</td>
<td>67673</td>
</tr>
<tr>
<td>Clean Reads Q30 (%)</td>
<td>89.87</td>
<td>89.88</td>
<td>Novel Gene Number</td>
<td>2034</td>
<td>2040</td>
</tr>
<tr>
<td>Clean Reads Ratio (%)</td>
<td>91.36</td>
<td>92.06</td>
<td>Total Transcript Number</td>
<td>76288</td>
<td>79518</td>
</tr>
<tr>
<td>Total Genome Mapping Ratio (%)</td>
<td>43.34</td>
<td>49.68</td>
<td>Known Transcript Number</td>
<td>70220</td>
<td>73307</td>
</tr>
<tr>
<td>Uniquely Genome Mapping Ratio (%)</td>
<td>2.29</td>
<td>2.89</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3. Exploration of Novel Transcripts via mRNA Sequencing

One of the key advantages of RNA sequencing is the identification of novel genes and/or transcripts [40]. In this study, we identified a total of 12,582 novel transcripts, out of which 9238 and 3344 were coding and noncoding transcripts, respectively. Among the coding transcripts, 2229 novel genes without any known features and 7009 novel isoforms with previously unknown splicing events for known genes were obtained.

3.4. Identification of DEGs of *T. zhukovskyi* Roots Involved in B Toxicity Stress Response

Though a total of 74120 genes were differentially regulated, after applying the criteria of log2 fold change ≥ 1 and FDR ≤ 0.001, a total of 5992 genes were determined to be significantly differentially expressed in *T. zhukovskyi* roots under B toxicity stress. Among these significant DEGs, 4313 and 1679 were down- and up-regulated genes, respectively (Figure 1).

![Figure 1](image_url)
3.5. Functional Grouping and Gene Ontology (GO) Enrichment Analysis of Differentially Expressed Genes

Gene Ontology analysis was used to determine the functions of *T. zhukovskyi* by categorizing them according to various ontologies. In molecular function, cellular component, and biological process, the DEGs were grouped into 13, 16, and 23 categories respectively (Figure 2, Supplementary Figure S2). In the biological process grouping, the majority of the genes were engaged in the cellular process, followed by metabolic process, response to stimulus, and biological regulation. In the cellular component grouping, membrane, cell, cell part, membrane part, and organelle were the most prominent GO terms. Catalytic activity and binding were the most frequent terms in the molecular function grouping.

![Gene Ontology classification of DEGs in the roots of *T. zhukovskyi* (Tz) genotype grown under highly toxic B (TB 10 mM) treatment relative to those grown in control (3.1 μM B) treatment. X axis signifies the number of DEGs in the molecular function, cellular component, and biological process classifications. Y axis signifies Gene Ontology term.]

**Figure 2.** Gene Ontology classification of DEGs in the roots of *T. zhukovskyi* (Tz) genotype grown under highly toxic B (TB 10 mM) treatment relative to those grown in control (3.1 μM B) treatment. X axis signifies the number of DEGs in the molecular function, cellular component, and biological process classifications. Y axis signifies Gene Ontology term.
3.6. KEGG Pathway Classification and Enrichment Analysis of Differentially Expressed Genes

Genes typically interact with each other to facilitate certain biological functions. These biological functions can be understood by employing pathway-based analysis. The pathway enrichment analysis of DEGs was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The analysis revealed that 5483 DEGs showed pathway annotation and were categorized into five main KEGG classes comprising 129 KEGG pathways (Supplementary Figure S3). Among these pathways, biosynthesis of secondary metabolites, plant–pathogen interaction, metabolic pathways, phenylpropanoid biosynthesis, RNA transport, and MAPK signaling pathway (plant) were the pathways with the highest number of DEGs (Table 4). In Level 2, most of the DEGs were involved in the biosynthesis of other secondary metabolites, global and overview maps, carbohydrates metabolism, translation, signal transduction, environmental adaptation, and amino acid metabolism. The KEGG pathway enrichment showed a maximum enrichment ratio in phenylalanine metabolism followed by nitrogen metabolism (Figure 3).

Table 4. The 15 most enriched KEGG pathways identified in the roots of *T. zhukovskyi* (Tz) genotype grown under boron toxicity. Pathway ID is the ID of the pathway. No. of genes is the number of DEGs annotated to a particular pathway.

<table>
<thead>
<tr>
<th>Pathway ID</th>
<th>Pathway</th>
<th>No. of Genes</th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ko01100</td>
<td>Metabolic pathways</td>
<td>1395</td>
<td>Metabolism</td>
<td>Global and overview maps</td>
</tr>
<tr>
<td>ko01110</td>
<td>Biosynthesis of secondary metabolites</td>
<td>949</td>
<td>Metabolism</td>
<td>Global and overview maps</td>
</tr>
<tr>
<td>ko00940</td>
<td>Phenylpropanoid biosynthesis</td>
<td>497</td>
<td>Metabolism</td>
<td>Biosynthesis of other secondary metabolites</td>
</tr>
<tr>
<td>ko03013</td>
<td>RNA transport</td>
<td>327</td>
<td>Genetic info</td>
<td>Translation</td>
</tr>
<tr>
<td>ko04626</td>
<td>Plant–pathogen interaction</td>
<td>288</td>
<td>Organismal systems</td>
<td>Environmental adaptation</td>
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Figure 3. KEGG pathway functional enrichment of DEGs of the roots of *T. zhukovskyi* (Tz) genotype grown under highly toxic B (TB 10 mM) treatment relative to those grown in control (3.1 μM B) treatment. X axis represents rich factor signifying the enrichment factor. The larger the enrichment factor, the more significant the enrichment. Y axis signifies the pathway name. The color shows the q value (high: white, low: blue), and a lower q value specifies a more significant enrichment. DEG number is indicated by point size.

3.7. DEGs Associated with Transcription Factors

Transcription factors (TFs) govern gene expression by adhering to genes’ specific cis-acting elements. As compared to control, B toxicity stress caused differential expression of 339 TFs belonging to 42 TF families in *T. zhukovskyi* roots. MYB, MYB-related, WRKY, Tify, AP2-EREBP, bZIP, bHLH, and NAC were the families with the highest percentages of differentially expressed TFs. It has already been established that these transcription factors contribute to the way plants react to abiotic stress (Figure 4).
Figure 4. Transcription factor families of the DEGs of the roots of *T. zhukovskyi* (Tz) genotype grown under highly toxic B (TB 10 mM) treatment relative to those grown in control (3.1 μM B) treatment.

3.8. Validation of DEGs by RT-qPCR Analysis

The expression profile of three candidate boron-toxicity-regulated genes was determined by employing RT-qPCR to confirm the RNA sequencing results (Table 1). All three selected genes showed consistent results between RNA sequencing and RT-qPCR. Among the selected genes, two genes were up-regulated and one was down-regulated in *T. zhukovskyi* roots in B toxic growth condition in comparison with the control. Based on the obtained RT-qPCR results, the TzG1 (TraesCS5A02G234200) gene showed a several-fold significant increase in expression; TzG2 (TraesCS3D02G325400) revealed 3-fold up-regulation under B toxicity as compared to control. TzG3 (TraesCS7A02G555600) was significantly down-regulated approximately 4-fold under B toxic growth conditions (Figure 5).
4. Discussion

The genetic diversity of the contemporary wheat gene pool has been severely degraded by the process of domestication, making it prone to several biotic and biotic stress conditions [2]. In this scenario, it is necessary to thoroughly explore different wheat gene pools and determine their level of tolerance to different stress conditions [3]. Moreover, identification of genes of these neglected gene pools involved in tolerance and transferring them to modern cultivars can be an efficient way of developing stress tolerance in today’s genotypes. *T. zhukovskyi* is one of those neglected wheat species that has not been screened at all for B toxicity tolerance. Here, we discuss the changes in the growth and transcriptome of B-toxicity-tolerant *T. zhukovskyi* genotype, PI296968, under high B supply.

4.1. *T. zhukovskyi* Showed a Higher B Toxicity Tolerance Than Bolal 2973

*T. zhukovskyi* PI296968 genotype showed less physiological effects of high B in comparison with the boron-tolerant bread wheat genotype Bolal 2973. The B tolerance in wheat genotypes is frequently determined by root–shoot growth characteristics [3,33,37–39,41]. Most of the measured growth parameters such as shoot length (SL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW) showed a much lesser influence of B toxicity in comparison with Bolal 2973. This was similar to the previously reported studies where growth parameters of wild genotypes were less affected by B toxicity in comparison to the tolerant cultivar [3]. SDW and RDW have been extensively employed to determine the B toxicity tolerance in wheat genotypes [3,38,41,42]. In line with our study, Khan et al. [3] also determined a clear increase in SDW and a lesser decrease in RDW of B-tolerant experimental genotypes from other wheat gene pools in comparison with the check cultivar, Bolal. Moreover, in line with our study, Torun et al. [41] considered the wheat varieties with greater SDW as the tolerant ones. The RDW of the *T. zhukovskyi* genotype was more affected by B toxicity than the SDW, in accordance with the results obtained by Kalayci et al. [43] on wheat cultivars.

4.2. Gene Ontology Classification

The GO classification of up-regulated and down-regulated genes showed that the most enriched GO terms in the biological process grouping were metabolic and cellular processes; in cellular components, the most enriched GO terms were membrane, cell, and cell part; and in molecular function, the most enriched GO terms were binding activity and catalytic activity (Figure 2). The GO enrichment results showed that in cellular
components, genes associated with protein–DNA complex (GO:0032993), nucleosome (GO:0000786), DNA packaging complex (GO:0044815), extracellular region (GO:0005576), and chromatin (GO:0000785) were significantly enriched in B toxicity. In molecular function, genes related to antioxidant activity (GO:0016209), acting on peroxide as an acceptor (GO:0016684), oxidoreductase activity, peroxidase activity (GO:0004601), and protein heterodimerization activity (GO:0046982) were enriched. In the biological process grouping, the enriched genes included genes associated with response to oxidative stress (GO:0006979), reactive oxygen species metabolic process (GO:0072593), hydrogen peroxide metabolic process (GO:0042743), and hydrogen peroxide catabolic process (GO:0042744). These results were in line with the previous studies conducted on B toxicity tolerance in wheat [25].

4.3. Transcription Factors

Due to their function as chief regulators of numerous stress-responsive genes, transcription factors (TFs) are potential candidates for genetic engineering to produce crops that are stress-tolerant [44]. Numerous families such as bZIP, NAC, WRKY, MYB, and AP2/EREBP comprise TFs that regulate a variety of abiotic stresses, and certain TF genes have also been altered to enhance stress tolerance in crop plants. In this study, TFs of MYB and MYB-related families were maximally enriched in T. zhukovskyi roots under B toxicity stress (Figure 4). The MYB TFs are widely dispersed in plants and have been found to have key functions in numerous physiological and biochemical processes, such as hormone synthesis, signal transduction, primary and secondary metabolism, cell cycle, and plant responses to diverse biotic and abiotic stressors. Similar to our results, two Myb TFs of Arabidopsis thaliana, AtMYB13 and AtMYB68, developed tolerance to high B in wild-type yeast [45], showing their role in B homeostasis. The responsive roles of R2R3 MYB TFs towards B toxicity have also been determined in barley [46]. In line with our experiment, in a study conducted by Kayihan et al. [25], two MYB-related genes (ta.8661.1.s1_at and ta.5405.1.s1_x_at) were differentially regulated in the roots of a B-tolerant wheat variety in B stress.

One of the biggest TF families is made up of the plant-specific WRKY TFs. WRKY TFs have been determined to be engaged in several abiotic and biotic stresses via controlling the signal transduction pathway of plant hormones. The significant enrichment of 48 WRKY transcription factors in T. zhukovskyi roots under B toxicity in our study was in accordance with several previous studies [25,47]. Kayihan et al. [25] observed the significant down-regulation of WRKY40 and WRKY53 in roots of both B-tolerant and B-susceptible wheat genotypes in high B. Feng et al. [47] determined that down-regulation of the AtWRKY47 TF led to B toxicity tolerance in plants. Similarly, in our study, all the WRKY TFs showed down-regulation under high B. Interestingly, in contrary to previous studies, one of the transcripts of the WRKY TF family (TraeCS3D02G226300.1) showed up-regulation with a log2 fold change of 4.4 under B toxicity and should be further studied for its functional characterization.

NAC TFs are plant-specific transcriptional regulators with a well-established involvement in the regulation of abiotic stresses such as cold, salinity, and drought in plants [48]. In this study, 11 differentially regulated genes from the NAC gene family were found to be significantly enriched under high B. This was in line with the results obtained by Ochiai et al. [49] in rice where the repression of the NAC-like TF gene named BORON EXCESS TOLERANT1 (BET1) conferred tolerance to B toxicity. A NAC domain TF (HM07L17r_at) was 2-fold up-regulated in barley leaves under 10 mM boric acid treatment [50]. One of the major TF families in plants, the basic leucine zipper (bZIP) gene family, is engaged in several biological activities, involving development and growth along with functions in biotic and abiotic stress tolerance [51]. In our study, 16 differentially regulated genes of the bZIP TF family were found to be enriched under B toxicity. A 2-fold elevation of the At1g03770 TF of the bZIP TF family was observed in the roots of A. thaliana under B toxicity [52]. The C2H2 zinc finger protein based TF gene showed
around 2-fold up-regulation in barley leaves under B toxic conditions [50]. The basic helix–loop–helix (bHLH) family TFs help to control the production of flavonoids, which in turn helps to maintain ROS homeostasis. A total of 13 bHLH family proteins were significantly enriched in *T. zhukovskyi* roots under B toxic growth conditions, most of which were down-regulated. Two genes related to the bHLH TF family were found to up- and down-regulated in B-sensitive and B-tolerant wheat cultivars, respectively [25]. A number of TF families enriched in our study in *T. zhukovskyi* roots under high B such as NAC, bHLH, and MYB have also been found to be expressed under combined boron toxicity and salinity (BorSal) stress, which is a deadly stress combination for agricultural crops [53,54].

### 4.4. Transporters

It has been widely demonstrated that transporter proteins are crucial for maintaining B homeostasis in tissues [9,55]. The expression of genes encoding transporters, such as nodulin-26-like intrinsic proteins (NIPs) and B exporters, is tightly controlled via high B in the growth medium. In addition to facilitating the passage of water through biological membranes, aquaporins provide a pathway for uncharged tiny molecules such as ions, solutes, and glycerol [56]. In our study, three aquaporin NIPs, aquaporin NIP2-2 (TraesCS7D02G188800), aquaporin NIP3-1 (TraesCS1D02G176000), and aquaporin NIP1-3-like (TraesCS7A02G553000), which are homologs of *Aegilops tauschii* were down-regulated under B toxicity in *T. zhukovskyi* roots. The borate efflux transporters are known to be regulated by the content of B in the tissues. The two major-effect quantitative trait loci for boron tolerance in wheat, Bo1 and Bo4, were found to be mediated by root-specific boron transporter genes (*Bot-B5b*, *Bot(Tp4A)-B5c*, and *Bot-B5c*) by Pallotta et al. [33]. In our study, three borate efflux transmembrane transporters (TraesCS5B02G091200, TraesCS5D02G097600, and TraesCS3D02G127300) were significantly down-regulated under high B supply. This was in line with the outcomes obtained in *Citrus macrophylla* W [57], barley [50], and wheat [25], where different NIPs were down-regulated under toxic B.

The flow of sulfate into cells and subcellular compartments is regulated by sulfate transporters, which are essential membrane proteins. Contrary to the results obtained by Öztürk et al. [55] in *Puccinellia distans* (*P. distans*), two sulfate transporters in our study were up-regulated under high B. One was sulfate transporter 2 (TraesCS4B02G264100), and the other was sulfate transporter 3 (TraesCS7D02G084100); they are homologs to *Sorghum bicolor* and *Oryza sativa* Japonica, respectively. Sulfate transporters help plants to deal with abiotic stresses by offering sulfate for cysteine synthesis during ABA biosynthesis [58,59]. Some oxyanions, such as chromate and molybdate, are also transported by sulfate transporters [60,61].

ABC transporters are found to play a role in B homeostasis in different plant species via allowing a variety of substrates to traverse biological membranes, internal detoxification of B, and exporting the B–anthocyanin complexes [25,50]. In our study, 24 and 39 ABC transporters were up- and down-regulated, signifying their possible function in facilitating B homeostasis of *T. zhukovskyi* roots under B toxic conditions. Similarly, ABC transporters previously showed up-regulated expression in B-susceptible barley [50], *P. distans* [55,62], and wheat [25] under high B and down-regulated expression in *P. distans* [55,62]. Not only B transporters, sulfate transporters, and ABC transporters but also several peptide/histidine transporters, proton-coupled amino acid transporters, molybdate transporters, sugar transporters, nitrate/nitrite transporters, sodium borate transporters, ammonium transporters, zinc transporters, and metal transporters were significantly enriched and differentially regulated under high B in *T. zhukovskyi* roots.

### 4.5. Enriched KEGG Pathways

Among the KEGG pathways, phenylalanine metabolism followed by nitrogen metabolism pathways showed the most significant enrichment. However, phenylpropanoid
biosynthesis, biosynthesis of secondary metabolites, and metabolic pathways showed a maximum number of enriched DEGs under B toxic conditions (Figure 3). The biosynthesis of secondary metabolites consisted of 191 up-regulated and 758 down-regulated genes. Several abiotic stress conditions have been linked to the activation of biosynthesis of secondary metabolites in stressed plants [63]. Several secondary plant metabolites, such as indole alkaloids, anthocyanins, phenylpropanoids, and flavonoids, are reserved in plant vacuoles and are known to be associated with enhanced activity of ABC transporters [64,65]. Our results were different from those obtained by Yıldırım and Uyulaş [66] in black poplar where all the genes involved in secondary plant metabolisms were down-regulated under high B. The phenylpropanoid pathway, involving the synthesis of secondary metabolites, acts as the initiation point for the production of several compounds such as lignans, flavonoids, and coumarins that support the combating of stress conditions [67]. In our study, B toxicity led to up- and down-regulation of 75 and 422 genes, respectively.

4.6. Genes Studied in RT-qPCR

The WG1 gene (TraesCS5A02G234200) of T. zhukovskyi, whose up-regulation was confirmed through RT-qPCR (Figure 5), is a homolog of the Triticum dicoccoides 1-aminocyclopropane-1-carboxylate oxidase (ACO) 1-like (LOC119301783) gene that showed Gene Ontology enrichment in metal ion binding and oxidoreductase activity. In enriched KEGG pathways, this gene is involved in cysteine and methionine metabolism, biosynthesis of secondary metabolites, and metabolic pathway. Contrary to our study, the gene was found to be down-regulated under senescence and nitrogen stress [68]. ACO is one of the main enzymes involved in the ethylene biosynthesis pathway, thus having a contribution to adaptation towards different abiotic stresses such as drought and salinity along with a role in developmental growth [69–72].

The WG2 gene (TraesCS3D02G325400) of T. zhukovskyi, which showed enhanced expression under B toxicity (Figure 5), is a homolog of the T. aestivum WAT1-related protein At5g07050-like (LOC123065817) gene that revealed Gene Ontology enrichment in plasma membrane and transmembrane transporter activity. In enriched KEGG pathways, this gene is involved in the metabolic pathway, phenylpropanoid biosynthesis, biosynthesis of secondary metabolites, RNA polymerase, and purine and pyrimidine metabolism. Plant WAT1 (walls are thin1) proteins are necessary for secondary cell-wall formation [73]) and providing tolerance to vascular pathogens [74,75]. A WAT1-related protein that is an auxin transporter was identified as taking part in regulating drought stress in several species such as peanut roots and oaks [76,77]. In line with our study, up-regulation of WAT1-related protein was observed in providing resistance to alkaline stress in wild jujube roots [78].

The WG3 gene (TraesCS7A02G555600) of T. zhukovskyi, which showed down-regulation under B toxicity (Figure 5), is a homolog of the Triticum aestivum mixed-linked glucan (MLG) synthase 2-like (LOC123147229) gene that revealed Gene Ontology enrichment in integral component of membrane, cellulose synthase (UDP-forming) activity, cell wall organization, and cellulose biosynthetic process. MLGs are specific to the cereal grasses order, Poales, and are known to be related to cellulose microfibrils required for cell growth [79]. Contrary to our study, MLG synthase showed up-regulation under drought stress in pearl millet [80].

5. Conclusions

In this study, root transcriptome sequencing and RT-qPCR expression analysis of a boron-toxicity-tolerant T. zhukovskyi genotype, PI296968, grown under control and B toxic conditions was performed. The outcomes suggested crosstalk among several pathways in T. zhukovskyi in response to boron toxicity stress. The differential response included significant regulation of several candidate genes involved in biological regulation, metabolic processes, cellular processes, responses to stimulus, membranes, cells,
catalytic activity, and binding activity. Several *T. zhukovskyi* transcription factors of different families, including the MYB and MYB-related, WRKY, NAC, bZIP, and bHLH families, were significantly enriched under B toxicity. The highest number of DEGs was observed in the phenylpropanoid biosynthesis, RNA transport, plant–pathogen interaction, and MAPK signaling pathways; however, phenylalanine metabolism and nitrogen metabolism were the pathways with maximum enrichment. The B-tolerant *T. zhukovskyi* genotype studied in this experiment can be used as a source of B-tolerant germplasm in breeding programs, and the identified B toxicity stress responsive candidate genes could facilitate additional omics studies in wheat and other cereal crops. The study is the first in the literature on B toxicity tolerance of a *Triticum zhukovskyi* genotype, and further research including transgenic experiments and subcellular characterization is required to functionally characterize the identified high-B-responsive genes to confirm their role in providing B toxicity tolerance. Our findings support the use of different genes from *Triticum zhukovskyi* to enlarge the limited genetic diversity of domesticated wheat. The results described here could be used to improve wheat’s tolerance to high B via molecular breeding or genetic engineering.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102421/s1. Figure S1: Pictures showing the plants of the two genotypes, Bolal 2973 and *T. zhukovskyi* (PI296968), grown in a hydroponic system under highly toxic B (10 mM B) just before the harvest (7th day after B treatment); Figure S2: GO classification of up-regulated and down-regulated genes of roots of the *T. zhukovskyi* (Tz) genotype grown under highly toxic B (TB 10 mM) treatment relative to those grown in control (3.1 μM B) treatment; Figure S3: KEGG pathway classification of DEGs of roots of the *T. zhukovskyi* (Tz) genotype grown under highly toxic B (TB 10 mM) treatment relative to those grown in control (3.1 μM B) treatment. X axis represents number of DEGs. Y axis represents functional classification of KEGG. There are five classes for KEGG pathways: Organismal Systems, Metabolism, Genetic Information Processing, Environmental Information Processing, and Cellular Processes. Table S1: Means of the RL (root length), SL (Shoot length), RFW (root fresh weight), SFW (shoot fresh weight), RDW (root dry weight), and SDW (shoot dry weight) of PI296968 (*T. zhukovskyi*) and Bolal 2973 (*T. aestivum*) grown under control (3.1 μM B) and highly toxic B (10 mM B).

**Author Contributions:** A.P. and M.K.K. conceived and designed the study and wrote the manuscript. A.P. and M.K.K. performed the experiment and analysis. M.B. and M.H. helped in editing the manuscript. M.B., M.H., A.T., and S.G. contributed intellectually to the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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