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Original article

Relationships between some transcription factors and concordantly expressed drought stress-related genes in bread wheat

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ABSTRACT

The challenge of climate change makes it mandatory to improve tolerance to drought stress in bread wheat (*Triticum aestivum*) via biotechnological approaches. Drought stress experiment was conducted followed by RNA-Seq analysis for leaves of two wheat cultivars namely Giza 168 and Gemmiza 10 with contrasting genotypes. Expression patterns of the regulated stress-related genes and concordantly expressed TFs were detected, then, validated via qPCR for two loss-of-function mutants in *Arabidopsis* background harboring mutated genes analogue to those in wheat. Drought-stress related genes were searched for concordantly expressed TFs and a total of eight TFs were shown to coexpress with 14 stress-related genes. Among these genes, one TF belongs to the zinc finger protein CONSTANS family and proved via qPCR to drive expression of a gene encoding a speculative TF namely zinc transporter 3-like and two other stress related genes encoding tryptophan synthase alpha chain and asparagine synthetase. Known functions of the two TFs under drought stress complement those of the two concordantly expressed stress-related genes, thus, it is likely that they are related. This study highlights the possibility to utilize metabolic engineering approaches to decipher and incorporate existing regulatory frameworks under drought stress in future breeding programs of bread wheat.

1. Background

Wheat grain contributes a portion of daily intake of up to 50% in countries like Egypt and Turkey (Seleiman et al., 2010, Shewry and Hey 2015, Shewry 2018, Seleiman et al., 2021). In terms of nutritional value, wheat grain is high in specific proteins (gluten), dietary fibers and B vitamins, which makes wheat one of the most important staple foods of humanity (Ding et al., 2021). Therefore, its ability to withstand abiotic stresses partly due to climate changes is a global demand (Seleiman and Abdel-Aal 2018, Ding et al., 2021, Ding et al., 2021). Environmental stresses, especially drought stress, are major causes of crop losses where yield and its attributes are the most affected traits under stress (Batool et al., 2020, Roy et al., 2021, Sharma et al., 2022). Earlier records of annual yield losses due to drought or water deficit reached 50% for major crops including wheat (Chaves and Oliveira 2004). Nonetheless, drought stress tolerance is the ability of plants to lessen detrimental effects of drought (Farooq et al., 2014). Drought stress is accompanied with several changes at the morphological and physiological levels (Hafez and Seleiman 2017, Sallam et al., 2019, Seleiman et al., 2019, Seleiman et al., 2021). Scoring changes at the molecular level might allow researchers to manipulate stress tolerance mechanisms to make plants able to withstand such stresses via approaches like genetic transformation and metabolic engineering. As wheat genome size (16,000 Mbp) is among the highest across plant species (Consortium et al., 2018), consequences of improving any economically feasible trait via genetic transformation are uncertain. However, a number of transgenic wheat lines have proven to exhibit high levels of tolerance to drought stress due to overexpressing the barley *HVA1* gene for seed desiccation tolerance (Sivamani et al., 2000). Promising records of stability and homozygosity of the transgene were shown across generations in the field (Bahieldin et al., 2005). However, no information is available with regard to appearance of this genetically modified wheat in the market place.

In terms of metabolic engineering approaches, researchers can provoke a given endogenous cellular physiological process(s) to produce a desired compound or metabolite. This can be done after knowing the regulatory elements that drive/regulate expression of

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a given stress-related gene. RNA-Seq datasets can detect expression patterns of stress-related genes along with their coexpressed regulatory elements namely transcription factors (TFs) (Roider et al., 2009, Serin et al., 2016). Previous efforts to detect TFs concordantly expressed with heat-stress related genes demonstrated useful information that can be utilized to further improve the ability of plants to tolerate both heat and water deficit stresses (Obaid et al., 2016). Until recently, this approach is used successfully to detect concordant expression of TFs and genes related to polyphyllin biosynthesis in the plant species *Paris polyphylla* (Gao et al., 2022).

Transcription factors (TFs) are proteins or *cis*-acting elements mostly harbor DNA-binding domains (DBDs). TFs bind to regulatory sequences of DNA (enhancers or silencers) existing upstream of target genes (e.g., promoter region) in order to manipulate/regulate their expression rates (Hong 2016). In eukaryotes, TFs are made of two types. The first is called general transcription factors (GTFs) that directly bind to core region of gene promoter at the DBD region to allow binding of RNA polymerase. TFs of this type include TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH that are named based on chromatographic elution profiles and their order of discovery (Thomas and Chiang 2006). However, there are TFs with no DBD domain and can bind to DNA regulatory proteins or other TFs to form transcriptional complexes to enhance/repress activity of RNA polymerase based on cell's requirements. This other TF type is called specific transcription factors (STFs).

This study aims to detect transcription factors concordantly expressed with drought stress-related genes along with their expression patterns across time of drought stress in two Egyptian wheat cultivars namely Giza 168 and Gemmiza 10 and explore the possibility for these TFs to drive expression of these stress-related genes.

2. Materials and methods

2.1. Drought stress experiment

Two wheat cultivars with contrasting genotypes in terms of level of drought tolerance were used in a randomized complete block drought stress experiment. The first cultivar is the drought tolerant high-yielding Giza 168 (GZ168) and the second is the drought sensitive Gemmiza 10 (GM10) cultivar (Al-Naggar et al., 2015, Abdelghany et al., 2016). Before conducting the experiment, surface-sterilized seeds of the two cultivars were washed with sterile distilled water and put to germinate in half-strength Hoagland solution for 9 days. Growth conditions were 22 ± 2 °C temperature, 16 h/8h light/dark photoperiod and 450 µmol $m^{-2}s^{-1}$ light intensity. Then, generated seedlings were treated with 20% PEG-6000 (Baloglu et al., 2014) for 0 h, 2 h and 12 h, and leaf samples were harvested in liquid nitrogen and frozen at −20 °C.

2.2. RNA isolation and next-generation RNA sequencing

Total RNAs of replicated leaf samples of the two wheat cultivars were isolated using Trizol (Invitrogen, Life Tech, Grand Island, NY, USA). Then, RNAs were treated with RNase-free DNase (Promega Corporation, Madison, WI, USA) in the presence of 1 U/uL of RNasin® Plus RNase Inhibitor (Promega Corp.) for 2 h at 37 °C to degrade contaminating DNAs. DNA contamination in RNA samples was checked by PCR to amplify *actin* gene of the original RNA samples and results were negative. Then, 30-ug RNA (400 ng/ul) samples were shipped to Beijing Genomics Institute (BGI), Shenzhen, China, for deep sequencing and generation of transcriptomic datasets of ≥ 100 million reads per sample. Generated RNA-Seq raw data were deposited in SRA database of the NCBI (https://www.ncbi.nlm.nih.gov/sra, SRA no. PRJNA306536). High-quality paired-end RNA-Seq clean reads were mapped to the most recent reference *Triticum aestivum* genome (https://plants.ensembl.org/Triticum_aestivum) of the International Wheat Genome Sequencing Consortium (IWGSC) (Consortium et al., 2018) using HISAT2 (v2.2.1) based on Burrows-Wheeler transform and FM index and other default parameters (Kim et al., 2015). Genome assembly was refined using optical maps as recently described (Zhu et al., 2021). Then, genome-guided transcriptome assembly of Trinity (v2.15.0) (Grabherr et al., 2011) was performed and a merged binary alignment map (BAM) file was generated with a minimum contig length of 200 bp. Cluster analysis and differential expression with fold change of ≥ 4 (log 2/$fpkm^{+1}$) and false discovery rate (FDR) of ≥ 10^{-3} were done as previously described (Bahieldin et al., 2015). FPKM-derived read counts were compared for the two cultivars at the three time points using a likelihood ratio test (Casella and Berger 2021). Generated transcripts were annotated against blast-2-GO (version 2.3.5, https://www.blast2go.org/) and GO terms were obtained using default parameters. The PlantTFDB (https://planttfdb.gao-lab.org/index.php?sp = Tae) was searched for TFs in wheat (*Triticum aestivum*) to be located in the generated clusters. Principle component analysis was drawn using EdgeR package of Trinity *de novo* RNA-Seq transcript assembler (Haas et al., 2013) to detect genetic distances among samples and groups.

2.3. Relationships between TFs and coexpressed genes using a model plant system

Mutant *Arabidopsis* seeds of two transcription factors were obtained from *Arabidopsis* Biological Resource Center (ABRC), Ohio State University (https://abrc.osu.edu/). These two mutants include a homozygous mutated (CS175) *CO1* gene (AT5G15840) encoding CONSTANS/BBX1/CO/B-box domain protein 1 (https://abrc.osu.edu/stocks/4328) in Ler (Landsberg erecta) background and a knockout homozygous T-DNA insertion mutated (SALK_043732) *ZIP3* gene (ID At2g32270) encoding zinc transporter 3 (https://abrc.osu.edu/stocks/526393) in Col (Columbia) background. Wild type (WT) seeds of the two *Arabidopsis* backgrounds were used as controls in qPCR. Surface-sterilized seeds of the two mutant plant types in two replicates along with their respective WTs were germinated in a growth chamber for 2 wks under standard growth conditions (Bahieldin et al., 2018). Then, drought stress experiment with 0 h, 2 h and 12 h drought stress time points was conducted for *Arabidopsis* mutant and WT plants and RNA samples were isolated as described earlier for wheat cultivars.

Then, qPCR was conducted as previously described (Bahieldin et al., 2015) in order to detect expression levels of selected genes in wheat genotypes and their analogues in *Arabidopsis* (Ler & Col) backgrounds. These genes encode two transcription factors CO1 and ZIP3 along with the possibly coexpressed stress-related genes encoding tryptophan synthase alpha chain-like (e.g., *trpA* gene), asparagine synthetase [glutamine-hydrolyzing]-like (e.g., *ASNS* gene) and COP1-interacting protein 7-like (e.g., *COP1* gene). Primers were designed using Netprimer software (https://www.premierbiosoft.com/netprimer/index.html) following standard procedure (Table S1), with *actin* and *ubiquitin* used as the two reference house-keeping genes in wheat, while Plant UBX domain-containing protein 7 (PUX7) and E2 ubiquitin-conjugating enzyme 21 (UBC21) in *Arabidopsis* as described (Škiljaica et al., 2022). Statistical analysis in terms of multiple comparison and detetion of error bars were conduced by Duncan multiple range test (Duncan 1955).

3. Results

Transcriptomic data were used in order to detect concordantly expressed transcription factors (TFs) with drought stress-related genes of two bread wheat (*Triticum aestivum*) cultivars that have contrasting performance under drought stress. Expression profiling at statistical level was detected in the two types of transcriptomes across drought stress time points, e.g., 0 h, 2 h and 12 h.

3.1. Differential transcriptome signatures of the two contrasting genotypes

Principle component analysis (PCA) indicated high similarity within replicated transcriptome samples of each cultivar and each time point, while complete separation was detected between transcriptome signatures of the two cultivars across drought stress time points, and among time points across cultivars. The results indicated that transcriptomes of GZ168 were located in the positive side of PC1 across time points, while those of GM10 were located in the negative side (Fig. 1). In terms of the three time points across cultivars, transcriptomes of 0 h time point were located at ~ 0 value of PC2, while those of 2 h and 12 h time points were, respectively, located in the positive and negative sides of PC2. These data support the existence of long genetic distance between transcriptome signatures of the two contrasting genotypes, on the one hand, and among those of the three time points, on the other hand. Heatmap generated from hierarchical clustering with Pearson correlation - as the distance metric - supported PCA results, where genetic distances for transcriptomes of the two genotypes across and among time points align with those of PCA (Fig. 2). In terms of GZ168, transcriptomes at 0 h and 2 h time points were closer than that of either time point, and that at 12 h time point. For GM10, transcriptomes at 0 h and 12 h time points were closer than those of either one, and that at 2 h time point (Fig. 2). According to the results of PCA and heatmap, we expected to see discrete differences in transcriptomes of the two genotypes within and across time points in terms of structure and gene expression levels.

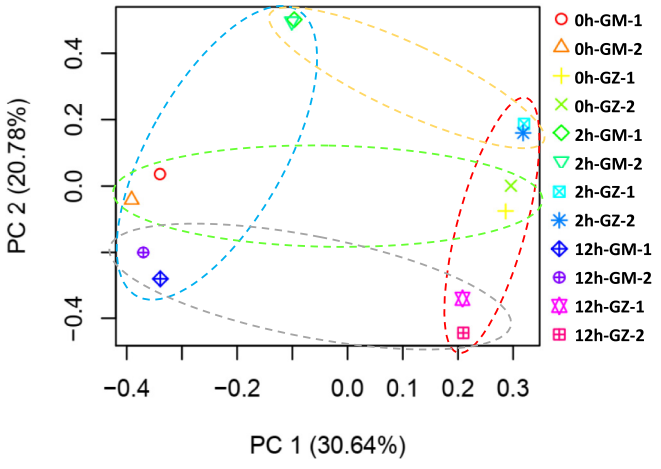

Fig. 1. Principle component analysis (PCA) describing the interrelation among transcriptomes of the replicated samples of the two contrasting genotypes, e.g., GZ (drought tolerant) and GM (drought-sensitive), at the three time points of drought stress, e.g. 0, 2 h & 12 h, in *T. aestivum*. GZ = Giza168, GM = Gimmeza10. Blue and red dotted circles, respectively, refer to GM10 and GZ168 samples across different time points. Green, orange and gray dotted circles, respectively, refer to 0 h, 2 h and 12 h time point of drought stress across the two contrasting genotypes.

3.2. Gene ontology of upregulated transcripts of the drought tolerant genotype

Gene ontology (GO) in terms of percentages of genes in different subcategories of each of the three functional categories "biological process", "molecular function" and "cellular component" was done using blast2GO (https://www.blast2go.org/) as shown in Fig. 3. The resulted GO terms were based on RNA-Seq data for clusters of upregulated transcripts of the wheat drought tolerant genotype GZ168 (Tables S2 & S3). The most abundant subcategories of "biological process" category were "metabolic process", "cellular process", "response to stimulus" and "signaling", while those of "molecular function" category were "catalytic activity", "binding", "transport activity" and "antioxidant activity" and those of "Cellular component" category were "membrane", "cell" and "Cell parts" (Fig. 3). Almost all of these subcategories refer to the plethora of abiotic stress responses and mechanisms by which drought stress-tolerant genotypes usually perform under abiotic stresses.

3.3. Differential expression of drought stress-related genes in transcriptomes of the two contrasting genotypes

Cluster transcriptomic analysis generated > 28,000 regulated transcripts assigned to 881 clusters for the two genotypes across the three time points (Figure S1 & Table S2). Then, transcripts related to drought stress that can be used as molecular markers of drought stress tolerance were searched. They included transcripts that promote photosynthesis and stomatal responses, on the one hand, and enzymes detoxifying reactive oxygen species (ROS), on the other hand. The first group of transcripts included those encoding ribulose bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll *a*-b binding proteins (CAB), while the second group included transcripts encoding peroxidase (POD) and catalase (CAT). Interestingly, the ROS detoxifying enzyme namely superoxide dismutase (SOD) was not differentially expressed in the two wheat cultivar under drought stress (Table S2).

In terms of the first group of genes, the results for 56 transcripts encoding Rubisco indicated the generation of six expression patterns across different clusters (Figures S2-S7 & Table S4). These patterns include transcripts that were highly expressed in the drought stress-tolerant genotype GZ168 regardless of drought stress compared with those of GM10 (Figure S2). Other expression patterns included transcripts that were upregulated in GZ168 at 2 h (Figure S3) and 12 h (Figure S4) time points. Other patterns included upregulation of transcripts of the two genotypes at 2 h time point (Figure S5) and upregulation of the two genotypes at 2 h time point, while at 12 h time point only for GZ168 (Figure S6). The sixth pattern included transcripts that were downregulated in the two genotypes at 12 h time point (Figure S7). Almost all these patterns, except the last one, are in favor to show higher expression rates of transcripts of the drought stress-tolerant genotype GZ168 than those of the drought stress-sensitive genotype GM10 across time. The results for over 150 transcripts encoding chlorophyll *a*-b binding proteins (CAB) collectively showed upregulation of the two genotypes at 2 h time point, while downregulation for those of GM10 at 12 h time point (Figure S8 & Table S5). We can conclude that transcripts of genotype GM10 that influence stomatal response and photosynthesis cannot hold expression for 12 h, which means that the latter two important processes might be retarded earlier in the day in this genotype.

In terms of the second drought stress tolerance group of genes, ~45 peroxidase encoding transcripts in seven expression patterns across different clusters were regulated under stress (Figures S9-S15 & Table S6). These patterns included transcripts with higher expression level of the drought stress-tolerant genotype GZ168 regardless of drought stress compared with those of GM10 (Fig-

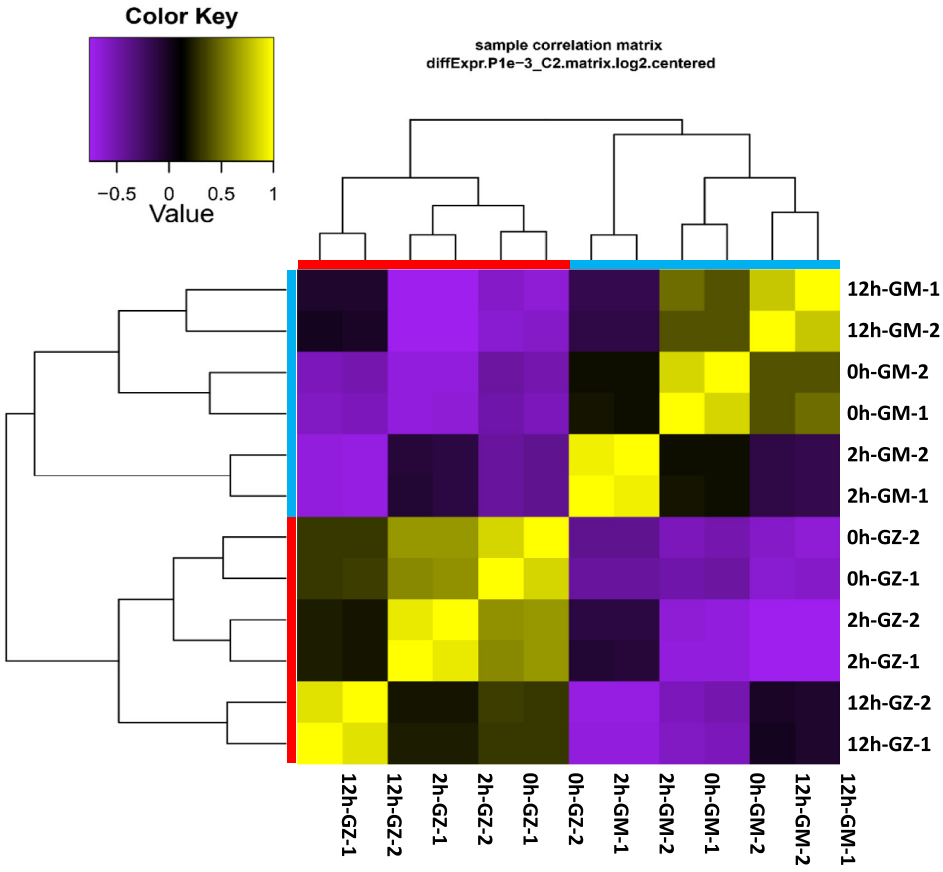

Fig. 2. Heat map referring to hierarchical cluster analysis to describe interrelation among transcriptomes of the replicated samples of the two contrasting genotypes GZ168 (drought tolerant) and GM10 (drought sensitive) at different time points, e.g. 0, 2 h & 12 h, in *T. aestivum*. GZ = Giza168, GM = Gimmeza10. Blue and red lines, respectively, refer to GM and GZ bidirectional clusters across the three different time points (e.g., 0 h, 2 h & 12 h) of drought stress.

ure S9). Other expression patterns included transcripts that were upregulated in GZ168 at 2 h (Figure S10) and 12 h (Figure S11) time points. Other patterns included concurrent upregulation of transcripts of GZ168 at 0 h and 2 h time points (Figure S12), one the one hand, and at 2 h and 12 h time points (Figure S13), on the other hand. There is a pattern with one transcript that was upregulated in GZ168 and GM10 backgrounds at 12 h time point (Figure S14). The seventh pattern showed upregulation of transcripts of the two genotypes at 2 h time point, while at 12 h time point only for GZ168 (Figure S15). The results for the 12 transcripts encoding catalase were shown to be upregulated in GZ168 at 12 h time point (Figure S16 & Table S7). We can conclude that almost all patterns of the two sets of transcripts of the second group are in favor to show higher expression rates in the drought stress-tolerant genotype GZ168 than those in the drought stress-sensitive genotype GM10. Interestingly almost similar patterns of expression were reached for transcripts encoding Rubisco and those encoding POD. This observation refers to the possible share of transcription factors that drive expression of these two sets of genes under drought stress. This speculation can be investigated by searching transcription factors (TFs) known for their influence on these two types of transcripts and make a functional genomics analysis to prove whether these TFs concurrently drive expression of genes of the two sets or the similarity of expression patterns is coincidental.

3.4. Concordant expression of TFs and drought stress-related genes

Out of the 881 clusters, 136 clusters with discrete expression patterns were selected for further analysis. These clusters were assigned to 13 expression patterns across genotypes and drought stress time points (Table S3). Again, there are two patterns where transcript expression is higher in one genotype at the three time point regardless of drought stress. Expression patterns with upregulation of transcripts of the drought tolerant GZ168 at 2 h (cluster 536) and 12 h (clusters 38, 338, 469 & 474) were searched for TFs concordantly expressed with drought stress-related genes. Successful results were reached in clusters 38 (Figs. 4-6 & Table S8), 338 (Fig. 7 & Table S8) and 536 (Fig. 8 & Table S8). Cluster 38 showed three patterns (subclusters) of concordant expression of TFs and stress-related genes. The first showed concordant expression of two TFs and five stress-related transcripts (Fig. 4), while two TFs and three transcripts for the second pattern (Fig. 5 & Table S8) and two TFs and two transcripts for the third (Fig. 6 & Table S8). Cluster 338 indicated concordant expression of one TF and three transcripts (Fig. 7 & Table S8), while one TF and one transcript for cluster 536 (Fig. 8 & Table S8). Thus, there is a total of eight TFs that possibly drive expression of one or more of the 14 stress-related transcripts. This speculation was validated for two TFs via a functional genomics approach.

3.5. Validating relationships of selected TFs with their possibly driven stress-related genes

Before we proceeded to validate relationships of TFs and concordantly expressed stress-related genes, we approached to validate RNA-Seq data of wheat transcriptomes via qPCR. Validation included genes of subcluster 38/2 that encode two TFs and three stress-related genes (Tables S2 & S8) and results assured that

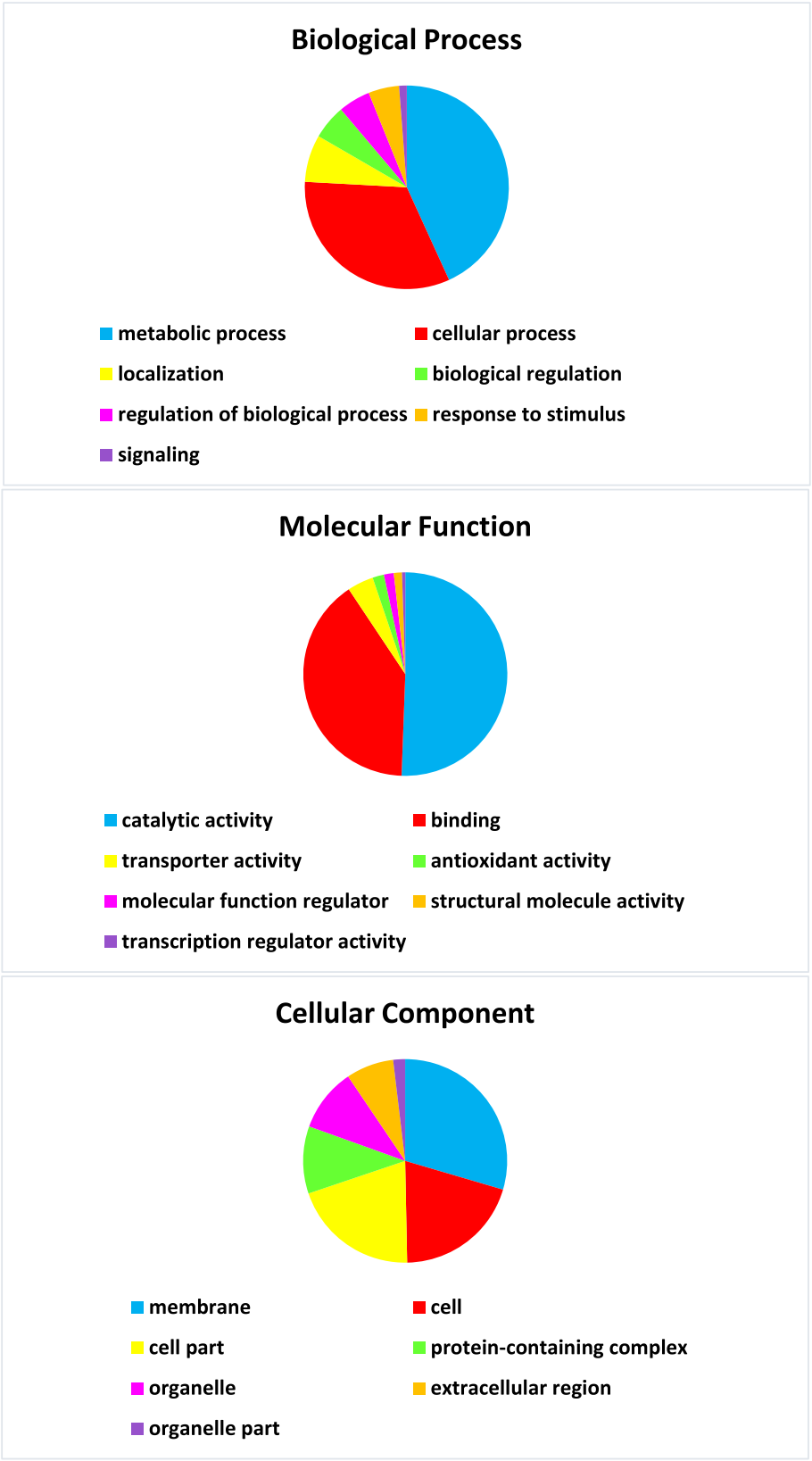

Fig. 3. Gene ontology (GO) analysis describing the most abundant level 2 GO terms of the three main categories "biological process", "molecular function" and "cellular component" based on clusters with upregulated transcripts in drought tolerant wheat genotype GZ168 under drought stress (see Table S3). BLASTX against the non-redundant (NR) protein database was used for GO term mapping and annotation.

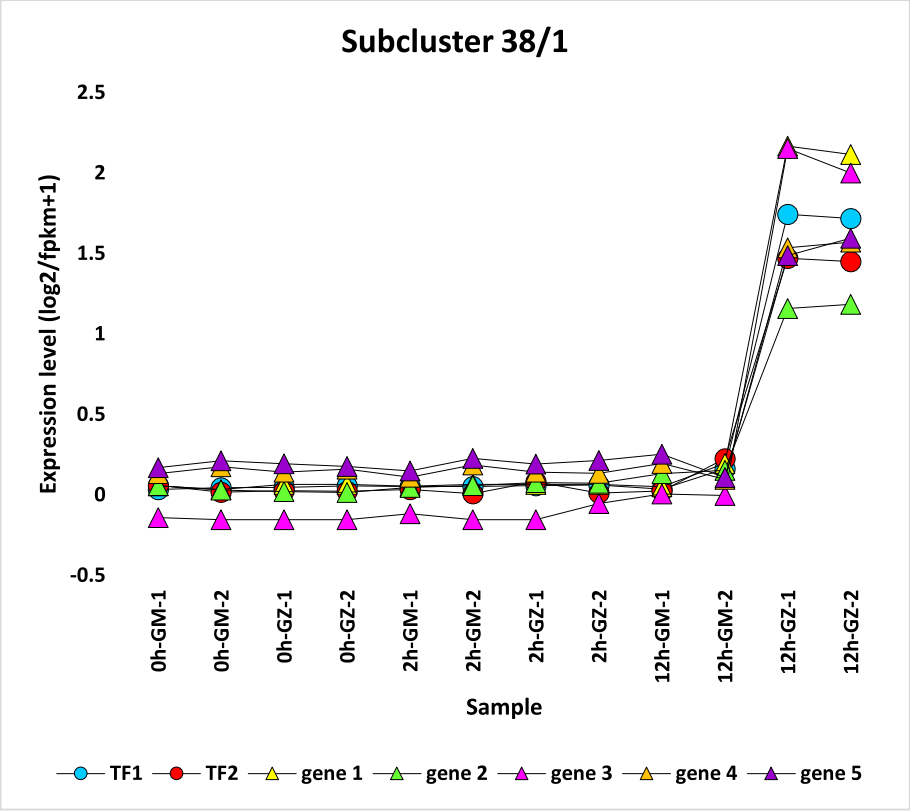

Fig. 4. Expression pattern of two upregulated transcription factor (TF1 & TF2) in GZ168 transcriptome at 12 h time point of drought stress and concordantly expressed with five genes (nos. 1–5) of subcluster 38/1 under drought stress across time (0, 2 & 12 h). Detailed cluster analysis data are shown in Table S8. TF1 = ethylene-responsive transcription factor RAP2-13-like (RAP2), TF2 = AP2-like ethylene-responsive transcription factor (AP2), gene 1 = aquaporin TIP1-1-like (*TIP1*), gene 2 = aquaporin TIP4-1 (*TIP4*), gene 3 = dehydrin DHN4-like (*DHN4*), gene 4 = dehydrin DHN3 (*DHN3*), gene 5 = dehydrin DHN3-like (*DHN3*-like).

wheat qPCR data align with those of wheat RNA-Seq data (Figure S17).

In terms of validating relationships of TFs and concordantly expressed stress-related gene, we selected *Arabidopsis* genes analogues to those of subcluster 38/2 of wheat transcriptome for the test. The term "mutated" refers to gene(s), while the term "mutant" refers to plant(s) harboring the mutated genes. Two mutant lines harboring mutated versions of genes encoding two transcription factors (TFs) in *Arabidopsis* were used. These two genes in subcluster 38/2 in RNA-Seq data of tolerant wheat genotype showed upregulation at 12 h time point of drought stress (Fig. 5 & Table S8). Analogues in *Arabidopsis* encode zinc finger protein CONSTANS-LIKE 1-like^{Ler} or CO1^{Ler}, whose mutated version of the encoding gene exists in *Arabidopsis* Landsberg erecta (Ler) background, and zinc transporter 3-like^{Col} or ZIP3^{Col}, whose mutated version of the encoding gene exists in *Arabidopsis* Colombia (Col) background.

Firstly, drought stress experiment was done for the two *Arabidopsis* mutant plants (Mu-CO1^{Ler} & KO-ZIP3^{Col}). The respective wild type plants (WT^{Ler} & WT^{Col}) harboring the two WT genes (*CO1*^{Ler} & *ZIP3*^{Col}, respectively) were also used in the validation. The qPCR results indicated that the mutated version of either gene, e.g., Mu-*CO1*^{Ler} or KO-*ZIP3*^{Col} showed retarded expression across the three time points of drought stress, while respective WT version of either gene (*CO1*^{Ler} or *ZIP3*^{Col}), respectively, showed upregulation at 12 h time point (Fig. 9). The qPCR results to detect relationship between the two TFs under drought stress at 0 h, 2 h and 12 h time points indicated that expression of *ZIP3*^{Ler} was retarded when *CO1*^{Ler} gene was knocked out, while expression of *CO1*^{Col} was not affected when *ZIP3*^{Col} gene was mutated (Fig. 10).

The qPCR results to detect the possible influence of the two *Arabidopsis* TFs in driving expression of three *Arabidopsis* drought stress-related genes that are analogues to those concordantly expressed in subcluster 38/2 of RNA-Seq data in wheat are shown in Figure S18. These three *Arabidopsis* genes encode tryptophan synthase alpha chain-like^{At} (*trpA*^{At} gene), asparagine synthetase-like^{At} (*ASNS*^{At} gene) and COP1-interacting protein 7-like^{At} (*COP1*^{At} gene). The qPCR results for zinc finger protein CONSTANS-LIKE 1-like^{Ler} (CO1^{Ler}) indicated that expression of *trpA*^{Ler} and *ASNS*^{Ler} genes was retarded at 12 h time point of drought stress when expression of *CO1*^{Ler} gene was retarded (Figure S18). While, the results of qPCR for TF zinc transporter 3-like^{Col} (ZIP3^{Col}) indicated that expression of *ASNS*^{Col} was retarded at 12 h time point of drought stress when expression of *ZIP3*^{Col} gene was retarded (Figure S18). Expression of *COP1*^{At} seems not to be affected when expression of either gene encoding the respective TF was retarded, thus not analyzed further.

4. Discussion

The two bread wheat (*Triticum aestivum*) cultivars that have contrasting performance under drought stress, e.g., GZ168 (tolerant) and GM10 (sensitive), were utilized in the present study mainly to detect relationships between transcription factors (TFs) and concordantly expressed drought stress-related genes under the stress. As a prerequisite, it was important to support prior information regarding performance of the two genotypes under drought stress at the molecular level and see that expression of important genes for drought stress tolerance is feasibly higher in

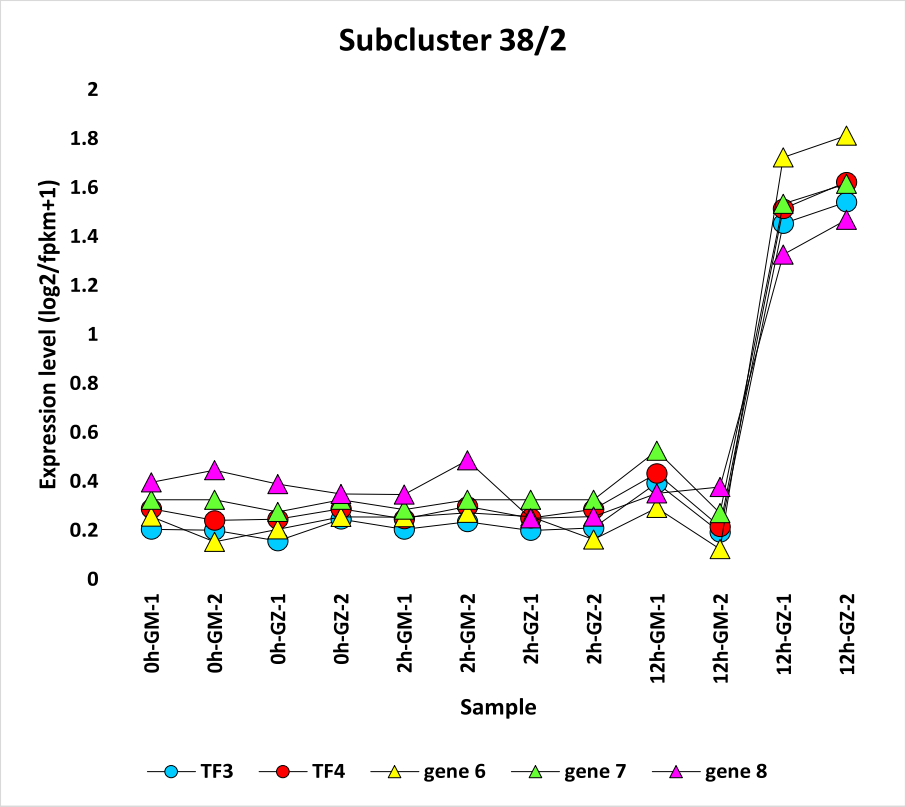

Fig. 5. Expression pattern of two upregulated transcription factor (TF3 & TF4) in GZ168 transcriptome at 12 h time point of drought stress and concordantly expressed with three genes (nos. 6–8) of subcluster 38/2 under drought stress across time (0, 2 & 12 h). Detailed cluster analysis data are shown in Table S8. TF3 = zinc finger protein CONSTANS-LIKE 16-like (CO16), TF4 = zinc transporter 3-like (ZIP3), gene 6 = tryptophan synthase alpha chain-like (*trpA*), gene 7 = asparagine synthetase [glutamine-hydrolyzing]-like (*ASNS*), gene 8 = COP1-interacting protein 7-like (*COP1*).

the tolerant genotype. Such relationships are hard to prove in bread wheat as its germplasm contains three genetically-related genomes namely A, B and C. Thus, it is likely that a given gene to be knocked out or knocked down in a functional genomics experiment in bread wheat should have analogue(s) on homoeologous chromosome(s) of the other two genomes. Therefore, we thought to use the model diploid plant *Arabidopsis* that harbors a small genome (~135 Mb) in order to test our speculative relationships between TFs and coexpressing drought stress-related genes.

4.1. Gene ontology (GO) analysis

In the present study, the most important GO terms of "biological process" category are "metabolic process", "cellular process", "response to stimulus" and "signaling" (Fig. 3). These processes refer to the most important actions that take place when the drought tolerant wheat cells are exposed to a given type of abiotic stresses. Transcription factors (TFs) in the selected expression patterns of Table S3 were proven to participate in these processes. For example, the TF zinc finger protein CONSTANS (CO1) of subcluster 38/2 (Tale S8) was proven to participate in the processes "metabolic process", "signal transduction" and "response to heat stimulus" (https://www.uniprot.org/uniprotkb/Q39057/entry). CO1 is regulated under drought stress as a response to external stimuli. In terms of GO category of "molecular function", the drought tolerant wheat genotype is expected to perform "catalytic activity", "binding", "transport activity" and "antioxidant activity" (Fig. 3). These functions seem to be very important for plants to cope with a type of abiotic stresses like drought. Again, CO1 helps perform "binding", "DNA binding" and "DNA-binding transcription factor activity". These functions support its possible role in driving drought stress-related genes. In terms of "Cellular component" category of Fig. 3, "membrane", "cell" and "Cell parts" seem to be the most active cell compartments under drought stress in the tolerant genotype GZ168. CO1, as a TF, is more effective in the nucleus (https://www.uniprot.org/uniprotkb/Q39057/entry), thus, other inducer or putative TF genes might be effective in the membrane. For example, zinc transporter 3 (ZIP3), a speculated TF, was proven to participate in the biological processes "transport" and "cellular process", in the molecular function "transporter activity" and acts in cellular components "membrane" and "plasma membrane" (https://www.uniprot.org/uniprotkb/Q7XLD4/entry). These processes are important for transferring important minerals like zinc that is a core inducer of a possible category of TFs with zinc finger protein domains, like CO1 (Hara et al., 2017). Thus, it was important to know whether ZIP3 transports Zn to the cell that, in turn, induces expression of CO1, or CO1 induces ZIP3 to transport Zn to the cell to be translated into several important functions under drought stress. All TFs to be analyzed in the present study exist in the PlantTFDB, except for ZIP3 that is not considered as a TF as it functions in the membrane, not the nucleus. But, based on its action in promoting zinc-induced expression of genes performing several important functions, we thought to test its direct influence on inducing expression of the coexpressed stress-related genes, which might be indirect influence on these genes via induction of a given Zn-induced TF.

4.2. Differential expression of stress-related genes in the two contrasting genotypes

Several wheat cultivars were bred to be tolerant to drought stress using approaches like molecular breeding, marker-assisted selection and water budgeting (Ahmad et al., 2018). Physiological responses to drought stress in a plant like wheat involve stomatal

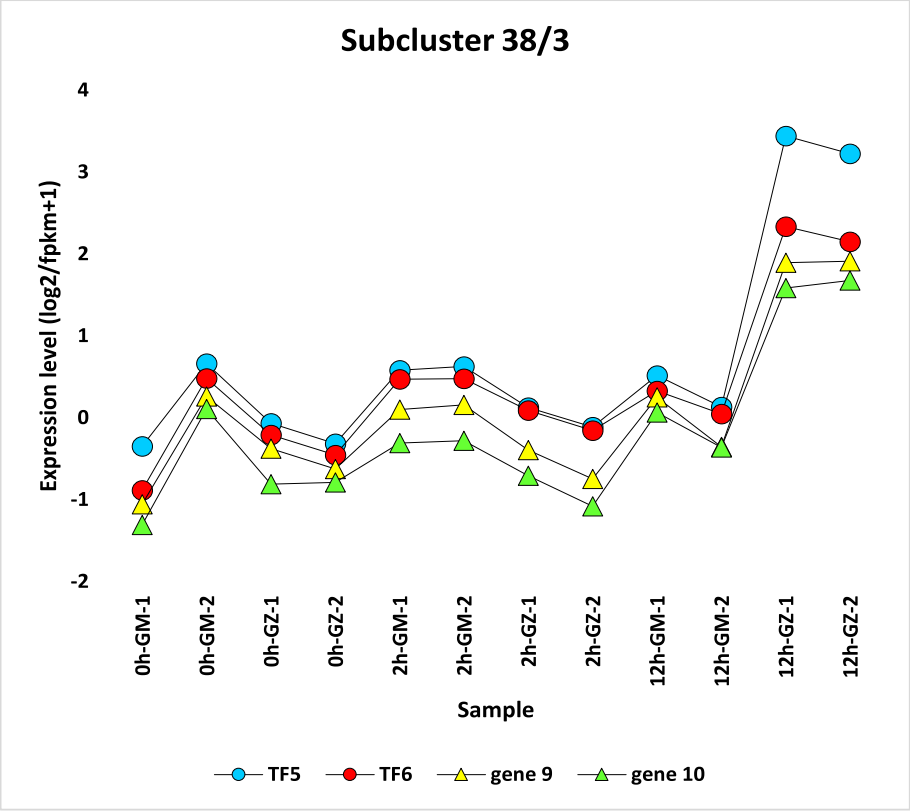

Fig. 6. Expression pattern of two upregulated transcription factor (TF5 & TF6) in GZ168 transcriptomes at 12 h time point of drought stress and concordantly expressed with two genes (no. 9 & 10) of subcluster 38/3 under drought stress across time (0, 2 & 12 h). Detailed cluster analysis data are shown in Table S8. TF5 = ethylene-responsive transcription factor 4-like (ERF4), TF6 = ethylene-responsive transcription factor 1-like (ERF1), gene 9 = beta-amylase 1 (*BAM1*), gene 10 = indole-3-pyruvate monooxygenase YUCCA1-like (*YUCCA1*).

closure when sun rises to avoid water loss by transpiration (Gurumurthy et al., 2019, Sharma et al., 2022). This action limits plant's ability to take up enough CO_2 to make photosynthesis required for its survival and growth (Yang et al., 2006). Adverse physiological effects other than stomatal responses involve the decrease in ribulose-1,5 – bisphosphate carboxylase/oxygenase (Rubisco) activity; an action that negatively influences CO_2 availability in chloroplasts and retards occurrence of efficient photosystem II (PSII) (Flexas et al., 2006). Thus, drought tolerant genotypes manage to orchestrate such responses based on their genetic makeup. In case of the drought tolerant wheat cultivar GZ168, a battery of genes encoding ribulose bisphosphate carboxylase/oxygenase (Rubisco) comprising a number of expression patterns were mostly upregulated across the day (Figures S2-S7). Some of these genes act constitutively at higher rate compared with that of the drought sensitive cultivar GM10 regardless of drought stress time points (Figure S2). Other genes in GZ168 respond shortly after drought stress (Figure S3), while others act before sunset (Figure S4) to maintain continuous occurrence of photosynthesis in an orchestrated fashion. Action of such a battery of genes (Figures S3 & S4) secures continuous supply of CO_2 required for photosynthesis. Unlike GZ168, most of the Rubisco-encoding genes in GM10 seem not to hold their expression for 12 h (Figure S6), thus, GM10 likely cannot stand drought stress all day long. To support our claim on the differential response of Rubisco enzyme in the two contrasting genotypes, the results of > 150 genes encoding light-harvesting chlorophyll *a*-b binding protein (LHCB) indicated that their expression declined in GM10 at 12 h time point possibly due to the scarce of Rubisco activity at that time of the day (Figure S8). Genes encoding chlorophyll *a*-b binding (CAB) proteins act downstream those encoding Rubisco in order to promote occurrence of photosystem II (PSII) (Flexas et al., 2006).

LHCBs proteins were also previously reported to participate in guard cell signaling and in modulating reactive oxygen species (ROS) homeostasis in response to drought stress in *Arabidopsis* (Xu et al., 2012). Wheat has both enzymatic and nonenzymatic activity to detoxify ROS (Abid et al., 2016). There are several antioxidant protective enzymes that alleviate the negative effects of water scarce on stability and function of cell membrane structure (Liu et al., 2015). These enzymes include superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Gill and Tuteja 2010, Arbona et al., 2013). Transcripts of the first enzyme showed no regulation in the two contrasting wheat genotypes under drought stress (Table S2). In terms of transcripts encoding POD, a number of expression patterns were generated under drought stress (Figures S9-S15). Among expression patterns, a large number of transcripts in the drought tolerant GZ168 cultivar showed higher constitutive expression than in the drought sensitive GM10 (Figure S9). The other transcripts of GZ168 showed expression patterns similar to those encoding Rubisco, where their upregulation across the day, e.g., 2 h & 12 h (Figures S10 & S11), secures the absence of toxic effects induced by ROS due to the stress. Late upregulation (at 12 h time point) is the expression pattern of all regulated catalase enzymes in transcriptomes of the tolerant wheat cultivar GZ168, while almost no regulation in transcriptomes of GM10 (Figure S16). Hydrogen peroxide (H_2O_2) is one type of ROS that is known as a destructive molecule of plant cells (Abid et al., 2016). H_2O_2 is known to be detoxified to O_2 and H_2O by the two enzymes CAT and POD. When these two enzymes are concurrently enriched inside the cell, low levels of the two ROS

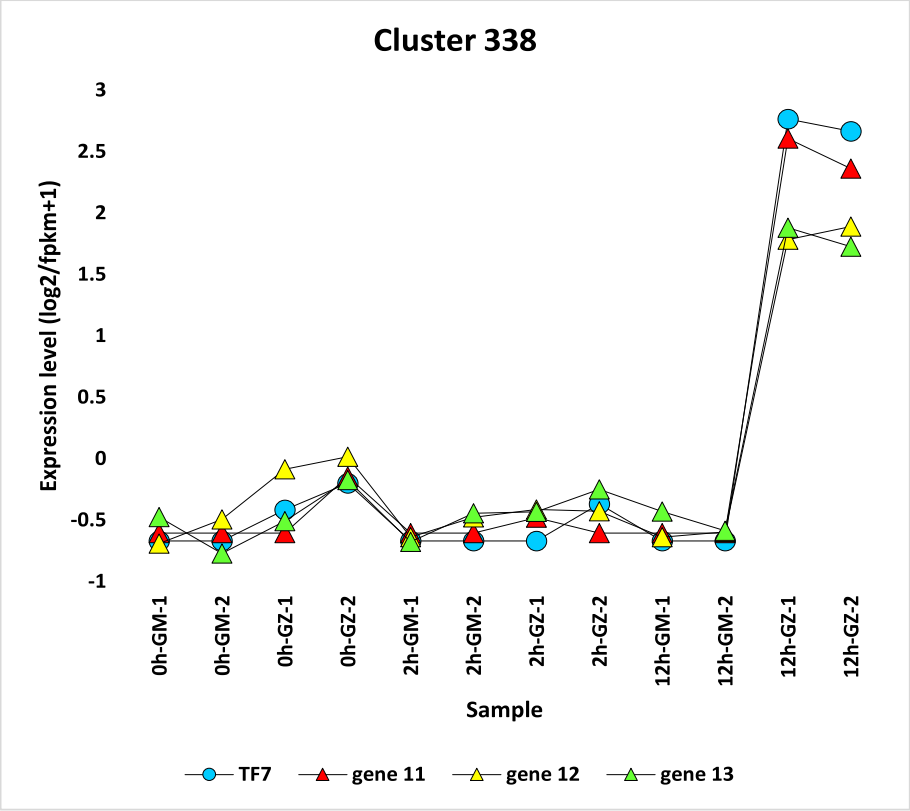

Fig. 7. Expression pattern of an upregulated transcription factor (TF7) in GZ168 transcriptome at 12 h time point of drought stress and concordantly expressed with three genes (nos. 11–13) of cluster 338 under drought stress across time (0, 2 & 12 h). Detailed cluster analysis data are shown in Table S8. TF7 = zinc finger protein CONSTANS-LIKE 9-like (CO9), gene 11 = cold-shock protein CS120-like (*CSP120*), gene 12 = tryptophan synthase beta chain 2-like (*trpB*), gene 13 = GDSL esterase/lipase (*GELP*).

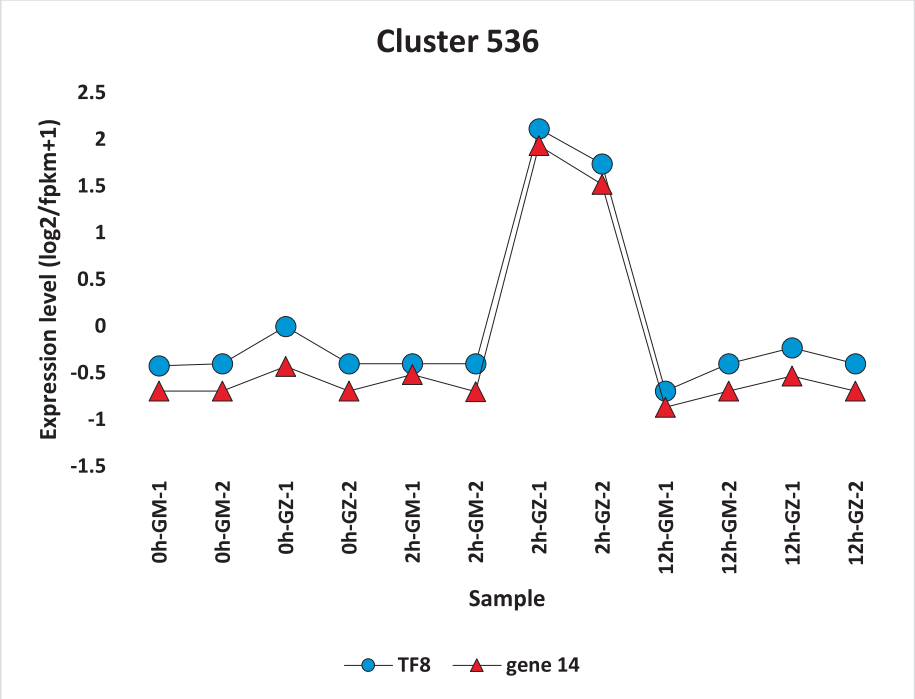

Fig. 8. Expression pattern of an upregulated transcription factor (TF8) in GZ168 transcriptome at 2 h time point of drought stress and concordantly expressed with two genes (nos. 14 & 15) of cluster 536 under drought stress across time (0, 2 & 12 h). Detailed cluster analysis data are shown in Table S8. TF8 = transcription factor bHLH112-like, gene 14 = peroxidase 2-like (*POD2*). Gene 14 is also included in Figure S10.

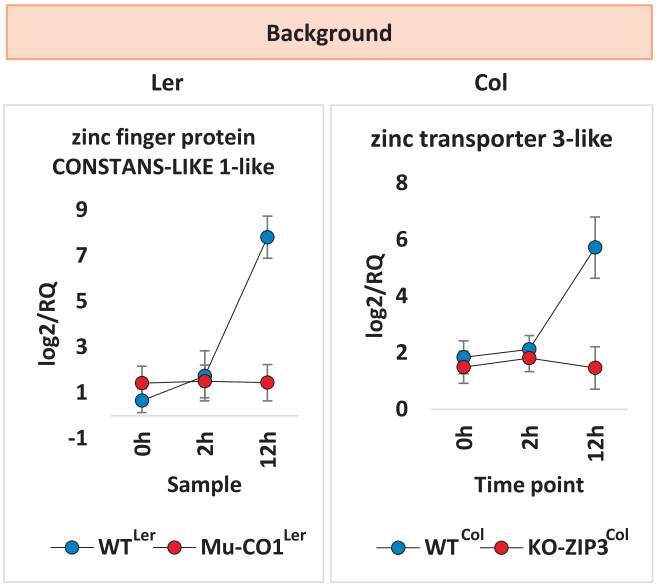

Fig. 9. Comparisons of expression pattern referring to upregulation at 12 h time point of drought stress in Arabidopsis via qPCR for each of the two genes encoding two transcription factors^{At} (TFs) analogue to those of subcluster 38/2 in GZ168 transcriptomes^{Ta}. The first comparison involved *CO1*^{Ler} gene in its wild type^{At} (or *WT*^{Ler}) version existing in the wild type plant (WT^{Ler}) versus its homozygous mutated^{At} gene (Mu-*CO1*^{Ler}) version existing in mutant plant (Mu-CO1^{Ler}) of Arabidopsis Ler background. The second comparison involved *ZIP3*^{Col} gene in its wild type^{At} (or *WT*^{Col}) version existing in the wild type plant (WT^{Col}) versus its homozygous mutated^{At} gene (KO-*ZIP3*^{Col}) version existing in mutant plant (KO-ZIP3^{Col}) of Arabidopsis Col background. The First TF gene encodes zinc finger protein CONSTANS-LIKE 1-like^{Ler} (CO1^{Ler}), while the second encodes zinc transporter 3-like^{Col} (ZIP3^{Col}). Col = Colombia, Ler = Landsberg erecta, At = *Arabidopsis thaliana*, Ta = *Triticum aestivum*. Error bars refer to standard errors (SE).

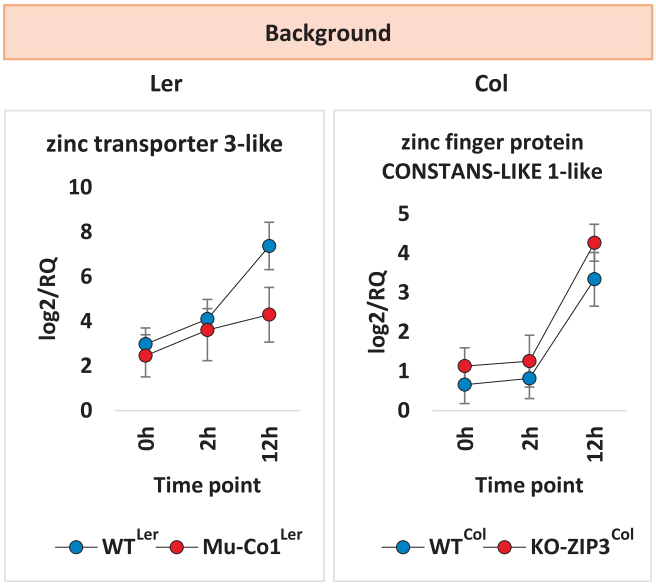

Fig. 10. Comparisons of expression pattern via qPCR for each of the two genes encoding the two transcription factors (TFs) ZIP3^{Ler} and CO1^{Col}. The first comparison involved *ZIP3*^{Ler} gene in the wild type^{At} version existing both in the wild type plant (WT^{Ler}) and in mutant plant (Mu-CO1^{Ler}) of Arabidopsis Ler background. The second comparison involved *Co1*^{Col} gene in the wild type^{At} version existing both in the wild type plant (WT^{Col}) and in mutant plant (KO-ZIP3^{Col}) of Arabidopsis Col background. The First TF gene encodes zinc transporter 3-like^{Ler} (ZIP3^{Ler}) in Ler background, while the second encodes zinc finger protein CONSTANS-LIKE 1-like^{Col} (CO1^{Col}) in Col background. Col = Colombia, Ler = Landsberg erecta, At = *Arabidopsis thaliana*. Error bars refer to standard errors (SE).

types O_{2-} and H_2O_2 exist (Sharma et al., 2022). The data for these two enzymes in the present study along with those for Rubisco and LHCB validate our previous claim of the differential drought tolerance level of the two wheat genotypes.

4.3. Validating relationships between TFs and concordantly expressed stress-related genes

As no knockout lines are available in hexaploid bread wheats, we used two mutant lines harboring mutated loss-of-function versions of genes encoding two TFs in *Arabidopsis*. These two genes are analogues to those in subcluster 38/2 in our RNA-Seq data of GZ168 that showed upregulation at 12 h time point of drought stress (Fig. 5 & Table S8). The two TFs are zinc finger protein CONSTANS-LIKE 1-like^{Ler} or CO1^{Ler}, whose mutated version of the encoding gene exists in *Arabidopsis* Landsberg erecta (Ler) background, and zinc transporter 3-like^{Col} or ZIP3^{Col}, whose mutated version of the encoding gene exists in *Arabidopsis* Colombia (Col) background. Drought stress experiment of the two *Arabidopsis* mutants (Mu-CO1^{Ler} & KO-ZIP3^{Col}) along with their respective wild type (WT^{Ler} & WT^{Col}) plants was done in a way similar to that of bread wheat with the aim to detect possible relationships between the two genes encoding these TFs, on the one hand, and between each of the two genes, e.g., *CO1*^{At} or *ZIP3*^{At}, and those analogues to the three coexpressed genes of subcluster 38/2 in wheat RNA-Seq data, on the other hand (Fig. 5 & Table S8).

Mutant plants of the two TFs were particularly chosen for validating relationships for two reasons. The first is that mutated version of either gene, e.g., Mu-*CO1*^{Ler} or KO-*ZIP3*^{Col}, expectedly showed retarded expression during qPCR across the three time points of drought stress, while respective WT version of either gene, e.g., *CO1*^{Ler} or *ZIP3*^{Col}, expectedly showed upregulation at 12 h time point of drought stress (Fig. 9). In other words, the WT versions of the two genes performed similar to their analogues in the tolerant genotype GZ168 under drought stress (Fig. 5 & Table S8), while the mutated versions of the two genes showed almost no expression at 12 h time point of drought stress, similar to their analogues in the sensitive genotype GM10 (Fig. 9). The second reason for choosing genes encoding these two TFs is that mutant seeds harboring the mutated versions of genes *Co1* and *ZIP3* were in stock albeit in two different *Arabidopsis* backgrounds, e.g., Ler and Col, on the one hand, and mutated genes are in a homozygous state, on the other hand.

The qPCR results, to detect possible relationship between the two TFs under drought stress at 0 h, 2 h and 12 h time points, indicated that expression of *ZIP3*^{Ler} gene was retarded when *CO1*^{Ler} gene is mutated (Mu-*CO1*^{Ler}), while expression of *CO1*^{Col} gene was not affected when *ZIP3*^{Col} gene is knocked out (KO-*ZIP3*^{Col}) (Fig. 10). Then, we assume that transcription factor CO1 might drive expression or functionally act upstream of *ZIP3* gene. To validate the latter speculation, we have tested the possible influence of the two TFs via qPCR in driving expression of the three drought stress-related genes *CO1*^{At}, *ZIP3At* and *COP1*^{At} (Figure S18). In terms of the mutant plant Mu-CO1^{Ler}, the results of qPCR indicated that expression of *trpA*^{Ler} and *ASNS*^{Ler} genes was retarded at 12 h time point of drought stress. In terms of the mutant plant KO-ZIP3^{Col}, the results of qPCR indicated that expression of the *ASNS*^{Col} gene is the only among the three stress-related coexpressed genes to be retarded at 12 h time point of drought stress (Figure S18). Thus, it seems that expression of *ASNS*^{At} gene is likely driven, either directly or indirectly, by *ZIP3*^{At} gene, while transcription factor CO1^{At} likely drives expression of *ZIP3*^{At} as well as two, out of the three, stress-related coexpressed genes. Based on these results, we speculate that CO1^{At} directly drives expression of *ZIP3* gene, while indirectly drives expression of *trpA*^{At} gene as other TF likely exists in the middle. In other words, *CO1* gene might act upstream

of *ZIP3* gene that, in turn, acts directly or indirectly upstream of *ASNS* gene.

Members of zinc transporter precursor or ZIP family act in transporting Zn^{2+} and other divalent metal cations, like Fe^{2+}, in allowing cellular uptake of Zn, in intracellular trafficking and in Zn biofortification of grains (Ajeesh Krishna et al., 2020). In *Arabidopsis*, there are TFs belonging to basic-region leucine zipper (bZIP) family involved in the upregulation of ZIP transporters during Zn deficiency as they harbor a Zn-sensor made of a histidine-rich motif (Assunção et al., 2013, Castro et al., 2017). We assure that CO1 also make the same function as that of bZIP. All of these TFs are also involved in the regulation of abiotic stress responses (Corrêa et al., 2008), which supports recent claim that Zn homeostasis is required under drought stress (Umair Hassan et al., 2020). The latter researchers claimed that Zn application improves stomatal regulation, water use efficiency, photosynthesis, and the production of osmolytes like proline and antioxidant enzymes, thus, a better performance under drought stress,; characteristics that secure surivival of plant cell under abiotic stress conditions (Chaves and Oliveira 2004, Gupta et al., 2020). The wheat transcription factor $ZIP3^{Ta}$ was recently proven to be highly expressed in leaves and roots under Zn defeciency (Evens et al., 2017, Ajeesh Krishna et al., 2020). In rice, $ZIP3^{Os}$ was proven to positively support grain filling and yield (Meng et al., 2018) and contribute to high Zn efficiency under various environmental conditions (Chen et al., 2008).

In terms of the transcription factor family zinc finger protein CONSTANS or CO, it is known to encode proteins structurally similar to those of zinc finger transcription factors. Wheat genome harbors seven members of this TF family on charomosomes 5BL, 6DL, 7AS and 7DS (https://planttfdb.gao-lab.org/family.php?sp = Tae&fam = CO-like). Thus, it is not surprising that bZIP and CO possibly share similar functions. Members of CONSTANS family also regulate genes involved in proline biosynthesis (https://www.uniprot.org/uniprotkb/Q39057/entry) as *P5CS2*, a paralog gene encoding Δ1-pyrroline-5-carboxylate synthetase for proline accumulation, was proven to be an early target of CONSTANS (Wenkel et al., 2006). The CCT domain of this TF is involved in light signaling (Wenkel et al., 2006); an important process to promote photosynthesis. Various *P5CS* genes generally play distinct roles in stress regulation and providing proline as an osmolyte for cell's osmoprotection under abiotic stresses like drought and salt (Székely et al., 2008, Wei et al., 2016). In the present study, the two TFs of zinc finger protein CONSTANS-like 9 and 16 were regulated and likely and indirectly participate in zinc homeostasis in addition to their role in light signaling during photosynthesis and in proline accumulation under drought stress. Besides the PCR results, the latter characteristics of CO family members complement those of ZIP family members, thus, justify relationship between the two types of TF.

Functional analysis in the present study indicated that CO1 in *Arabidopsis* promotes expression, not only of *ZIP3* gene, but also of two stress-related genes (Table S8). In terms of the *trpA* gene, the encoded enzyme catalyzes the last step in tryptophan biosynthesis. This amino acid is known for its important role in auxin production and subsequent signal transduction cascades that help plant cells approach the "Plant hormone and signal transduction" pathway to promote growth under stress conditions (Abulfaraj et al., 2022). Expression of *ASNS* gene in wheat leaves is involved in asparagine biosynthesis and metabolism as a response to sulfur deficiency (Gao et al., 2016, Postles et al., 2016). The encoded enzyme bidirectionally generates asparagine from aspartic acid and vice versa (Lomelino et al., 2017). In wheat grain, the favorite direction is the biosynthesis of aspartic acid to avoid accumulation of acrylamide (Raffan and Halford 2019). However, the two downstream reactions result in the production of oxaloacetate (OAA). This action activates alternative oxidase (AOX) pathway and malate/OAA shuttle to alleviate drought-induced photoinhibition and restore optimized PSII efficiency and photosynthetic electron transport chain (Hu et al., 2019). Thus, this shuttle results in the transfer of NADH from cytosol into mitochondria, thus, operates under low physiological NADH concentrations via the action of OAA/malate antiporter (Pastore et al., 2003). Overall, AOX is involved in photosynthesis and metabolic homeostasis in the cell under drought stress by reducing oxygen to water (Saha et al., 2016). The latter characteristics mostly complement those of CO16 and ZIP3, thus, relationship with *ASNS* gene is likely.

Interestingly, the *CO9* gene of cluster 338 also coexpressed with *trpB* gene (Fig. 7). The α and β subunits of tryptophan synthetase form an αββα complex that structurally forms a tunnel for release of indole (Xie et al., 2002). Expression pattern of this cluster is the same as that of cluster 38 (Fig. 6), thus, we assume the data of the two clusters in terms of the relationship between either TFs, e.g., CO16 or CO9, and either stress-related genes, e.g., *trpA* or *trpB*, respectively, validates one another (Figs. 5 and 7, respectively). In terms of the other stress-related genes of cluster 338, the gene encoding cold-shock protein CS120-like (*CSP120*) was shown to reduce intracellular freezing damage in wheat by hydrogen-bonding (https://www.uniprot.org/uniprotkb/P46525/entry). However, analogue to the latter gene, encoding a cold-shock domain protein 3 (CSP3), was reported to participate in drought stress tolerance in *Arabidopsis* (Kim et al., 2013). The authors indicated that *CSP3* gene is induced under drought stress and is regulated by abscisic acid (ABA). Their results indicated that 2-wk-old seedlings, with loss-of-function version of the mutated gene, could not withstand drought stress for 5d, compared with WT. Interestingly, the authors also indicated that the drought stress-related genes, whose expression was retarded in the mutant plant ($Mu-CSP3^{At}$), included analogues of *ASNS* (Fig. 5) and *POD* (Figure S11) genes of the present study. The latter three genes were upregulated under drought stress in the wheat tolerant genotype of the present study, indicating possible influence of *CS120* gene of cluster 338 (Fig. 7) on regulating expression of the two aforementioned *ASNS* and *POD* genes especially that expression pattern of the three genes is upregulation at 12 h time point of drought stress (Figs. 5, 7 & S11). Overall results confirm possible involvement of transcription factors CO9 and CO16, individually or collectively, in driving expression of genes in clusters 38/2 and 338. In terms of the *GELP* gene encoding GDSL-type esterase/lipase protein (GELPs) of cluster 338 (Fig. 7), recent reports indicated that this protein represents a collection of hydrolyzing enzymes with a conserved GDSL motif at their N-terminus (Su et al., 2020). GELPs are widely distributed in the majority of biological systems and participate in regulating plant growth and development in bread wheat (Watkins et al., 2019), especially under drought stress (Hong et al., 2008). Su and colleagues (2020) indicated that overexpression of GELP in *Arabidopsis* resulted in higher chlorophyll content and in the osmoprotectant proline level, while decreased level of H_2O_2. The characteristics of the three stress-related genes of cluster 338, e.g., *trpB*, *CSP120* and *GELP*, mostly complement those of *CO9* gene, thus, justify possible relationship.

The two TFs of subcluster 38/1, namely ethylene-responsive transcription factor RAP2-13 (or RAP2-13) and AP2-like, coexpress with two genes encoding aquaporin (AQP) tonoplast intrinsic proteins (TIP) 1–1-like and 4–1, in addition to three genes encoding dehydrins 3 and 4 (DHN3, DHN3-like & DHN4) (Fig. 4). Two other TFs of the same family, namely ethylene-responsive transcription factors 1 and 4 (ERF1 & ERF4) of subcluster 38/3 were shown to coexpress with two stress-related genes encoding β-amylase 1 (BAM1) and indole-3-pyruvate monooxygenase YUCCA1-like (Fig. 6). The families of AP2 and ERF transcription factors were reported to regulate several developmental and stress-responsive

pathways (Li et al., 2015). In wheat, two TFs - analogues to the four TFs of the present study - namely TaPIE1 and TaERF3, promote stomatal conductance as well as reduced chlorophyll degradation and H_2O_2 formation, while promote increased proline content under drought stress (Zhu et al., 2014, Phukan et al., 2017).

The genes encoding TIP1-1-like and TIP4-1 are among the most abundant water-transporting aquaporins (AQPs) in plant vacuolar membrane (tonoplast) (Kapilan et al., 2018). TIPs participate in the bidirectional intracellular water movement/permeability and flow of water in addition to small solutes and gases across the membranes (Nozaki et al., 2008). These actions secure the rapid osmotic adjustment of the cytoplasm and maintenance of intracellular turgor pressure. In barley, these two genes were proven to participate in the adaptation to drought stress at seedling stage (Kurowska et al., 2019), similar to the results of the wheat GZ168 genotype. In addition, dehydrins represent a group of late embryogenesis abundant II (LEA-II) proteins (Kosová et al., 2014). Expression of genes encoding dehydrins is induced under salt and drought stresses and induced by abscisic acid (ABA). Dehydrins have protective effects on lactate dehydrogenase (LDH) and β-glucosidase activities under dehydration condition (Drira et al., 2013). LDH, in turn, confers drought stress tolerance in plants by maintaining cellular homeostasis (Jain et al., 2020). It provides energy by detoxifying the stress-induced toxic metabolites and maintains low levels of ROS and other toxic metabolites under drought stress. While, β-glucosidase participates in the degradation of β-glucosides and cellulose of plant soil debris (Martinez and Tabatabai 1997, Gil-Sotres et al., 2005)towards the production of glucose, which is a crucial carbon energy source for the growth and activity of rhizospheric microbes that help plant stand the stress (Merino et al., 2016). β-amylase 1 (BAM1) is among the family of β-amylases that biodegrade starch in guard cells and sustain stomata opening (Zanella et al., 2016). During degradation, maltose is released from the non-reducing end of the polysaccharide chain. This reaction takes place in mesophyll cells under osmotic stress, thus, BAM1 is predicted to be more active during photosynthesis (Sparla et al., 2006) and released maltose sustains biosynthesis of proline (and soluble sugars like glucose) that acts in alleviating the oxidative stress (Zanella et al., 2016). Indole-3-pyruvate monooxygenase YUCCA1-like supports the action of tryptophan synthetase in indole-3-acetic acid (IAA) biosynthesis as it functions at the last step of "Tryptophan metabolism" pathway. IAA was recently reported to improves plant drought tolerance via activating genes for biosynthesizing abscisic acid (ABA) and jasmonic acid (JA) and those for inhibiting senescence (Zhang et al., 2020). The characteristics of the stress-related genes of subclusters 38/1 and 38/3 (e.g., *TIP1*, *TIP4*, *DHN3*, *DHN3*-like, *DHN4*, *CSP120*, *trpB* & *GELP*) largely complement those of the four TFs (e.g., PAP2, AP2, ERF1 & ERF4), thus, we claim that the chance of relationship involving some of them is high.

The results of cluster 536 indicate coexpression of the transcription bHLH112-like and a drought stress-related gene encoding peroxidase 2-like (*POD2*) (Fig. 8 & Table S8). A recent report indicated that bHLH112 in *Arabidopsis* improves drought tolerance in plants as it promotea accumulation of less reactive oxygen species (ROS), while high activities of the antioxidant enzymes POD and CAT under stress (Li et al., 2021). This TF also promotes low water loss rate and participates in ABA biosynthesis. It was proven experimentally that bHLH112 induces POD promoter (Li et al., 2021). These data complement our speculation of the relationship between the genes encoding bHLH112 and POD2 under drought stress especially that they were upregulated at 2 h time point of drought stress in the GZ168 (Fig. 8 and Table S8). Expression pattern of the 12 genes encoding CAT in wheat was upregulation at 12 h time point (Figure S16), thus, unlikely connects with bHLH112 that was upregulated at 2 h time point.

5. Conclusion

Differential expression of drought-stress related genes was detected between two wheat cultivars with contrasting performance under the stress. These genes were searched for concordantly expressed transcription factors (TFs) and results were validated for two TFs using loss-of-function genomic approach. One TF of the zinc finger protein CONSTANS family was proven to drive expression of a gene encoding a speculative TF namely zinc transporter 3-like (ZIP3) and two other stress-related genes encoding tryptophan synthase alpha chain and asparagine synthetase. Based on qPCR results and known functions of the two TFs under drought stress that complement those of the two stress-related genes, it is likely that they are related. These results validates those of other generated cases of concordant expression between TFs and stress-related genes in other clusters. This study scopes the light on the possible use of metabolic engineering approaches referring to regulatory frameworks under drought stress in breeding programs of bread wheat in the future.

Data availability statement:

Supplemental data can be accessed at: https://drive.google.com/drive/folders/1kIWqOVAshA0zZ9Bz0-ziAvPDHyXjDJET?usp=sharing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

All the supplemental data can be accessed from the link "https://drive.google.com/drive/folders/1kIWqOVAshA0zZ9Bz0-ziAvPDHyXjDJET?usp=sharing".

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103652.

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