INSILICO COMPARATIVE ANALYSIS OF DROUGHT TOLERANCE-RELATED GENES AFFECTING THE PHYSIOLOGICAL TRAITS OF DURUM WHEAT

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Durum wheat yield efficiency has remained approximately low in arid ambient notwithstanding considerable breeding progress. Development of advanced cultivars accompanying drought tolerance-related physiological traits derived from disparate and supplementary genetic pools results in increased grain yield. Therefore, the aim of the present research was to evaluate the major physiological traits related to the genetic potential of drought tolerance in *T. turgidum* through comparative EST analysis of an unstressed library with 3064 EST and a drought-stressed library with 4515 EST. Preliminary data were collected from the Harvard University database. To find similarities between the two libraries, all EST sequences were assembled using EGassembler software and then analyzed using X-blast by CLCbio software against a non-redundant protein database. Over 120 differentially ESTs were detected by the Audic-Claverie statistics of IDEG6 software. All significant differential unigenes were attributed to seven functional categories via the MapMan comparative classification tool. The results revealed that there are significant differences in gene expression of tolerant wheat which affect major physiological processes, culminating in maximizing the assimilate conversion amount and enhancement of carbon capture under drought stress, including increased photosynthetic efficiency, enhanced growth, retaining membrane integrity, and mitigation of any toxic metabolite. These results provide significant indices for appraising drought-tolerant *T. turgidum* for use in crop breeding programs in arid regions.

Keywords: physiological traits, expressed sequence tags (EST), functional catalogs, drought-tolerant cultivars.

INTRODUCTION

Drought is a significant yield-limiting factor in crop production, particularly in Iran where approximately 80% of the country is located in arid and semi-arid regions. A yearly production average of *T. turgidum* is 0.6 million tons in Iran during the crop season 2017–2018 and drought may even result in 30 to 50 % yield loss annual in rainfed areas (Heidari *et al*., 2019). For adjusting complex physiological processes in response to drought stress, plants adopt a variety of cellular and

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molecular regulatory mechanisms which are regulated by both ABA-dependent and ABA-independent pathways, whether for short-term responses to prevent water loss via transpiration from guard cells or for long-term adaptations to acquire stress tolerance at the whole-plant level by regulating stress-responsive gene expression (Takahashi *et al*., 2018). Development of advanced cultivars accompanying drought tolerance-related physiological responses derived from supplementary and disparate genetic pools results in increased grain yield. These responsive genes can be considered as a promising horizon in the appraisal of plant species in order to make effective use of tolerant varieties in breeding programs in arid regions of Iran. The limited success of traditional breeding and present physiological approaches indicates that there is an urgent need for efficient computational strategies. EST analysis is known to be a prompt and efficient means of distinguishing the enormous sets of gene sequences that are expressed in a condition-specific manner in a broad range of tissues and organisms. An EST is a unique sub-sequence of a cDNA within the coding region of a gene that is used for identifying full-length genes in order to eliminate long, costly, and time-consuming steps in sequencing the whole-genome and serves as a landmark for allocating genes to specific physiological traits (Adams *et al*., 1991). Comparing ESTs can provide information on physiological response modulation. Aimed to provide access to sequence data and bioinformatics based tools for data mining, various plant specific EST databases have been established which presently contain collections of plant EST from about 200 species include more than 3.8 million sequences. There are comprehensive analysis pipelines to organize and annotate ESTs with several computational tools for preprocessing, clustering, assembly into contiguous segments known as contigs, and annotation of functional biological information (Ranganathan *et al*., 2009). Gao *et al*. (2008) performed a comparative EST analysis of two cDNA libraries consisting of 2,500 ESTs from the seedling leaves of a drought-tolerant chickpea cultivar under PEG-treated and non-treated conditions to identify the water-stress-induced genes. Based on IDEG6 online software analysis, they deduced that 92 genes were differentially expressed, and these genes were involved in diverse biological processes, such as metabolism, transcription, signal transduction, protein synthesis, and others. Rudd (2003) described the genes involved in abiotic stresses in bean libraries and their results showed that 30% of total ESTs had no similarity to non-redundant protein databases and were introduced as new genes. Deokar *et al*. (2011) generated a single complementary DNA (cDNA) clone to identify differential ESTs in drought-tolerant and susceptible genotypes in chickpea and showed more than 50% of the identified genes are associated with drought stress. Heidari *et al*. (2012) using EST library analysis in crop plants such as bread wheat and rice through IDEG6 and Mapman classification tools, reported the most important genes involved in the physiology of drought stress, including dehydrin, phosphatase, glutathione s-transferase, LEA, lipid transferase, and metallothionein. The present study was performed with the aim of appraising the major physiological traits-associated genes' expression in drought response in *T. turgidum*.

MATERIALS AND METHODS

Leaf's EST preliminary data from drought tolerant cultivar OS-GA of *T. turgidum* includes unstressed (control) and drought-stressed conditions were collected from the Harvard University database. The DFCI gene index web pages (http://compbio.dfci.harvard.edu/tgi/) provide access to a lot of resources for ESTs and gene sequences for over 114 species (Antonescu *et al*., 2010). In the preprocessing step, poor-quality sequences or sequences with less than 100 bp, and vector sequences were trimmed from the raw single-pass sequences using the VecScreen database (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) with a cutoff matching percentage of N≥95 (Schäffer *et al*., 2018). Here, we sought a query sequence for segments that match any sequence in UniVec (a specialized non-redundant vector database) using BLAST with parameters for optimal detection of vector contamination. In the processing step, the cDNA sequences were assembled into clusters using EGassembler software (http://egassembler.hgc.jp/) for constructing contigs with the parameters set at 95% identity over 40 bp. Contigs consist of two or more ESTs, and singletons consist of only one EST (Masoudi Nejad *et al*., 2006). To find the similarities between the two libraries, all EST sequences were assembled by Egassembler software and analyzed using X-blast by CLCbio Workbench software (installed on a Linux system using the installer script which allows special control of the software) against a non-redundant protein database with an E-value≤10^{-5}. IDEG6 software (http://telethon.bio.unipd.it/ideg6) and Audic-Claverie statistics were used to identify genes with differential expression among libraries (Romualdi *et al*., 2003). Generally, cDNA libraries encompass a lot of different expressed genes and existence of a certain cDNA is known as a scarce event which estimate through Poisson distribution. For an EST indicating a small part of a lot of n clones in a library, the probability of the existence of x tags of the similar gene will be measured through the Poisson distribution parametrized by $\lambda \geq 0$ of Equation 1 (Tiňo, 2009).

$$\text{Equ 1: } P(X = x \mid \lambda) = e^{-\lambda} \frac{\lambda^x}{x!}$$

The parameter λ implies the EST number of a certain type (tag) per n clones in the cDNA library. Under the null hypothesis of not differentially expressed genes, it is presumed that the tag count x in one library comes from the similar underlying Poisson distribution P(·|λ) as the tag count y in the other library, when comparing two libraries. Under the null hypothesis that the counts of tag are made from the similar but unknown poisson distribution, the pragmatic gadget of the Audic-Claverie method is a distribution P(y|x) upon counts of tag y in a library informed by the count of tag x in the one another library (Equation 2). P(y|x) is generated through Bayesian averaging in infinite mixture of whole feasible Poisson distributions (Tiňo, 2009).

Equ 2: $P(Y \mid X) = \frac{1}{2^{x+y+1}} \frac{(x+y)!}{x!y!}$

The sequences of contigs and singletons of each library were analyzed by the X-blast program against the Arabidopsis information resource downloaded from the TAIR database (ftp://ftp.arabidopsis.org) (Bassel *et al*., 2011). The comparative classification tool of GoMapMan (http://www.gomapman.org) was used to categorize functional catalogs. GoMapMan is a web tool for gene functionality annotations in the sciences of plants. It was expanded to simplify betterment, visualization, and stabilization of annotations of gene amidst several plant species (Ramšak *et al*., 2014). Mapman outputs are used to describe various catalogs in libraries that can identify useful catalogs in several sample experiments. IDEG6 software and the Audic-Claverie test were used to find functional differential catalogs in libraries.

RESULTS

ASSEMBLY OF CONTIGS AND EST ANNOTATION

3064 and 4515 ESTs were obtained from the unstressed (control) (UCT) and drought-stressed (DST) *T. turgidum* leaf libraries, respectively after eliminating unwanted and vector sequences. The number of constructed contigs and singletons is given in Table 1. The result of X-blast searches using CLCbio software revealed that in the (UCT) library, 15% of ESTs and in (DST), (16%) ESTs had very weak homology (E-value $>10^{-5}$) against the NCBI non-redundant protein database or there was no similar sequence with them, while other ESTs showed high or moderate homology (E-value$<10^{-5}$). Based on the results of this study, sequences that failed to show significant homology to the public database protein are good candidates to be considered as new genes under drought stress. Many contigs and singletons were matched to unknown or hypothetical functions of proteins (results not shown).

FUNCTIONAL CLASSIFICATION AND GO ONTOLOGY

In the present study, 120 unisequences, including contigs and singletons, showed statistically significant differences (E-value$\leq 10^{-5}$) and most of these (over 87%) were upregulated in response to drought. The “only unisequences” that had very significant expression differences have been shown in Table 2 (E-value$\leq 10^{-150}$). Annotated ESTs matched with other organisms (outside of the Plantae kingdom’s spectrum) are not given in Table 2. Well-annotated significant differential unigenes based on IDEG6 software results were divided into seven functional categories (Fig. l). Besides, based on subcellular localization, the identified proteins were categorized into eleven groups (Fig. l). Since the protein

function is generally associated to its subcellular localization, the subcellular localization prediction will be effective for deducing functions of the protein. According to the results of this study, most of the subcellular localization happened in the chloroplast and cytosol. This prediction includes the subcellular localization of photosynthetic proteins in chloroplast, transport in plasma membrane and integral component, the subcellular localization of proteins related to stress and defense in cytosol and endoplasmic reticulum, proteins of transcription regulatory pathways in nucleus, detoxification proteins in chloroplast and extracellular as well as proteins of glycolysis in cytosol.

Table 1

The number of ESTs, contigs and singletons in each library
and determining the hit associated with sequences

Library name	UCT*	DST
Unisequences	3064	4515
Contigs	412 (954 EST)	564 (1890 EST)
Singletons	2110 (69%)	2625 (58%)
Contigs with distinct hit	338 (82%)	488 (87%)
Singletons with distinct hit	1866 (88%)	2127 (81%)

*Unstressed (Control) *T. turgidum* (UCT), Drought-stressed *T. turgidum* (DST)

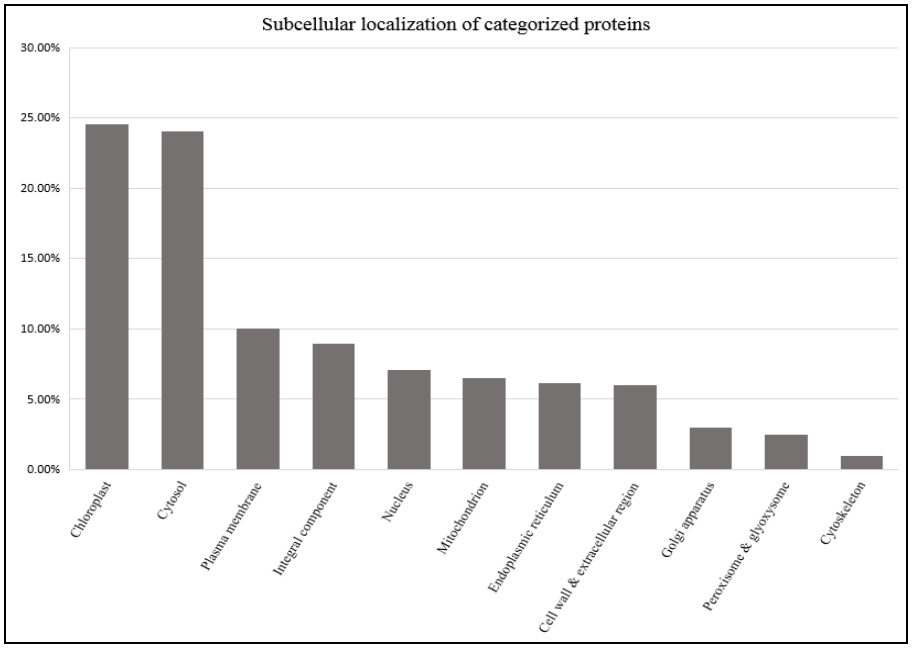

Fig l. Distribution of GO categories in biological process.

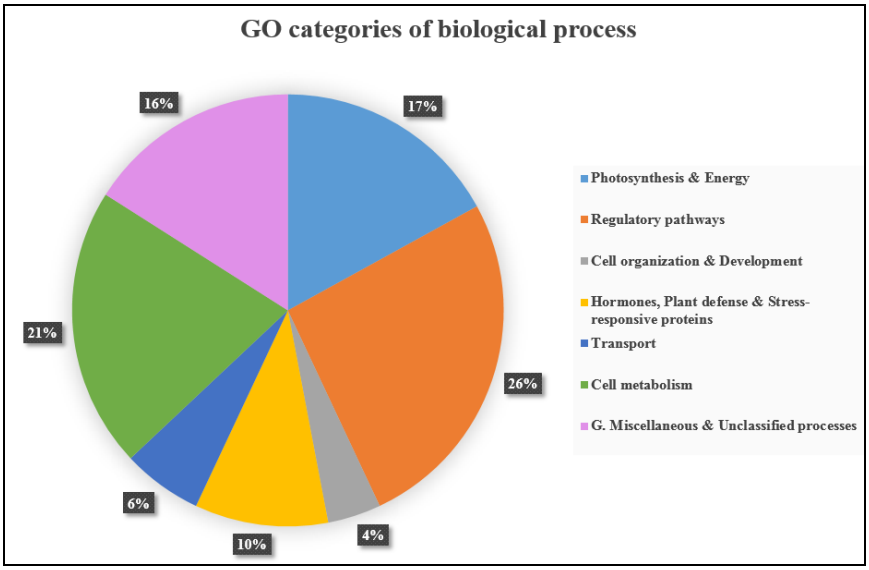

Fig 2. Subcellular localization of categorized proteins in *T. turgidum* under drought stress.

DISCUSSION

PHOTOSYNTHESIS AND ENERGY-RELATED GENES IN *T. TURGIDUM*

17% of differential ESTs were annotated for photosynthesis and energy processes. In the present study, the majority of ESTs involved in photosynthesis were downregulated during the stress, including PSII cytochrome b559 8kDa subunit (*psbE*). The results were in agreement with Wang *et al.* (2020), who found that in Alfalfa under drought stress, there were significantly downregulated proteins *psbE*. Ashraf and Karim (1991) observed a sharp decline in the levels of chlorophyll in plants upon exposure to drought stress, which is attributed to decreased enzymes related to chlorophyll function as the inhibition of photosynthesis is a primary physiological consequence of drought stress. Besides, we identified a unique oxygen-evolving enhancer protein 1-1 (*OEEP*) under drought stress. This result is in accordance with the results from Nouri *et al.* (2015) indicating the upregulation of *OEEP* protein under abiotic stress. However, inconsistent evidence of this protein's expression has been observed in the results of Zadražnik *et al.* (2019), which may indicate the condition- or species-dependent expression of this protein. The upregulation of ESTs annotated to respiration and energy generation such as fructose-bisphosphate aldolase 2 (*FBA2*) was observed in drought stress. It is reported that *FBA* is a main enzyme associated with the Krebs cycle, glycolysis and gluconeogenesis, playing a role in responses to abiotic stresses and in regulating growth and development processes (Rodrigues *et al.*, 2009).

REGULATORY PATHWAYS-RELATED GENES

The largest number (26%) of differential EST affected by the stress were annotated to regulatory pathways. ESTs involved in DNA synthesis and repair remarkably changed in drought-stressed *T. turgidum* compared to unstressed controls. EST annotated to DEAD-box ATP-dependent RNA helicase (*RH*) was upregulated in drought stress. RH is implicated in RNA processing and metabolism. This protein has important roles in diverse cellular functions, e.g., plant growth and development, and in response to biotic and abiotic stresses (Vashisht and Tuteja, 2006). Recently, Gu *et al*. (2014) demonstrated the relevant role of the RH on the growth and stress response in Arabidopsis. Vashisht and Tuteja (2006) reported that RH plays a key function in stabilizing plant growth under drought conditions by adjusting stress-induced pathways. EST analysis revealed that 8% of the drought-responsive proteins are attributed to protein synthesis, processing, and degradation functions, which are the fundamental processes for coping with drought stress. In the present study, expression of some ribosomal proteins that are involved in protein synthesis increased under drought stress, such as 50S ribosomal protein (*RP*). Xu *et al*. (2013) reported that *RP* genes are upregulated in response to abiotic stresses. A similar conclusion has been drawn that expression of *RP* was induced at high and low temperatures in rice. However, the mechanism of their action during abiotic stress has not been described (Kim *et al*., 2004). Furthermore, the proteins functioning in protein folding and processing showed increased change among the libraries. Heat shock proteins (HSPs) and other molecular chaperones were increased. HSPs have a role in retaining normal protein folding and renaturation of the stress-denatured proteins. The role of HSPs in protein folding in drought tolerance has been largely discussed (Timperio *et al*., 2008). Aspartic proteinase (WAP1) as a degradation-related protein was increased in the drought stress library. Proteases (peptidases) catalyzing the hydrolysis of peptide bonds in proteins generally increased in drought-tolerant plant species and decreased in drought-sensitive plant species. The essential role of proteases in plant drought tolerance has been well addressed previously by Miazek *et al*. (2008). Nevertheless, there is conflicting evidence regarding the role of proteases in drought tolerance in the literature. Transcription factor MYB showed change during the drought stress. A large number of studies have discerned stress-responsive transcription factors (TFs) that belong to this TF family that mediate plant tolerance responses to abiotic stress. Functional studies have shown that MYB is involved in ambient responses and plays a key regulatory role in the cell cycle, cell differentiation, and leaf morphogenesis (Kobayashi *et al*., 2015). 4% of differential ESTs were annotated for signal transduction. The genes involved in signaling components are highly upregulated under drought stress, including calcium-dependent protein kinases (*CDPK*) and calmodulin. Drought perception is subsequently followed by the generation of secondary molecule signaling such as protein kinases and phosphatases (Tuteja and Sopory, 2008).

It has been well-established that a specific calcium (Ca^{2+}) signature is generated in response to environmental stimuli. The Ca^{2+} changes are primarily perceived by several Ca^{2+} sensors, such as calmodulin, to initiate various cellular responses (Kudla *et al*., 2010). Zhu (2002) showed that CDPK positively regulates ABA responses and they were also able to phosphorylate AREB/ABF TFs in an ABA dependent manner in *Z. mays*. The results of the present study show the positive role of signaling pathways affecting the physiological processes of *T. turgidum* in creating drought tolerance. ESTs annotated to antioxidant detoxification and redox genes such as Catalase-1 (*CAT1*) were upregulated and accounted for 6% of the differentially expressed ESTs under drought stress conditions. This study demonstrated that mechanisms of defense in tolerant *T. turgidum* have evolved towards antioxidant production to detoxify the ROS and defend the plant against oxidative damage. In agreement with present results, Heidari *et al.* (2018) showed that there is an increase in the accumulation of catalase in Durum wheat to combat drought and reported that more antioxidant detoxification enzymes are generated in drought tolerant cultivars.

CELL ORGANIZATION AND DEVELOPMENT-RELATED GENES

Under drought stress, 4% of ESTs encoding cell organization and development-related genes were identified. We found that the abundance of the cytoskeleton-related protein annotated to expansin (EXPA2) was upregulated under drought stress. EXPs are associated with diverse plant processes of development, including plant leaf and root growth (Goh *et al*., 2012). It has been reported that EXPs are associated with environmental stress tolerance in various plant species. The overexpression of a wheat EXP, TaEXPB23, enhanced drought and oxidative stress tolerance (Li *et al*., 2015). The present study shows the role of EXPs in cell organization and the development process in *T. turgidum* stress tolerance.

HORMONES, PLANT DEFENSE AND STRESS RESPONSIVE PROTEINS-RELATED GENES

In the present study, 10% of the differential EST were annotated as hormones, plant defense, and stress-responsive proteins. The genes encoding allene oxide synthase (*AOS*) were expressed during drought stress. AOS catalyzes the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides of free fatty acids, participating in several critical processes of plant morphogenesis such as cell division, enlargement, and growth (Rodrigues *et al*., 2009). Another gene that was upregulated in drought stress was Pyrroline 5-carboxylate synthetase (*P5CS1*), a bifunctional enzyme able to catalyze the conversion of glutamate to delta pyrroline-5-carboxylate, which is then reduced to proline. It has been shown that proline accumulation correlates with drought stress tolerance in plants

(Szabados and Savouré, 2010), so the upregulation of *P5CS1* may also lead to increased drought tolerance in *T. turgidum* through modulating several cellular processes ranging from metabolism to transport, growth, development, and stress response.

TRANSPORT-RELATED GENES

A large number of ESTs were annotated for transport (6%). Glutamate receptor (*GLR*) was upregulated under drought stress. It has been shown that these transporters have remarkable features that confer tolerance to drought stress. The upregulation of other transporters (i.e. amino acids, calcium, sugars, sucrose, cyclic nucleotide transporters) indicates their important functions in different physiological processes, particularly maintaining relative water content, which is crucial to coping with water deficit. The study of transporters associated with osmolytes and ions paves the way for the assessment of new drought-tolerant cultivars in *T. turgidum*.

CELL METABOLISM-RELATED GENES

Most ESTs annotated to metabolism group were mainly involved in carbohydrates, amino acids, lipids, nucleotide and secondary metabolism and account for 21% of differential ESTs. In carbohydrate metabolism, beta amylase (*BAM1*) was upregulated in response to drought stress. *BAM 1* in mesophyll cells contributes to diurnal starch turnover under osmotic stress (Zanella *et al*., 2016). ESTs annotated to secondary metabolic pathways affected by the stress with a predominant increase in expression of carotenoid biosynthesis genes, i.e., phytoene synthase (*Psy*), were upregulated in response to drought. *Psy* is a significant regulatory enzyme in carotenoid biosynthesis. Carotenoids play vital roles in drought acclimation through involvement in signaling and neutralizing oxidative stress (Uarrota *et al*., 2018). These enzymes and their change of expression are presently highlighted for evaluating their possible protecting roles in drought stress response. ESTs annotated to biosynthetic enzymes of fatty acid, i.e., 12 oxo-phytodienoic acid reductase 2 (*OPR2*) and lipoxygenase (*LOX*), are altered under dehydration conditions. Physiological studies have discerned that the metabolism of fatty acids is altered in drought stress response. Mou *et al*. (2019) showed that expression of *TaOPRs* is induced by various stresses and phytohormones. The *OPRs* are FMN dependent oxidoreductases with limited roles in plants. Rather than serving purely as an antioxidant, Dong *et al*. (2013) believe that *TaOPR1* functions as a signaling compound related to the ABA-mediated signaling regulation in abiotic stress response. *LOX* catalyzes the hydroperoxidation of polyunsaturated fatty acids and thus is the first step in the synthesis of fatty acid metabolites in plants. Gigon *et al*. (2004) showed that expression of *LOX* was induced by water deficits. In the present study, the changes in the gene expression involved in amino acid metabolism were

also found. Amino acids have pivotal functions in plant metabolism and development. In this regard, an increase in the expression of branched-chain amino acid aminotransferase (*BCAT*) has been detected in drought-stressed *T. turgidum*, which plays a role in the amino acid interconversion mechanism and aminotransferase reactions. *BCATs* play a crucial role in the metabolism of branched-chain amino acids (BCAAs). They catalyze the last step of the synthesis and the first step of the degradation of the branched chain amino acids, including valine, isoleucine, and leucine. An accumulation of BCAAs was observed under drought stress conditions in Arabidopsis. The participation of *BCAT*s in response to drought stress has been reported in Arabidopsis and other plant species (Batista-Silva *et al*., 2019). Increased levels of BCAAs under drought conditions lead to protein degradation or trigger a defense mechanism that stimulates the catabolic activity of *BCAT* and prevents the accumulation of toxic metabolites, which maintains the pool of free BCAAs at low and nontoxic levels, as shown in sugarcane (Ali *et al*., 2019).

MISCELLANEOUS AND UNCLASSIFIED PROCESSES

16% of the significant differential ESTs remained miscellaneous and unclassified functions, which were regulated under drought stress. A catalog of ESTs, attributed to miscellaneous, is associated with different metabolic processes having moderately great alteration in expression. But the unclassified genes are interesting as they harbor the potential to provide drought adaptation and therefore serve as novel drought tolerance genes.

CONCLUSION

Drought stress results in photosynthesis impairment, decreased CO2 assimilation rate, plant growth and development restrictions, increased ROS, structural and membrane injuries by altering biological functions and metabolic activity, eventually leading to reduced yield. However, some cultivars of plants have evolved several cellular and molecular mechanisms to overcome drought stress. Effective traits can be considered as a promising horizon in the appraisal of plant species (Heidari *et al*., 2020). This study revealed that tolerant *T. turgidum* possibly improves photosynthetic efficiency by regulating the expression of genes involved in carbohydrate metabolism (through *BAM1*) and secondary metabolic pathways (*Psy*). In a way, through *BAM1*, tolerant wheat remobilizes its reserve of starch to release sugars, other metabolites, and eventually energy, contributing to alleviating the stress. *BAM1* is an essential gene for plant fitness with an important function for the productivity of plants under ambient stress conditions (Zanella *et al*., 2016). On the other hand, tolerant wheat recruits carotenoids natural pigments (through upregulated

Psy) that are consider as substantial components in photosynthetic processes encompassing harvesting light energy, protection to photosynthetic apparatus against ROS and acts as signaling component that effect growth, development and respond to drought stress (Isaacson *et al*., 2002; Farré *et al*., 2010). Therefore, through enhancement in water soluble carbohydrates, it results in enhanced grain numbers and a greater source for filling of grain under drought stress. We identified five candidate genes in *T. turgidum* affecting growth and development processes under drought stress, including *FBA2*, RNA helicase, *MYB*, *EXP*, and *AOS*. Whilst *FAB2* plays a vital role in the response of tolerant wheat to drought by affecting growth processes and enhancing respiration, this gene may also play a role in tolerating drought stress in gluconeogenesis-associated processes. Because gluconeogenesis pathways are considered a primary metabolism that establishes a new homeostasis under drought stress in plants, providing a key interface between sugar, lipid, and amino acid metabolism (Caruso *et al*., 2009). Besides, results suggest that *T. turgidum* adopts mechanisms to retain the normal physiological function of plants through maintaining the correct protein folding via *HSP* and to preserve cell compartmentation and maintain membrane integrity through changed fatty acid metabolism since lipids are constituent substances of the membrane and their composition alteration may contribute to drought tolerance. It defends against the elevated levels of toxic metabolites in cells under drought stress by modulating the amino acid metabolism (*BCAT*), secondary metabolites (*OPR2*) and other regulatory pathways, i.e., intracellular enzymatic antioxidant. This study provides important indices for evaluating *T. turgidum* as a drought-tolerant plant for use in crop breeding programs in arid regions.

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