DIFFERENTIAL RESPONSE OF DROUGHT STRESS-RESPONSIVE GENES AMONG CONTRASTING GENOTYPES OF BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract. This study investigates the transcriptomic responses of two wheat cultivars, GZ168 (drought-tolerant) and GM10 (drought-sensitive), to drought stress induced by 20% polyethylene glycol (PEG) treatment at 0h, 2h, and 12h. RNA-Seq data were analyzed to identify differentially expressed genes (DEGs) and enriched KEGG pathways. Hierarchical clustering revealed distinct transcriptomic profiles between the cultivars, with GM10 exhibiting transient changes, while GZ168 demonstrating sustained transcriptional regulation under PEG-induced drought. Key DEGs encoding enzymes involved in carbohydrate, amino acid, and lipid metabolism were identified, highlighting metabolic pathways associated with drought tolerance. Specifically, the drought-tolerant GZ168 showed enrichment of acetyl-CoA, long-chain acyl-CoA, tryptophan, indole acetate, amylose, starch, and glucose as core intermediate crosstalking metabolites. These metabolic shifts were associated with the upregulation of key enzymes such as acetyl-CoA synthetase, long-chain acyl-CoA synthetase, tryptophan synthase, and starch synthase. This transcriptomic data provides insights into the molecular mechanisms underlying drought tolerance in wheat. We recommend leveraging these findings in wheat breeding programs by targeting key enzymes and metabolic pathways identified in GZ168 to enhance drought resilience in other wheat cultivars through targeted metabolic engineering approaches.
Keywords: *RNA-Seq, Acyl-CoA, tryptophan, melatonin, ABA, IAA, glucose*

Introduction

Bread wheat (*T. aestivum*), possessing a hexaploid genome structure (genomes AABBDD, 2n = 6x = 42, genome size = 16,000 Mbp), is a vital crop in global agriculture, providing an essential food source for both humans and animals (Guan et al., 2020; Walkowiak et al., 2020). Shown by its widespread presence, wheat is highly important, being the most widely cultivated cereal crop globally, covering approximately 218 million hectares (Ding et al., 2021; Grote et al., 2021; Abulfaraj, 2023). Wheat provides a substantial proportion of daily nutritional needs, contributing up to 50% of dietary intake in many regions worldwide (Shewry, 2018; Seleiman et al., 2021). However, drought stress significantly impacts wheat productivity, reducing grain yield and quality (Roy et al., 2021; Sharma et al., 2022). Tolerant plant germplasm offers a promising strategy to mitigate the impacts of abiotic stresses (Amini et al., 2023). Climate change and global warming pose serious threats to the global food system, particularly in regions at risk of hunger and malnutrition, increasing the vulnerability of staple crops such as wheat and rice to changing climates (Javadi et al., 2023; Qin et al., 2023).

Among environmental stresses, drought stands out as the most harmful problem affecting agricultural productivity (Wani, 2023). Water scarce, shown by lack of rain and/or groundwater during pivotal phases of the plant growth cycle, creates a mix of obstacles preventing optimal grain yield in cereals, notably wheat (Zovko et al., 2019; Skendžić et al., 2023). Under such stress, the phenomenon of stomatal closure, initiated

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by abscisic acid (ABA), emerges as a pivotal adaptive response, reducing cell water loss. However, this adaptive mechanism also leads to a decrease in CO_2 assimilation and subsequent metabolic processes pivotal to plant growth and development. Noteworthy among the problems affecting normal cell function under drought stress is the reduction of signal transduction cascades and the stopping of critical protein synthesis pathways crucial for sustaining growth amidst adversity (Wu et al., 2022; Movahedi et al., 2023; Wang et al., 2024a). These harmful changes affect cell metabolism, causing fluctuations in essential macromolecules such as carbohydrates, lipids, and proteins, all of which are pivotal orchestrators of stress adaptation responses (Ahmad et al., 2018; Liang et al., 2023). Notably, lipids assume a pivotal role in fortifying cellular integrity and resilience, serving as reservoirs of stored energy and signalling molecules pivotal for orchestrating adaptive responses to adverse conditions (Zhao et al., 2021; Liang et al., 2023). Meanwhile, carbohydrate mobilization reserves, including starch and soluble sugars, emerges as a key strategy facilitating metabolic homeostasis in the face of adversity (Prathap and Tyagi, 2020; Li et al., 2021; Raza et al., 2023). Moreover, select proteins emerge as indispensable architects of the signal transduction apparatus, ready at the forefront of stress-responsive cascades (Wu et al., 2022; Wang et al., 2024b).

Other crucial drought adaptation mechanisms warranting consideration include the accumulation of osmoprotectants, like proline, glycine betaine, and trehalose that play vital roles in maintaining cellular turgor and protecting cellular structures from dehydration-induced damage (Sharma et al., 2019; Seleiman et al., 2021; Raza et al., 2023). Furthermore, the activation of specific transcription factors, such as those belonging to the AP2/ERF, bZIP, and MYB families, orchestrates the expression of numerous stress-responsive genes (Hrmova and Hussain, 2021). This leads to the synthesis of protective proteins and the modulation of metabolic pathways. Additionally, epigenetic modifications, including DNA methylation and histone modifications, contribute to long-term acclimation to drought stress by altering gene expression patterns (Ashapkin et al., 2020; Sun et al., 2021; Guarino et al., 2022). These epigenetic modifications can persist through generations, potentially conferring transgenerational drought tolerance. For instance, in rice, stable methylation changes in stress-responsive genes have been observed to be passed on to progeny for multiple generations (Guarino et al., 2022). Abscisic acid (ABA) functions as a pivotal phytohormone orchestrating drought acclimation responses in plants, exerting its regulatory influence not solely through the induction of stomatal closure, but also by fostering root system proliferation and potentiating the expression of genes encoding stress-protective proteins (Haghpanah et al., 2024). Incorporating these additional mechanisms provides a more holistic understanding of the intricate drought response in plants.

Further worsening the effects of drought stress are multifaceted disruptions to essential physiological processes encompassing chlorophyll content, gas exchange dynamics, water potential gradients, cell turgidity, antioxidant defense systems, and pivotal metabolite biosynthetic pathways (Khaleghi et al., 2019; Ahmad et al., 2024). In light of these challenges, the imperative to fortify cultivated crops against abiotic stresses, including drought, becomes a global urgency (Chaudhry and Sidhu, 2022). Deciphering the intricacies of plant adaptive strategies under such adversities and harnessing biotechnological modalities to augment crop resilience stands as a crucial necessity (Chaudhry and Sidhu, 2022; Abulfaraj, 2023).

This study aims to conduct a comprehensive RNA-Seq analysis on two wheat varieties that exhibit contrasting responses to drought conditions. The primary objective is to

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unravel the metabolic processes that enable certain wheat genotypes to better withstand water scarcity. By scrutinizing these sophisticated biological mechanisms, our research seeks to identify key genes and pathways associated with drought resilience, elucidate the molecular basis of differential drought responses between the two cultivars, and generate valuable knowledge that can be applied to wheat improvement programs. Furthermore, this investigation aspires to harness the acquired insights to engineer more resilient wheat cultivars capable of enduring extended periods of water deficit.

Materials and methods

Growth condition and induction of drought stress

The experimental protocol was instituted in 2015 at King Abdulaziz University, Jeddah, Saudi Arabia, employing a randomized complete block design to scrutinize two divergent Egyptian bread wheat cultivars concerning their drought resilience levels. The chosen cultivars encompassed a drought-tolerant/high-yielding Giza 168 cultivar alongside its counterpart, the drought-sensitive Gemmiza 10 cultivar (Al-Naggar et al., 2015; Abdelghany et al., 2016). Commencing with the germination phase, seeds were germinated within a greenhouse milieu supplemented with Hoagland solution at half-strength, allowing germinated seedlings to undergo a preliminary incubation period of 9 days. Subsequently, the seedlings were transitioned to a full-strength nutrient solution for a duration of 12 days, maintaining growth parameters at a constant temperature of 22 ± 2°C, under a 16-hour photoperiod, and an illuminance of 450 μmol μmol $m^{-2}s^{-1}$. Thereafter, a randomized complete block design was performed for three-week-old seedlings of both cultivars, with each under two replicates, where seedlings underwent treatment with 20% polyethylene glycol (Baloglu et al., 2014) across varying durations (0 h, 2 h, and 12 h) within the light phase. Subsequent to treatment, leaf samples (six from each cultivar) were meticulously harvested, promptly subjected to flash-freezing, and maintained at -20°C until downstream processing.

RNA sequencing and bioinformatics

Total leaf RNA extraction was performed for each wheat cultivar in two replicates across the three time points 0h, 2h, and 12h, utilizing the Trizol kit (cat. no. 12183555), following established protocols (Abulfaraj, 2023). The extracted RNA samples underwent RNase-free/DNase treatment (Invitrogen, cat. no. 89836) to eliminate DNA contaminants, subsequently validated via standard PCR targeting the housekeeping actin gene. The PCR amplification was executed using *actin*-specific primers, generating a 369-bp amplicon, with a PCR regimen comprising 40 cycles following an initial denaturation step. Gel electrophoresis showed absence of any PCR products, thus no DNA contamination. Subsequently, 30 μg of RNA from each sample was cryopreserved in dry ice and dispatched to BGI, China, to conduct RNA-Seq analysis. The generated RNA-Seq raw data (≥ 100 million reads per sample) were archived in the SRA database of the NCBI (National Center for Biotechnology Information) and received no. PRJNA306536. Post-retrieval reads were mapped against a reference genome of *Triticum aestivum*, as curated by the IWGSC, with subsequent refinement of assembly employing standard procedure (Kim et al., 2015; Walkowiak et al., 2020; Zhu et al., 2021; Abulfaraj, 2023).

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Following *de novo* assembly of transcriptomes via Trinity (v2.15.0) RNA-Seq transcript assembler, contigs exceeding 200 bp in length were retained for subsequent analyses. Differential gene expression analysis was executed utilizing EdgeR (R version 2.1.5, Robinson et al., 2010), filtering candidates with log $2/fpkm^{+1}$ and FDR (False Discovery Rate) of 10-3 or more. Comparison of FPKM (Fragments Per Kilobase of transcript per Million) read counts was done as described (Casella and Berger, 2021). Differential gene expression profiles were subjected to Blastx analysis with an E-value cut off of $1e^{-5}$, with significant Pearson correlation ascertained through permutation analysis. The identified encoding genes were subsequently assigned to pathways within KEGG (Kyoto Encyclopedia of Genes and Genomes) database to discern enriched enzymes/proteins across distinct expression patterns. Enriched enzymes/proteins were annotated via manual curation utilizing the KEGG pathway database.

Results

Transcriptome data overview

The transcriptomic landscape of two distinct wheat (*Triticum aestivum*) cultivars, characterized by differential drought stress tolerance, was carefully scrutinized across three temporally defined stress intervals (0h, 2h, and 12h). Importantly, the accuracy of the RNA-Seq datasets was corroborated through validation via real time PCR, as detailed in our recent study (Abulfaraj, 2023). Hierarchical clustering heat maps were instrumental in discerning the relative distances between disparate transcriptomes (*Fig. 1*). Notably, a discernible separation between the transcriptomic clusters of the two contrasting genotypes was evident, irrespective of the imposed drought stress conditions; whereas, the sub-clusters of each genotype exhibited a correlation with the duration of the drought stress treatment. Intriguingly, the transcriptomic landscape of the GM10 cultivar for 12h exhibited a notable convergence with its 0h counterpart, suggestive of transient regulatory changes in response to short-term drought exposure, followed by a restoration of baseline expression levels. In contrast, the drought-tolerant GZ168 cultivar demonstrated sustained transcriptional regulation, with a plethora of transcripts exhibiting differential expression persisting up to the 12h time point.

Differentially expressed genes (DEGs)

Employing an algorithmic approach delineated in previous investigations, cluster analysis facilitated the identification of a substantial cadre comprising 881 clusters. Among these, 30 clusters exhibiting discrete expression patterns and a fold change threshold of ≥4 across the three delineated stress times (0h, 2h, and 12h) were selectively chosen for in-depth scrutiny (*Fig. S1* and *Table S1*). These clusters encompassed six distinct expression patterns, denoted as 2h up (expression pattern 1), GZ-2h up (expression pattern 2), GM-2h up (expression pattern 3), GZ-2h/12h up (expression pattern 4), 12h up (expression pattern 5), and GZ-12 up (expression pattern 6), with the respective cluster distribution depicted in *Fig. S1*.

Noteworthy among these differentially expressed genes (DEGs) were those encoding enzymes/proteins integral to various KEGG pathways, spanning four principal categories as shown in *Table S2*. Cumulatively, these functional categories encompassed over 300 genes across the contrasting wheat genotypes. Of particular interest were 38 genes encoding enzymes/proteins implicated in KEGG pathways related to drought stress,

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which were subjected to comprehensive analysis (*Table S3*), while expression profiling for the encoded enzymes/proteins at the different time points of drought stress for the two cultivars are shown in *Figs. 2-7*.

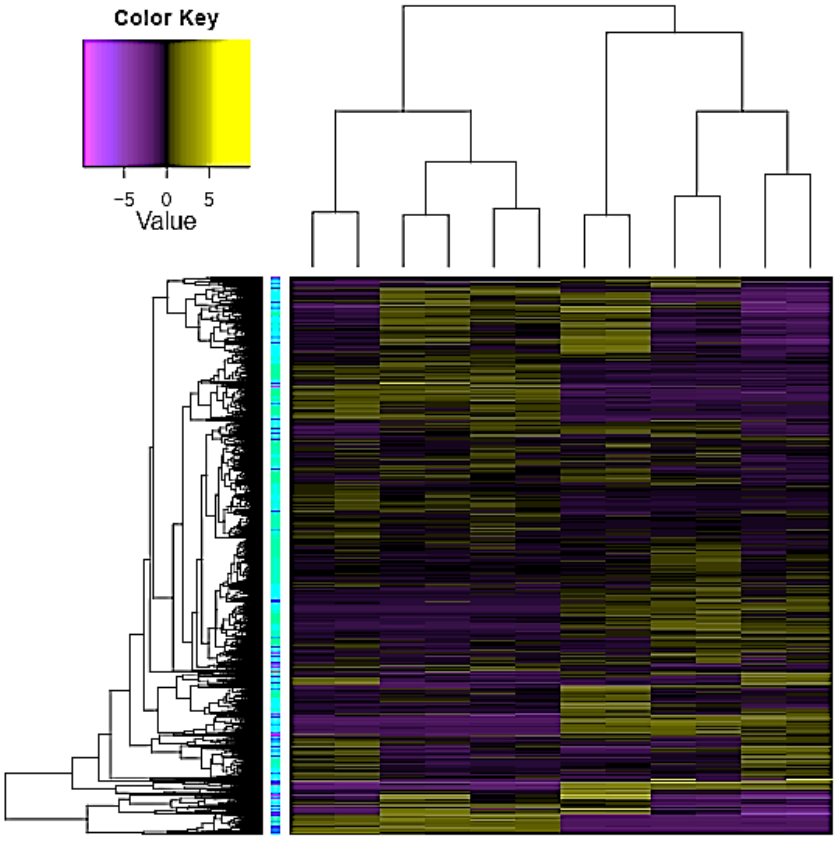

Figure 1. *Hierarchical cluster analysis of gene expression to describe relationships among leaf transcriptomes of the two bread wheat (T. aestivum) contrasting genotypes GZ168 (drought-tolerant) and GM10 (drought-sensitive) at time points of 0, 2h and 12h of drought stress. GZ = Giza168, GM = Gimmeza10. 1 = GZ-12h-1, 2 = GZ-12h-2, 3 = GZ-2h-1, 4 = GZ-2h-2, 5 = GZ-0h-1, 6 = GZ-0h-2, 7 = GM-2h-1, 8 = GM-2h-2, 9 = GM-0h-1, 10 = GM-0h-2, 11 = GM-12h-1, 12 = GM-12h-2*

The results in *Fig. 2* highlights upregulated genes at the 2h time point of drought stress (Expression pattern 1) across leaf transcriptomes of GZ168 and GM10. Drought stress-related genes in this figure include those encoding UDPglucose 6-dehydrogenase (EC 1.1.1.22) (Cluster 5), shikimate kinase (EC 2.7.1.71) (Cluster 5), glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) (Cluster 2), beta-fructofuranosidase (EC 3.2.1.26) (Cluster 13), L-arabinokinase (EC 2.7.1.46) (Cluster 2), caffeic acid 3-O-methyltransferase (EC 2.1.1.4) (Cluster 28), aldehyde dehydrogenase (NAD+) (EC 1.2.1.3) (Cluster 9), two-component response regulator ARR-A family (A-ARR) (K14492) (Cluster 28), xylan 1,4-beta-xylosidase (EC 3.2.1.37) (Cluster 24), phytochrome-interacting factor 4 (TF) (K16189) (Cluster 28), 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha (EC 1.2.7.11) (Cluster 4), and glycine hydroxymethyltransferase (EC 2.1.2.1) (Cluster 13).

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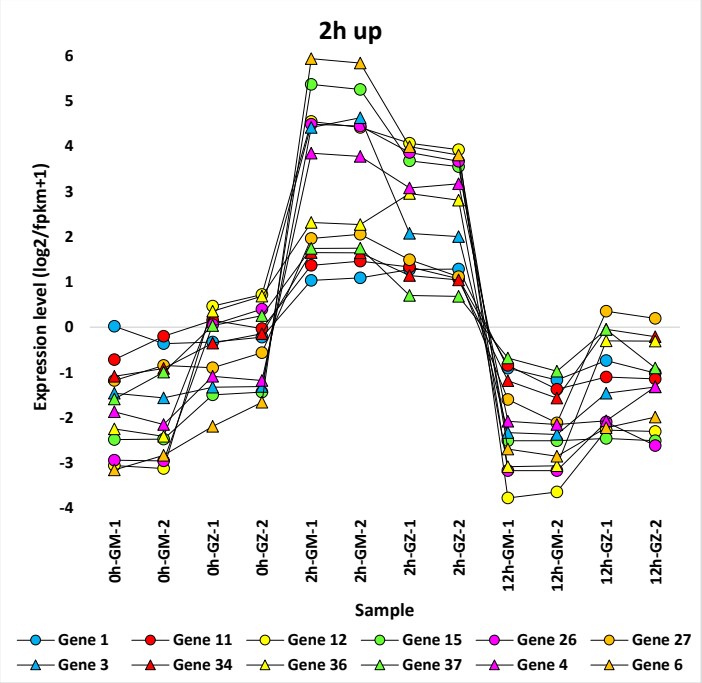

Figure 2. *Upregulated genes (12) at 2h time point of drought stress (Expression pattern 1) across leaf transcriptomes of the two contrasting wheat (T. aestivum) genotypes GZ168 (drought-tolerant) and GM10 (drought-sensitive). Gene 1 = encoding UDPglucose 6-dehydrogenase (EC 1.1.1.22) (Cluster 5), Gene 11 = encoding shikimate kinase (EC 2.7.1.71) (Cluster 5), Gene 12 = encoding glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) (Cluster 2), Gene 15 = encoding beta-fructofuranosidase (EC 3.2.1.26) (Cluster 13), Gene 26 = encoding L-arabinokinase (EC 2.7.1.46) (Cluster 2), Gene 27 = encoding caffeic acid 3-O-methyltransferase (EC 2.1.1.4) (Cluster 28), Gene 3 = encoding aldehyde dehydrogenase (NAD+) (EC 1.2.1.3) (Cluster 9), Gene 34 = encoding two-component response regulator ARR-A family (A-ARR) (K14492) (Cluster 28), Gene 36 = encoding xylan 1,4-beta-xylosidase (EC 3.2.1.37) (Cluster 24), Gene 37 = encoding phytochrome-interacting factor 4 (TF) (K16189) (Cluster 28), Gene 4 = encoding 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha (EC 1.2.7.11) (Cluster 4), Gene 6 = encoding glycine hydroxymethyltransferase (EC 2.1.2.1) (Cluster 13). Detailed cluster analysis and KEGG pathway datasets for these genes are shown in Tables S1 and S2*

While, the results in *Fig. 3* present upregulated genes at the 2h time point of drought stress (Expression pattern 2) specifically in the drought-tolerant GZ168 cultivar. Key genes in this expression pattern include those encoding starch synthase (EC 2.4.1.21) (Cluster 23), ATP citrate (pro-S)-lyase (EC 2.3.3.8) (Cluster 17), acetyl-CoA synthetase (EC 6.2.1.1) (Cluster 17), long-chain acyl-CoA synthetase (EC 6.2.1.3) (Cluster 23), malate dehydrogenase (EC 1.1.1.40) (Cluster 22), phosphoethanolamine methyltransferase (EC 2.1.1.103) (Cluster 22), 3-oxoacyl-[acyl-carrier-protein] synthase II (EC 2.3.1.179) (Cluster 17), granule-bound starch synthase (EC 2.4.1.242) (Cluster 30), enoyl-[acyl-carrier protein] reductase I (EC 1.3.1.9/1.3.1.10) (Cluster 22), 3-oxoacyl-[acyl-carrier-protein] synthase III (EC 2.3.1.180) (Cluster 17), and 1,4-alpha-glucan branching enzyme (EC 2.4.1.18) (Cluster 17).

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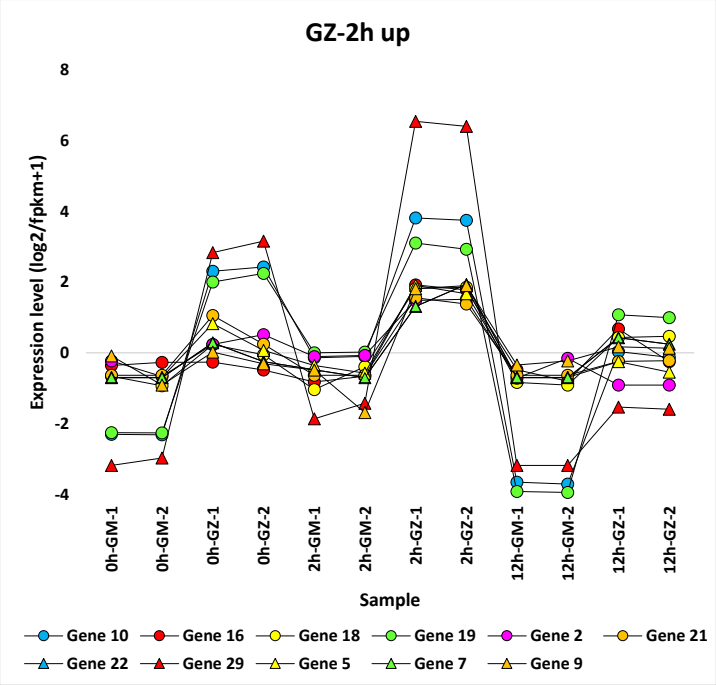

Figure 3. *Upregulated genes (11) at 2h time point of drought stress (Expression pattern 2) in leaf transcriptomes of the drought-tolerant wheat (T. aestivum) cultivar GZ168. Gene 10 = encoding starch synthase (EC 2.4.1.21) (Cluster 23), Gene 16 = encoding ATP citrate (pro-S)-lyase (EC 2.3.3.8) (Cluster 17), Gene 18 = encoding acetyl-CoA synthetase (EC 6.2.1.1) (Cluster 17), Gene 19 = encoding long-chain acyl-CoA synthetase (EC 6.2.1.3) (Cluster 23), Gene 2 = encoding malate dehydrogenase (EC 1.1.1.40) (Cluster 22), Gene 21 = encoding phosphoethanolamine methyltransferase (EC 2.1.1.103) (Cluster 22), Gene 22 = encoding 3-oxoacyl-[acyl-carrier-protein] synthase II (EC 2.3.1.179) (Cluster 17), Gene 29 = encoding granule-bound starch synthase (EC 2.4.1.242) (Cluster 30), Gene 5 = encoding enoyl-[acyl-carrier protein] reductase I (EC 1.3.1.9/1.3.1.10) (Cluster 22), Gene 7 = encoding 3-oxoacyl-[acyl-carrier-protein] synthase III (EC 2.3.1.180) (Cluster 17), Gene 9 = encoding 1,4-alpha-glucan branching enzyme (EC 2.4.1.18) (Cluster 17). Detailed cluster analysis and KEGG pathway datasets for these genes are shown in Tables S1 and S2*

Coversally, *Fig. 4* displays upregulated genes at the 2h time point of drought stress (Expression pattern 3) in the drought-sensitive GM10 cultivar. These genes encode phospholipase D1/2 (EC 3.1.4.4) (Cluster 25), Ras-related C3 botulinum toxin substrate 1 (Rac/Rac1) (K04392) (Cluster 25), glycerol-3-phosphate acyltransferase (EC 2.3.1.15/2.3.1.198) (Cluster 25), and jasmonic acid-amino synthetase (JAR1) (EC 6.3.2.52) (Cluster 25). The results in *Fig. 5* demonstrate upregulated genes at both 2h and 12h time points of drought stress (Expression pattern 4) in the drought-tolerant GZ168 cultivar. The identified genes encode tryptophan synthase (EC 4.2.1.20) (Cluster 7), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (EC 4.2.1.17/1.1.1.35/1.1.1.211) (Cluster 7), and tryptophan aminotransferase (EC 2.6.1.27) (Cluster 7).

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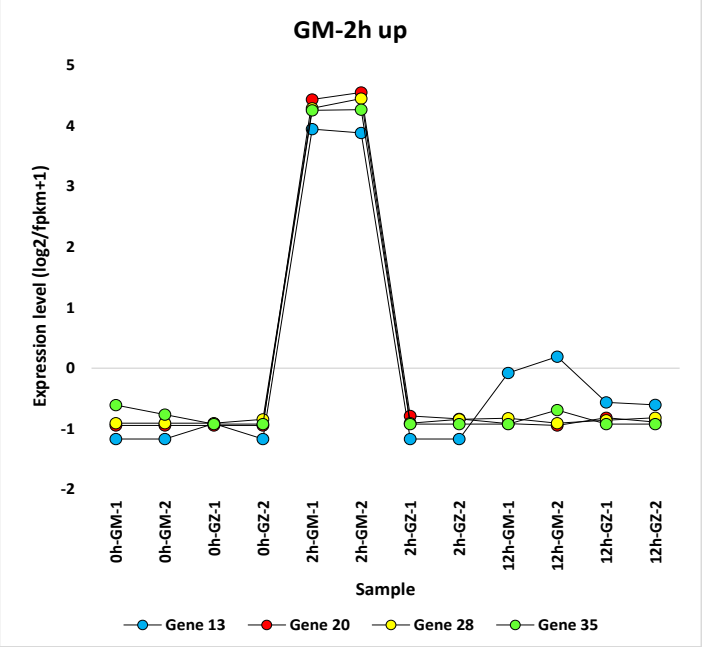

Figure 4. *Upregulated genes (4) at 2h time point of drought stress (Expression pattern 3) in leaf transcriptomes of the drought-sensitive wheat (T. aestivum) cultivar GM10. Gene 13 = encoding phospholipase D1/2 (EC 3.1.4.4) (Cluster 25), Gene 20 = encoding Ras-related C3 botulinum toxin substrate 1 (Rac/Rac1) (K04392) (Cluster 25), Gene 28 = encoding glycerol-3-phosphate acyltransferase (EC 2.3.1.15/2.3.1.198) (Cluster 25), Gene 35 = encoding jasmonic acid-amino synthetase (JAR1) (EC 6.3.2.52) (Cluster 25). Detailed cluster analysis and KEGG pathway datasets for these genes are shown in Tables S1 and S2*

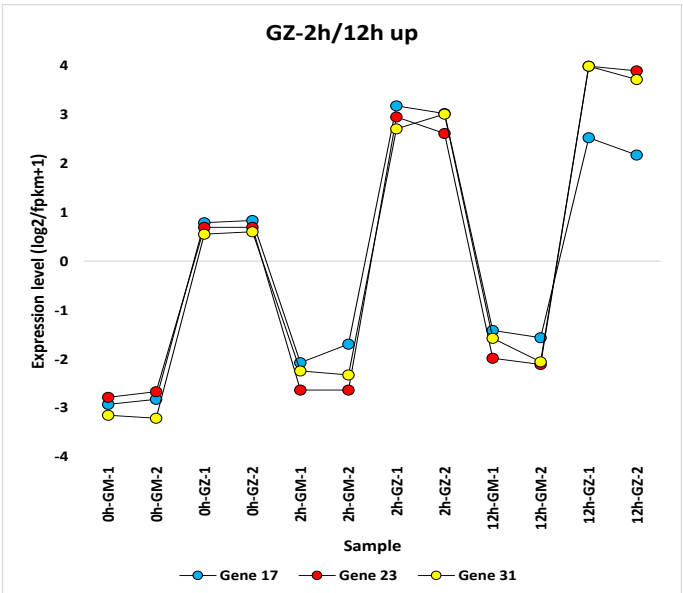

Figure 5. *Upregulated genes (3) at 2h/12h time points of drought stress (Expression pattern 4) in leaf transcriptomes of the drought-tolerant wheat (T. aestivum) cultivar GZ168. Gene 17 = encoding tryptophan synthase (EC 4.2.1.20) (Cluster 7), Gene 23 = encoding enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (EC 4.2.1.17/1.1.1.35/1.1.1.211) (Cluster 7), Gene 31 = encoding tryptophan aminotransferase (EC 2.6.1.27) (Cluster 7). Detailed cluster analysis and KEGG pathway datasets for these genes are shown in Tables S1 and S2*

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However, *Fig. 6* indicates an upregulated gene encoding sucrose synthase (EC 2.4.1.13) (Cluster 8) at the 12h time point of drought stress (Expression pattern 5) across both GZ168 and GM10 cultivars, while *Fig. 7* presents seven upregulated genes at the 12h time point of drought stress (Expression pattern 6) specifically in the drought-tolerant GZ168 cultivar. These genes encode beta-glucosidase (EC 3.2.1.21) (Cluster 11), indole-3-pyruvate monooxygenase (EC 1.14.13.168) (Cluster 11), hexosaminidase (EC 3.2.1.52) (Cluster 18), auxin influx carrier (AUX1 LAX family) (AUX1) (K13946) (Cluster 11), auxin-responsive protein IAA (AUX/IAA) (Cluster 18), SAUR family protein (SAUR) (K14488) (Cluster 18), and basic endochitinase B (EC 3.2.1.14) (Cluster 11).

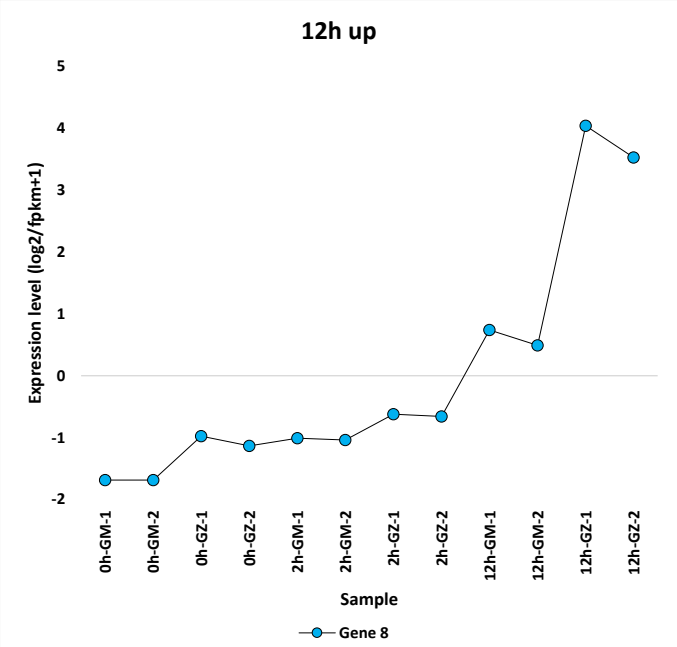

Figure 6. *Upregulated gene at 12h time point of drought stress (Expression pattern 5) across leaf transcriptomes of the two contrasting wheat (T. aestivum) genotypes GZ168 (drought-tolerant) and GM10 (drought-sensitive). Gene 8 = encoding sucrose synthase (EC 2.4.1.13) (Cluster 8). Detailed cluster analysis and KEGG pathway datasets for this gene are shown in Tables S1 and S2*

Subsequent scrutiny elucidated that the selected enzymes/proteins were affiliated predominantly with the "Metabolism" and "Environmental Information Processing" KEGG categories. Within the "Metabolism" domain, subcategories referring to amino acid, energy and lipid, and carbohydrate metabolism harbored enriched pathways, each contributing to distinct facets of drought stress resilience (*Tables S4-S7*). Additionally, the "Environmental Information Processing" subcategory encapsulated pathways crucial to drought stress adaptation (*Table S8*). Comprehensive profiling of these pathways, encompassing 14 distinct categories, underscored their intricate modulation across the delineated time points of drought stress exposure (*Figs. S2-S31*). Notably, three discernible drought tolerance mechanisms emerged, characterized by a profusion of enriched crosstalking enzymes/proteins, emblematic of pivotal steps implicated in fortifying the resilience of each genotype (*Figs. 8-10*).

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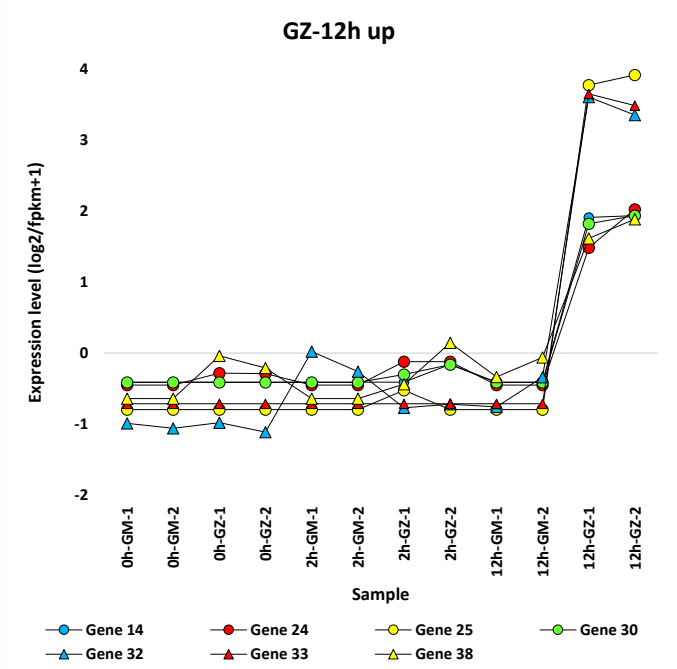

Figure 7. *Upregulated genes (7) at 12h time points of drought stress (Expression pattern 6) in leaf transcriptomes of the drought-tolerant wheat (T. aestivum) cultivar GZ168. Gene 14 = encoding beta-glucosidase (EC 3.2.1.21) (Cluster 11), Gene 24 = encoding indole-3-pyruvate monooxygenase (EC 1.14.13.168) (Cluster 11), Gene 25 = encoding hexosaminidase (EC 3.2.1.52) (Cluster 18), Gene 30 = encoding auxin influx carrier (AUX1 LAX family) (AUX1) (K13946) (Cluster 11), Gene 32 = auxin-responsive protein IAA (AUX/IAA) (Cluster 18), Gene 33 = encoding SAUR family protein (SAUR) (K14488) (Cluster 18), Gene 38 = encoding basic endochitinase B (EC 3.2.1.14) (Cluster 11). Detailed cluster analysis and KEGG pathway datasets for these genes are shown in Tables S1 and S2*

Figure 8 illustrates the enrichment of acetyl-CoA and long-chain acyl-CoA as two core intermediate crosstalking metabolites to confer drought stress tolerance in the drought-tolerant wheat cultivar GZ168. These chemical reactions occurred via crosstalking of six KEGG pathways namely "Glycolysis / Gluconeogenesis" (*Figs. S9* and *S10* and *Table S5*), "Citrate cycle (TCA cycle)" (*Figs. S11* and *S12* and *Table S5*), and "Pyruvate metabolism" (*Figs. S19* and *S20* and *Table S5*), "Carbon fixation in photosynthetic organisms" (*Fig. S21* and *Table S6*), "Fatty acid biosynthesis" (*Fig. S22* and *Table S7*), "Fatty acid degradation" (*Figs. S23-S25* and *Table S7*). Key encoded enzymes include aldehyde dehydrogenase (NAD+) (EC 1.2.1.3), 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha (EC 1.2.7.11), acetyl-CoA synthetase (EC 6.2.1.1), ATP citrate (pro-S)-lyase (EC 2.3.3.8), malate dehydrogenase (EC 1.1.1.40), 3-oxoacyl-[acyl-carrier-protein] synthase II (EC 2.3.1.179), 3-oxoacyl-[acyl-carrier-protein] synthase III (EC 2.3.1.180), enoyl-[acyl-carrier protein] reductase I (EC 1.3.1.9/EC 1.3.1.10), long-chain acyl-CoA synthetase (EC 6.2.1.3), and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (EC 4.2.1.17/EC 1.1.1.35/EC 1.1.1.211).

Figure 9 depicts the enrichment of tryptophan and indole acetate (IAA) as core intermediate crosstalking enzymes/proteins to confer drought stress tolerance in the drought-tolerant wheat cultivar GZ168. These chemical reactions occurred via crosstalking of four KEGG pathways namely "Glycine, serine and threonine metabolism"

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(*Figs. S2* and *S3* and *Table S4*), "Tryptophan metabolism" (*Figs. S4-S6* and *Table S5*), "Phenylalanine, tyrosine and tryptophan biosynthesis" (*Figs*. *S7* and *S8* and *Table S5*) and "Plant hormone signal transduction" (*Figs*. *S29-S31* and *Table S8*). Key encoded enzymes include glycine hydroxymethyltransferase (EC 2.1.2.1), tryptophan synthase (EC 4.2.1.20), aldehyde dehydrogenase (NAD+) (EC 4.2.1.20), indole-3-pyruvate monooxygenase (EC 1.14.13.168), tryptophan aminotransferase (EC 2.6.1.27), shikimate kinase (EC 2.7.1.71), caffeic acid 3-O-methyltransferase (EC 2.1.1.4), auxin-responsive protein IAA (AUX/IAA, K14484), SAUR family protein (SAUR, K14488), auxin influx carrier (AUX1 LAX family) (AUX1, K13946).

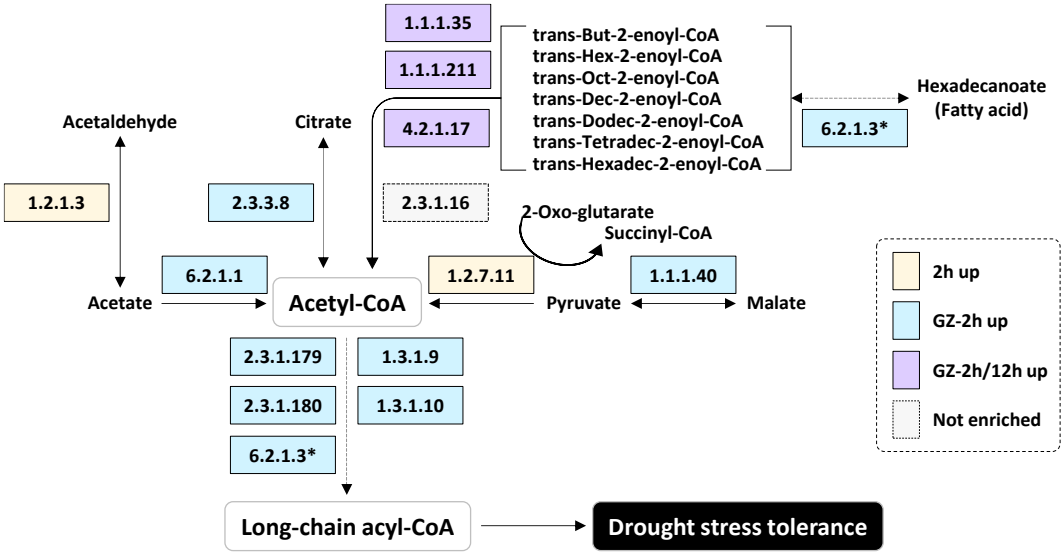

Figure 8. *Enrichment of acetyl-CoA and long-chain acyl-CoA as core intermediate crosstalking metabolites to confer drought stress tolerance in the drought-tolerant wheat (T. aestivum) cultivar GZ168. These chemical reactions occurred via crosstalking of six KEGG pathways namely "Glycolysis / Gluconeogenesis" (Figs. S9 and S10 and Table S5), "Citrate cycle (TCA cycle)" (Figs. S11 and S12 and Table S5), and "Pyruvate metabolism" (Figs. S19 and S20 and Table S5), "Carbon fixation in photosynthetic organisms" (Fig. S21 and Table S6), "Fatty acid biosynthesis" (Fig. S22 and Table S7), "Fatty acid degradation" (Figs. S23-S25 and Table S7). 1.2.1.3 = aldehyde dehydrogenase (NAD+), 1.2.7.11 = 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha, 6.2.1.1 = acetyl-CoA synthetase, 2.3.3.8 = ATP citrate (pro-S)-lyase, 1.1.1.40 = malate dehydrogenase, 2.3.1.179 = 3-oxoacyl-[acyl-carrier-protein] synthase II, 2.3.1.180 = 3-oxoacyl-[acyl-carrier-protein] synthase III, 1.3.1.9/1.3.1.10 = enoyl-[acyl-carrier protein] reductase I, 6.2.1.3 = long-chain acyl-CoA synthetase, 4.2.1.17/1.1.1.35/1.1.1.211 = enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase*

Figure 10 illustrates the enrichment of amylose, starch, and glucose as core intermediate crosstalking metabolites to confer drought stress tolerance in the drought-tolerant wheat cultivar GZ168. These chemical reactions occurred in the KEGG pathway "Starch and sucrose metabolism" (*Figs. S13-S16* and *Table S5*). Key encoded enzymes are glucose-1-phosphate adenylyltransferase (EC 2.7.7.27), beta-fructofuranosidase (EC 3.2.1.26), granule-bound starch synthase (EC 2.4.1.242), 1,4-alpha-glucan branching enzyme (EC 2.4.1.18), starch synthase (EC 2.4.1.21), sucrose synthase (EC 2.4.1.13), beta-glucosidase (EC 3.2.1.21), glucose-1-phosphate phosphodismutase (EC 2.7.1.41) (not enriched).

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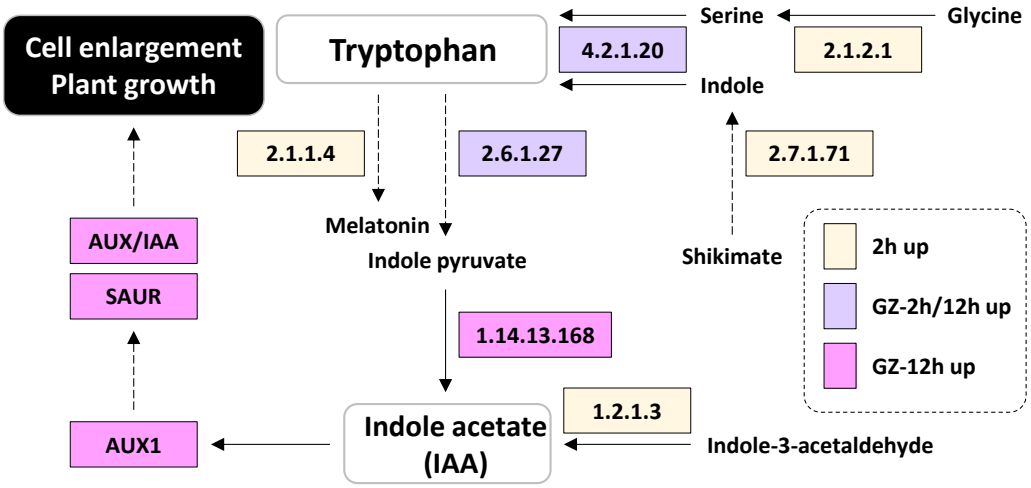

Figure 9. *Enrichment of tryptophan and indole acetate (IAA) as core intermediate crosstalking enzymes/proteins to confer drought stress tolerance in the drought-tolerant wheat (T. aestivum) cultivar GZ168. These chemical reactions occurred via crosstalking of four KEGG pathways namely "Glycine, serine and threonine metabolism" (Figs. S2 and S3 and Table S4), "Tryptophan metabolism" (Figs. S4-S6 and Table S5), "Phenylalanine, tyrosine and tryptophan biosynthesis" (Figs. S7 and S8 and Table S5) and "Plant hormone signal transduction" (Figs. S29-S31 and Table S8). 2.1.2.1 = glycine hydroxymethyltransferase, 4.2.1.20 = tryptophan synthase, 1.2.1.3 = aldehyde dehydrogenase (NAD+), 1.14.13.168 = indole-3-pyruvate monooxygenase, 2.6.1.27 = tryptophan aminotransferase, 2.7.1.71 = shikimate kinase, 2.1.1.4 = caffeic acid 3-O-methyltransferase, AUX/IAA (K14484) = auxin-responsive protein IAA, SAUR (K14488) = SAUR family protein, AUX1 (K13946) = auxin influx carrier (AUX1 LAX family)*

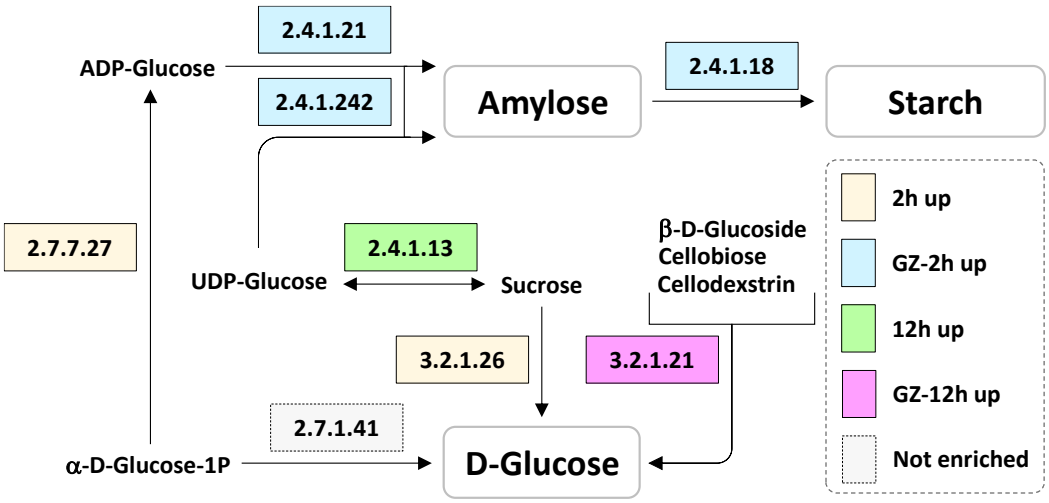

Figure 10. *Enrichment of amylose, starch and glucose as core intermediate crosstalking metabolites to confer drought stress tolerance in the drought-tolerant wheat (T. aestivum) cultivar GZ168. These chemical reactions occurred in the KEGG pathway "Starch and sucrose metabolism" (Figs. S13-S16 and Table S5). 2.7.7.27 = glucose-1-phosphate adenylyltransferase, 3.2.1.26 = beta-fructofuranosidase, 2.4.1.242 = granule-bound starch synthase, 2.4.1.18 = 1,4-alpha-glucan branching enzyme, 2.4.1.21 = starch synthase, 2.4.1.13 = sucrose synthase, 3.2.1.21 = beta-glucosidase, 2.7.1.41 = glucose-1-phosphate phosphodismutase (not enriched)*

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These findings elucidate key molecular determinants underpinning drought tolerance mechanisms in wheat cultivars, thereby illuminating avenues for targeted intervention strategies aimed at bolstering crop resilience amidst climatic adversity.

Discussion

Egypt's agricultural landscape, heavily reliant on the Nile River, faces increasing water demands and climate change impacts, exacerbating drought conditions in regions where wheat cultivars are commonly grown (El-Hashash et al., 2022). The two cultivars in question, Giza 168 (GZ168) and Gemmiza 10 (GM10), were developed through breeding programs and named after the regions where they were cultivated, reflecting distinct agro-ecological zones within Egypt. These regions are characterized by varying levels of environmental stresses, particularly in terms of water availability and disease prevalence. Giza, the origin of GZ168, experiences more severe water scarcity compared to Gemmiza. Consequently, GZ168 was specifically bred to exhibit enhanced drought tolerance, enabling it to thrive under conditions of limited water resources. In contrast, Gemmiza, the birthplace of GM10, faces a different set of challenges. This region is more susceptible to leaf rust, a fungal pathogen that can significantly impact wheat yields. As a result, GM10 was selectively bred to possess heightened resistance to leaf rust, making it better equipped to withstand this common disease threat in the Gemmiza region.

To provide a stronger rationale for our choice of these two wheat cultivars, we emphasize that GZ168 and GM10 were strategically selected due to their contrasting performance under drought stress, as demonstrated in previous studies. Al-Naggar et al. (2015) utilized morphological traits alongside molecular markers (SSR) to assess drought tolerance in advanced wheat families, while Abdelghany et al. (2016) focused on evaluating yield and related components in Egyptian wheat cultivars under water stress conditions in a specific geographic region. The prior research clearly indicates a differential response to drought between these cultivars, making them an ideal system for investigating the genetic and metabolic processes underlying drought tolerance, which is the focus of this study. Extensive research on other plant species has capitalized on RNA-Seq methodologies, utilizing analogous temporal parameters, e.g., 0h, 2h, and 12h time points, to characterize the differential expression profiles of genes under conditions of either saline or drought-induced stress (Bahieldin et al., 2015, 2018; Hajrah et al., 2017; Zhang et al., 2023).

The main point of elucidating molecular mechanisms underpinning drought stress tolerance in the present investigation depends on discerning the differential expression patterns of stress-related genes pivotal in orchestrating critical steps within various metabolic pathways. An additional criterion for dissecting the nuanced performance discrepancies between the two contrasting wheat genotypes pertains to the potential manifestation of negative regulatory responses within specific genes under stress conditions, aimed at modulating cellular growth rates optimally. Moreover, the timing of gene expression within a given genetic circuit assumes paramount importance, with upstream genes necessitating earlier or concurrent expression vis-à-vis downstream counterparts to ensure an orchestrated regulatory cascade. Furthermore, if a critical step within a pathway is bidirectional, discerning the prevailing directionality mandates robust downstream gene expression unidirectionally at elevated levels, thereby designating the reaction direction as "favorable." In the present study, we posit the existence of three principal genetic circuits, underpinned by timely and favorable gene expression patterns,

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culminating in beneficial responses (*Figs. 8-10*). Detailed elucidation of these mechanisms, alongside other pivotal pathways contributing to drought stress tolerance, ensues.

Acetyl CoA and long-chain Acyl-CoA as core metabolites

Central to the orchestrated interplay of metabolites and pathways fostering drought tolerance mechanisms is the biosynthesis of long-chain acyl-CoA (*Fig. 8*). The long-chain acyl-CoA has been previously implicated in boosting abscisic acid (ABA) signalling pathways, precipitating pivotal responses such as stomatal closure in plant guard cells, as previously reported (Du et al., 2013). This aligns with recent findings in wheat, where genes involved in long-chain fatty acid production for cuticular wax deposition, including fatty acyl-CoA reductases, were found to be enriched under drought stress conditions (Onyemaobi et al., 2021). The importance of ABA signaling in drought response is further supported by studies in drought-tolerant wheat varieties, which maintain stomatal conductance longer under drought conditions (Onyemaobi et al., 2021).

Upstream reactions leading to long-chain acyl-CoA biosynthesis encompass multiple routes, each culminating in the enrichment of the intermediary substrate, acetyl CoA. Notably, differential enrichment patterns were observed across the contrasting genotypes, with certain routes favoring the drought-tolerant GZ168 cultivar, indicative of its enhanced capacity for acetyl CoA production. This genotype-specific response is consistent with recent research in other plant species. For instance, in willows, females exhibit greater drought tolerance and benefit more from exogenous acetic acid-improved drought tolerance than males, possibly due to enhanced acetyl-CoA biosynthesis and utilization (Xia et al., 2024). Of particular interest are four putative routes governing acetyl CoA biosynthesis, each entailing distinct enzymatic transformations. Differential enrichment of these enzymes, including long-chain acyl-CoA synthetase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase of the first route, and ATP citrate (pro-S)-lyase of the second route, underscores the genetic underpinnings of drought resilience in GZ168 (*Fig. 8*). This complex network of enzymatic pathways aligns with recent studies highlighting the importance of acetyl-CoA in drought tolerance. For example, overexpression of acetyl-CoA synthetase (ACS) in willows has been shown to reprogram fatty acids, increase lysine acetylation levels, and improve drought tolerance (Xia et al., 2024).

The third route involves enrichment of two enzymes, the first, e.g., aldehyde dehydrogenase (NAD+), was enriched in wheat cultivars after 2h, while the second, e.g., acetyl-CoA synthetase, was enriched only in GZ168 after 2h with acetaldehyde acting as the initial substrate. Thus, it is unlikely that this route is ultimately wired in the drought sensitive cultivar GM10. This differential expression of acetyl-CoA synthetase in drought-tolerant varieties is consistent with findings in Arabidopsis, where the modulation of acetic acid pathway genes, including PDC1 and ALDH2B7, has been shown to enhance drought stress tolerance (Rasheed et al., 2018). Intriguingly, the bidirectional enrichment of malate dehydrogenase suggests a favorable directionality towards acetyl CoA biosynthesis, further boosting the drought tolerance phenotype of GZ168, while the second, e.g., 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha, was unidirectionally enriched in the wheat cultivars after 2h with malate used as the initial substrate. Based on our criterion, malate dehydrogenase will likely favor the direction of acetyl CoA biosynthesis (*Fig. 8*). As a conclusion of the four routes, it is likely that the drought-tolerant genotype (G168) will promote the production of acetyl

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CoA more effectively as compared with GM10. This metabolic flexibility in drought-tolerant genotypes is mirrored in other studies, such as the genome-wide association study of drought tolerance in wheat, which identified multiple genetic loci associated with drought tolerance traits (Nouraei et al., 2024).

Downstream biosynthesis of long-chain acyl-CoA from acetyl CoA involves a cascade of enzymatic reactions, with selective enrichment observed exclusively in the tolerant cultivar after 2h (*Fig. 8*). Enzymes involved in these reaction are 3-oxoacyl-[acyl-carrier-protein] synthase II, 3-oxoacyl-[acyl-carrier-protein] synthase III, enoyl-[acyl-carrier protein] reductase I and long-chain acyl-CoA synthetase. Downstream biosynthesis of long-chain acyl-CoA from acetyl CoA involves a cascade of enzymatic reactions, with selective enrichment observed exclusively in GZ168 after 2h (*Fig. 8*). The latter enzymes were enriched only in GZ168 after 2h (*Fig. 8*). This route was not wired in GM10, thus, downstream production of the end product, e.g., long-chain acyl-CoA, is unlikely in the drought sensitive genotype. This genotype-specific enrichment of long-chain acyl-CoA synthetase aligns with recent studies in poplar, where long-chain acyl-CoA synthetases were found to promote resistance to abiotic stresses (Wei et al., 2022). Notably, long-chain acyl-CoA synthetase intensively participates in energy storage and serves as a signalling molecule orchestrating plant responses to abiotic stresses, as evidenced by studies in transgenic Arabidopsis overexpressing the gene encoding long-chain acyl-CoA that was resulted in a reduced water loss due to the reduced permeability of the plasma membrane (Zhang et al., 2018, 2020; Zhao et al., 2019, 2021; Geng et al., 2024). This role of long-chain acyl-CoA synthetase in drought tolerance is further supported by studies in cotton, where overexpression of the GhACX3 gene, involved in acyl-CoA metabolism, enhanced drought and salt stress tolerance in Arabidopsis (Shiraku et al., 2021).

Additionally, melatonin has been implicated in analogous regulatory mechanisms in rice, underscoring its potential to ameliorate adverse environmental conditions (Khan et al., 2024). The synthesis of long-chain acyl-CoA is intricately intertwined with six crosstalking pathways (*Figs. S9-S25* and *Tables S5-S7*). These pathways collectively underpin the metabolic framework governing drought tolerance mechanisms, underscoring the multifaceted nature of stress adaptation strategies in wheat. This complex interplay of metabolic pathways in drought response is consistent with recent proteomic studies in tea plants, which revealed that drought stress triggers changes in multiple pathways, including lignin biosynthesis and flavonoid metabolism (Gu et al., 2020). Such findings underscore the need for a systems-level approach to understanding and enhancing drought tolerance in crops.

Elucidation of tryptophan and indole acetate (IAA) as central metabolites

In the complex milieu of wheat stress tolerance mechanisms, the biosynthesis pathways of tryptophan and indole acetate (IAA) emerge as pivotal processes (Etesami and Glick, 2024; Wang et al., 2024a,b) (*Fig. 9*). Expounding upon this, our investigation unveils two distinct routes for tryptophan biosynthesis. The former pathway manifests a tandem enzymatic cascade, featuring glycine hydroxymethyltransferase and tryptophan synthase, with discernible enrichments observed at the 2-hour time point across both genotypic variants. However, the latter route, albeit encompassing the same tryptophan synthase, exhibits shikimate kinase as a rate-limiting precursor (Wu et al., 2022). In the complex milieu of wheat stress tolerance mechanisms, the biosynthesis pathways of tryptophan and indole acetate (IAA) emerge as pivotal processes (*Fig. 9*). Expounding

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upon this, our investigation unveils two distinct routes for tryptophan biosynthesis. The former pathway manifests a tandem enzymatic cascade, featuring glycine hydroxymethyltransferase and tryptophan synthase, with discernible enrichments observed at the 2-hour time point across both genotypic variants. However, the latter route, albeit encompassing the same tryptophan synthase, exhibits shikimate kinase as a rate-limiting precursor (Itam et al., 2020), accentuates its indispensable role in orchestrating stress responses through the coordination of tryptophan and abscisic acid (ABA), pivotal for modulating stomatal conductance and enhancing plant resilience under adverse conditions (Liu et al., 2022).

Tryptophan, as a metabolic precursor to melatonin, assumes a multifaceted role in stress amelioration, as corroborated by recent studies elucidating its facilitative influence on plant development or growth amidst adversities (Khan et al., 2024). Melatonin's function as an osmolyte in regulating stomatal dynamics (Khan et al., 2024) Melatonin's function as an osmolyte in regulating stomatal dynamics (Wang et al., 2024a). Furthermore, our findings delineate the enrichment of caffeic acid 3-O-methyltransferase at the 2-hour time point in both genotypes, accentuating its pivotal role in melatonin biosynthesis. Intriguingly, the metabolic flux towards indole acetate under drought conditions exhibits a distinct pattern, primarily enriched in GZ168. This metabolic avenue involves sequential enzymatic actions mediated by tryptophan aminotransferase and indole-3-pyruvate monooxygenase, indicative of the genotype-specific responses to stress. Moreover, the alternate route for IAA biosynthesis, catalyzed by aldehyde dehydrogenase (NAD+), signifies a concerted effort towards stress adaptation, particularly enriched in the drought-tolerant genotype. Notably, the pivotal role of IAA in stress mitigation is underscored by its modulation of the "Plant hormone signal transduction" pathway (*Fig. 9*), wherein key proteins such as AUX1 (K13946), AUX/IAA (K14484), and SAUR (K14488) orchestrate a cascade of events culminating in enhanced stress tolerance and growth promotion, predominantly observed in GZ168. Enrichment of this route in GZ168 gives it the advantage to tolerate the stress more properly than GM10. Auxin influx carrier (AUX1) is known to elicit a battery of metabolites including auxin/indole acetic acid (AUX/IAA) as well as the small a*uxin*-up RNA (SAUR) (*Fig. 9*). In “Plant signal hormone transduction” pathway, auxin response factor (or ARF) binds promoter elements of the auxin-responsive gene *SAUR* to block its expression, while auxin-responsive protein AUX/IAA binds ARFs to alleviate this negative action (Luo et al., 2018; Zhang et al., 2023). Then, SAUR can act as a stimulator of shoot elongation in the stress-tolerant wheat genotype as previously indicated (Ren and Gray, 2015; Xiong et al., 2019; Chen et al., 2023; Huang et al., 2023).

Elucidation of these metabolic pathways implicates the involvement of four KEGG interconnected pathways, namely "Glycine, serine and threonine metabolism" (*Figs. S2* and *S3* and *Table S4*), "Tryptophan metabolism" (*Figs. S4-S6* and *Table S5*), "Phenylalanine, tyrosine and tryptophan biosynthesis," (*Figs. S7* and *S8* and *Table S5*) and "Plant hormone signal transduction" (*Figs. S29-S31* and *Table S8*), thereby providing a comprehensive framework for understanding the genetic underpinnings of stress tolerance in wheat.

Starch, amylose and glucose as principal metabolites amidst drought stress

Within the framework of drought tolerance mechanisms, starch and the soluble sugar glucose emerge as pivotal constituents indispensable for plant resilience (Swami et al., 2024) (*Fig. 10*). Amidst drought-induced demands, plant cells manifest a pronounced

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demand for the accumulation of these carbohydrates, depicting a nuanced interplay between complexity and simplicity. This dynamic manifests as a shuttle reaction, wherein starch undergoes metabolic flux to engender glucose, and conversely, glucose yields starch, with amylose occupying an intermediary stance. The orchestration of this reaction trajectory pivots on the strategic enrichment of enzymes along divergent routes. The initial route predicates upon UDP-glucose as the pivotal intermediary substrate, compared with the second route reliant upon α-D-glucose-1P (*Fig. 10*). Enzymatic entities along these pathways exhibit bidirectional activity, with one avenue fostering starch biosynthesis while its counterpart catalyzes glucose synthesis.

The former route of enzymatic enrichment entails the participation of granule-bound starch synthase, which catalyzes starch biosynthesis in GZ168 at the 2-hour temporal juncture. Conversely, sucrose synthase instigates bidirectional catalysis across both genotypes after 12h. However, the unidirectional activity of beta-fructofuranosidase after 2h suggests a proclivity towards glucose biosynthesis via sucrose synthase (*Fig. 10*). Downstream cascades of the former trajectory implicate 1,4-alpha-glucan branching enzyme utilizing amylose as the substrate, while the latter pathway engages beta-fructofuranosidase with sucrose as its substrate (*Fig. 10*). Granule-bound starch synthase (GBSS) is indeed involved in starch biosynthesis, specifically in the synthesis of amylose (Wang et al., 2023). Notably, enzymes facilitating starch biosynthesis enrichment exclusively manifest in GZ168 at the 2-hour interval, whereas those pivotal for glucose biosynthesis exhibit enrichment across both genotypes at the 12-hour interval, including sucrose synthase and beta-fructofuranosidase (invertase) at the 2-hour time point. The latter enzyme catalyzes the hydrolysis of sucrose into fructose and glucose (Malek et al., 2020). This temporal disjunction indicates a less viable prospect for the latter route's efficacy in glucose generation, as the enrichment of upstream enzymes, exemplified by sucrose synthase after 12h, precedes the downstream enzyme enrichment, such as beta-fructofuranosidase after 2h.

In the context of the secondary route of enzyme enrichment, glucose-1-phosphate adenylyltransferase or ADP glucose pyrophosphorylase orchestrates starch biosynthesis across both genotypes at the 2-hour time point, while glucose-1-phosphate phosphodismutase drives biosynthesis of glucose yet remains unenriched in either genotype across temporal delineations (*Fig. 10*). Subsequent steps of starch biosynthesis feature a triad of enzymes—granule-bound starch synthase, starch synthase, and 1,4-alpha-glucan branching enzyme enriched in GZ168 at the 2-hour interval, employing ADP-glucose as the primary substrate. Conversely, the downstream pathway of glucose biosynthesis remains unenriched in both wheat genotypes (*Fig. 10*). Thus, the prospects of glucose synthesis via these delineated routes appear improbable. However, the tolerant genotype manifests alternative routes at the 12-hour time point for glucose biosynthesis, facilitated by the enrichment of beta-glucosidase, with β-D-glucoside, cellobiose, and cellodextrin as initial substrates (Badoni et al., 2025).

Additional regulatory pathways encompass the "Amino sugar and nucleotide sugar metabolism" pathway, featuring four enriched enzymes across both wheat cultivars at the 2-hour time point, including UDP glucose 6-dehydrogenase, L-arabinokinase, xylan 1,4-beta-xylosidase, and glucose-1-phosphate adenylyltransferase, with two enzymes enriched solely in GZ168 at the 12-hour time point, namely hexosaminidase and basic endochitinase B (*Figs. 10*, *S17* and *S18*). The latter enzyme, categorized within the CAZy (Carbohydrate-Active enZymes) class of glycosyl hydrolases (GH), catalyzes chitin degradation to yield N-acetylglucosamine (GlcNAc), thereby contributing to plant

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defense against biotic and abiotic stresses. While its functional significance remains incompletely elucidated, its pivotal role in stress mitigation is underscored by existing literature (Liu et al., 2020; Capovilla et al., 2023; He et al., 2023; Xuan et al., 2024).

Moreover, within the "Glycerophospholipid metabolism" pathway, three enzymes are enriched, with phospholipase D1/2 and glycerol-3-phosphate acyltransferase enriched in GM10 after 2h, and phosphoethanolamine methyltransferase enriched in GZ168 after 2h (*Figs. S26* and *S27*). While the former two enzymes lack conclusive association with drought stress, the latter catalyzes a pivotal step in choline biosynthesis, a precursor to membrane phospholipids, thereby implicating its potential significance amidst stress response (Sagaro and Amenta, 2023; Sakuragi and Nagata, 2023). Furthermore, the "Ras signalling" pathway evinces the enrichment of the Ras-related C3 botulinum toxin substrate 1 (Rac1) in the drought-sensitive genotype GM10 at the 2-hour time point (*Fig. S27*), indicative of its role as a small GTPase enzyme transducing extracellular signals to elicit downstream cellular responses, including actin cytoskeleton modulation (Le and Li, 2022; Gaffke et al., 2023). Under adverse environmental conditions necessitating stomatal closure to mitigate transpiration, the stress phytohormone ABA inactivates GTPase, facilitating stomatal closure. The enrichment of small GTPase in the drought-sensitive genotype implies suboptimal ABA levels, thereby abating GTPase inhibition and preserving stomatal functionality amidst adverse conditions (Feiguelman et al., 2018).

While our study provides valuable insights into drought tolerance mechanisms, it is important to recognize that controlled experiments may not fully capture the complexity of field environments. Laboratory drought simulations often lack the dynamic fluctuations in temperature, humidity, and soil moisture that plants experience in natural settings (Wang et al., 2024c). A recent research emphasizes the need for careful design and interpretation of drought experiments to ensure their relevance to real-world scenarios (Moshelion et al., 2024). To address these limitations and validate our findings, future studies should incorporate field trials under realistic environmental conditions. This approach would allow for the assessment of plant responses to drought stress in the presence of other abiotic and biotic factors, as recently suggested (Wang et al., 2024). Additionally, integrating advanced phenotyping techniques and multi-omics approaches in field trials could provide a more holistic understanding of plant drought responses and enhance the translational potential of laboratory findings to agricultural applications (Moshelion et al., 2024).

Practical applications of the findings of the present study in terms of the biosynthesis of long-chain acyl-CoA and its role in ABA signaling present a promising target for enhancing drought tolerance in cultivated plants, as demonstrated by studies showing that modulation of related pathways can improve water use efficiency and drought tolerance (Liu et al., 2023). Additionally, manipulating acetyl-CoA biosynthesis, as observed in GZ168, aligns with successful metabolic engineering approaches where overexpression of acetyl-CoA synthetase reprogrammed fatty acid metabolism and improved drought responses (Xia et al., 2024). These findings provide a foundation for developing drought-tolerant wheat varieties through targeted breeding and genetic engineering, potentially leading to more resilient crops in water-limited environments. We assume outcomes of this study moght have broader implications for agricultural policies aimed at enhancing food security in drought-prone regions. The identification of metabolic pathways linked to drought tolerance also highlights opportunities for integrating climate-smart agricultural practices (CSA), such as improved crop varieties and soil fertility

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management, which have been shown to enhance household dietary diversity and resilience in drought-affected areas.

Conclusion

In summation, differential enrichment of select constituents within stress-responsive crosstalking pathways elucidates the nuanced performance of plant genotypes under drought stress. Enrichment of pivotal metabolites within these pathways delineates a mechanistic framework facilitating efficacious drought tolerance strategies. Key candidates among these pivotal metabolites, including long-chain acyl-CoA, acetyl CoA, tryptophan, IAA, SAUR, starch, and glucose, underscore the potential targets for crop amelioration through metabolic engineering or genetic intervention strategies. We assume this study has successfully met its stated aims and objectives, making significant contributions to crop resilience strategies. By focusing on metabolic pathways and enzymatic reactions involved in drought tolerance, the research has provided valuable insights that align with objectives of developing climate-resilient crop varieties, promoting sustainable farming practices, and fostering collaboration among stakeholders to enhance agricultural resilience in the face of climate change.

Interest statement. The author declares no conflict of interest.

Data availability statement. Supplemental data can be accessed at: https://drive.google.com/drive/folders/1pe8wrylwj7EvRB7kTrHA_IAewP1E0wlS?usp=share_link.

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