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RNA-Seq-Based Transcriptome Analysis of Barley (Hordeum vulgare) Reveals Molecular Responses to Drought Resilience

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ABSTRACT

Drought is a major abiotic stress that significantly impairs plant physiological processes and development, ultimately leading to substantial crop yield losses. As a complex quantitative trait, drought tolerance is governed by multiple genes and intricate metabolic pathways. However, the molecular mechanisms and associated morphological and physiological adaptations to drought stress in barley remain poorly understood. In this study, we employed an integrative approach combining morpho-physiological assessments and RNA-Seq-based transcriptome analysis to identify core drought-responsive genes and regulatory networks in the barley (Hordeum vulgare) cultivar 'Giza 134'. Field trials under water-deficit conditions revealed significant reductions in crop growth rate, relative water content, leaf area duration, flag leaf area, chlorophyll (Chl) a, b, and total chlorophyll (a + b), net photosynthesis rate, and key yield components. Conversely, drought stress caused notable increases in the chlorophyll a/b ratio, stomatal resistance, and proline accumulation. Transcriptomic analysis identified 2,462 differentially expressed genes (DEGs), with 1,555 genes upregulated and 907 downregulated under drought conditions. Comparative analysis of gene expression profiles highlighted three critical metabolic pathways—carbohydrate metabolism, iron ion binding, and oxidoreductase activity—as potentially involved in the plant's drought response. Several drought-induced marker genes were found to be associated with key physiological functions, including chlorophyll biosynthesis, photosynthesis, light harvesting, gibberellin biosynthesis, and iron homeostasis, along with distinct cis-regulatory elements. Overall, our findings provide novel insights into the molecular and physiological mechanisms underlying barley's response to water-deficit stress.

INTRODUCTION

Drought is a major abiotic stress that adversely affects plant physiology and development, ultimately causing significant yield losses in crops (Farooq et al., 2009; Tuberosa, 2012). As a complex quantitative trait, drought tolerance is regulated by multiple genes and diverse metabolic pathways (Shinozaki & Yamaguchi-Shinozaki, 2007; Gupta et al., 2020). Despite progress in crop improvement, the molecular and physiological mechanisms underlying drought resilience remain insufficiently understood (Fang & Xiong, 2015).

In this study, a combination of morpho-physiological assessments and RNA-Seq-based transcriptome analysis was employed to investigate the drought response in the barley (*Hordeum vulgare*) cultivar 'Giza134'. Field evaluations under drought conditions revealed a marked reduction in crop growth rate, relative water content, leaf area duration, flag leaf area, chlorophyll (Chl) a, b, and a + b concentration, net photosynthesis, and yield components. Conversely, drought stress significantly increased the Chl a/b ratio, stomatal resistance, and proline concentration (Anjum et al., 2011).

Transcriptomic profiling identified 2,462 differentially expressed genes (DEGs), of which 1,555 were upregulated and 907 were downregulated under water-deficit stress. Comparative analysis highlighted key metabolic pathways—including carbohydrate metabolism, iron ion binding, and oxidoreductase activity—as potentially involved in drought tolerance (Huang et al., 2021; Wu et al., 2017). Several drought-induced marker genes were associated with diverse physiological functions, such as photosynthesis, chlorophyll biosynthesis, light harvesting, gibberellin biosynthesis, and iron homeostasis, as well as cis-regulatory elements (Li et al., 2022).

The insights from this study offer valuable molecular resources for the identification of gene-associated markers that can accelerate the development of drought-resilient barley cultivars. Moreover, these findings enhance the current understanding of drought response mechanisms in barley, emphasizing the importance of integrative transcriptomic approaches to improve crop resilience (Seki et al., 2002; Bevan et al., 2017). Given the increasing frequency and severity of drought events due to climate change, such research is critical (IPCC, 2021).

Barley, a globally important cereal crop, serves as a model organism for studying stress responses due to its relatively high tolerance to abiotic stresses (Baum et al., 2007). With the recent availability of a high-quality reference genome and advancements in omics technologies, transcriptome analysis has become a powerful tool for exploring drought-responsive genes and

their regulatory networks (Mascher et al., 2017). Gene expression under drought stress is primarily controlled at the transcriptional level, regulated by key transcription factors such as ABF, AP2/ERF, DREB, NAC, MYB, and others (Yamaguchi-Shinozaki & Shinozaki, 2006; Nakashima et al., 2012). Additionally, hormone-mediated pathways, particularly those involving abscisic acid (ABA) and gibberellins (GA), play critical roles in stress signal transduction (Ullah et al., 2017).

Despite numerous studies, understanding the full spectrum of transcriptional responses to long-term drought stress in barley remains limited. This study, therefore, contributes to bridging that gap by identifying core genes and regulatory elements associated with drought tolerance over a prolonged stress period. Such knowledge is essential for developing improved barley cultivars that are capable of maintaining high productivity under water-limited conditions.

STUDY OBJECTIVES

- To investigate the transcriptomic responses of barley (*Hordeum vulgare* cv. Giza 134) under drought stress using RNA-Seq technology.
- To identify differentially expressed genes (DEGs) and key regulatory pathways associated with drought resilience.
- To uncover potential gene-associated markers and cis-regulatory elements for improving drought tolerance in barley cultivars.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Hordeum vulgare cv. Giza 134 a high-yielding, disease-resistant spring barley variety with an average yield of 4600 kg/ha, was used in this study. Seeds were obtained from the Barley Research Department of the Egyptian Agricultural Research Center (ARC).

GROWTH CHAMBER EXPERIMENT

Seeds were surface sterilized with 0.5% (v/v) 10% sodium hypochlorite (NaOCl) and Tween 20 for 15 minutes, followed by thorough rinsing with distilled water. Sterilized seeds were sown in pots (23 × 23 × 19 cm) containing JA Kumiai King Soil (Agr. Japan Co., Ltd.), with four plants per pot. The pots were incubated in darkness at 25°C for three days. Upon coleoptile emergence, seedlings were transferred to a growth chamber (SANYO) set to a photoperiod of 14 h light at 27°C and 10 h dark at 25°C. Seedlings were maintained at 40–50% field water capacity (FWC) for 10 days.

The experiment followed a completely randomized design (CRD) with three replicates and 12 seedlings per treatment at the Institute of Plant Genetic Resources, Kyushu University, Japan. For drought treatment, one group received regular watering (well-watered, WW), while another was treated with 30% polyethylene glycol (PEG-600), simulating water-deficit (WD) stress (osmotic potential = −1.03 MPa, Michel and Kaufmann, 1973) for 15 days. At the end of the treatment, root and shoot tissues from five plants per treatment were flash-frozen in liquid nitrogen and stored at −80°C for RNA extraction and physiological analyses.

FIELD EXPERIMENT

Field trials were conducted at the Gemmeiza Research Station, Egypt (31°09′ N, 30°94′ W) during the 2021/2022 and 2022/2023 growing seasons using a randomized complete block design (RCBD) with three replicates. Each plot (4.2 m^2) contained six rows spaced 0.2 m apart and 3.5 m long. Two irrigation treatments were applied: well-watered (WW, 4538.2 m^3/ha) and water-deficit (WD, 1359.66 m^3/ha). Upon physiological maturity, plants from a 2 m^2 central area in each plot were harvested for yield measurements. Meteorological data (precipitation, humidity, temperature) were recorded throughout the growing season.

PHENOTYPIC, PHYSIOLOGICAL, AND BIOCHEMICAL MEASUREMENTS

Traits measured included:

- **PHYSIOLOGICAL**: Crop growth rate (CGR), relative water content (RWC), leaf area duration (LAD), leaf water potential (ΨL), flag leaf area (FLA), net photosynthesis rate (PN), stomatal resistance (SR), and proline content.
- **BIOCHEMICAL**: Chlorophyll a, chlorophyll b, and total chlorophyll content.
- **AGRONOMIC**: Plant height (PH), spike length (SL), number of grains per spike, and number of spikes per square meter.

Measurements were taken from three randomly selected plants per plot. CGR was calculated as $(\text{dry weight}_2 - \text{dry weight}_1)/\text{days}$ (Watson, 1952). RWC (%) was determined using $(FW - DW)/(TW - DW) \times 100$ (Barrs and Weatherley, 1962). LAD was calculated between 60 and 90 days after sowing (Parsons and Hunt, 1981). Statistical differences were assessed using Student's t-test.

RNA EXTRACTION AND SEQUENCING

Total RNA was extracted from 100 mg of frozen leaf tissue using the RNeasy® Plant Mini Kit (QIAGEN). RNA quality and quantity were determined using a Nanodrop 2000

spectrophotometer. Libraries were sequenced on the DNBSEQ platform using 150-bp paired-end reads.

TRANSCRIPTOME ANALYSIS

Raw reads were quality-filtered using Cut adapt to remove adapters and low-quality sequences. Clean reads were aligned to the barley reference genome (IBSC_v2) using HISAT2 (v2.2.0). Transcript assembly was performed using String Tie (v2.2.1), and differential expression analysis was conducted with NOISeq, with a q threshold of 0.95. Genes with $\log_2$ fold change ≥ 1.5 or ≤ -1.5 and probability <0.7 were designated as differentially expressed genes (DEGs). DEGs were annotated using BARLEYMAP, More x Genome Map, and the James Hutton iSelect platform.

FUNCTIONAL ANNOTATION AND ENRICHMENT ANALYSIS

Gene Ontology (GO) enrichment analysis was performed using Shiny GO, applying p-value and FDR cutoffs of 0.05. KEGG pathway enrichment was carried out using the PANTHER classification system. Expression data were visualized using hierarchical clustering (pheatmap R package v1.0.12) and volcano plots (GraphBio).

CIS-REGULATORY MOTIF AND TRANSCRIPTION FACTOR ANALYSIS

Promoter regions (1 kb upstream of TSS) of DEGs were analyzed for over-represented motifs using the RSAT suite and position-specific scoring matrices (PSSMs). Motif enrichment was modeled using Markov chain analysis. Motifs were mapped relative to genomic reference positions using the RAST toolkit. Transcription factors (TFs) associated with enriched motifs were identified using Plant TFDB 4.0, and cross-species comparisons were made to assign TF families.

RESULTS

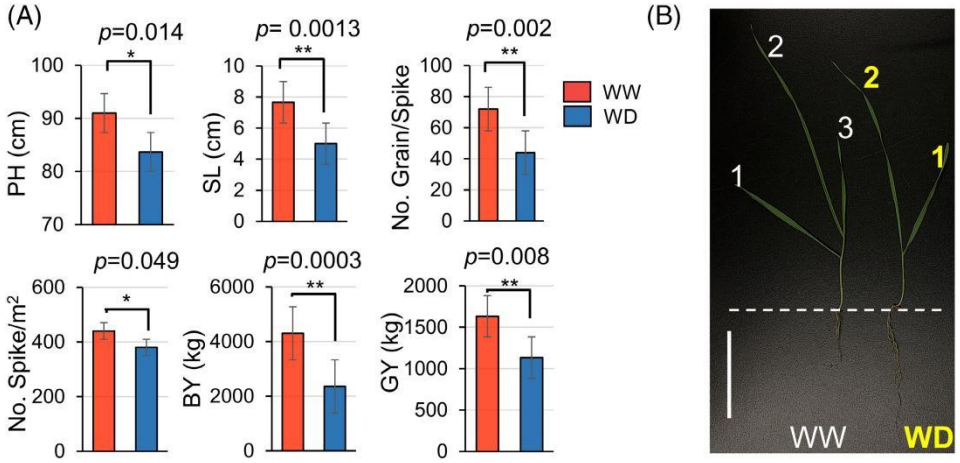
MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO WATER DEFICIT (WD) STRESS

The morphological, physiological, and yield-related traits of *Hordeum vulgare* cv. Giza 134 were significantly affected by water deficit (WD) stress compared to well-watered (WW) conditions. A significant reduction ($p < 0.01$ or $p < 0.05$) in crop growth rate (CGR) was observed under WD. Similarly, relative water content (RWC), leaf area duration (LAD), flag leaf area (FLA), chlorophyll a, b, and total chlorophyll (a + b) content, as well as net photosynthetic rate (PN), were significantly decreased under WD conditions ($p < 0.01$).

Interestingly, the chlorophyll a/b ratio increased under WD, suggesting a disturbance in

PSI:PSII balance. Net photosynthesis rate was reduced by 23.5 μmol m^{-2} s^{-1}. Proline content in WD-stressed plants increased by 29.8% compared to WW, indicating osmotic adjustment. Additionally, stomatal resistance was elevated under WD conditions.

Yield-related parameters, including plant height, spike length, number of grains per spike, number of spikes per m^2, biological yield, and grain yield, all significantly decreased under WD stress. However, no significant differences were detected in leaf water potential (ΨL). These results confirmed that water deficit stress had a pronounced negative effect on both plant growth and yield components in Giza 134.



TRANSCRIPTOMIC RESPONSE OF GIZA 134 TO WD STRESS

Transcriptome sequencing of leaf blades under WW and WD conditions yielded approximately 15.2 GB of data per lane. After quality filtering and adapter removal, high-quality reads were used for differential gene expression analysis.

FUNCTIONAL ANNOTATION OF DIFFERENTIALLY EXPRESSED GENES (DEGS)

Gene Ontology (GO) analysis revealed that significant DEGs under WD stress were classified into three major categories: biological process (BP), cellular component (CC), and molecular function (MF). In total, 20 BP, 5 CC, and 32 MF functional groups were significantly upregulated (FDR-adjusted $p < 0.05$).

A total of 338, 156, and 407 DEGs were enriched in BP, CC, and MF categories, respectively. Co-expression analysis identified 39 genes commonly expressed in all three GO categories. Additionally, 57 BP, 13 CC, and 42 MF terms were significantly enriched in the overall GO classification. Enriched BP terms with high -log10 FDR values (>8) included "defense response", "response to biotic stimulus", and "photosynthesis, light harvesting in photosystem I".

KEGG PATHWAY ENRICHMENT AND PROTEIN CLASS ANALYSIS

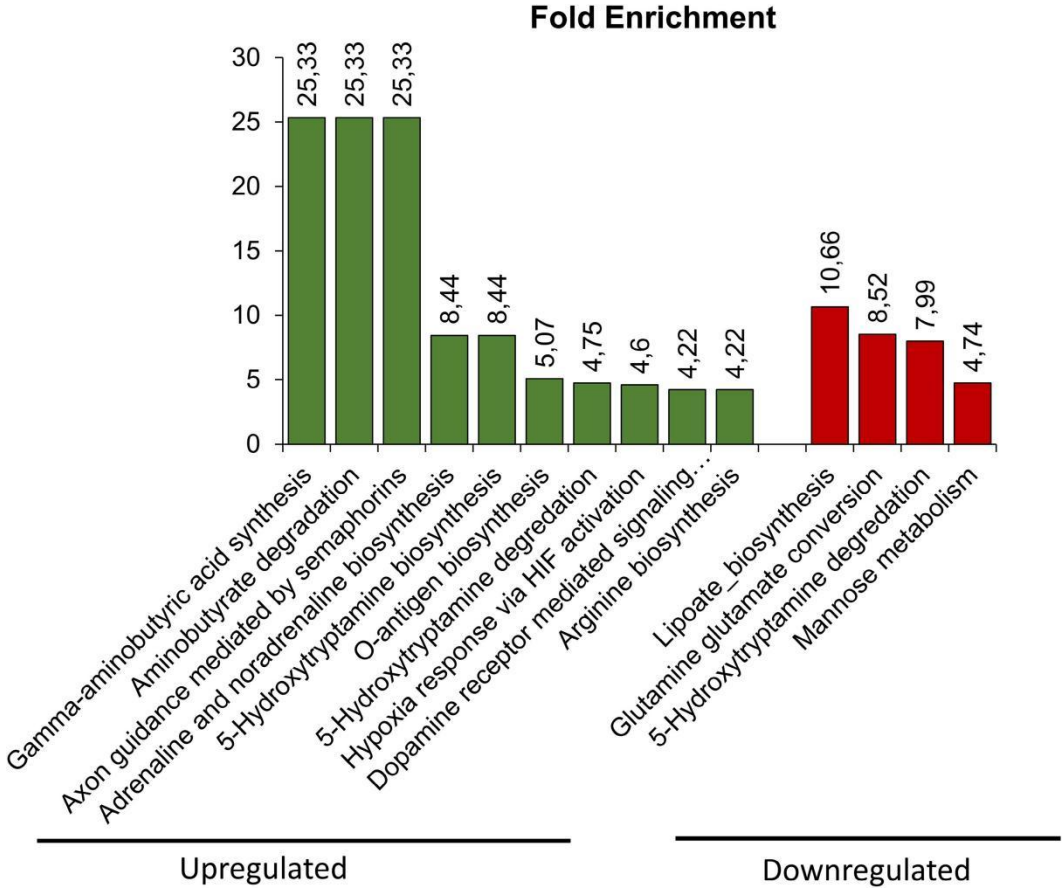


FIG.1 KEGG ENRICHMENT METABOLIC PATHWAY ANALYSIS OF SIGNIFICANT DEGS UNDER WATER DEFICIT STRESS.

KEGG pathway analysis revealed that 1555 upregulated and 907 downregulated DEGs under WD stress were significantly enriched in 10 and 4 pathways, respectively (FDR < 0.05) (Table S4). The most significantly enriched upregulated pathways included "gamma-aminobutyric acid synthesis", "aminobutyrate degradation", and "axon guidance mediated by semaphorins". "Lipoate biosynthesis" was significantly downregulated.

The "5-hydroxytryptamine degradation" pathway was commonly enriched under both WW and WD conditions. Upregulated DEGs were frequently associated with dehydrogenase activity, NAD-dependent epimerase/dehydratase, and pyridoxal phosphate-related enzymes. Downregulated pathways included "lipoate-protein ligase" and "mannose-6-phosphate isomerase". Protein classification of DEGs based on PANTHER indicated a dominance of gene-specific transcriptional regulators, DNA-binding transcription factors, oxidoreductases, and metabolite interconversion enzymes. These findings suggest that transcriptional

reprogramming and stress-related metabolic shifts underlie the WD stress adaptation in Giza 134 barley.

DISCUSSION

PHYSIOLOGICAL AND MORPHOLOGICAL ADAPTATIONS UNDER WATER DEFICIT STRESS

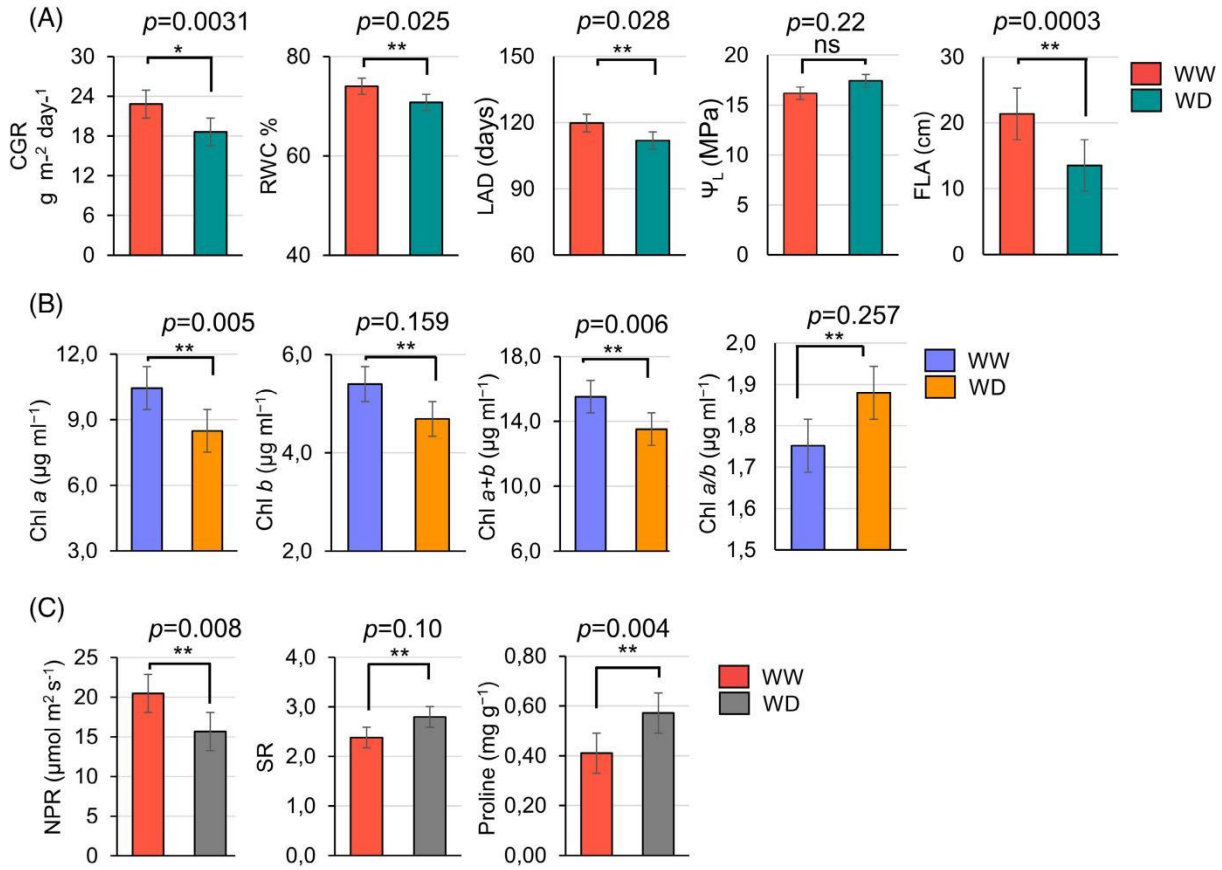
Drought stress significantly affects plant growth, development, and productivity. In this study, Giza 134 barley plants subjected to water deficit (WD) conditions exhibited marked morpho-physiological changes, including a significant reduction in crop growth rate, relative water content (RWC), flag leaf area (FLA), leaf area duration (LAD), chlorophyll content, and photosynthetic rate. These physiological responses align with previously reported studies that demonstrated the adverse effects of drought on photosynthesis, biomass accumulation, and reproductive traits in barley and other cereal crops (Hasanuzzaman et al., 2018; Askarnejad et al., 2021; Elakhdar et al., 2022).

The increase in stomatal resistance observed under WD stress suggests a drought avoidance mechanism that limits transpiration by reducing stomatal aperture. Concurrently, the significant accumulation of proline under WD conditions supports its well-established role as an osmoprotectant that contributes to cellular osmotic balance, membrane stabilization, and scavenging of reactive oxygen species (ROS) (Bandurska et al., 2017). These findings support the notion that barley utilizes both avoidance and tolerance strategies to mitigate water deficit stress, with proline accumulation and stomatal closure serving as key adaptive responses.

MOLECULAR INSIGHTS IN TO DROUGHT STRESS TOLERANCE

Transcriptome profiling under WD stress revealed substantial changes in gene expression, with 1,555 genes upregulated and 907 downregulated in Giza 134 barley plants. These differentially expressed genes (DEGs) were found to be associated with a broad range of biological processes and molecular functions including photosynthesis, hormone signaling, lipid transport, carbohydrate metabolism, and antioxidant defense mechanisms.

Gene Ontology (GO) enrichment analysis indicated that many of the DEGs were involved in abscisic acid (ABA) signaling, lignin catabolism, lipid transport, and oxidoreductase activity—processes known to play essential roles in drought stress adaptation. In particular, the upregulation of genes related to ABA response highlights the central role of this hormone in mediating drought-induced gene expression, including those involved in stomatal regulation, osmolyte accumulation, and stress signaling.



The KEGG pathway analysis further supported these findings, identifying key enriched pathways such as gamma-aminobutyric acid (GABA) synthesis, aminobutyrate degradation, and semaphorin-mediated axon guidance as significantly upregulated under WD stress. Conversely, pathways such as lipoate biosynthesis were downregulated, indicating a reprioritization of metabolic processes under water limitation.

Furthermore, the classification of DEGs revealed a high representation of transcriptional regulators, oxidoreductases, and enzymes involved in metabolite interconversion. These genes likely play central roles in mediating the complex network of transcriptional reprogramming and metabolic shifts required for drought stress adaptation.

INTEGRATION OF TRANSCRIPTOMIC AND PHYSIOLOGICAL DATA

The integration of transcriptomic and morpho-physiological data provides a comprehensive understanding of drought tolerance in Giza 134 barley. Physiological traits such as reduced chlorophyll content and increased proline levels were corroborated by the differential expression of genes involved in chlorophyll biosynthesis, osmotic regulation, and stress-responsive signaling. This supports the hypothesis that drought tolerance is governed by

coordinated physiological changes and gene regulatory networks. These insights reveal not only the stress responses at the cellular level but also identify candidate genes and pathways that may serve as targets for genetic improvement. The observed gene expression patterns reflect a reconfiguration of metabolic and developmental priorities, shifting from growth promotion to survival mechanisms under drought conditions.

CONCLUSION

This study investigated the physiological and molecular responses of the barley cultivar Giza 134 to water deficit stress through integrated morpho-physiological assessment and transcriptome analysis. Water deficit stress significantly impaired growth and photosynthetic parameters, including crop growth rate, chlorophyll content, leaf water content, and yield components. Adaptive responses included increased stomatal resistance and elevated proline accumulation, indicative of drought avoidance and osmotic adjustment mechanisms. Transcriptomic analysis revealed 2,462 differentially expressed genes under WD conditions, with significant enrichment in pathways related to ABA signaling, carbohydrate metabolism, lipid transport, and oxidoreductase activity. These gene expression changes underpin the complex regulatory mechanisms involved in drought stress tolerance. Functional annotation and enrichment analyses identified key candidate genes and pathways involved in stress adaptation. Notably, genes associated with chlorophyll concentration, iron ion binding, hormone regulation, and light harvesting were significantly modulated, suggesting their roles in maintaining physiological integrity under drought stress. The identified drought-responsive genes and regulatory elements offer valuable targets for future genetic and biotechnological interventions aimed at improving drought tolerance. These findings contribute to the broader goal of developing resilient barley cultivars adapted to arid and semi-arid environments.

In summary, the integration of physiological and transcriptomic data provides novel insights into the drought adaptation mechanisms in barley. The results of this study lay a strong foundation for further functional genomics research and marker-assisted selection strategies to enhance drought tolerance in barley breeding programs.

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