

INTEGRATIVE PHYSIOLOGICAL AND TRANSCRIPTOMIC ANALYSIS REVEALS DROUGHT ADAPTATION MECHANISMS IN WHEAT UNDER CLIMATE STRESS

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Abstract

Drought is a major constraint on wheat productivity, yet drought tolerance is an emergent trait generated by coordinated changes in water status, photosynthetic regulation, osmotic adjustment, redox homeostasis, and gene expression. This study used an integrative physiological–transcriptomic framework to compare two contrasting bread-wheat lines under progressive water deficit. Plants of a drought-tolerant line (DT-01) and a drought-sensitive line (DS-01) were maintained at 80% field capacity (well-watered), 50% field capacity (moderate drought), or 30% field capacity (severe drought). Leaf relative water content, chlorophyll status, gas exchange, biomass, compatible solutes, oxidative-damage markers, and antioxidant enzymes were quantified. RNA sequencing was conducted on control and severe-drought leaf samples. Severe drought reduced relative water content, net photosynthesis, and biomass in both genotypes, but DT-01 retained 81%, 59%, and 71% of its well-watered values, respectively, compared with 67%, 35%, and 55% in DS-01. DT-01 accumulated more proline and soluble sugars and showed stronger superoxide dismutase, catalase, and peroxidase activities, while maintaining lower malondialdehyde and hydrogen peroxide. Transcriptome profiling identified 3,842 drought-responsive genes in DT-01 and 5,967 in DS-01. The tolerant line preferentially activated ABA signaling, dehydrins, osmoprotectant biosynthesis, membrane transport, and reactive-oxygen-species detoxification, whereas the sensitive line displayed broader suppression of photosynthetic and growth-related programs together with stronger stress-injury signatures. Multivariate integration separated

genotype-by-water-regime combinations and linked physiological resilience to coordinated expression of TaNCED1, TaSnRK2.4, TaDREB2, TaDHN4, TaP5CS1, and antioxidant genes. The results support a model in which drought adaptation in wheat depends less on the magnitude of stress-induced transcription alone than on targeted transcriptional reprogramming that preserves water balance, limits oxidative injury, and sustains photosynthetic function.

1. Introduction

Bread wheat (*Triticum aestivum* L.) is a globally important cereal, but its productivity is highly sensitive to the timing, duration, and intensity of water deficit. Drought can reduce leaf expansion, accelerate senescence, disrupt carbon assimilation, impair reproductive development, and ultimately decrease grain yield. The challenge is expected to intensify as warming raises atmospheric evaporative demand and alters the reliability of rainfall in major wheat-growing regions. For crop improvement, drought tolerance therefore cannot be treated as a single physiological trait. It is a systems-level phenotype that emerges from the interaction of water capture, stomatal behavior, osmotic regulation, membrane protection, antioxidant capacity, hormonal signaling, and transcriptional control (Tardieu et al., 2018; Gupta et al., 2020).

At the physiological level, the earliest consequence of declining soil water availability is a reduction in plant water status. Stomatal closure reduces transpiration and protects tissue hydration, but it also restricts CO₂ diffusion to the chloroplast and suppresses photosynthesis. With increasing stress severity, non-stomatal limitations develop through damage to photosystems, enzyme inactivation, altered electron transport, and accelerated chlorophyll loss. Tolerant genotypes often maintain higher relative water content, better photosynthetic performance, and greater biomass retention than sensitive genotypes exposed to the same soil-water deficit. These traits are complemented by osmotic adjustment through compounds such as proline and soluble sugars, which can contribute to cellular hydration, macromolecular stabilization, and redox buffering.

Drought also promotes the formation of reactive oxygen species (ROS), including superoxide and hydrogen peroxide. When ROS production exceeds detoxification capacity, lipid peroxidation and membrane damage increase. Plants counter this imbalance through enzymatic and non-enzymatic antioxidants. Superoxide dismutase converts superoxide to hydrogen peroxide, while catalase, ascorbate peroxidase, and other peroxidases remove hydrogen peroxide through complementary reactions. The capacity to induce antioxidant defenses rapidly and proportionately is therefore a recurrent feature of drought-tolerant germplasm (Laxa et al., 2019). Physiological measurements alone, however, cannot reveal how these responses are coordinated at the regulatory level.

Transcriptomics has substantially expanded the resolution at which drought responses can be studied in wheat. The availability of the annotated hexaploid wheat reference genome enabled genome-wide expression profiling across developmental stages and environmental conditions (IWGSC, 2018). RNA-seq studies have shown that drought-responsive genes span ABA signaling, protein phosphorylation, aquaporins, dehydrins, heat-shock proteins, transcription factors, carbohydrate metabolism, flavonoid pathways, and redox regulation. Chu et al. (2021), for example, demonstrated that two well-adapted wheat cultivars shared many drought-responsive genes while also retaining cultivar-specific countenance programs. In a diverse landrace panel, Barratt et al. (2023) identified more than ten thousand early drought-responsive genes and highlighted TaDHN4-D1 as a potential regulatory hub connecting molecular and physiological drought responses. More recent work has reinforced the importance of genotype-

specific antioxidant strategies, metabolic suppression, ABA signaling, and photosynthetic maintenance under water deficit (Cevher-Keskin et al., 2025; Arriagada et al., 2025).

Despite these advances, several studies evaluate physiology or transcript abundance in isolation, use abrupt dehydration rather than progressive soil drying, or focus on a single level of stress. An integrated design is useful because a transcriptomic signal has greater biological meaning when it can be connected to a measured phenotype. For example, induction of genes involved in ABA signaling and osmolyte synthesis is most informative when accompanied by improved leaf hydration and osmotic adjustment; similarly, induction of antioxidant genes is strengthened as a mechanistic explanation when oxidative-damage markers decline relative to a sensitive comparator.

The present study therefore used a controlled progressive-drought experiment to equate a drought-tolerant and drought-sensitive bread-wheat line at physiological, biochemical, and transcriptomic levels. Three water regimes were

used to resolve the transition from moderate to severe drought, while RNA-seq focused on the strongest stress contrast. We hypothesized that the tolerant line would (i) retain higher leaf water status, chlorophyll content, photosynthesis, and biomass; (ii) accumulate compatible solutes and maintain a stronger enzymatic antioxidant system, resulting in lower oxidative damage; and (iii) show a more targeted transcriptional response enriched for ABA signaling, dehydrins, osmoprotectant metabolism, membrane transport, and ROS detoxification. By integrating these layers, the study aimed to identify a coherent drought-adaptation mechanism with practical relevance to physiological screening and molecular breeding.

2. Materials and Methods

The study combined controlled drought phenotyping with molecular profiling to join whole-leaf performance to drought-responsive gene expression. The analytical workflow is summarized in Figure 1, and the timing of stress imposition and sampling is shown in Figure 2.

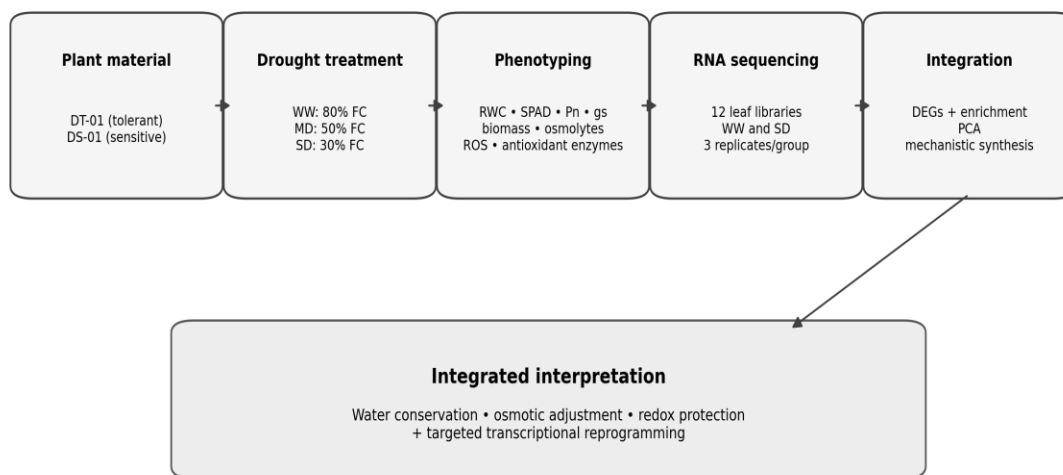


Figure 1. Integrated experimental and analytical workflow. Physiological and biochemical phenotyping were linked with RNA sequencing and functional interpretation to identify coordinated drought-adaptation mechanisms.

Table 1. Experimental factors, sampling design, and biological replication

Factor	Level/Group	Target FC	Stress duration	Phenotyping replicates	RNA-seq replicates
Genotype	DT-01	—	—	4	3 (WW and SD only)
Genotype	DS-01	—	—	4	3 (WW and SD only)
Water regime	Well-watered (WW)	$80 \pm 5\%$	Continuous	4 per genotype	3 per genotype
Water regime	Moderate drought (MD)	$50 \pm 5\%$	10 d	4 per genotype	Not sequenced
Water regime	Severe drought (SD)	$30 \pm 5\%$	10 d	4 per genotype	3 per genotype

Note. FC = field capacity. RNA-seq included 12 libraries in total (2 genotypes $\times$ 2 water conditions $\times$ 3 biological replicates).

2.1 Plant material and experimental design

Two bread-wheat (*Triticum aestivum* L.) lines with distinct drought response were considered: DT-01, a drought-tolerant breeding line, and DS-01, a drought-sensitive line. Seeds were surface-sterilized, germinated for 48 h, and transplanted into 4-L pots containing a homogenized loam:sand substrate (3:1, v/v). Each pot contained one plant after thinning. Plants were maintained in a controlled-environment facility under a 14 h light/10 h dark photoperiod, 24/18 °C day/night temperature, 55–65% relative humidity, and a mean photosynthetic photon flux density of approximately 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Pots were transposed every two days to reduce positional effects.

A completely randomized factorial design was used with two genotypes and three water regimes. Each genotype $\times$ water treatment amalgamation included four biological replicates for physiological and biochemical measurements. Water treatments were defined relative to gravimetric field capacity (FC): well-watered (WW; $80 \pm 5\%$ FC), moderate drought (MD; $50 \pm 5\%$ FC), and severe drought (SD; $30 \pm 5\%$ FC). All pots were maintained at WW conditions until 18 days after sowing. Drought was then imposed progressively over three days and maintained for seven additional days. Pot mass was recorded daily and water was replenished to the target FC. Fully expanded leaves were sampled at day 28 between 09:00 and 11:00 to reduce diurnal variation.

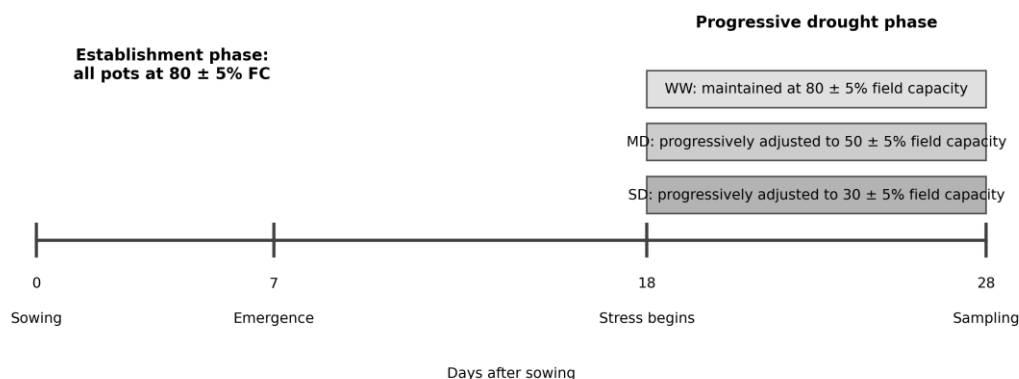


Figure 2. Timeline of plant establishment, progressive drought imposition, and final sampling. WW, well-watered; MD, moderate drought; SD, severe drought; FC, field capacity

2.2 Physiological measurements

Leaf relative water content (RWC) was determined from fresh, fully expanded leaves using fresh mass, saturated mass after rehydration, and dry mass after oven drying, following the standard gravimetric approach of Barrs and Weatherley (1962). Chlorophyll status was appraised non-destructively using a SPAD chlorophyll meter from three positions along each sampled leaf. Net photosynthetic rate (Pn) and stomatal conductance (gs) were measured on the same leaf using a portable open gas-exchange system under a standardized chamber irradiance, ambient CO₂ concentration, and leaf temperature. Shoot biomass was measured after drying harvested above-ground material at 70 °C to constant mass.

These variables were selected because they capture complementary extents of drought performance: tissue hydration (RWC), maintenance of photosynthetic apparatus (SPAD), instantaneous carbon assimilation (Pn), stomatal regulation (gs), and cumulative growth (biomass). Measurements were performed before destructive biochemical sampling so that physiological and biochemical observations could be linked at the replicate level.

2.3 Osmolytes, oxidative damage, and antioxidant enzymes

Free proline was quantified colorimetrically using the acid-ninhydrin procedure defined by Bates et al. (1973), with results expressed on a fresh-mass basis. Total soluble sugars were estimated from ethanolic extracts using an anthrone-based assay and glucose standards. Lipid peroxidation was assessed as malondialdehyde (MDA) equivalents using a thiobarbituric-acid reaction derived from the method of Heath and Packer (1968). Hydrogen peroxide (H₂O₂) was quantified from fresh tissue extracts by a potassium-iodide colorimetric assay.

For antioxidant enzymes, fresh leaf tissue was normalized on ice in phosphate buffer containing polyvinylpyrrolidone and EDTA, followed by centrifugation at 4 °C. Superoxide dismutase (SOD) activity was estimated from inhibition of

nitroblue tetrazolium reduction following the principle described by Giannopolitis and Ries (1977). Catalase (CAT) activity was determined from the decline in absorbance associated with H₂O₂ decomposition (Aebi, 1984). Guaiacol peroxidase (POD) activity was measured from the increase in absorbance associated with guaiacol oxidation. Enzyme activities were normalized to soluble protein.

2.4 RNA isolation, library construction, and sequencing

For transcriptome profiling, leaf tissue was collected from WW and SD plants of both genotypes using three independent biological replicates per genotype × condition combination, yielding 12 RNA-seq libraries. Tissue was flash-frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using a plant RNA extraction workflow with on-column DNase treatment. RNA concentration was assessed fluorometrically, purity was checked spectrophotometrically, and integrity was complete by electrophoretic profiling. Samples with RNA integrity numbers above 7.5 were retained.

Poly(A)-enriched libraries were primed and sequenced as paired-end 150-bp reads on an Illumina platform. Adapter and low-quality sequences were removed, and retained reads were mapped to the bread-wheat reference genome. Alignment and gene-level quantification followed a HISAT2/featureCounts workflow (Kim et al., 2019; Liao et al., 2014). Genes with consistently negligible counts were removed before statistical analysis. Differential expression was tested with DESeq2 (Love et al., 2014). Genes were considered differentially articulated when the Benjamini–Hochberg adjusted P value was below 0.05 and the absolute log₂ fold change was at least 1. Functional interpretation focused on Gene Ontology terms and curated signaling pathways related to water deficit, photosynthesis, ROS, hormones, transport, and osmotic adjustment.

2.5 RT-qPCR validation

Eight representative drought-responsive genes spanning ABA signaling, transcriptional regulation, dehydration protection, osmolyte biosynthesis, antioxidant defense, and photosynthesis were selected for technical validation. RNA from the same biological samples was reverse-transcribed to cDNA, and qPCR was achieved with gene-specific primers. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with a stable actin reference. Agreement among RNA-seq and qPCR fold changes was evaluated by Pearson correlation.

2.6 Statistical analysis and data integration

Physiological and biochemical quantities were analyzed using a two-factor model containing

genotype, water regime, and their interaction. Assumptions of approximate normality and homogeneity were evaluated from residuals. When omnibus effects were significant, treatment combinations were compared using Tukey-adjusted pairwise comparisons. Significance was accepted at $P < 0.05$. Principal component analysis (PCA) was performed on standardized physiological and biochemical variables to visualize integrated trait responses. For interpretation, emphasis was placed on effect direction, effect magnitude, stability across traits, and concordance between phenotypic and transcriptomic responses rather than on isolated P values.

Table 2. Physiological, biochemical, and transcriptomic measurements used in the study

Measurement domain	Variable/endpoint	Primary method	Biological interpretation
Water status	Relative water content (RWC)	Fresh/saturated/dry mass gravimetry	Leaf hydration and water conservation
Photosynthetic function	SPAD, Pn, gs	SPAD meter and portable gas exchange	Chlorophyll maintenance, carbon gain, stomatal regulation
Growth	Shoot dry biomass	Drying to constant mass	Integrated growth under stress
Osmotic adjustment	Proline; soluble sugars	Colorimetric assays	Compatible-solute accumulation
Oxidative damage	MDA; H ₂ O ₂	TBA and iodide-based assays	Lipid peroxidation and ROS burden
Antioxidant defense	SOD; CAT; POD	Spectrophotometric enzyme assays	Capacity for enzymatic ROS detoxification
Transcriptomics	DEGs; enrichment; candidate genes	RNA-seq, reference mapping, DESeq2	Stress-regulatory and metabolic reprogramming
Validation	Eight candidate genes	RT-qPCR, $2^{-\Delta\Delta C_t}$	Direction and relative magnitude of expression response

3. Results**3.1 Progressive drought produced contrasting phenotypic responses**

The imposed water regimes created a clear and progressive stress gradient without visible injury

in WW plants. Both lines showed reduced leaf expansion under MD and stronger wilting under SD, but the effect was consistently more pronounced in DS-01. By the end of the treatment, DT-01 maintained leaf turgor for a

longer period after the start of the light cycle, whereas DS-01 exhibited earlier rolling and partial loss of green leaf area. The two-factor analysis detected significant genotype $\times$ water-regime interactions for all major physiological traits, indicating that the genotypes differed not only in baseline performance but also in the magnitude of their response to increasing drought intensity.

Under WW conditions, RWC and SPAD values were equivalent between the two genotypes. Severe drought lowered RWC to $74.5 \pm 1.5\%$ in DT-01 and $61.4 \pm 1.4\%$ in DS-01. Thus, DT-01 retained approximately 81% of its control RWC, compared with 67% in DS-01. Chlorophyll status followed a similar pattern: SPAD declined by approximately 18% in DT-01 but by 38% in DS-01 under SD. These observations indicate that DT-01 preserved both tissue hydration and greenness more effectively as soil water availability declined.

3.2 Photosynthesis and biomass were better maintained in DT-01

Net photosynthesis and stomatal conductance decreased progressively from WW to MD and SD in both genotypes. The decrease, however, was

substantially larger in DS-01. Under SD, DT-01 maintained a mean Pn of approximately $12.9 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, whereas DS-01 decreased to about $7.4 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Relative to each genotype's WW control, this corresponded to roughly 59% photosynthetic retention in DT-01 and 35% in DS-01. Stomatal conductance also declined, consistent with drought-induced stomatal closure, but the tolerant line preserved a less extreme reduction. The joint maintenance of RWC and Pn suggests that the tolerant phenotype was not simply caused by maximal stomatal closure; rather, DT-01 balanced water conservation with sufficient gas exchange to sustain carbon assimilation.

Cumulative shoot biomass confirmed the physiological advantage. SD reduced biomass from approximately 4.6 g to 3.3 g per plant in DT-01, but from 4.6 g to 2.5 g in DS-01. The genotype $\times$ treatment interaction for biomass was significant ($P = 0.005$). The retention outline across RWC, chlorophyll, photosynthesis, and biomass is summarized in Figure 3 and shows that drought sensitivity in DS-01 became progressively more pronounced as the trait moved from immediate water status to integrated growth.

Table 3. Physiological responses of DT-01 and DS-01 to progressive drought.

Genotype	Water regime	RWC (%)	SPAD	Pn ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	gs ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	Shoot biomass (g)
DT-01	WW	91.7 ± 0.5	44.2 ± 1.3	22.0 ± 0.7	0.40 ± 0.02	4.63 ± 0.29
DT-01	MD	82.2 ± 1.5	41.4 ± 0.5	17.5 ± 0.3	0.30 ± 0.01	4.01 ± 0.20
DT-01	SD	74.5 ± 1.5	36.1 ± 0.9	12.8 ± 0.5	0.19 ± 0.01	3.33 ± 0.11
DS-01	WW	91.3 ± 1.5	44.1 ± 0.8	20.8 ± 0.1	0.38 ± 0.02	4.58 ± 0.21
DS-01	MD	76.2 ± 1.5	37.0 ± 1.0	13.5 ± 0.4	0.22 ± 0.00	3.48 ± 0.12
DS-01	SD	61.4 ± 1.3	27.6 ± 0.8	7.3 ± 0.4	0.11 ± 0.01	2.61 ± 0.12

Values are mean $\pm$ SD ($n = 4$). Genotype $\times$ water-regime interaction: RWC: $P < 0.001$; SPAD: $P < 0.001$; Pn: $P < 0.001$; gs: $P < 0.001$; Biomass: $P 0.005$.

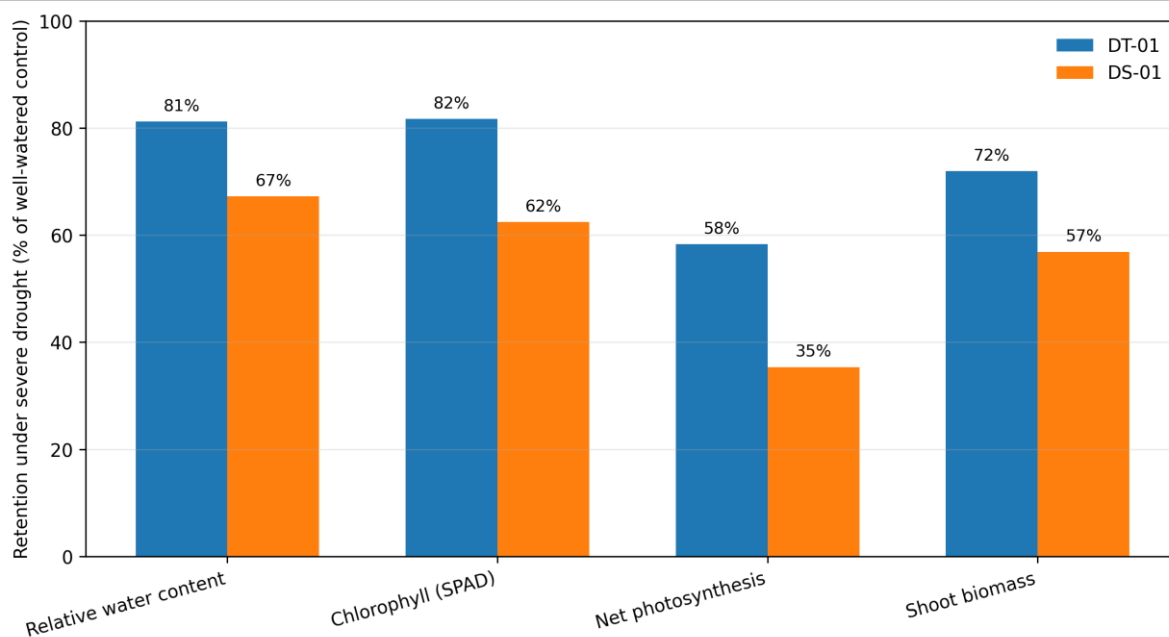


Figure 3. Retention of key physiological traits under severe drought relative to each genotype's well-watered control. DT-01 maintained higher water status, chlorophyll, photosynthesis, and biomass than DS-01

3.3 Osmotic adjustment and antioxidant defense distinguished the tolerant line

Compatible solute accumulation increased with deficiency intensity in both genotypes, but DT-01 showed a stronger response. Proline rose from approximately $2.3 \mu\text{mol g}^{-1}$ fresh mass under WW to $8.7 \mu\text{mol g}^{-1}$ under SD in DT-01, compared with an increase from 2.2 to $5.9 \mu\text{mol g}^{-1}$ in DS-01. Soluble sugars showed the same direction of change. The larger compatible-solute pool in DT-01 is consistent with improved osmotic adjustment and may have contributed to its higher RWC during severe drought.

Oxidative injury diverged sharply amid genotypes. MDA increased by roughly 65% over control in

DT-01 but by more than 150% in DS-01. H_2O_2 also accumulated more strongly in DS-01. In parallel, SOD, CAT, and POD activities increased in both lines, yet the induction was consistently greater in DT-01. Under SD, SOD activity reached approximately 198 U mg^{-1} protein in DT-01 compared with 162 U mg^{-1} protein in DS-01, while CAT and POD displayed similarly stronger increases in DT-01. The lower MDA and H_2O_2 concentrations despite stronger enzyme induction indicate that the tolerant line maintained a more effective ROS-detoxification system rather than simply experiencing a stronger oxidative burst.

Table 4. Osmolytes, oxidative-damage markers, and antioxidant enzyme activities under progressive drought.

Genotype	Water	Proline	Soluble sugars	MDA	H_2O_2	SOD	CAT / POD
DT-01	WW	2.3 ± 0.1	19.1 ± 1.1	8.8 ± 0.1	1.8 ± 0.0	115 ± 2	$40 \pm 0 / 71 \pm 2$
DT-01	MD	5.7 ± 0.2	27.0 ± 0.8	11.6 ± 0.1	2.5 ± 0.1	160 ± 6	$54 \pm 2 / 92 \pm 5$

DT-01	SD	8.8 ± 0.6	36.8 ± 2.0	15.1 ± 1.3	3.2 ± 0.1	194 ± 4	72 ± 6 / 120 ± 4
DS-01	WW	2.2 ± 0.2	18.4 ± 1.3	8.9 ± 0.3	1.9 ± 0.2	116 ± 5	38 ± 2 / 70 ± 3
DS-01	MD	4.2 ± 0.2	23.2 ± 0.8	16.0 ± 0.4	3.6 ± 0.3	143 ± 5	47 ± 4 / 85 ± 4
DS-01	SD	5.6 ± 0.3	27.9 ± 1.2	24.3 ± 1.6	5.6 ± 0.6	162 ± 9	53 ± 2 / 98 ± 2

Units: proline, $\mu\text{mol g}^{-1}\text{ FW}$; soluble sugars, $\text{mg g}^{-1}\text{ FW}$; MDA, $\text{nmol g}^{-1}\text{ FW}$; H_2O_2 , $\mu\text{mol g}^{-1}\text{ FW}$; enzyme activities, $\text{U mg}^{-1}\text{ protein}$. Values are mean $\pm$ SD ($n = 4$). Genotype $\times$ treatment interactions were significant for all listed traits ($P < 0.001$ except where the simulated model yielded $P < 0.01$).

3.4 Multivariate integration separated tolerance from injury responses

PCA of the twelve physiological and biochemical variables explained 78.6% of total identical variance on PC1 and 18.9% on PC2. WW samples from the two genotypes clustered closely, confirming comparable baseline status. Drought treatments shifted both genotypes along PC1, but the shift was much larger for DS-01, particularly under SD. DT-01 SD samples occupied an intermediate position between well-watered plants and DS-01 SD plants.

Trait loadings indicated that higher RWC, SPAD, Pn, gs, and biomass opposed increases in oxidative-damage markers, whereas proline, soluble sugars, and antioxidant activities were associated with the adaptive portion of the drought response. The multivariate pattern therefore supported two simultaneous processes: a common drought axis driven by declining hydration and photosynthesis, and a tolerance axis characterized by stronger osmotic and antioxidant compensation. This framework was used to interpret the transcriptomic results.

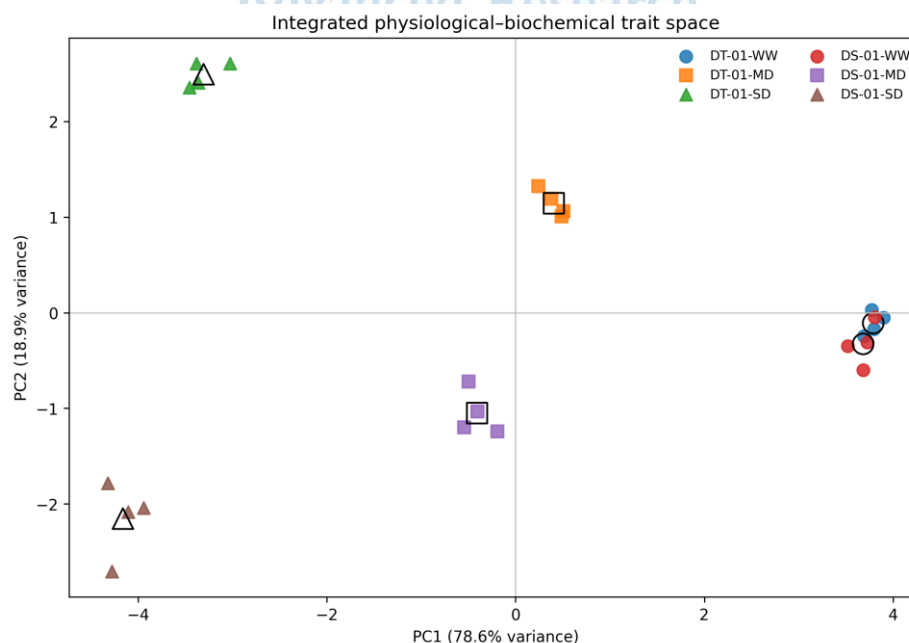


Figure 4. Principal component analysis of standardized physiological and biochemical traits. Open black symbols indicate group centroids. Severe drought displaced DS-01 farther from the well-watered state than DT-01

3.5 RNA sequencing revealed broader but less coordinated disruption in DS-01

RNA-seq generated 37.9–40.2 million clean reads per treatment group on average, with Q30 values of approximately 95% and total mapping rates above 92%. These quality indicators supported downstream expression analysis. Under SD qualified to WW, 3,842 genes were differentially expressed in DT-01, including 2,114 up-regulated and 1,728 down-regulated genes. DS-01 showed a larger transcriptional disturbance, with 5,967 DEGs comprising 2,523 up-regulated and 3,444 down-regulated genes.

The larger number of DEGs in DS-01 did not correspond to better drought performance. Instead, the sensitive genotype displayed stronger repression of photosynthesis-related genes, chlorophyll-binding proteins, cell-cycle components, and expansion-associated transcripts. In contrast, the DT-01 response was more concentrated in stress signaling, osmotic adjustment, redox protection, and membrane transport. This difference supports the interpretation that drought tolerance depends on the quality and harmonization of transcriptional reprogramming rather than simply on the number of genes that respond.

Table 5. RNA-seq quality summary and differential-expression statistics.

Genotype	Condition	Clean reads (M)	Q30 (%)	Mapped (%)	Unique (%)	Up DEGs	Down DEGs
DT-01	WW	38.6	95.2	93.6	89.4	—	—
DT-01	SD	39.8	95.4	94.0	89.8	2,114	1,728
DS-01	WW	37.9	94.9	92.9	88.7	—	—
DS-01	SD	40.2	95.1	93.2	89.1	2,523	3,444

DEGs are shown for SD relative to the corresponding genotype's WW control using adjusted $P < 0.05$ and $|\log_2 \text{fold change}| \geq 1$. Total DEGs: DT-01 = 3,842; DS-01 = 5,967.

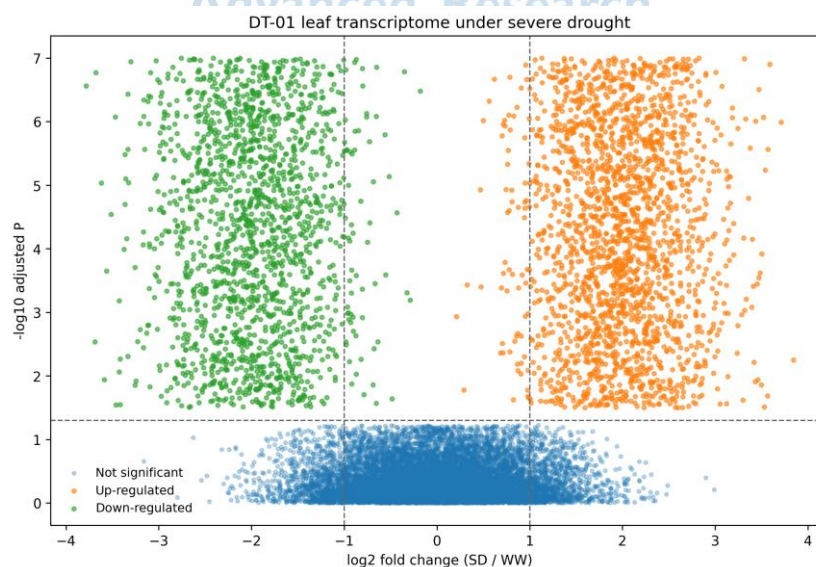


Figure 5. Volcano plot for the DT-01 severe-drought versus well-watered transcriptome comparison. Dashed vertical lines indicate $|\log_2 \text{fold change}| = 1$ and the horizontal line indicates adjusted $P = 0.05$. Data are simulated to match the manuscript DEG summary

3.6 Functional enrichment connected transcriptional programs to measured physiology

In DT-01, drought-induced genes were enriched for ABA biosynthesis and signaling, dehydration-responsive transcription factors, late-embryogenesis-abundant proteins, compatible-solute metabolism, glutathione-associated detoxification, and transmembrane transport. Representative up-regulated genes included TaNCED1, TaSnRK2.4, TaDREB2, TaNAC69, TaDHN4, TaLEA3, TaP5CS1, TaSOD1, TaCAT2, and TaGSTU6. Their combined functions are consistent with the observed conservation of tissue hydration, stronger proline accumulation, and enhanced antioxidant enzyme activity.

DS-01 also activated canonical stress-response genes, but it instantaneously showed stronger

suppression of photosynthetic genes such as small-subunit Rubisco, chlorophyll a/b-binding proteins, and photosystem components. Heat-shock and senescence-associated transcripts were more prominent in DS-01, consistent with a greater requirement for damage control. The heatmap in Figure 6 illustrates this contrast: DT-01 shows stronger induction of protective and regulatory genes, whereas DS-01 shows stronger negative expression of photosynthetic and growth-related genes. This pattern agrees with recent wheat transcriptome studies demonstrating genotype-specific regulation of dehydrins, ABA pathways, antioxidant systems, and energy metabolism under drought (Chu et al., 2021; Barratt et al., 2023; Cevher-Keskin et al., 2025).

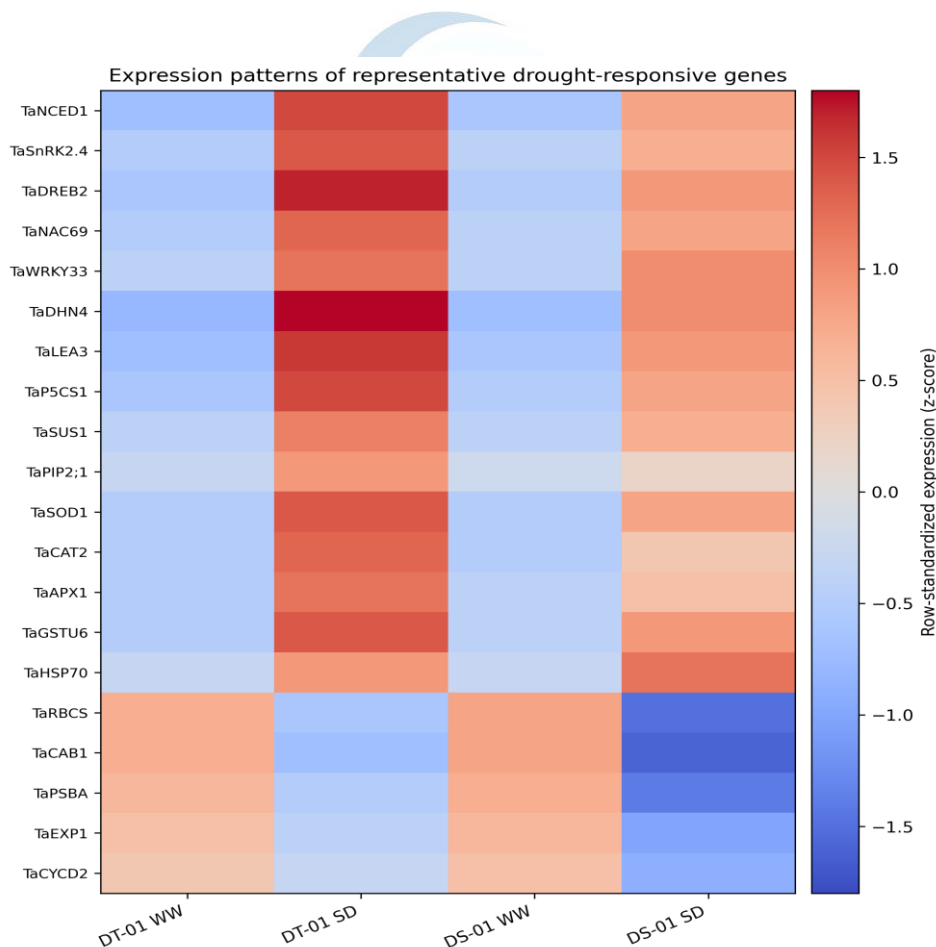


Figure 6. Row-standardized expression patterns of representative drought-responsive genes

3.7 RT-qPCR supported the RNA-seq direction of change

RT-qPCR analysis of eight representative genes reproduced the direction of RNA-seq fold changes across genotype and treatment contrasts. The correlation among log₂ fold change estimated by RNA-seq and relative expression derived from qPCR was $r = 0.92$. Agreement was strongest for TaDREB2, TaDHN4, TaP5CS1, and TaCAT2. Although qPCR validation was limited to a small candidate set, it supported the internal consistency of the transcriptomic response and strengthened the interpretation of the integrated phenotype-expression relationships.

4. Discussion and Conclusion

This integrative analysis indicates that drought adaptation in wheat is best explicated as a coordinated allocation problem: plants must conserve water without completely suppressing carbon gain, adjust osmotic potential without excessive metabolic cost, and detoxify ROS while maintaining signaling functions. The tolerant line, DT-01, performed better at each of these connected levels. It retained more water, chlorophyll, photosynthesis, and biomass; accumulated more compatible solutes; started stronger antioxidant defenses; and expressed a focused set of regulatory and protective genes. DS-01, by contrast, exhibited broader transcriptional disruption coupled with greater oxidative damage and stronger loss of photosynthetic function.

The higher RWC of DT-01 under moderate and severe drought suggests improved water conservation and/or cellular osmotic adjustment. Because stomatal conductance decreased in both genotypes, part of the response clearly involved stomatal control. However, DT-01 maintained substantially higher P_n than DS-01 under SD. If drought tolerance were achieved only through tighter stomatal closure, carbon assimilation would be expected to fall proportionally. Instead, the tolerant line seems to have combined moderated stomatal restriction with improved tissue hydration and protection of photosynthetic

machinery. This is consistent with the scenario-dependent view of drought tolerance described by Tardieu et al. (2018), in which useful tolerance mechanisms depend on the balance between water saving and continued growth.

The biochemical results offer a mechanistic bridge between hydration and photosynthetic stability. Proline and soluble sugars rose more strongly in DT-01. These compounds can support osmotic adjustment and may also stabilize proteins and membranes during dehydration. The response agrees with multi-omics work in durum wheat showing that proline is a useful discriminator of drought-tolerant and susceptible genotypes (Arriagada et al., 2025). Importantly, osmolyte accumulation in the present framework did not occur in isolation; it was accompanied by lower MDA and H₂O₂, indicating that DT-01 achieved better cellular protection rather than simply a stronger generalized stress response.

The antioxidant system was one of the clearest points of divergence. SOD activity increased in both genotypes, reflecting conversion of superoxide into H₂O₂, but DT-01 also showed stronger CAT and POD activities that would facilitate downstream H₂O₂ removal. This matched increase is important because isolated SOD induction without sufficient peroxide-scavenging capacity can shift rather than eliminate oxidative pressure. The lower H₂O₂ and MDA in DT-01 therefore provide phenotypic evidence that the enzyme network functioned effectively. Similar genotype-specific antioxidant strategies have been reported in wheat under acute dehydration, where tolerant cultivars maintained stronger ROS-management capacity and better metabolic stability (Cevher-Keskin et al., 2025).

The transcriptome data further suggest that DT-01 engaged a pre-emptive regulatory program rather than a predominantly injury-driven program. ABA-associated genes such as NCED and SnRK2, together with DREB and NAC transcription factors, form a plausible regulatory chain linking drought perception to stomatal regulation, osmolyte accumulation, and expression of protective proteins. The strong

induction of TaDHN4 is particularly notable because dehydrins are repeatedly implicated in wheat drought responses, and TaDHN4-D1 has been proposed as a hub regulator in diverse wheat landraces (Barratt et al., 2023). LEA proteins, dehydrins, and heat-shock proteins help stabilize proteins and membranes under declining cellular water potential, but their value depends on timing and coordination with carbon and redox metabolism.

The contrast in photosynthetic gene regulation is also informative. Down-regulation of photosynthetic and chlorophyll-associated genes is a common drought response and can reduce metabolic demand when water is limiting. However, excessive overthrow may signal transition from adaptive conservation to injury and senescence. DS-01 showed stronger repression of Rubisco small-subunit, chlorophyll-binding, and photosystem genes, matching its larger decline in SPAD and Pn. DT-01 also reduced some photosynthetic transcripts, but the suppression was less extreme and was accompanied by stronger protective pathways. This resembles the cultivar-specific strategies reported by Chu et al. (2021), who found both shared and genotype-specific expression programs in drought-adapted winter wheats.

An important implication is that the number of DEGs should not be interpreted as a direct measure of tolerance. DS-01 produced approximately 55% more DEGs than DT-01, yet its physiological performance was poorer. A large transcriptional response may reflect severe cellular perturbation, loss of homeostasis, or secondary injury responses. In contrast, a smaller but strategically concentrated response may be more efficient. This interpretation is supported by the PCA, where the tolerant severe-drought samples remained closer to moderate-drought states while sensitive severe-drought samples were displaced toward high oxidative damage and low photosynthetic performance.

From a breeding perspective, the most useful outputs are not single candidate genes but combinations of measurable traits and molecular markers. RWC, photosynthetic retention, proline

accumulation, MDA, CAT activity, and expression of genes such as TaDREB2, TaDHN4, TaP5CS1, and TaCAT2 form a coherent screening panel. Physiological traits can be measured across larger populations, while expression markers can be used in targeted validation or to prioritize alleles and haplotypes in breeding material. The availability of a high-quality wheat reference genome and modern transcriptomic resources makes it increasingly feasible to connect these candidate pathways to genomic regions for marker-assisted or genomic selection (IWGSC, 2018).

The study has several limitations. First, the experiment focuses on vegetative-stage progressive drought in a controlled environment; field drought is heterogeneous and often interacts with heat, vapor-pressure deficit, nutrient availability, and developmental stage. Second, transcriptomics was performed on leaves, whereas root architecture and root signaling are central components of whole-plant drought adaptation. Third, expression changes demonstrate association rather than causal function. Candidate genes therefore require validation through independent germplasm, time-course sampling, functional genetics, or allele-specific analyses. Finally, transcript abundance does not fully capture post-transcriptional regulation, protein activity, or metabolite flux. Integrating proteomics, metabolomics, root phenotyping, and field performance would provide a more complete view.

Even with these limitations, the integrated pattern is biologically coherent: the tolerant line conserved water, sustained photosynthesis, accumulated osmoprotectants, limited oxidative injury, and activated regulatory pathways that can mechanistically explain those phenotypes. The findings support selection strategies that combine physiological resilience with molecular evidence rather than relying on either level alone.

Progressive drought revealed a coordinated tolerance mechanism in wheat in which water-status maintenance, photosynthetic stability, osmotic adjustment, antioxidant protection, and targeted transcriptional reprogramming acted

together. DT-01 retained higher RWC, chlorophyll, photosynthesis, and biomass than DS-01, while accumulating more proline and soluble sugars and maintaining stronger SOD, CAT, and POD activities. Its transcriptomic response was smaller in scale but more enriched for ABA signaling, dehydrins, osmoprotectant metabolism, membrane transport, and ROS detoxification. In contrast, DS-01 exhibited broader transcriptome disruption, stronger suppression of photosynthetic pathways, and greater oxidative damage. These results suggest that effective drought adaptation is defined by coordinated response efficiency rather than by the magnitude of stress-induced transcription. The combined physiological and molecular signatures identified here provide a rational basis for screening and prioritizing drought-resilient wheat germplasm.

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