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Genotypic variation in physiological, biochemical, and transcriptional responses to drought stress in spring barley at an early growth stage

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Abstract: Drought is a major abiotic stress limiting barley (*Hordeum vulgare* L.) productivity. We evaluated 17 spring barley genotypes at the early leaf development stage under controlled laboratory conditions with optimal and drought treatments, integrating physiological, biochemical, and molecular traits. Drought reduced relative water content (−1.3% to −3.2%), plant height (−14.7% to −29.6%), and dry biomass (−2.3% to −24.9%), while inducing strong proline accumulation (+23.6% to +454%) and pigment loss (chlorophyll *a* −10.1% to −79.5%; carotenoids −6.2% to −70.9%). Principal component and discriminant analyses identified plant height and chlorophyll *a* as the most reliable discriminators, whereas relative water content was less predictive of the species. Multivariate stratification separated tolerant (Argument, Exalis, Slaven, Malz, Valis), intermediate (Laudis 550, Tango, Kompakt, LG Belcanto, SK Levitus), and sensitive (Kangoo, LG Tosca, LG Flamenco, Karmel, Bojos, Nitran, Tadmor) groups of genotypes. Gene expression profiling of 12 genotypes revealed a modest induction of *HvABF2* (1.77-fold), moderate upregulation of *HvSOD1* (1.82-fold) and *HvAPX1* (2.28-fold), and the strongest response in *HvP5CS* (3.29-fold), which did not consistently correlate with tolerance. Tolerant genotypes combined growth stability, pigment retention, and moderate osmotic adjustment, whereas sensitive genotypes relied on excessive proline accumulation, resulting in severe pigment and growth penalties. Overall, drought tolerance in barley at the early growth stage emerged from the coordinated regulation of growth, photoprotection, and stress-gene activation, providing a foundation that can guide the selection of genotypes for subsequent validation under field conditions and future breeding programmes.

Keywords: abscisic acid signalling; antioxidant enzymes; drought stress genes; high-stress environment; water deficit; osmoprotectant; pigment stability

The increasing frequency and severity of droughts under climate change pose a fundamental threat to global food security by constraining the productivity of major cereal crops (Langridge 2018). Barley (*Hordeum vulgare* L.) is a vital global commodity for both feeding and malting (Newton et al. 2011), yet

it is also an exceptionally resilient crop, cultivated across a wide range of marginal, high-stress environments (Grando and Gomez Macpherson 2005). This resilience has made barley a model system for dissecting adaptive strategies to abiotic stress, particularly drought.

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Plants respond to water deficit through a complex and integrated cascade of physiological, biochemical, and molecular processes (Cai et al. 2020, George et al. 2025). These adaptive strategies are commonly classified as escape, avoidance, or tolerance mechanisms. Escape involves accelerating the life cycle to complete reproduction before the onset of terminal drought. Avoidance strategies aim to maintain a favourable internal water status (high relative water content, RWC) despite external water deficit, through mechanisms such as developing extensive root systems for enhanced water uptake or implementing efficient stomatal control to minimise transpirational water loss. Tolerance strategies, in contrast, enable plants to endure low tissue water potential through protective biochemical and cellular mechanisms (Kishor et al. 2014, Sallam et al. 2019, Elakhdar et al. 2022, Nakashima et al. 2025).

Among the biochemical responses, proline is one of the most widely reported metabolites accumulating under drought and other abiotic stresses. Beyond its classical role as a compatible osmolyte, studies emphasise its multifunctionality, including protection of proteins and membranes, maintenance of redox homeostasis, and signalling roles in stress recovery and programmed cell death (Szabados and Savaure 2009). In barley, the accumulation of proline under drought conditions has been demonstrated to stabilise photosynthetic performance, growth, and metabolism, while also enhancing lateral root development, highlighting its role in supporting plant productivity during water deficits (Frimpong et al. 2021). However, the functional significance of proline remains ambiguous: elevated levels are often found in stress-sensitive genotypes, suggesting that its accumulation may indicate stress severity rather than confer tolerance (Bandurska et al. 2017, Cai et al. 2020).

Drought stress also leads to overproduction of reactive oxygen species (ROS), which damage cellular membranes and degrade pigments (Samanta et al. 2024). Effective tolerance, therefore, depends on the activation of antioxidant systems and the protection of photosynthetic machinery, reflected by the stability of chlorophylls and carotenoids (Sallam et al. 2019). Integrating these biochemical indicators with growth traits, such as biomass and plant height, provides a more reliable framework for discriminating between tolerant and sensitive genotypes. Indeed, recent high-throughput screening of barley germplasm has highlighted shoot biomass, water relations, and

osmotic adjustment as key predictors of drought resilience (Cai et al. 2020).

Recent advances in genomics have shed further light on the molecular determinants of barley drought tolerance. Genome-wide association studies (GWAS) conducted on the International Barley Core Selected Collection have revealed that drought responses are highly genotype-specific, identifying 20 single-nucleotide polymorphisms (SNPs) and 41 candidate genes significantly associated with shoot water content under drought stress, confirming water balance as a reliable discriminator among tolerant and sensitive accessions (Xiong et al. 2023). In particular, genes associated with osmotic regulation, ROS detoxification, and abscisic acid (ABA) signalling have emerged as critical molecular determinants of drought adaptation (Ferdous et al. 2015, Bandurska et al. 2017, Alexander et al. 2019). These findings underscore the importance of integrating molecular and phenotypic markers to gain a more comprehensive understanding of drought tolerance mechanisms.

This study aimed to comprehensively evaluate drought responses in a set of 17 spring barley genotypes at the early leaf development stage using an integrative approach. Physiological traits (relative water content, plant height, and dry biomass), biochemical markers (proline, chlorophyll *a*, and carotenoids), and, for a subset of 12 genotypes, transcriptional responses of selected drought-related genes (*HvP5CS*, *HvABF2*, *HvSOD1*, and *HvAPX1*) were assessed under controlled optimal and drought conditions. By combining these datasets, we sought to identify genotype-specific response patterns and to stratify genotypes into tolerant, intermediate, and sensitive groups. This approach was designed to provide a framework for screening and selecting drought-resilient barley germplasm suitable for breeding and adaptation to water-limited environments.

MATERIAL AND METHODS

Plant material and growth conditions. Seventeen genotypes of spring barley (*Hordeum vulgare* L.) with diverse genetic backgrounds and contrasting physiological behaviour were selected to assess physiological and biochemical responses under controlled environmental conditions. The tested set comprised locally bred and international cultivars: Argument (SVK), Exalis (SVK), Karmel (SVK), Kompakt (SVK), Malz (CZE), Nitran (SVK), SK Levitus (SVK), Slaven (SVK), Tango (FRA), Valis (SVK), Bojos (CZE), Laudis

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550 (CZE), LG Belcanto (NLD), LG Flamenco (NLD), LG Tosca (NLD), Kangoo (NLD), and the Syrian landrace Tadmor. Seeds were sown in 300 mL plastic pots (25 seeds per pot) filled with a peat-based substrate (KEKKILA Brown OPM 025 W R0332, Vantaa, Finland), with three independent biological replicates per genotype and treatment combination. Plants were grown in a phytotron chamber under a 16/8 h day/night photoperiod and a temperature of 16–18 °C for the day and 12–14 °C for the night.

Drought stress treatment. Two watering regimes were applied: a well-watered control (optimal) and a drought-stressed treatment (drought). Soil moisture was measured using the WET-2 Sensor (Delta-T Devices, Cambridge, UK). In the control variant, substrate moisture was maintained between 40–45% volumetric water content (VWC), which corresponded to 65–73% of field capacity (FC) measured on the fully saturated soil. In the drought stress treatment, the initial soil moisture during germination was 35%. During the 2nd week, the humidity of the substrate gradually decreased and, between the 14th and 24th day, it was maintained at 17–20% (corresponding to 27–32% of FC). Plants were checked and watered every 2–3 days to maintain the optimal humidity levels. The experiment was terminated after 24 days at the three-leaf stage (BBCH 13; Zadoks et al. 1974).

Morpho-physiological traits measurements. At the end of the experiment, five plants per replicate were randomly selected for morphological assessment. Plant height was measured from the base to the tip of the longest leaf using a ruler. The aerial parts were excised and oven-dried at 60 °C to constant weight to determine dry biomass (DW).

Relative water content (RWC) was assessed following the protocol of Sade et al. (2015). Fully expanded leaf blades (6–10 cm) were used to obtain fresh weight (FW), turgid weight (TW; after floating in 5 mmol CaCl₂ for 8 h), and dry weight (DW; 60 °C, 72 h). RWC was calculated as:

$$\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Determination of proline and photosynthetic pigments. Proline content was quantified according to Bates et al. (1973), with the only modification being the use of ethanol extraction. Leaf tissue (50 mg) was homogenised in ethanol, reacted with acid-ninhydrin, and the absorbance was measured at 512 nm using an INNO Microplate reader (LTK, Seongnam-si, Republic of Korea). Proline concentration was calculated

from an L-proline standard curve (0.025–1.5 mmol) and expressed as millimoles per litre (mmol).

Photosynthetic pigment determination followed the method of Lichtenthaler and Wellburn (1983). The remaining portion of the same leaf blade used for proline measurement (i.e., unused tissue from the same sampled leaf) was extracted in 80% (v/v) acetone, homogenised, and centrifuged (4 000 rpm, 3 min, 4 °C). The absorbance of the supernatant was measured at 665, 646, and 470 nm using an INNO Microplate reader (LTK, Seongnam-si, Republic of Korea), the same instrument used for proline quantification. Chlorophyll *a* (Chl *a*, mg/g FW) and total carotenoids (Car, mg/g FW) were calculated as:

Chl *a*:

$$C_{chlA} = 12.21 \times A_{663} - 2.81 \times A_{646}$$

Car:

$$C_{car} = \frac{(1000 \times A_{470} - 3.27 \times C_{chlA} - 104 \times C_{chlB})}{229}$$

RNA isolation, cDNA synthesis, and quantitative RT-PCR. Total RNA was extracted from 100 mg of frozen leaf tissue using the NucleoSpin® RNA Plant kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's protocol, including on-column DNase digestion to remove genomic DNA. RNA was eluted in 60 µL RNase-free water and stored at –80 °C. RNA purity and concentration were determined spectrophotometrically (A260/A280 ratio), and integrity was verified by agarose gel electrophoresis.

First-strand cDNA was synthesised from 1 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) with Random Hexamer primers in a final volume of 20 µL, according to the manufacturer's instructions. The reaction contained 5 × Reaction Buffer, 20 U RiboLock RNase Inhibitor, 10 mmol dNTP Mix, and 200 U RevertAid Reverse Transcriptase. The cDNA synthesis program consisted of 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 5 min in GeneAmp PCR System 9700 (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). Quantitative PCR (qPCR) was performed on an ABI PRISM® 7000 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) in 96-well MicroAmp optical plates. Target genes included *HvABF2* (abscisic acid-responsive binding factor 2), *HvP5CS* (Δ¹-pyrroline-5-carboxylate synthetase, proline biosynthesis), *HvSOD1* (superoxide dismutase), and

HvAPX1 (ascorbate peroxidase), along with the reference gene *HvACT* (Table 1). Reaction mixtures (25 µL) contained 12.5 µL SYBR Green PCR Master Mix, 150 nmol of each primer, and 25 ng cDNA template. The thermal profile was: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each assay included a no-template control (NTC) and a minus-reverse transcriptase control (RT-). Standard curves were generated from five 10-fold serial dilutions of pooled cDNA (5–50 000 pg/µL) in duplicate. PCR amplification efficiency (E) for each primer pair was calculated from the slope of the standard curve as $E = 10^{-1/\text{slope}}$ (Pfaffl 2001) and expressed as $\%E = (E - 1) \times 100$ (Bustin et al. 2009). Primer specificity was verified by melt-curve analysis and the absence of non-specific amplification or primer-dimer formation. Amplification efficiency and linearity were evaluated prior to gene expression analysis using standard serial dilutions. All qPCR reactions were run with three biological replicates and two technical replicates per sample. Relative expression levels

were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001, Schmittgen and Livak 2008), normalising target gene C_t values to *HvACT*. Stability of the reference gene *HvACT* was evaluated using NormFinder. Gene expression analysis was performed on a subset of 12 barley genotypes, selected from 17 to represent the full range of physiological and biochemical responses observed in the experiment. The number of genotypes analysed was limited by the capacity of a single qPCR plate, which enabled all samples, including target and reference genes, to be amplified under identical thermal and reaction conditions. This approach was chosen to minimise inter-run and inter-plate variability, in accordance with MIQE recommendations (Bustin et al. 2009).

Statistical evaluation. All statistical analyses were performed to evaluate the effects of drought stress in comparison with optimal watering conditions across physiological, biochemical, and molecular parameters. Normality (Shapiro-Wilk) and homogeneity of variance (Levene's test) were assessed separately for each genotype-treatment comparison. Depending

Table 1. Primer sequences and characteristics for quantitative RT-PCR analysis of *Hordeum vulgare* genes evaluated in this study. Forward (F) and reverse (R) primer sequences are given in 5'–3' orientation, together with the corresponding amplicon size, melting temperature (T_m), GC content (%GC), amplification efficiency, and regression coefficient (R^2) from the standard curve

Gene	Name (GenBank ID)	Primer F/R (5'–3')	Amplicon (bp)	T_m (°C)	%GC	Efficiency (%)	R^2	Reference
Actin	<i>HvACT</i> (AY145451)	F: GCTGGAGATGA TGCGCCAAGG R: GCGCCTCATCA CCAACATAAGC	112	60	61.9 54.55	92.71	0.9986	Bandurska et al. (2017)
Abscisic acid- responsive binding factor 2	<i>HvABF2</i> (AK363330)	F: AGAGGCGCATG ATCAAGAAC R: AGTTTTGCTAC CTCGGCTTC	94	60	50.0 50.0	107.23	0.9871	Alexander et al. (2019)
Δ^1 -pyrroline-5- carboxylate synthetase	<i>HvP5CS*</i> (AK249154)	F: GAGGTGATAATG GTCACGTCCGG R: GCACGCCTTTC CATCCAGCTCC	126	60	56.52 63.64	94.92	0.9998	Bandurska et al. (2017)
Superoxide dismutase 1	<i>HvSOD1</i> (AK363344.1)	F: CTTGAAGGACA CCGACTTGC R: CTCAAAAAGCC AAATGACAGTG	141	60	55.0 40.91	108.20	0.9883	Ferdous et al. (2015)
Ascorbate peroxidase 1	<i>HvAPX1</i> (AJ006358)	F: CGGAGCTTTTG AGTGGTGACA R: CCGCAGCATA TTTCTCCACAA	107	60	52.38 47.62	108.68	0.9926	Alexander et al. (2019)

*Designed to anneal on exon 3

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on these assumptions, either an independent two-sample Student's *t*-test (for normally distributed and homoscedastic data) or a Mann-Whitney U test (for non-normal or heteroscedastic data) was applied using Statgraphics 18-X64 (Statgraphics Technologies, Inc., The Plains, Virginia). This statistical software was also used to assess the overall effects of genotype (17 levels), treatment (optimal *vs.* drought), and their interaction using a two-way ANOVA (Type III sums of squares) for all physiological and biochemical traits. For multivariate analyses, data were first subjected to z-score transformation to standardise variables and allow direct comparison across traits with different scales. Principal component analysis (PCA) and Pearson correlation matrices were computed using PAST v4.17 (Hammer et al. 2001), with correlation significance determined at $P < 0.05$. Additional multivariate methods, including pairwise PERMANOVA tests and Multivariate Exploratory ROC Analysis using partial least squares discriminant analysis (PLS-DA) as the classification method, were performed using MetaboAnalyst 6.0 (Pang et al. 2024). For the PLS-DA in the Biomarker Analysis module, a PLS-DA built-in was used as the Feature ranking method, and a metadata table framework (multifactorial approach) was employed, which considers both treatment effects (optimal *vs.* drought) and genotype effects. In this approach, the importance of each variable was represented by the selected frequency (%), indicating how consistently each trait was identified as discriminative across repeated cross-validations. To evaluate the magnitude of stress-induced changes, percentage differences between drought and optimal conditions were calculated for each genotype. These values were used for hierarchical clustering (using Euclidean distance and Ward's linkage) and heatmap analysis. Gene expression data were analysed using the $\Delta\Delta C_t$ method with *HvACT* as a reference gene (Livak and Schmittgen 2001). Violin plots of fold changes were generated in RAWGraphs 2.0 (Mauri et al. 2017), while gene-level associations were further examined through Pearson correlation analyses on z-score transformed percentage differences.

RESULTS

Physiological and biochemical responses to drought. Drought stress significantly affected the physiological performance of all 17 barley genotypes. Relative water content (RWC, Figure 1A) decreased from an average of 97.8% under optimal conditions to

95.7% under drought, with genotype-specific reductions ranging from -1.3% (Bojos) to -3.2% (Kangoo). Plant height (Figure 1B) was strongly inhibited, with the average decreasing from 242 mm under optimal conditions to 189.7 mm under drought, representing relative losses from -14.7% (SK Levitus) to -29.6% (Kangoo). Aboveground biomass production, measured as dry weight (DW, Figure 1C), dropped from 0.156 g to 0.137 g, ranging from minimal reductions in LG Belcanto (-2.3%) and Tadmor (-2.6%) to severe declines in Nitran (-24.9%) and Karmel (-19.6%). Overall, drought reduced hydration, shoot elongation, and biomass accumulation; however, the magnitude of these effects was strongly genotype-dependent, revealing contrasting resilience among cultivars.

Drought induced strong biochemical shifts across genotypes, with proline levels increasing on average 2.5-fold (from 0.121 in optimal to 0.307 mmol in drought conditions, Figure 2A). The most pronounced accumulation occurred in Kangoo (+454%), LG Tosca (+279%), LG Flamenco (+212%), and Tadmor (+203%), whereas Exalis (+44%), Nitran (+47%), and Valis (+58%) showed only minor increases. Chlorophyll *a* content (Figure 2B) declined by $\sim 36\%$ (from 3.76 to 2.37 mg/g FW), with the greatest losses in LG Tosca (-79%), Kangoo (-56%), and LG Flamenco (-54%), while Slaven (-10%), Argument (-12%), and Malz (-14%) maintained higher pigment stability. Carotenoids (Figure 2C) decreased by $\sim 31\%$ (from 1.00 to 0.67 mg/g FW), ranging from severe reductions in LG Tosca (-71%) and Karmel (-62%) to near stability in Malz (-6%), Tango (-13%), and Laudis 550 (-16%). Collectively, the data highlight contrasting strategies: some genotypes combined high proline induction with severe pigment loss, whereas others exhibited modest osmotic adjustment while retaining photosynthetic pigments.

Two-way ANOVA (Table 2) confirmed a significant main effect of drought treatment on all traits ($P < 0.0001$), indicating that the imposed water deficit consistently affected physiological and biochemical performance. Genotype had a significant effect on all parameters except RWC, indicating that leaf hydration was comparatively uniform across genotypes. In contrast, proline, chlorophyll *a* and carotenoids showed significant genotype \times treatment interactions, reflecting genotype-specific plasticity in osmotic adjustment and photoprotection. Growth-related traits (plant height and dry biomass), together with relative water content, showed no significant genotype \times treatment interaction, indicating that the magnitude of drought-induced reductions in these traits was relatively uniform among genotypes.

Principal component analysis (PCA) clearly separated genotypes by treatment along PC1, accounting for 65.6% of the variance (Figure 3A). PERMANOVA confirmed highly significant differences ($P = 0.001$; Figure 3B). Higher proline accumulation drove separation toward drought, while higher RWC, plant height, DW, and pigment contents aligned with optimal conditions. PC2 (15.0%) differentiated genotypes by pigment stability, distinguishing tolerant accessions (Slaven, Exalis, Valis, Argument) from highly stressed ones such as Kangoo (strong proline accumulation) and LG Tosca (severe pigment loss). The partial least squares discriminant analysis (PLS-DA, Figure 3C) achieved

excellent classification performance, as indicated by cross-validated ROC curves with AUC values consistently above 0.99 (95% CI = 0.972–1.000), identifying plant height and chlorophyll *a* as the most consistent discriminators, followed by proline, carotenoids, and biomass. According to PLS-DA, RWC was excluded from the set of significant predictors, suggesting that its variation was less decisive for treatment separation. Correlation patterns shifted markedly under drought (Figure 3D). Height and DW biomass remained positively associated ($r = 0.69$, $P = 0.002$), but proline showed negative correlations with both height ($r = -0.51$, $P = 0.035$) and RWC ($r = -0.55$, $P = 0.021$), re-

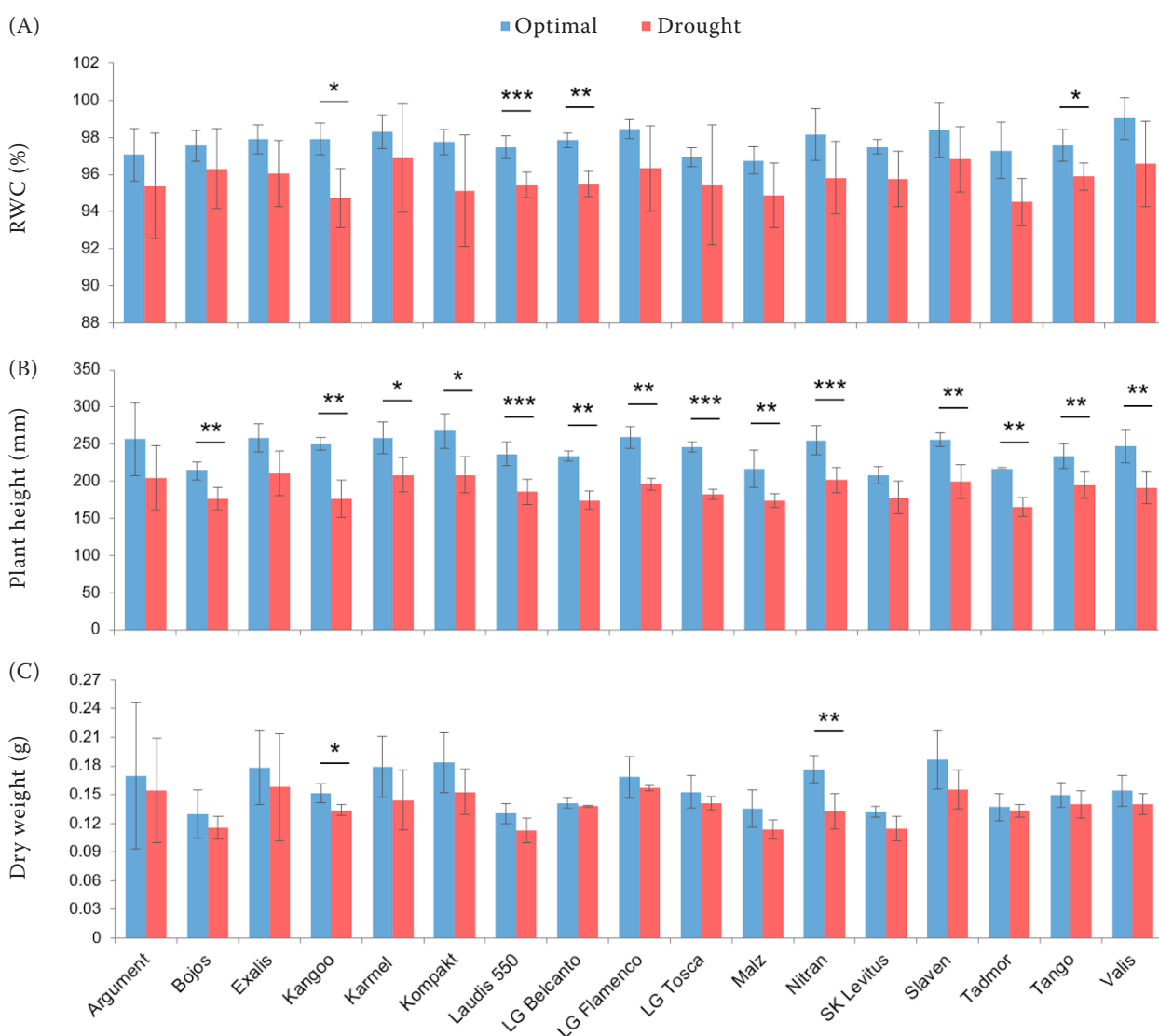


Figure 1. Effects of drought stress on physiological traits in 17 barley genotypes. (A) Relative water content (RWC); (B) plant height, and (C) aboveground biomass expressed as dry weight (DW) under optimal (blue) and drought (red) conditions. Data are presented as means \pm standard deviation. Asterisks indicate statistically significant differences between treatments based on independent two-sample Student's *t*-tests or Mann-Whitney U tests, depending on data normality and variance homogeneity: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

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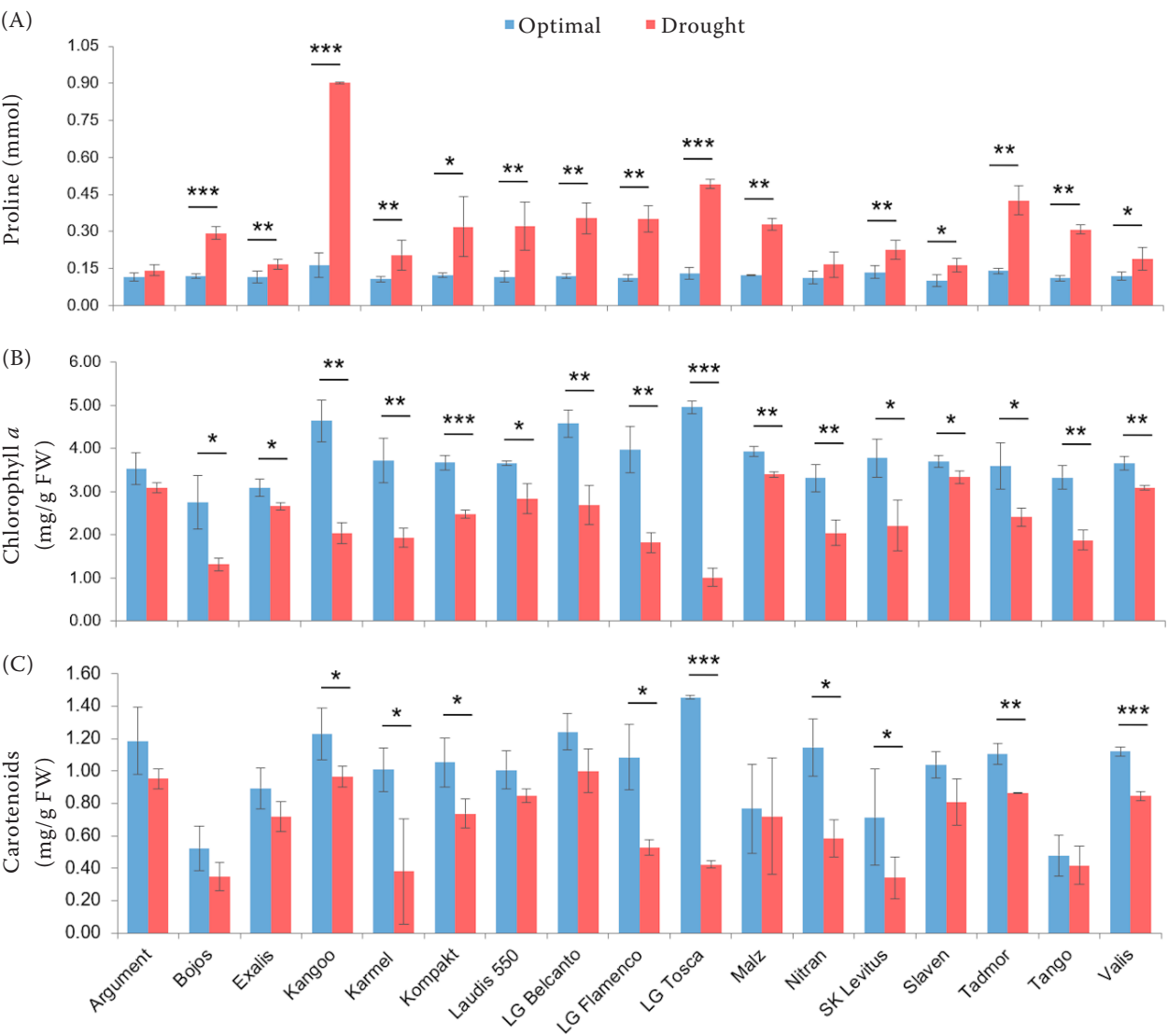
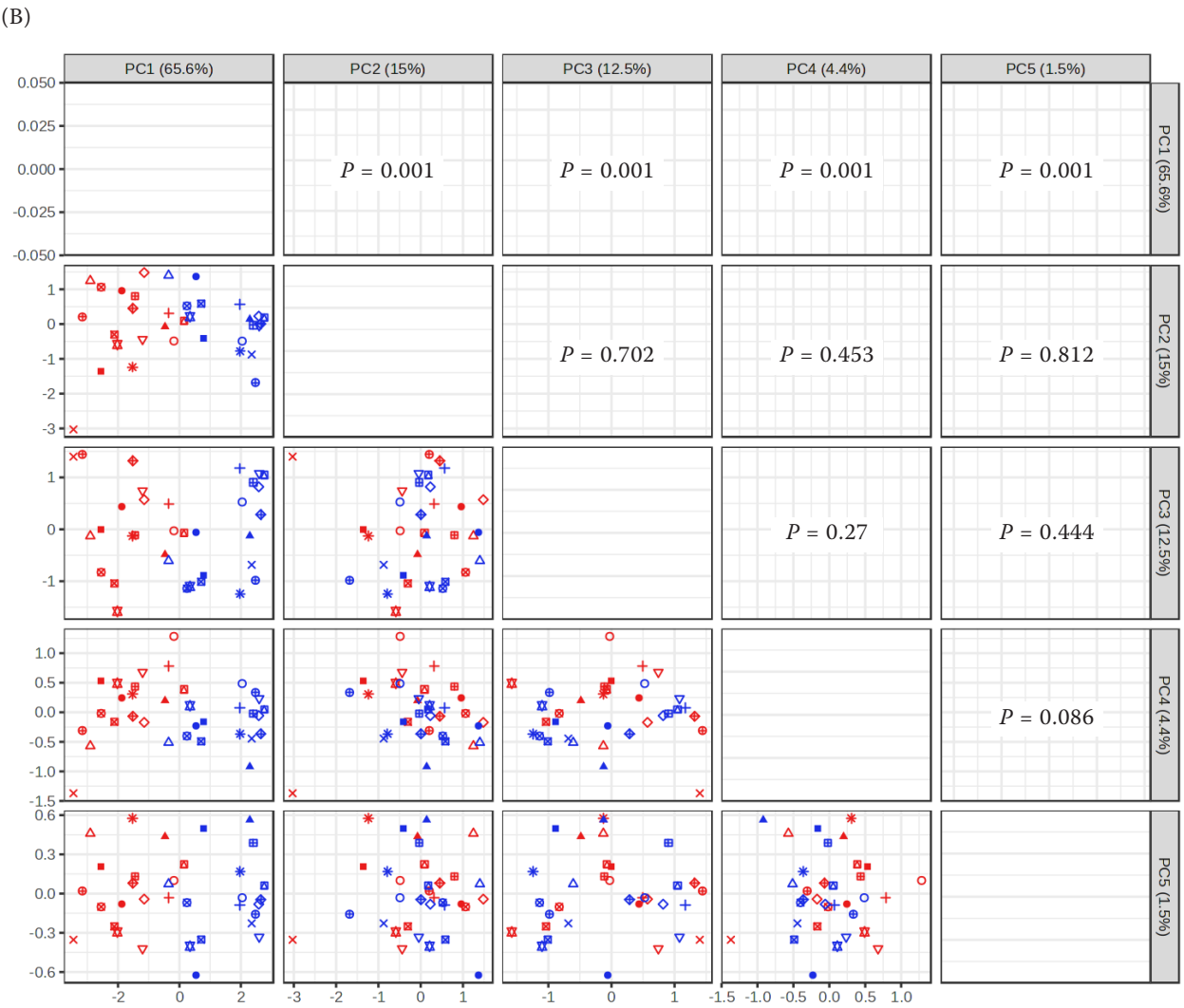
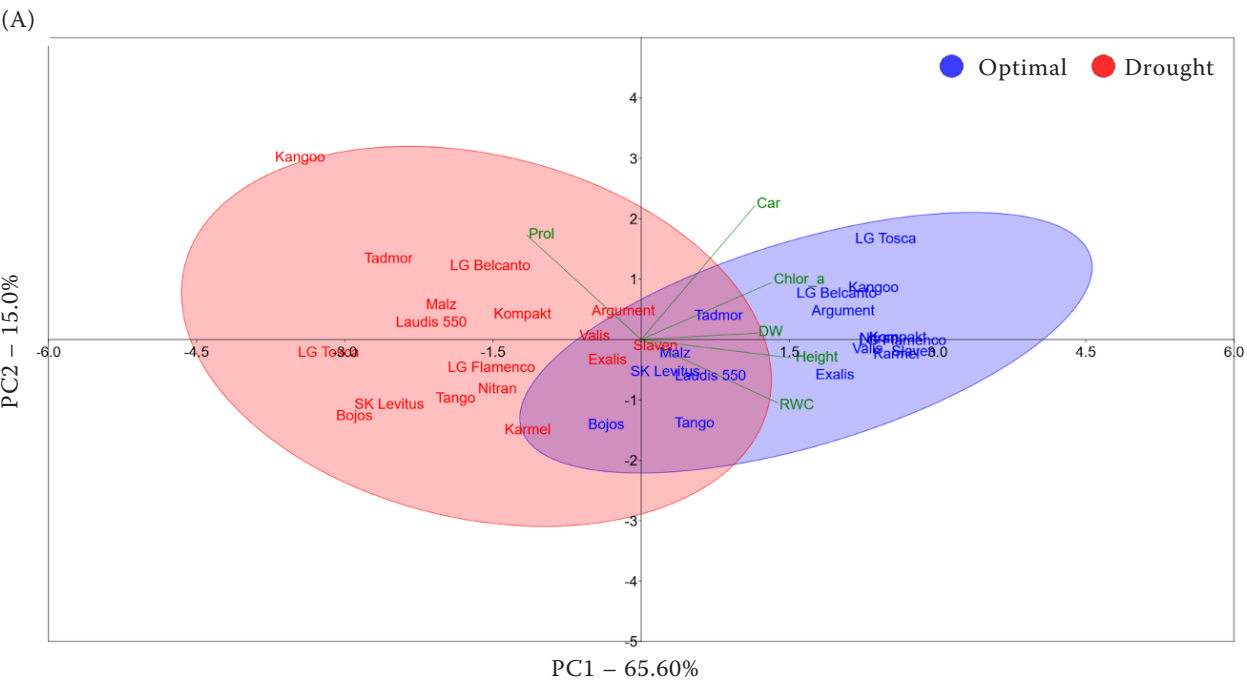


Figure 2. Biochemical responses of 17 barley genotypes to drought stress. (A) Leaf proline concentration; (B) chlorophyll *a* content, and (C) carotenoid content under optimal (blue) and drought (red) conditions. Data are presented as means ± standard deviation. Asterisks indicate statistically significant differences between treatments based on independent two-sample Student's *t*-tests or Mann-Whitney U tests, depending on data normality and variance homogeneity: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; FW – fresh weight

flecting the cost of osmotic adjustment. Chlorophyll *a* *P* = 0.004), but pigment stability was reduced in and carotenoids remained strongly correlated (*r* = 0.66, high-proline genotypes.

Table 2. Summary of two-way ANOVA *P*-values for the effects of genotype, treatment, and genotype × treatment on physiological and biochemical traits

Parameter	Genotype	Treatment	Genotype × treatment
Relative water content	0.2352	< 0.0001	0.9966
Height	< 0.0001	< 0.0001	0.8634
Dry weight	< 0.0001	< 0.0001	0.9877
Proline	< 0.0001	< 0.0001	< 0.0001
Chlorophyll <i>a</i>	< 0.0001	< 0.0001	< 0.0001
Carotenoids	< 0.0001	< 0.0001	0.0080



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(C)

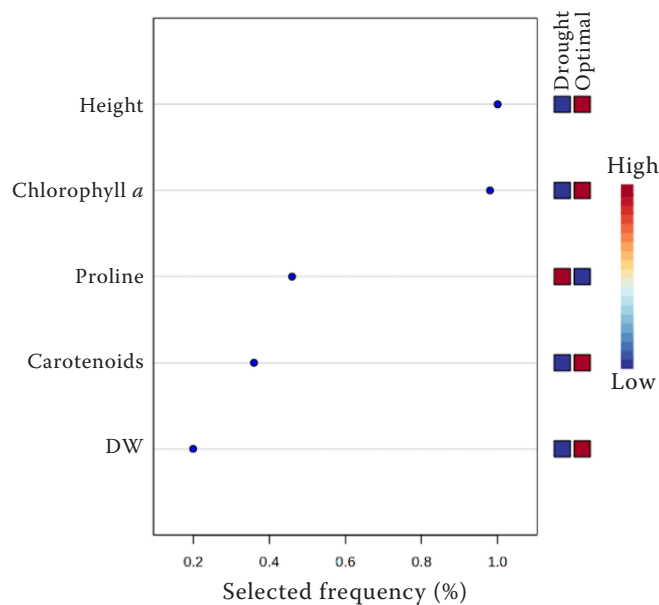
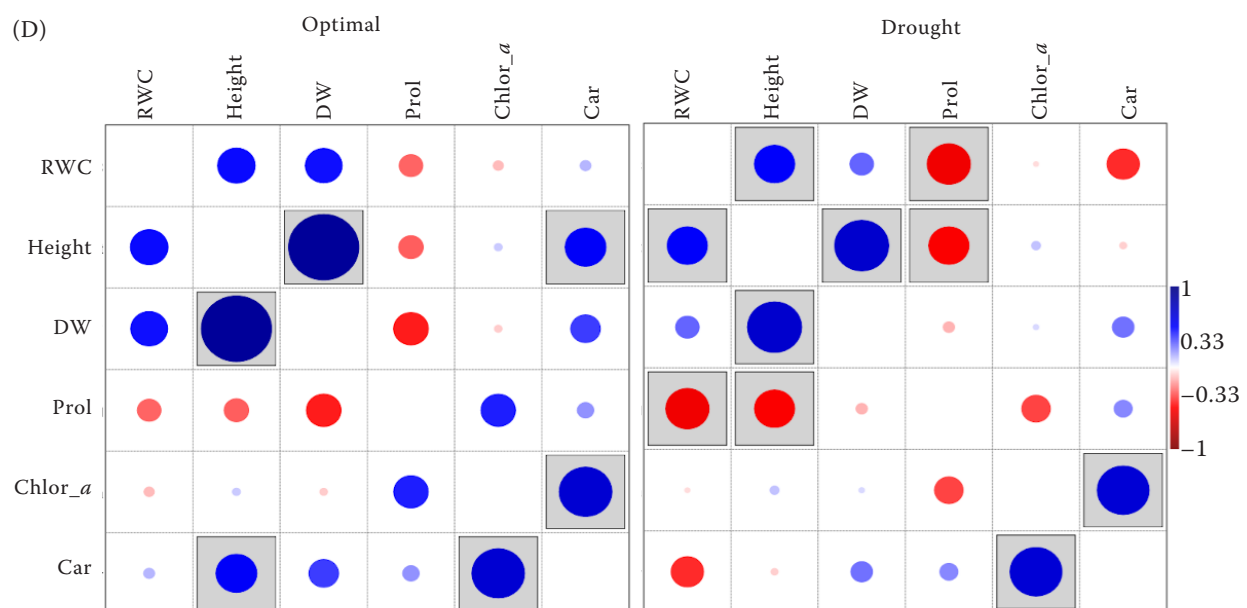


Figure 3. (A) Principal component analysis (PCA) biplot of 17 barley genotypes under optimal (blue) and drought (red) conditions with 95% confidence ellipses. Green vectors represent trait loadings; (B) PERMANOVA scatterplots of principal components with P -values for treatment separation; (C) PLS-DA ranking of discriminative traits based on selection frequency (%), and (D) Pearson correlation matrices of physiological and biochemical traits under optimal (left) and drought (right) conditions; circle size/colour indicates correlation strength and direction (blue – positive, red – negative; framed – significant, $P < 0.05$). RWC – relative water content; height – plant height; DW – aboveground dry weight; Prol – proline; Chlor_ a – chlorophyll a ; Car – carotenoids

(D)

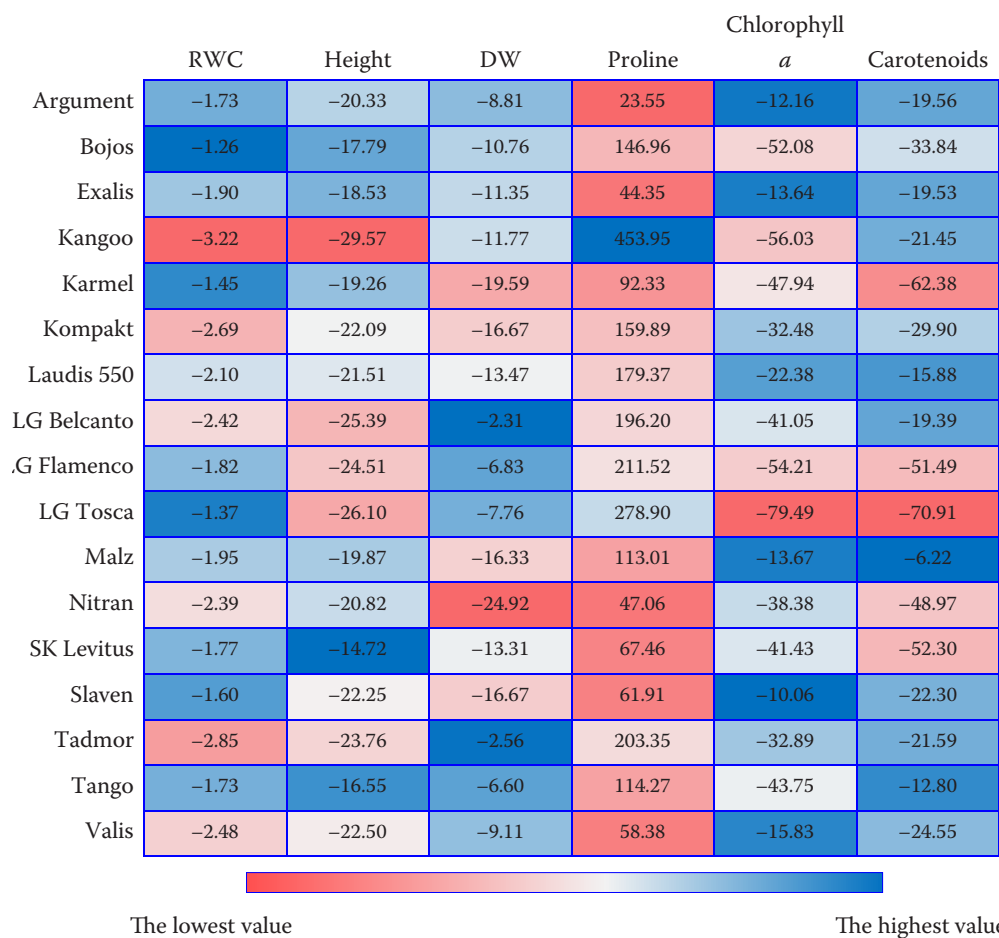


Drought-induced percentage differences varied strongly among genotypes (Figure 4A). Proline showed the most pronounced increases (+24% in Argument to +454% in Kangoo), while RWC declined only slightly (–1.3% to –3.2%). Growth traits were more affected, with plant height reduced by 14–30% and dry weight by 2–25%. Pigments consistently decreased (chlorophyll a –10% to –79%; carotenoids –6% to –71%), indicating impaired photoprotection in sensitive genotypes. PCA of percentage changes explained 73% of total variance (Figure 4B). PC1 separated genotypes by a trade-off between growth/pigment stability and proline accumulation, while PC2

captured additional variation in RWC and pigments. Kangoo and LG Tosca represented extreme drought responses (excessive proline or pigment loss), whereas Tango, Kompakt, Nitran, and Laudis 550 clustered near the centre, reflecting balanced reductions across traits. Genotypes such as Malz, Argument, Exalis, and Slaven aligned with chlorophyll a , suggesting a tolerance strategy based on pigment retention. Despite variability, most cultivars clustered within a common drought-response space, as indicated by the 95% confidence ellipse.

Gene expression responses to drought. For gene expression, we analysed 12 genotypes (a subset of

(A)



(B)

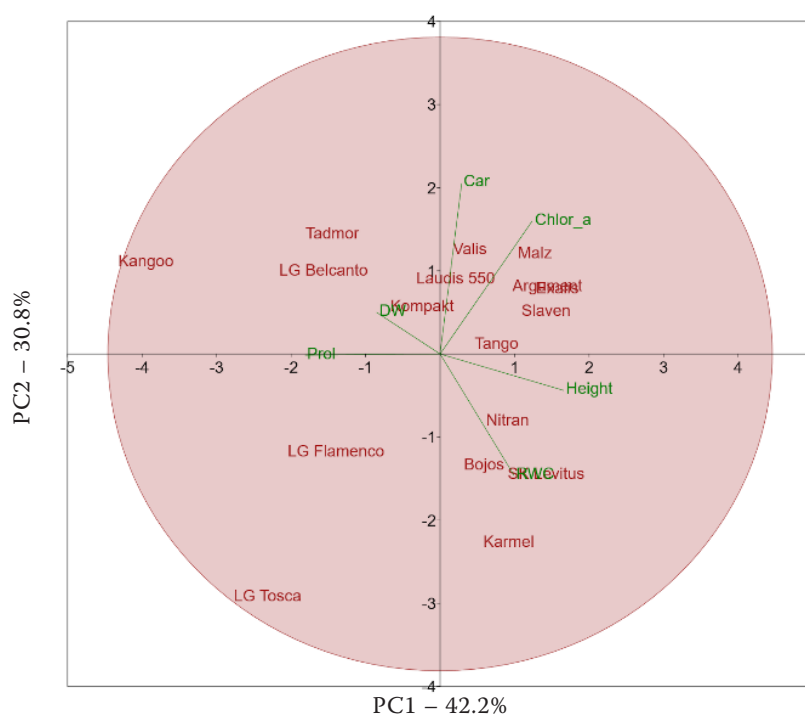


Figure 4. Percentage changes in physiological and biochemical traits under drought stress. (A) Heatmap of relative differences (%) between drought and optimal conditions; blue – higher, red – lower values (scaled per trait), and (B) principal component analysis (PCA) of percentage differences with 95% confidence ellipse; green vectors indicate trait loadings. RWC – relative water content; height – plant height; DW – aboveground dry weight; Prol – proline; Chlor_ *a* – chlorophyll *a*; Car – carotenoids

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those used for physiological and biochemical traits), representing the diversity of responses observed in the larger collection. All primer pairs used for RT-qPCR exhibited acceptable amplification performance, with efficiencies ranging from 93–109% and $R^2 \geq 0.98$. The reference gene *HvACT* showed high expression stability under experimental conditions, as confirmed by NormFinder (stability value = 0.12), supporting its suitability for data normalisation. Expression analysis (Figure 5) revealed distinct transcriptional regulation of four drought-responsive genes. *HvP5CS*,

a key enzyme in proline biosynthesis, showed the strongest and most consistent induction (2–8-fold), with the highest upregulation in Tango. *HvABF2*, an ABA-responsive factor, displayed variable patterns: it was significantly induced in some genotypes (Laudis 550, Tango), but remained unchanged or was even downregulated in others (e.g. Bojos, Exalis, LG Flamenco, Malz, Nitran). Antioxidant-related genes exhibited genotype-dependent regulation: *HvSOD1* was upregulated in Kangoo and Tango, but significantly downregulated in Bojos and Exalis, whereas

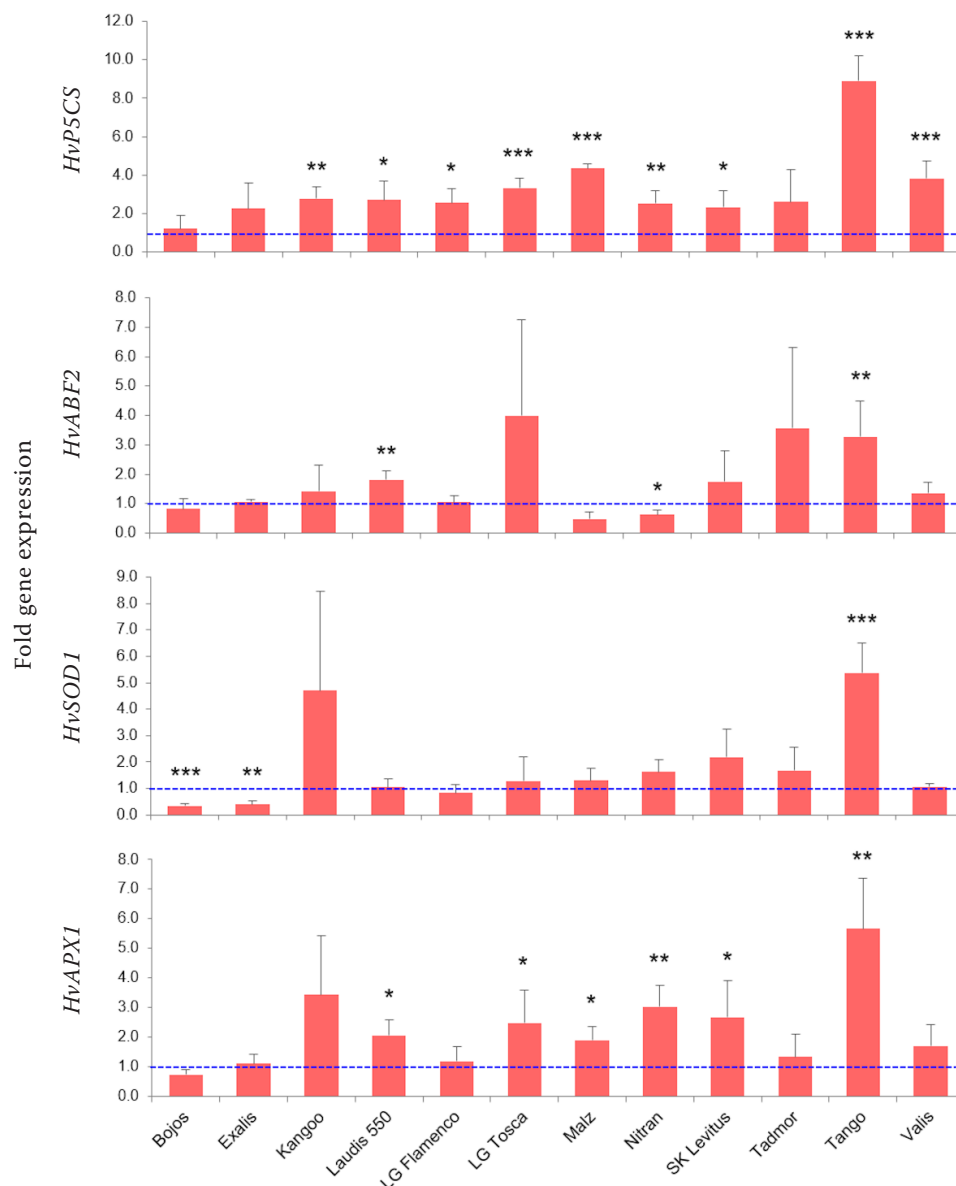
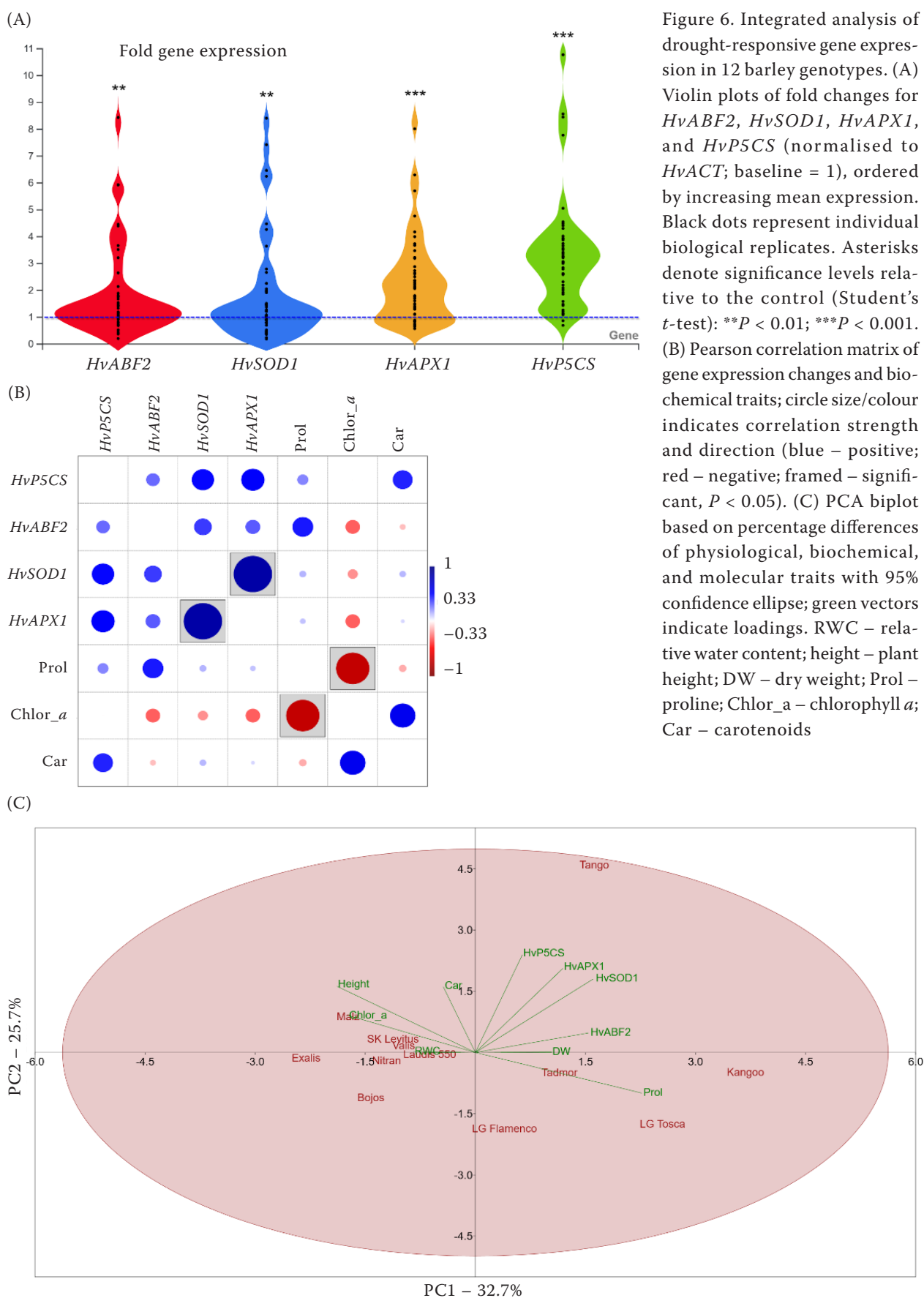


Figure 5. Relative expression of drought-responsive genes (*HvP5CS*, *HvABF2*, *HvSOD1*, *HvAPX1*) in 12 barley genotypes. Expression was normalised to *HvACT* and calculated by the $2^{-\Delta\Delta C_t}$ method. Bars represent means \pm standard deviation. The dashed blue line at 1.0 indicates the expression level of the respective genes under optimal conditions (set as baseline fold-change = 1). Asterisks indicate statistically significant differences from this control (Student's *t*-test): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

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HvAPX1 was significantly induced across several accessions (Laudis 550, LG Tosca, Malz, Nitran, SK Levitus, and Tango). Overall, these results highlight differential reliance on osmotic adjustment and antioxidant defences in barley drought responses.

Combined analysis across genotypes confirmed the significant upregulation of all four genes (Figure 6A). *HvP5CS* showed the strongest induction, in some cases exceeding 10-fold, followed by *HvAPX1* and *HvSOD1*, whereas *HvABF2* was moderately but variably induced. Correlation analysis (Figure 6B) revealed a tight link between antioxidant genes (*HvSOD1* and *HvAPX1*, $r = 0.85$, $P < 0.001$), while *HvP5CS* showed moderate but non-significant associations with both antioxidant genes and a weak, non-significant association with proline levels. Notably, proline content correlated negatively with chlorophyll *a* ($r = -0.73$, $P < 0.01$), indicating that excessive osmotic adjustment was coupled with pigment loss. PCA (Figure 6C) explained 58.4% of the variance, separating genotypes along a gradient from strong osmotic and antioxidant activation or proline accumulation (Tango, Kangoo, LG Tosca) to pigment and growth stability (Malz). Genotypes such as Laudis 550, Valis, and SK Levitus occupied intermediate positions, reflecting balanced stress responses. Despite genotype-specific variability, most cultivars clustered within a shared drought-response space, underscoring common adaptive mechanisms.

Comprehensive classification of drought tolerance. The integrative evaluation of physiological, biochemical, and molecular traits enabled the comprehensive classification of barley genotypes according to their drought-response strategies (Table 3). Genotypes were classified into three categories based on combined criteria: (i) tolerant – characterised by minor reductions in relative water content ($\leq -2\%$), moderate to low losses in biomass and plant height (≤ -15 – 23%), limited pigment degradation (≤ -20 – 30%), and balanced or moderate proline accumulation ($< \sim 115\%$); (ii) sensitive – exhibiting severe pigment loss ($> -30\%$), pronounced biomass and height reductions ($> -20\%$), and/or extreme proline accumulation ($> \sim 200$ – 300%), often accompanied by poor growth maintenance; (iii) intermediate – showing mixed responses, i.e., maintaining stability in one or two traits (e.g., biomass or pigments) but with a significant decline in others. According to these criteria, tolerant genotypes combined stable biomass production with pigment retention or moderate osmotic adjustment. In contrast, sensitive genotypes displayed severe pig-

ment loss, excessive or inefficient osmotic responses, and/or pronounced biomass penalties. Across the evaluated set, maintenance of growth-related traits, particularly plant height and biomass, consistently separated more resilient genotypes from sensitive ones. Conversely, genotypes showing the highest proline accumulation frequently exhibited stronger growth and pigment penalties. These data indicate that although proline accumulation was a prominent drought response across genotypes, elevated proline levels were not consistently associated with improved drought tolerance in the evaluated set. Genotypes showing intermediate responses maintained partial stability in some traits, such as growth or pigment content, while simultaneously displaying pronounced declines in others. Taken together, the applied classification reflects the heterogeneity of drought responses across physiological, biochemical, and molecular traits observed among the analysed barley genotypes.

Additional hierarchical clustering supported, but did not fully reproduce, the genotype pre-classification into tolerant, intermediate, and sensitive groups. The heatmap (Figure 7A) from physiological and biochemical parameters highlighted clear contrasts: tolerant genotypes showed smaller reductions in pigments and RWC, along with moderate height loss, while sensitive genotypes showed severe pigment degradation, growth decline, or excessive proline accumulation. Intermediate lines displayed mixed profiles, maintaining stability in some traits (e.g., DW, height) but marked reductions in others. The dendrogram (Figure 7B) grouped tolerant accessions (Exalis, Argument, Valis), while sensitive types (Kangoo, LG Tosca, LG Flamenco, Tadmor) formed separate branches, sometimes joined by intermediates such as LG Belcanto. Other intermediates clustered variably, with some closer to tolerant (Malz, Slaven) and others nearer to sensitive (Nitran, Karmel, Bojos). Notably, Laudis 550 clustered with tolerant genotypes, and Nitran and Kompakt also joined this group at higher hierarchical levels. These discrepancies likely reflect that clustering in Figure 7 was based solely on physiological and biochemical traits, allowing for the inclusion of all 17 genotypes. In contrast, our final stratification (Table 3) integrated molecular data as well. This revealed disproportionate gene induction in Laudis 550 and Nitran, and substantial penalties in physiological traits in Kompakt, underscoring that integrated evaluation provides a more complete assessment of drought tolerance.

Table 3. Classification of 17 spring barley genotypes into tolerant, intermediate, and sensitive groups based on physiological, biochemical, and molecular responses to drought stress

Group	Genotype	Key responses under drought (relative to optimal)
Tolerant	Argument	Height –20.3%, DW –8.8%, Chl <i>a</i> –12.2%, Car –19.6%, proline +23.6%; stable growth (lower DW decline), very good chlorophyll retention; low proline increase → "more energy efficient" response.
	Exalis	Height –18.5%, DW –11.4%, Chl <i>a</i> –13.6%, Car –19.5%, proline +44.3%; good pigment stability, small-moderate growth decline, slight increase in proline; in PCA close to "optimal" directions; genes: APX1 slightly ↑, SOD1 ↓ – molecularly mixed, phenotype strong.
	Slaven	Height –22.3%, DW –16.7%, Chl <i>a</i> –10.1% (best), Car –22.3%, proline +61.9%; excellent pigment stability despite moderate growth loss; medium proline. Suitable "pigment stabiliser".
	Malz	Height –19.9%, DW –16.3%, Chl <i>a</i> –13.7%, Car –6.2% (best), proline +113%; very good photoprotection (best carotenoid protection, stable chlorophyll); medium growth; genes: APX1 ↑, SOD1 slightly ↑.
	Valis	Height –22.5%, DW –9.1%, Chl <i>a</i> –15.8%, Car –24.6%, proline +58.4%; stable biomass and pigments, moderate osmotic (proline) response; genes: APX1 ↑, ABF2 slightly ↑; supports a "balanced" strategy.
Intermediate	Laudis 550	Height –21.5%, DW –13.5%, Chl <i>a</i> –22.4%, Car –15.9%, proline +179%; moderate stress, relatively good pigment retention, DW moderate decrease; PCA does not place it among the extremes; genes: ABF2/APX ↑.
	Tango	Height –16.6%, DW –6.6% (very good), Chl <i>a</i> –43.8% (worse), Car –12.8% (good), proline +114%; growth maintained, carotenoids too; but chlorophyll decreases more → medium tolerant (growth robust type); genes: strong induction of several markers and still good growth.
	Kompakt	Height –22.1%, DW –16.7%, Chl <i>a</i> –32.5%, Car –29.9%, proline +160%; moderate across all traits.
	LG Belcanto	Height –25.4% (worse), DW –2.3% (best), Chl <i>a</i> –41.1%, Car –19.4%, proline +196%; biomass stable, pigments degraded.
	SK Levitus	Height –14.7% (best), DW –13.3%, Chl <i>a</i> –41.4%, Car –52.3%, proline +67%; growth stable, pigments weak; genes: SOD/APX ↑ intermediate.
Sensitive	Kangoo	Height –29.6%, DW –11.8%, Chl <i>a</i> –56%, Car –21.5%, proline +454% (extreme); "hyper-osmoprotective" profile with significant chlorophyll loss; extreme point in PCA; genes: SOD/APX very strongly ↑ – reactive/"crisis" response.
	LG Tosca	Height –26.1%, DW –7.8%, Chl <i>a</i> –79.5% (worst), Car –70.9% (worst), proline +278.9%; extreme pigment degradation, high proline; phenotypically clearly sensitive; genes: multiple markers ↑.
	LG Flamenco	Height –24.5%, DW –6.8%, Chl <i>a</i> –54.2%, Car –51.5%, proline +212%; large pigment losses, high proline; DW decreases less, but photoprotection fails.
	Karmel	Height –19.3%, DW –19.6%, Chl <i>a</i> –47.9%, Car –62.4%, proline +92%; strong pigment degradation and biomass.
	Bojos	Height –17.8%, DW –10.8%, Chl <i>a</i> –52.1%, Car –33.8%, proline +147%; severe pigment loss; genes: ABF2 ↓, SOD ↓, APX ↓ – antioxidant system rather suppressed → underlines sensitivity.
	Nitran	Height –20.8%, DW –24.9% (worst), Chl <i>a</i> –38.4%, Car –49%, proline +47%; largest biomass loss, pigment instability, despite moderate proline, the phenotype declines; genes: APX significantly ↑, SOD ↑.
	Tadmor	Height –23.8%, DW –2.6% (low), Chl <i>a</i> –32.9%, Car –21.6%, proline +203%; despite small DW loss, combination of large height loss, moderate pigment loss, and high proline shows "costly" adaptation; rather sensitive pole in multivariate spaces; genes: multiple ↑, but phenotype not robust.

Values indicate percentage differences under drought relative to optimal conditions. Height – plant height; DW – aboveground dry weight; Chl *a* – chlorophyll *a*; Car – carotenoids; APX1 – ascorbate peroxidase 1; SOD1 – superoxide dismutase 1; ABF2 – abscisic acid responsive binding factor 2

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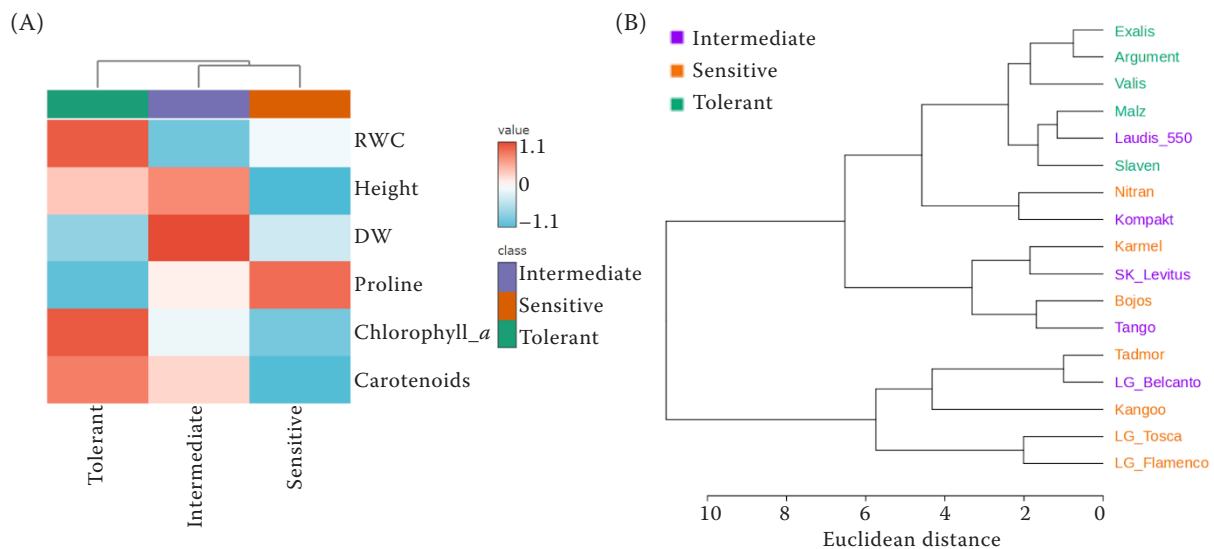


Figure 7. Heatmap and hierarchical clustering of barley genotypes under drought and optimal conditions. Genotypes were pre-classified into tolerant, intermediate, and sensitive groups based on integrated physiological, biochemical, and transcriptional traits (Table 3). (A) Heatmap of relative drought-induced changes in relative water content (RWC), height, dry weight (DW), proline, chlorophyll *a*, and carotenoids, with values shown as z-score standardised (−1.1 to +1.1). (B) Dendrogram (Euclidean distance, Ward's linkage) illustrating multivariate clustering and the continuum from tolerant to sensitive responses

DISCUSSION

Our stratification of genotypes into tolerant, intermediate, and sensitive groups is consistent with recent multi-environment studies. Töpfer et al. (2025) similarly identified three response clusters and reported a negative correlation between excessive proline accumulation and grain biomass ($r = -0.66$), confirming that high osmotic adjustment does not ensure yield stability. This aligns with our observation that sensitive genotypes (Kangoo, LG Tosca) accumulated large amounts of proline but suffered severe penalties in terms of pigment and growth, whereas tolerant types (Argument, Valis) combined moderate proline levels with pigment retention and stable biomass. Additionally, Töpfer et al. (2025) reported that growth-related traits such as plant height are highly heritable and relatively stable across environments, supporting their suitability as indicators of drought tolerance. In the same study, sensitive genotypes were characterised by elevated proline content, reinforcing our observation that excessive proline accumulation was frequently associated with drought sensitivity rather than tolerance. Bandurska (2022) emphasised the distinction between biological tolerance (the ability of plants to survive under dehydration through avoidance or tolerance strategies) and agricultural tolerance (the capacity

to sustain growth and yield under stress), which is particularly relevant as our tolerant genotypes combined efficiency with stability in some traits, while sensitive ones displayed costly responses unlikely to support productivity. The view that moderate proline induction is beneficial is supported by Szabados and Saviouré (2009), who linked proline metabolism to redox balance, and by Bandurska et al. (2017), who showed its regulation via P5CS and ABA. In contrast, Ferioun et al. (2023) classified the Moroccan cultivars with the highest proline accumulation as tolerant, interpreting this as the activation of detoxification pathways. Together, these findings suggest that proline contributes to drought responses but is not a stand-alone determinant of tolerance; rather, its role depends on integration with hormonal and redox regulation.

Cai et al. (2020) screened over 400 barley genotypes and showed that drought-tolerant lines maintained higher shoot biomass and RWC, with osmotic adjustment emerging as the most discriminative trait. However, they also noted that sensitive genotypes exhibited disproportionately strong osmotic responses, consistent with our finding that excessive proline accumulation does not guarantee tolerance to osmotic stress. In our dataset, RWC did not emerge as a key predictor in PLS-DA, likely due to methodological differences in trait ranking methods, i.e. selection

frequency in our study *vs.* VIP scores in Cai et al. (2020), which may explain this deviation despite its recognised biological relevance. Moreover, *HvP5CS* expression in our dataset correlated only weakly with proline content, indicating that transcriptional activation of the glutamate pathway does not necessarily result in proportional accumulation of metabolites. Deng et al. (2013) similarly reported that proline was not consistently associated with drought tolerance in Tibetan hulless barley, suggesting that alternative compatible solutes (e.g., glycine betaine or other compatible solutes) may compensate for stress responses. These findings reinforce that neither proline levels nor *HvP5CS* expression alone provides a reliable marker of drought tolerance, which instead depends on integrated osmolyte and stress-response networks. This interpretation is consistent with the multifunctional role of proline, as described by Szabados and Savouré (2009), who highlighted that high proline levels can also be observed in stress-sensitive mutants. The biological role of proline extends beyond osmotic adjustment to include redox regulation, signalling, and control of programmed cell death. Accordingly, genotypes showing intermediate responses in our study maintained partial stability in selected traits, such as growth or pigments, while remaining vulnerable in others. This reinforces the view that drought tolerance represents a complex, multilevel trait that requires an integrated assessment across physiological, biochemical, and molecular dimensions, rather than relying on a single marker (Sallam et al. 2019).

Among the four stress-related genes in our dataset, *HvABF2* exhibited the lowest average increase in expression in drought-stressed plants, with a 1.77-fold expression under drought relative to the control (1.0). This gene encodes an ABA-responsive transcription factor and is part of the ABA signalling pathway, which is essential for plant responses to drought stress (Al-Sayaydeh et al. 2024). ABA functions as a phytohormone mediating plant responses to drought stress by regulating stomatal conductance, gene expression, and photosynthetic efficiency (Collin et al. 2025). Despite its central regulatory role, the relatively weak induction of *HvABF2* in our dataset suggests that this transcriptional pathway may not have been the predominant driver of drought tolerance differences among the studied genotypes. Instead, tolerance appeared to rely more strongly on traits such as pigment stability and balanced osmotic adjustment, supported by the

higher expression levels of antioxidant-related genes (*HvAPX1*, *HvSOD1*). This interpretation aligns with previous findings that ABA-responsive factors can act in concert with, rather than independently of, downstream protective mechanisms (e.g., antioxidant enzymes, osmolyte accumulation), and that modest ABA induction may be sufficient to activate baseline stress responses (Yoshida et al. 2009). In contrast, genes encoding antioxidant enzymes showed moderately higher induction under drought stress. On average, *HvSOD1* reached a 1.82-fold increase and *HvAPX1* a 2.28-fold increase compared to control plants. The *HvSOD1* gene encodes the superoxide dismutase (SOD) enzyme, which is vital in plant stress responses by catalysing the dismutation of superoxide radicals into oxygen and hydrogen peroxide, thereby protecting against oxidative damage (Abu-Romman and Shatnawi 2011). Meanwhile, *HvAPX1* encodes a peroxisomal ascorbate peroxidase, an enzyme that helps detoxify reactive oxygen species and enhances tolerance to environmental stresses, such as heat and salinity (Shi et al. 2001). Finally, the strongest induction was observed for *HvP5CS*, with a 3.29-fold increase relative to the control. This gene encodes Δ^1 -pyrroline-5-carboxylate synthetase, the key enzyme in proline biosynthesis, confirming its pivotal role in osmotic adjustment under drought stress (Szabados and Savouré 2009). However, our results indicated that although *HvP5CS* exhibited the highest induction among the analysed genes, its significant upregulation did not necessarily lead to improved drought tolerance.

Taken together, our findings emphasise that drought tolerance in barley at the early vegetative leaf development stage cannot be attributed to a single parameter such as proline accumulation, but rather to the coordinated regulation of multiple physiological, biochemical, and molecular processes. Genotypes classified as tolerant maintained pigment stability and moderate osmotic adjustment while activating antioxidant defences, thereby avoiding the energetic costs associated with excessive stress responses. In contrast, sensitive genotypes showed disproportionate osmotic adjustment and pigment degradation, indicating stress severity rather than resilience. The observed variability in transcriptional activation of drought-related genes, particularly within the antioxidant and ABA signalling pathways, highlights the importance of genotype-specific regulatory strategies. Although these responses were characterised under controlled laboratory conditions, they provide

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a mechanistic foundation for subsequent field-level validation. Overall, the integrative traits identified in this study may serve as useful early-stage markers for guiding future breeding and for selecting germplasm with potential drought resilience under agronomically relevant environments.

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