

Identification of marker genes for drought stress tolerance in wheat (*Triticum aestivum*) through carbon isotope composition

Sepideh Jafarian^{a,b,c}, Manuel Geyer^a, Franz Buegger^b, Barbro Winkler^b, Georg Gerl^b, Klaus F.X. Mayer^{b,c}, Nadia Kamal^{b,c}, Jörg-Peter Schnitzler^{b,*}, Lorenz Hartl^{a,**}, Manuel Spannagl^{b,d,***}

^a LfL, Bavarian State Research Center for Agriculture (LfL), Institute for Crop Science and Plant Breeding, Freising, 85354, Germany

^b Helmholtz Center Munich, German Research Center for Environmental Health, Plant Genome and Systems Biology (PGSB), Neuherberg, 85764, Germany

^c Technical University of Munich, School of Life Science, Freising, 85354, Germany

^d Center for Crop and Food Innovation, Food Future Institute, Murdoch University, Murdoch, WA, 6150, Australia

HIGHLIGHTS

- $\delta^{13}\text{C}$ enabled iWUE assessment across a Bavarian MAGIC wheat population.
- Six QTLs for $\delta^{13}\text{C}$ were identified on chromosomes 1D, 2B, 4B, 4D, 6A and 6B.
- Contrasting wheat lines revealed distinct drought-responsive phenotypes.
- Transcriptomics uncovered ABA, stomatal and energy metabolism pathways.
- Eighteen candidate genes provide targets for improving wheat iWUE.

ARTICLE INFO

Keywords:

Drought stress
Carbon isotope composition ($\delta^{13}\text{C}$)
Intrinsic water-use efficiency
Quantitative trait loci
Marker genes

ABSTRACT

Improving drought adaptation in wheat requires robust physiological traits that can be genetically dissected and translated into breeding tools. Carbon isotope composition ($\delta^{13}\text{C}$) is a stable proxy for intrinsic water-use efficiency (iWUE) and enables the identification of genetic factors underlying drought-stress responses. In this study, $\delta^{13}\text{C}$ was measured in the Bavarian MAGIC Wheat (BMW) population, and quantitative trait locus (QTL) mapping at the drought-prone Morgenrot field site identified six significant loci on chromosomes 1D, 2B, 4B, 4D, 6A, and 6B. Based on phenotypic extremes at this site, eight genetically diverse lines with contrasting $\delta^{13}\text{C}$ /iWUE values (four high $\delta^{13}\text{C}$ and four low $\delta^{13}\text{C}$) were selected for detailed characterization. These extreme lines were evaluated under controlled greenhouse conditions using high-throughput phenotyping under both well-watered and drought-stress treatments.

Transcriptomic analyses revealed pronounced drought-induced expression changes, with enrichment of genes involved in abscisic acid signaling, stress perception, stomatal regulation, and energy metabolism. To identify robust molecular targets, QTL mapping results were integrated with differential gene expression (DGE) and weighted gene co-expression network analysis in a multi-layer framework. This approach prioritized 18 high-confidence candidate genes located mainly on chromosomes 1D, 4B, 4D, 6A, and 6B. These genes are embedded in regulatory networks dominated by transcription factors such as WRKY and NAC. Collectively, this study provides genetically anchored candidate genes for iWUE and drought adaptation, offering valuable resources for marker-assisted selection in wheat breeding.

* Corresponding author.

** Corresponding author.

*** Corresponding author. Helmholtz Center Munich, German Research Center for Environmental Health, Neuherberg, 85764, Germany.

E-mail addresses: joergpeter.schnitzler@helmholtz-munich.de (J.-P. Schnitzler), Lorenz.Hartl@lf.l.bayern.de (L. Hartl), manuel.spannagl@helmholtz-munich.de (M. Spannagl).

<https://doi.org/10.1016/j.agrcom.2026.100158>

Received 21 December 2025; Received in revised form 31 July 2026; Accepted 9 August 2026

Available online 11 August 2026

2949-7981/© 2026 The Authors. Published by Elsevier B.V. on behalf of Beijing Academy of Agriculture and Forestry Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Drought stress poses a major threat to global wheat production, affecting approximately 42% of cultivation areas and increasingly challenging food security under ongoing climate change [1]. To cope with water limitation, wheat plants rely on coordinated physiological responses, including stomatal regulation, osmotic adjustment, and altered canopy architecture, which collectively influence water loss and carbon assimilation [2,3]. Central to these responses is intrinsic water-use efficiency (iWUE), largely determined by stomatal conductance and its regulation under stress [4]. Genetic regulators such as a leucine-rich repeat receptor-like kinase (ERECTA) and GT-2 LIKE 1, a trihelix transcription factor (GTL1), modulate stomatal density and transpiration efficiency, thereby directly influencing iWUE and drought adaptation [5,6].

At the physiological level, carbon isotope discrimination ($\Delta^{13}\text{C}$) provides a direct measure of long-term photosynthetic gas exchange and reflects the ratio of intercellular to atmospheric CO_2 (c_i/c_a), which is closely linked to stomatal behavior and iWUE. However, direct measurement of $\Delta^{13}\text{C}$ is impractical in large breeding populations. Therefore, carbon isotope composition ($\delta^{13}\text{C}$), which is inversely related to $\Delta^{13}\text{C}$, is widely used as a measurable proxy [7–10]. Because $\delta^{13}\text{C}$ integrates stomatal conductance and carbon assimilation over time, it shows a strong positive association with iWUE and has been reported to exhibit moderate to high heritability in wheat, making it a robust and genetically tractable trait for improving drought tolerance [8,11,12].

Consequently, $\delta^{13}\text{C}$ represents a stable and genetically tractable trait for improving drought resilience in wheat breeding programs. In this context, genes involved in abscisic acid (ABA) biosynthesis and signaling

further contribute to drought adaptation by regulating stomatal behavior and water conservation, reinforcing the central role of stomatal control in maintaining productivity under water-limited conditions [13].

Multi-parent Advanced Generation Inter-Cross (MAGIC) populations [14] have further enhanced Quantitative trait locus (QTL) resolution and allelic diversity, enabling the discovery of novel loci associated with $\delta^{13}\text{C}$ and consequently with iWUE [15]. Building on this progress, the present study aims to (I) identify key genes and molecular markers associated with iWUE through $\delta^{13}\text{C}$ in wheat; (II) identify drought-adapted wheat lines for breeding applications; and (III) explore genetic diversity related to drought resistance. We integrated phenotyping and transcriptomics data through QTL mapping, differential gene expression (DGE), and co-expression network analysis to identify candidate genes underlying iWUE under drought stress, supporting the development of climate-resilient wheat cultivars (Fig. 1).

2. Materials and methods

2.1. Plant material and population description

The MAGIC wheat population BMWpop used in this study was developed by the Bavarian State Research Center for Agriculture (LfL) [14]. The population was created from eight founder lines (Supplementary Fig. 1). Founders were selected to maximize diversity based on: (i) variation in disease resistance, quality, and agronomic traits; (ii) origin from diverse breeding programs; and (iii) importance within their quality class (Supplementary Table 5). All founders originated from German breeding programs except ‘Ambition’ (Nordic Seed, Denmark).

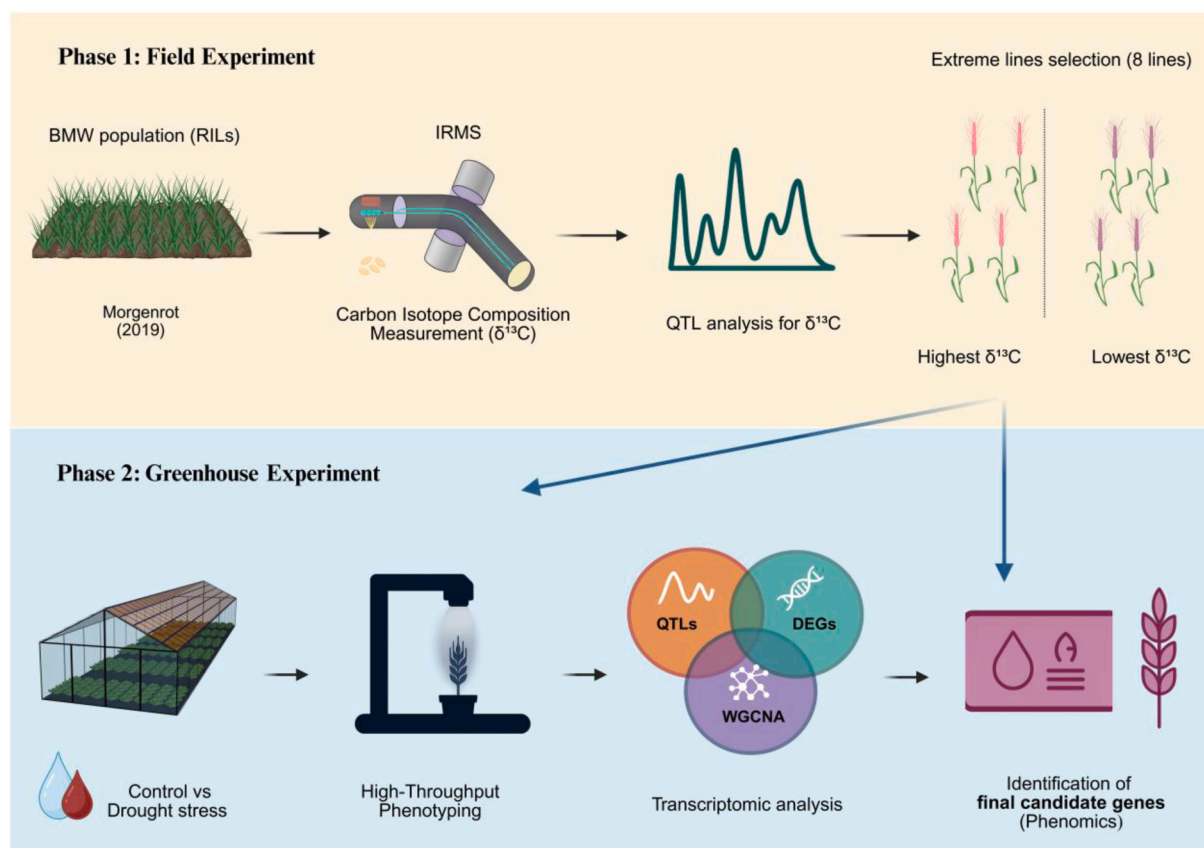


Fig. 1. Schematic overview of the experimental design and analytical workflow. $\delta^{13}\text{C}$ was quantified in the BMW population from the field trial to map $\delta^{13}\text{C}$ -associated QTLs. Ten extreme lines (five high-iWUE/low $\delta^{13}\text{C}$ and five low-iWUE/high $\delta^{13}\text{C}$), selected from the Morgenrot trial station in 2019, were used for a controlled greenhouse drought experiment with high-throughput phenotyping and F-2 RNA-seq. Differential expression, Weighted Gene Co-expression Network Analysis (WGCNA), and QTLs were integrated to prioritize high-confidence candidate genes for iWUE and drought response.

The crossing scheme followed standard MAGIC procedures. Briefly, four two-way crosses were produced using one ear per plant to generate F_1 seed. From each two-way cross, one F_1 was advanced; two ears per plant were then intercrossed to generate four-way crosses. Of the 32 four-way crosses obtained, 16 independent plants were selected and intercrossed to produce eight F_1 populations encompassing four reciprocal combinations. Subsequently, four eight-way crosses were hybridized with their four reciprocal eight-way counterparts to establish 16 eight-way intercross combinations.

F_1 seed of the BMWpop was advanced to F_6 by single-seed descent, followed by two generations of bulk propagation in the field. The final population comprised 394 RILs (recombinant inbred lines) [14] (Supplementary Fig. 1). These lines are the basis of the QTL analysis.

2.2. Field trials and environmental conditions

A field trial was conducted at the Morgenrot site in 2019, located in the German federal states of Saxony-Anhalt and Lower Saxony. This site experienced water limitation and was chosen to represent drought-prone condition in this region. Grain samples of the RILs were retrieved from the Morgenrot field trial conducted by the breeding companies SaatZucht Josef Breun GmbH & Co. KG, Syngenta Seeds GmbH, and Strube D&S GmbH, respectively. Plot sizes were 13.5 m², 9 m² and 6 m². The field trial was laid out as an alpha lattice, consisting of two complete blocks and 86 incomplete blocks. BMWpop lines were grown in two replications, whereas the parental line Julius and the standard RGT Reform were grown in 14 and 18 replications. All other parents were replicated four times. The amount of nitrogen fertilizer is legally restricted to a total of 250 kg N ha⁻¹ of fertilizer and mineralized soil N in the spring, together at a yield level of 100 dt/ha.

At maturity, grain was harvested and used for $\delta^{13}\text{C}$ analysis. This population-wide screen of a multi-parent RIL panel provided a genetically diverse platform to test responses relevant to iWUE under drought.

2.3. DNA extraction and genotyping

For each of the 394 RILs and the eight founders, ten primary leaves were harvested, pooled, and freeze-dried. Genomic DNA was extracted following Plaschke et al. [16]. All lines (parents duplicated) were genotyped by TraitGenetics (Gatersleben, Germany) using the 15K + 5K Infinium iSelect array comprising 17,267 SNPs, which integrates markers from the 90K iSelect array [17] and the 820K Axiom array [18].

2.4. Marker filtering and QTL mapping procedures

Genotypic data were filtered in R using synbreed v0.12-6 [19]. Markers were removed if missing data $\geq 10\%$ or minor allele frequency (MAF) $\leq 1\%$. After QC, 10,842 SNPs remained from the initial 20k. To construct a non-redundant set for mapping, one SNP per locus on the linkage map was retained, yielding 2804 SNPs for composite interval mapping [14].

After the DNA extraction, genotyping, and marker filtration steps, QTL detection was conducted using composite interval mapping as implemented in the R package mpMap [20]. Probabilities of identity by descent with each of the eight founders were estimated at 1-centimorgan intervals and at all marker loci. Genome-wide significance thresholds for QTL detection were established using 10,000 permutations of phenotypic data. The number of cofactors included in the model was set equal to the number of QTL identified in an initial scan using simple interval mapping. QTL support intervals were defined as the genomic regions in which the $-\log_{10}(p)$ value was within one unit of its maximum. The linkage map used for QTL mapping was described in detail by Stadlmeier et al. [14]. Founder allele effects were estimated using a single-QTL regression model in which discrete founder alleles were included as independent variables. When none of the eight founder allele probabilities at a given locus exceeded 0.5, the allelic descent was classified as

unknown. The effect of each founder allele was calculated as the mean phenotype of all lines identical by descent for that allele, centered relative to the overall mean.

2.5. Design and implementation of the greenhouse experiment (plant material, growth condition, stress induction, and sampling)

Eight selected wheat lines, derived from BMWpop RILs from the MAGIC population [14] (Supplementary Fig. 1), were cultivated in the greenhouse of the Environmental Simulation research unit (EUS) at Helmholtz Munich, in a facility for high-throughput shoot phenotyping (FitnessSCREEN). These varieties were grown under controlled conditions and subjected to a defined drought stress for the purpose of the study. The selected winter wheat varieties were first germinated in 200 mL pots. After germination, the young plants underwent a vernalization process, where they were placed in a phytotron chamber (ExposCREEN) at the Helmholtz Zentrum München, German Research Center for Environmental Health (HMGU) and exposed to cold temperatures (+4 °C) for a period of 8 weeks. This step was crucial for the development of winter wheat varieties. Plants were grown in 5.5-L pots filled with a standardized peat-based horticultural substrate consisting of raised bog peat (Hochmoortorf; degree of decomposition H2–H5) and raw clay, amended with carbonated lime and mineral fertilizer. The substrate had a pH of 5.8 (CaCl₂) and a salinity of 2.0 g L⁻¹ (KCl), with an organic matter content of approximately 70%. Basal fertilization was provided via an NPK fertilizer (15–7–22 + 6 MgO), supplying nitrogen as nitrate (10%) and ammonium (5%), phosphorus as water-soluble P₂O₅ (7%), potassium as K₂O (22%), and magnesium (6% MgO), supplemented with sulfur and micronutrients (B, Cu, Fe, Mn, Mo, Zn). The substrate was mixed with quartz sand (grain size 0.6–1.2 mm) in a 1:1.2 (w/w) ratio prior to pot filling.

The part for the shoot phenotyping system had a total of 195 storage spaces for pots (39 carriers, each with 5 pots of 5.5 L and 1 plant per pot, which were automatically moved between the measuring cabin, the irrigation station, and the storage sections via transport and storage routes (Supplementary Fig. 6). A scale in the measuring cabin enabled the determination of pot weight, which served as a control variable for the irrigation system. For each line, 19–20 pots (replications) were grown up to BBCH stage 31 under well-watered conditions. All the plants were randomly distributed among the carriers and pots (Completely Randomized Design (CRD)). Leaf samples of all lines were taken from the leaves (F-2) at the anthesis stage (BBCH 65–69, depending on the genotypes, when 50% of the anthers were out). They were used for transcriptome analysis using NGS (see next chapter) and were stored at –80 °C.

2.6. Drought stress treatment

At the beginning of the experiment, all plants of each cultivar were grown under the same water regime. At the time when all the plants had reached BBCH stage 31, half of the plants were kept under well-watered conditions, while the other half were subjected to drought stress. Drought was induced by withholding irrigation until the end of the experiment. Thereafter, plants received the amount of (deionized) water needed to reach the desired target weight every 3 to 4 days, which corresponded to 70% of pot capacity in the control treatment and 40% of pot capacity in the drought treatment. Pot weights were monitored automatically on a daily basis using the scale in the measuring cabin, enabling precise and continuous control of soil moisture levels throughout the experiment.

2.7. Phenotyping of morphological traits

Agronomic traits were assessed every other day on the High-Throughput Phenotyping (HTP) platform. Carriers were automatically transported to the measurement station. Plants were individually

scanned by a multispectral 3D dual scanner (PlantEye F500, Phenospex, Heerlen, Netherlands) mounted 1.35 m above the plot edge and moved over the plants. The PlantEye produced a 3D point cloud from which 3D plant models were created using the x, y, and z coordinates (voxels) of each point. From the 3D models, all plant parameters, including plant height, leaf area, and digital biomass (height $\times$ leaf area), were calculated and stored in the HortControl software (Phenospex, Heerlen, Netherlands). To eliminate possible positional effects in the greenhouse, the carriers were repositioned in the greenhouse cabin after each scan. At the beginning of the drought treatment, the number of pots per tray was reduced to three to avoid overlapping leaves and to allow good separation of individual plants.

Plants were harvested at maturity (BBCH $\geq$ 92). Morphological parameters of the above-ground parts (maximum plant height, number of shoots, weight of ears, etc.) were determined manually. Spikes were discarded, and the total grain yield per plant was determined. Thousand-grain weight was determined on a set of 100 grains for each plant.

2.8. Analysis of ^{13}C stable isotope and elemental analysis of C and N

Grain from each line was milled to fine flour for homogenization. Approximately 1.3–1.7 mg of flour was weighed into zinc capsules for isotope analysis. Measurements of $^{13}\text{C}/^{12}\text{C}$ and elemental C and N were performed on an Isotope Ratio Mass Spectrometer (IRMS; Delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled to an Elemental Analyzer (Euro EA, EuroVector, Milano, Italy) following established procedures [21].

The $\delta^{13}\text{C}$ values of the samples were expressed relative to the international VPDB standard:

$$\delta^{13}\text{C} (\text{‰ VPDB}) = \left(\frac{R_{\text{sample}} - R_{\text{VPDB}}}{R_{\text{VPDB}}} \right) \times 1000,$$

where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the flour sample and $R_{\text{VPDB}} = 0.0111802$ [22].

2.9. Calculation of iWUE

Intrinsic water-use efficiency was derived from $\delta^{13}\text{C}$ measurements obtained from seed flour samples using the R package “isocalcR” [23]. Mean air temperature during the grain-filling period (1 July–7 August 2020; average 19.67 °C) was calculated from daily temperature records collected in the greenhouse. Corresponding climate data are provided in Supplementary Fig. 5.

2.10. Data analysis and statistics

All statistical analyses were conducted in R (v4.3.1; R Core Team, 2023; <https://www.R-project.org/>). A generalized linear model (GLM) was applied to evaluate the effects of treatment (control vs. drought), iWUE/ $\delta^{13}\text{C}$ groups (high vs. low), and their interaction on phenotypic traits during key growth stages, BBCH 60–92 (Biologische Bundesanstalt, Bundessortenamt, and Chemische Industrie), in the greenhouse. Residual diagnostics confirmed model assumptions, and Tukey's HSD test ($p < 0.05$) was used for post hoc comparisons.

2.11. Transcriptomic analysis

2.11.1. RNA extraction, library construction, sequencing, and quality control

To investigate transcriptional differences associated with iWUE, RNA-seq analysis was conducted on eight wheat lines representing phenotypic extremes of $\delta^{13}\text{C}$ (four high-iWUE and four low-iWUE). For each genotype and treatment (control and drought), 4–5 biological replicates were included, resulting in a total of 65 leaf samples. This

extreme-phenotype design was employed to maximize biological contrast and enhance the detection power of differential gene expression.

All plants were grown under controlled greenhouse conditions, and leaf samples were collected at a synchronized developmental stage (BBCH 65) to minimize biological and developmental variability. Total RNA was extracted using an optimized protocol provided by the Helmholtz Center Munich (EUS department). RNA purity ($A_{260}/_{280}$ and $A_{260}/_{230}$ ratios) and concentration were assessed using a NanoPhotometer® N60/N50 (Implen GmbH, Munich, Germany). RNA integrity was confirmed by Novogene, with RIN values above 5 and predominantly ranging between 8 and 10, indicating high-quality RNA suitable for sequencing.

Library preparation was performed using poly(A) enrichment, and sequencing was conducted on the Illumina platform, generating approximately 20 million paired-end reads (150 bp) per sample. The inclusion of multiple biological replicates per genotype and treatment enabled robust estimation of within-group variance and increased confidence in downstream differential expression and co-expression analyses. Raw sequencing data were delivered via cloud storage in FASTA format, ensuring accessibility and secure data handling.

The Fastp version was utilized for a quality control process of read trimming and quality assessment, generating a summary report from MultiQC to ensure reliability and accuracy in downstream transcriptomic analyses. Fastp was applied to remove adapter sequences and to trim low-quality bases as well as to filter out short reads from raw reads before pre-processing for further downstream analysis. It also provided detailed quality metrics, including per-base quality scores, GC content distribution, sequence duplication rates, and the identification of overrepresented sequences. To aggregate the results and provide a holistic overview of the dataset quality, MultiQC was used to consolidate the Fastp output into a single report.

2.11.2. Sequence mapping and differentially expressed gene identification

The cleaned sequence reads were then mapped against the bread wheat reference genome, International Wheat Genome Sequence Consortium (IWGSC) RefSeq v1.1, using kallisto version 3.27.0 [24]. A kallisto index was generated from the IWGSC RefSeq v1.1 transcriptome using the default k-mer length ($k = 31$). The derived expression values were normalized and output as transcript-level abundance estimates (TPM, estimated counts, and effective lengths). The transcript-level data were aggregated to gene-level counts using the tximport package in R (version 4.3.1), ensuring compatibility with downstream analyses. Gene-level counts were analyzed using the DESeq2 package [25] (version 1.42.1), which began with the creation of a DESeqDataSet and proceeded with normalization to correct for differences in library sizes. Size-factor normalization was applied to correct for sequencing depth, and variance-stabilizing transformation (VST) was used for visualization and clustering. DGE analysis was performed to identify genes with significant changes between drought and control as well as between high-iWUE and low-iWUE lines under drought stress conditions. Genes were filtered based on a threshold of $\log_2$ fold change ≥ 1 or ≤ -1 and a false discovery rate (FDR) ≤ 0.05 .

2.11.3. Functional annotations and pathway enrichment

Functional annotation for the bread wheat reference genome was performed using Mercator4 (v5.0) [26] to assign MapMan hierarchical functional categories (BIN and sub-BIN levels). These annotations were then integrated with the differentially expressed genes (DEGs) identified from our analysis, using the clusterProfiler package in R (v4.6) [27]. Enrichment analyses were conducted for each functional level. DEGs were tested for enrichment in these categories, with a significance threshold of $p \leq 0.1$, adjusted for multiple testing using the Benjamini-Hochberg method.

2.11.4. Weighted Gene Co-Expression Network Analysis (WGCNA)

Gene co-expression networks were constructed using R (version 4.3.1) and the WGCNA package [28] (version 1.73) based on variance-stabilized expression values from leaf (F-2) RNA-seq data. As input, we used the union of all genes (73,697 genes) that were significantly differentially expressed in the control vs. drought stress contrast (see differential expression analysis). Pairwise Pearson correlations were computed to create an adjacency matrix using an unsigned network, allowing detection of both positively and negatively co-expressed gene sets. A soft-thresholding power of $\beta = 8$ was determined using the “pickSoftThreshold” function according to scale-free topology criteria. The adjacency matrix was then transformed into a topological overlap matrix (TOM), and genes were clustered using TOM-based dissimilarity.

Modules were identified using the dynamic tree cut algorithm with a minimum module size of 30 genes. To refine the module structure,

modules with highly similar eigengenes were merged using a cut height of 0.3, which corresponds to an eigengene correlation threshold of approximately 0.7. For each resulting module, the module eigengene (first principal component) was calculated and correlated with treatment (control vs. drought), iWUE group (high vs. low) using Pearson correlation. Modules with $p < 0.05$ were considered significantly associated with a trait. Each module was assigned a unique color label for visualization (e.g., pink), while genes that did not fit into any co-expression cluster were placed into the “grey” module.

2.12. Phenomics and transcriptomics integration: bridging the gap for trait discovery

To unravel the molecular underpinnings of iWUE in wheat, we integrated phenotypic and transcriptomic datasets. This approach combined QTL mapping results from the MAGIC population, which

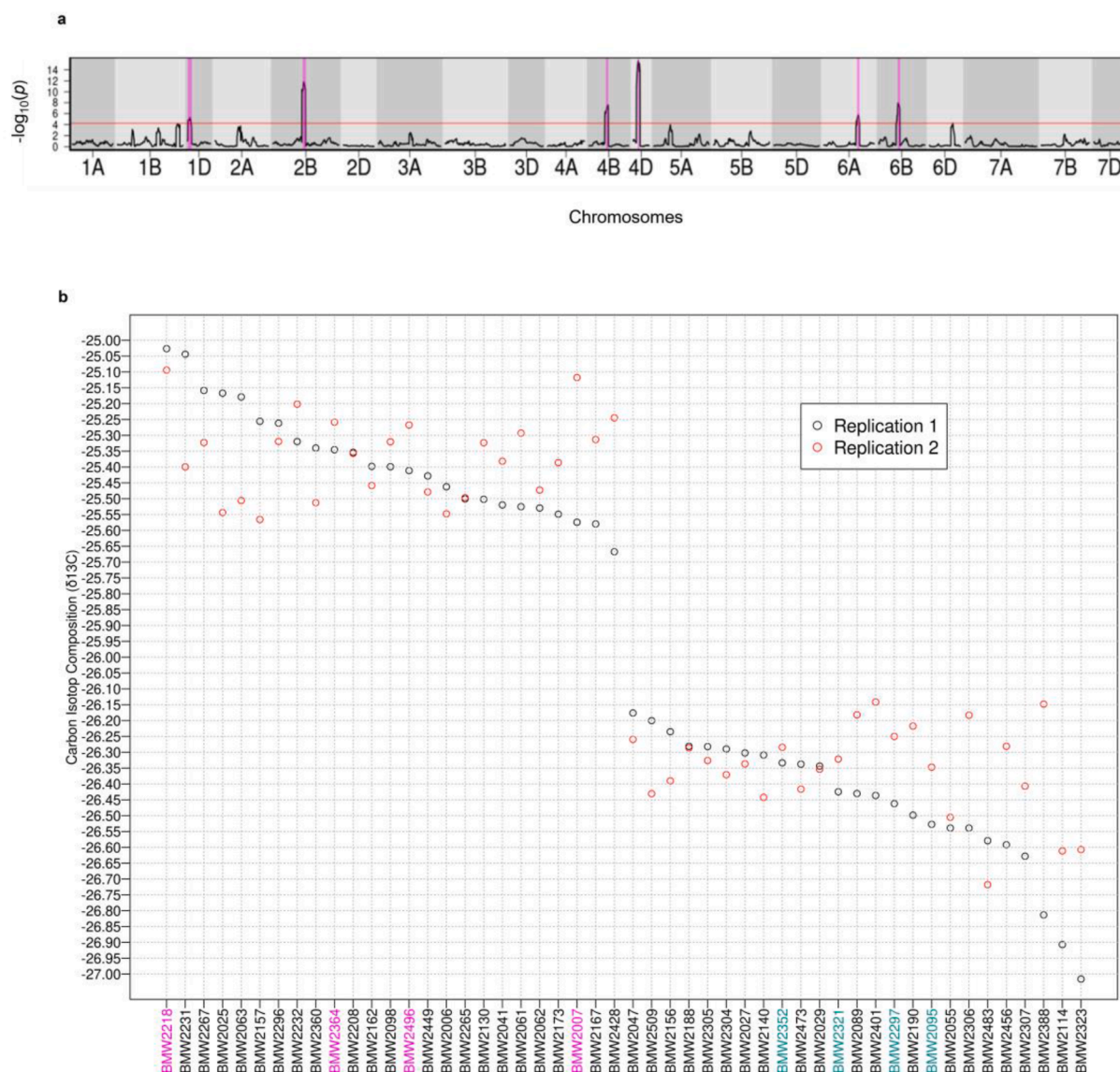


Fig. 2. Identification and selection of contrasting wheat genotypes. (a) Genome-wide $-\log_{10}(P)$ profiles for $\delta^{13}\text{C}$ measured at Morgenrot in 2019. Horizontal dashed lines indicate the genome-wide significance threshold. Colored bars represent QTL support intervals corresponding to significant loci on chromosomes 1D, 2B, 4B, 4D, 6A, and 6B associated with variation in $\delta^{13}\text{C}$ and iWUE. (b) Technical replicate consistency for $\delta^{13}\text{C}$ among the 50 most extreme lines at Morgenrot. Measured $\delta^{13}\text{C}$ values for two independent laboratory replicates are shown for each genotype (Replication 1: black circles, Replication 2: red circles). Lines are ordered from highest to lowest $\delta^{13}\text{C}$ values based on Replicate 1. The tight clustering of replicate measurements for most genotypes demonstrates high analytical precision and validates $\delta^{13}\text{C}$ as a robust physiological trait suitable for QTL mapping and subsequent selection of contrasting lines for greenhouse experiments. Genotypes highlighted in magenta and cyan correspond to the final eight extreme lines (four highest, four lowest $\delta^{13}\text{C}$) selected for transcriptomic analysis.

identified six QTLs associated with carbon isotope composition (a proxy for iWUE). These results were integrated with DGE analysis and WGCNA. By overlaying these datasets, we identified genes that were not only differentially expressed but also part of significant co-expression modules and located within the identified QTL regions.

Such an integrative approach enabled the identification of high-confidence candidate genes that are most likely to contribute to iWUE under drought stress. We present a robust framework that connects phenomics and transcriptomic insights to narrow down gene candidates, paving the way for functional validation and application in breeding programs aimed at enhancing drought resilience in wheat.

3. Results

3.1. $\delta^{13}\text{C}$ results from the Bavarian MAGIC Wheat (BMW) population under field conditions

The BMW population, developed by the Bavarian State Research Center for Agriculture (LfL) in Germany [14], is derived from eight diverse winter wheat founder lines and offers extensive allelic diversity and high recombination. Its 394 recombinant inbred lines (RILs) provide a powerful resource for both gene discovery and applied breeding under challenging environments (Supplementary Fig. 1). In this study, the BMW population was used to investigate drought tolerance in bread wheat via $\delta^{13}\text{C}$, a robust physiological proxy for iWUE [11,12]. A field trial was conducted at the Morgenrot in 2019. The site, located in Sachsen-Anhalt, Germany, faced varying water limitations and was selected to simulate drought-prone conditions in its respective region (Fig. 1).

$\delta^{13}\text{C}$ was measured in wheat lines at the Morgenrot site using an Isotope Ratio Mass Spectrometer, and QTL mapping was performed to identify genomic regions associated with $\delta^{13}\text{C}$. A total of six QTLs were detected on chromosomes 1D, 2B, 4B, 4D, 6A, and 6B in the Morgenrot site, highlighting them as targets for breeding drought-tolerant wheat (Fig. 2a, Supplementary Table 1). Following the identification of these QTLs, a set of extreme lines was selected based on the $\delta^{13}\text{C}$ results obtained at this site. Selection was based on the following criteria: (i) Phenotypic extremes, selecting lines with the highest and lowest $\delta^{13}\text{C}$ values from the Morgenrot site; (ii) Allelic status at height-related loci, ensuring only lines with wild-type *Rht* alleles were retained to avoid confounding; and (iii) Genome-wide genetic distance and measurement consistency, ensuring selected lines were genetically diverse and showed stable $\delta^{13}\text{C}$ values in replicates. Eight contrasting wheat lines (four with high $\delta^{13}\text{C}$ and four with low $\delta^{13}\text{C}$) were selected following these criteria (Fig. 2b). These lines constitute the basis of the greenhouse experiment, where they served as the biological material for transcriptomic analysis aimed at understanding the molecular basis underlying their observed contrasting iWUE phenotypes.

3.2. Comparative phenotyping of extreme wheat lines with respect to iWUE

Selected extreme lines were cultivated under two contrasting irrigation regimes in the fully automated high-throughput phenotyping facility at Helmholtz Munich: well-watered conditions and controlled drought stress. All pots were standardized to an initial weight of approximately 4.2 kg. Drought stress was imposed by maintaining pot weights below predefined thresholds that were gradually increased (2.8–3.7 kg) over the course of the experiment to ensure consistent water limitation while avoiding excessive wilting. Phenological variation among genotypes was controlled by monitoring plant development from growth stage BBCH 31 [29] onward.

A comprehensive set of phenotypic traits was quantified: (i) leaf architectural parameters (e.g., leaf angle, leaf inclination, leaf area index), light penetration depth, and digital biomass were measured automatically using an integrated shoot phenotyping system; (ii) traits

such as number of shoots, number of seeds, dry biomass, and thousand-kernel weight (TKW) were measured manually.

3.3. Statistical analysis of phenotypic traits at BBCH 60–92

The analysis during the interval from flowering to ripening stage revealed that both treatment (drought vs. control) and $\delta^{13}\text{C}$ /iWUE group (high vs. low) had significant effects on multiple traits, particularly those related to biomass accumulation and TKW.

Significant interaction effects between treatment and $\delta^{13}\text{C}$ group were also observed for traits such as leaf inclination (LIC), light penetration depth (LPD), leaf area projection (LAP), and leaf angle, indicating that the response of certain phenotypes to drought stress differed depending on their $\delta^{13}\text{C}$ group (Table 1).

In contrast, traits related to reproductive output, such as the number of tillers, fully and partially filled ears, were more strongly influenced by treatment alone, with less evidence of interaction or $\delta^{13}\text{C}$ group effect. However, the number of mature seeds was affected by the type of treatment and the iWUE group, although no interactions were observed. These findings highlight the phenotypic plasticity of the genotypes belonging to different $\delta^{13}\text{C}$ groups.

Detailed results of the statistical analyses, including estimated marginal means (EMMs), standard errors (SE), and Tukey post hoc groupings for all the traits measured in the experiment, are provided in Supplementary Table 2.

3.3.1. Drought stress effects on plant growth parameters

Significant association was observed between treatment and iWUE group for both digital biomass and TKW, indicating contrasting responses of high- and low-iWUE lines under drought conditions (Fig. 3a and b). Under well-watered (control) conditions, high-iWUE lines produced significantly higher digital biomass than low-iWUE lines, with means of approximately 4×10^7 and 3.5×10^7 , respectively (Fig. 3a). While drought stress resulted in a substantial reduction of biomass across both groups, high-iWUE lines maintained relatively higher biomass than low-iWUE lines.

Similarly, a pronounced interaction between treatment and iWUE group was observed for TKW. Under control conditions, high-iWUE lines had a significantly greater TKW (~35 g) compared to low-iWUE lines (~26 g), suggesting a potential yield advantage under favorable conditions. Under drought stress, however, the difference between the groups became less pronounced, with both high- and low-iWUE lines exhibiting similar TKW values (~29–26 g) (Fig. 3b).

The temporal dynamics of canopy architectural traits under drought stress revealed pronounced differences between wheat lines with high and low $\delta^{13}\text{C}$ /iWUE. High-iWUE lines, which have higher $\delta^{13}\text{C}$, consistently exhibited greater leaf angle values throughout most developmental stages (Fig. 3c), indicating a wider angle between the leaf blade and the stem. Although high-iWUE (also higher $\delta^{13}\text{C}$) lines had a greater leaf angle, they showed lower leaf inclination (LIC) during the reproductive stage, reflecting reduced lamina bending and resulting in a more erect and vertically aligned canopy under drought stress (Fig. 3d).

3.3.2. Trait correlation analysis across developmental stages (flowering to full ripening)

A correlation analysis was performed for all measured traits across the growth stages from flowering (BBCH 60) to full ripening (BBCH 92). Such a holistic approach allowed insights into how phenotypic traits interrelate in critical growth phases and, by the same virtue, into patterns and dependencies that might determine overall plant performance and resilience under fluctuating conditions.

The Pearson correlation matrix highlights how drought stress alters relationships among traits (Fig. 3e). For instance, there is a strong negative correlation between the leaf angle and leaf inclination under both control (green, lower triangle) and drought conditions (pink, upper triangle). $\delta^{13}\text{C}$ shows strong correlations with water-use traits, like

Table 1
Summary of *p*-values from GLM analysis testing the effects of treatment, iWUE, and their interaction (Treatment (control vs. stress) × $\delta^{13}\text{C}$ /iWUE groups) on agronomic traits measured during grain filling to ripening stages (BBCH 69–92).

SOV	Biomass (3D)	Biomass (Dry)	Max-Height	Height	TKW	LA	LAI	Lic	Leaf angle	LPD	LAP
Treatment	0.0000 ***	1.04E-70 ***	4.08E-293 ***	0.00000 ***	0.02100 *	0.000 ***	0.000 ***	6.66E-38 ***	1.99E-41 ***	5.99E-20 ***	0.00000 ***
$\delta^{13}\text{C}$ /iWUE	2.12E-51 ***	1.06E-07 ***	1.57E-113 ***	2.35E-89 ***	9.64E-06 ***	1.09E-14 ***	1.12E-25 ***	0.03220 *	0.01498 ***	2.08E-99 ***	9.49E-14 ***
Treatment*iWUE	1.34E-28 ***	0.09086 ns	0.17845 ns	0.21599 ns	0.02671 *	0.2105 ns	0.2088 ns	0.01314 *	0.00215 **	0.00918 **	0.03189 *

SOV	Tillers	Fertile tillers	Unfertile tillers	Total ear	Filled ears	Partially filled ears	Mature seeds	Poor-quality seeds
Treatment	4.16E-25 ***	2.97E-29 ***	0.41889 ns	2.55E-27 ***	4.36E-30 **	2.48E-05 ***	6.88E-93 ***	0.00787 **
$\delta^{13}\text{C}$ /iWUE	0.86200 ns	0.51650 Ns	0.09838 ns	0.87088 ns	0.37609 ns	0.09830 ns	0.00232 **	0.21784 *
Treatment*iWUE	0.24200 ns	0.92370 Ns	0.93040 ns	0.984671 ns	0.38518 ns	0.63887 ns	0.97144 ns	0.47390 ns

Note: Significant effects are indicated by asterisks. Traits include biomass (3D imaging and dry), thousand kernel weight (TKW), canopy characteristics (LA: leaf area, LAI: leaf area index, LPD: light penetration depth, LAP: leaf area projection, Lic: leaf inclination, leaf angle), and maximum and average height.
Note: Significance levels are denoted as follows: ****p* < 0.001, ***p* < 0.01, **p* < 0.05, ns = not significant.

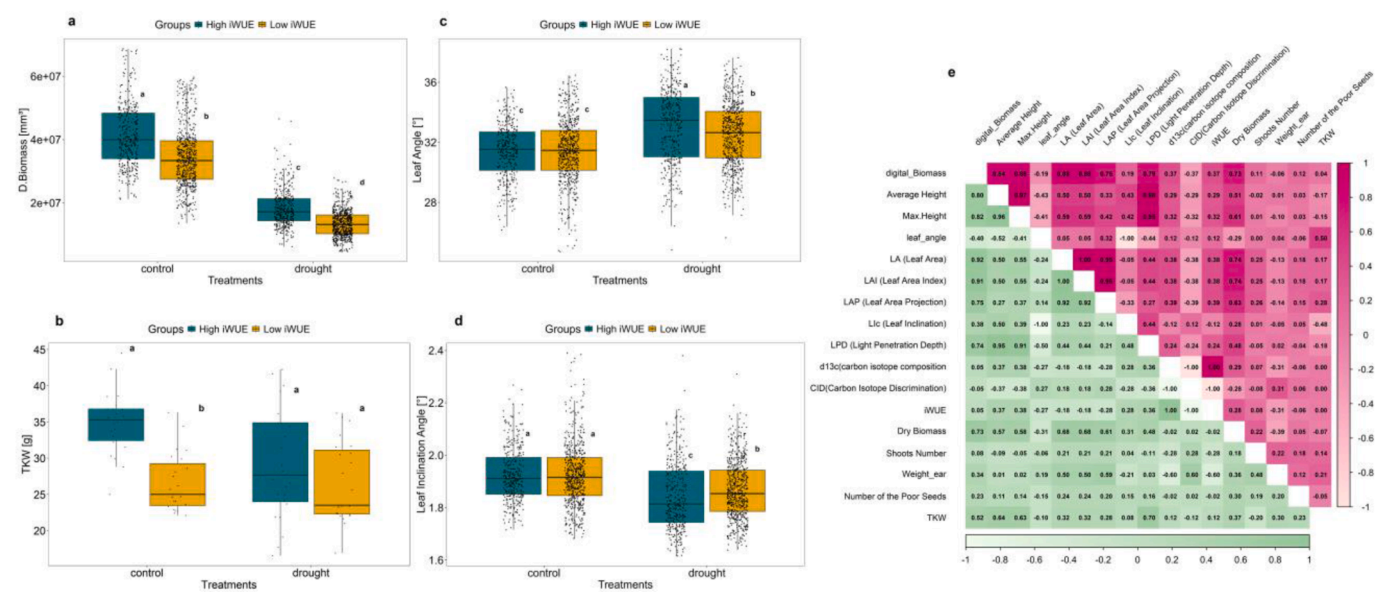


Fig. 3. Phenotypic variation and trait correlations in contrasting iWUE wheat groups. (a) Digital biomass, (b) TKW, (c) Leaf angle variation, and (d) Lic dynamics across treatments (control vs. drought stress) in high- and low-iWUE groups. A GLM and Tukey's HSD post hoc test were performed to identify significant differences between groups and treatments. Common letters above bars indicate no significant differences between groups or treatments (*p* > 0.05). (e) Trait correlation matrix across all measured phenotypes from flowering to full ripening (BBCH 60–92). Pearson correlation coefficients illustrate the relationships among physiological, architectural, and yield-related traits under combined control (green, lower triangle) and drought (pink, upper triangle) conditions. Color intensity represents correlation strength. Each number quantifies the strength of the linear relationship between two traits, indicating whether the relationship is positive or negative.

iWUE, under drought conditions, suggesting its potential as a marker for iWUE and drought tolerance in wheat.

3.4. Principal Component Analysis (PCA) of gene expression profiles

Transcriptomic data were generated from the second leaf below the flag leaf (F-2 leaf) tissue collected under drought and control conditions at the flowering stage (BBCH 65) for all eight genotypes. To ensure strict comparability across genotypes, developmental staging was monitored daily and samples were harvested only once each line had completed BBCH 65. All leaf samples were collected within the same time window, ensuring uniform developmental and temporal sampling across treatments and genotypes. RNA sequencing reads were mapped to the *Triticum aestivum* cv. Chinese Spring reference genome (IWGSC RefSeq v1.1) [30] and normalized gene expression values were obtained. Normalized and variance-stabilized expression values were used to explore major

sources of variance among samples. An initial PCA including both control and drought condition samples, as well as $\delta^{13}\text{C}$ groupings, confirmed clear separation between control and stress treatment, underscoring the strong impact of drought on transcriptional profiles (Supplementary Fig. 2).

The PCA plot displayed in Fig. 4a focuses exclusively on drought-stress samples to assess transcriptional differences between the high-iWUE and low-iWUE groups. PC1 and PC2 explained 23% and 10% of the variance, respectively. Replicates from the same iWUE group showed a tendency to cluster, indicating partially shared transcriptional responses within each group. However, the overlap between the two groups suggests that the iWUE contrast is driven by a comparatively limited set of genes rather than a genome-wide shift in expression, consistent with a polygenic and network-based regulation of $\delta^{13}\text{C}$ under drought.

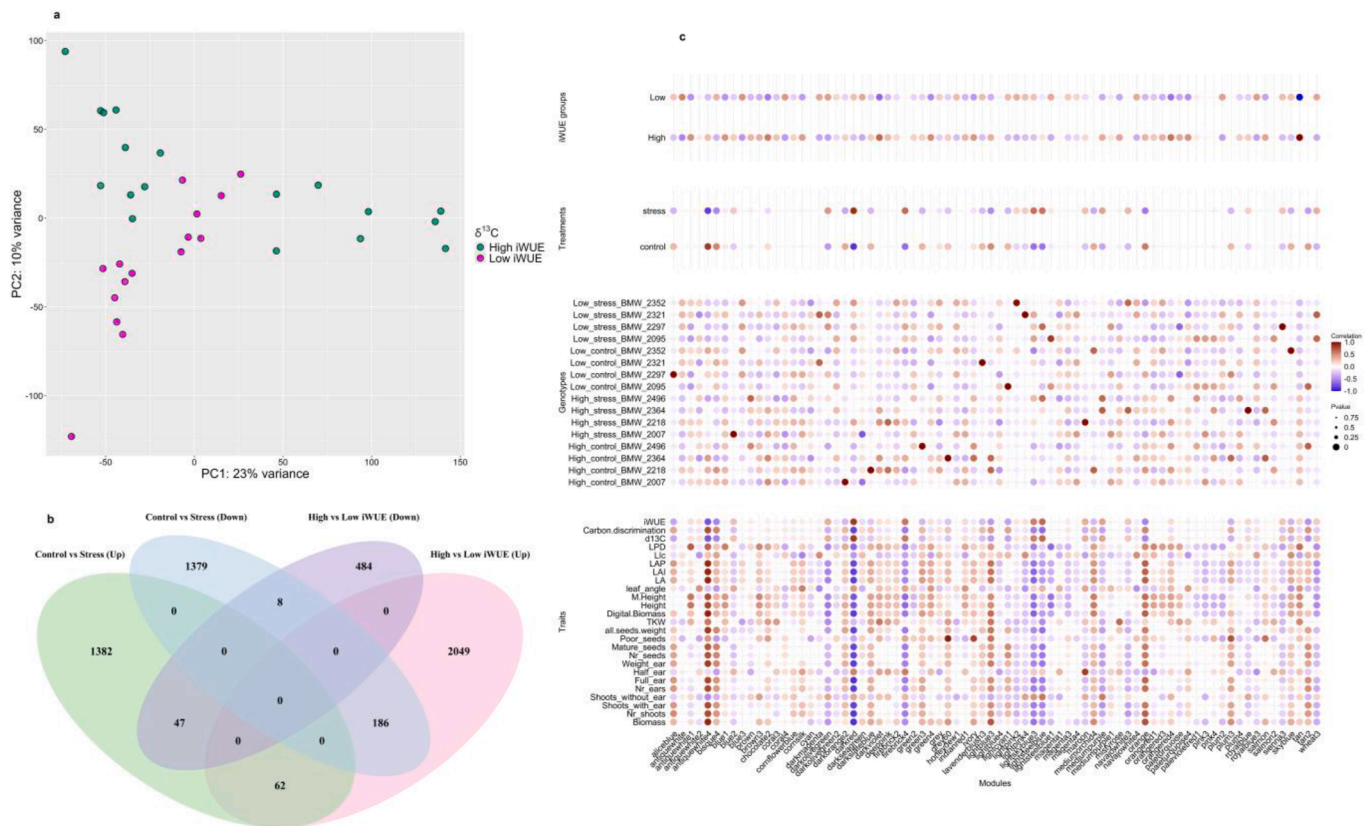


Fig. 4. Transcriptomic characterization of contrasting iWUE wheat groups. (a) PCA of high and low- $\delta^{13}\text{C}$ wheat lines under drought stress. Variance-stabilized expression data show partial separation of high (green) and low (purple) iWUE groups along PC1 (23% variance) and PC2 (10% variance). While samples do not form completely discrete clusters, the grouping tendency indicates that drought induces shared transcriptional adjustments within each iWUE class. (b) DEGs contrast across key comparisons. The four-way Venn diagram depicts the overlap between DEGs identified in (i) control vs. drought (upregulated), (ii) control vs. drought (downregulated), (iii) high vs. low-iWUE under drought (upregulated), and (iv) high vs. low-iWUE under drought (downregulated). 62 genes were commonly upregulated, and 8 genes were commonly downregulated in both comparisons. These shared DEGs represent strong candidates for mediating drought-induced physiological differences between the two iWUE groups. (c) WGCNA module-trait association map. The heatmap displays Pearson correlations between module eigengenes and phenotypic traits, treatments, genotypes, and iWUE classes. Color intensity reflects correlation strength (red = positive; blue = negative), and point size denotes significance level (larger dots = lower p -values). These graphs underscore the modular architecture of drought-induced gene regulation and support the identification of biologically meaningful co-expression groups underlying iWUE.

3.5. DGE analysis identifies candidate genes associated with drought stress response

Understanding plant responses to drought stress is key to developing more resilient crops. Our research identifies genes involved in this process by analyzing gene expression profiles under drought and control conditions. For this purpose, DGE analysis was performed across two main comparisons: (i) control vs. drought stress, and (ii) high- vs. low-iWUE lines under drought conditions.

In the control vs. drought comparison, genes were classified as either upregulated or downregulated in response to drought stress. In parallel, differential expression between high- and low-iWUE lines under drought conditions identified genes associated with variation in iWUE. These four DEG categories (up- and downregulated in each comparison) are summarized in Fig. 4b.

The Venn diagram illustrates the overlap between these comparisons, highlighting genes that are consistently differentially expressed in both drought response and $\delta^{13}\text{C}$ /iWUE contrast. In total, 2761 genes were differentially expressed between drought and control conditions. Additionally, 2533 genes were differentially expressed between high- and low- $\delta^{13}\text{C}$ lines under drought conditions. Among these, 62 genes were commonly upregulated and 8 genes were commonly downregulated in both comparisons.

These overlapping DEGs likely represent key regulators linking drought response and $\delta^{13}\text{C}$, suggesting their involvement in shared stress-responsive pathways contributing to improved iWUE in wheat.

3.6. Functional enrichment analysis of differentially expressed genes

To understand the biological implications of these changes (genes differentially expressed in high vs. low $\delta^{13}\text{C}$ (iWUE) groups under drought stress conditions), we performed a functional enrichment analysis.

Under drought stress conditions, stress-responsive genes were found upregulated, particularly those involved in ABA signaling and transcriptional regulation, while downregulated genes were related to photosynthesis, such as RuBisCO and the light-harvesting complex II (LHC-II) complex. This indicates a shift from growth to survival strategies, with enhanced stress response and reduced photosynthetic activity helping the plants conserve resources during water deficit (Supplementary Fig. 3).

For the comparison of high- $\delta^{13}\text{C}$ vs. low- $\delta^{13}\text{C}$ (shown by grouping in iWUE) under drought conditions, the results of the enrichment analysis reveal that the majority of upregulated genes in lines exhibiting high $\delta^{13}\text{C}$ are significantly associated with key biological functions, including growth regulation (WRKY transcription factors (TFs)/WRKY33 TFs), cell wall stability (WAK/WAKL TFs), metabolic reprogramming (regulated

by SnRK1 kinase regulatory system), and the most notably, stomatal regulation (controlled by LRR-XII protein kinase) (Supplementary Fig. 4). These findings suggest that the identified genes play a pivotal role in the plant's adaptive responses to water-limited environments, enhancing overall iWUE.

3.7. Exploring gene co-expression modules under drought stress

To characterize coordinated transcriptional responses associated with drought stress and $\delta^{13}\text{C}$, WGCNA was performed. This approach enabled the identification of gene modules based on shared expression patterns across samples and the evaluation of their associations with phenotypic traits, treatment conditions, and $\delta^{13}\text{C}$ groups.

A total of 72 co-expression modules were identified, reflecting the complex regulatory architecture underlying drought response (Fig. 4c). Several modules exhibited significant correlations with key traits, including $\delta^{13}\text{C}$, biomass, plant height, and leaf architectural parameters, suggesting that distinct gene networks contribute to phenotypic variation under both control and drought conditions.

Furthermore, module-trait relationships revealed clear differentiation between stress and control treatments, as well as between high- and low- $\delta^{13}\text{C}$ groups, indicating the presence of gene networks specifically associated with drought responsiveness and variation in iWUE. In Fig. 4c, color intensity represents the strength and direction of correlations (red = positive; blue = negative), while dot size corresponds to statistical significance, with larger dots indicating stronger associations.

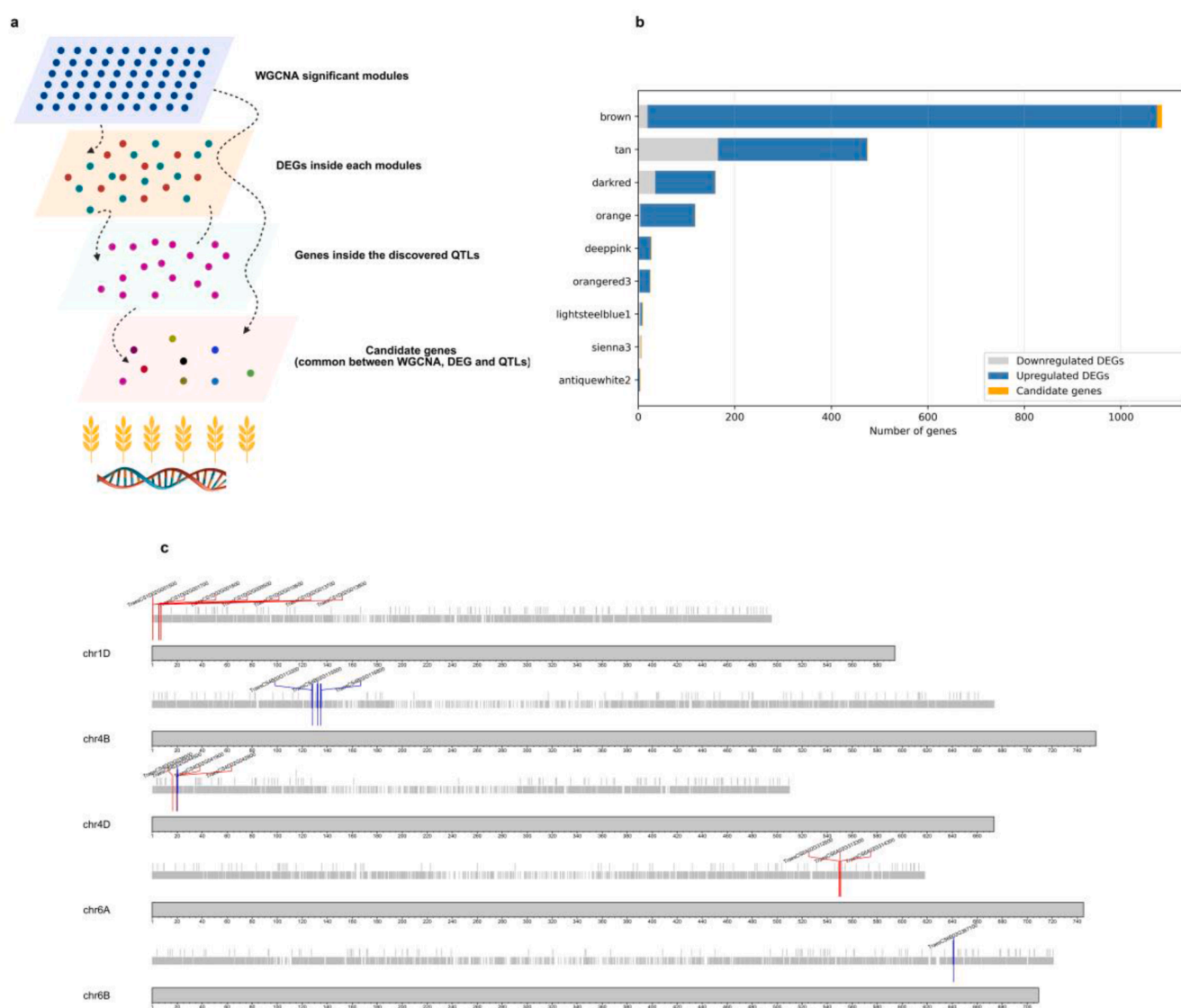


Fig. 5. Integrative identification of candidate genes associated with iWUE. (a) Schematic overview of the multi-layered integrative framework used to identify candidate genes. The approach combines WGCNA, DGE analysis, and QTL mapping to prioritize genes that are differentially expressed, belong to trait-associated co-expression modules, and are located within QTL regions associated with water-use efficiency. Genes shared across all three layers were retained as final candidate genes. (b) Distribution of DEGs within WGCNA modules containing at least one final candidate gene. Bars represent the total number of upregulated (blue) and downregulated (grey) DEGs per module. Candidate genes meeting all three selection criteria (QTL co-localization, differential expression, and module membership) are highlighted in orange. (c) Genomic distribution of high-confidence candidate genes across wheat chromosomes. Candidate genes retained after multi-layer filtering were mapped to their physical positions on chromosomes 1D, 4B, 4D, 6A, and 6B using the IWGSC RefSeq v1.1 reference genome. Red markers denote upregulated candidate genes, while blue markers denote downregulated genes.

These findings highlight the presence of coordinated transcriptional programs underlying drought adaptation and iWUE-related traits.

3.8. Unraveling key candidate genes for iWUE in wheat: integrating QTL mapping, DGE, and WGCNA results

To identify robust candidate genes underlying observed variation in $\delta^{13}\text{C}$, we integrated the results from QTL mapping, DGE, and WGCNA. This multi-layered approach allows the pinpointing genes that are (i) differentially expressed, (ii) part of significant co-expression modules, and (iii) located within the QTL regions associated with $\delta^{13}\text{C}$ (Fig. 5a). Importantly, this integrative framework ensures that the selected candidate genes are not only physically localized within QTL intervals identified at the Morgenrot site, but also functionally supported by transcriptional and network-level evidence. Many of these genes are involved in key drought-responsive processes, including stomatal regulation and energy metabolism, thereby providing a mechanistic basis for the observed QTL effects on $\delta^{13}\text{C}$ and iWUE.

WGCNA identified several trait-associated modules containing both up- and downregulated DEGs. For visualization, modules harboring at least one final candidate gene are shown in Fig. 5b, with upregulated DEGs depicted in blue and downregulated DEGs in grey. Candidate genes that meet all three selection criteria (QTL co-localization, differential expression, and module membership) are highlighted in orange, illustrating their relative contributions within each module. In total, 18 candidate genes were identified across nine distinct modules, supporting their potential regulatory relevance.

3.9. Genomic localization and functional annotation of candidate genes

The candidate genes, which are associated with drought stress resistance and $\delta^{13}\text{C}$, were mapped onto wheat chromosomes according to their physical positions in the reference genome. These genes are located on chromosomes 1D, 4B, 4D, 6A, and 6B. The red and blue markers (up- and down-regulated genes, respectively) indicate the locations of these candidate genes, with implications for their involvement in controlling certain key physiological and phenotypic responses to water stress (Fig. 5c). This extended visualization offers an integrated overview of the localization of candidate genes, which is important for understanding their functional relevance in drought resistance and iWUE.

To aid the use and further validation of the identified candidate genes associated with $\delta^{13}\text{C}$ and drought stress resistance, we provide an integrated table (Supplementary Table 3) including alternative names retrieved from KnetMiner 5.7 [31]. This table also contains corresponding orthologs that provide insights into the evolutionary conservation and potential functions of these genes across species.

4. Discussion

Drought stress threatens global crop productivity by affecting growth, development, and yield. Plants adapt via morphological, physiological, and biochemical changes, including water-use efficiency, biomass allocation, and reproductive strategies. Reproductive success is key to survival, making genotype-specific responses crucial for breeding under water limitation.

Under drought stress, TKW did not differ between high- and low- $\delta^{13}\text{C}$ /iWUE groups, despite pronounced differences in vegetative and canopy-related traits. Lines with higher $\delta^{13}\text{C}$ maintained greater aboveground biomass and canopy integrity, indicating enhanced vegetative performance and improved stress buffering rather than increased reproductive output. This suggests that under sustained drought, plants prioritize the maintenance of photosynthetically active tissues, enabling continued carbon assimilation while limiting yield penalties.

Although drought stress was imposed continuously from the vegetative stage (BBCH 31) until maturity, reproductive traits such as TKW

appeared comparatively buffered. This likely reflects the strong physiological regulation of sink strength and grain filling in wheat, where moderate but prolonged water limitation can be compensated by sustained source activity and efficient remobilization of assimilates. As a result, high $\delta^{13}\text{C}$ alone did not translate into increased grain weight, underscoring the role of sink capacity and assimilate partitioning in determining yield under drought.

In contrast, leaf architectural traits showed clearer drought adaptation, with high- $\delta^{13}\text{C}$ lines exhibiting more erect leaves and reduced inclination. This canopy configuration is associated with lower transpirational demand, improved light distribution, and reduced thermal stress, and has been linked to enhanced water interception under water-limited conditions [32,33], supporting its functional relevance for drought resilience independent of grain yield.

At the genetic level, previous studies have demonstrated that $\delta^{13}\text{C}$ and related traits are controlled by a complex, polygenic architecture with strong environmental dependency. QTL associated with $\delta^{13}\text{C}$ and transpiration efficiency have been reported across multiple chromosomes, particularly on 2B and group 4 chromosomes (including 4B, 4D), suggesting partially conserved genomic regions underlying water-use efficiency [34,35].

Consistent with these findings, we detected significant QTLs on chromosomes 2B, 4B, and 4D, supporting the reproducibility of these loci across genetic backgrounds. Importantly, the use of lines carrying wild-type alleles at major height loci minimized confounding effects of plant stature, indicating that the signal on chromosome 4D and 4B reflects independent genetic control of $\delta^{13}\text{C}$ rather than indirect architectural effects. Beyond these conserved regions, additional loci identified on chromosomes 6A and 6B suggest further genetic contributions to drought adaptation, particularly under severe water-limited conditions. Notably, we also detected a QTL on chromosome 1D, a region associated with $\delta^{13}\text{C}$ and water-use efficiency. This expands the current understanding of the genetic architecture underlying iWUE and highlights the potential of underexplored genomic regions as targets for breeding.

Transcriptomic analysis identified 18 candidate genes associated with drought adaptation and iWUE, many of which were embedded in transcriptional networks dominated by stress-responsive transcription factors. In our study, abscisic acid (ABA)-related functions were enriched among candidate genes associated with iWUE under drought stress. Several candidates were linked to ABA-responsive and stomatal regulatory pathways, suggesting coordinated drought acclimation. Supporting this, enhanced ABA perception has been shown to increase iWUE in wheat, as demonstrated by overexpression of the ABA receptor gene *TaPYL1-1B* [36].

Here, we highlight selected up- and down-regulated candidate genes with reported roles in iWUE, stomatal regulation, and drought-responsive processes. Detailed functional annotations and descriptions of the remaining candidate genes are provided in the Supplementary Functional Description Table (Supplementary Table 4). *TraesCS1D02G001700* (INRPK1) encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) that functions as a central signaling hub in wheat drought stress response [31]. It is embedded in a WRKY-regulated network (e.g., TaWRKY18, TaWRKY51, TaWRKY54, TaWRKY46), linking it directly to drought tolerance pathways supported by experimental evidence [37–39]. Functionally, it integrates stress and hormone signaling (salicylic acid and jasmonic acid pathways) with developmental regulation, particularly root architecture traits (root length and lateral root formation) that enhance water uptake. At the physiological level, it is associated with regulation of transpiration and chlorophyll content, thereby connecting drought signaling with water-use efficiency and maintenance of photosynthetic capacity under stress [31].

TRAESCS4D02G038000 likely functions as a transcriptional regulator acting as a central hub in wheat drought stress networks, integrating signals from WRKY and NAC transcription factors (e.g., TaWRKY46, TaWRKY51, TaWRKY54, TaNAC061 [31]). Through these

interactions, it contributes to key drought-adaptive mechanisms, including stomatal closure to reduce transpiration [37] and enhanced root architecture for improved water uptake [38]. Among the down-regulated genes, *TraesCS4B02G116800* (OFP8) encodes an OVATE Family Protein acting as a transcriptional repressor involved in plant growth regulation and hormone signaling. It is functionally linked to brassinosteroid (BR) signaling, where OFP8 modulates plant architecture and development through interactions with key kinases in rice [40].

TraesCS6B02G367100 (TaRIPK) encodes a receptor-like cytoplasmic kinase (RLCK) involved in stress and MAPK signaling pathways. It regulates key traits such as root development and stomatal behavior [39–41], linking environmental signals to water uptake and transpiration control.

While the integrative framework applied in this study enabled the identification of high-confidence candidate genes, certain limitations should be considered when interpreting these results. QTL detection was performed using data from a single field environment (Morgenrot, 2019), representing drought-prone conditions. Given the well-known influence of genotype $\times$ environment interactions on quantitative traits such as $\delta^{13}\text{C}$ and iWUE, the identified loci may reflect environment-specific genetic effects rather than universally stable QTLs. Accordingly, these loci should be interpreted as drought-relevant QTLs under water-limited conditions. While cross-location phenotypic correlations indicate a genetic component underlying $\delta^{13}\text{C}$ variation, formal validation of QTL stability across environments will require multi-environment mapping in future studies. Furthermore, our identified candidate genes represent prime targets for future experimental validation, using approaches such as quantitative PCR, gene editing, or functional characterization in controlled systems.

5. Conclusion

This study used $\delta^{13}\text{C}$ as a proxy for iWUE in a winter wheat MAGIC population to identify key phenotypic traits and candidate genes associated with drought resistance. Wheat lines with higher $\delta^{13}\text{C}$ were found to be linked to improved drought tolerance, including optimized leaf architecture, sustained biomass, and enhanced canopy greenness, under water-limited conditions. Transcriptomic analysis showed increased expression of genes involved in ABA and JA signaling, pattern-triggered immunity, photosynthesis, and strigolactone-mediated root development, along with decreased expression of genes regulating leaf inclination and ABA sensitivity. This suggests a coordinated genetic and physiological response that balances water conservation with carbon assimilation. The identification of candidate genes and QTLs for improved water use efficiency facilitates the development of markers for this important trait and its use in marker-assisted selection (MAS). Our findings highlight specific molecular pathways and phenotypic strategies that support drought resilience, offering a valuable foundation for breeding wheat varieties with better water-use efficiency and productivity under drought stress.

Data availability

Sequencing data have been submitted to EMBL-EBI (<https://www.ebi.ac.uk/biostudies>) under the accession number E-MTAB-17102.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this manuscript, the authors used ChatGPT (version 5.5) for language polishing, literature search assistance, and R code development. No AI tools were used for candidate gene identification, data analysis, experimental design, scientific interpretation, or any other core research process. The authors reviewed and edited all AI-generated content thoroughly, verified the accuracy of all information,

and assume full responsibility for the integrity and scientific validity of the final manuscript.

CRedit authorship contribution statement

Sepideh Jafarian: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Funding acquisition, Formal analysis, Data curation. **Manuel Geyer:** Software, Investigation, Formal analysis, Data curation. **Franz Buegger:** Resources, Investigation. **Barbro Winkler:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis. **Georg Gerl:** Resources. **Klaus F.X. Mayer:** Supervision, Funding acquisition. **Nadia Kamal:** Writing – review & editing, Supervision. **Jörg-Peter Schnitzler:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Lorenz Hartl:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Manuel Spannagl:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We gratefully acknowledge BayKlimaFit 2 for funding this research through the Bavarian State Ministry for the Environment and Consumer Protection (Bayerisches Staatsministerium für Umwelt und Verbraucherschutz). We also acknowledge the Bavarian State Research Center for Agriculture (LfL), Freising, for providing the Bavarian MAGIC Wheat (BMW) population and access to field trial facilities. Furthermore, we thank the breeding companies Strube D&S GmbH, Saatzucht Josef Breun GmbH & Co. KG, and Syngenta AG for conducting field trials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.agrcom.2026.100158>.

References

- [1] Ortiz R. Climate change: can wheat beat the heat? *Agric Ecosyst Environ* 2008;126: 46–58.
- [2] Ashraf M. Inducing drought tolerance in plants: recent advances. *Biotechnol Adv* Jan. 2010;28(1):169–83.
- [3] Ashe P, Shaterian H, Akhlov L, Kulkarni M, Selvaraj G. Contrasting root and photosynthesis traits in a large-acreage Canadian durum variety and its distant parent of Algerian origin for assembling drought/heat tolerance attributes. *Front Chem Dec.* 2017;5:121.
- [4] Pernicová N, Hlaváčová M, Findurová H, Čáslavský J, Urban O, Klem K, et al. Grain carbon isotopes indicate the ability of wheat plants to maintain enhanced intrinsic water-use efficiency even after short-term exposure to high temperatures and drought. *Plant Physiol Biochem Dec.* 2023;205(108155):108155.
- [5] Yoo CY, Pence HE, Jin JB, Miura K, Gosney MJ, Hasegawa PM, et al. The arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. *Plant Cell Dec.* 2010;22(12):4128–41.
- [6] Hafeez A, Ali S, Javed MA, Iqbal R, Khan MN, Çiğ F, et al. Breeding for water-use efficiency in wheat: progress, challenges and prospects. *Mol Biol Rep Mar.* 2024;51(1):429.
- [7] Farquhar GD, O'Leary MH, Berry JA. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust J Plant Physiol Apr.* 1982;9(2):121–37.
- [8] Farquhar GD, Richards RA. Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Aust J Plant Physiol Dec.* 1984;11(6): 539–52.

- [9] Condon AG, Richards RA, Rebetzke GJ, Farquhar GD. Breeding for high water-use efficiency. *J Exp Bot Nov.* 2004;55(407):2447–60.
- [10] Seibt U, Rajabi A, Griffiths H, Berry JA. Carbon isotopes and water use efficiency: sense and sensitivity. *Oecologia Mar.* 2008;155(3):441–54.
- [11] Condon AG, Richards RA. Broad sense heritability and genotype $\times$ environment interaction for carbon isotope discrimination in field-grown wheat. *Aust J Agric Res Aug.* 1992;43(5):921–34.
- [12] Condon AG, Richards RA, Rebetzke GJ, Farquhar GD. Improving intrinsic water-use efficiency and crop yield. *Crop Sci* 2002;42(1):122–31.
- [13] Yacoubi I, Gadaleta A, Mathlouthi N, Hamdi K, Giancaspro A. Absciscic acid-stress-ripening genes involved in plant response to high salinity and water deficit in durum and common wheat. *Front Plant Sci Feb.* 2022;13:789701.
- [14] Stadlmeier M, Hartl L, Mohler V. Usefulness of a multiparent advanced generation intercross population with a greatly reduced mating design for genetic studies in winter wheat. *Front Plant Sci Dec.* 2018;9:1825.
- [15] Chen H, Xie W, He H, Yu H, Chen W, Li J, et al. A high-density SNP genotyping array for rice biology and molecular breeding. *Mol Plant Mar.* 2014;7(3):541–53.
- [16] Plaschke J, Ganai MW, Röder MS. Detection of genetic diversity in closely related bread wheat using microsatellite markers. *Theor Appl Genet Nov.* 1995;91(6–7):1001–7.
- [17] Wang S, Wong D, Forrest K, Allen AJ, Chao S, Huang BE, et al. Characterization of polyploid wheat genomic diversity using a high-density 90,000 single nucleotide polymorphism array. *Plant Biotechnol J Aug.* 2014;12(6):787–96.
- [18] Winfield MO, Allen AM, Burridge AJ, Barker GLA, Benbow HR, Wilkinson PA, et al. High-density SNP genotyping array for hexaploid wheat and its secondary and tertiary gene pool. *Plant Biotechnol J May* 2016;14(5):1195–206.
- [19] Wimmer V, Albrecht T, Auinger H-J, Schön C-C. Synbreed: a framework for the analysis of genomic prediction data using R. *Bioinformatics Aug.* 2012;28(15):2086–7.
- [20] Huang BE, George AW. R/mpMap: a computational platform for the genetic analysis of multiparent recombinant inbred lines. *Bioinformatics Mar.* 2011;27(5):727–9.
- [21] Simon J, Dong F, Buegger F, Rennenberg H. Rhizospheric NO affects N uptake and metabolism in Scots pine (*Pinus sylvestris* L.) seedlings depending on soil N availability and N source. *Plant Cell Environ May* 2013;36(5):1019–26.
- [22] Werner RA, Brand WA. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Commun Mass Spectrom* 2001;15(7):501–19.
- [23] Mathias J. isocalcR: isotope calculations in R. <https://CRAN.R-project.org/package=isocalcR>; 2023. 25.12.17.
- [24] Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol May* 2016;34(5):525–7.
- [25] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):550.
- [26] Bolger M, Schwacke R, Usadel B. MapMan visualization of RNA-seq data using Mercator4 functional annotations. *Methods Mol Biol* 2021;2354:195–212.
- [27] Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innovation* 2021;2(3):100141.
- [28] Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinf Dec.* 2008;9(1):559.
- [29] Meier U. Growth stages of mono- and dicotyledonous plants. In: Bleiholder H, Feller C, editors. *Entwicklungsstadienmono- und dikotyler Pflanzen*. Berlin: Blackwell; 2018. p. 11–153.
- [30] International Wheat Genome Sequencing Consortium. Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science* 2018;361(6403):eaar7191.
- [31] Hassani-Pak K, Singh A, Brandizi M, Hearnshaw J, Parsons JD, Amberkar S, et al. KnetMiner: a comprehensive approach for supporting evidence-based gene discovery and complex trait analysis across species. *Plant Biotechnol J Aug.* 2021;19(8):1670–8.
- [32] Merrium S, Ali Z, Habib-Ur-Rahman M, Hakeem S, Khalid MA. Leaf rolling and leaf angle improve fog capturing and transport in wheat; adaptation for drought stress in an arid climate. *Bot Stud May* 2022;63(1):13.
- [33] Hakeem S, Ali Z, Saddique MAB, Merrium S, Arslan M, Habib-Ur-Rahman M. Leaf wettability and leaf angle affect air-moisture deposition in wheat for self-irrigation. *BMC Plant Biol Feb.* 2023;23(1):115.
- [34] Rebetzke GJ, Condon AG, Farquhar GD, Appels R, Richards RA. Quantitative trait loci for carbon isotope discrimination are repeatable across environments and wheat mapping populations. *Theor Appl Genet Dec.* 2008;118(1):123–37.
- [35] Acuña-Galindo MA, Mason RE, Subramanian NK, Hays DB. Meta-analysis of wheat QTL regions associated with adaptation to drought and heat stress. *Crop Sci Mar.* 2015;55(2):477–92.
- [36] Mao H, Jian C, Cheng X, Chen B, Mei F, Li F, et al. The wheat ABA receptor gene TaPYL1-1B contributes to drought tolerance and grain yield by increasing water-use efficiency. *Plant Biotechnol J May* 2022;20(5):846–61.
- [37] Ma J, Gao X, Liu Q, Shao Y, Zhang D, Jiang L, et al. Overexpression of TaWRKY146 increases drought tolerance through inducing stomatal closure in *Arabidopsis thaliana*. *Front Plant Sci Nov.* 2017;8:2036.
- [38] Hu Z, Wang R, Zheng M, Liu X, Meng F, Wu H, et al. TaWRKY51 promotes lateral root formation through negative regulation of ethylene biosynthesis in wheat (*Triticum aestivum* L.). *Plant J Oct.* 2018;96(2):372–88.
- [39] Li L, Zang X, Liu J, Ren J, Wang Z, Yang D. Integrated physiological and weighted gene co-expression network analysis reveals the hub genes engaged in nitrate-regulated alleviation of ammonium toxicity at the seedling stage in wheat (*Triticum aestivum* L.). *Front Plant Sci Nov.* 2022;13:1012966.
- [40] Yang C, Shen W, He Y, Tian Z, Li J. OVATE family protein 8 positively mediates brassinosteroid signaling through interacting with the GSK3-like kinase in rice. *PLoS Genet June* 2016;12(6):e1006118.
- [41] Lee D, Bourdais G, Yu G, Robatzek S, Coaker G. Phosphorylation of the plant immune regulator RPM1-INTERACTING PROTEIN4 enhances plant plasma membrane H⁺-ATPase activity and inhibits flagellin-triggered immune responses in *Arabidopsis*. *Plant Cell July* 2015;27(7):2042–56.