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The drought-induced plasticity of mineral nutrients contributes to drought tolerance discrimination in durum wheat

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ABSTRACT

Drought is a major challenge for the cultivation of durum wheat, a crucial crop for global food security. Plants respond to drought by adjusting their mineral nutrient profiles to cope with water scarcity, showing the importance of nutrient plasticity for plant acclimation and adaptation to diverse environments. Therefore, it is essential to understand the genetic basis of mineral nutrient profile plasticity in durum wheat under drought stress to select drought-tolerant varieties. The research study investigated the responses of different durum wheat genotypes to severe drought stress at the seedling stage. The study employed an ionomic, molecular, biochemical and physiological approach to shed light on distinct behaviors among different genotypes. The drought tolerance of SVEMS16, SVEVO, and BULEL was related to their capacity of maintaining or increasing nutrient's accumulation, while the limited nutrient acquisition capability of CRESO and S.CAP likely resulted in their susceptibility to drought. The study highlighted the importance of macronutrients such as SO_4^{2-}, NO_3^-, PO_4^{3-}, and K^+ in stress resilience and identified variant-containing genes potentially influencing nutritional variations under drought. These findings provide valuable insights for further field studies to assess the drought tolerance of durum wheat genotypes across various growth stages, ultimately ensuring food security and sustainable production in the face of changing environmental conditions.

1. Introduction

Durum wheat (*Triticum durum* Desf.), a widely cultivated crop, is crucial for human nutrition, providing 20% of daily caloric intake and carbohydrates, proteins, vitamins, and minerals (Buffagni et al., 2020). Despite its adaptability to various climatic conditions, durum wheat cultivation in the Mediterranean area faces increasing environmental stressors, particularly drought (Aprile et al., 2013). Drought is a significant threat to crop production in many regions due to climate change (Zhang et al., 2018). Drought's impact on wheat has drawn attention from researchers and agricultural stakeholders due to its critical role in global food security, particularly with the increasing world population (Buffagni et al., 2020). Therefore, development of drought-tolerant wheat varieties is crucial for mitigating the impact of climate change on food production (Farooq et al., 2009).

Drought negatively impacts plant growth by reducing CO_2 assimilation, inducing oxidative stress and nutritional imbalances due to limited nutrient uptake at the root level, particularly nitrate, phosphorus, and potassium (Farooq et al., 2009). Thus, disrupting plant nutrient allocation and altering the concentration of nutrients in plant tissues (Nieves-Cordones et al., 2019). To maintain growth and productivity, plants can cope with drought stress by regulating ion homeostasis through nutrient uptake and utilization strategies accumulating an appropriate quantity of salts and water (Nieves-Cordones et al., 2019; Ghaffari, 2022). Particularly, inorganic cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) and anions (Cl^-, PO_4^{3-}, NO_3^-, and

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SO_4^{2-}) have a significant role in osmotic adjustment and ion homeostasis in response to osmotic stresses.

On the other hand, drought stress often leads to changes in root growth and morphology to enhance water uptake (Kou et al., 2022), and the production of antioxidants and osmolytes (De Carvalho et al., 2013). Indeed, proline accumulation in wheat plays a pivotal role in osmotic adjustment as plant adaptation to water scarcity (Maghsoudi et al., 2018; Quagliata et al., 2023). This amino acid can be synthesized through the enzyme pyrroline-5-carboxylate synthetase (P5CS) from glutamate (Meena et al., 2019). Interestingly, due to the overexpression of the *P5CS* gene, higher yield was associated with higher proline accumulation in wheat plants under severe drought stress (Maghsoudi et al., 2018). Nevertheless, some studies have reported a positive correlation between proline contents and stress tolerance, while others have found higher proline levels in less tolerant genotypes under stress conditions (Arteaga et al., 2020).

At the molecular level, plants activate several genetic responses to protect themselves against stress-related damage and enhance drought tolerance (Lawlor, 2013). The accumulation of dehydrins is considered an important strategy to resist water-deficit conditions (Abedini et al., 2017). Moreover, transcription factors (TFs) from the WRKY family, as well as those from the APETALA2/ethylene-responsive (AP2/ERF) family, such as SHINE1 and dehydration-responsive element-binding (DREB) members, have been extensively studied for their implication in stress tolerance. Furthermore, some of the genes encoding WRKY and DREB TFs have been used in transgenic approaches aimed at enhancing stress tolerance in model and crop plants (Wang et al., 2016). Among the AP2/ERF superfamily in Arabidopsis, the SHINE TFs regulate the expression of several wax biosynthesis genes to enhance drought resistance (Djemal and Khoudi, 2015).

Under drought conditions, plants increase the secretion of root exudates, such as organic acids, soluble sugars, amino acids, and other compounds, which enhance their drought resistance (Chen et al., 2022). Moreover, root exudates can improve soil nutrient availability and plant growth by altering the physical, chemical, or biological characteristics of the rhizosphere (Zhao et al., 2020).

The study of genetic diversity related to the nutritional plasticity in durum wheat is crucial in developing drought-tolerant genotypes. Natural variations in genes that respond to drought stress can affect their function, and studying genome-wide polymorphisms can reveal distinct tolerance responses among genotypes. Genetic screening methods like genotyping-by-sequencing (GBS) effectively navigate large genomes like durum wheat by reducing genome complexity using restriction enzymes, enabling the identification of novel variants (Pootakham, 2023).

This study explores the relationships between genetic variability among durum wheat genotypes, stress tolerance, and their capacity to exhibit nutrient plasticity in response to drought. Defining drought tolerance is a challenging issue, as the criteria for evaluating it depend on various factors such as the timing, duration, and intensity of drought stress, climatic conditions, measurement time, and plant phenological stages. Given that drought stress can reduce plant nutrient uptake, the plant's ability to effectively withstand stress may be linked to its ability to maintain nutrient uptake and homeostasis under drought. Therefore, the efficiency of water and nutrient transport across cell layers plays a crucial role in tolerance mechanisms (Bárzana and Carvajal, 2020). This aspect is even more important and critical during the plant seedling stage, as the processes involved in resource acquisition (water and nutrients) are fundamental for the establishment of the plants (Mahmood et al., 2022). Thus, this study aimed to characterize drought-induced plasticity in mineral nutrients by severe drought in durum wheat genotypes specifically at seedling stage. Recently, Quagliata et al. (2023) identified distinct clusters of drought-tolerant and sensitive durum wheat genotypes, characterized by deep-rooting and shallow-rooting systems. From those clusters, six tetraploid wheat genotypes - SVEVO, SVEMS16, SVEMS1, CRESO, ETRUSCO, and S.CAP (Senatore Capelli) - and one hexaploid tritordeum genotype (BULEL), differing for tolerance degree to drought and root architecture, were selected. Understanding genetic factors and physiological mechanisms involved in this phenomenon can help develop more resilient durum wheat genotypes, ensuring food security and sustainability in water-limited conditions.

2. Materials and methods

2.1. Plant materials

In this study, we selected seven wheat genotypes from 37 previously characterized for their sensitivity or tolerance to drought (Quagliata et al., 2023). The selected genotypes include SVEMS1 and BULEL (sensitive genotypes), CRESO, SVEVO, and SVEMS16 (tolerant), and ETRUSCO and S.CAP (intermediate performance). Seeds of six tetraploid *Triticum durum* genotypes (SVEVO, SVEMS16, SVEMS1, CRESO, ETRUSCO, and S.CAP) and one hexaploid tritordeum genotype (BULEL) were provided by the University of Tuscia (Italy) (Quagliata et al., 2023).

2.2. Plant growth and stress conditions

Seeds of the seven wheat genotypes were germinated on filter paper saturated with distilled water and incubated in the dark at 28 °C for four days. Seedlings were transplanted into plastic pots (3 seedlings per pot) containing 600 mL of a nutrient solution (Astolfi et al., 2018) and grown hydroponically under controlled conditions (26 °C, photoperiod of 16h light/8h dark and relative humidity of 65%). After 7 days, half of the plants were exposed for 7 days to drought stress (S) by adding polyethylene glycol PEG 6000 (10% w:v) to the medium, while the remaining half were kept in the control solution (C). To minimize nutrient depletion, all hydroponic solutions were renewed daily. Subsequently, plants were sampled, and their roots were gently blotted with paper towels after washing with distilled water. Shoots were separated from roots, and the fresh tissues were frozen in liquid N_2 and stored at −80 °C or analyzed immediately.

2.3. Physiological and biochemical measurements

Samples from the seven wheat genotypes were used for physiological and biochemical measurements. Chlorophylls and carotenoids were extracted from the shoot tissues of plants grown either under control or drought stress conditions as previously described (Parmagnani et al., 2022). For liquid chromatography of chlorophylls and carotenoids, a High-Performance Liquid Chromatography (HPLC) (1200 HPLC, Agilent Technologies, Santa Clara, CA, USA) coupled to a Diode Array Detector (DAD) was used to analyze samples. The molecules were separated, identified, and quantified as previously described (Pumilia et al., 2014).

Concerning stable carbon isotope discrimination ($\delta^{13}C$) analyses, leaf samples were processed and $\delta^{13}C$ values of the samples were measured using a Flash 2000 HT elemental analyzer coupled, via a ConFLo IV Interface, with a Delta V Advantage isotope ratio mass spectrometer (IRMS), interconnected to the software Isodat 3.0 (Thermo), according to Bononi et al. (2022).

Lipid peroxidation of root and shoot tissues was quantified by measuring the concentration of malondialdehyde (MDA) using the thiobarbituric acid (TBA) reactive metabolite method, as described in the study by Astolfi et al. (2005).

Proline concentration of root and shoot tissues was determined following the method described by Arteaga et al. (2020).

Cation and anion concentrations were determined in root and shoot tissues by capillary electrophoresis (Agilent 7100, Agilent Technologies, Santa Clara, CA, USA) (Fiorilli et al., 2022).

2.4. RNA extraction and qRT-PCR analysis

Total RNA was extracted and purified from roots and shoots of the

seven studied wheat genotypes, and first-strand cDNA synthesis was carried out as described by Maghrebi et al. (2021). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis of drought-responsive genes (*TdDHN15.3, TdP5CS, TdWRKY2, TdDRF1, TdSHN1,* and *TdPIP2.1*) and genes involved in sulfur homeostasis (*TdSULTR1;1*, *TdSULTR1;3*, *TdSAT1*, and *TdOASTL1*) was performed on first-strand cDNA in a 20 μL reaction mixture containing GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) and the specific primers, using QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative transcript level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the expression of the *TdActin* gene as a reference. Primers for qRT-PCR are listed in Table S1.

2.5. Collection and extraction of root exudates

For root exudates, we selected four representative genotypes of the main cluster identified using the hierarchical clustering analysis of the previous studied parameters. The root exudates of wheat seedlings of selected genotypes (BULEL, SVEMS16, SVEMS1, and CRESO) were collected according to Astolfi et al. (2020). After growing the wheat seedlings in a hydroponic medium, the roots were rinsed twice with distilled water for 1 min to remove any residual of the nutrient solution. Following the rinsing, roots were placed in the final sampling solution (50 ml Milli-Q water) and kept under glasshouse conditions for the entire sampling period (8 h). Finally, the Milli-Q water was collected, conserved at −20 °C, and lyophilized for the downstream analysis. The metabolomic profile was evaluated by using Ultra-High-Pressure Liquid Chromatography coupled to a Quadrupole-Time-Of-Flight mass spectrometer (UHPLC-QTOF-MS; Agilent, Santa Clara) as described by Cesco et al. (2021).

2.6. Genotyping-by-sequencing (GBS) and variant calling analysis

The DNA of the seven genotypes was extracted from the leaves with the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The DNA control quality, the genotyping-by-sequencing (GBS) library preparation, enzymatic digestion, and sequencing were performed by CD Genomics (45-1 Ramsey Road, Shirley, NY, USA). The restriction enzymes used for the DNA digestion were *PstI/Msp*I. The Sequencing-Illumina Novaseq system was used for sequencing (5 M PE150 reads).

The variant calling analysis was performed in a high-performance computing (HPC) cluster with 24 nodes Bull x440, 192 GB of RAM per node, and a total of 960 cores (Computing Center, University of Córdoba, Córdoba, Spain). First, the low-quality paired-end reads, and adapters were trimmed with fastp with default parameters (including the –detect_adapter_for_pe option) (Chen et al., 2018). The trimmed reads were mapped to the Svevo.v1 genome (Maccaferri et al., 2019) with BWA-mem (Li and Durbin, 2009). The quality control of the mapping step was performed with samtools (Danecek et al., 2021). The duplicates were marked with samtools before the variant calling analysis, which was performed with GATK software (Van der Auwera and O'Connor, 2020). The genomic Variant Call Format (gVCF) files were obtained by running the GATK HaplotypeCaller command with the -ERC GVCF option for all samples. The resulting gVCF files were combined (CombineGVCFs) to perform joint genotyping of the multi-sample gVCF (GenotypeGVCFs). The single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) were selected separately from the VCF (SelectVariants). The resulting VCFs were subjected to a hard-filtering process (VariantFiltration and SelectVariants –exclude-filtered): (i) SNPs: QD < 2.0, FS > 60.0, MQ < 40.0, SOR >3.0, MQRankSum < −12.5, ReadPosRankSum < −8.0; (ii) InDels: QD < 2.0, FS > 200.0, SOR >10.0, ReadPosRankSum < −20; and then were filtered by Genotype Quality (GQ) annotation allowing to keep variant sites if any genotype had a GQ upper than 20 with the SnpSift filter command (Cingolani et al., 2012a). The variants were annotated by snpEff (Cingolani et al., 2012b) to focus on variants directly affecting protein functionality.

2.7. Analysis of common variants between genotypes with similar responses under drought stress

The variant list was simplified by grouping variations that potentially affect protein activity under the same gene ID. This process provided a catalog of genes with or without variants for each genotype. Subsequently, we assigned a quantitative score to genes exhibiting variants, considering the phenotypic traits associated with each genotype. Conversely, genes lacking any modifications were assigned a null value. The scoring metric was computed as the binary logarithm (base 2) of the fold change observed in plants subjected to PEG6000 treatment relative to their control counterparts. We summed the scores of the seven distinct genotypes analyzed for each gene. Specifically, we focused on genes displaying extreme scores, a consequence of the presence of variants across multiple genotypes. These extreme-scoring genes were selected from the upper 5% with the highest scores and the lower 5% with the lowest scores. Next, selected genes were subjected to a gene ontology (GO) enrichment analysis using the gprofiler2 R package (Kolberg et al., 2020). The *P*-value for each term was adjusted by the Benjamini-Hochberg procedure to reduce the number of false positives, and a threshold of 0.05 or 0.001 was used for data interpretation. The heatmap containing GOs results was performed with the pheatmap R package (Kolde and Kolde, 2015). The Manhattan plots were constructed with ggplot2 (Wickham, 2011), and the upset plots with the UpSetR package for the sets representation and their intersections (Conway et al., 2017).

2.8. Data analyses

To describe the variation between the two experimental conditions, the logarithmic scale (base 2) of each ratio stress/control was calculated. Heatmap representations were performed on the group average clustering method and Euclidean distance method in NCSS software (NCSS, Kaysville, UT, USA) using the GraphPad Prism 8.0.1 (GraphPad Software).

For root exudate, the untargeted metabolomics analyses were conducted following the methodology outlined by Cesco et al. (2021). The chemometric interpretation of the metabolomic dataset was performed using Mass Profiler Professional B.12.06 software (Agilent, Santa Clara, CA, USA) for alignment, normalization and baselining. Subsequently, multivariate unsupervised hierarchical cluster analysis was employed on fold-change values to visualize similarities among treatments (HCA, Euclidean distance, Ward's linkage rule). Thereafter, the supervised modeling by orthogonal projections to latent structures discriminant analysis (OPLS-DA) multivariate modeling was carried out, and fitness parameters (goodness of fit: R2Y; goodness of prediction: Q^2Y; cross-validation: CV-ANOVA, $p < 0.01$), permutation test for overfitting ($n = 200$), and Hotelling's T2 (95% and 99 % confidence limit) for outliers were evaluated in SIMCA 16 software (Umetrics, Malmo, Sweden). Finally, a Volcano plot ($\alpha = 0.05$, Bonferroni multiple testing correction; fold change ≥1.2) was used for pairwise comparisons to identify differential compounds.

3. Results

3.1. Drought tolerance discrimination of durum wheat genotypes throughout physiological, biochemical, and molecular drought-related parameters

The variation in drought-induced responses in durum wheat genotypes was investigated by considering several physiological, biochemical, and molecular parameters. Drought significantly affected the biomass production of some genotypes, while others were not significantly affected (Fig. 1A and B). Only the BULEL genotype displayed a

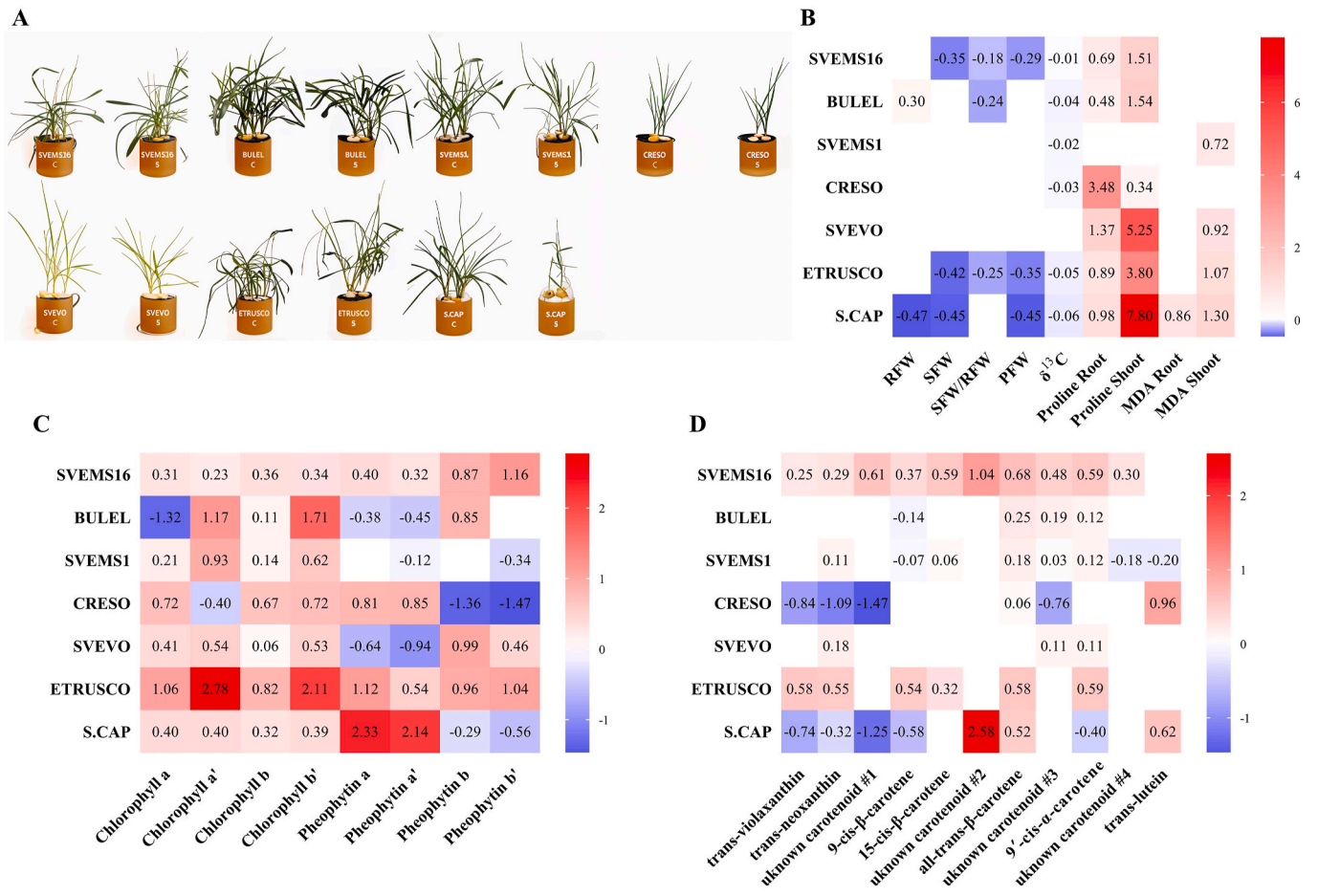

Fig. 1. **(A)** Comparison of the growth of seven durum wheat genotypes under control (C) and drought stress (S) conditions at early growth stages. Heatmap of relative physiological traits of *Triticum durum* plants in response to drought stress: **(B)** Relative biomass, $\delta^{13}C$, Proline, and MDA content **(C)** Relative chlorophylls and **(D)** carotenoids content was calculated as the ratio of stressed/control plants. Data are expressed as Log_2 fold-change. Only values significant for $p < 0.05$ are given, with the color gradient indicating values relative to control plants between Blue = low and Red = high. Blank cells in heatmap corresponded to non-significant variations in biomass or Proline and MDA content compared to control plants. Fresh weight of roots (RFW), shoot (SFW), whole plant (PFW), and SFW/RFW ratio, $\delta^{13}C$ (stable carbon isotope discrimination), proline, malondialdehyde (MDA), chlorophylls, and carotenoids of different durum wheat genotypes grown under control (C) and drought stress (S) conditions.

significant increase in root fresh weight (RFW) when subjected to drought stress, while the shoot (SFW) and whole plant (PFW) biomass were not affected compared to control plants. On the other hand, drought stress did not significantly affect root, shoot, or whole plant biomass of SVEMS1, CRESO, or SVEVO. Conversely, stress conditions significantly affected the fresh weights of roots and shoots of the S.CAP genotype. Notably, drought stress reduced the SFW/RFW ratio of SVEMS16, BULEL, and ETRUSCO genotypes compared to the control (Fig. 1B–Table S2).

The $\delta^{13}C$ variations among wheat genotypes are linked to variations in water use efficiency (Blum, 2009). We observed that plants grown under drought stress showed higher $\delta^{13}C$ values, indicating stomatal conductance changes, except for SVEVO's carbon isotope composition, which remained independent of growing conditions (Fig. 1B–Table S3).

Drought stress triggers the generation of reactive oxygen species (ROS), leading to lipid peroxidation evaluated by quantifying malondialdehyde (MDA) levels (Tirani and Haghjou, 2019). Drought stress increased MDA content in shoots of four genotypes, indicating severe lipid peroxidation, while water stress did not affect MDA content at the root level, except in S.CAP (Fig. 1B–Table S4). We then found that drought stress effectively induces proline accumulation in both root and shoot of six genotypes (Fig. 1B–Table S4), highlighting the plant's adaptive strategy to drought stress (Szabados and Savouré, 2010).

HPLC analysis detected functional chlorophylls (chlorophyll *a* and *b*) and their degradation products, including early-phase (chlorophyll *a*' and b'), medium-phase (phaeophytin a and b), and late-phase (phaeophytin a' and b') products (Fig. 1C–Table S5). BULEL and SVEVO varieties grown under drought stress had lower intermediate and late chlorophyll degradation products (−28% and −44%, respectively). The CRESO, ETRUSCO, and S.CAP varieties showed higher chlorophylls' medium and late degradation products under drought stress conditions compared to control conditions (51%, 110%, and 166%, respectively). Moreover, SVEMS1 and SVEMS16 showed reduced alterations in degradation product content. Regarding carotenoids, 11 compounds were detected, 7 identified, and 4 with a UV/Vis spectrum remain unknown (Fig. 1D–Table S6). BULEL and SVEVO showed only small variations in carotenoid content, while the other varieties had higher total carotenoid contents under drought stress, with low levels of trans-violaxanthin and trans-neoxanthin in CRESO and S.CAP.

In this study, we analyzed the expression of the drought-responsive genes linked to plant drought tolerance (Zhu et al., 2012; Szabados and Savouré, 2010; Yamaguchi-Shinozaki and Shinozaki, 2006). Under drought stress conditions, the expression of *TdDHN15.3* and *TdP5CS* was enhanced in SVEMS16, SVEMS1, and ETRUSCO shoot tissues, while it was increased in the root tissue of BULEL and SVEMS1 (Fig. S1). Similarly, *TdSHN1* genes showed a significantly higher expression only in the root of BULEL and SVEMS1. Moreover, the most pronounced down-regulation of *TdP5CS*, *TdWRKY2*, and *TdDRF1* in root tissues was

observed in CRESO and S.CAP genotypes. Remarkably, SVEMS1 showed a particularly interesting genetic response, being the only genotype with upregulated *TdDRF1* compared to the other genotypes under drought stress.

A clustered heatmap was generated (Fig. 2) and identified three main clusters (A-C), representing upregulated (cluster A, and B), and down-regulated (cluster C) relative expression of the *TdP5CS*, *TdDRF1*, and *TdWRKY2* genes in root tissues. Cluster A and B including SVEMS16, SVEMS1, ETRUSCO, and BULEL showed higher relative expression compared to other genotypes, while cluster C including CRESO, SVEVO, and S.CAP revealed a lower transcript abundance.

3.2. Drought-induced plasticity of inorganic ions content in durum wheat genotypes

To investigate the impact of water stress on the nutritional status of durum wheat genotypes, inorganic cations and anions content was measured in root and shoot tissues (Table 1a, b; Fig. S2).

Under drought stress, a significant increase of NO_3^- and PO_4^{3-} content was observed in the root of SVEVO. Similarly, this genotype exhibited increased content of SO_4^{2-} in the shoot. Moreover, a significant increase in SO_4^{2-} content was observed also in the shoot of BULEL. The imposition of drought stress resulted in higher K^+ content in the shoot of SVEVO, SVEMS1, and BULEL. Remarkably, only the S.CAP genotype showed a decrease in both K^+ and Cl^- content in root and shoot (Table 1 a, b; Fig. S2). Moreover, a clustered heatmap on inorganic ion content was performed and identified three main clusters (A–C) (Fig. 3). Cluster B included BULEL and SVEVO, which could be identified as the more tolerant genotypes, showing higher accumulation of SO_4^{2-}, NO_3^-, PO_4^{3-}, K^+ in both tissues with respect to other clusters. On the contrary, cluster C includes CRESO and S.CAP, which could be considered as the most sensitive genotypes, shows the lower accumulation of SO_4^{2-}, NO_3^-, PO_4^{3-}, K^+ in both tissues. Another main cluster, A, was identified, including SVEMS16, SVEMS1, and ETRUSCO which demonstrate a moderate tolerance by exhibiting maintained levels of SO_4^{2-}, NO_3^-, PO_4^{3-}, and K^+ in both tissues. Among nutrients displaying higher drought-induced variation in durum wheat genotypes, SO_4^{2-} allows for discrimination of the clusters identified in Fig. 3. Therefore, the effect of drought stress on SO_4^{2-} uptake and assimilation pathways was investigated by analyzing the expression of the genes coding for major transporters (*TdSULTR1;1* and *TdSULTR1;3*) and the enzymes involved in sulfur assimilation (*TdOASTL1* and *TdSAT1*) (Fig. S3). Interestingly, only BULEL showed an upregulation of the relative transcript abundance of *TdSULTR1;1* in the root tissues under drought conditions. At the shoot level, only the S.CAP genotype displayed an increased transcript level. Additionally, drought stress increased the *TdSULTR1;3* relative expression level in the root of BULEL and SVEMS16. After drought stress, only SVEMS16 exhibited an increased *TdOASTL1* and *TdSAT1* expression levels in root tissues, while only SVEVO showed a significant increase in the expression level of both genes at shoot tissues. Remarkably, the expression of all studied genes involved in sulfur homeostasis were significantly decreased in the root of S.CAP and CRESO under drought stress. Moreover, a clustered heatmap was performed on the relative expression of genes involved in sulfur homeostasis (Fig. 4) and identified two main clusters (A and B), representing genotypes displaying upregulation (cluster A) and down-regulation (cluster B) of these genes, especially in root tissue. Cluster A, including SVEMS16, BULEL, SVEVO, SVEMS1, and ETRUSCO, showed higher relative expression compared to other genotypes, while cluster B including CRESO, and S.CAP revealed lower transcript abundance.

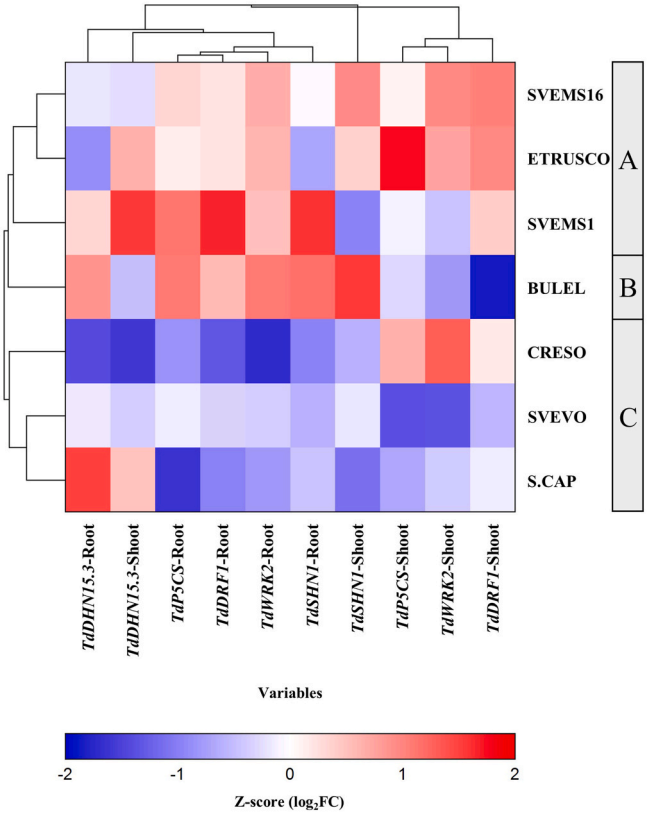

Fig. 2. Clustered heatmap representing hierarchical clustering of the relative expression of drought-responsive genes across seven wheat genotypes based on the Z-score of Log_2 fold-change values in response to drought stress (group average method and Euclidean distance measure). The 3 main clusters considered (A–C) were based on relative expression changes. The color bar describes the gradient of Z-score of Log_2 fold changes in response to drought stress.

3.3. Drought-induced variation of root exudates in durum wheat genotypes displaying differential S uptake and assimilation patterns

Drought-induced SO_4^{2-} uptake and assimilation modulation showed differential sulfur partitioning between root and shoot, suggesting differential modulation of root metabolism among genotypes which potentially affect root exudate synthesis. To further investigate this point, the exudate profiling of selected genotypes (BULEL, SVEMS16, SVEMS1, and CRESO) exposed to drought stress (S) was investigated (Fig. 5). Two different sub-clusters were obtained from the hierarchical clustering analysis, resulting from the interaction between plant genotype and treatment (C or S), separating control plants of SVEMS16 and BULEL from the rest of the genotypes under both conditions (Fig. 5A). In addition, the OPLS-DA supervised model (Fig. 5B) showed that stress treatment significantly impacted the exudomic profile of all genotypes showing that drought stress elicited marked effects on the metabolomic profile of the root exudates.

Several compounds have been identified with differential accumulation in stressed plants compared with control ones depending on the genotype. Specifically, results showed that the exudomic profiles of SVEMS16 and BULEL displayed larger variation than other genotypes under drought stress (119 and 81 compounds, respectively), and the analysis suggested that this effect was driven by 16 and 14 key metabolites for SVEMS16 and BULEL, respectively. However, CRESO displayed a very different exudation ability (31 compounds). The key compounds identified are reported in Supplemental material ES1 and Fig. S4. Moreover, each genotype exhibited a distinct signature in its exudate profile when subjected to drought stress. Particularly, 41, 17, 16, and 11 up-exuded metabolites were specific to SVEMS16, BULEL, SVEMS1, and CRESO, respectively. SVEMS16 and BULEL plants produced a common 37 up-exuded metabolites most of which were identified as glucosinolates, anthocyanins, cinnamates, flavanones, flavones, flavonoids, glycosides, and phenols (Fig. 5D–Supplemental material ES1). According to Fig. 5C, CRESO plants presented the lowest number

Table 1a
Root nutritional status. Anion and cation concentration in root tissues of different durum wheat genotypes grown under control (C) and drought stress (S) conditions. Data are presented as the mean ± SE (n = 5) and bold values indicate significant differences between control and stressed plants: *$p < 0.05$; Student's t-test. Anions (chloride (Cl^-), sulfate (SO_4^{2-}), nitrate (NO_3^-), phosphate (PO_4^{3-})) and cations (potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+})).

Genotypes	Conditions	Cl^-(mg g^{-1} DW)	SO_4^{2-}(mg g^{-1} DW)	NO_3^-(mg g^{-1} DW)	PO_4^{3-} (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	Na^+ (mg g^{-1} DW)	Ca^{2+} (mg g^{-1} DW)	Mg^{2+} (mg g^{-1} DW)
SVEMS16	C	4.09 ± 0.17*	0.99 ± 0.07*	41.55 ± 2.79*	11.79 ± 0.37*	**53.02 ± 4.28***	3.35 ± 0.40*	1.73 ± 0.21*	**1.78 ± 0.12***
	S	4.22 ± 0.34*	0.99 ± 0.03*	36.26 ± 3.23*	09.65 ± 1.55*	**39.48 ± 1.27***	2.39 ± 0.36*	1.64 ± 0.17*	**1.16 ± 0.10***
BULEL	C	4.20 ± 0.66*	1.56 ± 0.18*	**41.45 ± 2.89***	**11.38 ± 0.94***	**32.65 ± 1.44***	1.71 ± 0.25*	**2.16 ± 0.22***	**1.95 ± 0.27***
	S	4.87 ± 0.44*	1.77 ± 0.13*	**31.66 ± 2.49***	**08.53 ± 0.93***	**40.66 ± 1.83***	1.89 ± 0.36*	**1.64 ± 0.03***	**1.12 ± 0.04***
SVEMS1	C	3.90 ± 0.27*	1.49 ± 0.24*	35.09 ± 2.76*	13.63 ± 0.80*	48.55 ± 8.52*	**3.04 ± 0.39***	**3.16 ± 0.27***	**2.05 ± 0.24***
	S	3.49 ± 0.54*	1.36 ± 0.12*	32.45 ± 4.09*	13.72 ± 2.56*	35.20 ± 3.76*	**1.22 ± 0.22***	**2.07 ± 0.16***	**1.34 ± 0.05***
CRESO	C	2.50 ± 0.45*	1.75 ± 0.28*	**38.74 ± 5.12***	18.08 ± 2.82*	**54.54 ± 7.59***	1.21 ± 0.19*	**2.60 ± 0.11***	**1.62 ± 0.10***
	S	3.03 ± 0.41*	1.40 ± 0.14*	**21.78 ± 1.06***	12.64 ± 1.77*	**36.74 ± 1.49***	1.56 ± 0.55*	**1.38 ± 0.14***	**2.09 ± 0.17***
SVEVO	C	3.29 ± 0.17*	1.34 ± 0.07*	**22.65 ± 1.77***	**10.76 ± 0.76***	42.68 ± 3.64*	3.10 ± 0.69*	3.40 ± 0.43*	1.87 ± 0.16*
	S	3.62 ± 0.25*	1.54 ± 0.09*	**33.35 ± 2.48***	**14.68 ± 0.96***	46.45 ± 2.01*	2.77 ± 0.77*	3.21 ± 0.21*	2.02 ± 0.14*
ETRUSCO	C	4.58 ± 0.44*	1.43 ± 0.03*	**31.99 ± 2.77***	13.72 ± 0.45*	59.44 ± 2.90*	2.41 ± 0.59*	3.76 ± 0.28*	2.24 ± 0.15*
	S	4.00 ± 0.41*	1.33 ± 0.04*	**23.74 ± 1.69***	11.42 ± 0.99*	47.74 ± 8.03*	2.61 ± 0.93*	3.79 ± 0.46*	1.96 ± 0.21*
S.CAP	C	**4.72 ± 0.42***	**2.97 ± 0.29***	**39.90 ± 5.68***	**19.58 ± 0.79***	**46.79 ± 4.88***	2.16 ± 1.00*	4.21 ± 1.04*	**1.88 ± 0.23***
	S	**3.04 ± 0.06***	**1.25 ± 0.08***	**04.44 ± 0.15***	**08.71 ± 0.88***	**20.75 ± 5.80***	1.07 ± 0.42*	3.24 ± 0.62*	**1.44 ± 0.22***

Table 1b
Shoot nutritional status. Anion and cation concentration in shoot tissues of different durum wheat genotypes grown under control (C) and drought stress (S) conditions. Data are presented as the mean ± SE (n = 5) and bold values indicate significant differences between control and stressed plants: *$p < 0.05$; Student's t-test. Anions (chloride (Cl^-), sulfate (SO_4^{2-}), nitrate (NO_3^-), phosphate (PO_4^{3-})) and cations (potassium (K^+), sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+})).

Genotypes	Conditions	Cl^-(mg g^{-1} DW)	SO_4^{2-}(mg g^{-1} DW)	NO_3^-(mg g^{-1} DW)	PO_4^{3-} (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	Na^+ (mg g^{-1} DW)	Ca^{2+} (mg g^{-1} DW)	Mg^{2+} (mg g^{-1} DW)
SVEMS16	C	8.69 ± 0.60*	**2.59 ± 0.16***	57.86 ± 2.33*	31.80 ± 1.80*	75.22 ± 8.19*	2.38 ± 0.12*	4.28 ± 0.19*	2.06 ± 0.13*
	S	8.70 ± 0.20*	**2.21 ± 0.05***	53.11 ± 3.00*	28.60 ± 1.54*	78.14 ± 8.95*	2.76 ± 0.22*	4.34 ± 0.81*	2.12 ± 0.11*
BULEL	C	**8.58 ± 0.47***	**2.67 ± 0.10***	41.14 ± 3.33*	22.61 ± 1.43*	**54.42 ± 3.93***	1.47 ± 0.05*	**4.53 ± 0.31***	1.76 ± 0.16*
	S	**6.75 ± 0.05***	**3.17 ± 0.13***	42.10 ± 3.28*	23.69 ± 2.01*	**66.11 ± 1.75***	2.04 ± 0.57*	**3.55 ± 0.22***	1.83 ± 0.22*
SVEMS1	C	7.55 ± 0.60*	3.12 ± 0.35*	40.48 ± 3.50*	24.52 ± 0.64*	**44.62 ± 4.06***	1.92 ± 0.31*	5.55 ± 0.72*	**2.22 ± 0.13***
	S	7.97 ± 0.56*	2.71 ± 0.12*	40.53 ± 1.26*	28.34 ± 1.85*	**57.97 ± 2.10***	2.38 ± 0.41*	4.67 ± 0.13*	**1.77 ± 0.05***
CRESO	C	8.00 ± 0.54*	**3.47 ± 0.18***	**52.50 ± 5.47***	32.58 ± 5.64*	47.08 ± 6.80	2.19 ± 0.12*	4.53 ± 0.29*	1.73 ± 0.33*
	S	5.97 ± 0.96*	**2.43 ± 0.23***	**34.16 ± 5.07***	24.83 ± 3.37*	57.87 ± 2.97*	1.98 ± 0.41*	3.94 ± 0.11*	1.34 ± 0.10*
SVEVO	C	5.71 ± 0.62*	**3.28 ± 0.16***	27.99 ± 3.69*	15.44 ± 1.72*	**52.91 ± 5.78***	**3.08 ± 0.37***	5.26 ± 1.00*	2.10 ± 0.24*
	S	5.24 ± 0.16*	**3.86 ± 0.14***	27.08 ± 1.41*	16.26 ± 1.45*	**76.16 ± 6.78***	**4.48 ± 0.52***	6.21 ± 0.24*	2.42 ± 0.16*
ETRUSCO	C	6.79 ± 0.13*	3.75 ± 0.06*	29.70 ± 4.22*	13.65 ± 1.46*	58.48 ± 9.36*	3.69 ± 0.57*	4.57 ± 0.94*	1.98 ± 0.21*
	S	7.56 ± 0.55*	3.58 ± 0.22*	28.24 ± 1.09*	15.54 ± 0.75*	86.80 ± 14.29*	2.70 ± 0.86*	5.86 ± 0.97*	2.53 ± 0.32*
S.CAP	C	**8.32 ± 0.34***	**3.66 ± 0.03***	**31.65 ± 1.44***	**24.04 ± 1.27***	**100.84 ± 5.74***	2.18 ± 0.47*	**8.72 ± 0.78***	**4.06 ± 0.34***
	S	**6.14 ± 0.27***	**2.86 ± 0.07***	**14.29 ± 0.98***	**12.37 ± 0.43***	**060.22 ± 8.62***	3.38 ± 1.00*	**4.74 ± 0.49***	**2.00 ± 0.21***

of up-accumulated metabolites, with 11 compounds identified as amino acids, flavonoids, and lignans suggesting that CRESO has lower plasticity in managing root exudates under drought stress. In addition, 13, 5, 4, and 9 specific metabolites were down-accumulated in SVEMS16, BULEL, SVEMS1, and CRESO respectively (Fig. 5E–Supplemental material ES1) under drought stress conditions. From the total of down-accumulated metabolites in SVEMS16 and BULEL, two were commonly found in the root exudate comprising 6-methylthiohexyldesulfoglucosinolate (glucosinolate) and 5-tricosenylresorcinol (stilbene) (Fig. 5E).

3.4. Genetic variants in durum wheat genotypes

To explore the genetic diversity among genotypes, a genotyping-by-

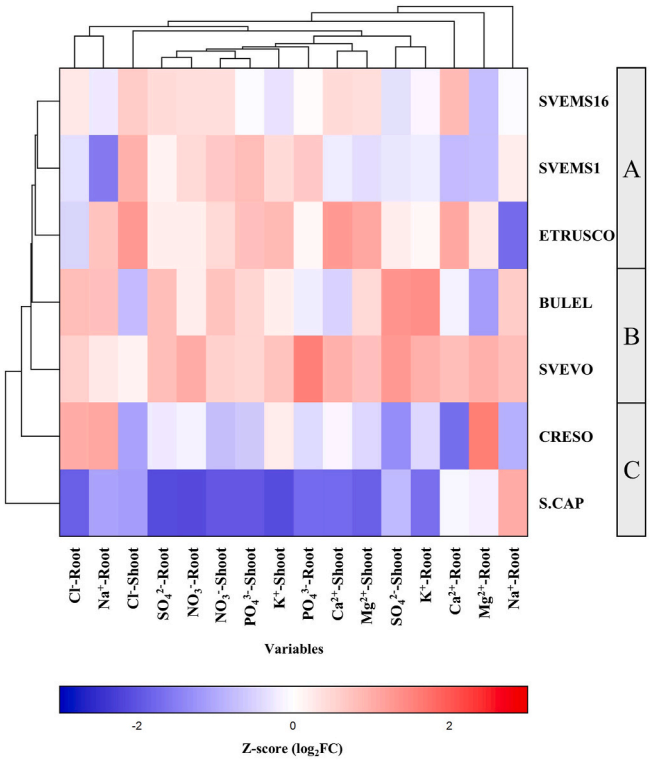

Fig. 3. Clustered heatmap representing hierarchical clustering of inorganic ion contents across seven wheat genotypes based on the Z-score of Log_2 fold-change values in response to drought stress (group average method and Euclidean distance measure). The 3 main clusters considered (A–C) were based on inorganic ion content changes. The color bar describes the gradient of Z-score of Log_2 fold changes in response to drought stress.

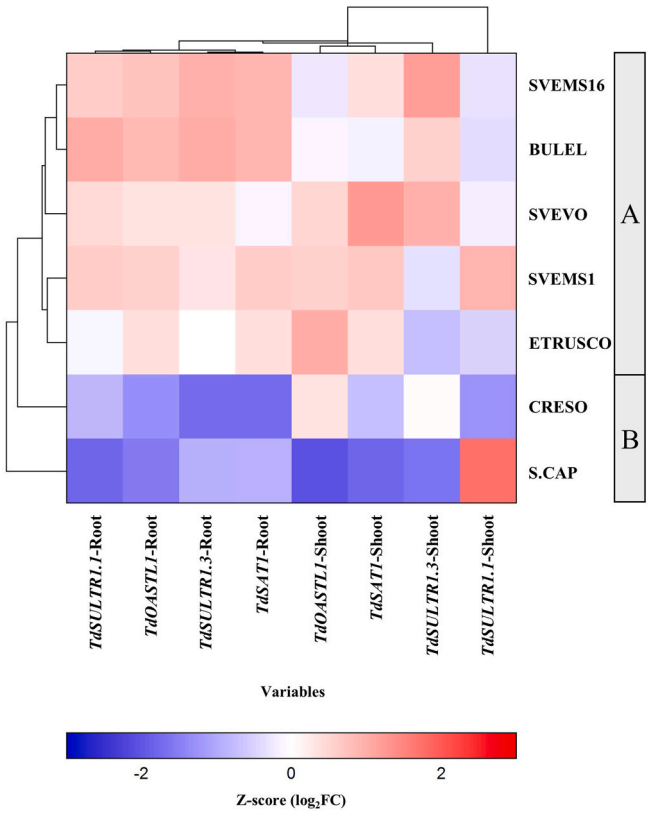

Fig. 4. Clustered heatmap representing hierarchical clustering of the relative expression of genes involved in sulfur homeostasis across seven wheat genotypes based on the Z-score of Log_2 fold-change values in response to drought stress (group average method and Euclidean distance measure). The 2 main clusters considered (A and B) were based on relative expression changes. The color bar describes the gradient of Z-score of Log_2 fold changes in response to drought stress.

sequencing (GBS) analysis was performed. The genome-wide variants found in the genotypes were mainly SNPs with a higher abundance in chromosomes 2 and 6 of the durum wheat B subgenome (Fig. S5A). The insertions and deletions comprised only 7.82% of the total variants, with a median length of 3 and 2, respectively (Fig. S5B). The largest insertion and deletion had lengths of 163 bp and 198 bp, respectively. The insertion was located on chromosome 3B and was conserved in CRESO and S.CAP. A high proportion of the identified variants were in the intergenic regions or within the 5000 bp up/down-stream of durum wheat genes (Fig. 6A). Notably, a large number of variants were in the protein-coding genes, leading to frameshifts, the introduction of stop codons, missense variations, or the loss of the start codon, among others (Fig. 6A). Focusing on variants affecting protein-coding regions and their up/down-stream regions, we conducted a gene ontology (GO) enrichment analysis to obtain a functional profile for each genotype. The resulting profile differences allowed the clustering of the seven genotypes into four groups (Fig. 6B). BULEL showed a distinctive enriched GO profile, with terms such as "response to stress" being less enriched, indicating a lower proportion of variants in these genes. Conversely, terms like "ABC-type transporter activity" were more enriched in BULEL (Fig. 6D). Although some variants were conserved across genotypes, the majority of them were unique for each genotype (Fig. S5C).

3.5. *Identification of common gene variants of genotypes with comparable nutrient plasticity under drought stress*

As denoted, there were significant differences in traits between wheat genotypes under control and stress conditions. The aim was to explore natural variation in the genotypes to identify common molecular variants that contribute to osmotic stress resistance. This could help to identify genes containing variants associated with important traits that are common across genotypes. To do so, we focused on variants directly affecting protein functionality from the list of variants identified through GBS analysis. In our approach, we specifically excluded upstream_gene_variants, downstream_gene_variants, and intron_variants. Our focus remained on variants with effects on protein-coding genes, encompassing categories such as conservative_inframe_deletion, conservative_inframe_insertion, disruptive_inframe_deletion, disruptive_inframe_insertion, frameshift_variant, missense_variant, splice_acceptor_variant, splice_donor_variant, splice_region_variant, start_lost, stop_gained, and stop_lost (Supplemental material ES2). Focusing on genes showing variants likely affecting protein functionality (from now on, called as VPFs), reduced the number of genes containing variants to work with to around 7500 genes. The VPFs were connected with measured parameters by assigning a score based on the trait value variation under stress conditions (PFW; SO_4^{2-} in Root and Shoot; K^+ in Root and Shoot; NO_3^- in Root and Shoot; PO_4^{3-} in Root and Shoot; Proline in Root and Shoot; MDA in Root and Shoot). For each trait, the VPFs matrix was scored based on the logarithmic fold change of each trait value in stressed versus control plants. This way VPFs present in several genotypes responding in similar ways to stress conditions for each trait resulted in extreme scores, *i.e.,* very negative or very positive. Conversely, GPVs present in genotypes not responding in similar ways to stress, resulted in scores close to 0. Therefore, we identified the VPFs with the extreme scores, indicating the presence of the variants in multiple genotypes responding in the same direction to the stress: (i) highest scores related to an increase in traits value; (ii) lowest scores corresponding to a decrease in traits value.

The VPFs common to genotypes with high increase or decrease in

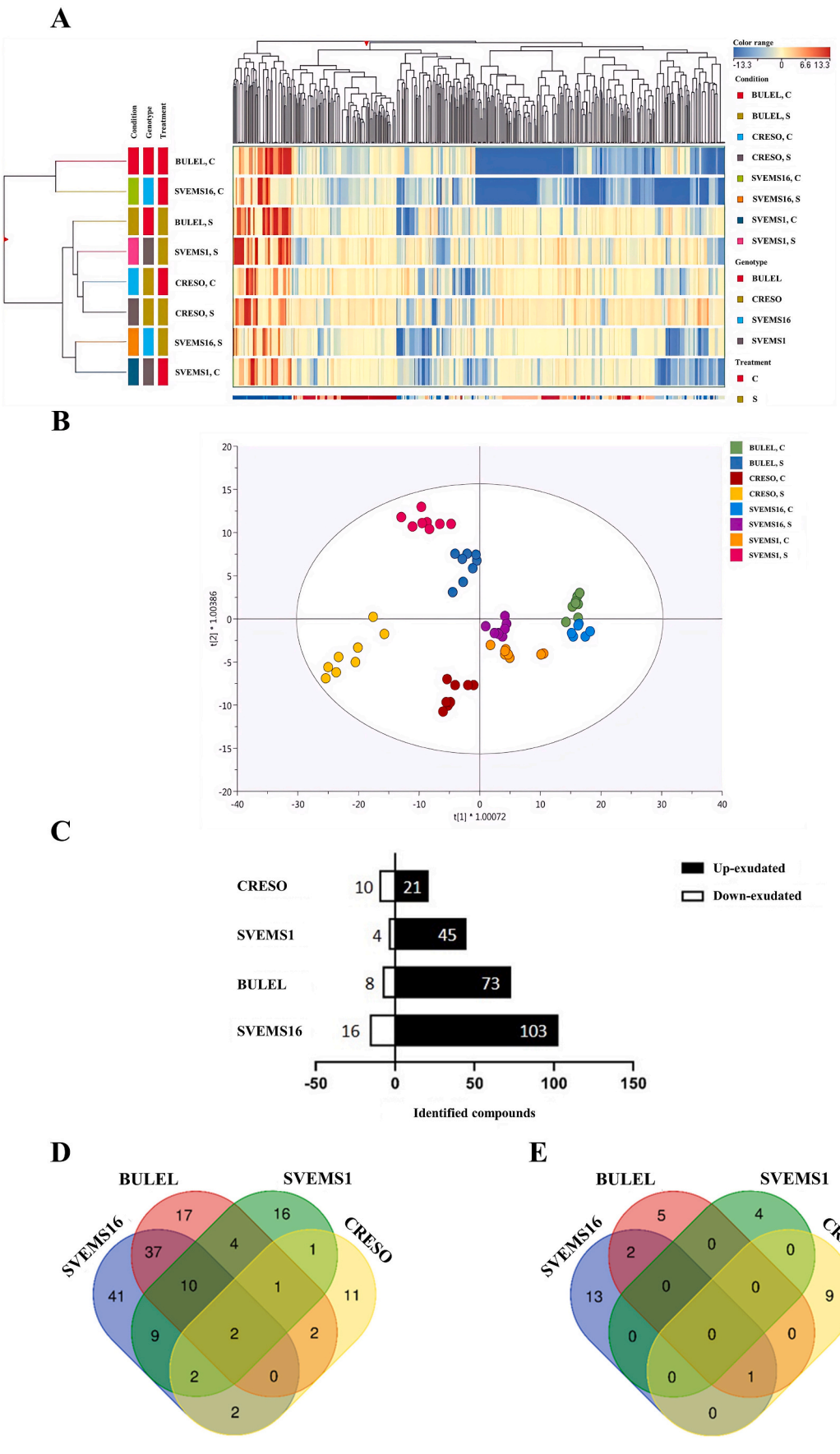

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Fig. 5. Multivariate statistics and interpretations of metabolites identified in the root exudates of durum wheat genotypes grown hydroponically under control (C) and drought stress (S) conditions. **(A)** Unsupervised hierarchical clustering analysis (HCA) based on fold-change heatmaps (Euclidean distance, Ward's linkage rule). Metabolites were obtained by Ultra-High-Pressure Liquid Chromatography Coupled to a Quadrupole-Time-Of-Flight Mass Spectrometer (UHPLC-ESI/QTOF-MS) untargeted analysis, and their intensities were used to build up the fold-change heatmap. **(B)** Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) supervised modeling carried out from untargeted metabolomics profiles of root exudates (correlation $R^2Y = 0.92$, prediction ability $Q^2Y = 0.79$). **(C)** Number of up-exuded and down-exuded metabolites per genotype under drought stress, Venn diagram of the metabolites identified differentially **(D)** Up-exuded and **(E)** Down-exuded under drought stress.

each trait were subjected to separate functional analysis. Among the GOs enriched (Supplemental material ES2), there were some of them over-represented for most traits ($P_{adj} < 0.001$) in both the set of VPFs related to traits value decrease (Fig. 7A) and VPFs related to traits value increase (Fig. 7B). These GOs corresponded to anion/ion/ADP binding, response to stress/stimulus, and defense response. Interestingly, these GOs were enriched for ion-related traits in the set of VPFs associated with trait decrease and for proline and MDA in the set related to trait increase, except for K^+ in shoots (Fig. 7A).

To discover common mechanisms in the stress response involving the PFW and ion concentrations, we identified common elements among the set of VPFs connected with each trait (Fig. 8). Interestingly, we identified groups of traits characterized by a high number of common VPFs, such as the decrease in NO_3^-_Root, PO_4^{3-}_Root, and PO_4^{3-}_Shoot (94 genes), or the decrease in PFW, SO_4^{2}_Shoot, K^+_Root, PO_4^{3-}_Root, NO_3^-_Root and NO_3^-_Shoot (68 genes). At the same time, we also observed clusters of GPVs associated with a single trait, such as K^+_Shoot, Proline_Shoot, and MDA_Shoot with more than 200 VPFs each (Fig. 8A). Similarly, we observed similar association in the increase of PFW, SO_4^{2-}_Root, K^+_Root and SO_4^{2}_Shoot (208 genes), Proline_Shoot, Proline_Root and MDA_Shoot (147 genes) and a peak of 1361 common VPFs between the increase of PO_4^{3-}_Root and NO_3^-_Root traits value (Fig. 8B). We selected these two groups of common genes related to the increase in ion/nutrient concentration, along with the first two sets mentioned previously for the decrease-related genes. For these sets of genes, the functional study did not retrieve any significant GOs after the enrichment analysis ($-Log_{10}(P_{adj}) > 1.3$); however, the top 20 terms with the highest $-Log_{10}(P_{adj})$ values were represented for each set in a heatmap (Fig. 8C). In this heatmap, there were no terms with cell values higher than zero in more than one set, except for adenyl nucleotide binding and purine nucleotide binding, which were present among the decrease-related unique genes in the K^+_Shoot set and the increase-related genes in the PO_4^{3-}_Root and NO_3^-_Root set (Fig. 8C).

4. Discussion

Plant drought tolerance is influenced by various metabolic and physiological changes, necessitating comprehensive understanding of plant responses, particularly the balance between water and nutrients. In this study, different wheat genotypes were characterized for many physiological, biochemical, and molecular traits.

Drought impacts stomatal conductance, limiting photosynthesis and reducing biomass production (Ghotbi-Ravandi et al., 2014). Similar to Quagliata et al. (2023), the different wheat genotypes showed varying degrees of drought tolerance, ranging from S.CAP, showing the highest decrease in biomass production, to SVEMS16, showing the lowest decrease. Moreover, SVEMS16, BULEL, and ETRUSCO adjusted biomass allocation under drought by decreasing the shoot/root ratio and thus focusing on root growth at the expense of above-ground growth (Poorter et al., 2011).

The observations were confirmed at the physiological level by determining the leaf's carbon stable isotope composition ($\delta^{13}C$), a reliable indicator of stomatal conductance (Rasheed et al., 2012). S.CAP showed the highest increase in $\delta^{13}C$ during drought, indicating greater stomatal closure, reducing transpiration rate and CO_2 availability for carboxylation sites compared to other genotypes (Richards et al., 2002). The other genotypes displayed a lower variation in $\delta^{13}C$, suggesting that they could possess different traits enabling the plant to mitigate the adverse impacts of drought stress. For instance, the accumulation of K^+ in SVEVO, SVEMS1, and BULEL shoots may aid plants in tolerating drought conditions (Table 1; Fig. 2) by regulating stomatal dynamics and osmoregulation (Sardans and Peñuelas, 2015). Accordingly, the SVEVO genotype was able to keep its stomata open during drought as it exhibited negligible changes in $\delta^{13}C$ under stress.

The impact of drought stress was extremely evident in S.CAP, where the reduction in shoot and root biomass was associated with the highest increase in MDA concentration in both root and shoot (Fig. S6), indicating a low antioxidant ability and drought tolerance (Marcińska et al., 2012). Additionally, S.CAP exhibited the greatest increase in proline accumulation when exposed to drought, suggesting high drought susceptibility (Arteaga et al., 2020). The drought response mechanism of this genotype involved the stomatal closure, leading to a reduction in nutrient translocation and their accumulation in the shoot and subsequently affecting plant growth, while increasing the accumulation of osmo-compatible solutes. It has been suggested that plants primarily synthesize proline in leaves, which can then be transported to the root to cope with water stress (Wang et al., 2022). In the experimental conditions, S.CAP seems to exhibit a decreased proline translocation. In contrast, CRESO showed higher proline content in root with a minimal increase in leaf content, indicating that this genotype likely sustains proline transport to the root to withstand drought. Despite the higher proline levels in S.CAP genotype, a lower expression of *TdP5CS* gene was observed (Fig. 1B,S1, and S7), suggesting that proline accumulation is not solely dependent on increased biosynthesis via the P5CS pathway. Additionally, the two homologues of genes encoding for P5CS (*TRITD1Av1G174960* and *TRITD1Bv1G160830*) exhibited variants in the upstream regulatory region in S.CAP. These variants could potentially be associated with the gene's low expression in this genotype. These findings align with previous reports indicating that enhanced proline import from other sources could contribute to accumulation without impacting biosynthesis genes (Wang et al., 2015). Moreover, regulatory mechanisms, such as post-transcriptional or post-translational modifications, may influence proline metabolism and accumulation in drought-sensitive genotypes (Strizhov et al., 1997; Wang et al., 2015).

Furthermore, our study reveals that BULEL, SVEMS16, and SVEVO genotypes maintained stable carotenoid levels under stress, where BULEL and SVEVO showed lesser chlorophyll degradation products, while are not affected in SVEMS16. These results suggest that plants can maintain carotenoid homeostasis, which in turn ensures the chemical stability of chlorophyll *a* and *b* (Yang et al., 2021). In contrast, CRESO, ETRUSCO, and S.CAP showed increased total carotenoid content (Table S6) and chlorophyll degradation products (Table S5) indicating that stress overcame the enhanced carotenoid production. Interestingly, CRESO and S.CAP were found more vulnerable to stress as revealed by the decrease in stomatal conductance, chlorophyll degradation, and carotenoid accumulation, and down-regulation of drought-responsive genes. On the other hand, despite their variability in the stomatal closure, SVEMS1, BULEL, and SVEMS16 actively coped with drought by up-regulating drought-responsive genes, limiting chlorophyll degradation and maintaining or increasing carotenoid contents under stress.

Exposure to drought stress can cause altered nutrient concentration in plant tissues where inorganic ions play a crucial role in osmotic homeostasis to cope with stress (Nieves-Cordones et al., 2019). Therefore, the impact of drought stress on plant nutritional status was evaluated to investigate the link between stress tolerance and the ability to modulate nutrient homeostasis among different genotypes (Table 1, Fig. S2). The

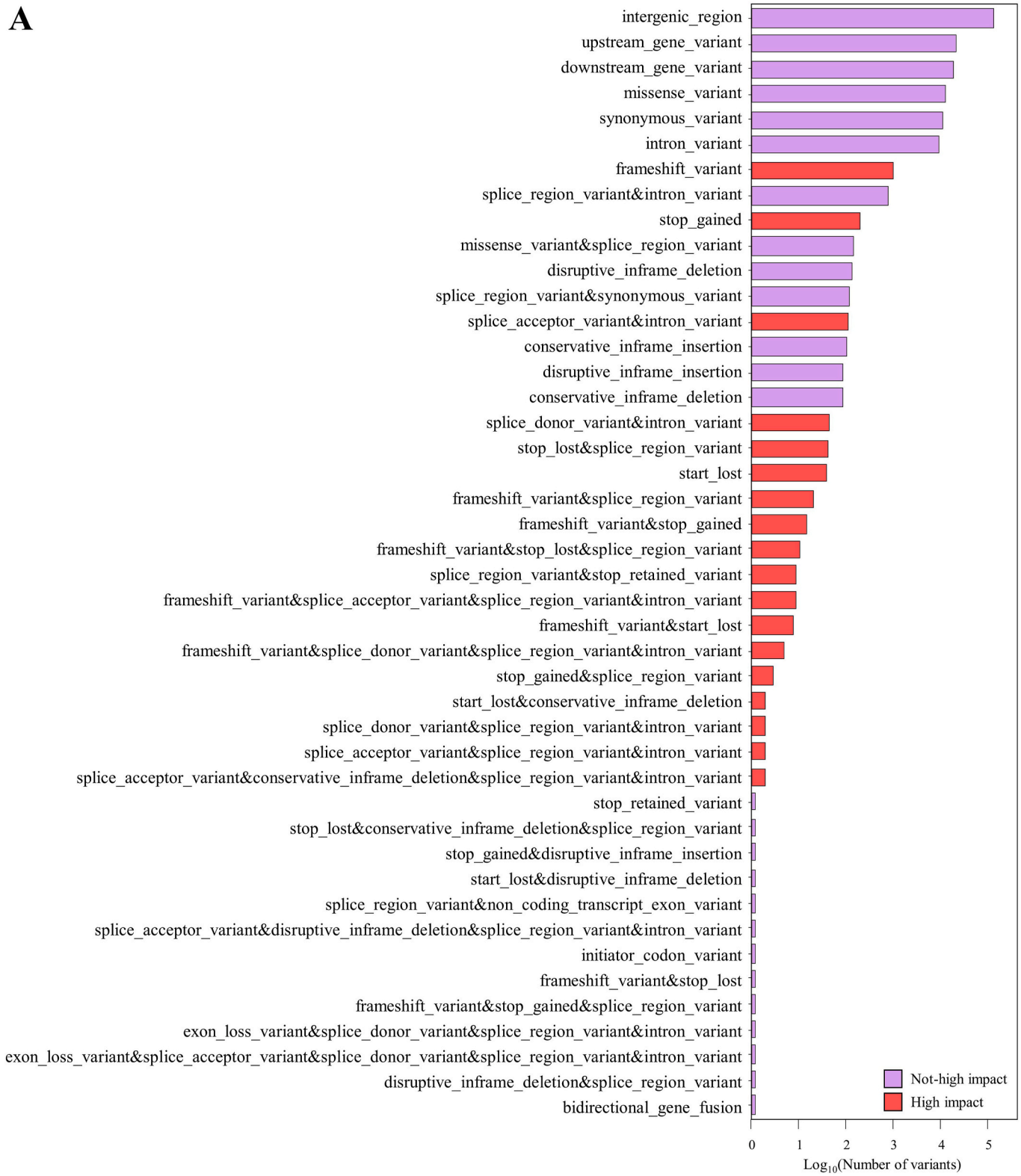

Fig. 6. **(A)** The Log_{10} (Number of variants) based on their effect. The impact level of the effects was highlighted. **(B)** Gene Ontology (GOs) enriched in each genotype based on the variants found in the protein-coding genes and their flanking regions.

clustered heatmap separates the genotypes based on a few discriminants (SO_4^{2-}, NO_3^-, PO_4^{3-}, and K^+) in both organs. Cluster A included genotypes (SVEMS16, ETRUSCO, and SVEMS1) in which the ion concentration did not change under stress, while cluster B included those (SVEVO and BULEL) showing ion accumulation under stress. Finally, CRESO and S. CAP, belonging to cluster C, showed reduced ion accumulation under drought stress (Fig. 3). Additionally, Franco-Navarro et al. (2015) suggested that the accumulation of Cl^- is a potential strategy to withstand

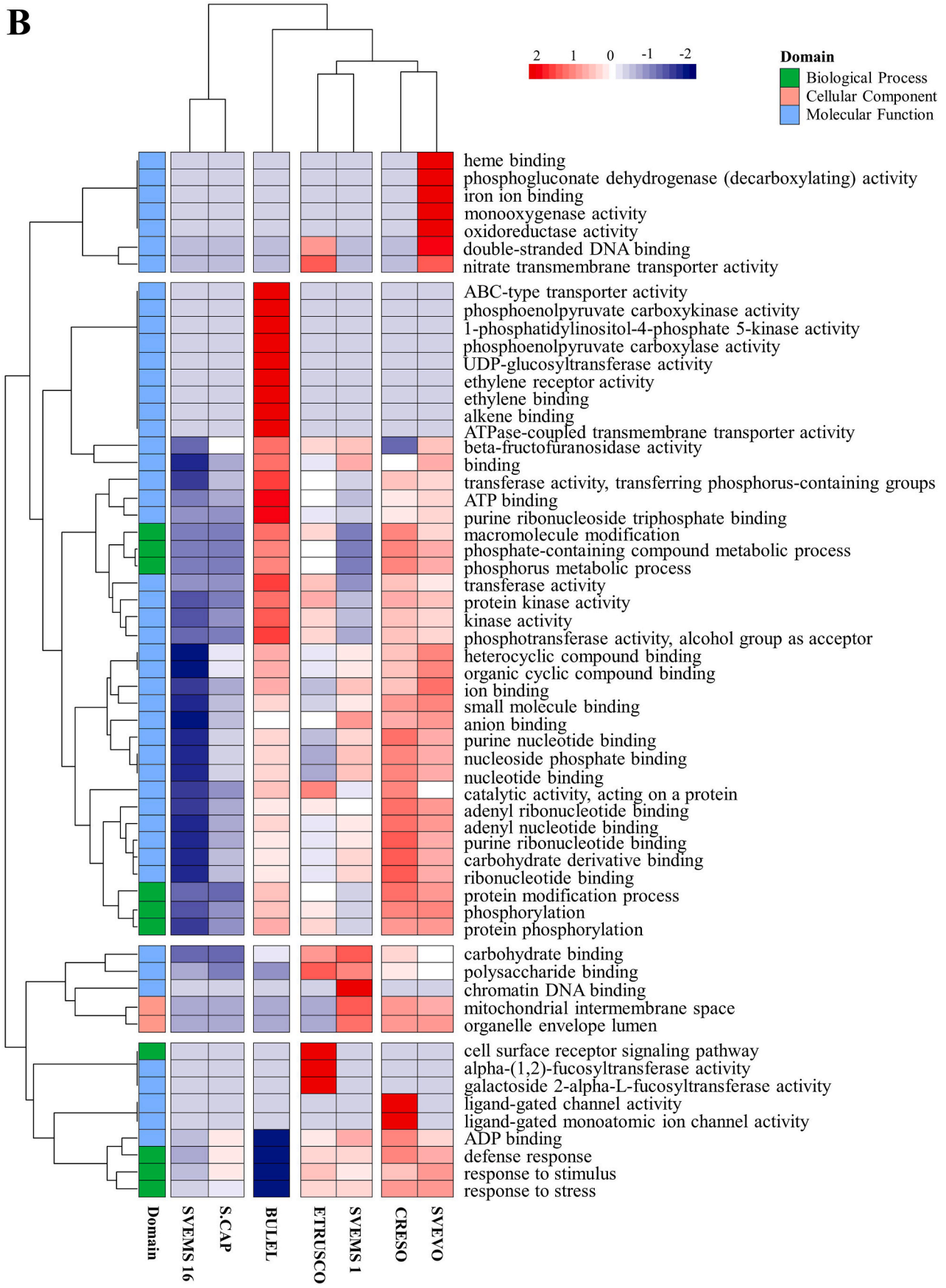

Fig. 6. (*continued*).

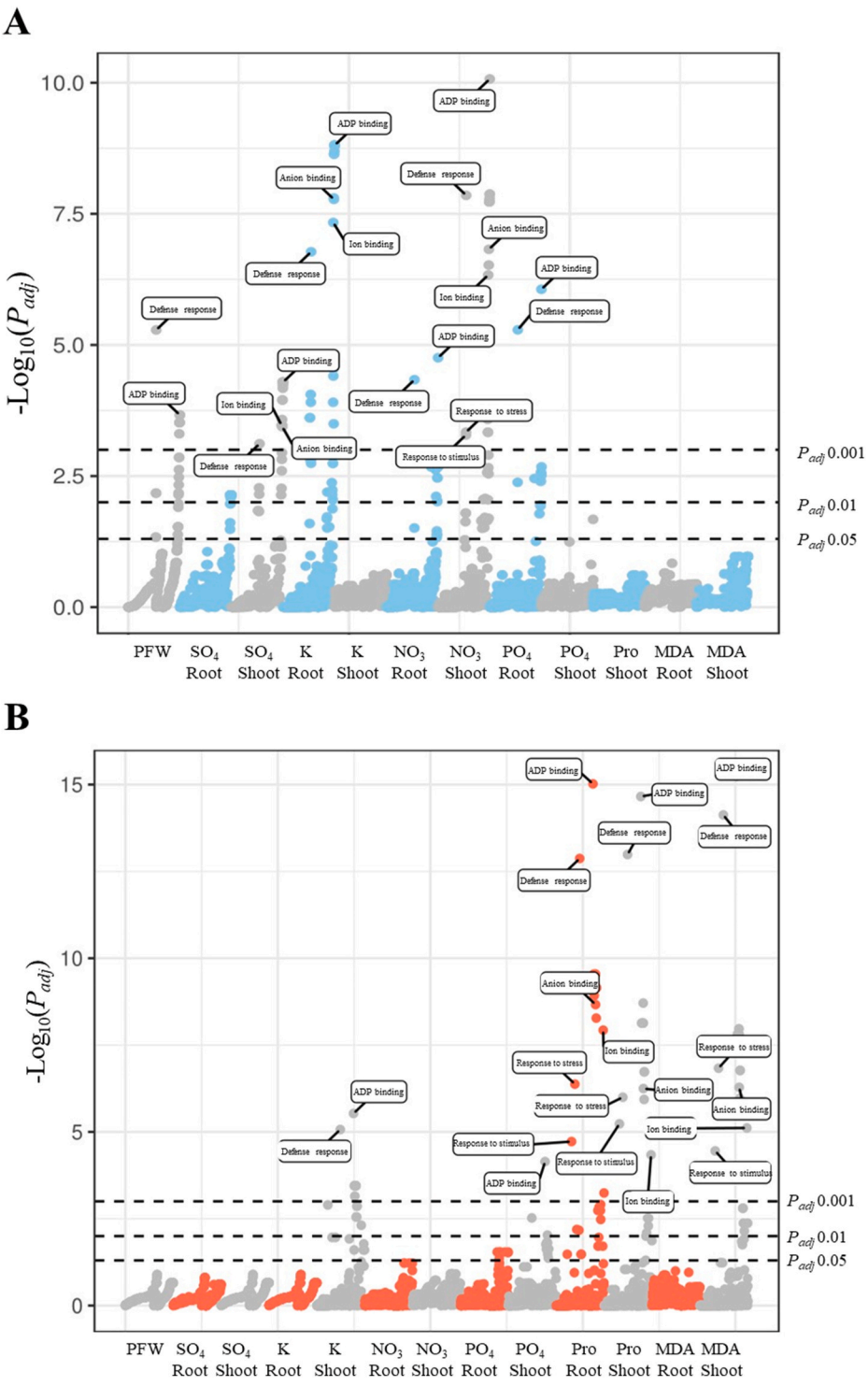

Fig. 7. Gene Ontology (GO) enrichment analysis of the variant-containing-genes related to **A)** the decrease and **B)** the increase of each trait under drought stress. The dashed lines represent the thresholds of P_{adj}-values considered in the analysis. Gene Ontology (GO) terms such as ADP/ion/anion binding, defense response, and response to stress/stimulus were indicated in the plots. The complete list of enriched Gene Ontology (GOs) per set of genes is available in Supplemental file_ES2.

water deficit and to improve plant WUE. In the present study, the significant decrease in Cl^- content in S.CAP, associated with reduced biomass production and essential nutrients accumulation (NO_3^-, PO_4^{3-}, SO_4^{2-}, and K^+) under drought, supports this idea. On the other hand, most genotypes' organs exhibited relatively stable Cl^- concentration under drought, enabling them to withstand drought deficit. Interestingly, SVEVO had a greater ability to take up and accumulate both NO_3^- and PO_4^{3-} in roots (Table 1, Fig. S2). It is well known that plants regulate osmotic balance by adjusting the content of inorganic anions, which act as osmolytes in their cells (Courbet et al., 2019). Accordingly, under sulfur deficiency, NO_3^- and PO_4^{3-} accumulate in the vacuole to compensate for the remobilization of vacuolar SO_4^{2-} to young tissues and maintain plant growth (Sorin et al., 2015). The accumulation of SO_4^{2-} in plant tissues under drought, except for CRESO and S.CAP (Fig. 3), is crucial for stress tolerance through the synthesis of S-containing antioxidant or protective molecules (Ahmad et al., 2016), but also for

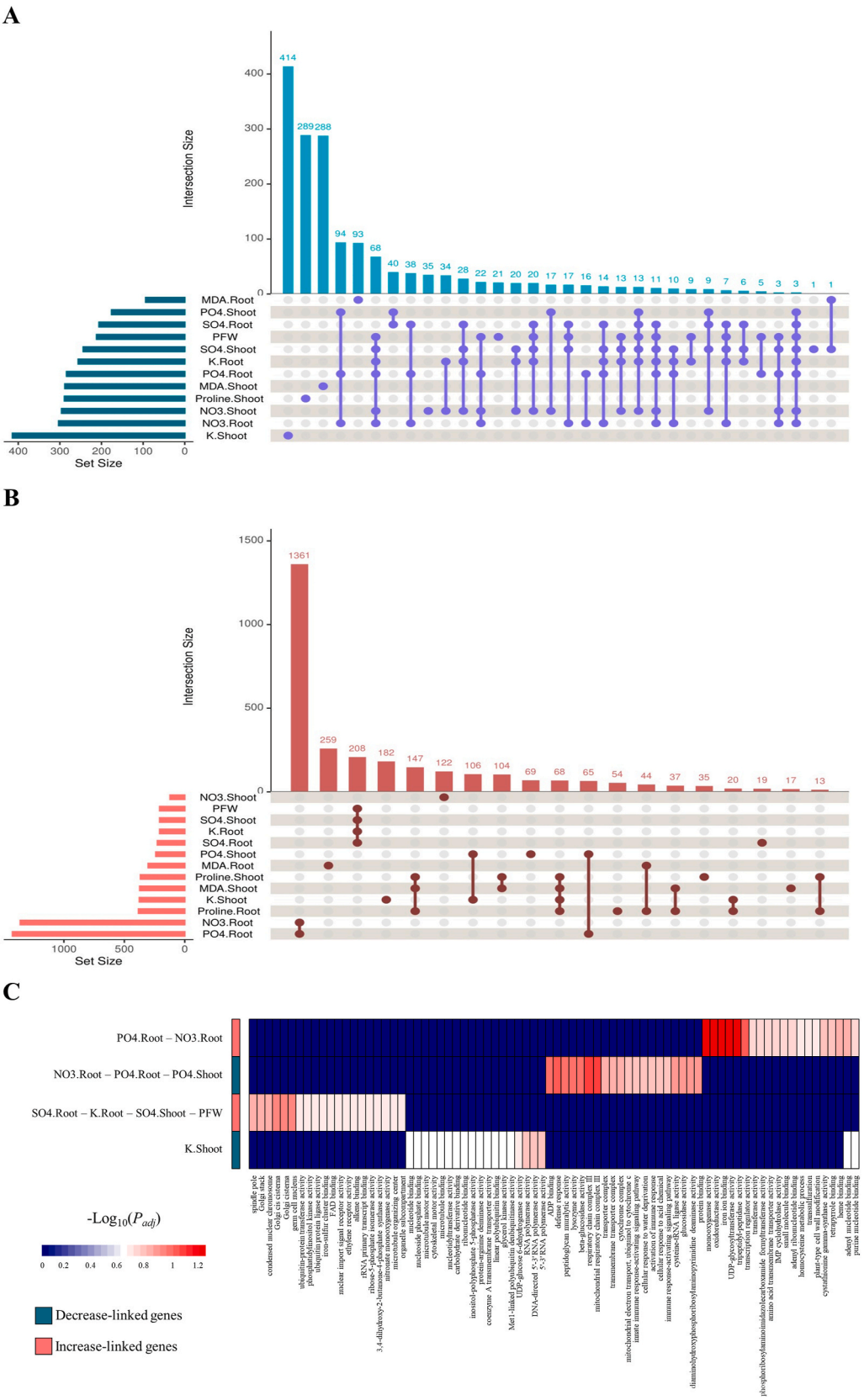

Fig. 8. Upset plot with the common variants-containing genes among traits in relation to **(A)** the decrease or **(B)** increase of ion/nutrient concentration under drought stress. **(C)** Heatmap with the $-\text{Log}_{10}(P_{adj})$ (Benjamini-Hochberg procedure) values obtained from the Gene Ontology (GO) enrichment analysis of gene sets selected from the upset plots.

stomatal regulation (Usmani et al., 2020).

Notably, several genes containing variants (*i.e.*, genes affected by variants located in their protein-coding region) related to sulfur homeostasis and metabolism are over-represented in the genes' sets connected to combinations of nutrient-related traits such as NO_3^-, PO_4^{3-}, K^+, and SO_4^{2-} (Supplemental material ES3). However, the variation of SO_4^{2-} alone was related to a few variant-encoding genes: 18 at the root level (including MYB TFs, sulfite reductase, amino acid transporters, and sugar transporters) and 2 genes at the shoot level with aspartic-type endopeptidase-related activity (Supplemental material ES4). In durum wheat plants, sulfur nutrition is essential for the quality of dough and baking due to its impact on the composition of grain proteins (Klikocka et al., 2016). Thus, we investigated the transcriptional regulation of the sulfate uptake, translocation, and assimilation pathway (Figs. S3 and 4). The high-affinity sulfate transporter *SULTR1;1* is a highly sulfur-responsive gene (Ciaffi et al., 2013), up-regulated in maize plants under drought stress, and affecting sulfate uptake by roots and sulfur distribution within cells and tissues in common wheat (Büchner et al., 2010). Accordingly, the relative transcript abundance of *TdSULTR1;1* increased under drought in BULEL roots and to a greater extent in S.CAP shoots, suggesting an increased sulfate uptake likely driven by a higher drought-induced sulfate demand in the former, and an increased sulfate remobilization and transport within the plant in the latter. On the other hand, the expression of *TdSULTR1;3* was up-regulated by drought in the roots of both BULEL and SVEMS16. SULTR1; 3 is responsible for loading sulfate into the phloem and allocating it to sink organs (Ciaffi et al., 2013), and may also provide sulfur for protective mechanisms like antioxidant response (Henriet et al., 2021). Interestingly, under drought stress the higher expression of *SULTR1;3* in the root of BULEL was associated with higher sulfate concentration in the shoot as compared to the control. The S assimilatory pathway involves the synthesis of cysteine, a crucial step facilitated by *O*-acetylserine (thiol) lyase (OASTL), which combines sulfide and O-acetylserine, synthesized by serine acetyltransferase (SAT) (Ahmad et al., 2016). Both *TdSAT1* and *TdOASTL1* are preferentially expressed in shoots (Ciaffi et al., 2013) since the sulfate reduction pathway is thought to occur mainly in leaves (Howarth et al., 2008). Drought stress resulted in the up-regulation of both *TdSAT1* and *TdOASTL1* in root tissues of SVEMS16, most likely to favor the assimilation of sulfate absorbed by roots or translocated from shoot to the root, thus allowing the production of sulfur-containing compounds, such as cysteine and glutathione with antioxidant activity. On the other hand, at the shoot level, the expression of *TdOASTL1* was up-regulated in SVEVO, ETRUSCO, CRESO, and SVEMS1. Interestingly, SVEMS16, ETRUSCO, BULEL, and SVEMS1, showing higher accumulation of SO_4^{2-}, NO_3^-, PO_4^{3-}, and K^+ in both organs, belonged to the cluster with up-regulated genes involved in sulfur homeostasis. On the other hand, CRESO and S.CAP, with lower accumulation of these elements in both tissues, belonged to the cluster with down-regulated genes involved in sulfur homeostasis.

Under drought stress, sulfur and S-containing compounds modify root exudates to recruit beneficial microbes and enhance plant stress resistance (Ulrich et al., 2022). Considering the crucial role of sulfur metabolism for drought-induced responses in plants (Chan et al., 2013), we investigated the exudomic profile of some genotypes displaying a differential modulation of sulfur metabolism: BULEL and SVEMS16 (high induction in root), SVEMS1 (moderate induction in both root and shoot), and CRESO (low induction). Under drought, there is a differential partitioning of sulfur assimilation between root and shoot genotypes, with higher root sulfur assimilation observed in BULEL and SVEMS16. Fig. S4 confirmed this relationship where more significant alteration was observed in the genotypes with highly induced sulfur assimilation in the roots (BULEL and SVEMS16), while CRESO exudome was less modulated by drought stress. Lin et al. (2023) found that root exudate composition reflects the plant's response to drought. Noteworthy, chemical similarity enrichment revealed moderate diversity in stress response metabolites, with sulfur-containing and related compounds being the most abundant, including amino acids, glycosides, glucosides, glucosinolates, and phenolics. Ulrich et al. (2022) suggested that the release of S-containing compounds in root exudates, often secondary metabolites, is related to drought severity. Under drought, the SVEMS16 genotype released 41 metabolites, including 4-hydroxycoumarin, 3-hydroxymugineic acid, 4-hydroxycoumarin, and gallic acid 4-O-glucoside. The 4-hydroxycoumarin is an allelopathic compound known to enhance nutrient acquisition by interacting with root microbiota specifically in the acquisition of iron by chelation and/or reduction of Fe (III) to Fe (II) (Harbort et al., 2020). Accordingly, 3-hydroxymugineic acid is known to be excreted by grasses to bind Fe in the root environment favoring its acquisition under Fe deficiency and drought stress conditions (Kumari et al., 2024). Moreover, gallic acid 4-O-glucoside is known to help plants deal with stressful conditions by controlling water influx, thus improving drought tolerance (Pratyusha, 2022). This suggests that when SVEMS16 experiences drought, it induces the release of metabolites facilitating nutrient uptake from the soil, including iron. Additionally, organic acids, including gluconic acid, have been detected in root exudates of wheat plants, and their production increases under drought stress (Yahya et al., 2021) most likely to help plants to resist osmotic stress by solubilizing phosphate (Chen et al., 2022). The up-exuded metabolites of the BULEL genotype under drought included malvidin 3-O-glucoside (anthocyanins), apigenin 6,8-di-C-glucoside (apigenin), and quercetin (flavonol) known to shape root microbiome to cope with drought (Wang et al., 2022). Overall, the modulation of root exudate profile is a well-known adaptive mechanism that plants use to respond to various stresses (Kopřivová and Kopriva, 2022). It has been observed that exudation profiles are influenced by plant mineral nutrition, including sulfate availability, due to the strong link between soil sulfatase activity and microbiome function. For instance, phenolic metabolism, glucosinolate profile, and sulfur assimilation are correlated in Arabidopsis (Pant et al., 2014) and durum wheat (Bernardo et al., 2019) plants. In our experiments, glucosinolate accumulation in root exudates was directly linked to sulfur availability, particularly in BULEL and SVEMS16 genotypes, while SVEMS1 and CRESO have intermediate and negligible accumulation, respectively. The presence of glucosinolates in the wheat exudome was unexpected and further investigations are required to fully understand this finding.

Most of the genomic variants found in the durum wheat genotypes were in the intergenic regions, potentially affecting the regulation of gene expression (Marques and Ponting, 2014). A smaller proportion of variants were in the protein-coding region, leading to frameshift mutations by nucleotide insertions or deletions. These mutations may result in non-functional or even detrimental protein products (Maki, 2002). Most variants were unique to a single genotype, providing valuable information for genotyping, especially in terms of drought tolerance. The GBS analysis facilitated the identification of the common variants among genotypes with the largest trait variations, focusing on the biological relevance of the sets of genes containing them. We aimed at exploring the natural variation characterizing the seven wheat genotypes (as representative of a wheat population previously characterized) to identify common molecular mechanisms involved in drought stress resistance. The GO terms revealed significant enrichment in terms related to response to stress and anion binding, suggesting that the genetic variability could lead to the observed phenotype plasticity among genotypes. Notably, common variant-containing genes related with the variation in NO_3^- and PO_4^{3-} content at the root level were identified. Among these, we found genes encoding for transporters (Fig. S8B) linked to regulating water transport in plants. It has been reported that under drought stress, aquaporins (encoded by PIP genes) interact with nutrient transporters to enhance tolerance (Fig. S8C) (Bárzana et al., 2020). PIP expression is controlled by both external and internal NO_3^- concentrations, and the up-regulation of PIP expression is linked with increased root hydraulic conductivity stimulated by NO_3^- (Li et al., 2016). Accordingly, in this work, the expression of the PIP2.1 gene was down-regulated in CRESO and S.CAP showing low nutrient content

(Fig. S8A).

Our study found that SVEMS16 and SVEVO plants exhibited drought tolerance by showing a low or unaffected variation in transpiration, along with high levels of carotenoids and chlorophylls, and the expression of drought-responsive genes. In terms of nutrients, SVEMS16 was able to maintain its nutritional status even under drought through increased root assimilation. In contrast, SVEVO maintained or even increased the content of nutrients by inducing shoot S assimilation. Despite their similar drought-induced responses, these genotypes have different genotypic backgrounds, with many variant-containing genes in SVEVO belonging to several GO-enriched terms. This observation suggests a different molecular mechanism underlying their ability to face drought.

Other genotypes, namely BULEL, S.CAP, and CRESO displayed significant variation in $\delta^{13}C$ when exposed to drought, suggesting that their response is based on stomata closure. In the case of S.CAP and CRESO, the response was accompanied by a down-regulation of drought-responsive genes, alteration of the carotenoid profile, an increase of chlorophyll degradation, and MDA accumulation. Furthermore, there was a decrease in transcript abundance of *TdP5CS*, *TdDRF1*, and *TdWRKY2* genes in root tissue, as well as genes involved in sulfur homeostasis. This cluster was also found to have a lower accumulation of SO_4^{2-} NO_3^-, PO_4^{3-}, K^+ in both tissues and Ca^{2+} and Mg^{2+} in shoot tissues. Ultimately, this resulted in lower biomass production (RFW, SFW, and PFW), indicating a drought-sensitive behavior of these genotypes. On the other hand, BULEL displayed a more efficient regulation of drought-induced responses compared to other genotypes. BULEL up-regulated drought responsive genes (*TdP5CS*, *TdDRF1*, and *TdWRKY2*) and genes involved in sulfur homeostasis, maintained carotenoid and chlorophyll levels, and increased concentrations of SO_4^{2-}, NO_3^-, PO_4^{3-}, K^+ in both tissues, and Ca^{2+} and Mg^{2+} in shoot tissue, resulting in higher biomass production (RFW, SFW, and PFW). Notably, BULEL is genotypically different from the other genotypes, and it has variant-containing genes related to ethylene metabolism. Ethylene and its metabolic process activate plant responses to water deficit (Habben et al., 2014) acting in parallel or synergistically with abscisic acid (ABA) to enhance its biosynthesis (Müller, 2021). Such observations highlight possible new routes of investigation to unravel drought-induced responses in durum wheat.

5. Conclusion

This study provided evidence about the importance of considering the drought-induced plasticity in mineral nutrients to unravel drought tolerance in durum wheat genotypes, specifically at seedling stage. Previous studies using morphological and biochemical traits, classified CRESO, SVEVO, and SVEMS16 as tolerant, SVEMS1 and BULEL as sensitive, and ETRUSCO and S.CAP as intermediate genotypes (Quagliata et al., 2023). Our work identified that the variability of key macronutrients SO_4^{2-}, NO_3^-, PO_4^{3-}, and K^+ during drought was potentially linked to the genetic diversity of the durum wheat genotypes analyzed. This connection was supported by the presence of a high number of variant-containing genes linked to these macronutrients, with many genes shared among the nutrients showing an increase, while others were unique to each nutrient showing a decrease.

Among the genotypes studied, only SVEMS16 and SVEVO demonstrated effective drought resistance by sustaining/maintaining nutrient acquisition and limiting drought-induced damage. Interestingly, under drought, BULEL showed increased levels of some nutrients (such as SO_4^{2-} and K^+) which are associated with a high number of variant-containing genes, suggesting a potential genetic adaptation. In addition, BULEL maintained the expression of PIP2; 1 gene at control levels and produced a high number of root exudates compounds, similar to SVEMS16. In contrast, CRESO, despite no change in biomass, exhibited cellular damage and relied on proline accumulation in roots. Additionally, CRESO showed limited transpiration and nutrient accumulation, potentially indicating a state of nutrient starvation hindering its proper development under drought stress. This effect was even more pronounced in S.CAP, which displayed enhanced damage (MDA accumulation and chlorophyll degradation) and significant reduction of nutrient content. Notably, the reduced expression of the PIP2; 1 gene in CRESO and S.CAP genotypes suggests impaired water uptake under drought. This further explains their limited number of root exudates, reflecting overall metabolic limitation. Finally, despite experiencing enhanced damage, ETRUSCO maintained nutrient content, while SVEMS1 preserved levels of chlorophyll, carotenoid in leaves, and nutrients content in roots.

Based on these findings, while SVEMS16, SVEVO, and BULEL exhibit distinct drought responses, they all maintain or increase nutrients content at the seedling stage. This suggests their potential for further field studies to assess their drought tolerance degree across subsequent phenological stages. In this context, ETRUSCO and SVEM1 genotypes seem to display an intermediated behavior, and further efforts are needed to discriminate their drought tolerance degree. In contrast, the limited nutrient acquisition observed in CRESO and S.CAP during the seedling stage suggests they might be less effective for crop establishment and plant growth under drought conditions. Overall, this study highlights the importance of considering drought-induced plasticity in mineral nutrients for a more comprehensive understanding of drought tolerance in durum wheat seedlings.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109077.

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