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Fine scale gene expression dynamics at the foliar level reveal grapevine strategies to cope with recurrent drought stress

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Abstract

Background The frequency of consecutive and often leading to extreme drought events is increasing in the Mediterranean region, shaping the physiological strategies that grapevine plants employ to cope with recurring water stress. This study evaluates the effects of two recurrent drought cycles, each followed by rewatering, on potted plants of *Vitis vinifera* L. cv. Sauvignon blanc.

Results Physiological measurements were combined with transcriptomic analysis, performed on leaf samples collected every six hours during the last day of drought cycle and subsequent rewatering. RNA-Seq results showed temporal and cycle-dependent dynamics in genes associated with carbohydrate metabolism, abscisic acid, and photosynthesis. By analyzing gene expressions throughout the day in both drought cycles, we observed conserved and distinct transcriptional patterns, with different processes activated or repressed depending on the time of day and the cycle (drought or recovery). Conserved genes such as galactinol synthase and dehydrin were present in both cycles most of the time. On the other hand, it was observed that plants change from immediate defense and stress mitigation in the first drought cycle to more centered on metabolic changes and acclimative adjustments during the second drought and at the end of the day.

Conclusions These findings suggest that grapevines adjust their molecular responses after an initial drought episode, consistent with acclimation or possible stress-priming mechanisms, useful to better cope with subsequent water deficits. This fine-scale temporal perspective provides insight into the strategies that *Vitis vinifera* implement to survive recurring drought, with relevance for improving grapevine management under climate change.

Keywords RNA-Seq, *Vitis vinifera*, Environmental stress, Gas-exchange, Carbohydrate metabolism

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Introduction

Mediterranean regions are projected to face significant temperature increases, prolonged episodes of intense drought, greater exposure to ultraviolet radiation, and a higher frequency of extreme weather events [1–4]. Droughts and rising temperatures are increasingly impacting the global viticulture industry, as water shortages are closely linked to reduced grapevine growth, lower yields, and declines in fruit quality [5–9]. Studies demonstrated that the non-structural carbohydrates (NSC), including starch, are significantly reduced in grapevine leaves and petioles under drought stress [10–12]. Plants produce carbohydrates through photosynthesis, and abiotic factors can influence the dynamics of NSC reserve storage in grapevines [13]. These reserves, accumulated in perennial organs, provide energy and carbon for growth and sustain early-season vegetative development until photosynthesis becomes the main carbon source, usually around flowering [14], but also play a role in osmotic adjustment during drought [15, 16].

Gene expression analysis has been applied to reconstruct the regulatory networks affected by water stress at the organ and whole plant level, enhancing our understanding of transcriptional regulation under drought conditions [8, 17–19]. In grapevine leaf responses to water deficit, it was demonstrated that prolonged water stress triggers rapid metabolite changes, accompanied by significant alterations in the expression of genes involved in abscisic acid (ABA) and carbohydrate metabolism, particularly those related to sucrose synthesis and catabolism, starch metabolism, and sugar transport, highlighting the critical role of NSC in drought acclimation [20].

Grapevines frequently face recurrent drought episodes, yet the regulatory mechanisms that enable them to cope with repeated water-deficit events remain poorly understood. While previous studies have described physiological and biochemical adjustments occurring during consecutive drought cycles, the present work aims to uncover the molecular programs that orchestrate these responses. Here, we combine physiological measurements with hourly RNA-Seq profiling to examine how grapevine leaves respond to recurrent drought cycles, with a particular focus on the temporal dynamics of key pathways activated or repressed. We hypothesize that recurrent drought events elicit distinctive transcriptional patterns - particularly in genes involved in carbohydrate metabolism, ABA signaling, and photosynthesis - that are associated with the altered physiological and biochemical responses observed under drought stress. By resolving these fine-scale temporal expression changes, this study provides new insight into the molecular strategies that support grapevine acclimation to recurring drought, offering relevant implications for better understanding the physiological responses at the organ level thus enabling the adoption of proper adaptation strategies for viticulture under increasing climate variability.

Materials and methods

Plant material and experimental setup

Experiments were conducted on 30 L potted 8-year-old grapevines (*Vitis vinifera* cv. Sauvignon Blanc, clone 108) grafted onto Kober 5BB rootstock (the grafted vines were provided by a national commercial nursery) at the "L. Toniolo" Experimental Farm, University of Padua, Italy, during summer 2024. Two treatment groups were established: well-watered control plants (20 plants) and drought-stressed plants (30 plants) subjected to two cycles of water stress (D1 and D2), each followed by a rehydration period of 6 days (R-D1 and R-D2; Supplementary Figure S1) performed by modulating irrigation provision. In both cycles water supply was stopped until reaching a stem water potential (Ψ_{stem}) of -1.3 MPa and soil water content was daily determined gravimetrically at 18:00 solar time, by weighing each pot using an electronic scale. The quantity of water adequate to reach/maintain the desired soil water content was supplied twice a day, in the early morning (6:00 h) and in the evening (20:00 h). Substrate composition, environmental conditions, and vine management are described in detail in Canton et al. [10] (Supplementary Figure S2).

Physiological analyses

In each drought cycle, single-leaf gas exchanges were measured with an open-system (LI-6800, LI-COR Inc.) when plants reached the maximum drought and after six days of rewatering. Measurements were conducted using a 6 cm^2 leaf cuvette equipped with a Multiphase Flash Fluorometer (6800–01 A). Each leaf was subjected to at least 20-min of acclimation at CO_2 concentration of 400 µmol CO_2 mol^{-1} and a constant saturating photosynthetic photon flux density (PPFD) of 1500 µmol of photons m^{-2} s^{-1} before starting the measurements. Leaf temperature was maintained at 30 °C, with relative humidity (RH) ranging between 50% and 70%, resulting in a vapor pressure deficit (VPD) of approximately 1.5 kPa inside the chamber. Measurements were performed on at least three fully expanded leaves per treatment, between 11:00 h and 14:00 h solar time.

The stem water potential (Ψ_{stem}, MPa) was measured using a Scholander-type pressure chamber (model PMS-600, PMS Instruments). For each measurement, six randomly chosen, sun-exposed, and fully expanded leaves per treatment were excised from the scions with a scalpel blade and immediately placed in the chamber. Ψ_{stem} measurements were conducted from 12:00 h until 13:00 h solar time. To prevent transpiration and allow equilibration with the stem water potential, leaves used for Ψ_{stem}

measurements were enclosed in opaque plastic bags for at least 1 h prior to excision.

ABA quantification

To monitor daily regulation of ABA levels, ABA concentrations in leaves from drought-stressed and well-watered plants were quantified during both the D1 and D2 cycles, with samples collected at 03:00 and 15:00 solar time. ABA was quantified by ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS), as described by Müller & Munné-Bosch [21]. In short, after samples were repeatedly extracted with methanol using ultrasonication (Branson 3800 ultrasonic cleaner, Bransonic), supernatants were collected, pooled and filtered with hydrophobic PTFE filters of 0.22 µm (Phenomenex, Torrance, CA, United States) prior to UHPLC-ESI-MS/MS analysis. High-performance liquid chromatography was coupled to a triple quadrupole mass spectrometer (QTRAP 6500, AB Sciex, Concord, Ontario, Canada), the flow rate was set at 0.5 mL min^{-1} and a LUNA C18 column (Phenomenex Inc., United States [1,6 µm, 100 × 2,1 mm]) was used. Quantification was made considering recovery rates for each sample by using the deuterium-labeled ABA and calibration curves for each analyte were generated using MultiQuantTM 3.0.1 software.

RNA sequencing

RNA extraction and library preparation

For RNA-seq analysis, fully expanded leaves from the middle portion of the shoot were collected to ensure consistency in developmental stage. A total of three biological replicates consisting of tissue pooled from three plants per treatments and control were used. All samples were immediately frozen in liquid nitrogen, and stored at – 80 °C until RNA extraction.

RNA extraction, library preparation, sequencing and mapping were performed at Biomarker Technologies (BMK; Germany). Total RNA was extracted from plant leaves using the RNAprep Pure Plant Kit (Tiangen, Beijing, China), following the manufacturer's instructions. Sampling was performed every six hours at different time points: 3:00 (T3), 9:00 (T9), 15:00 (T15), and 21:00 (T21) during the D1, R-D1, D2, and R-D2 phases to assess the dynamics of gene transcription throughout the day and across the drought cycles. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq platform and paired-end reads were generated.

RNA-Seq analysis

Raw sequencing data (FASTQ format) were filtered to obtain clean reads, retaining only sequences with a perfect match or a single mismatch. In addition to read-level filtering, sample-level quality control was performed using FastQC to assess sequencing quality metrics across all samples. These included base quality distribution, GC content, adapter contamination, and duplication levels. No samples were excluded at this stage, as all libraries showed consistent quality metrics. The reads were then annotated based on the reference genome of *Vitis vinifera* (V3, liftoff on T2T) from Grapedia (https://grapedia.org/files-download/) using Hisat2 [22]. Transcript assembly and quantification were subsequently performed with StringTie [23] Differential expression analysis of two conditions/groups was performed using DESeq2 [24]. DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. Raw count matrices were filtered to retain only genes with an average count ≥ 10 in at least one experimental group. A DESeq2 dataset was constructed using the experimental group as the design factor, and pairwise comparisons between drought and watered groups were performed (~ treatment * time * cycle). Genes with an absolute log2 fold change ≥ 1 and an adjusted p-value < 0.001 were assigned as differentially expressed (DEGs). DEGs with FPKM ≥ 1 in at least one condition were retained for downstream analysis. DEGs were classified as upregulated or downregulated based on the sign of the log2 fold change.

Principal component analysis (PCA) was performed on variance-stabilized expression values of all retained genes after filtering to explore dataset structure and sources of variation. Raw counts were normalized using variance-stabilizing transformation (VST) to account for sequencing depth and heteroscedasticity. PCA was conducted on the transposed normalized matrix, with samples represented in the reduced-dimensional space defined by principal components. To identify genes driving major variation, PCA loadings were calculated and Euclidean distances from the origin in PC1-PC2 space were computed. Genes were classified by dominant loading direction (PC1/PC2 positive or negative), and those above the 95th percentile were highlighted.

Clustering analysis

For each experimental cycle, only differentially expressed genes from pairwise drought-watered comparisons were retained. Expression values were averaged across replicates for each treatment-time combination and Z-score normalized across genes. K-means clustering was applied to the normalized matrix using Euclidean distance from cluster centroids. The optimal number of clusters was

determined with the elbow method, examining the within-cluster sum of squares for k = 1–10. GO enrichment analysis was performed for genes differentially expressed across experimental cycles. Gene Ontology (GO) annotations for *Vitis vinifera* were downloaded from Ensembl Plants (https://plants.ensembl.org/index.html. For each cycle and regulation group, GO enrichment was performed in R using the topGO package [25] with the Biological Process ontology, applying Fisher's exact test ($p \leq 0.05$) to identify significantly enriched GO terms.

Statistical analysis

The experiment followed a completely randomized design. Statistical analyses were performed using R software (version 4.3.1). Since the data did not meet the assumption of normality, differences between treatments were evaluated using the non-parametric Kruskal–Wallis test. When significant effects were detected, pairwise comparisons were conducted with Dunn's test applying the sequential Bonferroni correction. Statistical significance was set at $p \leq 0.05$. In addition, a one-way ANOVA was applied to assess treatment differences at each time point for intrinsic water use efficiency (WUEi), followed by Tukey's post hoc test when significant effects were observed ($p \leq 0.05$). For ABA, a one-way ANOVA was performed across all experimental groups (combining cycle, time, and treatment), and Tukey's HSD post hoc test was applied for pairwise comparisons among all groups ($p \leq 0.05$).

Results

Physiological data

In this study, we combined physiological measurements with RNA-Seq analyses to characterize the leaf responses of grapevine undergoing two successive drought stress cycles of identical intensity (Ψ_{stem} -1.3 MPa), followed by a 6-day rewatering period. We focused on characterizing the differences and similarities between both stress cycles and the hourly effect on gene expression, whereas the recovery phases were not the primary focus of this study.

To evaluate plant water status under drought stress conditions and provide physiological context for gene expression, we monitored key grapevine traits during the two consecutive drought cycles, including leaf gas exchange (net photosynthesis, A_n; stomatal conductance, g_s), stem water potential (Ψ_{stem}), intrinsic water-use efficiency (WUEi; Table 1) and abscisic-acid levels (ABA; Fig. 1). While part of these physiological traits has been previously characterized (A_n, g_s and Ψ_{stem}) ([10]), here they are summarized to provide physiological context for the transcriptomic analysis. Watered plants consistently maintained A_n rates of around 10 µmol CO_2 µmol m^{-2} s^{-1} throughout the experiment. In contrast, drought-stressed plants showed a pronounced decline in A_n during drought episodes, falling nearly to 0 µmol CO_2 µmol m^{-2} s^{-1} at peak stress. However, these drought-treated plants displayed a strong recovery capacity in both drought cycles, with A_n returning to near watered levels following 6-days of rewatering, although recovery was slightly greater after D2 than D1. In well-watered plants, g_s remained stable, ranging between 0.12 and 0.15 mol H_2O m^{-2} s^{-1}, whereas drought-stressed plants exhibited near-zero g_s values during dry periods, indicating that the plants experienced conditions leading to complete stomatal closure. However, during rewatering phases, g_s returned to baseline levels in R-D1 (0.096 mol m^{-2} s^{-1}) but showed only a weaker recovery in R-D2 (0.109 mol m^{-2} s^{-1}), remaining below the corresponding well-watered values.

Ψ_{stem} was used as a key parameter to control drought treatment imposition. While Ψ_{stem} remained steady at around −0.5 MPa in watered plants, in stressed plants it dropped to approximately − 1.3 MPa during both D1 and D2 drought cycles. The WUEi was similar between

Table 1 Physiological measurements in grapevine leaves under watered and drought conditions across multiple drought-rewatering cycles. Parameters measured include net assimilation rate (A_n, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), stem water potential (Ψ_{stem}, MPa) and intrinsic water use efficiency (WUEi) in leaves of plants under watered and drought conditions. Measurements include the first drought phase (D1), rewatering (R-D1) and subsequent second drought period (D2) and its corresponding recovery phase (R-D2). Values represent the mean ± SE. Significant differences between watered and drought stressed groups at each time point are indicated by different lowercase letters according to Dunn test with sequential Bonferroni correction, except for WUEi where Tukey's test was applied ($p \leq 0.05$; $n = 3$ leaves per treatment)

Cycle	Treatment	A_n (µmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	Ψ_{stem} (MPa)	WUEi (µmol CO_2 mol^{-1} H_2O)
D1	Drought	0.38 ± 0.16*b*	0.002 ± 0.000*b*	-1.26 ± 0.02*b*	84.70 ± 19.82*a*
	Watered	10.11 ± 0.40*a*	0.136 ± 0.012*a*	-0.17 ± 0.03*a*	76.53 ± 5.28*a*
R-D1	Drought	7.63 ± 0.61*b*	0.096 ± 0.005*a*	-0.61 ± 0.01*b*	79.22 ± 6.51*a*
	Watered	10.30 ± 0.67*a*	0.136 ± 0.015*a*	-0.45 ± 0.02*a*	77.10 ± 7.10*a*
D2	Drought	0.77 ± 0.54*b*	0.005 ± 0.003*b*	-1.37 ± 0.08*b*	133.23 ± 18.00*a*
	Watered	10.12 ± 0.93*a*	0.162 ± 0.007*a*	-0.80 ± 0.05*a*	62.61 ± 4.14*b*
R-D2	Drought	8.33 ± 0.27*a*	0.109 ± 0.002*b*	-0.68 ± 0.04*a*	76.80 ± 3.82*a*
	Watered	9.10 ± 0.44*a*	0.149 ± 0.003*a*	-0.56 ± 0.04*a*	61.34 ± 3.86*b*

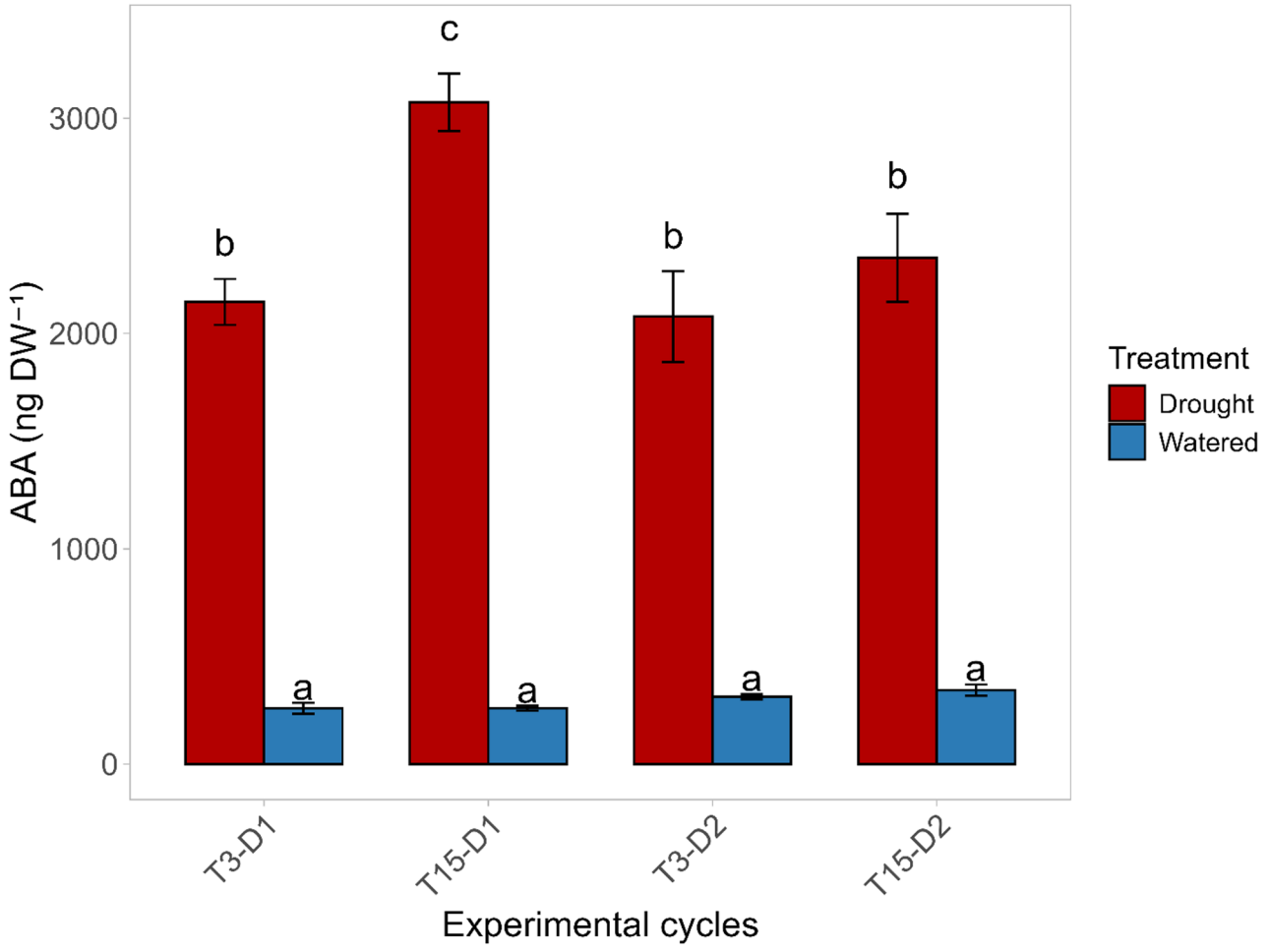

Fig. 1 Endogenous contents of abscisic acid (ABA) in leaves during drought-stress cycles drought 1 (D1) and drought 2 (D2) at 3:00 h (T3) and at 15:00 h (T15) in drought (red bars) and watered samples (blue bars). Values represent the mean ± SE. Significant differences between treatments are indicated by different lowercase letters according to Tukey's test ($p \leq 0.05$; $n = 3$ leaves per treatment)

drought stressed and watered plants in the D1 cycle, showing values around 80 µmol CO_2 mol^{-1} H_2O. However, during the D2 cycle, drought-stressed plants exhibited higher WUEi values both under stress and after rewatering (R-D2), showing values around 133 and 77 µmol CO_2 mol^{-1} H_2O, respectively.

ABA analysis revealed higher ABA levels in drought-stressed plants compared to watered plants at both 3:00 (T3) and 15:00 (T15) during drought cycles D1 and D2 (Fig. 1). In D1 drought-stressed plants, a clear time-dependent effect on ABA accumulation was observed, with highest levels measured at T15. Conversely, no evident time effect could be observed in watered and D2 stressed plants.

Changes in leaf transcript levels in response to repeated water stress

To investigate the transcriptional changes occurring in grapevine leaves during the two consecutive drought stress cycles, a genome-wide transcriptomic analysis was performed using the RNA-Seq approach at each pair-wise comparison between drought stressed versus watered samples at each time-point (T3, T9, T15 and T21) and each drought cycle (D1, R1, D2, R2; Table 2). High-quality reads were mapped on the *Vitis vinifera* V3 liftoff on T2T reference genome [26]. On average, 39.5 million (93.29%) paired-end reads per sample were successfully mapped (Supplementary Table S1).

To obtain a detailed overview of the global transcriptional variation among samples before identifying differentially expressed genes, we first performed a Principal Component Analysis (PCA) based on normalized gene expression values (Fig. 2). PCA revealed that the first two PCs explained 47.7% of the total transcriptional variance". The PCA score plot (PC1 vs. PC2) showed that drought stressed and watered samples formed distinct clusters at their respective time points during the re-watering cycles, whereas during the drought cycles the stressed samples were shifted upward along the axis of PC2 (Fig. 2A). In addition, the samples clustered distinctly

Table 2 Overview of the RNA-seq experimental design. Summary of the experimental factors including drought cycles (D1, D2), phases (Stress, Recovery), sampling time points (3, 9, 15, and 21 h), and treatment status (Stress vs. Control). For each condition, three independent biological replicates were sequenced, for a total of 96 samples

Cycle	Phase	Treatment Status	Time Points (h)	Replicates	Group IDs
D1	Stress	Drought (S) vs. Control (C)	3, 9, 15, 21	3	C3S1, S3S1, C9S1, S9S1, C15S1, S15S1, C21S1, S21S1
R1	Recovery	Drought (S) vs. Control (C)	3, 9, 15, 21	3	C3S1, S3S1, C9S1, S9S1, C15S1, S15S1, C21S1, S21S1
D2	Stress	Drought (S) vs. Control (C)	3, 9, 15, 21	3	C3S1, S3S1, C9S1, S9S1, C15S1, S15S1, C21S1, S21S1
R2	Recovery	Drought (S) vs. Control (C)	3, 9, 15, 21	3	C3S1, S3S1, C9S1, S9S1, C15S1, S15S1, C21S1, S21S1

according to the time of day, with those collected at T3 and T9 tending to be positioned along the positive axis of PC1, and those collected at T15 and T21 located toward the negative axis of PC1 (Fig. 2A).

To better underline the key genes responsible for major variations among treatments, PCA loadings were calculated and Euclidean distances from the origin in PC1-PC2 space were computed (Fig. 2B and Supplementary Table S2). Genes were further classified according to their dominant loading direction (PC1/PC2, positive(+) or negative(-)), and those placed above the 95th percentile were highlighted. Focusing on the top 100 out of the 509 genes of the PC2 + group (red dots; Fig. 2B and Supplementary Table S2), several genes resulted being related to carbohydrate transport and metabolism, such as polygalacturonase GH28 (*Vitvi08g01857*), sugar transporter ERD6-like 6 (*Vitvi18g00970*), xylose isomerase (*Vitvi11g01300*), UDP-glucosyltransferase (*Vitvi03g00533*), 1,4-alpha-D-glucan malt hydrolase (*Vitvi05g00357*), cellulase (*Vitvi19g01878*) and sucrose synthase (Susy, *Vitvi07g00353*).

Other interesting carbohydrate-related genes were found among the top 100 most negatively correlated with PC2- (green dots, Fig. 2B). These included a sucrose-phosphate synthase (*Vitvi05g01193*), a trehalose-phosphate synthase 1 (*Vitvi10g00674*), and a beta-amylase (*Vitvi12g00558*). In addition, genes involved in photosynthesis (e.g., PSBX—photosystem II subunit X, *Vitvi11g01345*) and in signal reception or transduction (e.g., a leucine-rich repeat family protein, *Vitvi10g01164*; and a serine/threonine-protein kinase receptor ARK3, *Vitvi10g02152*) were also strongly downregulated in water-stressed vines.

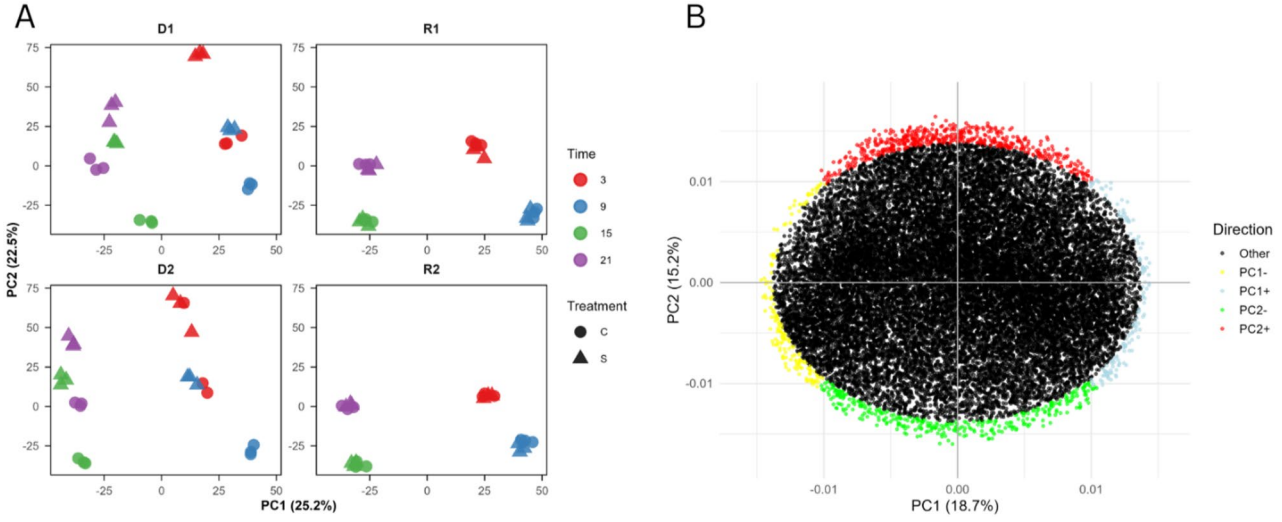

Fig. 2 PCA of samples and genes during drought–rewatering cycles. **A** Distribution of drought-stressed (S) and well-watered (C) samples across different hours of the day (3, 9, 15 and 21) during Drought 1 (D1), Rewatering 1 (R1), Drought 2 (D2), and Rewatering 2 (R2). **B** PCA based on variance-stabilized transformed expression values of all expressed genes, with gene loadings indicated to highlight major contributors to the observed variation

Subsequently, by using Deseq2 and a threshold of 0.001 on the adjusted *p*-value and log2fold-change ≥ |1| (corresponding to 2 fold-change variations in expression level), we identified 5396, 122, 7159 and 75 differentially expressed genes (DEGs) at each drought-rewatering cycle (D1, R1, D2, R2) respectively (Fig. 3A and Supplementary Table S3 and S4). Only DEGs with an FPKM value ≥ 1 in at least one condition were retained. In D1, most differentially expressed genes were detected at T3, followed by T15, T9, and T21. In contrast, in D2, the highest number of DEGs was observed at T15, followed by T21, T9, and T3, likely reflecting the impact of higher daytime temperatures (Supplementary Figure S2). To assess the potential contribution of higher temperature in D2, we also compared control samples between D1 and D2 at each time point; this analysis identified a limited number of differentially expressed genes that represent not only drought- but also temperature-driven responses (Supplementary Table S5). Interestingly, during both recovery cycles, differential gene expressions between watered and drought-stressed samples were minimal, suggesting a rapid transcriptional adjustment upon rewatering. Therefore, we chose to concentrate on drought-stress cycles.

The genes from D1 and D2 were separated in a Venn diagram to better understand which genes were unique or shared between both drought cycles (Fig. 3B Supplementary Table S6). Among the 5396 genes from D1, 3645 overlapped with those in D2. This indicates that approximately 68% of the DEGs identified in the first drought cycle were also regulated in the second cycle D2, suggesting a conserved set of drought-responsive genes; while the unique genes to each cycle may reflect cycle-specific adaptive mechanisms. To further refine this analysis, we separated the DEGs into up- and down-regulated genes for each drought cycle (Supplementary Figure S3 and Supplementary Table S6). Among the up-regulated DEGs, 33% were shared between the two stress cycles, whereas this proportion increased to 39.8% for down-regulated DEGs. These results indicate that, in addition to a shared core response, a substantial fraction of genes is regulated in a cycle-dependent manner, with genes specific to D1 not being activated/repressed in D2 and, conversely, D2-specific genes not being required during D1. Focusing on the top twenty genes with the highest fold-change in D1 and D2 averaged across all daily time points, we identified five genes that are in common between the two stress cycles (Supplementary Table S7). These genes include galactinol synthase (*Vitvi05g00170*), dehydrin 1b (*Vitvi04g01368*), ATP-dependent zinc metalloprotease (FTSH 6, *Vitvi14g01942*), and two heat shock

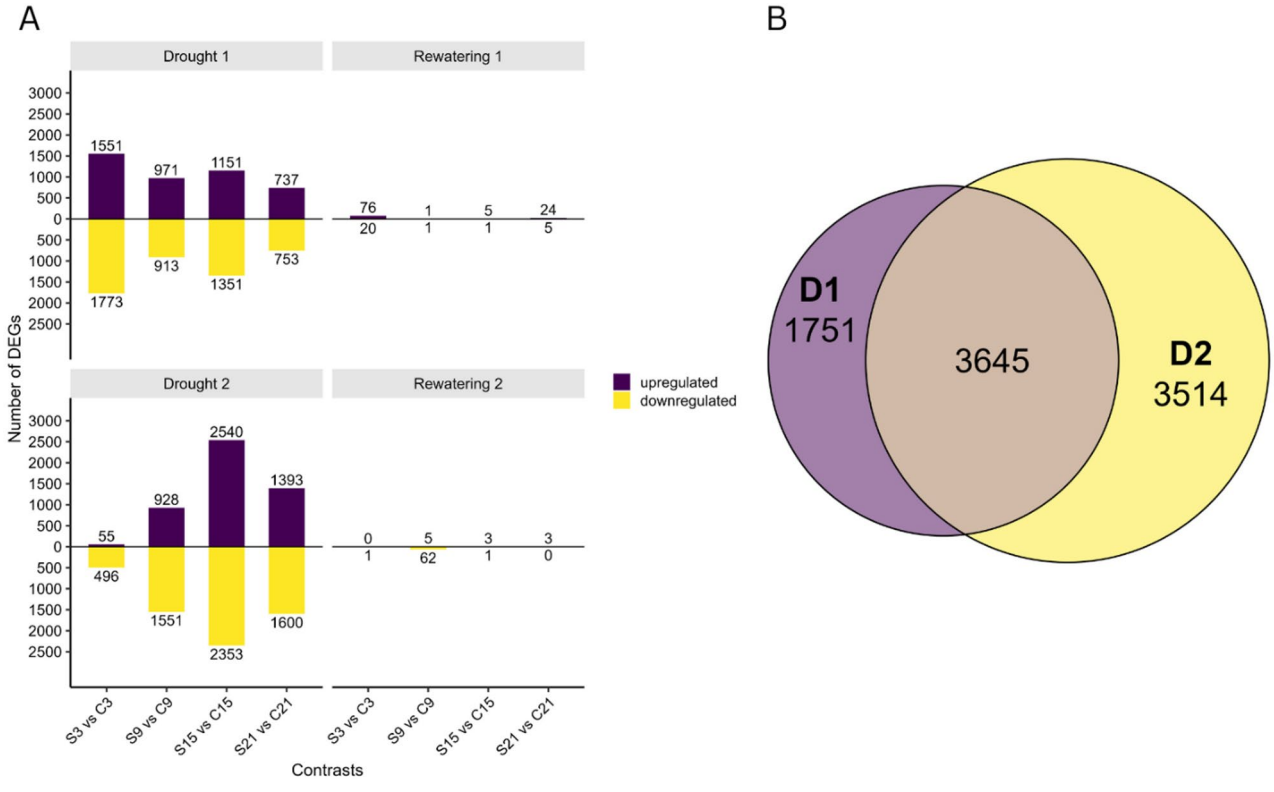

Fig. 3 Distribution of DEGs during drought–rewatering cycles. **A** Histogram showing the number of differentially expressed genes in pairwise comparisons between drought-stressed (S) and watered (C) samples at each time point (3, 9, 15 and 21) during Drought 1 (D1), Rewatering 1 (R1), Drought 2 (D2), and Rewatering 2 (R2). **B** Venn diagram illustrating the unique and shared DEGs between the two drought cycles

proteins (*Vitvi04g01795* and *Vitvi12g02138*), highlighting their consistent involvement in the stress response. On the other hand, genes that exhibited high modulation in D1 but did not reach the top twenty threshold in D2 include expansin-like B1 (*Vitvi00g02157*), glycerophosphodiester phosphodiesterase (GDPD1, *Vitvi14g01500*), osmotin-like protein (*Vitvi06g00440*) and galactinol synthase (*Vitvi05g00204*). In D2, across the twenty top genes, the following three out of seven heat shock proteins were found: raffinose synthase (*Vitvi11g00513*), osmotin-like protein TPM-1 (*Vitvi02g01409*) and galactinol synthase (*Vitvi14g00065*) (the complete list can be found in the Supplementary Table S7).

To investigate the functions and pathways associated with the drought cycles D1 and D2, we performed a GO enrichment analysis using the topGO package with the Biological Process ontology, separately analyzing up- and downregulated genes (Fig. 4 and Supplementary Table S8). Several enriched GO terms were shared between D1 and D2 upregulated genes, including *response to abiotic stimulus* (GO:0009628), *response to temperature stimulus* (GO:0009266), *response to osmotic stress* (GO:0006970), *response to heat* (GO:0009408), and *response to reactive oxygen species* (GO:0000302; Fig. 4AC). In contrast, some terms were specific to D1 upregulated genes, such as *small molecule metabolic process* (GO:0044281), *organic acid metabolic process* (GO:0006082), and *carboxylic acid metabolic process* (GO:0019752), while others were specific to D2, including *response to stimulus* (GO:0050896) and *galactose metabolic process* (GO:0006012; Fig. 4A).

For downregulated genes, D1 and D2 shared GO terms related to photosynthesis, including *photosynthesis* (GO:0015979), *photosynthesis - light reaction* (GO: 0019684), and *photosynthesis - light harvesting* (GO:0009765; Fig. 4BD). D1-specific downregulated terms included *water transport* (GO:0006833), *fluid transport* (GO:0042044), and *xyloglucan metabolic process* (GO:0010411), whereas D2-specific terms included *fructose 1,6-bisphosphate metabolic process* (GO:0030388), *sucrose biosynthetic*

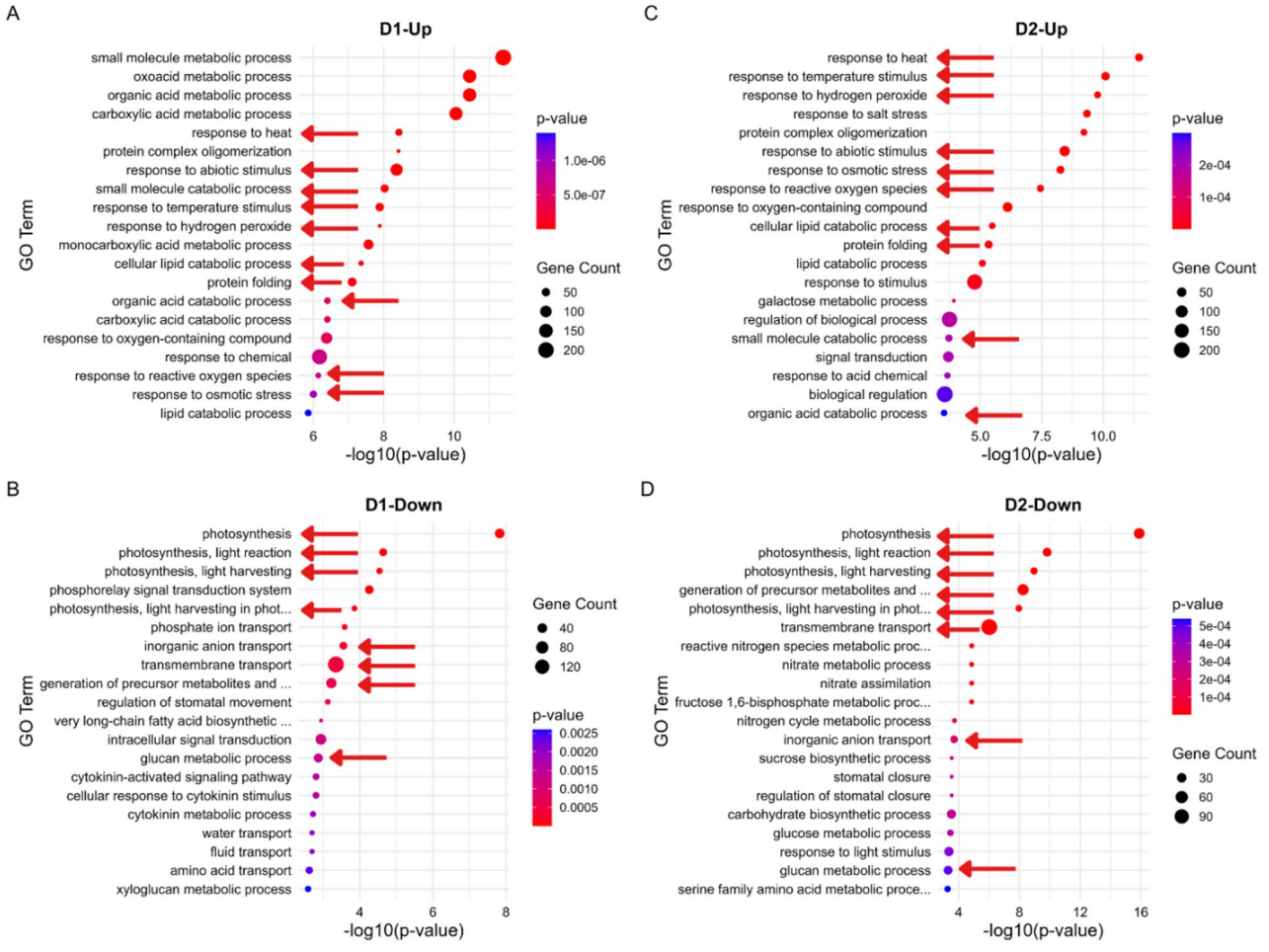

Fig. 4 GO enrichment analysis of genes responding to different drought-rewatering cycles such as Drought 1 (D1) and Drought 2 (D2). GO terms are shown separately for upregulated and downregulated genes, highlighting the biological processes affected under each condition. Red arrows indicate the GO terms that are commonly upregulated (**A**, **C**) and downregulated (**B**, **D**) in both D1 (**A**, **B**) and D2 (**C**, **D**)

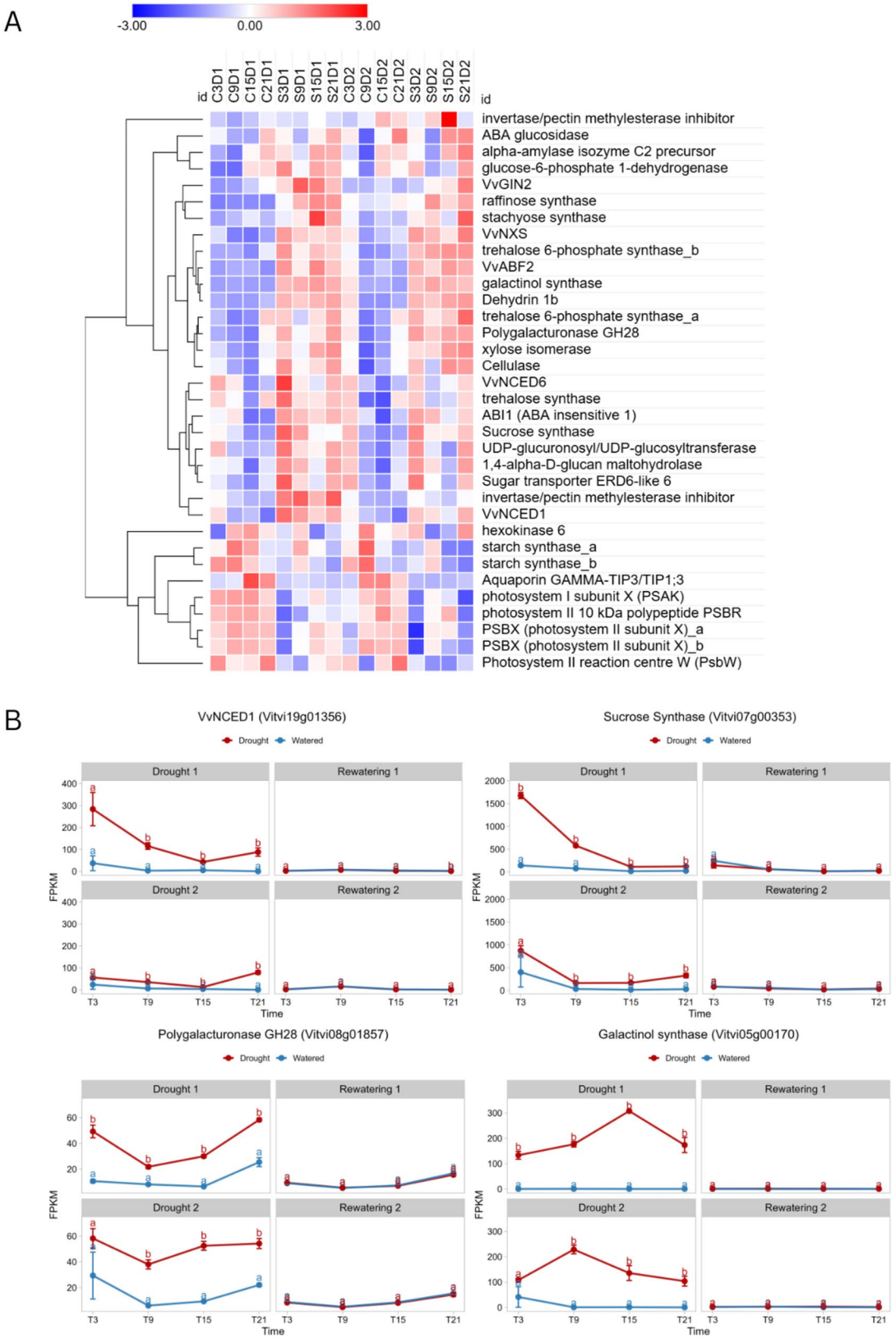

Fig. 5 (See legend on next page.)

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Fig. 5 Gene expression of selected genes related to drought response, including carbohydrate metabolism, ABA signaling, and photosynthesis. **A** Heat map of gene expression shows the z-score scaled gene expression levels (fragments per kilobase of transcript per million mapped reads) of samples in watered (C) and drought-stress (S) samples in drought 1 (D1) and drought 2 (D2) cycles. **B** Line plot showing four selected genes during drought-rewatering cycles in all four analyzed hours (T3, T9, T15 and T21). Values represent the mean ± SE. Significant differences between treatments at each time point are indicated by different lowercase letters according to Tukey's test ($p \leq 0.05$; $n = 3$). Gene labels refer to individual transcripts; genes encoding similar proteins are distinguished by suffixes (e.g., a, b), and no aggregation was performed

process (GO:0005986), *stomatal closure* (GO:0090332), *carbohydrate biosynthetic process* (GO:0016051), *glucose metabolic process* (GO:0006006), and *response to light stimulus* (GO:0009416).

Based on physiological analyses, the GO enrichment results and the PCA loadings highlighting genes with the greatest contribution to the stress effect, we focused on selected pathways relevant to drought response, including carbohydrate metabolism, ABA signaling, and photosynthesis. These pathways involved key genes in energy regulation, stress signaling, and photosynthetic performance, and were therefore prioritized for further analysis.

In addition to the carbohydrate-related genes highlighted by the PCA loadings as major contributors to the stress effect in both D1 and D2 cycles, we observed further differentially expressed genes involved in carbohydrate metabolism in drought samples in both cycles D1 and D2 (Fig. 5A).

Important upregulated genes included vacuolar invertase 2 (*VvGIN2*, *Vitvi02g00512*), raffinose synthase (*Vitvi14g01717*), trehalose synthase (*Vitvi10g00625*), trehalose 6-phosphate synthase _a and b (*Vitvi17g00778 and Vitvi01g00793*), alpha-amylase isozyme C2 precursor (*Vitvi01g00932*), glucose-6-phosphate 1-dehydrogenase (*Vitvi14g01605*), and stachyose synthase (*Vitvi07g00431*), whereas others were downregulated, such as starch synthase_a and b (*Vitvi02g00250* and *Vitvi15g00885*), aquaporin GAMMA-TIP3/TIP1 (*Vitvi06g01346*), and hexokinase 6 (*Vitvi06g01272*; Fig. 5AB).

Among ABA-related genes, we identified differential expression across multiple functional categories of the pathway. Key genes involved in ABA biosynthesis, including neoxanthin synthase (VvNXS, *Vitvi14g01185*) and the 9-cis-epoxycarotenoid dioxygenases VvNCED6 (*Vitvi05g00963*) and VvNCED1 (*Vitvi19g01356*), were upregulated under drought conditions. In addition, genes associated with ABA mobilization, such as ABA glucosidase (*Vitvi17g00234*), and ABA signaling components, including ABA-responsive element-binding protein 2 (VvABF2, *Vitvi18g00784*) and ABA insensitive 1 (*Vitvi11g00270*), were also upregulated (Fig. 5AB). These transcriptional changes are consistent with the observed increase in ABA levels under drought conditions, suggesting that enhanced ABA biosynthesis and mobilization contribute to hormone accumulation. Most photosynthesis-related genes were downregulated, including photosystem II reaction center W (PsbW, *Vitvi14g00489*), photosystem I subunit X (PSAK, *Vitvi10g00882*), photosystem II subunit X (PSBX_a and b, *Vitvi04g01806* and *Vitvi11g01345*), and photosystem II 10 kDa polypeptide (PSBR, *Vitvi19g01479*; Fig. 5AB).

Distinct gene expression clusters define temporal responses to drought

We further investigated the differential gene expression driven by the drought stress associated with distinct hours of the day. To this end, we performed hierarchical clustering of all DEGs, grouping genes in 30 clusters separately for D1 and D2 (Supplementary Figure S4 and Supplementary Table S9). Based on the average expression profile of each cluster, we selected 8 clusters for D1 and 11 clusters for D2 that exhibited pronounced hourly dynamics - that is, clusters showing a modulation peak at least at one time point during the day (Fig. 6) - including 1147 and 2836 genes, respectively. In both D1 and D2, several clusters comprised genes for which the expression difference from the watered plants was greatest at T15. However, the patterns differed at other times: in D1, the greatest divergence also occurred at T3, whereas in D2, it occurred at T21. In detail, we observed that in D1 cycle, several drought-stressed genes were up-regulated at T3 (groups 20 and 27), at T9 (group 18), and at T15 (groups 3 and 17), and down-regulated at T3 (groups 10 and 16) and at T15 (group 25). In D2 cycle, we observed a strong up- regulation at T15 (groups 5, 13, 21) and T21 (groups 22 and 26). The down-regulated genes were observed at T9 (group 17 and 27), at T15 (groups 16, 20 and 23) and at T21 (group 24).

To better understand the functions and pathways of those genes, we performed a GO enrichment analysis for each selected cluster revealing time- and cycle-specific functional patterns (Supplementary Table S10 and S11). Focusing on GO terms corresponding to D1 early-morning (T3), we noticed that down-regulated genes were mainly associated with cell wall metabolism, defense, and hormone signaling, while up-regulated genes were enriched in stress responses and hypoxia-related processes. At midday (T15), up-regulated genes were predominantly involved in oxidative and osmotic stress responses, heat stress, regulation and response to ethylene and hormone-mediated signaling pathways.

Similarly, in D2, midday (T15) up-regulated genes were also enriched in ethylene signaling but differed from D1

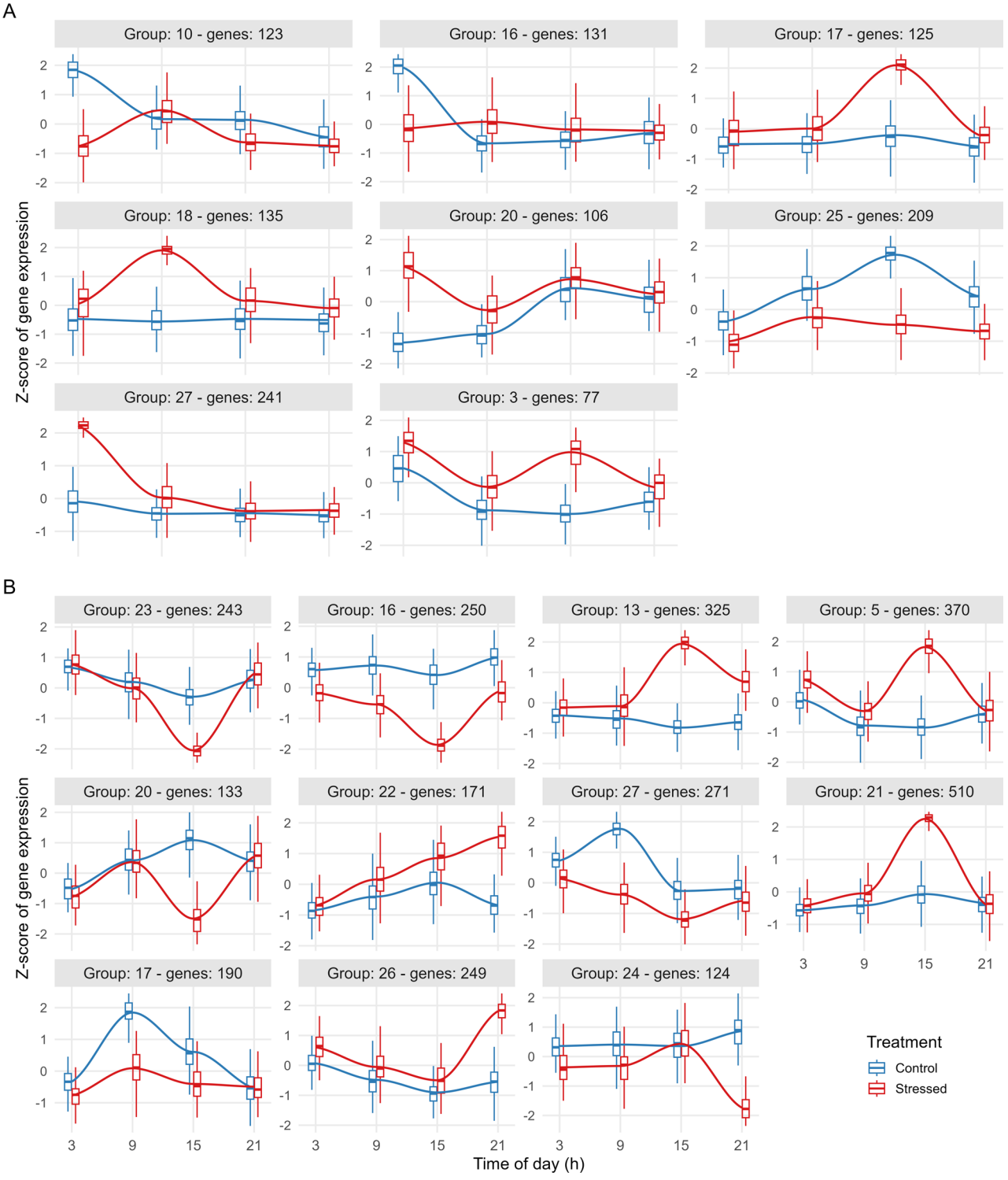

Fig. 6 Selected DEG clusters for D1 (Drought 1) and D2 (Drought 2) cycles. The clusters for D1 (**A**) and D2 (**B**) display clear hourly expression patterns, highlighting temporal dynamics of gene expression over the course of the day (3, 9, 15 and 21)

in their association with stress responses, leaf development, and polysaccharide metabolism. At evening (T21), D2 up-regulated genes were mainly associated with sugar and amino acid metabolism.

These results indicate temporal and cycle-specific transcriptional dynamics, with distinct biological processes activated or repressed depending on the time of day and drought cycle. It is important to note that leaf

samples were collected at the same position of the shoot, as reported in material and methods, to minimize the difference in plant phenological stage between D1 and D2.

Focusing on the top twenty most highly up-regulated genes at T3, T9, T15, and T21, we observed that several genes were consistently present across multiple time points and in both drought cycles (Supplementary Table S12). For instance, galactinol synthase (*Vitvi05g00170*) and dehydrin 1b (*Vitvi04g01368*) were present among the top twenty genes at all four time points in both D1 and D2 cycles, except for the T3 time point, where they ranked in the top twenty only in D1. In fact, the T3 top list consists solely of D1 cycle genes, coinciding with a period of very weak transcriptional modulation in D2 (Fig. 3A). Other genes expressed in more than one specific time of day are osmotin (*Vitvi02g01409*), present at T3 in D1 and at T15 in D2, VvNAC17 (*Vitvi19g00270*), present at T3 in D1 and at T9 in D2, NCED1 (*Vitvi19g01356*) expressed at T9 in D1 and at T21 in both D1 and D2, Heat shock protein (*Vitvi12g02138*) expressed at T9 in D2, at T15 in both D1 and D2 and at T21 at D2, and expansin-like B1, expressed at T3 and T9 in D1 and at T21 in D2. In addition to those genes, we can also identify other interesting genes that are in the top twenty only for one specific hour of the day. The time points T15 and T21, for example, are marked by several other Heat shock proteins.

Discussion

The present work integrates physiological measurements with transcriptome analyses to investigate grapevine leaf responses to recurrent drought cycles, with a particular focus on the hourly dynamics of gene expression. The results focused on fine-scale temporal changes, showing important genes related to carbohydrate metabolism, ABA and photosynthesis pathways that are differentially modulated between two consecutive cycles of drought stress. We hypothesize that, at least in part, the differences observed between the two drought cycles may reflect changes in plant responses following the first stress event. However, because the experimental design did not include plants exposed only to the second drought cycle (D2), it is not possible to directly assess the occurrence of stress memory or priming. Therefore, this interpretation remains tentative, and the results should be considered as consistent with acclimation. As expected, ABA levels were higher in stressed plants than in watered plants during both cycles, with evident effect of sampling time only in D1. This pattern is consistent with the g_s measurements, which showed reduced values during the drought-stress periods (Table 1). Similar results were observed also from Degu et al. and Tombesi et al. [20, 27] both of which support the hypothesis that hydraulic signals are responsible for triggering stomatal closure, whereas ABA plays a key role in maintaining low g_s thereafter. Molecular studies have demonstrated that, despite being very species-specific, ABA signaling is regulated by the circadian clock. Indeed, comparative profiling of circadian-clock-controlled and ABA-responsive genes revealed that about 40% of the latter are under circadian regulation in *Arabidopsis thaliana* [28]. The clock not only influences ABA-responsive transcription, but also controls ABA biosynthesis, with ABA production and concentration showing strong daily changes peaking in the afternoon [29]. This suggests that the circadian clock may enhance plant responsiveness to heat, drought, and osmotic stress during daylight hours by timing ABA accumulation [30, 31].

We did not observe stress memory related to physiological parameters in D2. However, most evidence dealing with stress memory, is sustained by experiments on annual plants in controlled environments and during short periods of time. In *Aptenia cordifolia*, ABA accumulation differed in plants exposed to drought twice compared with those experiencing it only once. After the first drought cycle, ABA levels rose about 40-fold and then returned to their original pre-stress values during recovery. When the second drought occurred, plants that have been stressed before produced even higher ABA levels than those encountering drought for the first time [32] under controlled growth conditions and clearly defined drought cycles. Zamorano et al. [33] observed a positive effect of previous season drought stress imprint in grapevine cv. Cabernet sauvignon exposed to a drought event lasting beyond the winter recess, involving contrasting growth responses, photosynthetic performance and soil water use, when they were grown outdoors in large pots. Vines were grown outdoors in large pots and were exposed to 67 consecutive days of drought during the first season and 41 consecutive days of drought during the second season.

In contrast to these studies, in the present experiment the two drought cycles (D1 and D2) occurred in different periods characterized by distinct environmental conditions, with D1 taking place under cooler temperatures. Therefore, the absence of control subjected to a single drought event measured simultaneously with D2 limits a direct assessment of stress memory effects. Consequently, the physiological responses observed during D2 may reflect not only the legacy of a previous drought exposure but also the influence of the prevailing environmental conditions, which may partly explain the discrepancies with studies reporting persistent stress memory after recovery.

Most molecular responses took place mainly during drought cycles with just a few genes differentially expressed during the recovery cycles. This result was not entirely surprising, since also Dal Santo et al. [17] reported a small number of expressed genes during

rewatering in Sangiovese, an anisohydric cultivar like Sauvignon blanc. This suggests that probably the return to the normal state was more passive than driven by transcriptional changes, without the need for new gene activation. The distribution of DEGs during D1 and D2 cycles was slightly different. In D1 the number of DEGs was similar between the different hours of the day, with the higher number at T3. In D2, instead, a pattern was observed that reflected the increasing temperature during the day, peaking at T15. Because temperatures were milder in D1 at T15, this could have prevented the identification of a clear hourly pattern like that seen in D2, which occurred during a very hot, typical summer day (Supplementary Fig. 2) with plants experiencing maximum stress at T15, enhancing the differences between watered and stressed plants. Additionally, at T3 in D2, plants were experiencing the second cycle of stress and may have already activated an acclimation mechanism. Combined with the fact that during the night plants in general do not need to maintain a high active transcription program active, this could explain the relatively low number of DEGs at T3 in D2.

One interesting finding was the identification in PC2 + of several genes among those associated with carbohydrate transport and metabolism (Fig. 2B). The positive loadings on PC2 were associated with the drought condition, as stressed samples shifted upward along the PC2 positive axis during the drought cycles. Genes such as polygalacturonase GH28 are known for their ability to degrade pectin in the primary cell wall and middle lamella [34]. In grapevine leaves, polygalacturonases probably function in cell wall remodeling to help the tissue to cope with water deficit and mediate stress-related signaling. In fact, pectin degradation by polygalacturonases may release short-chain oligomers (oligogalacturonides). These are potent elicitors of defense responses and could act as DAMPs (Damage-Associated Molecular Patterns) [35–37]. Furthermore, the production of oligogalacturonides aligns with the fascinating hypothesis of hydrogels production osmotically active as proposed by Zwieniecki et al. [38] and recently reprised by Vuerich et al. [16]. However, its specific mechanism of action has not yet been elucidated in leaves, which provides a direction for future research on the molecular mechanism of drought tolerance in plants.

Several interesting genes emerged for their high association with stress conditions in both D1 and D2 cycles (Fig. 5A). Among them, we observe an upregulation of Susy, invertases, raffinose synthase and galactinol synthase. Susy is unique among sucrose-metabolizing enzymes as it catalyzes both the reversible formation of sucrose from fructose and UDP-glucose and the cleavage of sucrose, in the presence of UDP, into fructose and UDP-glucose. In addition to UDP, Susy can use other nucleotide diphosphates-particularly ADP-for sucrose cleavage, although typically with lower affinity and efficiency [39]. Susy enzymes have pivotal roles in a large range of plant metabolic processes, such as sucrose distribution between plant source and sink tissues [40–43], starch biosynthesis, cellulose synthesis in secondary cell wall [44], nitrogen fixation [45] and response to abiotic stresses [42, 46]. Plants accumulate sugars-including sucrose, glucose, galactose, maltose, raffinose, and fructose-which help regulate cellular osmotic balance and provide an energy reserve during stress. These sugars function as osmolytes, modulating osmotic potential, and act as osmoprotectants to safeguard plant cells [47].

A major role in sucrose metabolism under stress is played by invertases. In plants, invertases are classified into two types based on their optimal pH: neutral or alkaline invertases, and acid invertases [48]. Acid invertases are localized either in the cell wall, where they exist in tightly bound forms, or in the vacuole, where they are present as soluble forms [49]. Vacuolar invertases are generally considered to play a key role in controlling cell expansion through osmotic regulation [50, 51]. By hydrolyzing sucrose into two hexose molecules, they effectively double the osmotic contribution, thereby promoting water influx and driving cell expansion [49]. In poplar trees, cell wall acidic invertases enzymes were upregulated in plants under drought stress [52]. Authors suggest that the biological control of physiological activity in response to stress is linked to an accumulation of monosaccharides in the xylem.

Oligosaccharides also play a crucial role in plant stress responses. Galactinol, a plant-derived disaccharide, has been extensively documented to enhance drought tolerance by acting both as an osmoprotectant and as a scavenger of reactive oxygen species (ROS) [53, 54]. Galactinol synthases play a central role in its biosynthesis, catalyzing the transfer of galactosyl groups from UDP-galactose (UDP-Gal) to myo-inositol [55]. Consequently, the overexpression of galactinol synthases genes has become an important approach for improving drought resilience in plants [53, 56]. They are a key enzyme in the synthesis of raffinose family oligosaccharides (RFOs). In grapevine, genes encoding galactinol-sucrose galactosyltransferase 1 (RFS), were significantly induced in leaves under drought stress in "Shine Muscat" (*Vitis labruscana* × *Vitis vinifera*). In addition, galactinol synthase was found upregulated also in leaves of *Vitis champinii* cv. Ramsey under drought stress [57]. Overexpression of galactinol synthases in plants grown in controlled conditions or in the field leads to significant increases in drought tolerance resulting in increased yields [58, 59]. In addition to galactinol synthases, raffinose synthases are responsible for raffinose biosynthesis [60, 61]. Using myo-inositol and UDP-galactose,

galactinol synthase catalyzes the production of galactinol [62]; whereas raffinose uses sucrose and galactinol to synthesize raffinose [60]. Moreover, alpha-amylase catalyzes the hydrolysis of α-1,4 glycoside linkages in large α-linked polysaccharides, especially starch, to release malto-oligosaccharides. Together, all these enzymes work synergistically to degrade disaccharides and polysaccharides into monosaccharides, thereby contributing to osmotic regulation and helping plants maintain cellular homeostasis under stress conditions.

Multiple drought-tolerance mechanisms help sustain normal photosynthesis during water deficit by supporting osmotic adjustment with sugars as discussed, but also accumulating amino acids (such as proline, betaine, and glycine) and by producing protective metabolites like trehalose and raffinose [63]. According to Tamayo et al. [64] drought stress led to a general rise in metabolite levels-including sugars and polyols-in two grapevine cultivars, with organic acids accumulating more in leaves than in roots. Additionally, Lin et al. [65] found in their study that the expression levels of transcripts associated with sugar and organic acid metabolism were significantly higher in the leaves of plants under drought stress. Our results also showed that most photosynthesis-related genes were downregulated in stressed plants (Fig. 5A) correlating with the leaf photosynthetic rate that was near to 0 μmol m^{-2} s^{-1} (Table 1). This behavior was also found by Lin et al. [65], where photosynthesis-relevant genes in leaves were downregulated in the two cultivars of grapevine under drought stress, indicating that there was a significant reduction in photosynthesis-related genes.

Gene expression dynamics throughout the day in both D1 and D2 cycles revealed temporal and cycle-specific transcriptional patterns, with distinct biological processes being activated or repressed depending on the time of the day and drought cycle (Fig. 6). Genes such as galactinol synthase (already discussed) and dehydrin 1b did not appear to be influenced by the time of the day, as they were consistently upregulated across all four time points analyzed. The same genes were also found in the top list of both D1 and D2 cycles. Dehydrins (DHN), classified as group II LEA proteins, are produced in many plant species in response to dehydration-related stresses including drought, low temperatures, and high salinity [66, 67]. These LEA/DHN proteins perform osmotic adjustment in multiple ways such as sequestering ions, stabilizing membranes, and/or by functioning as chaperones [68]. It is generally accepted that dehydrins function to protect cells from damage caused by stress-induced dehydration [69]. Apart from the role of osmotic adjustment, dehydrins are involved in ion sequestering and control of solute concentration in the cytoplasm under drought conditions [70]. Dal Santo et al. [17] by examining physiological and molecular perturbations in the leaf and berry in Montepulciano and Sangiovese cultivars, found similar genes involved in drought stress. Genes encoding dehydrins, osmotines, heat shock and a large amount of carbohydrate related genes such as VvGIN2 and galactinol synthases were upregulated under drought conditions.

Water stress affects not only carbon metabolism but also nitrogen metabolism and further differences between the D1 and D2 cycles were observed in D2 plants, which were enriched in GO terms related to nitrate metabolic processes and nitrate assimilation. Huffaker et al. [71] suggested that shutting off the nitrate assimilation pathway at the first step would reduce energy requirements during periods of stress and prevent accumulation of nitrite and ammonium. Results that were also confirmed by Bertamini et al. [72] in grapevine cv. Riesling under water stress.

Overall, focusing on the transcriptional differences between the D1 and D2 drought cycles and on the hourly effects during the day under stress, our analysis reveals that some genes, such as galactinol synthase and dehydrin 1b, are conserved across both cycles, as they remain highly expressed for most of the time in D1 and D2. In contrast, plants appear to shift from immediate defense and stress mitigation responses during the first drought cycle, characterized by the activation of stress- and hypoxia-related genes, toward more metabolically oriented and adaptive adjustments during the second drought cycle and toward the end of the day, including the activation of sugar and amino acid metabolism. In this context, Ding et al. [73] described four distinct classes of dehydration stress memory genes in *Arabidopsis thaliana*, including genes involved in the enhanced synthesis of protective and damage-repairing proteins, coordination of photosynthetic processes, osmotic readjustment, and the interaction between dehydration stress and hormone signaling pathways. Genes associated with responses to salt stress and general stimuli were upregulated exclusively in D2 and may therefore belong to these functional categories. Transcriptional memory genes are activated during the initial dehydration event but exhibit altered expression levels upon recurrent stress, thereby contributing to the fine-tuning of transcriptional responses under repeated dehydration conditions. Consistent with this framework, our results reveal a dynamic and time-dependent reprogramming of gene expression across successive drought cycles.

Conclusion

Examining daily gene expression pattern dynamics in both the D1 and D2 cycles revealed clear temporal and cycle-dependent transcriptional dynamics, with different biological processes being activated or suppressed depending on the time of day and the drought cycle. This

evidence supports the idea that plants dynamically reconfigure their stress responses, transitioning from immediate defense and mitigation on the first day (D1) toward metabolic reprogramming and adaptive adjustments on the second day (D2). This aligns with the concept of stress priming, whereby prior experience with environmental cues can prepare an organism for future challenges, as shown by Hilker et al. [74]. Further research is needed to clarify the subtle differences in plant behavior during D2 as they acclimate to recurrent drought stress.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
Monica Canton: conceptualization, investigation, data curation, formal analysis, visualization, and writing of the original draft, as well as review and editing. Franco Meggio: conceptualization, investigation, review and editing. Francesco Mirone: investigation and visualization. Valentino Casolo: visualization, review and editing. Sergi Munné-Bosch: investigation, visualization and editing. Andrea Pitacco: funding acquisition, supervision, and conceptualization. Giovanni Battista Tornielli: supervision, visualization, review and editing.

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Data availability
All sequenced data produced were deposited in the European Nucleotide Archive (ENA) with the identifier: PRJEB102784.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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