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Aquaporin translation tunes plant water transport to external conditions in grapevine

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ABSTRACT

Water stress challenges global crop productivity, particularly for perennial species such as grapevines, where effective water management is crucial for berry quality and yield. Aquaporins, a family of water channel proteins, play a key role in regulating water transport within plant cells, affecting water uptake and redistribution. Although the transcriptional response of aquaporin genes to water stress in grapevines has been documented, their translational regulation remains less explored. This study investigates the transcriptional and translational dynamics of three Plasma Membrane Intrinsic Proteins and three Tonoplast Intrinsic Proteins in leaves and roots of a grafted 'Pinot Noir' on 'Kober 5BB' rootstock during water deficit conditions and recovery. Aquaporin translation analyzed by polysome profiling and co-sedimentation analysis of their transcripts highlighted that water stress had a general negative effect, although significant only for *VviTIP1-3*. Conversely, recovery measured at 6 h after rewatering was characterized by a boost of translation reactivation for all but one aquaporins. Transcriptional profiling of the same aquaporins revealed significant down-regulation at prolonged stress in roots, highlighting the contribution of aquaporins to osmoregulation and drought tolerance. Moreover, transcriptional modulation resembles a long-term adaptative response to limit water loss. In the leaf, only two specific genes, *VviPIP2-5* and *VviTIP2-1*, were modulated during water deficit and even more during recovery and positively correlated with stomatal conductance and leaf water potential. They represent important regulators of water homeostasis and good candidates for breeding programs. This study uncovered an additional level of aquaporin post-transcriptional control finely tuning vines to changing external conditions.

1. Introduction

Grapevine is one of the most economically important crops widely cultivated, with over 7.3 million hectares of vineyard area in total and over 74 million tons of production every year in the world (Food and Agriculture Organization of the United Nations, 2023). In the context of climate change, this important agricultural sector is facing challenges as the temperature is getting warmer and the precipitation frequency and intensity are becoming difficult to predict (Yuan et al., 2023). Despite its adaptation to dry environments, excessive water stress not only impairs growth and productivity but also negatively affects berry quality, such as organic acid content, pH, and titratable acidity (Gupta et al., 2020; Hewitt et al., 2023). However, plants can deploy various physio-biochemical and anatomical adaptations to cope with stressful conditions, including stomatal closure, modified root growth and architecture, shifts in metabolic pathways, and altered physiological responses (Farooq et al., 2024).

Water is transported from roots to leaves *via* three primary pathways: apoplastic, symplastic, and transcellular, ultimately releasing it into air-filled substomatal cavities (Steudle, 2000). Depending on different species, growth conditions, and developmental stages, these pathways contribute differently to overall water flow in all parts of the plants

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(Hachez et al., 2006; Sack and Holbrook, 2006). Aquaporins are deeply involved in the inter- and intra-cellular water transport, in roots, leaves as well as in fruits. By regulating water and ion homeostasis, they participate in mechanisms of vines adaptation to water deficit (Sabir et al., 2021). Aquaporins are major integral proteins that facilitate the movement of water and other small molecules such as H_2O_2 (Bienert et al., 2007), CO_2 (Ermakova et al., 2021), and ions (Tyerman et al., 2021) through the membranes.

In most organisms, aquaporins are encoded by a large gene family, comprising as many as 13 genes in humans (Azad et al., 2021) and 35 genes in *Arabidopsis* (Johanson et al., 2001). In *Vitis vinifera* L., 33 aquaporin genes were identified in the reference genome and categorized into 5 subfamilies: Plasma Membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), NIPs (nodulin-like proteins), SIPs (small basic intrinsic proteins), and XIPs (uncharacterized intrinsic proteins) (Wong et al., 2018). These families include 6 PIP1 genes (*VviPIP1-1*, *VviPIP1-2a*, *VviPIP1-2b*, *VviPIP1-2c* pseudogene, *VviPIP1-3*, *VviPIP1-4*), 5 PIP2 genes (*VviPIP2-3*, *VviPIP2-4*, *VviPIP2-5*, *VviPIP2-7*, *VviPIP2-9* pseudogene), 11 TIP genes (*VviTIP1-1*, *VviTIP1-2*, *VviTIP1-3*, *VviTIP1-4*, *VviTIP2-1*, *VviTIP2-2*, *VviTIP2-3*, *VviTIP3-1*, *VviTIP4-1*, *VviTIP5-1*, *VviTIP5-2*), 8 NIP genes (VviNIP1-2, VviNIP4-1, VviNIP5-1, VviNIP6-1, VviNIP7-1, VviNIP8-1, VviNIP9-1a pseudogene, VviNIP9-1b pseudogene), 2 XIP genes (VviXIP2-1, VviXIP2-2) and 1 SIP gene (VviSIP2-1) (Wong et al., 2018).

Aquaporin expression regulation has been studied in grapevine roots, leaves and fruits during well-watered and drought conditions and correlated with physiological parameters. In Table 1 previous results on the modulation of six aquaporins in root and leaf of grapevine cultivars (cvs.) is summarized, indicating the gene name according to the Vitis Gene Catalogue (Navarro-Payá et al., 2022) but reporting as synonyms the names used in the original articles. Pou and collaborators (2013) found that in 'Chardonnay' leaves, the expression of *VvTIP2;1* and *VvPIP2;1* correlated with leaf hydraulic conductance, with *VvTIP2;1* showing a close link to stomatal conductance (Pou et al., 2013). Shelden and colleagues (2017) extended these observations by showing that in both cv. 'Chardonnay' and 'Grenache', considered anisohydric and isohydric cultivars, respectively, the expression of *VvPIP2;1* and *VvPIP2;2* was influenced by leaf and stem water potential (Shelden et al., 2017). They also noted that *VvTIP2;1* and *VvTIP1;1* were downregulated under water stress in 'Chardonnay' petioles and 'Grenache' leaves, respectively. Zarrouk et al. (2016) observed that in 'Touriga Nacional', *VvPIP2;1* and *VvTIP2;2* were downregulated in water-stressed roots, consistent with reduced hydraulic conductivity, but *VvPIP2;1* and *VvTIP1;1* were upregulated in leaves without correlation to leaf hydraulic conductivity, indicating aquaporin genes regulation can be tissue-specific (Zarrouk et al., 2016). Finally, for some aquaporins their gene expression was quantified during recovery, showing a prevalent up-regulation (Vandeleur et al., 2009; Pou et al., 2013; Zarrouk et al., 2016).

Recently, transcriptomic studies performed in wild species and hybrids used in rootstock breeding programs provided insights into the molecular mechanisms responsible for the different degrees of observed tolerance to water deficit (Vitulo et al., 2014; Corso et al., 2015; Khadka et al., 2019; Cochetel et al., 2020). These studies analyzed both leaf and root samples, highlighting different responses in the two organs. In viticulture, rootstocks have been widely used to enhance scion resilience to abiotic stresses like drought and salt, and biotic stresses like pathogens and fungi. The reciprocal influence between rootstock and scion in water transport mediated by aquaporin in the transcellular pathway has been reported (Tramontini et al., 2013; Koc et al., 2023). Moreover, a quantitative trait locus (QTL) analysis revealed that aquaporins can be candidate genes for drought tolerance (Marguerit et al., 2012). Rootstock 'Kober 5BB' is a *Vitis* hybrid (*Vitis berlandieri* x *Vitis riparia*) commonly used in viticulture and confers good vigor. Still, it is not considered a drought-tolerant rootstock (Minio et al., 2022).

Besides the abundance of gene expression data measured either by qPCR or transcriptomics, much less information is available regarding translation of mRNA into proteins, which actually represents another important regulatory step (Urquidi Camacho et al., 2020; Wu et al., 2024). Plants utilize translational control to reversibly adjust protein synthesis to a wide variety of environmental stresses (hypoxia, heat) or conditions (darkness), as well as during developmental processes (Guo et al., 2023). One of the approaches developed for exploring translational control of gene expression is polysome profiling. It has been used to investigate metal ion stress (Sormani et al., 2011), high salinity stress (Matsuura et al., 2010), and hypoxia stress in *Arabidopsis* (Branco-Price et al., 2008). This involves lysing cells to preserve ribosome-mRNA complexes, then using sucrose gradient centrifugation to separate these complexes by their co-sedimentation coefficient, followed by RNA extraction and quantitative PCR analysis to evaluate

Table 1
Modulation of some aquaporins in grapevine cultivars in previous studies.

Gene symbol	Gene synonym	Tissue	Modulation during water stress	Modulation during recovery	Cultivar	Reference
VviPIP1-1	VvPIP1; 1	Root	up	not mod.	Chardonnay (anisohydric)	Vandeleur et al. (2009)
VviPIP1-1	VvPIP1; 1	Root	not mod.	up	Grenache (isohydric)	Vandeleur et al. (2009)
VviPIP1-1	VvPIP1; 1	Leaf	not mod.	not mod.	Chardonnay	Pou et al. (2013)
VviPIP2-5	VvPIP2; 1	Leaf	down	up	Chardonnay	Pou et al. (2013)
VviPIP2-5	VvPIP2; 1	Leaf	up	not mod.	Touriga Nacional (anisohydric or nearly isohydric)	Zarrouk et al. (2016)
VviPIP2-5	VvPIP2; 1	Petiole	down	/	Chardonnay	Shelden et al. (2017)
VviPIP2-5	VvPIP2; 1	Root	down	down	Touriga Nacional	Zarrouk et al. (2016)
VviPIP2-7	VvPIP2; 2	Leaf	down	up	Chardonnay	Pou et al. (2013)
VviPIP2-7	VvPIP2; 2	Leaf	down	/	Chardonnay and Grenache	Shelden et al. (2017)
VviPIP2-7	PIP2; 2	Root	not mod.	not mod.	Grenache and Chardonnay	Vandeleur et al. (2009)
VviTIP1-3	VvTIP1; 1	Leaf	down	/	Chardonnay	Pou et al. (2013)
VviTIP1-3	VvTIP1; 1	Leaf	up	up	Touriga Nacional	Zarrouk et al. (2016)
VviTIP1-3	VvTIP1; 1	Petiole Leaf	up	/	Chardonnay	Shelden et al. (2017)
VviTIP1-3	VvTIP1; 1	Root	up	not mod.	Touriga Nacional	Zarrouk et al. (2016)
VviTIP2-1	VvTIP2; 1	Leaf	down	up	Chardonnay	Pou et al. (2013)
VviTIP2-1	VvTIP2; 1	Leaf	down	down	Touriga Nacional	Zarrouk et al. (2016)
VviTIP2-1	VvTIP2; 1	PetioleLeaf	down	/	Chardonnay and Grenache	Shelden et al. (2017)
VviTIP2-1	VvTIP2; 1	Root	not mod.	not mod.	Touriga Nacional	Zarrouk et al. (2016)
VviTIP2-2	VvTIP2; 2	Root	down	down	Touriga Nacional	Zarrouk et al. (2016)

Not mod.: not modulated in the corresponding study;/: the information is not available.

translation efficiency (Morita et al., 2013). It has been used to investigate the mechanism of exoribonuclease XRN4-mediated degradation of polysome-bound mRNA in *Arabidopsis* seedlings after heat stress (Merret et al., 2015), or to study light-harvesting complex chloroplastic proteins translation in *Arabidopsis* plants treated with different light intensities by measuring the ratio between polysomal versus total mRNA (Floris et al., 2013).

This study explores the translational regulation of six aquaporins - three PIPs (PIP1-1, PIP2-5, PIP2-7) and TIPs (TIP1-3, TIP2-1, TIP2-2) - previously functionally characterized and transcriptionally modulated during water stress and recovery, in *Vitis vinifera* cvs. 'Pinot Noir' grafted on 'Kober 5BB', an élite variety of our territory, has been chosen as plant material. In a first experiment, three different water stress and recovery durations were analyzed in terms of physiological parameters and aquaporins transcriptional modulation. Then, a second experiment was performed to study aquaporin translational activity during water deficit conditions and very early responses upon rewatering, by successfully applying the polysome profiling technique to a perennial crop.

2. Materials and methods

2.1. Phylogenetic analysis

To create a phylogenetic tree with the maximum likelihood method, MAFFT (Katoh et al., 2019) was used for sequence alignment and IQ-TREE (Minh et al., 2020) for tree construction. The aquaporin protein sequences of *Arabidopsis thaliana* were retrieved from the *Arabidopsis thaliana* Araport11. The aquaporin protein sequences of *Vitis vinifera* were retrieved from the PN40024 telomere-to-telomere (T2T) genome (v5 (Shi et al., 2023),) (grapegenomics.com, last accessed on December 15, 2024). In addition, aquaporin protein sequences were also retrieved from the 'Pinot Noir' ENTAV115 clone and 'Kober 5BB' genome by BLAST (grapegenomics.com, last accessed on December 15, 2024). Before the analysis, the gene models were analyzed using the Jbrowse2 (Diesh et al., 2023) tool with all the annotations of the *Vitis* genome. The alignment was performed using the command: *mafft –auto input_sequences.fasta > aligned_sequences.fasta*. The alignment quality was verified using Jalview (Waterhouse et al., 2009). Subsequently, the phylogenetic tree was constructed with IQ-TREE using the command: *iqtree -s aligned_sequences.fasta -m MFP -bb 1000 -alrt* 1000 -nt *AUTO*. In this command, -m MFP selects the best-fit model, and -bb 1000 and -alrt 1000 conduct 1000 bootstrap and SH-aLRT tests, respectively. The output files were reviewed and visualized with iTOL (Letunic and Bork, 2024), selecting only high bootstrap values (70–100 %) to ensure well-supported branches that likely represent true evolutionary relationships. The analysis was made as suggested by Grimplet and collaborators in 2014 (Grimplet et al., 2014). Gene names were adopted from the most recent version of the Vitis Gene Catalogue (Navarro-Payá et al., 2022) (https://grapedia.org/wp-content/uploads/2025/02/grapevine_catalogue_v3.xlsx, last accessed on December 15, 2024).

2.2. Transcriptomic meta-analysis

Four BioProjects related to water stress were selected from the SRA (Sequence Read Archive) database: PRJNA516950 (Cochetel et al., 2020), PRJNA429560 (Khadka et al., 2019), PRJNA226228 (Vitulo et al., 2014), and PRJNA226229 (Corso et al., 2015). The resulting experiments were downloaded in SRA format, converted to fastq format using *fastq-dump*, trimmed with *Fastp* software (Chen et al., 2018), and mapped to the grapevine PN40024.v5 genome using *STAR* (Dobin et al., 2013). Raw counts were computed with *featureCounts* (Liao et al., 2014) on the PN40024.v5 genome annotation. The final analysis was performed using Transcripts Per Kilobase Million (TPM) values from all RNA-seq experiments, normalized to log2(TPM+1).

2.3. Plant materials and growth conditions

One-year grapevine cuttings of 'Pinot Noir' clone ENTAV115 (*Vitis vinifera* L.) grafted on 'Kober 5BB' (*Vitis berlandieri* x *Vitis riparia*) rootstocks were used. In the water stress experiment conducted in August 2023, 60 plants were placed in 3-L pots containing loam-based potting compost (Ter-compost, Italy) with a thin layer of pumice at the bottom and placed in the greenhouse at Fondazione Edmund Mach (FEM), located at San Michele all'Adige, Trento, Italy (46°11′26.8″ N 11°08′08.1″ E). The temperature (°C) and relative humidity (RH%) in the greenhouse were 25–30 °C and 50–70 % respectively, as measured by continuously recording using two data loggers (Tinytag Ultra 2, Gemini Data Logger, UK) on the East and West sides. In the water stress experiment conducted in March 2024, 20 plants were repotted with the same substrate as before in 5-L pots and placed in the same greenhouse, while the temperature and relative humidity were 20–25 °C and 45–65 %. Before the stress treatment, plants were fertilized and regularly watered twice a week; lateral shoots and fruits were removed to maintain one vertical shoot. The top shoots were removed from the plants to normalize growth, leaving 12 leaves on the plants.

2.4. Experimental design and samples collection

Plants were randomly allocated on the bench (Supplementary Fig. 1a and b), the weight of all plants (including soil, pot, and stick) was set to 1550 g in August 2023 (and 2700 g in March 2024) and the pots were wrapped in a transparent plastic bag to prevent water leakage from the pot. In August 2023, control plants were watered every day with 100 g of water to supplement water loss occurred over the previous 24 h (well-watered group) while the other plants were not watered for either 10, 15 or 20 days. A gradual decrease in pot weight mirrored the progressive reduction of water availability. Rewatering consisted in providing 500 g of water on the first day and then replacing transpired water, as for the control plants. In March 2024, control plants were watered every day with 75 g of water while the other plants were not irrigated for 19 days and then rewatered in the same way as in 2023.

The third/forth fully expanded mature leaves (5th-6th from the shoot tip) were collected from three plants of each experimental group (Supplementary Fig. 1). In the experiment conducted in August 2023, sampling after rewatering was performed at the time-points indicated in Supplementary Fig. 1c. Critical time-points were selected on the basis of the transpiration curve and used for aquaporin gene expression analysis by qPCR as indicated in Fig. 1a. Leaf water potential was measured at certain time-points immediately after leaf detachment, and then leaves were wrapped in aluminium foil and rapidly frozen in liquid nitrogen (N_2). Root samples of well-watered and water-stressed plants were collected in biological triplicates on days 0, 4, and 20. Fine roots were carefully detached from the root system, thoroughly washed with tap water, and flash-frozen in a 50 mL Falcon tube in liquid N_2. All collected samples were stored at −80 °C for subsequent analyses. In the experiment conducted in March 2024, leaves at the third node were collected on days 19–20 of the experiment (Fig. 1b). Leaves from each treatment were collected from four biological replicates, rapidly frozen in liquid N_2, and kept at −80 °C for subsequent analyses.

2.5. Physiological parameters measurements

To monitor the whole plant transpiration (E), the total pot weight was measured daily at 9:00 a.m. using a scale (Ohaus Defender 3000). Transpiration on day *n* of the experiment was calculated as the difference between the weight on day *n* and the weight on the previous day (*n-1*). Leaf water potential (Ψ_L, n = 3) was measured every two days during the experiment between 10:00 a.m. and 12:00 p.m. (midday leaf water potential). For this analysis, two fully expanded and light-exposed leaves were detached from the plant and used for the analysis via a pressure chamber (Pump-up Chamber, PMS Instrument Company, USA). When a

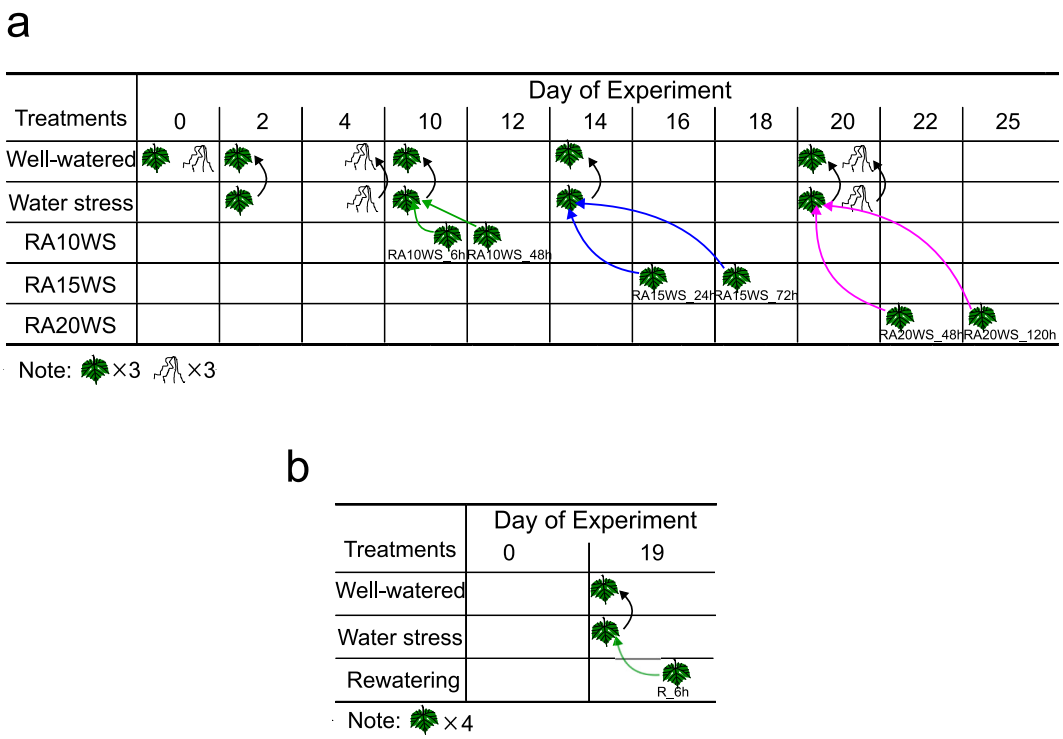


Fig. 1. Sampling description and experimental design. The tissue (root or leaf) and time of sample collection of the first experiment performed in August 2023 (a) and the second one performed in March 2024 (b) are shown. Black arrows indicate the comparison between water-stressed and well-watered samples used in the gene expression analyses, while green, blue and pink arrows indicate the comparison between re-watered and water-stressed samples. The number of biological replicates is indicated below the scheme. RA10WS: rewatering after 10 days of water stress; RA15WS: rewatering after 15 days of water stress, RA20WS: rewatering after 20 days of water stress. 6h_RA10WS: 6 h after rewatering after 10 days of water stress, 48h_RA10WS: 48 h after rewatering after 10 days of water stress; 24h_RA15WS: 24 h after rewatering after 15 days of water stress, 72h_RA15WS: 72 h after rewatering after 15 days of water stress; 48h_RA20WS: 48 h after rewatering after 20 days of water stress, 120h_RA20WS: 120 h after rewatering after 20 days of water stress; R_6h: 6 h after rewatering.

small water droplet appeared at the cut surface of the petiole, the corresponding air pressure (bar) was recorded. The water potential in leaves was expressed in megapascals (MPa), where 1 bar equals 0.1 MPa. Stomatal conductance (g_s) was measured in the morning from 10:00 a.m. to 12:00 p.m. with an LI-600 porometer (LI-COR Bioscience, Lincoln, NE, USA). A single fully expanded and light-exposed leaf from the upper section of each plant was chosen, and one measurement was taken per plant on different days, all of them under natural lighting conditions. The length of the newly grown shoot on the top bud was measured on different days of the experiment, starting on day 11, with three plants for each condition.

2.6. Total RNA extraction and reverse transcription

Approximately 50 mg of leaf and 500 mg of root material were ground to a fine powder under liquid N_2. Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA integrity was assessed using agarose gel electrophoresis, while Nanodrop was used for quantification and quality check. Genomic DNA contamination was removed by DNase I treatment using the TURBO DNA-*free* ™ Kit (Thermo Fisher, USA). The complementary DNA (cDNA) was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, USA) and Oligo$(dT)_{18}$ (#k1622, Thermo Fisher, USA), following the manufacturer's instructions.

2.7. Primer design

Primers spanning exon-exon junctions were designed using Primer3 (https://primer3.ut.ee/, version 4.1.0) to amplify amplicons between 120 bp and 145 bp in length with T_m value between 58 °C and 62 °C and GC content not higher than 55 % (Supplementary Table S1). The primers were aligned against the genome of 'Pinot Noir' (*Vitis vinifera* L.) (PN40024 v5, https://www.grapegenomics.com/pages/PN40024/jbrowse2.php) and the genome of 'Kober 5BB' (*Vitis berlandieri* x *Vitis riparia*) (version 1.0, https://www.grapegenomics.com/pages/VKober5BB/blast.php), respectively, to verify they anneal to targets in all the genetic backgrounds analyzed.

2.8. Quantitative PCR and data analysis

Primer efficiency was determined using a serial dilution of one leaf cDNA, and the efficiencies of all the primers except for *VviPIP1-1* were within 90–110 % (details see Supplementary Table S2). All cDNA samples were diluted 20-fold before quantitative PCR (qPCR). 1 μL of cDNA, 2 μL of 2.5 μM mixed forward and reverse primers, 6.25 μL of 2X qPCRBIO SyGreen mix (PCRBIOSYSTEMS, UK), and 3.25 μL of PCR grade water were added in a 12.5 μL reaction system. PCR thermocycling was performed as the following program: 1 cycle at 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 s and 62 °C for 25 s. Before the melting curve analysis, PCR products were heated to 95 °C for 15 s and then subjected to the melting curve analysis between 62 °C and 95 °C at 0.5 °C increments for 15 s. Each reaction was performed in duplicate. Fold changes were calculated following the 2-ΔΔCt method (Livak and Schmittgen, 2001), either as water-stressed versus well-watered samples or rewatered versus water-stressed samples. *VviGAPDH* was used as the reference gene in both cases.

2.9. Polysome profiling

Polysome profiling was performed as described in (Bernabò et al., 2017). Leaf samples of well-watered, water-stressed, and 6 h after rewatering (R_6h) groups were collected during the experiment

conducted in March 2024 and ground to a fine powder in liquid N_2. For sucrose gradient preparation, solutions were prepared using a specific buffer composition (40 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, pH 8.4) with varying sucrose concentrations. Sucrose gradients were prepared in 12 ml tubes by overlaying 5.5 mL of 50 % (w/V) sucrose buffer and filling the tube with 15 % (w/V) sucrose buffer. The gradient was formed by keeping the tube horizontally for 120 min at 4 °C. Approximately 200 mg of each powdered sample was lysed in 1200 μL of lysis buffer (160 mM Tris-HCl, 80 mM KCl, 40 mM $MgCl_2$, 0.6 U/μL RNase inhibitor, 0.005 U/μL DNase I, 1 mM dithiothreitol, 200 μg/mL cycloheximide, 0.5 % IGEPAL, 2.5 % PVP-40, pH 8.4) to prepare the cytoplasmic lysates. The lysate was incubated on ice for 20 min and then subjected to four rounds of centrifugation at 4 °C, 20,854 g for 20 min to remove tissue debris, nuclei, chloroplasts, and mitochondria. Following centrifugation, approximately 850 μL of clear supernatant was transferred to a 2 mL thick-walled ultracentrifuge tube (Beckman Coulter, USA) and ultracentrifuged at 4 °C, 436,000 g for 67 min using a TLA100.2 rotor (Beckman Coulter, USA) to precipitate ribosomal subunits, ribosomes, and polysomes. Next, the pellet was vigorously resuspended with 600 μL buffer (160 mM Tris-HCl, 80 mM KCl, 40 mM $MgCl_2$, 200 μg/mL cycloheximide, 1 mM dithiothreitol, 0.005 U/μL DNase I, 0.6 U/μL RNase inhibitor, pH 8.4). For density-based separation, 500 μL of the clear supernatant (excluding undissolved pellets) were layered onto a sucrose gradient (15 %–50 %, w/V) and subjected to ultracentrifugation at 4 °C, 274,000 g, for 1 h 40 min in a Beckman Optima LE-80K ultracentrifuge using SW60 Ti rotor (Beckmann, USA). Sucrose fractions were collected using a Teledyne Isco model 160 gradient analyzer equipped with a UA-6 UV/VIS detector to measure the absorbance at 254 nm. Plotting absorbance vs fraction number yields a polysome profile. The fractions were either used immediately for RNA extraction or stored at −80 °C for future use.

2.10. Fraction of ribosomes in polysomes

The Fraction of Ribosomes in Polysomes (FRP) was calculated from the polysome profile curve. FRP(%) is calculated using the ratio of the area under the curve for polysomes to the sum of the areas for polysomes and 80S ribosomes, using the following formula (Bernabò et al., 2017):

$$\%FRP = \frac{A_{polysomes}}{A_{polysomes} + A_{80S}} * 100$$

where A is the area under the curve, %FRP is the Fraction of ribosomes in polysomes.

2.11. Co-sedimentation analysis of target mRNA

To analyze the co-sedimentation profiles of mRNAs, RNA was extracted from each sucrose fraction. Sucrose fraction from polysome profiling was treated with 2.5 μL of 20 mg/mL proteinase K (Thermo Fisher Scientific) and 50 μL of 10 % (V/V) sodium dodecyl sulfate (SDS) solution at room temperature and incubated at 37 °C for 1 h and 45 min to digest protein contaminants. After incubation, 130 μL of acid-phenol/chloroform/isoamyl alcohol (125/24/1, pH 4.5) (Invitrogen) was added and mixed thoroughly. Next, samples were centrifuged at 4 °C, 12,750 g for 10 min. The upper aqueous phase containing RNA was carefully transferred to a new nuclease-free 2 mL Eppendorf tube, ensuring the bottom phase was not disturbed. 500 μL of 100 % isopropanol and 1 μL of glycol blue (to visualize the RNA pellets) were added to the sample and mixed thoroughly and stored at −80 °C overnight. After overnight precipitation, the RNA pellets were collected by centrifugation at 4 °C, 15,300 g for 45 min. The supernatant was then discarded, and the pellets were carefully dried. Subsequently, pellets were washed with 500 μL of 80 % (V/V) ethanol in diethylpyrocarbonate (DEPC)-treated water (RNase-free) and centrifuged at 4 °C, 12,750 g for 10 min. The supernatant was discarded, and the pellets were carefully dried again. Finally, the RNA pellets were dissolved in 11 μL of DEPC-treated water (RNase-free). The quality of the RNA was assessed by measuring 260/230 and 260/280 absorbance ratios using Nanodrop. The remaining RNA was aliquoted into two 200 μL tubes and stored at −80 °C for downstream analyses.

cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. Quantitative PCR was performed using exactly 1 μL of cDNA from each fraction following the procedures described in the previous section. Each fraction was analyzed in a single reaction. The threshold was set at 40 amplification cycles, and the amplification efficiencies of all primers were assumed to be 100 %. The distribution of mRNA in each fraction was expressed as a percentage across the nine fractions, calculated as follows:

$$\%(mRNA)_n = \frac{\left(2^{40-Cq_{mRNA}}\right)_n}{\sum_{n=1}^{n=9}\left(2^{40-Cq_{mRNA}}\right)_n} * 100$$

where n represents the fraction number.

2.12. Statistical analysis

Statistical analysis was performed with Excel (Microsoft, USA) and R studio. For gene transcriptional modulation, data have been expressed as Log2 of the fold change of water-stressed vs. well-watered samples or samples collected after recovery vs. the corresponding stressed sample, as shown in Fig. 1a. The same comparisons were made to study translational modulation (Fig. 1b). In the physiological study, both water-stressed and re-watered plants were compared to the well-watered plants. Data represent mean ± SEM (n = 3). Statistical significance was assessed using a two-sided, unpaired Student's t-test. Violin plot visualization was made using package 'ggplot' for the log2(TPM+1) values. The normality of transcriptomic data in the meta-transcriptomic analysis was assessed with the Shapiro-Wilk test and the Wilcoxon signed-rank test was used to compare the root and leaf groups.

2.13. Software

Co-sedimentation profiles and representative polysome profiles were visualized using Origin Pro 2021 (OriginLab, USA). Results from physiological experiments and gene expression analyses were plotted with GraphPad Prism 8.0 (Dotmatics, USA) and Excel (Microsoft, USA). All result images were organized using Inkscape (v1.3.1).

3. Results

3.1. Phylogenetic characterization of Vitis Aquaporins

To properly identify and characterize PIP and TIP aquaporin genes in the rootstock and scion, the genes identified in previous versions of the *Vitis vinifera* reference genome (Shelden et al., 2009; Wong et al., 2018) were searched in the latest PN40024 T2T genome (v5) (Shi et al., 2023) and named according to the last version of the *Vitis* Gene Catalogue (Navarro-Payá et al., 2022). The pseudogene *VviPIP1-2c* was not confirmed in the v5 grapevine genome and thus was not considered further in our analysis. *VviPIP2-9* was confirmed as a pseudogene, while the genes *VviTIP2-2* and *VviTIP2-3*, previously identified as distinct genes, were the same gene in v5, called *VviTIP2-2*. The retrieved sequences were used for a BLAST search against the genomes of 'Pinot Noir' clone ENTAV115 and 'Kober 5BB', a hybrid of *Vitis berlandieri* and *Vitis riparia* (Minio et al., 2022) (Supplementary Table S3). Multiple alignments and phylogenetic analysis allowed us to properly identify the homologs in the different genetic backgrounds and classify them into two sub-groups within the PIPs family (called PIP1 and PIP2) and five sub-groups within the TIPs family, with the corresponding paralogous

genes identified for *Arabidopsis thaliana* (Fig. 2).

No orthologs were found in 'Pinot Noir' ENTAV115 and 'Kober 5BB' for the pseudogene *PIP2-9*. The tandem duplicates *VviPIP1-2a* and *VviPIP1-4* (Wong et al., 2018) were retrieved as a single gene in 'Kober 5BB', named *PIP1-2a*, and were not retrieved in 'Pinot Noir' ENTAV115. In addition, *VviPIP2-4*, *VviTIP1-1*, *VviTIP1-4*, and *VviTIP3-1* were not identified in 'Pinot Noir' ENTAV115.

3.2. Transcriptomic meta-analysis of Vitis aquaporin genes during water stress

To complement the information available for the six aquaporins reported in Table 1, public RNA-seq data from rootstocks under water stress were retrieved (Supplementary Table S4). PIPs and TIPs expression levels extracted from these datasets were visualized as violin plots in Fig. 3 and Supplementary Fig. 2. Two *non-vinifera* species ('Riparia Gloire' and 'Ramsey'), cv. 'Cabernet Sauvignon' and one natural hybrid ('SC2') - characterized by different tolerance to drought stress - were analyzed by sampling root and leaves from potted plants at 7 and 14 days of moderate water deprivation (Cochetel et al., 2020). A similar experimental and sampling design has been applied to *Vitis riparia* Michx., a wild American species widely used as rootstock and scion (Khadka et al., 2019). Corso et al. (2015) examined two rootstocks, the drought tolerant 'M4' hybrid and the less tolerant '101.14' hybrid. In this case, water stress was induced by progressively lowering the soil field capacity in pots from 80 % (control) to 30 % along 10 days and analyzing gene expression in roots and leaves at 2, 4, 7 and 10 days, described as mild, moderate and severe stress according to measured physiological parameters (Corso et al., 2015).

Comparing expression values in leaf and root tissues, allowed to identify genes with significantly higher expression in the roots (*VviPIP1-1*, *VviPIP2-3*, *VviPIP2-4*, *VviPIP2-7*, and *VviTIP2-1*, *VviTIP2-2*, *VviTIP1-4*), in the leaf (*VviTIP1-1*), root-specific (*VviTIP2-2*), very low expressed (*VviTIP3-1*, *VviTIP5-1*, *VviTIP5-2*) or equally highly expressed (*VviPIP1-3, VviPIP1-4, VviPIP2-5, VviTIP1-3, VviTIP1-2, VviTIP4-1)* in the two tissues (Supplementary Table S5). The low expression in roots and leaves of *VviTIP3-1*, *VviTIP5-1*, and *VviTIP5-2* is supported by a previous study showing the preferential expression in flower structures for *VviTIP5-1* and *VviTIP5-2* and in bud, stem, and berry for *VviTIP3-1* (Wong et al., 2018).

Focusing on aquaporins modulation during water deficit stress, we can see different profiles. *VviPIP1-1* and *VviTIP2-1* are modulated only in roots at 14 or 10 days of water deficit in all the genotypes. *VviPIP2-5* and *VviTIP1-3* are modulated in both leaf and root at prolonged stress, with some exceptions. *VviPIP2-7* is modulated only in leaf in three genotypes and late time-points. *VviTIP2-1* is the most extensively modulated (15

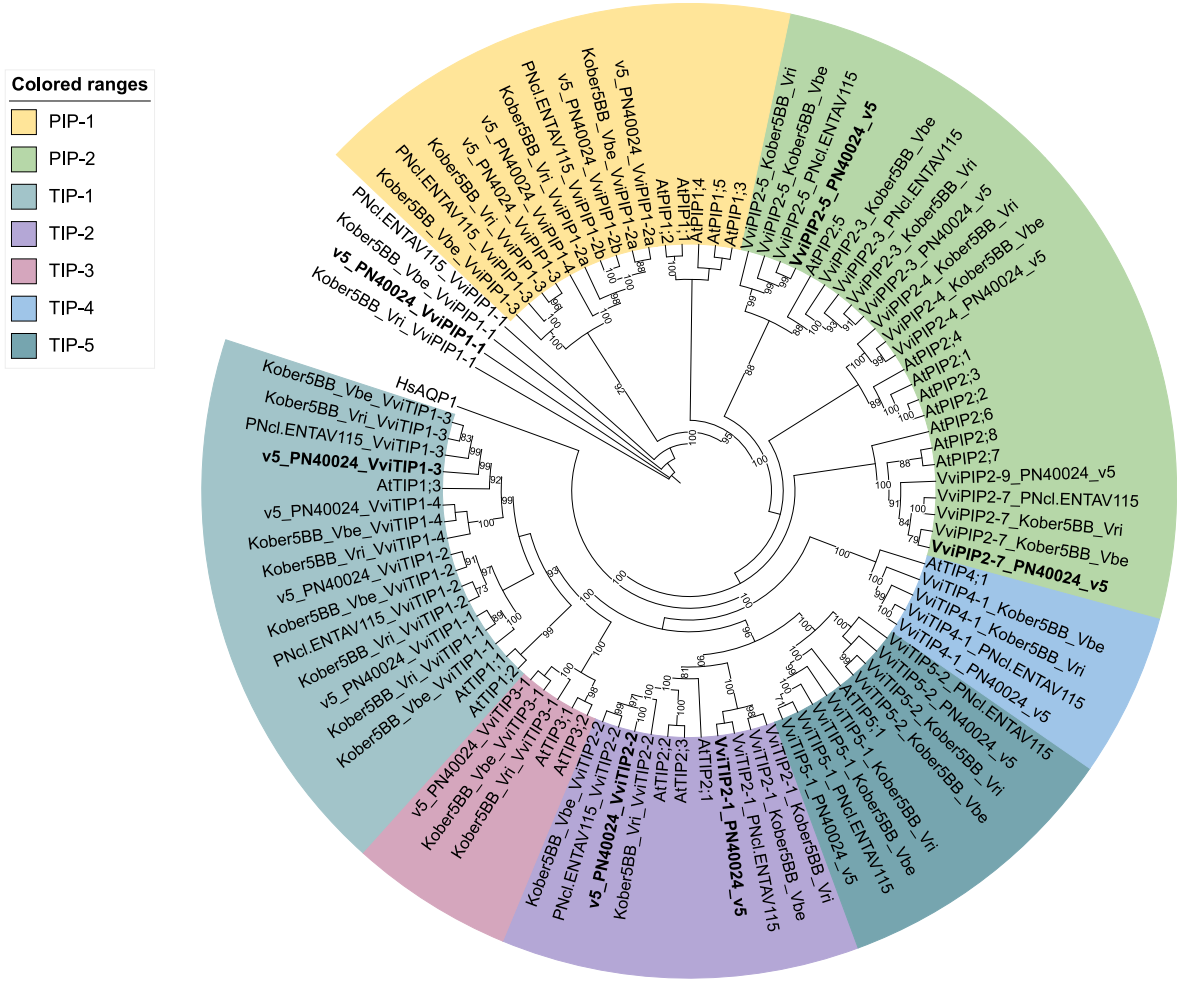


Fig. 2. Phylogenetic analysis of TIP and PIP genes in *Vitis* and *Arabidopsis thaliana*. Circular phylogenetic tree depicting the evolutionary relationships of aquaporins, particularly PIPs and TIPs, from *Arabidopsis thaliana*, PN40024 reference genome, 'Kober 5BB' (*Vitis berlandieri* × *Vitis riparia*), and 'Pinot Noir' clone ENTAV115 genomes. It was conducted using the maximum likelihood method and a thousand bootstraps, selecting those with at least 70 % bootstraps as statistically relevant. The groups identified in the four studied genomes are represented in colors listed in the legend. Branches are labeled with identifiers such as "Kober5BB_Vbe", "Kober5BB_Vri", and "PNcl.ENTAV115" corresponding to *Vitis berlandieri*, *Vitis riparia*, and 'Pinot Noir' clone, respectively. Human AQP1 was used as an outgroup. The genes analyzed in the qPCR and polysome profiling analyses are highlighted in bold.

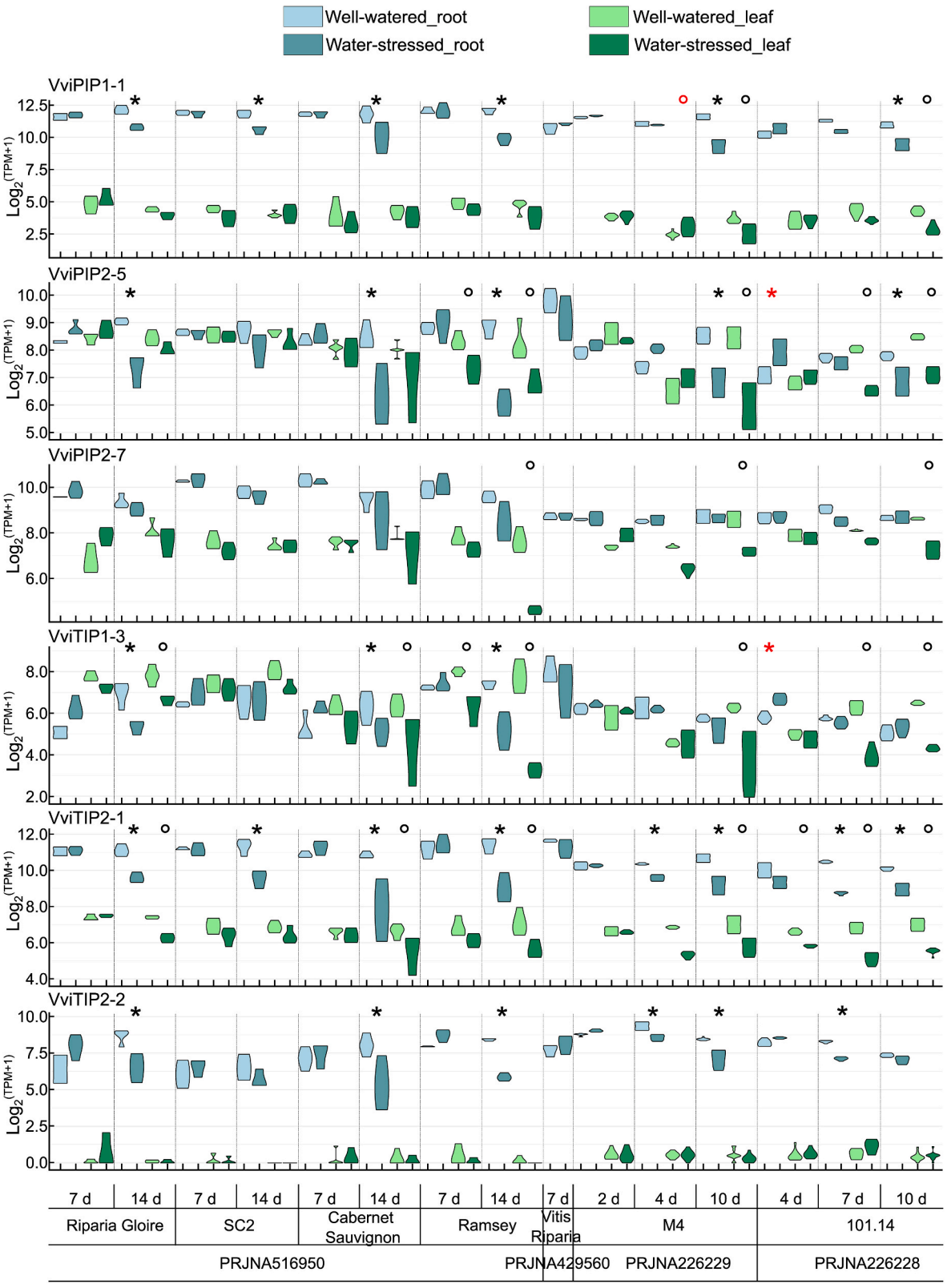


Fig. 3. Visualization of selected aquaporin gene expression levels. Data were taken from published studies performed in different *Vitis* genotypes under control and water stress conditions. The data on roots are represented in blue, and those on leaves are represented in green. Lighter and darker colors indicate well-watered and water-stress samples, respectively. The expression level is expressed as log2(TPM+1). Violin plots have been used to represent data distribution of replicates. Project, genotype and duration of the water deficit stress are indicated under the graphs. Asterisk and circle symbols indicate statistically significant modulation in root and leaf, respectively, as reported in the supplementary tables of the corresponding articles (Supplementary Table S4). Black and red colors indicate down and up-regulation.

comparisons out of 29). A general down-regulation at prolonged stress duration is observed for most of the aquaporin.

3.3. Physiological characteristics were significantly altered by water stress

A first experiment of water stress and recovery was performed on one-year grapevine cuttings of 'Pinot Noir' clone ENTAV115 grafted on 'Kober 5BB' rootstock to gain insight into the physiological behavior of this specific plant-environment system and the transcriptional modulation in leaf and roots of the six aquaporins selected from the literature as water stress-related. Potted vines were subjected to three durations of water deficit (10, 15 and 20 days) with subsequent recovery by restoration of daily watering. Several physiological parameters and ambient factors (temperature and humidity) were measured (Fig. 4a). The environment in the greenhouse was rather stable with temperature 25–30 °C and relative humidity 50–70 % throughout the experiment (Fig. 4b). Well-watered plants showed stable transpiration rates, averaging around 100 g per day. However, water-stressed plants exhibited a

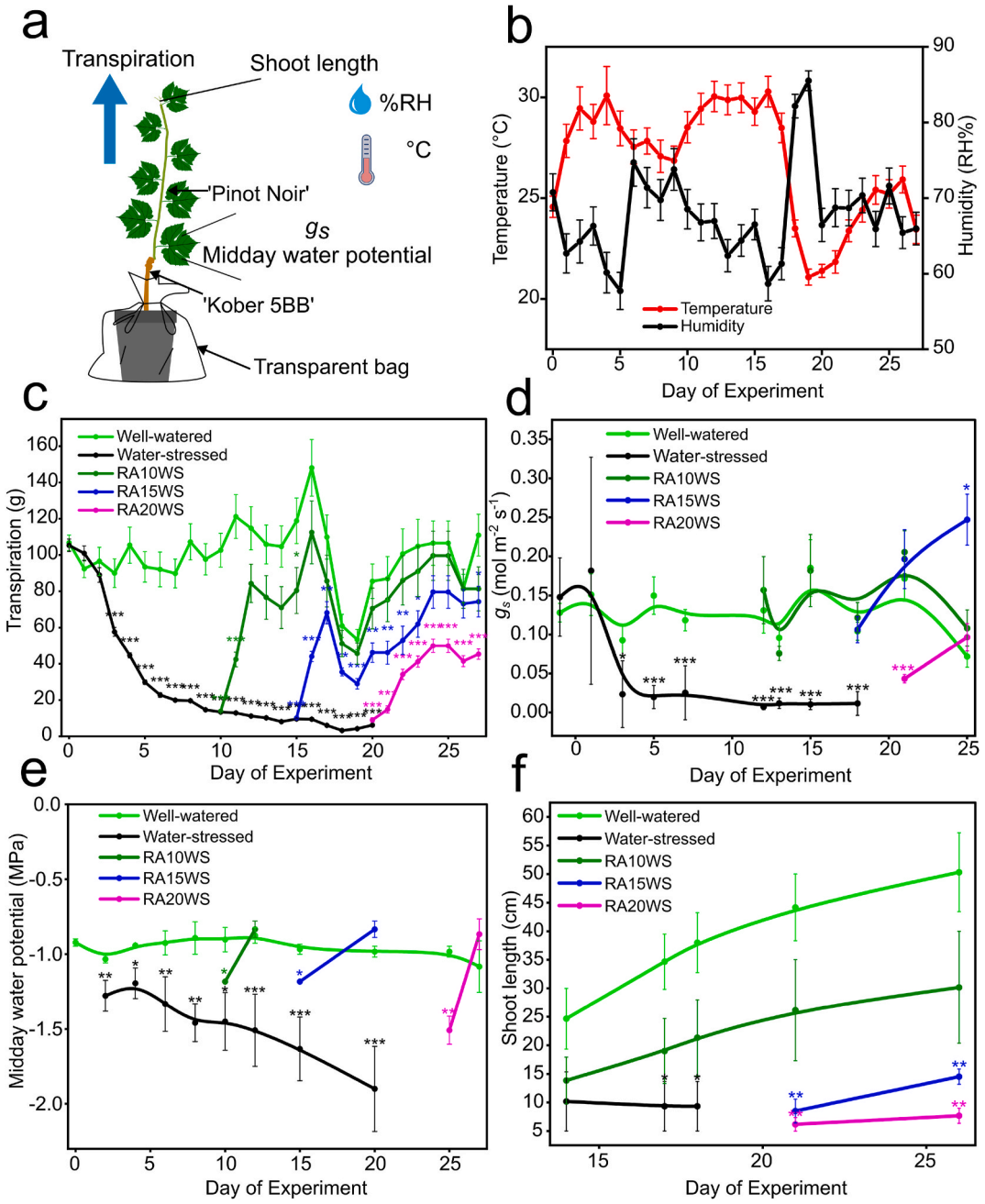


Fig. 4. Physiological responses of *Vitis vinifera* cv. 'Pinot Noir' grafted on 'Kober 5BB' under varying hydration regimes. (**a**) Schematic representation of the physiological parameters evaluated in this work. (**b**) Mean air temperature (°C) and relative humidity (%) within the greenhouse. Data are presented as mean ± SEM (n = 48). (**c**) Whole-plant daily transpiration (g). Each data point represents the mean ± SEM from at least three biological replicates. (**d**) Stomatal conductance (g_s; mol m^{-2} s^{-1}) of leaves. Data are shown as mean ± SEM from at least three biological replicates. (**e**) Midday leaf water potential (MPa). Data are mean ± SEM (n = 3). (**f**) Shoot elongation from the apical bud (cm). Data are mean ± SEM (n = 3). For panels (**d**) to (**f**), data points were interpolated using spline curves. Statistical analysis for panels (**c**) to (**f**) was performed using a two-sided, unpaired Student's t-test, where * denotes $p < 0.05$ and ** denotes $p < 0.01$. Water-stressed and recovery samples were compared to well-watered samples. Treatments are denoted as follows: RA10WS, RA15WS, and RA20WS, indicating rewatering after 10, 15, or 20 days of water stress, respectively.

significant reduction in transpiration since day 3 ($p < 0.001$), dropping to approximately 20 g around day 6 and remaining low throughout the experiment (Fig. 4c). Plants started wilting on day 8, eventually reaching severe drought symptoms where more than half of the leaves were dropped (Supplementary Fig. 3a). Different recovery was observed according to the duration of the stress: plants rewatered after 10 days (RA10WS) restored full transpiration within 2 days ($p < 0.05$), whereas those rewatered after 15 days (RA15WS) required 8 days ($p < 0.05$), and those rewatered after 20 days (RA20WS) exhibited incomplete recovery by the conclusion of the experiment ($p < 0.001$) (Fig. 4c). Phenotypic analyses corroborated these findings, demonstrating that increased stress severity was associated with progressively delayed recovery rates in plants (Supplementary Figs. 3b, c, d). This pattern was mirrored in stomatal conductance, which significantly decreased by day 3 ($p < 0.05$) in water-stressed plants and remained low (around 0.03 mol m^{-2} s^{-1}). Well-watered plants maintained a g_s of approximately 0.12 mol m^{-2} s^{-1}, while the RA10WS, RA15WS, and RA20WS plants showed relatively quick recovery of g_s after 2, 3, and 5 days of rewatering, respectively (Fig. 4d).

The midday water potential in well-watered plants was stable at around −1 MPa throughout the experiment. However, water-stressed plants experienced a decline to around −1.3 MPa after 2 days of water stress ($p < 0.01$) and gradually reached the lowest point of around −2 MPa by the end of the experiment ($p < 0.001$). In line with transpiration and g_s results, the water potential in RA10WS, RA15WS, and RA20WS plants recovered to a level similar to well-watered plants after 2, 5, and 7 days of rewatering, respectively (Fig. 4e). As expected, plants subjected to water stress exhibited stunted shoot growth, with some plants displaying complete cessation of shoot growth. In contrast, shoots in RA10WS plants continued to grow, albeit at a slightly shorter length than in well-watered plants. The RA15WS and RA20WS plants, while also exhibiting cessation of growth, demonstrated slow or no recovery after rewatering ($p < 0.01$) (Fig. 4f).

3.4. Transcriptional modulation of aquaporin genes

To study the transcriptional modulation of the six water-stress related aquaporins in our plant-environment system, their expression has been analyzed at critical time-points during stress and recovery in leaves and roots by qPCR from total cytoplasmic RNA. Concerning the water stress condition, leaf samples collected on days 0, 2, 10, 14, and 20 were analyzed to measure events from early to prolonged water deficit conditions, corresponding to increasing degrees of stress. Root samples were collected on days 0, 4, and 20 to minimize plant disruption during the study. Not all aquaporin genes were modulated to the same extent in response to water stress. For instance, in the leaves the expression levels of *VviPIP1-1*, *VviPIP2-7*, and *VviTIP1-3* remained unchanged throughout the experiment. In contrast, *VviPIP2-5* was 2-fold down-regulated at day 10 and 4-fold at day 14, while *VviTIP2-1* was 4-fold down-regulated at day 20 (Fig. 5). *VviTIP2-2* was not determined in leaves, as its expression is highly root-specific as observed in the meta-transcriptomic analysis.

In the root samples from 'Kober 5BB' rootstock, four out of six genes,

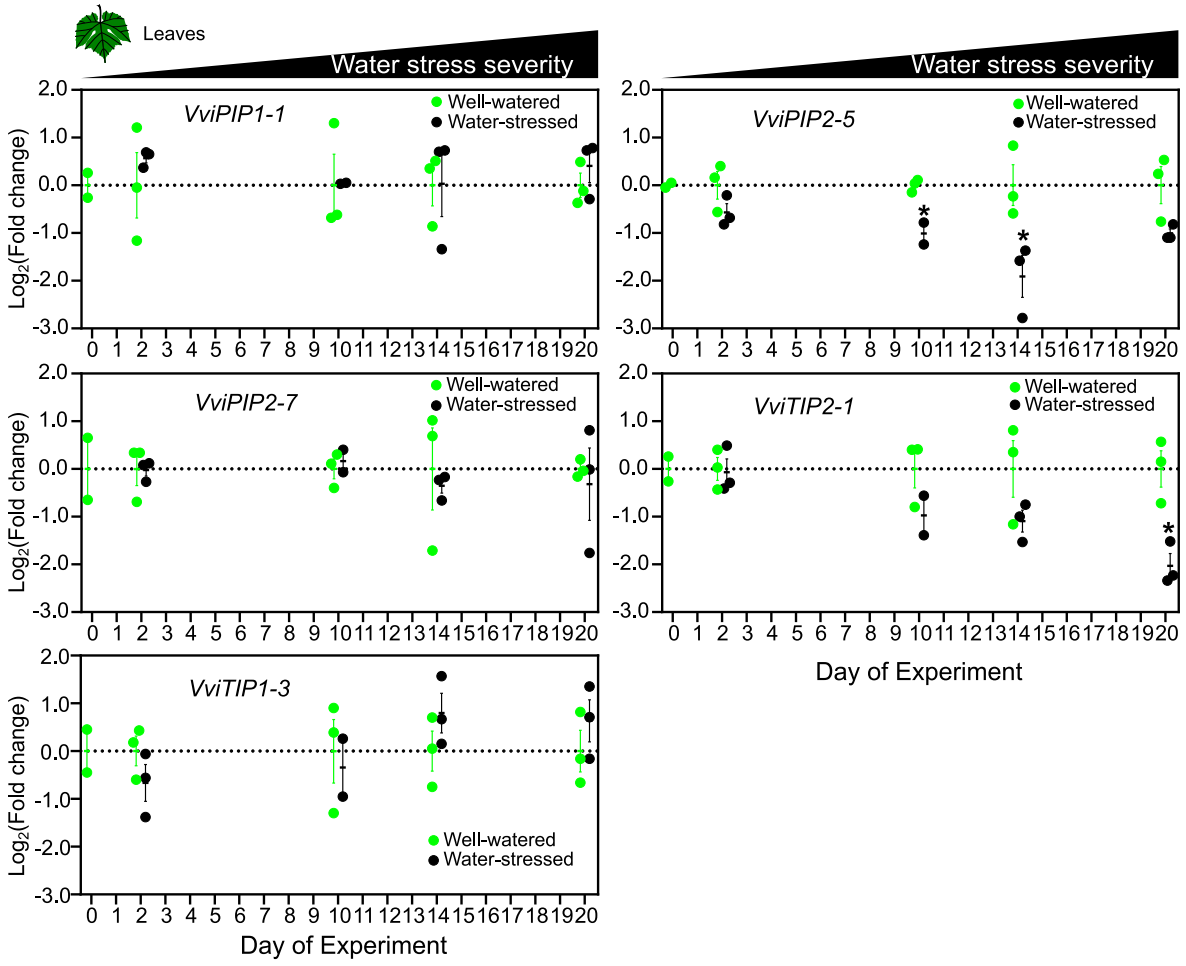


Fig. 5. Analysis of aquaporin gene transcript modulation in *Vitis vinifera* leaves under well-watered and water deficit stress conditions. Modulation of aquaporin gene transcripts in leaves under water stress. Data are presented as Log2 fold change of water-stressed samples relative to well-watered samples. Data represent mean ± SEM (n = 3). Statistical significance was assessed using a two-sided, unpaired Student's t-test, where * indicates $p < 0.05$.

VviPIP2-5, *VviTIP2-1*, *VviTIP2-2*, and *VviTIP1-3*, were significantly and highly down-regulated at 20 days of water stress (Fig. 6). *VviPIP1*-1 was highly down-regulated as well, but didn't pass the statistical test due to a p-value of 0.06.

Then, the modulation of aquaporin gene expression at the transcriptional level was tested in the leaf samples after rewatering to gain insight into their role in water stress recovery (Fig. 7). The selection of the samples was based on the transpiration curve (Fig. 4c), to identify one time-point at the initial rise and one at the plateau of the curve, corresponding to a complete transpiration recovery. After 10 days of water deficit, recovery was measured at 6 and at 48 h: none of the analyzed genes were significantly modulated (Fig. 7a). After 15 days of water deficit, recovery was measured at 24 and 72 h: *VviPIP2-5* and *VviTIP2-1* exhibited significant up-regulation after 24 h of recovery, while. *VviPIP2-7* and *VviTIP1-3* showed a down-regulation, significant at both time-points for the former and at 72 h for the latter (Fig. 7b–c). Finally, after 20 days of water deficit, transcriptional modulation was measured at 48 h and 120 h (5 days): *VviPIP2*-5 and *VviTIP2-1* showed a significant and remarkable up-regulation (between 5.5 and 8 fold) at both time-points.

3.5. Translation analysis of aquaporin genes

To investigate global and aquaporin-specific translation activities in grapevine leaves under water deficit stress and recovery, a second water deprivation experiment was conducted in March 2024 (Supplementary Fig. 4). Despite the different environmental conditions, the average air temperature was around 23 °C (Supplementary Fig. 5a), the samples were collected at a water stress level similar to that used for the transcriptional analysis as evidenced by decreased water availability in the soil, decreased transpiration, decreased midday water potential in leaves, and decreased stomatal conductance (Supplementary Fig. 5b–e). We evaluated global translation using polysome profiling and the calculation of the Fraction of Ribosomes in Polysomes (FRP) (Fig. 8a). Despite similar FRP values in well-watered and water-stressed plants, rewatering resulted in the significant increase in FRP values and thus translation activity (Fig. 8b and c).

Next, we performed a co-sedimentation analysis of aquaporin mRNA across the polysome profile to study the relative association of transcripts with polysomes. We found that water deprivation stress induced a general reorganization of ribosomes and mRNA (Fig. 9). In fact, we observed a shift of mRNA co-sedimentation from polysomal fractions towards lighter fractions in almost all cases and was statistically supported for *VviTIP1-3* and the two housekeeping genes *VviGAPDH* and *VviActin* (Fig. 9). After rewatering, the relative distribution of most aquaporins and the two housekeeping mRNAs shifted from non-polysomal fractions to polysomal fractions in a statistically significant way, suggesting that translation was restored. Only *VviPIP1-1* was not modulated in either stress or recovery conditions.

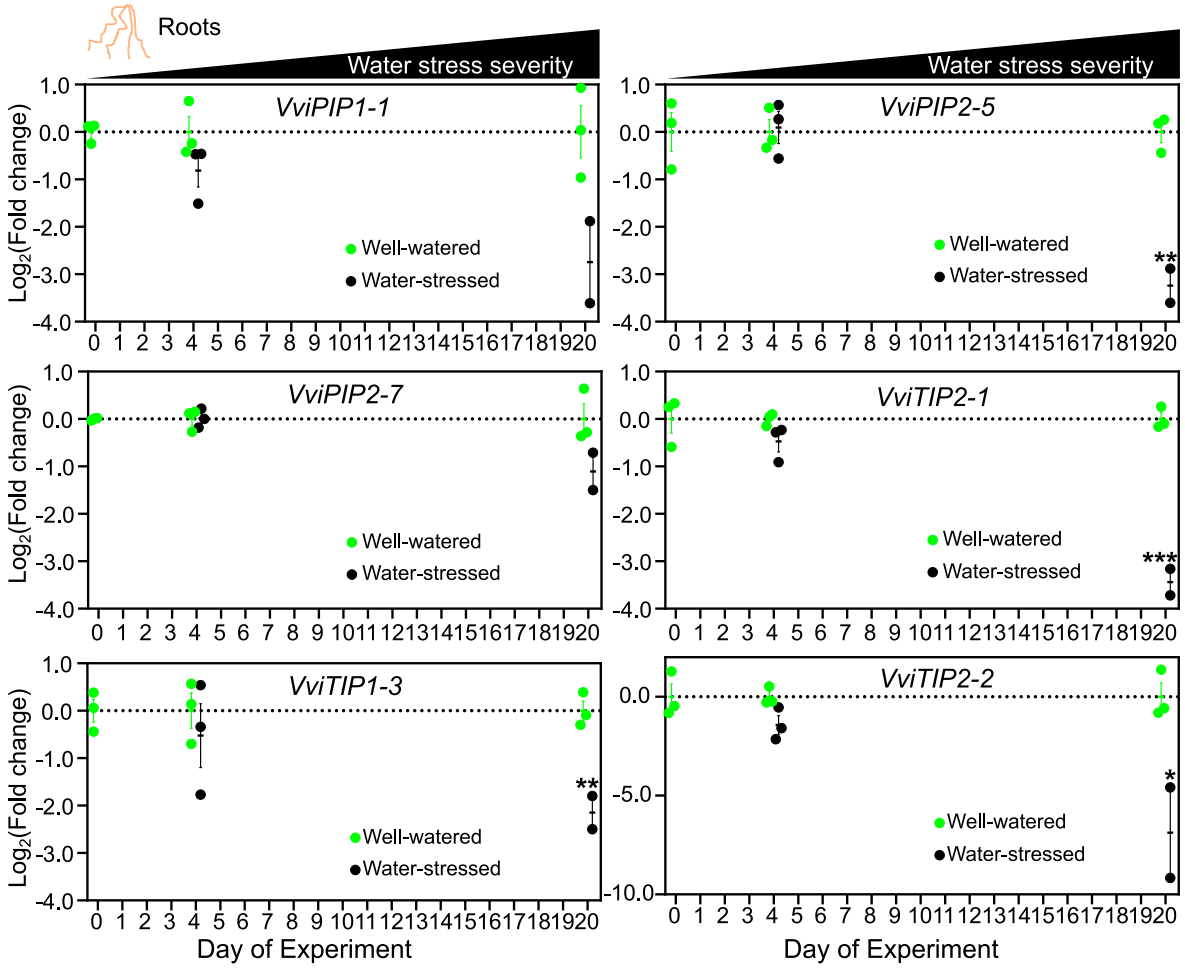


Fig. 6. Transcript modulation analysis of aquaporin genes in *Vitis vinifera* roots under well-watered and water deficit stress conditions. Modulation of aquaporin gene transcripts in roots. Fold change is expressed as the Log2 transformation of the ratio of water-stressed samples relative to well-watered samples. Data represent mean ± SEM (n = 3). Statistical significance was assessed using a two-sided, unpaired Student's t-test, where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

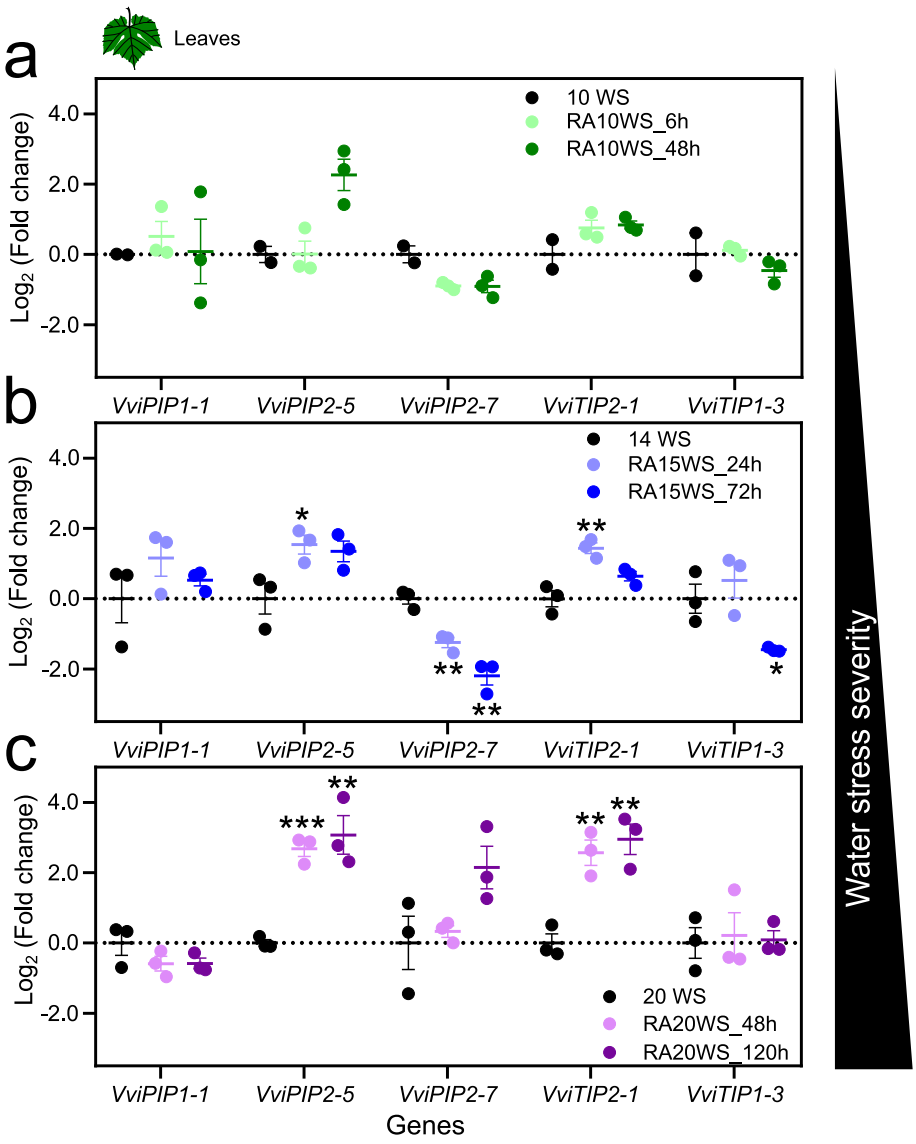


Fig. 7. Modulation of aquaporin transcripts in *Vitis vinifera* leaves under water stress and subsequent rewatering conditions. (a) Modulation of aquaporin gene transcripts in the RA10WS group (rewatered after 10 days of water stress). Fold change is shown as the Log2 transformed ratio of 6 h (6h_RA10WS) and 48 h (48h_RA10WS) post-rewatering samples compared to 10 days of water-stressed samples (WS). (**b**) Modulation of aquaporin gene transcripts in the RA15WS group (rewatered after 15 days of water stress). Fold change is shown as the Log2 transformed ratio of 24 h (24h_RA15WS) and 72 h (72h_RA15WS) post-rewatering samples compared to 14 WS samples. (**c**) Modulation of aquaporin gene transcripts in the RA20WS group (rewatered after 20 days of water stress). Fold change is shown as the Log2 transformed ratio of 48 h (48h_RA20WS) and 120 h (120h_RA20WS) post-rewatering samples compared to 20 WS samples. Data represent mean ± SEM (n = 3). Statistical significance for these panels was assessed using a two-sided, unpaired Student's t-test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.

3.6. The transcription and translation of VviPIP2-5 and VviTIP2-1 were significantly modulated under water stress and recovery conditions

Aquaporin transcriptional and translational modulation in root and leaf samples has been summarized in Fig. 10. Concerning transcription, at 2 and 4 days, when the physiological parameters were still decreasing, not significant modulation was observed. At day 10, when transpiration and stomatal conductance reached their lower plateau, *VviPIP2-5* was down-regulated in the leaf, together with *VviTIP2-1* at day 20. At day 20, when also water leaf potential reached its minimum, *VviPIP2-5* and all the three TIPs were down-regulated in roots, even more strongly than in leaves. Rewatering stimulated a faster and stronger modulation of aquaporins: positive for *VviPIP2-5* and *VviTIP2-1*, and negative for *VviPIP2-7* and *VviTIP1-3*. Focusing on aquaporin specific mRNA translation, only for *VviTIP1-3* a significant reduction of the fraction of mRNA found in polysome was observed after 19 days of water deprivation while a general increase was measured after only 6 h of water availability restoration.

4. Discussions

While aquaporin gene expression during water stress and recovery has been extensively studied at the transcriptional level in grapevine, significant gaps remain in understanding their post-transcriptional or translational regulation. Several studies have reported discrepancy between aquaporin transcript and protein levels under water stress, such as in *Arabidopsis* (Kammerloher et al., 1994), maize (Bárzana et al., 2014), and broccoli (Muries et al., 2011). Our results clearly show that the process of translation as measured by FRP values obtained from polysome profiling at prolonged water deficit was not significantly affected, but different trends were observed at the mRNA-specific level. The translation of the two housekeeping genes *VviGAPDH* and *VviACTIN*,

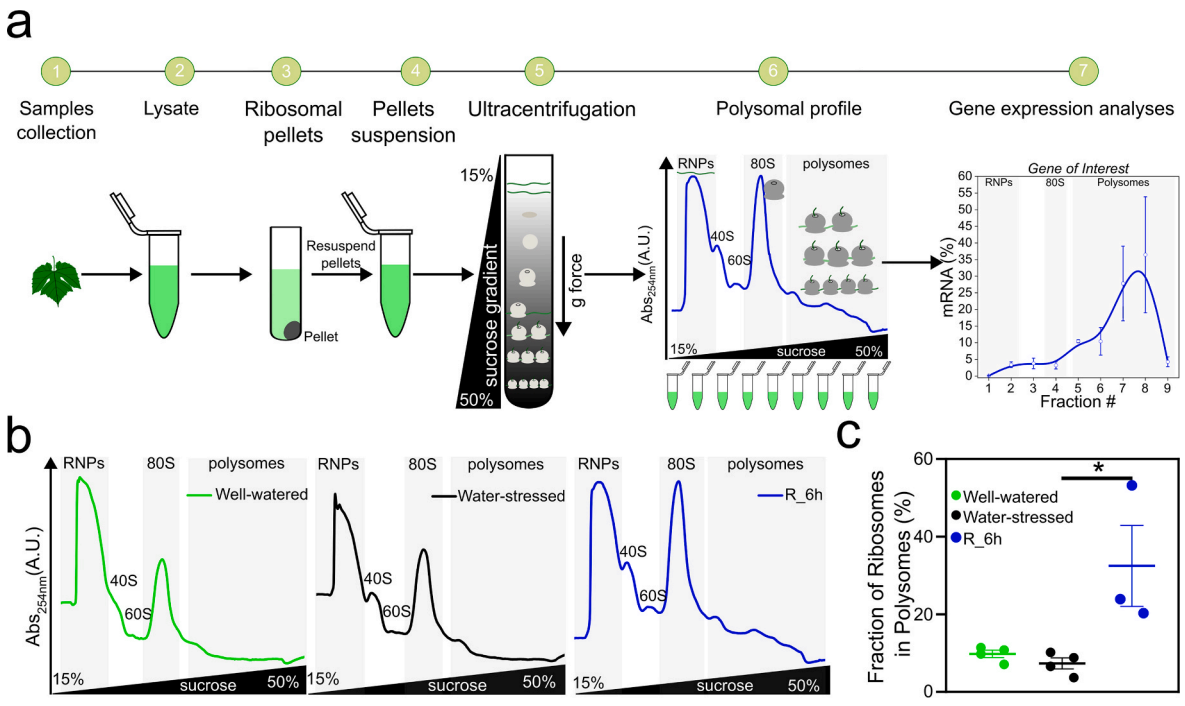


Fig. 8. Global translation in *Vitis vinifera* is significantly influenced by water status. (**a**) Schematic diagram depicting the key steps in polysome profiling and subsequent analyses. (**b**) Representative polysome profiles from leaf samples under well-watered, water-stress (19 days), and rewatering conditions (R_6h). Key components, including ribonucleoproteins (RNPs), 40S, 60S, and 80S ribosomal subunits, and polyribosomes (polysomes) are indicated. (**c**) The fraction of ribosomes in polysomes (FRP) obtained from polysome profiling of well-watered, water-stressed, and recovery samples. Data represent mean ± SEM (n ≥ 3 biological samples). Statistical significance was determined using a two-sided, unpaired Student's t-test, where * denotes $p < 0.05$. Water-stressed samples were compared to well-watered samples and recovery samples were compared to water-stressed samples.

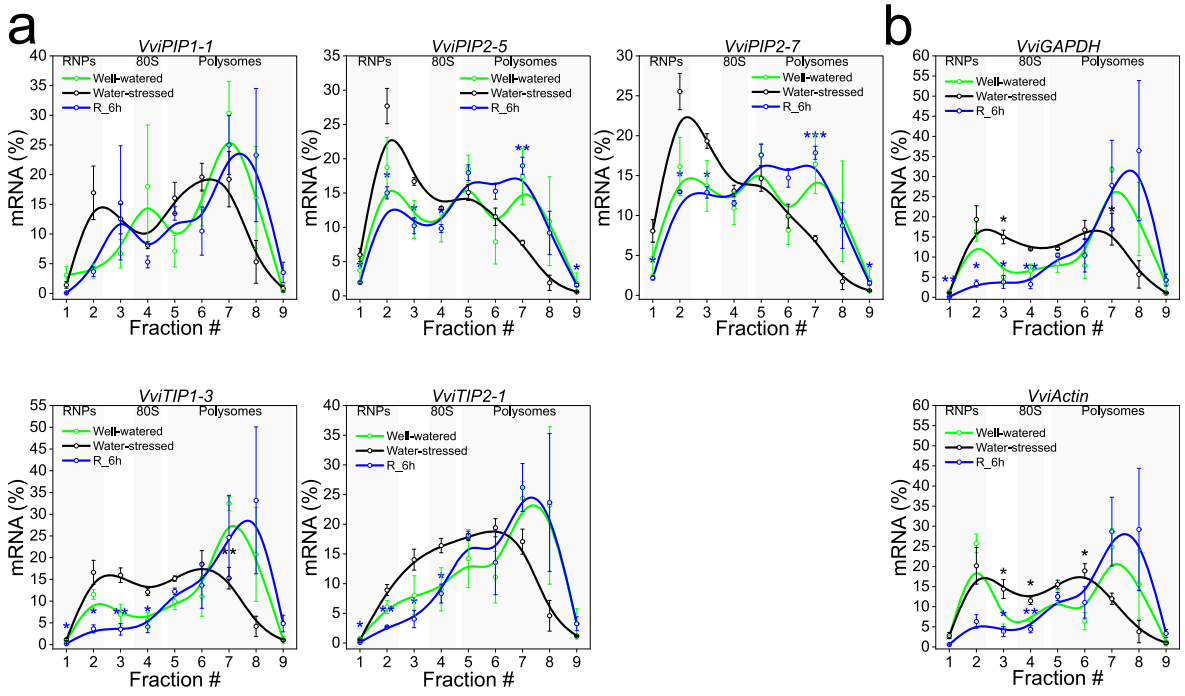
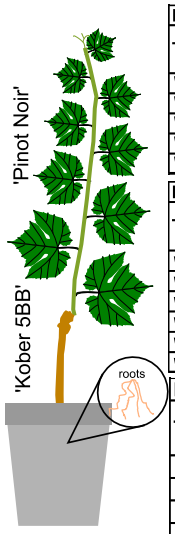


Fig. 9. The translation of aquaporin and housekeeping genes was significantly altered by plant water status in *Vitis vinifera*. Relative co-sedimentation profiles of selected aquaporin genes (**a**) and two housekeeping genes (**b**) across sucrose gradient fractions from well-watered (green lines), water-stressed (black lines), and 6 h after rewatering (blue lines) conditions. For both panels (**a**) and (**b**), data are presented as mean ± SEM (n = 3). Water-stressed samples were compared to well-watered samples and recovery samples were compared to water-stressed samples. Statistical significance was evaluated using a two-sided, unpaired Student's t-test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.



Leaf	Log2(FC) during water stress			
Transcription	2 days	10 days	14 days	20 days
VviPIP1-1	0.57	0.04	0.03	0.41
VviPIP2-5	-0.57	**-1.01**	**-1.91**	-1.00
VviPIP2-7	-0.02	0.17	-0.35	-0.32
VviTIP1-3	-0.67	-0.35	0.80	0.63
VviTIP2-1	-0.07	-0.98	-1.09	**-2.03**

Leaf	Log2(FC) during recovery					
Transcription	10 days + 6 h	10 days +2 days	15 days + 1 day	15 days + 3 days	20 days + 2 days	20 days + 5 days
VviPIP1-1	0.52	0.08	1.15	0.53	-0.59	-0.58
VviPIP2-5	0.01	**2.26**	**1.54**	1.35	**2.68**	**3.08**
VviPIP2-7	**-0.90**	-0.91	**-1.25**	**-2.20**	0.33	2.15
VviTIP1-3	0.12	-0.46	0.52	**-1.45**	0.22	0.09
VviTIP2-1	0.75	0.84	**1.44**	0.64	**2.57**	**2.95**

Root	Log2(FC) during water stress	
Transcription	4 days	20 days
VviPIP1-1	-0.81	-2.75
VviPIP2-5	0.09	**-3.24**
VviPIP2-7	0.01	-1.11
VviTIP1-3	-0.52	**-2.15**
VviTIP2-1	-0.47	**-3.44**
VviTIP2-2	-1.42	**-6.88**

Log2(FC)

-3
0
3

Leaf	Fraction of mRNA in polysomes (%)		
Translation	control	water stressed	recovery (6 hour)
VviPIP1-1	69	61	76
VviPIP2-5	57	37	**63**
VviPIP2-7	55	34	**60**
VviTIP1-3	75	**54**	**89**
VviTIP2-1	76	60	**85**

Fig. 10. Overview of aquaporin transcriptional and translational modulation during water deficit stress and recovery in a 'Pinot Noir'/'Kober 5BB' grafted vine. Aquaporin genes transcriptional modulation in leaf and root samples is represented as Log2 transformed fold change of water-stress vs. well-watered conditions and re-watered vs. water-stress conditions. Data are coloured according to the modulation value and direction (up-modulation in red, down-modulation in green). Bold numbers represent statistical significance of the modulation ($p < 0.05$). Translational activity has been quantified as the mRNA fraction associated with polysomes (Fraction #5–9), and green and red are qualitatively used to highlight reduction during water stress and increase during rewatering. Bold numbers represent statistically significant modulation, when at least one fraction comparison shown in Fig. 9a passed the *t*-test.

together with *VviTIP1-3*, was significantly down-regulated, coherent with a previous heat stress study performed in *Arabidopsis* for *ACTIN2* and ribosomal protein S9 (*RPS9*) (Yángüez et al., 2013). However, the pronounced shift towards mRNA association with ribonucleoproteins (RNPs) observed during water stress in the case of *VviPIP2-5* and *VviPIP2-7* mRNAs, even if not statistically supported due to high data dispersion, may suggest their sequestration into stress granules, which are membraneless compartments storing mRNAs during stress conditions and making it readily available for translation upon recovery (Urquidi Camacho et al., 2020; Wu et al., 2024). *VviPIP1-1* and *VviTIP2-1* maintained polysome association during water stress, in agreement with some dehydration-stress related genes, such as dehydrin and other ABA inducible genes, in *Arabidopsis*, actively translated during water stress despite a general reduction in translation (71 % of the genes) (Kawaguchi et al., 2004).

The prompt reactivation of all aquaporin translation (except *VviPIP1-1*) measured at 6 h after water supply and preceding the transcriptional up-regulation observed for specific isoforms, provides strong evidence for the involvement of post-transcriptional control mechanisms in aquaporin synthesis, regulating water cell-to-cell transport in the leaf. Phosphorylation, membrane trafficking and turnover (reviewed in (Verdoucq et al., 2014; Yepes-Molina et al., 2020)) as well as miRNA-mediated post-transcriptional regulation (Xie et al., 2015) contribute to define aquaporin real amount and activity, and thus membrane water permeability. Due to its fast kinetics upon recovery, translational activity regulation may represent an additional post-transcriptional mechanism fine-tuning aquaporins to changing external conditions.

The comparison of transcriptional profiles of aquaporin genes here measured in leaf and root of 'Pinot Noir' cultivar grafted on 'Kober 5BB' rootstock with cultivars characterized by different stomatal sensitivity ('Chardonnay', 'Grenache' and 'Touriga Nacional') and rootstocks with different degrees of tolerance to water deprivation ('Riparia Gloire', 'Ramsey', the hybrid 'CS2', 'M4' and '101.14') raised interesting considerations about their roles in water transport. In the roots of 'Kober 5BB' and all considered rootstocks, a significant down-regulation has been observed at later time-points (10, 14 or 20 days in the different experiments) for all the TIPs and PIPs, except for *VviPIP2-7*. The latter has been characterized in *Xenopus* oocytes as a water channel with low basal activity, positively regulated by *VviPIP1-1* (Vandeleur et al., 2009). Our meta-analysis suggests that in roots *VviPIP1-1* is the transcriptionally regulated element (also in 'Kober 5BB' even if with a slightly not significant p-value of 0.06), regulating post-translationally *VviPIP2-7*, highlighting again the multi-level control of aquaporin-mediated water transport. Moreover, the strong down-modulation of TIPs in root likely contributed to maintaining cell turgor upon prolonged water deficit and soil osmolarity increase by preventing water efflux from the vacuole. Remarkably, the post-translational regulation of *VviTIP2-1* has been characterized in yeast, showing that this isoform is activated by internal membrane pressure, thus tuning its activity to vacuole osmotic pressure (Leitão et al., 2014).

Concerning 'Pinot Noir' leaf, only *VviPIP2-5* and *VviTIP2-1* were downregulated during water deficit and promptly and strongly up-regulated during recovery, as reported for 'Chardonnay' (Pou et al., 2013) but not for 'Touriga Nacional' (Zarrouk et al., 2016). For these genes, transcriptional and translational profiles are coherent (despite the different kinetics), supporting a *bona fide* increase in their protein abundance. As reported above, VviTIP2-1 activity is also regulated by vacuolar osmotic pressure, boosting the effectiveness of restoring cell turgor optimal conditions upon water availability. *VviPIP2-5* and *VviTIP2-1* expression has been previously reported to correlate with leaf hydraulic conductance and *VviTIP2-1* with stomatal conductance (Pou et al., 2013). Our study confirms a strong association with stomatal conductance and leaf water potential during periods of water deficit and subsequent recovery.

According to aquaporins modulation, especially evident during recovery, grafted 'Pinot Noir' seems to adopt a rather conservative behaviour during water deficit, but showing a high responsivity to improved water availability. The steep reduction in stomatal conductance, transpiration, and leaf water potential observed within 5 days of water deprivation and then maintained at minimal values until the water was supplied again, indicated that leaves quickly regulated stomatal opening to reduce water loss. The characteristic of maintaining a stable leaf water potential by swiftly closing stomata when soil moisture drops is termed isohydric, however it is highly influenced by phenological development; differences in rootstock, soil type and

environmental conditions (Gambetta et al., 2020). Previous reports classified 'Pinot Noir' as a near-anisohydric cultivar (Griesser et al., 2015; Gutiérrez-Gamboa et al., 2019), although a behavioural shift towards a more conservative behaviour in particular phenological conditions has been already observed (Poni et al., 1993). In our study, pot-grown grafted 'Pinot Noir' without reproductive sinks displayed a generalized high stomatal sensitivity under reduced soil water availability, thus showing a near-isohydric dynamic. The progressive increase in stomatal resistance is generally associated with a reduction in carbon assimilation and, therefore, in photosynthesis (Lawson and Blatt, 2014), in line with the arrest of shoot growth observed in our study.

While this study focused on a select group of aquaporins that may not encompass the full complexity of the aquaporin family within grapevines, future research could benefit from a broader analysis including more aquaporin genes along with proteomic data to correlate better transcriptional and translational changes with protein functionality. Here, we report that *VviPIP2-5* and *VviTIP2-1* are differentially regulated in the leaf of 'Pinot Noir' at both transcriptional and translational according to water availability. These are, therefore, promising candidate genes to be applied in breeding programs for drought tolerance, utilizing new genetic technologies. Tonoplast water permeability control in the root apparatus seems like a conserved mechanism of osmoregulation to maintain cell turgor and viability in dry soils. Moreover, this study shows the application of polysome profiling on a perennial crop such as grapevine shedding light on the relevance of translational regulation of stress-related genes, such as aquaporins, to better describe the molecular mechanisms underlying drought resilience.

CRediT authorship contribution statement

Lubin Guan: Writing – original draft, Investigation. **Alvaro Vidal Valenzuela:** Visualization, Formal analysis, Data curation. **Gaurav Sharma:** Methodology. **Michele Faralli:** Writing – review & editing, Supervision. **Mirko Moser:** Writing – review & editing, Supervision. **David Navarro-Payá:** Writing – review & editing, Supervision. **Claudio Moser:** Supervision, Conceptualization. **Gabriella Viero:** Writing – review & editing, Supervision, Conceptualization. **Elena Baraldi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Stefania Pilati:** Writing – review & editing, Supervision.

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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.

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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.2025.110298.

Data availability

Data will be made available on request.

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