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Translational Control of Gene Expression in Plant Development and Stress Response

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Declaration

I, Lubin Guan, hereby declare that this thesis is my work with due acknowledgment of other materials used. I further state that the thesis has not been submitted for any award at any university other than the University of Bologna.

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List of Abbreviations

Abbreviation	Full name	Abbreviation	Full name	Abbreviation	Full name
µL	microlitre	eIF2	Eukaryotic Initiation Factor 2	NADH	Nicotinamide adenine dinucleotide + hydrogen
10X	10 times	eIF2B	Eukaryotic Initiation Factor 2B	NIPs	Nodulin-like proteins
18S	18S ribosomal RNA	eIF2α	Eukaryotic Initiation Factor 2α	ORF	Open reading frame
28S	28S ribosomal RNA	eIF3	Eukaryotic Initiation Factor 3	OST1	Open Stomata 1
2X	2 times	eIF4A	Eukaryotic Initiation Factor 4A	PABP	Poly A binding protein
40S	Eukaryotic small ribosomal subunit	eIF4B	Eukaryotic Initiation Factor 4B	PCR	Polymerase chain reaction
43S PIC	43S preinitiation complex	eIF4E	Eukaryotic initiation factor 4E	PIP	Plasma membrane intrinsic protein
48S PIC	48S preinitiation complex	eIF4F	Eukaryotic initiation factor 4F	PIP1	Plasma membrane intrinsic protein 1
5X	5 times	eIF4G	Eukaryotic initiation factor 4G	PIP2	Plasma membrane intrinsic protein 2
60S	Eukaryotic large ribosomal subunit	eIF5	Eukaryotic initiation factor 5	PIP2F	Plasma membrane intrinsic protein 2;4
80S	Eukaryotic ribosome	eIF5B	Eukaryotic initiation factor 5B	PTGS	Post-transcriptional gene silencing
ABA	Abscisic acid	eIFiso4E	Eukaryotic initiation factor (iso) 4E	PVP	Polyvinylpyrrolidone
ABCE1	ATP binding cassette E1	eIFiso4F	Eukaryotic initiation factor (iso)4F	RA10DS	Rewatering after 10 days of drought stress
ACO3	1- amino-cyclopropane-1-carboxylic acid oxidase 3	eRF1	Eukaryotic translation termination factor 1	RA15DS	Rewatering after 15 days of drought stress
AGO1	Argonaute 1	eRF3	Eukaryotic translation termination factor 3	RA19DS	Rewatering after 19 days of drought stress
AGO10	Argonaute 10	*FBW2*	F-box and WD-repeat domain-containing 2	RA20DS	Rewatering after 20 days drought stress

Abbreviation	Full name	Abbreviation	Full name	Abbreviation	Full name
AP2	Apetala 2	FEM	Fondazione Edmund Mach	RACK1	Receptor for Activated C Kinase 1
AOC3	Allene oxide cyclase 3	FRP	Fraction of Ribosomes in Polysomes	RISC	RNA-induced silencing complexes
AQP1	Aquaporin 1	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	RNA	Ribonucleic acid
AQPs	Aquaporins	GCN2	General control nonderepressible 2	RNP	Ribonucleic particles
ATP	Adenosine triphosphate	GDP	Guanosine diphosphate	RP	Ribosomal protein
BCA	Bicinchoninic acid	GTP	Guanosine-5'-triphosphate	RT	Reverse transcription
BSA	Bovine serum albumin	GTS	Germination translation shift	SDS	Sodium dodecyl sulfate
CI	Confidence interval	HAI	Hours after imbibition	SEM	Standard error of the mean
Cis-NATs	Cis-natural antisense transcripts	HCl	Hydrochloric acid	SIP	Small basic intrinsic protein
CO_2	Carbon dioxide	HRP	Horseradish peroxidase	SWI/SNF	SWItch/Sucrose Non-Fermentable
CTK	Cytokinin	HSP90	Heat shock protein 90	TBS	Tris-buffered saline
DCL1	Dicer-like 1	HTS	Hydration translation shift	TBST	Tris-buffered saline with 0.1% Tween 20
DEPC	Diethyl pyrocarbonate	IRES	Internal ribosome entry site	TIP	Tonoplast intrinsic proteins
DNA	Deoxyribonucleic acid	JA	Jasmonate	TIP1	Tonoplast intrinsic proteins 1
dNTPs	Deoxyribonucleotide triphosphates	KCl	Potassium chloride	TIP2	Tonoplast intrinsic proteins 2
DTT	Dithiothreitol	LARP6C	La-related proteins 6C	TPM	Transcript per million
ECL	Enhanced chemiluminescence	MGD2	Monogalactosyldiacylglycerol synthases 2	TRAP	Translating ribosome affinity purification
EDTA	Ethylenediaminetetraacetic acid	mL	milliliter	UBC9	Ubiquitin-conjugating enzyme 9
eEF1A	Eukaryotic translation elongation factor 1A	MON1	MONENSIN SENSITIVITY1	UTR	Untranslated region
eEF2	Eukaryotic elongation factor 2	Mpa	Megapascal	UV	Ultraviolet
eIF1	Eukaryotic translation initiation factor 1	MS	Murashige and Skoog	XIP	Uncharacterized intrinsic proteins
eIF1A	Eukaryotic initiation factor 1A	N_2	Nitrogen	Ψ_L	Leaf water potential

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Abstract

According to the central dogma of molecular biology, RNA levels should directly correlate with protein expression. However, empirical data from both plant and animal systems often reveal a weak correspondence between these molecular entities. This study reevaluates transcriptomic and proteomic datasets from *Arabidopsis thaliana* cotyledons and hypocotyls to illustrate instances of uncoupled transcript and protein levels.

This phenomenon extends beyond developmental biology into stress response mechanisms. Analysis of the aquaporin gene *AtPIP2F* in *Arabidopsis* demonstrates a similar disjunction between RNA and protein expression, paralleled by the homologous gene *VviPIP2;2* in *Vitis vinifera* (grapevine), where aquaporins play a pivotal role in managing hydraulic conductance and root water uptake under water stress conditions. Despite these parallels, the underlying reasons for the weak correlation in gene expression remain elusive.

To delve into the regulatory mechanisms, polysome profiling was employed on *Arabidopsis* seedlings and *Vitis vinifera* leaves, focusing on translational control during development and in response to drought stress, respectively. The results indicate significant translational differences between cotyledons and hypocotyls of *Arabidopsis*, yet individual gene translation remained consistent across developmental stages. In *Arabidopsis*, AGO1 deficiency causes malformed leaves and sterile flowers. AGO1, regulated by miR168, plays a role in RNA silencing. miR168 binds to polysomes at day 6 but not at day 10, yet AGO1 protein levels decrease by day 10, suggesting complex regulation. In *Vitis vinifera*, the translation of aquaporin genes *VviPIP2-5* and *VviTIP2-1* was notably suppressed under drought conditions but showed recovery upon rehydration, underscoring the role of translational regulation in stress adaptation.

This thesis elucidates the intricacies of post-transcriptional regulation, providing insights into the discordance between transcript and protein levels, and highlighting novel layers of gene expression control in plant development and stress response.

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1 General Introduction

1.1 Gene expression

Gene expression is the process by which information encoded in a gene is transformed into a functional product, such as proteins or non-coding RNAs, which ultimately influence an organism's phenotype (Buccitelli & Selbach 2020). In eukaryotic cells, DNA serves as a template for the synthesis of RNA molecules, which are categorized into coding messenger RNA (mRNA) and non-coding RNA. While mRNA primarily functions as a protein production template, most non-coding RNAs do not lead to protein synthesis (Li & Liu, 2019). In the case of mRNA, after transcription, the initial RNA transcripts, known as pre-mRNAs, undergo several processing steps, including exon splicing (Zhan et al., 2015), the addition of a 5' cap (7-methylguanosine (m^7G) added to the 5' end of pre-mRNA (Ramanathan et al., 2016), and a 3' poly-A tail (Munoz-Tello et al., 2015; Passmore & Coller 2022). Following these processing steps, various RNA-binding proteins, export adaptors, and export receptors are recruited to facilitate mRNA export to the cytoplasm (Köhler and Hurt, 2007). Once in the cytoplasm, mature mRNAs serve as templates for protein synthesis by ribosomes (Köhler and Hurt, 2007), where polypeptides are folded into their functional three-dimensional structures (Anfinsen, 1972). Additionally, some proteins are transported to specific locations to carry out their functions (Neumann et al., 2003). These processes are critical in shaping an organism's phenotype. Frequently, these steps occur simultaneously—for example, transcription can coincide with RNA localization, and translation can happen alongside protein degradation to conserve energy (Jewett et al., 2009; Li et al., 2014) or to rapidly respond to environmental changes (Dahan et al., 2011; Bentley, 2014; Liu and Qian, 2014). Among these processes, translation plays a central role in governing protein production (Merchante et al., 2017).

1.1.1 Translation

Translation in eukaryotes consists of three main steps: initiation, elongation, and termination (Roy and Arnim, 2013). The initiation phase is particularly significant for the regulation of translation and

has been extensively studied (Jackson et al., 2010). It begins with the formation of a 43S preinitiation complex (PIC). During this process, the 40S ribosomal subunit assembles with initiation factor proteins (eIF1A, eIF1, eIF3, and eIF5) and interacts with the ternary complex, which includes eIF2, GTP, and the initiator methionyl-tRNA (Met-tRNA$^{Met}_i$) (Sonenberg and Hinnebusch, 2009). Among these factors, eIF3 is the largest, consisting of 12 subunits that serve as a scaffold for eIF1, eIF1A, eIF2, and eIF5. Together, they form the 43S PIC (Figure 1.1) (Hinnebusch et al., 2016; Bhardwaj et al., 2019). Simultaneously, the mRNA's 5' cap is recognized by eIF4E, which partners with eIF4G to create a complex known as eIF4F (Miras et al., 2017). Subsequently, eIF4A and eIF4B are recruited to the mRNA to link poly A binding protein (PABP) with eIF4G (Mayberry et al., 2009; Liu & Goss, 2018). This interaction connects the 5’ and 3’ ends of the mRNA, resulting in a closed-loop structure (Figure 1.1) (Wells et al., 1998; Amrani et al., 2008). This closed-loop enhances their efficient translation, facilitates ribosome recycling, and increases mRNA stability by protecting mRNAs from degradation via exonucleases (Browning and Bailey-Serres, 2015; Passmore and Coller, 2022). The closed-loop mRNA is likely attached to the 43S PIC through interactions between eIF4G and eIF3, forming the 48S preinitiation complex (48S PIC, also known as the scanning complex) (Figure 1.1) (Bhardwaj et al., 2019; Raabe et al., 2019). The loading of mRNA into the 40S subunit is facilitated by helicase eIF4A, with assistance from RNA-binding protein eIF4B in an ATP-dependent manner that unwinds the closed loop (Browning and Bailey-Serres, 2015). The small proteins eIF1A and eIF1 aid in recognizing the start codon (Nanda et al., 2013; Martin-Marcos et al., 2014). At this stage, eIF5 promotes GTP hydrolysis by eIF2 (Singh et al., 2012; Jennings et al., 2013). Following this, the initiation factors dissociate from the 40S subunit, allowing large GTPase eIF5B to recruit and bind a 60S subunit to form the complete 80S ribosome, thereby completing the initiation phase (Figure 1.1) (Dennis et al., 2009; Hinnebusch, 2014).

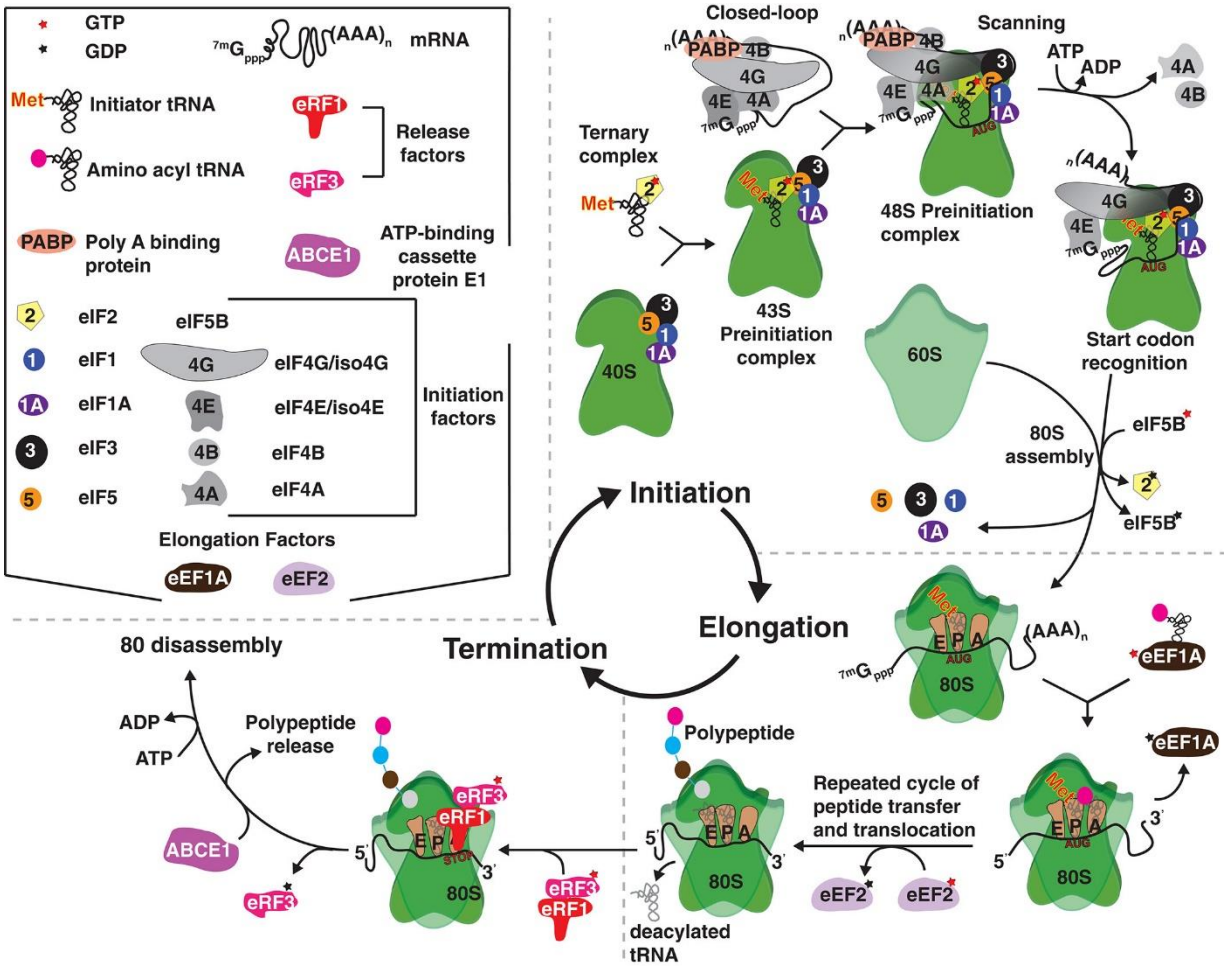

Figure 1.1. The main translation steps in eukaryotes: initiation, elongation, and termination. The main factors involved are indicated. Information in detail is seen in the text. Taken from (Urquidi Camacho et al., 2020).

Elongation is another critical stage, as in other eukaryotes, the scanning ribosome recognizes the AUG start codon better when it appears in a specific “Kozak”-sequence context, which in eudicot plants is AANAUGGC and in monocotyledonous plants is GCNAUGGC (Sugio et al., 2010; Gupta et al., 2016). The elongation factor eEF1A delivers a charged aminoacyl-tRNA to the aminoacyl site (A-site) of the 80S ribosome. When a correct codon-anticodon match occurs, GTP hydrolysis by eEF1A takes place along with the release of eEF1A-GDP (Gromadski et al., 2007; Mateyak and Kinzy, 2010; Dever et al., 2018). After, the peptide bond formation occurs, facilitated by the ribosome's peptidyl

transferase activity, elongation factor eEF2 uses GTP hydrolysis to translocate the mRNA-tRNA complex by one codon (three nucleotides) (Jørgensen et al., 2003). Consequently, uncharged tRNA moves to the exit site (E-site) while tRNA carrying the peptide shifts to the peptidyl site (P-site), making the A-site available for incoming charged tRNAs (Dever and Green, 2012; Doerfel et al., 2013). The coding sequence is translated into a protein through successive cycles of aminoacyl-tRNA binding, peptide bond formation, and translocation of uncharged tRNA into the E-site (Butcher & Jan 2016).

Termination occurs when one of three stop codons (UAA, UGA, or UAG) enters the A-site and triggers termination via a release factor complex composed of eRF1-eRF3-GTP (Tuite and Stansfield, 1994; Frolova et al., 1996; Merkulova et al., 1999; Nyikó et al., 2017). The eRF3-GTP binds to eRF1 which acts as a GTP dissociation inhibitor (Elakhdar et al., 2019). When the ribosome reaches a stop codon on mRNA, this complex is recruited to the A-site, blocking further entry of any new aminoacyl-tRNA complexes from eEF1A (Dever and Green, 2012; Jackson et al., 2012). At this site, GTP hydrolysis by eRF3 triggers its release and opens up binding for recycling factor ABCE1 (Preis et al., 2014). This process frees the polypeptide from the P-site while a final ATP hydrolysis by ABCE1 facilitates the disassembly of the components of the 80S ribosome for reuse in subsequent rounds of protein synthesis (Figure 1.1) (Pisarev et al., 2010; Schuller and Green, 2018).

1.1.2 Peculiarities of plant translation

Plants are categorized as eukaryotes, yet their translation processes exhibit distinct differences compared to other eukaryotes, such as mammals (Castellano and Merchante, 2021). A significant characteristic of plant translation is the presence of a unique and abundant isoform of the cap-binding complex known as eIFiso4F, which consists of eIFiso4E and eIFiso4G (Patrick et al., 2014; Kropiwnicka et al., 2015; Khan and Goss, 2018; Castellano and Merchante, 2021). Although the eIFiso4F complex is smaller than the eIF4F complex (approximately 80 kD compared to 180 kD), it is more prevalent in plants (Patrick and Browning, 2012). The proteins eIF4G and eIFiso4G exhibit different preferences for mRNA sequences and interact variably with distinct isoforms of poly(A) binding protein (PABP) (Gallie and Browning, 2001; Gallie, 2018). Consequently, these complexes may preferentially bind specific

sets of mRNAs and regulate unique physiological responses. For instance, eIFiso4G has been shown to select mRNAs with unstructured sequences, which are typically associated with translation during hypoxic conditions (Gallie and Browning, 2001; Branco-Price et al., 2005). Recent studies have also indicated that eIFiso4G plays a role in managing specific mRNAs related to chloroplast functions, hypoxia, and abscisic acid (ABA) signaling (Bi et al., 2019; Cho et al., 2019; Lellis et al., 2019). Another important factor is general control non-derepressible-2 (GCN2), a widespread protein kinase that phosphorylates the α subunit of the eukaryotic initiation factor eIF2. This phosphorylation inhibits the conversion of eIF2-GDP to eIF2-GTP, thereby blocking the initiation of new protein synthesis cycles and reducing overall protein biosynthesis (Zhang et al., 2008). In contrast to other eukaryotic systems, GCN2 in plants does not necessarily inhibit translation (Izquierdo et al., 2018; Zhigailov et al., 2020). Additionally, unlike mammals, the recycling of eIF2-GDP to eIF2-GTP mediated by eIF2B appears unnecessary in plants (Zhigailov et al., 2020). Notably, GCN2 plays a crucial role in mediating the phosphorylation of eIF2α, which is essential for regulating plant growth and responses to various stresses, both biotic and abiotic (Zhang et al., 2008; Lageix et al., 2008; Faus et al., 2015; Li et al., 2018; Liu et al., 2019; Berrocal-Lobo et al., 2020). Furthermore, plant ribosomal proteins have diversified through multiple rounds of genome duplication. In *Arabidopsis* alone, there are 80 families of ribosomal proteins (RPs), comprising two to six genes (Barakat et al., 2001; Browning and Bailey-Serres, 2015; Scarpin et al., 2023). This extensive variety of potential RP combinations within ribosomes is believed to enhance the heterogeneity of ribosomal properties and facilitate the specific translation of mRNAs in response to diverse internal and external signals (Browning and Bailey-Serres, 2015).

1.1.3 Factors influencing translation: focus on mRNA sequence structures

The intricate structures of mRNA play a crucial role in translational regulation, with specific sequence features significantly influencing this process (Figure 1.2) (Kawaguchi and Bailey-Serres, 2005).

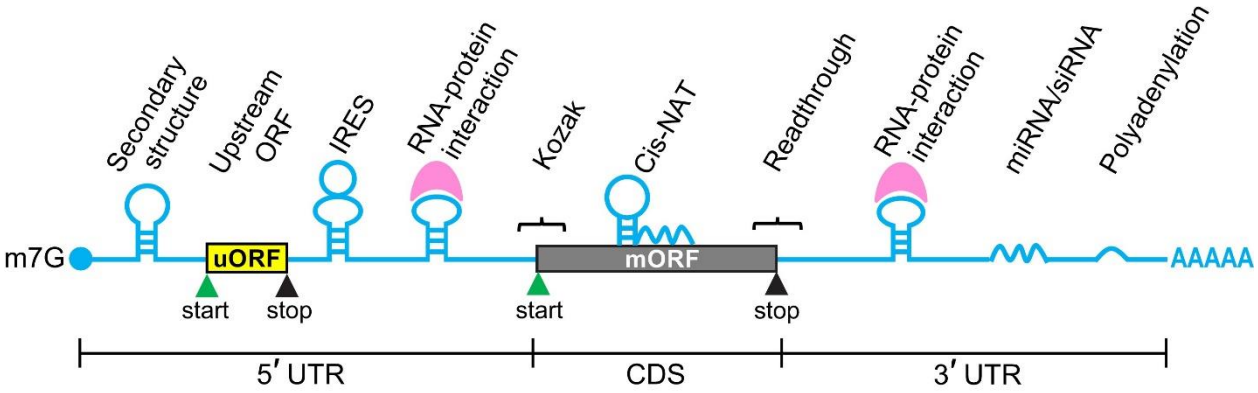

Figure 1.2. mRNA sequence features contribute to translational regulation in mRNA. A typic mRNA with 5' UTR and 3' UTR is shown, and several important innate sequence features are highlighted. ORF: open reading frame. uORF: upstream ORF. IRES: Internal ribosome entry site. Cis-NATs: Cis-natural antisense transcripts. mORF: main ORF. Taken from (Wu et al., 2024).

One key element is the Kozak consensus sequence found in eukaryotes, in plants, as well as in other eukaryotes, the Kozak consensus sequence is 5' -A/GNNAUGG- 3', meaning that the upstream third nt (-3) of the AUG is enriched for A or G, and the downstream fourth nt (+4) is enriched for G (Kozak, 1981; Sugio et al., 2010; Lei et al., 2015). However, there are some discrepancies in the sequences between mammals and plants (Lütcke et al., 1987; Nakagawa et al., 2008) and even within the plant kingdom (Gupta et al., 2016). Another important feature is the presence of upstream open reading frames (uORFs) located within the 5' untranslated region (5' UTR) of mRNA, which are found across all eukaryotes (Tanaka et al., 2016; Zhang et al., 2021). When ribosomes scan mRNA and encounter an uORF, the translation of that uORF can hinder the progression of ribosomes to the main open reading frame (mORF), potentially leading to reduced translation of the mORF (Ribone et al., 2017; van der Horst et al., 2020; Gage et al., 2022). In plants, numerous cases have been documented where translation is regulated by uORFs, conferring various agronomic benefits such as increased fruit sweetness (Sagor et al., 2016; Xing et al., 2020), enhanced fertilizer acquisition efficiency (Guo et al., 2022), and augmented pathogen resistance (Xu et al., 2017b). In addition to Kozak sequences and uORFs, other sequence features are critical for translational regulation. These include internal ribosome

entry sites (IRES) (Cui et al., 2015; Wang et al., 2022), RNA secondary structure (Cho et al., 2018; Chung et al., 2020), sequences bound by RNA-binding proteins (Billey et al., 2021; Chen et al., 2022), readthrough events (Nyikó et al., 2017; Sahoo et al., 2022; Skuzeski et al., 1991), G-content in the poly(A) tail (Chang et al., 2014; Zhao et al., 2019), and cis-natural antisense RNAs (cis-NATs) (Deforges et al., 2019; Reis et al., 2021). Furthermore, the binding sequences for small interfering RNAs (miRNAs and siRNAs) are vital for translational regulation. These small RNAs associate with ARGONAUTE (AGO) proteins to form RNA-induced silencing complexes (RISCs), which target specific mRNA sequences for translational repression (Brodersen et al., 2008; Bologna and Voinnet, 2014). Recent studies indicate that miRNAs mediate target gene repression through both mRNA cleavage and translational inhibition (Iwakawa and Tomari, 2013; Yang et al., 2021). Ribo-seq analyses have shown that predicted miRNA targets exhibit reduced translation efficiency in species such as *Arabidopsis* and tomato (Liu et al., 2013; Wu et al., 2019). Notably, miRNAs and siRNAs are essential for plant development and adaptation to stress conditions (Wu et al., 2020).

1.2 Plant development: focus on model plant *Arabidopsis thaliana*

Arabidopsis thaliana, a model organism in plant biology, undergoes a well-defined life cycle that includes four main stages: germination, vegetative growth, reproduction, and senescence (Meinke et al., 1998). The life cycle of *Arabidopsis* typically spans 6 to 8 weeks under optimal conditions (Boyes et al., 2001). The germination of *Arabidopsis* seeds is a critical developmental transition influenced by environmental factors such as moisture and temperature (Fait et al., 2006). This process can be broken down into several stages: imbibition, radicle emergence, and cotyledon hypocotyl emergence (Gallardo et al., 2001; Holdsworth et al., 2008). During seed germination, plants also have DNA damage repairing mechanism to ensure proper seedling development (Waterworth et al., 2022). After germination, *Arabidopsis* enters the vegetative growth phase characterized by rapid leaf development and biomass accumulation (Xu et al., 2016; Werner et al., 2021; Zhou et al., 2023). After about three weeks of vegetative growth, *Arabidopsis* begins transitioning to reproductive growth. This transition is irreversible;

once flowering begins, the plant allocates resources toward reproduction (Araki, 2001; J. Zhao et al., 2023; Wang et al., 2024). Reproductive development in *Arabidopsis* involves several key stages: inflorescence emergence, flowering, pollination and seed development, and seed dispersal (Ó'Maoiléidigh et al., 2014; Wils and Kaufmann, 2017). Once the reproductive phase is concluded, senescence starts. In *Arabidopsis*, senescence is a highly regulated process where the nutrients are moved from the senescent organs to mature fruit or other live organs (Kim et al., 2018; Schippers et al., 2015; Kim et al., 2018). In summary, *Arabidopsis thaliana*'s life cycle encapsulates a series of well-defined stages from germination through growth and reproduction to senescence. Each phase is intricately linked with environmental cues and internal regulatory mechanisms that ensure successful development and reproduction (Boyes et al., 2001; Koornneef and Meinke, 2010).

1.2.1 Translational regulations in plant development

The global translation changes throughout the entire life cycle of plants control the developmental transitions (Bailey-Serres, 1999; Basbouss-Serhal et al., 2015; Merchante et al., 2017; Jang et al., 2019). In general, the polysome association decreased as plants aged and therefore decreased general translation (Mason et al., 1988; Yamasaki et al., 2015). More importantly, translation during plant development and growth is stage- and cell-type-specific. Several studies using translating ribosome affinity purification (TRAP) assay (Zanetti et al., 2005) with tissue-specific promoters were employed to examine the translatomes of specific cell populations, flower developmental domains, and germinating pollen tubes in *Arabidopsis*. These studies revealed cell type-specific differences in the populations of mRNAs being translated (Mustroph et al., 2009; Jiao and Meyerowitz, 2010; Lin et al., 2014). The effects on translation during seed germination were investigated by comparing dormant versus non-dormant seeds in sunflower and *Arabidopsis*. Polysomal loading increases after imbibition and is higher in non-dormant seeds, although the amount of total RNA does not differ between the two seed populations, showing that germination is mainly regulated at the translational level (Layat et al., 2014; Basbouss-Serhal et al., 2015). Bai et al. (2017) analyzed total and polysomal RNA populations, along with polysome profiles, across five germination stages (0 to 72 hours after imbibition, HAI). They

identified two major phases of translation regulation: the hydration translation shift (HTS) from 0 to 6 HAI and the germination translation shift (GTS) from 48 to 72 HAI, which correspond to critical points in the seed-to-seedling transition. These phases are regulated by distinct mechanisms, evidenced by different transcription-to-translation ratios and mRNA sequence features (Bai et al., 2017). In their later studies, they confirmed that *Arabidopsis* selectively translates mRNA in dormant and non-dormant states (Bai et al., 2020, 2018). A comparable phenomenon of selective mRNA translation during different developmental stages is observed in pollen. Pollen lacking LARP6C, an evolutionarily conserved RNA-binding protein, exhibits delayed germination and guidance defects related to sperm delivery to the ovule. In dry pollen, LARP6C binds to transcripts encoding proteins involved in lipid synthesis and homeostasis, vesicular trafficking, and polarized cell growth. It also forms cytoplasmic granules containing poly(A) binding protein, potentially serving as storage sites for translationally silent mRNAs. In germinated pollen, LARP6C may influence the quantities and distribution of storage lipids and vesicular trafficking by targeting MGD2. This research supports the notion that plants transition through developmental stages by regulating the translation of specific mRNAs (Billey et al., 2021).

miRNAs play a crucial role in regulating gene expression by effectively repressing translation or cleaving target mRNAs associated with the RNA-induced silencing complex (RISC) at the 3' UTR. The initial study on an *AtAGO1* gene mutant in *Arabidopsis thaliana* revealed a squid-like morphology, characterized by rosette leaves lacking leaf blades, filamentous cauline leaves, and infertile flowers (Bohmert et al., 1998). Subsequent research established that *At*AGO1 is essential for post-transcriptional gene silencing (PTGS) in plants (Fagard et al., 2000). Notably, PTGS was found to be particularly sensitive to the absence of the *At*AGO1 gene, as evidenced by some ago1 mutants that exhibited milder phenotypic defects while remaining fertile despite PTGS deficiencies (Morel et al., 2002). Further investigations revealed that miR168 targets and cleaves *AGO1* mRNA (Rhoades et al., 2002). In transgenic plants with reduced complementarity between miR168 and *AtAGO1* mRNA, researchers observed both developmental defects and decreased miRNA accumulation. This finding suggests an autoregulatory mechanism between miR168 and *AtAGO1* mRNA that is vital for

developing *Arabidopsis* seedlings (Vaucheret et al., 2004, 2006). A recent study indicated that the passenger strand of miR168 functions as a molecular buffer to modulate *At*AGO1 protein levels (Dalmadi et al., 2021). Additionally, RACK1, a core ribosomal protein of the eukaryotic small (40S) ribosomal subunit, directly interacts with SERRATE, a zinc finger protein that is one of the protein complexes in nuclear dicing bodies (D-bodies) ensuring the processing of pri-miRNAs (Speth et al., 2013). These studies collectively shed light on this autoregulatory mechanism.

Research has increasingly shown that miR168 binds to the conserved PAZ, PIWI, MID domains, and N terminal of AGO1 to facilitate mRNA cleavage (Baumberger and Baulcombe, 2005; Zhang et al., 2006; Mi et al., 2008; Frank et al., 2012). This cleavage likely occurs on polyribosomes at the endoplasmic reticulum (Lanet et al., 2009; Li et al., 2013). Importantly, the transcriptional regulation of miR168 and the post-transcriptional control of AGO1 homeostasis are thought to play significant and conserved roles in stress responses (Li et al., 2012). A relevant study demonstrated that nuclear AGO1 associates with small RNAs and SWI/SNF complexes specifically bound to stimulus-responsive genes, promoting the transcription of genes involved in jasmonate (JA) signaling pathways and responses (Liu et al., 2018). In tomato (*Solanum lycopersicum*), both miR168 and AGO1 have been implicated in modulating downstream miRNA pathways in response to low K^+ stress through CTK/ABA signaling and root growth modulation, thereby contributing to plant development under such stress conditions (Liu et al., 2020). In rice (*Oryza sativa*), suppressing miR168 expression has been shown to enhance yield, flowering time, and immunity (Wang et al., 2021). Interestingly, miRNAs are capable of binding not only to the 3' UTR but also to the 5' UTR of target mRNAs, silencing translation by obstructing ribosome recruitment or movement (Iwakawa and Tomari, 2013). More recently, it has been discovered that miRNAs bind to AGO1 upon their production in the nucleus before being transported to the cytoplasm by AGO1 in association with the chaperone protein HSP90 (Bologna et al., 2018).

In summary, there exists an autoregulatory mechanism between miR168 and AGO1 that is critical for plant development, particularly in *Arabidopsis thaliana*. Any disruption in this mechanism can lead to developmental defects (Z. Zhao et al., 2023).

1.3 Abiotic stresses and biotic stresses in plants

Plants face various stresses throughout their life cycles, which can be categorized into abiotic and biotic stresses. Abiotic stresses include water scarcity, heat shock, extreme cold, hypoxia, salinity, heavy metals, and reactive oxygen species, while biotic stresses encompass threats from bacteria, insects, fungi, and viruses (Zhang et al., 2022; Nawaz et al., 2023; Du et al., 2024). To cope with these challenges, plants have developed the ability to selectively absorb essential resources, store them for later use, generate energy for cellular functions, repair tissues, facilitate communication among different plant parts, manage structural assets under changing conditions, and adapt their development to thrive in diverse environments (Patel and Mishra, 2021; Munns and Millar, 2023). However, climate change is exacerbating these stresses by raising global temperatures and altering regional climates, leading to increased aridity in some areas and heavier rainfall in others. These environmental challenges can weaken plant immunity and create favorable conditions for pathogens (Velásquez et al., 2018). Consequently, crop yields and food security are increasingly threatened by these intensified stresses (Chaudhry and Sidhu, 2022; Son and Park, 2022). Since plants cannot relocate to escape stressors, adaptive changes driven by alterations in gene expression are vital for their survival under extreme conditions. This regulation of gene expression is particularly crucial for plants compared to animals. Additionally, plant protein translation mechanisms are adjusted in response to various stresses (Spriggs et al., 2010; Echevarría-Zomeño et al., 2013; Zlotorynski, 2022). Therefore, understanding the translation mechanisms that contribute to stress tolerance and engineering these processes in key crops is essential for sustainable agriculture. More importantly, aquaporin channels on the plasma membrane and endomembrane are critical as plants need water to combat osmotic stress caused by water deficit.

1.3.1 Role of aquaporins in drought stress adaptation

Most of the water utilized by plants is absorbed from the soil through the roots and subsequently transported to the shoots, where it is lost to the atmosphere via transpiration from the leaves (Prieto et al., 2012; Scharwies and Dinneny, 2019). This long-distance water transport occurs through dead xylem

vessels, which are encased in a sheath of living cells that regulate the flow of water into and out of the xylem (Figure 1.3) (Venturas et al., 2017). The movement of water through and along the walls outside a cell's plasma membrane is referred to as apoplastic flow. This mechanism allows water to travel through many interconnected cells, such as those found in the root cortex, leaf mesophyll, bundle sheath extensions, or epidermis (Figure 1.3) (Venturas et al., 2017). However, certain barriers block this pathway, notably the root endodermis (Steudle, 2000; Zimmermann et al., 2000) and the bundle sheath surrounding veins in the shoots and leaves (Figure 1.3) (Canny, 1995, 1988; Hachez et al., 2008). At these sites, water must enter the cells to bypass these barriers. To do so, water must cross the plasma membrane via what is known as the transcellular path. Given that the plasma membrane is relatively impermeable to water, aquaporins (AQPs) serve as channels that facilitate most of this water movement (Figure 1.3) (Reuss, 2012). Additionally, water can also navigate between cells while remaining in the cytosol through plasmodesmata pores connecting adjacent cells through so-called symplastic flow (Figure 1.3) (Groszmann et al., 2017). For stomatal guard cells the transcellular pathway is the sole route to exchange water and CO2, as the plasmodesmata connecting guard cells to surrounding cells become nonfunctional upon the maturation of stomatal guard cells (Willmer and Sexton, 1979; Oparka and Roberts, 2001). As a result, aquaporins (AQPs) play a crucial role in regulating the opening and closing of stomata (Figure 1.3).

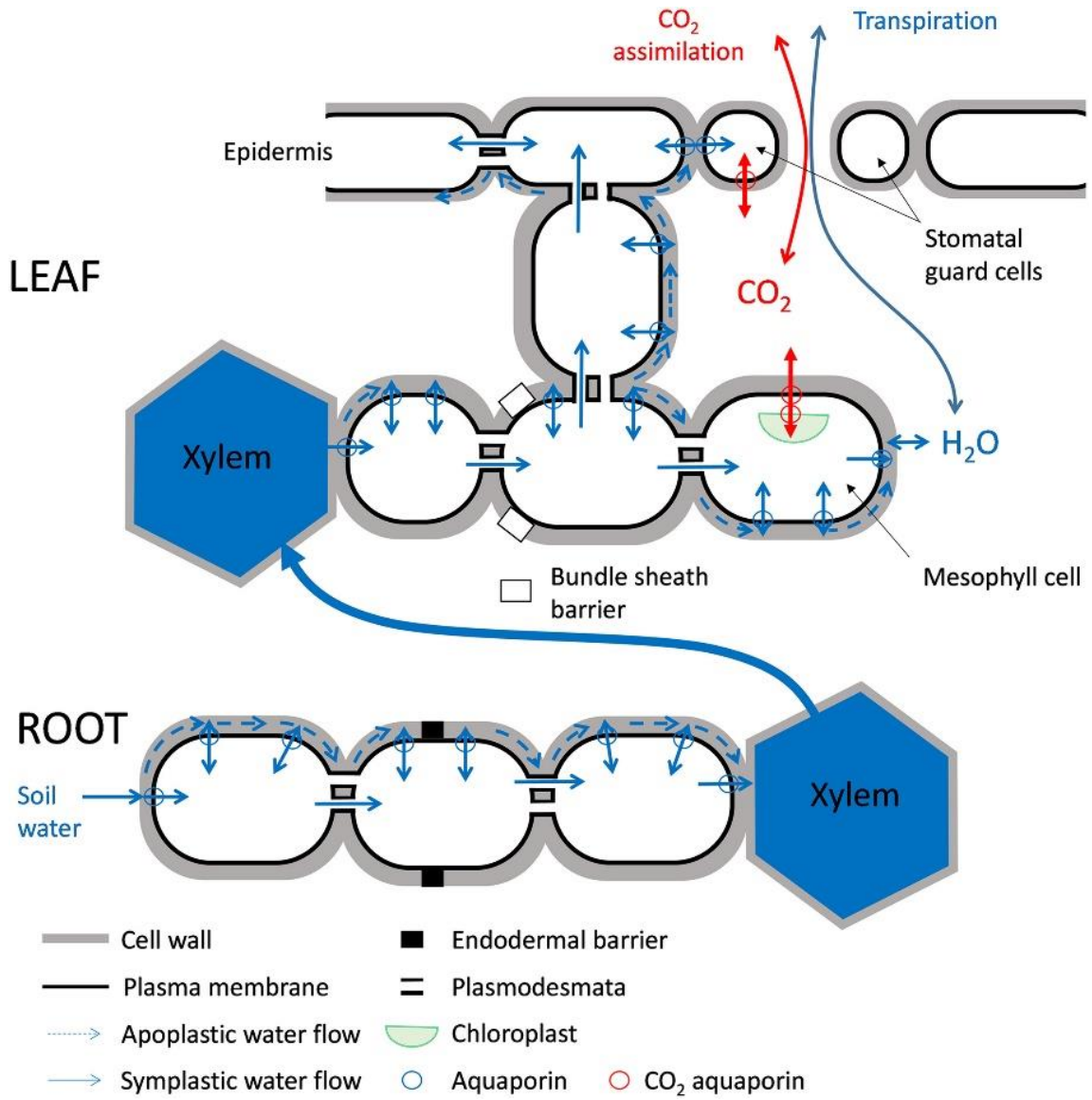

Figure 1.3. Diagrammatic illustration of water flowing from the soil through the plant to the atmosphere and CO_2 diffusion between the leaf and atmosphere. Water is transported via three pathways: apoplastic (along cell walls), symplastic (via plasmodesmata), and transcellular (across the cell membranes) pathways. Water needs to cross plasma membranes at several points along the way through aquaporins, which allow bidirectional flow. Water entry and exit from the vascular system are isolated from apoplastic flow by the endodermis in roots and the bundle sheath in leaves. Mature guard cells have no symplastic connection to adjacent epidermal cells. Leaf mesophyll and stomatal guard cells have aquaporins that enhance the permeability of the plasma membrane and

chloroplast envelope to CO_2. Taken from (Groszmann et al., 2017).

AQPs are categorized into seven subfamilies in plants: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and X intrinsic proteins (XIPs) (Ahmed et al., 2021; Tang et al., 2023). Their transcriptional regulation is diverse, with expression being tissue-specific and significantly affected by drought stress (Smart et al., 2001; Jang et al., 2004; Alexandersson et al., 2005, 2010). In response to drought, plants close their stomata to minimize transpiration, leading to a down-regulation of AQP gene expression (Pirasteh-Anosheh et al., 2016; Buckley, 2019). This stomatal closure results in a decrease in intercellular CO_2 partial pressure (C_i), which subsequently reduces photosynthesis (Engineer et al., 2016; Pankasem et al., 2024). Notably, *At*PIP1;4, which enhances membrane CO_2 permeability (Li et al., 2015), was one of the few AQPs consistently upregulated during drought stress experiments in *Arabidopsis* (Jang et al., 2004; Alexandersson et al., 2005, 2010). Similar result was observed in C_4 plant *Setaria viridis* where, however, *Si*PIP2;7 facilitate CO_2 diffusion (Ermakova et al., 2021). In tobacco, while the water-permeable AQPs *Nt*PIP1;1 and *Nt*PIP2;1 were down-regulated under drought conditions, the CO_2-permeable but weakly water-permeable *Nt*AQP1 was up-regulated (Mahdieh et al., 2008). The activity of AQPs was also affected by specific post-translational modifications, such as phosphorylation which regulates their gating and trafficking in response to environmental stimuli (Kapilan et al., 2018; Tang et al., 2023). For instance, phosphorylation of PIP2;1 at the C-terminus (e.g., Ser280 and Ser283) occurs during osmotic stress treatments in *Arabidopsis* (Qing et al., 2016). Additionally, phosphorylation at Ser283 is essential for the trafficking of AtPIP2;1 from the endoplasmic reticulum to the plasma membrane (Chevalier and Chaumont, 2015; Prado et al., 2019). Furthermore, the ABA-activated protein kinase SnRK2.6 (also known as Open Stomata 1 or OST1) phosphorylates PIP2;1 at Ser-121, enhancing its water transport activity and facilitating stomatal closure (Grondin et al., 2015).

Over the years, researchers have investigated how dehydration affects global translation

across various species such as maize (Hsiao, 1970; Lei et al., 2015), soybean (Bensen et al., 1988; Mason et al., 1988), Tobacco (Kawaguchi et al., 2003), and *Arabidopsis* (Kawaguchi et al., 2004; Kawaguchi and Bailey-Serres, 2005). These studies used a range of techniques from one-dimensional western blots to ribosome profiling. Results consistently indicated a reduction in polysomal loading and protein synthesis during water stress, with recovery noted upon rehydration. This implies that despite an overall decline in translation rates, most stress-induced mRNAs continued to be effectively translated (Kawaguchi et al., 2004). The extent of polysomal decrease and the duration required for recovery varied depending on the severity of stress, the species examined, and the specific tissue analyzed. However, there is a notable lack of data concerning the translational or post-transcriptional regulation of aquaporins (Yepes-Molina et al., 2020). Interestingly, some aquaporin genes can be silenced by miRNAs indicating the post-transcriptional control mechanisms in aquaporin gene expression (Sade et al., 2014).

1.4 Tools to investigate translational regulation in the plant (i.e. polysome profiling and ribosome profiling)

Numerous methods exist for investigating translation in plant research (Mazzoni-Putman and Stepanova, 2018), with polysome profiling and ribosome profiling being central techniques discussed in this thesis. Polysome profiling, the first genomic technique employed to study translation (Figure 1.4a), involves the separation of mRNAs bound by varying numbers of ribosomes through a sucrose gradient via ultracentrifugation. The process begins with preparing a 5-10 mL sucrose gradient (20%-60%, Figure 1.4a), followed by layering 0.5-1 mL of cell extract and centrifuging at high speed for 3-4 hours. After centrifugation, the gradient solution is passed through an absorbance recorder to generate a polysome profile, from which ten to twelve fractions (0.8-1 mL each) are collected for isolating different RNA and protein populations. The distribution of specific mRNAs across these fractions can be quantified using reverse-transcription PCR, microarrays, or RNA-sequencing (RNA-seq), allowing for the estimation of their relative translational activity. This powerful technique has facilitated numerous discoveries in the field (Coate et al., 2014; Missra et al., 2015; Bai et al., 2017; Zhang et al., 2017).

Despite its utility, polysome profiling is a tedious and time-consuming process (6-9 hours), requiring access to a suitable ultracentrifuge rotor and centrifuge, as well as a substantial amount of tissue material, which can be limited. Additionally, this method does not provide precise information on ribosome occupancy on mRNAs, and the resolution of polysome separation tends to decrease as the number of bound ribosomes increases (Figure 1.4a). In contrast, ribosome profiling (also known as Ribo-seq; Figure 1.4b) addresses these limitations. Ribo-seq involves digesting ribosome-bound mRNAs with ribonucleases and sequencing the resulting ribosome-protected mRNA fragments—commonly referred to as ribosome footprints—which are typically 28 to 30 nucleotides in length (Ingolia et al., 2009; Brar and Weissman, 2015). Sequencing these footprints reveals the precise quantity and position of ribosome occupancy on mRNAs throughout the transcriptome. Translation efficiency can be inferred by normalizing Ribo-seq data against RNA-seq read density. Furthermore, high-quality Ribo-seq data exhibit strong three-nucleotide periodicity in ribosome footprints, consistent with translating ribosomes moving every three nucleotides along coding regions. This periodicity can be utilized to identify actively translated regions (Ingolia et al., 2009). The first ribosome profiling studies in *Arabidopsis* emerged about a decade ago, focusing on translational control of light responses (Liu et al., 2013) and chloroplast translation (Zoschke et al., 2013). Optimizing Ribo-seq resolution led to the discovery of previously unannotated translated open reading frames (ORFs), significantly expanding the *Arabidopsis* translatome landscape and enhancing genome annotation (Hsu et al., 2016). Ribo-seq has also been employed to investigate translational responses to various environmental stimuli, including hypoxia, hormonal responses, pathogen interactions, and phosphate deficiency in *Arabidopsis* (Juntawong et al., 2014; Merchante et al., 2015; Bazin et al., 2017; Xu et al., 2017a). Additionally, it has been used to characterize translatomes in several crops such as maize (*Zea mays*), soybean (*Glycine max*), tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and wheat (*Triticum aestivum*) (Chotewutmontri and Barkan, 2016; Guo et al., 2023; Lei et al., 2015; Li and Liu, 2020; Shamimuzzaman and Vodkin, 2018; Sotta et al., 2022; Wu et al., 2019; Zhu et al., 2021). However, the short length of ribosome footprints poses challenges for mapping capabilities,

making it difficult to implement Ribo-seq in polyploid genomes where short reads are less likely to map uniquely (Jiang et al., 2022). Despite this limitation, Ribo-seq has recently been successfully performed in hexaploid bread wheat (Guo et al., 2023).

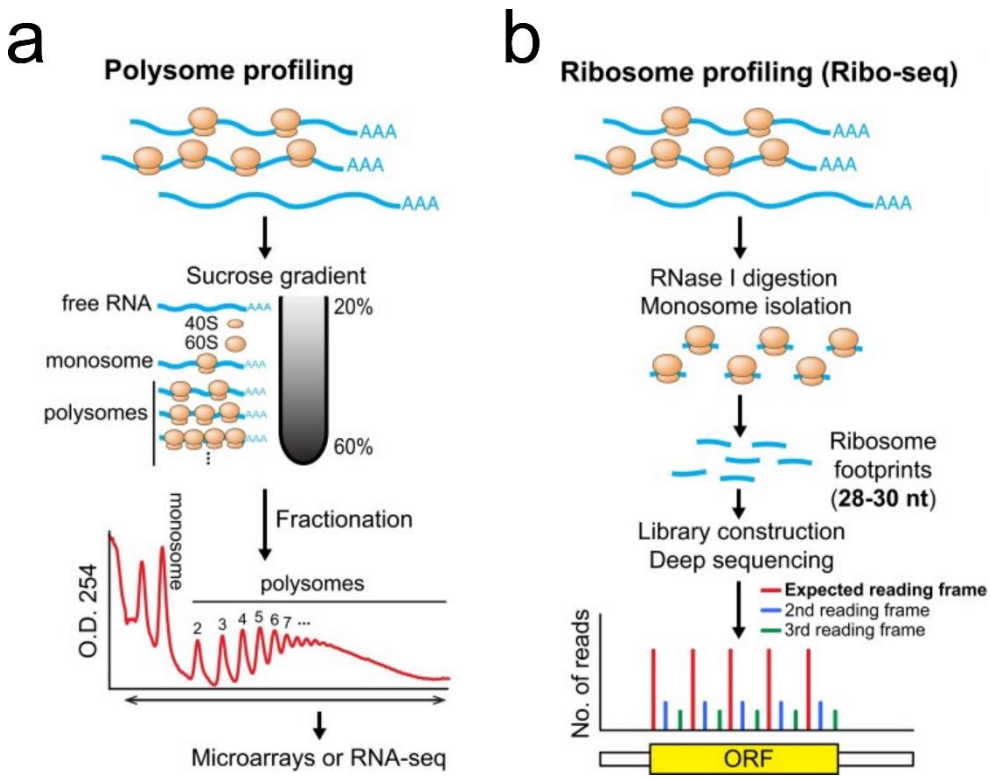

Figure 1.4. Methods to investigate translation. (**a**). polysome profiling. (**b**). ribosome profiling. Taken from (Wu et al., 2024).

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1.6 Objectives

This thesis has two main objectives. The first was to investigate translational regulation during the development of *Arabidopsis* seedlings. The observed low correlation between mRNA and protein levels may be due to translational control, making it essential to study mechanisms such as miRNA-mediated regulation, as miRNAs play a key role in controlling the translation of their target genes. The second objective was to examine the translation of aquaporin-encoding genes in grapevine (*Vitis vinifera* L.) under drought conditions followed by rewatering. Aquaporins, channel-forming transmembrane proteins present in plasma and intracellular membranes across all eukaryotes and most prokaryotes, are of particular interest. In the first study, *AtPIP2F* (also known as *AtPIP2;4*), a member of the plasma membrane intrinsic protein subfamily, demonstrated uncoupled transcription and translation. Phylogenetic analysis of aquaporin protein sequences has also shown a close evolutionary relationship between *AtPIP2F* in *Arabidopsis thaliana* and *VviPIP2;2* in grapevine (*Vitis vinifera* L.) (Figure 1.5). The results from both studies will be presented in two main chapters of this thesis, and manuscripts are being prepared for submission to peer-reviewed journals.

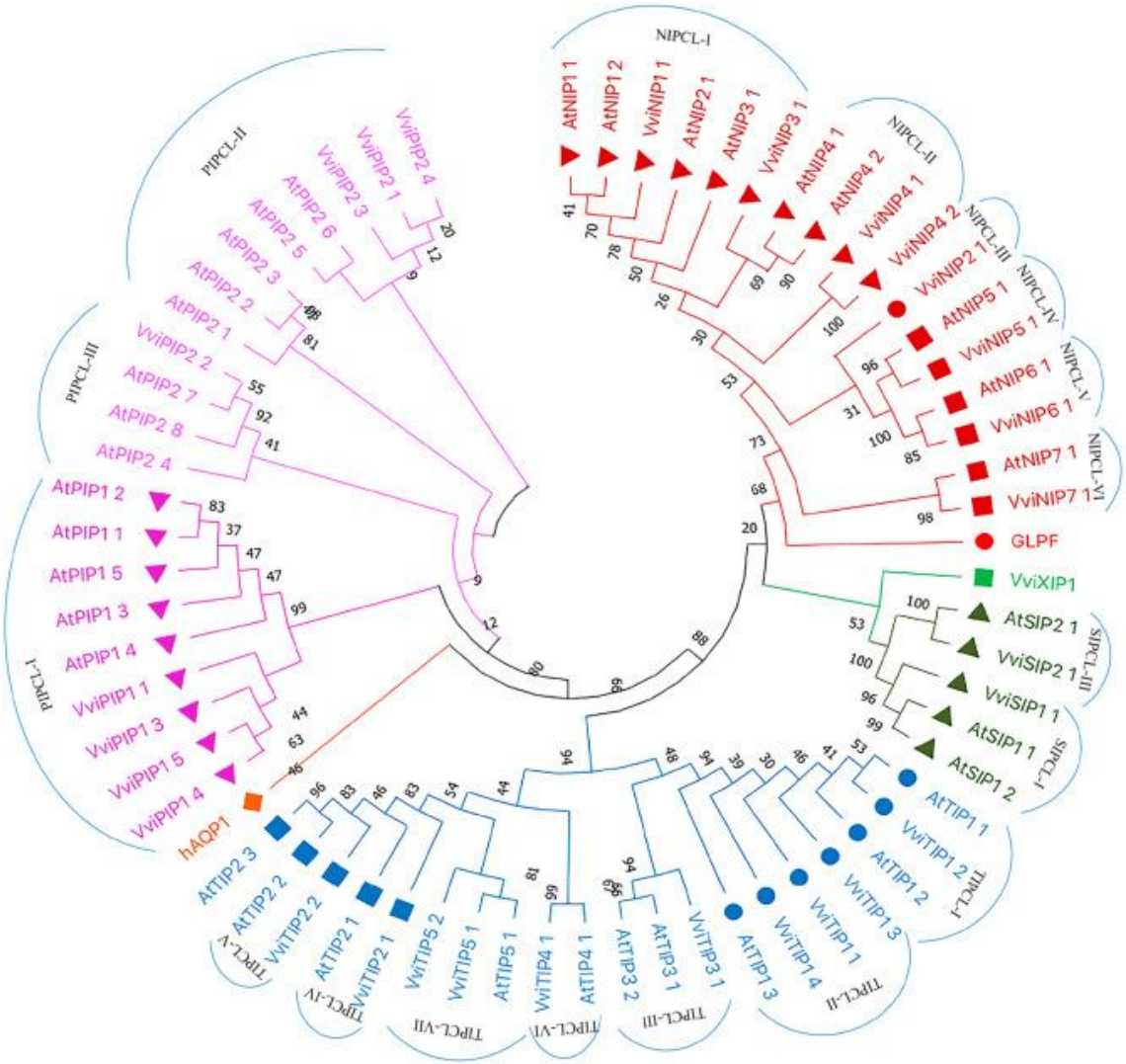

Figure 1.5. The phylogenetic tree containing *Vitis vinifera* L. aquaporins and the previously identified *Arabidopsis thaliana* aquaporins. Human AQP1 (hAQP1) and E. coli GLPF (a glycerol facilitator protein) are also included as outgroups. The tree was generated by the Neighbor-Joining method on 66 amino acid sequences. The bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the taxa analyzed. *At*PIP2;4 and *Vvi*PIP2;2 were clustered into the PIPCL-III group indicating a close evolutionary relationship. Taken from (Sabir et al., 2021).

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2 miRNA-Mediated Translational De-Repression of *AGO1* mRNAs in *A. thaliana*

2.1 Abstract

Transcriptome and proteome analyses offer valuable and distinct insights into gene expression; however, they often fail to reveal unknown yet potentially significant post-transcriptional and translational mechanisms in both animals and plants. In this study, we investigated the interaction between mRNAs and miRNAs using polysome profiling while simultaneously examining transcriptional, translational, and protein changes across various tissues during the development of *Arabidopsis thaliana*. Our analysis of mRNA recruitment to polysomes at different developmental stages indicated that fluctuations in this recruitment are not solely driven by transcriptional changes. Notably, during the first 12 days of development, we observed shifts in the association of miR168 with polysomes without corresponding changes in the recruitment of its target mRNA, *AtAGO1*. The dissociation of miR168 from polysomes leads to the alleviation of its repression on *AtAGO1* translation, resulting in increased protein levels at later growth stages. These findings suggest an unidentified post-transcriptional regulatory mechanism influencing *At*AGO1 protein levels during plant development. This research emphasizes the complexity of translational regulation in plants and highlights the crucial role of miR168 and other post-transcriptional mechanisms in sustaining *AtAGO1* gene expression throughout development.

2.2 Introduction

The formation of a multicellular organism necessitates the synchronized processes of cell division, growth, and specialization to establish organized tissues, distinct organs, and a complex body structure (Byrne, 2009). This intricate development hinges on the precise regulation of gene expression, which is governed by transcriptional, post-transcriptional, translational, and post-translational mechanisms (Byrne, 2009). In plants, particularly during seed and pollen development, RNA granules and translational controls facilitate the transition between dormant and metabolically active states

(Urquidi Camacho et al., 2020). Additionally, the allocation of resources throughout the daily light-dark cycle is closely tied to the regulation of cellular protein synthesis (Liu et al., 2012; Urquidi Camacho et al., 2020). For higher eukaryotes, changes in gene expression during developmental transitions and cell fate specification must be well-coordinated to trigger appropriate differentiation programs while inhibiting unsuitable ones (Kaufmann et al., 2010). The ribosome serves as the fundamental translational machinery within cells and plays a significant regulatory role in plant development (Byrne, 2009; Weis et al., 2015). Variations in ribosomal protein levels can affect transcript sensitivity and influence developmental phenotypes (Byrne, 2009). Research utilizing polysome and ribosome profiling in *Arabidopsis thaliana* has revealed that the translation efficiency of mRNAs varies among different transcripts and dynamically shifts under various conditions such as seed germination (Bai et al., 2017), tissue development (Yamasaki et al., 2015), circadian cycles and heat stress (Bonnot and Nagel, 2021), although the underlying molecular mechanisms remain largely unexplored.

MicroRNAs (miRNAs) play a crucial role in the post-transcriptional and translational regulation of mRNA, influencing significant developmental transitions and cell fate determination across various tissues (Kaufmann et al., 2010; Yu et al., 2019). *MIR* genes are transcribed into primary microRNAs (pri-miRNAs), which are processed by Dicer-like 1 (DCL1) to produce miRNA/miRNA* duplexes (Kurihara and Watanabe, 2004; Shang et al., 2023; Yan et al., 2024). One strand of this duplex, known as the guide miRNA, is incorporated into ARGONAUTE 1 protein (AGO1), the miRNA hub (Z. Zhao et al., 2023), forming the RNA-induced silencing complex (RISC) alongside other proteins (Baumberger and Baulcombe, 2005). The AGO1-loaded complex can either cleave target mRNA through post-transcriptional gene silencing (PTGS) or bind without cleavage to inhibit translation (Kidner and Martienssen, 2005). In *Arabidopsis thaliana*, deficiencies in the *AtAGO1* gene result in developmental anomalies such as malformed leaves and sterile flowers (Bohmert et al., 1998). Its protein AGO1 is crucial for PTGS in plants (Fagard et al., 2000; Morel et al., 2002). Notably, miR168 specifically targets *AtAGO1* mRNA; this interaction is vital for maintaining proper plant architecture (Rhoades et al., 2002; Vaucheret et al., 2004; Dalmadi et al., 2019). In transgenic *Arabidopsis* where the complementarity

between miR168 and *AtAGO1* mRNA was diminished, which resulted in increased *AtAGO1* mRNA level and thus increased AGO1 protein level, researchers have observed developmental defects and increased accumulation of miR168 (Vaucheret et al., 2004). On the other hand, excess of miR168 can be stabilized by AGO1 protein, indicating the autoregulatory loop between miR168 and *AtAGO1* mRNA (Vaucheret et al., 2006; Dalmadi et al., 2021). Interestingly, both miR168 and AGO1 proteins co-sediment within polysomal fractions (Lanet et al., 2009), suggesting that the translational efficiency of *AtAGO1* mRNA can be modulated within polysomes by both miR168 and AGO1 proteins.

In this study, to test whether miR168 influences *AtAGO1* mRNA translational efficiency, we used polysome profiling to isolate mRNA-ribosome complexes through sucrose gradients. Studying the association of mRNAs with non-translating ribosomes and ribonucleic particles (RNPs) or with translating polysomes with different numbers of ribosomes per mRNA provides insights into translation efficiency (Lecampion et al., 2016; Panda et al., 2017). This method offers a snapshot of active mRNA translation and was utilized to compare transcriptional, translational, and protein levels of multiple mRNAs, aiding in understanding post-transcriptional regulations during *Arabidopsis* development (Chassé et al., 2017; Mergner et al., 2020).

2.3 Materials and Methods

2.3.1 Plant materials, growth conditions, and sample collection

Arabidopsis (*Arabidopsis thaliana*) seeds (wild type, ecotype: Columbia-0) were thoroughly sterilized with 50% (v/v) commercial bleach with gentle agitation for 10 min and then washed several times using sterile water. After sterilization, approximately 100 seeds were evenly spread on ½ Murashige and Skoog (MS) medium (0.22% (w/v) MS macronutrients, 1% (w/v) sucrose, 0.8% (w/v) Agar). Next, seeds were placed at 4 °C in the dark for 24 hours for stratification. Next, imbibed seeds were put in a 22 °C growth chamber where the light period was set up as 16 h light, and 8 h dark. From the day the seeds were put in a growth chamber, around 100 seedlings were collected using a sterilized tweezer on days 4, 6, 7, 10, 13, and 16 respectively (hereinafter referred to as 4 d, 6 d seedlings, etc.) and flash frozen in liquid N_2. Samples were stored at -80°C for further experiment. In the case of

cotyledons and hypocotyl dissections and corresponding analyses, seeds were sowed in rows onto square Petri dishes with ½ MS medium to easily separate cotyledons and hypocotyl. Then, Petri dishes were put vertically in the growth chamber. At 4d, cotyledons and hypocotyl were separated and collected using a sterilized scissor and a sterilized tweezer respectively, and flash frozen in liquid N_2. Samples were stored at -80°C for further experiment. In this study, seedlings were small, approximately 100 seedlings were collected for each replicate.

2.3.2 Stereomicroscopy

Seedlings grown in Petri dishes were retrieved from the growth chamber at 4, 6, 7, 10, 13, and 16 days. Without relocating the seedlings, images of individual whole seedlings were captured directly under a Leica stereo microscope (Leica MZ16F, Switzerland) equipped with a Leica DFC490 camera. Using the live image acquisition mode in Leica Application Suite (LAS) v4.13 software, the dish containing the seedlings was placed on the base of the stereomicroscope. The desired seedling was located and centered in the frame by adjusting the focus and moving the dish. Optimal images were then acquired and saved to the specified file location.

2.3.3 Polysome profiling

Polysome profiling was carried out according to Bernabò et al (2017) with small changes (Bernabò et al., 2017). Seedlings were reduced to fine powder using a pestle and mortar with liquid N_2. Around 150 mg of sample were used and lysed in 700 µL of lysis buffer (160 mM Tris-HCl, 80 mM KCl, 40 mM $MgCl_2$, 0.6 U/µL RNase inhibitor, 1 mM dithiothreitol, 200 µg/mL cycloheximide, 0.005 U/µL DNase I, 0.5% IGEPAL, pH 8.4). The samples were mixed thoroughly with lysis buffer and incubated on ice for 20 min. After 20 min incubation on ice, the lysate was centrifuged twice at 4 °C, 12,750 g for 10 min to remove tissue debris, nuclei, chloroplasts, and mitochondria. Next, 600 µL of supernatant were loaded onto a sucrose gradient (15–50% sucrose (w/v) in 40 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, pH 8.4). The sucrose gradient was ultracentrifuged for 1 h 40 min at 4 °C, 40,000 rpm using SW41 swinging bucket rotor in a Beckman Optima LE-80K ultracentrifuge. Following ultracentrifugation, by slowly pumping a 50% sucrose solution from the bottom of the gradient, the gradient was forced to

pass through an optical unit where the UV absorbance at 254 nm was continuously measured using an ISCO UA-6 UV detector to generate an absorbance profile. The gradients were collected in 12 aliquots, except for the first fraction which contains approximately 600 µL of liquid, and the other fractions contain approximately 1,000 µL of liquid. The fractions were either used immediately for RNA extraction or stored at -80 °C for future use.

The fraction of ribosomes in polysomes (FRP) was calculated from polysome profiles as the ratio between the area under the curve of polysomes and the sum of the area under the curve of polysomes peaks and the area of the 80S peaks (Bernabò et al., 2017) as follows:

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

Where A is the area under the curve of the profile, %FRP is the Fraction of Ribosomes in Polysomes expressed as a percentage.

2.3.4 Co-sedimentation profile of mRNAs along the sucrose gradient, and quantitative PCR on cytoplasmic RNA

The extraction of RNA was performed as described in the protocol from Lauria et al (2020) (Lauria et al., 2020). Total RNA was purified from pooled sucrose fractions (a sum of 10% of the total volume of each fraction) and the remaining liquid was used to extract RNA from individual fractions. Pooled and individual fractions were added with 5 µL of 20 mg/mL proteinase K and 100 µL of 10% (v/v) SDS respectively and mixed thoroughly. The mixed solutions were incubated at 37°C for 1 hr and 45 min to remove potential protein contaminants. After the incubation, the solutions were placed on ice, and 130 µL of acid-phenol/chloroform/isoamyl alcohol (pH 4.5, 125:24:1, Invitrogen) was added for phase separation, followed by thorough mixing. The solutions were then centrifuged at 4 °C, 12,750 g for 10 min. The upper aqueous phase was carefully transferred to a new nuclease-free 2 mL Eppendorf tube, ensuring the bottom phase was not disturbed. Next, the transferred aqueous phase was added approximately 1000 µL of 100% isopropanol and 1 µL of glycol blue (to visualize the RNA pellets), followed by thorough mixing and placed at -80°C overnight to precipitate RNA. After overnight

precipitation, the RNA pellets were collected by centrifugation at 4 °C, 15,300 g for 45 min. The RNA pellets were air-dried under the hood for 10 min to evaporate excess liquid. The RNA pellets were subsequently washed with 500 µL of 80% (v/v) ethanol in DEPC-treated water (RNase-free) and centrifuged at 4 °C, 12,750 g for 10 min. The supernatant was discarded, and the RNA pellets were air-dried under the hood for 10 min. Finally, the RNA pellets were dissolved in 11 µL of DEPC-treated water (RNase-free). The quality of the RNA was assessed by measuring the 260/230 and 260/280 absorbance ratios using a Nanodrop. The remaining RNA was aliquoted and stored at -80 °C for downstream analyses. For cDNA synthesis from total RNA, 500 ng of RNA was used, while in the case of RNA from individual fractions, the cDNA was synthesized using the same volume throughout the entire profile (1 µL, corresponding to 40 ng to 500 ng of RNA) according to Lauria et al., 2020. Reverse transcription was performed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) with Oligo $(dT)_{18}$ primer. Quantitative PCR (qPCR) was performed using qPCRBIO SyGreen Mix (PCRBIOSYSTEMS) in CFX Connect Real-Time PCR Detection System (Bio-Rad). The following PCR procedure was employed: 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds and 62 °C for 25 seconds, before the melting curve analysis, PCR products were heated to 95 °C for 15 seconds and then subjected to the melting curve analysis between 62 °C and 95 °C at 0.5 °C increments for 15 seconds. The primers used are listed in the Supplemental Table. For fold change of each gene in total RNA, *AtMON1* was used as the reference gene for normalization. Fold changes were calculated by the following equations:

The arithmetic means of the Cq values of duplicates of the gene of interest and *AtMON1* were calculated respectively: $\overline{Cq_{gene\ of\ interest}} = \frac{Cq_1+Cq_2}{2}$, and $\overline{Cq_{MON1}} = \frac{Cq_1+Cq_2}{2}$.

The difference between the arithmetic means of the gene of interest and the reference gene *AtMON1* in the samples was calculated: $\Delta Cq_{sample} = \overline{Cq_{gene\ of\ interest}} - \overline{Cq_{MON1}}$.

The mean of ΔCq of reference samples was calculated: $\overline{\Delta Cq_{reference}} = \frac{\sum_1^n (Cq_{gene\ of\ interest} - Cq_{MON1})}{n}$, n=biological replicate.

Next, ΔΔCq was calculated: $\Delta\Delta Cq = \Delta Cq_{sample} - \overline{\Delta Cq_{reference}}$.

The efficiency of primers determined above was within 90-110% and therefore, for simplifying calculation, set at 100%. So, the fold changes were calculated by the following equation: $Fold\ change = 2^{-\Delta\Delta Cq}$.

To obtain the co-sedimentation profiles of mRNAs along the sucrose gradient, a method referred to (Panda et al., 2017) with some changes. Briefly, the relative percentage of transcript in each fraction was calculated by the following equation:

$$\%(mRNA)_n = \frac{(2^{40-Cq_{mRNA}})_n}{\sum_{n=1}^{n=12}(2^{40-Cq_{mRNA}})_n} * 100\%$$

Where n = fraction, mRNA is the gene of interest.

All experiments were performed in two or three technical replicates and at least 3 biological replicates.

2.3.5 Analysis of microRNA expression level and co-sedimentation by stem-loop reverse transcription quantitative PCR

The analyses of microRNA expression level and co-sedimentation were carried out using the method described in Varkonyi-Gasic et al, 2011 (Varkonyi-Gasic and Hellens, 2011). Briefly, 1 µM specific stem-loop primer of microRNA (see Supplemental Table 1, stem-loop RT primer) was prepared for reverse transcription by heating at 65°C for 5 min and immediately cooling down on ice for 2 min. Next, 500 ng of total RNA and 1 µL of RNA extracted from each sucrose fraction (40 ng to 500 ng) from 6 d and 10 d *Arabidopsis* seedlings that containing microRNA were reverse transcribed using 1 µL specific stem-loop primers generated above in a 20 µL reaction system that contained 1x first strand buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.25 mM dNTPs, 0.2 U/µL RNaseOUT™ (Cat. no. 10777-019), 2.5 U/µL SuperScript™ III Reverse Transcriptase (Cat. No. 18080-093). The reverse transcription was carried out as follows: 16°C for 30min, 60 cycles of 30°C for 30 s, 42°C for 30 s, 50°C for 1 s, following the final step 85°C for 5min. Then, the synthesized complementary DNA was used for quantitative PCR (qPCR) using qPCRBIO SyGreen Mix (PCRBIOSYSTEMS) in CFX

Connect Real-Time PCR Detection System (Bio-Rad). The cDNA was denatured at 95 °C for 2 min, followed by 40 cycles of 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 seconds and then subjected to the melting curve analysis between 62 °C and 95 °C at 0.5 °C increments for 15 seconds. Each condition was performed in three to four technical replicates and at least three biological replicates. The obtained Cq values were used to calculate the fold change of each miRNA using the same delta/delta Ct method as described previously (Livak and Schmittgen, 2001). *AtMON1* was used as the reference gene for normalization. Transcript distribution in each fraction was calculated by following the equation (Lauria et al., 2020):

$$\%(miRNA)_n = \frac{(2^{40-Cq_{miRNA}})_n}{\sum_{n=1}^{n=12}(2^{40-Cq_{miRNA}})_n} * 100\%$$

n is the fraction number.

2.3.6 Total protein extraction and quantification

Total protein extraction followed the method outlined by Conlon and Salter (2007) (Conlon and Salter, 2007). Approximately 100 whole seedlings were collected at 6, 10, 12, and 14 days, then flash-frozen in liquid nitrogen. The frozen seedlings were ground into a fine powder using a mortar and pestle with liquid nitrogen. For each sample, 40 mg of the powder was lysed in 200 µL of extraction buffer (125 mM Tris-HCl, 1% SDS, 10% glycerol, 50 mM DTT, pH 8.8). The lysates were thoroughly homogenized by vortexing for 30 seconds. Afterward, the homogenates were centrifuged at 12,750g for 10 minutes at room temperature, and 180 µL of the supernatant containing total protein was collected and stored at -20°C for further analysis. Protein concentration was determined using the bicinchoninic acid (BCA) method, following the protocol from the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). The total protein was diluted 80-fold with MilliQ water. In parallel, nine BSA standard solutions were prepared with concentrations of 2,000 µg/mL, 1,500 µg/mL, 1,000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 25 µg/mL, and 0 µg/mL. Subsequently, 10 µL of the diluted protein samples and 10 µL of each BSA standard solution were mixed with 190 µL of BCA reagent (prepared by combining reagent A and reagent B in a 1:49 ratio) in duplicate respectively. Both the unknown protein samples and BSA

standards were added to the wells of a 96-well cell culture plate. The plate was then covered with aluminum foil and incubated at 37°C for 30 minutes. After incubation, the absorbance at 575 nm was measured using a plate reader. A regression analysis was performed based on the absorbance readings of the BSA standards to create a standard curve, and the protein concentrations of the samples were calculated using the slope and intercept from the regression plot.

2.3.7 Western blotting

The western blot analysis for detecting *At*AGO1 was conducted as per the protocol outlined by Huang et al. (2019) (Huang et al., 2019). In brief, 80 µg of total protein was combined with 3.75 µL of 4x SDS sample buffer (Laemmli buffer: 250 mM Tris, 8% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.4% (w/v) Bromophenol blue, with 200 mM DTT freshly added) and MilliQ water, making up a final volume of 15 µL. The protein samples were denatured at 95 °C for 5 minutes, then loaded onto an 8% polyacrylamide gel. Electrophoresis was carried out with a voltage progression of 80 V for 10 minutes, 100 V for another 10 minutes, and 120 V until the front reached the gel's edge. Proteins were transferred to a nitrocellulose membrane using an electroblotting system at 4°C, 200 mA for 90 minutes. The membrane was stained with Ponceau S (Thermo Fisher, USA) for 5 minutes with gentle agitation and subsequently washed twice with MilliQ water to remove excess stain. Next, the total protein signal on the membrane was acquired using the ChemiDoc imaging system (Bio-Rad). The membrane was then blocked at room temperature for 4 hours in TBST (Tris-buffered saline with 0.1% Tween 20) containing 3% non-fat milk (Blotto, Santa Cruz Biotechnology, USA). Primary antibody incubation (rabbit anti-AGO1, AS09 527, Agrisera, Sweden, 1:1000 dilution in 3% non-fat milk in TBST) was carried out overnight at 4°C. Next, the primary antibody was removed, and the membrane was washed three times in TBST to remove excess primary antibody. The hybridization with secondary antibody (1:2000 dilution in 3% no-fat milk in TBST, mouse anti-rabbit IgG-HRP, sc-2357, Santa Cruz Biotechnology, USA) conjugated with HRP (Horseradish peroxidase) was performed at room temperature with a gentle agitation for 90 min. Next, the membrane was washed in TBST twice and in TBS (Tris-buffered saline) once, each time with 10 min of gentle agitation. Then, the membrane was

developed by 1,200 µL (600 µL of reagent 1 and 600 µL reagent 2) of Pierce™ ECL western blotting substrate mix (Thermo Fisher, USA). Next, the protein signal of interest was obtained using the ChemiDoc imaging system (Bio-Rad). Finally, ImageJ software (v1.53t, USA) was used to quantify the intensity of AGO1 protein, and the intensity of the Ponceau staining membrane (used as the total protein level). The relative AGO1 protein level (fold change) was normalized by comparing it to the average AGO1 protein level at 6d. The equation is followed below:

$$Fold\ Change = \frac{\text{AGO1}\ protein\ level}{average\ \text{AGO1}\ protein\ level\ at\ 6d}$$

Where AGO1 is Argonaut 1 protein.

2.3.8 Statistical analysis and software

The two-sided and unpaired Student's T-test was used to perform statistical analyses using Excel (Microsoft). ImageJ (v1.53t, USA) was used to evaluate the AGO1 protein level and total protein level by measuring intensities of AGO1 bands and ponceau stained membrane. OriginPro 2021 was used to plot co-sedimentation profiles and representative polysome profiles. GraphPad Prism 8.0 was used to plot the results of the Fraction of Ribosomes in Polysomes (FRP). Inkscape (v1.3.1) was used to assemble and embellish representative polysome profiles.

2.3.9 Linear regression plotting

Expression data on transcriptome and proteome levels were obtained from the tissue atlas expression values generated by Mergner J. and colleagues (Supplemental Table 1) (Mergner et al., 2020). The iBAQ and TPM values for each gene in the atlas were retrieved for the cotyledon and hypocotyl tissues. The scatterplots were generated in Python (v 3.11.4) using the matplotlib library (v 3.7.1). Regression analysis was carried out on the plotted datasets using the stats model. api (v 0.14.0) applying the ordinary least squares (OLS) method to the linear regression model. The confidence intervals were calculated with alpha=0.05.

2.4 Results

2.4.1 Uncoupling of transcription and translation in cotyledon and hypocotyl

Over the past decade, evidence has increasingly shown that transcriptomics and proteomics alone are inadequate for fully capturing the complex post-transcriptional and translational dynamics that underlie organisms' responses to environmental changes and developmental processes (Schwanhäusser et al., 2011; Tebaldi et al., 2012; Buccitelli and Selbach, 2020). In this study, we began by re-analyzing previously published transcriptome and proteome data from 7 d *Arabidopsis thaliana* cotyledon and hypocotyl tissues (Supplemental Figure S2.1a, b) (Mergner et al., 2020). As anticipated, we found a strong correlation between mRNA and protein levels in both tissues (Supplemental Figure S2.1c, d), although some exceptions suggest the presence of post-transcriptional regulatory mechanisms. We focused on eleven genes (Table 2. 1) selected based on their steady-state mRNA and protein abundances, which either aligned with or deviated from a linear correlation (Supplemental Figure S2.1c, d).

Table 2.1. Detailed information on selected genes.

Category	Gene name	Accession	Description
Coupled genes	*AtF21M12.13*	AT1G09750	Eukaryotic aspartyl protease family protein.
	AtPetA	ATCG00540	Photosynthetic electron transfer A.
	AtF17M5.140	AT4G33380	Dimethylallyl, adenosine tRNA methylthiotransferase.
	AtMON1	AT2G28390	SAND family protein.
	AtAGO1	AT1G48410	Encodes an RNA Slicer that selectively recruits microRNAs and siRNAs.
Uncoupled genes	*AtACO3*	AT1G12010	Encodes a protein that appears to have 1-amino-cyclopropane-1-carboxylic acid oxidase activity based on mutant analyses. The mRNA is cell-to-cell

			mobile.
	AtF27J15.9	AT1G49140	NADH dehydrogenase ubiquinone 1 beta subcomplex subunit 10-B-like protein (Complex I subunit NDUFS6).
	AtPIP2F	AT5G60660	A member of the plasma membrane intrinsic protein subfamily PIP2. When expressed in yeast cells can conduct hydrogen peroxide into those cells.
	AtAOC3	AT3G25780	Allene oxide cyclase 3.
Unknown gene	*AtUBC9*	AT4G27960	Ubiquitin-conjugating enzyme 9.

To investigate whether these mRNAs are subject to tissue-specific post-transcriptional control, we compared the absolute differences between transcriptome and proteome levels (Figure 2.1b). Our analysis categorized these genes into three groups: i) coupled genes, which display linear changes in both mRNA and protein levels (*AtPetA*, *AtF21M12.13*, *AtMON1*, *AtF17M5.140*, and *AtAGO1*); ii) uncoupled genes, which show poor correlation (*AtACO3*, *AtPIP2F*, *AtAOC3*, and *AtF27J15.9*); and iii) genes with unknown correlation due to a lack of signal in the proteomics data (*AtUBC9*) (Figure 2.1b). This re-analysis leads us to hypothesize that the expression of genes such as *AtACO3*, *AtPIP2F*, *AtAOC3*, and *AtF27J15.9* is regulated by post-transcriptional mechanisms when comparing the two tissues, as their mRNA levels do not correspond with their protein levels. To validate this hypothesis and potentially identify new post-transcriptional controls of gene expression, we selected two case studies for comparison: i) 4 d hypocotyls and cotyledons, and ii) whole seedlings during various stages of early development (Figure 2.1c, stage 1). We then performed total RNA extraction to assess transcriptional levels of the selected genes and utilized polysome profiling to examine mRNA engagement with ribosomes in polysomes (Figure 2.1c, stages 2-5). Polysome profiling enabled us to separate actively translated mRNAs bound by ribosomes from nuclei, chloroplasts, and mitochondria-free cytoplasmic lysate (Figure 2.1c, stage 2). Following sucrose gradient separation into multiple

fractions (Figure 2.1c, stage 3), RNA extraction followed by qPCR allowed for relative quantification of mRNA association with light or heavy polysomes (Figure 2.1c, stage 5). Specifically, the initial portion of the profile—representing the top of the gradient—contains cellular components with low sedimentation coefficients. This section primarily consists of free cytoplasmic RNA and ribonucleic particles (RNPs), along with small (40S) and large (60S) ribosomal subunits as well as monosomes (80S). In contrast, the final section corresponds to polysomes—mRNAs associated with multiple actively translating ribosomes—characterized by a higher sedimentation coefficient (Figure 2.1c, stage 4). This method has been effectively employed to estimate the relative percentage of mRNAs bound by multiple ribosomes, assess the translational status of these mRNAs, evaluate changes in mRNA or non-coding RNA (ncRNA) associations with polysomes (Lauria et al., 2020; Tebaldi et al., 2018), and further elucidate the underlying regulatory mechanisms.

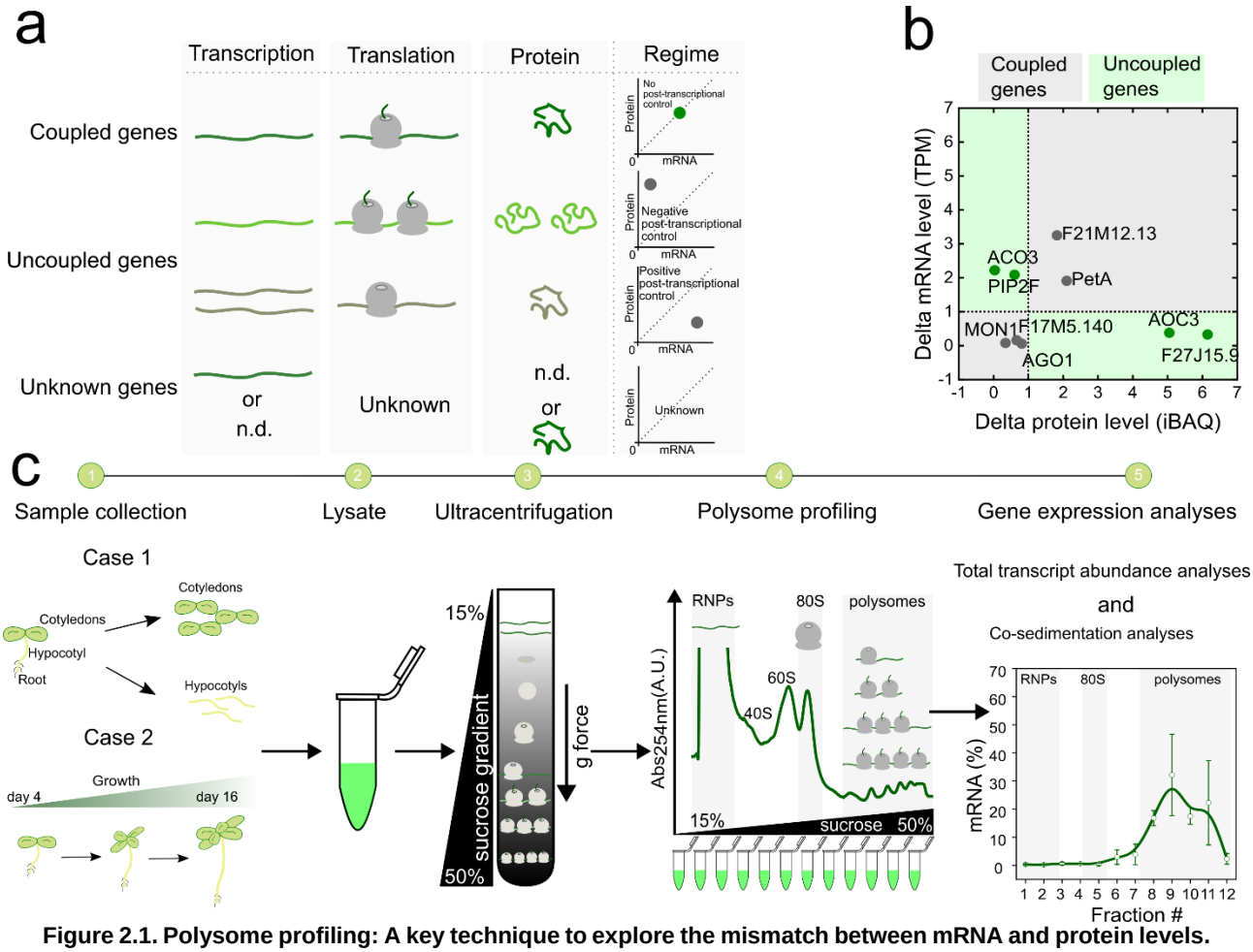

Figure 2.1. Polysome profiling: A key technique to explore the mismatch between mRNA and protein levels. (**a**) Hypothesis of potential translation patterns. The numbers of transcripts and proteins represent the hypothesized quantitative characteristics. n.d.: not determined. (**b**) Analysis of mRNA versus protein levels in 7-day-old Arabidopsis seedlings' cotyledons and hypocotyls. Based on data from Mergner et al., 2020. TPM: Transcripts per million. iBAQ: Intensity-based absolute quantification. (**c**) Overview of the experimental setup for this study. Abs_{254nm}: absorbance at 254 nm. RNPs: ribonucleoproteins. 80S: ribosome. A.U.: arbitrary units.

2.4.2 Relative lower global translational activity but with higher mRNA abundance in hypocotyl indicating translational repression

First, we analyzed the chromatograms from the polysome profiling of 4 d hypocotyls and cotyledons to estimate translation efficiency, expressed as the Fraction of Ribosomes in Polysomes (FRP) (Figure 2.2a, b, c). Our findings revealed that the translation efficiency in cotyledons is slightly,

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yet significantly, higher than in hypocotyls. To determine whether the selected genes are differentially expressed between the two tissues, we extracted total RNA from pooled cytoplasmic fractions and conducted a differential expression analysis on four uncoupled genes (*AtACO3*, *AtPIP2F*, *AtAOC3*, and *AtF27J15.9*), three coupled genes (*AtF21M12.13*, *AtF17M5.140*, and *AtMON1*) and the unknown gene *AtUBC9*. The coupled genes showed no significant changes between the two tissues, while the uncoupled genes exhibited higher transcript levels in hypocotyls on day 4 (Figure 2.2d), which aligns with observations made on day 7 for *AtPIP2F* and *AtACO3* (Figure 2.2e) (Mergner et al., 2020). Given the lower FRP value in the hypocotyl (Figure 2.2b), we hypothesized that post-transcriptional and/or translational regulations might be at play in this tissue. To explore this hypothesis, we performed qPCR to analyze the distribution of each mRNA along the sucrose gradient fractions (Figure 2.2f). This analysis enabled us to accurately assess mRNA engagement with ribosomes in polysomes and identify any reorganizations in mRNA distribution across non-translating RNPs, 40S preinitiation complexes, monosomes, and actively translating polysomes. Our results indicated that all mRNAs were robustly engaged in translation, as evidenced by a higher percentage found in fractions 7-12, which correspond to polysomal fractions. Despite a significant transcriptional upregulation of uncoupled genes (Figure 2.2d), we did not observe notable differences in the relative distribution of mRNAs between cotyledons and hypocotyls (Figure 2.2f, Supplemental Figure S2.2c). These findings imply that higher mRNA levels in hypocotyls do not necessarily lead to increased translation and support our hypothesis regarding the presence of post-transcriptional controls within this tissue.

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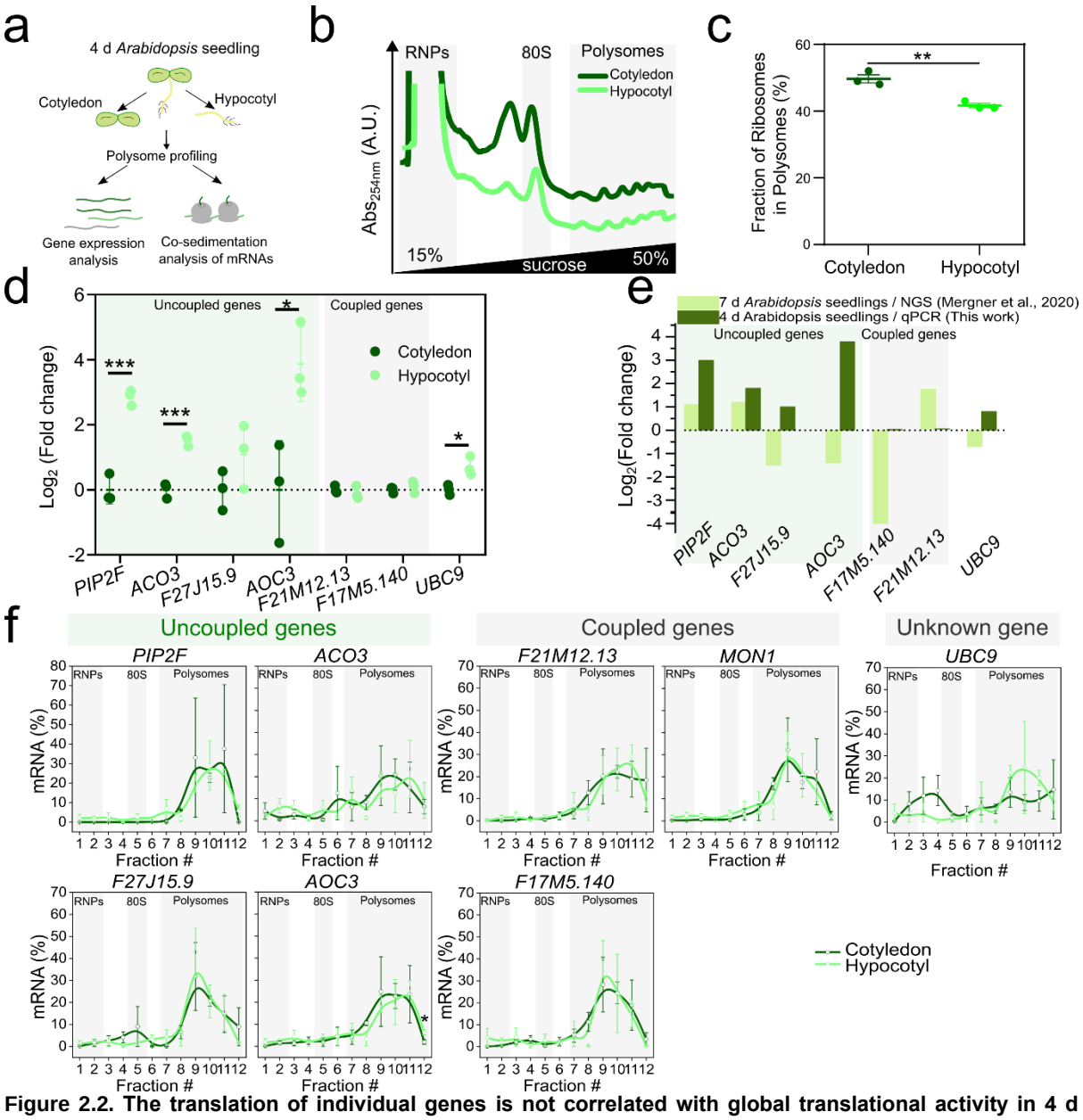

Figure 2.2. The translation of individual genes is not correlated with global translational activity in 4 d *Arabidopsis* cotyledon and hypocotyl. (**a**) Experimental workflow of polysome profiling on 4 d *Arabidopsis* cotyledon and hypocotyl. (**b**) Representative polysome profiles of cotyledon and hypocotyl were collected from 4 d *Arabidopsis* seedlings grown on ½ MS solid medium. RNPs, 80S, and polysomes are highlighted. (**c**) Fraction of ribosomes in polysomes obtained from polysome profiling of 4 d *Arabidopsis* cotyledon and hypocotyl. (**d**) Relative transcript abundance in cotyledon and hypocotyl, which the transcript abundance in cotyledon was used as a control. Gene *AtMON1* was used as the reference gene. (**e**) The comparison of transcript fold changes in hypocotyl

compared to cotyledon in Mergner et al., 2020 and this work. (**f**) Relative co-sedimentation profiles of selected genes along the sucrose gradient fractions of the cotyledon (green lines) and hypocotyl (light green lines). A spline curve was used to connect the data points between fractions. Data in (**c**), (**d**), and (**f**) are mean ± SEM from three independent experiments. The two-sided and unpaired Student's t-test was used to test the significant difference. Asterisks denote the significant difference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abs_{254nm}: Absorbance at 254 nm UV. RNPs: ribonucleic particles. 80S: ribosome.

2.4.3 Relative lower global translational activity in 10 d *Arabidopsis* seedlings compared to 6 d *Arabidopsis* seedlings

Next, we turned our attention to our second case study (Figure 2.3a) and conducted polysome profiling analysis on seedlings at various growth stages: 4, 6, 7, 10, 13, and 16 days (Figure 2.3b). From these profiles, we calculated the FRP value as an estimate of translation efficiency as translation efficiency is largely determined by polysomal fractions (Figure 2.3c) and found that global translational activity remained relatively stable across the investigated stages, with a notable decrease observed in the 10 d and 13 d seedlings compared to the 6 d seedlings. These results suggest the presence of stage-specific translational or post-transcriptional regulations. As a result, we focused on examining the transcriptional and translational levels of several previously mentioned transcripts in 6 d and 10 d seedlings, as these stages exhibited the most variability. At the transcription level, all selected mRNAs showed no significant differences in transcript levels between the 6 d and 10 d stages, except for *AtACO3* (Figure 2.3d). Analyzing the distribution of transcripts across the polysome profile fractions revealed that while *AtMON1* showed no change in co-sedimentation, *AtF21M12.13*, *AtF17M5.140*, and *AtPIP2F* shifted from lighter RNPs and 40S or 80S fractions to polysomal fractions by day 10 (Figure 2.3e), indicating a positive regulation of translation. In contrast, *AtUBC9* exhibited an increase in its relative abundance in the RNPs and polysome-free fraction, suggesting translational inhibition during the transition from day 6 to day 10. A similar translational inhibition can be inferred for *AtACO3*, which showed decreased engagement with polysomal fractions during this period.

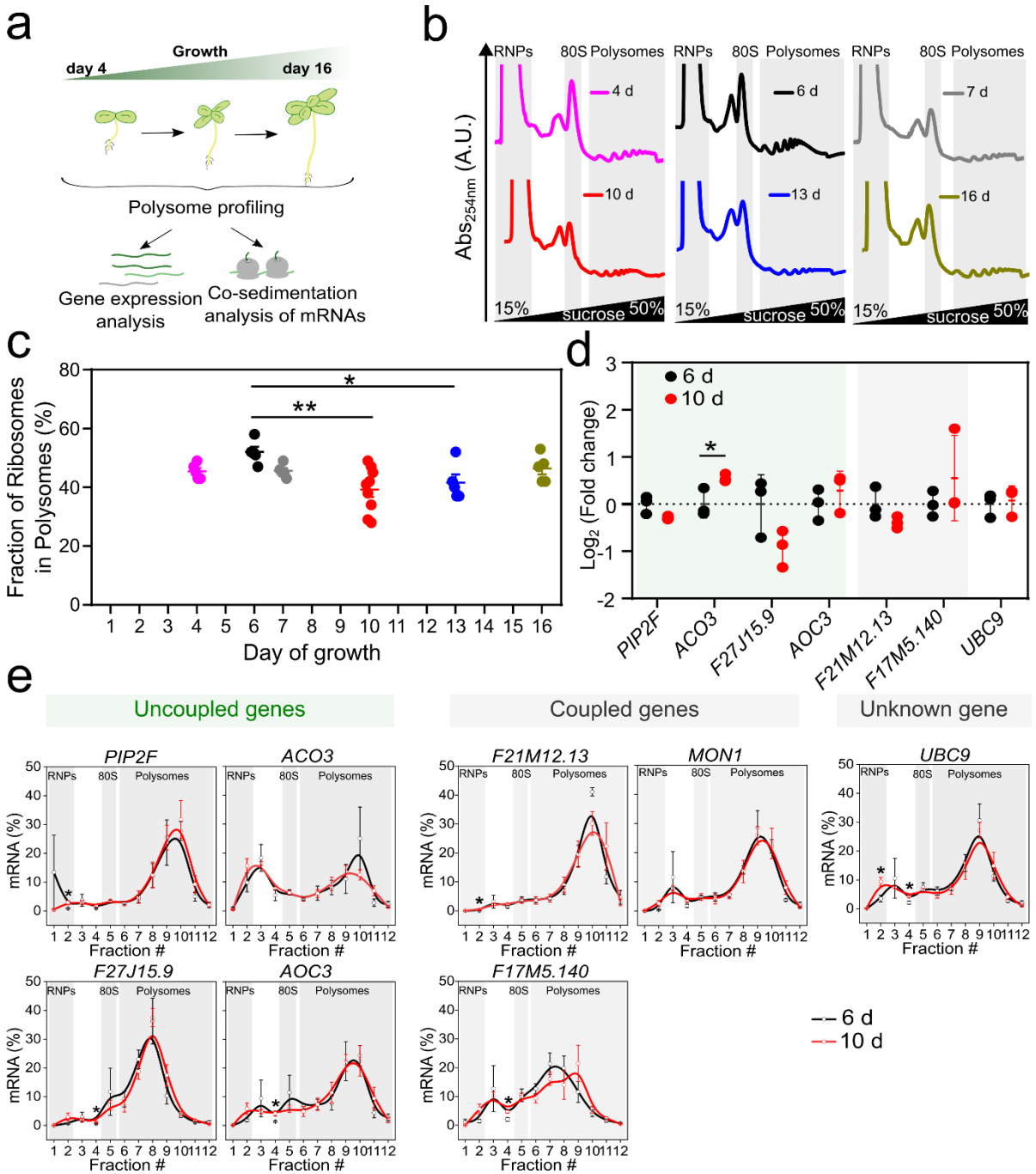

Figure 2.3. The discrepancy between global translational activity and translation of individual genes was also observed in 6 d and 10 d *Arabidopsis* seedlings. (**a**) Experimental workflow of polysome profiling on varying development stages of *Arabidopsis* seedlings. (**b**) Representative profiles of investigated stages of *Arabidopsis* seedlings. (**c**) Fraction of ribosomes in polysomes obtained from polysome profiling of investigated stages of

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Arabidopsis seedlings. The data represent the mean ± SEM (n = 5 - 9 independent experiments). (**d**) Total transcript abundance in the cytoplasm compared to 6 d using *AtMON1* as the reference gene. (**e**) Relative co-sedimentation profiles of selected genes along the sucrose gradient fractions of the 6 d *Arabidopsis* seedlings (black lines) and 10 d *Arabidopsis* seedlings (red lines). The data are mean ± SEM from four independent experiments. A spline curve was used to connect the data points between fractions. The two-sided, unpaired Student's t-test was used to test the significant differences in (**c**), (**d**), and (**e**). Asterisks denote the significant difference: * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. Abs_{254nm}: Absorbance at 254 nm. RNPs: ribonucleic particles. 80S: ribosome.

2.4.4 The interplay of miR168 and *AtAGO1* and *At*AGO1 homeostasis are crucial for plant development

To investigate specific post-transcriptional control of gene expression, we focused on miR168 and its target, *AtAGO1* mRNA. Maintaining proper levels of *At*AGO1 protein is essential for ensuring appropriate plant architecture (Bohmert et al., 1998). In *Arabidopsis*, the translation efficiency of *AtAGO1* mRNA is regulated by the miR168-induced RNA silencing complex, which includes *At*AGO1 itself (Vaucheret et al., 2004, 2006; Dalmadi et al., 2021). Notably, we observed a decrease in global translational activity in 10 d seedlings compared to those at 6 days. Previous studies have indicated that miR168 is found in polysomal fractions and likely interacts with *At*AGO1 protein, thereby repressing *AtAGO1* mRNA translation (Lanet et al., 2009). We assessed transcriptional changes of both *AtAGO1* and miR168 in 6 d and 10 d seedlings and examined the relative distribution of miR168 and *AtAGO1* mRNA across the sucrose gradient. Our primary objective was to evaluate changes in total *At*AGO1 protein levels under potential post-transcriptional regulations (Figure 2.4). The transcriptional analysis revealed no significant changes in either miR168 or *AtAGO1* mRNA levels (Figure 2.4a). The co-sedimentation profile indicated that miR168 predominantly associates with polysomal fractions in 6 d seedlings, consistent with findings from Lanet et al. (2009). However, in 10 d *Arabidopsis* seedlings, the profile of miR168 shifted towards lighter fractions, peaking within the RNP fractions (Figure 2.4b). Importantly, this change in miR168 distribution did not correspond to a similar alteration in the association of *AtAGO1* mRNA with polysomes (Figure 2.4c). These results suggest that miR168 has

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been released from the complex associated with polysomal RNA by day 10. The association or release of miR168 from its mRNA targets did not align with changes in transcription levels for either miRNA or its target mRNA; however, this process may influence target protein levels.

To test this hypothesis, we measured the dynamics of *At*AGO1 protein levels from day 6 to day 14 (Figure 2.4d). We selected this timeframe because any observable changes on polysomes may take several days to manifest at the protein level due to the multiple steps involved from translation to functional protein (Munro et al., 2023) and the time required for protein degradation (Hausser et al., 2013; Ando et al., 2017). Supporting our hypothesis, after a slight decrease in *At*AGO1 protein levels at day 10, we observed a two-fold increase by days 12 and 14 (Figure 2.4e). These findings indicate a previously unidentified post-transcriptional control mechanism affecting gene expression dynamics during *Arabidopsis thaliana* development. Key events appear to occur on polysomes involving the release of miR168-mediated inhibition of *AtAGO1* mRNA translation, leading to an increase in total *At*AGO1 protein levels without changes in *AtAGO1* mRNA levels or its association with polysomes (Figure 2.4f).

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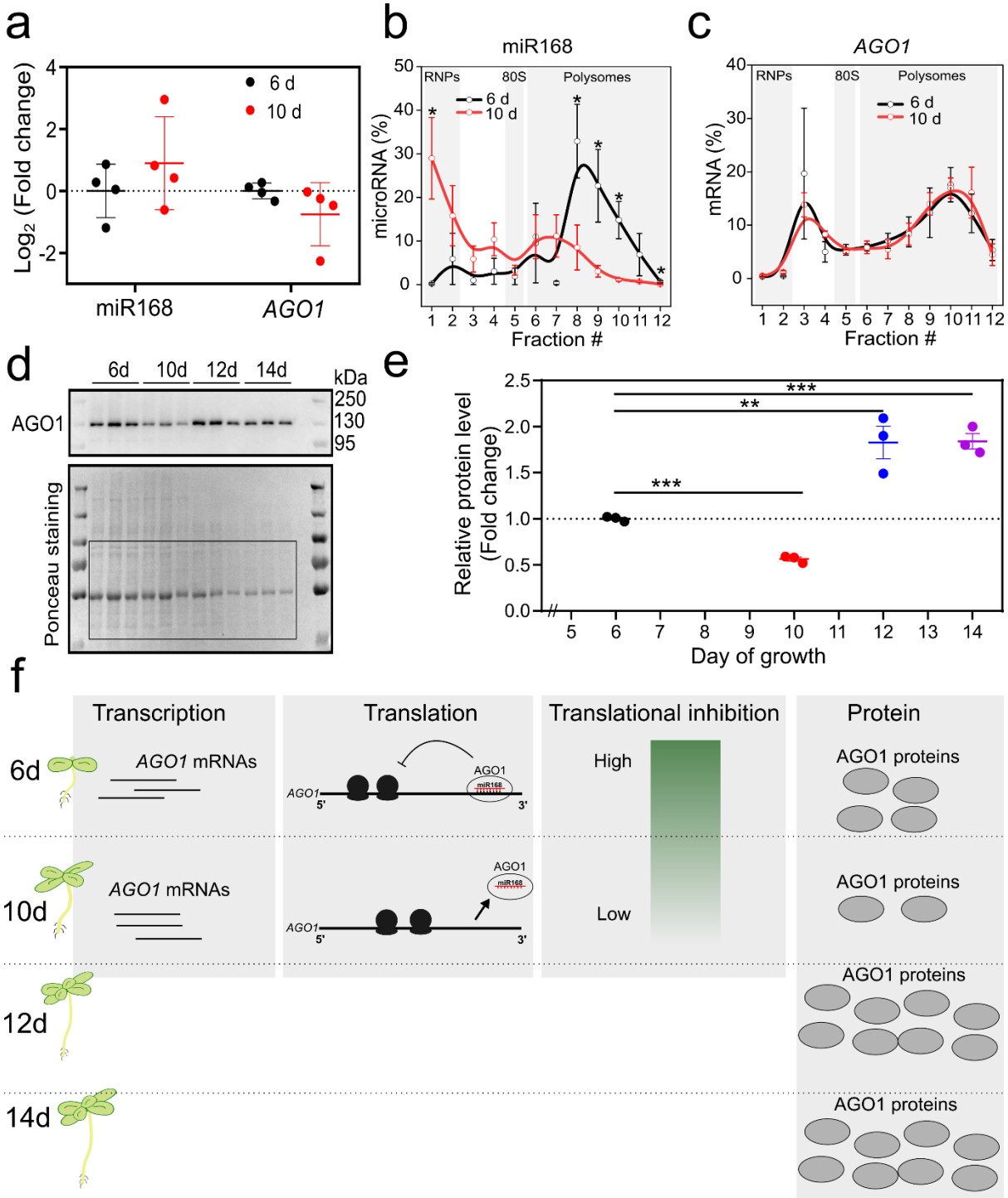

Figure 2.4. Polysome profiling was used to investigate the post-transcriptional regulations caused by miR168. (**a**) Relative transcript abundance of miR168 and its target *AtAGO1* compared to 6 d *Arabidopsis* seedlings using *AtMON1* as the reference gene. (**b**), (**c**) Relative co-sedimentation profile of miR168 (**b**) and AGO1 (**c**) along

the sucrose gradient fractions of 6 d (black line) and 10 d (red line) *Arabidopsis* seedlings. (**d**) Approximately 100 *Arabidopsis* seedlings at 6, 10, 12, and 14 d were used to detect AGO1 protein level using western blot. Three replicates for each stage. A black rectangular frame highlighted the total protein level used for normalization. (**e**) Relative AGO1 protein level at varying stages normalized to protein level at 6 d. (**f**) A schematic diagram shows miR168 and *AtAGO1* autoregulatory mechanisms. The numbers of transcripts and proteins represent the quantitative characters. A spline curve was used to connect the data points between fractions in (**b**) and (**c**). The graphs in (**a**), (**b**), and (**c**) show the mean ± SEM from four independent experiments, and (**e**) show the mean ± SEM from three independent experiments. The two-sided and unpaired Student's t-test was used to do statistical analyses in (**a**), (**b**), and (**e**). Asterisks denote the significant difference: * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. RNPs: ribonucleic particles. 80S: ribosome.

2.5 Discussion

This study highlights that focusing solely on transcription or post-transcription cannot fully unravel the complexities of gene expression regulation during plant development (Vogel and Marcotte, 2012). Recent research has increasingly spotlighted the regulation of translational activity in plants, driven by mounting evidence of a weak correlation between mRNA and protein levels (Bailey-Serres et al., 2009; von Arnim et al., 2014). We initiated our investigation by examining the relationship between transcriptome and proteome data from the cotyledons and hypocotyls of *Arabidopsis* seedlings. While a majority of mRNAs and proteins exhibit a strong correlation, notable exceptions exist. This variance can be attributed to biological factors such as translation rates, protein half-life, and transport mechanisms, as well as technical aspects like sequencing depth and mass spectrometry sensitivity (Liu et al., 2016).

To explore the low correlation observed in specific genes, we utilized polysome profiling to investigate potential post-transcriptional regulatory mechanisms. Our findings revealed that both correlated and uncorrelated genes did not display significant differences in transcript distribution along sucrose gradients in cotyledons and hypocotyls (Figure 2.2f, Supplemental Figure S2.2c). When comparing the association of various transcripts with polysomes in 6- and 10 d *Arabidopsis* seedlings,

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minor variations in transcript-polysome associations were observed (Figure 2.3e, Supplemental Figure S2.3). However, these transcripts did not correlate with global translation in either cases (Figures 2, 3), suggesting that regulatory controls extend beyond transcription and involve the reprogramming of mRNA engagement with polysomes (Traubenik et al., 2020; Billey et al., 2021).

Consequently, we shifted our focus to microRNAs (miRNAs) and their targets to uncover new potential post-transcriptional controls during seedling development (Li et al., 2017). We selected miR168 and its target *AtAGO1* mRNA as a pertinent case study due to their critical interaction for maintaining proper plant architecture (Rhoades et al., 2002; Vaucheret et al., 2004; Dalmadi et al., 2019). The regulation of AGO1 protein is intricately controlled through several mechanisms: (i) miR168-directed AGO1-RISC modulates *AtAGO1* mRNA expression (Vaucheret et al., 2004), (ii) AGO1 cleavage generates secondary siRNAs that further inhibit AGO1 protein levels (Mallory and Vaucheret, 2009), (iii) co-transcriptional regulation of *AtAGO1* and *MIR168* genes promotes miR168 stabilization by AGO1 protein (Vaucheret et al., 2006), and (iv) two *MIR168* genes produce distinct transcripts where *MIR168a* produces 21-nt miR168 and *MIR168b* produces 21-nt and 22-nt miR168, and only the 21-nt miR168 is preferentially stabilized by AGO1, adding another layer of regulation (Vaucheret, 2009). Note that 21-nt miR168 was used in this study. This multifaceted regulation ensures the effective operation of the RNA silencing pathway. An excess of miR168 acts as a molecular buffer, adjusting AGO1 protein levels in response to fluctuations in the miRNA pool (Dalmadi et al., 2021).

Our analysis revealed that the total abundance of miR168 and *AtAGO1* mRNA was comparable in both 6 d and 10 d *Arabidopsis* seedlings (Figure 2.4a). However, there was a significant difference in miR168 distribution across sucrose gradients (Figure 2.4b). In 6 d seedlings, predominant miR168 was found within polysomal fractions, indicating possible translational repression of *AtAGO1* mRNA. By day 10, miR168 dissociated from polysomes, likely alleviating the translational repression on its targets. Consistent with these observations, we noted that while *At*AGO1 protein levels slightly decreased at day 10, they surged significantly by days 12 and 14. This may reflect a delay in *At*AGO1 protein reaching a new steady state (Buccitelli and Selbach, 2020). The asynchronous relationship between miR168's

association with polysomes and *At*AGO1 protein levels suggests additional factors influencing *At*AGO1 protein levels during the transition from days 6 to 10. For instance, the loss of *ZLL*, which encodes ZWILLE/PINHEAD/AGO10 protein, along with FBW2 protein, a protein that is involved in ubiquitin-mediated protein degradation, led to increased AGO1 levels without affecting its mRNA levels (Mallory et al., 2009; Earley et al., 2010). Moreover, Furthermore, AGO10, AGO1's closest paralog, interacts with miR168 to antagonize AGO1 accumulation, contributing to its homeostasis (Iki et al., 2018). Additionally, certain virus-encoded proteins directly affect AGO1 levels by influencing miRNA or siRNA pathways (Zhang et al., 2006; Várallyay et al., 2010). Altogether, achieving the homeostasis of AGO1 is a complex process not only dependent on fine-tuning from miR168 but also on other proteins including endogenous and exogenous ones.

In conclusion, maintaining *At*AGO1 homeostasis is a complex process influenced not only by the fine-tuning effects of miR168 but also by various endogenous and exogenous proteins. Our findings suggest that we likely identified a previously unknown post-transcriptional control mechanism affecting *AtAGO1* mRNA levels occurring on polysomes independently of transcriptional regulation.

2.6 Conclusions

This study demonstrated that the hypocotyl exhibits lower global translational activity compared to the cotyledon in 4 d *Arabidopsis* seedlings. Notably, the levels of transcripts for certain uncoupled genes—specifically *AtACO3*, *AtPIP2F*, *AtAOC3*, and *AtF27J15.9*—were higher in the hypocotyl, suggesting potential post-transcriptional regulation in this region. However, polysome profiling and RT-qPCR analyses revealed no significant differences in the translational activity of these genes. Additionally, the same methodologies were applied to assess translation across different developmental stages. The findings indicated that 10 d *Arabidopsis* seedlings exhibited significantly lower global translational activity compared to their 6 d counterparts. However, no significant difference in translational activity of individual genes was observed between the 6 d and 10 d seedlings. Further investigations, including assessments of protein degradation rates, are necessary to clarify the mechanisms underlying this translational repression. Collectively, these results imply that additional

regulatory processes, such as mRNA degradation or protein degradation, may also influence gene expression. Among these regulatory factors, miRNAs stand out due to their ability to degrade target mRNA or inhibit its translation. Previous research has identified an autoregulatory mechanism between miR168 and its target *AtAGO1*, which is vital for plant development; disruptions in this mechanism can result in developmental abnormalities (Vaucheret et al., 2006, 2004). This study found that while total transcript levels of miR168 and *AtAGO1* were comparable in 6 d and 10 d seedlings, miR168 was primarily located in polysomal fractions in 6 d *Arabidopsis* seedlings but transitioned to non-polysomal fractions in 10 d *Arabidopsis* seedlings (Figure 2.4a, b). Additionally, *AtAGO1*'s transcript distribution also shifted from polysomal to non-polysomal fractions at both developmental stages (Figure 2.4c). These observations suggest that miR168 may suppress the translation of *AtAGO1* in 6 d seedlings. However, unexpectedly, *At*AGO1 protein levels were significantly lower in 10 d seedlings compared to those in 6 d seedlings. This discrepancy may indicate a more complex regulatory relationship between miR168 and *AtAGO1* rather than a simple competitive dynamic. Notably, *At*AGO1 protein levels increased in later stages (12 d and 14 d), potentially due to the alleviation of miR168 repression by day 10. Further studies are warranted to explore the discordance between the mRNA levels of miR168 and its target mRNA and protein levels.

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2.8 Supplemental materials

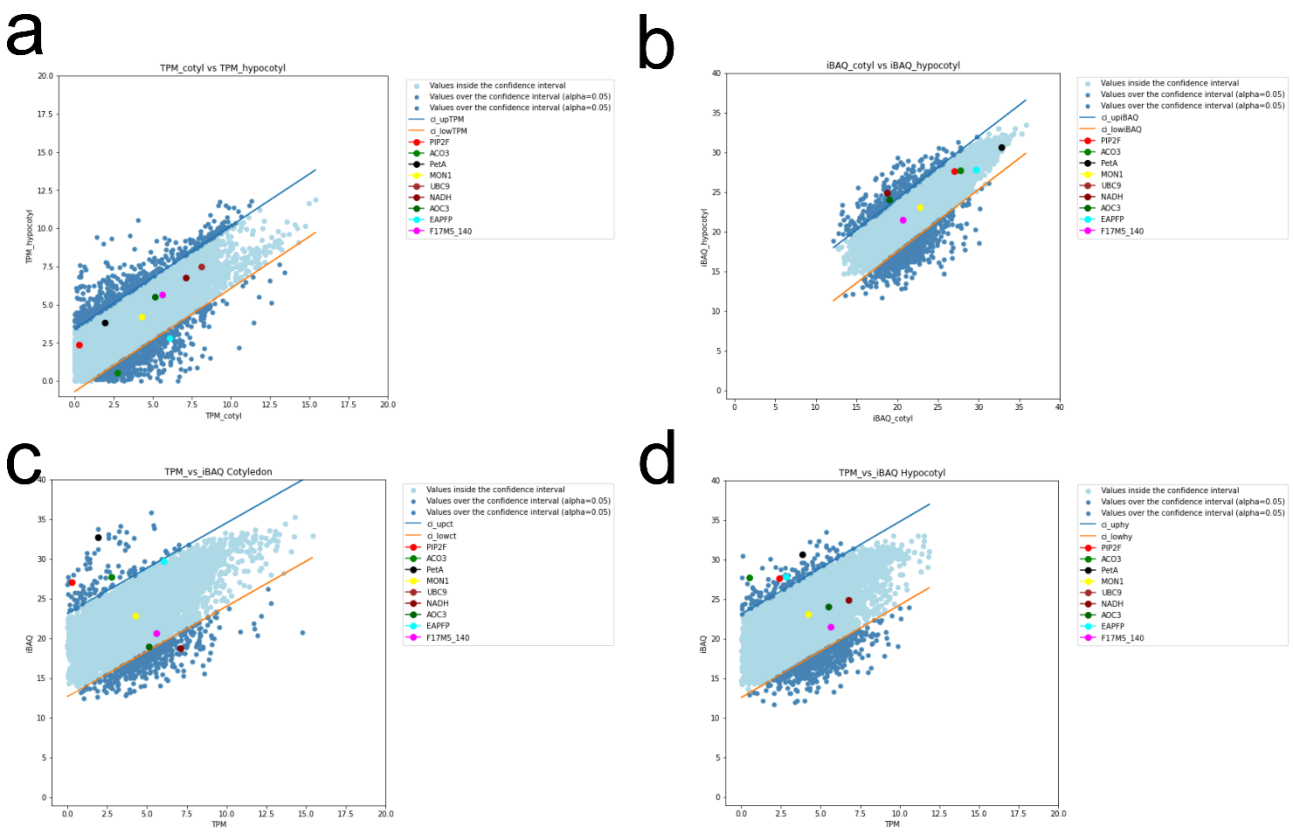
2.8.1 Supplemental tables

Supplemental table S2.1. Primers used in this study.

Gene Name	Accession	Strand	Sequence (5'->3')
AtPIP2F	AT5G60660	Forward primer	ATGACGTACTTGGTCGTCCC
		Reverse primer	GATCCCAAGCGAAATGCTCG
AtF21M12.13	AT1G09750	Forward primer	ACCACCGCAAGGACTAATGG
		Reverse primer	TAAAGTGATGGACGGCGAGG
AtACO3	AT1G12010	Forward primer	TCTCCGGCAACATCTCTTGTGG
		Reverse primer	GAATGTCTCAACCACAGCCACC
AtPetA	ATCG00540	Forward primer	TATTCTTGCTCCAGACCCTGC
		Reverse primer	ATTATCCCTCCTGCCGTAGC
AtMON1	AT2G28390	Forward primer	TGGCGACTTCAGATTCGAGG
		Reverse primer	CACCGCTAAATTCGGTGTGG
AtUBC9	AT4G27960	Forward primer	TCACAATTTCCAAGGTGCTGC
		Forward primer	AGGACAGTATTTGTGTCAGCCC
AtF17M5.140	AT4G33380	Forward primer	CAAGCATGAGGTTTCCACTGC
		Reverse primer	AGCTCTTTAGCTTCCTCCAGC
AtF27J15.9	AT1G49140	Forward primer	GTGTTACCGCGTCGAAGGC
		Reverse primer	GCTACTGGCTTAGGACCGTG
AtAOC3	AT3G25780	Forward primer	ACCGAAAACTCCAGACCAAGTAAG
		Reverse primer	AAGCTCTGTTGGTTTCTTGCCG
AtAGO1	AT1G48410	Forward primer	CCACCGCAGAGACAATCAGT
		Reverse primer	ACCTGGGTAGGACTCACCTC
AtAP2	AT4G36920	Forward primer	GCCTCGACGAACCAAGTGTT
		Reverse primer	GCAGCCAATTTTGATGAGGAGT

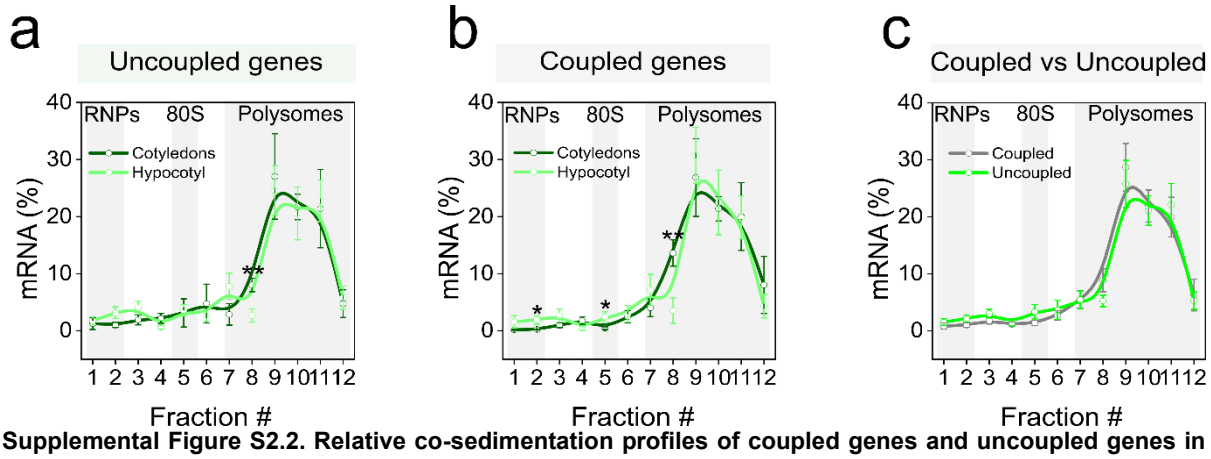
		Stem-loop RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTC GCACCAGAGCCAACTTCCCG
ath-miR168	MI0000210 (miRBase Accession)	Specific Forward primer	GTTTTCGCTTGGTGCAGGT
		Universal Reverse primer	GTGCAGGGTCCGAGGT

2.8.2 Supplemental figures



Supplemental Figure S2.1. Linear regression analysis of transcriptome and proteome data of cotyledon and hypocotyl (Mergner et al., 2020). (**a**) The comparison of the transcriptome in cotyledons and hypocotyl. Two arbitrary lines (blue and orange) were used to indicate confidence interval (CI) (α=0.05). Genes that were distributed outside of CI were colored in dark blue and genes that were distributed inside of CI were colored in light blue. Genes that will be investigated furthermore were colored in different colors: *AtPIP2F* (red), *AtACO3* (light green), *AtPetA* (black), *AtMON1*(yellow), *AtUBC9* (dark red), *AtF27J15.9* (dark red), *AtAOC3* (dark green), *AtF21M12.13* (turquoise), *AtF17M5.140* (rhodamine red). (**b**) The comparison of the proteome in cotyledons and

hypocotyl. Two arbitrary lines (blue and orange) were used to indicate confidence interval (CI) (α=0.05). The colorization was the same as (**a**). Proteins that were distributed outside of CI were highlighted in dark blue while ones that were inside of CI were highlighted in light blue. Proteins that will be investigated furthermore were highlighted in different colors: *AtPIP2F* (red), *AtACO3* (light green), *AtPetA* (black), *AtMON1*(yellow), *AtUBC9* (dark red), NADH (*AtF27J15.9*) (dark red), *AtAOC3* (dark green), EAPFP (*AtF21M12.13*) (turquoise), *AtF17M5.140* (rhodamine red). (**c**) The comparison of transcriptome and proteome in cotyledons. The colorization was the same as (**a**) and (**b**). (**d**) The comparison of transcriptome and proteome in hypocotyl. The colorization was the same as (**a**) and (**b**).



Supplemental Figure S2.2. Relative co-sedimentation profiles of coupled genes and uncoupled genes in cotyledon and hypocotyl of 4 d *Arabidopsis* seedlings. (**a**) Relative co-sedimentation profile of uncoupled genes along the sucrose gradient fractions of polysome profiling on cotyledon (green lines) and hypocotyl (light green lines). (**b**) Relative co-sedimentation profile of coupled genes along the sucrose gradient fractions of polysome profiling on cotyledon (green lines) and hypocotyl (light green lines). (**c**) Relative co-sedimentation profile of uncoupled genes (light green lines) and coupled genes (gray lines) along the sucrose gradient fractions. All the data are mean ± SEM of the transcript percentage in the fraction. The two-sided and unpaired Student's t-test was used to test the significant difference. The asterisks were used to denote the statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. RNPs: ribonucleic particles. 80S: ribosome.

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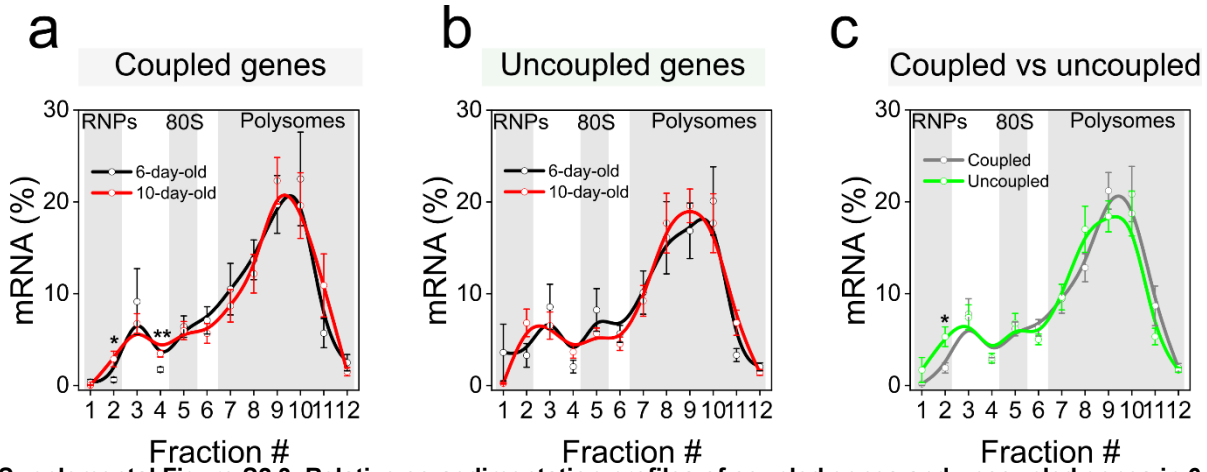

Supplemental Figure S2.3. Relative co-sedimentation profiles of coupled genes and uncoupled genes in 6 d and 10 d *Arabidopsis* seedlings. (**a**) Relative co-sedimentation profile of coupled genes along the sucrose gradient fractions of the 6 d *Arabidopsis* seedlings (black lines) and 10 d *Arabidopsis* seedlings (red lines). (**b**) Relative co-sedimentation profile of uncoupled genes along the sucrose gradient fractions. (**c**) Relative co-sedimentation profile of uncoupled genes (light green lines) and coupled genes (gray lines) along the sucrose gradient fractions. All the data are mean ± SEM among the transcript percentage of uncoupled genes and coupled genes. The two-sided and unpaired Student's t-test was used to test the statistical significance. The asterisks were used to denote the significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. RNPs: ribonucleic particles. 80S: ribosome.

3 Translational Regulation of Aquaporin Genes in Grapevine During Drought Stress and Recovery

3.1 Abstract

Drought stress is a major challenge for crop productivity worldwide, especially in perennial species like grapevine (*Vitis vinifera* L.), where efficient water management is vital for productivity and berry quality. As climate change exacerbates water scarcity and alters precipitation patterns, understanding plant responses to drought becomes increasingly important for developing sustainable agricultural practices. Aquaporins, a family of water channel proteins, play a central role in regulating water transport within plant cells, influencing water uptake and redistribution during drought. While their

transcriptional responses to drought are documented, the translational regulation of aquaporin genes, particularly in grapevines, remains underexplored. In this study, we investigated the transcriptional modulation of six aquaporin genes, three PIPs and three TIPs in leaves (Pinot noir) and roots (Kober 5BB) during various days of drought stress and subsequent rewatering. In parallel, we also investigated the translation of six aquaporin genes under well-watered, drought-stressed, and rewatered conditions in grapevine by polysome profiling. By combining physiological measurements, transcript abundance analysis, and polysome profiling, we show that drought stress suppresses the global translation, while rewatering reactivates it. Focusing on aquaporins, *VviPIP2-5* and *VviTIP2-1* exhibit significant regulation at the transcriptional level both in leaves and roots, and at the translational levels in leaves. These findings provide new insights into how grapevines manage water transport at the molecular level during and after drought stress and present potential targets for enhancing drought tolerance in grapevine breeding programs. Importantly, this study offers the first evidence of translational control in the regulation of aquaporin gene expression in grapevine.

3.2 Introduction

In plants, roots absorb water and minerals from the soil, creating a gradient in water potential, which drives water uptake (Scharwies and Dinneny, 2019; Yu et al., 2024). Water moves through the root's cortex via three main pathways: the apoplastic (through cell walls), symplastic (through plasmodesmata), and transcellular (across cell membranes) routes (Steudle and Peterson, 1998; Steudle, 2000; Oparka and Roberts, 2001). Upon reaching the endodermis, the Casparian strip acts as a selective barrier, ensuring that water must pass through the living cells before entering the vascular system (Venturas et al., 2017; Dinneny, 2019). Inside the xylem, water moves upward through cohesion and adhesion forces, allowing it to reach the leaves despite gravity (Steudle, 2001). The water eventually exits the plant through stomata, tiny pores regulated by guard cells, where it evaporates into the atmosphere, driven by differences in water vapor concentration (Lin et al., 2015). Plants are continually challenged by environmental factors, with drought stress being one of the most significant threats to growth and productivity (Gupta et al., 2020). To cope with water scarcity, plants optimize their

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water use by adjusting physiological parameters such as transpiration, stomatal conductance, and water potential (Farooq et al., 2009; Zhang et al., 2020; Nagahatenna et al., 2022).

Aquaporins are channel-forming transmembrane proteins found in both plasma and intracellular membranes across all eukaryotes and most prokaryotes and are vital for regulating water movement in vascular plants (Chaumont et al., 2001; Hachez et al., 2006; Gomes et al., 2009; Heinen et al., 2009). These specialized water channel proteins control water influx and efflux in plant cells (Takata et al., 2004; Chaumont and Tyerman, 2014; Moshelion et al., 2015; Afzal et al., 2016; Singh et al., 2020). In plants, aquaporins govern both water uptake in roots and water loss through stomata in leaves (Hachez et al., 2012; Perrone et al., 2012a; Sade et al., 2014; Vandeleur et al., 2014; Grondin et al., 2015). In *Vitis vinifera* L., researchers have identified 33 aquaporin genes categorized into five 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; Sabir et al., 2021). Among these, PIPs and TIPs are essential for maintaining cellular water homeostasis, with PIPs localized to the plasma membrane and TIPs to the vacuolar membrane, a crucial water-storage organelle in plant cells (Maurel et al., 2015; Sun et al., 2024).

Climate change has exacerbated drought conditions globally, particularly in regions like the Mediterranean (Seager et al., 2014), by decreasing precipitation and increasing evaporative losses, which amplify soil water deficits (Mukherjee et al., 2018). Human activities have further worsened the problem (Kelley et al., 2015), complicating drought monitoring and forecasting (Yuan et al., 2023). Understanding how grapevines adapt to such variable water conditions—especially by examining aquaporin gene expression (Galmés et al., 2007; Perrone et al., 2012b; Pou et al., 2013; Zarrouk et al., 2016)—provides valuable insights for viticulture. Recognizing grapevine drought tolerance limitations is critical for effective irrigation management (Chaves et al., 2010; Sack and Scoffoni, 2012).

However, inconsistencies between aquaporin transcript and protein levels have been noted in various studies (Fox et al., 2017; Yepes-Molina et al., 2020; Byrt et al., 2023), as seen in species like broccoli (*Brassica oleracea*) (Muries et al., 2011) and maize (*Zea mays* L.) (Marulanda et al., 2010),

suggesting that translational regulation may significantly influence aquaporin function. While aquaporin gene expression has been extensively studied at the transcriptional level, significant gaps remain in understanding their post-transcriptional and translational regulation during water stress and recovery.

This study aims to explore the transcriptional and translational dynamics of three PIPs and three TIPs in grapevines under well-watered, drought-stressed, and rewatered conditions. Grapevines were subjected to drought stress for 10, 15, and 20 days, followed by rewatering, to simulate the variable drought stress periods characteristic of climate change. Alongside molecular analyses, we also assessed key physiological responses—such as transpiration rates, stomatal conductance, water potential, and shoot growth—to better understand how aquaporins mediate water transport during drought and recovery. Our findings reveal distinct regulatory patterns across tissues and conditions, providing new insights into the complex role of aquaporins in maintaining water homeostasis during stress. Notably, this research also sheds light on potential translational regulation mechanisms involved. Grafting onto drought-tolerant rootstocks is a widely used method to improve grapevine (*Vitis vinifera* L.) drought tolerance in viticulture (Tramontini et al., 2013; Zhang et al., 2016; Bonarota et al., 2024). Identifying which aquaporin genes contribute to this tolerance remains an open question, but *VviTIP2-2* emerges as a potential candidate. It is expressed in the roots of the Kober 5BB rootstock (*Vitis berlandieri* × *Vitis riparia*), but not in the leaves of *Pinot noir* (*Vitis vinifera* L.), highlighting its possible role in rootstock-mediated drought tolerance.

3.3 Materials and Methods

3.3.1 Plant materials, growth conditions, experimental design

Drought stress and rewatering experiments on grapevines were conducted twice, in August 2023 and March 2024, in the greenhouse at Fondazione Edmund Mach (FEM), located in San Michele all'Adige, Trento, Italy (46°11′26.8″ N 11°08′08.1″ E).

In the August 2023 drought stress experiment, a total of 60 grafted vines of Pinot noir (*Vitis vinifera* L.) grafted on Kober 5BB (*Vitis berlandieri* x *Vitis riparia*) rootstocks were planted in 3-liter pots.

The roots were immersed in water to maintain moisture and were trimmed right before being potted. On 23rd June 2023 plants were potted using loam-based potting compost (Ter-compost, Italy) with a thin layer of pumice at the bottom. Then, during the growing, all the plants were fertilized at least once. All the plants were regularly watered twice a week, and lateral shoots and fruits were removed to maintain one vertical shoot on rootstocks via stickers. To reach homogeneous growing states, the top shoot was removed and around 12 leaves were left on the plant. Before the experiment commenced, plants were either withheld from water or watered until the total weight (including plant, soil, pot, and sticker) reached approximately 1550 g. On 9 August 2023 (the day preceding the commencement of the experiment), the pots were wrapped in transparent plastic bags to prevent the loss of water through evaporation and drainage from the soil. They were then randomly divided into two groups: a well-watered group and a drought-stressed group. The well-watered group, comprising 27 plants, was irrigated daily to compensate for the water lost due to transpiration over the preceding 24 hours. The drought-stressed group, comprising 33 plants, was further divided into the following subgroups: Ten plants were deprived of water for 10 days (RA10DS group), eight plants for 15 days (RA15DS group), and nine plants for 20 days (RA20DS group). Subsequently, the plants were irrigated daily following the designated drought stress period. The remaining six plants were subjected to drought stress for root sampling, with three plants deprived of water for four days and another three for 20 days. Following this, their roots were sampled, and the entire plants were removed from the experiment. Temperature and relative humidity in the greenhouse were continuously recorded every 30 minutes using two data loggers (Tinytag Ultra 2, Gemini Data Logger, UK), one placed on a plant on the east side and the other on a plant on the west side.

In the March 2024 drought stress experiment, 20 plants from the August 2023 drought stress experiment were reused. To induce new shoot growth, the old shoots with leaves were completely removed, leaving only one bud per plant. On 19th February 2024, the plants were transferred to 6-litre pots, supplemented with additional loam-based potting compost (Ter-compost, Italy), and fertilized twice before the experiment commenced. The plants managed to ensure homogeneous growth, each with

only one shoot growing on the rootstock. As in the previous experiment, the total weight of the system (including plant, soil, and pot) was adjusted to approximately 2700 g before the start of the experiment. On 14th March (which was set as day -1 of this experiment), pots were wrapped in transparent plastic bags to stop evaporation and drainage from the soil. Four of the 20 plants were allocated to the well-watered group, while the remaining 16 plants were assigned to the drought-stressed group. The well-watered group was watered daily to replace the water lost due to the transpiration over the last 24 hours, while the drought-stressed group was deprived of water for 19 days. Of the drought-stressed plants, 8 were rewatered after 19 days of drought stress (RA19DS group), while the remaining 8 were withheld from water until the experiment concluded. Temperature and relative humidity in the greenhouse were continuously recorded using a data logger (EL-USB-2-LCD+, EasyLog, UK).

3.3.2 Physiological parameters measurements

To monitor the whole plant transpiration (E), the total pot weight, including the plant, soil, pot, plastic bag, and sticker, was measured daily at 9:00 am 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*) as:

$$E_n = W_{n-1} - W_n$$

Where *E* represents transpiration (g), *n* is the day of the experiment, and *W* denotes the weight of the pot.

Leaf water potential (Ψ_L, n = 3) was measured every two days during the experiment between 10:00 am and 12:00 pm. For this analysis, two fully expanded and light-exposed leaves were detached from the plant, the petiole of each leaf was trimmed to create a flat surface and then the whole leaf was tightly sealed inside a metal pressure chamber (Pump-up Chamber, PMS Instrument Company, USA) with the cut surface of the petiole being outside of the chamber. Air pressure was gradually applied to the leaf by pumping air into the chamber. When a 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.

All measurements of stomatal conductance (g_s) were conducted in the morning between 10:00 am and 12:00 pm using an LI-600 porometer (LI-COR Bioscience, Lincoln, NE, USA). Measurements were performed on various days throughout the experiment under natural light conditions. To assess the stomatal conductance of the entire plant, readings were taken from leaves located at both the top and bottom positions of the plant. Stomatal conductance was calculated using the following equation:

$$g_s = \frac{1}{(\frac{1}{g_{tw}} - \frac{1}{g_{bw}})}$$

Where g_s is the stomatal conductance, g_{tw} is the total conductance to water vapor, and g_{bw} is the boundary layer conductance to water vapor. Both g_{tw} and g_{bw} were obtained directly from the LI-600 porometer measurements.

To monitor the growth status of plants under three conditions—well-watered, drought-stressed, and rewatered after drought stress—the length of newly grown shoots was measured starting from day 11 of the experiment (the top shoot of all the plants were removed at the beginning of the experiment) to the end of the experiment. The shoots emerging from the highest bud were tagged and measured using a tape measure from the base of the shoot till the end by carefully straightening the shoot as much as possible on the tape measure, 2-8 biological replicates were chosen at each time of measuring.

3.3.3 Samples collection

In the experiment conducted in August 2023, leaf samples were collected every two days from the biological triplicates of each experimental group. For the well-watered group, leaves were collected from day 0 to day 22 of the experiment. In the drought-stressed group, leaf samples were collected from day 2 to day 20. In the three rewatering groups, leaf samples were collected at specific intervals following rewatering. Specifically, in the RA10DS group, leaves were collected at 6, 48, 96, 144, and 288 hours post-rewatering. In the RA15DS group, leaves were collected at 24, 72, 120, and 168 hours post-rewatering. In the RA20DS group, leaves were collected at 48 and 120 hours post-rewatering. After detachment, leaf water potential was measured, and the leaves were then wrapped in aluminum foil and rapidly frozen in liquid N_2. Root samples from biological triplicates of the well-watered group

were collected on days 0, 4, 10, and 20 of the experiment. In the drought-stressed group, root samples were collected on days 4, 10, 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 from four replicates in the well-watered group were collected on day 19 of the experiment. In the drought-stressed group, leaf samples were collected on day 19 and day 19 plus 6 hours respectively. For the RA19DS group, leaves from four replicates were collected at 6- and 24-hour post-rewatering (referred to as 6hrs_RA19DS and 24hrs_RA19DS, respectively). The leaves were promptly wrapped in aluminum foil, frozen in liquid N_2, and stored at -80 °C for downstream analyses.

3.3.4 Total RNA extraction, reverse transcription, primer efficiency determination, quantitative PCR

Total RNA was extracted from selected samples to analyze aquaporin gene expression based on transpiration data. Specifically, leaf samples from the well-watered group were collected on days 0, 2, 10, 14, and 20 of the experiment. Root samples from the well-watered group were collected on days 0, 4, and 20. For the drought-stressed group, leaf samples were collected on days 2, 10, 14, and 20, while root samples were collected on days 4 and 20. In the RA10DS group, leaf samples were collected 6 and 48 hours post-rewatering. In the RA15DS group, samples were collected at 24 and 72 hours post-rewatering. For the RA20DS group, leaf samples were collected at 48 and 120 hours post-rewatering.

Approximately 50 mg of leaf material 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 quality was assessed using agarose gel electrophoresis to confirm the presence of 28S and 18S rRNA bands, with an ideal intensity ratio of 28S to 18S being 2:1. Additionally, Nanodrop was used to measure the 260/230 and 260/280 absorbance ratios, both of which should be approximately 2. Any potential genomic DNA contamination in the RNA samples was removed by DNase treatment using the TURBO DNA-*free* ™ Kit (Thermo Fisher). Briefly,

a 20 µL reaction mixture containing 1 µL of TURBO DNase™ Enzyme (2 U/µL), 2 µL of 10X TURBO DNase™ Buffer, 1 µg of total RNA, and DEPC-treated water (RNase-free, was added accordingly) was prepared in a 1.5 mL Eppendorf tube. Genomic DNA was digested during a 30-minute incubation at 37 °C. Following this, 2 µL of DNase Inactivation Reagent was added at room temperature, with occasional mixing for 5 minutes. The DNase Inactivation Reagent was precipitated by centrifugation at 10640 g for 1.5 minutes, and 10 µL of supernatant (around 450 ng total RNA since another 12 uL liquid was discarded) was carefully transferred into a new 0.2 mL PCR tube.

The complementary DNA (cDNA) was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen) and Oligo(dT)$_{18}$ (Thermo Fisher, #k1622). In brief, 1 µL of 100 µM oligo(dT)$_{18}$ (Thermo Fisher, k1622) was added to the 10 µL total RNA (approximately 450 ng) in a 0.2 mL PCR tube. The RNA secondary structure was denatured by heating at 65 °C for 5 minutes, followed by rapid cooling on ice for at least 1 minute and brief centrifugation. Subsequently, a mixture containing 1 µL of 10 mM dNTPs, 4 µL of 5X First strand buffer (containing 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3), 1 µL of 0.1 M DTT, 0.8 µL of 200 U/µL Superscript III Reverse Transcriptase, 2.2 µL DEPC-treated water (RNase-free) was added into the reaction system. The cDNA was synthesized in a PCR thermocycler using the following program: 60 minutes at 50°C, followed by 15 minutes at 70°C. The synthesized cDNA was transferred to a 1.5 mL tube and stored at -20 °C.

The primers for aquaporin genes used in this study were derived from three aquaporin gene expression studies (Pou et al., 2013; Zarrouk et al., 2016; Shelden et al., 2017). The gene names were updated following (Wong et al., 2018). 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. New 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% (Table 1).

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Table 3.1. Primer sequences of selected genes for qPCR. Note that *VviActin* and *VviGAPDH* were used for gene expression normalization.

Gene symbol	Vitvi ID	Forward sequence (5'->3')	Reverse sequence (5'->3')	References
VviTIP2-2	Vitvi02g00568	GAACCCAGCTGT CACCTTTG	GTCGGTATGCTC TCCCCATT	(Zarrouk et al., 2016; Wong et al., 2018)
VviTIP2-1	Vitvi09g00329	CACAGGAGGCTT GACGACT	CCAGTGAGCCCT TCTTCGG	(Pou et al., 2013; Zarrouk et al., 2016; Shelden et al., 2017; Wong et al., 2018)
VviTIP1-3	Vitvi13g00255	CGGGCATGGCTT TTAACAAG	CAGATATGTTCG CACCCACC	(Pou et al., 2013; Zarrouk et al., 2016; Shelden et al., 2017; Wong et al., 2018)
VviPIP1-1	Vitvi13g00012	TGGTGCGGGTGT AGTGAAGG	AGACAGTGTAGA CAAGGACGAAGG	(Shelden et al., 2017; Wong et al., 2018)
VviPIP2-5	Vitvi13g00605	GCAGCCTTCTAC CACCAGTT	TTCTTCACCCCCA AAAACAG	(Shelden et al., 2017; Wong et al., 2018)
VviPIP2-7	Vitvi03g00155	GCAACCCCACCA ACTAAAAA	GGGGCTCTCATC ATCAACAT	(Shelden et al., 2017; Wong et al., 2018)
VviActin	Vitvi04g01613	TGGAATGGTGAA GGCTGGAT	GGGCTTCATCAC CAACATAGG	(Shelden et al., 2017; Wong et al., 2018)
VviGAPDH	Vitvi17g01598	GAAGCCAGTCAC CGTCTTTG	ACCTTCTTAGCAC CACCCTT	(Wong et al., 2018)

Primer efficiency was determined using a 10-fold serial dilution of stock cDNA. Five serial dilutions: stock cDNA, 1/10, 1/100, 1/1000, and 1/10,000 diluted cDNAs were prepared and subjected to quantitative PCR. In a 12.5 µL reaction mixture, 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), 3.25 µL of PCR grade water were combined. PCR thermocycling was performed using a CFX96 real-time PCR system with

the following program: 1 cycle at 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds and 62 °C for 25 seconds. Before the melting curve analysis, PCR products were heated to 95 °C for 15 seconds and then subjected to the melting curve analysis between 62 °C and 95 °C at 0.5 °C increments for 15 seconds. Each reaction was performed in duplicate. The average Cq values for each dilution were plotted against the logarithm of the cDNA concentration. The slope of the resulting linear regression curve was used to calculate primer efficiency using the following formula:

$$Efficiency = \left(10^{\frac{-1}{slope}} - 1\right) * 100$$

Where Efficiency is the primer efficiency expressed as a percentage.

All cDNA samples were diluted 20-fold prior to quantitative PCR. In a 12.5 µL reaction mixture, 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), 3.25 µL of PCR grade water were combined. PCR thermocycling was performed as previously described. Each reaction was performed in duplicate. In drought-stressed samples versus well-watered samples, gene expression levels were expressed as fold changes relative to well-watered samples. For drought-stressed samples versus rewatering samples, gene expression levels were compared to drought-stressed samples. *VviGAPDH* was used as the reference gene in both cases. Fold changes were calculated using the following equations:

The arithmetic means of the Cq values of duplicates of the gene of interest and *VviGAPDH* were calculated as follows: $\overline{Cq_{gene\ of\ interest}} = \frac{Cq_1 + Cq_2}{2}$, and $\overline{Cq_{VviGAPDH}} = \frac{Cq_1 + Cq_2}{2}$.

The difference between the arithmetic means of the gene of interest and the reference gene *VviGAPDH* was calculated as: $\Delta Cq = \overline{Cq_{gene\ of\ interest}} - \overline{Cq_{VviGAPDH}}$.

The mean ΔCq of the reference samples was calculated as: $\overline{\Delta Cq_{reference}} = \frac{\sum_1^n (Cq_{gene\ of\ interest} - Cq_{VviGAPDH})}{n}$, n represents the biological replicate.

Next, ΔΔCq was calculated: $\Delta\Delta Cq = \Delta Cq - \overline{\Delta Cq_{reference}}$.

The efficiencies of primers were within 90-110% and therefore were set at 100% to simplify calculations. Fold changes were then calculated using the following equation: $Fold\ change = 2^{-\Delta\Delta Cq}$.

3.3.5 Polysome profiling

Polysome profiling was performed as described protocol with modifications to suit grapevine tissues (Bernabò et al., 2017). Leaf samples from triplicates of the well-watered, drought-stressed, and 6-hour post-rewatering (6hrs_RA19DS) groups were collected during an experiment conducted in March 2024. The collected leaves were ground to a fine powder in liquid N_2. Approximately 200 mg of each powdered sample was lysed in 12,00 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). With this lysis buffer, the nuclei membranes were kept intact, and therefore, the cytoplasmic contents were extracted. The lysate was incubated on ice for 20 minutes and then subjected to four rounds (to precipitate the chloroplast as much as possible before ultracentrifugation, the rounds of centrifugation can be scaled up) of centrifugation at 4 °C, 20,854 g for 20 minutes 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 subjected to ultracentrifugation at 4 °C, 100,000 rpm for 67 minutes using TLA100.2 rotor (Beckman Coulter, USA) to precipitate mRNAs and ribosomes. 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) sucrose solution containing 40 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, pH 8.4) and subjected to ultracentrifugation at 4 °C, 40,000 rpm for 1 hour and 40 minutes using an SW41 swinging bucket rotor in a Beckman Optima LE-80K ultracentrifuge. Following ultracentrifugation, the gradient was forced to pass through an optical unit, where the UV absorbance at 254 nm was measured using an ISCO UA-6 UV detector. This was achieved by slowly pumping a 50% sucrose solution from the bottom of the gradient, thereby generating an absorbance profile. The gradients were collected in nine aliquots, each containing approximately 500 µL of liquid. The fractions were either used immediately for RNA extraction or stored at -80 °C for future use.

The Fraction of Ribosomes in Polysomes (FRP) was calculated from the absorbance profiles. This was expressed as a percentage by determining the ratio of the area under the curve (A) for polysomes to the sum of the areas under the curve 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.

3.3.6 RNA extraction, reverse transcription, and quantitative PCR for co-sedimentation analysis

Each fraction was treated with 2.5 µL of 20 mg/mL proteinase K and 50 µL of 10% (v/v) SDS solution at room temperature, followed by thorough mixing. The samples were then incubated in a 37 °C water bath for 1 hour and 45 minutes to digest potential protein contaminants. After the incubation, the samples were placed on ice, and 130 µL of acid-phenol/chloroform/isoamyl alcohol (pH 4.5, 125:24:1, Invitrogen) was added to each fraction, followed by thorough mixing for RNA extraction. The fractions were then centrifuged at 12,750 g for 10 minutes at 4 °C. The aqueous phase was carefully transferred to a new nuclease-free 2 mL Eppendorf tube, ensuring the bottom phase was not disturbed. The total RNA was precipitated overnight by adding approximately 500 µL of 100% isopropanol and 1 µL of glycol blue (to visualize the RNA pellets) to the transferred aqueous phase, followed by thorough mixing. After overnight precipitation, the RNA pellets were collected by centrifugation at 15,300 g for 45 minutes at 4 °C. The supernatant was then discarded, and the tubes were carefully dried using tweezers wrapped in clean tissue paper, ensuring the RNA pellets remained undisturbed. The RNA pellets were subsequently washed with 500 µL of 80% (v/v) ethanol in DEPC-treated water (RNase-free) and centrifuged at 12,750 g for 10 minutes at 4 °C. The supernatant was discarded, and the tubes were dried again using tweezers wrapped in clean tissue paper. The RNA pellets were air-dried for 10 minutes under a hood. 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, both ratios should be approximately 2. 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). To remove potential genomic DNA contamination, each fraction was treated in a 10 µL reaction system containing 1 µL of RNA, 1 µL of 10X Reaction Buffer, 0.2 µL of 1 U/µL DNase I, and 7.8 µL of nuclease-free water, and incubated at 37 °C for 30 minutes. Subsequently, 1 µL of 50 mM EDTA was added to each fraction, and the samples were heated at 65 °C for 10 minutes to inactivate DNase I. First-strand cDNA synthesis was then carried out in a 20 µL reaction system consisting of 1 µL Oligo $(dT)_{18}$ primer, 4 µL 5X Reaction Buffer, 1 µL 20 U/ µL RiboLock, 2 µL 10 mM dNTP mix, 1 µL 200 U/µL reverse transcriptase (Revert Aid M-MuLV, Thermos Fisher). The reaction was incubated at 42 °C for 60 minutes, followed by incubation at 70 °C for 5 minutes to terminate the reaction. The synthesized cDNA was stored at -20 °C for further analyses.

Quantitative PCR was performed using exactly 1 µL of cDNA from each fraction. The 10 µL reaction mixture consisted of 1 µL of cDNA, 0.4 µL of 10 µM mixed forward and reverse primers, 5 µL of 2X qPCRBIO SyGreen mix (PCRBIOSYSTEMS, UK), and 3.6 µL of PCR grade water. PCR thermocycling was performed in a CFX96 real-time PCR system with the following settings: an initial cycle of 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 5 seconds and 62 °C for 25 seconds. Before melting curve analysis, PCR products were heated at 95 °C for 15 seconds, followed by melting curve analysis between 62 °C and 95 °C in 0.5 °C increments for 15 seconds. 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 of a single biological sample, calculated as follows:

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

Where *n* represents the fraction number.

3.3.7 Statistical analyses and software

The two-sided and unpaired Student's t-test was used to perform statistical analyses. Origin Pro 2021 was used to plot co-sedimentation profiles and representative polysome profiles. GraphPad Prism 8.0 and Excel were used to plot the results of the physiological experiment and gene expression analyses. Inkscape (v1.3.1) was used to arrange all the images of the results.

3.4 Results

3.4.1 Physiological responses to drought stress

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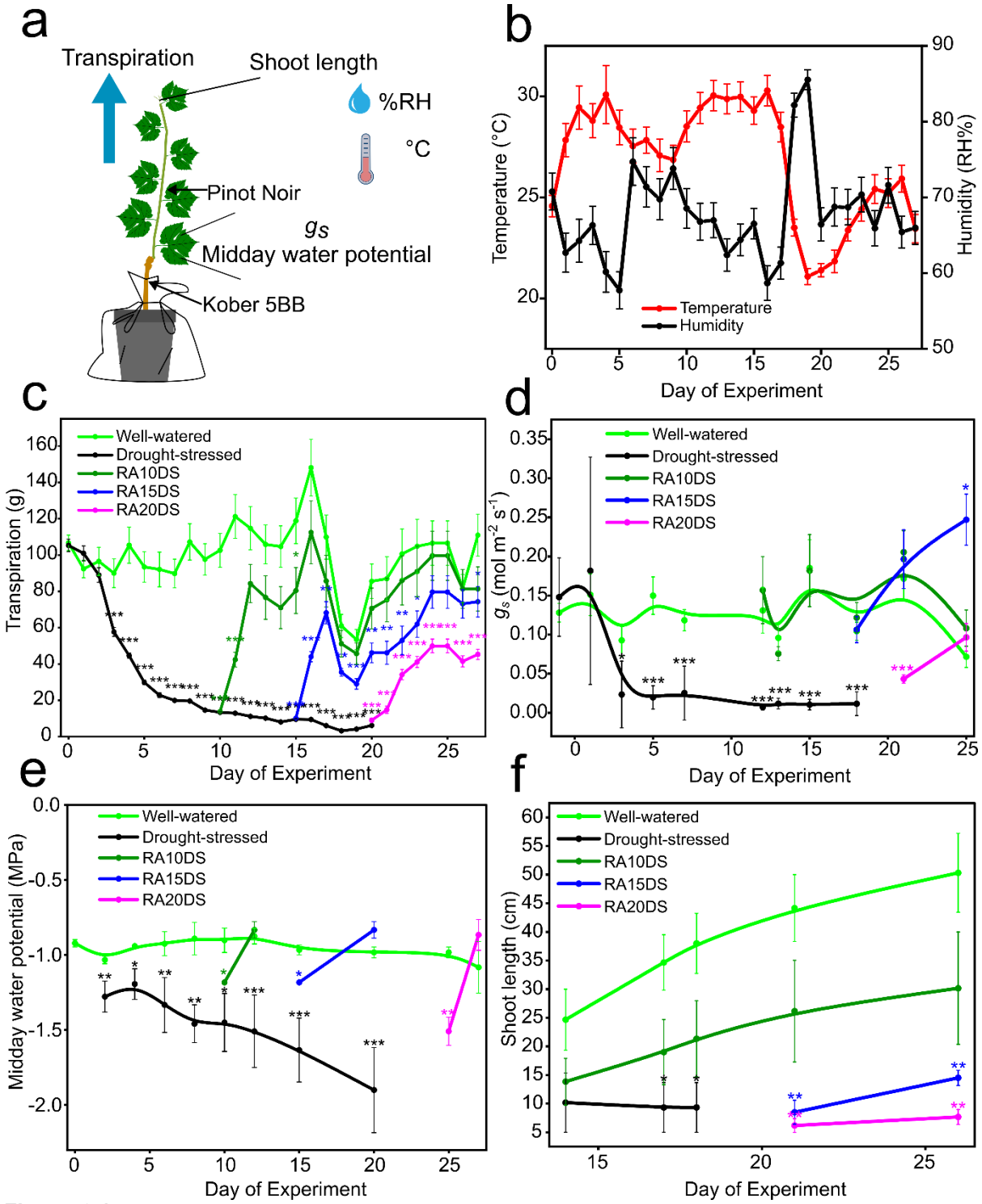

Figure 3.1. Growth conditions for grapevine, physiological responses under well-watered, drought-stressed, and rewatering. (**a**) Schematic diagram representing measurements of physiological parameters. The

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grapevines were the Pinot noir grafted on rootstock Kober 5BB. (**b**) Mean air temperature and humidity in the greenhouse throughout the experiment. The data are mean ± SEM (n=48, each n was a technical replicate and originated from the mean of the data collected from two dataloggers located at the East and West of the experimental bench respectively that continuously recorded temperature and humidity every 30 minutes throughout the experiment). (**c**) Daily whole-plant transpiration data throughout the experiment. (**d**) Stomatal conductance (g_s) data of top leaves of plants. (**e**) Midday water potential data was measured from leaves randomly selected from plants. (**f**) The length of shoots grown from the top bud. In (**c**), (**d**), (**e**), (**f**), RA10DS, RA15DS, and RA20DS represent plants that were rewatered after 10, 15, and 20 days of drought stress respectively. A spline curve was used to connect the data points in (**d**), (**e**), and (**f**). The data are mean ± SEM (collected from at least three biological replicates). Statistical significance was tested by a two-sided and unpaired Student's t-test. The data on drought-stressed and rewatering plants were compared to well-watered plants. The arterials were used to show the significant difference where the * represents $p < 0.05$, the ** represents $p < 0.01$, and the *** represents $p < 0.001$.

We subjected 60 one-year Pinot noir (*Vitis vinifera* L.) grapevine cuttings grafted on Kober 5BB (*Vitis berlandieri x Vitis riparia)* to varying duration of water deficit over 27 days. Of these, 27 were watered daily, while the remaining 33 were withheld from water for different durations before rewatering: 10 days (RA10DS), 15 days (RA15DS), and 20 days (RA20DS). Temperature and humidity are the most important ambient factors that influence the plant transpiration demand. It is noteworthy that when the temperature was elevated, the humidity was relatively low, and conversely, as the temperature increased, the humidity decreased in this study (Figure 3.1b). Well-watered plants showed stable transpiration rates, averaging around 100 g per day (Figure 3.1c). However, drought-stressed plants exhibited a significant reduction in transpiration since day 3 ($p < 0.001$), dropping to approximately 20 g around day 6 and remaining low throughout the experiment (Figure 3.1c). Plants started wilting on day 8 and dropping leaves, eventually reaching a severe drought symptom where more than half of the leaves were dropped, and the leaves were wilted at midday (Supplemental Figure S3.1a). Upon rewatering, the RA10DS plants restored full transpiration after two days ($p < 0.05$), while RA15DS plants took eight days ($p < 0.05$), and RA20DS plants did not fully recover by the end of the experiment (7 days) ($p < 0.001$) (Figure 3.1c). This pattern was mirrored in stomatal conductance, which significantly

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decreased by day 3 ($p < 0.05$) in drought-stressed plants and remained low (around 0.03 mol m^{-2} s^{-1}) (Figure 3.1d). Well-watered plants maintained a g_s of approximately 0.12 mol m^{-2} s^{-1}, while the RA10DS, RA15DS, and RA20DS plants showed relatively quick recovery of g_s after 2, 3, and 5 days of rewatering, respectively. After rewatering, plants slowly recovered, but the more severe drought they experienced, the slower and less likely recovered evidenced by the fewer leaves on RA15DS and RA20DS plants (Supplemental Figure S3.1b, c, d).

The midday water potential in well-watered plants was stable at around -1 MPa throughout the experiment (Figure 3.1e). However, drought-stressed plants experienced a decline to around -1.3 MPa after 2 days of drought stress ($p < 0.01$) and gradually reached the lowest point around -2 MPa by the end of the experiment ($p < 0.001$) (Figure 3.1e). In line with transpiration and g_s results, the water potential in RA10DS, RA15DS, and RA20DS plants recovered to a level similar to well-watered plants after 2, 5, and 7 days of rewatering, respectively (Figure 3.1e). As anticipated, plants subjected to drought stress exhibited stunted shoot growth, with some plants displaying complete cessation of shoot growth (Figure 3.1f). In contrast, shoots in RA10DS plants continued to grow, albeit at a slightly shorter length than in well-watered plants. The RA15DS and RA20DS plants, while also exhibiting cessation of growth, demonstrated slow or no recovery after rewatering ($p < 0.01$) (Figure 3.1f).

3.4.2 Transcriptional modulation of aquaporin genes

Given the importance of aquaporins in regulating water transport under stress conditions, we investigated the transcriptional response of six aquaporin genes in leaves and roots respectively. These genes encoding for the channel proteins localized on the plasma membrane (*VviPIP1-1*, *VviPIP2-5*, *VviPIP2-7*) and the tonoplast membrane (*VviTIP1-3*, *VviTIP2-1*, *VviTIP2-2*) (Table 1). Leaf samples were collected at critical time points: day 0, day 2, day 10, day 14, and day 20. Root samples were collected on days 0, 4, and 20 to minimize plant disruption during the study.

Not all aquaporin genes exhibited a transcriptional response to drought stress. For instance, the transcript levels of *VviPIP1-1*, *VviPIP2-7*, and *VviTIP1-3* remained unchanged in the leaves throughout the experiment (Figure 3.2b). In contrast, *VviPIP2-5* and *VviTIP2-1* showed a gradual

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decline in transcript abundance after day 10 of the experiment (Figure 3.2b). *VviTIP2-2* was not determined in leaves.

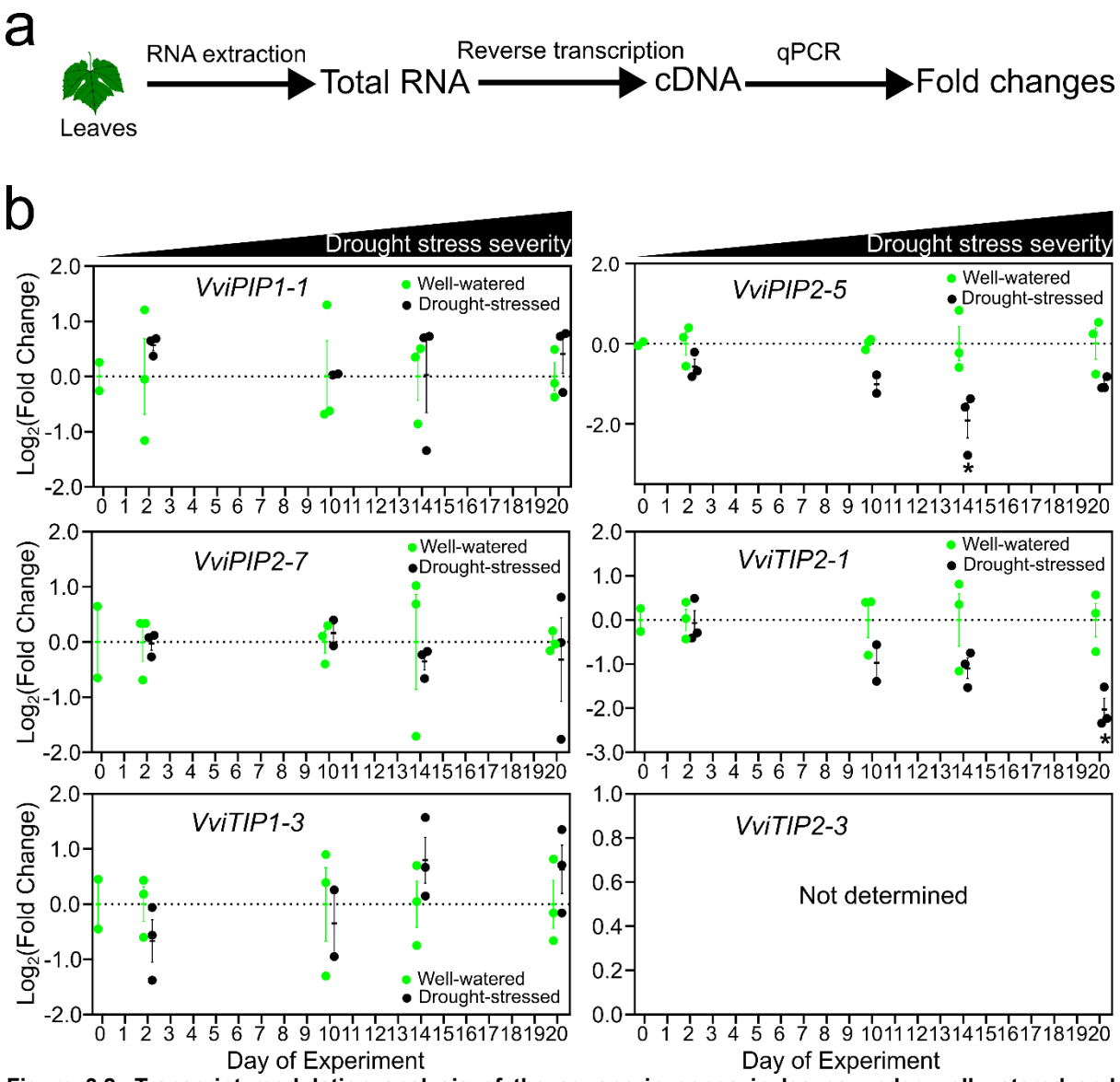

Figure 3.2. Transcript modulation analysis of the aquaporin genes in leaves under well-watered and drought-stressed conditions. (**a**) Experimental procedures: total RNA was extracted from leaves. (**b**) Transcript modulation is expressed as the log2 of the ratio between the drought-stressed over the well-watered samples normalized relative quantities (NPQ). Data was collected from triplicates at each stage of the experiment. Statistical

significance was tested by a two-sided and unpaired Student's t-test and the asterisk * represents $p < 0.05$. The transcript of *VviTIP2-2* was not determined in leaves.

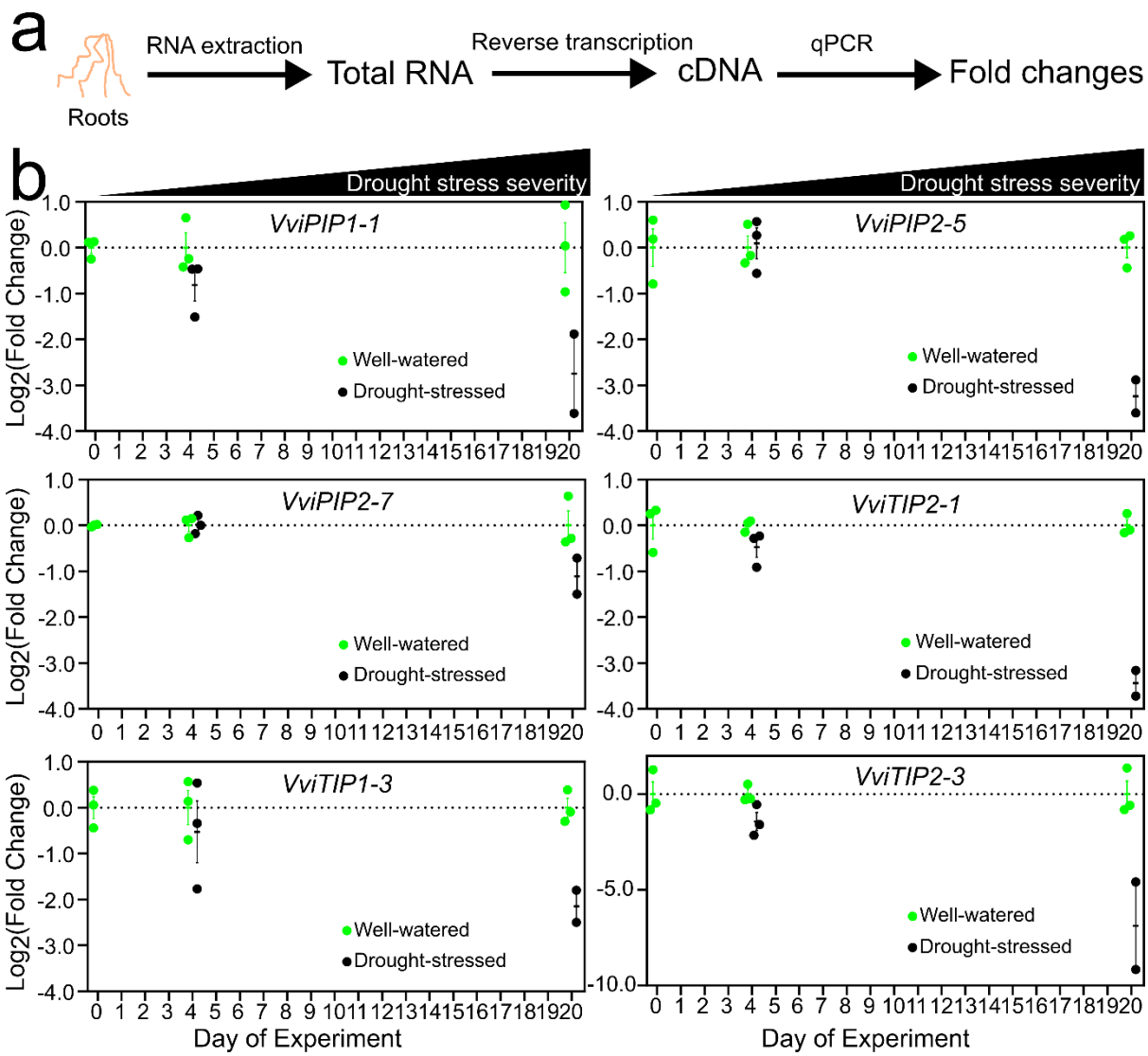

Figure 3.3. Transcript modulation analysis of the aquaporin genes in roots under well-watered and drought-stress conditions. (**a**) Experimental procedures: total RNA was extracted from roots. (**b**) Transcript modulation is expressed as the log2 of the ratio between the drought-stressed over the well-watered samples normalized relative quantities (NPQ). Data were collected in triplicate at each stage of the experiment. Statistical significance was tested by a two-sided and unpaired Student's t-test.

In roots, transcriptional changes were observed later, with significant reductions by day 20. Of note, *VviTIP2-2* was exclusively expressed in roots (Figure 3b).

Given that rewatering effectively restored physiological functions (Figure 3.1), we proceeded to examine aquaporin gene expression after rehydration. Transcript levels of *VviPIP1-1* remained unaltered, irrespective of the degree of drought stress. Likewise, *VviTIP1-3* expression was unaffected in RA10DS and RA20DS plants with one exception in RA15DS plants after 72 hours of rewatering. However, *VviPIP2-7* exhibited a notable decrease in expression, particularly in RA15DS plants ($p < 0.01$). In contrast, *VviPIP2-5* and *VviTIP2-1* exhibited a significant upregulation within 48 hours of rewatering (Figure 3.4b, c, d).

It is noteworthy that the RA10DS, RA15DS, and RA20DS plants exhibited a gradient of drought stress, which was classified as mild, moderate, and severe, respectively. The transcriptional responses were found to be influenced by the differing levels of drought stress. Following 48 hours of rewatering, a significant transcript abundance difference was observed in rewatering plants compared to drought-stressed plants. However, at an earlier stage, such as 6 hours after rewatering, no significant difference was observed (Figure 3.4 b, c, d).

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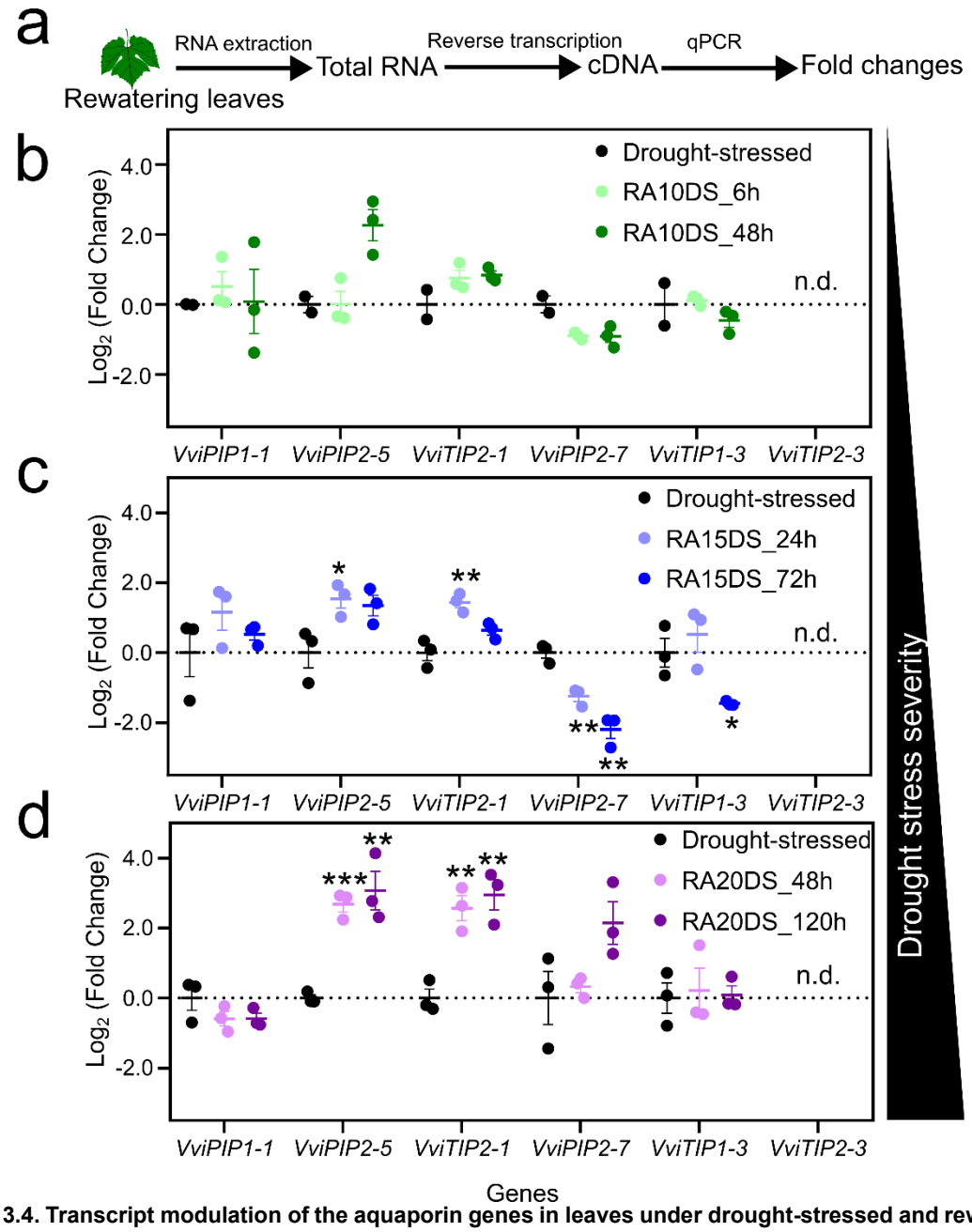

Figure 3.4. Transcript modulation of the aquaporin genes in leaves under drought-stressed and rewatering conditions. (**a**) Experimental procedures: total RNA was extracted from leaves collected from rewatering plants. (**b**) Transcript modulation is expressed as the log2 of the ratio between NRQs of leaf samples collected at 6 and 48 hours after rewatering over the leaf samples collected from plants withheld from water for 10 days. (**c**) Transcript modulation is expressed as the log2 of the ratio between NRQs of leaf samples collected at 24 and 72 hours after rewatering over the leaf samples collected from plants withheld from water for 14 days. (**d**) Transcript modulation

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is expressed as the log2 of the ratio between NRQs of leaf samples collected at 48 and 120 hours after rewatering over the leaf samples collected from the plants withheld from water for 20 days. In (**b**), (**c**), and (**d**), samples were collected in triplicate. Statistical significance was tested by the two-sided and unpaired Student's t-test and the asterisk * represents $p < 0.05$. The transcript of *VviTIP2-2* was not determined in leaves.

3.4.3 Translation analyses of aquaporin genes

To investigate global translation and translation of individual aquaporin genes under drought stress and rewatering conditions, a second drought experiment was conducted in March 2024. This was to collect fresh samples, given that polysomes are susceptible to degradation. The drought stress was estimated to be at a similar level, evidenced by decreased water availability in the soil (Supplemental Figure S3.3d), decreased transpiration (Supplemental Figure S3.3b), decreased midday water potential in leaves (Supplemental Figure S3.3c), and decreased stomatal conductance (Supplemental Figure S3.3e) in drought-stressed plants. We evaluated the translation of aquaporin genes using polysome profiling (Figure 3.5a). As anticipated, despite similar global translational activity between well-watered and drought-stressed plants, rewatering resulted in a significant enhancement of translation, as evidenced by the significantly increased fraction of ribosomes in polysomes (Figure 3.5b, c).

Next, we did co-sedimentation analysis across the polysome profile, which RT-qPCR was conducted in each fraction collected in polysome profiling. Our findings indicate that drought stress inhibits the translation of specific aquaporin genes, particularly *VviPIP2-5* and *VviPIP2-7*. This is evidenced by the presence of mRNA predominantly distributed in non-polysomal fractions in drought-stressed plants, in contrast to the predominantly polysomal fractions observed in control plants (Figure 3.6a). Interestingly, even housekeeping genes such as *VviGAPDH* and *VviActin* exhibited repressed translation under drought conditions (Figure 3.6b). After rewatering, the translation levels of all genes were significantly increased within 6 hours except for *VviPIP1-1*, evidenced by the distribution of mRNA shift from non-polysomal fractions to polysomal fractions (Figure 3.6a, b).

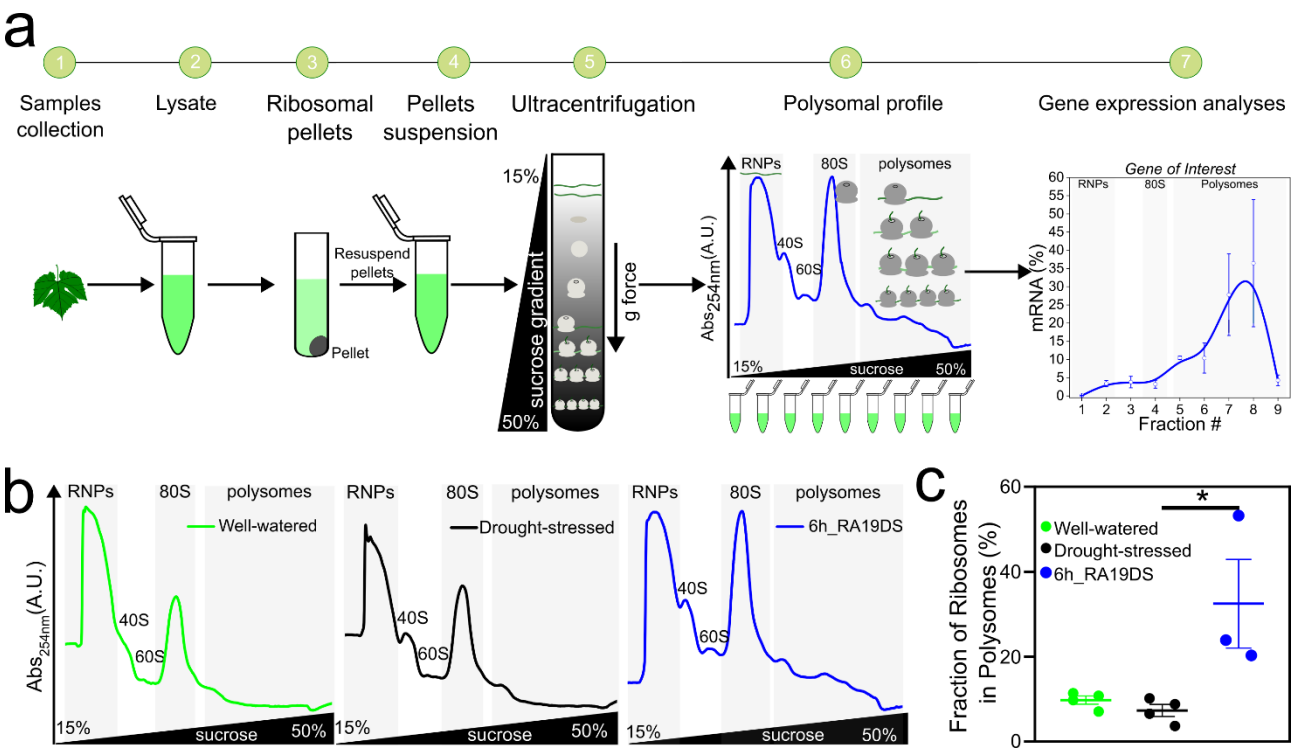

Figure 3.5. The global translation of grapevine was significantly altered by water status. (**a**) The procedure of polysome profiling and downstream analysis. (**b**) Representative polysome profiles were obtained from samples of well-watered, drought-stressed, and after 6 hrs of rewatering leaves. (**c**) The fraction of ribosomes in polysomes was calculated from polysome profiles. The data was expressed as percentages. Statistical analysis was performed by the two-sided and unpaired Student's t-test, and the asterisk * represents $p < 0.05$.

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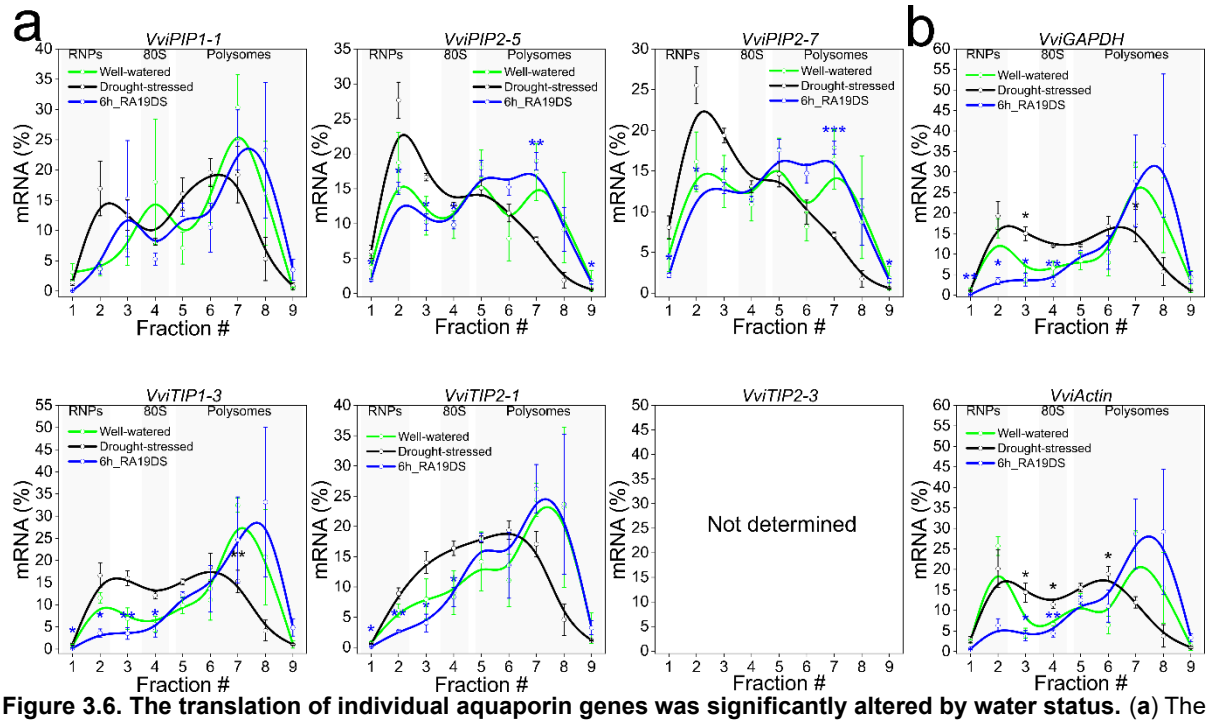

Figure 3.6. The translation of individual aquaporin genes was significantly altered by water status. (**a**) The relative co-sedimentation profiles of the aquaporin gene family indicated mRNA distribution in each sucrose fraction. The transcript of *VviTIP2-2* was not determined in the fractions. (**b**) The relative co-sedimentation profiles of two housekeeping genes *VviGAPDH* and *VviActin*. Rewatering leaf samples in (**a**) and (**b**) were collected from grapevines subjected to 19 days of drought stress and then rewatering. Samples were collected 6 hrs after rewatering. The data were expressed as percentages of mRNA in a single fraction among 9 fractions. The data are mean ± SEM from triplicates. Statistical analysis was performed by the two-sided and unpaired Student's t-test, and the asterisks were used to show the statistical significance where * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

3.5 Discussions

This study demonstrates that plants can adapt to adverse environmental conditions, such as drought stress, at both physiological and cellular levels (Ilyas et al., 2021; Kim et al., 2024). The regulation of aquaporin gene expression at the translational level is crucial for this adaptation to drought stress.

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Drought stress significantly alters the water conditions for plants (Yu et al., 2024). As soil moisture decreases, the water potential in the soil becomes lower than that in plant roots, compelling plants to reduce their root water potential. Consequently, the water potential in leaves also declines as soil water availability diminishes (Figure 3.1e). This reduction in leaf water potential enhances water absorption and transport, helping to alleviate water stress (Gupta et al., 2020). To mitigate further water loss, plants must close their stomata, which slows transpiration (Figure 3.1c, d) (Schweiger et al., 2023). The closure of stomata leads to a decrease in CO_2 assimilation and halts shoot growth, signaling a shift from growth to stress response (Figure 3.1f) (Farooq et al., 2024). However, these physiological parameters can recover after rewatering, with the recovery rate dependent on the severity of the drought stress. These findings align with previous reports indicating that drought stress restricts plant growth through mechanisms such as stomatal closure, reduced transpiration, lowered water potential, and ultimately growth cessation (Scharwies and Dinneny, 2019; Zhang et al., 2020). Drought also significantly impacts grapevine physiology and fruit yield (Chaves et al., 2010; Flexas et al., 2010; Dietz et al., 2021).

At the cellular level, aquaporin gene expression is also affected by drought conditions. For instance, the transcript levels of *VviPIP2-5* and *VviTIP2-1* in leaves decreased as drought stress intensified (Figure 3.2b), but these levels increased upon rewatering. Similar trends were observed in *Nicotiana glauca* (Smart et al., 2001), and *Arabidopsis thaliana* (Martre et al., 2002). Notably, more severe drought conditions correlated with more pronounced changes in aquaporin transcript abundance during rewatering, underscoring the role of aquaporins in alleviating water stress (Figure 3.4b, c, d) (Gautam and Pandey, 2021; Tang et al., 2023). Aquaporins significantly influence the hydraulic conductivity of whole plants (Sade et al., 2014; Ding et al., 2020). The nomenclature system for aquaporin genes in *Vitis vinifera* L. has been standardized (Grimplet et al., 2014). In this study, we utilized coding sequences from three aquaporin gene expression studies (Pou et al., 2013; Zarrouk et al., 2016; Shelden et al., 2017), while adhering to another published study for gene names (Wong et al., 2018). For example, *VviPIP2-5* was previously reported as *VviPIP2;1* and exhibited behavior

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consistent with earlier findings involving three days of water deprivation where transcript abundance slightly decreased in drought-stressed plants during early morning hours (Shelden et al., 2017). Similarly, *VviTIP2-1* showed comparable behavior to prior reports (Pou et al., 2013; Zarrouk et al., 2016; Shelden et al., 2017). These results indicate a conserved protective mechanism involving adjustments in gene expression, including aquaporin genes, under fluctuating environmental conditions (Figure 3.7) (Zhang et al., 2014; Afzal et al., 2016; Patel and Mishra, 2021).

While many studies have concentrated on transcriptional changes in aquaporins during drought conditions (Fox et al., 2020; Yepes-Molina et al., 2020; Byrt et al., 2023), it is important to note that mRNA abundance does not always correlate with protein levels in plants facing varying growth conditions such as water or salt stress or changes in light exposure (Yepes-Molina et al., 2020; Byrt et al., 2023). This research is among the first to highlight aquaporin gene expression regulation at both translational levels. Notably, although global translation levels in drought-stressed plants were similar to those in well-watered plants, a significant increase was observed after six hours of rewatering (Figure 3.5c). The stable translation status of fully expanded leaves may explain the similar global translation levels observed in drought-stressed plants since these samples were less influenced by environmental conditions (Merchante et al., 2017). Furthermore, transcripts of *VviPIP2-5* and *VviTIP2-1* were primarily found in non-polysomal fractions under drought stress but shifted to predominantly polysomal fractions upon rewatering, indicating reactivation of translation (Figure 3.6a). These findings suggest that these two genes play a significant role in adapting to drought stress (Figure 3.7). Importantly, the response of translation was quicker than that of transcription: the translation was altered while transcript levels remained unchanged after six hours of rewatering. This discrepancy may clarify why there is often a low correlation between aquaporin mRNA and protein levels (Suga et al., 2002; Lopez et al., 2003; Hachez et al., 2012).

Notably, not all aquaporin genes exhibited uniform transcriptional responses in leaves, reflecting the complex regulatory landscape governing aquaporin gene expression in grapevines (Fox et al., 2017; Kapilan et al., 2018). For instance, transcripts of *VviPIP1-1* and *VviTIP1-3*, where *VviTIP1;1*

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is used in other studies, remained unchanged across well-watered, drought-stressed, and rewatered conditions throughout the experiment (Figure 3.2b; Figure 3.4b, c, d), suggesting these genes may not respond to drought stress. Additionally, *VviPIP2-7* (previously named *VviPIP2;2*) demonstrated a unique pattern by not showing significant changes under well-watered and drought-stressed conditions but exhibiting an overall decrease upon rewatering except for RA20DS plants where transcript levels remained similar (Figure 3.2b; Figure 3.4b, c, d). This indicates that while *VviPIP2-7* may not respond directly to drought stress, its decreased transcript level warrants further investigation (Pou et al., 2013; Zarrouk et al., 2016; Shelden et al., 2017). Lastly, *VviTIP2-2* exhibited tissue-specific expression localized to roots rather than leaves (Figure 3.2b; Figure 3.3b; Figure 3.4b, c, d), corroborating its previously reported tissue-specific expression in *cv.* Touriga Nacional (*Vitis vinifera L.*) (Zarrouk et al., 2016). Interestingly, despite these variations in transcriptional behavior among different genes under drought conditions, their translational responses were notably coherent. Except for *VviPIP1-1*, translation was significantly inhibited under drought stress compared to well-watered conditions but surged after six hours of rewatering. Consistent with total transcript analysis results, *VviTIP2-2* was not detected in polysomal transcript analysis further confirming its tissue-specific expression profile (Figure 3.6a).

Many studies have compared PIPs and TIPs' roles in regulating radial transcellular water transport and osmoregulation within roots (Kjellbom et al., 1999; Maurel et al., 2008, 2002). In this study, root samples collected on day four when stress levels were low showed no changes in transcript abundance across all genes examined. However, a general decline was noted by the end of the experiment (Figure 3.3b), suggesting an intriguing consistency in expression patterns that merits further exploration, especially given the complexity observed in leaf expression patterns. Future investigations could examine root expression under higher drought stress levels, such as on day ten, and during rewatering phases since roots are directly impacted by water deficits leading to the initial damage (Cuneo et al., 2021).

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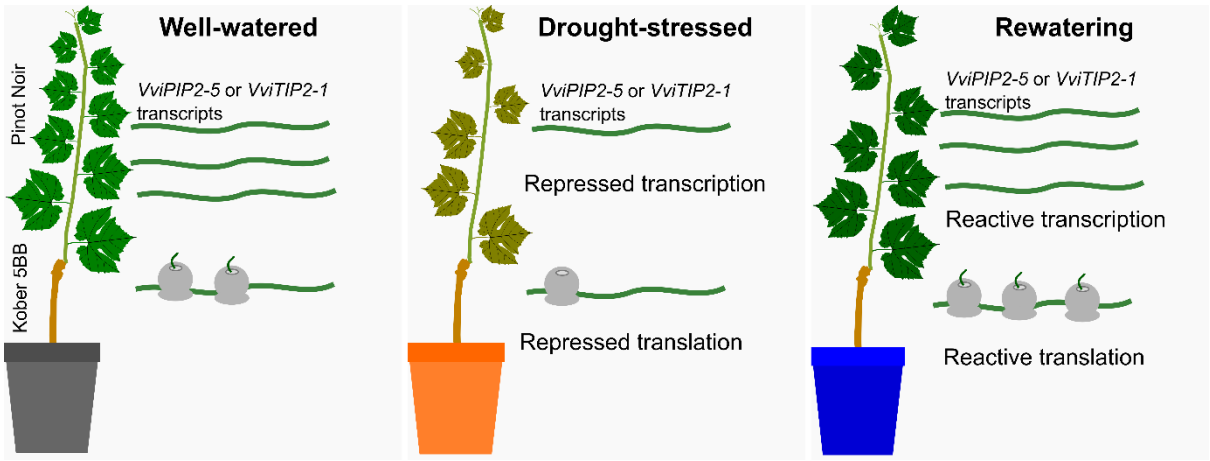

Figure 3.7. Transcription and translation were significantly affected by environmental stress. A schematic diagram shows that transcription and translation were affected by water status. The upper parts are Pinot noir scions, and the bottom part are Kober 5BB rootstocks. The grey pot represents the well-watered setting, the yellow pot represents the drought-stressed setting, and the blue pot represents rewatering after the drought-stressed setting.

In summary, these findings indicate that translational regulations of aquaporin gene expression are equally important as transcriptional changes during drought adaptation processes (Figure 3.7) (Yepes-Molina et al., 2020; Zhang et al., 2022).

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 encompassing various aquaporin genes along with proteomic data to better correlate transcriptional and translational changes with protein functionality (Paluch-Lubawa and Polcyn, 2024). We propose concentrating on *VviPIP2-5* and *VviTIP2-1* for engineering drought resistance in grapevines due to their demonstrated decrease in transcript abundance during drought but an increase upon rewatering. Their co-sedimentation profiles further suggest active translation following rewatering which aids water movement when soil moisture is restored (Kjellbom et al., 1999; Johansson et al., 2000; Takata et al., 2004; Mahdieh et al., 2008; Tyerman et al., 2009). However, breeding efforts aimed at enhancing drought resistance should be approached cautiously since increased water transport does

not necessarily guarantee survival during severe droughts (Martinez-Ballesta and Carvajal, 2014). The focus should instead be on achieving a balance between efficient water transport and other stress response mechanisms like growth cessation during prolonged periods of drought (Ding et al., 2020). Another promising avenue for future research involves exploring plant "stress memory," which largely relies on epigenetic modifications and translational reprogramming mechanisms (Sharma et al., 2022; Jin et al., 2024). Investigating these aspects through ribosome profiling could yield new insights into how plants adapt to drought stress at molecular levels (Lei et al., 2015). Ultimately, this study sheds light on the translational regulation of aquaporins within grapevines and sets the stage for future inquiries into molecular mechanisms underlying drought resilience at this level. Our findings could significantly contribute to developing grapevine varieties capable of withstanding increasing climate challenges.

3.6 Conclusions

This study provides the first evidence that environmental cues can influence aquaporin gene expression, particularly at the post-transcriptional level. In plants, aquaporins function as crucial water channels located on both plasma membranes and endomembranes, with their encoding genes showing dynamic transcriptional changes in response to varying water availability. Specifically, the transcripts of *VviPIP2-5* and *VviTIP2-1* were found to decrease under drought stress but recover upon rewatering. Notably, the translation of these genes was repressed during drought conditions, aligning with observed shifts in global translational activity. Additionally, some aquaporin genes exhibit tissue-specific expression; for instance, *VviTIP2-2* appears to be primarily expressed in roots. Future research could involve knocking out these two genes to further investigate their translational regulatory networks and elucidate their roles in plant responses to environmental stressors.

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3.8 Supplemental materials

3.8.1 Supplemental figures

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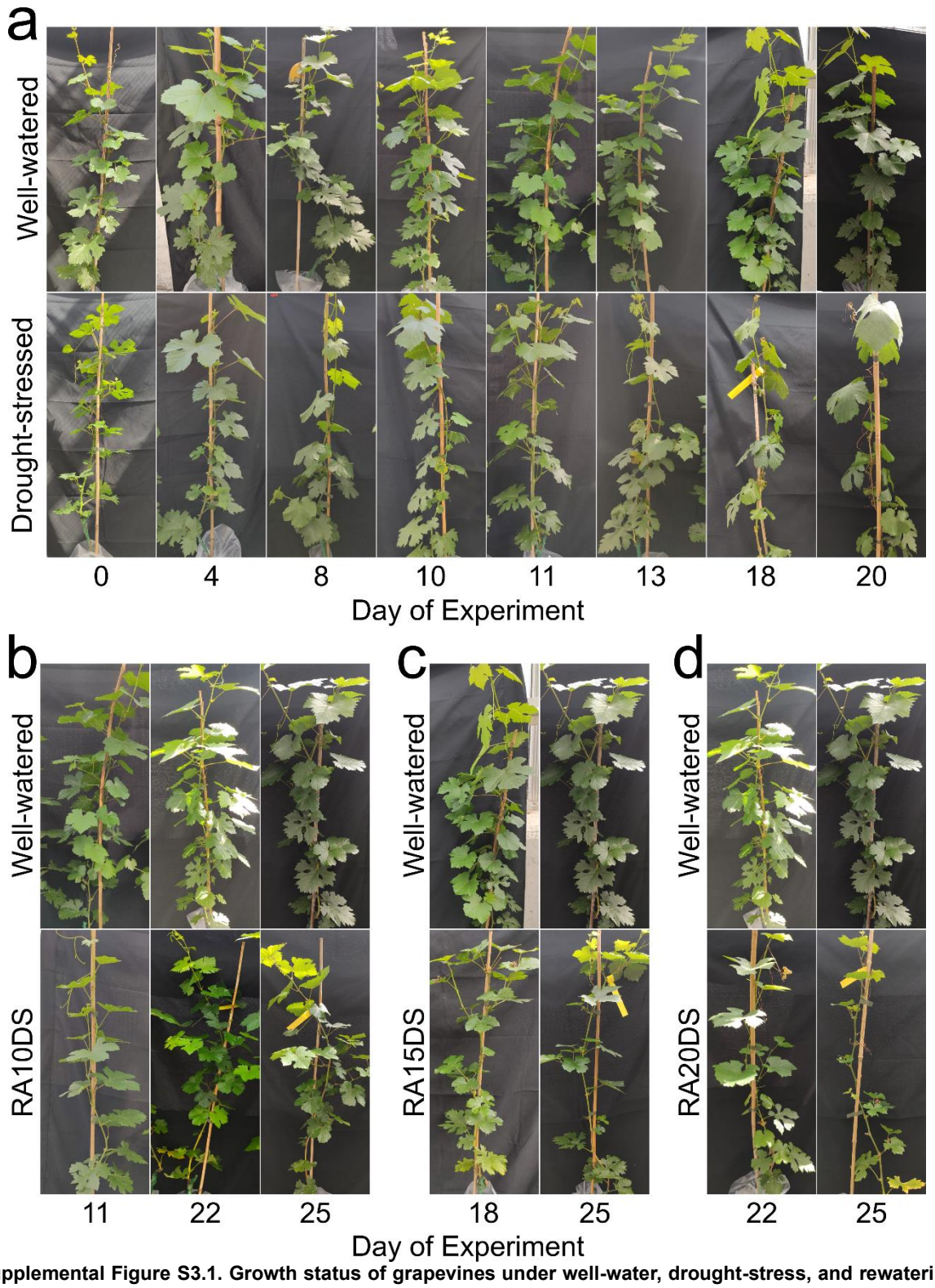
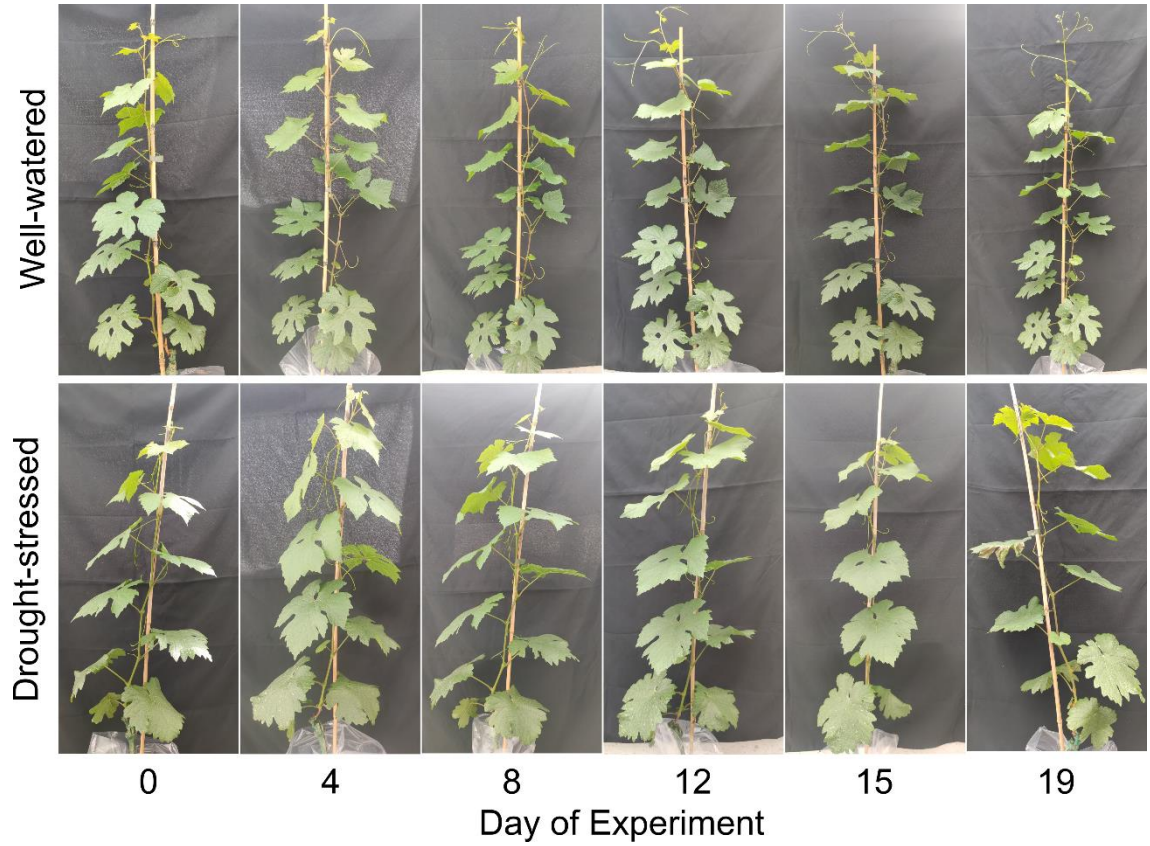


Supplemental Figure S3.1. Growth status of grapevines under well-water, drought-stress, and rewatering

conditions in the August 2023 drought stress experiment. (**a**) Well-watered plants versus drought-stressed plants. The leaves in drought-stressed plants were getting less and wilted at midday. (**b**), (**c**), (**d**) Well-watered plants versus rewatering plants, among them, RA10DS, RA15DS, and RA20DS represent plants that were rewatered after 10, 15, and 20 days of drought stress respectively. They represent mild, moderate, and severe drought stress respectively.



Supplemental Figure S3.2. Growth status of grapevines under well-watered and drought-stressed conditions in the March 2024 drought stress experiment. The top panels are well-watered plants, and the bottom panels are drought-stressed plants. The drought-stressed plants show significant drought-stress symptoms such as scorched and wilted leaves.

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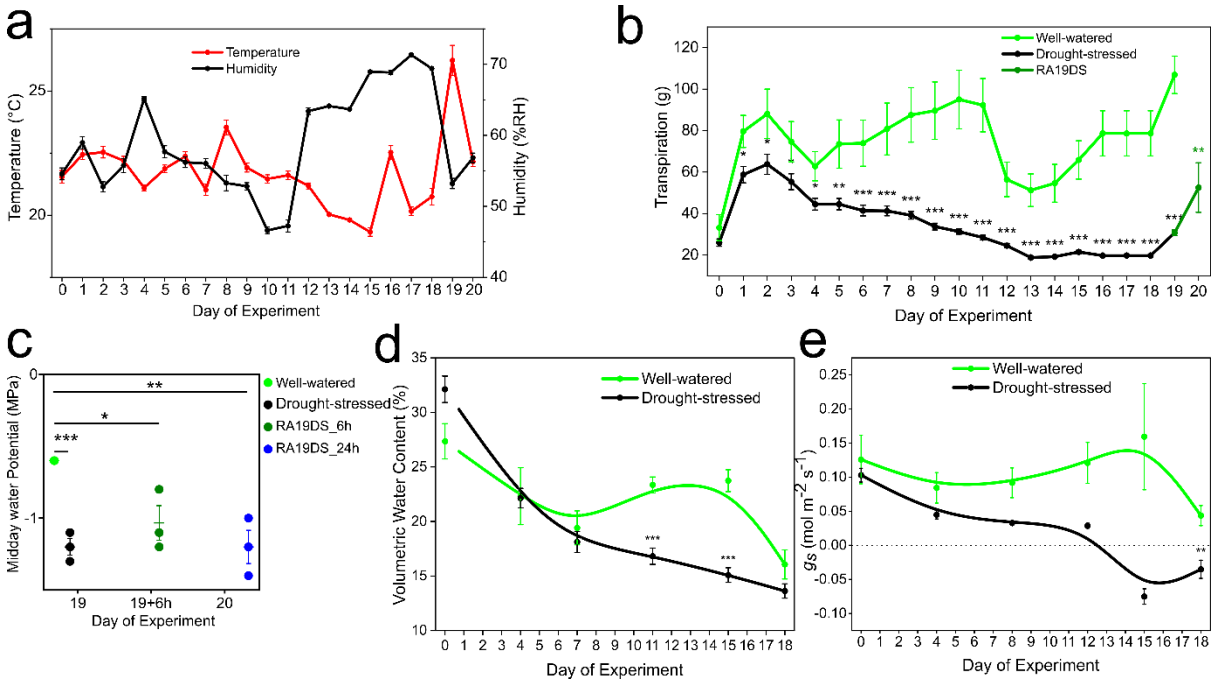

Supplemental Figure S3.3. Physiological response of grapevines in March 2024 drought stress experiment under well-watered, drought-stressed, and rewatering conditions. (**a**) Mean air temperature and humidity in the greenhouse throughout the experiment. Data represent mean ± SEM (n=288, each n was a technical replicate and originated from the data collected from the data logger that continuously recorded temperature and humidity every 5 minutes throughout the experiment). (**b**) Whole plant transpiration throughout the experiment. Data are mean ± SEM (n=4 biological replicates in the well-watered group, n=16 biological replicates in the drought-stressed group, and n=3 biological replicates in the RA19DS group). (**c**) Midday water potential in the leaves. Data are mean ± SEM (n=3). (**d**) Volumetric water content in the soil. (**e**) Stomatal conductance (g_s) of the top leaves. Data in (**d**) and (**e**) are mean ± SEM (n=4 in the well-watered group, n=16 in the drought-stressed group). Statistical analyses of all the data were performed by two-sided and unpaired Student's t-test. The asterials were used to show the statistical significance where * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$.

4 Concluding Remarks

A notable observation across various eukaryotes, including plants, is the low correlation between mRNA and protein levels in different tissues, developmental stages, and environmental

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conditions, a phenomenon likely attributed to the complex regulatory mechanisms governing the translation process.

In this thesis, I primarily employed polysome profiling to address this aspect and clarify the cellular process underneath the varying correlations between mRNA and protein levels detected for specific genes in the cotyledons and hypocotyls of *Arabidopsis* seedlings. Polysome profiles of 4 d *Arabidopsis* seedlings indicated that for the selected genes the hypocotyl had lower overall translational activity compared to the cotyledon, despite the higher transcript levels found in this tissue. Polysome profiling and qPCR analyses on these genes revealed no significant differences in their translational activity. This suggested that other regulatory processes, such as mRNA degradation or protein degradation, may concur to regulate the expression of these genes.

Moreover, polysome profiling and qPCR were used to assess the translational status of these genes during various developmental phases. The results indicated that 10 d *Arabidopsis* seedlings had significantly lower translational activity compared to their 6 d counterparts, even though total transcript levels remained similar—except for *AtACO3*, which showed a higher transcript level in 10 d seedlings. The translational status of *AtACO3* also was repressed, as its mRNA distribution shifted from polysomal to non-polysomal fractions. Further experiments, such as measuring protein degradation rates, will be needed to elucidate the detailed mechanisms behind this translational repression.

Among the regulatory factors influencing translation, miRNAs are particularly noteworthy. Previous studies have established an autoregulatory mechanism between miR168 and its target *AtAGO1*, which is crucial for plant development; any disruption in this mechanism can lead to developmental defects (Vaucheret et al., 2006, 2004). This thesis revealed that although total transcript levels of miR168 and *AtAGO1* were similar in 6 d and 10 d seedlings, miR168 was predominantly found in polysomal fractions at day 6 but shifted to non-polysomal fractions by day 10. *AtAGO1* transcript distribution also transitioned from polysomal to non-polysomal fractions at both stages. These results suggest that miR168 is a translation repressor for *AtAGO1* in 6 d seedlings. However, unexpectedly, the *At*AGO1 protein level was significantly lower in 10 d seedlings compared to 6 d ones. A possibility

underneath this phenomenon is that a more intricate regulatory relationship exists between miR168 and *AtAGO1*, rather than a straightforward competitive dynamic. Interestingly, *At*AGO1 protein levels increased in later stages (12 d and 14 d), possibly due to the release from miR168 repression at day 10.

The transcript level and protein level of *AtPIP2F*, encoding channel-forming transmembrane proteins, Plasma Intrinsic membrane Protein 2; 4, were found not correlated in cotyledons and hypocotyls in the above *Arabidopsis* study. However, no evidence shows the post-transcriptional or translational regulations of this gene despite the significantly different global translational activity in cotyledons and hypocotyls. This is possibly due to no environmental cues in *Arabidopsis* seedlings growing. We thus shift our focus to the same gene family in grapevine (*Vitis vinifera* L.). Drought stress represents a significant abiotic challenge for plants, with its duration and frequency becoming increasingly unpredictable due to climate change. Aquaporins serve as essential water channels located on both plasma membranes and endomembranes. Plants possess a greater number of aquaporin-encoding genes compared to animals. While transcriptional and post-translational regulations of aquaporins have been well-documented, their translational regulation remains less understood. In this thesis, I examined both transcriptional and translational aspects of six aquaporin-encoding genes associated with plasma membrane and tonoplast membranes. My findings indicate that aquaporin translation is suppressed during drought conditions but reactivated upon rewatering. Notably, VviPIP2-5 demonstrated downregulation of both transcription and translation during drought stress, with subsequent upregulation following rewatering. Future experiments could involve knocking out this gene to further explore its regulatory network.

In conclusion, the study of translation is crucial as it ultimately determines the final product of gene expression. Ongoing research into translation holds great promise for uncovering exciting discoveries within plant biology.

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