



ORIGINAL RESEARCH

Chemical activation of ABA signaling in grapevine through the iSB09 and AMF4 ABA receptor agonists enhances water use efficiency

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Abstract

Grapevine (*Vitis vinifera* L.) is the world's third most valuable horticultural crop, and the current environmental scenario is massively shifting the grape cultivation landscape. The increase in heatwaves and drought episodes alter fruit ripening, compromise grape yield and vine survival, intensifying the pressure on using limited water resources. ABA is a key phytohormone that reduces canopy transpiration and helps plants to cope with water deficit. However, the exogenous application of ABA is impractical because it suffers fast catabolism, and UV-induced isomerization abolishes its bioactivity. Consequently, there is an emerging field for developing molecules that act as ABA receptor agonists and modulate ABA signaling but have a longer half-life. We have explored the foliar application of the iSB09 and AMF4 agonists in the two grapevine cultivars cv. 'Bobal' and 'Tempranillo' to induce an ABA-like response to facilitate plant adaptation to drought. The results indicate that iSB09 and AMF4 act through the VviPYL1-like, VviPYL4-like, and VviPYL8-like ABA receptors to trigger stomatal closure, reduce plant transpiration, and increase water use efficiency. Structural and bioinformatic analysis of VviPYL1 in complex with ABA or these agonists revealed key structural determinants for efficient ligand binding, providing a mechanistic framework to understand receptor activation by the ligands.

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Physiological analyses further demonstrated that iSB09 has a more sustained effect on reducing transpiration than ABA, and agonist spraying of grapevine leaves protected PSII during drought stress. These findings offer innovative approaches to strengthen the vine's response to water stress and reduce plant consumptive water use under limited soil water conditions.

1 | INTRODUCTION

Grapevine (*Vitis vinifera* L.) and its derived products constitute one of the most relevant fruit crops in the world (7.5 million cultivated hectares, >50% of them in Europe), and wine exports annually contribute >6 billion euros to the EU (OIV, 2023). Despite its condition as a commodity crop, the wine sector faces unprecedented challenges, given that it must satisfy a growing demand for wines of greater typicity and quality through sustainable viticulture in the current context of climate change (Hannah et al., 2013; Gambetta et al., 2020). Global warming threatens grape yield and quality wine production in mid-latitude wine-growing regions (Hannah et al., 2013; Van Leeuwen et al., 2024). The increase in temperature and drought episodes in these regions accelerate the phenology of the vine and modify the ripening and composition of grapes and wines (Gambetta et al., 2020). Indeed, a recent analysis of the impact of climate change on viticulture reveals that traditional wine regions in the Mediterranean area could be at risk because of drought and heat waves associated with climate change (Van Leeuwen et al., 2024). Adaptation to climate change will require changes in grape plant material and vineyard management (Van Leeuwen et al., 2024). Mechanisms to adapt vines to drought episodes involve limiting transpiration or increasing soil water foraging through the root system (Van Leeuwen et al., 2019; Seleiman et al., 2021). This could be achieved by choosing varieties with lower transpiration rates or rootstocks that promote deeper rooting, respectively (Gambetta et al., 2020; Van Leeuwen et al., 2024). Additionally, proper vineyard management optimizing irrigation, canopy size, exposed leaf area, and vineyard density can support these adaptation mechanisms (Miras-Avalos and Intrigliolo, 2017; Van Leeuwen et al., 2024).

Absciscic acid (ABA) is an essential hormone in regulating different processes of plant development, fruit ripening, and the plant response to abiotic stress (Cutler et al., 2010; Pilati et al., 2017). ABA is critical in regulating stomatal aperture and, thus, transpiration (Schroeder et al., 2001; Cotellet and Leonhardt, 2019; Buckley et al., 2019). Therefore, the acquisition of an efficient ABA signaling pathway during evolution was decisive in coping with water deficit and adapting to drought stress (Sun et al., 2020; Hewage et al., 2020). Furthermore, ABA signaling is required for root hydrotropism and the promotion of root growth in response to low air humidity detected by the plant's aerial part (Dietrich et al., 2017; Miao et al., 2021; Rowe et al., 2023). As a result, both genetic and chemical activation of ABA signaling can be used to enhance plant drought tolerance (Gonzalez-Guzman et al., 2014; Cao et al., 2017; Yang et al., 2019; Vaidya et al., 2019;

Lozano-Juste et al., 2023). The application of ABA may be an appropriate strategy to promote the grapevine's response to stress, increasing the production and quality of the grapes (Murcia et al., 2017). Additionally, several studies have shown that ABA initiates and regulates ripening, sugar accumulation, and color development in non-climacteric berries such as grapes (Pilati et al., 2017). For example, one of the roles of ABA is its ability to increase the production of anthocyanins, which are mainly responsible for the color of grapes (Pilati et al., 2017). During grape berry ripening, endogenous ABA concentration increases dramatically (Coombe and Hale, 1973; Pilati et al., 2017). Exogenous ABA treatment might increase the grape skins' anthocyanin, phenolic content, and antioxidant properties, increasing their nutritional value (Ferrara et al., 2013; Villalobos-Gonzalez et al., 2016; Pilati et al., 2017).

ABA is perceived by the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) gene family of ABA receptors (Park et al., 2009; Ma et al., 2009; Rodriguez et al., 2019). The binding of ABA to ABA receptors leads to the formation of ternary complexes with group A protein phosphatases type 2C (PP2C), thus inhibiting these PP2Cs, which are key repressors of ABA signaling (Rubio et al., 2009). Upon the inhibition of PP2Cs, the activity of protein kinases, called SnRK2s, is released, which additionally requires the action of B2/B3-type RAF-like MAP3Ks as upstream activating kinases (Cutler et al., 2010; Lozano-Juste et al., 2022). ABA-activated SnRK2s phosphorylate various downstream targets, leading to the activation of the plant stress response (Cutler et al., 2010). Identification of ABA receptors in *V. vinifera* has been reported by several groups, which have provided information on the expression of these genes in different tissues and their response to abiotic stress. Unfortunately, it has also led to a divergent nomenclature that makes it difficult for data interpretation (Li et al., 2012; Boneh et al., 2012; Zhao et al., 2020; Zhang et al., 2021). Moreover, the number of ABA receptors identified in these works varies, which can be solved with the arrival of a high-quality reference genome assembly termed telomere-to-telomere (T2T) that enables a complete annotation of the ABA receptor family (Shi et al., 2023).

A critical ABA-dependent response for drought adaptation is the induction of stomatal closure in water scarcity situations to reduce water loss through transpiration. Drought-induced increase in endogenous ABA is very efficient in reducing water loss, and the priming of this response would be beneficial to anticipate a plant response and delay soil water consumption (Helander et al., 2016). This can be achieved for example through ABA receptor agonists that show

long-lasting effects and can be applied before the stress occurs to reduce soil water consumption (Helander et al., 2016; Sanchez-Olvera et al., 2024). In contrast, the exogenous application of ABA is not very practical due to the fragility of its structure, which is sensitive to ultraviolet light, and its rapid catabolism through different pathways (Gao et al., 2016; Han et al., 2017). Therefore, there is an emerging field for developing molecules that act as ABA receptor agonists and show a longer half-life than ABA (Hewage et al., 2020). The application of these molecules can modulate ABA signaling in a timely, dynamic, and exogenous manner, and their persistence after exogenous application is higher than that of ABA (Vaidya et al., 2019). In this work, we aim to explore the use of ABA receptor agonists in grapevine, specifically iSB09 and AMF4, as possible substitutes for ABA. iSB09 and AMF4 are ABA receptor agonists that showed their efficacy in regulating transpiration in model plants such as *Arabidopsis thaliana* (*Arabidopsis*) and *Solanum lycopersicum* (tomato; Cao et al., 2017; Lozano-Juste et al., 2023; Jimenez-Arias et al., 2023; Sanchez-Olvera et al., 2024). Therefore, we decided to explore their use in the two commercial grapevine cultivars cv. 'Bobal' and 'Tempranillo' and examine their physiological effect on plant transpiration and water use efficiency.

2 | MATERIALS AND METHODS

2.1 | Plant material, infrared (IR) thermography and gas exchange devices

This work used *V. vinifera* L. cultivars 'Bobal' and 'Tempranillo Tinto' and four sets of experiments with different grapevine plants were performed.

For group 1, *V. vinifera* L. (cv. 'Bobal') plants were grown from pruned wood cuttings in forestry trays in controlled conditions (temperature $26^{\circ}\text{C} \pm 2$ and humidity 65%) to a height of 12 cm until the four-leaves-stage or phenological stage BBCH 14 (Figure 3A) at the phytotrons of the IBMCP (Valencia, Spain). They were grown under long day conditions (16:8 h light/dark) with a temperature of 26/24°C (day/night) and a photosynthetic photon flux density (PPFD) of $300 \mu\text{mol m}^{-2} \text{sec}^{-1}$. IR thermography and gas exchange analyses were conducted on fully developed leaves. IR images were taken using a FLIR E95 camera to quantify leaf temperature. Stomatal conductance (gs), and transpiration rate (E) were measured using a portable LI-600 porometer/fluorometer (LI-COR). Measurements were conducted at an ambient CO_2 concentration ($400 \mu\text{mol CO}_2 \text{mol}^{-1}$ air) and a PPFD of $300 \mu\text{mol m}^{-2} \text{sec}^{-1}$.

For group 2, *V. vinifera* L. (cv. 'Bobal') plants were transplanted from the forestry trays to 6 L pots and grown to the nine or more unfolded leaves-stage or phenological stage BBCH 19 (Figure 4). With these plants, photosynthesis (A_N), stomatal conductance (gs), and transpiration rate (E) were measured using an infrared gas analyzer (LI-6800, LI-COR) on three to four sun-exposed leaves of the same plant. Measurements were performed at an ambient CO_2

concentration ($400 \mu\text{mol CO}_2 \text{mol}^{-1}$ air), a PPFD of $1000 \mu\text{mol m}^{-2} \text{sec}^{-1}$ (optimized with a light curve), and an airflow of 500 mL min^{-1} .

For group 3, at the Centro de Investigaciones sobre Desertificación (CIDE, Moncada, Spain), nine grape plants (*Vitis vinifera* L. cv. Bobal) were transplanted into 6 L pots filled with soil and organic substrate (Figure 3E and 6) when they were about 50–70 cm tall. The plants were grown at 27°C in a greenhouse with a cooling system throughout the experiment. Treatments were applied when the plants had six unfolded leaves (phenological stage BBCH 16) or until their lateral shoots were more than 5 cm long (phenological stage BBCH 23). Gas exchange analyses were conducted on fully developed leaves (10 leaves per plant), and the stomatal conductance (gs), transpiration rate (E), electron transport rate (ETR) in photosystem II (PSII) and PSII quantum efficiency (Φ PSII) were measured using a portable LI-600 porometer/fluorometer (LI-COR). Measurements were conducted at an ambient CO_2 concentration ($400 \mu\text{mol CO}_2 \text{mol}^{-1}$ air) and a PPFD range of $1300\text{--}1400 \mu\text{mol m}^{-2} \text{sec}^{-1}$.

For group 4, six grapevine plants (*V. vinifera* L., cv Tempranillo) grafted on rootstock 110R were grown in 30 L pots filled with soil and organic substrate under field conditions at the Instituto de Ciencias de la Vid y del Vino (ICVV) experimental field (coordinates: $42^{\circ} 26' 40.6176''$ N, $2^{\circ} 30' 49.8276''$ W, Logroño, Spain). Seven-year-old plants were irrigated daily from April to mid-July, receiving around 5.3 L of water per plant and day. The field-grown plants were treated with mock or agonist solution on a sunny day on the 10th of July 2023 at the BBCH phenological stage 77, with green and hard berry bunches before the onset of ripening, and when all shoot apexes were fully grown. Gas exchange measurements were conducted at the indicated times post-treatment on the same plants and were performed on 3 to 4 sun-exposed leaves per plant, between 12:00 and 13:00 h, using an open gas exchange system (LI-6400; LI-COR). All measurements were performed with a CO_2 concentration in the cuvette of $400 \mu\text{mol CO}_2 \text{mol}^{-1}$ air and a PPFD range of $1300\text{--}1400 \mu\text{mol m}^{-2} \text{sec}^{-1}$. Temperature and vapor pressure deficit (VPD) were not controlled. Net photosynthesis (A_N), stomatal conductance (gs), and transpiration rate (E) were measured, and intrinsic water use efficiency (WUEi) was calculated as the ratio between A_N and gs.

2.2 | Treatments

Foliar treatments were done on both the adaxial and abaxial leaf surfaces carefully sprayed on each plant leaf.

For group 1, plants were grown under well-watered (WW) conditions, and we tested the effect of 50 μM iSB09 and 100 μM ABA on stomatal function using IR thermography (Figure 3A). Quantification of leaf temperature by thermal imaging was performed on fully expanded leaves by analyzing 10 sections per leaf (upper leaf) and at least 12 plants per treatment were analyzed ($n = 2$ replicates). Quantification was done 24 h after spraying using the FLIR tool software. The average plant temperature $\pm$ standard deviation of all the

plants for each treatment was calculated and used to report the increase in temperature produced by the agonist or ABA treatment.

For group 2, the plants were grown under WW conditions and we tested the effect of 30 μ M iSB09 and 30 μ M ABA using an infrared gas analyzer LI-6800 on 3–4 sun-exposed leaves of the same plant as indicated above ($n = 2$ replicates, three plants per treatment; Figure 4).

For group 3, the plants were grown under WW conditions (Figure 3E) or subjected to progressive drought (Figure 6) with a single irrigation at 25% FC (field capacity) in the middle of the experiment. Under WW conditions, three individual plants per treatment were mock- (0.1% DMSO and 0.05% Tween20), 30 μ M iSB09- and 30 μ M ABA-treated, and physiological measurements were performed at 96 h and 1 week after foliar spraying (Figure 3B). The agonist and ABA stock solutions (10 mM in DMSO) were dissolved in distilled water and 0.05% of Tween20 as an adjuvant. The progressive drought/single irrigation experiment was conducted for 15 days and only one irrigation with 25% FC was performed after 6.5 days. Stomatal conductance and chlorophyll fluorescence were measured with an LI-600 porometer/fluorometer before treatment and at 24 h, 96 h, 7, 8, and 15 days. A total of 30 measurements were taken per plant on healthy leaves.

For group 4, the plants were fully treated with 20 μ M of AMF4 (dissolved in 0.1% DMSO and 0.05% Tween80 in distilled water) or with a mock treatment (0.1% DMSO and 0.05% Tween80 in distilled water) by spraying all leaves and shoot apices individually. A total volume of 1 L of either mock or 20 μ M AMF4 solution was applied to the mock- or AMF4-treated plants. Treated and control plants were sprayed simultaneously from 9:30 to 10:30 AM. Physiological parameters were measured using an open gas exchange system (LI-6400; LI-COR).

2.3 | Cloning of ABA receptors and PP2C inhibition assays

We have used the PN40024 T2T reference genome (Shi et al., 2023; T2T.v5.1 annotation) to identify the sequences of ABA receptors through a BLAST search in <https://grapedata.org/genomes/> using as query the *Arabidopsis thaliana* ABA receptors (TAIR). As a result, nine ABA receptors were identified in the PN40024.T2T genome of *V. vinifera* L. (Shi et al., 2023). We selected the coding sequences of Vitvi05_01chr02g11700/Vvi02g00695 (PYL1-like), Vitvi05_01chr08g07160/Vvi08g00768 (PYL4-like-b), Vitvi05_01chr16g18290/Vvi16g01226 (PYL8-like-a), Vitvi05_01chr02g01490/Vvi02g00119 (PYL8-like-b) and Vitvi05_01chr15g17650/Vvi15g0097 (PYL8-like-c) and ordered synthetic genes (GeneArt Gene Synthesis, Thermofisher) in the pMK vector backbone. The coding sequence of the receptor was NcoI-EcoRI subcloned into the *E. coli* expression vector pETM11. His-tagged VviPYL1-like, VviPYL4-like-b, VviPYL8-like-a, VviPYL8-like-b, VviPYL8-like-c ABA receptors and Δ N-HAB1 PP2C were purified using

a Ni-NTA affinity chromatography as described by Santiago et al., (2009). Protein purification was evaluated using SDS-PAGE, followed by Instant Blue staining. To test receptor activity, PP2C inhibition assays were conducted. Phosphatase activity was measured using pNPP (25 mM) as a substrate, 1 mM MnCl_2 and 1 μ M of the PP2C and 2 μ M of each receptor. Proteins were incubated with the indicated agonists for 10 minutes at room temperature, and the pNPP substrate was added to start the duplicated reactions in at least two independent experiments. The absorbance of the hydrolysis product was monitored at 405 nm for 20 minutes in a ViktorX5 plate reader.

2.4 | Protein purification, crystallization, structure solution and refinement of VviPYL1:ABA complex

Escherichia coli BL21 (DE3) cells were transformed with pETM11-VviPYL1 and grown at 37°C to an optical density of 0.7 at 600 nm. Then, 0.3 mM IPTG was added and the cells were harvested after incubation overnight at 16°C. The pellet was resuspended in lysis buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 40 mM imidazole, 1 mM DTT), sonicated and clarified by centrifugation for 45 min at 10 000 g. The soluble fraction was loaded on a His-Trap HP column (Cytiva), and VviPYL1 was eluted using 500 mM imidazole. After His-tag cleavage by the tobacco etch virus protease, the protein was loaded on a Superdex 200 10/300 GL column, previously equilibrated with 50 mM Tris, pH 8.5, 150 mM NaCl, and 1 mM DTT buffer. Fractions corresponding to dimeric VviPYL1 were pooled and concentrated to 16 mg ml^{-1} . Crystals of VviPYL1 were obtained using the vapor diffusion sitting drop method, with a protein concentration of 16 mg ml^{-1} , under the precipitant solution 0.1 M sodium acetate, pH 5.0, 0.2 M magnesium chloride, 20% PEG 6000, at 18°C. A protein:precipitant volume ratio of 1:1 was used to obtain crystals of VviPYL1. Crystals of the VviPYL1:ABA complex were obtained by adding 2 mM ABA (Biosynth) to the VviPYL1 crystal droplet. Crystals were cryoprotected in the crystallization solution using 20% PEG 400, mounted on a fiber loop, and flash-frozen in N₂(l). A dataset of 2700 diffraction images was collected in an ALBA synchrotron (see details in Supplementary Table 1). The diffraction data were processed with XDS (Kabsch, 2010) and merged with AIMLESS from the CCP4 package to solve the crystal structure (Collaborative Computational Project, Number 4, 1994; Winn et al., 2011). The crystal structure of CsPYL1 in complex with ABA (PDB code 5MMX; Moreno-Alvero et al., 2017) was employed to phase the diffraction data of the VviPYL1:ABA crystal and solve its structure. The final model of VviPYL1:ABA was obtained after performing multiple cycles of restrained refinement with PHENIX (Adams et al., 2010) and manual building with COOT (Emsley and Cowtan, 2004). The stereochemistry of the crystal structure was verified with MolProbity. The figures showing the structural models of the VviPYL1 receptor in complex with the ligands were produced using PyMOL (Version 2.5.5 Schrödinger, LLC), employing the experimental structure of VviPYL1:ABA and the PDB structures 8AY3 (Lozano-Juste et al., 2023) and 5VSR (Cao et al., 2017) to display the agonists iSB09 and AMF4, respectively.

2.5 | iSB09 and AMF4 synthesis

Synthesis of iSB09, i.e., N-((1-ethyl-4-methyl-2-oxo-1,2-dihydroquinolin-6-yl) methyl)benzenesulfonamide), has been described previously by Lozano-Juste et al., (2023). The synthesis of abscisic acid mimic-fluorine derivative 4 (AMF4), i.e., N-((2-oxo-1-propyl-1,2,3,4-tetrahydroquinolin-6-yl)-1-(2,3,5,6-tetrafluoro-4-methylphenyl) methanesulfonamide) was conducted following the protocols specified in the literature (Cao et al., 2017).

2.6 | Analysis of ABA receptor expression in grapevine public transcriptomic data

The data presented here were generated in the context of the GeneCards app within the Vitis Visualization platform (<http://vitviz.tomsbiolab.com>). Briefly, metadata for public RNA-Seq experiments (Illumina platform) assigned to *Vitis* sp. were first obtained from Sequence Read Archive (SRA)-NCBI. The data were then curated to select all SRA libraries (i.e., runs) corresponding to leaf and root tissues. A total of 3772 and 367 runs were used to evaluate expression of ABA receptors in leaves and roots, respectively, across any condition. All of these runs were downloaded from SRA, trimmed using fastp (default parameters + - -cut_front_window_size 1 --cut_front_mean_quality 30 --cut_front_cut_tail_window_size 1 cut_tail_mean_quality 30 --cut_tail -l 20) and aligned to the 12Xv2 genome using STAR (default parameters + - -runMode alignReads --limitOutSJcollapsed 8000000 --limitIObufferSize 220000000). Count matrices were obtained using featureCounts (-t "exon" -C -g "featurecounts_id") using the VCOST.v3 annotation and summarizing counts at the gene level, even if only counting reads within exons. Expression data was normalized to transcripts per kilobase of exon model per million mapped reads (TPM) values.

2.7 | Statistical analysis of the means between two groups or among three groups

One-way ANOVA tests were applied to analyze the differences among treatments, followed by a Tukey's HSD test, a multiple comparison test to identify specific groups. When indicated, the Student's *t*-test was used to compare agonist-treated samples to their corresponding mock-treated line or for direct comparisons involving only two groups.

3 | RESULTS

3.1 | An update on the gene family encoding ABA receptors in *Vitis vinifera* L.

Several studies reported the identification of ABA receptors in the grapevine genome, leading to a divergent nomenclature (Li et al., 2012; Boneh et al., 2012; Zhao et al., 2020; Zhang et al., 2021).

Using the latest reference genome published recently (Shi et al., 2023), which was assembled as a telomere-to-telomere (T2T) gap-free reference genome, we screened for the PYR/PYL/RCAR gene family of ABA receptors using *Arabidopsis thaliana* receptors as a query. As a result, we found nine genes encoding putative ABA receptors, named VviPYLs, agreeing with previous surveys performed by Zhang et al., 2021 (Figure S1). However, their amino acid sequence alignment suggests that one of them, named below as VviPYL4c, is a pseudogene because it lacks critical residues for the ABA receptor function, i.e., those forming gate and latch loops (Figure S1). This likely explains the different number of genes considered by Zhao et al., (2020) and Zhang et al., (2021), eight and nine, respectively. We provide the chromosomal location of the nine VviPYL genes across the cv. 'PN40024' T2T gap-free assembly of the grapevine genome (Figure S2).

Previous nomenclature published by Li et al., (2012), Boneh et al., (2012), Zhao et al., (2020), and Zhang et al., (2021) are very divergent, and no consensus was achieved for the naming of VviPYLs. For example, VviPYL1 (Zhao et al., 2020) and VviPYL9 (Zhang et al., 2021) refer to the same gene. Indeed, no correspondence exists in gene naming among Li et al., (2012), Boneh et al., (2012), Zhao et al., (2020), and Zhang et al., (2021) (Table S1). In the attempt to add functional information to grape PYL gene names, we constructed a phylogenetic tree, excluding the VviPYL4c pseudogene, and drew up relationships according to their proximity to each *Arabidopsis* PYL subfamily (Figure 1). Grape ABA receptors were classified into three subfamilies, as already shown in pioneering studies in *Arabidopsis*, namely PYL1-like (subfamily III, dimeric receptors), PYL4-like (subfamily II), and PYL8-like (subfamily I; Ma et al., 2009; Park et al., 2009; Rodriguez et al., 2019). Therefore, we named grape genes accordingly to facilitate a correspondence with the *Arabidopsis* subfamilies (Figure 1). Additionally, we included the correspondence with the 12X.2_VCost.v3_cds nomenclature to provide unequivocal naming of the VviPYLs (Figure 1; Table S1; GRAPEDIA portal). Next, we examined the expression levels of these receptors in public RNA-seq data, particularly in leaves and roots, given the focus of this work on abiotic stress responses (Figure 2). As a result, we found that expression of PYL-like genes was predominant in leaves and roots, but PYL1-like and PYL4-like expression was also relevant compared to PYL8-like genes, particularly in roots. Conversely, VviPYL4c and VviPYL11 expression was almost undetectable in leaves and roots (Figure 2).

3.2 | Regulation of grapevine transpiration by ABA receptor agonists under well-watered (WW) conditions

ABA receptor agonists can activate the ABA signaling pathway on demand and prime adaptive responses in plants before they experience drought or water deficit conditions. Therefore, they are promising molecules for use in agriculture in the current context of climate change. However, most ABA receptor agonists were initially developed using *Arabidopsis* and not crop plant ABA receptors as targets. Therefore, translational studies must validate their efficacy upon

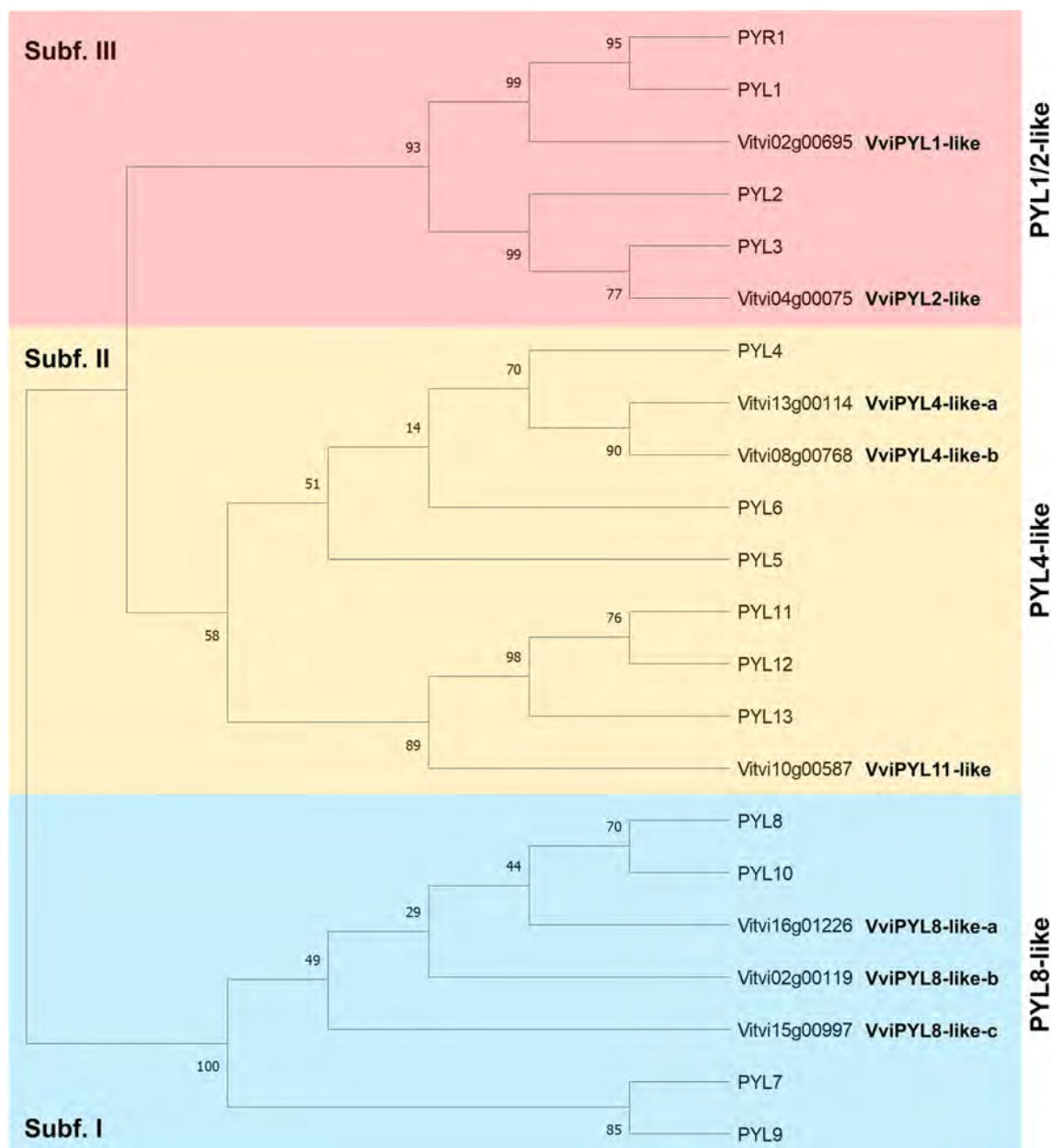
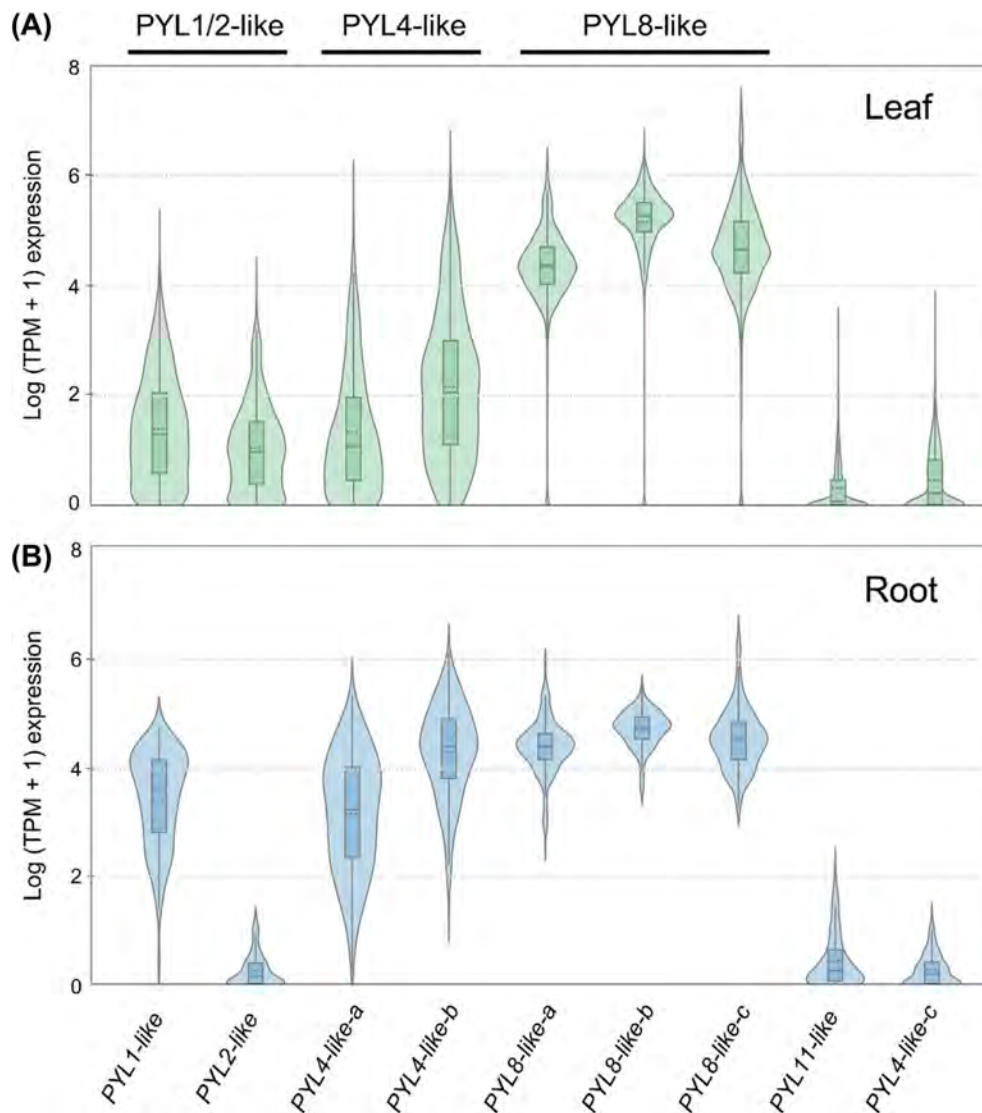


FIGURE 1 Phylogenetic relationships among PYL family members from *Vitis vinifera* and *Arabidopsis thaliana*. A maximum-likelihood tree was built from full protein sequences (MEGAx), using an LG model and 1000 bootstraps. Clades represent subfamilies I (PYL8-like), II (PYL4-like) and III (PYL1-like), respectively. Protein sequences were translated from VCOST.v3 lift-off gene models in the T2T genome assembly, found in the GRAPEDIA portal. Bootstrap values are indicated in each tree node.

exogenous application in different crops. We used IR thermography to test the effect of 50 μ M iSB09 and 100 μ M ABA foliar application on the stomatal aperture in the Bobal grapevine variety grown at the CIDE facilities (Figure 3A-B). Leaf temperature is a good readout of stomatal aperture and transpiration and can be measured by IR thermography. We detected a 4°C temperature increase in iSB09-treated plants after 24 h of iSB09 application, whereas a lower increase (1.2°C) was recorded for ABA-treated plants (Figure 3B). Next, direct transpiration measurements were performed using a LI-600 porometer at 96 h and 1 week after spraying with 30 μ M iSB09 or 30 μ M ABA under well-watered conditions (Figure 3C-E). At a 30 μ M

concentration, a significant reduction in plant transpiration was recorded after iSB09 application but not after ABA application (Figure 3C-D). Next, we used an IRGA LI-6800 device to obtain g_s , E , A_N , and WUE physiological parameters at 3 and 96 hours after the foliar application of the agonist under optimal irrigation conditions (Figure 4A-B). We recorded a reduction in g_s , E , and A_N upon spraying with iSB09, whereas the ABA effect vanished at 96 h after spraying (Figure 4B). Although g_s and E values were very low 3 h after the treatment, A_N was not affected as much as the other parameters. As a result at 96 h, iSB09 reduced only 20–25% of A_N , while WUE was approximately 3-fold higher (Figure 4B). We also tested another

FIGURE 2 Leaf and root expression of grape *PYL* genes across public transcriptomic data. RNA-Seq raw data (Illumina) corresponding to (A) leaf (3772 runs) and (B) root (367 runs) tissues were downloaded from the SRA-NCBI and reanalyzed. Violin plots show the overall frequency distribution of data points, while the inner boxplots mark interquartile ranges, mean (dotted line), and median (solid line) values.



ABA agonist in the Tempranillo grapevine variety grown outdoors at the ICVV facilities called AMF4 at 20 μ M concentration (Figure 5A-C). We also recorded a reduction of g_s , E, and A_N at 3 and 96 hours after the foliar application of the agonist under WW conditions (Figure 5A-B). Interestingly, the instantaneous water-use efficiency was higher in AMF4- compared to mock-treated plants (Figure 5 A-B).

3.3 | iSB09 treatment protects PSII after drought stress followed by rehydration

Once we established that iSB09 was effective in WW conditions in grapevine, we performed a progressive drought experiment for 15 days to test the effect of iSB09 on several physiological parameters (Figure 6). During this experiment, 30 μ M iSB09 was applied at the start of the drought period, and we introduced a single irrigation step at 25% field capacity (FC) in the middle. At 24 h after iSB09 application, we measured a reduction in g_s and E compared to mock-treated plants, which might lead to reduced soil water consumption.

At 96 h after the start of the drought experiment, both iSB09- and mock-treated plants showed a sharp reduction in g_s and E (Figure 6A-B), which led to a drop in the PSII parameter (Figure 6C). At 6.5 days, we watered at 25% FC, and physiological parameters were recorded at 7 d. Interestingly, plants treated with iSB09 at the start of the experiment could recover and showed higher g_s and E than mock-treated plants. Accordingly, the PSII and ETR parameters were higher in iSB09- than in mock-treated plants at 8 and 15 days (Figure 6C-D).

3.4 | Grapevine PYL1-like, PYL4-like and PYL8-like ABA receptors are activated by iSB09 and AMF4 agonists

ABA signaling is a universal pathway conserved in higher plants. However, ABA receptors show subtle variations among different plant species and might display different sensitivities to chemical ligands. iSB09 and AMF4 were developed by biochemical and bioinformatic screening using Arabidopsis ABA receptors (Lozano-Juste et al., 2023; Cao

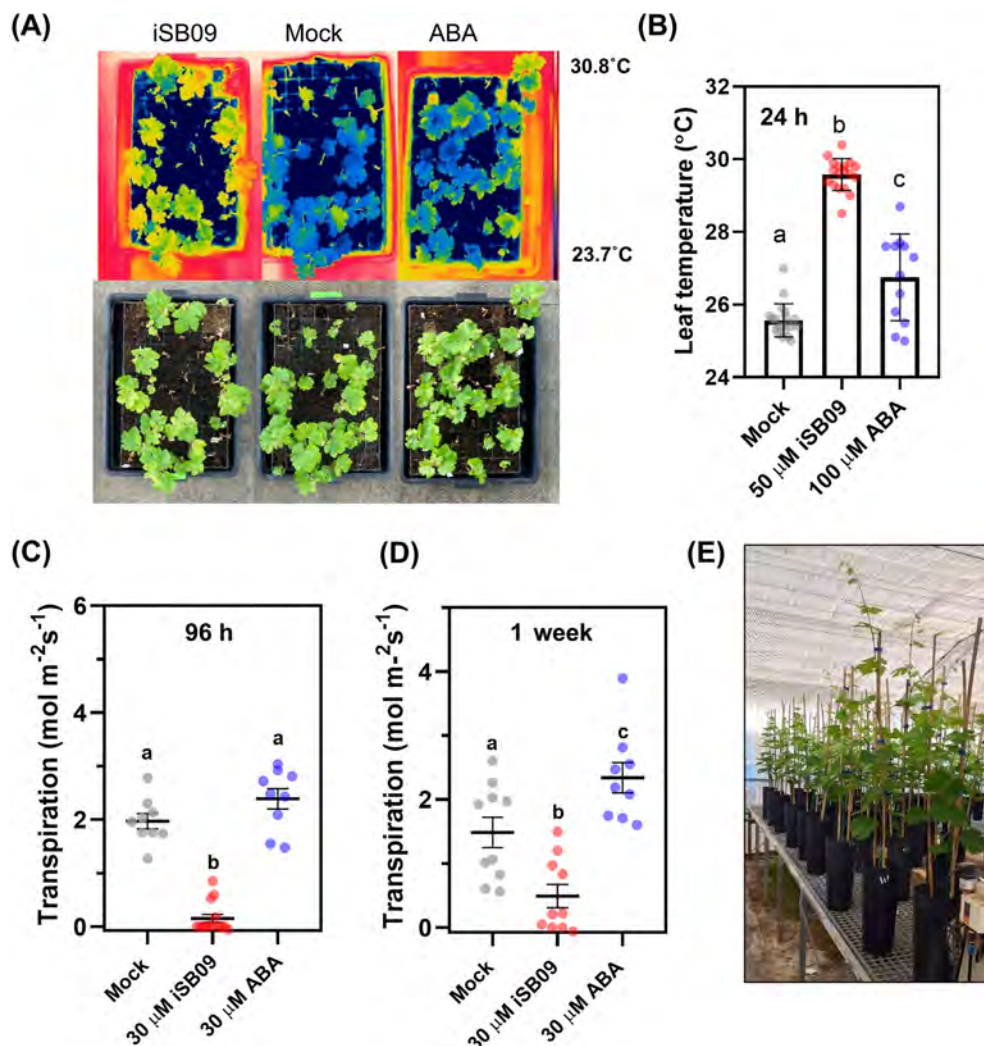


FIGURE 3 IR thermography and LI-600 measurements show that iSB09 treatment induces stomatal closure in well-watered Bobal grapevine plants. (A) iSB09 treatment is more effective than exogenous ABA treatment. IR thermography reveals a higher increase in leaf temperature in 50 μM iSB09- than in 100 μM ABA-treated plants at 24 h posttreatment. (B) Imaging quantification was conducted using FLIR Thermal Studio; bars show the mean ± SD of leaf temperature, and points represent individual data ($n = 2$ replicates, 12 plants per treatment). (C, D) The effect of iSB09 on transpiration persists for at least one week after foliar spraying. Transpiration measurements at (C) 96 h and (D) one week after mock- or foliar spraying treatment with 30 μM ABA or 30 μM iSB09 solution. Bold lines show the mean ± SD, representing individual values as points ($n = 2$ replicates, three plants per treatment). Different letters indicate a significant difference. (E) Photograph of plants used in (C) and (D).

et al., 2017). Therefore, testing these molecules with crop ABA receptors is necessary to obtain a molecular understanding of the agonist effect. To this end, we expressed five grapevine ABA receptors in *E. coli*, i.e., VviPYL1, VviPYL4b, VviPYL8a, VviPYL8b, VviPYL8c, representing the three subfamilies of ABA receptors, i.e., PYL1-like, PYL4-like and PYL8-like, purified them by Ni-NTA affinity chromatography, and performed PP2C inhibition assays with ABA, iSB09 and AMF4 (Figure 7A–B). Thus, to study the iSB09/AMF4 mechanism of action in grapevine, we tested the effect of these agonists in combination with PYL1-like, PYL4-like, or PYL8-like grapevine receptors. We conducted PP2C inhibition assays using 10 μM ABA, iSB09, or AMF4 (Figure 7A–B). PYL1-like and PYL4-like grapevine receptors were sensitive to iSB09/AMF4, inhibiting the ΔN-HAB1 PP2C activity by more than 80% and 50%, respectively (Figure 7B). Depending on the receptor considered, the PYL8-like receptors showed a specific sensitivity to iSB09/AMF4 (Figure 7A). For example, in combination with these agonists, the phosphatase HAB1 was inhibited by approximately 40–50% in the case of VviPYL8c, which is the most expressed receptor both in leaves and roots (Figure 7A). Therefore, these results suggest that the iSB09/AMF4 effect in grapevine is mediated jointly by PYL1-like, PYL4-like, and PYL8-like receptors.

3.5 | Structural insights on agonist binding to ABA receptors

To get structural insights into ligand binding to grapevine ABA receptors, we solved the crystal structure of VviPYL1 in complex with ABA (Figure 7C; Table S2). The VviPYL1:ABA structure shows that the ketone group of ABA points towards the Trp lock, the gate loop is open and the latch loop is closed, as occurs for CsPYL1:ABA (Moreno-Alvero et al., 2017). The Lys96 of VviPYL1, essential for ABA binding, establishes a hydrogen bond with ABA, and there are also some additional water-mediated interactions. Upon overlapping the structures of iSB09 and AMF4 with the crystallographic structure of VviPYL1:ABA, both ligands fit perfectly into the pocket, establishing the same interactions as ABA with the protein residues (Figure 7C–D; Figure S3). Thus, they can also interact with the conserved Lys96 of VviPYL1 and reproduce the critical binding of the ABA's carboxylate to this residue. On the other hand, with both ABA and iSB09 there is a water-mediated interaction with Glu178, which is not observed in the case of AMF4. However, an additional interaction with Glu131 occurs in AMF4 (Figure 7C, right). Accordingly, a similar pattern of interactions is generated for all ligands, which would

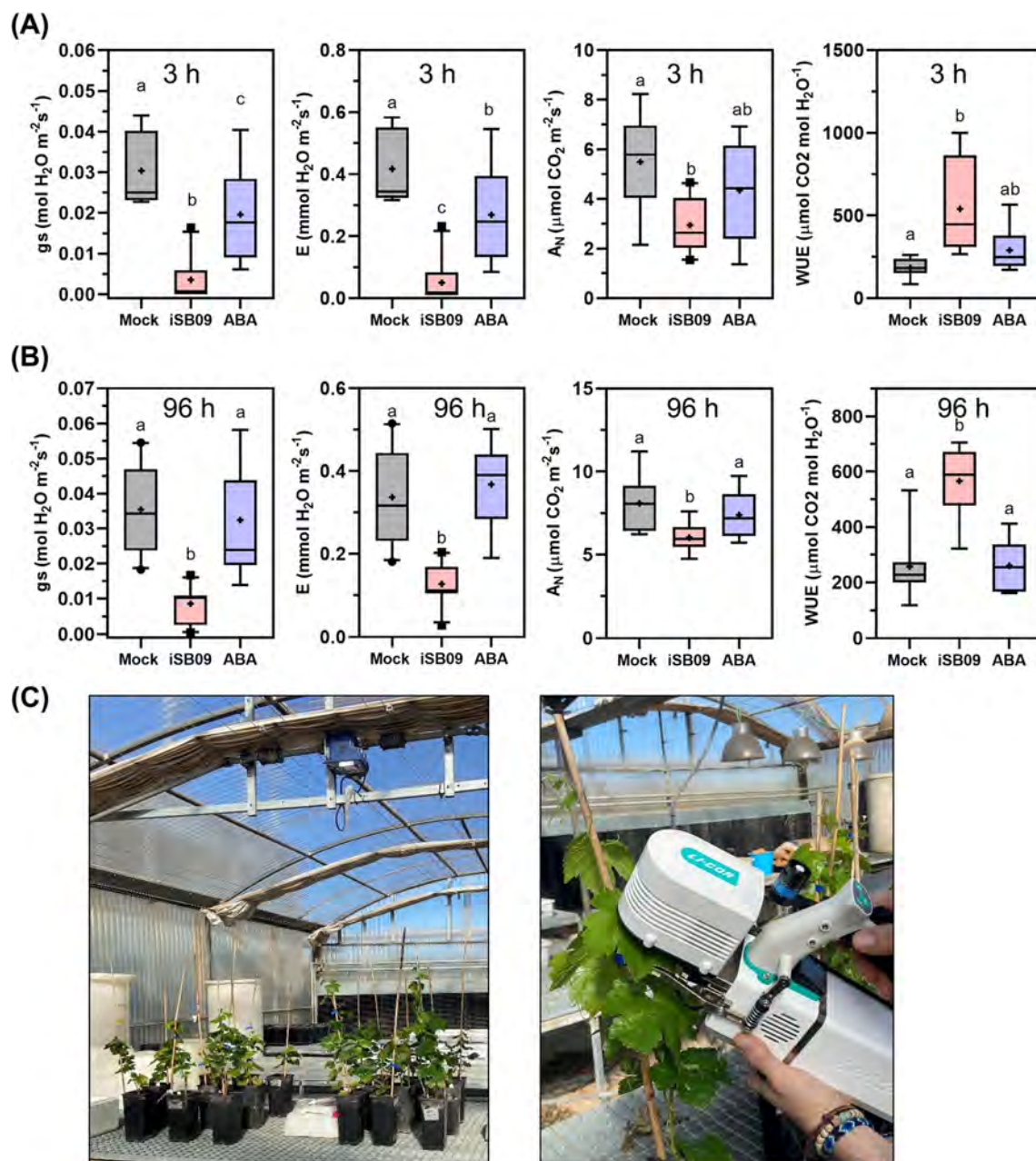


FIGURE 4 Physiological measurements in the Bobal grapevine cultivar under well-watered conditions reveal increased water use efficiency after agonist application. (A, B) The effect of mock, 30 μM iSB09, or 30 μM ABA treatment on stomatal conductance (gs), photosynthesis (A_N), leaf transpiration (E), and water use efficiency (WUE; A_N/gs) was determined at 3 (A) and 96 (B) hours after the foliar application of the agonist under optimal irrigation conditions. N = 2 replicates, three plants per treatment. Different letters indicate a significant difference. (C) Photographs of the vines in the greenhouse and the LI-6800 device used for the physiological measurements.

explain the similar inhibition of the phosphatase by VviPYL1 in their presence. The coordination of the iSB09 molecule into the ligand binding pocket of VviPYL1 displays the ligand binding pattern determined previously for other crop receptors (Moreno-Alvero et al., 2017; Lozano-Juste et al., 2023). For example, the *Citrus sinensis* (sweet orange) CsPYL1-iSB09-HAB1 or *Solanum lycopersicum* (tomato) SIPYL1-ABA complexes, indicating that different crop receptors show a similar binding to this agonist (Moreno-Alvero et al., 2017; Lozano-Juste et al., 2023).

4 | DISCUSSION

The most important effects of climate change that can already be observed in the Mediterranean and other wine-growing regions located in mid-latitude regions are water scarcity and the rise in temperature (Van Leeuwen et al., 2024). Grapevine is a woody crop with production quality standards heavily linked to specific geographical locations i.e., terroirs, which implies that vineyard growers need measures to adapt to climate change (Van Leeuwen et al., 2024). Water

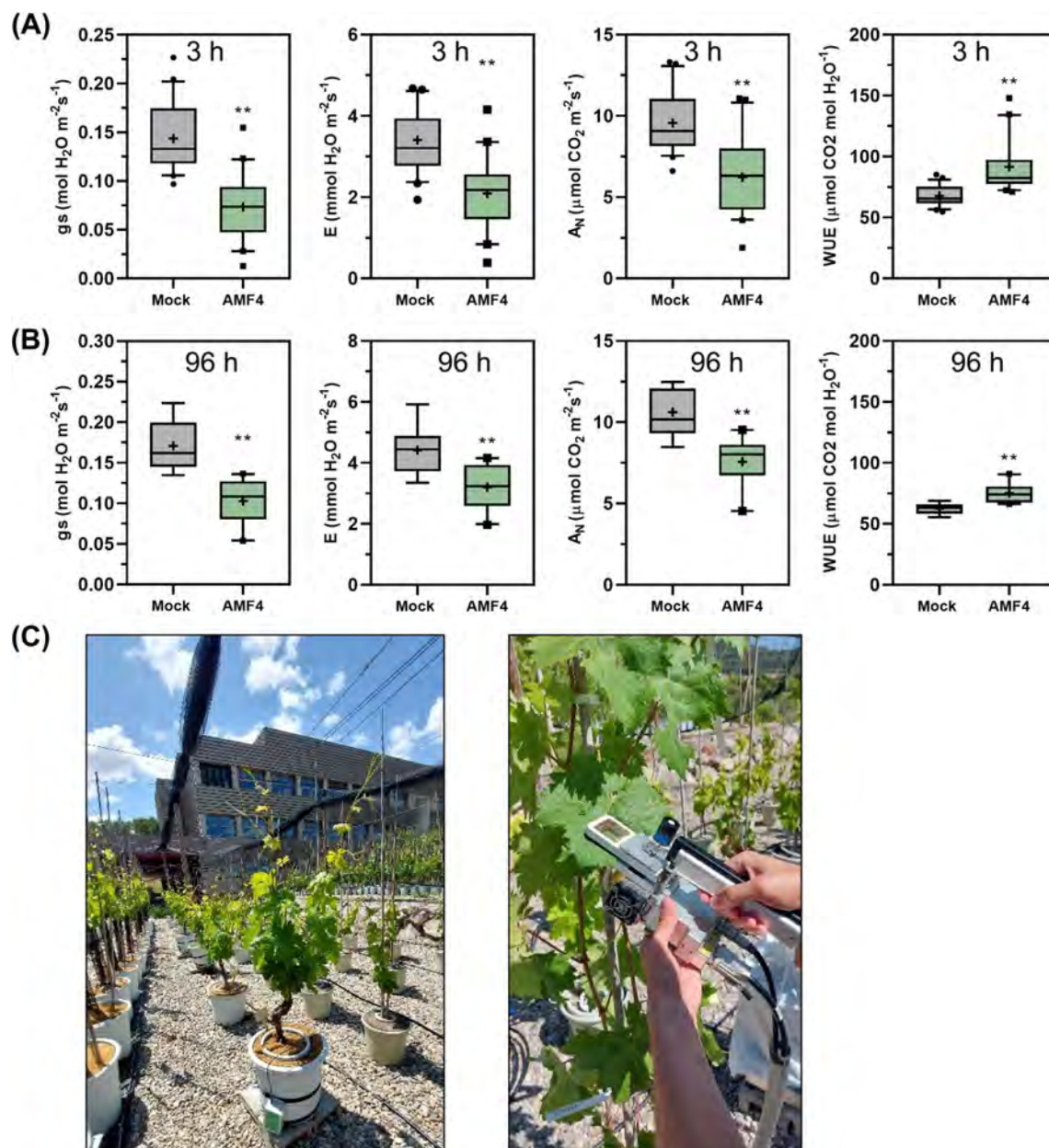


FIGURE 5 Physiological measurements in the Tempranillo grapevine cultivar under well-watered conditions reveal increased water use efficiency after 20 μM AMF4 application. (A, B). The effect of mock or AMF4 treatment on stomatal conductance (g_s), leaf transpiration (E), photosynthesis (A_N), and water use efficiency (WUE; A_N/g_s) was determined at 3 (A) and 96 (B) hours after the foliar application of the agonist under optimal irrigation conditions. $N = 2$ replicates, three plants per treatment. Student's t -test was used to compare agonist-treated to their corresponding mock-treated samples. The asterisks indicate $p \leq 0.01$. (C) Photographs of the vines grown outdoors at ICVV and the LI-6400 device used for the physiological measurements.

availability affects both vegetative and reproductive grape development. Although grapevine is considered a moderate drought-resistant crop, both high-quality production and plant survival require the availability of irrigation systems that are being implemented progressively in an increasing percentage of vineyard surfaces more and more (Gambetta et al., 2020; OIV, 2023). However, much of the world's grape production is still in regions characterized by warm and dry summers, where grapevines are exposed to drought and not irrigated (Gambetta et al., 2020). Facing water scarcity requires varietal

innovation, which involves the selection of rootstocks, varieties, and clones that are better adapted to climate change requirements. This might lead to choosing varieties with lower transpiration rates or rootstocks adapted to soil water foraging (Gambetta et al., 2020). As a complementary approach, chemical control of plant water use through the activation of ABA signaling on demand might be a promising strategy.

Different studies have been performed to understand the differences in drought tolerance between existing grapevine varieties

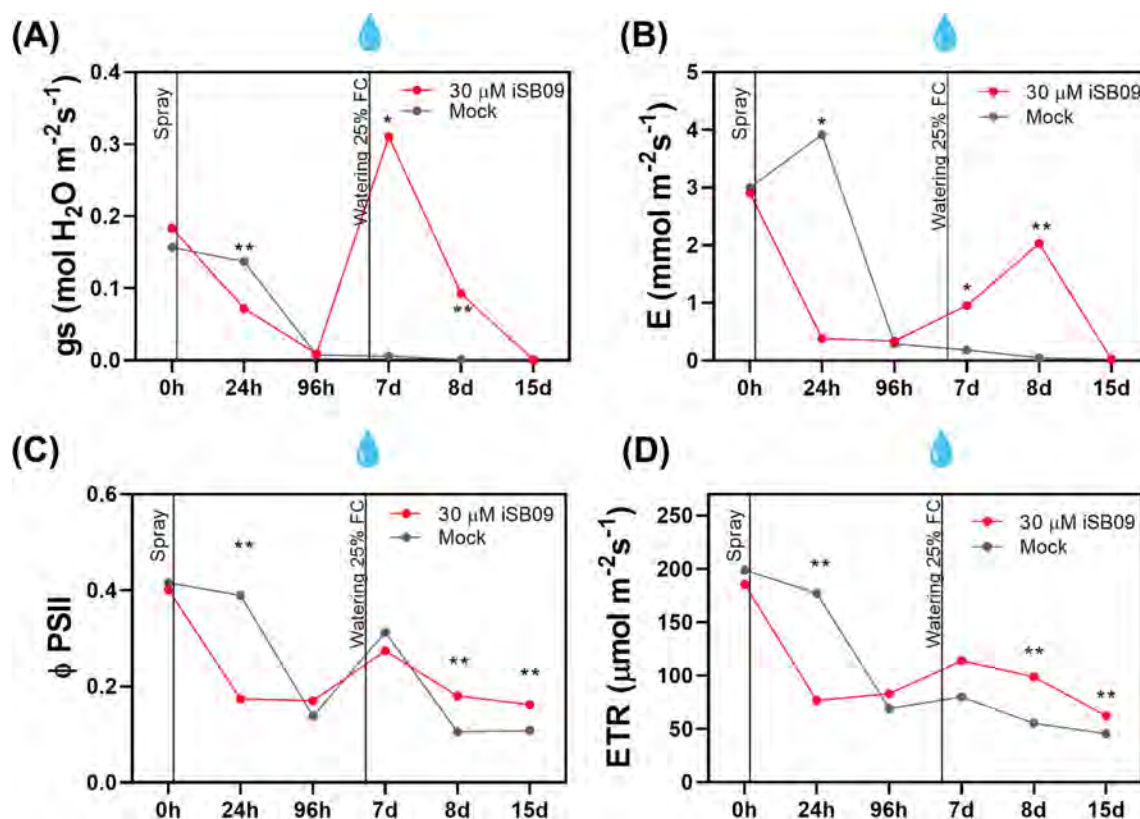


FIGURE 6 iSB09 treatment protects PSII after drought stress followed by rehydration. Measurements of (A) g_s , (B) E , (C) Φ PSII and (D) ETR in mock and 30 μM iSB09-treated Bobal grapevine plants under greenhouse conditions. Three plants per treatment were grown in greenhouse conditions for 2.5 months. At 0 h, plants were sprayed with mock (0.1% DMSO and 0.05% Tween20) or 30 μM iSB09. On the 6.5 days, plants were watered with 25% of field capacity (blue drop). Continuous measurements were taken with an LI-600 porometer (LI-COR). Points show the mean of four measures per plant (12 measurements per treatment) at each time, and asterisks reveal statistical significance between agonist- and mock-treated plants in a two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$).

(Medrano et al., 2003; Flexas et al., 2010; Gambetta et al., 2020). A general consensus from these studies is that grapevines regulate stomatal conductance to protect against severe damage, such as leaf cavitation or shedding (Hochberg et al., 2017; Dayer et al., 2020). Stomatal closure reduces transpiration and avoids critical water potential in the leaf tissues that might lead to cavitation (Gambetta et al., 2020). Therefore, it seems that approaches aimed at regulating stomatal conductance under water deficit might be promising in agriculture. The physiological results obtained in this work and the structural analysis of ABA, iSB09, and AMF4 in the ligand binding pocket of VviPYL1 indicate that ABA receptor agonists initially designed to regulate stomatal conductance in *A. thaliana* can be effectively applied to woody perennial crops.

The ligand binding pattern of iSB09 and AMF4 displays two key features for efficient binding to VviPYL1. First, the hydrogen bond between the SO_2 moiety of iSB09/AMF4 and the conserved Lys96 residue of VviPYL1. Second the orientation of the ketone group to form the hydrogen bond network that constitutes the Trp lock (Melcher et al., 2009; Moreno-Alvero et al., 2017; Figure 7C). The conserved Lys96 residue binds the ABA's carboxylate through a salt bridge, whereas in iSB09 and AMF4, the SO_2 moiety performs an analogous role (Figure 7C). The Trp lock involves a central water

molecule, which establishes hydrogen bonds with the ligand, the PP2C and the receptor (Melcher et al., 2009; Miyazono et al., 2009). Specifically, with the carbonyl oxygen of iSB09 or AMF4 ligands (reminiscent of the ABA's ketone group), the side chain of a conserved Trp residue from the PP2C, and the backbone of the gate and latch loops from the receptor (Melcher et al., 2009; Miyazono et al., 2009). iSB09 was reported to be a suitable agonist of the dimeric subfamily III of ABA receptors, e.g. AtPYL1 and CsPYL1, and monomeric receptors from subfamily II, e.g. AtPYL4 and AtPYL5 (Lozano-Juste et al., 2023). AMF4 has a similar activity range than iSB09 in Arabidopsis and was recently reported to activate PYL1-like and PYL4-like tomato receptors (Cao et al., 2017; Jimenez-Arias et al., 2023). Either PYL1-like or PYL4-like ABA receptor activation elicits stomatal closure; therefore, both iSB09 and AMF4 can regulate transpiration through the activation of these receptors (Okamoto et al., 2013; Pizzio et al., 2013; Mega et al., 2019). However, PYL8-like receptors also contribute to the quantitative regulation of stomatal aperture (Gonzalez-Guzman et al., 2012). Although iSB09 and AMF4 activate mainly PYL1-like and PYL4-like Arabidopsis receptors, ABA receptors of crops might show a different sensitivity to these agonists. For example, the PYL8-like receptor from *Phoenix dactylifera*, Pd27, was also sensitive to agonists such as quinabactin, cyanabactin, and AMF4 (Garcia-Maquilon

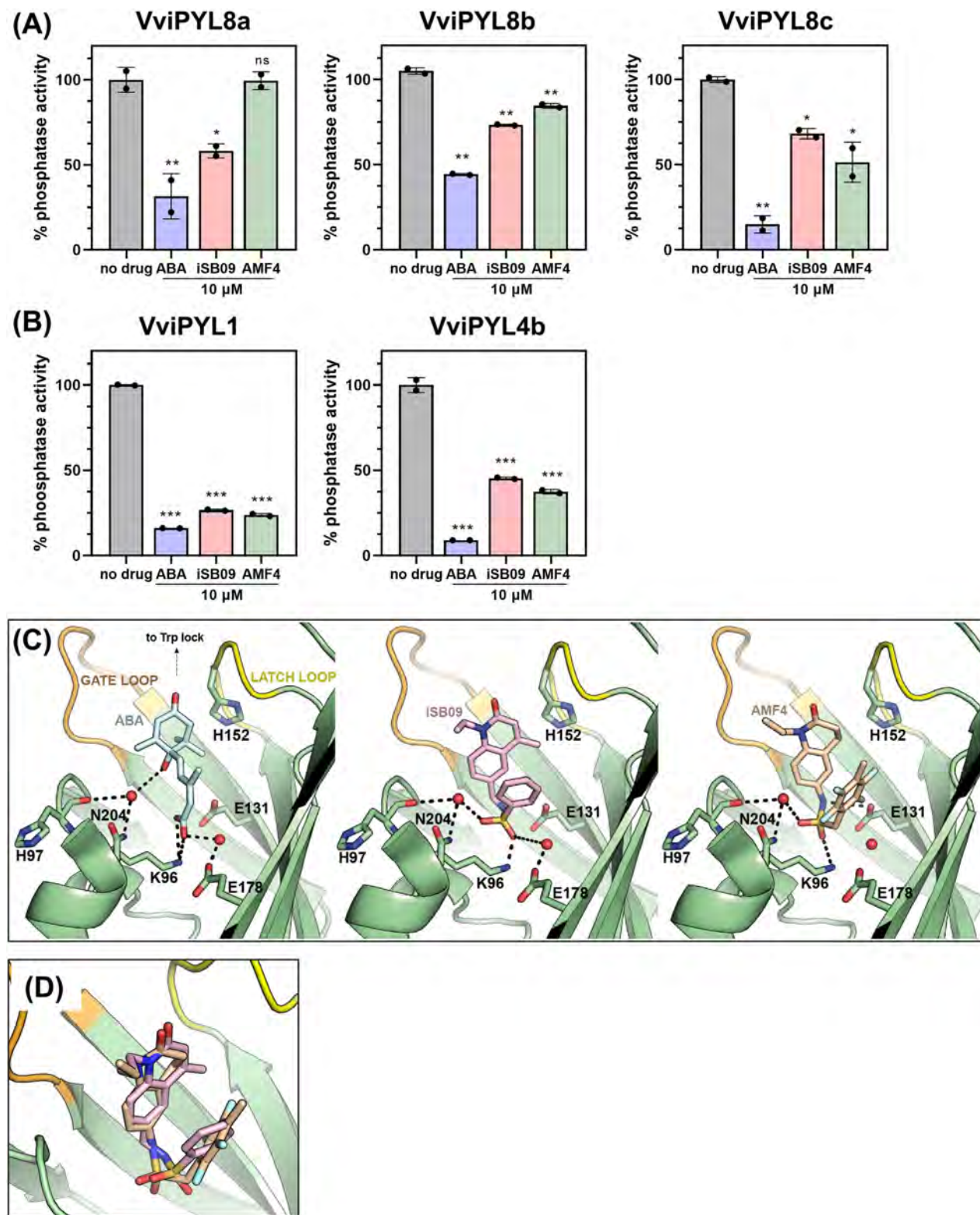


FIGURE 7 PP2C inhibition assays reveal that iSB09 and AMF4 activate VviPYL1-like, VviPYL4-like and VviPYL8-like ABA receptors. (A, B) In vitro PP2C assays in which the indicated *Vitis vinifera* receptors were incubated with Δ N-HAB1 PP2C in the absence (no drug, 0.1% DMSO) or presence of 10 μ M ABA, iSB09, or AMF4. Bars show mean $\pm$ SD, and asterisks represent different levels of statistical significance (* $p \leq 0.05$, *** $p \leq 0.001$) in a one-tailed t-test analyzing each drug against no drug treatment. (C) The pattern of interactions of ABA (light blue, left), iSB09 (pink, middle), and AMF4 (wheat, right) into the VviPYL1 pocket (green). The experimental structure of VviPYL1:ABA fulfills all the necessary requirements for proper ABA binding within the pocket. The VviPYL1:ABA complex (PDB code 9GNM) shows structural similarity to the CsPYL1:ABA complex (PDB code 5MMX) reported by Moreno-Alvero et al. (2017). (D) Superposition of the iSB09 (pink) and AMF4 (wheat) agonists into the ligand binding pocket of the VviPYL1 receptor (green).

et al., 2021). In contrast, these molecules do not activate AtPYL8 (Garcia-Maquilon et al., 2021). Therefore, our results with VviPYL8 receptors agree with those of the PYL8-like Pd27 receptor and indicate that testing the agonist molecules with several crop receptors is required to understand their effect comprehensively.

Stomatal regulation involves biochemical and hydraulic signals, and the best-known stomatal biochemical regulator so far is ABA (Buckley et al., 2019). Because of ABA's shortcomings, i.e. rapid catabolism and light-induced isomerization into inactive *trans*-ABA (Gao et al., 2016; Han et al., 2017), an emerging field exists for the development of ABA receptor agonists that show long-lasting effects for the regulation of stomatal conductance (Helander et al., 2016; Lozano-Juste et al., 2023). Under well-watered conditions, treatment with iSB09 in the Bobal variety or AMF4 in the Tempranillo variety initially led to a reduction in transpiration and a decrease in photosynthesis. In particular, photosynthesis was proportionally less affected in iSB09 than in AMF4-treated plants, although the differential vine genotype and growth conditions might also contribute to this effect. However, the instantaneous water use efficiency increased in both agonist-treated plants (Figures 4 and 5). Interestingly, in a progressive drought stress experiment followed by irrigation deficit (25% FC), agonist-treated plants recovered higher E, ETR, and quantum efficiency of photosystem II (Φ PSII) after rehydration, demonstrating a protective mechanism of photosystem II and electron transport rate that, coupled with the maintenance of E, should translate into higher A_N (Figure 6). Our results suggest that the delay in soil water consumption likely induced by the agonist treatment, together with the induction of ABA-responsive genes, exerted a protective role on the Φ PSII and ETR. For example, those are genes encoding enzymes involved in ROS scavenging or osmolyte biosynthesis. In grapevines, the photosynthesis machinery appears to be tolerant to water deficit, probably through molecular mechanisms that prevent ROS damage and involve ABA signaling. Therefore, the beneficial effect of the agonist might facilitate photosynthesis protection through the coordinated activation of the ABA response (Medrano et al., 2003). Additionally, the present results could have a practical significance for limiting water use in those periods of the growing season when soil water availability is high, i.e. in spring, to save water for later in the season when severe water deficit could be particularly detrimental for grapevine performance.

In summary, this work shows that applying ABA-receptor agonists can regulate key drought-tolerant traits such as the maximal transpiration rate and stomatal regulation. However, the activation of ABA signaling might also regulate other core physiological traits for drought tolerance of grapevine, such as the turgor loss point in leaves and root architecture (Gambetta et al., 2020). For example, ABA signaling can modify the expression or activity of aquaporins and osmolyte-synthesizing enzymes that determine osmotic pressure and water potential in leaves (Deluc et al., 2009; Grondin et al., 2015; Savoi et al., 2016 and 2017; Sanchez-Olvera et al., 2024). Moreover, ABA favors primary root elongation at low water potential and is required for hydrotropism (Spollen et al., 2000; Dietrich et al., 2017; Miao et al., 2021). Structural effects of ABA on the composition of the primary and secondary cell wall and the synthesis of suberin in the root also favor the adaptive response to water deficit

(Pizzio et al., 2024; Canto-Pastor et al., 2024). Finally, future transcriptional studies with ABA receptor agonists are required to comprehensively analyze their mechanism of action in grapevine and their influence on grape composition.

AUTHOR CONTRIBUTIONS

M.B., R.F.G., A.P., P.C-B., A.A., D.S.I and P.L.R. were responsible for the conceptualization. The methodology was performed by M.B., R.F.G., A.P., M.R-M., J.L.B., C.M., L.D., P.C-B., G.A.P., D.N-P., J.T.M., J.M. M-Z., A.A., D.S.I. and P.L.R. The investigation was done by M.B., R.F.G., A.P., M.R-M., J.L.B., C.M., L.D., P.C-B., G.A.P., D.N-P., J.T.M., J.M. M-Z., A.A., D.S.I. and P.L.R. The visualizations were performed by M.B., R.F-G., A.P., M.R-M., J.L.B., C.M., L.D., P.C-B., G.A.P. and D.N-P. P.L.R. wrote the original draft. The reviewing and editing got input from all the authors. The supervision and the funding obtention was done by R.F.G., A.P., P.C-B., A.A., D.S.I. and P.L.R. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data supporting our findings are available in the manuscript file or from the corresponding author upon request. Crystallization and structural data have been deposited in the PDB database under accession number 9GNM. The functional annotation for VviPYL1, VviPYL4b, VviPYL8a, VviPYL8b and VviPYL8c has been deposited in the Gene Reference Catalogue found at the Grape Genomics Encyclopedia portal (<http://grapedia.org/>).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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