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RESEARCH ARTICLE

# Amur grape *VaMYB4a* mediates grapevine cold tolerance *via* dual regulation of *CBF–COR* and ABA pathways

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**Highlights**

- *VaMYB4a* integrates *CBF-COR* and ABA signaling to enhance cold tolerance in grapevines.
- CRISPR/Cas9 knockout and overexpression studies reveal *VaMYB4a*'s dual regulatory mechanisms.
- ABA exerts a dual role, dynamically modulating *VaMYB4a*-mediated cold tolerance.

**Abstract**

Cold stress represents a critical constraint on crop productivity, particularly in temperate climates. Despite the established role of abscisic acid (ABA) in cold stress responses, the precise mechanisms through which transcription factors mediate ABA-dependent cold tolerance remain elusive. Here, we identify *VaMYB4a*, a *MYB* transcription factor from *Vitis amurensis* Rupr. (Amur grape), as a key regulator of cold tolerance. It integrates ABA signaling with the *CBF* (C-repeat binding factors)-*COR* (cold-regulated) pathway to orchestrate cold stress adaptation. Through a combination of overexpression and CRISPR/Cas9-mediated knockout lines in *Arabidopsis thaliana*, grape callus, and *Vitis vinifera* L. seedlings, we demonstrate that *VaMYB4a* enhances freezing tolerance by promoting osmotic regulation, reactive oxygen species (ROS) scavenging, and stomatal closure. VaMYB4a functions as a homo-dimer, with its C-terminal domain being essential for transcriptional activation. Mechanistically, VaMYB4a directly upregulates *CBF* and *COR* genes while fine-tuning ABA signaling components such as *ABI1* and *ABF4*. Notably, ABA exhibits a dual role: enhancing *VaMYB4a*-mediated freezing tolerance under short-term stress but attenuating its effects during prolonged cold exposure, revealing an intricate regulatory crosstalk between cold and hormonal pathways. Our work not only advances the molecular understanding of cold adaptation but also provides a promising genetic target for developing stress-resilient grape varieties to mitigate the impacts of climate change.

**Keywords:** *Vitis amurensis* Rupr., *VaMYB4a*, CRISPR/Cas9, ABA signaling, *CBF-COR* pathway, cold tolerance

## 1. Introduction

Cold stress is a major abiotic factor that negatively impacts plant growth, development, and productivity, particularly in temperate regions. Plants have evolved various mechanisms to cope with cold stress, including changes in gene expression, metabolite accumulation, and physiological adaptations (Yadav 2010; Miura and Furumoto 2013; Eremina *et al.* 2016). Among these mechanisms, abscisic acid (ABA) plays a crucial role in modulating stress responses, including cold stress, by regulating stomatal movement, osmotic balance, and antioxidant activities (Waadt *et al.* 2022). ABA signaling also activates several transcription factors (TFs), including MYB TFs, which are involved in regulating cold-responsive genes that enhance tolerance to low

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temperatures (Mantyla *et al.* 1995; Gusta *et al.* 2005; Buskirk and Thomashow 2006). Despite extensive research, the precise molecular mechanisms by which ABA regulates cold tolerance, especially through specific transcription factors like MYB, remain incompletely understood.

In recent years, TFs have emerged as key regulators in plant stress responses (Singh *et al.* 2002; Chen and Zhu 2004). Among them, MYB TFs have been extensively studied for their roles in various abiotic stresses, including cold stress (Dubos *et al.* 2010; Zhang *et al.* 2017, 2024; Luo *et al.* 2023; Ren *et al.* 2023; Xu *et al.* 2023; Zheng *et al.* 2023). One of the key pathways through which plants respond to cold stress involves the activation of TFs like CBFs, which regulate the expression of *COR* genes (Zhu 2016). These *COR* genes play a crucial role in enhancing plant cold tolerance by protecting cellular structures and maintaining cellular function under stress conditions (Fowler and Thomashow 2002; Maruyama *et al.* 2004; Zhao *et al.* 2016). However, the precise transcriptional regulation mechanisms downstream of ABA signaling in cold stress responses remain partially unclear. Recent studies have highlighted the involvement of MYB TFs in plant stress responses (Guo *et al.* 2017; Wang P *et al.* 2019; Yao *et al.* 2020; Liu *et al.* 2021, 2024; Wang *et al.* 2023). The MYB family, one of the largest and most diverse families of TFs in plants, plays significant roles in regulating secondary metabolism, cell morphogenesis, and stress responses (Dubos *et al.* 2010; Yan *et al.* 2019; Gu *et al.* 2020; Yao *et al.* 2020; Yang *et al.* 2023). In particular, the MYB4 subfamily has been implicated in cold stress tolerance through ABA signaling pathways (Vannini *et al.* 2004; Chen H *et al.* 2020; Li *et al.* 2020). Studies have shown that MYB4 regulates the biosynthesis of phenolic compounds, which are crucial for plant defense against various stresses, including cold stress (Jin *et al.* 2000; Colquhoun *et al.* 2011; Cavallini *et al.* 2015). By integrating signals from the *CBF* pathway, ABA signaling, and ROS scavenging, MYBs orchestrate adaptive responses to freezing stress. For example, MYB15 in *Arabidopsis* and its homolog in tomato enhance freezing tolerance *via inducer of CBF expression 1* (*ICE1*)-mediated regulation of *CBF* expression (Zhang *et al.* 2022; Xin *et al.* 2024), while *MdMYB23* in apple and *PbrMYB5* in pear support ROS detoxification and metabolic adjustments such as proanthocyanidin and ascorbic acid (AsA) accumulation to mitigate cold damage (An *et al.* 2018; Xing *et al.* 2018). Conversely, *OsMYB30* in rice suppresses cold tolerance by repressing stress-responsive genes (Lv *et al.* 2017). Post-translational modifications further refine MYB activity, as shown by *MdMYB308L* in apple, where ubiquitination regulates its function under cold stress (An *et al.* 2019). These findings highlight the regulatory versatility of MYBs in cold stress adaptation, establishing them as promising targets for improving crop resilience.

ABA, a key plant hormone, plays a critical role in regulating plant growth, metabolic activities, and adaptive responses to diverse stress factors (Chen K *et al.* 2020). One of its key functions is to promote seed maturation and prolong seed dormancy, ensuring the seed germination under favorable growth conditions (Koornneef *et al.* 2002; Nonogaki 2014; Sun *et al.* 2015). It is worth noting that ABA also regulates plant responses to multiple abiotic stresses by modulating stomatal movement, water transport, and other stress-relieving mechanisms (Sah *et al.* 2016; Xie *et al.* 2017; Wang *et al.* 2022). ABA also serves as a core regulator of cold stress, with several transcription factor families rely on ABA-dependent signaling pathway to participate in cold stress responses. More specifically, MdABI4 interacts with ICE1 and JAZ1 to regulate ABA signaling-mediated cold tolerance in apple (An *et al.* 2022). MicroRNA162 modulates stomatal conductance under low-night temperature stress through the ABA signaling pathway in tomato (Li *et al.* 2023). However, how ABA controls physiological and biochemical changes in grapevines in response to low temperature stress, and whether ABA has a complex regulatory role in modulating grapevine responses to cold stress, remains unclear.

Grapevines (*Vitis* spp.), being particularly sensitive to low temperatures, suffer from reduced yield and quality in cold climates (Fennell 2004). The ability to enhance cold tolerance in grapevines is, therefore, a crucial goal for viticulture, especially in regions prone to harsh winters. In *Vitis amurensis* Rupr., a cold-hardy grapevine species, the MYB transcription factor *VaMYB4a* has been identified as a potential regulator of cold stress response (Yu *et al.* 2022). Although VaMYB4a has been identified as a potential regulator of cold tolerance in *V. amurensis*, its functional characterization, particularly its interaction with ABA signaling pathways, remains insufficiently understood. Elucidating this relationship is crucial for uncovering the molecular mechanisms underlying VaMYB4a's role in cold stress tolerance and its regulatory dynamics within ABA signaling networks.

Our study aims to elucidate the function of *VaMYB4a* in enhancing cold tolerance *via* ABA signaling pathways in Amur grape (*V. amurensis*). By investigating the expression patterns, regulatory mechanisms, and physiological impacts of *VaMYB4a* under cold stress and ABA treatment, we aim to contribute to the understanding of the molecular basis of cold tolerance in grapevines. Understanding the role of *VaMYB4a* in cold stress tolerance has significant implications for viticulture, particularly in regions prone to sudden cold spells. This research can significantly contribute to the development of grape varieties with enhanced cold tolerance, thereby stabilizing yields and improving crop resilience. Additionally, the insights gained from this study can inform broader agricultural practices and crop improvement strategies aimed at mitigating the impacts of abiotic stress. Ultimately, this work advances our understanding of plant stress biology and offers potential applications in crop breeding programs focused on enhancing stress tolerance.

## 2. Materials and methods

### 2.1. Plant materials, growth conditions

*Vitis amurensis* Rupr. cv. 'Zuoshan-1' was cultivated in a greenhouse under controlled environmental conditions, with a temperature range of 22–28°C, humidity maintained at 60–70%, and a 16 h light/8 h dark photoperiod. Callus cell cultures of *V. vinifera* cv. 'Chardonnay' were generated and propagated as previously described (Ya *et al.* 2023). Seeds

of the *cipk3* mutant (SALK_137779C), sourced from the Nottingham Arabidopsis Stock Centre (NASC, University of Nottingham, UK), were cultivated in growth chambers under the following conditions: 22°C with a 16-h light/8-h dark photoperiod, and 70% relative humidity. The *cipk3* mutant line was in the Columbia-0 (Col-0) background.

### 2.2. Cold treatment conditions

For the *Arabidopsis thaliana*, seedlings were exposed to freezing temperatures for 12 h, with two treatment groups: non-cold-acclimated (NA) at –5°C and cold-acclimated (CA) at –7°C. Following the cold treatment, seedlings were transferred to normal growth conditions (22°C, 16-h light/8-h dark photoperiod) for 1 to 3 d to recover. For grape callus, callus cultures were pre-cultured on proliferation medium $MEL_5$ (Ya *et al.* 2023) under normal conditions (25°C, dark) for 5 d. The plates were then subjected to cold treatment at 10°C for 10 d in the dark. For tissue-cultured grapevine seedlings, both untransformed (UT) and transgenic seedlings were exposed to –5°C for 1.5 h, followed by recovery under normal growth conditions (25°C, 16-h light/8-h dark photoperiod) for 3 d. Each treatment was performed with at least 10 biological replicates per group, and all experiments were repeated three times with independent biological samples.

### 2.3. Protein expression profile assay

For the expression pattern assay, potted *V. amurensis* cv. 'Zuoshan-1' plants were placed in an incubator chamber at –2°C for cold treatment, with or without pretreatment with 50 μmol $L^{-1}$ ABA (Solarbio, Beijing, China) for 12 h before cold exposure. The middle and upper parts of one to two mature leaves were selected for collection, and samples were taken at 0, 3, 6, 12 and 24 h after the –2°C freezing treatment. All samples were immediately frozen in liquid nitrogen and stored at –80°C until further use. Total protein was extracted from the leaves using a protein extraction buffer (50 mmol $L^{-1}$ Tris-HCl, pH=8.0, 2% SDS, 5% *β*-mercaptoethanol, 10% glycerol) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were denatured by heating at 95°C for 5 min before loading onto the gel. The separated proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with blocking buffer (3% skim milk powder and 1% BSA) at 4°C for 2 h, followed by washing three times for 10 min each with TBST. Membranes were then incubated with VaMYB4a-specific antibody (ABclonal, Wuhan, China, 19000445A-1-2) at 4°C for 2 h, followed by incubation with Goat Anti-Rabbit IgG (H+L) (Transgene, Beijing, China) at 4°C for 1 h. The protein bands were visualized using the Super ECL Plus Kit (Proandy, Xi'an, China). Actin served as an internal loading control and was detected using an anti-Actin antibody (ABL1055, Abbkine, Wuhan, China). Densitometric analysis of the protein bands was conducted using ImageJ software to quantify the relative protein expression levels normalized to Actin.

### 2.4. Gene expression analysis

Total RNA was isolated from transgenic *Arabidopsis thaliana* and grape callus treated at 4°C for 3 and 12 h, using the OMEGA Plant RNA Kit (OMEGA Bio-tek, Norcross, GA, USA). Following DNase I treatment to remove genomic DNA, RNA concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using the PrimeScript™ Reverse Transcription Kit (Takara Biotechnology Ltd., Dalian, China), following the manufacturer's protocol. Reverse transcription quantitative PCR (RT-qPCR) was performed using 2× SYBR Green qPCR Mix (Aidlab, Beijing, China) on a qTOWER2.2 thermal cycler (Analytik Jena AG, Thuringia, Germany), with the following amplification conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s. *VvActin01* (GenBank accession no. XM_002282480) and *AtActin01* (TAIR: AT2G37620) were employed as reference genes for normalization. Gene expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the *Actin* reference gene. Primer sequences are listed in Appendix A, and their specificity was validated through melting curve analysis.

### 2.5. Yeast two-hybrid assay (Y2H)

The full-length coding sequence of *VaMYB4a* and its truncated forms (VaMYB4a$^{1–112}$ and VaMYB4a$^{113–252}$) were cloned into the GAL4 DNA-binding domain vector pGBKT7 and the activation domain vector pGADT7, respectively. The colonies were verified through sequencing to ensure correct insertion and reading frame. Various pairs of bait (BD-VaMYB4a, BD-VaMYB4a$^{1–112}$, BD-VaMYB4a$^{113–252}$) and prey constructs (AD-VaMYB4a and AD empty vector) were co-transformed into *Saccharomyces cerevisiae* strain Y2H-Gold using the lithium acetate transformation method, following the manufacturer's protocol (Clontech, Takara, Clontech Laboratories Inc., CA). Transformants were first plated on selective medium (SD/–Leu/–Trp) and incubated at 30°C for 3 d to confirm successful transformation.

To test for protein-protein interactions, transformed colonies were then transferred to quadruple dropout medium (SD/–Leu/–Trp/–His/–Ade) supplemented with 200 ng $mL^{-1}$ aureobasidin A (AbA), 30 mmol $L^{-1}$ 3-Amino-1,2,4-triazole (3-AT) and 4 mg $mL^{-1}$ X-α-Gal at 30°C. The presence of protein-protein interactions was indicated by the growth of colonies and a blue color change, which suggests the activation of the *GAL4* reporter gene. The experiment included positive controls with known interacting partners and negative controls with empty vectors (EV) to confirm the specificity of the interaction.

### 2.6. BiFC assay

The full-length coding sequences of *VaMYB4a* and its truncated forms were fused to the N-terminal and C-terminal fragments of yellow fluorescent protein (YFP) and cloned into the pBI221-cYFP and pBI221-nYFP vectors, respectively. The pBI221-cYFP and pBI221-nYFP vectors are widely used for BiFC assays in *Arabidopsis*, and they contain selection markers for ampicillin resistance to facilitate transformation and selection of positive transformants. The construction was

verified by sequencing to ensure correct insertion of the YFP fragments.

*Arabidopsis* protoplasts were isolated from three-week-old seedlings and transiently transformed with the BiFC constructs using the polyethylene glycol (PEG) method. Protoplasts were incubated for 16 h at 22°C after transformation, and fluorescence signals were observed at 22 h post-transformation using a Leica fluorescence stereo microscope (TCS SP8, Leica Microsystems, Wetzlar, Germany). The excitation wavelength for YFP was set to 488 nm, and emission was captured at 520–550 nm. For each construct, a control sample was included with the EV to detect background fluorescence. To ensure reproducibility, each treatment was performed in triplicate. The presence of fluorescence signal at the site of interaction was considered positive evidence of protein–protein interactions.

### 2.7. Generation of *VaMYB4a* transgenic lines

The full-length *VaMYB4a* gene was cloned into the pHZM03::EGFP vector under the control of the *CaMV35S* promoter. The pHZM03::EGFP vector is a widely used expression vector for plant transformation, containing a strong promoter for constitutive expression and an enhanced green fluorescent protein (EGFP) tag for visual screening. The vector was transformed into *Agrobacterium tumefaciens* strain GV3101 (*pSoup-p19*) using electroporation and verified by sequencing.

Transformation of *Arabidopsis* plants was performed using the floral-dip method, following the protocol described by Clough and Bent (1998). Briefly, the flowering plants were dipped into an *Agrobacterium* suspension containing $OD_{600}$=1.0 for 5–10 s, and then incubated under normal growth conditions (22°C, 16-h light/8-h dark cycle) for 48 h. Transgenic plants were selected on 1/2 MS medium containing 50 μg $mL^{-1}$ kanamycin, and T3 homozygous lines (OE-*VaMYB4a* #1, #2, and #3) were used for further analysis.

For grapevine callus transformation, the *35S::VaMYB4a*:*:EGFP* fusion expression vector was introduced into 'Chardonnay' leaf-derived calli by *Agrobacterium tumefaciens* (EHA105-*pSoup*)-mediated transformation. The procedure followed Wu *et al.* (2024) with some modifications. Specifically, the calli were immersed in the *Agrobacterium* suspension (diluted in 1/2 MS medium to $OD_{600}$=1.0) and subjected to ultrasonication for 4 min followed by vacuum infiltration at 0.8 MPa for 6 min. After a 2-d co-culture period at 22°C in the dark, the calli were screened on $MEL_5$ medium containing 30 μg $mL^{-1}$ kanamycin and 200 μg $mL^{-1}$ Timentin for two months in the dark. Transgenic 'Chardonnay' seedlings were successfully regenerated following the methods of Zhang *et al.* (2023). Positive transformants were first identified by Western blot. RT-qPCR was then used to quantify *VaMYB4a* expression in transgenic seedlings. For Cas9 lines, the genomic regions harboring the Cas9 target sites were PCR-amplified using gene-specific primers (Appendix A) and confirmed by sequencing.

### 2.8. Freezing tolerance assay

For *A. thaliana*, 14-d-old wild-type (WT) and OE-*VaMYB4a* seedlings grown on 1/2 MS medium at 22°C were subjected to two freezing treatments: non-cold-acclimated (NA) –5°C for 1 h, and cold-acclimated (CA) 4°C for 3 d followed by –7°C for 1 h. The seedlings were then returned to normal growth conditions (22°C, 16-h light/8-h dark cycle) for 2 d before being photographed. The survival rates were recorded 7 d after freezing treatments. Ion leakage rates, an indicator of membrane damage, were measured by incubating leaf discs in deionized water and quantifying solution conductivity using a conductivity meter (CPC-505, Fengmei, Beijing, China), following the protocol by Ding *et al*.(2015).

For grapevine calli, UT and transgenic calli were pre-cultured at 22°C for 5 d, with or without 50 μmol $L^{-1}$ ABA, and then exposed to 10°C in darkness for cold treatment. After 10 d, images were taken, and fresh weights were measured. The ABA concentration and treatment duration were optimized in preliminary experiments to ensure maximal treatment efficacy without causing unnecessary stress.

For grapevine seedlings (*V. vinifera* cv. 'Chardonnay'), UT, OE-*VaMYB4a* and Cas9-*VaMYB4a* lines were pretreated with 50 μmol $L^{-1}$ ABA for 12 h, then exposed to –5°C for 1.5 h in a RuMED low temperature chamber (KBS-P350, Laatzen, Germany). After the freezing treatment, seedlings were returned to normal growth conditions (22°C, 16-h light/8-h dark cycle) for 3 d. Each treatment included five biological replicates and three technical replicates. Fresh weight and visible phenotypes were recorded to assess recovery.

### 2.9. ABA sensitivity assay

Approximately 80 seeds from WT and OE-*VaMYB4a* lines were sown on 1/2 MS medium with varying ABA concentrations (0, 0.5, 0.75, 1.0, 1.5 and 2.0 μmol $L^{-1}$). The seeds were stratified at 4°C for 3 d to break dormancy and then incubated at 22°C under long-day conditions (16-h light/8-h dark). Germination rates were recorded daily for 7 d, and the ratio of green cotyledons was assessed on day 10. Seeds were considered to have germinated when the radicle emerged and at least one cotyledon was visible.

For ABA-induced stomatal closure, fully expanded leaves from three-week-old WT and OE-*VaMYB4a* plants were detached and incubated in 10 mmol $L^{-1}$ MES-KOH (pH=6.15) for 4 h, followed by treatment with different ABA concentrations (0, 1 and 10 μmol $L^{-1}$) for 2 h (Kim and Kim 2013). Stomatal apertures were measured under a light microscope, and at least 30 stomata per treatment per genotype were measured. Stomatal aperture was assessed with ImageJ software, where the ratio of the inner width to the outer length of each guard cell pair was used for calculation.

### 2.10. Physiological and biochemical analyses

To quantify osmotic regulation and oxidative stress responses, multiple assays were performed. *Arabidopsis thaliana* seedlings were subjected to freezing treatments at –5 or –7°C for 1 h. Grapevine callus was pre-cultured at 22°C for 5 d (with or without ABA) and then treated at 10°C for 10 d under dark conditions. Grapevine seedlings were treated at –5°C for 1.5 h. After treatment, physiological and biochemical parameters were assessed using commercial kits

(Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's protocol: malondialdehyde (MDA, A003-1-2), soluble sugars (A145-1-1), proline (A107-1-1), superoxide dismutase (SOD, A001-3-2), and peroxidase (POD, A084-3-1).

ROS accumulation was visualized using the nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining. For NBT staining, leaves and callus were incubated in staining solution (0.05% (w/v) NBT, 50 mmol $L^{-1}$ $Na_2HPO_4$, 0.05% Tween 20, pH=7.8) for 2 h at 37°C in the dark, followed by destaining with 95% ethanol at 70°C to remove chlorophyll. The formation of blue formazan indicated superoxide anion ($O_2^{-}$·) accumulation (Rao and Davis 1999). For DAB staining, leaves and callus were incubated in DAB solution (1 mg $mL^{-1}$ DAB, 10 mmol $L^{-1}$ $Na_2HPO_4$, 0.05% Tween 20, pH=3.8) for 6 h at 28°C. After staining, the tissues were bleached in boiling ethanol and images were taken to visualize $H_2O_2$ accumulation (Ramel *et al.* 2009). Both assays were repeated in triplicate, and the ROS accumulation levels were analyzed qualitatively by visual inspection. Quantitative measurements of ROS scavenging capacity were obtained by assessing SOD and POD activities using commercial kits (Nanjing Jiancheng Bioengineering Institute, China), following the manufacturer's protocol. Soluble sugar content was measured using the anthrone-sulfuric acid method. Proline content, a key osmoprotectant, was determined following Bates *et al.* (1973) using ninhydrin-based colorimetric quantification. MDA content was measured to assess lipid peroxidation as an indicator of cellular damage, using an assay kit (A003-1-2, Nanjing Jiancheng Bioengineering Institute).

### 2.11. Statistical analysis

Data analysis was performed using SPSS software (version 24.0, USA). Statistical significance between different groups was assessed using one-way analysis of variance (ANOVA) combined with Student's *t*-test. Significance thresholds were set at $P<0.01$. All experiments were conducted with a minimum of three biological replicates, and data are expressed as mean±SD.

## 3. Results

### 3.1. ABA synergistically enhances *VaMYB4a* expression to improve cold tolerance in *V. amurensis*

ABA is a crucial phytohormone that regulates plant responses to abiotic stress, and it can induce stress-related protein expression under cold stress (Zhu 2016). In this study, Western blot analysis was conducted to explore the effect of exogenous ABA on the expression pattern of VaMYB4a under freezing treatment. The VaMYB4a-specific antibody was used to detect native VaMYB4a expression under freezing conditions (–2°C) and a combination of freezing stress (–2°C) with ABA (50 μmol $L^{-1}$ for 12 h) in 'Zuoshan-1'. Freezing stress alone significantly altered the VaMYB4a expression profile, with a marked increase in protein levels at 3 h, a rapid decline at 6 h, followed by a resurgence at 12 and 24 h (Fig. 1-A). This temporal pattern indicates a rapid and sustained induction of VaMYB4a in response to freezing temperatures. Notably, the combination of freezing stress and ABA treatment further amplified VaMYB4a expression, with a substantial increase in protein levels as early as 3 h, and more pronounced bands at 6 and 12 h. This synergistic effect suggests that ABA acts as a potent enhancer of VaMYB4a expression under cold stress. The combined treatment led to the most significant accumulation of VaMYB4a, implying that ABA signaling may potentiate the cold stress response through upregulation of VaMYB4a. Quantitative analysis further corroborated these findings (Fig. 1-B), revealing a pivotal role for *VaMYB4a* in mediating cold stress tolerance. Moreover, our results emphasize ABA as a key regulatory factor, shedding light on the molecular mechanisms that govern cold tolerance in *V. amurensis*.

### 3.2. VaMYB4a forms a homo-dimer through its C-terminal domain

Notably, in addition to the expected 30 kD band, VaMYB4a exhibited a specific band at approximately 60 kD. The intensity of the 60 kD band was significantly higher than that of the 30 kD band, suggesting that VaMYB4a may function through

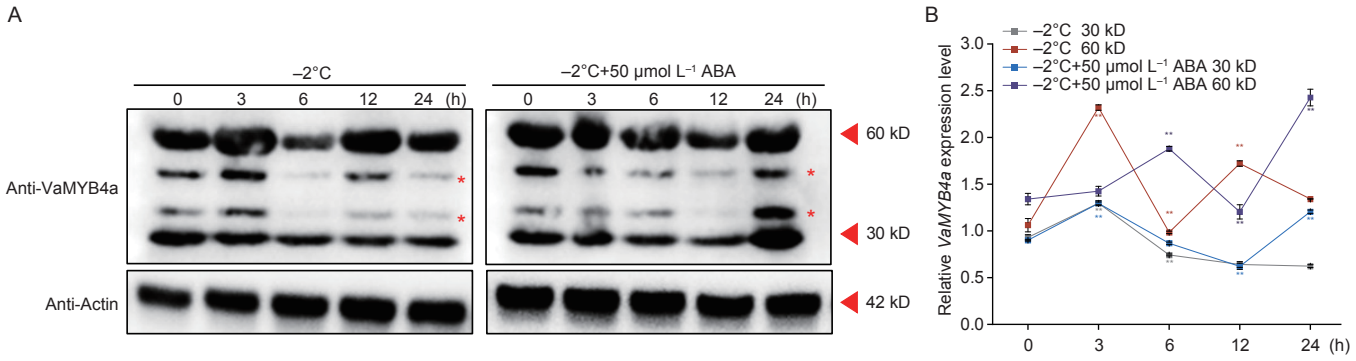



**Fig. 1 Effect of ABA treatment on VaMYB4a expression under freezing treatment.** A, expression pattern analysis of VaMYB4a protein in 'Zuoshan-1' leaves under cold stress (–2°C) and combined cold stress with ABA treatment (50 μmol $L^{-1}$ for 12 h). Total proteins were extracted at the indicated time points and subjected to immunoblotting using an anti-VaMYB4a antibody. Actin was used as a loading control. The VaMYB4a protein band (~30 kD) shows increased expression in the presence of ABA, suggesting an ABA-induced upregulation during cold stress. The asterisk denotes a non-specific band detected by the antibody. B, Western blot analysis was quantified using ImageJ and normalized against an internal loading control (Actin). Each experiment was independently repeated three times, with three replicates per experiment. Data are mean±SD ($n$=3). **, $P<0.01$.

homo-dimerization (Fig. 1-A). To explore this further, a Y2H assay was performed. Constructs of full-length VaMYB4a, and its truncated forms (VaMYB4a$^{1–112}$, containing the DNA binding domain, and VaMYB4a$^{113–252}$, containing the C-terminal regulatory region), were fused to GAL4 binding domain (BD) and activation domain (AD) vectors. Recombinant vectors were co-transformed into the Y2H-Gold yeast strain, and the transformed cells were cultured on SD/–Trp/–Leu selective medium. Afterward, the cells were plated onto SD/–Trp/–Leu/–His/–Ade/X-α-gal/3-AT medium containing 30 mmol $L^{-1}$ 3-AT to monitor protein–protein interactions.

As shown in Fig. 2-A, yeast cells co-expressing BD-VaMYB4a$^{113–252}$ and AD-VaMYB4a grew well on both selective (SD/–Trp/–Leu) and screen media (SD/–Trp/–Leu/–His/–Ade/X-α-gal/3-AT), indicating that VaMYB4a forms homodimers in yeast, mediated by its C-terminal regulatory region (113–252 amino acids). This demonstrates that VaMYB4a is capable of forming a homo-dimer. Positive controls (Po, pGBKT7–53 and pGADT7-T) grew well on both media, confirming the assay's functionality. Negative controls (Ne, pGBKT7-Lamine and pGADT7-T) did not show growth, verifying the specificity of the assay. Internal controls, such as BD-VaMYB4a+pGADT7, BD-VaMYB4a$^{1–112}$+pGADT7, and BD-VaMYB4a$^{113–252}$+pGADT7, also failed to grow on the screen media, further supporting the accuracy of the assay. Further analysis with truncated constructs revealed that VaMYB4a$^{113–252}$ could still form dimers, while VaMYB4a$^{1–112}$ could not, suggesting that the C-terminal domain (amino acids 113–252) is crucial for dimerization.

To further confirm the homo-dimerization of VaMYB4a in planta, a BiFC assay was conducted using *Arabidopsis* protoplast. Constructs of VaMYB4a and its truncated forms were fused to the N-terminal and C-terminal fragments of YFP and transiently expressed in *Arabidopsis* protoplast. As shown in Fig. 2-B, co-expression of CE-VaMYB4a and NE-VaMYB4a resulted in a strong fluorescence signal in the nucleus, confirming the interaction between VaMYB4a proteins. Similarly, co-expression of CE-VaMYB4a$^{113–252}$ and NE-VaMYB4a also produced a yellow fluorescence signal, while CE-VaMYB4a$^{1–112}$ and NE-VaMYB4a did not, consistent with the Y2H results. This indicates that the C-terminal domain is necessary for the dimerization of VaMYB4a. The control combination (CE-YFP and NE-VaMYB4a) did not produce any fluorescence, confirming the specificity of the interaction. This dimerization is essential for VaMYB4a's function and may play a critical role in its regulatory mechanisms under cold stress and ABA treatment.

### 3.3. Overexpression of *VaMYB4a* enhances freezing tolerance in *Arabidopsis* by reducing cellular damage under cold stress

To assess the role of *VaMYB4a* in freezing tolerance, we constructed a *VaMYB4a* overexpression vector (*35S::VaMYB4a*::GFP), which includes a *GFP* reporter gene for easy detection. Compared to WT protoplasts, a strong GFP signal was observed in the nucleus of *Arabidopsis* mesophyll protoplasts transiently transformed with *35S::VaMYB4a*::GFP (Appendix B-a and b). The recombinant vector was then transformed into *Agrobacterium tumefaciens* (GV3101--*pSoup-p19*) for subsequent genetic transformation (Appendix B-c).

To rapidly determine the function of *VaMYB4a* in cold resistance, we generated transgenic *Arabidopsis* lines overexpressing *VaMYB4a* (OE-*VaMYB4a* lines #1, #2, and #3). Seedlings of WT and OE-*VaMYB4a* at 14 d old were subjected to freezing stress under two conditions: NA and CA (4°C for 3 d). The phenotypes of these plants were observed and recorded (Fig. 3-A). Under NA conditions, 14-d-old seedlings grown at 22°C were exposed to –5°C for 1 h. The WT plants exhibited significant damage, with extensive wilting

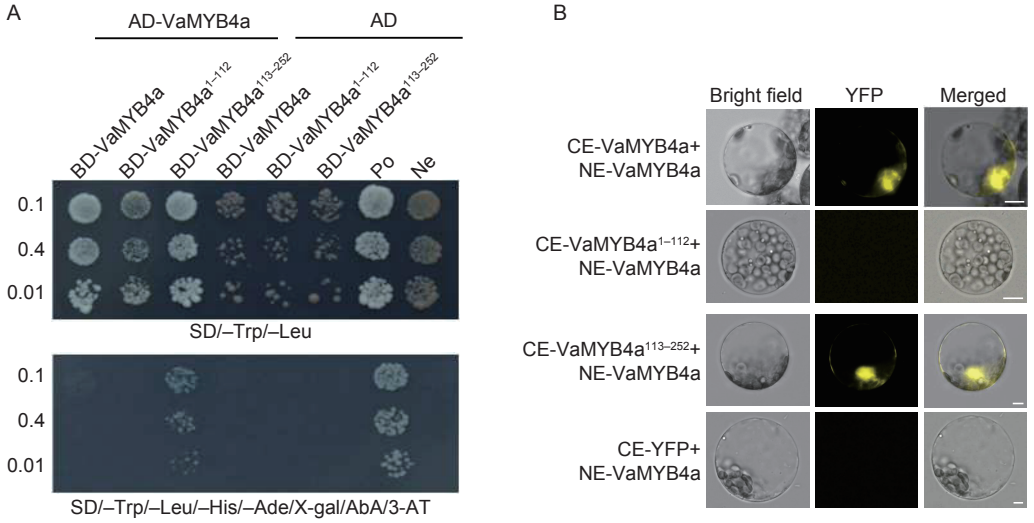



**Fig. 2 VaMYB4a forms a homo-dimer through its C-terminal domain.** A, yeast two-hybrid assay showing that VaMYB4a forms a homo-dimer. pGBKT7-53 and pGADT7-T (Clontech) were used as positive controls (Po) for protein–protein interaction. Negative controls (pGBKT7-Lamine+pGADT7-T, Ne) and internal control (BD-VaMYB4a+AD, BD-VaMYB4a$^{1–112}$+AD and BD-VaMYB4a$^{113–252}$+AD) demonstrated that VaMYB4a, VaMYB4a$^{1–112}$ and VaMYB4a$^{113–252}$ were not self-activating. B, BiFC assay confirms that VaMYB4a interacts with itself in the nucleus of *Arabidopsis* protoplast, bars=10 μm. Each sample included at least three biological replicates.

and chlorosis, whereas the OE-*VaMYB4a* lines displayed less severe symptoms, indicating enhanced freezing tolerance. Under CA conditions, 14-d-old seedlings were first acclimated at 4°C for 3 d before being subjected to –7°C for 1 h. The WT plants showed severe damage, while the OE-*VaMYB4a* lines maintained better growth and a greener appearance, demonstrating the positive effect of *VaMYB4a* on freezing tolerance after cold acclimation.

The survival rates of the WT and OE-*VaMYB4a* lines were quantified following freezing treatments. As shown in Fig. 3-B, the survival rates of the OE-*VaMYB4a* lines were significantly higher than those of the WT plants under both NA and CA conditions. Specifically, after exposure to –5°C under NA conditions, the survival rates of OE-*VaMYB4a* lines #1, #2, and #3 were approximately 60, 65, and 70%, respectively, compared to 40% in WT plants. Under CA conditions, after freezing at –7°C, the survival rates of the OE-*VaMYB4a* lines were around 70, 75, and 80%, respectively, compared to 50% in WT plants. These results indicate that overexpression of *VaMYB4a* significantly enhances the freezing tolerance of *Arabidopsis*.

Ion leakage assays were conducted to measure cellular damage in WT and OE-*VaMYB4a* lines after freezing treatments. As shown in Fig. 3-C, ion leakage was significantly lower in the OE-*VaMYB4a* lines compared to the WT plants under both NA and CA conditions. Specifically, under NA conditions, the ion leakage in WT plants was approximately 60%, whereas it was reduced to 40, 35, and 30% in OE-*VaMYB4a* lines #1, #2 and #3, respectively. Under CA conditions, ion leakage in WT plants was around 50%, whereas it was only 30, 25 and 20% in OE-*VaMYB4a* lines #1, #2 and #3, respectively. These data suggest that *VaMYB4a* overexpression reduces cellular damage during freezing stress. Collectively, overexpression of *VaMYB4a* in *Arabidopsis* enhanced its resistance to freezing stress, further supporting its role as a positive regulator of freezing tolerance.

### 3.4. *VaMYB4a* enhances cold stress tolerance in *Arabidopsis* by modulating ROS scavenging, *CBF-COR* pathway and ABA signaling

To elucidate the role of *VaMYB4a* in the cold stress response,

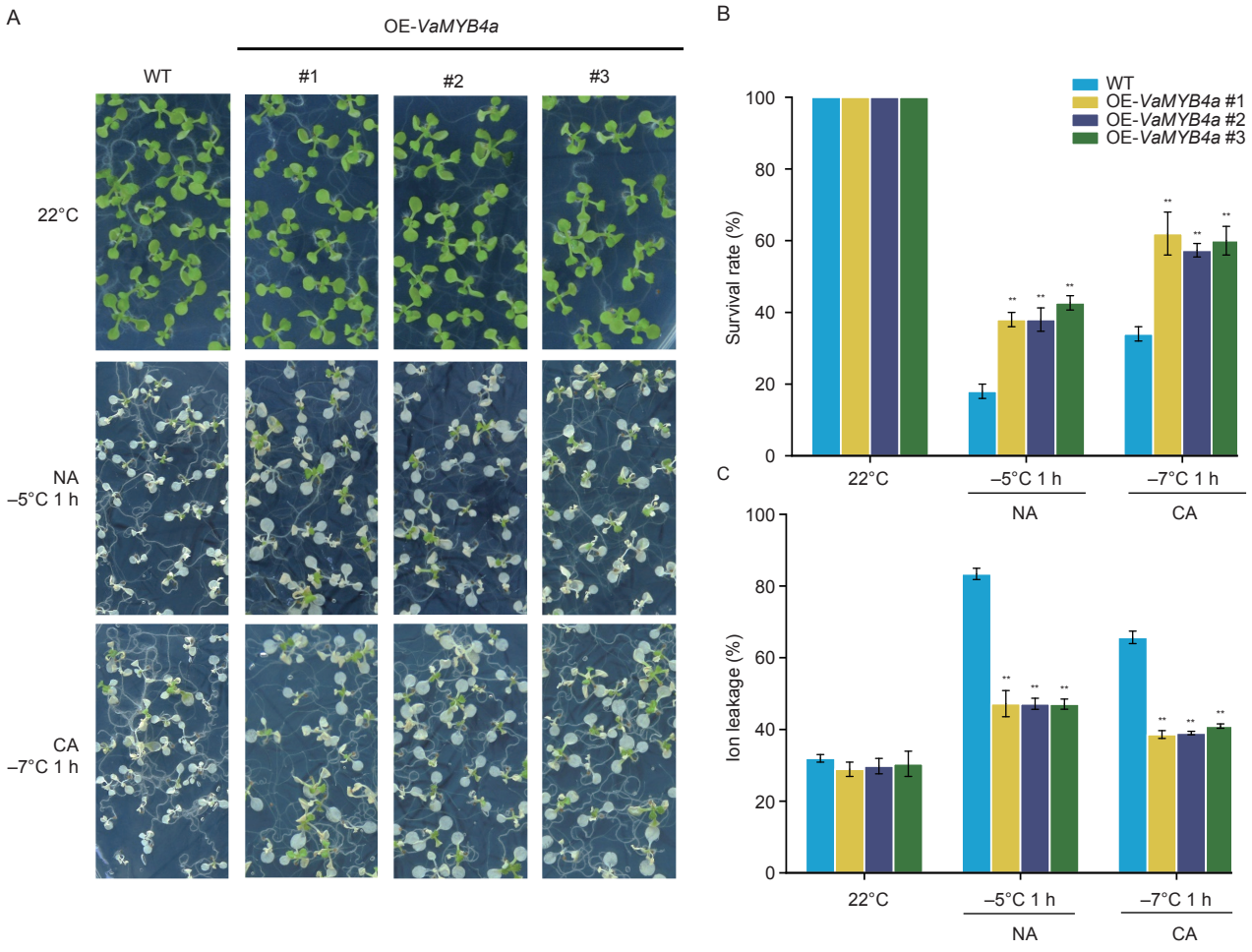



**Fig. 3 *VaMYB4a* positively regulates freezing tolerance in *Arabidopsis*.** A, phenotypes analysis. B, survival rate. C, ion leakage of the WT and OE-*VaMYB4a* transgenic lines (#1, #2 and #3). 14-d-old seedlings grown on 1/2 MS medium at 22°C were subjected to a gradient freezing treatment at –5°C for 1 h under non-cold acclimation (NA) conditions. For cold acclimation (CA), seedlings were pretreated at 4°C for 3 d, followed by freezing at –7°C for 1 h. Data are presented as the mean±SD of three independent experiments, each with three technical replicates. Asterisks indicate significant differences from the wild type under the same treatment (**, $P$<0.01, Student's $t$-test, $n$=3).

various physiological and biochemical parameters were assessed in WT and OE-*VaMYB4a* transgenic *Arabidopsis* lines subjected to cold treatment (4°C for 3 d). The MDA content, an indicator of lipid peroxidation and cellular damage, was significantly lower in the OE-*VaMYB4a* lines compared to WT under cold stress conditions (Fig. 4-A). This reduction in MDA levels suggests enhanced protection against cold-induced lipid peroxidation in the transgenic lines.

Concurrently, the soluble sugar content, which contributes to osmoprotection and energy supply under stress, was significantly higher in OE-*VaMYB4a* lines compared to WT plants (Fig. 4-B). This indicates an improved adaptive response to cold stress in the transgenic lines. ROS production was monitored using DAB and NBT staining to visualize the accumulation of $H_2O_2$ and $O_2^{-}\cdot$, espectively. Under cold stress, WT plants exhibited more intense staining, indicative of higher ROS accumulation, while OE-*VaMYB4a* lines showed reduced staining, suggesting decreased oxidative damage (Fig. 4-C and D). Consistent with the DAB and NBT staining results, enzyme activity measurements of SOD and POD also indicated that VaMYB4a enhances freezing resistance by modulating ROS scavenging enzyme activity. Specifically, the SOD activity was approximately 1.5-times higher in the transgenic plants compared to WT plants under cold acclimation conditions. Similarly, POD enzyme activity in OE-*VaMYB4a* lines was approximately 1.66 times higher than that of WT plants under freezing treatment (Fig. 4-E and F). Furthermore, the expression levels of key cold-regulated genes within the *CBF-COR* pathway (*AtCBF1*, *AtCBF2*, *AtCBF3*, *AtCOR47* and *AtKIN1*) were significantly upregulated in OE-*VaMYB4a* lines compared to WT plants under cold treatment (Fig. 4-G). This upregulation indicates that *VaMYB4a* positively regulates these genes, thereby enhancing the cold response mechanism in *Arabidopsis*.

Conversely, the expression levels of ABA-responsive genes (*AtABI2*, *AtRD22* and *AtRD29B*) were significantly lower in the OE-*VaMYB4a* lines than in WT under cold stress, suggesting a complex regulatory role of *VaMYB4a* in ABA signaling. Notably, *VaMYB4a* specifically upregulates the *ABA-responsive element binding factor 4* (*ABF4*) under low temperature (Fig.4-H). These findings suggest that VaMYB4a plays a crucial role in cold stress responses by modulating both the *CBF-COR* pathway and ABA signal transduction, providing new insights into the intricate regulatory networks that govern plant cold tolerance.

### 3.5. *VaMYB4a* enhances ABA sensitivity in seed germination and early seedling growth in *Arabidopsis*

In our previous studies, we identified that VaCIPK18, an upstream protein kinase of VaMYB4a, positively regulates plant responses to low-temperature stress and shares significant phylogenetic similarity with *Arabidopsis AtCIPK03* (85.0% sequence similarity) (Yu *et al.* 2022). Additionally, we obtained *cipk3* mutants, which exhibited heightened sensitivity to ABA stress compared to WT plants. These mutants displayed reduced seed germination rates and significantly lower green seedling rates (Yu *et al.* 2022) (Appendix C-a–d). Based on these findings, we hypothesized that *VaMYB4a* might share similar biological functions in response to ABA stress.

To investigate the role of *VaMYB4a* in ABA sensitivity during seed germination and early seedling growth, WT and OE-*VaMYB4a* seeds were incubated on 1/2 MS medium supplemented with various concentrations of ABA. The seeds were first stratified at 4°C for 3 d before being transferred to 22°C for germination. The phenotypic responses and germination rates were subsequently observed and recorded.

Under increasing ABA concentrations, the early growth of OE-*VaMYB4a* seedlings was more strongly inhibited compared to WT seedlings (Fig. 5-A). At 10 d after transfer to 22°C, a noticeable reduction in the presence of green cotyledons was observed in the OE-*VaMYB4a* lines, particularly at higher ABA concentrations. In contrast, WT plants maintained a higher proportion of green cotyledons (Fig. 5-B). This suggests that OE-*VaMYB4a* seedlings are more sensitive to ABA, with early growth inhibition being more pronounced than in WT seedlings.

The germination rates of WT and OE-*VaMYB4a* seeds were also tracked over a period of 6 d under varying ABA concentrations (0, 0.5, 0.75, 1.0, 1.5 and 2.0 μmol $L^{-1}$). At 0 μmol $L^{-1}$ ABA, both WT and OE-*VaMYB4a* lines exhibited similar germination rates, reaching nearly 100% by d 6 (Fig. 5-C). However, as ABA concentrations increased, the germination rate of OE-*VaMYB4a* lines was significantly reduced compared to WT. Specifically, at 0.5, 0.75, 1.0, 1.5, and 2.0 μmol $L^{-1}$ ABA (Fig. 5-D–H), OE-*VaMYB4a* seeds showed markedly lower germination rates, indicating their increased sensitivity to ABA. These results demonstrate that *VaMYB4a* overexpression enhances ABA sensitivity, resulting in reduced seed germination and early seedling growth under ABA treatment.

### 3.6. Overexpression of *VaMYB4a* enhances stomatal closure in response to ABA-induced stress in plant

To investigate the effect of *VaMYB4a* overexpression on ABA-induced stomatal closure, WT and OE-*VaMYB4a* lines (#1, #2, and #3) were subjected to ABA treatment. The stomatal apertures were measured after treatment with various concentrations of ABA (0, 1 and 10 μmol $L^{-1}$). Photomicrographs of guard cells revealed that under control conditions (0 μmol $L^{-1}$ ABA), both WT and OE-*VaMYB4a* lines exhibited similar stomatal apertures (Fig. 6-A). However, upon ABA treatment, the stomata of OE-*VaMYB4a* lines closed to a greater extent compared to WT. At 1 μmol $L^{-1}$ ABA, the stomatal apertures of OE-*VaMYB4a* lines were significantly smaller than those of WT plants. This trend was even more pronounced at 10 μmol $L^{-1}$ ABA, where OE-*VaMYB4a* lines exhibited substantial stomatal closure, indicating an enhanced response to ABA-induced stress.

Quantitative measurements of stomatal apertures, expressed as the width-to-length ratio, further confirmed these observations (Fig. 6-B). In the absence of ABA, no significant differences were observed between the stomatal apertures of WT and OE-*VaMYB4a* lines. However, at 1 μmol $L^{-1}$ ABA, the aperture ratio was significantly reduced in OE-*VaMYB4a* lines compared to WT. At 10 μmol $L^{-1}$ ABA, the stomatal aperture in OE-*VaMYB4a* lines was further

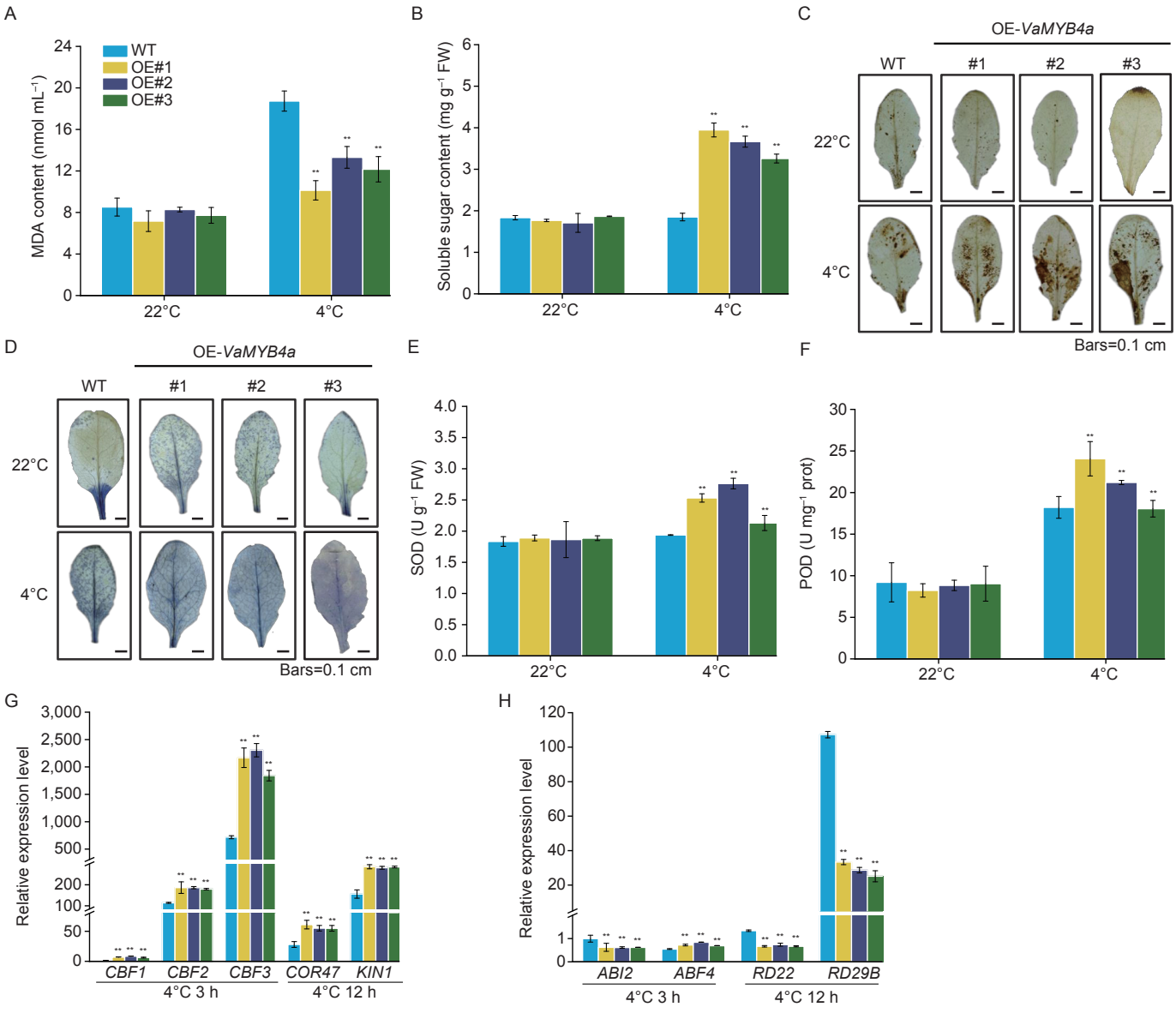



**Fig. 4 *VaMYB4a* enhances cold stress response in *Arabidopsis* through regulation of reactive oxygen species (ROS) scavenging and abscisic acid (ABA) signaling pathways.** A and B, comparative analysis of malondialdehyde (MDA; A) and soluble sugar (B) levels in WT and OE-*VaMYB4a* transgenic lines (#1, #2, and #3) under cold treatment (4°C for 3 d). C and D, peroxidase (POD) and superoxide dismutase (SOD) activity in WT and OE-*VaMYB4a* transgenic lines (#1, #2 and #3) visualized by DAB (C) and NBT staining (D) to monitor ROS production in cold-treated *Arabidopsis*. Bars=0.1 cm. Images were digitally extracted for comparison. E and F, analysis of SOD (E) and POD (F) activities in OE-*VaMYB4a* lines under normal (22°C) and cold stress (4°C) conditions for 3 d. G, *VaMYB4a* positively regulates the expression of *CBF* (*C-repeat binding factor*) and *COR* (*Cold-responsive*) genes. Expression levels of cold-regulated genes within the *CBF-COR* pathway (*AtCBF1–3*, *AtCOR47* and *AtKIN1*) were assessed in transgenic *Arabidopsis* plants exposed to 4°C for 3 and 12 h, respectively. H, *VaMYB4a* upregulates the expression of key genes in the ABA signaling pathway (*AtABI2*, *AtABF4*, *AtRD22* and *AtRD29B*) under cold stress (4°C for 12 h). Data are presented as means±SD of three biological replicates. Statistical significance was determined by *t*-test (**, $P<0.01$).

diminished, showing a highly significant difference from the WT. These results indicate that *VaMYB4a* overexpression enhances the sensitivity of stomatal closure in response to ABA, highlighting the significant role of VaMYB4a in ABA-mediated stress responses.

### 3.7. *VaMYB4a* overexpression confers cold tolerance in grape callus, but ABA pretreatment inhibits antioxidant defense and stress resistance

Based on our previous studies regarding the role of *VaMYB4a* in cold and ABA stress signaling in *Arabidopsis*, we hypothesized that *VaMYB4a* may serve as a key regulator in the crosstalk between these two stress pathways. However, there are significant differences in the regulatory mechanisms of cold stress between model plants like *Arabidopsis* and perennial fruit trees such as grapevines. Thus, findings from *Arabidopsis* cannot be directly extrapolated to grapevine. To address this, we used grape callus tissues overexpressing *VaMYB4a* as a model system to evaluate the impact of exogenous ABA application on *VaMYB4a*-mediated cold

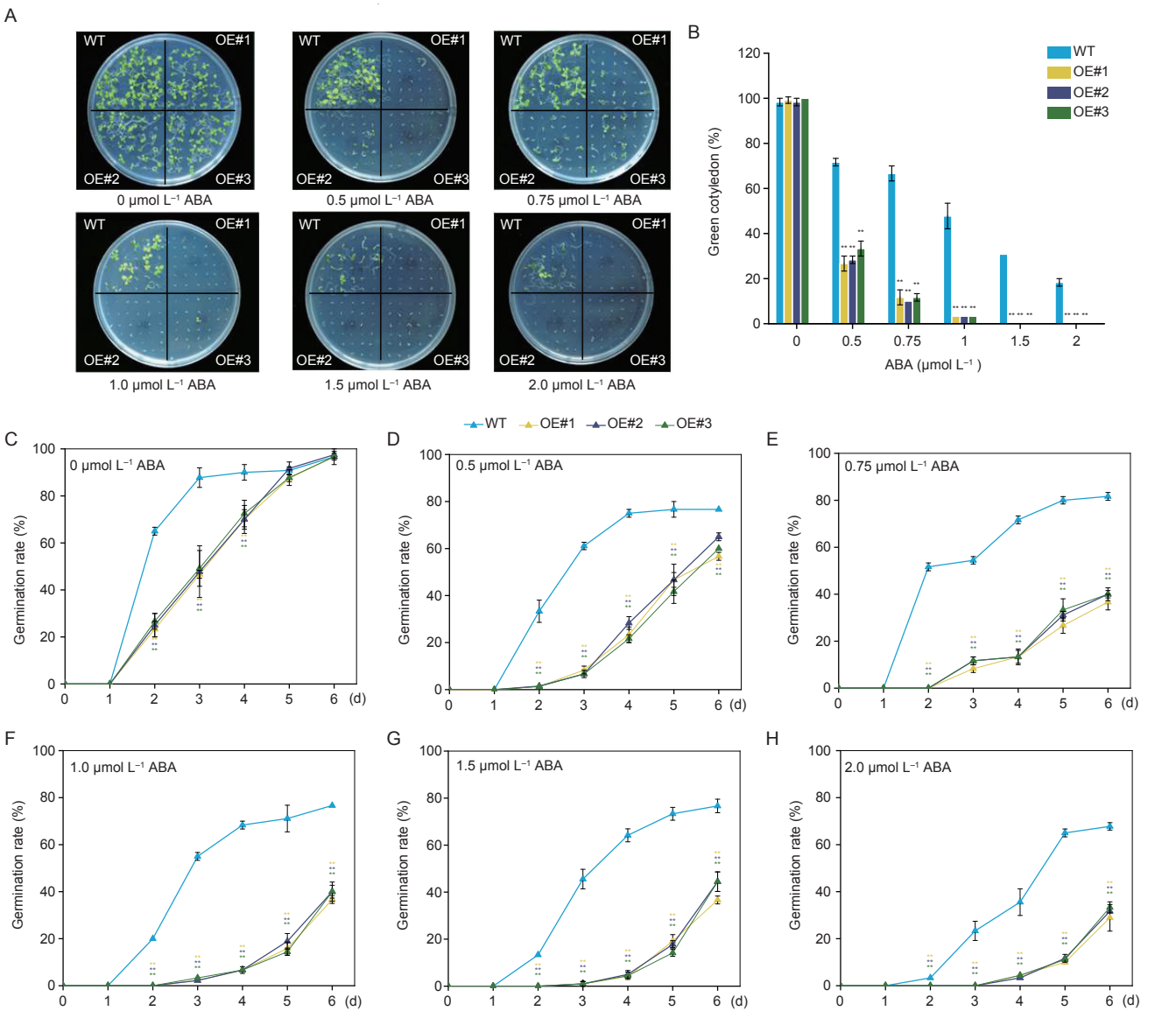



**Fig. 5** ***VaMYB4a*** **overexpression enhances seed sensitivity to abscisic acid (ABA) during germination.** A, inhibition of early seedling growth by ABA in OE-*VaMYB4a* and WT (Col-0) plants. Seeds were sown on 1/2 MS agar medium supplemented with varying concentrations of ABA and incubated at 4°C for 3 d, followed by transfer to 22°C for germination. Images were captured 10 d after transfer to 22°C. B, green cotyledon ratio of WT and OE-*VaMYB4a* seedlings under ABA stress. Seeds were grown on 1/2 MS medium containing the indicated concentrations of ABA (0, 0.5, 0.75, 1.0, 1.5 and 2.0 μmol $L^{-1}$) for 10 d. Photographs were taken on d 10. C–H. germination time course of WT and OE-*VaMYB4a* seeds on 1/2 MS medium supplemented with different concentrations of ABA, measured in days after incubation at 22°C. Results in (B) to (H) are presented as means±SD of three independent experiments. At least 50 seeds per genotype were measured per replicate. Asterisks denote significant differences (**, $P$<0.01, Student's $t$-test).

tolerance (for confirmation of *VaMYB4a* overexpression in grape callus, see Appendix D.).

To assess the effect of ABA on *VaMYB4a*-mediated cold resistance, we examined the growth and physiological responses of UT and OE-*VaMYB4a* grape callus under cold stress, both with and without ABA pretreatment. Phenotypic analysis revealed that OE-*VaMYB4a* callus maintained at 22°C showed robust growth compared to UT callus. Under chilling stress (10°C), OE-*VaMYB4a* callus growth was significantly reduced, although it still performed better than UT callus. However, when ABA was added alongside cold stress, the growth of OE-*VaMYB4a* callus was significantly impaired, exhibiting a browning phenotype. Despite this, the OE-*VaMYB4a* callus still outperformed the UT callus under these combined stress conditions (Fig. 7-A). When cold stress (10°C) was combined with 50 μmol $L^{-1}$ ABA, both UT and OE-*VaMYB4a* callus displayed severe growth inhibition and an intensified browning phenotype, suggesting that prolonged ABA exposure compromises the cold tolerance conferred by VaMYB4a. Quantitative analysis of fresh weight corroborated these findings, revealing significant reductions in fresh weight for both UT and OE-*VaMYB4a* callus under combined cold and ABA stress. However, the fresh weight of OE-*VaMYB4a* callus remained notably higher than that of UT

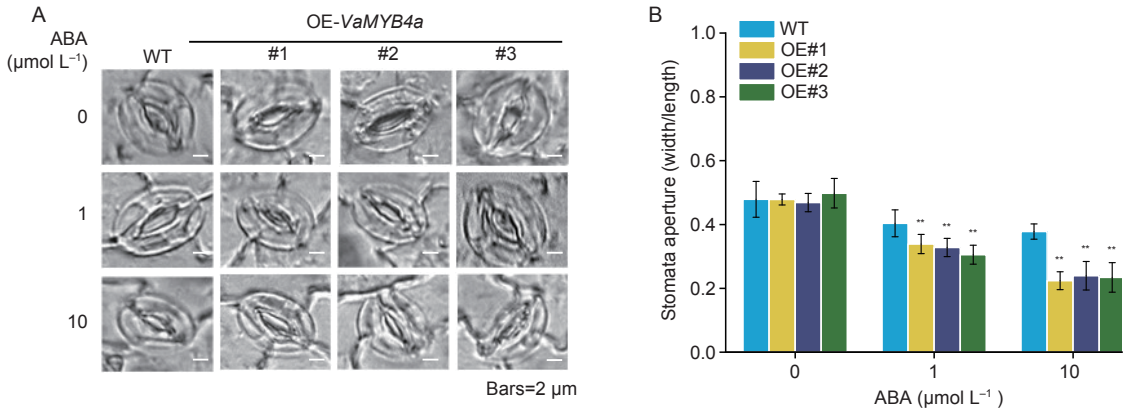



**Fig. 6 Abscisic acid (ABA)-induced stomatal closure is mediated by *VaMYB4a* overexpression.** A, stomatal closure in response to ABA in OE-*VaMYB4a* lines. WT and OE-*VaMYB4a* lines (T3, #1, #2 and #3) were pre-incubated in a solution promoting stomatal opening for 4 h, followed by treatment with ABA solutions (0, 1.0 or 10 μmol $L^{-1}$) for 2 h. Guard cells were visualized using bright-field microscopy. B, quantification of stomatal aperture in WT and OE-*VaMYB4a* seedlings after ABA treatment. Stomatal aperture was expressed as the ratio of stomatal width to length, with approximately 30 stomata analyzed per genotype. Data are presented as mean±SD. Statistical significance between WT and OE-*VaMYB4a* lines was determined using a *t*-test (**, $P<0.01$).

(Fig. 7-B).

To further investigate whether VaMYB4a regulates grapevine callus responses to cold stress by modulating antioxidant enzyme activity, we first performed qualitative analysis using NBT and DAB staining. Under normal conditions, ROS levels were low in both UT and OE-*VaMYB4a* callus. Cold stress significantly increased ROS accumulation in callus tissues, but the ROS levels in OE-*VaMYB4a* callus were markedly lower than those in UT callus, indicating that VaMYB4a positively regulates the antioxidant defense system under cold stress. When exogenous ABA was applied, ROS levels in both UT and OE-*VaMYB4a* callus increased compared to those observed under cold stress alone. However, ROS accumulation remained significantly lower in OE-*VaMYB4a* callus than in UT callus (Fig. 7-C and D). To further assess the impact of ABA on antioxidant defenses, we measured the activities of POD and SOD. As shown in Fig. 7-E and F, OE-*VaMYB4a* callus exhibited approximately 40 and 27% higher POD and SOD activities, respectively, compared to UT callus under cold stress, suggesting an enhanced antioxidant defense. However, the addition of ABA under cold stress conditions significantly reduced the activities of both enzymes in OE-*VaMYB4a* callus, indicating that prolonged exogenous ABA treatment inhibits the *VaMYB4a*-mediated antioxidant response. We also measured proline and soluble sugar contents as indicators of stress tolerance. Both proline and soluble sugar levels were significantly higher in OE-*VaMYB4a* callus under cold stress compared to UT callus. After ABA pretreatment, the levels of proline and soluble sugars in *VaMYB4a*-overexpressing callus decreased, though they remained significantly higher than in UT callus under the same treatment conditions (Fig. 7-G and H). These results indicate that *VaMYB4a* positively regulates grapevine callus responses to cold stress by enhancing antioxidant enzyme activity and osmotic regulator accumulation. However, exogenous ABA application suppresses the cold tolerance conferred by *VaMYB4a*.

### 3.8. *VaMYB4a* enhances cold tolerance in grape callus by regulating the *CBF-COR* pathway and modulating ABA signaling under cold stress

To elucidate the molecular mechanisms by which *VaMYB4a* enhances cold tolerance, we analyzed the expression of key genes involved in the *CBF-COR* pathway and ABA-dependent stress responses in UT and OE-*VaMYB4a* grape callus under three different conditions: control (22°C), cold stress (4°C), and cold stress combined with ABA treatment (4°C+50 μmol $L^{-1}$ ABA). RT-qPCR analysis revealed that the expression levels of *CBF1*, *CBF2*, *CBF3* and *CBF4* were significantly upregulated in OE-*VaMYB4a* callus compared to UT callus under cold stress (4°C) (Fig. 8-A). This suggests that *VaMYB4a* positively regulates these cold-responsive genes. However, the addition of ABA (4°C+50 μmol $L^{-1}$ ABA) led to a marked reduction in the expression of these *CBF* genes in both UT and OE-*VaMYB4a* callus. Although the transgenic lines still exhibited higher expression levels than the UT callus, the expression was notably lower than that observed under cold stress alone. These results suggest that ABA treatment dampens the *VaMYB4a*-induced upregulation of the *CBF* genes.

Furthermore, VaMYB4a modulates the expression of key genes in the ABA signaling pathway during cold stress. Specifically, VaMYB4a downregulated the expression of *ABI1*, *ABF1* and upregulated the expression of *ABI2* under cold stress (Fig. 8-B). Under combined cold and ABA stress conditions, VaMYB4a's regulatory effect on *ABI1* and *ABF1* was attenuated, while its effect on *ABI2* was enhanced. This indicates that *VaMYB4a* plays a crucial role in fine-tuning ABA signaling, particularly under cold stress conditions.

### 3.9. *VaMYB4a* enhances freezing tolerance in grape by modulating ABA signaling and accumulating osmoprotectants under freezing stress

To investigate the role of *VaMYB4a* in ABA-induced freezing tolerance, we generated transgenic grape plants overexpressing *VaMYB4a* (OE-*VaMYB4a*) and knockout

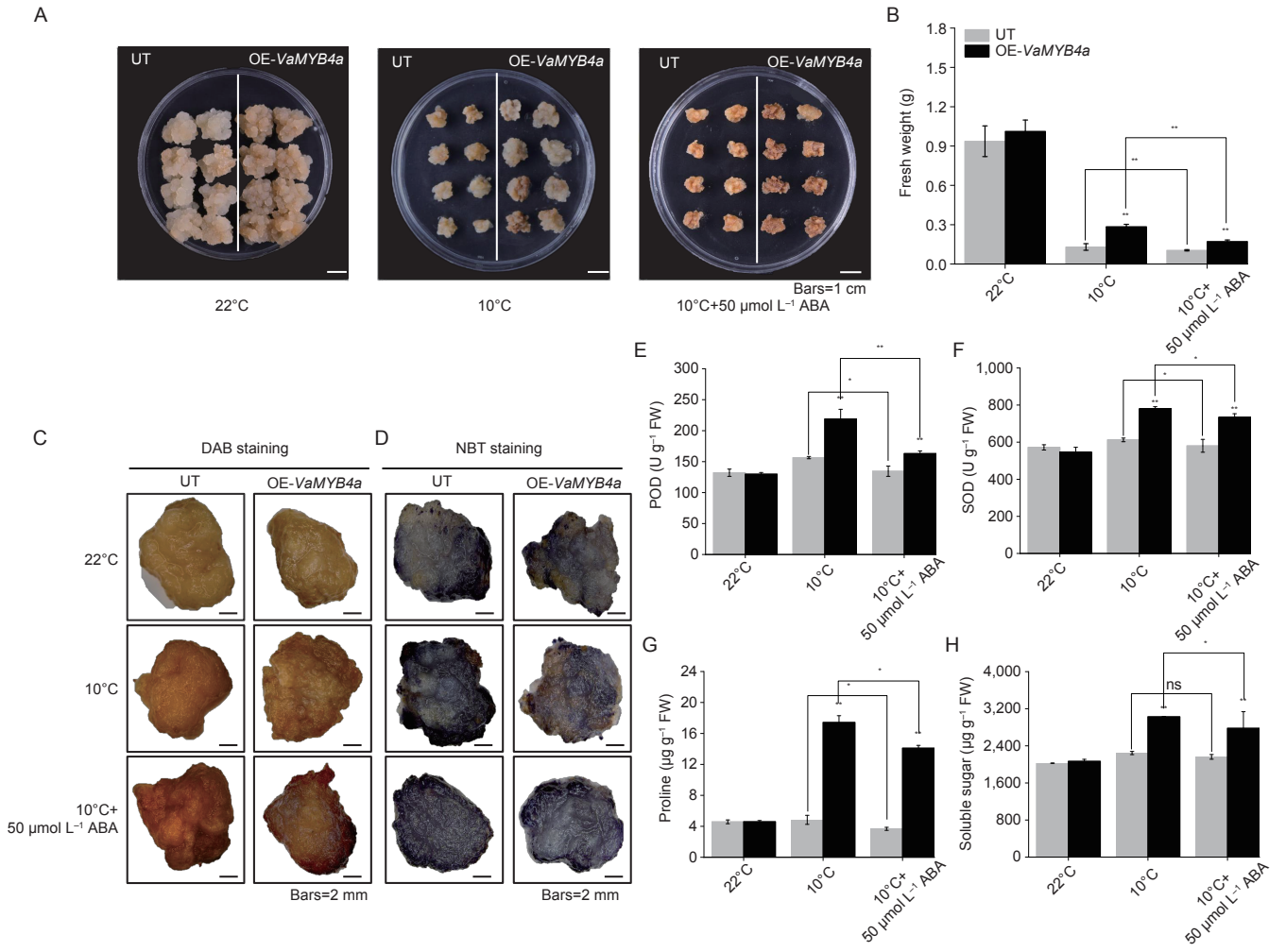



**Fig. 7 Abscisic acid (ABA) inhibits *VaMYB4a*-mediated cold tolerance in grape callus.** A, appearance of 5-d-old grape callus grown at 22 or 10°C for 10 d, with or without ABA (50 μmol $L^{-1}$). UT, untransformed; OE-*VaMYB4a*, *VaMYB4a*-overexpressing callus. B, fresh weights of the grape callus shown in (A). C and D, DAB (C) and NBT (D) staining were used to assess ROS production. Callus were immersed in DAB and NBT staining solutions for 12 h, followed by decolorization in a gradient of ethanol (70, 80 and 90%). Images were digitally extracted for comparison. E and F, analysis of peroxidase (POD) and superoxide dismutase (SOD) activities in OE-*VaMYB4a* callus under normal (25°C), cold stress (10°C), and cold+ABA stress (10°C+50 μmol $L^{-1}$ ABA) conditions for 3 d. G, proline content in UT and OE-*VaMYB4a* callus. H, soluble sugar content in UT and OE-*VaMYB4a* callus under normal, cold, and cold+ABA treatment conditions. Data are presented as the means±SD of three biological replicates. Comparison between UT and OE-*VaMYB4a* callus was represented as * $P$<0.05; ** $P$<0.01. And the asterisk with horizontal line indicated that the significantly differences between cold stress and cold+ABA combined stress.

lines using CRISPR-Cas9 (Cas9-*VaMYB4a*). The constructs for overexpression and CRISPR-Cas9-mediated knockout are shown in Fig. 9-A and B, respectively. Western blot analysis confirmed successful integration and expression of *VaMYB4a* in the transgenic lines (OE-*VaMYB4a* #99 and OE-*VaMYB4a* #99A; Cas9-*VaMYB4a* #1 and Cas9-*VaMYB4a* #11; Fig. 9-C and D). Additionally, RT-qPCR analysis showed that *VaMYB4a* expression was significantly elevated in the overexpression lines compared to UT and knockout plants, while expression in the knockout lines was reduced by approximately 80 and 91.3% compared to the UT and OE lines, respectively (Appendix E). To identify whether the *VaMYB4a* gene of the positive transgenic plants had been edited, gene-specific primers (Appendix A) were used to amplify the DNA sequence containing the target site by PCR, and then Sanger sequencing was used to identify the mutants. Compared with UT, the Cas9-*VaMYB4a* transgenic seedlings (#1 and #11) occurred 4 or 5 bp point mutation, respectively (Fig. 9-E).

Phenotypic analysis under normal conditions (22°C) revealed no significant growth differences between the OE-*VaMYB4a*, Cas9-*VaMYB4a*, and UT plants (Fig. 9-F). However, under freezing stress (–5°C for 1.5 h), OE-*VaMYB4a* plants exhibited improved tolerance with less visible damage compared to UT and Cas9-*VaMYB4a* plants. However, the Cas9 lines (#1 and #11) with the lowest expression levels of *VaMYB4a* exhibited cold sensitivity. Under combined freezing and ABA treatment (–5°C+50 μmol $L^{-1}$ ABA), all transgenic lines showed improved cold tolerance compared to UT plants, with the overexpression lines exhibiting the strongest tolerance, while the gene-edited lines remained cold-sensitive. We further evaluated proline content, a key stress marker, and found that OE-*VaMYB4a* plants accumulated significantly more proline under both

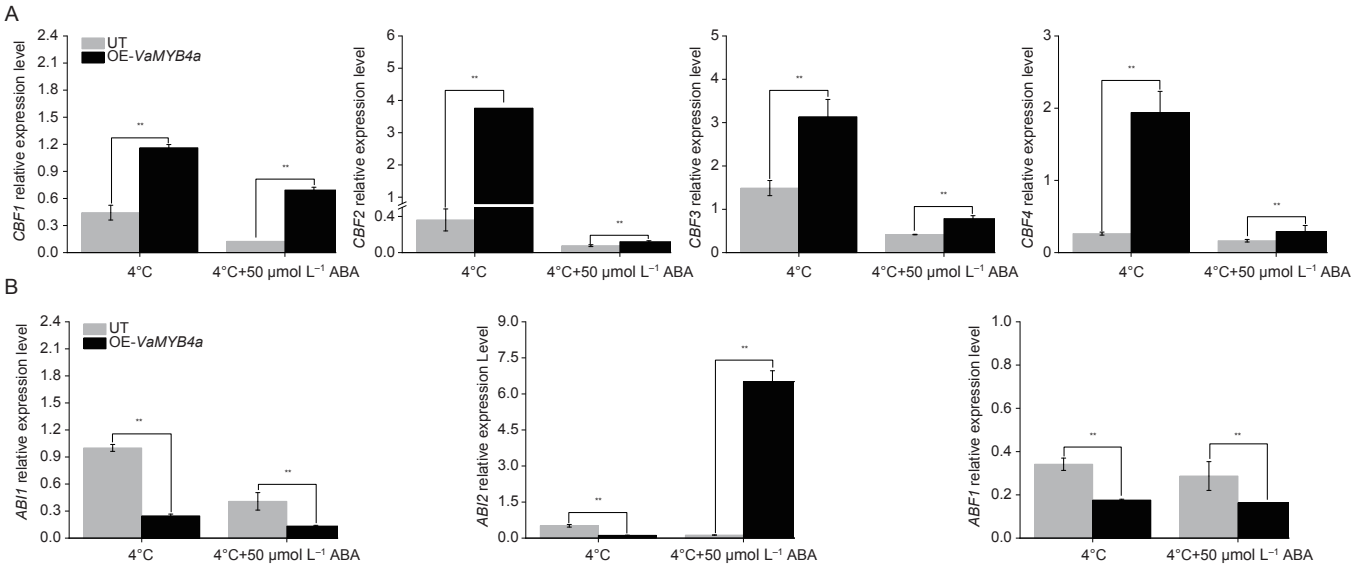



**Fig. 8 *VaMYB4a* regulates the expression of genes in the *CBF–COR* pathway and ABA (abscisic acid)-dependent signaling pathway.** A, expression levels of cold-regulated genes in the *CBF-COR* pathway (*CBF1*, *CBF2*, *CBF3* and *CBF4*) in OE-*VaMYB4a* callus under cold stress (4°C, 3 h), and combined cold+ABA treatment (4°C+50 μmol $L^{-1}$ ABA, 3 h). B, expression levels of ABA-dependent stress-related genes (*ABI1*, *ABI2* and *ABF1*) in OE-*VaMYB4a* callus compared to UT (untransformed) controls. Error bars represent the standard deviation (SD) from three biological replicates. Asterisks denote statistically significant differences in gene expression levels between transgenic lines and UT controls (**, $P<0.01$, Student's *t*-test; $n=3$).

freezing stress and combined freezing and ABA treatment than UT and Cas9-*VaMYB4a* plants (Fig. 9-G). Similarly, soluble sugar content, which provides osmoprotection, was also significantly higher in the OE-*VaMYB4a* lines under these stress conditions (Fig. 9-H).

### 3.10. *VaMYB4a* enhances ROS scavenging and antioxidant enzyme activity under cold stress and ABA treatment in grape seedlings

To further investigate the role of *VaMYB4a* in ROS scavenging under cold stress, we analyzed transgenic 'Chardonnay' seedlings overexpressing *VaMYB4a* (OE-*VaMYB4a*) and knockout lines generated *via* CRISPR-Cas9 (Cas9-*VaMYB4a*). The plants were subjected to three treatments: normal conditions (22°C), freezing stress (–5°C for 1.5 h), and combined freezing stress with ABA treatment (–5°C+50 μmol $L^{-1}$ ABA for 1.5 h). NBT and DAB staining were used to visualize ROS production. Under normal conditions, ROS levels were low in all lines (Fig. 10-A and B). Upon exposure to cold stress, ROS accumulation significantly increased, as indicated by intense staining, particularly in the UT and Cas9-*VaMYB4a* lines. However, in the OE-*VaMYB4a* lines, ROS accumulation was considerably lower, suggesting an enhanced capacity for ROS scavenging. Furthermore, the combined treatment of cold and ABA (–5°C+50 μmol $L^{-1}$ ABA for 1.5 h) led to further reduction of ROS in OE-*VaMYB4a* lines, indicating that ABA supplementation promotes ROS scavenging in these plants.

To validate the results from NBT and DAB staining, we measured the enzyme activities of SOD and POD under the same conditions. As shown in Fig. 10-C and D, knockout of *VaMYB4a* (Cas9-*VaMYB4a* lines) led to significantly decreased enzyme activities of SOD and POD under freezing stress (–5°C for 1.5 h). In contrast, the overexpression lines exhibited an increase in the activity of both enzymes, with the combined cold and ABA treatment resulting in even higher enzyme activities across all genotypes. Notably, the OE-*VaMYB4a* lines consistently showed significantly higher SOD and POD activities compared to the UT and Cas9 lines.

### 3.11. *VaMYB4a* overexpression enhances stomatal closure under cold and ABA-induced osmotic stress in grape plants

To investigate the role of *VaMYB4a* in regulating stomatal closure under cold and ABA-induced osmotic stress, stomatal apertures in UT, OE-*VaMYB4a* and Cas9-*VaMYB4a* grape plants were analyzed under three conditions: control (22°C), cold stress (4°C) and combined cold stress with ABA treatment (4°C+50 μmol $L^{-1}$ ABA). Microscopic examination of guard cells revealed that under normal conditions (22°C), all lines exhibited similar stomatal apertures (Fig. 11-A). Upon cold stress (4°C), partial stomatal closure occurred in all lines, with the OE-*VaMYB4a* plants showing a more pronounced reduction in aperture compared to UT and Cas9-*VaMYB4a* plants. When the plants were subjected to combined cold and ABA treatment (4°C+50 μmol $L^{-1}$ ABA), the OE-*VaMYB4a* lines displayed significantly enhanced stomatal closure, whereas UT and Cas9-*VaMYB4a* lines showed a less marked response.

Quantitative analysis of stomatal apertures, expressed as the width-to-length ratio, confirmed these observations (Fig. 11-B). Under control conditions, there were no significant differences in stomatal aperture between the genotypes. Cold stress led to a reduction in stomatal aperture, with the

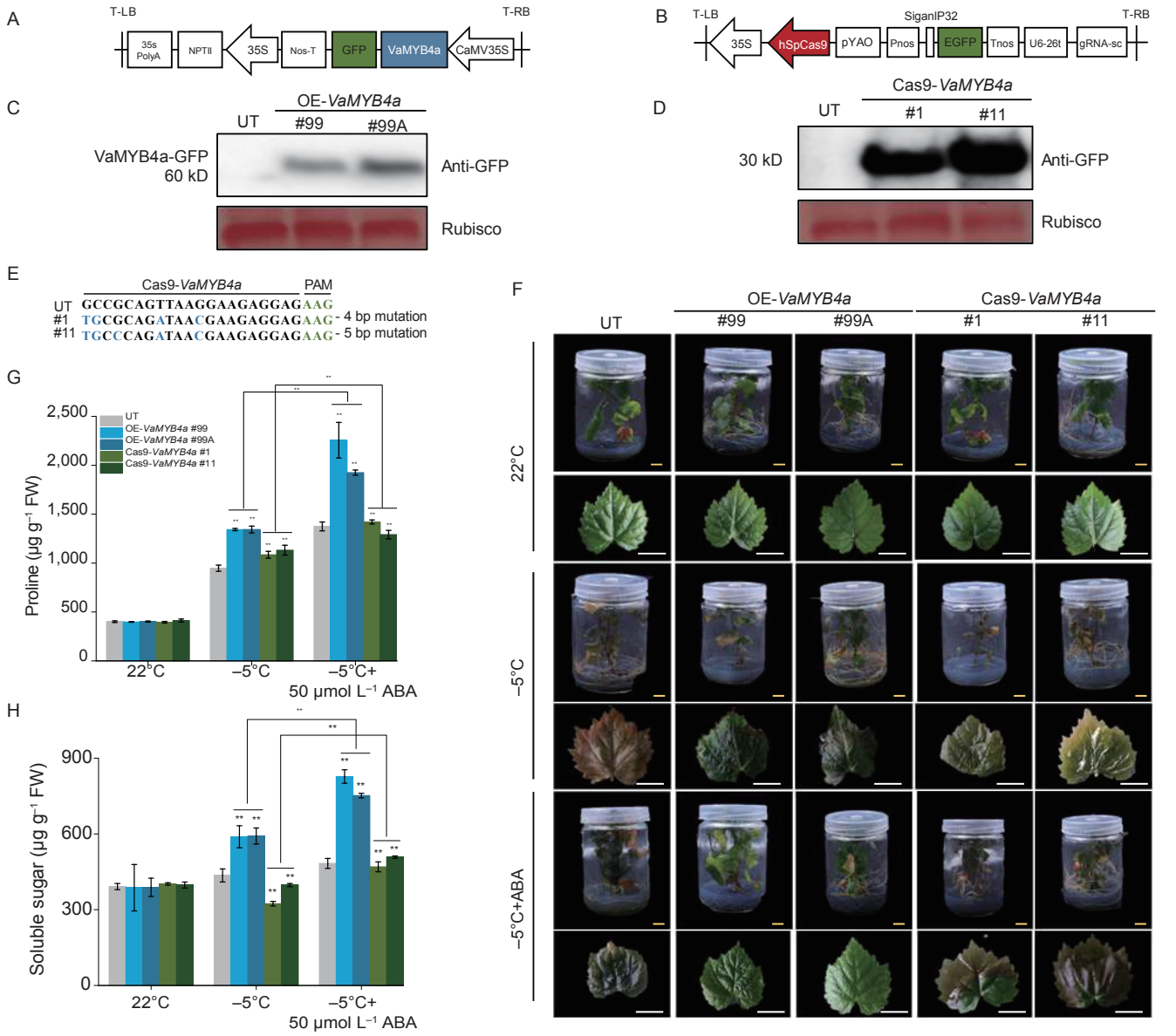



**Fig. 9 *VaMYB4a* enhances freezing tolerance through abscisic acid (ABA)-dependent signaling pathways.** A and B, schematic diagram of the recombinant overexpression and CRISPR-Cas9 vector. C and D, Western blot analysis of transgenic plants to confirm stable integration of the constructs (OE-*VaMYB4a*, *VaMYB4a* overexpression; Cas9-*VaMYB4a*, pKSE401-pYAO-hpCas9-*VaMYB4a*). E, Sanger sequencing analysis of mutation patterns at the *VaMYB4a* target sites. Sequences that could not be paired are shown in blue, while the PAM sequence is highlighted in green. F, phenotypic analysis of transgenic plants after freezing treatment (–5°C for 1.5 h) with or without ABA pretreatment (50 μmol $L^{-1}$). Yellow bar=0.3 cm, white bars=1.0 cm. Images were digitally extracted for comparison. G and H, proline (G) and soluble sugar (H) content in transgenic seedlings under normal (22°C), cold stress (–5°C) and cold+ABA stress (–5°C+50 μmol $L^{-1}$ ABA) conditions for 6 h. This experiment was repeated three times, with three replicates per genotype. **, $P$<0.01. Data were represented as mean±SD, and *t*-test was performed for comparison between UT (untransformed), OE (overexpressed) lines and Cas9 lines under the same treatment condition. The asterisk with horizontal line indicated that the significant differences between freezing treatment and freezing treatment+ABA combined stress. Asterisk on the horizontal line indicates significance when the grape seedlings under freezing treatment and freezing treatment+ABA treatment were compared.

OE-*VaMYB4a* lines showing a more substantial decrease compared to the UT and Cas9-*VaMYB4a* lines. The addition of ABA further reduced stomatal apertures in OE-*VaMYB4a* lines, indicating that *VaMYB4a* overexpression enhances stomatal sensitivity to ABA-induced closure.

## 4. Discussion

Our study reveals that the transcription factor *VaMYB4a* plays a pivotal role in enhancing cold tolerance in Amur grape through ABA-mediated signaling pathways. We show that *VaMYB4a* is upregulated in response to both cold stress and ABA treatment, where it forms a homo-dimer that orchestrates key cold stress responses and enhances freezing tolerance in grapevines and *Arabidopsis*. These findings provide new insights into the molecular mechanisms underpinning cold tolerance in grapevines, positioning *VaMYB4a* as a critical regulator within the ABA-dependent stress response network.

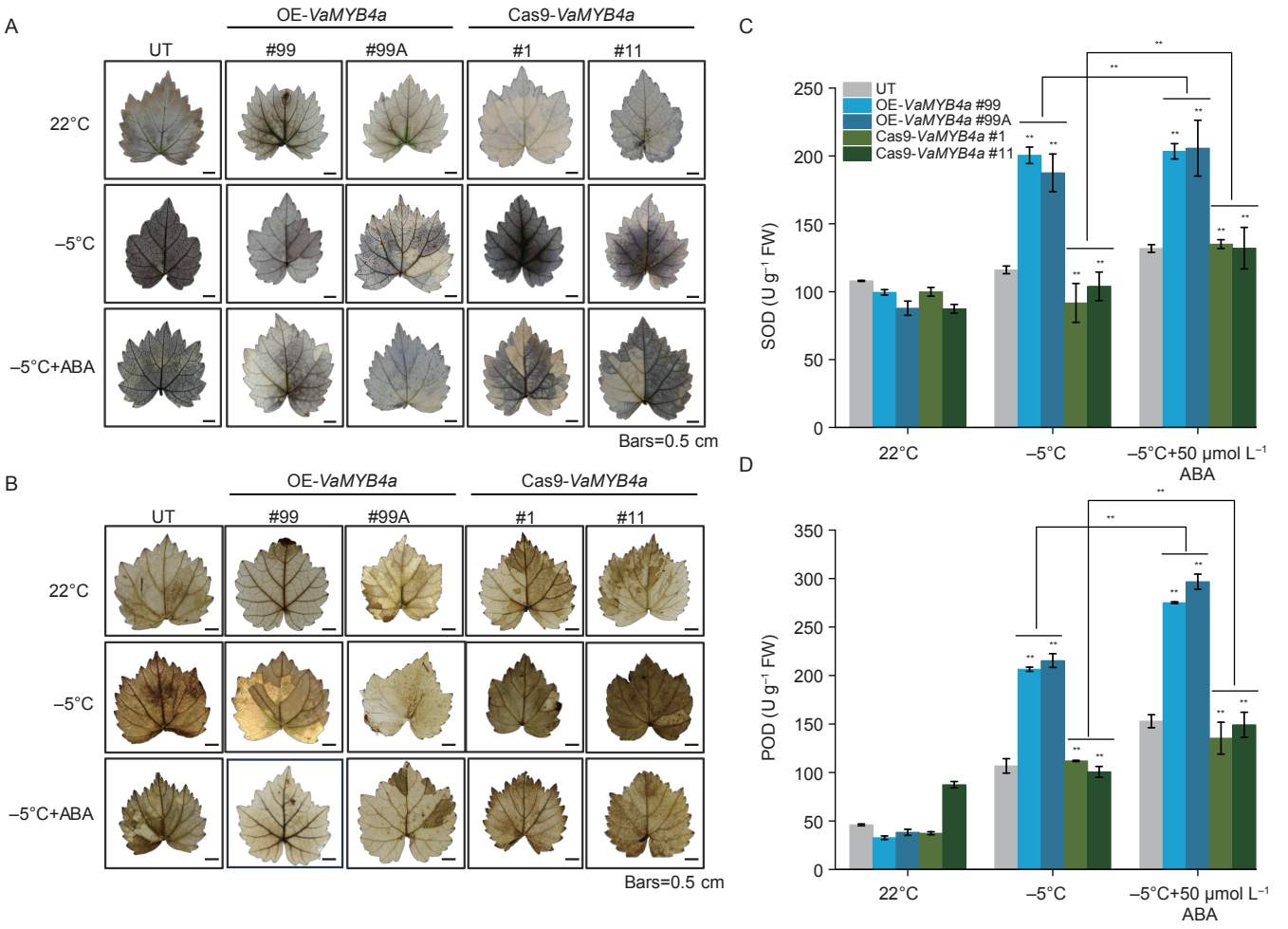



**Fig. 10 Exogenous abscisic acid (ABA) promotes reactive oxygen species (ROS) scavenging enzyme activity in *VaMYB4a* transgenic 'Chardonnay' seedlings under freezing treatment.** A and B, NBT (nitroblue tetrazolium) and DAB (3,3'-diaminobenzidine) staining detect accumulation of superoxide anion ($O_2^{\cdot-}$) and $H_2O_2$ (hydrogen peroxide), respectively. Transgenic (OE-*VaMYB4a*: #99 and #99A; Cas9-*VaMYB4a*: #1 and #11) and UT (Untransformed) seedlings were exposed to –5°C for 1.5 h, with or without ABA treatment, before staining. Samples were then cleared in a series of gradient ethanol solutions (100, 90 and 75%) and representative images were presented. Five seedlings were analyzed for each treatment. Images were digitally extracted for comparison. C and D, analysis of SOD (C) and POD (D) activities in OE-*VaMYB4a* (#99 and #99A) and Cas9-*VaMYB4a* (#1 and #11) seedlings under normal (22°C), freezing stress (–5°C), and freezing+ABA stress (–5°C+50 μmol $L^{-1}$ ABA) conditions for 1.5 h. Each experiment was performed with three biological replicates, each containing three technical replicates. Significant statistical differences are represented as ** ($P$<0.01) for comparison between UT, OE lines and Cas9 lines. The asterisk with horizontal line indicated the significantly differences between freezing treatment and freezing treatment+ABA combined stress. Asterisk on the horizontal line indicates significance when the grape seedlings under freezing treatment and freezing treatment+ABA treatment were compared (**, $P$<0.01; $n$≥3),

### 4.1. VaMYB4a positively regulates cold stress through *CBF* and ABA-dependent pathway

The *CBF-COR* signaling pathway is central to cold acclimation and plays a crucial role in plant responses to freezing stress (Cook *et al.* 2004). Previous studies have highlighted that *CBF* genes are rapidly activated by low temperatures, driving the expression of protective proteins that help safeguard plants from the detrimental effects of freezing stress (Nakashima and Yamaguchi-Shinozaki 2005; Agarwal *et al.* 2006). In addition to the *CBF* pathway, the ABA signaling pathway also plays a critical role in cold stress responses (Mittler and Blumwald 2015). For instance, SlPIF4 has been shown to promote ABA biosynthesis and *CBF* expression (Wang F *et al.* 2019), whereas GhGTG1 enhances cold tolerance by increasing ABA sensitivity (Wei *et al.* 2023). Furthermore, the expression of *CBF* genes is also regulated by the ABA signaling pathway (Zhou *et al.* 2010). Notably, both ABA and low temperatures act synergistically to upregulate the expression of *CBF/DREB1* transcription factors, thereby enhancing cold hardiness in grapevine dormant buds (Rubio *et al.* 2018). In *Hevea brasiliensis*, ABA enhances cold stress responses by positively regulating cold-regulated genes within the *ICE-CBF* signaling cascade (Wang *et al.* 2021). Additionally, the MYB96-HHP module integrates both ABA-dependent and ABA-independent signals to activate the *CBF* pathway, thereby ensuring efficient adaptation to cold stress (Lee and Seo 2015).

In this study, we dissected the function of *VaMYB4a* in regulating cold stress responses in grapevines by utilizing *Arabidopsis*, grape callus, and stable transgenic grapevine

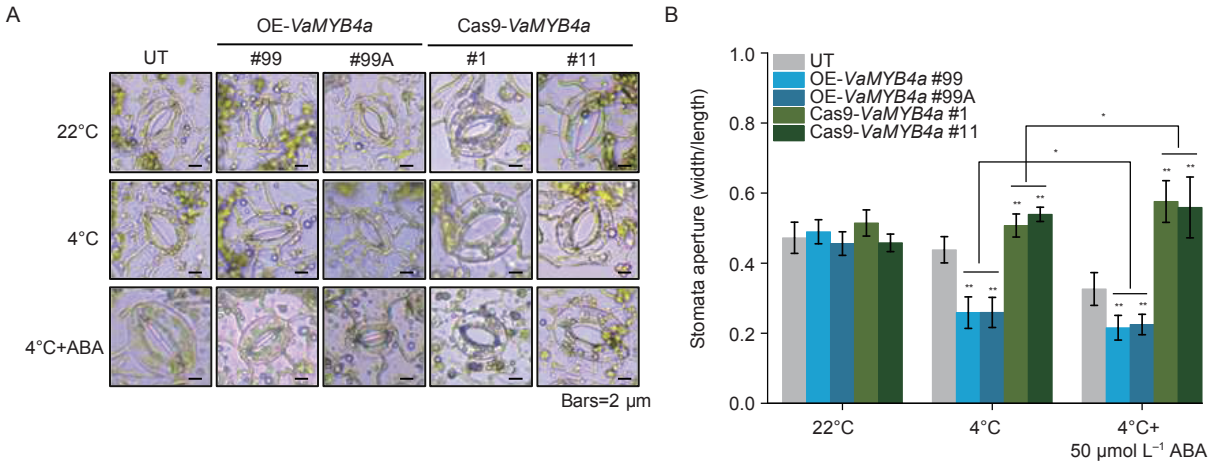



**Fig. 11 Exogenous abscisic acid (ABA) promotes stomatal closure under osmotic stress in *VaMYB4a* transgenic grape lines.** A, representative images of stomatal morphology in UT (untransformed) and transgenic seedlings at 22°C and 4°C, with or without ABA treatment. ABA-mediated stomatal closure was observed in OE-*VaMYB4a* grape lines (#99 and #99A), Cas9-*VaMYB4a* grape lines (#1 and #11), and UT plants. Seedlings were first immersed in a stomatal opening solution for 4 h, followed by treatment at 4°C for an additional 8 h, with or without 50 μmol $L^{-1}$ ABA. Guard cells were imaged using bright-field microscopy. B, measurement of stomatal aperture. Stomatal aperture in transgenic (OE-*VaMYB4a*: #99 and #99A; Cas9-*VaMYB4a*: #1 and #11) and UT leaves was calculated as the ratio of stomatal width to length. Seedlings were immersed in stomatal opening solution for 4 h and then treated at 4°C for another 8 h, with or without 50 μmol $L^{-1}$ ABA. Approximately 30 stomata were analyzed per genotype. Data are presented as mean±SD from three independent experiments, with significant differences indicated by $^{*}$, $P<0.05$ and $^{**}$, $P<0.01$.

lines. The *Arabidopsis* model allowed for rapid functional validation, leveraging its well-characterized cold stress pathways. While informative, *Arabidopsis* does not fully capture grapevine-specific mechanisms, necessitating the use of grape callus, which offers a species-relevant system for assessing *VaMYB4a* under mild cold stress. Although grape callus provides valuable insights, its undifferentiated nature limits its ability to fully represent in planta responses. Thus, we confirmed the role of *VaMYB4a* in regulating freezing tolerance in stable transgenic grapevines, which provided a more comprehensive and biologically relevant context.

Our findings demonstrate that *VaMYB4a* positively regulates cold tolerance in grapevines through both the *CBF–COR* and ABA-dependent pathways. The overexpression of *VaMYB4a* in grapevines enhances the expression of key cold stress genes, reinforcing its dual role in modulating cold acclimation. The integration of data from *Arabidopsis*, grape callus, and transgenic grapevines provides a robust framework for understanding VaMYB4a's function, with each system offering unique insights into the molecular mechanisms underlying cold stress tolerance. *VaMYB4a* is a critical regulator of grapevine cold stress responses, acting through both *CBF* and ABA-dependent pathways. Future studies should explore its long-term effects in field conditions and further elucidate its interactions with other transcription factors involved in grapevine stress adaptation.

### 4.2. Dual roles of ABA in regulating *VaMYB4a*-mediated cold stress responses

ABA, a well-known stress hormone, plays a pivotal role in plant resistance to abiotic stress by coordinating a range of physiological processes (Wani 2015). It is widely acknowledged that various abiotic stresses can trigger endogenous ABA accumulation, which in turn promotes stomatal closure to minimize water loss (Tuteja 2007; Hong *et al.* 2013). Recent studies, however, suggest that ABA may also exert negative effects on plant growth and stress responses. For instance, CBL9 (Pandey *et al.* 2004), CIPK3 (Kim *et al.* 2003) and other abiotic stress regulators (Gong *et al.* 2004; Liu *et al.* 2016; Wei *et al.* 2023) display ABA-sensitive phenotypes during seed germination. In addition, upstream kinases in the ABA signaling pathway, such as TOR and SnRK (Wang *et al.* 2021), as well as key receptor proteins like the ABA receptor PYLs (Lim and Lee 2020; Nai *et al.* 2022; Ren *et al.* 2022), play positive regulatory roles in cold stress responses. Conversely, members of the ABI gene family exhibit diverse functions in cold tolerance (Yan *et al.* 2012; Chen *et al.* 2018; De *et al.* 2021; An *et al.* 2022; Li *et al.* 2024), underscoring the complexity of ABA's regulatory role in cold stress adaptation.

Our findings demonstrate that overexpression of *VaMYB4a* results in heightened ABA sensitivity during early seedling development and in the regulation of stomatal closure. This suggests that *VaMYB4a* may play a crucial role in modulating ABA-mediated stress responses. In line with this, the key component of the ABA signaling pathway, ZmABI5, has been shown to negatively regulate abiotic stress responses by promoting chlorophyll degradation and reducing the activity of ROS scavenging enzymes under stress (Yan *et al.* 2012). Similarly, EsABI5 (De *et al.* 2021) and OsABI5 (Guo *et al.* 2023) have been described as negative regulators of stress tolerance. These studies highlight the complex regulatory role ABA plays in plant responses to abiotic stress. An unexpected observation in our study was the inhibition of *VaMYB4a*-mediated cold resistance by ABA under prolonged mild cold stress (10°C for 10 d) in grape callus. In contrast,

a short-term ABA treatment (50 μmol $L^{-1}$ for 12 h) enhanced *VaMYB4a*-mediated freezing tolerance (–5°C for 1.5 h) in grape seedlings. This dual role suggests a sophisticated regulatory mechanism wherein *VaMYB4a* modulates stress responses differently depending on the physiological context. The combined use of callus and seedling systems was pivotal for comprehensively elucidating the dual effects of ABA on *VaMYB4a*-mediated cold tolerance. Callus tissues allowed for detailed dissection of the biochemical mechanisms underpinning ABA's inhibitory effects, whereas seedlings provided critical physiological validation at the whole-plant level. This integrative approach offered a robust framework for analyzing ABA's dual role, effectively bridging molecular insights with organismal responses. Future investigations could refine this approach by consolidating analyses into a single system, such as seedlings, to enhance experimental coherence and translational relevance. Interestingly, VaMYB4a also regulates the expression of ABA-responsive genes during cold stress. The combined effect of ABA and cold stress appears to amplify this regulatory interaction, providing a mechanism that warrants further investigation.

Moreover, the formation of a VaMYB4a homo-dimer, a critical structural feature for its function, was an intriguing finding. This dimerization may influence VaMYB4a's stability, subcellular localization, or interactions with other proteins and DNA sequences, potentially providing a mechanistic basis for its regulatory activity under stress conditions (Klumpp *et al.* 2015). Further studies focusing on the structural dynamics of VaMYB4a and its interaction networks could shed light on the molecular mechanisms underpinning its role in stress tolerance.

### 4.3. The potential regulatory role of *VaMYB4a* under cold stress and cold combined ABA stress

While our study provides valuable insights into the role of *VaMYB4a* in grapevine cold tolerance, it does not fully address potential interactions with other transcription factors and signaling pathways. For example, the interplay between MYB transcription factors and other key regulatory families, such as bHLH and WRKY, could offer a more comprehensive understanding of the plant's cold stress response (Xie *et al.* 2017; An *et al.* 2018; Jiang *et al.* 2022; Zheng *et al.* 2023). Additionally, the specificity of ABA responses remains an important area for further investigation. Although VaMYB4a is shown to enhance ABA sensitivity, the precise mechanisms by which ABA modulates VaMYB4a activity and its downstream gene expression remain to be clarified. Identifying the specific ABA signaling components that directly interact with VaMYB4a could shed light on the underlying regulatory network governing these interactions.

Our study advances the understanding of the molecular mechanisms underlying cold tolerance in grapevines, particularly by highlighting the role of VaMYB4a within the ABA signaling pathways. As depicted in the proposed model (Fig. 12), exogenous ABA activates the ABA signaling pathway, which in turn triggers *VaMYB4a*-mediated stomatal closure and the upregulation of ABA-responsive genes, thereby reducing water loss during cold stress. Concurrently, *VaMYB4a* positively regulates cold-responsive genes within the *CBF* pathway (e.g., *CBF1-4*), thereby enhancing cold tolerance. This dual regulatory function of *VaMYB4a* offers significant insights into grapevine cold acclimation, with particular relevance for viticulture in regions prone to harsh winter conditions.

Future research should focus on expanding these findings to field conditions, exploring the broader applicability of VaMYB4a in natural environments. Identifying additional regulatory components involved in *VaMYB4a*-mediated stress responses, as well as investigating its interactions with other transcription factors and signaling pathways, will be critical

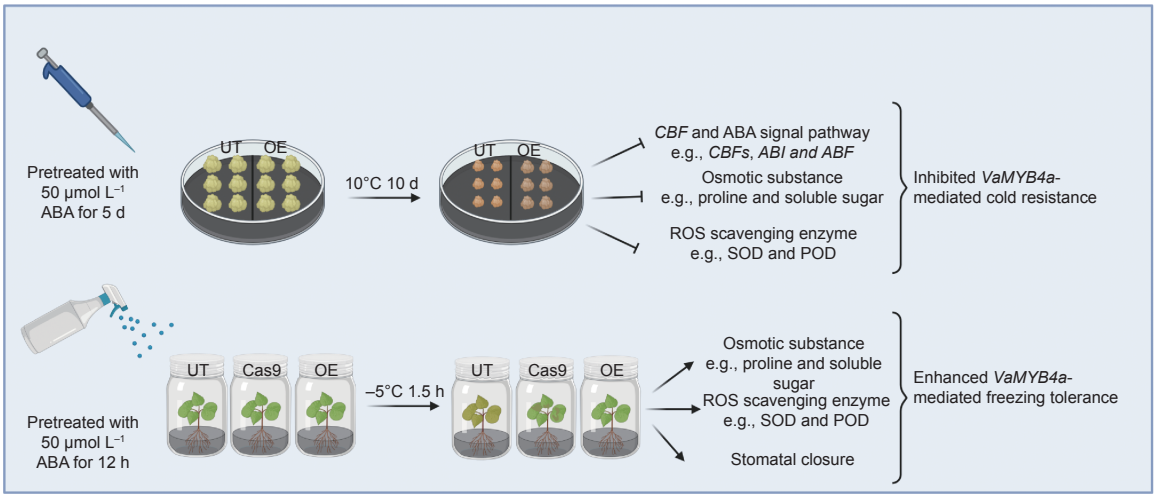



**Fig. 12 Dual effects of exogenous abscisic acid (ABA) on VaMYB4a-mediated cold tolerance.** This schematic illustrates the timing-dependent effects of exogenous ABA on *VaMYB4a*-mediated cold tolerance. Prolonged ABA treatment (50 μmol $L^{-1}$ for 5 d) inhibits *VaMYB4a*-mediated cold resistance by downregulating the expression of key genes in the *CBF* (*C-repeat Binding Factors*)-*COR (Cold-Regulated)* pathway and ABA (abscisic acid)-dependent signaling pathway, while promoting osmotic substance accumulation and enhancing ROS scavenging enzyme activity. Conversely, short-term ABA pretreatment (50 μmol $L^{-1}$ for 12 h) enhances *VaMYB4a*-mediated freezing tolerance by increasing osmotic substance accumulation, boosting ROS (Reactive oxygen species) scavenging enzyme activity, and inducing stomatal closure to mitigate water loss. Arrows indicate positive regulation, while barred lines represent negative regulation.

for further understanding its role in abiotic stress tolerance. By addressing these gaps, this work lays the groundwork for developing grapevine varieties with enhanced resilience to abiotic stresses, contributing to more stable and sustainable viticulture practices.

## 5. Conclusion

In this study, we demonstrate that *VaMYB4a* expression is significantly induced by both cold stress and ABA, resulting in enhanced freezing tolerance in grapevine. Overexpression of *VaMYB4a* in *Arabidopsis*, grapevine callus, and transgenic grapevine seedlings led to increased accumulation of osmotic regulators, heightened activity of ROS scavenging enzymes, and enhanced stomatal closure under cold stress. A key finding of this work is that VaMYB4a forms a homo-dimer *via* its C-terminal regulatory domain, a feature that is crucial for its function both in yeast and plant cells. Furthermore, we reveal a complex dual regulatory role of ABA in *VaMYB4a*-mediated cold tolerance, indicating an intricate interplay between ABA and cold stress signaling pathways. These findings significantly advance our understanding of the molecular mechanisms that underlie cold tolerance in grapevine, particularly the role of *VaMYB4a* in coordinating responses to both ABA and cold stress. As global climate change increases the frequency and severity of freezing events, the molecular insights presented here provide valuable guidance for breeding or genetically engineering grapevine varieties with improved cold resilience. Future research should focus on examining the long-term stability and performance of *VaMYB4a*-modified grapevines under field conditions, as well as exploring the synergistic interactions between VaMYB4a and other key cold-regulated transcription factors. Such studies could uncover additional layers of regulatory complexity, offering new opportunities to enhance stress tolerance in grapevine and other crops.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

**Appendices** associated with this paper are available at https://doi.org/10.1016/j.jia.2025.09.005

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