Journal of Experimental Botany
doi:10.1093/jxb/erx136

RESEARCH PAPER

VpPUB24, a novel gene from Chinese grapevine, *Vitis pseudoreticulata*, targets VpICE1 to enhance cold tolerance

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Received 30 November 2016; Editorial decision 30 March 2017; Accepted 31 March 2017

Editor: Christine Foyer, Leeds University

Abstract

The ubiquitination system plays important roles in the degradation and modification of substrate proteins. In this study, we characterize a putative U-box type E3 ubiquitin ligase gene, *VpPUB24* (plant U-box protein 24), from Chinese wild grapevine, *Vitis pseudoreticulata* accession Baihe-35-1. We show that *VpPUB24* is induced by a number of stresses, especially cold treatment. Real-time PCR analysis indicated that the *PUB24* transcripts were increased after cold stress in different grapevine species, although the relative expression level was different. In grapevine protoplasts, we found that VpPUB24 was expressed at a low level at 22 °C but accumulated rapidly following cold treatment. A yeast two-hybrid assay revealed that VpPUB24 interacted physically with VpICE1. Further experiments indicated that VpICE1 is targeted for degradation via the 26S proteasome and that the degradation is accelerated by VpHOS1, and not by VpPUB24. Immunoblot analyses indicated that VpPUB24 promotes the accumulation of VpICE1 and suppresses the expression of VpHOS1 to regulate the abundance of VpICE1. Furthermore, VpICE1 promotes transcription of *VpPUB24* at low temperatures. We also found that VpPUB24 interacts with VpHOS1 in a yeast two-hybrid assay. Additionally, over-expression of *VpPUB24* in *Arabidopsis thaliana* enhanced cold tolerance. Collectively, our results suggest that VpPUB24 interacts with VpICE1 to play a role in cold stress.

Key words: Chinese wild grapevine, cold stress, ICE1, transcriptional regulation, ubiquitin ligase, U-box protein.

Introduction

Temperature has major influences on plant growth and development. A number of temperature-regulated signaling networks have been identified. One such pathway is the ICE (INDUCER OF CBF EXPRESSION)-CBF/DREB1 (C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1) pathway, which is induced by exposure to low temperatures (Chinnusamy *et al.*, 2007; Hua, 2009; Knight & Knight, 2012; Jung *et al.*, 2014; Kazan, 2015; Zhao *et al.*, 2015). In this signaling cascade, the ICE1 protein, a helix-loop-helix (HLH)-type transcription factor, has been studied widely and shown to be involved in a range of aspects of plant growth and development, including stomatal development

(Kanaoka *et al.*, 2008), defense against bacteria (Zhu *et al.*, 2011), flowering (Lee *et al.*, 2015), and, especially, cold tolerance (Chinnusamy *et al.*, 2003; Lee *et al.*, 2005; Dong *et al.*, 2006; Miura *et al.*, 2011). Another component of the pathway, HOS1 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1), is a RING-type E3 ubiquitin ligase that mediates the degradation of ICE1 via the 26S proteasome (Dong *et al.*, 2006). Meanwhile, SIZ1 (SAP and Miz1) specifically interacts with ICE1 to maintain its stability, thereby enhancing cold tolerance (Miura *et al.*, 2007). In addition, JAZ (JASMONATE ZIM-DOMAIN) proteins have been shown to regulate the ICE-CBF/DREB1 cascade in *Arabidopsis thaliana* by repressing the transcription regulatory function of ICE1 (Hu *et al.*, 2013). However, OST1 (OPEN STOMATA 1) kinase phosphorylates ICE1 and enhances cold tolerance (Ding *et al.*, 2015). Other studies have shown that *BAP1* (*BON1-ASSOCIATED PROTEIN1*) is directly regulated by ICE1, and influences growth and also programmed cell death (Hua, 2001; Yang *et al.*, 2006, 2007; Zhu *et al.*, 2011). The transcription factor CBF/DREB1 acts downstream of the ICE genes. Three CBF genes (*CBF1*, *CBF2*, and *CBF3*) have been identified in *A. thaliana* (Zarka *et al.*, 2003; Park *et al.*, 2015; Zhao *et al.*, 2015). Additionally, the R2R3-type MYB transcription factor, *MYB15*, regulates the *CBF* genes in response to cold stress (Agarwal *et al.*, 2006). Finally, several cold-related genes, such as *COR15*, *COR47*, and *RD29A*, are induced by the ICE-CBF/DREB1 cascade, thereby promoting freezing tolerance (Wang & Hua, 2009; Park *et al.*, 2015).

The ubiquitination system plays important roles in the degradation of proteins. It involves three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Of these, E3 catalyzes the polyubiquitination of proteins, thereby targeting them for degradation by the 26S proteasome (Smalle & Vierstra, 2004; Vierstra, 2009). In *A. thaliana*, more than 1300 E3 ubiquitin ligase genes have been identified and classified into four types (HECT, RING/U-box, SCF, and APC), and more than 60 of these genes belong to the U-box family (Vierstra, 2009; Yee & Goring, 2009; Gao *et al.*, 2013). A number of plant U-box type (PUB) E3 proteins involved in growth and defense in *A. thaliana* have been studied. For example, *AtPUB13* has been reported to regulate flowering time, and its expression is seen to be induced by flg22, a bacterial peptide that can trigger the plant immune system (Lu *et al.*, 2011; Li *et al.*, 2012). *AtPUB10* regulates root growth by affecting the stability of *MYC2* (Jung *et al.*, 2015), and *AtPUB18*, *AtPUB19*, *AtPUB22*, *AtPUB23*, and *AtPUB24* participate in drought stress responses (Cho *et al.*, 2008; Bergler & Hoth, 2011; Liu *et al.*, 2011; Seo *et al.*, 2012). *AtPUB22* and *AtPUB23* mediate the degradation of AtEXO70B2, thereby suppressing defense responses that are induced by pathogen-associated molecular pathogens (PAMPs) (Stegmann *et al.*, 2012). Finally, *AtPUB17* has been shown to play a role in cell death and defense (Yang *et al.*, 2006). In species other than *A. thaliana*, some other PUB E3 proteins have been identified as taking part in a range of processes, including defense responses: in hot pepper (*Capsicum annuum*), *PUB1* expression is upregulated by a broad spectrum of abiotic stresses and bacterial pathogens (Cho *et al.*, 2006); in *Medicago truncatula*, *PUB1* suppresses nodulation (Mbengue *et al.*, 2010); in rice (*Oryza sativa*), *Spotted leaf 11* (*SPL11*) suppresses innate immunity (Zeng *et al.*, 2004; Liu *et al.*, 2015) and *OsPUB15* reduces defense responses (Park *et al.*, 2011). However, studies of U-box type E3 ubiquitin ligase in grapevine species have seldom been reported.

Grapes are one of the most important fruit crops. The cultivars of the European grapevine (*Vitis vinifera* L.) are grown worldwide, and are known for their excellent qualities both for winemaking and for table fruit. However, many *V. vinifera* cultivars exhibit low resistance to abiotic and biotic stresses (Wang *et al.*, 1995; Cao *et al.*, 2010; Cadle-Davidson *et al.*, 2011). China is one center of origin of grapes, and a Chinese wild grapevine species, *Vitis pseudoreticulata*, shows good levels of resistance to a range of abiotic and biotic stresses. This species has been used to study plant responses to stresses such as powdery mildew, drought, and cold (Wang *et al.*, 1995; Cao *et al.*, 2010; Li *et al.*, 2014; Xu *et al.*, 2014*a*; Zhang *et al.*, 2015). These studies have led to the identification of transcription factors involved in responses to powdery mildew, salinity, and low temperature (Li *et al.*, 2010; Zhu et al., 2012*a*, 2012*b*; Peng *et al.*, 2013; Zhu *et al.*, 2013). Our group has also identified a Ring-type ubiquitin ligase, EIRP1, from *V. pseudoreticulata*, which interacts with the transcription factor WRKY11 to positively regulate defenses against powdery mildew (Yu *et al.*, 2013*b*). The ICE-CBF/DREB1 cascade has also been investigated in a number of other grape genotypes (Tillett *et al.*, 2012; Li *et al.*, 2014; Rahman *et al.*, 2014; Xu *et al.*, 2014*a*, 2014*b*). These studies include one with another Chinese wild grape species, *Vitis amurensis*, where it was reported that *VaPUB* is involved in cold hardiness (Jiao *et al.*, 2014).

In the present study, working with *V. pseudoreticulata*, we characterize the mode of action of a putative U-box type E3 ubiquitin ligase, *VpPUB24*. We also consider its role in plant responses to a range of abiotic and biotic stresses, in particular cold treatment. Additionally, we elucidate its association with the ICE-CBF/DREB1 signaling pathway.

Materials and methods

Plant materials

Grapevines, including two Chinese wild species, *V. pseudoreticulata* (accession Baihe-35-1) and *Vitis yeshanensis* (cv. yanshan-1), the American species *Vitis riparia*, and two cultivars of the European species *V. vinifera* (cvs. 'Cabernet Sauvignon' and 'Red Globe') were propagated by tissue culture and transplanted as previously described (Yu *et al.*, 2013*b*). The Asian grapevine *V. amurensis* (cv. Zuoshan-1) was obtained from hardwood cuttings. All young plants were transplanted into pots filled with soil and each was covered with a 250 ml transparent plastic cup and placed in a growth chamber (22 °C; photoperiod 16:8 h) for 25 d, after which the cup was removed. The plants were then cultured for a further 30 d prior to imposing the treatments. Plants of thale cress (*A. thaliana*) and tobacco (*Nicotiana benthamiana*) were grown in a growth chamber under the same environmental conditions.

Cold treatment

Cold treatment was carried out as previously described (Jiao *et al.*, 2014; Xu *et al.*, 2014*b*; Karimi *et al.*, 2015) with some modifications.

Briefly, the grapevines were transferred from the 22 °C growth chamber to a –2 °C growth chamber. Leaf samples were collected immediately (0 h) and again after 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h. Analysis of cold-treated transgenic *A. thaliana* used the methods described by Hu *et al.* (2013). Freezing tolerance assays employed 12-d-old seedlings of *A. thaliana*. The survival rates and relative electrolyte leakage were quantified. Seedlings of *A. thaliana* were grown in soil for 30 d, then subjected to cold treatment at 4 °C, and leaves were then sampled for real-time (RT)-PCR analysis.

Yeast two-hybrid assay

The cDNA library from *V. pseudoreticulata* was constructed using the Make Your Own 'Mate & Plate™' Library System (Clontech). Primers used to amplify the genes are listed in Supplementary Table S1 at *JXB* online. The *VpPUB24* ORF sequence was cloned into pGBKT7 as the bait for screening. To confirm protein interactions, the amplified ORFs of the *VpHOS1*, *VpJAZ1*, and *VpJAZ4* sequences were inserted into the pGBKT7 vector and the *VpICE* genes were cloned into the pGADT7 prey vector. After co-transformation of the prey and bait plasmids, yeast cells grown on SD/-Trp/-Leu media were suspended in sterile water and then placed on SD/-Trp/-Leu/-Ade/-His/Aba/x-α-Gal media for interaction confirmation.

Subcellular location and bimolecular fluorescence complementation assay

For identifying the subcellular location of VpPUB24, we first fused the $P_{VpPUB24}$ fragment to the ORF of *VpPUB24*, generating $P_{VpPUB24}$-*VpPUB24* PCR products. Next, the $P_{VpPUB24}$-*VpPUB24* PCR products were introduced into the pBI221 vector and tagged with green fluorescent protein (GFP) to generate $P_{VpPUB24}$-*VpPUB24-GFP*. The ORFs were then inserted into the pCAMBIA2300-GFP vector, generating the over-expression vectors *35S-VpICE1-GFP*, *35S-VpICE2-GFP*, and *35S-VpICE3-GFP*. The full-length *AtHY5* (AT5G11260) sequence was cloned from *A. thaliana* Col-0 and inserted into the pCAMBIA2300-mCherry vector, generating *35S-AtHY5-mCherry*. For bimolecular fluorescence complementation (BiFC) assays, the coding sequences of *VpPUB24* and *VpHOS1* were cloned into the pSPYNE vector to generate pSPYNE/VpPUB24, pSPYNE/PUB24$^{\Delta ARM}$, and pSPYNE/VpHOS1, respectively. The ORFs of VpICE1 and VpICE1$^{1–411}$ were inserted into the pSPYCE vector to generate pSPYCE/VpICE1 and pSPYCE/VpICE1$^{1–411}$. The PCR primers are listed in Supplementary Table S1. The various plasmids were co-transformed or transformed individually into grapevine or Arabidopsis protoplasts using the polyethylene glycol (PEG) method (Yu *et al.*, 2013*b*), and protoplasts were treated with 50 μM MG132, 20 h after transformation. Prior to observation of transformed protoplasts, cells were treated at 4 °C for 4 h.

GUS activity measurement

For transient gene expression in grapevine leaves, the *VpPUB24* promoter was cloned and introduced into the pC0380GUS vector to generate $P_{VpPUB24}$-*GUS*. The P_{35S}-*GUS* vector was used as a positive control and the empty vector *pC0380GUS* (P_0-*GUS*) as a negative control. Leaf transformation was carried out using the vacuum infiltration method (Xu *et al.*, 2011; Yu *et al.*, 2013*a*). After infiltration, the leaves were incubated in a 22 °C chamber for 48 h followed by treatment with either powdery mildew, jasmonate, salicylic acid, or cold (4 °C). All treated leaves were harvested 24 h after treatment and analyzed for β-glucuronidase (GUS) activity. The tobacco leaf infiltration assay was carried out as previously described by Liu *et al.* (2010). The detection of grapevine and tobacco leaf GUS activity was as described by Xu *et al.* (2011).

RT-PCR analysis

Extraction of RNA and subsequent cDNA synthesis were carried out using the PrimeScript RT reagent kit with gDNAEraser (Takara) as previously described by Yu *et al.* (2013*b*). RT-PCRs were carried out using SYBR Premix Ex Taq™II and an IQ5 Real-Time PCR Detection System (Bio-Rad), according to the manufacturer's instructions. Three replicates were analyzed for each sample and the *VvActin* gene was used as an internal control. All the primers used are listed in Supplementary Table S2.

Co-immunoprecipitation assay

A co-immunoprecipitation (Co-IP) assay was carried out using the PEG-mediated *A. thaliana* protoplast transformation method (Liu *et al.*, 2010; Yoon & Kieber, 2013). Briefly, DNA sequences encoding *VpPUB24* were introduced into the *pCAMBIA2300-GFP* vector to generate *35S-VpPUB24-GFP*. Meanwhile, the *VpICE1* sequence was introduced into *pCAMBIA1307-Myc*, generating *35S-Myc-VpICE1*. The different combinations of plasmids were transformed into grapevine protoplasts. Transformed cells were incubated for 20 h at 22 °C and then at 4 °C for 4 h, prior to harvest. Proteins were extracted from 750 μl of transformed cells using Co-IP buffer, as described by Yoon and Kieber (2013) with a supplement of 50 μM MG132. GFP antibody was added to the protein extract mixture and incubated for 3 h at 4 °C, and then 50 μl protein A+G agarose beads were added for another 2–3 h of incubation to capture the target complex. Finally, the agarose beads were collected by centrifugation at 12,000 *g* for 5 s and washed three times with cold 1×PBS buffer (pH 8.0). Proteins were eluted from the pelleted beads with 60 μl 5×loading buffer and boiled for 5 min for use in immunoblot analyses.

Protein degradation assay in vivo

The degradation of ICE1 protein in tobacco leaves was assessed as previously described (Liu *et al.*, 2010; Bueso *et al.*, 2014). *Agrobacterium tumefaciens* GV3101 carrying the *35S-Myc-ICE1* vector were infiltrated into tobacco leaves, which were then incubated for 48 h at 22 °C and treated at 4 °C for 12 h before harvesting. For the MG132 treatment, 50 μM MG132 was infiltrated at 24 h after infiltration with *Agrobacterium*. Various co-infiltration combinations were analyzed with different ratios of the *Agrobacterium* cultures. The infiltrated tobacco plants were maintained at 22 °C for 48 h and leaves were harvested after 4 °C treatment for 12 h. Leaves were ground in liquid nitrogen and extracted with lysis buffer. Extracts were incubated on ice for 15 min and then centrifuged at 20,000 *g* for 15 min at 4 °C. The concentration of total protein was measured by the Bradford method (Bradford, 1976). The supernatant was then subjected to western blot analysis. The leaf samples were also used for semi-quantitative RT-PCR analysis and tobacco *Actin8* (*ACT8*) was used as the internal control.

Results

Identification of the U-box type E3 ubiquitin ligase gene VpPUB24

In grapevines, there are 56 predicted U-box type E3 ubiquitin ligases (Du *et al.*, 2009; Gao *et al.*, 2013). We first observed the expression of *VpPUB24* in the Chinese native wild *V. pseudoreticulata* and found that it was induced by powdery mildew (data not shown). First, the full-length cDNA of *VpPUB24* (GenBank accession number KU296025) was cloned. The VpPUB24 encoded a 411 amino acid protein with an N-terminal U-box domain and C-terminal ARM repeats. This shared 99% amino acid identity with the predicted protein encoded by *V. vinifera* PUB24, which is located on chromosome 6 of the Pinot Noir genotype (Fig. 1A). As shown in Fig. 1B, alignment analysis indicated that *Theobroma cacao* TcPUB24 and *Gossypium raimondii* GrPUB24 also

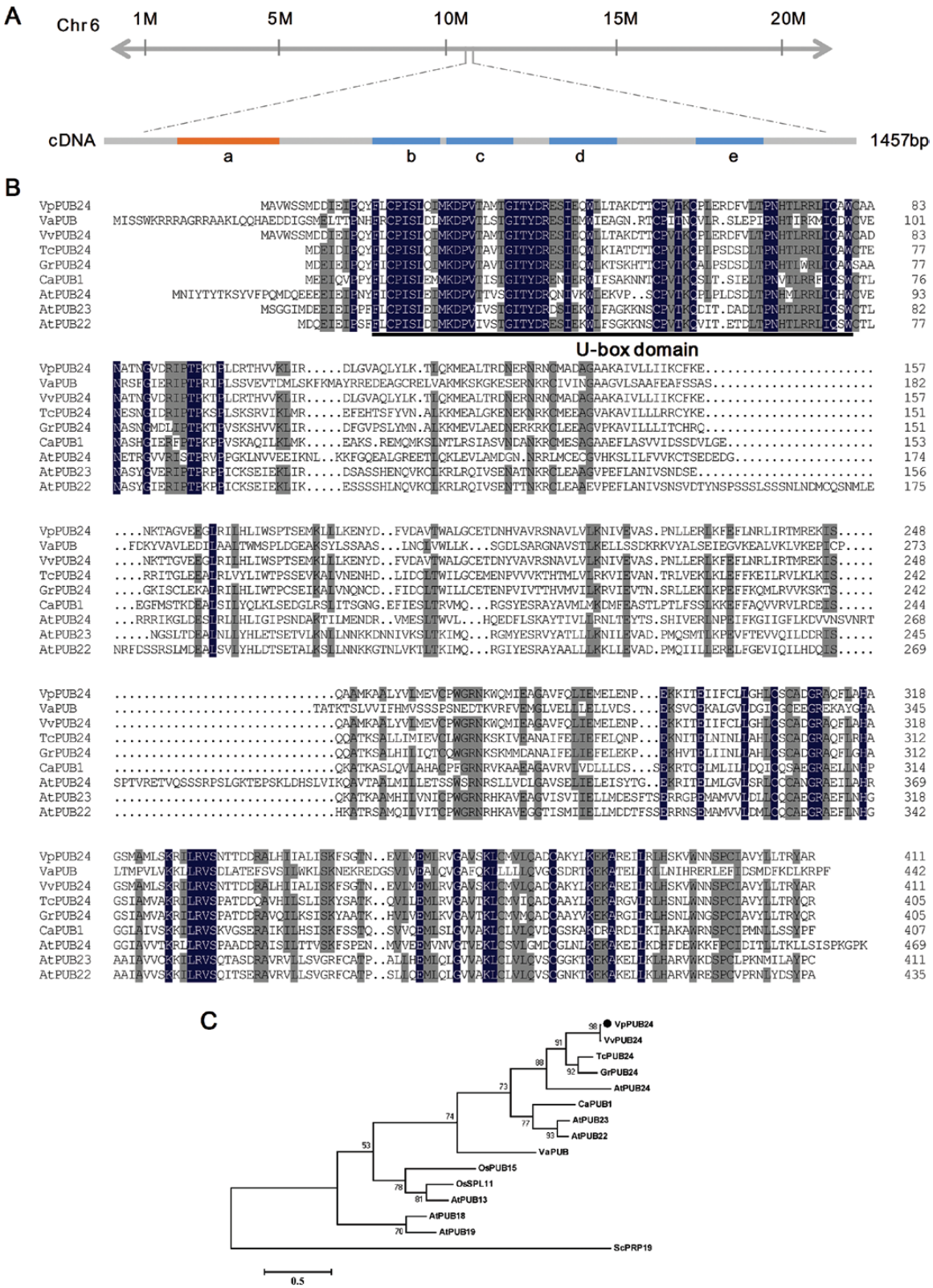

Fig. 1. Sequence analysis of the U-box type E3 ubiquitin ligase gene *VpPUB24*. (A) Schematic diagram of the position and structure of *VpPUB24*. The U-box domain is indicated with a and the ARM domain is indicated with b–e. (B) Sequence analysis of VpPUB24. The solid black line indicates the conserved U-box domain and the consensus sequence is shown in black. Dashes show gaps in the alignment. The accession numbers are as follows: VpPUB24 (KU296025), VvPUB24 (XP_010651653), VaPUB (KM243344), TcPUB24 (XP_007015663), GrPUB24 (XP_012477445), CaPUB1 (DQ211901), AtPUB22 (At3g52450), AtPUB23 (At2g35930), AtPUB24 (At3g11840). (C) Cluster analysis of VpPUB24. Different U-box type E3 ubiquitin ligases from *Vitis pseudoreticulata*, *Vitis vinifera*, *Vitis amurensis*, *Oryza sativa*, *Theobroma cacao*, *Gossypium raimondii*, and *Arabidopsis thaliana* were used for the phylogenetic analyses. The accession numbers are as follows: OsSPL11 (AAT94161), OsPUB15 (BAD09539), AtPUB13 (At3g46510), AtPUB18 (At1g10560), AtPUB19 (At1g60190). ScPRP19 (YLL036C) from *Saccharomyces cerevisiae* was used as a rooting outlier. (This figure is available in colour at *JXB* online.)

show high sequence identity to VpPUB24 (65% and 64%, respectively). *V. amurensis* VaPUB is a U-box E3 ubiquitin ligase that has been reported to enhance cold tolerance when overexpressed in *A. thaliana* (Jiao *et al.*, 2014). However, we determined that this protein had only 33% sequence identity with VpPUB24. *C. annuum CaPUB1* has also been shown to respond to cold stress; the corresponding protein also had a relatively low sequence identity with VpPUB24 (43%) (Cho *et al.*, 2006). Of the *A. thaliana* proteins, VpPUB24 was most homologous to AtPUB24 (43% identity) and shared similar identity percentages with AtPUB23 and AtPUB22 (42% and 38%, respectively). Finally, the other three *A. thaliana* U-box proteins included in this analysis (AtPUB13, AtPUB18, and AtPUB19), as well as the two *O. sativa* U-box proteins (OsSPL11 and OsPUB15), had ≤26% identity to VpPUB24 (Fig. 1C).

We also cloned the promoter sequences of *VpPUB24*. A 1611 bp promoter fragment of *VpPUB24* ($P_{VpPUB24}$; KU296032) was isolated from *V. pseudoreticulata* and by analysis of promoter sequences we found that there were some *cis*-acting elements involving defense and stress. These *cis*-acting elements included two ABRE elements involved in abscisic acid responsiveness, three HSE elements involved in heat stress responsiveness, three LTR elements involved in low-temperature responsiveness, two TC-rich repeats involved in defense and stress responsiveness, one TCA element involved in salicylic acid responsiveness, and five E-box (CANNTG) binding sites within a 850 bp region at the 3′-terminal of $P_{VpPUB24}$, which was predicted to be the MYC-recognition element (Supplementary Fig. S1). Meanwhile, $P_{VpPUB24}$ was fused with the GUS reporter gene for promoter activity analysis in *V. pseudoreticulata* leaves. The GUS activity assay indicated that *VpPUB24* is regulated by a range of stresses—powdery mildew, salicylic acid, jasmonate, and, especially, cold (4 °C) (Fig. 2A, B). We also cloned five homologous genes of *VpPUB24* from other grapevine species (*V. vinifera* cvs. 'Cabernet Sauvignon' and 'Red Globe', *V. riparia*, *V. yeshanensis* yanshan-1, and *V. amurensis* Zuoshan-1) and found that their amino acid sequences all shared 100% identity with VpPUB24 (Supplementary Fig. S2).

Expression of VpPUB24 *is induced by cold treatment*

We further investigated the expression pattern of *PUB24* in response to cold treatment among different grapevine genotypes. Previous studies have suggested that the cold hardiness of the European grape cultivar 'Cabernet Sauvignon' is ranked 'low', while that of *V. pseudoreticulata* is ranked 'medium/high' and that of *V. amurensis* Zuoshan-1 is ranked 'high' (Cao *et al.*, 2010; Zhang *et al.*, 2015). To investigate the expression pattern of *PUB24* in different grapevine leaves after cold stress, we treated plants at –2 °C and found that the leaves from 'Cabernet Sauvignon' were almost dry at 24 h; in *V. pseudoreticulata* Baihe-35-1 leaf death occurred at 36 h, and in Zuoshan-1 serious leaf damage was observed at 60 h (Fig. 2C). Next, an RT-PCR assay was performed. In 'Cabernet Sauvignon', we found that the abundance of the *PUB24* transcript increased rapidly and peaked at 1 h but then decreased gradually, reaching a trough at 8 h after cold treatment. In leaves of *V. pseudoreticulata*, the trend of *PUB24* transcript abundance was similar to that in 'Cabernet Sauvignon', but the relative expression levels were obviously higher at 0.5 h and 1 h. In *V. amurensis* Zuoshan-1, *PUB24* expression peaked after 0.25 h of cold treatment, then gradually reduced to the normal level and peaked again at 24 h after cold treatment (Fig. 2D).

Next, the full length of *VpPUB24* was fused to the GFP tag and was driven by its own promoter $P_{VpPUB24}$. After the construct was transformed into grapevine protoplast, we found that at 22 °C the fluorescent signals produced by VpPUB24-GFP were weak and were expressed in only a few protoplast cells, but when protoplast cultures were exposed to 4 °C for 4 h, fluorescence was clearly observed throughout the field of view (Fig. 3A) and we observed the VpPUB24-GFP protein in all grapevine protoplasts (Fig. 3B). Immunoblot analysis also confirmed that VpPUB24 abundance was lower at 22 °C than at 4 °C. The expression of 35S-GFP was not influenced at either 22 °C or 4 °C (Fig. 3C). These results suggest that *VpPUB24* expression is sensitive to low temperatures.

Identification of VpICE1 as a VpPUB24 interacting protein

To better understand the function of VpPUB24, a yeast two-hybrid assay was used to screen for interacting proteins, based on a *V. pseudoreticulata* cDNA library. The *VpPUB24* ORF was inserted into the pGBKT7 vector and used as the bait. We found five independent clones encoding ICE1 in the candidate interacting protein. To confirm the interaction, we cloned the full length of *VpICE1* (KU296026), which encoded 516 amino acids. *VpICE1* was most homologous to two grapevine ICE1 proteins from *V. vinifera* and *V. riparia* (each exhibited 99% sequence identity). In *A. thaliana,* AtICE1 (AT3G26744; 52% identity) and AtICE2 (AT1G12860; 52% identity) were most closely related to VpICE1 (Supplementary Fig. S3A, B). Using sequence alignment analysis, we observed that VpICE1 had the conserved HLH domain and the C-terminal ACT domain. A comparison of VpICE1 with ICE1 proteins from other species identified VpICE1 Ser426 (corresponding to AtICE1 Ser403) as a highly conserved residue, since all the ICE1 proteins contained a serine residue at this position. We also noted that the AtSUMO protein-binding site lysine residue (Lys393) was conserved, but in *V. pseudoreticulata* the lysine residue was replaced by an arginine residue (Arg416) (Supplementary Fig. S3A).

To confirm the interaction between VpPUB24 and VpICE1, VpICE1 was introduced to the pGADT7 prey vector and different deletion fragments of *VpPUB24* gene were cloned into the pGBKT7 bait vector. The bait and prey plasmids were co-transformed into yeast cells for confirmation of the protein interaction. We found that when the U-box domain ($PUB24^{1–94}$) was deleted, $PUB24^{95–411}$ still interacted with VpICE1 (Fig. 4A). Furthermore, deletion of the VpICE1 N-terminal region, to leave only the C-terminal ACT domain ($ICE1^{411–516}$), did not affect the interaction (Fig. 4B). These results indicate that the VpPUB24 ARM domain interacted with the VpICE1 ACT domain.

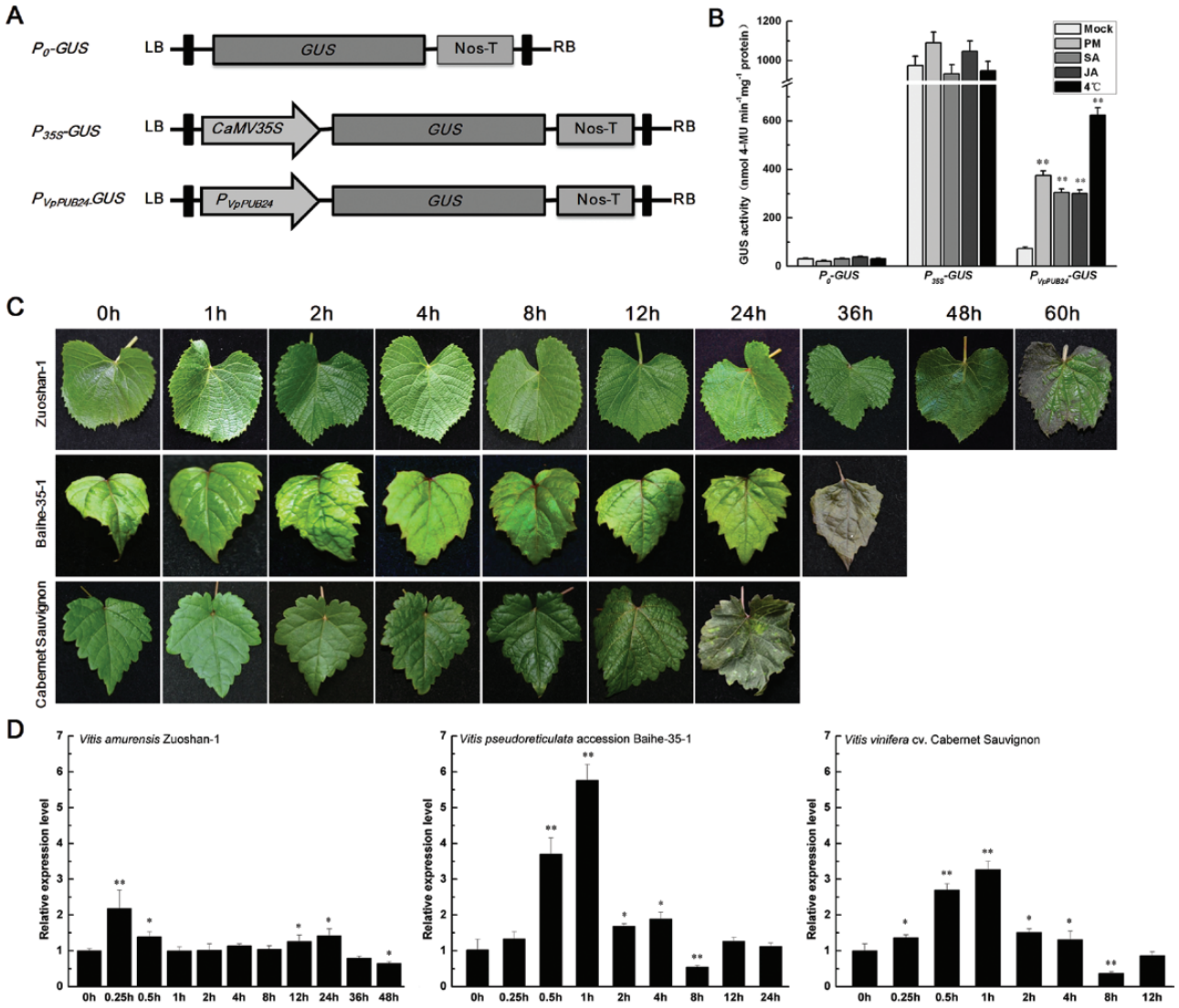

Fig. 2. Activity analysis of the *VpPUB24* promoter and the expression of *PUB24* in various grapevine species under cold treatment. (A) Schematic diagram of the vector construct. The *VpPUB24* promoter ($P_{VpPUB24}$) was cloned into pC0380GUS and fused with the GUS reporter. P_0-*GUS* (empty pC0380GUS vector) was used as a negative control and P_{35S}-*GUS* (*CaMV35S-pC0380GUS*) as a positive control. *GUS*, β-glucuronidase; LB, left border; NOS-T, Nos terminator; RB, right border. (B) Activity of the *VpPUB24* promoter induced by different stressors. The vector $P_{VpPUB24}$-*GUS* was transiently expressed in *Vitis pseudoreticulata* accession Baihe-35-1 leaves by the *Agrobacterium*-mediated method, and the leaves were then exposed to powdery mildew, salicylic acid (SA), jasmonate (JA), and cold (4 °C). GUS activity in leaf extracts was measured using a microplate spectrophotometer. (C) Leaf phenotype of different grapevine species after cold treatment at –2 °C. Plants were treated in a –2 °C chamber. The morphology of the leaves indicates the different sensitivities among three examined grapevine species at different time points (images show the leaf phenotype after cold treatment for 0, 1, 2, 4, 8, 12, 24, 36, 48, and 60 h). During cold treatment, leaves from *Vitis vinifera* cv. 'Cabernet Sauvignon' changed from normal to dry from 0–24 h; in *V. pseudoreticulata* accession Baihe-35-1 leaf death occurred at 36 h; in *V. amurensis* Zuoshan-1, serious leaf damage was apparent at 60 h. At least 10 grape plants of each species were treated and the experiment was repeated three times with similar results. (D) *PUB24* responded to cold treatment in a range of cold-resistant grapevines. Three grapevine genotypes (*V. amurensis* Zuoshan-1, *V. pseudoreticulata* accession Baihe-35-1, and *V. vinifera* cv. 'Cabernet Sauvignon') were used in an RT-PCR analysis. The grapevines were exposed to –2 °C and leaf samples were harvested at different times for RT-PCR analysis using *VvActin* as a control. For B and D, significance was assessed by a least significant difference test (**P*<0.05, ***P*<0.01). Error bars indicate the SD from three independent experiments. (This figure is available in colour at *JXB* online.)

To validate the interaction of VpPUB24 and VpICE1 *in vivo*, a BiFC assay was carried out using grapevine protoplasts and the PEG-mediated method. Fluorescence was observed only when pSPYNE/VpPUB24 was co-expressed with pSPYCE/VpICE1 (Fig. 5A). Furthermore, we also carried out a Co-IP analysis using grapevine protoplasts. In this assay, VpPUB24 was fused with a GFP tag on the C-terminal, while VpICE1 was fused with a Myc tag on the N-terminal. The Co-IP assay was carried out using a GFP antibody. Western blot analysis revealed that Myc-tagged VpICE1 co-immunoprecipitated with GFP-tagged VpPUB24 (Fig. 5B). These results confirm that VpPUB24 interacted physically with VpICE1.

We also carried out a BLAST search of the Pinot Noir grape genome browser database (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) using the VpICE1 amino acid sequence, and identified three VpICE1 homologs: VvICE1, VvICE2, and VvICE3. Using the homology cloning strategy, the full length *VpICE2* (KU296027) and *VpICE3*

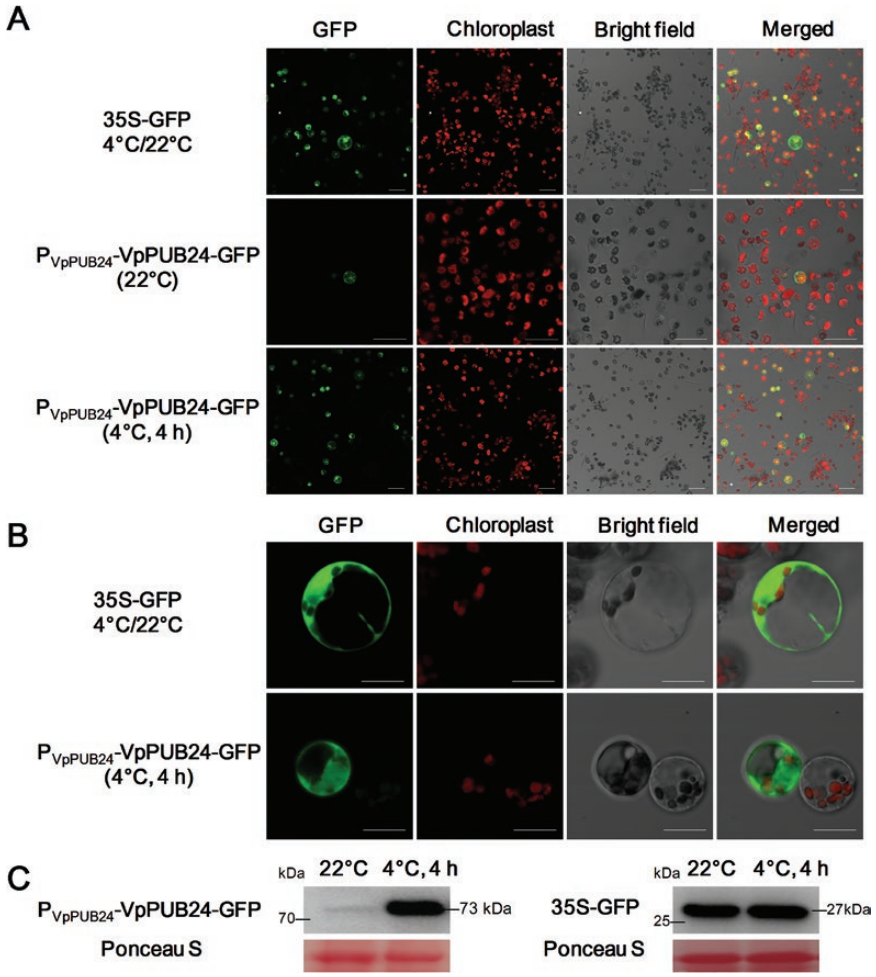

Fig. 3. Expression of VpPUB24 protein was induced by cold treatment. (A) Expression of *VpPUB24-GFP* was induced by cold treatment in grapevine protoplasts. The full-length open reading frame of *VpPUB24* was fused with green fluorescent protein (GFP) and driven by its own promoter $P_{VpPUB24}$. 35S-GFP was used as a positive control. The plasmids were transformed into grapevine protoplasts. Protoplasts were treated at 4 °C for 4 h, prior to imaging with a fluorescence microscope. Bars=50 μm. (B) Subcellular location of VpPUB24 in grapevine protoplast. Bars=10 μm. (C) Western blot showing the accumulation of VpPUB24 in protoplasts incubated at 4 °C for 4 h. After transformation of the indicated plasmid into grapevine protoplast, cells were cultured for 20 h at 22 °C and then treated at 4 °C for 4 h. Protoplast cells cultured at 22 °C or 4 °C were harvested at the same time and submitted for western blot analysis. A Ponceau S-staining band was used as a loading control. The molecular masses of marker proteins (kDa) are indicated at the left of each panel. (This figure is available in colour at *JXB* online.)

(KU296028) from *V. pseudoreticulata* were then cloned. The corresponding predicted proteins were found to have conserved amino acid domains, including a long N-terminal domain, an HLH domain in the middle, and a C-terminal ACT domain (Supplementary Fig. S4). A yeast two-hybrid assay indicated that VpPUB24 interacted with VpICE1, VpICE2, and VpICE3 (Fig. 5C), suggesting that the interaction between VpPUB24 and ICEs may be conserved in grapevine species.

Subcellular location of VpICE proteins

To determine the subcellular location of the three VpICE proteins, each was co-expressed in *A. thaliana* protoplasts with the *AtHY5* (*ELONGATED HYPOCOTYL 5*; AT5G11260) gene, which encodes a known nuclear protein (Li & He, 2016). The VpICE1 signal (GFP fluorescence) was seen to overlap with the AtHY5 signal (mCherry), indicating that VpICE1 accumulated in the nucleus. A similar analysis of the other two ICE proteins (VpICE2 and VpICE3) revealed that they were also located in the nucleus (Fig. 6A).

ICE1 *is induced by cold treatment in different cold-resistant grapevines*

We further investigated *ICE1* expression in the various grapevine species after cold treatment. Using RT-PCR analysis, we found that the quantity of *ICE1* transcripts increased rapidly after cold treatment in different grapevines (Fig. 6B–D). In the grapevines *V. amurensis* Zuoshan-1, *V. pseudoreticulata* Baihe-35-1, and *V. vinifera* cv. 'Cabernet Sauvignon', *ICE1* transcripts showed a similar trend before 12 h, but the relative expression levels and the time point of second increase in transcript levels differed among the species. In Zuoshan-1, the *ICE1* transcript level decreased after 12 h and returned to normal at 48 h. These results indicated that *ICE1* was induced by cold treatment in the three grapevine species examined.

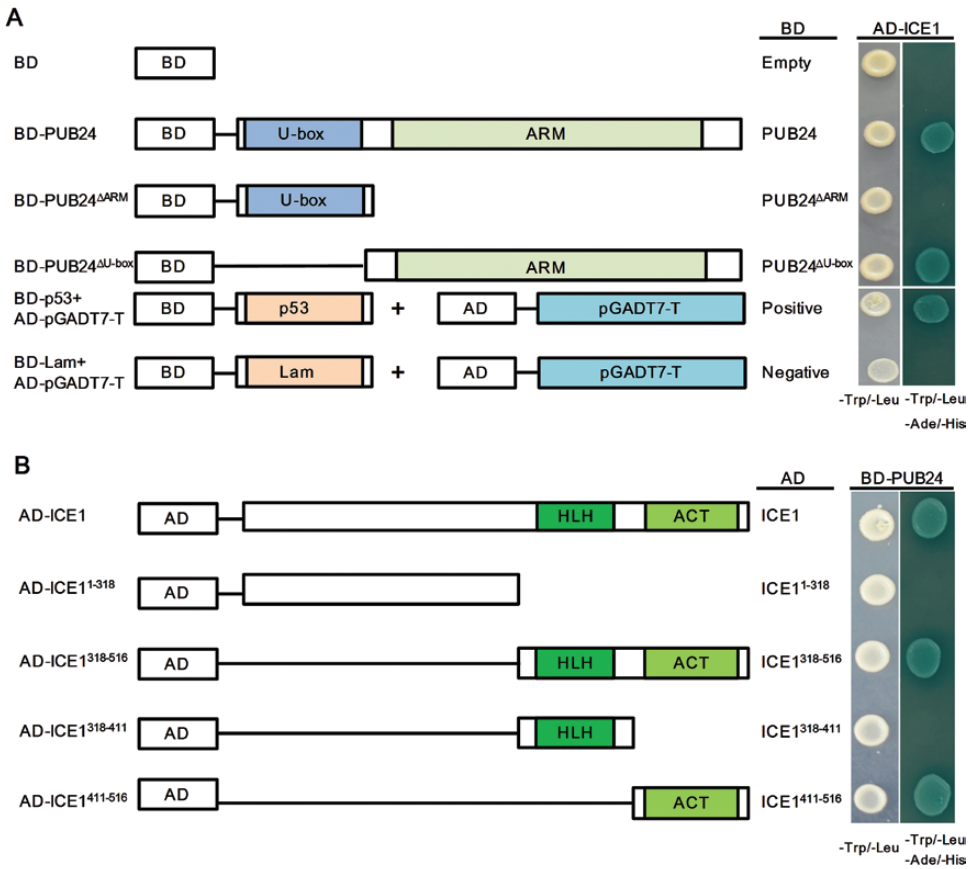

Fig. 4. Interaction between VpPUB24 and VpICE1 in the yeast two-hybrid assay. (A) The ARM domain of VpPUB24 interacted with transcription factor VpICE1 in the yeast two-hybrid assay. The pGADT7-VpICE1 plasmid was co-transformed with pGBKT7-PUB24, pGBKT7-PUB24$^{1–94}$, and pGBKT7-PUB24$^{95–411}$ individually in yeast Y2H Gold cells. The empty pGBKT7 vector co-transformed with pGADT7-VpICE1 was used as a control. pGADT7-T was co-transformed with pGBKT7-p53 and pGBKT7-Lam to generate positive and negative controls, respectively. Left panel: Schematic diagram of the full-length and deletion constructs of VpPUB24. Right panel: Interacting confirmation of different plasmid co-transformations. (B) Interaction between the C-terminal ACT domain of ICE1 and PUB24 in the yeast two-hybrid assay. The pGBKT7-PUB24 plasmid was co-transformed with pGADT7-VpICE1, pGADT7-VpICE1$^{1–318}$, pGADT7-VpICE1$^{318–516}$, pGADT7-VpICE1$^{318–411}$, and pGADT7-VpICE1$^{411-516}$ individually in yeast Y2H Gold cells. Left panel: Schematic diagram of the full-length and deletion constructs of VpICE1. Right panel: Interacting confirmation of different plasmid co-transformation. In A and B, after co-transformation of the prey and bait plasmids, yeast cells from the SD/-Trp/-Leu medium were dissolved in sterile water and placed on SD/-Trp/-Leu/-Ade/-His/Aba/x-α-Gal media for interaction confirmation. (This figure is available in colour at *JXB* online.)

Degradation of VpICE1 is mediated by the 26S proteasome but not accelerated by over-expression of VpPUB24

AtICE1 has previously been shown to be degraded by the 26S proteasome after cold treatment (Chinnusamy *et al.*, 2003; Dong *et al.*, 2006). To determine whether VpICE1 is similarly degraded, we carried out degradation assays using *Agrobacterium*-infiltrated tobacco leaves. When the leaves were infiltrated with 50 μM MG132 (a 26S proteasome-specific protease inhibitor) the degradation of VpICE1 was substantially inhibited. These results indicated that the degradation of VpICE1 occurred via the 26S proteasome (Fig. 7A). Additionally, since *VpPUB24* was expected to encode an E3 ubiquitin ligase, we investigated whether it promoted the degradation of VpICE1. Co-infiltration experiments were carried out using varying amounts of *Agrobacterium* expressing Myc-VpICE1 and VpPUB24-GFP, with 35S-GFP being used as a control. The abundance of Myc-tagged VpICE1 protein showed some accumulation along with an increasing ratio of GFP-tagged VpPUB24. However, this phenomenon was not found in samples coexpressing 35S-GFP and Myc-ICE1 (Fig. 7B). These results suggest that VpICE1 was degraded by the 26S proteasome, but that the degradation was not promoted by VpPUB24. In fact, VpPUB24 protein promoted the accumulation of VpICE1.

VpICE1 promotes the expression of VpPUB24 *under cold treatment*

ICE1 is a key transcription factor in the regulation of the response to cold stress in many plant species. Because we found that *VpPUB24* expression was rapidly induced by cold temperatures (Figs. 2 and 3) and VpPUB24 was seen to interact with VpICE1 (Fig. 4), we reasoned that VpICE1 may act directly to promote *VpPUB24* transcription. We used a tobacco leaf co-infiltration assay to study the relationship between $P_{VpPUB24}$ and *VpICE1*. GUS activity was used to monitor the activity of expression of the *VpPUB24* promoter. As shown in Fig. 7C, in accordance with our previous experiment, $P_{VpPUB24}$ was at a low level under room temperature conditions (22 °C) but its promoter activity was significantly

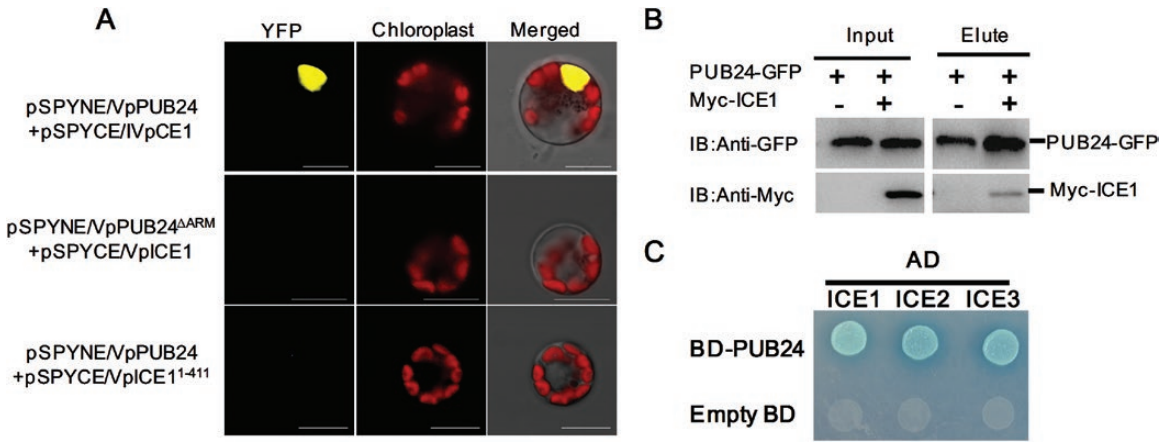

Fig. 5. VpPUB24 interacts with the transcription factor VpICE1 *in vivo*. (A) A BiFC assay confirmed that VpPUB24 interacted with VpICE1. The different plasmid combinations (pSPYNE/VpPUB24 and pSPYCE/VpICE1, pSPYNE/PUB24$^{\Delta ARM}$ and pSPYCE/VpICE1, pSPYNE/VpPUB24 and pSPYCE/VpICE1$^{1-411}$) were co-transformed into grapevine leaf protoplasts. Yellow fluorescent protein (YFP) fluorescence was observed when pSPYNE/VpPUB24 was coexpressed with pSPYCE/VpICE1. YFP fluorescence was observed after transformation for 20 h and then exposure to 4 °C for 4 h. Bars=10 μm. (B) A Co-IP assay confirmed that VpPUB24 physically interacted with VpICE1. The full length of VpPUB24 and VpICE1 were fused with a GFP and Myc tag, respectively, then the indicated plasmids were co-expressed in grapevine protoplasts. Protoplast cells were cultured at 22 °C for 20 h and then exposed to 4 °C for 4 h before the Co-IP assay. The Myc-tagged VpICE1 was immunoprecipitated by GFP-tagged VpPUB24. (C) ICE genes interact with PUB24 in a yeast two-hybrid assay. All the members of the ICE family interact with PUB24 in the yeast two-hybrid assay. Three similar genes (*ICE1*, *ICE2*, and *ICE3*) were cloned into the pGADT7 vector and co-transformed with pGBKT7-PUB24, respectively. Empty pGBKT7 with or without pGADT7-VpICE1 were used as negative controls. Yeast was placed on SD/-Trp/-Leu/-Ade/-His/Aba/x-α-Gal media for the confirmation assay. (This figure is available in colour at *JXB* online.)

increased after cold treatment. Furthermore, co-infiltration of *VpICE1* with $P_{VpPUB24}$ caused a significant increase in GUS activity compared with the $P_{VpPUB24}$ line. These results indicated that at 22 °C VpICE1 had little influence on the expression of *VpPUB24*, but during cold treatment it played a clear role in promoting the expression of *VpPUB24*.

VpHOS1 interacts with VpICE1 and promotes the degradation of VpICE1

Our results suggest that the degradation of VpICE1 is related to the ubiquitination system, but that VpPUB24 does not promote the degradation of VpICE1. Previous studies with *A. thaliana* revealed that an E3 ubiquitin ligase, AtHOS1 (AT2G39810), mediates the degradation of AtICE1 (Dong *et al.*, 2006). We therefore cloned a homolog of *AtHOS1* from *V. pseudoreticulata*, called *VpHOS1* (KU296029). This is predicted to share 54% amino acid identity with AtHOS1. A yeast two-hybrid assay showed that VpHOS1 interacted with VpICE1 (Fig. 8A). We further confirmed this interaction using a BiFC assay (Fig. 8B).

To further investigate the relationship between *VpHOS1* and *VpICE1* in grapevine, *VpHOS1* was cloned into the *pCAMBIA2300-GFP* vector and an *Agrobacterium*-mediated transient expression assay was carried out in tobacco leaves. Immunoblot analysis indicated that increasing the proportion of GFP-tagged VpHOS1 accelerated the degradation of Myc-tagged VpICE1. We conclude that VpHOS1 promotes the degradation of VpICE1 in grapevine, in the same way as in *A. thaliana* (Fig. 8C).

HOS1 *expression was different after cold treatment in various cold-resistant grapevines*

To further study how *HOS1* responds to low temperatures, we carried out RT-PCR experiments to measure *HOS1* transcripts after cold treatment. The results indicated that in 'Cabernet Sauvignon', *HOS1* transcripts increased to a relative high level rapidly after cold treatment; in *V. pseudoreticulata*, the expression of *HOS1* increased only slightly. Notably, in *V. amurensis*, *HOS1* expression stayed at a relatively stable level or declined at 2 h but then increased at 8 h and peaked at 12 h (Fig. 8D). These results suggest that *HOS1* responds to cold treatment but there are differences in response among the grapevine species examined here.

VpPUB24 stabilizes VpICE1 and inhibits the accumulation of VpHOS1 under cold treatment

Both VpPUB24 and VpHOS1 interact with VpICE1, but they play opposite roles in the regulation of VpICE1. To further investigate the function of VpPUB24 in stabilizing the amount of VpICE1, we carried out a transient expression assay in tobacco leaves by co-expressing *VpHOS1* and *VpICE1* with *VpPUB24*. In this study, the concentration of *Agrobacterium* lines harboring expression vectors for *VpHOS1* and *VpICE1* was held constant with a culture of OD_{600} ≈0.4, and the ratio of the relative concentration of *Agrobacterium* expressing *VpPUB24* was gradually increased from 1 to 4. The plant materials were maintained at 22 °C for 2 d and then placed at 4 °C for 12 h. In the immunoblot analyses, anti-Myc was used to detect the expression levels of VpICE1 and anti-GFP was used to detect VpPUB24 and VpHOS1. We observed that while the abundance of VpPUB24 increased, VpICE1 levels were relatively stable and the abundance of VpHOS1 showed a downward trend (Fig. 9A). Furthermore, a yeast two-hybrid assay confirmed that VpPUB24 interacted with VpHOS1 (Fig. 9B). These results indicate that when plants are stimulated by cold temperatures, VpPUB24 acts to stabilize VpICE1 levels by inhibiting the accumulation of VpHOS1.

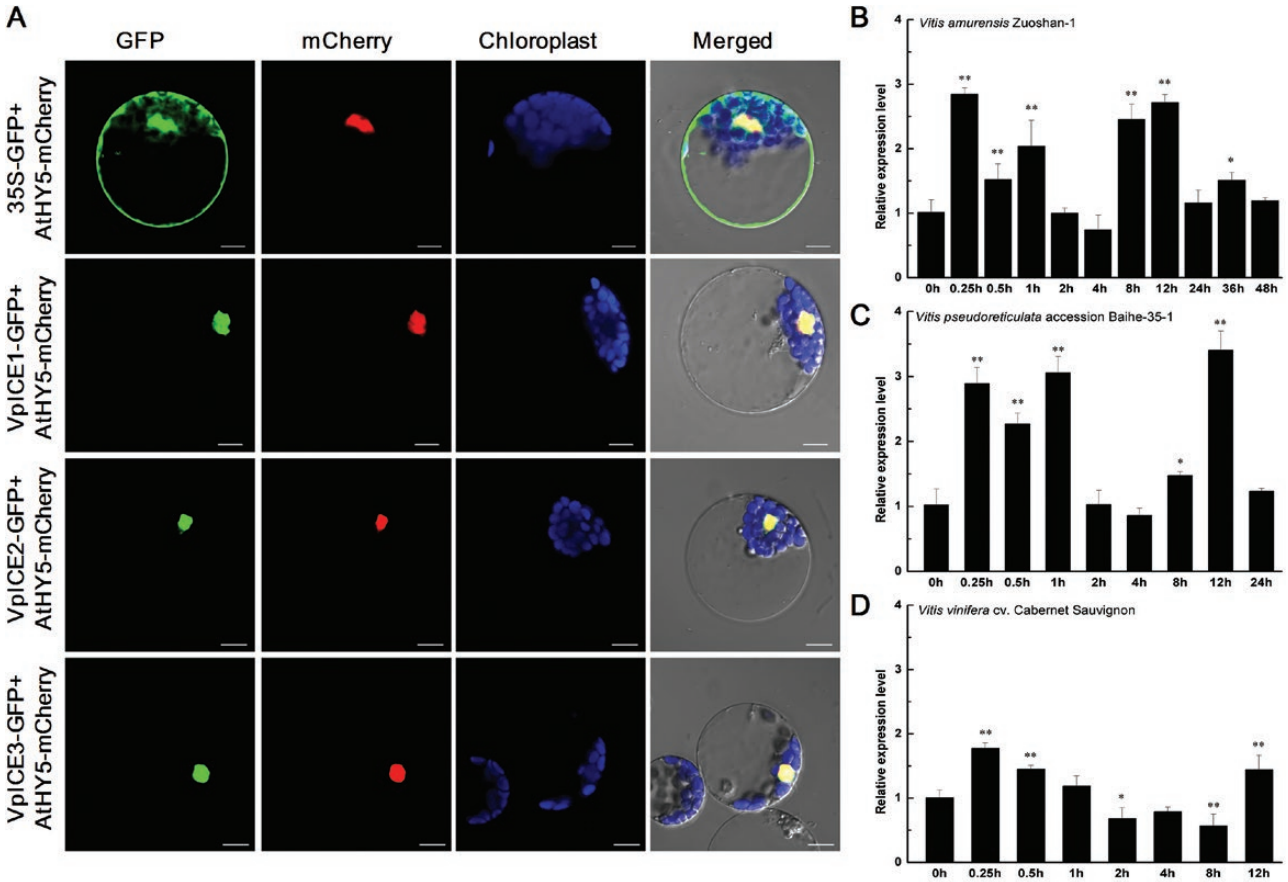

Fig. 6. Subcellular location of the transcription factor VpICEs, and response of *ICE1* to cold treatment in different grapevine species. (A) Subcellular location of transcription factor ICE proteins in *Arabidopsis thaliana* protoplasts. VpICEs (VpICE1, VpICE2 and VpICE3) and the *A. thaliana* nuclear protein AtHY5 were combined with GFP and mCherry individually and plasmids were co-transformed in protoplasts. After incubating the protoplasts at room temperature for 20 h, fluorescence was observed. Protoplasts expressing GFP driven by the *CaMV35S* promoter were used as a positive control. Bars=10 μm. (B–D) *ICE1* responds to cold treatment in various grape genotypes. Three grape cultivars (*V. amurensis* Zuoshan-1, *V. pseudoreticulata* accession Baihe-35-1 and *V. vinifera* cv. 'Cabernet Sauvignon') were used in RT-PCR analysis. The grapevines were treated at –2 °C and leaf samples were harvested at different times for RT-PCR analysis, using *VvActin* as a control. Significance was assessed by a least significant difference test (*P<0.05, **P<0.01). Error bars show the SD from three independent experiments. (This figure is available in colour at *JXB* online.)

Overexpression of VpPUB24 *in* A. thaliana *enhances freezing tolerance*

Finally, to further study the function of *VpPUB24* in cold tolerance, we generated transgenic *Arabidopsis* lines that overexpressed *VpPUB24*. Seedlings from three independent T3 lines (OE#2, OE#3, and OE#7) were subjected to cold treatment. First, we carried out RT-PCR and semi-quantitative PCR tests to measure the transcripts of *VpPUB24* in the transgenic Arabidopsis plants and found that *VpPUB24* was highly expressed in the three transgenic lines but not in the wild-type (Col-0) plants (Supplementary Fig. S5). In the freezing treatment assay we observed that the *VpPUB24*-overexpressing lines had a higher survival rate than the Col-0 plants (Fig. 10A, B). Consistent with this observation, relative electrolyte leakage in the transgenic seedlings was also significantly lower than in the wild-type plants (Fig. 10C). This suggests that heterologous expression of *VpPUB24* in *A. thaliana* enhanced cold tolerance. Furthermore, RT-PCR analysis supported these findings, as the transcript levels of *ICE1*, *ICE2*, *CBF3*, *COR47*, *COR15A*, and *RD29A* were all higher in the overexpression lines than in the wild-type plants after treatment at 4 °C (Fig. 11). Additionally, the expression of the *CBF* genes increased more rapidly and reached higher levels in the transgenic plants (Fig. 11). We conclude that the expression of *VpPUB24* promotes the expression of both the *ICE* and *CBF* genes, thereby inducing the expression of downstream genes in order to enhance cold tolerance in Arabidopsis.

Discussion

Plants have evolved a number of mechanisms to respond to cold stress. One of these is the ICE-CBF/DREB1 signaling cascade, which has been shown in *A. thaliana* to play important roles in signal transduction following cold stress (Zhao *et al.*, 2015). In this study, we characterized a U-box protein encoding gene, *VpPUB24*, from Chinese wild grapevine, whose expression is rapidly induced by cold treatment. Our results indicate that VpPUB24 physically interacts with VpICE1 and causes a decrease in the expression of VpHOS1, thereby regulating cold response.

Originally, VpPUB24 was considered a putative U-box type E3 ubiquitin ligase. Using promoter analysis, we found

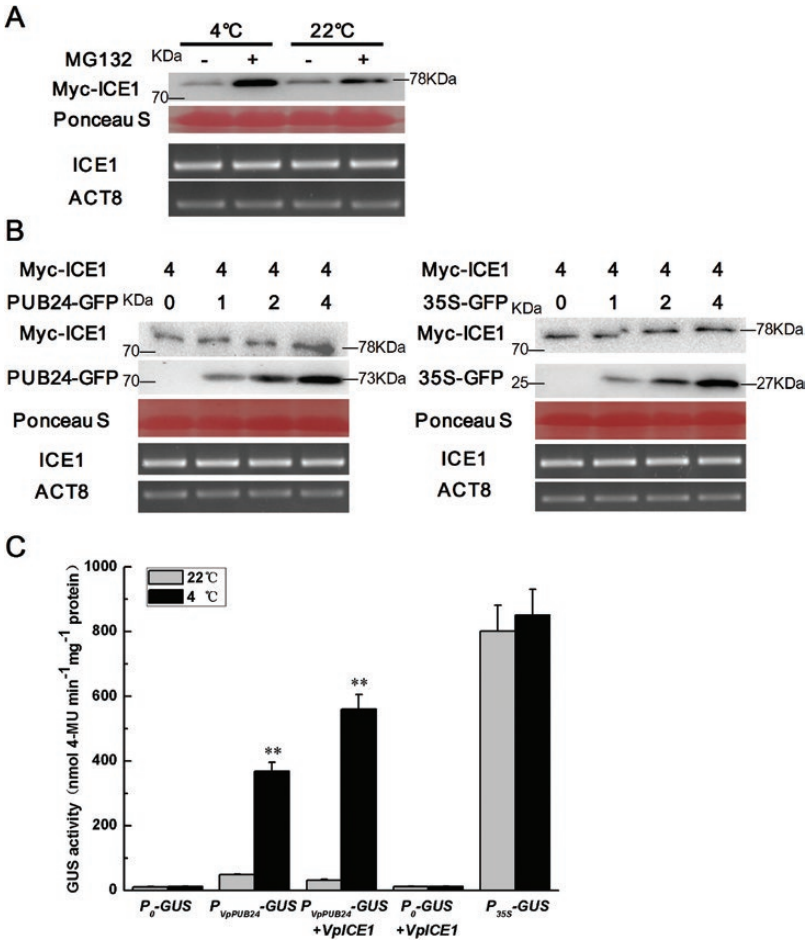

Fig. 7. (A, B) The U-box type E3 ubiquitin ligase gene *VpPUB24* promotes the accumulation of ICE1. The molecular masses of marker proteins (kDa) are indicated at the left of each panel. A Ponceau S-staining band was used as loading control. The expression of *ICE1* and *ACT8* mRNA was analyzed by semi-quantitative RT-PCR. (A) Degradation of VpICE1 via the plant 26S-proteasome pathway. Myc-ICE1 was expressed in *Nicotiana benthamiana* leaves by *Agrobacterium* infiltration. After 24 h, half of the transformed leaves were infiltrated with 50 μM MG132 and cultivated for an additional 24 h. The plantlets were then exposed to 4 °C or 22 °C for 12 h and leaves were harvested for western blot analysis. (B) VpPUB24 promotes the accumulation of VpICE1 protein levels under low temperature. Numbers above each panel refer to the ratio of the relative *Agrobacterium* concentrations used in the different co-infiltration experiments. Cell extracts were used for immunoblot analyses. Anti-GFP antibody was used to detect VpPUB24-GFP and 35S-GFP, and anti-Myc was used to detect Myc-VpICE1. (C) VpICE1 promotes the expression of VpPUB24 under cold treatment. Tobacco leaves were infiltrated with *Agrobacterium* cultures carrying the indicated plasmid combinations and the plants were cultivated at 22 °C for 2 d before transfer to 4 °C. After treatment at 4 °C for 12 h, the samples were collected for GUS activity analysis. P_0-*GUS* (empty pC0380GUS vector) was used as a negative control and P_{35S}-*GUS* (*CaMV35S-pC0380GUS*) as a positive control. All the tests were repeated three times, and significance was assessed by a least significant difference test (*P<0.05, **P<0.01). Error bars indicate the SD from three independent experiments. (This figure is available in colour at *JXB* online.)

that *VpPUB24* expression was induced by biotic and abiotic stresses, and especially by cold treatment (Fig. 2A, B). Next, in grapevine protoplasts, we confirmed that the accumulation of VpPUB24 protein was strongly affected by cold treatment (Fig. 3). Previous studies have identified a number of U-box type ubiquitin ligases that are involved in cold responses. Hot pepper (*C. annuum*) *CaPUB1* has been shown to be induced by a range of abiotic stresses, including cold temperatures (Cho *et al.*, 2006). Interestingly, *AtPUB18*, *AtPUB19*, *AtPUB22*, and *AtPUB23* from *A. thaliana* all decreased tolerance to drought stress, although they were rapidly induced by cold stress (Cho *et al.*, 2008; Seo *et al.*, 2012). Recently, *VaPUB* from *V. amurensis* has also been shown to be involved in cold hardiness, and its heterologous expression in *A. thaliana* is reported to enhance cold tolerance (Jiao *et al.*, 2014). Although many PUB proteins have been reported to be involved in cold stress, their regulatory mechanisms are not clear. In the present study, we screened the target protein VpICE1 as the interacting protein of VpPUB24 by the yeast two-hybrid assay and the following experiments confirmed their interacting relationship (Figs. 4 and 5). Previous studies have identified four *ICE* genes in grapevine (Rahman *et al.*, 2014). After alignment analysis, we cloned three *ICE* genes and found that the three VpICE genes had conserved HLH and C-terminal ACT domains. In the yeast two-hybrid assay we found that all the *ICE* genes could interact with VpPUB24 (Fig. 5C). Therefore, the VpPUB24 may act as a regulator of *VpICE* genes to activate the ICE1-CBF/DREB1 cascade.

In *A. thaliana*, ICE1 has been shown to control the expression of the downstream genes *CBF3*/*DREB1a* in response to low temperatures and to be degraded by an E3 ubiquitin ligase, HOS1 (Dong *et al.*, 2006; Miura *et al.*, 2007; Zhao

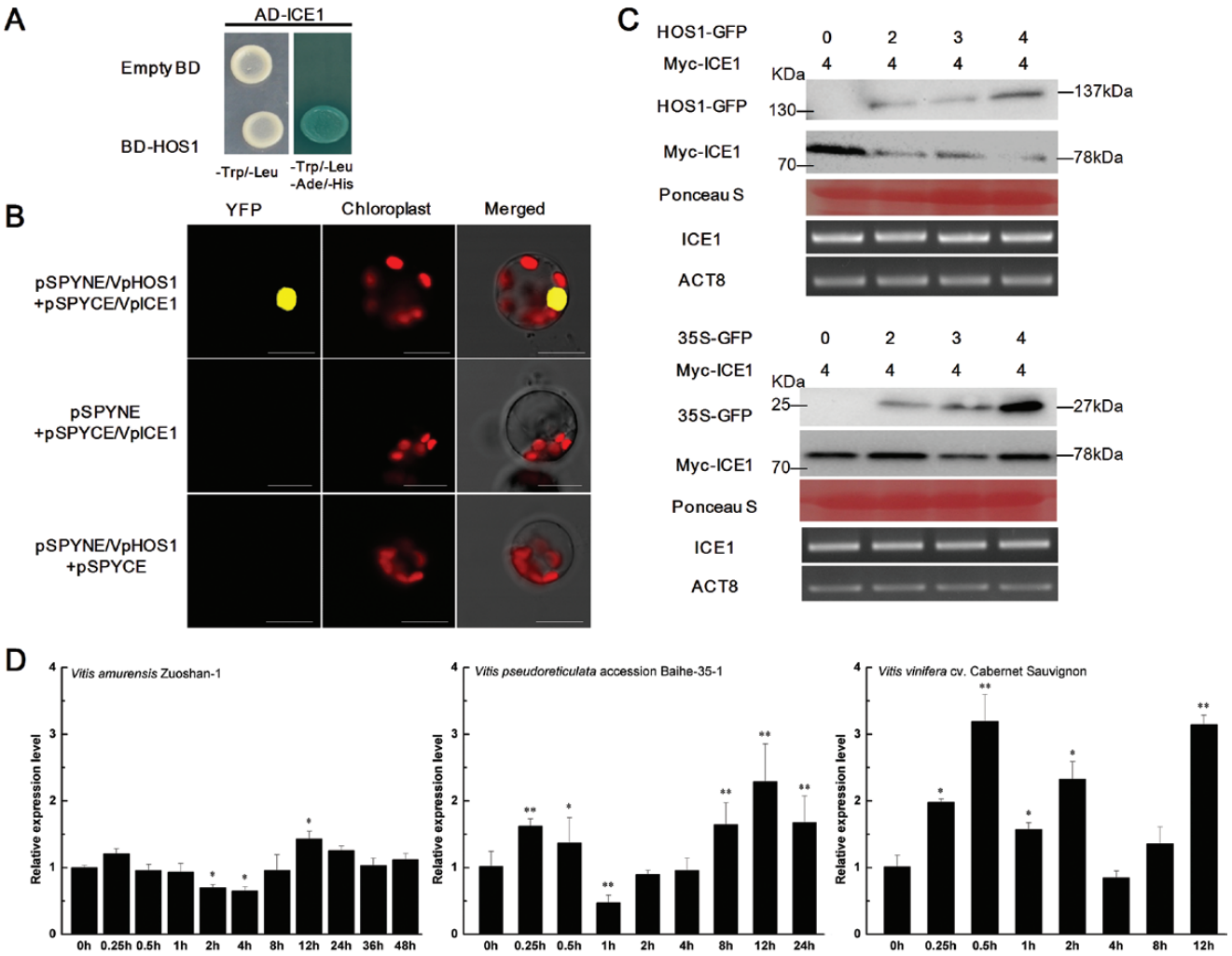

Fig. 8. VpHOS1 interacts with the transcription factor VpICE1 and mediates the degradation of VpICE1. (A) Interaction of VpHOS1 with VpICE1 in a yeast two-hybrid assay. pGADT7-VpICE1 and pGBKT7-VpHOS1 plasmids were co-transformed into yeast Y2H Gold cells. The empty pGBKT7 vector co-transformed with pGADT7-VpICE1 was used as a control. Yeast was placed on SD/-Trp/-Leu/-Ade/-His/Aba/x-α-Gal media for the confirmation assay. (B) A BiFC assay was carried out to confirm the interaction between VpHOS1 and VpICE1 in grapevine protoplasts. Plasmids carrying pSPYNE/VpHOS1 and pSPYCE/VpICE1 were transformed into protoplasts with the indicated combinations. The transformed protoplasts were incubated at 4 °C for 2 h prior to analysis. Bars=10 μm. (C) VpHOS1 mediated the degradation of VpICE1 *in vivo*. Different amounts of *Agrobacterium* culture carrying HOS1-GFP plasmid were co-infiltrated into tobacco leaves with an equal proportion of an *Agrobacterium* culture carrying a Myc-ICE1 encoding plasmid. The plants were exposed to 4 °C for 12 h prior to harvesting the leaves. Leaf extracts were used for immunoblot analyses. Anti-GFP antibody was used to detect VpHOS1-GFP and 35S-GFP, and anti-Myc was used to detect Myc-VpICE1. The molecular masses of marker proteins (kDa) are indicated at the left of each panel. A Ponceau S-staining band was used as a loading control. *ICE1* and *ACT8* gene mRNA expression was analyzed by semi-quantitative RT-PCR. (D) Response of *HOS1* to cold treatment in different grapevines. Three grapevine varieties (*V. amurensis* Zuoshan-1, *V. pseudoreticulata* accession Baihe-35-1, and *Vitis vinifera* cv. 'Cabernet Sauvignon') were used in an RT-PCR analysis. The grapevines were treated at –2 °C and leaf samples were harvested at different times for RT-PCR analysis, using *VvActin* as a control. Significance was assessed by a least significant difference test (**P*<0.05, ***P*<0.01). Error bars show the SD from three independent experiments. (This figure is available in colour at *JXB* online.)

et al., 2015). *ICE1* homologs have been identified in many plant species. Sequence analyses indicate that the *A. thaliana* ICE1 Ser403 is conserved in ICE1 proteins from other species. It has been suggested that this residue plays a role in stabilizing ICE1. *A. thaliana* SIZ1 has also been shown to stabilize ICE1 by sumoylation of Lys393, and the conversion of Lys393 to Arg393 blocks this sumoylation (Miura *et al.*, 2007, 2011). Therefore, the ICE1 protein was regulated by the collaboration of AtSIZ1 and AtHOS1. Here we found some diversity among the ICE proteins, since this Lys residue was replaced by Arg in the grapevine species *V. pseudoreticulata*, *V. vinifera*, *V. riparia*, and *V. amurensis* (Supplementary Fig. S3A). The presence of Arg at position 416 (corresponding to *A. thaliana* Lys393) in these grapevine species may explain the different modes of regulation, where *VpPUB24* may have developed a similar function to *SIZ1*. Furthermore, by protein degradation assay we found that the degradation of VpICE1 is inhibited by infiltration with the 26S proteasome-specific protease inhibitor MG132 (Fig. 7A). We hypothesize that VpICE1 Ser426 (corresponding to AtICE1 Ser403) is involved in transactivation and polyubiquitination of the protein, as is the case in *A. thaliana*, and that HOS1 may compete with PUB24 to modify ICE1.

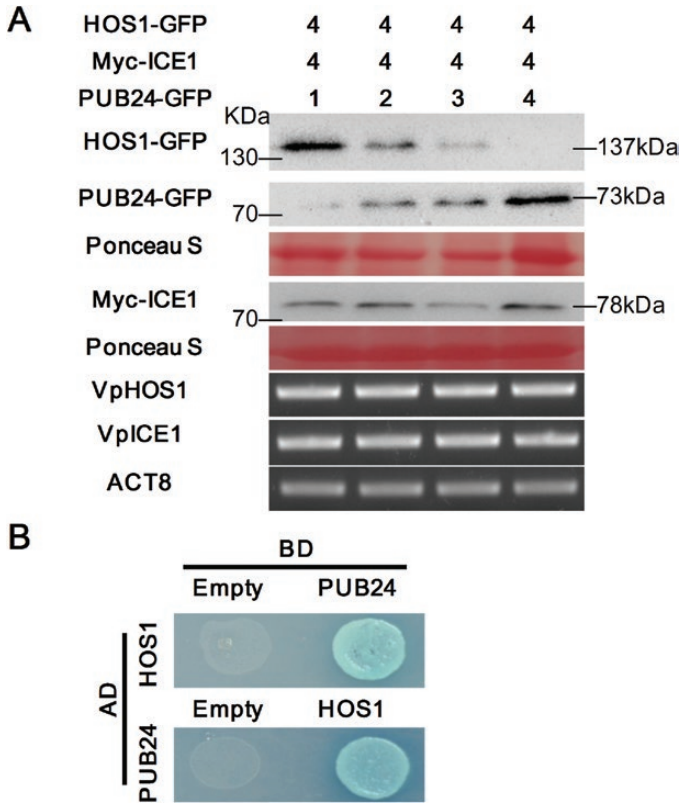

Fig. 9. VpPUB24 promotes the accumulation of the transcription factor VpICE1 by inhibiting the expression of VpHOS1. (A) VpHOS1 and VpPUB24 coordinate and regulate VpICE1 protein levels. Different ratios of *Agrobacterium* cultures harbouring vectors to express PUB24-GFP, Myc-ICE1, and HOS1-GFP were co-infiltrated in tobacco leaves and the plants were exposed to 4 °C for 12 h prior to harvesting the leaves. Leaf extracts were used for immunoblot analyses. Anti-GFP antibody was used to detect VpHOS1-GFP and VpPUB24-GFP, and anti-Myc was used to detect Myc-VpICE1. The molecular masses of marker proteins (kDa) are indicated at the left. A Ponceau S-staining band was used as a loading control. The *HOS1*, *ICE1* and *ACT8* gene mRNA expression was analyzed by semi-quantitative RT-PCR. (B) Interaction of VpPUB24 with VpHOS1 in yeast. To confirm the interaction of VpPUB24 and VpHOS1, the pGADT7-VpHOS1 plasmid was co-transformed with pGBKT7-PUB24 and empty pGBKT7 vector, respectively. The pGADT7-VpPUB24 plasmid was co-transformed with pGBKT7-VpHOS1 or empty pGBKT7 vector. Yeast was placed on SD/-Trp/-Leu/-Ade/-His/Aba/x-α-Gal media for the confirmation assay. (This figure is available in colour at *JXB* online.)

Ubiquitin ligases are known to play important roles in the degradation of target proteins. Because VpPUB24 is a putative E3 ubiquitin ligase, we initially considered that PUB24 might promote the degradation of VpICE1. Interestingly, we found that VpPUB24 increased the accumulation of VpICE1 (Fig. 7B). We determined that VpPUB24 did not promote the degradation of VpICE1, but rather acted to stabilize VpICE1 levels. Thus, we conclude that VpPUB24 is a previously unreported factor involved in the regulation of the ICE1-CBF/DREB1 cascade. To further investigate the degradation of VpICE1, we cloned *VpHOS1*. Previous studies have shown that the amino acid sequence of HOS1 is highly conserved among plant species, and the rice protein OsHOS1 also interacts with ICE1, supporting a close relationship (Lourenço *et al.*, 2013). Consistent with a previous study, we observed that HOS1 interacts with VpICE1 and mediates ICE1 degradation (Fig. 8A–C). In the following tobacco leaf infiltration assay, the results indicated that the levels of VpHOS1 protein show an opposite trend to those of VpPUB24 and at the same time the VpICE1 protein level is stabilized (Fig. 9A). Taken together, these results suggest that *PUB24* enhances cold tolerance by stabilizing the level of ICE1 protein, while HOS1 plays a negative role in this process. A yeast two-hybrid assay further confirmed that VpPUB24 interacted with VpHOS1 (Fig. 9B). Hence, we conclude that VpPUB24 is likely a component of the VpICE1 regulatory complex, which plays roles in the regulation of VpHOS1 and VpICE1. In addition, we cloned five homologous genes of *VpPUB24* from other grapevine species and found that their amino acid sequences shared 100% identities with one another (Supplementary Fig. S2). Therefore, PUB24 may play similar roles in various grapevine species.

The grapevine species examined here exhibit substantially different levels of cold resistance (Zhang *et al.*, 2015). Using RT-PCR analyses, we found that in the three grapevines examined transcripts of *PUB24* and *ICE1* increased rapidly after cold treatment, although their expression levels were different (Figs. 2D and 6B–D). Interestingly, *HOS1* transcripts increased and peaked rapidly in 'Cabernet Sauvignon', but in *V. pseudoreticulata* increased only slightly and in *V. amurensis* even declined after cold stress (Fig. 8D). These results indicate that in different grapevines, the relative expression levels of *PUB24*, *ICE1*, and *HOS1* show some difference in response to cold stress.

Previous studies have indicated that ICE1 is the key regulator in the ICE-CBF/DREB1 cascade. AtICE1 specifically binds to the E-box (CANNTG) *cis*-acting element of transcription factor CBF3 promoter, and in the promoter of CBF3 there are five E-box sites within 1 kb (Chinnusamy *et al.*, 2003). Promoter analysis suggests that in the promoter region of *VpPUB24* there were five E-box binding sites within 850 bp (Supplementary Fig. S1). Furthermore, we coexpressed $P_{VpPUB24}$ and *35S-VpICE1* in tobacco leaves and found that at 22 °C, VpICE1 had little influence on the expression of *VpPUB24*. However, under cold treatment, VpICE1 played a positive role in promoting the expression of *VpPUB24* (Fig. 7C). Therefore, PUB24 promotes the accumulation of VpICE1, and VpICE1 promotes the transcription of *VpPUB24*. Together, the collaboration between VpPUB24 and VpICE1 caused VpICE1 to be accumulated under cold conditions.

Using the yeast two-hybrid assay, we confirmed that the C-terminal ACT domain of all *VpICE* genes interacted with *VpPUB24*. It has previously been shown in *A. thaliana* that under cold stress, AtJAZ1 and AtJAZ4 repress the transcriptional regulatory function of ICE1 by interacting with its C-terminal ACT domain (Hu *et al.*, 2013). We also explored the relationship between VpICE1 and two grapevine genes encoding JAZ proteins, *VpJAZ1* (KU296030) and *VpJAZ4* (KU296031), which are homologous to *AtJAZ1* and *AtJAZ4*, respectively. Our results demonstrate that *VpJAZ1* indeed interacts with the *VpICE* genes (Supplementary Fig. S6). In contrast, VpPUB24 promotes the accumulation of ICE1. Therefore, *VpPUB24* plays the opposite role to *JAZ1* under

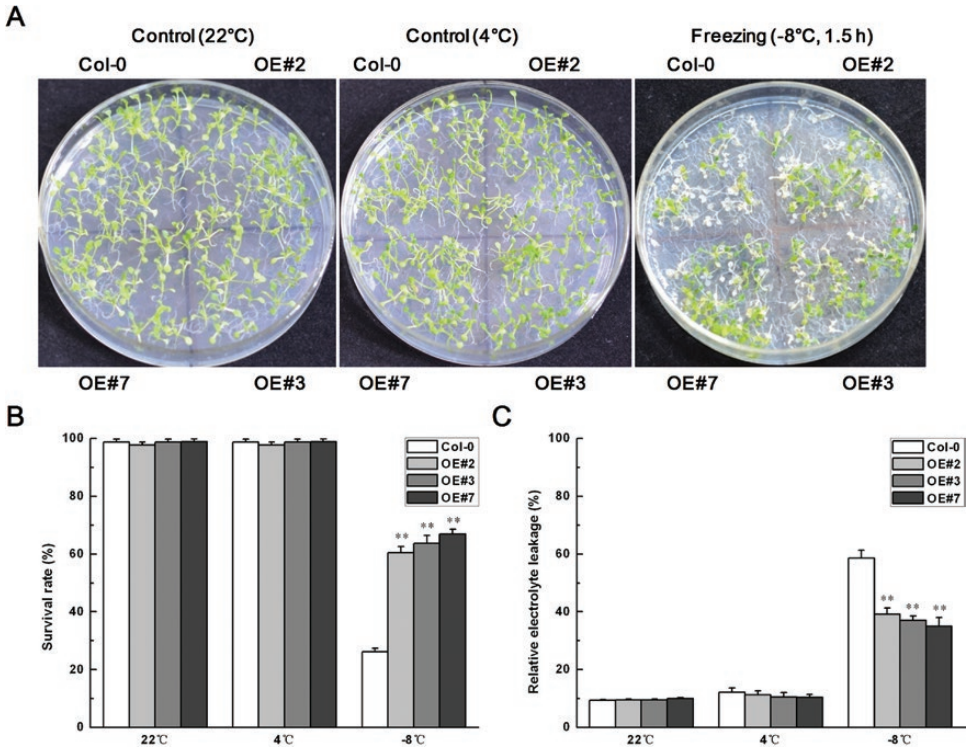

Fig. 10. Overexpression of the U-box type E3 ubiquitin ligase gene *VpPUB24* in *Arabidopsis thaliana* enhances cold tolerance. (A) Phenotype of 12-d-old wild-type *A. thaliana* and three *VpPUB24* overexpression lines (OE#2, OE#3, and OE#7) with or without freezing treatment (control at 22 °C). Seedlings (12-d-old) grown on 1/2 MS agar medium were treated at 4 °C for 1 d and then placed in a chamber set to cool at –1 °C per hour. When the temperature reached –8 °C, plates were exposed for 1.5 h. After the freezing treatment, the seedlings, including control seedlings, were incubated at 4 °C in the dark for 10 h, followed by recovery at 22 °C for 4 d. The experiments were carried out three times with similar results and at least 100 seedlings of each genotype were tested. (B) Survival rate and (C) relative electrolyte leakage of seedlings after exposure to freezing temperatures. Significance was assessed by a least significant difference test (**P*<0.05, ***P*<0.01). Error bars show the SD from three experiments. (This figure is available in colour at *JXB* online.)

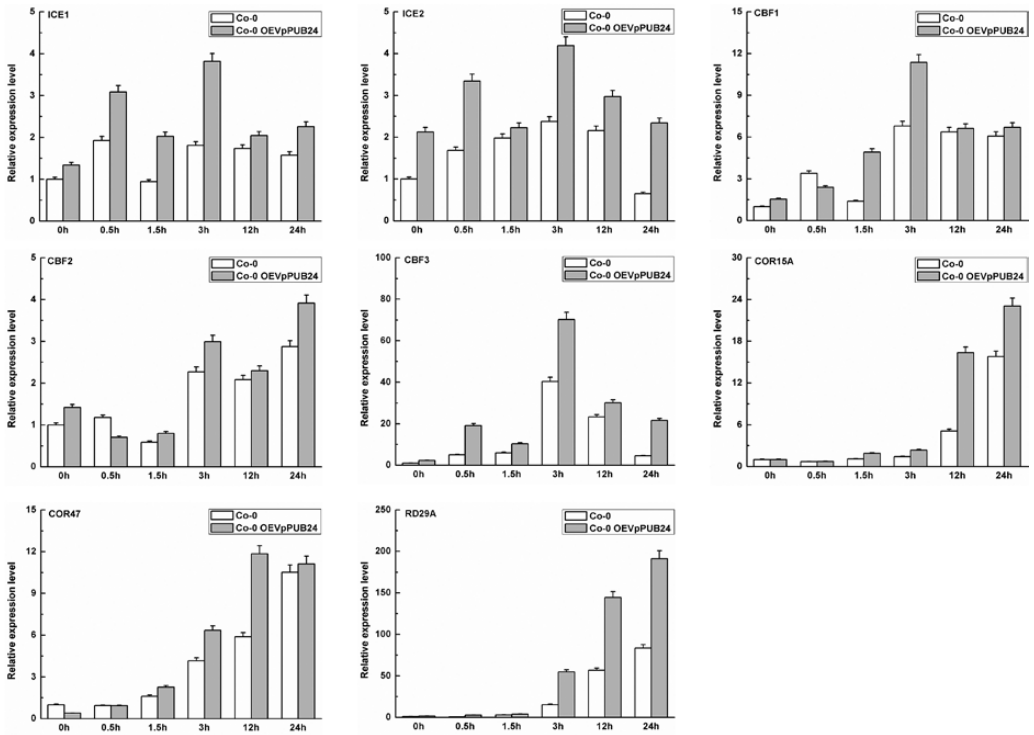

Fig. 11. RT-PCR analysis of cold-related genes in *VpPUB24* transgenic *Arabidopsis thaliana* at 4 °C. Seedlings 12-d-old grown on MS medium were transplanted into soil and cultivated at 22 °C for 30 d and then used for cold treatment. Different genotypes grown at 22 °C were transferred to a 4 °C growth chamber and samples were harvested at the times indicated. For the RT-PCR assay, the *AtActin* gene was used as internal control. Error bars show the SD from three independent experiments.

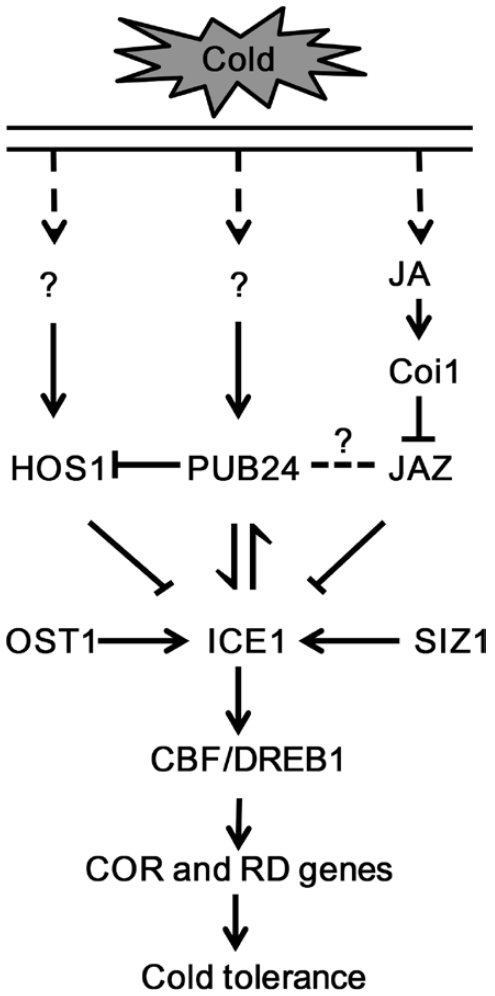

Fig. 12. Hypothetical pathway of cold stress response regulation by the U-box protein VpPUB24. Under cold stress, VpPUB24 is rapidly induced and physically interacts with VpICE1 in the nucleus. While VpPUB24 stabilizes the level of VpICE1 protein, VpPUB24 also suppresses the expression of HOS1 protein, thereby increasing the transcription of *CBF* genes, resulting in enhanced cold hardiness. VpPUB24 also interacts with the ACT domain of the VpICE1 protein, which is also the interaction target of JAZ protein. Therefore, VpPUB24 may compete with JAZ protein to interact with VpICE proteins. In the ICE-CBF/DREB1 cascade, the three proteins VpPUB24, VpICE1, and VpHOS1 work together to regulate the cold stress response.

cold stress, and there may thus be a competitive interaction between VpPUB24 and VpJAZ1. Additionally, we found the abundance of VpHOS1 protein decreased to some extent when VpPUB24 was highly expressed, and a yeast two-hybrid assay indicated that there was an interaction between VpHOS1 and VpPUB24. Therefore, the relationship between *HOS1* and *PUB24* is also worth further investigation.

In Arabidopsis, the downstream genes of the ICE-CBF/DREB1 cascade are *COR* and *RD* genes such as *COR15*, *COR47*, and *RD29A* (Wang & Hua, 2009; Park *et al.*, 2015). In our study, ectopic overexpression of *VpPUB24* in Arabidopsis improved freezing tolerance significantly, and in an RT-PCR analysis we found that the transcript levels of *CBF3*, *COR47*, *COR15A*, and *RD29A* were higher in the overexpression lines than in the wild-type after cold treatment at 4 °C (Fig. 11). These results indicate that in the overexpression lines, because of the high expression of *VpPUB24*, the abundance of internal ICE transcripts may accumulate faster and to higher levels than in wild-type plants. This effectively promoted the accumulation of the downstream protein.

In summary, we hypothesize that under normal temperatures, PUB24 is present at low levels in grapevines. The internal *PUB24* and *ICE1* are induced rapidly and their expression level showed some difference among different grapevine species, which may be because their sensitivity to cold stress (and, hence, their response) differed. In this process, PUB24 protein promotes the accumulation of ICE1 and, at the same time, restrains the accumulation of HOS1. Furthermore, VpPUB24 interacts with the ACT domain of the VpICE1 protein, which is the interaction domain of the JAZ protein. Therefore, VpPUB24 may compete with JAZ protein to interact with VpICE proteins. In addition, the transcription factor ICE1 activates the promoter of *PUB24*, which causes ICE1 to accumulate still further. OST1 and SIZ1 also act as positive regulators in the ICE-CBF/DREB1 cascade, which may promote the accumulation of ICE1 in grapevines. Finally, ICE1 activates the expression of the downstream *COR* and *RD* genes (Fig. 12). In short, VpPUB24 interacts with the ICE-CBF/DREB1 cascade to regulate the cold response.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Sequences of the primers used in vector construction.

Table S2. Primers used for RT-PCR.

Fig. S1. Sequence analysis of the *PUB24* promoter from Chinese wild grapevine *Vitis pseudoreticulata*.

Fig. S2. Alignment of *PUB24* in different grapevine species.

Fig. S3. Sequence analysis of VpICE1.

Fig. S4. Alignment of the *ICE* gene family in Chinese wild grapevine *Vitis pseudoreticulata*.

Fig. S5. Overexpression of *VpPUB24* in *Arabidopsis thaliana*.

Fig. S6. Interaction between VpICE1 and VpJAZs in the yeast two-hybrid assay.

Acknowledgements

The research was undertaken with grants from the National Science Foundation of China (grant no. 31171924) and the Project for Innovative Research Team of Grape Germplasm Resource and Breeding of Shaanxi Province (no. 2013KCT-25). The authors thank Prof. Jocelyn Rose (Plant Biology Section, School of Integrative Plant Science and Institute of Biotechnology, Cornell University, Ithaca, NY, USA) for helpful editing of the manuscript. The authors specifically thank Dr Alexander (Sandy) Lang from RESCRIPT Co. (New Zealand) for useful comments and language editing, which have greatly improved the manuscript.

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