Molecular Cloning and Characterization of the *HOS1* Gene from ‘Muscat Hamburg’ Grapevine

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ABSTRACT. Cold stress is an important factor that limits grape (*Vitis* sp.) production around the world. The high expression of osmotically responsive genes 1 (*HOS1*) protein acts as a repressor of cold-responsive genes in plants. To increase understanding of mechanism regulating cold tolerance in grape, we isolated and characterized a novel *HOS1* gene, designated *VvHOS1* from ‘Muscat Hamburg’ grapevine (*Vitis vinifera*). Real-time polymerase chain reaction (PCR) analysis revealed that the expression of *VvHOS1* could be induced by the application of exogenous abscisic acid and various abiotic environmental conditions such as low temperature, drought, and salinity. Moreover, *VvHOS1* expression could also be induced by cold plus drought conditions (4 °C, 10% polyethylene glycol 6000). In addition, overexpression of *VvHOS1* in arabidopsis (*Arabidopsis thaliana*) decreased the plants’ tolerance to cold, drought, and salt as well as negatively regulated the expression level of two stress-responsive genes, *AtRD29A* and *AtCOR47*. The results obtained in this study should help us to elucidate the function of *VvHOS1* and understand the cold-responsive pathway in grapevine.

Cold stress, including chilling (0 to 15 °C) and freezing (less than 0 °C), is one of the limiting environmental factors affecting plant growth (Levitt, 1980). To adapt to a cold environment, many plants have evolved a cold acclimation process when exposed to low non-freezing temperatures mediated by complex and elaborate signaling networks (Guy, 1990; Tang et al., 2006; Thomashow, 1999). This process is associated with the accumulation of compatible osmolytes and the stability of biomembranes (Orvar et al., 2000; Suzuki et al., 2000; Suzuki and Mittler, 2005). For the past several years, considerable attention has been devoted to the transcriptional activation of

the positive cold-responsive genes such as C-repeat binding factor (*CBF*) and its downstream genes: responsive to desiccation 29A (*RD29A*), cold-regulated 15A (*COR15A*), cold-regulated 47 (*COR47*), etc. A large number of genes that respond to cold acclimation have been identified in a number of plants (Fursova et al., 2009; Ganeshan et al., 2008; Knight et al., 2009; Lee and Thomashow, 2012; Medina et al., 2011; Provart et al., 2003; Thomashow, 2010; Xiao et al., 2006). Water-deficit stress such as drought and high salinity results in a marked reduction in crop productivity on as much as half of the irrigated land in the world. Signal transduction pathways triggered by various stresses, including drought and high salt content, share a number of signaling components that transduce the signal into downstream processes, which subsequently endow resistance to such stresses. In contrast, continuous activation of the plant’s cold-responsive genes is metabolically expensive and may result in permanent damage to the cellular components of the plant itself; thus, the expression of the cold-responsive genes must be controlled by negative regulation to maintain the balance of gene expression in

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the plant. Transcriptional repression and negative regulation are also important components of the cold-response regulatory cascades (Thiel et al., 2004). Therefore, the investigation of negative regulatory factors is extremely important in elucidating these pathways.

Components of the CBF-dependent signaling pathway are negatively regulated by upstream transcription factors. A negative regulator of this pathway, *HOS1*, was identified in arabidopsis. Plants with a mutated form of this gene, *Hos1-1*, exhibit enhanced expression of the cold-responsive genes (Ishitani et al., 1998). *HOS1* encodes an E3 ubiquitin ligase containing a RING-finger motif, and this gene product regulates both cold acclimation and vernalization in arabidopsis (Lazaro et al., 2012). *HOS1* protein is localized in the cytoplasm at normal growth temperatures but translocates to the nucleus in response to cold treatments, thereby causing the cold-regulated nucleocytoplasmic partitioning activity that negatively regulates cold signal transduction (Lee et al., 2001). During cold acclimation, *HOS1* mediates the ubiquitination and degradation of *ICE1* to reduce the CBF-induced activation of many cold-responsive genes and thus to decrease the plant's cold tolerance (Dong et al., 2006). In fact, *ICE1* has been identified as a target of the *HOS1* ubiquitin ligase. *ICE1*, the upstream transcriptional activator of *CBF*, can control the expression of *CBF* and its downstream cold-responsive genes in arabidopsis (Chinnusamy et al., 2003; Zarka et al., 2003). The *AtICE1* gene encodes a MYC basic helix-loop-helix (bHLH) transcription activator, which binds to the MYC *cis*-elements (CANNTG) in the promoter of the *CBF3* gene to induce its expression (Miura et al., 2007). However, *ICE1* has little effect on the cold induction of the *CBF1* and *CBF2* genes. Furthermore, *HOS1* also negatively regulates vernalization and flowering time by decreasing *FLOWERING LOCUS C (FLC)* expression (Ishitani et al., 1998; Lazaro et al., 2012), which is a central repressor of flowering induction by vernalization (Michaels and Amasino, 1999; Sheldon et al., 2000). Overexpression of the U-box-containing E3 ubiquitin ligase gene *AtCHIP* in arabidopsis rendered plants more sensitive to both low- and high-temperature treatments (Yan et al., 2003). The arabidopsis DREB2A-interacting proteins, DRIP1 and DRIP2, function as RING E3 ubiquitin ligases and negatively regulate drought stress-responsive gene expression by marking DREB2A to be degraded by the 26S proteasome (Qin et al., 2008). Another RING domain E3 ubiquitin ligase, *AtRGLG2*, negatively regulates the drought stress response by mediating the transcriptional activity of *AtERF53* in arabidopsis (Cheng et al., 2012). Overexpression of the arabidopsis RING-H2 gene *XERICO* results in hypersensitivity to salt and osmotic stresses and exogenous abscisic acid (ABA) during germination and early seedling growth (Ko et al., 2006). To gain insight into the biotic and abiotic stress resistance mechanisms in grape, in this study, we isolated and characterized a novel *HOS1* gene and investigated its response to various abiotic stresses.

Grapevine is the most widely planted fruit crop worldwide and is cultivated in all continents except Antarctica (Mullins et al., 1992). However, cold stress limits the geographical distribution and productivity of grape and results in considerable economic losses. As one of the most important cultivars, Muscat Hamburg is largely cultivated in the world as a result of its interesting fruit quality. Although cold-hardiness is found in 'Muscat Hamburg', which can withstand midwinter temperatures to -22°C (Fennell, 2004), these plants need to be buried in soil to protect the vine in winter in northern China, where

the minimal temperature in winter is much higher than the previous critical temperature and this burial is an obligatory practice for all the cultivars of *V. vinifera*. This phenomenon should be explained by low temperature plus dry climate in winter. Therefore, the molecular mechanisms of cold tolerance are important for the grape industry. To increase our understanding of the molecular mechanism of this plant's response to cold stress, it is necessary to confirm the molecular nature of the cold-responsive genes and to elucidate the regulatory network. The identification of genes that regulate cold acclimation in grape cultivars could be useful for molecular breeding. In this study, we isolated the *VvHOS1* gene from 'Muscat Hamburg' and characterized its expression patterns under various abiotic stress conditions and after the application of exogenous ABA. It was also shown that ectopic overexpression of *VvHOS1* in transgenic arabidopsis plants resulted in decreased tolerance to cold, drought, and salt.

Materials and Methods

PLANT MATERIALS. Tissue culture seedlings of 'Muscat Hamburg', the clonal plants germinated and grown in vitro, were cultured on half-strength B5 basal medium (Gamborg et al., 1968) containing $30\text{ g}\cdot\text{L}^{-1}$ sucrose, $1.25\text{ g}\cdot\text{L}^{-1}$ potassium nitrate (KNO_3), $12.5\text{ mg}\cdot\text{L}^{-1}$ inositol, and $0.2\text{ mg}\cdot\text{L}^{-1}$ indole-3-acetic acid in a growth chamber for 1 month at a constant temperature of 26°C under a cycle of 16 h of light with an intensity of $100\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 8 h of darkness.

RNA EXTRACTION AND FIRST-STRAND cDNA SYNTHESIS. Total RNA was extracted from the shoot apex with the first two young leaves of tissue culture seedlings of 'Muscat Hamburg' using a Column Plant RNAout 2.0 Kit (90404-50; Tiandz, Beijing, China). All protocols followed the manufacturer's instructions. The quality of the extracted RNA was determined by agarose gel electrophoresis, and the concentration was quantified using a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific, Waltham, MA). First-strand cDNA was synthesized from $1\text{ }\mu\text{g}$ high-quality total RNA treated with DNaseI (RQ1; Promega, Fitchburg, WI) in a $20\text{-}\mu\text{L}$ reaction volume using random hexamer primers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols.

ACQUISITION AND SEQUENCING OF THE FULL-LENGTH *VvHOS1* cDNA. *AtHOS1* (At2g39810) was used to find the homologous fragment of the *V. vinifera HOS1* gene in the *Vitis* genome, which is available in the Grape Genome Browser database (Genoscope, 2009). A pair of gene-specific primers P1 (Table 1) were designed based on this sequence to amplify this fragment. The fragment was amplified using the following program: one cycle of 30 s at 94°C , 35 cycles of 5 s at 94°C , 15 s at 54°C , 20 s at 72°C , and one cycle of 2 min at 72°C . The resulting PCR product was separated on a 1.2% agarose gel and extracted using a TIANGel Midi Purification Kit (Tiangen, Beijing, China). Purified fragments were then cloned into the pGEM T-easy vector (Promega) and transformed into *Escherichia coli* DH5 α cells. Positive colonies were confirmed through PCR analysis and sequencing of the inserted DNA (Sunny, Shanghai, China).

For the 3'-RACE protocol, first-strand cDNA was reverse-transcribed using a SMARTerTM RACE cDNA Amplification Kit (Clontech, Mountain View, CA) using 3'-CDS as a primer according to the manufacturer's instructions with some modifications. To obtain the 3' end of the *VvHOS1* gene, 3'-RACE was

Table 1. Primer sequences used in this study for polymerase chain reaction analysis in ‘Muscat Hamburg’ grapevine and arabidopsis.

No.	Name	Oligonucleotides (5' → 3')	Note
P1	HOS1f HOS1r	gaactcatcctaagatgcacaa catcaatggcactacttaacagg	For acquisition of <i>VvHOS1</i> fragment from ‘Muscat Hamburg’
P2	HOS1-3f1	ttacgggtggtctgggcatgatggg	Nested primers for 3' RACE of <i>VvHOS1</i>
P3	HOS1-3f2	gaagcagtcattgacgctcgggt	
P4	HOS1-5r1	caccaagccctccggatacaaaaga	Nested primers for 5' RACE of <i>VvHOS1</i>
P5	HOS1-5r2	gtcaccagtgtctccatccaatcc	
P6 ^z	HOS1fs HOS1rs	aaac <u>gagctc</u> aacactcattcacacatttatcatt acgc <u>ctcgacc</u> catgtaattgagaagcgtagc	For full-length cloning of <i>VvHOS1</i> from ‘Muscat Hamburg’
P7	HOS1s HOS1a	caaggaaaagcagttacggg ctctatcattctatccaccaagcc	For qRT-PCR analysis of <i>VvHOS1</i> in ‘Muscat Hamburg’ and transgenic arabidopsis
P8	18SrRNAs 18SrRNAa	cgaagcatttgccaaggat ccctggctcgcatcgtttat	For qRT-PCR analysis of the reference genes in ‘Muscat Hamburg’
P9	EF1αs EF1αa	gaactgggtgcttgatagc aaccaaaatatccggagtaaaaga	For qRT-PCR analysis of transgenic arabidopsis
P10	AtRD29As AtRD29Aa	gaactccggtctctctgc caatctccgtactctcca	
P11	AtCOR47s AtCOR47a	agcgatgaagaagtgagga acactggtaccgggatgga	
P12	AtACTINs AtACTINa	ctgcaccaagcagcatga ccgatccagacactgtacttcctt	

^zThe underlined bases in primers are the restriction sites.

qRT-PCR = quantitative reverse transcription–polymerase chain reaction.

performed according to the instructions of the SMARTer™ RACE Kit. Briefly, two nested gene-specific primers, P2 and P3 (Table 1), were designed according to the candidate *VvHOS1* gene fragment amplified previously. The UPM primer mix provided by the RACE kit, including 0.4 μM UPML and 2 μM UPMS, was used as an antisense primer during the first round of PCR. The 10 μM NUP primer provided by the RACE kit was used as an antisense primer for the second round of PCR. The first-round PCR reaction was performed in a total volume of 50 μL, including 35.3 μL double-distilled H₂O, 5 μL 10× Ex Taq buffer, 1 μL 10 mM dNTP mix, 2.5 μL diluted 3' RACE-ready cDNA, 5 μL UPM primer mix, 1 μL 10 μM P2 primer, and 0.2 μL 5 U·μL⁻¹ Ex Taq DNA polymerase. The touch-down PCR program was one cycle of 30 s at 94 °C, five cycles of 10 s at 94 °C, 30 s at 69 °C, 1.5 min at 72 °C, five cycles of 10 s at 94 °C, 30 s at 67 °C, 1.5 min at 72 °C, 20 cycles of 30 s at 94 °C, 10 s at 65 °C, 1.5 min at 72 °C, and one cycle of 3 min at 72 °C. Subsequently, the second-round PCR was carried out using a 50-fold dilution of the first-round PCR product as a template and the NUP and P3 oligos (Table 1) as primers. The touch-down PCR program was one cycle of 30 s at 94 °C, five cycles of 10 s at 94 °C, 30 s at 70 °C, 1.5 min at 72 °C, five cycles of 10 s at 94 °C, 30 s at 67 °C, 1.5 min at 72 °C, 20 cycles of 30 s at 94 °C, 10 s at 64 °C, 1.5 min at 72 °C, and one cycle of 3 min at 72 °C. The PCR product was separated on a 1.2% agarose gel. The purified fragments were cloned into the pGEM T-easy vector and then transformed into *E. coli* (DH5a) cells. White colonies were picked and the inserted DNA sequenced.

First-strand cDNA for the 5' RACE reaction was synthesized using PowerScript Reverse Transcriptase (Clontech) using the 5' linker (SMARTer IIA oligonucleotide) and the 5' CDS primer provided by the RACE kit. Nested PCR was carried out using a Advantage 2 PCR Kit (Clontech). Two antisense gene specific primers, P4 and P5 (Table 1), were designed for the 5' RACE reaction. Touch-down PCR was also implemented in both rounds of the PCR program. The purified product of the

nested PCR reaction was sequenced. The detailed procedure was similar to that described for the 3' RACE reaction.

The primer pair P6, including the sense primer HOS1fs located in the 5' UTR (*Sac*I site is underlined) and the antisense primer HOS1rs located in the 3' UTR region (*Sal*I site is underlined), was designed for reverse transcription PCR (RT-PCR) (Table 1). The first-strand cDNA used for amplifying the candidate *VvHOS1* gene fragment was also used as a template to isolate the full-length cDNA of *VvHOS1* from ‘Muscat Hamburg’. The PCR reaction was carried out in a total volume of 50 μL, including 33 μL double-distilled H₂O, 10 μL 5× HF buffer, 1 μL 10 mM dNTPs, 1 μL 10 mM sense and antisense primers, 1.5 μL DMSO, 2 μL cDNA template, and 0.5 μL 2 U·μL⁻¹ Phusion DNA Polymerase (NEB, Ipswich, MA). The PCR was amplified using the following program: one cycle of 30 s at 98 °C, 35 cycles of 5 s at 94 °C, 15 s at 56 °C, 1.5 min at 72 °C, and one cycle of 6 min at 72 °C. The resulting PCR product was separated on 1.2% agarose gels. The purified positive fragments were cloned into the pGEM T-easy vector and then transferred into *E. coli* (DH5a). White colonies were picked out and the inserted DNA was sequenced.

BIOINFORMATICS ANALYSIS. The verified full-length cDNA sequence was translated into amino acid sequences using the Expert Protein Analysis System server [ExPASy (Swiss Institute of Bioinformatics, 1993)]. The theoretical isoelectric point (pI) and molecular weight were also predicted by the ExPASy ProtParam tool. The bHLH-ZIP domain was predicted using the web-based protein database Simple Modular Architecture Research Tool [SMART (Schultz et al., 1998)]. Related protein sequences were retrieved from the GenBank database using the BLASTP algorithm at the National Center for Biotechnology Information (NCBI). *VvHOS1* protein sequence was used as a query sequence and several homologous HOS1 proteins from different plants were selected based on their score. A multiple protein sequence alignment was performed using the ClustalW program (Thompson et al., 1994) using the default

parameters. For phylogenetic and sequence alignment analysis, the HOS1-like protein sequences from other plant species were obtained from the GenBank database, including *Arabidopsis* (NP_181511), *Arabidopsis lyrata* (XP_002879823), *Populus trichocarpa* (XP_002304293), *Ricinus communis* (EEF30910), *Poncirus trifoliata* (ACY92092), *Oryza sativa* (AFK10207), and *Physcomitrella patens* (XP_001780235). The phylogenetic tree generated using the HOS1 protein sequences was constructed using the neighbor-joining (NJ) method of the MEGA software [Version 4.0 (Tamura et al., 2007)].

REAL-TIME PCR ANALYSIS. For the expression analysis, 1-month-old tissue culture seedlings, fully adapted to growing in a growth chamber at 26 °C under a 24-h light intensity of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, were transferred to another chamber set at 4 °C with a constant light intensity of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for the cold treatment. Drought, salinity, and ABA treatments were initiated when the tissue culture plants were transferred from the solid half-strength B5 medium to a liquid half-strength B5 medium supplemented with 10% polyethylene glycol (PEG) 6000, 100 μM NaCl, or 100 μM ABA, respectively. The plantlets were also placed in liquid half-strength B5 medium containing 10% PEG 6000 under 4 °C to mimic a cold plus drought environment. Samples of the shoot apex with the first two young leaves were collected at 0, 0.5, 1, 2, 4, 8, 24, and 48 h. Samples were immediately frozen in liquid N₂ and stored at -80 °C until analysis. For each tested tissue, three biological replicates were collected by harvesting samples from three different plants.

Total RNA was extracted using a Column Plant RNAout 2.0 Kit and digested with RNase-free DNaseI (RQ1) to eliminate the remaining traces of genomic DNA. First-strand cDNA was synthesized from 1 μg total RNA in a 20- μL reaction volume using a M-MLV Reverse Transcriptase Kit (Invitrogen). *VvHOS1* gene fragments were amplified using the gene-specific primer pair P7 (Table 1). The *18S rRNA* and *EF1 α* genes (AF207053 and BQ799343, respectively) were used as reference genes in this analysis by qbaseplus software (Helleman et al., 2007) and were amplified using primer pairs P8 and P9 (Table 1), respectively. The reaction mixture contained 5 μL of 2 $\times$ SYBR Green Master Mix (Roche, Basel, Switzerland), 2.6 μL double-distilled H₂O, 0.2 μL each primer (10 μM), and 2 μL diluted cDNA template. The PCR was performed in a StepOne Plus real-time PCR instrument (Applied Biosystems, Foster City, CA) using the following conditions: one cycle of 5 min at 95 °C, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and one cycle of 95 °C for 10 min. The fluorescent products were read at the last step of each cycle. To confirm the specificity of the primers, a melting curve was constructed after the final PCR cycle. Furthermore, the real-time PCR products were analyzed on a 1.2% agarose gel to confirm the presence of a single amplicon and were later sequenced. The three biological and technical replicates were performed. Reaction mixtures without cDNA templates were used as negative controls to evaluate the specificity of each real-time PCR. The Ct values and the real-time PCR efficiencies were obtained using LinRegPCR (Ruijter et al., 2009) and the normalized relative quantities and SES for each sample were obtained by qbaseplus (Helleman et al., 2007). The relative fold difference for *VvHOS1* expression in various abiotic condition was calculated on the basis of the normalized relative quantities obtained previously with the normalized relative quantity of the untreated samples as one. The ΔCt value for each sample was obtained by subtracting mean Ct (*VvHOS1*) from mean Ct (reference), where mean Ct

for *VvHOS1* is the average cycle number at which a reaction reaches a specified fluorescence level from all biological and technique replications. The relative expression data were analyzed using the Eq. $2^{-\Delta\text{Ct}}$.

The expression levels of two endogenous stress response genes *AtRD29A* and *AtCOR47* in two T₄ transgenic lines and wild-type *Arabidopsis* were examined using real-time PCR. For this purpose, the third and fourth rosette leaves were harvested from 4-week-old wild-type and T₄ transgenic *Arabidopsis* lines (OE1 and OE2) between 1100 and 1200 HR (i.e., 3 to 4 h after starting the light period), frozen in liquid N₂, and stored at -80 °C pending RNA extraction. cDNA preparation and reaction conditions were as described previously. Real-time PCR was also conducted using a StepOnePlus qPCR machine and the SYBR GreenI Master Mix (Roche) with gene-specific primers for *AtRD29A* (P10, Table 1), *AtCOR47* (P11, Table 1) and *VaCBF4* (cbf4s and cbf4a). The relative expression level of each gene was calculated on the basis of the *Arabidopsis* reference gene *AtACTIN* with the primer pair P12 (Table 1). The expression level of each gene in the wild-type *Arabidopsis* was set to 1. For *VvHOS1*, the amplification on 10 pg plasmid DNA (pCambia 1301s-35S-*VvHOS1*) was set to 1.

VECTOR CONSTRUCTION AND ARABIDOPSIS TRANSFORMATION. The coding region of *VvHOS1* was amplified using the primer pair P6 (Table 1). The digested PCR product was ligated into the pCambia 1301s vector, which is a modified form of the pCambia1301 vector (donated by Y. Zhou), between the corresponding restriction sites under the control of the CaMV 35S promoter. The recombinant plasmid was sequenced to confirm the correct insertion and electroporated into *Agrobacterium tumefaciens* strain GV3101 cells. The genetic transformation of *Arabidopsis* was performed using the floral dip method (Clough and Bent, 1998). Transgenic *Arabidopsis* plants were selected on solid half-strength Murashige-Skoog medium [MS (Murashige and Skoog, 1962)] containing 50 $\mu\text{g}\cdot\text{L}^{-1}$ hygromycin B. T₁ transgenic plants were confirmed by RT-PCR analysis using the primer pair P6 (Table 1) for *VvHOS1*. T₄ plants were used for the stress tolerance experiments.

ANALYSIS OF COLD, DROUGHT, AND SALT TOLERANCE IN TRANSGENIC ARABIDOPSIS PLANTS. Abiotic stress tests were carried out as previously described (Dong et al., 2006) with some modifications. *Arabidopsis* seedlings (wild-type and T₄ transgenic lines OE1 and OE2) were grown for 12 d on half-strength MS medium in a growth chamber under a 16-h light (22 °C) and 8-h dark cycle (20 °C). The seedlings were later transferred to half-strength MS medium containing different concentrations of PEG 6000 (0%, 4%, and 8%) or NaCl (0, 20, and 40 mM) to assess drought and salt tolerance. The seedlings were subjected to stress for 5 d under the same environmental conditions as described previously. Cold stress was performed by transferring the 12-d-old plants into the chamber at three different temperatures (0, -2, and -4 °C) for 8 h and then returning to normal conditions for 5 d.

Results

SEQUENCE ISOLATION AND ANALYSIS OF *VvHOS1*. A candidate gene fragment from *V. vinifera* with a length of 461 bp was selected from the Grape Genome Browser database. This fragment had high sequence identity with the *AtHOS1* gene (At2g39810). Based on the RT-PCR and RACE analysis, a novel gene, designated *VvHOS1* (KC524503), was isolated from

‘Muscat Hamburg’. The full length of *VvHOS1* is 3118 bp with an open reading frame of 2931 bp and encodes a deduced protein of 976 amino acids with a theoretical isoelectric point of 5.74 and a molecular mass of 109.8 kDa.

VvHOS1 sequence homology was verified by using the BLAST algorithm on the NCBI server. The results revealed that *VvHOS1* protein exhibits a high sequence identity with the HOS1-like proteins from other plant species such as *Arabidopsis* (53%, NP_181511), *A. lyrata* (56%, XP_002879823), *P. trichocarpa* (64%, XP_002304293), *R. communis* (63%, EEF30910), *P. trifoliata* (64%, ACY92092), *O. sativa* (56%, AFK10207), and *P. patens* (41%, XP_001780235).

Furthermore, a multiple sequence alignment was performed using the ClustalW program. Alignment analysis revealed that *VvHOS1* protein contains a conserved RING finger domain in its N-terminal region (Supplementary Fig. 1). The RING finger motif can be described as a series of conserved cysteine and histidine residues and has a consensus sequence of Cys-X₂-Cys-X₍₉₋₃₉₎-Cys-X₍₁₋₃₎-His-X₍₂₋₃₎-Cys/His-X₂-Cys-X₍₄₋₄₈₎-Cys-X₂-Cys (C₃HC₄), where X can be any amino acid residue (Saurin et al., 1996). According to these results, we concluded that the predicted protein is the HOS1 protein from ‘Muscat Hamburg’.

To investigate the phylogenetic relationship between *VvHOS1* and other HOS1-like proteins, a phylogenetic tree was constructed (Supplementary Fig. 2) using the NJ method of the MEGA software (Version 4.0). The phylogenetic tree included three groups. The dicotyledonous species ‘Muscat Hamburg’, *Arabidopsis*, *A. lyrata*, *P. trichocarpa*, *R. communis*, and *P. trifoliata* were clustered into the first group, and the monocotyledon *O. sativa* solely occupied the second group.

The non-vascular plant moss *P. patens* composed the third group. The phylogenetic tree suggested that *VvHOS1* may have a closer relationship to the *PtHOS1* protein.

***VvHOS1* EXPRESSION IS INDUCED BY COLD, DROUGHT, HIGH SALT, AND COLD PLUS DROUGHT CONDITIONS AS WELL AS EXOGENOUS ABA.** To understand the function of *VvHOS1* better, we investigated *VvHOS1* transcript levels in shoot apices of 1-month-old ‘Muscat Hamburg’ seedlings, which had been subjected to exogenous 100 μ M ABA, or various stresses such as low temperature (4 °C), drought (10% PEG 6000), high salt (100 mM NaCl), and cold plus drought conditions (4 °C, 10% PEG 6000). Real-time PCR analysis indicates that *VvHOS1* gene expression in ‘Muscat Hamburg’ could be induced by exogenous 100 ABA application and various abiotic stresses (Fig. 1). In response to cold stress, *VvHOS1* transcripts accumulation declined at 30 min after initiating cold stress; however, *VvHOS1* expression had nearly recovered to its previous untreated level (0 h) at 1 h after cold stress. This rapid and transient reduction in *VvHOS1* message in response to brief cold treatments was consistently observed in independent experiments. After recovering from the transient decrease, *VvHOS1* transcript level reached its maximal level, 2 h after the cold stress, expression decreased gradually to its minimum value by 24 h, and recovered a little nearly to the untreated value at 48 h after treatment (Fig. 1A). After ABA treatment, *VvHOS1* mRNA transcripts increased slightly at 0.5 h, decreased from 1 to 4 h, and recovered nearly to the untreated level by 8, 24, and 48 h after ABA induction (Fig. 1B). *VvHOS1* mRNA abundance declined at 0.5 h after exposure to drought conditions but later peaked with a 2-fold increase over the untreated level after 1 h; expression subsequently decreased gradually, reaching the minimum expression level at 4 h; *VvHOS1*

Fig. 1. Expression patterns of *VvHOS1* gene from ‘Muscat Hamburg’ grapevine in response to various treatments under a 24-h light intensity of 100 μ mol·m⁻²·s⁻¹: (A) low-temperature stress (4 °C), (B) 100 μ M exogenous abscisic acid (ABA) for exogenous ABA induction, (C) drought stress [10% polyethylene glycol (PEG) 6000], (D) salt stress (100 mM NaCl), and (E) 10% PEG 6000 at 4 °C mimicking cold plus drought condition. The expression level of *VvHOS1* gene in samples grown under normal growth conditions was set to 1. The bars represent SD from three biological replicates.

Fig. 2. Expression patterns of *VvHOS1*, *AtRD29A*, and *AtCOR47* in wild-type (WT) and transgenic arabidopsis plants (lines OE1 and OE2) under normal growth conditions. Parallel reactions using *AtACTIN* were carried out to normalize the amounts of added template. The expression level of each gene in the wild-type control was set as 1. For *VvHOS1*, the amplification on 10 pg plasmid DNA (pCambia 1301s-35S-*VvHOS1*) was set as 1. The bars refer to SD of three biological replicates. Different upper case letters indicate significant differences at $P < 0.05$ according to least significant difference multicomparison analysis.

mRNA expression levels returned to nearly the untreated level by 8 h, declined slightly at 24 h, and recovered to 1.5-fold of the untreated level by the end of treatment (Fig. 1C). After salt treatment, *VvHOS1* expression increased at 0.5 h and decreased at 1 h; expression peaked at 4 h after treatment and gradually decreased by 24 h; and expression recovered a little at the end (48 h; Fig. 1D). Cold plus drought stress induced the expression of *VvHOS1* slightly at 0.5 h after application of the stress; the expression then declined at 2 h, peaked with a 2-fold increase over the untreated level by 4 h, and gradually decreased to minimum levels from 4 to 48 h (Fig. 1E).

COLD, DROUGHT, AND SALT TOLERANCE OF TRANSGENIC ARABIDOPSIS PLANTS EXPRESSING *VvHOS1*. To assess the effect of *VvHOS1* overexpression on endogenous gene expression, the expression of two stress response genes, *AtRD29A* and *AtCOR47*, were examined by real-time PCR in 4-week-old wild-type and T₄ transgenic lines (OE1 and OE2) as shown in Figure 2. The expression of endogenous gene *AtRD29A* and *AtCOR47* was significantly lower in *VvHOS1*-overexpression plants than wild-type arabidopsis under normal growth conditions. These results demonstrate that *VvHOS1* overexpression may have a negative impact on the expression of downstream stress response genes.

To investigate the biological function of *VvHOS1* gene in plant stress responses, two T₄ transgenic lines (OE1 and OE2) overexpressing *VvHOS1* under the control of the 35S promoter were selected to assess how *VvHOS1* affects cold, drought, or salt tolerance. Seeds from both wild-type and transgenic lines were grown on half-strength MS agar medium plates for 12 d. A portion of the seedlings was exposed to three different temperatures (0, -2, and -4 °C) for 8 h and then grown in normal growth conditions for a further 5 d to assess their cold tolerance. The *VvHOS1*-overexpression seedlings (OE1 and OE2) were less tolerant to low temperature than the wild type, particularly at -4 °C (Fig. 3A–B). For example, exposure to -4 °C killed nearly 55% of the transgenic lines (OE1 and OE2) but only 44% of wild-type plants. Another portion of the seedlings was transferred to half-strength MS medium plates supplemented with

three different concentrations of PEG 6000 (0%, 4%, and 8%) or NaCl (0, 20, and 40 mM). Five d after being exposed to drought or high salinity conditions, the growth of both the wild-type and transgenic lines were inhibited. However, the survival rate of wild-type plants was higher than that of transgenic plants [OE1 and OE2 (Fig. 3C–F)]. The lower survival rates of the *VvHOS1*-expression lines (OE1 and OE2) were significantly different from that of the wild-type control (WT) after subjecting to low temperatures (0, -2, and -4 °C), different concentrations of PEG 6000 (4% and 8%), and different concentrations of NaCl (20 and 40 mM). However, the survival rates of the 35S-*VvHOS1* plants (OE1 and OE2) was not significantly different from that of the WT control under normal growth conditions (i.e., no PEG 6000 or NaCl). These results show that the seedlings overexpressing *VvHOS1* were less tolerant to low temperature, drought conditions, and high salt than the wild-type plants.

Discussion

‘Muscat Hamburg’ is a *V. vinifera* grape cultivar that was derived by crossing ‘Schiava Grossa’ with ‘Muscat of Alexandria’ (Crespan, 2003). ‘Muscat Hamburg’ is a black table grape that is highly appreciated for its beautiful bunches and its fair muscat flavor and can also be used to produce wine. For these valuable characteristics, breeders have used ‘Muscat Hamburg’ as a maternal parent to obtain new table grape cultivars. In this study, a negative regulator of cold-responsive genes, designated *VvHOS1*, was identified from ‘Muscat Hamburg’ using RT-PCR and RACE methods. Multiple sequence alignments showed that *VvHOS1* has a highly conserved RING finger domain in its N-terminal region (Supplementary Fig. 1). The RING finger motif is a small zinc-binding domain found in proteins of many eukaryotes. The proteins that contain this domain have been identified as functional E3 ubiquitin ligases, which interact with the E2 ubiquitin conjugating enzyme to degrade specific target proteins (Joazeiro and Weissman, 2000). In many metabolic processes, numerous proteins are ubiquitinated and degraded, which means that the existence of a large number of RING finger proteins may be needed to control the highly selective protein degradation process. For example, there are more than 1300 genes predicted to encode putative E3 subunits with one family containing almost 700 members (Smalle and Vierstra, 2004). As previously reported, the *AtHOS1* gene encodes a RING finger protein that functions as an E3 ubiquitin ligase; this ligase physically mediates the ubiquitination and degradation of ICE1 (Dong et al., 2006). Therefore, *HOS1* acts as a negative regulator of cold-responsive genes because inactivation of ICE1 reduces the CBF-induced activation of many cold-responsive genes (Chinnusamy et al., 2003; Lee et al., 2005). *VvHOS1* shared high sequence identity with the *HOS1* protein from arabidopsis (53%) according to the BLASTP analysis, suggesting that *VvHOS1* may also interact with ICE1 and regulate the expression of cold-responsive genes. In addition, phylogenetic analysis shows that *VvHOS1* is closely related to the *HOS1*-like proteins of *P. trifoliata* (Supplementary Fig. 2).

In this article, the expression of *VvHOS1* after different treatments was analyzed by real-time PCR. *VvHOS1* expression was induced by low temperatures, as reported for *HOS1* in arabidopsis, and the expression pattern observed for *VvHOS1* was similar to that of *AtHOS1* when the plants were subjected to low temperatures (Lee et al., 2001). It is interesting that *VvHOS1* transcript abundance was transiently down-regulated

Fig. 3. Analysis of the stress tolerance and quantification of the survival rates for wild-type plants (WT) and transgenic arabidopsis plants overexpressing *VvHOS1*. Twelve-d-old seedlings of the transgenic plants (OE1 and OE2) and the wild-type plants (WT) were subjected to –4 °C for 8 h for cold stress and grown for a further 5 d in normal growth conditions (A), 8% polyethylene glycol (PEG) 6000 for 5 d drought stress (C), and 40 mM NaCl for 5 d salt stress (E). Quantification of the survival rates for WT and *VvHOS1*-overexpression transgenic plants (35S-*VvHOS1*) after being subjected to 8-h low-temperature treatment at 0, –2, and –4 °C (B); different concentrations of PEG 6000 (0%, 4%, and 8%) for drought stress (D); and different concentrations of NaCl (0, 20, and 40 mM) for salt stress (F). The bars represent SD of three biological replicates, and different upper case letters indicate significant differences at $P < 0.05$ according to least significant difference multicomparison analysis.

by cold stress (Fig. 1A). Because the genetic role of *HOS1* is to attenuate cold signaling, the transient down-regulation of the *HOS1* transcript may be important to allow the amplification of the cellular cold signals (Dong et al., 2006). During cold acclimation, the expression of a large number of genes is altered in plants (Benedict et al., 2006; Ishitani et al., 1998; Lee et al., 2001; Thomashow, 1999). This rapid transient reduction in expression and the extended duration of *VvHOS1* expression in

response to cold treatment suggests that *VvHOS1* may help activate the cold-responsive genes during cold acclimation in grape, as previously reported (Dong et al., 2006; Lee et al., 2001).

The transcript levels of *VvHOS1* also fluctuated after ABA application or after exposure to drought or high salinity (Fig. 1B–D); this expression pattern differs from that of *AtHOS1*. Although *AtHOS1* transcript abundance was not substantially

influenced by ABA, NaCl, or PEG treatments, the expression patterns of *AtHOS1* under these stresses were also unknown (Lee et al., 2001). To date, no study has focused on the effects of cold plus drought conditions on the gene expression patterns in grape. Thus, we analyzed the expression of *VvHOS1* under cold plus drought conditions; this stress is the most common one that grape encounters during winter in the major grape-producing areas in northern China. The results of the real-time PCR analysis suggest that cold plus drought conditions could also induce *VvHOS1* expression (Fig. 1E). Based on these findings, we suggest that *VvHOS1* expression is also regulated by exogenous ABA as well as drought, salt, and cold plus drought conditions, indicating that there is substantial cross-talk in the various stress signal transduction pathways in plants. In short, *VvHOS1* may be involved in ABA-dependent and ABA-independent signal transduction pathways and thereby affects the stress response of ‘Muscat Hamburg’.

Ectopic expression of *VvHOS1* resulted in decreased survival of the transgenic plants (35S-*VvHOS1*) after exposure to freezing temperatures, which was similar to the effects of over-expressing *AtHOS1* in arabidopsis (Dong et al., 2006). Meanwhile, the expression levels of *AtRD29A* and *AtCOR47* were also decreased in 35S-*VvHOS1* plants. Because the arabidopsis RING E3 ligase RGLG2, DRIP1, and DRIP2 proteins all negatively regulate the plant drought stress response (Cheng et al., 2012; Qin et al., 2008), it is possible that the over-expression of *VvHOS1* may reduce a plant’s tolerance to drought. The sensitivity of the 35S-*VvHOS1* plants to salt stress is similar to that of transgenic arabidopsis overexpressing the RING-H2 gene *AtXERICO* during early seedling growth (Ko et al., 2006). In this experiment, all of the T₄ transgenic plants tested (Fig. 3) were hypersensitive to cold, drought, and salt stresses compared with the WT plants.

Our findings indicate that expression of *VvHOS1* can be induced by a variety of abiotic stress conditions and the application of the signaling molecule ABA. Ectopic expression of *VvHOS1* in arabidopsis plants decreased tolerance to cold, drought, and salt stress and suppressed the expression of *AtRD29A* and *AtCOR47*. In conclusion, *VvHOS1* may be a negative regulator of the stress response in ‘Muscat Hamburg’. Further investigations are necessary to understand the exact nature of the function of *VvHOS1* protein and the effects of its overexpression on more cold-responsive genes, which may provide a novel approach to regulating plants’ tolerance to different environmental stresses.

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Supplementary Fig. 1. Amino acid sequence alignment of the HOS1-like proteins from *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Populus trichocarpa*, *Ricinus communis*, *Poncirus trifoliata*, *Oryza sativa*, *Physcomitrella patens*, and *Vitis vinifera* ‘Muscat Hamburg’. Identical amino acids are shaded. The conserved RING finger domain is underlined. The conserved cysteine and histidine residues are indicated by asterisks.

Supplementary Fig. 2. The phylogenetic tree of the close homologues of PtrHOS1 and other HOS1-like proteins found in plants. The solid diamond represents VvHOS1. The polypeptides used in the analysis include VvHOS1 from *Vitis vinifera* ‘Muscat Hamburg’ (AGH20655.1), AtHOS1 from *Arabidopsis thaliana* (NP_181511), AlHOS1 from *Arabidopsis lyrata* (XP_002879823), PtHOS1 from *Populus trichocarpa* (XP_002304293), RcHOS1 from *Ricinus communis* (EEF30910), PtrHOS1 from *Poncirus trifoliata* (ACY92092), OsHOS1 from *Oryza sativa* (AFK10207), and PpHOS1 from *Physcomitrella patens* (XP_001780235). Construction of the phylogenetic tree was performed using the neighbor-joining algorithm in MEGA 4.0 software. The numbers at each node represent the bootstrap values for 1000 replicates.