Introduction

Grapevine (Vitis L.) is one of the most widely cultivated fruit crops worldwide, and is of great economic importance. However, grape production is often severely limited by various biotic and abiotic stresses [1–5]. For example, low temperature stress greatly restricts the geographic range of grapevines cultivation, and decreases berry yield and quality. Significantly, the grapevine cultivars that currently dominate the market in terms of acreage and production of premium wines are derived from the species Vitis vinifera, but they tend to be very sensitive to low winter temperatures [6]. Thus, enhancing low temperature tolerance of grapevine is of great practical importance.

In this context, the current study focuses on Vitis amurensis Rupr., a wild grape species that is native to China and is extremely cold-tolerant [7], withstanding freezing temperature as low as ~40°C [8]. V. amurensis therefore has great potential as an experimental system to identify mechanisms of cold tolerance and as a germplasm resource for grapevine cold-resistance breeding. Some highly cold-resistant Vitis cultivars have previously been produced using classical breeding methods; however, resistance to cold stress is a multigenic trait, which limits the effectiveness of using traditional breeding [9]. Therefore, understanding the mechanisms underlying tolerance and adaptation to cold stress could potentially lead to the development of new strategies for improving the yield of cold sensitive agronomic plants and expanding the geographic areas of production.

Cold acclimation has been extensively studied, resulting in considerable evidences at the molecular level that cold stress triggers a multitude of physiological responses [10]. Cold responses are complex and highly regulated via activation of signaling pathways and numerous genes encoding proteins that act directly in stress tolerance. To date, numerous cold-regulated (COR) genes have been functionally identified, such as antifreeze proteins, later embryo abundant (LEA) proteins, molecular chaperones and...
enzymes involved in detoxification and biosynthesis of osmoprotectants [9], [11–13]. Ectopic expression of some COR genes have been reported to result in improved cold tolerance, including studies with tobacco [14–16], rice [17], [18], strawberry [19] and Arabidopsis [20], [21]. Conversely, the expression of Cicer arietinum or spinach LEA proteins in tobacco did not induce any significant changes in freezing tolerance [15], [22]. Such findings are not surprising since a clear increase in freezing tolerance is rarely obtained by expressing a single cold-induced gene, even if the end product is directly related to development of freezing tolerance.

Recent studies have shown that Arabidopsis cold stress tolerance can be enhanced by modulating the signaling pathways triggered by low temperature stress [23]–[25]. Such pathways include the CBF (C-repeat binding factor), also known as DREB (dehydration responsive element binding)/ICE (inducer of CBF expression) signaling pathway, which have also been characterized in other plant species [25]–[27]. It was reported that increased expression of the entire battery of CBF genes resulted from overexpressing the Arabidopsis transcriptional activator CBF1 [23], [24], [28], [29]. The underlying mechanism involves the CBF protein binding to the CRT/DRE regulatory element located in the promoters of the target genes, thereby inducing cold response metabolic pathways and enhancing cold tolerance. Jago-Ottsen et al. [23] further found that constitutive overexpression of CBF1 induces expression of the COR genes in non-acclimated Arabidopsis plants and increased freezing tolerance at a whole plant level, an effect that was not observed by expressing COR1A alone. This further suggests that freezing tolerance is a multigenic trait involving genes with additive effects. Overexpression of Arabidopsis CBF genes, which reside at the nodes of regulatory networks in cold responses, were able to improve chilling/freezing tolerance in different plant species, or homologs from other plant species could enhance the freezing tolerance of transgenic Arabidopsis [30], [31]. Other known regulatory components include an upstream transcription factor, ICE1 (inducer of CBF Expression 1), which encodes a MYC-type transcription factor that positively regulates CBF2, and which plays a critical role in cold acclimation [32]. Additionally, the Arabidopsis gene AtICE2, a positive regulator belonging to the bHLH family, has been shown to activate AtCBF1 [33].

Since the discovery of the Arabidopsis ICE genes, homologs have subsequently been found in a variety of crop species, including wheat, rice, banana, tea, trifoliate orange and grapevines [34]–[44]. In addition, ICE-like proteins have been overexpressed in transgenic plants and shown to increase stress tolerance [34], [35], [37]. For example, overexpression of an ICE-like gene from V. amurensis in tobacco was reported to result in a increased cold tolerance [42], and Arabidopsis has been shown to become more tolerant of cold, drought and salt stresses when expressing the V. vinifera ICE1a or -1b genes [43]. In this current study, we investigated whether two members of the ICE gene family from a highly cold-tolerant accession of V. amurensis differ functionally from those previously identified from other Vitis species, or whether they play similar roles in activating multiple components of cold acclimation responses.

### Materials and Methods

**Plant materials, growth conditions, and cold treatment**

Two-year-old Chinese wild-growing Vitis amurensis accession ‘Heilongjiang Seedling’ potted plants developing from stem cuttings were grown in a greenhouse under natural photoperiod. For the cold treatment, plants with a uniform growth status were transferred to a chamber (LT-BIX120L, LEAD-Tech (SHANG-HAI) SCIENTIFIC INSTRUMENT CO., LTD, China) at 0°C with a 16 h photoperiod (200 μmol m⁻²s⁻¹ light) and 8 h dark. Unstressed plants were used as controls (0 h). Leaves from unstressed (control) and cold-treated V. amurensis plants were harvested at time points 0, 1, 3, 6, 12, 24, and 48 h and immediately frozen in liquid nitrogen, prior to RNA extraction. More than 3 plants were collected and pooled for each time point, and the sampling was in triple for biological replicates.

**Structural features, phylogenetic tree and expression analysis**

Total RNA derived from grapevine leaves were isolated using the plant RNA kit (Omega Bio-teko, Doraville, GA, USA), and first-strand cDNA synthesis was synthesized from 2 μg of total RNA using PrimeScript RT Reagent Kit according to manufacturer’s manual (TaKaRa, Dalian, China). Two partial lengths of V. amurensis ICE cDNA fragments were amplified by PCR using degenerate primers 5′-TGGACTSSTCTCTCGTGTCTKCT-CC-3′ and 5′-TCCTCGCCTCTCTCWWCTCAACCSGA-3′, 5′-CCTYCACTGGBKGACGCCMACKCT-3′ and 5′-GGAAAAACYTGTGARACAGCTGATRCMG-3′, which were designed based on the known nucleotide sequence of ICE homologs from other plant species. Isolation of the full length DNA sequences was carried out using the SMART RACE Kit (Clontech, Palo Alto, CA, USA). Amplification was performed at 94°C for 3 min; 27 cycles of 94°C for 30 s, 55 to 58°C for 30 s, and 72°C for 1 min; followed by 5 min at 72°C. The cDNA pools for 3′ and 5′ RACE were generated from total RNA extracted from cold-stressed leaves of V. amurensis, using the RNAprep Pure Plant Kit (Omega). Subsequently, a nested PCR was performed with the prepared cDNA pool using the adaptor primer UPm and VaICE1 gene-specific primer (GSP) 5′-CATCACAATGGTCTTGATGKGGCAGACCCAGG-3′ for 3′ RACE, 5′-CCTATGATCAGCGCTTGTGGAATGATGCTGACCCCGGA-3′ for 5′ RACE; and the VaICE2 gene-specific primer: 5′-GCCGCGTGTCAGACGACTGGGAAGCTTTAGG-3′ for 3′ RACE, 5′-ATCGCTGCGACTGGAGTTTGTGCAGAACGGC-3′ for 5′ RACE. Amplicons were cloned into the pMD19-T vector (TakaRa) for sequencing and the full-length cDNAs of ICE homologs were predicted by comparing and aligning the 5′- and 3′- RACE amplified sequences, using BioEdit software (Version 7.0.1). The putative full-length cDNAs were amplified by primers designed from the extreme 5′ and 3′ ends and 5′-RACE Ready cDNA as template, cloned to pMD-19-T (TaKaRa) to generate pMD-VaICE1, and pMD-VaICE2, and verified by sequencing. Chromosomal location prediction was performed using the BLAT server (http://www.genoscope.cns.fr/cgi-bin/vitis/webBlat) at the Genoscope Genome Browser. The molecular weights (MW) and isoelectric points (pI) of the corresponding proteins were predicted with the ProtParam tool (http://www.expasy.ch/tools/pi_tool.html). Nuclear localization signals were predicted based on the predicted protein sequence using the online server (http://www.predictprotein.org/) and homolog searches were conducted with the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular model building of bHLH-ZIP domain of VaICE1 or VaICE2 was carried out using SWISS-MODEL server (http://swissmodel.expasy.org). Sequence alignment was performed using the DNAMAN software (Version 6.0, Lynnon Biosoft) and the phylogenetic tree was constructed with MEGA 5.1 software [45] using the neighbor-joining method.

To determine the expression profiles of VaICE1 and VaICE2 in V. amurensis and Arabidopsis plants, total RNAs were extracted as described above. After treatment with RNase-free DNase, the first-
strand cDNA was synthesized using the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa). Semi-quantitative RT-PCR was performed to assess the expression of VaICE1 and VaICE2 over a cold stress time course. The VaICE1 cDNA was amplified using the primers 5'-ATGTTACCCAGTGCAAGCGTG-3' and 5'-CTACAGCATACCGTGAAGCGTC-3', and the VaICE2 cDNA was amplified using the primers 5'-ATGCTGTGCAAGTGGAACCGTGTC-3' and 5'-CTACAGCATACCGTGAAGCGTG-3'. Grapevine GAPDH (GenBank accession no. CB979647) was used as a leading control using the following primers: 5'-TTCTGGTTGAGGGCATTTCGA-3' and 5'-CCACGACTTTCACTCGTACAA-3'. Arabidopsis Actin2 (AT3G18780) was served as reference control using the following primers: 5'-CTTTGGAACCAAGCAGATGA-3' and 5'-CCGATCCAGACACTGATCTCTTCT-3'. Three replicates were performed for each semi-quantitative RT-PCR reaction.

Subcellular localization and transactivation activity assay

To construct green fluorescent protein (GFP) translational fusion vectors, full-length VaICE1 and VaICE2 cDNAs were PCR-amplified with the primers 5'-ggattcCATATGATGTTACCACGTGCAAGCAGCCTG-3' (EcoR I) and 5'-gggtcGGATCCCTACAGCATACCGTGGAAGCCTG-3' as vector backbone (pGBKT7) and 5'-gggtcGGATCCCTACAGCATACCGTGGAAGCCTG-3' (Xho I) and 5'-ccgTCTCGAGCTACAGCATACCGTGGAAGCCTG-3' in-frame upstream of the of GFP reporter gene in the pBII21 vector. The empty vector containing only GFP sequence was used as a positive control. The isolated plasmids were transformed into Agrobacterium tumefaciens C58C1 (positive control) and pGBKT7 (negative control), were transformed into floral dip method [48]. T1 seeds were collected from individual plants and screened on 50 mg/mL kanamycin-contained medium to analyze the segregation of the resistant phenotype. T2 kanamycin-resistant seeds were harvested from non-segregating families and confirmed the kanamycin-resistant phenotypes. T3 homozygous lines were validated by RT-PCR using the primers as above described in grapevine expression analysis, and further used for all experiments.

Stress tolerance assay

For the cold treatment, surface-sterilized T3 homozygous seeds of vector-carrying control and VaICE1/2-overexpressing Arabidopsis lines were germinated and grown on MS medium supplemented with 50 mg/L kanamycin for one week, then transferred to pots containing a mixture of perlite: sand: peat (1:1:1, v/v) for two weeks. The three-week-old seedlings of the transgenic plants overexpressing VaICE1 and VaICE2 (n = 50) and control plants (n = 50) were transferred to the chamber (LT-BIX120L) at 0°C under the condition as above-described in grapevine for 0, 6, 12, 24, or 48 h. The freezing treatment assay was performed by transferring 3-week-old seedlings (n = 50) grown in pots to the pre-chilled chamber (LT-BIX120L) at −6°C for 8 h under continuous dim light conditions (2.5 μmol m−2 s−1) and subsequently returning them to normal conditions. Survival rates of plants were evaluated after 7 days. The freezing treatment experiment was performed in triplicate.

Determination of electrolyte leakage, and malondialdehyde and proline content

Three-week-old seedlings from the empty vector control Arabidopsis plants (n = 50), VaICE1 and VaICE2 T3 transgenic lines (n = 50) were subjected to the cold stress treatment described above and leaves were harvested to determine three biochemical features associated with cold tolerance: electrolyte leakage was assessed by ion leakage analysis as previously described [49]; proline content was determined using acid-ninhydroxid reagent and acetic acid [50]; and malondialdehyde (MDA) content was measured using the thio-barbituric acid (TBA) method as previously described [51].

Analysis of cold-responsive genes regulated by VaICE1 or VaICE2

Total RNA was extracted from vector-carrying control and T3 transgenic Arabidopsis leaves treated at 0°C for 0, 3, 12, 24 and 48 h using the plant RNA kit (Omega) following the manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA using the PrimeScript RT Reagent Kit with the oligo (dT)18 primer, according to the manufacturer’s instructions (TakaRa) and quantitative real-time PCR was performed using the SYBR Premix Ex Taq II kit (TakaRa) on an iCycler iQ5 thermal cycle (Bio-Rad). The reactions were carried out in triplicate in 96-well plates (25 μl/well) in a mixture containing 12.5 μl 2 x SYBR Premix Ex Taq II, 1 μl each of primer (10 μM in stock), 1 μl template cDNA and 9.5 μl ddH2O. Two-step real-time PCR reactions were performed under the following conditions: 95°C for 10 s, followed by 40 cycles of denaturation at 94°C for 15 s, annealing and extension at 57°C for 30 s, and data acquisition at
57°C for 15 s. Three replicate PCR amplifications were performed for each sample. Transcription levels of AtICE1, AtICE2, AtCBF1, AtCBF2, AtCBF3, AtCOR15A, AtCOR47, ArRD29A and AtKIN1 were measured in VaICE1 or VaICE2 overexpressing or empty vector control plants. The amount of transcript for each gene, normalized to the internal reference Atactin2, was analyzed using the 2-ΔΔCt method [52]. Oligos used for real-time PCR were: 5′-GTGGTGGAGGCTATTT-3′ and 5′-TGGTGGAGGCTATTT-3′ for AtICE1; 5′-GACGTGGACTCCATCCGTTGACACTAC-3′ and 5′-GTCACAATCGTCTGCTGGACACTAC-3′ for AtCBF1; 5′-TGTTCTGCTGGACAAG-3′ and 5′-ATCCCTTCGCTGCTGGACTTTTAC-3′ for AtICE2; 5′-GAGCTTGTTGGAGGCTATTT-3′ and 5′-AGCTTGTTGGAGGCTATTT-3′ for AtICE2. Data analysis shown was obtained from three biological replicates.

**Results**

**Characterization of two ICE homologs from *V. amurensis***

Two ICE-orthologs, designated VaICE1 (GenBank accession no. KC815984) and VaICE2 (GenBank accession no. KC815985), were isolated from leaves of the cold-tolerant Chinese wild-growing *V. amurensis* accession ‘Heilongjiang Seedling’. The complete cDNA of VaICE1 is 1,949 bp, comprising a 97 bp 5′UTR, 301 bp 3′UTR and a 1,551 bp ORF that was predicted to encode a 516 amino acid protein with a MW of 55.5 kDa and a pI of 5.05. A sequence alignment showed that the complete cDNA of VaICE2 is mapped on chromosome 1, spanning 2714 bp, while VaICE2 is 98.38 kDa and a pI of 5.30. The complete cDNA of VaICE2 was 2,108 bp, comprising a 214 bp 5′UTR, 277 bp 3′UTR and a 1,617 bp ORF that was predicted to encode a 538 amino acid protein with a MW of 58.38 kDa and a pI of 5.05. A sequence alignment showed that these two proteins differ significantly from each other (60% amino acid similarity). Chromosome location of the two ICE homologs in the *V. vinifera* cv. Pinot Noir clone P40024 genome suggested that VaICE1 is mapped on chromosome 1, spanning 2714 bp, while VaICE2 was mapped on chromosome 14 and spans 4234 bp (Fig. 1A). Analysis of structural properties revealed that the predicted VaICE1 or VaICE2 protein possesses the typical features of ICE proteins, including a serine-rich region (S-rich), a basic helix-loop-helix (bHLH) domain, an ICE-specific domain [38], a zipper region (ZIP) and an ACT_ UUT-ACR-like domain (Fig. 1B). An interPro scan suggested that both proteins belong to the MYC-like bHLH family of transcription factors, and in support of this, they contain nuclear location signals (NLSs) at position 12–46 aa for VaICE1 and 339–362 aa for VaICE2 (Fig. 1B). The predicted three dimensional structures of the bHLH-ZIP domain of VaICE1 and VaICE2 were distinct, and were more similar to the structures of DIMER (PDB ID: 1r05) and HETERO DIMER (PDB ID: 2q2l), respectively (Fig. 1C).

Phylogenetic analysis of various ICE amino acid sequences indicated the existence of two major groups (Fig. 2A). VaICE4 is phylogenetically distinct from the other groups in which they could be clearly classified into dicot and monocot specific subgroups. Our phylogetic data demonstrated VaICE1 and VaICE2 fell into different clades of the dicot subgroup, with VaICE1 being closely related to the previously reported VaICE14 (98.7% identity) from *V. amurensis* [42], VaICE1 (97.7% identity) from *V. riparia* [44], whereas VaICE2 being closely similar to VaICE2 (99.4% identity) from *V. riparia* [44], VaICE1a (98.9% identity) and VaICE1e (98.7% identity) from *V. vinifera* [43]. In addition, VaICE1b shared 98.7% identity with VaICE3, which belongs to another clade of dicot subgroup. Comparison of VaICE1 and their homologous AtICE1 and AtICE2 from *Arabidopsis*, as determined by 49–60% sequence similarity, revealed they share highly conserved regions in the bHLH DNA binding domain and ACT domain in their C-terminal regions (Fig. 2B). In contrast, only a moderate sequence conservation was found in the ZIP domains between *Vitis* and *Arabidopsis* ICE proteins. A potential sumoylation site previously identified in *Arabidopsis* ICE proteins [53] was also observed in VaICE2, and the S-rich region, which has been suggested to be a site of phosphorylation [32], [34] was present in either VaICE1 or VaICE2. Finally, the R236 residue that substituted with H236 in the *Arabidopsis* ice1 mutant [32], causing a loss of ICE function, was shown to be present in the two *V. amurensis* ICE proteins (Fig. 2B). To gain insight into the biological functions of VaICE1 and VaICE2, semi-quantitative RT-PCR was used to examine their expression profiles in leaves of *V. amurensis* over a cold stress time-course (Fig. 2C). VaICE2 transcript levels were constant over the time course, while a rapid induction of VaICE1 expression was observed after 1 h, followed by a gradual decline from 3 h to the minimum level after 12 h, and finally a return to background levels at the end of the time course. These results suggest that VaICE1 and VaICE2 may be involved in cold stress responses.

**VaICE1/VaICE2 is nuclear-localized and can act as transcriptional activators in transient assays**

Sequence analysis showed that VaICE1 and VaICE2 have putative NLSs, suggesting that they target the mature proteins to the nucleus. To test this, VaICE1 and VaICE2 were transiently expressed as translational fusions at the N terminus of GFP in onion (*Allium cepa*) epidermal cells. Confocal imaging showed that GFP alone (control) was present in the cytoplasm and nucleus, as expected, whereas cells transformed with either VaICE1::GFP or VaICE2::GFP showed strong fluorescence exclusively in the nucleus (Fig. 3A).

Yeast one hybrid assays were carried out to determine whether VaICE1 and VaICE2 possess transactivation activity. As shown in Fig. 3B, yeast transformants with pGAL1 (positive control), pGBK17-VaICE1 and pGBK17-VaICE2 grew well on the SD/Trp- medium, but also grew normally on the SD/Trp-/His-/Ade-medium, and exhibited fairly strong β-galactosidase activity. In contrast, transformants carrying the negative control (pGBK17) did not grow on the SD/Trp-/His-/Ade-medium and could not show β-galactosidase activity. Thus, VaICE1 and VaICE2 have transactivation activities in yeasts.

**Over-expression of VaICE1 or VaICE2 in Arabidopsis increases cold tolerance**

To determine whether VaICE1 or VaICE2 enhances cold stress tolerance, *Arabidopsis* WT-type Col-0 was transformed with constructs carrying empty vector, VaICE1- or VaICE2-coding regions under the control of constitutive 35S promoter (Fig. 4A). Expression of VaICE1 or VaICE2 transgens in these T2 lines was confirmed by semi-quantitative RT-PCR, and two VaICE1 (L2 and L5) and two VaICE2 transgenic T3 homozygous lines (L1 and L6) with similar transcript levels (Fig. 4B) were selected for further analyses. These two independent transgenic lines over-expressing VaICE1 and VaICE2, together with the corresponding empty vector-transformed control lines, were subjected to a whole plant...
freezing assay to evaluate plant survival after freezing treatment (Fig. 4C). The freezing test consisted of exposing 3-week-old transgenic and control plants to a temperature of $-26^\circ C$ for 8 h, before recovery at normal temperatures. The VaICE1 and VaICE2 transformed plants exhibited less freezing damage and increased survival rates compared with empty vector control plants after a 7 d recovery period (Fig. 4D). Only $5\%$ of the control plants (L1 and L2) survived the treatment, while the VaICE1-overexpressing plants exhibited survival rates of 91% for L2 and 81% for L3, and 85% for L1 and 95% for L6 in VaICE2-overexpressing plants (Fig. 4D). No obvious differences in phenotype were observed between the empty vector control plants and VaICE1 or VaICE2 transgenic plants grown under normal conditions. These results suggest that VaICE1 or VaICE2 overexpression leads to enhanced freezing tolerance.

Over-expression of VaICE1 or VaICE2 affects electrolyte leakage as well as proline and MDA metabolism

To investigate the physiological and biochemical factors that might contribute to the improved cold tolerance of the transgenic plants, MDA and proline content were evaluated over a time course of exposure to cold stress ($0^\circ C$), as well as electrolyte leakage, which is commonly used as an index of membrane injury [23], [55]. A representative experiment comparing the empty vector control L1 and 35S::VaICE1 L2 and 35S::VaICE2 L6 transgenic lines is shown in Fig. 5. Under non-acclimation conditions, electrolyte leakage of all tested lines did not vary greatly i.e. between 22% in the control (L1) and 21% in the VaICE1-(L2) and VaICE2-(L6) overexpressing lines (Fig. 5A). Electrolyte leakage increased with the prolonged cold stress and reached 47% in the control and 40% and 39% in VaICE1 and VaICE2 lines, respectively, after 48 h.

Proline accumulation in plants has been associated with a wide variety of environmental stresses, and confers stress tolerance by facilitating osmotic adjustment, protecting proteins and membranes and quenching reactive oxygen species [56], [57]. Significantly, for this study, increases in proline levels have been well-documented in Arabidopsis and other plants during cold acclimation [58], [59]. Similar changes were observed in both VaICE1 and VaICE2 overexpressing Arabidopsis lines (Fig. 5B), since
similar levels of proline were detected in the transgenic and wild type plants without cold stress, but substantial proline accumulation occurred in cold-treated VaICE1 and VaICE2 transgenic lines. After 48 h of cold stress, the proline contents in the leaves of VaICE1 or VaICE2 transgenic lines were ∼2.3-fold higher than those of the control plants (Fig. 3B).

MDA is also considered an indicator of plant oxidative stress and structural integrity of the membranes in response to low temperature [60], [61]. Under non-acclimation conditions, all tested lines had similar MDA levels ranging from 1.76–2.11 mg/g FW (Fig. 5C). However, MDA accumulation was considerably less in the VaICE1 or VaICE2 transgenic lines than in the controls during the cold stress time-course. These results indicated that VaICE1 and VaICE2 overexpression resulted in increased levels of proline, but decreased levels of MDA and reduced levels of electrolyte leakage.

Figure 2. Phylogenetic tree, alignment and expression profiles of VaICE1,2. (A) Phylogenetic tree based on the deduced amino acid sequences of ICE from a range of plant species. Neighbor-joining tree, with the following predicted ICE protein sequences, with GenBank accession numbers listed in parentheses: V. amurensis (VaICE1, AGP04217; VaICE2, AGP04218; VaICE14, ADY17816), V. vinifera (VvICE1, AF149627; VvICE1a, AGQ03811), V. riparia (VrICE1, AGG34704; VrICE2, AIA58705; VrICE3, AIA58706; VrICE4, AIA58707), Arabidopsis thaliana (AtICE1, NP_189309; AtICE2, NP_172746), Brassica napus (BnICE1, AEL33687), Brassica rapa subsp. Chinensis (BrICE1, ACB69502), Capsella bursa-pastoris (CbICE1, AAS79350), Camellia sinensis (CsICE, ACT90640), Eucalyptus camaldulensis (EciCE, AEF68776), Eucalyptus globules (EgICE1, AEF33833), Extrema salisugineum (EsICE, ACT68317), Glycine max (GmICE1, ACJ92111), Hordeum vulgare (HvICE2, ABA25896), Malus x domestica (Mdhlh1, ABSS0251), Oryza sativa (OsICE1, Os11g023700; OsICE2, Os01g0928000), Populus trichocarpa (PtICE1, ABN58427), Raphanus sativus (RsICE1, AEF68771), Triticum aestivum (TaICE41, ACB69501; TaICE87, ACB69502), Zea mays (ZmICE1, ACG46593), was produced by ClustalX 2.0 alignment followed by tree construction using MEGA 5.0 with 100 bootstrap tests. The branch support values are indicated. The length of the scale bar corresponds to 5 substitutions per site. (B) Comparison of ICE amino acid sequences from V. amurensis (VaICE1,2) and Arabidopsis thaliana (AtICE1,2). Deduced amino acid sequences were aligned using ClustalW. Sequences and accession numbers are shown for the following: Vitis amurensis (VaICE1, KC815984; VaICE2, KC815985) and A. thaliana (AtICE1, AAP14668; AtICE2, AAO63441). Residues in black and gray regions indicate identical and similar residues, respectively, between isoforms. Four predicted domains are labeled: a S-rich motif, a basic-helix-loop-helix-leucine zipper (bHLH-ZIP) region, ICE-specific domain [38] and a ACT-UUR-ACR-like domain. The red triangles indicate the position of the mutation isolated by Chinnusamy et al. [32] and Kanaoka et al. [54], and the residue targeted for sumoylation by SIZ1[53]. Green triangle indicates E-box/N-box specificity site [69], blue asterisks indicate core residues for DNA binding sites [70], and red diamonds indicate dimerization interface/ polypeptide binding sites [70]. (C) Expression profiles of VaICE1 and VaICE2 during a cold stress time-course experiment. Semi-quantitative RT-PCR was used to determine VaICE1 and VaICE2 transcript levels in cold treated grapevine leaves at indicated times. Grapevine GAPDH was used as a loading control.

doi:10.1371/journal.pone.0102303.g002
ValICE1 or ValICE2 positively regulate cold-induced gene expression

To further identify molecular components associated with the ValICE1 and ValICE2 mediated stress tolerance that we observed in the 35S::ValICE1 L2 and 35S::ValICE2 L6 transgenic Arabidopsis lines, the expression patterns of genes in the ICE-CBF pathway were evaluated by real-time PCR (Fig. 6). Under control conditions, AtICE1 or AtICE2 expression was relatively low in either the empty vector control or ValICE1- and ValICE2-overexpressing plants, respectively. However, overexpression of ValICE1 or ValICE2 resulted in a reduction in transcript levels compared with the control during the cold stress time course. The possible effect of ValICE1 or ValICE2 on the expression of downstream genes through transcriptional regulation was also investigated (Fig. 6). Under control conditions, AtICE1 or AtICE2 expression was relatively low in either the empty vector control or ValICE1- and ValICE2-overexpressing plants, respectively. However, overexpression of ValICE1 or ValICE2 resulted in a reduction in transcript levels compared with the control during the cold stress time course.

The possible effect of ValICE1 or ValICE2 on the expression of downstream genes through transcriptional regulation was also investigated. Under control conditions, transcripts of none of the three CBF genes was detected in the control, but higher or similar level CBF transcript levels were present in the ValICE1 or ValICE2 overexpression lines. Expression of the CBF genes showed a general decline in the control plants starting at 12 h of cold stress. Starting at 3 h, CBF1 and CBF2 transcript levels were considerably greater in the transgenic lines than in the control plants, while a substantially greater expression in the transgenic lines was not observed for CBF3 until 24 h. Expression of the CBF downstream target genes (COR15A, COR47, KIN1 and RD29A) were also investigated. COR47 had extremely high expression in both transgenic lines and the vector control compared with the other genes, and COR15A exhibited higher expression in the transgenic lines compared with the control after 48 h. The KIN1 gene was expressed at considerably higher levels in the ValICE2 overexpression than that in the ValICE1 overexpression line and the vector control, while no substantial difference in RD29A expression was seen among the three different genotypes. These results suggest that ValICE1 and ValICE2 positively regulate the expression of the CBF genes by differentially controlling downstream genes in response to cold stress, which in turn likely contributes to freezing tolerance.

Discussion

In the current study, two putative ICE-orthologs, ValICE1 and ValICE2, from the highly cold-tolerant species *V. amurensis* were structurally analyzed and functionally tested. We report that these two genes encode MTF-type bHLH transcription factors that are nuclear-localized, and are candidate regulators of the CBF gene expression during cold stress. Reports on ICE-orthologs in three genotypes of *Vitis* species [42]–[44] might indicate that there are multiple ICE-like genes in grapevine. However, sequence comparisons revealed that none of the mRNA sequences that were previously reported [42], [43] completely matched the two sequences identified here (Fig. S1 in File S1). Thus, *Vitis* ICE3s were categorized on the basis of sequence similarity (Fig. S2A in File S1), phylogenetic clustering (Fig. S2B in File S1) and genetic locus (Table S1 in File S1). Our results provide evidence of the presence of 4 polymorphic loci for the ValICE1 and ValICE2, from which they are located in grapevine chromosome 1, whereas ValICE1, ValICE2, ValICE1a are alleles, mapping to chromosome 1 of *V. amurensis*.
Figure 4. Freezing tolerance evaluation of 35S:ValICE1,2 transgenic Arabidopsis plants. (A) A schematic map of the T-DNA region of 35S:ValICE1,2 fusion constructs employed for Arabidopsis transformation. RB, right border; LB, left borders; CaMV35S, Cauliflower mosaic virus 35S promoter; OCS, octopine synthetase terminator; NOS, nopaline synthase promoter; NPTII, Kanamycin resistance gene; NOS-T, nopaline synthase terminator. (B) RT-PCR was used to assess the transcript abundance of ValICE1 or ValICE2 in the transgenic Arabidopsis plants. Actin2 was used as a reference control. (C) 3-week-old plants were treated at −6°C for 8 h and then transferred back to normal conditions for recovery. Photographs were taken after 7 d of recovery. (D) Survival rates of plants exposed to −6°C. Average survival rates and standard errors were calculated using the results of three separate experiments with 50 seedlings per line for each freezing stress.

doi:10.1371/journal.pone.0102303.g004

Figure 5. Effect of ValICE1, 2 expression in Arabidopsis on levels of (A) electrolyte leakage, (B) proline and (C) malondialdehyde (MDA). Three-week-old Arabidopsis plants of vector-carrying control, ValICE1 L2, and ValICE2 L6 were grown at 0°C for the time indicated. Leaves were collected to assess electrolyte leakage and free proline and MDA. Each value is the mean ± SD of three replicates in case of electrolyte leakage, proline and MDA content (6 seedlings each) for the indicated time points.

doi:10.1371/journal.pone.0102303.g005
Additionally, the other independent cluster that VvICE1b and VvICE3 involved are alleles, which are located in chromosome 17. Of these three distinct categories, very few, and mostly conserved, substitutions were observed for each category, but it appears that VrICE4 is phylogenetically distinct from the above-mentioned three categories and located in chromosome 18. A detailed sequence assay on VrICE4 revealed that it lacked the typical S-rich motif of ICE proteins, presented 3 amino acid mutations (A-T, A-T, and E-D) in ICE-specific domain, and exhibited remarkable sequence difference in ACT_UUR_ACR-like (ACT) domain (Fig. S2A in File S1). Moreover, result from FLAGdb++ database prediction indicated that VrICE4 has the best match with GSVVG01009234001 with functional annotation as DNA binding protein rather than inducer of CBF expression 2 (Table S1 in File S1) when compared with other grapevine ICE proteins. Therefore, further investigation on VrICE4 is still required to confirm its role as ICE-like transcription factor.

Evidence from structural and phylogenetic analyses further suggested that VaICE1 and VaICE2 may have different properties when involved in cold stress. This is also suggested by their expression profiles (Fig. 2C), which showing that they are both constitutively expressed, but that the expression of VaICE1 is affected by cold treatments. With the availability of the reported expression data on grapevine ICE genes [42–44], a comparison of

Figure 6. Effect of VaICE1,2 overexpression in Arabidopsis on transcript levels of genes involved in the cold stress pathway. qRT-PCR analysis using leaves from control Arabidopsis L1, 35S::VaICE1 L2 and 35S::VaICE2 L6 plants. Three-week-old plants were grown at 0℃ for the time indicated. The tested genes were AtICE1, AtICE2, AtCBF1, AtCBF2, AtCBF3, AtCOR15A, AtCOR47, ATKIN1 and AtRD29A. Actin2 was used as a reference control. Each value is the mean of three replicates. doi:10.1371/journal.pone.0102303.g006
cold responses of these genes on different genotypes revealed that VaICE1, 2, VaICE1a, b and ViICE1-4 were all detected before and after cold treatment, which is most consistently observed to be constitutively expressed in other plants [32], [34], [40]. However, VaICE14 is a notable exception where the transcript was not detected under non-stress condition but under low temperature [43]. One possible explanation for such difference has been proposed [44], in which this discrepancy might be attributed to the different genotypes and sampling materials derived from different cultural ways. An additional effort in validation of the expression profile of this gene before and after cold stress in repeated trials is required to clarify its expression property.

Many of the physiological and biochemical changes that occur during cold acclimation are directly correlated to an up-regulation of COR gene expression, which is activated by the constitutive expression of the ICE transcription factor. Liu et al. [62] found that overexpression of AtICE1 in cucumber was sufficient to increase chilling tolerance and to simultaneously alter the levels of several cold responses associated factors (e.g., free proline, MDA and soluble sugars). Li et al. [43] discovered that the survival rates of VaICE1a and VaICE1b-overexpressing Arabidopsis lines were significantly higher than those of the wild type under cold stress. Similarly, overexpression of Solanum lycopersicum ICE1 was reported to improve tolerance of chilling stress in tomato plants, as indicated by differences in electrolyte leakage [37]. In our study, overexpression of either VaICE1 or VaICE2 in transgenic Arabidopsis plants increased freezing tolerance at the whole-plant level, based on survival rates (Fig. 4B, C), which correlated with increased accumulation of proline, and a reduction in MDA and electrolyte leakage (Fig. 5A-C). These results suggest that although an endogenous ICE-CBF pathway is present in Arabidopsis system and that enables the ectopic VaICE1,2 transgene expression to influence the plants' freezing-tolerance capacity. There are also considerable evidences to suggest that proline [63]–[65], MDA [14], [66], [67], and electrolyte leakage [25], [68], contribute to an enhancement of freezing tolerance. Our results confirmed that the greater tolerance of VaICE1 or VaICE2-overexpressing plants to cold stress positively linked to elevated proline levels, and negatively correlated to the reduction of MDA content as well as electrolyte leakage. However, these data did not reveal a difference between the VaICE1 and VaICE2 transgenic lines. It is important to note that plants overexpressing VaICE1 or 2 had similar levels of proline and MDA contents relative to the vector-carry controls under normal growth conditions (0 h), but upregulated in proline level and downregulated in MDA content under time-course cold stresses. We therefore assume that the commonly observed accumulation of free proline and MDA under non-acclimation are partially determined by proline biosynthesis gene (e.g. P5CS, d-1-pyrroline-5-carboxylate synthetase) or MDA reductase gene itself property with a relatively low expression, but also induced by the expression of a transcription factor under cold-stressed conditions. Additionally, ionic leakage from the cells is considered as an indicator that the semipermeable nature of the plasma membrane has been lost, at least transiently, in response to freezing. This is probably the main cause for the similar electrolyte leakage observed in controls and VaICE1-/VaICE2-overexpressing lines under non-stressed conditions.

We propose that our analyses of the VaICE1 or VaICE2 overexpressing Arabidopsis plants do not contradict the general cold acclimation model [32], [43], but rather suggest a more integrated circuitry involved in cold acclimation signaling. Chinnusamy et al. [32] reported that over-expression of AtICE1 increased the expression of AtCBF2, AtCBF3 and cold-regulated genes (CORs) under cold stress. In contrast, Fursova et al. [33] found that over-expression of AtICE2 only resulted in dominant changes in AtCBF1 transcription levels after cold acclimation, while Badawi et al. [34] showed that over-expression of wheat ICE genes in Arabidopsis induced a higher expression of AtCBF2, AtCBF3 and some COR genes only after cold acclimation. Our data suggest that the expression of AtICE1 or AtICE2 was slightly lower in the VaICE1 and VaICE2 overexpressing Arabidopsis plants. One possible explanation for this is that co-suppression of the endogenous gene occurred in the target plant, but we suggest that the V. amurensis and Arabidopsis ICE1 or ICE2 DNA nucleotide sequences are not highly homologous (64.6–69.9%). The observation that several downstream genes were significantly induced in VaICE1- or VaICE2-overexpressing lines might suggest that the constitutive expression of the transgenes affects the expression of the endogenous gene. Thus, as VaICE1 and VaICE2 mRNA levels are abundant in the transgenic plants, competition at a post-transcriptional level could explain this slight decrease in endogenous AtICE1 or AtICE2 transcript levels. We speculate that another signal transduction pathway may exist in the CBFI/DEREI gene regulatory network that is stimulated by VaICE1 or VaICE2. It should be noted that the activation of the expression of these downstream genes differed between the VaICE1 and VaICE2 overexpressing lines (Fig. 6). The enhanced tolerance of 35S:VaICE1 and 35S:VaICE2 plants coincides with an up-regulation of the stress-responsive genes CBF1, COR15A and COR47. However, some differences were seen in the expression patterns between the transgenic lines, and particular in the case of KIN1, which suggests functional differences between VaICE1 and VaICE2. In the case of VaICE1 and VaICE2 transgenic lines, both contribute to the modulation of AtIRD29A and AtCOR47 in response to cold stress [43]. Together with the above-mentioned examples, these differences in targeting to the downstream genes are likely due to a varying number of ICE homologs in different plant species [32], [33], [40]–[44]. However, it has not yet been established which of the identified grapevine ICE genes specifically control the different sets of cold-responsive genes. A detailed comparison of the expression of these ICE genes and their target genes through microarray analysis might help address this issue.

Taken together, VaICE1 and VaICE2 are two previously unreported ICE-like transcription factors from V. amurensis, whose regulatory roles in cold acclimation are suggested by the results presented here. Both genes may act as positive regulators to increase the levels of cold-responsive genes in transgenic lines under cold stress. Moreover, VaICE1 and VaICE2 influence cold stress-related factors such as electrolyte leakage, and levels of proline and MDA, thereby alleviating damage by ROS and enhancing osmotic protection. Our data may help elucidate the cold-acclimation pathways of Vitis species and more ultimately guide the design of strategies for improving the stress tolerance of agricultural crops.

Supporting Information

File S1 Contains the files: Figure S1 Multiple alignment of the mRNA sequences of ICE-homologous from different Vitis species. Identical nucleotide sequences are highlighted on a black background while white boxes indicate at least three identical nucleotides. The GenBank accession numbers are reported as follows: VaICE1 (KC815984, VaICE2 (KC815985), VaICE14 (HM231151), VaICE1 (JQ707298), VaICE1a (KC831748), VaICE1b (KC831749), VaICE1 (KF994961), VaICE2 (KF994962), VaICE3 (KF994963) and VaICE4 (KF994964). Figure S2 Protein sequence similarity and phylogenetic clustering of ICE from three different Vitis
**References**

1. Christen D, Susan S, Jermini M, Strasser RJ, Delfago G (2007) Characterization and early detection of grapevine (Vitis vinifera) stress responses to oesha disease by in situ chlorophyll fluorescence and comparison with drought stress. Environmental and Experimental Botany 66: 504–514.

2. Baldellou S, Guillamme S, Reinou GM, Krzacz G, Ghirbell A, et al. (2010) Isolation and expression analysis of salt induced genes from contrasting grapevine (Vitis vinifera L.) cultivars. Plant Science 179: 489–498.

3. C.F.A.L (2013) Abiotic stress effects on grapevine (Vitis vinifera). Journal of Plant Physiology 167: 812–819.

4. Zhang Y, Dami E (2012) Foliar Application of Absciscic Acid Increases Freezing Tolerance of Field-Grown Vitis vinifera Cabernet franc Grapes. American Journal of Enology and Viticulture 63: 377–384.

5. Liu L, Li H (2013) Review: Research progress in abscisic acid, Vitis amurensis Rupr. Canadian Journal of Plant Science 93: 565–575.

6. Wan Y, Schwenger Heidi, Li D, Simon CJ, Wang Y, et al. (2008) The ecor-geographic distribution of wild grape germplasm in China. VITIS 47: 77–80.

7. Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50: 571–599.

8. Lee B-H, Henderson DA, Zhu J-K (2005) The Arabidopsis Cold-Responsive Transcriptome and Its Regulation by CBF1. The Plant Cell Online: 17: 3155–3177.

9. Cushman JC, Bohent JH (2000) Genomic approaches to plant stress tolerance. Curr Opin Plant Biol 3: 117–124.

10. Fieo S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple stress pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14: 1675–1690.

11. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of genes involved in the stress response. Annu Rev Plant Biol 54: 449–474.

12. Fowler S, Thomashow MF (2002) Transcriptional activation of thermotolerance-related genes in grapevine (V. vinifera). Current Opinion in Plant Biology 6: 410–417.

13. Han M, Terasaka S, Fukaya T, Kubo T (2003) Enhancement of chilling resistance by overexpression of ABA-inducible genes in transgenic tobacco. Planta 217: 290–298.

14. Kaye C, Neven L, Hoq S, Maslak D, et al. (1998) Characterization of a gene for spinach CDPK19 and expression of two spinach cold-acclimation proteins in tobacco. Plant Physiol 116: 1367–1377.

15. Nakasuka T, Haruta KS, Pitaksutheepong C, Abe W, Kitaoka K, et al. (2008) Identification and characterization of R2R3 MYB and MHS transcription factors regulating anthocyanin biosynthesis in gentian flowers. Plant and Cell Physiology 49: 1818–1829.

16. Garg AK, Kim JK, Owens TG, Ranaolla AP, Choi YD, et al. (2002) Transgenic accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci U S A 99: 13709–13703.

17. Pramanik MH, Imari R (2005) Functional identification of a transgenic 6- phosphatase phosphatase gene that is involved in transient induction of transgenic biosynthesis during chilling stress in rice. Mol Plant Biol 58: 751–762.

18. Houde M, Dallaire S, N’Dong D, Sarhan A (2004) Overexpression of the acidic dehydrin WGR10 improves freezing tolerance in transgenic strawberry leaves. Plant Biotechnol J 2: 381–387.

19. Dai X, Xu Y, Ma Q, Xu W, Wang T, et al. (2007) Overexpression of an R2R3 MYB gene, OsMYB25, increases Tolerance to Freezing, Drought, and Salt stress in Transgenic Arabidopsis. Plant Physiol 143: 1739–1751.
41. Wang Y, Jiang CJ, Li YY, Wei CL, Deng WW (2012) CoRE1 and CoCBF1: two transcription factors involved in cold responses in Camellia sinensis. Plant Cell Rep 31: 27–34.

42. Dong C, Zhang Z, Ren J, Qin Y, Huang J, et al. (2013) Stress-responsive gene ICE1 from Ziziphus jujuba increases cold tolerance in tobacco. Plant Physiol Biochem 71: 212–217.

43. Li J, Wang L, Zhu W, Wang N, Xin H, et al. (2013) Characterization of two VvICE1 genes isolated from ‘Muscat Hamburg’ grapevine and their effect on the tolerance to abiotic stresses. Scientia horticulturae 165: 266–273.

44. Rahman MA, Mood MA, Nassuth A (2014): Grape contains 4 ICE genes whose expression includes alternative polyadenylation, leading to transcripts encoding at least 7 different ICE proteins. Environmental and Experimental Botany.

45. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28: 2731–2739.

46. Xu X, Chen C, Fan B, Shen Z (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRI1B, WRI4B, and WRI50 transcription factors. Plant Cell 18: 1310–1326.

47. Xu W, Zhang N, Jiao Y, Li R, Xiao D, Wang Z (2014). The grapevine basic helix-loop-helix (bHLH) transcription factor positively modulates CBF-pathway and confers tolerance to cold stress in Arabidopsis. Mol Biol Rep DOI: 10.1007/s11033-014-3404-2.

48. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743.

49. Weigel RR, Ba¨uscher C, Pfitzner AJ, Pfitzner UM (2001) NIMIN-1, NIMIN-2 and confers tolerance to cold-stress in Arabidopsis thaliana. FEBS Lett 461: 205–210.

50. Rudolph AS, Crowe HJ (1985) Membrane stabilization during freezing and salinity in Arabidopsis thaliana. Plant and soil 39: 205–207.

51. Sato Y, Masuta Y, Saito K, Murayama S, Ozawa K (2011) Enhanced chilling tolerance at the booting stage in rice by transgenic overexpression of the ascorbate peroxidase gene, OsAPX2. Plant cell reports 30: 399–406.

52. Liu L, Duan L, Zhang J, Zhang Z, Mi G, et al. (2010) Cucumber (Cucumis sativus L.) over-expressing cold-induced transcriptome regulator ICE1 exhibits changed morphological characters and enhances chilling tolerance. Scientia horticulturae 129: 24–33.

53. Carpenter JF, Crowe HJ (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25: 244–253.

54. Murre C, McCaw PS, Baltimore D (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and SIZ1 expression and freezing tolerance in Arabidopsis. Plant Cell Rep 30: 2177–2186.

55. Dexter ST, Tottingham WE, Graber LF (1932) Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiology 7: 63–78.