Expression of Basic Helix-Loop-Helix Transcripts During Low Temperature Treatments in Grapevines

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ABSTRACT The differential expression of six basic helix-loop-helix (bHLH) genes in response to low temperatures was studied by evaluating their mRNA levels in the buds and shoots of grapevines. Comparison of the amino acid sequences deduced from nucleotide sequences of the bHLH genes in *Vitis labruscana* transcripts revealed homologies ranging from 58.8% (VICEbHLH1 and VICEbHLH4) to 8.2% (VvbHLH and VICEbHLH3). Specific primers from unique regions of genes were obtained by alignment of nucleotide sequences and used to evaluate differential expression patterns of bHLH genes. The accumulation patterns of bHLH mRNAs were induced differentially and dependent on the treated temperatures, -20°C in the buds and -2°C and 4°C in the shoots of ‘Campbell Early’ and ‘Muscat Bailey A’ grapevines. The bHLH genes showed differential expression patterns in response to low temperatures. In ‘Campbell Early’, the expression of VICEbHLH1, VICEbHLH2, and VICEbHLH5 was up-regulated, while VICEbHLH3 was down-regulated in response to -20°C cold stress. In ‘Muscat Bailey A’, the expression of all genes was rapidly up-regulated, peaking at 6 h, then decreasing at 12 h after treatment.

Keywords Bud, Cold treatment, Transcription factor, *Vitis labruscana*

INTRODUCTION

Low temperature is a serious abiotic stress showing effects similar to drought, salinity, oxidative stress, and chemical toxicity. Cold stress, which includes chilling (<20°C) and freezing (<0°C) temperatures, adversely affects the growth and development of plants and significantly constrains the spatial distribution of plants and agricultural productivity. Some of the molecular and physiological changes that occur during cold acclimation are important to plant cold tolerance (Hsieh *et al.* 2004; Zhu *et al.* 2007).

Grape (*Vitis* spp.) is the most cultivated fruit crop in the world. Low temperature leads to a series of biochemical, molecular, and physiological changes in grape that cause adverse effects on their growth and production. Grapevines cultivated in cold regions during winter often suffer from various degrees of frost and freezing damage, which subsequently results in increased costs and limits further development of grape production (Zhang *et al.* 2013).

Basic helix-loop-helices (bHLH) are generally involved in the responses to various abiotic stresses in higher plants and comprise one of the largest families of transcription factors (TFs) (Ledent and Vervoort 2001). Induction of bHLHs has been reported in carpel margin development of *Arabidopsis* (Heisler *et al.* 2001), anthocyanin synthesis of petunia (Spelt *et al.* 2000) and dahlia (Ohno *et al.* 2011), the jasmonate signaling pathway of rice (Seo *et al.* 2011), alkaloid biosynthesis of *Coptis japonica* (Yamada *et al.* 2011), and cold treatment of apple (Feng *et al.* 2012). However, the relationship of the participation and function of bHLH TFs to low temperature response still remains largely unknown.

In this study, bHLH transcripts with different expression levels upon transcriptome analysis of *Vitis labruscana* subjected to freezing temperatures were obtained. Nucleotide and deduced amino acid sequences were then compared by alignment of bHLH genes, and their differential expression in response to cold stress was analyzed in ‘Campbell Early’

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and ‘Muscat Bailey A’ grapevines.

MATERIALS AND METHODS

Plant materials and cold treatment

One-year-old vine cane with latent buds of ‘Campbell Early’ (moderately tolerant to cold stress) and ‘Muscat Bailey A’ (MBA, sensitive to cold stress) grapevines were harvested, cut to small pieces with one bud, and used for cold treatment. Cold treatment of buds was carried out at -20°C in the dark for 0, 6, 12, and 24 h. Vines with green shoots were maintained under 16/8-h photoperiod at 4°C for 0, 1, 2, 3, and 4 weeks and at -2°C for 0, 24, 48, 72, and 96 h. All samples were collected at the indicated times, immediately frozen in liquid nitrogen and stored at -80°C until further use. Untreated samples were collected for use as a control. Five plants and 200 buds were used per treatment and each treatment was performed in triplicate.

RNA extraction and cDNA synthesis

Total RNA was extracted from grapevine buds using a modified version of the method described by Yang *et al.* (2008) and from grapevine leaves using a modified pine

tree method described by Chang *et al.* (1993). The yield and quality of total RNA were measured based on the absorbance at 230, 260, and 280 nm using a Nano Drop spectrophotometer (ND-1000, Technologies Inc., USA). First-strand cDNA was synthesized from the total RNA (500 ng) using a GoScriptTM reverse transcription system synthesis kit (Promega, Madison, USA) and subsequently used as a template for PCR.

Cloning of bHLH genes and sequence analysis

Among 35 DEGs identified upon transcriptome analysis of ‘Campbell Early’ treated with low temperature, six loci showing different expression level with more than 100 transcripts were selected for evaluation of their differential expression against low temperature. Specific primers of six bHLH genes were designed by alignment of the nucleotide sequences from the ‘Campbell Early’ transcripts and used to screen differential expression in response to low temperature of grapevines.

The ExPASy server was used to translate the nucleotide sequences into amino acid sequences (<http://www.expasy.org>). Nucleotide sequences were subjected to pairwise comparisons and multiple alignments of the deduced amino acid sequences were performed using the T-coffee (Notredame *et al.*

Table 1. Sequence of primers used for quantitative real-time PCR analysis.

Name	NABIC ^{z)} ID	Primer sequence
VICEbHLH1 ^{y)}	NS-0415-000001	5'-GGCACCAAAGACAAGAGCAAGT-3' 5'-GGTGTGGCTTTTCCTGCATAC-3'
VICEbHLH2	NS-0416-000001	5'-CCTTGCTGAAAGGGTCAGAAGA-3' 5'-CAAGCATTACTGCCTTCCCAGT-3'
VICEbHLH3	NS-0417-000001	5'-TCCCGTCTTTGTTGTTCCCTTA-3' 5'-CAGTCTGATGGGGATGGATAGC-3'
VICEbHLH4	NS-0418-000001	5'-AGAGAAGGGCAGGTGATGAAGA-3' 5'-CCGAGGATTTTCCTGTTCCTCT-3'
VICEbHLH5	NS-0419-000001	5'-ATTGAGCGAAAAGAGGATGTG-3' 5'-GCTTGTCCATGTTGGGAAAAAG-3'
VICEbHLH6	NS-0420-000001	5'-CTCTGCAGTCAAACCAGCTGAA-3' 5'-ATGCAGCTCCACCTTTGTCTTC-3'

^{z)}NABIC - National Agricultural Biotechnology Information Center, RDA, Korea.

^{y)}VICEbHLH1, VICEbHLH2, VICEbHLH3, VICEbHLH4, VICEbHLH5, and VICEbHLH6 : Basic helix-loop-helix (bHLH) genes from *Vitis labruscana* cv ‘Campbell Early’.

2000). Tree nodes were evaluated by bootstrap analysis, and a single tree was constructed using the neighbor-joining method in MEGA 4.0 (Tamura *et al.* 2007). Nucleotide and amino sequence data of the VICEbHLH1, VICEbHLH2, VICEbHLH3, VICEbHLH4, VICEbHLH5, and VICEbHLH6 were deposited in the National Agricultural Biotechnology Information Center (NABIC), Rural Development Administration, Korea database under the accession numbers NS-0415-000001, NS-0416-000001, NS-0417-000001, NS-0418-000001, NS-0419-000001, and NS-0420-000001, respectively.

Quantitative real-time PCR analysis

Real-time PCR was conducted on a C1000TM Thermal Cycler (CFX96TM Real-Time System, BioRad, CA, USA) using SYBR Premix Ex (SYBR Premix Ex Taq, TaKaRa Bio Inc., Osaka, Japan) as the fluorescent dye. Amplification was conducted by subjecting the samples to one cycle at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and

60°C for 30 s. Transcript levels were calculated using the standard-curve method and normalized against the grapevine actin gene (AB372563) as an internal control and non-treated samples as the reference sample, after which melting curves of the amplified products were recorded. For each gene, the reference sample was defined as the 1x expression level, and results were expressed as the fold increase of mRNA over the reference sample. All reactions were performed in triplicate to ensure consistency of the results.

RESULTS

Alignment of the amino acid sequences deduced from the nucleotide sequences of bHLH genes in *V. labruscana* transcripts is shown in Fig. 1. The homology of the amino acid sequence between each gene ranged from 58.8% (VICEbHLH1 and VICEbHLH4) to 8.2% (VvbHLH and

VICEbHLH1	129	-----RDN-ETTMTWPFSSSESPRSLKAKTTDEDSACHGGSENQD-----E-----D---R	169
VICEbHLH2	195	PRPPLNTSKSADGEQPKGLPWENSEFSEKQEEKKQKIDQNMSPNLRGKQ-----PNKHAKDNSSNG	255
VICEbHLH3	17	-----	16
VICEbHLH4	14	-----	13
VICEbHLH5	113	-----AADN-DLKMFSGLNS-----LEAQNVDSRYRSXGL---THHLSLPKNF-----PQ	153
VICEbHLH6	27	-----DNHMPXKLYL-----IDRAARDNAKAG	52
VICEbHLH1	170	ETK-T-----QTG-R-SHSTRR---SRAAAIHN-QSERRRDRINQKMKTLQKLVFNS SK-TDKASMLD	225
VICEbHLH2	256	EAPKE-----NY-IHVRARR---GQATNSHS-LAEVRREKISERMRLQLVFGCNKITGKAVMLD	312
VICEbHLH3	17	-----T-----VVG-R-LSPXKKNPGKVPKIHKAEREKLRDHLNVLFLELGNILESAQQNNGKACILT	74
VICEbHLH4	14	--K-R-----GTG-KSSVSSKR---SRAAAIHN-QSERKRRDKINQRMKTLQKLVFNS SK-TDKASMLD	68
VICEbHLH5	154	ITTIKFLHFQDSVPCKIRAKR---GCATHPRS-IAEVRVTRISERMKQLQELFNMMDKQNTADMLD	218
VICEbHLH6	53	STKKX-----KNA-SNETSEE---EKATDSHS-LAEARREKINARMKLLQELVFGCNKISGTALVLD	111
VICEbHLH1	226	EVIEYKQLQAQVQMMSVRNMPQ-MMMFPMGQ-----Q-----QLQMS---LLA-----	265
VICEbHLH2	313	EIINYVQSLQQQVEFLSMKLATVNPENIDIE-----R-----LLSKD-----ILNS-----	354
VICEbHLH3	75	DASRLRLDLAQVDCLKRDNAAL-LSESHYVTMEKXELREDNSALEAQIKKLQSE---LEE-----	131
VICEbHLH4	69	EVIEYKQLQAQVQMMXRMNMSF-MMMFMKLQ-----Q-----QLQMS---LMA-----	108
VICEbHLH5	219	LAVEYIKDLQKQVKTLDNT-----	237
VICEbHLH6	112	EIISHVQSLQRQVEFLSMRLAAVNPRIDFNLD-----S-----LLAPESGSLVDNFPSPMVV	163
VICEbHLH1	266	-----RMGMGVGL-GMGMGLDMSAV--PRAAPQT---LPSLLHANPVVA-A	305
VICEbHLH2	355	-----RGGSTSVL-GFGPGMSSSHFY--PHGISQG---TLPGI-----P	387
VICEbHLH3	132	-----RIRSKPAW-NSDPSQLDHNST--APQLPEDHLMFPVTDH---ASQT	171
VICEbHLH4	109	-----QMGMGMS PMGMGVDMNTIARPNVATTG---ISPLL-----H-P	145
VICEbHLH5	238	-----	237
VICEbHLH6	164	PLMWFVQANENRQFPYQQLWNDTLHQFPVW-GREE-XDPH-----	201
VICEbHLH1	306	TPTFFVPPFPVXXXXMPSSSQPKSDAGANAAPFLQDPYCAFLAQ-----SMNMDLYHKMAALYRQVNHK	369
VICEbHLH2	388	TP-----QFHSTQ-----TVWD--GELQSLQMG--FD	411
VICEbHLH3	172	APV-VGPFVFPVPLHQDLQTFQFPNA---AASNVSRRPHARYPSPDCWPSQILD--SQLKTPEQHE---	230
VICEbHLH4	146	TP--FLPLTSWD--VSGDRLF---A---APTMVPDPLAFLACQ--SQPMTMDAYSMAALYQHL---	198
VICEbHLH5	238	-----	237
VICEbHLH6	202	-N-----FIAPE-----H-----XLLS--YD	214

Fig. 1. Multiple amino acid sequence alignment of basic helix-loop-helix (bHLH). VICEbHLH1 (NS-0415-000001), VICEbHLH2 (NS-0416-000001), VICEbHLH3 (NS-0417-000001), VICEbHLH4 (NS-0418-000001), VICEbHLH5 (NS-0419-000001), and VICEbHLH6 (NS-0420-000001): bHLH proteins of *Vitis labruscana* cv. 'Campbell Early'.

VICEbHLH3). The amino acid sequences of VICEbHLH2, VICEbHLH5, VICEbHLH6 and VICEbHLH1 showed 39.6%, 29.3%, 26.5%, and 25.2% homology with those of VICEbHLH6, VICEbHLH2, VICEbHLH1 and VICEbHLH5, respectively (Table 2). The resulting phylogenetic tree of the deduced amino acid sequence of bHLH transcripts is shown in Fig. 2. The bHLH sequences were divided into

two groups, one including VvbHLH, VICEbHLH2, VICEbHLH5 and VICEbHLH6, and another including VICEbHLH1, VICEbHLH3, and VICEbHLH4.

Specific primers to each transcript were used to detect differential expression by real-time PCR of grapevine buds and leaves treated with various low temperatures. bHLH mRNAs showed differential expression patterns that were

Fig. 2. Phylogenetic tree of basic helix-loop-helix (bHLH) proteins from VvbHLH (JQ823168.1) and *Vitis labruscana* transcripts, VICEbHLH1 (NS-0415-000001), VICEbHLH2 (NS-0416-000001), VICEbHLH3 (NS-0417-000001), VICEbHLH4 (NS-0418-000001), VICEbHLH5 (NS-0419-000001), and VICEbHLH6 (NS-0420-000001).

Table 2. Identity of amino acid sequences compared to basic helix-loop-helix (bHLH) gene transcripts of *Vitis labruscana*.

	VvbHLH ²⁾	VICE ³⁾ bHLH1	VICE bHLH2	VICE bHLH3	VICE bHLH4	VICE bHLH5	VICE bHLH6
VvbHLH		18.5	36.2	8.2	17.5	17.8	25.2
VICEbHLH1	18.5		22.3	21.7	58.8	25.2	26.5
VICEbHLH2	36.2	22.3		18.8	25.6	29.3	39.6
VICEbHLH3	8.2	21.7	18.8		21.3	16.2	16.9
VICEbHLH4	17.5	58.8	25.6	21.3		16.1	25.6
VICEbHLH5	17.8	25.2	29.3	16.2	16.1		16.5
VICEbHLH6	25.2	26.5	39.6	16.9	25.6	16.5	

²⁾VvbHLH: originated from *V. vinifera*.

³⁾VICEbHLH1, VICEbHLH2, VICEbHLH3, VICEbHLH4, VICEbHLH5, and VICEbHLH6 : bHLH proteins of *Vitis labruscana* cv 'Campbell Early'.

dependent on various temperatures applied to ‘Campbell Early’ and ‘MBA’ (Fig. 3). In ‘Campbell Early’ buds treated with -20°C cold stress (Fig. 3A), the expression of VICEbHLH1, VICEbHLH2, and VICEbHLH5 was up-regulated, peaking at 12 h, while the expression of VICEbHLH3 was down-regulated, peaking at 6 h after treatment. In MBA (Fig. 3B), the expression of all tested genes was rapidly up-regulated, peaking at 6 h, then decreased at 12 h and increased again at 24 h after treatment. The mRNA level of VICEbHLH3 in the leaves of grapevines maintained at 4°C (Fig. 3C and D), was up-regulated, while VICEbHLH4 and VICEbHLH5 were slightly down-regulated at one week, then increased again, peaking at four and three weeks after treatment. The mRNA level of VICEbHLH1, VICEbHLH2, and VICEbHLH6 was down-regulated in ‘Campbell Early’ kept at 4°C , while the expression of VICEbHLH1 and VICEbHLH3 genes was down-regulated at one week and increased at four weeks in ‘MBA’ kept at 4°C .

Following -2°C cold treatment of the leaves of ‘Campbell Early’ grapevine, the expression of VICEbHLH3, VICEbHLH4, and VICEbHLH5 was slightly increased at one hour, peaking at 72 h (VICEbHLH3 and VICEbHLH4) and 96 h (VICEbHLH5) after treatment (Fig. 3E). The accumulation of VICEbHLH6 mRNA induced by cold stress (-2°C)

presented two peaks at 24 h and 72 h in ‘MBA’ grapevine (Fig. 3F). The VICEbHLH1 and VICEbHLH2 mRNA level were down-regulated and the VICEbHLH3 mRNA level was up-regulated, peaking at 72 h after cold treatment (-2°C).

DISCUSSION

Among various environmental stresses, low temperature is one of the most important factors limiting cultivation of plants. bHLH protein comprises a group of functionally diverse transcription factors found in plants. In the present study, six bHLH genes showed differential expression in response to various low temperatures that caused physiological damage and low productivity in grapevines. To examine the expression patterns of ‘Campbell Early’ bHLH genes, primers specific for unique regions in each gene were obtained using multiple sequence alignments. Quantitative PCR revealed that six bHLH genes were differently expressed in ‘Campbell Early’ transcripts.

Among the TFs, the bHLH motif-containing TFs are important regulatory components of the transcriptional networks. Expression of bHLH genes related to low temperature has been identified in various plants, including

Fig. 3. Expression of basic helix-loop-helix (bHLH) under various low temperatures in grapevines. Transcript levels were calculated using the standard curve method from triplicate data with the grapevine actin gene as an internal control and non-treated bud and leaves (at time zero) as a reference. The results represent the mean fold increase in mRNA level over non-treated leaves relative to the 1x expression level. Results are the means of triplicate data from three experiments. Vertical bars indicate the standard deviation of three replicates. A and B, expression following freezing treatment (-20°C) of buds for 0, 6, 12 and 24 h; C and D, expression following low treatment (4°C) of shoots for 0, 1, 2, 3, and 4 weeks; E and F, expression following freezing treatment (-2°C) of shoots for 0, 24, 48, 72, and 96 h. A, C, and E, ‘Campbell Early’; B, D, and F, ‘Muscat Baily A’. VICEbHLH1, VICEbHLH2, VICEbHLH3, VICEbHLH4, VICEbHLH5, and VICEbHLH6 : bHLHs genes from *Vitis labruscana* cv ‘Campbell Early’.

Arabidopsis (Penfield *et al.* 2005), apple (Xie *et al.* 2012), and *Poncirus trifoliata* (Huang *et al.* 2013). Xie *et al.* (2012) reported that apple bHLH TF MdbHLH3 is induced to regulate anthocyanin accumulation and fruit coloration at low temperature.

OsbHLH1 can be induced by low temperature and has been implicated in rice cold defense through an abscisic acid (ABA)-independent pathway (Wang *et al.* 2003). Up-regulation of the OsbHLH148 transcript level by low temperature resulted in activation of tolerance to drought (Seo *et al.* 2011). Feng *et al.* (2012) reported that the apple bHLH gene, MdCibHLH1 (Cold-Induced bHLH1), encodes an ICE-like protein, acts as a signal transduction component in the CBF pathway and is associated with cold tolerance in apple. Additionally, overexpression of MdCibHLH1 enhanced cold tolerance in transgenic *Arabidopsis*. In *Arabidopsis thaliana* plants, ICE2, which is a bHLH transcription factor and positive regulator, participates in the response to freezing through a cold acclimation-dependent pathway (Fursova *et al.* 2009). Although several bHLH genes were up-regulated by coldness, the transcript level of CsICE1 in *Camellia sinensis* was not altered by chilling treatment (Wang *et al.* 2012). These reports indicate that the bHLH genes respond differentially to abiotic stimuli in many plant models.

Buck and Atchley (2003) described 118 and 131 potentially unique bHLH proteins found in the *A. thaliana* and the *Oryza sativa* genomes, respectively. bHLH proteins are the second largest class of plant TFs, with 162 members in *Arabidopsis* and 167 members in rice (Carretero-Paulet *et al.* 2010; Feller *et al.* 2011). Most bHLH proteins identified were functionally characterized in model plants such as *Arabidopsis* and rice, and their roles include regulation of phytochrome signaling, flavonoid biosynthesis, fruit dehiscence, carpel, anther and epidermal cell development, hormone signaling and stress responses (Feller *et al.* 2011).

In this study, we analyzed nucleotide sequences and the amino acid sequences deduced from these nucleotides and compared the homologies among six bHLHs from *V. labruscana*. Additionally, we investigated the expression pattern of bHLH genes in response to various low temperature treatments in ‘Campbell Early’ and ‘MBA’ grapevines. The expression of bHLH transcripts differed following

cold treatment, suggesting that the bHLH gene may be involved in cold stress response of grapevine. Identification of the differential expression of the six bHLH transcripts tested in this study in response to different cold conditions will facilitate elucidation of mechanisms of tolerance to coldness, improvement of freezing tolerance, and development of critical molecular markers for selection of cold-hardy seedlings in grapevine breeding programs.

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REFERENCES

- Buck MJ, Atchley WR. 2003. Phylogenetic analysis of plant basic helix-loop-helix proteins. *J. Mol. Evol.* 56: 742-750.
- Carretero-Paulet L, Galstyan A, Roig-Villanova I, Martinez-Garcia JF, Bilbao-Castro JR, Robertson DL. 2010. Genome-wide classification and evolutionary analysis of the bHLH family of transcription factors in *Arabidopsis*, poplar, rice, moss, and algae. *Plant Physiol.* 153: 1398-1412.
- Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol.* 11: 113-116.
- Feller A, Machemer K, Braun EL, Grotewold E. 2011. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.* 66: 94-116.
- Feng XM, Zhao Q, Zhao LL, Qia Y, Xie XB, Li HF, Yao YX, You CX, Hao YJ. 2012. The cold-induced basic helix-loop-helix transcription factor gene *MdCibHLH1* encodes an ICE-like protein in apple. *BMC Plant Biol.* 12: 22.
- Fursova OV, Pogorelko GV, Tarasov VA. 2009. Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. *Gene* 429: 98-103.
- Heisler MG, Atkinson A, Bylstra YH, Walsh R, Smyth DR.

2001. SPATULA, a gene that controls development of carpel margin tissues in *Arabidopsis*, encodes a bHLH protein. *Development* 128: 1089-1098.
- Hsieh TH, Lee JT, Yang PT, Chiu LH, Chang YY, Wang YC, Chan MT. 2004. Heterology expression of the *Arabidopsis* C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. *Plant Physiol.* 135: 1145-1155.
- Huang XS, Wang W, Zhang Q, Liu JH. 2013. A basic helix-loop-helix transcription factor, PtrbHLH, of *Poncirus trifoliata* confers cold tolerance and modulates peroxidase-mediated scavenging of hydrogen peroxide. *Plant Physiol.* 162: 1178-1194.
- Ledent V, Vervoort M. 2001. The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis. *Genome Res.* 11: 754-770.
- Notredame C, Higgins DG, Heringa J. 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* 302: 205-217.
- Ohno S, Hosokawa M, Hoshino A, Kitamura Y, Morita Y, Park KI, Nakashima A, Deguchi A, Tatsuzawa F, Doi M, Iida S, Yazawa S. 2011. A bHLH transcription factor, DvIVS, is involved in regulation of anthocyanin synthesis in dahlia (*Dahlia variabilis*). *J. Exp. Bot.* 62: 5105-5116.
- Penfield S, Josse EM, Kannangara R, Gilday AD, Halliday KJ, Graham IA. 2005. Cold and light control seed germination through the bHLH transcription factor SPATULA. *Current Biol.* 15: 1998-2006.
- Seo JS, Joo J, Kim MJ, Kim YK, Nahm BH, Song SI, Cheong JJ, Lee JS, Kim JK, Choi YD. 2011. OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. *Plant J.* 65: 907-921.
- Spelt C, Quattrocchio F, Mol JN, Koes R. 2000. Anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. *Plant Cell* 12: 1619-1632.
- Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596-1599.
- Wang Y, Jiang CJ, Li YY, Wei CL, Deng WW. 2012. CsICE1 and CsCBF1: two transcription factors involved in cold responses in *Camellia sinensis*. *Plant Cell Rep.* 31: 27-34.
- Wang YJ, Zhang ZG, He XJ, Zhou HL, Wen YX, Dai JX, Zhang JS, Chen SY. 2003. A rice transcription factor OsbHLH1 is involved in cold stress response. *Theor. Appl. Genet.* 107: 1402-1409.
- Xie XB, Li S, Zhang RF, Zhao J, Chen YC, Zhao Q, Yao YX, You CX, Zhang XS, Hao YJ. 2012. The bHLH transcription factor MdbHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. *Plant Cell Environ.* 35: 1884-1897.
- Yamada Y, Kokabu Y, Chaki K, Yoshimoto T, Ohgaki M, Yoshida S, Kato N, Koyama T, Sato F. 2011. Isoquinoline alkaloid biosynthesis is regulated by a unique bHLH-type transcription factor in *Coptis japonica*. *Plant Cell Physiol.* 52: 1131-1141.
- Yang G, Zhou R, Tang T, Shi S. 2008. Simple and efficient isolation of high-quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives. *Prep. Biochem. Biotechnol.* 38: 257-264.
- Zhang J, Liu N, Niu R, Liu Y, Zhai H, Xu W, Wang Y. 2013. Construction of a cDNA library of the Chinese wild *Vitis amurensis* under cold stress and analysis of potential hardiness-related expressed sequence tags. *Genet. Mol. Res.* 12: 1182-1193.
- Zhu JH, Dong CH, Zhu JK. 2007. Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr. Opin. Plant Biol.* 10: 290-295.