

Expression Analysis of the *MdCibHLH1* Gene in Apple Flower Buds and Seeds in the Process of Dormancy

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Abstract

A bHLH transcription factor that is induced by low temperature was found in apple (*Malus × domestica* Borkh.). To understand the sequence characteristics of the gene, bioinformatics analysis was performed. Furthermore, gene expression patterns of the laminated apple seeds and lateral flower buds were analyzed during the period of dormancy release with semi-quantitative RT-PCR. Based on secondary structure predictions, the results showed that the *MdCibHLH1* protein structure mainly included α -helix and random coil, while β -sheet and extended strand content was less. Semi-quantitative RT-PCR analysis showed that the expression patterns of *MdCibHLH1* were similar in laminated apple seeds and lateral flower buds during the period of dormancy release. Before dormancy release, expression levels of *MdCibHLH1* were high and gradually decreased during the period of dormancy release. These results indicated that *MdCibHLH1* might play an important role during dormancy release in apple seeds and apple buds.

Keywords: apple; *MdCibHLH1*; dormancy; expression analysis

1. Introduction

The bHLH proteins comprise the second largest transcription factor (TF) family in plants. There are 162 genes encoding bHLH proteins in *Arabidopsis thaliana* (Bailey et al., 2003). bHLHs are composed of an N-terminal DNA binding domain and a C-terminal HLH region (Carretero-Paulet et al., 2010). They play pivotal roles in plant development (Heim et al., 2003), hormone signaling (Abe et al., 1997; Friedrichsen et al., 2002; Yin et al., 2005; Lee et al., 2006) and drought stress responses (de Pater et al., 1997; Smolen et al., 2002). The bHLH TFs in plants regulate the expression of downstream genes usually by binding to the E-Box (CANNTG) cis-acting elements at the promoter region of these genes in the form of homodimers or heterodimers (Feller et al., 2011). For example, Chinnusamy et al. (2003) found that *Arabidopsis* bHLH TF ICE1 (inducer of *CBF* expression 1) activated the expression of *CBF3* and its downstream target genes by binding to the E-box element present in the promoter of *CBF3* specifically to improve cold resistance of

the plant under cold stress. Fursova et al. (2009) isolated another bHLH TF ICE2 in *Arabidopsis thaliana* that could promote expression of the *CBF1/DREB1B* TF to improve cold resistance of the plant (Hu et al., 2013); JAZ (Jasmonate ZIM-domain) protein, as a plant jasmonic acid signal pathway inhibitor, can interact with bHLH TFs, ICE1 and ICE2, thus inhibiting expression of *CBF* and its downstream target genes to affect cold resistance in plants. Zhao et al. (2013) found that bHLH TF MaMYC2s interacts with MaICE1 and regulates expression of the downstream resistance genes to improve cold resistance in banana fruit. In addition, the photoperiod-related bHLH TFs, PIF4 and PIF7 negatively regulate the expression of *CBFs* by binding to *CBF1* and *CBF2* promoter region G-boxes (CACGTG) and the *CBF3* promoter E-box (Kidokoro et al., 2009; Lee and Thomashow, 2012). In rice, the bHLH TF rd22BP1 is able to bind to the *rd22* gene promoter E-box and then regulates its transcription and increases plant tolerance to abiotic stress (Abe et al., 1997). Overexpression of the *OsHHLH2* gene can increase the antioxidant capacity of transgenic plants in wild rice (Zhou et al.,

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2009). In addition, heterologous overexpression of the *Poncirus* bHLH TF, *PtrbHLH*, in tobacco and lemon can significantly enhance freezing tolerance. Further study found that the *PtrbHLH* gene can bind to the promoter of the *POD* gene and regulate its expression, which promotes clearance of ROS under low temperature stress (Huang et al., 2013).

The start and termination of dormancy in deciduous fruit trees is a gradual process. Temperature, photoperiod, and water affect bud dormancy and release; however, temperature is the main influential factor (Schmitz et al., 2014), especially for apple and peach trees. Physiological dormancy and release of fruit buds are related to metabolism, hormone regulation, absorption and transport of nutrients, and signal transduction. These processes are linked to the regulation of gene expression (Wang et al., 2008; Footitt et al., 2013; Fu et al., 2014). Overexpression of the *CBF1* gene enhanced cold tolerance in transgenic strawberries compared with controls (Yuan et al., 2006; Jin et al., 2007). In apple, heterologous overexpression of the *Prunus persica* *CBF1* gene (*PpCBF1*) not only increased plant tolerance to low temperatures but also showed a typical dormancy phenotype, which suggested that the *CBF* gene may be closely related to dormancy (Wisniewski et al., 2011). The laboratory prescreened a bHLH TF induced by low temperature. Transgenic *Arabidopsis*, tobacco, and apples were able to enhance cold tolerance (Feng et al., 2012). The findings of this study indicated that the bHLH TFs may be involved in dormancy release in apple by regulating CBF transcription expression.

In this study, we analyzed the expression of *MdCibHLH1* induced by low temperatures in apple flower buds and seeds in the process of dormancy and release, which provided a reference for revealing the mechanism of apple flower bud dormancy release on a molecular level.

2. Materials and methods

2.1. Plant material

Apple (*Malus × domestica* ‘Royal Gala’) flower buds were taken from Xiazhang Town, Tai’an City, Shandong Province at different stages of dormancy (December 10, 2012, February 12, 2013, March 6, 2013, March 23, 2013, March 30, 2013, April 6, 2013, and April 13, 2013). After the sample was taken, the flower buds were immediately frozen in liquid nitrogen for RNA extraction. The analysis of each type of sample was repeated five times.

The chosen apple seeds were placed to soak in water for 24 h and then were mixed with fine river sand (sand:seed = 4:1) at 4 °C cold stratification. The number of germinated seeds was observed and counted every 10 d.

2.2. Bioinformatics analysis of *MdCibHLH1*

The *MdCibHLH1* amino acid sequence, protein molecular weight, isoelectric point, and stability index were obtained with the help of Proteomics Server (<http://web.expasy.org/protparam/>). The hydrophobicity and transmembrane domain were analyzed using ProtScale (<http://web.expasy.org/protscale/>) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>). The tertiary structure and secondary structure of the proteins were

predicted using SWISS-MODEL (<http://swissmodel.expasy.org/>) and SOPMA (<http://npsa-pbil.ibcp.fr/>), respectively. The MEGA 4.0 program was used to construct phylogenetic trees for the bHLH proteins. Neighbor-joining (NJ) distance criteria were used for phylogenetic estimation.

2.3. Expression analysis of *MdCibHLH1* at different periods of dormancy

Total RNAs were extracted from different periods of apple flower buds and laminated apple seeds using RNAplant plus Reagent (Tiangen, China). First-strand cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, China) according to the manufacturer’s instructions.

The PCR reaction mixture contained 200 ng cDNA (Trans, China) in a 25 µL reaction volume. For the semi-quantitative RT-PCR reactions, the numbers of reaction cycles were 28–32. Each PCR product was analyzed with 1% agarose gel electrophoresis. The following primers were used in semi-quantitative RT-PCR: *MdCibHLH1*-F: 5'-ATGGACGACAGG GAGGAC-3', *MdCibHLH1*-R: 5'-GGAGGAGGAAGAGTCCA C-3'. *MdActin* was control.

2.4. Transformation of *Arabidopsis thaliana*

The full-length *MdCibHLH1* cDNA was cloned into the pBI121 transformation vector containing the CaMV 35S promoter and kanamycin resistance as a selection marker. The 35S:*MdCibHLH1*:GFP fusion construct was introduced into *Arabidopsis* using the floral dip method. Resultant seeds were plated on 1/2 MS culture containing 50 mg · L⁻¹ kanamycin. Three independent homozygous transgenic plants were used for further investigation.

3. Results

3.1. Bioinformatics analysis of *MdCibHLH1* protein

It was found that a bHLH TF was induced by low temperatures. Subsequently, its full-length cDNA was cloned using the RACE technique following EST-based *in silico* cloning; the gene was named *MdCibHLH1* (Cold-Induced bHLH1; NCBI accession number EF495202.1). The *MdCibHLH1* ORF was 1 596 bp in length and encoded a predicted protein containing 531 amino acid residues (Feng et al., 2012). Using the sequence analysis tools on the ExPASy Proteomics Server, the *MdCibHLH1* gene molecular weight was 57.37 kD and the pI was 5.51. The basic amino acid residues (Arg + Lys) were 54 and acidic amino acid residues (Asp + Glu) were 64. The formula could be written as C₂₄₆₀H₃₉₂₀N₇₂₈O₈₀₇S₂₄. The instability index in solution was 49.52, and the liposoluble index was 71.79. The total average hydrophilicity index was -0.544.

Based on the secondary structure prediction, the results showed that the *MdCibHLH1* protein mainly included α-helix and random coil structures, the β-sheet and extended strand content was less. The percentages of α-helix, random coil, β-sheets and extended strands were 27.31%, 58.38%, 3.58%, and 10.73%, respectively.

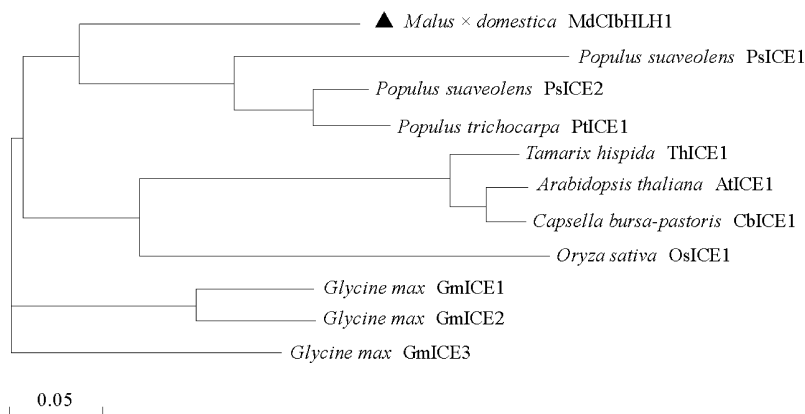


Fig. 1 Phylogenetic tree of *MdCibHLLH1* compared with bHLH proteins of other plants

To predict the *MdCibHLLH1* protein tertiary structure, we obtained the homologous sequence 1an4.1.C (2.9 Å) by searching the protein structure database (PDB), and then, based on a protein template, the predicted protein sequence backbone atom positions were adjusted to generate a protein tertiary structure model. Prediction information showed that the prediction model and template sequence homology was 34.62%.

3.2. Conserved functional domain and phylogenetic tree analysis of *MdCibHLLH1*

The predicted *MdCibHLLH1* protein was found to contain a conserved bHLH domain. In addition, the phylogenetic tree comparisons revealed that the *MdCibHLLH1*, *AtbHLH116* (*AtICE1*), and *AtbHLH033* (*AtICE2*) genes were aligned. Given the similarity of the *MdCibHLLH1* gene with the *Arabidopsis ICE* genes, we compared *MdCibHLLH1* with other known plant *ICE* genes such as *Arabidopsis thaliana*, *Capsella bursa-pastoris*, *Oryza sativa*, and *Populus suaveolens*. The phylogenetic tree and amino acid sequence comparison showed that the *MdCibHLLH1* protein clustered within the same clade as *PtICE1*, *PsICE1*, and *PsICE2*, which indicated it was the

closest relationship. In addition, the C-terminal of protein sequences all contained a Helix–Loop–Helix conservative region (Fig. 1). These results suggested that the functional domain of *MdCibHLLH1* exhibited high conservation with *ICE* proteins. It can be inferred that *MdCibHLLH1* is an *ICEs* homologous gene in apple.

3.3. Expression analysis of *MdCibHLLH1* in the period of flower bud dormancy release

The results of semi-quantitative RT-PCR showed that *MdCibHLLH1* expression levels were highly accumulated in dormant buds and gradually down-regulated during the period of dormancy release. *MdCibHLLH1* gene expression reached the minimum value when flower buds broke dormancy (March 23). However, from the flower buds breaking dormancy to cotyledons beginning to sprout (April 6), *MdCibHLLH1* gene expression increased. *MdCibHLLH1* gene expression levels were down-regulated and maintained at a low level in cotyledon expansion stage (April 13) (Fig. 2). It suggested that *MdCibHLLH1* gene had certain relationship with the maintenance and release of apple flower bud dormancy.

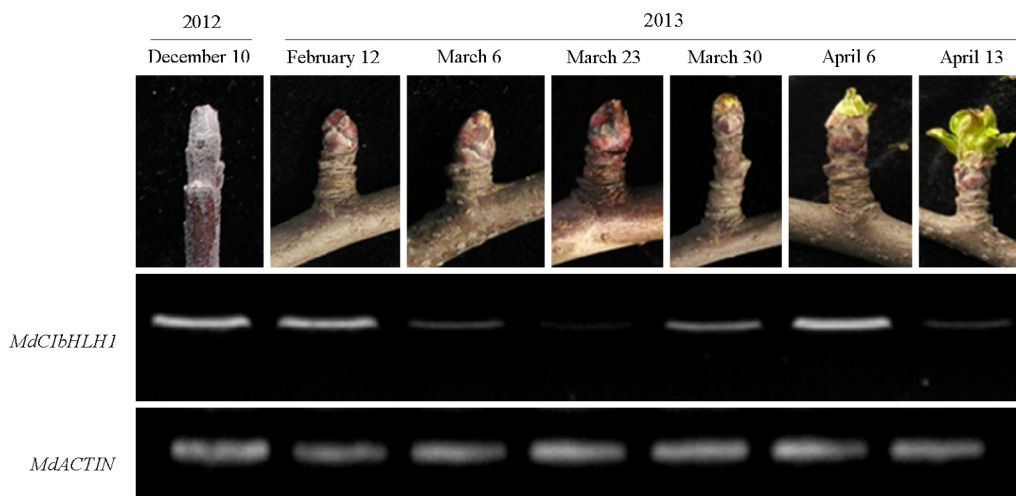


Fig. 2 Expression change of *MdCibHLLH1* in lateral apple flower buds during the period of dormancy release

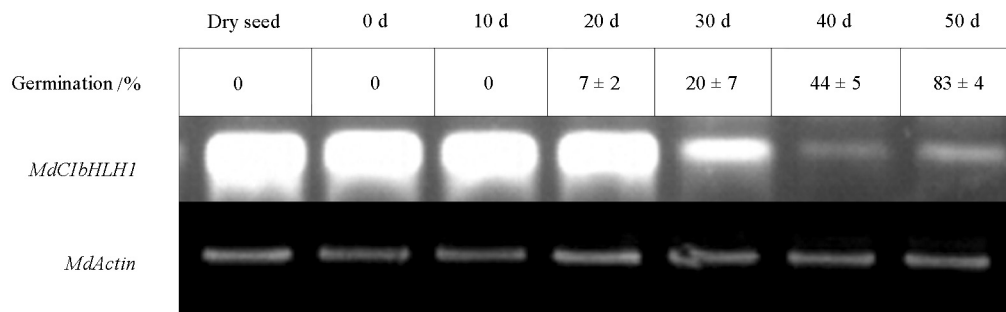


Fig. 3 Expression change of *MdCibHLH1* and germination of apple seeds in the stratification process

3.4. Expression analysis of *MdCibHLH1* in the process of seed stratification

The expression levels of *MdCibHLH1* were also characterized in the process of seed stratification. Apple seeds were ungerminated after stratification for 10 d and began to germinate after stratification for 20 d. The germination rates after stratification for 30 d, 40 d, and 50 d were 20%, 44%, and 83%, respectively. Semi-quantitative RT-PCR showed high transcript levels of *MdCibHLH1* in dry seeds that were gradually down-regulated with seed germination (Fig. 3). The results showed that the expression changes of *MdCibHLH1* during the period of seed germination were similar to the expression changes of the flower bud dormancy release process. This indicated that *MdCibHLH1* might also be involved in the process of seed dormancy release.

3.5. Phenotype identification of heterologous *MdCibHLH1* overexpression in *Arabidopsis*

To characterize further the functions of the *MdCibHLH1* gene, a construct containing *MdCibHLH1* driven by the CaMV 35S promoter was genetically transformed into *Arabidopsis*. Three homozygous transgenic lines, L1, L2, and L3, were used for further investigation. The transgenic seed germination rate, size, and the full extent were measured. Overexpression of the *MdCibHLH1* *Arabidopsis* seed germination rate was significantly lower than the wild type, and seeds were shriveled (Fig. 4 and Fig. 5).

These results further demonstrated that *MdCibHLH1* might affect dormancy.

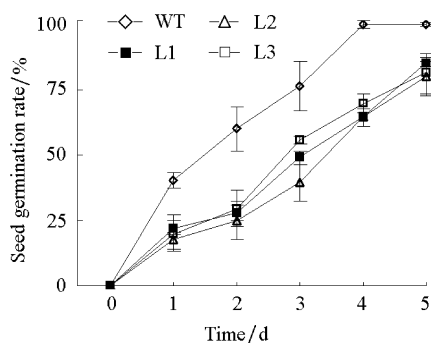


Fig. 4 The seed germination rate in *MdCibHLH1* transgenic *Arabidopsis*. WT: Wild-type; L1, L2 and L3: Transgenic *Arabidopsis* lines.

4. Discussion

In plants, bHLH proteins have been investigated for a long time. Studies of different species have indicated that bHLH proteins play an important role in plant stress tolerance. Nevertheless, the molecular mechanism of bHLH protein in cold resistance of perennial fruit trees is not clear. In this study, biological information analysis was carried out on the bHLH transcription factor, *MdCibHLH1*, which is induced by low temperatures (Feng et al., 2012). This study showed that *MdCibHLH1* had the same conserved bHLH domain as other bHLH transcription factors, and it exhibited high homology with other known plant *ICE* genes induced by low temperatures. In 2010, apple genome sequencing was completed and revealed that its genome was more complex than *Arabidopsis* (Velasco et al., 2010). Previous studies have also found that the *MdCibHLH1* gene is more complex than *ICE* genes in *Arabidopsis* (Feng et al., 2012). In *Arabidopsis*, *AtICE1* can bind to all four MYC recognition sites present in the promoter of the *AtCBF3* gene, and the role of MYC2 was shown to be the strongest (Chinnusamy et al., 2003). However, *MdCibHLH1* only binds to the MYC4 promoter in apple (Feng et al., 2012). This may be explained by an incomplete conservation between *MdCibHLH1* and the *AtICEs* in gene structure and regulation mechanisms.

In the model plant *Arabidopsis thaliana*, the bHLH transcription factor, PIL5 [PIF3-like 5 (PIL5)], as a molecular switch to regulate seed germination, connects the light and hormone signal pathways (Oh et al., 2004). In dark conditions, PIL5 not only can bind to the GA synthesis or decomposition gene (*GA3ox1*,



Fig. 5 The seed phenotype in *MdCibHLH1* transgenic *Arabidopsis*

GA3ox2, and *GA2ox2*) and the ABA synthesis or catabolism gene (*ABAI*, *NCED6*, *NCED9*, and *CYP707A2*) promoters, but can also bind to the promoter region of two *DELLA* genes (*GAI* and *RG1*) to regulate their transcriptional expression and subsequent synthesis or decomposition of GA and ABA to control seed germination (Oh et al., 2007). Later, researchers analyzed PIL5 downstream target genes using CHIP–chip and gene chip technology. The results showed that PIL5 can also bind to hormone-related transcription factors, including ABI3, ABI5, ARF18, BIM1, and JAZ1, participating in regulation of the abscisic acid, auxin, brassinosteroid, and jasmonic acid signaling pathways to affect seed germination (Oh et al., 2009). Since light can regulate seed dormancy through the bHLH transcription factor PIL5, *MdCibHLH1* may also be involved in dormancy release of apple seeds and flower buds. By analyzing *MdCibHLH1* expression in apple seeds and flower buds in different periods, the results showed that *MdCibHLH1* was involved in the dormancy release process of apple flower buds and seeds. In addition, the germination test of transgenic *Arabidopsis* seeds further showed that *MdCibHLH1* affected the process of dormancy. However, unlike stratification seeds, *MdCibHLH1* expression peaked at the late stage of flower bud dormancy release. This may be due to a late spring coldness phenomenon often appearing at the end of March to early April. The increased expression of *MdCibHLH1* is conducive to improve flower buds and other young tissue cold resistance.

It is well known that temperature, light, and hormones are key factors that affect seed germination. Under low temperature condition, the GA biosynthesis genes (*AtGA20ox1*, *AtGA20ox2*, and *AtGA3ox1*) are up-regulated, and the GA decomposition metabolism gene (*AtGA2ox2*) is down-regulated so that the content of active GAs within *Arabidopsis* seeds is increased, thus contributing to germination (Yamauchi et al., 2004). Apple seeds must undergo a certain period of low temperature stratification to germinate. However, the mechanism of stratification to break dormancy is still unclear. It has been suggested that the content of GA₃ and GA₇ in laminated apple seeds is increased, which was consistent with the time of seed germination. This showed that GA₃ and GA₇ play an important role in breaking dormancy and promoting seed germination (Dębska et al., 2013; El-Yazal et al., 2014). Preliminary analysis of the promoters of GA and ABA genes in apple showed that the promoter contained typical bHLH transcription factor binding elements (E-box and G-box). The inferred molecular mechanisms of *MdCibHLH1* involved in apple bud and seed dormancy release may be because it can bind to the promoter of ABA or GA synthesis genes, regulating the content of endogenous GA or ABA in flower buds or seeds, thereby affecting the flower bud or seed dormancy release. The shrunken phenotype of transgenic *Arabidopsis* seeds may also be due to changes caused by endogenous GA or ABA.

At present, the research on dormancy and germination has been mainly based on dormancy shallow model plants, such as *Arabidopsis thaliana* or rice. This cannot completely represent the woody plants that employ deep dormancy. The seed dormancy of woody plants is different from model plant seeds. In this study, *MdCibHLH1* was shown to play an important role

in regulation of dormancy and release in apple seeds and apple buds. It established a theoretical foundation for further study on the mechanisms of fruit dormancy and developing effective fruit trees cultivation measures.

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