March 2016. Horticultural Plant Journal, 2 (2): 61–66.

Horticultural Plant Journal

Available online at www.sciencedirect.com
The journal's homepage: http://www.journals.elsevier.com/horticultural-plant-journal

Expression Analysis of the *MdCIbHLH1* Gene in Apple Flower Buds and Seeds in the Process of Dormancy

REN Yiran, ZHAO Qiang, ZHAO Xianyan, HAO Yujin, and YOU Chunxiang *

National Key Laboratory of Crop Biology, National Research Center for Apple Engineering and Technology, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai'an, Shandong 271018, China

Received 7 December 2015; Received in revised form 20 January 2016; Accepted 1 March 2016

Available online 14 June 2016

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 *OsbHLH2* gene can increase the antioxidant capacity of transgenic plants in wild rice (Zhou et al.,

* Corresponding author. Tel: +86 538 8246692.
E-mail address: youchunxiang@126.com

Peer review under responsibility of Chinese Society for Horticultural Science (CSHS) and Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS)

The Chinese version of this paper is published in Acta Horticulturae Sinica. DOI:10.16420/j.issn.0513-353x.2015-0029

http://dx.doi.org/10.1016/j.hpj.2016.06.007

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 $C_{2460}H_{3920}N_{728}O_{807}S_{24}$. 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 MdCIbHIH 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.