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ARTICLE

Genome-Wide Identification, Expression Profiling and Protein-Protein Interaction Properties of the *BEL-Like Homeodomain* Gene Family in Apple

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ABSTRACT

BEL1-like homeodomain (BLH) family proteins are homeodomain transcription factors, which are found ubiquitously in plants and play important roles in regulating meristem and flower development. Although BLH proteins have been reported in some plant species, there is very little information available for plants in the *Malus* genus (e.g., apple tree:*Malus domestica*). In the present study, we identified 19 apple *MdBLH* genes. Phylogenetic analysis revealed that the *MdBLH* genes could be divided into five groups. Analysis of gene structure showed that *MdBLH* gene has four exons, and the third exon was 61 bp in length. Chromosomal location analysis suggested that the *MdBLH* genes are not distributed uniformly on 12 chromosomes. Eleven *MdBLH* genes were cloned by RT-PCR, and their expression patterns were also determined. Among them, the expression levels of *MdBLH4.1* and *MdBLH9.1* could be induced by sodium chloride stress, while the expression levels of *MdATH1.1*, *MdBLH8.1*, *MdBLH8.3,* and *MdBLH11.1* were down-regulated by such stress. Transcriptional levels of *MdATH1.1* and *MdBLH7.2* were down-regulated by mannitol stress. The result of yeast two-hybrid experiment showed that MdBEL1.1 interacted with apple ovate family proteins 6 (MdOFP6), and MdBLH3.1 interacted with the MdOFP4, MdOFP6, MdOFP13, and MdOFP16 proteins. Our results provide a strong theoretical basis and a valuable reference for analyzing of the biological functions of MdBLH proteins as transcription factors in apple growth, development, and stress and also for the construction of regulatory networks.

KEYWORDS

Apple; BEL-like homeodomain; gene cloning; expression analysis; interaction analysis

1 Introduction

The *homeobox* gene is a transcriptional regulator that encodes the homeodomain (HD), which is widely found in eukaryotes. The typical HD consists of 60 amino acids, which can form a 3-helical domain in space [1–3]. However, there are some exceptions, such as the TALE (Three Amino-acid Loop Extension) gene families, which encode an atypical DNA binding domain consisting of 63 amino acids, with three additional amino acid residues (P-Y-P) inserted between the first and second helices [3]. In plants, the TALE gene families include two gene subfamilies, which are known as homeodomain) and BLH, which

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is sometimes called BEL (BEL1-like homeodomain) [4]. The BLH protein consists of three domains: SKY, BEL, and homeobox KN. The SKY domain is a conserved region of about 17 amino acids at the N-terminal of the BEL protein [5]. The homeobox KN domain, which is located at the C-terminus of the BLH protein, is a typical homologous box region. It is a 60 amino acid helix-turn-helix DNA-binding domain involved in DNA binding and the formation of homologous protein dimers. Besides the 60 amino acids, there are three extra amino acids (P-Y-P) between the first and second helix [4]. The BEL domain is located between the SKY and the homeobox KN domains [4]. These three conserved domains are of great significance for the functioning of the BLH family proteins in plants. The SKY and BEL domains of the BLH proteins interact with the MEINOX domain of the KNOX proteins to form the BLH-KNOX heterodimer, which can be transferred from the cytoplasm to the nucleus. In the nucleus, their HD domains specifically bind to their respective target sequences to regulate the expression of downstream genes [4–7]. In addition, ovate family proteins (OFP) negatively regulate the complex formation of BLH-KNOX heterodimers, which results in relocating the heterodimers from the nucleus to the cytoplasm [8].

There are 13 BLH family genes in *Arabidopsis thaliana*, which play important roles in the development of plant meristems and flowers. For example, the BLH family members PENNYWISE (PNY/BLH9), POUNDFOOLISH (PNF/BLH8), and *ARABIDOPSIS THALIANA* HOMEOBOX 1 (ATH1) play important roles in the initiation, maintenance, and development of apical meristems and inflorescence formation [9–13]. SAWTOOTH1 (SAW1/BLH2) and SAWTOOTH2 (SAW2/BLH4) negatively regulate expression of the Class I *KNOX* gene *BREVIRUELLUS* (*BP*), which leads to leaf development defects [14]. The abnormal expression of *BLH1* affects the establishment of mature embryo sac cells fate [15]. Integument was transformed into carpel structure in the *bel1* mutant without BEL1 function [16]. In addition, SlBEL11 regulates the development of chloroplasts and chlorophyll synthesis in tomato fruits [17]. *RI* and *RIL1* genes of rice BLH regulate the maintenance of the inflorescence structure and meristems [18].

At present, most of the studies on *BLH* family genes are focused on *Arabidopsis*, while the related researches on apple (*Malus domestica*) *BLH* have not been reported. As one of the most important economic fruits grown in temperate regions worldwide, apples are deeply loved by people and used for cooking, fresh eating, and cider production. The improved draft of apple genomes provides an excellent platform for identifying and analyzing the functions of the BLH transcription factor family [19–21]. In this study, the high-quality 'GDDH13 v1.1' apple reference genome database was used to investigate and identify the BLH gene family. We analyzed conserved domains, evolutionary relationships, subgroup classification, gene structures, and chromosomal locations of the *MdBLH* family. In addition, we also examined gene cloning, stress and tissue expression, and protein interaction. These results not only provide a strong theoretical basis and reference for analyzing the biological function of the MdBLH transcription factor in apple growth, development and stress, but also provide a strong theoretical basis for the construction of regulatory networks.

2 Materials and Methods

2.1 Plant Materials and Stress Treatments

'Gala' (*Malus* × *domestica* cv. Gala) tissue culture seedlings were used as test material in the expression analysis under stress treatments. Under 14-h light/10-h darkness (24 ± 2)°C, 'Gala' tissue culture seedlings were grown on a subculture medium (MS medium + 0.2 $mg{\cdot}L^{-1}$ IAA+ 0.8 $mg{\cdot}L^{-1}$ 6-BA+30 $g{\cdot}L^{-1}$ sucrose + 7 $g{\cdot}L^{-1}$ agrose) and subcultured every 30 d; 150 $mM{\cdot}L^{-1}$ NaCl and 300 $mM{\cdot}L^{-1}$ mannitol were added to the subculture medium. 'Gala' tissue culture seedlings with the same growth status (about 20 d after the subculture) were inoculated in the subculture media with NaCl and mannitol as treatments, and the basic subculture medium was used as a control [22].

2.2 Identification of MdBLH Genes in Apple

Thirteen BLH amino acid sequences of *Arabidopsis* were downloaded from the *Arabidopsis* website [4,23]. We searched the apple genome functional database (https://www.rosaceae.org/species/malus/malus_x_domestica/genome_GDDH13_v1.1) using the BlastP program provided by the GDR database (https://www.rosaceae.org/blast/protein/protein); Protein BLAST Databases: [Apple Genome (GDDH13 V1.1) proteins], and then the nucleotide and amino acid sequence of the candidate genes were downloaded. The Pfam database (http://pfam.xfam.org/) and NCBI Conserved Domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to ensure that the above candidate genes contained the SKY, BEL and HD domains [22].

2.3 Analysis of Evolutionary Relationships, Gene Structure, Conserved Motifs, and Genome Location of MdBLH in Apple

Sequence analysis of MdBLH protein in apple was conducted by using the DNAMAN 6.0 software, and then evolution analysis was conducted by using the phylogenetic tree analysis software MEGA 6 (http://www.megasoftware.net) based on the NJ method (execution parameters: Poission correction, pairwise deletion and bootstrap (1000 repeats)) [24]. Structures of *MdBLH* and *Arabidopsis BLH* genes were downloaded from the PLAZA 3.0 comparative genome database (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/). The genomic location information for the *MdBLH* genes was obtained from the apple genome 'GDDH13 V1.1' database. The *MdBLH* genome localization was made using the MapInspect software (http://www.plant-breeding.wur.nl/UK/software_mapinspect.html). Tandem and fragment repeats were defined according to the method of Tian et al. [25]. MdBLH conserved motifs were identified using the online software MEME (http://meme-suite.org/tools/meme). Cis-elements in the *MdBLH* promoter were analyzed using the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Supplementary Sequence: A1) [22].

2.4 Gene Cloning of MdBLHs

RNA from fully expanded leaves of 'Zihong Fuji' (*Malus domestica* cv. Zihong Fuji) was extracted by a modified hot boric acid method, and then synthesized into cDNA using PrimeScript^{TM}1ST Strand cDNA Synthesis Kit (TaRaKa Company, Dalian, China) [22]. Specific primers (Tab. 1) were designed according to the *MdBLH* nucleotide sequence in the apple 'GDDH13 V1.1' genomic database for PCR amplification. The PCR reaction conditions were as follows: predenaturation at 94°C for 5 min; 94°C during 60 s, 56–62°C during 60 s, 72°C during 120 s; 35 cycles and elongation at 72°C for 10 min. PCR products were purified and cloned into the pMD18-T vector, recombinant plasmids were transformed into *E. coli* DH5α competent cells, and the positive clones were screened and sequenced (Supplementary Sequence: A2).

Table 1: List of primers for vector construction and PCR

Gene name	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
Complete ORF amplification		
MdATH1.2	ATGATGGATAACGAGATGTATAAT	TCAATTCACACCAAATTTGTGGT
MdBEL1.1	ATGGCAGGAGAACTGCTGAGTAGT	CTACCCTGCCAAGTCATGTAGCAAC
MdBLH1.1	ATGGCGACCTACTTTCATCACCA	TCACGCCACAAAGTCTGGTAACA
MdBLH3.1	ATGGCCACCTATTTCCAAAATTTG	TCAAGTAACGCGTGGGTATTGCC
MdBLH4.1	ATGGGCATAGTAACTTTACCGCCA	CTAACAGCCCCCGAAGTCTCTA
MdBLH7.1	ATGTCAACTTACTATGCTGGTTC	TCACACTACGAAATCACGTAATA
MdBLH7.2	ATGTCAACTTACTATGCTGGTTCG	TCACACTACGAAATCATGTAACAG

(Continued)

Table 1 (continued).

Gene name	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
MdBLH8.1	ATGGAGATGAGTGGCTTTAGGCAA	TTACCCCACATAATCACGTACCA
MdBLH8.3	ATGGCGGACGACGGCCTGGAAGC	TCACTTTCTCGCTTTAAGGCTTA
MdBLH9.1	ATGGCTGAGGGTTTCGAACCCTA	TCATCCCACAAAATCGTGCAAAA
MdBLH11.1	ATGGTGTCACAAGACTCACCTCCA	TTAATTCCCTGTCTGATCCGTGT
RT-qPCR		
MdATH1.2	ACCGAAGAAAGACCCGTCAA	AGATGTGCCCATTCCTTCCA
MdBEL1.1	TGACTTGGCAGGGTAGAAGG	TCATCACCATCTTGTTGCACA
MdBLH1.1	TTCAGCCTCTCACTCTTCCG	GGTCAAGCTTCTTCAAGGCC
MdBLH3.1	TTTTGGTTTCCGTCCCTCAA	GGGGTACGAGGGAAGTTTCT
MdBLH4.1	CGTGAAAAGAGAGGGAAGCG	TGGGAGTGGCGGTAAAGTTA
MdBLH7.1	GTCATACTCGGACGCATTGG	GTGCCACCAAGATGCGATAG
MdBLH7.2	AAAAGATGCTACGCCAATGC	CAATGCGTCCGAGTATGACC
MdBLH8.1	GACATGGGGTAGAAAGTGCC	TTCCGTTGCTTTTGTGTCGT
MdBLH8.3	AAGCGAGAAAGTGAGCACCT	TGACCTGTCTCTCGGAATATCT
MdBLH9.1	GCCTAGCCTCTCCTTTTCCA	GTTCGAAACCCTCAGCCATG
MdBLH11.1	CCATCATCTCGAAAGCCAGC	TGGGACATTCTTTCGCCAAG
MdMDH	CGTGATTGGGTACTTGGAAC	TGGCAAGTGACTGGGAATGA
Y2H-pGBT9		
MdOFP1	GCCGGAATTCCCGGGGATCCGTATGCAAAACACA	TTAGCTTGGCTGCAGGTCGACTCAATGCGGGCGGCG
MdOFP4	GCCGGAATTCCCGGGGATCCGTATGAAGTTGCCT	TTAGCTTGGCTGCAGGTCGACCTAAGACATTGTGAT
MdOFP5	GCCGGAATTCCCGGGGATCCGTATGGGCAAGAAA	TTAGCTTGGCTGCAGGTCGACTTAGTCGTCCTCGTC
MdOFP6	GCCGGAATTCCCGGGGATCCGTATGGGGAAGAAA	TTAGCTTGGCTGCAGGTCGACTTATTTAATCTGGTC
MdOFP11	GCCGGAATTCCCGGGGATCCGTATGGGAAACCAC	TTAGCTTGGCTGCAGGTCGACTTACTTGGACCCAAT
MdOFP13	GCCGGAATTCCCGGGGATCCGTATGCCTACAACA	TTAGCTTGGCTGCAGGTCGACCTAGTAGTCACGTGA
MdOFP14	GCCGGAATTCCCGGGGATCCGTATGGGGAATTAC	TTAGCTTGGCTGCAGGTCGACCATTTTGATGTGTGC
MdOFP16	GCCGGAATTCCCGGGGATCCGTATGGAAAACCGA	TTAGCTTGGCTGCAGGTCGACTCAAGATCCCCAGTT
MdOFP19	GCCGGAATTCCCGGGGATCCGTATGTCTTCATCC	TTAGCTTGGCTGCAGGTCGACTCACATGATCATCGG
MdOFP20	GCCGGAATTCCCGGGGATCCGTATGTCTTCTTCC	TTAGCTTGGCTGCAGGTCGACTCATATGATCATCGG
Y2H-pGAD424		
MdBEL1.1	AGATCGAATTCCCGGGGATCCGTATGGCAGGAGAA	CATAGATCTCTGCAGGTCGACCTACCCTGCCAAGTC
MdBLH1.1	AGATCGAATTCCCGGGGATCCGTATGGCGACCTAC	CATAGATCTCTGCAGGTCGACTCACGCCACAAAGTC
MdBLH3.1	AGATCGAATTCCCGGGGATCCGTATGGCCACCTAT	CATAGATCTCTGCAGGTCGACTCAAGTAACGCGTGG
MdBLH7.1	AGATCGAATTCCCGGGGATCCGTATGTCAACTTAC	CATAGATCTCTGCAGGTCGACTCACACTACGAAATC
MdBLH9.1	AGATCGAATTCCCGGGGATCCGTATGGCTGAGGGT	CATAGATCTCTGCAGGTCGACTCATCCCACAAAATC

2.5 Expression Analysis of MdBLH Genes

The tissue expression data for *MdBLH* genes was obtained using the GEO database in NCBI (https://www.ncbi.nlm.nih.gov/) with GEO accession no. GSE42873 [26]. These data included a set of expression arrays from 16 different apple tissues, with two biological replicates for each tissue. RNA in leaves of 'Gala' apple was extracted by RNeasy Plant Mini Kit (QIAGEN, China, Item No. 74903) and then cDNA was synthesized by using the reverse transcription system PrimeScript^{TM} 1ST Strand cDNA Synthesis Kit. Primers for qRT-PCR analysis were designed based on the 3 '-UTR or 5' -UTR of the *MdBLH* gene (Tab. 1). *MdMDH* was selected as the internal reference gene [27], and qRT-PCR was performed using the 3-step method in BIO-RAD IQ5 (USA). All qRT-PCR reactions were repeated three

times. *MdSOS2* and *MdSUT2*, as control genes, were demonstrated to respond to NaCl stress and osmotic stress, respectively [22,28,29]. The PCR reaction system was as follows: SYBR Green Master I 10 μL, upstream and downstream primers (5 $\mu mol \cdot L^{-1}$) 1 μL each, template 1 μL, and ddH_2O was added to obtain a total of 20 μL. The qRT-PCR conditions were 95°C for 3 min, then 40 cycles at 95°C for 10 s, 58°C for 30 s, 72°C for 15 s; after annealing to 55°C, temperature was increased 0.5°C every 7 s until 95°C, 81 cycles in total [22]. The $2^{-\Delta\Delta CT}$ method was used to analyze the experimental data [30].

2.6 Y2H Assay

The Y2H experiment was carried out based on the Matchmaker® Gold Yeast Two-Hybrid System (Clontech, Code No. 630489). Full-length cDNAs of *MdBLH* were amplified with designed primers (Supplementary File 3) and recombined with the pGAD424 (GAL4 activation domain, AD) vector. The amplified full-length cDNAs of *MdOFP* were recombined with the pGBT9 (GAL4 DNA-binding domain, BD) vector [22]. The recombinant plasmids were transformed into the yeast strain *Y2HGold* in different combinations and then coated in the screening medium SD/-Trp/-Leu for inverted cultivation at 30°C. Three days later, screening was conducted on a SD/-Trp/-Leu/-His/-Ade+X-α-Gal medium. Finally, the β-galactosidase activity was surveyed to represent the expression of the *LacZ* reporter gene. The empty pGAD424 plasmid together with the pGBT9-MdOFP co-transformed yeast were used as the negative control, and the pGAD424-MdVQ10 and PGBT9-MDWRKY52 co-transformed yeasts were used as the positive control (Tab. 1; Supplementary Sequence: A3) [31].

3 Results

3.1 Identification and Evolutionary Analysis of the Apple MdBLH Transcription Factor

A total of 24 *MdBLH*-related genes were searched in the 'GDDH13' database for studying the apple genome (Tab. 2). The SKY, BEL, and HD domains of the candidate protein were predicted through using the Pfam database and the NCBI Conserved Domains. Five candidate *MdBLH* genes were found to be incomplete, and 19 candidate *MdBLH* genes were identified with full information for further investigation.

MEGA 6 software was used to analyze the evolution of the full-length sequences of the BLH proteins in apple (19 sequences) and *Arabidopsis* (13 known sequences). We found that BLH proteins from apple and *Arabidopsis* could be classified into five categories (Fig. 1A) [5].

3.2 Analyses of Conserved Motifs and Gene Structure of the MdBLH Transcription Factor in Apple

Conserved motifs in the *BLH* gene family of apple and *Arabidopsis* were identified using online software MEME. Motif 4 represents the SKY domain, motifs 2 and 5 represent the BEL domains, and motifs 1 and 3 represent the HD domain (Fig. 1B). *BLH* gene family members in both apple and *Arabidopsis* contained the SKY, BEL, and HD conserved domains (Fig. 1B).

PLAZA3.0 comparative genome database was used to download structure maps of introns and exons of *BLH* genes in apple and *Arabidopsis* (Fig. 1C). *BLH* genes from both apple and *Arabidopsis* were composed of four exons, and the length of the third exon was 61 bp (31/32) (Figs. 1C and 1D).

(A) The phylogenetic tree was constructed with MEGA 6 software using full-length amino acid sequences from the 33 BLH and STM proteins of apple and *Arabidopsis*. (B) Conserved motifs of apple and *Arabidopsis* BLH proteins that were identified using the MEME program. Motifs 1–5 are indicated by different colored boxes. Motifs 1 and 3: HD domain; Motifs 2 and 5: BEL domain; Motif 4: SKY domain. (C) The exon-intron structure of apple and *Arabidopsis BLH* genes. Introns and exons are represented by grey lines and blue boxes, respectively. (D) Analysis of exon length distribution of apple and *Arabidopsis BLH* genes.

Table 2: Properties of *MdBLH* identified from the apple genome

Gene name	Apple gene ID	Chromosome location	ORF[a]	MW[b]	PI[c]
MdATH1.1	MD15G1061000	chr15:4102846..4104879	1755	65.535	6.18
MdATH1.2	MD08G1043600	chr08:3299278..3301362	1704	63.252	6.772
MdBEL1.1	MD06G1088700	chr06:21629196..21632398	2049	76.295	6.921
MdBEL1.2	MD14G1109800	chr14:17342131..17345340	3209	76.914	6.678
MdBLH1.1	MD12G1040700	chr12:4477054..4479851	2112	77.14	7.508
MdBLH1.2	MD14G1039700	chr14:3717198..3720077	2127	77.684	7.116
MdBLH3.1	MD08G1024200	chr08:1744124..1746986	1776	66.111	5.613
MdBLH3.2	MD15G1021800	chr15:1267638..1269877	1758	65.471	5.983
MdBLH4.1	MD02G1156800	chr02:13085028..13091529	2430	86.791	6.86
MdBLH4.2	MD15G1271200	chr15:23618809..23625111	2445	87.185	6.834
MdBLH7.1	MD05G1059800	chr05:10450588..10454594	2073	75.387	6.092
MdBLH7.2	MD10G1068000	chr10:9378840..9382888	2073	75.595	6.032
MdBLH8.1	MD11G1140600	chr11:13078622..13083776	2424	88.737	7.725
MdBLH8.2	MD03G1122400	chr03:11448491..11454379	2424	88.632	6.733
MdBLH8.3	MD07G1205600	chr07:28333255..28330041	1188	44.632	8.971
MdBLH9.1	MD12G1153600	chr12:23360953..23365134	1860	68.745	6.716
MdBLH9.2	MD04G1138100	chr04:22535482..22538649	1848	68.892	7
MdBLH11.1	MD15G1021900	chr15:1272749..1274416	1320	48.504	6.21
MdBLH11.2	MD08G1024300	chr08:1749890..1751566	1320	48.433	5.949

Notes: a: open reading frame; b: molecular weight; c: isoelectric point.

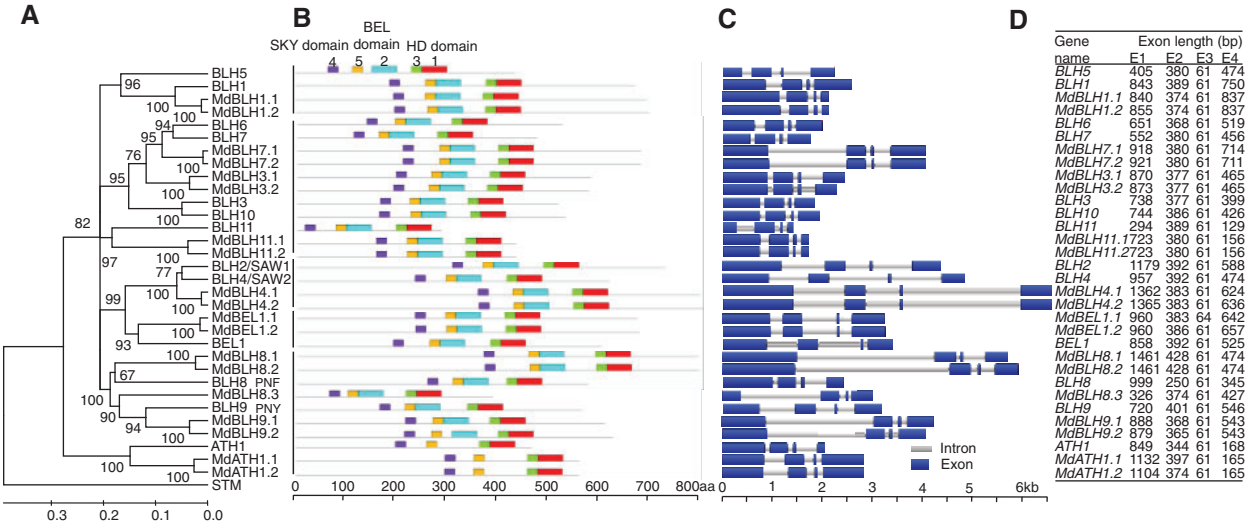

Figure 1: Analyses of phylogenetic relationships, conserved motifs, gene structures, and exon lengths of *BLH* genes in apple and *Arabidopsis* (A) The phylogenetic tree was constructed with MEGA 6 software using full-length amino acid sequences from the 33 BLH and STM proteins of apple and Arabidopsis. (B) Conserved motifs of apple and Arabidopsis BLH proteins that were identified using the MEME program. Motifs 1-5 are indicated by different colored boxes. Motifs 1 and 3: HD domain; Motifs 2 and 5: BEL domain; Motif 4: SKY domain. (C) The exon-intron structure of apple and Arabidopsis BLH genes. Introns and exons are represented by grey lines and blue boxes, respectively. (D) Analysis of exon length distribution of apple and Arabidopsis BLH genes

3.3 Analysis of Chromosome Localization of the Apple MdBLH Transcription Factor

The apple genome 'GDDH13' database was used to query related chromosome location information for the apple *MdBLH* gene family, and MapInspect software was used to map *MdBLH* genes on chromosomes. Nineteen apple *MdBLH* genes were mapped onto 12 chromosomes with inhomogeneity (Fig. 2). Chromosome 15 had the maximum with four *MdBLH* genes, Chromosome 8 followed with three, and Chromosomes 3, 4, 5, 6, 7, 10, 11, and 17 had the minimum of 1. Meanwhile, to elucidate the reasons for the expansion of *MdBLH* gene family, we examined gene replication events, which included tandem and segmental replication; *MdATH1.1/MdATH1.2*, *MdBLH1.1/MdBLH1.2*, *MdBLH3.1/MdBLH3.2*, *MdBLH4.1/MdBLH4.2*, *MdBLH7.1/MdBLH7.2*, *MdBLH8.1/MdBLH8.2*, *MdBLH9.1/MdBLH9.2,* and *MdBLH11.1/MdBLH11.2* undergone segmental replication (Fig. 2).

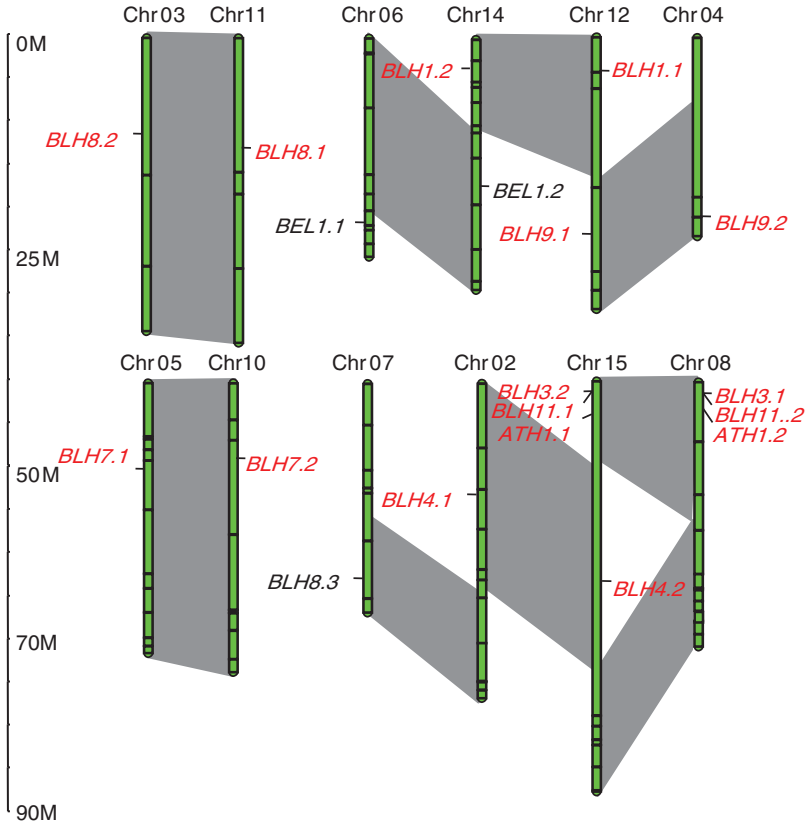

Figure 2: Chromosomal locations of apple *MdBLH* genes

The scale is in megabases (Mb). The red font group represents segmental duplication. The grey area represents genome-wide duplications.

3.4 Cloning and Sequence Analysis of the Apple MdBLH Gene from Cultivar 'Zihong Fuji'

Nineteen *MdBLH* genes were observed in apple based on genome-wide identification, and 11 of them were cloned using RT-PCR technology. Comparison of homologies showed that 11 deduced amino acid sequences of cloned MdBLH proteins contained SKY, BEL, and HD domains (Fig. 3).

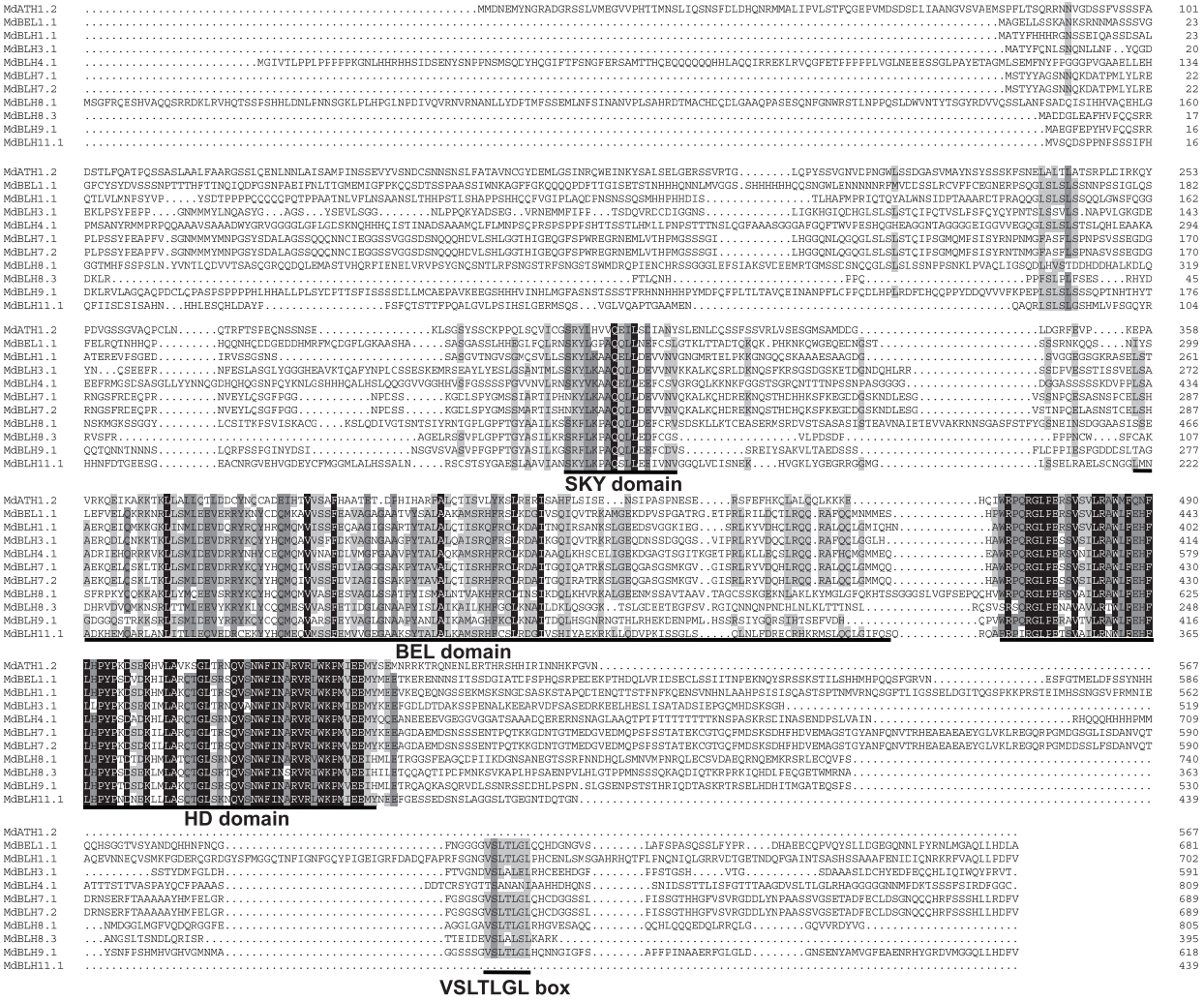

Figure 3: Comparison of homologies of the deduced amino acid sequence alignment of cloned MdBLH proteins

Black-highlighted residues are identical, and light gray-highlighted residues are similar in all proteins. Three conserved domains and one box were marked as indicated as SKY domain, BEL domain, HD domain, and VSLTLGL box.

The promoter sequences of 11 *MdBLH* genes were obtained by downloading the upstream 1500 bp sequences to the transcriptional initiation site of the 11 cloned *MdBLH* genes. The cis-acting elements of promoter sequences were analyzed by the PlantCARE database. In addition to the light response element, there were 12 different types of cis-acting elements, such as defense and stress responsiveness element, hormone responsiveness element, and so on (Fig. 4). These cis-acting elements responded to stresses such as anaerobic induction, low temperature, heat, drought, pathogen infections and hormones (such as MeJA, salicylic acid, auxin, gibberellin, ethylene and abscisic acid) (Fig. 4). That is, these cis-acting elements in the *MdBLH* promoters might play important roles in apple growth and development and the response to stress.

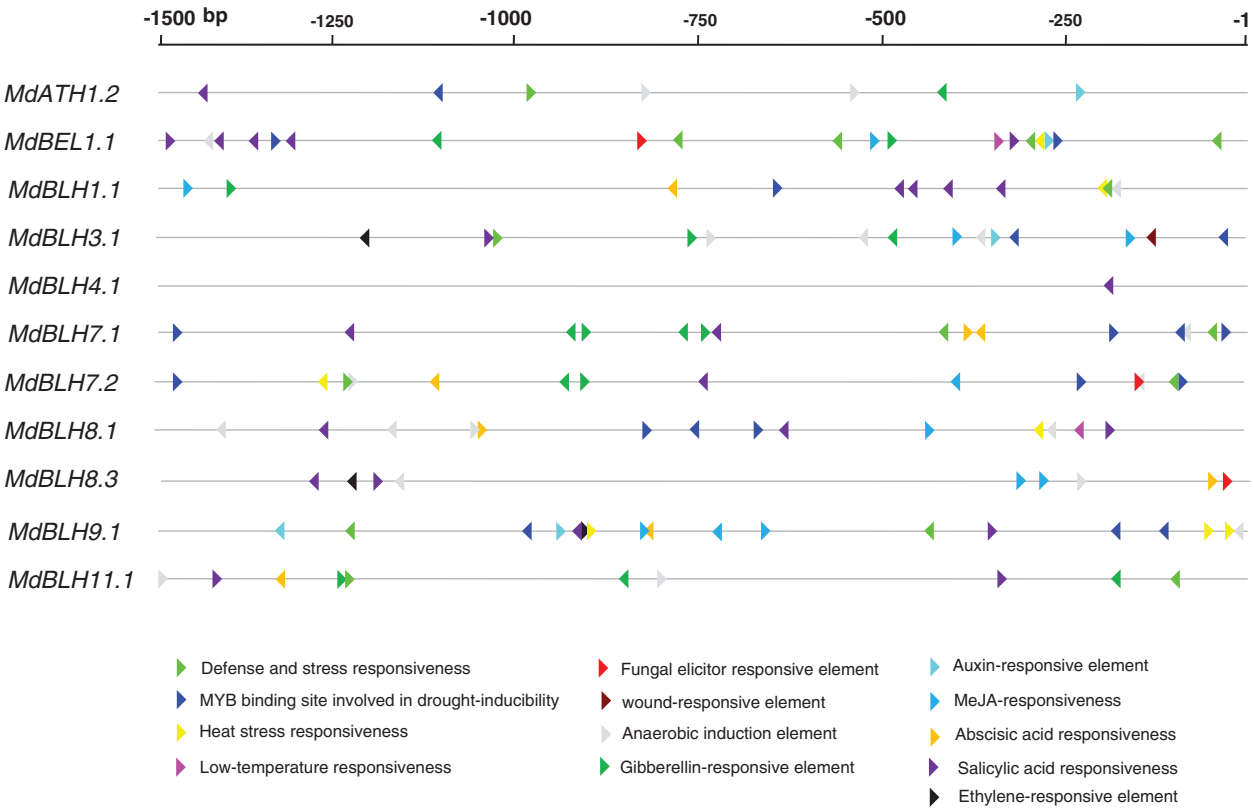

Figure 4: The cis-acting elements of 11 promoters in *MdBLH* genes

Different colored triangles represent different cis-acting elements. Bright green: Defense and stress responsiveness; Red: Fungal elicitor responsive element; Water green: Auxin-responsive element; Blue: MYB binding site involved in drought-inducibility; Crimson: wound-responsive element; Light blue: MeJA-responsiveness; Yellow: Heat stress responsiveness; Gray: Anaerobic induction element; Orange: Abscisic acid responsiveness; Bright purple: Low-temperature responsiveness; Green: Gibberellin-responsive element; Purple: Salicylic acid responsiveness; Black: Ethylene-responsive element.

3.5 Expression Analysis of BLH Transcription Factor in Apple

The transcription profiles of 16 different apple tissues (GSE42873) were downloaded from the GEO database from the NCBI website to detect the expression of *MdBLH* in different tissues (Fig. 5). We found that the *MdBLH* genes were expressed in all the tested tissues, and *MdBLH1.1* and *MdBLH7.1* showed relatively high expression levels in Leaf M49, Flower M74, Fruit M20 (100DAM), and Fruit M74 (harvest) tissues (Fig. 5).

The expression levels of the *MdBLH* genes in 'Gala' tissues from cultured seedlings under salt or mannitol treatments were analyzed further using qRT-PCR. Under the control treatment, the relative expression levels of *MdBLH* had no significant change within a 48 h period (Fig. 6A). Compared with the control group, the relative expression levels of *MdBLH4.1* and *MdBLH9.1* increased under sodium chloride treatment, reaching 3.5 and 2.3 times that of the control group at 48 h, respectively. Compared with the control group, the relative expression levels of *MDBLH1.1*, *MdBLH8.1*, *MdBLH8.3,* and *MdBLH11.1* significantly decreased, and the relative expression level of *MdBLH8.3* at 48 h was only 0.13 times of that of the control group (Fig. 6B). Compared with the control group, the relative

expression levels of *MdATH1.1* and *MdBLH7.2* decreased after the mannitol treatment, and decreased to 0.41 and 0.31 times of the control group at 24 h, respectively (Fig. 6C).

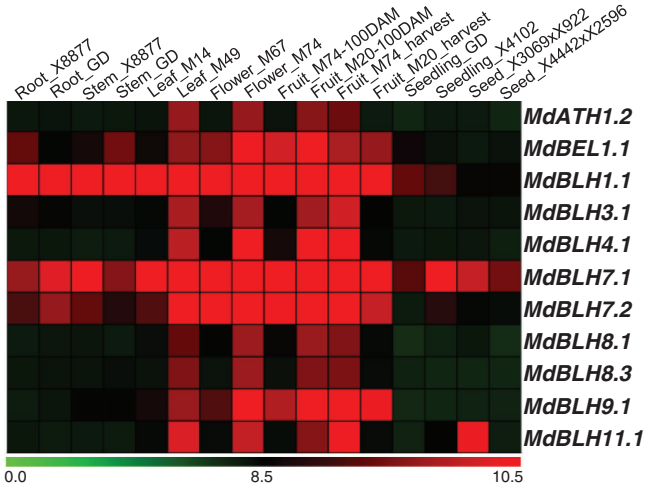

Figure 5: Expression profiles of *MdBLH* genes in various tissues in apple

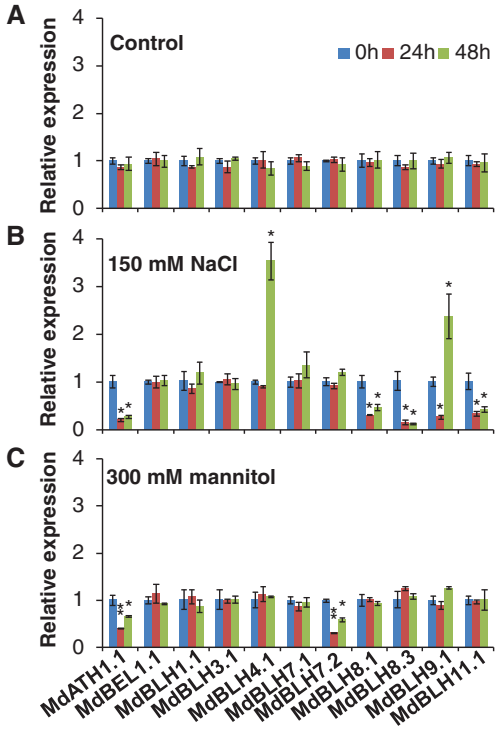

Figure 6: Expression analysis of *MdBLH*s in apple under (A) normal growth, (B) salt, and (C) mannitol treatments. Three independent biological replicates were used for calculations. Error bars indicate standard deviation. * and ** indicate statistically significant differences, as determined by Student's *t*-tests, at $p < 0.05$ and $p < 0.01$, respectively

3.6 Interactions between MdBLH and MdOFP Proteins

To investigate the interaction regulatory network of MdBLH with OFP proteins, a Y2H assay was performed to detect the interactions between MdBLH and MdOFP proteins. Five MdBLH full-length cDNA sequences were fused into the AD prey vector, and 10 MdOFP full-length cDNA sequences were fused into the BD bait vector. The fusion vectors of AD-MdBLH and BD-MdOFP were co-transformed into yeast cells, and the activity of β-galactosidase was tested by observing the expression of the *LacZ* reporter gene. The 10 BD-MdOFP fusion bait vectors, and the empty AD trap vectors, did not turn blue on a SD/-Trp/-Leu/-His/-Ade/X-α-gal minimal medium, which indicated that the 10 MdOFP proteins had no transcriptional self-activation activity (Fig. 7). But the co-transformed yeast cells of AD-MdBEL1.1 and BD-MdOFP6 and those of AD-MdBLH3.1 and BD-MdOFP4/MdOFP6/MdOFP13/MdOFP16 turned blue on the SD/-Trp/-Leu/-His/-Ade/X-α-gal minimal medium. These results indicated that the MdBEL1.1 protein interacts with the MdOFP6 protein, and the MdBLH3.1 protein interacts with the MdOFP4, MdOFP6, MdOFP13, and MdOFP16 proteins (Fig. 7).

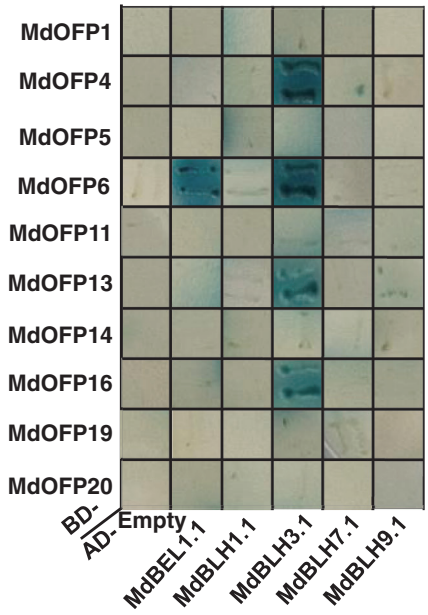

Figure 7: Interactions between MdBLH and MdOFP proteins in yeast cells

The AD-MdBLH fusion capture vectors were co-transformed with the BD-MdOFP fusion bait vectors into yeast cells. Positive interactions were indicated by the ability of cells to grow on synthetic dropout medium additive x-α-gal that lacked Leu, Trp, His, and Ade. The empty AD vector plus BD-MdOFP fusion vectors were used as negative controls.

4 Discussion

In recent years, the technology of sequencing and sequence assembly has been continuously improved and innovated, and the cost of next generation sequencing has dropped rapidly. Genome maps of high quality can be drafted for a variety of plants, which is very convenient for investigating specific agronomic traits of plants, accelerating plant breeding, increasing production, and improving resistance to abiotic and biotic stresses. We have now a deeper understanding of the *BLH* gene accompanied by successive identification of the BLH transcription factor families in *A. thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Zea mays*, *Solanum tuberosum*, *Vandenboschia speciosa*, *Brassica rapa*, *Daucus carota*, *Phyllostachys edulis*, *Pisum*

sativum, *Medicago truncatula*, *Camellia chekiangoleosa* and five legume species [2–5,32–36]. Extensive studies have shown that gene replication, including tandem, fragment and genome-wide replication, not only plays an important role in genome rearrangement and amplification, but also in gene function diversification and gene families expression [37]. Velasco et al. [19] found that genome-wide duplication occurred in the apple genome 60–65 million years ago, which resulted in the formation of 17 new chromosomes from the original nine ancestral chromosomes. Recently, Daccord et al. [21] reported an apple genome map (apple 'GDDH13 v1.1' genome) of the highest quality at present by integrating three different techniques. In our study, 19 *MdBLH* genes were identified in the apple 'GDDH13 v1.1' genome (Tab. 2). Eighteen members of the apple MdBLH family were located in genome-wide replication regions and, of those, *MdATH1.1/MdATH1.2*, *MdBLH1.1/MdBLH1.2*, *MdBLH3.1/MdBLH3.2*, *MdBLH4.1/MdBLH4.2*, *MdBLH7.1/MdBLH7.2*, *MdBLH8.1/MdBLH8.2*, *MdBLH9.1/MdBLH9.2,* and *MdBLH11.1/MdBLH11.2* (16/19; 84.2%) undergone segmental replication. Interestingly, members of the *MdBLH* family did not undergo tandem replication. Similar results were also found in soybeans, in which 89.1% of homeobox genes undergone segmental replication [32]. These results indicated that the event of segmental replication plays an important role in the extension of the BLH gene family in apple and soybean.

BLH transcription factors form either heterodimers with OFP proteins, and also interact with KNOX proteins, or form functional complexes with KNOX and OFP proteins, and then they participate in the regulation of plant growth and development [7,15,38–41]. For example, the heterodimers formed by BLH1 and KANT3 are involved in the normal development of embryo sacs and fate of cells in embryo sacs, and their activity is regulated by AtOFP5 [15]. Heterodimers that are formed by the interaction of BLH6 and KNAT7 proteins regulate secondary cell wall formation by inhibiting the expression of homeodomain-leucine zipper transcription factor *REVOLUTA/INTERFASCICULAR FIBERLESS1* (*REV/IFL1*) [38]. Functional complexes formed by BLH6-KNAT7 heterodimers interact with AtOFP1 and AtOFP4 that were involved in the regulation of secondary cell wall formation by increasing the transcriptional inhibitory activity of BLH6 [39]. The BLH3 protein interacts with the AtOFP1 protein to regulate the conversion from vegetative to reproductive growth in *Arabidopsis* [40]; OsOFP2 in rice interact with OsKNAT7, BLH6-like, and BLH-Like2 to regulate vascular development [41]. BLH12 and BLH14 in maize interact with KNOTTED1 to play an important role in the structural development of internode patterning and vein anastomosis [7]. In addition, the BELL1 protein in *Arabidopsis* interact with the MADS-box protein and SPOROCYTELESS to participate in the regulation of ovule development [16,42]. Therefore, the BLH transcription factor acts as a focal point to interact with multiple proteins, and it constitutes a complex network to regulate plant growth and development accurately and efficiently. In the present study, we found that MdBEL1.1 could interact with the MdOFP6 protein, and MdBLH3.1 interacted with MdOFP4, MdOFP6, MdOFP13, and MdOFP16. This indicates that MdBEL1.1 and MdBEL3.1 may play important roles in regulating multiple aspects of plant growth and development, and are fine-tuned regulated by MdOFP proteins. Moreover, we speculated that the interaction between the MdBLH3.1 protein and MdOFP4, MdOFP6, MdOFP13, and MdOFP16 may affect the activity of the BLH-KNOX heterodimers. This in turn will change the expression level of the target genes, leading to regulation of growth and development of apples. However, further experiments are needed to analyze the relevant molecular mechanisms of the MdBLH-MdKNOX-MdOFP complexes involved in regulating apple growth and development.

At present, there are few studies on the response mechanism of the BLH protein to biotic and abiotic stresses. *OsBIHD1*, a member of the BEL family in rice, was rapidly up-regulated after 6 h from inoculation with *Magnaporthe grisea* and participated in the response of rice disease resistance [43]. Overexpression of *OsBIHD1* in tobacco (*Nicotiana tabacum*) increases resistance to the tomato mosaic virus and to *Phytophthora parasitica* var. *nicotianae*; however, the transgenic tobacco plants overexpressing *OsBIHD1* showed more sensitivity to salt and oxidative stresses [44]. Recent studies have

shown that some *BEL* genes in chickpea roots and stems respond to stress treatment when plants are exposed to drought, salt, and cold conditions [32]. Under drought treatment, there are few *BEL* genes induced by stress in soybeans. A variety of *BEL* genes in soybeans were induced to different degrees by the pathogens *Heterodera glycines*, *Phakopsora pachyrhizi,* and *P. sojae* [32]. Overexpression of soybean *GmBLH4* in *Arabidopsis* changes leaf morphology and fruit pod length, improving the response ability to high temperature and high humidity [45]. In the present study, we found that *MdBLH4.1* and *MdBLH9.1* were induced by salt stress; *MdATH1.1*, *MdBLH8.1*, *MdBLH8.3,* and *MdBLH11.1* were inhibited by salt stress, and *MdATH1.1* and *MdBLH7.2* were inhibited by mannitol stress. These results indicated that MdBLH proteins also played a role in apple response to abiotic stress. The molecular mechanisms of *BLH* genes in regulating apple response to biotic and abiotic stresses should be investigated in future studies.

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Author Contributions: HL and QD collected the public dataset, performed bioinformatics analysis, and drafted the manuscript. QZ contributed to the bioinformatics analysis and preparation of all Figures and tables. QZ and HW conducted the experiments. QD and YX conceived this study and reviewed the manuscript. All authors have read and approved the final manuscript.

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References

1. Bürglin, T. R., Affolter, M. (2016). Homeodomain proteins: An update. *Chromosoma, 125(3),* 497–521. DOI 10.1007/s00412-015-0543-8.
2. Ruiz-Estévez, M., Bakkali, M., Martín-Blázquez, R., Garrido-Ramos, M. A. (2017). Identification and characterization of *TALE* homeobox genes in the endangered fern *Vandenboschia speciosa*. *Genes (Basel), 8(10),* 275. DOI 10.3390/genes8100275.
3. Dolgikh, A., Rudaya, E., Dolgikh, E. (2020). Identification of BELL transcription factors involved in nodule initiation and development in the legumes *Pisum sativum* and *Medicago truncatula*. *Plants, 9(12),* 1808. DOI 10.3390/plants9121808.
4. Yan, C., Hu, Z., Nie, Z., Li, J., Yao, X. et al. (2021). CcBLH6, a bell-like homeodomain-containing transcription factor, regulates the fruit lignification pattern. *Planta, 253,* 90. DOI 10.1007/s00425-021-03610-7.
5. Ghate, T. H., Sharma, P., Kondhare, K. R., Hannapel, D. J., Banerjee, A. K. (2017). The mobile RNAs, StBEL11 and StBEL29, suppress growth of tubers in potato. *Plant Molecular Biology, 93,* 563–578. DOI 10.1007/s11103-016-0582-4.
6. Ikeda, T., Tanaka, W., Toriba, T., Suzuki, C., Maeno, A. et al. (2019). *BELL1-like homeobox* genes regulate inflorescence architecture and meristem maintenance in rice. *The Plant Journal, 98(3),* 465–478. DOI 10.1111/tpj.14230.
7. Tsuda, K., Abraham-Juarez, M. J., Maeno, A., Dong, Z., Aromdee, D. et al. (2017). KNOTTED1 cofactors, BLH12 and BLH14, regulate internode patterning and vein anastomosis in maize. *The Plant Cell, 29(5),* 1105–1118. DOI 10.1105/tpc.16.00967.
8. Hackbusch, J., Richter, K., Muller, J., Salamini, F., Uhrig, J. F. (2005). A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. *Proceedings of the National Academy of Sciences of the United States of America, 102(13),* 4908–4912. DOI 10.1073/pnas.0501181102.

9. Smith, H. M. S., Hake, S. (2003). The interaction of two homeobox genes, BREVIPEDICELLUS and PENNYWISE, regulates internode patterning in the *Arabidopsis* in florescence. *The Plant Cell, 15(8),* 1717–1727. DOI 10.1105/tpc.012856.
10. Ragni, L., Belles-Boix, E., Günl, M., Pautot, V. (2008). Interaction of KNAT6 and KNAT2 with BREVIPEDICELLUS and PENNYWISE in *Arabidopsis* inflorescences. *The Plant Cell, 20(4),* 888–900. DOI 10.1105/tpc.108.058230.
11. Ung, N., Lal, S., Smith, H. M. (2011). The role of PENNYWISE and POUND-FOOLISH in the maintenance of the shoot apical meristem in *arabidopsis*. *Plant Physiology, 156(2),* 605–614. DOI 10.1104/pp.110.171462.
12. Khan, M., Tabb, P., Hepworth, S. R. (2012). *BLADE-on-PETIOLE1* and *2* regulate *Arabidopsis* inflorescence architecture in conjunction with homeobox genes KNAT6 and ATH1. *Plant Signaling & Behavior, 7(7),* 788–792. DOI 10.4161/psb.20599.
13. Xu, Y., Wang, Y., Wang, X., Pei, S., Kong, Y. et al. (2020). Transcription factors BLH2 and BLH4 regulate demethylesterification of homogalacturonan in seed mucilage. *Plant Physiology, 183,* 96–111. DOI 10.1104/pp.20.00011.
14. Kumar, R., Kushalappa, K., Godt, D., Pidkowich, M. S., Pastorelli, S. et al. (2007). The *Arabidopsis* BEL1LlIKE HOMEODOMAIN proteins SAW1 and SAW2 act redundantly to regulate KNOX expression spatially in leaf margins. *The Plant Cell, 19(9),* 2719–2735. DOI 10.1105/tpc.106.048769.
15. Pagnussat, G. C., Yu, H. J., Sundaresan, V. (2007). Cell-fate switch of synergid to egg cell in *Arabidopsis eostre* mutant embryo sacs arises from misexpression of the BEL1-like homeodomain gene *BLH1*. *The Plant Cell, 19(11),* 3578–3592. DOI 10.1105/tpc.107.054890.
16. Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S. et al. (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in *Arabidopsis*. *The Plant Cell, 19(8),* 2544–2556. DOI 10.1105/tpc.107.051797.
17. Meng, L., Fan, Z., Zhang, Q., Wang, C., Gao, Y. et al. (2018). BEL1-LIKE HOMEODOMAIN 11 regulates chloroplast development and chlorophyll synthesis in tomato fruit. *The Plant Journal, 94(6),* 1126–1140. DOI 10.1111/tpj.13924.
18. Ikeda, T., Tanaka, W., Toriba, T., Suzuki, C., Maeno, A. et al. (2019). BELL1-like homeobox genes regulate inflorescence architecture and meristem maintenance in rice. *The Plant Journal, 98(3),* 465–478. DOI 10.1111/tpj.14230.
19. Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A. et al. (2010). The genome of the domesticated apple (*Malus* × *domestica* borkh.). *Nature Genetics, 42(10),* 833–839. DOI 10.1038/ng.654.
20. Li, X., Ling, K., Zhang, J., Xie, Y., Wang, L. et al. (2016). Improved hybrid de novo genome assembly of domesticated apple (Malus x domestica). *Gigascience, 5(1),* 35. DOI 10.1186/s13742-016-0139-0.
21. Daccord, N., Celton, J. M., Linsmith, G., Becker, C., Choisne, N. et al. (2017). High-quality de novo assembly of the apple genome and methylome dynamics of early fruit development. *Nature Genetics, 49(7),* 1099–1106. DOI 10.1038/ng.3886.
22. Li, H., Dong, Q., Zhao, Q., Ran, K. (2019). Genome-wide identification, expression profiling, and protein-protein interaction properties of ovate family proteins in apple. *Tree Genetics & Genomes, 15,* 45. DOI 10.1007/s11295-019-1354-5.
23. Arnaud, N., Pautot, V. (2014). Ring the BELL and tie the KNOX: Roles for TALEs in gynoecium development. *Frontiers in Plant Science, 5,* 93. DOI 10.3389/fpls.2014.00093.
24. Li, H., Ran, K., Dong, Q., Zhao, Q., Shi, S. (2020). Cloning, sequencing, and expression analysis of 32 NAC transcription factors (MdNAC) in apple. *PeerJ, 8,* e8249. DOI 10.7717/peerj.8249.
25. Tian, Y., Dong, Q., Ji, Z., Chi, F., Cong, P. et al. (2015). Genome-wide identification and analysis of the MADS-box gene family in apple. *Gene, 555(2),* 277–290. DOI 10.1016/j.gene.2014.11.018.
26. Li, H., Dong, Q., Zhao, Q., Shi, S., Ran, K. (2020). Isolation, sequencing, and expression analysis of 30 AP2/ERF transcription factors in apple. *PeerJ, 8,* e8391. DOI 10.7717/peerj.8391.

27. Dong, Q., Duan, D., Zhao, S., Xu, B., Luo, J. et al. (2018). Genome-wide analysis and cloning of the apple stress-associated protein gene family reveals MdSAP15, which confers tolerance to drought and osmotic stresses in transgenic *arabidopsis*. *International Journal of Molecular Sciences, 19,* 2478. DOI 10.3390/ijms19092478.
28. Ma, Q. J., Sun, M. H., Liu, Y. J., Lu, J., Hu, D. G. et al. (2016). Molecular cloning and functional characterization of the apple sucrose transporter gene *MdSUT2*. *Plant Physiology & Biochemistry, 109,* 442–451. DOI 10.1016/j.plaphy.2016.10.026.
29. Dong, Q., Zheng, W., Duan, D., Huang, D., Wang, Q. et al. (2020). MdWRKY30, a group IIa WRKY gene from apple, confers tolerance to salinity and osmotic stresses in transgenic apple callus and *Arabidopsis* seedlings. *Plant Science, 299,* 110611. DOI 10.1016/j.plantsci.2020.110611.
30. Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods, 25(4),* 402–408. DOI 10.1006/meth.2001.1262.
31. Dong, Q., Zhao, S., Duan, D., Tian, Y., Wang, Y. et al. (2018). Structural and functional analyses of genes encoding VQ proteins in apple. *Plant Science, 272,* 208–219. DOI 10.1016/j.plantsci.2018.04.029.
32. Bhattacharjee, A., Ghangal, R., Garg, R., Jain, M. (2015). Genome-wide analysis of homeobox gene family in legumes: Identification, gene duplication and expression profiling. *PLoS One, 10(3),* e0119198. DOI 10.1371/journal.pone.0119198.
33. Que, F., Wang, G., Li, T., Wang, Y., Xu, Z. et al. (2018). Genome-wide identification, expansion, and evolution analysis of homeobox genes and their expression profiles during root development in carrot. *Functional & Integrative Genomics, 18(6),* 685–700. DOI 10.1007/s10142-018-0624-x.
34. Ruiz-Estévez, M., Bakkali, M., Martín-Blázquez, R., Garrido-Ramos, M. A. (2017). Identification and characterization of TALE homeobox genes in the endangered fern *Vandenboschia speciosa*. *Genes (Basel), 8(10),* 275. DOI 10.3390/genes8100275.
35. Khan, N., Hu, C., Khan, W. A., Wang, W., Han, K. et al. (2018). Genome-wide identification, classification, and expression pattern of homeobox gene family in *Brassica rapa* under various stresses. *Scientific Reports, 8(1),* 16265. DOI 10.1038/s41598-018-34448-x.
36. Xu, X., Lou, Y., Yang, K., Shan, X., Zhu, C. et al. (2019). Identification of homeobox genes associated with lignification and their expression patterns in bamboo shoots. *Biomolecules, 9(12),* 862. DOI 10.3390/biom9120862.
37. Cannon, S. B., Mitra, A., Baumgarten, A., Young, N. D., May, G. (2004). The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biology, 4,* 10. DOI 10.1186/1471-2229-4-10.
38. Liu, Y., You, S., Taylor-Teeples, M., Li, W. L., Schuetz, M. et al. (2014). BEL1-LIKE HOMEODOMAIN6 and KNOTTED ARABIDOPSIS THALIANA7 interact and regulate secondary cell wall formation via repression of *REVOLUTA*. *The Plant Cell, 26(12),* 4843–4861. DOI 10.1105/tpc.114.128322.
39. Liu, Y., Douglas, C. J. (2015). A role for OVATE FAMILY PROTEIN1 (OFP1) and OFP4 in a BLH6-KNAT7 multi-protein complex regulating secondary cell wall formation in *arabidopsis thaliana*. *Plant Signaling & Behavior, 10(7),* e1033126. DOI 10.1080/15592324.2015.1033126.
40. Zhang, L., Zhang, X., Ju, H., Chen, J., Wang, S. et al. (2016). Ovate family protein1 interaction with BLH3 regulates transition timing from vegetative to reproductive phase in *Arabidopsis*. *Biochemical & Biophysical Research Communications, 470(3),* 492–497. DOI 10.1016/j.bbrc.2016.01.135.
41. Schmitz, A. J., Begcy, K., Sarath, G., Walia, H. (2015). Rice ovate family protein 2 (OFP2) alters hormonal homeostasis and vasculature development. *Plant Science, 241,* 177–188. DOI 10.1016/j.plantsci.2015.10.011.
42. Bencivenga, S., Simonini, S., Benková, E., Colombo, L. (2012). The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in *Arabidopsis*. *The Plant Cell, 24(7),* 2886–2897. DOI 10.1105/tpc.112.100164.
43. Luo, H., Song, F., Goodman, R. M., Zheng, Z. (2005). Up-regulation of *OsBIHD1*, a rice gene encoding BELL homeodomain transcriptional factor, in disease resistance responses. *Plant Biology, 7(5),* 459–468. DOI 10.1055/s-2005-865851.

44. Luo, H., Song, F., Zheng, Z. (2005). Overexpression in transgenic tobacco reveals different roles for the rice homeodomain gene *OsBIHD1* in biotic and abiotic stress responses. *Journal of Experimental Botany, 56(420),* 2673–2682. DOI 10.1093/jxb/eri260.

45. Tao, Y., Chen, M., Shu, Y., Zhu, Y., Wang, S. et al. (2018). Identification and functional characterization of a novel BEL1-lIKE homeobox transcription factor GmBLH4 in soybean. *Plant Cell Tissue & Organ Culture, 134,* 331–344. DOI 10.1007/s11240-018-1419-4.

Appendix A

Supplementary Sequence: A1. Prediction of cis-acting elements in *MdBLH* promoters

Supplementary Sequence: A2. Genomic information for 11 cloned *MdBLH* genes

Supplementary Sequence: A3. Interactions of MdWRKY52 protein with MdVQ10 protein in yeast cells