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Identification and Functional Analysis of Transcription Factor NF-Y Family during Flower Bud Dormancy in *Prunus mume*

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Abstract: NF-Y transcription factor consists of three subsets, A, B, and C, which play various roles during biological processes in plants, particularly in growth and development, stress response, and microorganism interactions. The current study was exhaustively conducted on the NF-Y gene family in *Prunus mume* (Japanese apricot). We identified 27 *PmNF-Y* genes that were distributed on eight chromosomes of the Japanese apricot genome, and which were divided into three subgroups according to their phylogenetic relationship. Protein replication analysis showed that the Ka/Ks ratio of one pair of tandem repeats and two pairs of segmental repeats was less than 1. Cis-acting elements upstream of *PmNF-Y* genes were divided into four distinct categories: light response, growth metabolism, stress, and hormones, among which the numbers of hormone and light-responsive cis-acting elements were higher. RNA-seq analysis revealed that most of the genes were expressed with slight variation in various tissues. Interestingly, some genes were expressed differently during dormancy. During dormancy with exogenous hormones and low temperature, the qRT-PCR results showed that these differentially expressed genes had specific expression responses under GA_4, ABA, MeJA, and low-temperature treatments. Therefore, these findings could provide a novel theoretical foundation for future research into the function and molecular regulation mechanism of the NF-Y gene family in the *Prunus* species.

Keywords: *Prunus mume*; *PmNF-Y* gene; expression profile; bioinformatics

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1. Introduction

Nuclear factor Y (NF-Y), also known as CCAAT-box binding factor (CBF) or heme activator protein (HAP), is a heterotrimeric transcription factor complex in plants that has three distinct subsets: NF-YA (CBF-B, HAP2), NF-YB (CBF-A, HAP3), and NF-YC (CBF-C, HAP5). This transcription factor is highly conserved in evolution, and almost universal in eukaryotes [1]. The NF-YA protein contains a core conserved region consisting of 53 conservative amino acids. The core region consists of two conserved α-helices and a conserved gap sequence connecting the two α-helices. As with the NF-YB and NF-YC proteins, the primary role of their conserved domain is to bind to the CCAAT box and interact with the other two subsets [2–4].

Previous studies on NF-Y have proven that it can control flowering time by modulating the blossoming gene, FLOWERING LOCUS T (FT), and the NF-YA, YB, and YC subgroups, all of which play a crucial role in the regulation of the flowering period in plant species [5,6]. The overexpression of Arabidopsis NF-YA subsets (YA1, YA2, YA3, YA4, YA7, and YA10) can inhibit plant flowering time, suggesting that NF-YA subsets negatively regulate plant flowering time [7]. Both NF-YA/B/C and NF-YB-C-CO (CONSTANS) trimers can target the FT-bound promoter region [8]. The irregular time-dependent differential expression rate of CO and NF-Y transcription factors and the protein availability are believed to regulate FT

by the NF-YA/B/C and NF-YB/C-CO trimers that are involved in flowering processes. CO protein accumulates continually during the day and peaks at dusk/night [5,8]. Therefore, it can cooperate with NF-YA1/4 to produce the trimeric NF-YB/C-CO complex and induce FT transcription. The protein level of CO decreases gradually during the night, and the related NF-YB/C is substituted by nf-ya1/4 to form the NF-YA/B/C complex and inhibit the expression of FT [9]. Likewise, the overexpression of AtNF-YB1 inhibits the transcription of FT, delaying flowering [10]. NF-Y factor and the DELLA protein, a key component of the gibberellin pathway, also interact to control flowering in plants [11,12]. Arabidopsis *AtNF-YA2/B2/C9* has been reported to interact with DELLA and form polymer complexes. GA subsequently causes DELLA protein degradation and NF-Y complex release, allowing it to recruit REF6 and target the NFYBE (NF-Y binding element) cis-acting element binding to the SOC1 promoter region, causing H3K27me3 locus demethylation and SOC1 expression, as well as plant flowering [6].

Most fruit trees are woody perennial plants with flowers, which are important reproductive organs that affect fruit formation or production. However, the fruit production cycle is relatively stable/consistent, which could be affected further by the regulation of the flowering period. Hence, determining the flowering and production time is of economic benefit. In the previous decade, the efficient cultivation of fruit has generated substantial economic benefits and promoted industrialization, with flowering management playing an important role. As a result, further study into the distinct process of flowering regulation in woody fruit trees associated with bud dormancy is required. *Prunus mume* Sieb. et Zucc. is a deciduous woody fruit tree native to China which is economically significant due to its early flowering time and dual-purpose flowers and fruits. Flowering times vary greatly among its varieties, which have a high genetic diversity. In terms of flowering time, late and early flowering cultivars can be about 30 days. Temperature appears to have a significant influence on flowering time and period. As a result, the flowering period of the same variety in the same region might vary for about 40 days depending on the weather of the year [13], suggesting that *Prunus mume* blooming time is affected by genetic variation and environmental factors such as temperature. In view of all these, both genetic diversity and temperature are regarded as valuable resources for studying the flowering regulation mechanism of woody plant species. Therefore, a comprehensive genome-wide analysis and different hormone and temperature treatments were undertaken for an in-depth understanding of the NF-Y gene family's function and its effect in *Prunus mume*. The results of this current work will enhance our knowledge of the mechanism modulating flowering time in *Prunus mume* and lay the basis for further research on other *Prunus* species.

2. Materials and Methods

2.1. Plant Materials and Treatments

The plant materials were collected from the National Field Genebank for *Prunus mume* of Nanjing Agricultural University. The materials were from a 5-year-old early flowering cultivar, "Taoxingmei", with strong growth. The sampling period was after the leaves of "Taoxingmei" had fallen. Annual branches of 30–40 cm with full and substantial buds were collected. The branches were divided into four groups and replicated three times with five annual branches. The base of the branch was cut evenly and submerged at a height of 3 cm into 200 µmol/L of GA_4 solution, 200 µmol/L of ABA solution, 200 µmol/L of MeJA solution, and distilled water, respectively. After that, they were immediately put in a light incubator for cultivation. The conditions of GA_4, ABA, and MeJA treatment were: day and night temperature 21 ± 3 °C, light/dark hours 12/12 h, light intensity 55 mmol m^{-2} s^{-1}, and relative air humidity 70%, respectively. The conditions of the 4 °C treatment were: day and night temperature 4 ± 3 °C, light/dark hours 12/12 h, light intensity 55 mmol m^{-2} s^{-1}, and relative air humidity 70%, respectively. After every 3 days, 2 mm of the base of the branches was cut to expose the fresh injury to the media. After 0, 1, 3, and 5 days of treatment, the buds were collected from each treatment and stored at −80 °C for future use.

2.2. Identification and Characterization of NF-Y Family in Prunus mume

First, we downloaded the protein sequence data of *Prunus mume* from the NCBI database and obtained the HMM model (http://pfam.xfam.org/, accessed on 6 January 2021) database of NF-Y(PF00808) from PFAM. Subsequently, we used the HMM model of NF-Y as the query sequence to obtain relevant sequence data from *Prunus mume* protein sequence data (E value < 10^{-5}). Furthermore, the acquired sequence confirmed that it contained the fully conserved NF-Y domain on InterPro (http://www.ebi.ac.uk/interpro/, accessed on 6 January 2021) and SMART [14], and the chromosome location information of NF-Y was obtained simultaneously. The ExPaSy (http://web.expasy.org/protparam, accessed on 6 January 2021) online platform was utilized to analyze the physical and chemical properties of NF-Y family member proteins, whereas the BUSCA website (http://busca.biocomp.unibo.it/, accessed on 6 January 2021) was used to predict the subcellular localization of *PmNF-Y* family members.

2.3. Chromosomal Location and Synteny Analysis

For the chromosome localization analysis of *PmNF-Y* genes, we used the TBtools program [14]. Meanwhile, the tandem and segmented replication events of *PmNF-Y* family members were analyzed using MCScanX software (http://chibba.pgml.uga.edu/mcscan2/, accessed on 6 January 2021). Furthermore, we used the KaKs Calculator 2.0 [15] to estimate the Ka and Ks values of tandem and segmented replication gene pairs in order to determine their selection in the evolutionary process.

2.4. Analysis of Structure and Conserved Motifs of NF-Y Gene in Prunus mume

We used the GSDS tool [16] to analyze and draw the map of the gene structure of *PmNF-Y* gene family members. Using the MEME tool (http://meme-suite.org/tools/meme, accessed on 8 January 2021), the structure of the conserved domains of *PmNF-Y* proteins was analyzed. We set the maximum number of pattern discoveries to 10, the other parameters were the default values, and we viewed the results via TBtools software [14].

2.5. Analysis of Cis-Acting Elements in PmNF-Y Gene Upstream Promoter Region

The PlantCARE tool was used to identify the cis-acting components of the 2000 bp domain upstream of PmNF-transcription Y's initiation point in the *Prunus mume* genome [17]. These cis-acting elements were subsequently analyzed and classified.

2.6. Phylogenetic Analysis and Prediction of the Protein–Protein Interaction Network of the PmNF-Y Genes Family

A phylogenetic tree was built from the sequences of members of the *PmNF-Y* gene family of *Prunus persica, Prunus dulcis, Prunus armeniaca, Prunus mume,* and *Arabidopsis thaliana*. Protein sequences were aligned utilizing the MAFFT software with the default settings [18]. We constructed phylogenetic trees using the maximum likelihood (ML) method. The ML analysis was conducted using IQ-TREE [19], with ModelFinder software selecting the optimal model [20], and 1000 bootstrap replicates were performed. Members of the *PmNF-Y* gene family's protein–protein interactions were explored using the online database STRING (https://string-db.org/, accessed on 10 January 2021).

2.7. RNA-Seq and Quantitative qRT-PCR Analysis of PmNF-Y Gene

RNA-Seq analysis obtained clean reads by removing low-quality reads, reads containing adapters, and more than 10% anonymous nucleotides (N) from raw sequence data (raw reads). Subsequent analyses were conducted based on clean data with high quality and the clean reads were compared to the *Prunus mume* gene sequence reference data sets using Hisat2. The expression profiles of NF-Y genes in five different tissues—root, stem, leaf, flower bud, and fruit (presented in Table S1)—and the expression in the four different dormancy stages—paradormancy, endodormancy, ecodormancy, and dormancy release (presented in Table S2)—were mapped using the already-existing transcriptome

data from the NCBI Sequencing GEO database (accession number PRJNA172987 and PRJNA615074) [21,22]. Among them, the transcriptome data of different dormancy stages had three replicates in each stage, while the transcriptome data of different tissues had only one replicate in each tissue. The RSEM tool was used to calculate gene and transcript expression levels using the FPKM index (fragments per kilobase of transcript per million mapped reads) [23].

The total RNA of flower buds under GA_4, ABA, MeJA, and low-temperature treatments was extracted using an RNA extraction kit (FOREGENE, China), the concentrations were determined, and the quality was assured using agarose gel electrophoresis. We utilized the PrimeScript™ RT reagent Kit instructions in conjunction with the gDNA Eraser to synthesize cDNA. The ABI 7300 real-time PCR system (Applied Biosystems, Foster, CA, USA) and the SYBR green real-time PCR Master Mix (Dongbao, Osaka, Japan) were used for the qRT-PCR; the primers are listed in Table S3. The collected flower bud materials were made up of three independent biological replicates. Three technical replicates were performed for each biological replicate [24].

3. Results

3.1. Identification and Characterization of PmNF-Y Gene Family in Japanese Apricot

A total of 27 *PmNF-Y* genes were identified in the genome of *Prunus mume*, and family members of the *PmNF-Y* genes were named *PmNF-YA01* to *PmNF-YA05, PmNF-YB01* to *PmNF-YB13*, and *PmNF-YC01* to *PmNF-YC09* based on their sequence positions on eight chromosomes. The specific physicochemical properties of *PmNF-Y* genes are shown in Table 1. The proteins encoded by these 27 *PmNF-Y* genes range from 121 to 351 amino acids in length, and the maximum and minimum molecular weights are, respectively, 13301.29, and 38,208.65 Da. *PmNF-YC03* has the smallest isoelectric point, with 4.35, while *PmNF-YC01* has the largest, with 9.64. The different isoelectric points of these *PmNF-Y* proteins indicate that their acid–base characteristics are distinct. All protein GRAVY values are less than 0, suggesting that they are all hydrophilic proteins. Most *PmNF-Y* genes were located in the nucleus, whereas a few genes were sited in chloroplasts and the cytoplasm.

Table 1. Identification and physicochemical properties of the *NF-Y* gene family in the *Prunus mume* genome.

Gene Name	Gene ID	Chr Location	Protein					Subcellular Localization	Signal Pep
			Length	MW	pI	GRAVY	Aliphatic Index		
PmNF-YA01	LOC103325228	3	202	22,125.34	7.9	−0.957	57.08	Nucleus	No
PmNF-YA02	LOC103326841	3	351	38,168.4	6.7	−1.088	43.99	Nucleus	No
PmNF-YA03	LOC103327655	4	344	38,208.65	9.2	−0.682	62.91	Nucleus	No
PmNF-YA04	LOC103339547	8	321	35,232.58	9.36	−0.628	57.17	Nucleus	No
PmNF-YA05	LOC103340551	8	336	37,422.9	8.97	−0.633	65.03	Nucleus	No
PmNF-YB01	LOC103328056	1	156	17,228.58	8.69	−0.932	73.27	Nucleus	No
PmNF-YB02	LOC103332583	1	177	19,690.54	7.01	−0.976	55.65	Nucleus	No
PmNF-YB03	LOC103332591	1	121	13,397.32	7.78	−0.392	74.21	Chloroplast Nucleus	No
PmNF-YB04	LOC103338011	1	125	13,437.67	5.12	−0.908	41.44	Nucleus	No
PmNF-YB05	LOC103344608	1	178	19,906.28	5.27	−0.711	73.03	Nucleus	No
PmNF-YB06	LOC103322992	2	167	18,773.74	6.1	−0.998	62.57	Nucleus	No
PmNF-YB07	LOC103324365	3	271	29,604.61	6.38	−0.615	62.03	Nucleus	No
PmNF-YB08	LOC103326701	3	270	29,266.53	5.66	−0.688	55.41	Nucleus	No
PmNF-YB09	LOC103326707	3	198	21,263.44	6.52	−0.844	54.19	Nucleus	No
PmNF-YB10	LOC103326937	3	230	25,917.53	6.25	−0.957	64.87	Nucleus	No

Table 1. *Cont.*

Gene Name	Gene ID	Chr Location	Protein					Subcellular Localization	Signal Pep
			Length	MW	pI	GRAVY	Aliphatic Index		
PmNF-YB11	LOC103329260	4	247	25,666.21	7.09	−0.711	52.11	Nucleus	No
PmNF-YB12	LOC103332417	5	180	19,173.22	5.27	−0.696	60.78	Nucleus	No
PmNF-YB13	LOC103333886	6	176	19,826.99	6.39	−0.91	61.53	Nucleus	No
PmNF-YC01	LOC103325121	1	221	25,077.53	9.64	−1.294	50.32	Nucleus	No
PmNF-YC02	LOC103344662	1	262	29,161.91	5.89	−0.581	66.72	Cytoplasm Nucleus	No
PmNF-YC03	LOC103322124	2	309	34,672.45	4.35	−1.086	64.24	Nucleus	No
PmNF-YC04	LOC103322822	2	260	28,835.56	5.89	−0.499	73.62	Nucleus	No
PmNF-YC05	LOC103325736	3	280	31,364.25	5.76	−0.622	69.75	Nucleus	No
PmNF-YC06	LOC107880919	4	157	18,073.58	6.19	−0.568	77.01	Nucleus	No
PmNF-YC07	LOC103330752	5	293	31,620.66	4.84	−0.717	67.06	Nucleus	No
PmNF-YC08	LOC103337511	7	121	13,301.29	6.91	−0.273	77.36	Nucleus	No
PmNF-YC09	LOC103338075	7	238	26,082.18	5.14	−0.535	67.69	Cytoplasm Nucleus	No

3.2. Chromosomal Mapping and Synteny Analysis of PmNF-Y Genes

Figure 1 illustrates the chromosomal mapping of the 27 *PmNF-Y* genes. Among them, seven (*PmNF-YC01*, *PmNF-YB01*, *PmNF-YB02*, *PmNF-YB03*, *PmNF-YB04*, *PmNF-YB05*, and *PmNF-YC02*) were on Chr01, three (*PmNF-YC03*, *PmNF-YC04*, and *PmNF-YB06*) were on Chr02, seven (*PmNF-YB07*, *PmNF-YA01*, *PmNF-YC05*, *PmNF-YB08*, *PmNF-YB09*, *PmNF-YA02*, and *PmNF-YB10*) were on Chr03, three (*PmNF-YA03*, *PmNF-YC06*, and *PmNF-YB11*) were on Chr04, two (*PmNF-YC07* and *PmNF-YB12*) were on Chr05, one (*PmNF-YB13*) was on Chr06, two (*PmNF-YC08* and *PmNF-YC09*) were on Chr07, and two (*PmNF-YA04* and *PmNF-YA05*) were on Chr08. However, Chr01 and Chr03 contain the greatest number of *PmNF-Y* genes with 25.92%, while Chr06 contains the lowest with 3.70%.

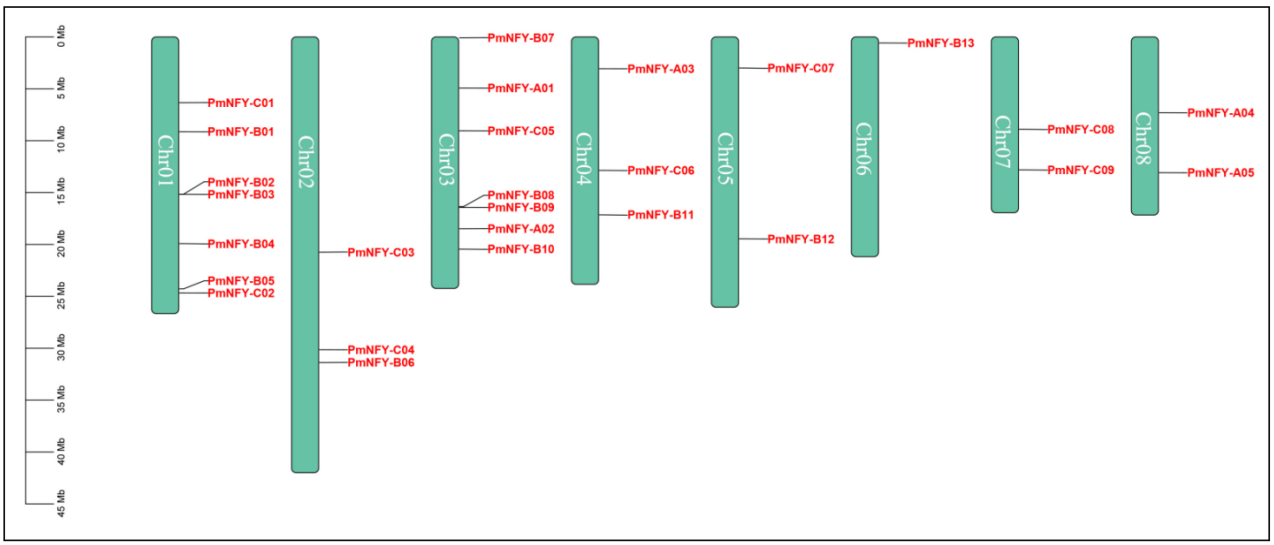

Figure 1. Distribution of *PmNF-Y* genes among 8 chromosomes. The scale on the left represents the length of the chromosome. The chromosome numbers are on each chromosome.

3.3. Gene Structural, Protein Conserved Domain, and Conserved Motif Analysis of PmNF-Y Genes

The phylogenetic tree was generated according to protein sequences of *PmNF-Y* genes to analyze their genetic evolution relationship. Meanwhile, we analyzed the conserved domains of the protein sequences of *PmNF-Y* genes (Figure 2A) and drew gene structure

maps (Figure 2B). It was found that the *PmNF-YB04* genes had the highest number of introns (six introns). However, twelve genes did not contain an intron (*PmNF-YB02*, *PmNF-YB05*, *PmNF-YB06*, *PmNF-YB09*, *PmNF-YB10*, *PmNF-YB011*, *PmNF-YC02*, *PmNF-YC03*, *PmNF-YC04*, *PmNF-YC05*, *PmNF-YC08*, and *PmNF-YC09*), three had one intron (*PmNF-YB03*, *PmNF-YB07*, *PmNF-YB13*), two had two introns (*PmNF-YB01* and *PmNF-YB08*), seven had four introns (*PmNF-YA01*, *PmNF-YA02*, *PmNF-YA03*, *PmNF-YA04*, *PmNF-YA05*, *PmNF-YC06*, and *PmNF-YC07*), and one had five introns (*PmNF-YB12*).

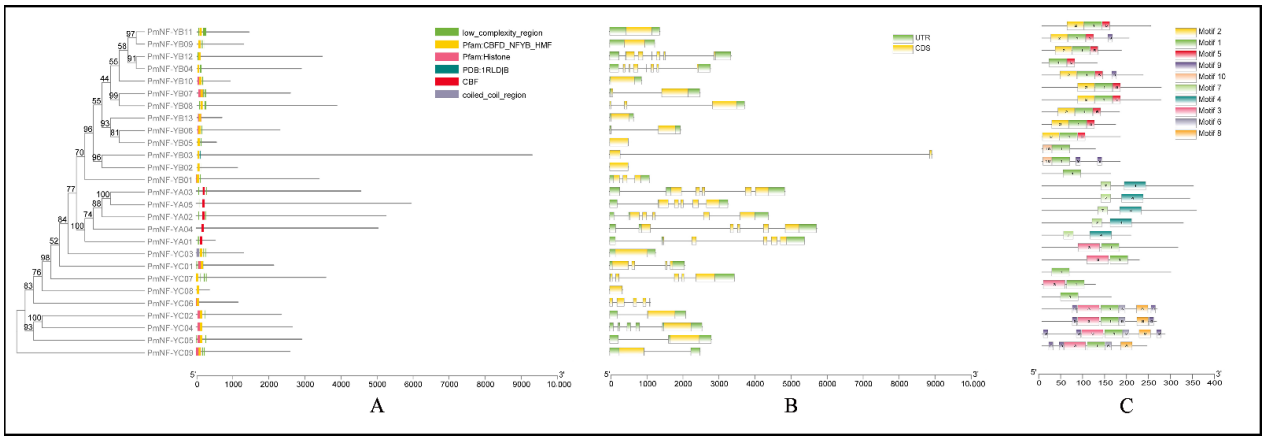

Figure 2. The protein conserved domains, gene structures, and conserved motifs of *PmNF-Y* genes based on phylogenetic relationships. (**A**) Distribution of protein conserved domains. (**B**) The *PmNF-Y* gene structures. (**C**) The *PmNF-Y* gene conserved motifs.

The analysis of conserved domains revealed that all *PmNF-YA* genes have the CBF domain, while *PmNF-YB* and *PmNF-YC* genes contain the CBFD NFYB HMF domains. Moreover, the analysis results of the conserved motifs of the *PmNF-Y* gene family via MEME revealed that all *PmNF-YA* proteins had motifs 4 and 7 (Figure 2C). In addition, all *PmNF-YB* proteins possessed motif 1, motif 2, and motif 5, except for *PmNF-YB01*, *PmNF-YB02*, *PmNF-YB03*, and *PmNF-YB04* (Figure 2C). The majority of *PmNF-YC* proteins possessed motif 1 and motif 3, except for *PmNF-YC06* and *PmNF-YC07*. Furthermore, *PmNF-YC05* contained the greatest number of motifs (7), whereas *PmNF-YB02*, *PmNF-YC06*, and *PmNF-YC07* contained the lowest number of motifs (1). Therefore, the types and numbers of different gene motifs varied significantly.

The synteny analysis of the *PmNF-Y* genes revealed that one pair of tandemly repeated sequences (*PmNF-YB02* and *PmNF-YB03*) occurred among the 27 *PmNF-Y* genes (Figure 3). Interestingly, we identified two pairs of segmental repeat genes (*PmNF-YB10* and *PmNF-YB11*, *PmNF-YB06* and *PmNF-YB13*).

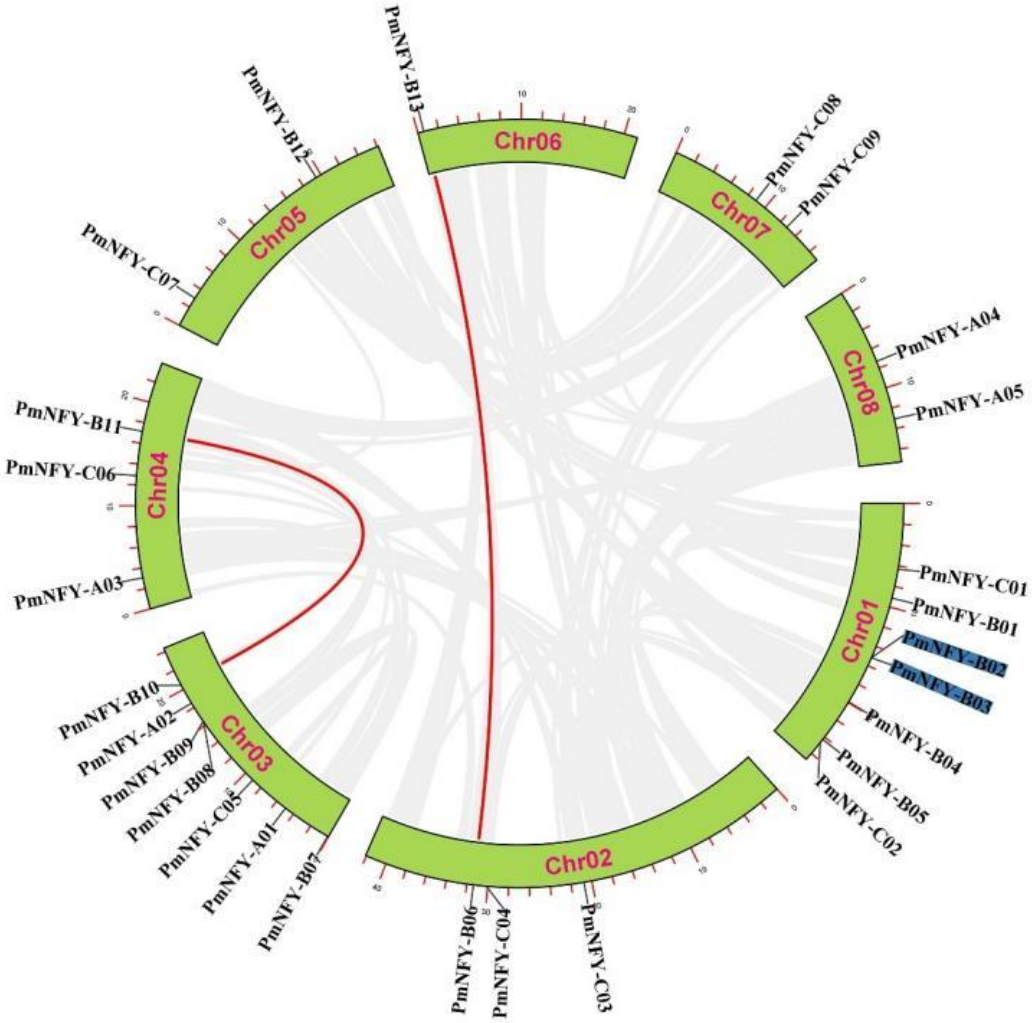

Figure 3. Synteny relationships of *Prunus mume NF-Y* genes. *PmNF-Y* gene duplication relationships. Genes that are tandemly repeated are labeled in blue, and genes that are segmentally duplicated are linked with red lines.

We assessed the Ka/Ks ratio of these tandem repeat and segmented repeat genes to see if they had been subjected to natural selection during evolution. *PmNF-Y* gene sequences had Ka/Ks values less than 1 (Table 2), signifying that they had undergone purifying selection during their evolutionary process.

Table 2. The Ka/Ks values of repeated *PmNF-Y* gene sequences.

Repeat Sequences	Ka	Ks	Ka/Ks	Type
PmNF-YB02/PmNF-YB03	0.310	0.3742	0.8305	Tandem
PmNF-YB10/PmNF-YB11	0.7122	NaN	NaN	Segmental
PmNF-YB06/PmNF-YB13	0.3622	NaN	NaN	Segmental

3.4. Cis-Acting Elements Analysis in PmNF-Y Gene Promoter Region

The promoters of 2000 bp regions in the upstream region of *PmNF-Y* were studied to better understand the transcriptional process/mechanism of the *PmNF-Y* gene. However, the *PmNF-Y* promoter region contains a large number of cis-acting elements that may be divided into four categories: light-response-related elements type, hormone-response-related elements type, stress-response-related type, and plant growth and development elements type (Figure 4B). The number of light-response-related elements is the greatest, with 344, followed by hormone-related response elements, with a total of 246. Among these,

there are 19 salicylic acid response elements, 96 abscisic acid response elements, 20 gibberellin response elements, 92 methyl jasmonate response elements, and 19 auxin response elements. The stress-related response elements came in third, with anaerobic induction, drought-inducibility, low-temperature responsiveness, defense and stress responsiveness, and anoxic specificity among them. Plant growth and development-related components were the weakest, including meristem expression, differentiation of the palisade mesophyll cells, zein metabolism regulation, endosperm expression, seed-specific regulation, circadian control, cell-cycle regulation, and flavonoid biosynthetic regulation (Figure 4A). Among all the members of the *PmNF-Y* gene family, the *PmNF-YC06* genes contained 63 cis-acting elements, and almost all of the gene promoter regions contained more than 15 cis-acting elements (Figure 4B).

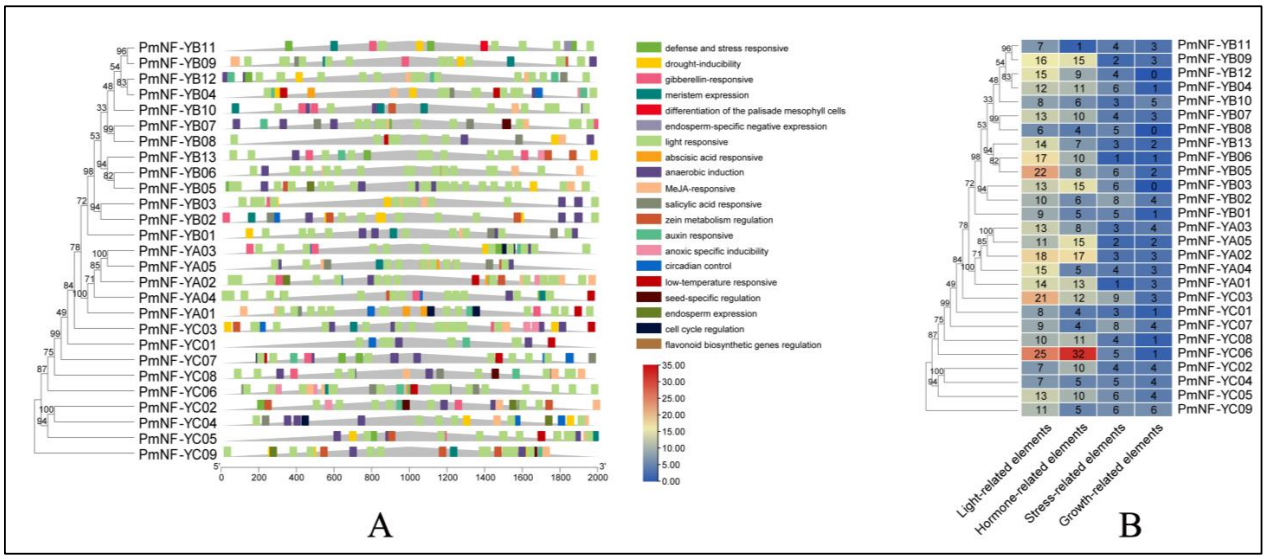

Figure 4. Predicted cis-acting elements in the promoters of *PmNF-Y* genes. (**A**) The 2000 bp sequences upstream of the 27 *PmNF-Y* genes were analyzed with PlantCARE. (**B**) Types and numbers of cis-acting elements in the promoter regions of *PmNF-Y* genes.

Moreover, among all the cis-acting elements, the number of different types of cis-acting elements varied greatly (Figure 5). The most abundant cis-acting elements are hormones, light, and the stress response; however, there are few types for plant growth and development. Hence, we concluded that the *PmNF-Y* plays a crucial role in the hormone, light, and stress responses in *Prunus mume*. The majority of the promoters comprised two cis-acting elements, GA and ABA, according to further examination. ABA, for instance, is currently assumed to have a major role in seed and bud dormancy. It also contains a chemical that prevents the release of bud dormancy and is believed to be responsible for initiating the "switch" of bud dormancy [25]. Gibberellin, as a dormancy breaker, can substitute some components of the cold-required conditions and promote/induce the early dormancy-breaking of fruit tree flower buds, with unexpected results [26]. This implies that members of the *PmNF-Y* family may be involved in plant dormancy modulation.

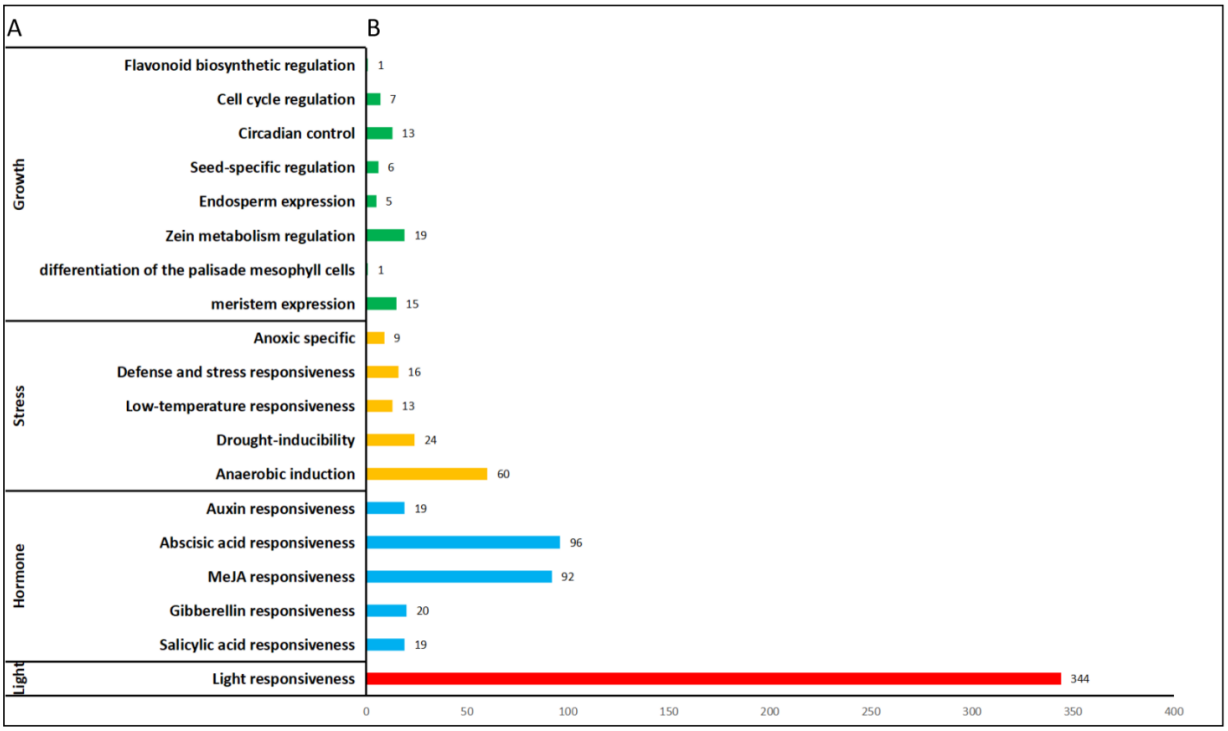

Figure 5. Statistics and classification of the cis-acting elements of *PmNF-Y*. (**A**) The cis-acting elements in the 2000 bp region upstream of *PmNF-Y* were divided into four groups according to their function. (**B**) The numbers of each cis-acting element of *PmNF-Y*.

3.5. Phylogenetic Tree of the PmNF-Y Genes

The phylogenetic tree of 158 NF-Y genes, including 27, 36, 34, 29, and 32 from *Prunus mume, Arabidopsis thaliana*, *Prunus salicina, Prunus armeniaca*, and *Prunus persica*, respectively, was constructed based on their amino acid sequences using IQ-TREE. On the basis/foundation of their sequences' similarities, these proteins, including 27 *PmNF-Y* in *Prunus mume*, were grouped into three groups (Figure 6). The integrated phylogenetic tree's classification results were essentially consistent with that generated via *PmNF-Y* protein sequences (Figure 1). Meanwhile, the conserved motif and protein conserved domain results indicate that the same subfamily has similar numbers and types of conserved motifs and protein conserved domains, which further supports the phylogenetic tree clustering results (Figure 1). The number of conserved motifs and protein conserved domains within the same subfamily suggests that they are relatively conserved and undertake/conduct similar functions.

3.6. Prediction of Protein–Protein Interaction Network

PmNF-Y gene regulatory relationships were predicted/hypothesized and elucidated. Almost every gene in the *PmNF-Y* gene family is a member of the regulatory network. In addition, three genes, *PmNF-YA03*, *PmNF-YB07*, and *PmNF-YC09*, interacted with regulatory network genes including *PmABI5*, *PmKEG*, *PmDR1-AC*, *PmATRX-like*, *PmB3-LEC2*, and *PmRGL2* (Figure 7).

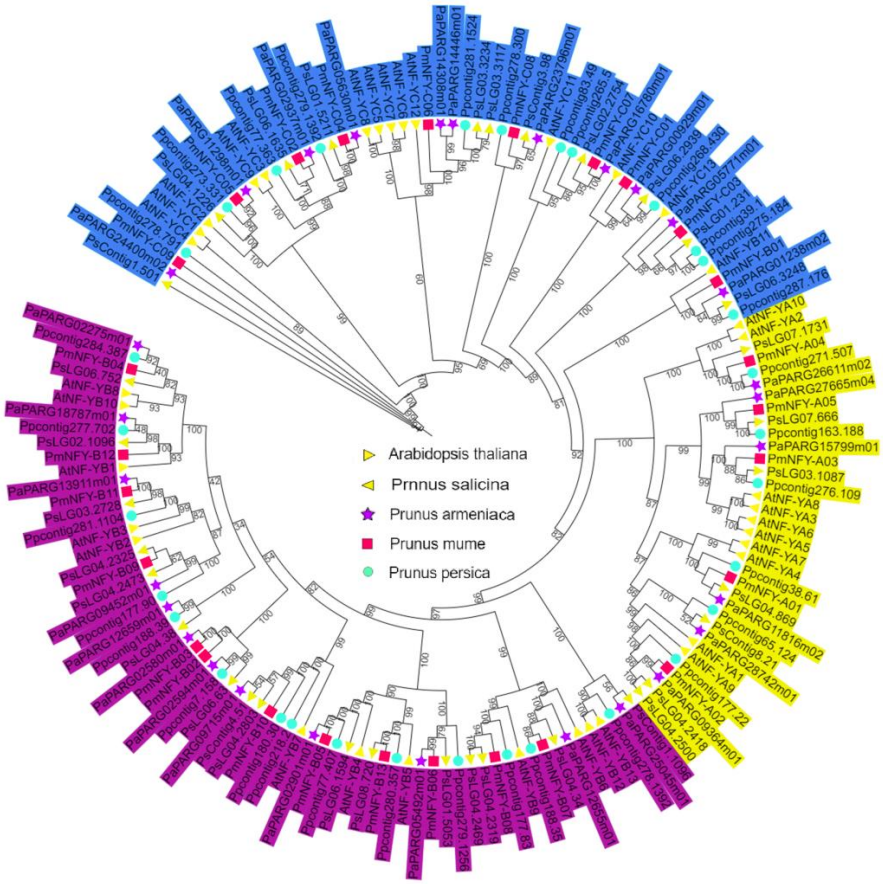

Figure 6. Phylogenetic analysis of NF-Y proteins from *Prunus mume* and four other plant species.

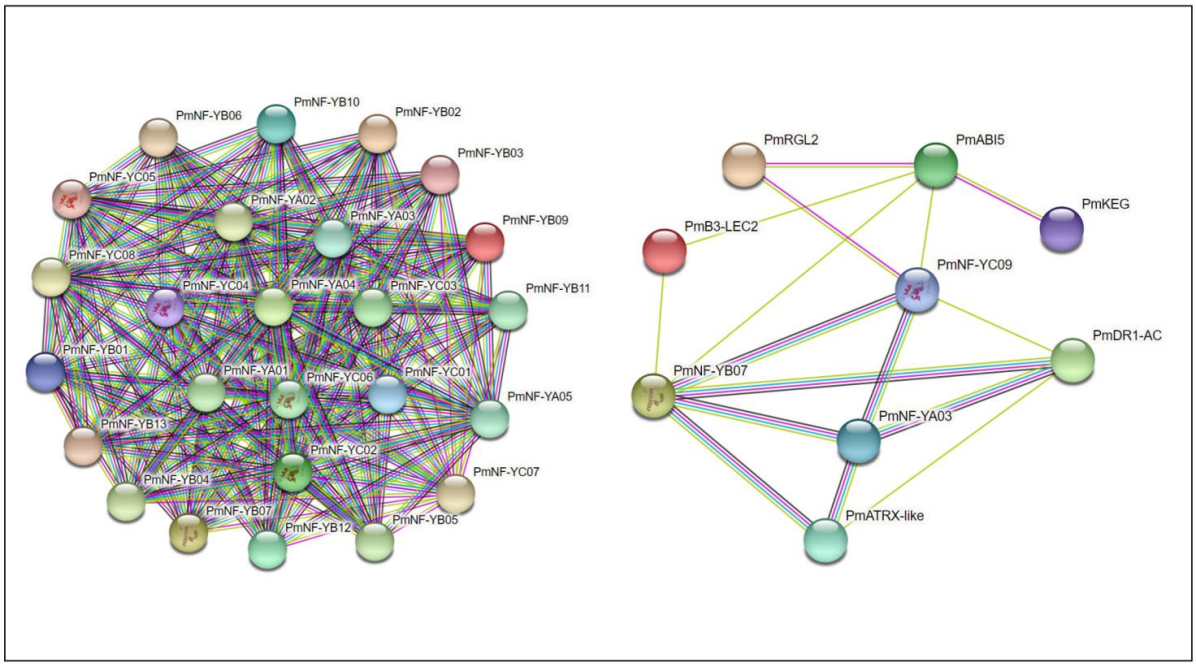

Figure 7. Prediction and analysis of protein–protein interactions of *PmNF-Y* genes.

3.7. Expression Profile Analysis of PmNF-Y Genes in Different Tissues and Dormancy Periods

Using the already-existing RNA-seq data, we investigated the expression levels of the identified 27 *PmNF-Y* genes in leaves, roots, buds, stems, and fruits to determine their

specific expression patterns in different tissues. The findings revealed that the expression levels of these genes differed significantly in *Prunus mume* tissues. However, the *PmNF-YC02* gene had the highest expression level in roots, whereas *PmNF-YB03*, *PmNF-YB07*, *PmNF-YB10*, and *PmNF-YC06* genes were not expressed in roots. The *PmNF-YA01* gene had the highest expression levels in the stem, while *PmNF-YB05*, *PmNF-YB07*, *PmNF-YB08*, *PmNF-YB10*, *PmNF-YC06*, and *PmNF-YC08* were not expressed in the stem. *PmNF-YB04* was found to be the highest-expressed in leaves, buds, and fruit, while *PmNF-YC06* was not expressed in all tissues. Furthermore, *PmNF-YB10* was exclusively expressed in the bud and fruit, whereas the *PmNF-YB08* gene was only expressed in the root.

Moreover, we used the already-existing RNA-seq data to determine the expression of *PmNF-Y* genes at distinct dormancy stages. The expression level of the *PmNF-YB04* gene was the highest at all four stages, as shown in Figure 8B, with no significant difference between them. At the paradormancy, ecodormancy, and endodormancy stages, the *PmNF-YC06* gene was not expressed. Four genes (*PmNF-YB06*, *PmNF-YB08*, *PmNF-YC06*, *PmNF-YC08*) were not expressed during dormancy breaking. The expression of *PmNF-YB07* and *PmNF-YA05* increased from the first to the second, third, and fourth stages. *PmNF-YB08*, *PmNF-YC08*, and *PmNF-YC09* expression levels initially increased at the first, second, and third stages, then decreased at the fourth stage. *PmNF-YA03*, *PmNF-YB10*, and *PmNF-YB13* expression levels, on the other hand, initially dropped/fell and subsequently rose. As a result of the significant differences in their expression levels at different stages of dormancy, these genes may play a key role in the control/modulation of the dormancy process.

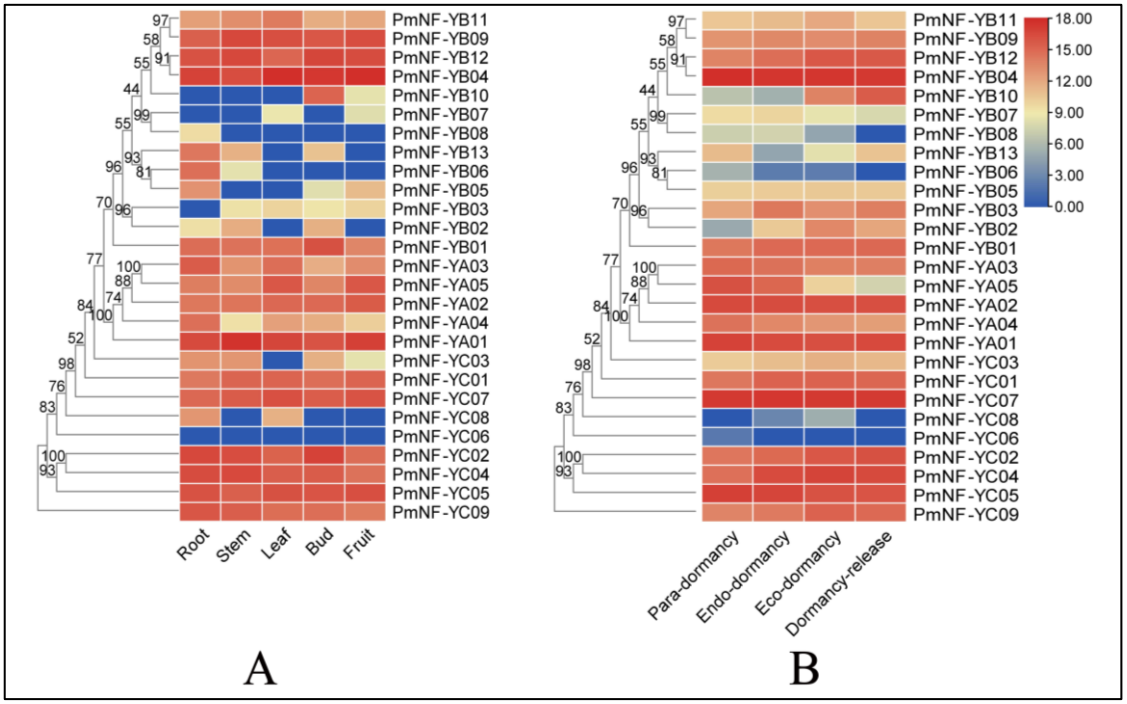

Figure 8. Expression levels of *PmNF-Y* genes in different tissues and at different stages of dormancy: (**A**) expression profiles of NF-Y genes in five different tissues; (**B**) expression profile of NF-Y genes in the four different dormancy stages.

3.8. Expression Pattern Analysis of PmNF-Y Genes under GA_4, ABA, MeJA, and Low-Temperature Treatments

To further study the role of *PmNF-Y* genes in the dormancy process, we screened the transcriptome for differentially expressed *PmNF-Y* genes at distinct stages of dormancy in the bud tissue and performed qRT-PCR to examine their expression levels under the 200 mol/L GA_4, 200 mol/L ABA, 200 mol/L MeJA, and 4 °C low-temperature treatments. However, under the GA_4 hormone treatment, the expression level of *PmNF-YA03*, *PmNF-YB07*,

and *PmNF-YC09* genes initially increased, then decreased, and then stabilized, whereas the expression levels of the *PmNF-YB08* and *PmNF-YB10* genes increased. On the fifth day, the expression levels of *PmNF-YB13* and *PmNF-YC08* increased significantly, whereas the expression level of the *PmNF-YA05* gene decreased. Under the ABA hormone treatment, the expression levels of the *PmNF-YA03* and *PmNF-YA05* genes also initially increased and then decreased, while the *PmNF-YB13*, *PmNF-YC08*, and *PmNF-YC09* genes initially increased, then decreased, and finally increased. Additionally, the expression level of the *PmNF-YB07* gene decreased, whereas the *PmNF-YB08* and *PmNF-YB10* genes increased. Under the MeJA hormone treatment, the expression levels of *PmNF-YA03*, *PmNF-YA05*, *PmNF-YB07*, and *PmNF-YB10* decreased and reached approximately zero. On the fifth day, the expression levels of *PmNF-YB13* increased significantly. Except for the *PmNF-YA03* gene, the levels of all other genes increased in response to the 4 °C low-temperature treatment. Eight members of the PmNF-Y gene family from flower buds (in the endodormancy stage) showed distinct expression responses to various exogenous hormones and low-temperature treatments. The results are presented in Figure 9.

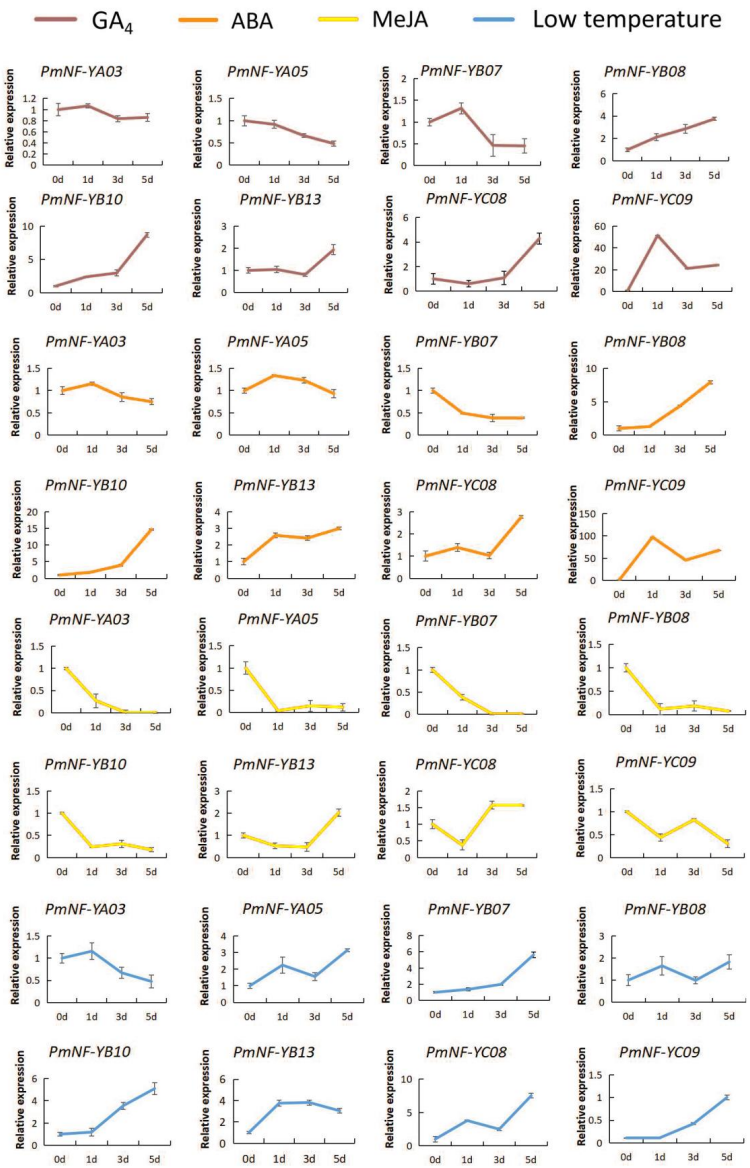

Figure 9. Expression analysis of the *PmNF-Y* genes in *Prunus mume* under GA_4, ABA, MeJA, and low-temperature treatments.

4. Discussion

NF-Y is encoded by a single gene in yeast and human genomes; however, in plants, NF-Y Subsets are encoded by a gene family of many genes, resulting in a large number of potential NF-Y genes. These distinct complexes may exhibit functional differentiation and redundancy due to their trimer composition. With the NF-YA and NF-YC subgroups, one subset of the NF-Y family forms a heterotrimer. To activate or regulate downstream gene expression, the heterotrimer complex interacts with the CCAAT box [27–29]. NF-YS had been reported to be associated with the regulation of plant growth and development processes as well as biological and abiotic stress responses, including embryonic development, seed maturation, post-embryonic development, flowering time, root growth and development, fatty acid synthesis, endoplasmic reticulum stress response, and others [30–34]. However, it has been reported in recent years that CO can substitute NF-YA and interact with NF-YB/NF-YC [35]. It forms a heterotrimer, specifically binds particularly to CCACA components, and is involved in flowering modulation [36,37]. Recent studies [38] have indicated that the *PtNF-YB1* gene can regulate flowering time. Moreover, the NF-Y complex composed of NF-YA2, NF-YB2/NF-YB3, and NF-YC3/NF-YC9 directly regulates the flowering regulation genes SOC1 and FT, and the complex can interact with the CO gene of the photoperiodic pathway and the DELLAS gene of the gibberellin inhibitor in the flowering regulation pathway to cooperatively regulate the flowering time of *Arabidopsis thaliana* [39]. As an inhibitor of GA signals, the DELLA protein, has been proven to be related to NF-Y in several studies/reports. At the same time studies in tobacco found that GA3 treatment elevated AsNF-YB3 levels, which were similarly influenced by ABA and MeJA [40]. The NF-Y complex has recently been discovered to be implicated in the GA-induced dormancy transition in Arabidopsis. RGA inhibits flowering by preventing the interaction between NF-Y with CO [36]. NF-YC and RGL2 synergistically restricted the GA-mediated germination of seeds, according to Liu et al. (2016). They also noted that distinct DELLA-NF-Y (C) combinations have played a role in various biological processes. Consequently, these genes and their interaction are necessary for the regulation of flowering time and dormancy, and they also play significant roles in several diverse biological processes in plant species.

In this current study, HMMER 3.0 was used to identify *PmNF-Y* genes, and a detailed list of 27 *PmNF-Y* genes was established in *Prunus mume* (Table 1). Their physicochemical features were analyzed to predict their subcellular location. Different *PmNF-Y* proteins vary substantially in molecular weight, and their structure and composition may vary, indicating that their functions could be different. The majority of *PmNF-Y* genes are located in the nucleus, with a few in the chloroplasts and cytoplasm. Transcription factors are essential controllers of gene expression in the nucleus. The replication of genes serves a crucial function in biological evolution [41]. However, its related analysis revealed one gene pair (*PmNF-YB02*/*PmNF-YB03*) as a tandem repeat and two gene pairs (*PmNF-YB10*/*PmNF-YB11*, *PmNF-YB06*/*PmNF-YB13*) as segmental repeats (Figure 3), indicating that tandem and segmental repeats additionally support the *PmNF-Y* gene family's evolution and diversity. Moreover, the segmental gene pairs' Ka/Ks values were calculated. Here, we noticed that the Ka/Ks ratio of all *PmNF-Y* gene pairs is less than 1, implying that they are subjected to significant purification selection during evolution and might evolve slowly, which could avoid/prevent the transmission of genetic variations or mutations. The 27 *PmNF-Y* genes were subdivided into three subfamilies based on the phylogenetic tree. The greater level of homology between the *PmNF-Y* protein sequences of various Rosaceae plants within the same subfamily indicates that the gene structure of the NF-Y protein in *Prunus* plants is highly conserved. However, there are significant disparities in the number of motifs, protein conserved domains, and gene structure amongst subfamilies, indicating that they have distinct biological functions in plant species. We predicted the interactions between genes within *PmNF-Y* and other related key genes and found that *PmNF-Y* genes were not only highly interconnected within the family, but *PmNF-YA03*, *PmNF-YB07*, and *PmNF-YC09* also interacted with *PmABI5*, *PmKEG*, *PmDR1-AC*, *PmATRX-*

like, *PmB3-LEC2*, and *PmRGL2*. ABI5 is an ABA-signaling controller factor that integrates several signals, controls the gene expression of stress responses, and controls a variety of mechanisms: lateral root development, seed germination, photosynthesis, and seedling growth [42–44]. Meanwhile, E3 ligase KEG has been found to regulate plant immunity in Arabidopsis [45]. However, the Aux/IAAs gene family member, DR1-AC genes, have been expressed during fruit development and ripening, which are regulated via ethylene and auxin [7]. *PmB3-LEC2* is involved in seed development, and miR395abcd's control of its target gene ATRX plays a positive protective role in the low-temperature response in cassava [46,47]. RGL2 contributes to GA-induced floral development, including stamen growth/expansion and anther dehiscence, as well as being a negative regulator of GA-mediated seed germination in Arabidopsis [48]. The upstream promoter region of the *PmNF-Y* gene analysis results revealed that cis-acting elements could be divided into four categories: light response, growth metabolism, stress, and hormones. The *PmNF-Y* gene plays a significant role in several stress and hormone responses, suggesting that the *PmNF-Y* gene could be involved in the growth and development of *Prunus mume*. Except for photo-response, most cis-acting elements are hormones, while GA, ABA, and MeJA are, respectively, the main hormones. GA, ABA, and MeJA have always been considered the most closely related hormones in the process of plant dormancy [26,49]. Plant hormones play an important role in the regulation of plant dormancy. In the previous research of our research group, we found that the content of GA was the highest in the period of flower bud dormancy release, and there was a significant difference in the four dormancy stages. It was speculated that GA was related to dormancy release: ABA content was the highest in the paradormancy stage, decreased in the endodormancy and ecodormancy stages, and was the lowest in the dormancy release stage. The content of JA was relatively low in the period of paradormancy and endodormancy, while the content of JA was significantly higher in the period of ecodormancy than that of endodormancy. The content of JA was the highest at the dormancy release stage, which indicated that JA had an inhibitory effect on the flower bud dormancy of Prunus mume. Moreover, the expression levels of several genes (*PmNF-YA03*, *PmNF-YA05*, *PmNF-YB07*, *PmNF-YB08*, *PmNF-YB10*, *PmNF-YB13*, *PmNF-YC08*, and *PmNF-YC09*) varied significantly at different stages of dormancy. Furthermore, specific expression responses to different exogenous hormones and low-temperature treatments were identified in eight *PmNF-Y* genes. Therefore, we hypothesized that these genes may exhibit diverse mechanisms and functions that regulate plant dormancy.

5. Conclusions

We identified 27 *PmNF-Y* genes in the *Prunus mume* genome. Based on their evolutionary relationship, we grouped them into three subgroups, *PmNF-YA*, *PmNF-YB*, and *PmNF-YC*. The genes contain one (1) pair of tandem repeats and two (2) pairs of segmental repeats, with a Ka/Ks of less than one (1). The studied *PmNF-Y* gene has four types of cis-acting elements upstream: light response, growth metabolism, stress, and hormones, with hormones and light response containing the most cis-acting elements. According to RNA-seq data, most genes' expression varied slightly in different tissues. *PmNF-YA03*, *PmNF-YA05*, *PmNF-YB07*, *PmNF-YB08*, *PmNF-YB10*, *PmNF-YB13*, *PmNF-YC08*, and *PmNF-YC09* genes varied during dormancy. The qRT-PCR results showed that differentially expressed genes responded specifically to GA_4, ABA, MeJA, and low temperature. The genome-wide identification of the NF-Y transcription factor helps to understand the evolution and function of the NF-Y gene family in *Prunus mume* and provides a basis for further functional studies of these genes in *Prunus* genus species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8121180/s1, Table S1: Expression profile analysis of *PmNF-Y* genes in different tissues; Table S2: Expression profile analysis of *PmNF-Y* genes in different dormancy periods; Table S3: Primer sequences used in qRT-PCR.

Author Contributions: Z.G. and T.S. designed and supervised the study. F.G., X.H., R.W., Y.M., C.M. and Y.B. performed the experiment. Z.N. and P.Z. prepared the materials. F.G., X.H. and W.T. performed data analysis. F.G. drafted the manuscript. D.C. edited the manuscript. F.G., S.S., D.C., T.S. and Z.G. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: This manuscript and Supplemental Materials include all data analyzed or generated during the present study. The *Prunus mume* NF-Y gene family sequences used in this study were downloaded from the NCBI Genbank (https://www.ncbi.nlm.nih.gov, accessed on 1 January 2021).

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Conflicts of Interest: All the authors declare no conflict of interest.

Abbreviations

ABA	Abscisic acid
GA	Gibberellic acid
MeJA	Methyl jasmonate
NF-Y	Nuclear transcription factor Y
Pm	*Prunus mume*
NCBI	National Center for Biotechnology Information

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