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Identification and Analysis of *Phosphatidylethanolamine-Binding Protein* Family Genes in the Hangzhou White Chrysanthemum (*Chrysanthemum morifolium* Ramat)

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Abstract: The Hangzhou White Chrysanthemum (*Chrysanthemum morifolium* Ramat) is one of the "Zhejiang eight flavors" in traditional Chinese medicine. The *phosphatidylethanolamine-binding protein* (PEBP) plays an important role in flowering and floral organ development. Even so, the biological role of PEBPs in the Hangzhou White Chrysanthemum has not been studied, which attracted us. Here, nine *CmPEBP* genes that contain the PF01161 domain were identified in the Hangzhou White Chrysanthemum for the first time, and their biological role in flowering was preliminarily studied. A phylogenetic analysis classified the *CmPEBP* genes into three subfamilies: MFT-like, TFL-like, and FT-like genes. The differential expression analysis was performed under different tissues and different stressors using qRT-PCR. It showed that each *CmPEBP* displayed tissue-specific expression patterns. Expression patterns in response to different temperatures and hormone stressors were investigated. They were finally demonstrated to be differentially expressed. *TFL-like* gene expression, which delayed reproductive growth, was upregulated under heat stress. Conversely, *FT-like* gene expression was upregulated under low temperatures. *CmFT1* expression could be inhibited by GA (gibberellin), 6-BA (benzylaminopurine), ET (ethylene), and MeSA (methyl salicylate) but could be activated by IAA (indole-3-aceticacid), ABA (abscisic acid), and SA (salicylic acid) in the dark, whereas *CmFT2* and *CmFT3* expression levels were upregulated by ET, MeJA (methyl jasmonate), and ABA but were downregulated by 6-BA, SA, and MeSA. GA, IAA, SA, and MeSA inhibited *CmTFL* gene expression under light and dark treatments. Further research on *CmPEBP* genes in the Hangzhou White Chrysanthemum could better determine their roles in flowering and floral organ development, especially in response to the prolonged spraying of exogenous hormones.

Keywords: *phosphatidylethanolamine-binding protein*; Hangzhou White Chrysanthemum; gene family; exogenous hormone; flowering regulation

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1. Introduction

The *phosphatidylethanolamine-binding protein* (PEBP) is found in all taxa, from bacteria to eukaryotes, and plays an important role in the flowering time of plants [1,2]. *PEBP* genes are ancient, widespread, and highly conserved; they are found in bacteria, animals, and plants [3–5]. The PEBP family is usually divided into three subfamilies, including FLOWERING LOCUS T (FT)-like proteins [6], TERMINAL FLOWER 1 (TFL1)-like proteins, and MOTHER OF FT AND TFL1 (MFT)-like proteins [7]. *FT* encodes a protein that is considered to be the florigen that is transported from the leaves to the shoot apical meristem (SAM) [8–10]. *TFL1* genes are expressed in the inflorescence meristems and delay the flowering time by negatively regulating inflorescence development [11]. FT is a floral activator, whereas TFL1 is a floral repressor [12]. Although the functions of FT-like and TFL1-like genes are opposite, both are involved in flowering regulation [13]. There is a likelihood that most plants possess FT-like/TFL-like modules, and FT-like/TFL-like

gene regulation varies from species to species [2,14–16]. In addition, *MFT-like* genes play a significant role in seed germination and development [17]. Most researchers consider the MFT-like subfamily to be an ancestor of the FT-like and TFL1-like subfamilies [18]. Four and two *MFT* homologous genes were found in mosses and ferns, respectively, but with no *FT* or *TFL1* homologous genes, indicating that the FT/TFL1-like module was the result of the evolution of seed plants [19]. Hence, the function of FT-like genes may be to promote flowering and floral organ development, whereas TFL-like genes are inhibitory. Additionally, MFT-like genes may simultaneously perform the functions of promotion and inhibition [14–16].

In recent years, individual *PEBP* genes have been studied in many plant species, including more than ten dicotyledons and monocotyledons [3,13,20]. In dicotyledons, such as *Arabidopsis thaliana* [21], *Solanum lycopersicum* [10,22], *Glycine max* [3], *Gossypium hirsutum* [20,23], *Cucumis sativus* [24], and *Populus tremula* [25], the PEBP family comprises 6–9 genes. For example, the *PEBP* family in *Arabidopsis* is composed of six genes: *FT*, *TFL1*, *BFT*, *ATC*, *TSF*, and *MFT* [21]. However, the number of PEBP family members in monocotyledons is about three times that of dicotyledons. *Zea mays* [7], *Oryza sativa* [9], *Triticum aestivum* [26,27], and *Oncidium* Gower Ramsey [28] have 17 to 30 *PEBP* genes, respectively. This complexity of the *PEBP* family genes in monocotyledons suggests that the functions of this family are more complex in monocotyledons than in dicotyledons. Notably, no significant difference was observed in the number of TFL-like members in plants. However, the number of FT-like members is much higher in monocotyledons (more than 10) than in dicotyledons (usually 1–2). In contrast, plants with numerous FT-like members have fewer MFT-like members. The sequences of the PEBP family members in these species have high homology with those of *A. thaliana*, and their mechanism of action in flowering regulation is also similar [3,4].

Plants have produced many repetitive genes through large-fragment and genome-wide duplication during evolution. Some functions are redundant, some genes are silent or nonfunctional, and some neofunctionalization genes have functions that are due to mutations [29,30]. DPDxP and GxHR, which presumably contribute to the conformation of the ligand binding pocket, are considered to be two highly conserved characteristic short motifs of PEBPs [31,32]. Six conserved ligand-binding sites in the PEBPs of different species form a pocket-like structure in which two amino acids are the key sites that determine the function of FT/TFL1 [33]. Hanzawa et al. [34] found that, after replacing the single amino acid Tyr^{85} in FT with His, the function of FT may be partially converted to that of TFL1 due to molecular interaction or structural changes. In addition, Ahn et al. [35] suggested that the coding amino acid sequence of PEBPs is divided into four segments (A–D) based on conservation; of these, the fourth segment (segment D) plays a crucial role in determining the FT and TFL1 protein functions. Segments B and C, which contain the LYN/IYN triplet conserved motif, are especially important for the determination of the functional specificity between FT and TFL1 [35]. Pin et al. [36] also found that the three amino acids (Tyr138, Gly141, and Trp142) and the exons in the B segment of *BvFT1* and *BvFT2*, which are two FT paralogs with antagonistic functions in sugar beets, led to changes in promoting and inhibiting activities. Thus, the identification and classification of the *PEBP* family genes have become more important.

The Hangzhou White Chrysanthemum (*Chrysanthemum morifolium* Ramat), a perennial subshrub, is one of the famous "Zhejiang eight flavors" in traditional Chinese medicine. It is planted separately by pressing branches from April to May. After removing the terminal bud (20 August), the vegetative-to-reproductive growth is rapid with the decrease in temperature and the shortening of sunshine. The Hangzhou White Chrysanthemum is planted in open air; thus, its planting environment is different from other Chrysanthemum species that are used as ornamentals or are cultivated for cut flowers. If artificial light control and shading are used to adjust the flowering period of the Hangzhou White Chrysanthemum, the production cost greatly increases [37]. The complex process of the flowering transition is coregulated by both external (environmental) and internal factors,

such as the photoperiod, the temperature, and endogenous hormone-mediated pathways, to ensure that flowering occurs at an appropriate time [38–40]. Plant endogenous hormones, which act as major internal influencing factors, participate in the whole life process of plants and govern these processes by controlling their transcriptional and translational networks [41–44]. The exogenous application of hormones, such as auxin and cytokinin, is likely performed to supplement the content of endogenous hormones and to regulate their functions [45]. Therefore, studying the response patterns of the PEBP family genes to exogenous hormones may provide guidance for the flower bud differentiation and floral organ development of the Hangzhou White Chrysanthemum.

With recent advances in sequencing technologies, a rapid increase in sequenced plant genomes has been accessed in the past few years. Additionally, genome sequence databases have provided researchers with a wealth of encoded information [46–49]. However, the PEBP gene family identified in the plant species genome is still uncharacterized, particularly in its function and regulation. Especially for polyploid plants with deficient genomes or complexes, comparative genomics is a very good method for mining family genes or transcription factors [46,47,49–51]. In this study, we used the sequence of the *PEBP* family genes in the genome data of *Arabidopsis* and thirteen other species (*Citrus clementina*, *C. sinensis*, *C. sativus*, *G. max*, *Medicago truncatula*, *O. sativa*, *Physcomitrella patens*, *P. trichocarpa*, *Prunus persica*, *Selaginella moellendorffii*, *Sorghum bicolor*, *Vitis vinifera*, and *Z. mays*) to identify all the potential PEBP family genes of the Hangzhou White Chrysanthemum for the first time. Furthermore, we used bioinformatics methods to analyze *CmPEBP*'s characteristics based on the results of the gene structures, physical and biochemical properties, phylogenetic relationships, and conserved motifs. The tissue-specific expression patterns and the transcriptional responses to various abiotic stressors (low and high temperatures and multihormone treatments) of identified *CmPEBP*s were analyzed to examine their possible involvement in flowering regulation. Our study can enable the investigation of the detailed molecular and biological functions of PEBP members as well as provide a reference for the application of exogenous hormones to control flowering in the Hangzhou White Chrysanthemum.

2. Materials and Methods

2.1. Plant Materials and Abiotic Treatments

Seedlings of the Hangzhou White Chrysanthemum cultivar "Wanxiaoyangju" (*C. morifolium* cv. Wanxiaoyangju) were selected as study samples. In April 2019, equally sized seedlings were planted in 8 × 10 cm pots containing garden soil and vermiculite without additional fertilizer. Plants were grown with a natural photoperiod under greenhouse conditions at the Extension Center for Agricultural Techniques of Tongxiang City, Zhejiang Province, China (120.45343 N, 30.661041 E). Subsequently, plants were transplanted to the greenhouse of China Jiliang University, with a 12 h light (300 μmol·m^{-2}·s^{-1})/12 h dark cycle at 25 °C and 75 ± 5% relative humidity. Reasonable pest control and fertilizer management measures were adopted during the growing period [52].

To determine tissue-specific expression patterns of *CmPEBP* genes, we collected the roots, stems, leaves, petioles, and shoot apices in the vegetative growth stage (19 August) and flowers instead of shoot apices in the reproductive growth stage (7 November). For high and low temperature treatments, plants grown in plant growth chambers (25 ± 2 °C) were transferred to chambers maintained at 38 °C or 4 °C for 24 h [53]. For hormone treatments, the surface of plants was evenly sprayed with a fresh working solution of 100 μM indole-3-acetic acid (IAA), benzylaminopurine (6-BA), gibberellin (GA), abscisic acid (ABA), salicylic acid (SA), methyl salicylate (MeSA), or methyl jasmonate (MeJA) [54,55]. For ethylene (ET) treatments, the surface of plants was evenly sprayed with a fresh working solution of 100 μM Ethrel. For fat-soluble hormones (IAA, GA, etc.), an appropriate amount of 95% ethanol was used to dissolve them, and then they were diluted with distilled water to reach the working solution concentration (the final concentration of ethanol was 1%). For water-soluble hormones (ABA, etc.), 1% ethanol distilled water was directly used as the

solvent for their preparation. The control (CK) used 1% ethanol as the working solution. Plants were divided into two groups: dark treatment group (light-interrupted) and light treatment group (light-exposed). The control group included nonstress-treated plants. The fifth and sixth mature leaves in these groups were obtained at 0, 4, 12, and 24 h after the experiment began. Controls were set up at each time point, and for all the above assays, plants of approximately equal sizes were selected for all treatments. Three independent biological replicates of each sample were harvested. The samples were treated with liquid nitrogen immediately after collection and were stored at −80 °C for RNA extraction.

2.2. RNA Isolation and cDNA Synthesis

The sample was ground in liquid nitrogen, and 100 mg of powder was transferred to a 1.5-mL RNase-free centrifuge tube, and then the RNAprep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China) was used to extract RNA [55]. All instruments used were sterilized with diethylpyrocarbonate liquid in advance, and the isolated RNA was stored at −80 °C. The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the concentration of isolated RNA, and the quality was assessed using 1.2% formaldehyde–agarose gel electrophoresis. Finally, first-strand cDNA of the sample was synthesized with the PrimeScript II 1st Strand cDNA Synthesis Kit (TAKARA, Tokyo, Japan). cDNA was diluted 10 fold for PCR amplification.

2.3. Identification of PEBP Family Genes

Using "PF01161 domain" and "*phosphatidylethanolamine-binding protein*" as keywords, we searched the genomic databases of 13 plant species at different evolutionary stages, including *A. thaliana*, *C. clementina*, *C. sinensis*, *C. sativus*, *G. max*, *M. truncatula*, *O. sativa*, *P. patens*, *P. trichocarpa*, *P. persica*, *S. moellendorffii*, *S. bicolor*, and *Z. mays* (https://phytozome.jgi.doe.gov/pz/portal.html (accessed on 15 February 2021). The strategy and steps of obtaining each gene of the *PEBP* family in the plant genome were the same as those of Pan et al. [55]. Based on transcriptome databases previously generated for the Chrysanthemum cv. "Wanxiaoyangju", all partial nucleotide sequences of the *CmPEBP* family were initially obtained. Using BLASTx (NCBI) and SeqMan in the DNASTAR package (DNASTAR, Inc., Madison, WI, USA), nucleotide sequences with more than 60% identity were identified and assembled [56]. By comparing them with known *PEBP* sequences, the candidate sequences were verified with ClustalX [57]. Using Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA), specific primer pairs (Table S1) were designed to clone the full-length cDNA sequences, and these were synthesized by Sangon Biotech Co. (Shanghai, China). SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) was used to isolate full-length cDNA according to the manufacturer's instructions [58]. The PCR product was purified and ligated into the pMD-20T vector (TAKARA, Tokyo, Japan) using the Axygen Gel Extraction Kit (Axygen, Union City, CA, USA). The plasmid constructs were sequenced by Sangon Biotech Co. The sequence accuracy was confirmed through sequencing. To confirm the potential *CmPEBP* genes, the SMART (http://smart.embl-heidelberg.de/ (accessed on 18 February 2021) [59], Pfam 31.0 (https://www.ebi.ac.uk/interpro/ (accessed on 18 February 2021) [60], and NCBI (http://www.ncbi.nlm.nih.gov/ (accessed on 18 February 2021) [61] databases were used.

2.4. Analysis of Sequences

Information on the length of coding and amino acid sequences was determined using DNAMAN and DNASTAR software packages. The molecular weights and theoretical isoelectric points (pIs) were predicted using the ProtParam tool (http://web.expasy.org/protparam/ (accessed on 19 February 2021) [62]. Both WoLF PSORT (http://www.genscript.com/wolf-psort.html (accessed on 19 February 2021) [63] and TargetP (http://www.cbs.dtu.dk/services/TargetP/ (accessed on 19 February 2021) [64] were used to predict subcellular localization of proteins. Full-length sequences of *PEBPs* of Hangzhou White Chrysanthemum and *Arabidopsis* were aligned using ClustalW with default pa-

rameters in BioEdit [57]. To determine the subgroup classification of *CmPEBP* genes, the phylogenetic tree and conserved motifs were assessed for CmPEBP-encoded proteins. *Arabidopsis* and *Z. mays PEBPs* were used to classify the tea plant proteins into groups using the neighbor-joining method with 1000 bootstrap replicates in MEGA 6.0 [65]. To find their conserved motifs, full-length amino acid sequences of *CmPEBP* genes were entered into the MEME analysis tool (http://meme.sdsc.edu/meme/website/meme.html (accessed on 20 February 2021) [66], setting the maximum number of motifs to 14. Each de novo detected motif was searched for using the InterPro database (https://www.ebi.ac.uk/interpro/ (accessed on 21 February 2021) [67] to find similarities with known domains. The consensus sequence was separately scanned in the InterPro database to determine the domains present in preidentified PEBPs.

2.5. Gene Expression Analysis Using qRT-PCR

Specific primers (Table S2) for qRT-PCR were designed using Primer Premier 5 and were synthesized by Sangon Biotech Co. (Shanghai, China). The StepOnePlus™ real-time PCR (Thermo Fisher Scientific, Foster City, CA, USA) was used for the quantitative analysis. ROX reference dye was mixed with TB SYBR Premix Ex Taq II (Tli RNase H Plus, TAKARA) in advance to normalize signal and ensure data integrity. The reaction volume of qRT-PCR was 25 µL, which contained 12.5 µL of SYBR Premix Ex Taq II (Tli RNase H Plus), 1 µL of forward primer, 1 µL of reverse primer, and 2 µL of cDNA template, and ddH_2O was added to make the volume 25 µL. The reaction program was performed under the following conditions: 95 °C for 30 s followed by 40 cycles at 94 °C for 5 s and 60 °C for 30 s. The mean and standard deviation values were calculated based on three independent biological replicates. *CmEF* was used as a reference gene to normalize the expression of related genes [68].

The relative gene expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method [69]. When calculating the expression level of different tissues, the expression level of the stem in the vegetative growth stage was calculated as 1, and log2 of the expression level was used to convert the heatmap. In the stress-treated plant samples, values were normalized to plant samples of the 0 h treatment and were expressed as log2 fold changes. In the histogram, values of tissues were normalized to those of bud tissues, whereas values for seed dehydration treatment samples were normalized to those of the 0 h treatment (expression = 1). Differences in gene expression levels were detected using Tukey's multiple range test at the 0.05 probability level. Three independent qRT-PCR experiments were performed on total RNA samples.

3. Results

3.1. Isolation and Identification of the PEBP Family Genes in the Chrysanthemum

Based on the transcriptome databases previously generated for the Chrysanthemum cv. "Wanxiaoyangju", more than 40 candidate *PEBP* genes were obtained. Several sequences were excluded because they shared an approximately 99% open reading frame identity with other candidate *CmPEBP* genes (data not shown), and nine genes were further analyzed. Each unique gene was named according to the *Arabidopsis* nomenclature. The coding sequences of all the *CmPEBP* genes were submitted to GenBank with the following accession numbers: *CmFT1* (OP617277), *CmFT2* (OP617278), *CmFT3* (OP617279), *CmTFL1* (OP617271), *CmTFL2* (OP617272), *CmTFL3* (OP617273), *CmTFL4* (OP617274), *CmMFT1* (OP617275), and *CmMFT2* (OP617276). The gene names, accession numbers, full-length sequences, and other bioinformatics analysis results are summarized in Table 1. The nucleotide sequence lengths of the identified *PEBP* family genes were between 507 and 531. The PEBP family gene-encoded proteins ranged from 167 (CmTFL2) to 176 (CmTFL3) amino acids (average of 172 amino acids), with a predicted molecular mass of 18.45–19.79 kDa and a pI ranging from 4.71 (CmMFT1) to 9.39 (CmTFL2). Most of the *CmPEBP* family genes were predicted to be located in the cytoplasm, nucleus, and chloroplast, and these predictions might be related to their functions [3,7,20,31].

Table 1. The 9 CmPEBP genes in the Chrysanthemum morifolium.

Gene Names	Accession Nos	Open Reading Frame (bp)	Number of Deduced Amino Acid (aa)	Molecular Weight (kDa)	Subcellular Location (WoLF PSORT/TargetP)	Theoretical pI	Groups
CmFT1	OP617277	525	174	19.26	chlo/cyto/nucl	6.83	Group A
CmFT2	OP617278	525	174	19.79	cyto/chlo/nucl/mito	8.64	Group A
CmFT3	OP617279	525	174	19.52	cyto/nucl/plas/chlo	6.82	Group A
CmTFL1	OP617271	507	168	19.04	cyto/nucl	8.96	Group B
CmTFL2	OP617272	504	167	19.14	cyto/nucl/extr	9.39	Group B
CmTFL3	OP617273	531	176	19.87	cyto/chlo/vacu	8.86	Group B
CmTFL4	OP617274	522	173	19.44	cyto	8.93	Group B
CmMFT1	OP617275	507	168	18.45	cyto/cysk	4.71	Group C
CmMFT2	OP617276	522	173	18.99	cyto/chlo	6.83	Group C

WoLF PSORT [63] and TargetP [64] were used to predict the subcellular localization of the 9 *CmPEBP* family genes; the most likely locations are listed. The abbreviation chlo represents chloroplast, cysk represents cytoskeleton, cyto represents cytoplasmic, extr represents extracell, mito represents mitochondrion, nucl represents nucleus, plas represents plasma membrane, and vacu represents vacuole. Groups were classified based on phylogenetic trees with their corresponding numbers in *Arabidopsis* and *Z. mays*.

3.2. Similarity and Phylogenetic Gene Structure Analysis

To further analyze the phylogenetic relationships between the *PEBP* family members in the Chrysanthemum and other species, a phylogenetic tree was generated based on the full-length *PEBP* sequences from *C. morifolium*, *Arabidopsis*, *C. clementina*, *C. sinensis*, *C. sativus*, *G. max*, *M. truncatula*, *O. sativa*, *P. patens*, *P. trichocarpa*, *P. persica*, *S. moellendorffii*, *S. bicolor*, *V. vinifera*, and *Z. mays*. From a functional point of view, the function of FT-like genes is to promote flowering and floral organ development, whereas that of TFL-like genes is inhibitory. Additionally, MFT-like genes have the functions of simultaneous promotion and inhibition [23,31]. All sequences could be divided into three subfamilies: MFT-like, TFL-like, and FT-like genes. Except for nonflowering plants (*P. patens* and *S. moellendorffii*), all the plants had the three subfamily members. No significant difference was observed in the number of TFL-like members between any of the plants except for soybean. Notably, the number of FT-like members was much higher in monocotyledons (more than 10) than in dicotyledons (usually 1–2). In contrast, plants with many FT-like members had fewer MFT-like members. MFT-like genes might be the evolutionary source of FT-like and TFL-like genes, and their differentiation might have occurred when flowering plants appeared. Meanwhile, the evolutionary rate of FT-like genes was faster than that of TFL-like genes. Similarly, FT-like genes changed in two directions: one was to reduce the number of redundant members (dicotyledons), and the other was to increase the number of members (monocotyledons). In addition to soybean and *M. truncatula*, the FT-like genes showed significant differences between dicotyledons and monocotyledons. The FT-like genes were more conservative in dicotyledons and were more diverse in monocotyledons, and they could be divided into three subgroups [3,20]. Therefore, the *CmPEBP* genes were classified into three subfamilies (Figure 1, Table 1). A multiple sequence alignment program was used to analyze the nine open reading frames and their encoded amino acid sequences. The results of their similarities are listed in Table S3. The nucleotide sequence similarities varied from 34.7% (*CmFT3* and *CmMFT1*) to 99.0% (*CmTFL1* and *CmTFL2*), whereas proteins exhibited similarities from 17.1% (*CmTFL4* and *CmMFT1*) to 93.7% (*CmFT2* and *CmFT3*).

The predicted amino acid sequences of the 9 *CmPEBP* and their corresponding sequences in 13 plants, including *Arabidopsis*, *C. clementina*, *C. sinensis*, *C. sativus*, *G. max*, *M. truncatula*, *O. sativa*, *P. patens*, *P. trichocarpa*, *P. persica*, *S. moellendorffii*, *S. bicolor*, and *Z. mays* were aligned using the ClustalW2 sequence alignment program. The phylogenetic tree was constructed using the MEGA6 software with the neighbor-joining tree method with 1000 bootstrap replicates. Three subgroups were shown in various colors, and the sequences of the Chrysanthemum, Arabidopsis, *S. moellendorffii*, and *P. patens* were highlighted with black dots, triangles, diamonds, and squares, respectively.

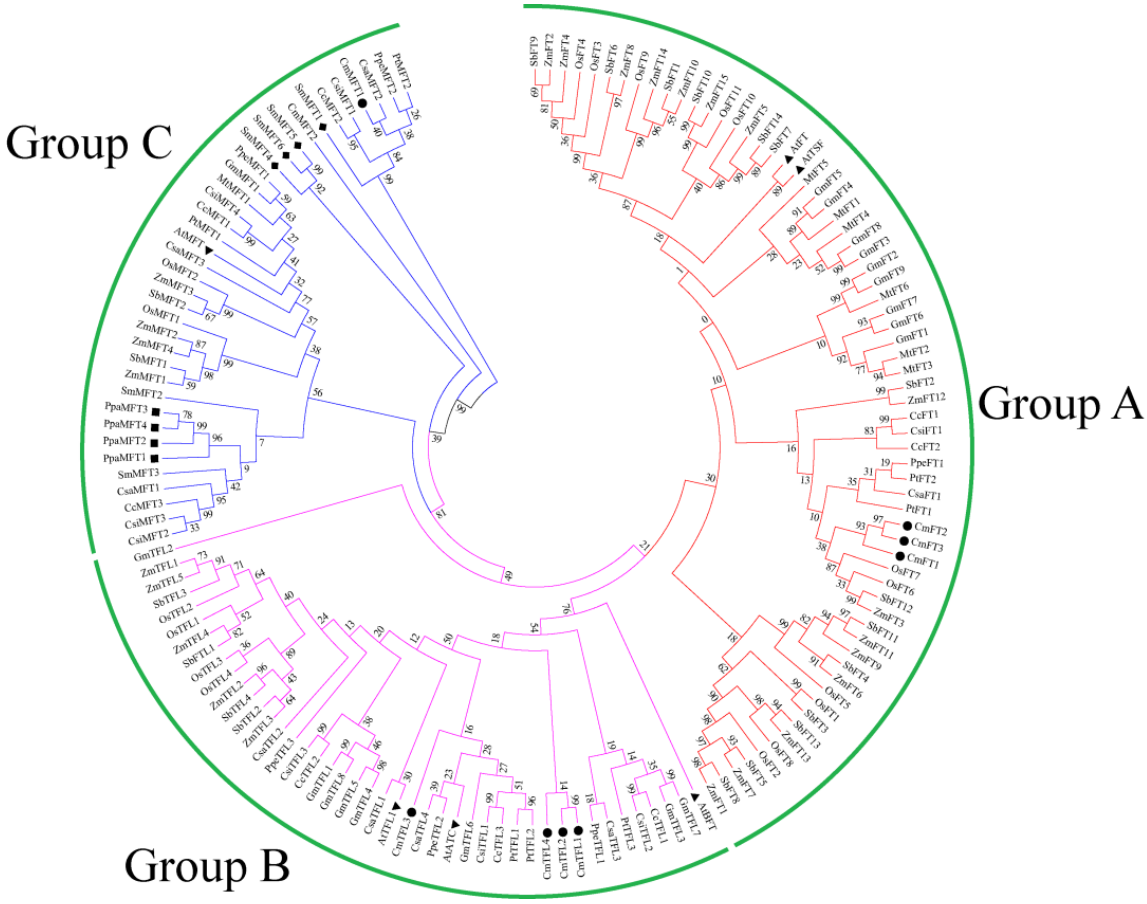

Figure 1. Phylogenetic analysis of putative PEBP family proteins in Chrysanthemum.

The alignment showed that these sequences contained the PBP (PF01161) domain (Figure 2). The proposed consensus sequences DPPxP and GxHR were highly conserved in the FT-like and TFL-like subfamilies (Figures 2, S1 and S2) but were only partially conserved in the MFT-like subfamily (Figures 2 and S3). The blue triangles indicate amino acid residues that interact with 14-3-3 proteins, which were conserved in the FT-like and TFL-like subfamilies but not in the MFT-like subfamily [18,32]. The key amino acid residues that were distinguishable among the FT-like (Y), TFL-like (H), and MFT-like (W) clades were present at position 85 of AtFT in the CmPEBP family proteins (excluding CmMFT2) (Figures 2 and S1–S3) [3,18,20]. However, after analyzing the amino acid sequences of the MFT-like clade in the 13 plants, the key amino acid residues might be Y and L in addition to W (Figure S3). Yang et al. found two highly conserved amino acid sequences, LGRQTVYAPGWRQN and LYN triad, which are considered to be determinant for FT activity and FT/TFL1 function [18]. However, these sequences were conserved only in some sequences of the FT-like subfamily. After comparing all the sequences of the LYN triad, the conserved sequences were mainly LYNL or IYNL in the FT-like subfamily and ENxL in the TFL-like subfamily, and no conservation was found in the equivalent position of the MFT-like subfamily (Figures S1–S3). According to the analysis results of the above conserved sequences and sites, the *CmPEBP* genes were divided into three subfamilies. These individual genes play a specific role in the flowering process and in floral organ development. Although the diversity of the binding sequences might be related to the diversity of the substrates, more research is needed to clarify this relationship.

The putative Chrysanthemum and Arabidopsis PEBP amino acid sequences were aligned using the ClustalW method and were edited in BioEdit. Identical and similar residues are shaded in black and gray, respectively. The proposed consensus sequence DPDxP, the GxHR motif, and I/LYN were highlighted with the boxes, respectively. The blue

triangles indicate amino acid residues that interact with 14-3-3 proteins. The red triangle indicates a key amino acid residue that may determine FT-like and TFL1-like functions.

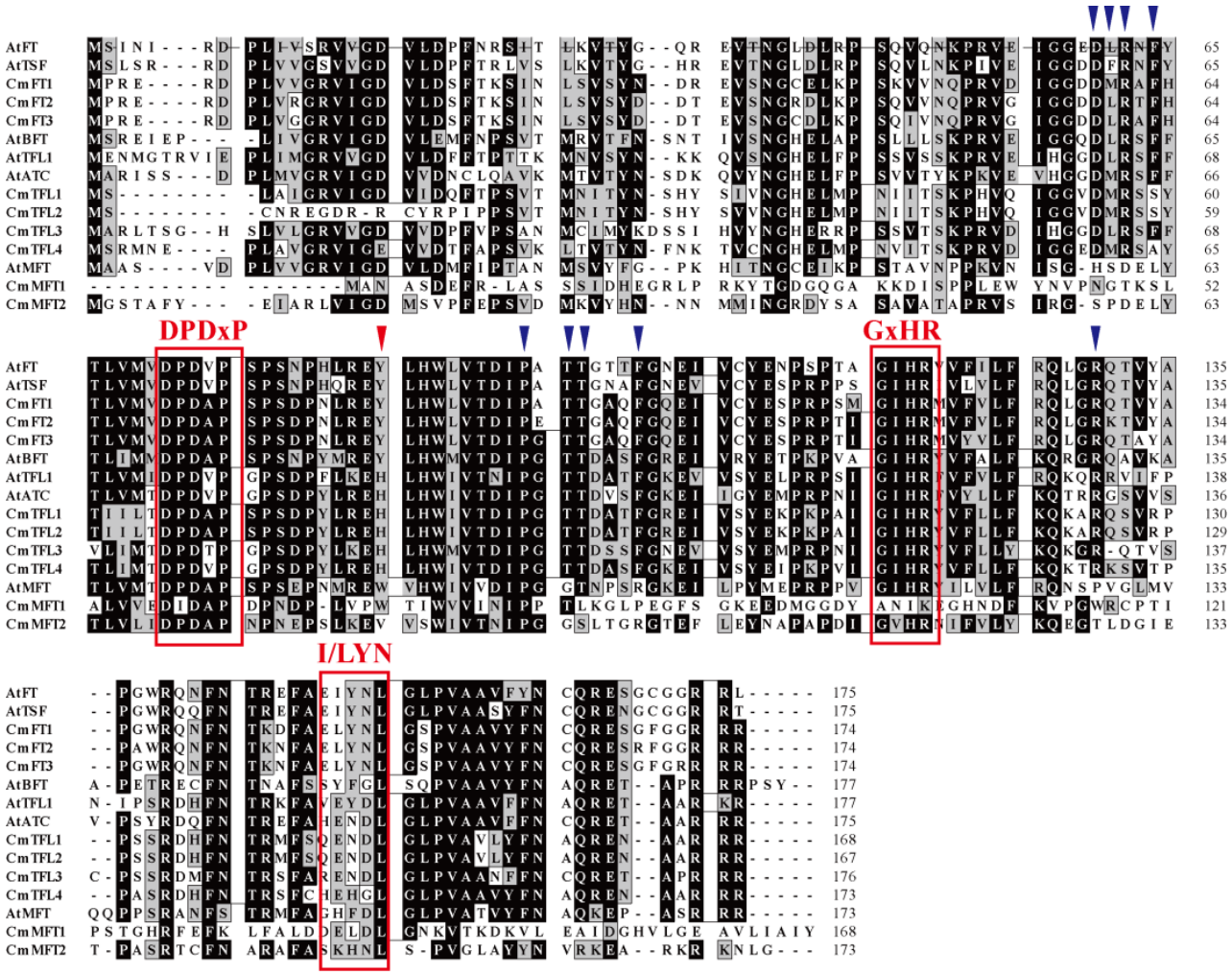

Figure 2. Alignment of the predicted amino acid sequences for the CmPEBP and AtPEBP genes.

A phylogenetic tree was constructed, and 14 discrete motifs were found, using AtPEBPs and ZmPEBPs to further assess and classify the CmPEBPs (Figure 3). The PBP domain might consist of motifs 1–5. Because of the lack of motifs 1–5, the CmMFTs were clearly distinguished, and because of the lack of motif 4, CmTFL2 was separated from the other TFL-like and FT-like members. Among all the CmPEBPs, motifs 6–14 were highly diversified. Thus, the CmFT proteins were relatively consistent, whereas the CmTFL proteins showed more diversified changes. The CmPEBPs could be classified into four clades when analyzed together with the motifs. Motif 1 might be contained in the proposed consensus sequences DPPxP and GxHR, whereas motif 2 might be contained in the LYN triad. These motifs might be involved in the binding of the substrate; thus, more studies are needed to clarify this relationship. The results of the MEME prediction suggested evolutionary conservation in the basal architecture of the PEBP family members.

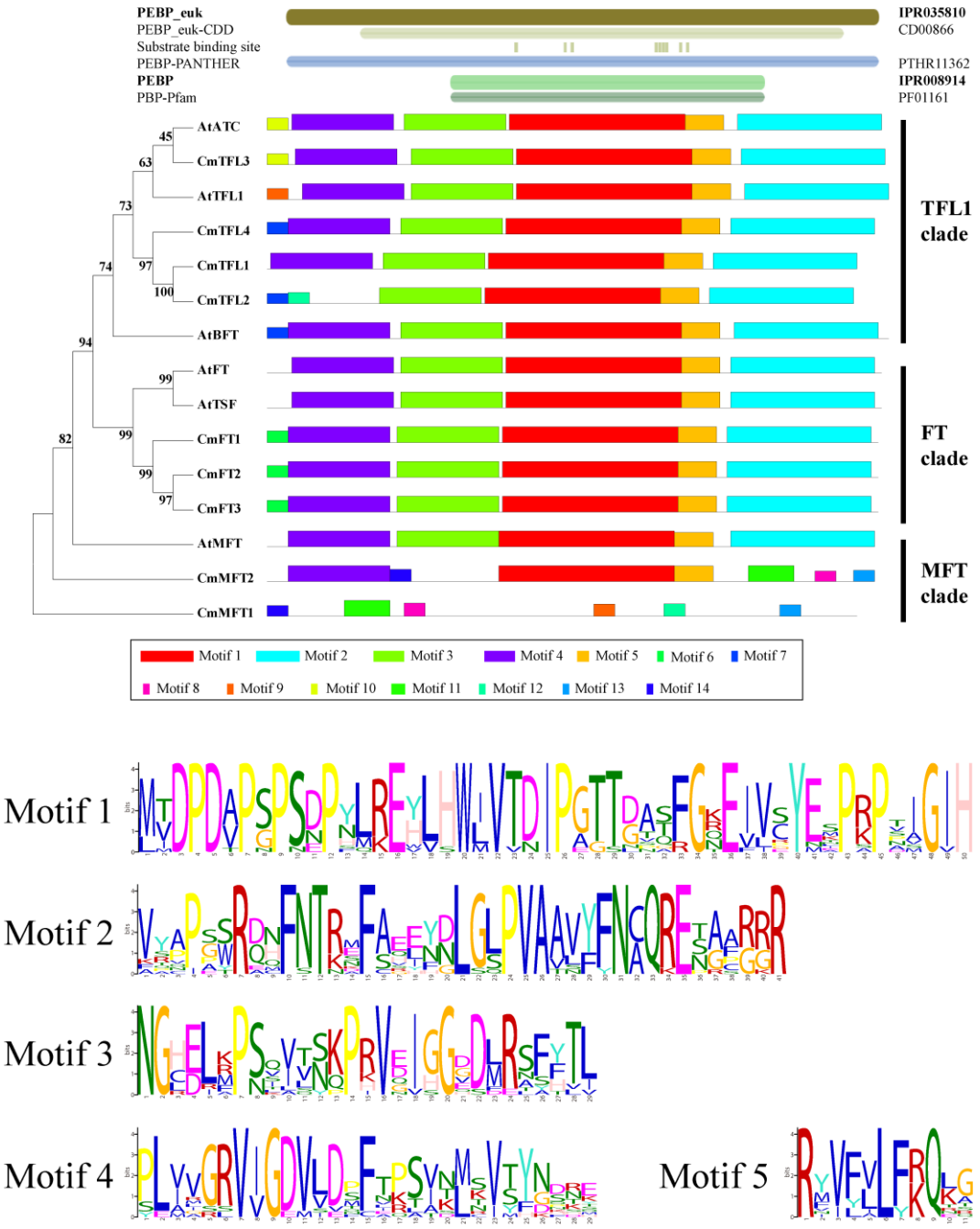

Figure 3. Conserved motif identification of PEBP proteins in Chrysanthemum and Arabidopsis. Interpro analysis revealed domains in previously defined PEBP proteins to be a part of *phosphatidylethanolamine-binding protein* family. De novo motif identification of PEBP proteins; motifs 1, 2, 3, 4, and 5 show resemblance to PBP (PF01161) domain.

3.3. Tissue-Specific CmPEBP Gene Expression

During flowering, the SAMs transform into floral meristems and then become floral organs, switching the plant from vegetative to reproductive growth [70]. There is a likelihood that most plants possess FT-like/TFL-like modules, but how the FT-like/TFL-like genes are regulated varies from species to species [16].

The tissue specificity of *CmPEBP* gene expression might be closely associated with the physiological and biochemical functions of each tissue. Therefore, analyzing *CmPEBP* gene expression in specific tissues and organs is important. The transcript abundance of nine *CmPEBP* genes in five tissues, including the roots, stems, leaves, petioles, and shoot apices,

was obtained. These genes were ubiquitously expressed in all the tissues with variable transcript levels (Figures 4 and S4). In the vegetative growth stage, most of the *CmPEBP* genes showed lower expression levels in the roots and leaves, while they showed higher expression levels in the petioles and shoot apices. In the reproductive growth stage, most of the *CmPEBP* genes (*CmFT1*, *CmFT2*, *CmFT3*, *CmMFT1*, *CmTFL1*, and *CmTFL2*) showed expression levels that were several folds lower in the leaf and petiole tissues, whereas some of the other *CmPEBP* genes (*CmTFL1*, *CmTFL2*, and *CmTFL4*) showed higher expression levels in the root and stem tissues compared with the other three tissue types. Overall, each *CmPEBP* gene showed tissue-specific expression patterns [18,31], although the levels of expression varied greatly.

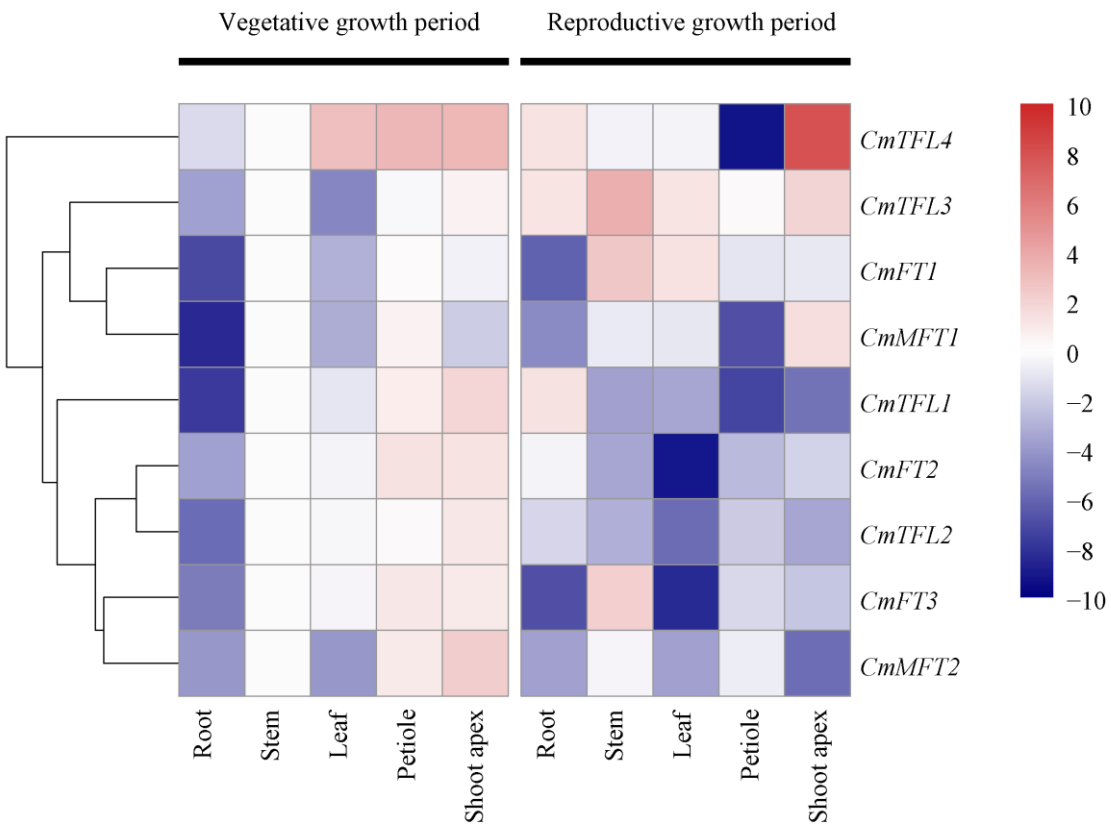

Figure 4. Tissue-specific expression of *CmPEBPs*. *CmPEBP* transcript abundance in root, stem, leaf, petiole, and shoot apex tissues was analyzed using qRT-PCR. The results were calculated using the $2^{-\Delta\Delta Ct}$ method and the *CmEF* housekeeping gene, and the average log2 values of three replicates were used to generate a heat map in R software. The intensity value bars are shown to the right of the heat maps. Bule represents low expression, white represents no significant difference in expression, and red denotes high expression.

3.4. Differential CmPEBP Gene Expression under High and Low Temperature Treatments

The regulation of flowering mainly includes the photoperiod pathway, vernalization pathway, and temperature pathway [71]. Temperature affects flowering time by influencing the development rate and vernalization [39]. High temperatures may reduce or delay flowering in the Chrysanthemum [38]. To understand the expression profiles of the *CmPEBP* genes under different temperature stressors, the qRT-PCR experiments were performed on the Chrysanthemum plants subjected to high (38 °C) or low (4 °C) temperature treatments. The differential expression levels of the *CmPEBP* genes were observed under two stressors (Figure 5). Because the expression levels of *CmMFT2* and *CmTFL4* in the leaves were low, the stressors of the leaves were not used for the *CmMFT2* and *CmTFL4* expression analyses.

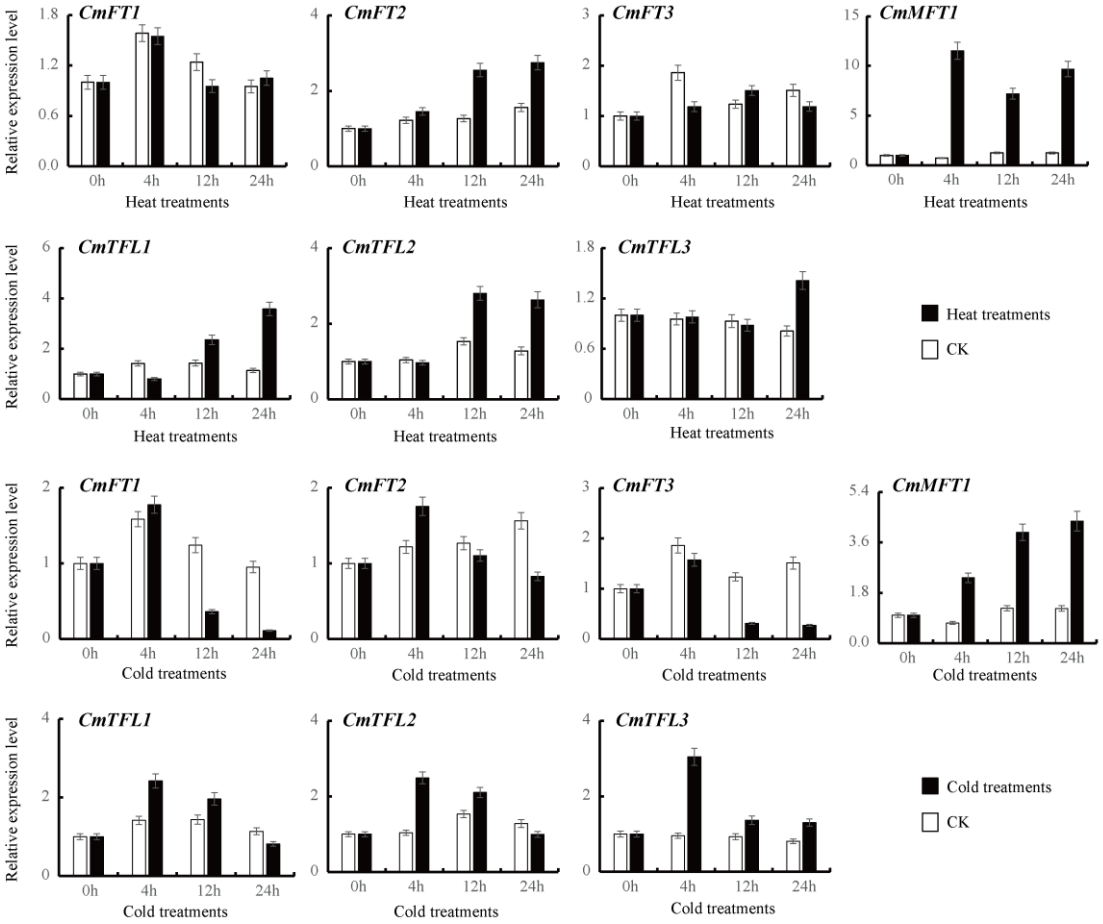

Figure 5. Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under heat (up) and cold stressors (down).

The genes under different temperatures had diverse expression patterns. The *TFL-like* genes were significantly upregulated in response to 24 h heat stress (Figure 5). Particularly, *CmTFL1* and *CmTFL2* showed expression levels that were several folds higher after 24 h heat stress. In contrast, the highest levels of *CmTFL1*, *CmTFL2*, and *CmTFL3* were observed at 4 h in response to cold stress. The transcript levels of the FT-like genes (*CmFT1* and *CmFT3*) were downregulated with cold stress, while the FT-like genes displayed no significant changes in response to heat stress. Notably, *CmMFT1* was upregulated at least five fold in response to heat and cold stress. Similar conclusions were drawn from previous studies [25,39].

3.5. Differential CmPEBP Gene Expression under Hormone Treatments

Hormones play an important role in the flowering process of plants [42–44,72]. For example, the IAA content is significantly lower in early-spring-flowering plants than in unflowered plants, whereas the content of GA_3 and ABA is higher [73]. To better understand the changes in the *PEBP* family genes in the Chrysanthemum under hormone treatments, a qRT-PCR experiment was performed. Plant materials were divided into dark and light treatment groups, and they were subjected to various hormone treatments, such as IAA (100 μM), 6-BA (100 μM), GA (100 μM), ABA (100 μM), ET (100 μM), SA (100 μM), MeSA (100 μM), and MeJA (100 μM). The differential expression levels of the *CmPEBP* genes were observed under the hormone treatments (Figure 6).

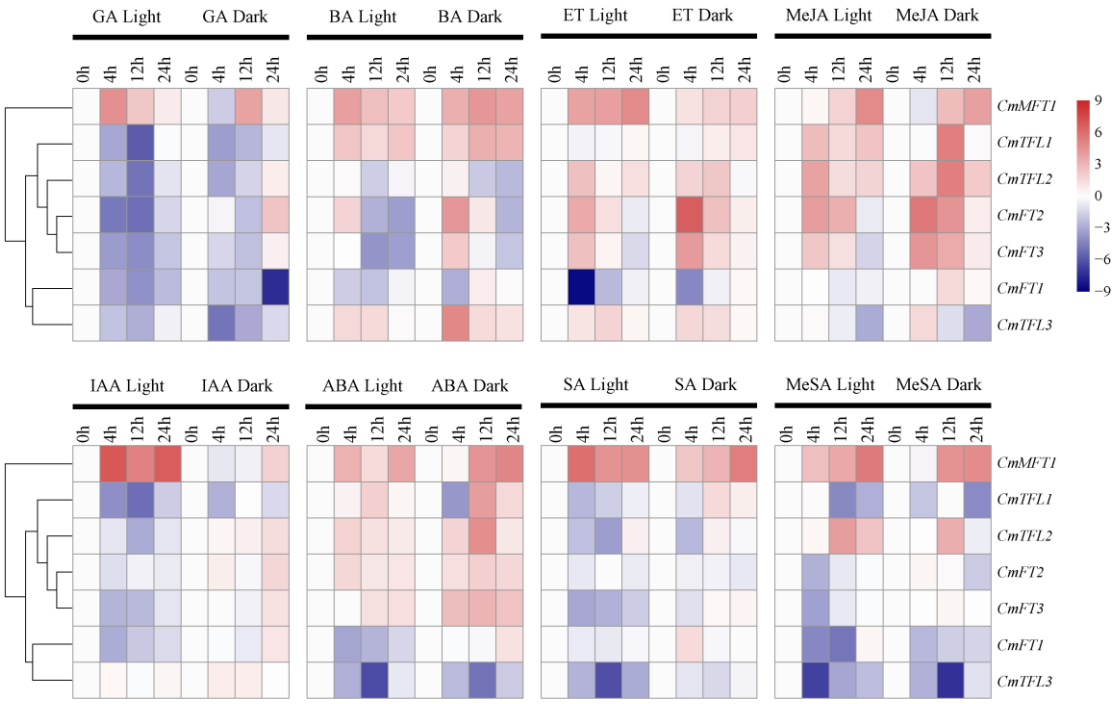

Figure 6. Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under eight hormone treatments. Gene expression analyses were examined through qRT-PCR using cDNA from fifth and sixth mature leaves in Chrysanthemum after treatment with eight hormones for 0, 4, 12, and 24 h, respectively. The results were calculated using the $2^{-\Delta\Delta Ct}$ method and the *CmEF* housekeeping gene, and the average log2 values of three replicates were used to generate a heat map in R software. The intensity value bars are shown to the right of the heat maps. Bule represents low expression, white represents no significant difference in expression, and red denotes high expression.

GA plays an important role in one of the major flowering pathways—the GA pathway—and exogenous GA can promote flowering in various plants [74]. Exogenous GA_3 could promote flower bud development and could finally improve the flowering quality of the tree peony [75]. Under GA stress (Figures 6 and S5), only *CmMFT1* was significantly upregulated during 24 h stress. In the light treatment group, almost all the expression levels of the *CmPEBP* genes dropped during the 24 h treatment period, with the same patterns of expression. They had the lowest regulation at 12 h, with a reduction of 7–50 times, followed by that at 4 h, and the lowest downregulation was at 24 h of GA treatment. In the dark treatment group, *CmFT2*, *CmFT3*, and *CmTFL2* were initially downregulated after GA stress, and then their expression increased with time (at 24 h). The remaining three genes (*CmFT1*, *CmTFL1*, and *CmTFL3*) were downregulated to varying degrees, especially *CmFT1*, as it was downregulated at least 170 fold at 24 h of GA stress. Overall, the *CmPEBP* genes were downregulated with the GA treatment except for *CmMFT1*.

Upon 6-BA treatment, the *CmTFL2* and *CmFT* genes displayed similar expression patterns in the light treatment group, and all four genes were downregulated by 6-BA (Figures 6 and S6). Simultaneously, the others (*CmMFT1*, *CmTFL1*, and *CmTFL3*) showed an upward trend under both the light and dark treatments. Among these, *CmMFT1* was greatly influenced by 6-BA during 24 h stress, and *CmTFL3* expression was at its maximum value at 4 h of dark treatment, reaching 30 times that of the control group. In addition, the expression levels of almost all the *CmPEBP* genes increased under the short-term 6-BA treatment.

Most of the genes in the Chrysanthemum, particularly *CmFT2*, *CmFT3*, *CmMFT1*, *CmTFL2*, and *CmTFL3*, were upregulated after ET stress in both the light and dark treatments (Figures 6 and S7). Among these, *CmFT2* and *CmFT3* had the same expression

patterns; gene expression under the dark treatment was usually greater than that under the light treatment, showing the highest level at 4 h followed by that at 12 h. The *CmFT1* gene was downregulated during the light and dark treatments, and the lowest levels were observed at 4 h in the light treatment group, dropping to 0.25% of the control group. Overall, ET had the effect of increasing the expression level.

Upon MeJA stress, the responses among the *CmPEBP* genes were different. In the dark treatment group, *CmTFL3* slipped to 42.98% of the control group at 12 h and declined to 12.68% at 24 h, whereas the other six genes showed various degrees of upregulation (Figures 6 and S8). The expression levels of *CmTFL1* and *CmTFL2* reached their highest values at 12 h of treatment, reaching about 40 times that of the control group, while that of *CmFT2* and *CmFT3* reached their highest values at 4 h. In the light treatment group, *CmMFT1*, *CmTFL1*, and *CmTFL2* showed an upward trend, and *CmMFT1* expression surged to 28 times that of the control group at 24 h.

IAA is a major growth hormone that stimulates plant growth in response to vernalization and has a dual function in flowering regulation [76]. Under the IAA treatment, *CmTFL2* and three *CmFT* genes showed similar expression patterns: their expression levels showed varying degrees of decline in the light treatment group but were first reduced and then climbed to two–three times that of the control group at 24 h in the dark (Figures 6 and S9). Meanwhile, *CmMFT1* maintained a high expression level under the light treatment which was 30–100 times that of the control group. *CmTFL1* was downregulated under exogenous IAA stress at 24 h, and the lowest level was observed at 12 h in the light treatment group. *CmTFL3* displayed no significant changes in response to IAA at 24 h in both the dark and light treatment groups.

Controversy remains over the role played by exogenous ABA at present. It has been mostly related to water stress, and one report suggested that exogenous ABA delays the flowering time in *Arabidopsis* [77]. ABA can promote the transcriptional upregulation of florigen genes [78]. Several genes, notably *CmMFT1*, *CmTFL2*, *CmFT2*, and *CmFT3*, were upregulated after ABA stress in both the light and dark treatments (Figures 6 and S10). In contrast, *CmTFL3* transcription was suppressed by ABA in both the dark and light during 24 h stress. *CmFT1* displayed different expression patterns: under light treatment, it was downregulated, but in the dark, it displayed no significant changes in its expression level compared with the control group. In contrast, ABA stress upregulated most of the *CmPEBP* genes, except for *CmFT1* and *CmTFL3*.

The application of exogenous SA affects many of the physiological processes of plants, including flowering; thus, SA is considered to be a new class of plant hormones. Since the 1990s, SA has been studied as a signaling molecule required for plant responses to stress [79]. Expression under the SA treatment was different from that under the ET and ABA treatments (Figures 6 and S11). Among the seven genes, only the *CmMFT1* gene was highly upregulated; a 60-fold increase in the expression level occurred at 4 h in the light treatment group, and it surged from 12 times compared with the control group (first 12 h) to 40 times at 24 h in the dark. *CmFT3* and the *CmTFL*s were suppressed in the light treatment group, especially *CmTFL3*, as it showed a 70-fold lower expression level after SA stress at 12 h. In the dark treatment group, they were downregulated by SA stress at 4 h but showed no significant changes compared with the control group after that time point. *CmFT1* and *CmFT2* displayed no significant changes in response to SA in both the light and dark treatment groups.

Gene expression under the MeSA treatment was comparable with that under the SA treatment, and only *CmMFT1* and *CmTFL2* exhibited highly sensitive upregulation in both the light and dark treatment groups (Figures 6 and S12). The upregulation of *CmMFT1* was more sensitive than that of *CmTFL2*, showing 44-fold higher expression in the light treatment group than in the control group at 24 h. Among the low expression genes, *CmTFL3* dropped to 0.96% of the control group under the light treatment at 4 h, and it slipped from 14.78% of the control group to 0.64% at 12 h.

4. Discussions

PEBPs play an important role in the flowering time of higher plants [1,2]. Plant endogenous hormones are major internal influencing factors that participate in the flowering time of higher plants [41–44]. Although extensive studies have been performed, knowledge of flowering time control is limited. Therefore, it is meaningful to study the relationship between *PEBP* family genes and endogenous hormones. Due to the complexity of polyploid plants and the deficiency of the genome of the Hangzhou White Chrysanthemum, knowledge on the *CmPEBP* family genes has remained relatively backward, which is similar to wheat [46–48,50]. In the present study, nine full-length *CmPEBP* coding genes were identified through homology studies using bioinformatics methods, and the results were consistent with the results of other species [3,4,7,9,18,19], all of which contain three subfamilies: MFT-like, TFL-like, and FT-like genes. These results agreed with those of other plants, indicating that the *PEBP* genes are conserved among species [3,4,18,19]. DPDxP and GxHR, which were found in the FT-like and TFL-like subfamilies, are considered to be two highly conserved characteristic short motifs of PEBPs [31,32]. Except for in the nonflowering plants (*P. patens* and *S. moellendorffii*), these sequences can be classified into three subfamilies, including A, B, and C. Most of the *CmPEBP* family genes were predicted to be located in the cytoplasm, nucleus, and chloroplast. Hou et al. [28] found that the FT protein was localized in the cellular endoplasmic reticulum. However, with the improvement and perfection of the Chrysanthemum genome database, further research is warranted on *CmPEBPs*, such as research on chromosomal localization and the exon–intron organization of the corresponding *CmPEBP* genes.

For group A, FT-like genes are an early target gene for CONSTANS (CO) activation, and their expression is activated by the direct transcription of CO induced by the photoperiod [15,80]. Long- and short-day plants perceive light signals through their leaves under the appropriate photoperiods, and then the CO protein initiates the activation of the *FT* gene in the leaves [15,26,80]. The FT protein is then transferred from the phloem to the SAM and activates flowering locus D (FD) gene expression in the SAM [8,26,35]. The expression of floral-meristem-specific genes, such as *AP1*, *LFT,* and, *SOC1*, is jointly activated by the FT and FD proteins, forming a protein complex that causes flowering [26,35,80]. To date, *Arabidopsis* contains two paralogous FT-like genes, *AtFT* and *AtTSF*, whereas rice contains eleven paralogous FT-like genes, *OsFT1* to *OsFT11* [3,20]. In the present study, a similar number of *CmFT*s were isolated and identified, including *CmFT1* to *CmFT3*. The FT-like genes also changed in two directions: one was to reduce the number of redundant members (dicotyledons), and the other was to increase the number of members (monocotyledons). The FT-like genes were more conservative in dicotyledons and were more diverse in monocotyledons, and they could be divided into three subgroups [3,20]. From the evolutionary tree, it can be clearly found that monocotyledons have more than 10 FT proteins at a higher level of classification and that they have all the member types of group A, whereas dicotyledons usually have only 1–2 FT proteins and only 1 of the member types of group A. FT-like genes plays an important role not only in the transformation and development of flowers but also in the regulation of tuber formation, seed germination, bud germination, branching, and stomatal opening [43,44]. More research may be needed in monocotyledonous plants.

For group B, TFL1 prolonged vegetative growth in *Arabidopsis* by maintaining the infinity of the SAM, thus delaying reproductive growth [16,24,28,35]. Conti et al. [43] showed that TFL-like proteins are synthesized in the leaves and are transported to the SAM to inhibit the expression of floral-meristem-specific genes such as *AP1*, *LFT*, and *SOC1*. TFL-like proteins are also encoded by multiple genes in all plant species. There are three TFL-like members in *Arabidopsis* and four TFL-like members in rice [3,9]. In this study, the number of TFL-like members in the plants was 3–5 with no significant difference except for in soybean. Four *CmTFL* isoforms were confirmed and sequenced through qRT-PCR amplification in the Hangzhou White Chrysanthemum. The proposed consensus sequences DPPxP and GxHR were also highly conserved in the TFL-like subfamily (Figures 2, S1 and S2).

We also found the conserved amino acid residues that interacted with 14-3-3 proteins, which were consistent with previous studies [18,20,23,31]. Two highly conserved amino acid sequences (LGRQTVYAPGWRQN and the LYN triad), which are closely related to FT activity and FT/TFL1 function [18,31], were also confirmed. After comparing all the sequences of the LYN triad, the conserved sequences were mainly LYNL or IYNL in the FT-like subfamily and ENxL in the TFL-like subfamily. These differences might be related to FT-like and TFL-like functions, and could also be used to distinguish, identify, and group PEBP members.

For group C, the MFT-like subfamily is the ancestor of the FT-like and TFL1-like subfamilies [17,19]. MFT-like genes play a significant role in seed germination and development [17]. The function of FT-like genes is to promote flowering and floral organ development, whereas that of TFL-like genes is inhibitory, and MFT-like genes perform the functions of simultaneous promotion and inhibition [23,31]. Four and two MFT homologous genes were found in mosses and ferns, respectively, but no FT-like or TFL-like homologous genes were found [3,20]. It was observed that, different from the FT-like and TFL-like subfamilies, the MFT-like subfamily still exists in nonflowering plants. In this study, two to three MFT-like members were present in dicotyledons, whereas the number was lower in monocotyledons (except for in maize). Two *CmMFT* isoforms were confirmed and sequenced in the Hangzhou White Chrysanthemum. Plants with many FT-like members have fewer MFT-like members [18,31]. The MFT-like subfamily may be the evolutionary source of the FT-like and TFL-like subfamilies, and its differentiation might have occurred when flowering plants appeared. Meanwhile, the evolutionary rate of the FT-like subfamily was faster than that of the TFL-like subfamily. The proposed consensus sequences DPPxP and GxHR were only partially conserved in the MFT-like subfamily (Figures 2 and S3). The conserved amino acid residues that interact with 14-3-3 proteins were not present, which was inconsistent with previous studies [18,20,23,31]. Similarly, no conservation was found in the equivalent position of the MFT-like subfamily after comparing all the sequences of the LYN triad (Figures S1–S3). In summary, the diversified sequence may be related to the binding of the substrate in all PEBPs, and, thus, more studies are needed to clarify this relationship.

In the present study, the expression levels of nine *CmPEBP* genes during the vegetative and reproductive growth stages were analyzed in the Hangzhou White Chrysanthemum tissues, and the expression levels varied greatly depending on the tissue type. The expression levels of the *CmPEBP* genes, such as *CmTFL1*, *CmTFL2*, *CmFT2*, *CmFT3*, and *CmMFT2*, were higher in the petiole and shoot apex tissues during the vegetative growth stage than during the reproductive growth stage. The *CmPEBP* genes (e.g., *CmTFL1* and *CmTFL2*) might play a key role in delaying reproductive growth. However, the expression levels of the *CmPEBP* genes, such as *CmFT1*, *CmTFL3*, *CmTFL4*, and *CmMFT1*, were lower in the shoot apex tissues during the vegetative growth stage than during the reproductive growth stage. From these results, FT proteins and *CmFT1* mRNA, which were synthesized in the stems and leaves, might have been transported to the SAM to bind FD to activate the expression of floral-meristem-specific genes, though more research is needed to prove this conjecture.

The temperature affects the flowering time by influencing the development rate and vernalization [39], and high temperatures reduce or delay flowering in the Chrysanthemum [38]. Thus, we identified candidate genes that may play important roles in response to low and high temperatures. TFL-like gene expression, which delays reproductive growth, was upregulated under heat stress but was only temporarily upregulated under cold stress. The expression level of the FT-like genes was upregulated under low temperatures but did not show a significant difference under high temperatures compared with the control group. *CmMFT1*, which was upregulated at least five fold in response to heat and cold stress, might have a unique function in specific developmental stages [17,19,23,31]. These results confirmed that our classification of the CmPEBP family genes was correct.

In addition to the plant biological clock, the flowering process is controlled by plant endogenous hormones [42–44,72]. The IAA content of early-spring-flowering plants is significantly lower than that of unflowered plants, whereas the content of GA_3 and ABA is higher [73]. The exogenous application of auxin and cytokinin is likely performed to supplement the content of endogenous hormones and to regulate their functions [45]. In the present study, the *CmPEBP* expression profiles were complex in response to eight exogenous hormones. For *CmFT* genes, the response patterns of different members to different hormones showed a certain degree of differentiation. Four hormones, GA, 6-BA, ET, and MeSA, inhibited *CmFT1* expression to varying degrees under the light and dark treatments, whereas IAA, ABA, and SA could stimulate *CmFT1* expression under the dark treatment. The expression patterns of *CmFT2* and *CmFT3* were similar, and their expression levels were upregulated after the ET, MeJA, and ABA treatments but were downregulated after the 6-BA, SA, and MeSA treatments. After treatment with GA and IAA, their expression was promoted in the light treatment group and was inhibited in the dark treatment group. For the *CmTFL* genes, the response patterns to different hormones were more differentiated than those of the *CmFT* genes, and they were less affected by the light and dark treatments. The GA, IAA, SA, and MeSA treatments inhibited *CmTFL* gene expression under the light and dark treatments. *CmTFL2* and *CmTFL3* gene expression could be activated with the ET treatment. After the MeJA and ABA treatments, the expression levels of *CmTFL1* and *CmTFL2* were upregulated, whereas that of *CmTFL3* was downregulated. However, the 6-BA treatment could upregulate *CmTFL1* and *CmTFL3* gene expression and could inhibit *CmTFL2* gene expression. Interestingly, all the hormones promoted the upregulation of *CmMFT1* expression. The *CmMFT1* expression level reached its highest value at 4 h of GA treatment and 24 h of MeJA, ABA, and MeSA treatment. After the ET, IAA, 6-BA, and SA treatments, gene expression in the light treatment group was upregulated by more than 10 times. Hence, the use of exogenous hormones can change the expression level of *CmFT*s and *CmTFL*s and can thereby promote or inhibit the flower bud differentiation and floral organ development of the Hangzhou White Chrysanthemum.

5. Conclusions

Nine *CmPEBP* genes, including three *CmFT*s, four *CmTFL*s, and two *CmMFT*s, were identified through comparative genomics analyses in the Hangzhou White Chrysanthemum. Furthermore, gene structure, physical and biochemical property, phylogenetic relationship, and conserved motif analyses on the *CmPEBP* family were conducted. They contained the PBP (PF01161) domain belonging to *PEBPs* and were phylogenetically clustered into three subfamilies: MFT-like, TFL-like, and FT-like genes. Each *CmPEBP* showed tissue-specific expression patterns, although the level of expression varied greatly. The TFL-like genes, which delay reproductive growth, were upregulated under heat stress. Instead, FT-like gene expression was upregulated under low temperatures. Based on the study of their expression patterns, we identified candidate genes that might play important roles in response to exogenous multihormone stress. *CmFT1* expression could be inhibited with the GA, 6-BA, ET, and MeSA treatments but could be activated with the IAA, ABA, and SA treatments in the dark, whereas the expression levels of *CmFT2* and *CmFT3* were upregulated after the ET, MeJA, and ABA treatments but were downregulated after the 6-BA, SA, and MeSA treatments. The GA, IAA, SA, and MeSA treatments inhibited *CmTFL* gene expression under the light and dark treatments. Our results provide a solid foundation for the further understanding of the role that PEBPs play in flowering and floral organ development and will provide the basis for future research on the functional characterization of the CmPEBP family in response to exogenous hormone stressors.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture13020374/s1, Table S1: RT-PCR of primers of *CmPEBP* genes; Table S2: qRT-PCR of primers of *CmPEBP* genes; Table S3: Comparison of the 9 CmPEBP ORFs and putative amino acid sequences; Figure S1: Predicted amino acid sequence alignment and phylogenetic tree of the *CmFT*s with the FT-like genes of other plant species; Figure S2: Predicted

amino acid sequence alignment and phylogenetic tree of the *CmTFLs* with the TFL-like genes of other plant species; Figure S3: Predicted amino acid sequence alignment and phylogenetic tree of the *CmMFTs* with the MFT-like genes of other plant species; Figure S4: Tissue-specific expression profiles of *CmPEBPs*; Figure S5: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under GA stressors; Figure S6: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under BA stressors; Figure S7: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under ET stressors; Figure S8: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under MeJA stressors; Figure S9: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under IAA stressors; Figure S10: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under ABA stressors; Figure S11: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under SA stressors; Figure S12: Expression profiles of *CmPEBP* genes in Chrysanthemum leaves under MeSA stressors.

Author Contributions: C.P. performed the majority of the experiments and conducted the data analysis; C.P., X.H., S.S., and L.Z. prepared and wrote the manuscript; C.P., X.H., L.Z., and S.S. participated in sample collection; X.H., L.Z., and S.S. were involved in the preparation of the RNA samples for the transcriptome data analysis. C.P., M.W., and B.H. designed this study. All authors have read and agreed to the published version of the manuscript.

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