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Identification and Functional Analysis of the Phosphatidylethanolamine-Binding Protein (PEBP) Gene Family in *Liriodendron* Hybrids

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Abstract: The plant phosphatidylethanolamine-binding protein (PEBP) gene family plays important roles in regulating flowering time and vegetative growth. Compared with its parents, *Liriodendron* hybrids (*Liriodendron chinense* (Hemsl.) Sarg. × *L. tulipifera* L.), have obvious heterosis in terms of higher seed germination, fast growth, bright flower colors, and long growth seasons. However, the genome-wide identification and functional analysis of PEBP genes that contribute to the heterosis of *Liriodendron* hybrids have not been studied. In this study, we characterized four members of expressed *LhPEBP* genes in *Liriodendron* hybrids and divided them into three subfamilies based on their phylogenetic relationships: *FT*-like (*LhFT*), *TFL1*-like (*LhTFL1*), and *MFT*-like (*LhMFT1* and *LhMFT2*). A functional analysis of Arabidopsis showed that the overexpression of *LhFT* significantly promoted flowering, and the *LhTFL1* gene induced a wide dispersion of the flowering timing. *LhMFTs* function differently, with *LhMFT2* suppressing flowering, while *LhMFT1* accelerates it and had a stronger promoting effect on the early stage of seed germination. Additionally, the seed germination of the *LhMFT* lines was relatively less influenced by ABA, while the transgenic *LhFT* and *LhTFL1* lines were sensitive to both ABA and GA3. These results provide valuable insights into the functions of *LhPEBP* genes in flowering and seed germination.

Keywords: *Liriodendron*; PEBP; *FLOWERING LOCUS T*; MFT; gene family

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1. Introduction

The phosphatidylethanolamine-binding protein (PEBP) gene family is widely distributed among plants, animals, and microorganisms [1], and functions as a critical regulator of diverse signal transduction pathways, governing growth and differentiation [2,3]. The plant PEBP gene family is closely associated with flowering and differentiation, serving as a key modulator of the transition from vegetative to reproductive growth and contributing to the determination of plant architecture [4,5]. The family is classified into three principal branches: *FLOWERING LOCUS T* (*FT*)-like, *TERMINAL FLOWER1* (*TFL1*)-like, and *MOTHER OF FT AND TFL1* (*MFT*)-like genes [6].

FT-like and *TFL1*-like genes are well studied and are mainly involved in plant flowering and phenological regulation [5,7,8]. *FT* and *TFL1* share sequence similarity, and the related proteins encoded by *FT* and *TFL1* have opposite effects on flowering [9]. The *FT* gene is a crucial regulator of flowering in plants, playing a pivotal role in integrating external and internal signals to modulate this developmental process [10,11]. Growing evidence has revealed that the *FT* homologous genes *HvFT1* in barley [12], *LsFT* in lettuce [13], and *RFT1* in rice [14] play crucial roles in plant flowering [15]. Notably, *FT*-like homologous

genes also perform diverse functions in different species with respect to growth, development [16,17], seed germination, and response to temperature changes associated with seasonal characteristics [18,19]. *TFL1* antagonizes *FT* [20]. The *TFL1* homologous gene family plays a pivotal role in regulating the transition, controlling the switch from vegetative to reproductive growth, and regulating flowering time and inflorescence architecture [4,21]. *TFL1* could inhibit the formation of flower primordia, thus delaying flowering [22]. Loss of *TFL1* accelerates flowering in apples [23] and causes continuous flowering in roses [24]. Similarly, the overexpression of mango *MiTFL1* genes in Arabidopsis delays flowering [25].

The *MFT* subfamily is the ancestor of the *FT* and *TFL1* subfamilies [26]. In contrast with FT-like and TFL1-like genes, the function of *MFT*-like genes is less characterized. It is generally believed that *MFT* homologs participate in the regulation of plant flowering and seed germination. *MFT* has partial *FT*-like activity [27,28], but it mainly plays a critical role in regulating seed germination via the abscisic acid (ABA) and gibberellic acid (GA) signaling pathways [29]. For example, soybean *GmMFT* [30], cotton *GhMFTs* [31], and rice *OsMFT2* [32] all inhibit seed germination by participating in GA and ABA signaling. In addition to its important roles in flowering and seed development, *MFT* is also involved in stress response and other functions. For example, *OsMFT1* promotes drought tolerance in rice [33], *MiMFT* enhances stress response [28], and *GmMFT* positively regulates seed content and seed weight [34].

Liriodendron L. (Magnoliaceae) is a genus of perennial temperate deciduous trees. Species in this genus are tall trees with straight trunks and beautiful flowers and leaves. It is a Tertiary relic genus and now comprises only two species, *Liriodendron chinense* and *L. tulipifera*, showing a typical discontinuous distribution pattern in East Asia and North America [35]. *L. chinense* is an endangered species in China with a low germination rate and poor natural regeneration, while the *Liriodendron* hybrid (*L. chinense* × *L. tulipifera*), an interspecific hybrid offspring of *L. tulipifera* and *L. chinense*, has obvious heterosis in many aspects. Compared with its parents, it has higher seed germination, fast growth, stress resistance, and a longer growing season [36]. To date, no identification or functional analysis of PEBP genes that are partially related to the heterosis of *Liriodendron* hybrids has been explored. Therefore, in the present study, we characterized the expressed PEBP family genes in *Liriodendron* hybrids and performed a functional analysis by overexpressing the PEBP genes in *Arabidopsis thaliana*. The results of this study will provide valuable information for elucidating the characteristics and functions of *LhPEBP* in *Liriodendron* hybrids.

2. Materials and Methods

2.1. Identification, Characteristics and Phylogenetic Analysis of LhPEBPs

The annotated genome data of *L. chinense* were obtained from Hardwood Genomics (https://treegenesdb.org/ (accessed on 3 June 2021)). Arabidopsis PEBP gene data were obtained from TAIR (http://www.arabidopsis.org/ (accessed on 10 June 2021)). Then, the HMM profile of the PEBP gene (PF01161) was downloaded from the Pfam website (http://pfam.xfam.org/family/PF01161 (accessed on 10 June 2021)) and used as a query to identify all PEBP-containing domains in *L. chinense* by retrieving against the genome (E-value $\leq$ 1E-5) using HMMER v3.3.2. Finally, all candidate PEBP genes were verified by Pfam (http://pfam.xfam.org/search (accessed on 15 June 2021)), CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (accessed on 15 June 2021)), and SMART (http://smart.emblheidelberg.de/ (accessed on 15 June 2021)) for further verification.

The protein sequences of *FT*, *TFL1*, and *MFT* from *A. thaliana*, *Populus alba*, and *Liriodendron* hybrids were initially aligned using muscle methods in Clustal W v. 2.1 [37]. The sequence differences and conserved motif analysis were performed in Jalview software v. 2.11.1.0 [38].

The 2000 bp upstream promoter sequences of PEBP genes were extracted from the *L. chinense* genomic database, which were used to search for putative cis-elements using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on

3 July 2021)). The physical distribution of cis-acting elements was visualized using TBtools v. 1.089 [39].

PEBP family proteins covering herbaceous angiosperms, tree species of gymnosperms and angiosperms, were downloaded from the NCBI database. The protein sequences were first aligned and then used to construct the phylogenetic tree by maximum-likelihood (ML) methods in MEGA 6 v. 7.0.26 [40]. The bootstrap replication value was set to 1000, and the substitution model was the JTT+G substitution model.

2.2. Plant Materials and RNA Extraction

The plant materials used in the experiment were young buds with mixed flowers and young leaves of *Liriodendron* hybrids from the campus of Jiangxi Academy of Sciences (28.698° N, 115.992° E) in May 2020. Samples were first frozen in liquid nitrogen and stored at −80 °C.

Total RNA was extracted using a TRIzol kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions, dissolved in sterile ultrapure water, digested with gDNase, assessed by 1% agarose gel electrophoresis, and finally stored at −80 °C.

2.3. LhPEBP Gene Cloning and Vector Construction

The expressed *LhPEBP* genes were first amplified by reverse transcription polymerase chain reaction (RT–PCR) methods as follows: the total RNA of the *Liriodendron* hybrid was used as a template and the first strand of cDNA was synthesized using the PrimeScript^{TM} II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The reaction system and conditions were set according to the kit instructions. The cDNA after the reverse transcription was used as a template for CDS amplification of *LhPEBPs*. The primer sequences are listed in the supplementary material (Table S1). The PCR products were purified by agarose gel electrophoresis, cloned, and inserted into the pMD 18-T vector (Takara) and confirmed by sequencing. Finally, the full-length coding sequences of *LhPEBP* genes were cloned and inserted into pBWA(V)BS expression vectors with the cauliflower mosaic virus 35S promoter.

Arabidopsis [Columbia-0 (Col-0)] was used in this study. The seeds were sown in plastic pots and grown in a phytotron under a long-day photoperiod (16 h of light/8 h of dark) at 24 °C. The constructed vectors were transformed into Col-0 by the Agrobacterium-mediated flower dip method. Transgenic Arabidopsis plants were screened by both spraying 15 mg/L ammonium-glufosinate on transgenic seedlings and PCR amplification verification using the corresponding primer sequences in Table S1. Seeds of transgenic lines were harvested and then sown in plastic pots under the above conditions and screened by ammonium-glufosinate and PCR amplification. The positive seeds were harvested and sown until T3 seeds were obtained.

2.4. Flowering and Germination Analysis in Arabidopsis

For flowering observation, T3 seeds of transgene lines overexpressing *LhPEBP* genes were sown in plastic pots and grown in a phytotron under a long day (LD) photoperiod pattern (16 h of light/8 h of dark) at 24 °C. The positive plants were screened by ammonium-glufosinate as described above.

For the germination analysis, T3 transgenic Arabidopsis seeds were first sterilized with 75% (*v*/*v*) alcohol for 8 min and washed three times with sterile water. The seeds were sown onto 1/2 MS. After three days of darkness at 4 °C, seeds were then transferred to a 16-h light/8-h dark photoperiod at 24 °C to examine seed germination. To explore the response to hormones, abscisic acid (ABA) and gibberellic acid (GA) were added to 1/2 MS at the following concentrations: GA3 1 μM and ABA 10 μM. Each type of transgenic line was represented by at least 100 seeds, and three independent experiments were conducted. Mutants with a loss-of-function in the MFT gene in Arabidopsis (*mft* mutants) and Col-0 were also sown as controls for flowering and germination analysis.

2.5. Data Collection and Statistical Analysis

The number of days from sowing to flowering for transgenic lines overexpressing *LhPEBP* genes, Col-0, and *mft* mutants of Arabidopsis was recorded. Additionally, the number of rosette leaves was recorded to compare flowering phenologies. Seed germination was also calculated under three different conditions: 1/2 MS, 1 μM GA3, and 10 μM ABA. To further clarify the germination progress, seed germination from day 1 to day 8 was recorded daily. Germination was defined as the number of germinated seeds divided by the total number of seeds sown.

All data were analyzed using SPSS v21.0. Significant differences were determined by ANOVA tests with a significance value of 0.05. All error bars in the figures represent the standard deviation (SD) values.

3. Results

3.1. Identification of PEBP Family Members in Liriodendron

Five PEBP genes were identified from the genome of *L. chinense*. Detailed information is shown in Table S1. The BLAST verification showed that the five PEBP genes belong to four types, including two *MFT*s, one *FT*, one *TFL1*, and one *BFT*. However, except for *LcMFT1* (*Lchi05661*), the other four PEBP family genes of *L. chinense* were incomplete. The published CDS of *FT* (*Lchi27713*) was only 267 bp, and the CDS of *TFL1* (*Lchi10509*), *MFT2* (*Lchi25487*), and *BFT* (*Lchi11903*) was only 327 bp, 321 bp, and 258 bp, respectively (Table S1). Therefore, we further located those genes in the genome data of *L. chinense* and then extracted the 2000 bp sequences upstream and downstream of the reported gene location. Finally, we found the CDS through FGENESH (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind (accessed on 20 June 2021)) and checked the domain by the CD-Search confirmation method. The full-length CDSs of the five PEBP gene family members of *L. chinense* were then obtained. We used primer3 software [41] (http://frodo.wi.mit.edu/primer3/ (accessed on 3 July 2021)) to design primers that covered the gene promoter and the terminator. Finally, four PEBP gene family genes of *Liriodendron* hybrids were successfully cloned from the mRNA, including one *FT*, one *TFL1*, and two *MFT* genes (Table 1), while the mRNA of the *BFT* gene failed to be amplified in either *L. chinense* or *Liriodendron* hybrids. The cDNA sequences of the four *LhPEBPs* were submitted to the NCBI database under the accession numbers OR347693-OR347696.

Table 1. Characterization of PEBP genes and proteins from *Liriodendron* hybrid.

Gene Name	Accession No.	CDS Length (bp)	Putative Protein Length (aa)	aa Identities to *Arabidopsis thaliana* (%)	aa Identities to *Populus alba* (%)
LhFT	OR347696	525	174	78.74	87.93
LhTFL	OR347695	522	173	75.72	79.19
LhMFT1	OR347693	522	173	70.52	69.94
LhMFT2	OR347694	525	174	63.79	64.94

CDS, coding sequence; aa, amino acid.

Nevertheless, we successfully amplified the gDNA sequences of *BFT* in all *Liriodendron* species, including one *Liriodendron* hybrid, one *L. tulipifera*, and two *L. chinense* individuals from the southern (JP) and northern (BZ) regions. We found that the gDNA sequences of *BFT* were 1114 bp for *Liriodendron* hybrids and 1115 bp for the two sister species (Table S2). The gDNA sequences of *BFT* were submitted to NCBI under the accession numbers OR347689-OR347692. The *BFT* gDNA sequences of *Liriodendron* hybrids showed a 98.30%, 99.55%, and 98.57% identity to *L. tulipifera* and the BZ and JP populations of *L. chinense*, respectively. The predicted amino acid sequence of *Liriodendron* hybrids was the same as that of *L. chinense* from BZ, while there were four amino acid differences between *Liriodendron* hybrids and *L. chinense* from JP and two amino acid differences between *Liriodendron* hybrids and *L. tulipifera*. A phylogenetic analysis of DNA sequences and amino

acids showed that *Liriodendron* hybrids have a closer relationship with *L. chinense* from the BZ population in the northern region (Figure S1).

3.2. Amino Acid Alignment and Conserved Domain Analysis

The *LhFT* cDNA encoded a 174-amino acid protein and shared a 78.74% and 87.93% amino acid sequence identity with the *FT* genes of *A. thaliana* and *Populus alba*, respectively (Table 1). *LhTFL1* has 173 amino acid residues and shares a 75.72% similarity to *A. thaliana* and a 79.19% similarity to *Populus PaTFL1.* The cDNAs of *LhMFT1* and *LhMFT2* encoded 173 and 174 amino acid proteins, respectively. *LhMFT1* had a 70.52% and 69.94% amino acid sequence identity to *A. thaliana* and *P. alba*, respectively, while *LhMFT2* shared a 63.79% and 64.94% amino acid sequence identity with *A. thaliana* and *P. alba*, respectively (Table 1).

The multiple amino acid sequence alignment showed that PEBP family proteins of *Liriodendron* hybrids have conserved DPDxP and GxHR motifs, which are critical for anion binding (Figure 1). Motif GxHR is localized in segment A and has a preference for the Ile residue in all three species. *LhFT* had the key amino acid residue Tyr84 (Y), but it was replaced by His85 (H) in *LhTFL1* and Trp (W) in *LhMFTs*, which are likely the most critical residues for distinguishing *FT* and *TFL1* activity. The conserved motif L/IYN in segment C was LYN for *LhFT*. The preferred Pro (P) residues in most *MFT* genes were found in both *LhMFTs* in segment D, and the remaining amino acid sequences in *LhMFT1* were more similar to those in *PaMFT* than to those in *LhMFT2* (Figure 1).

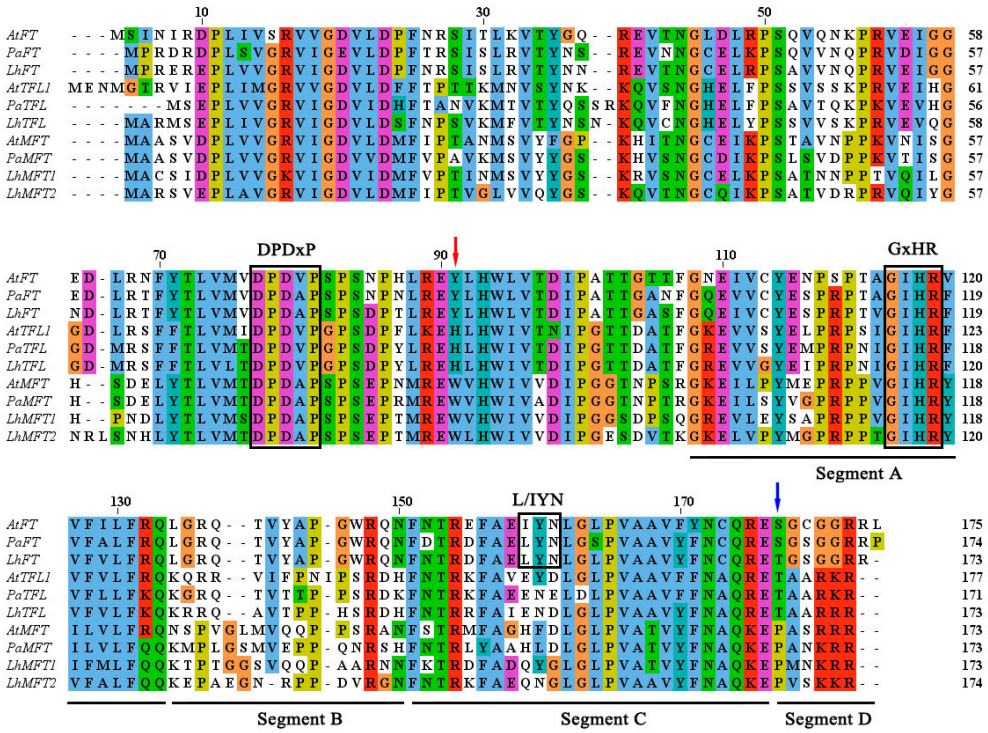

Figure 1. Multiple sequence alignment of PEBP family proteins. Amino acid sequences of the PEBP proteins were isolated from *Liriodendron* hybrids (*LhFT*, OR347696.1; *LhTFL1*, OR347695.1; *LhMFT1*, OR347693.1; *LhMFT2*, OR347694.1), *Arabidopsis thaliana* (*AtFT*, AAF03936.1; *AtMFT*, OAP13671.1; *AtTFL1*, AAB41624.1), and *Populus alba* (*PaFT*, TKR74523.1; *Pa*MFT, TKR98064.1; *PaTFL1*, TKR85832.1). Black boxes mark the conserved DPDxP and GxHR motifs. Underlines represent segments A, B, C, and D. The red arrow indicates the key amino acids distinguishing *FT*-like (Y), *TFL1*-like (H), and *MFT*-like (W) functions; the blue arrow indicates the preferred Pro (P) residue found in most *MFT* genes.

3.3. Cis-Elements Analysis in Promoter of the PEBP Genes

We conducted an analysis of the 2000 bp promoter sequences upstream of each PEBP gene in *L. chinense*. This analysis revealed multiple cis-acting elements that can be classified into four groups: hormone responses, light responsiveness, plant development, and stress responses. Among these regulatory elements, hormone-responsive elements are the most abundant, primarily dominated by MeJA-responsive and abscisic acid-responsive elements (Figure 2). Additionally, we identified gibberellin-responsive elements, auxin-responsive elements, and salicylic acid-responsive elements. The presence of light-responsive elements was also notable. Furthermore, the plant development elements included cell cycle regulation, circadian control, meristem expression, and seed-specific regulation. The stress-responsive elements comprised elements associated with drought stress, low-temperature response, anaerobic induction, defense, and other stress responses. It is noteworthy that all the genes contained abscisic acid-responsive and light-responsive elements in their promoters. However, the promoters of two *MFT* genes, namely *Lchi05661* and *Lchi25487*, possessed seed-specific regulation elements that were not found in the promoters of other *L. chinense* PEBP genes. Additionally, only the *FT* (*Lchi27713*) and *MFT1* (*Lchi05561*) elements featured GA-responsive elements in their promoter regions.

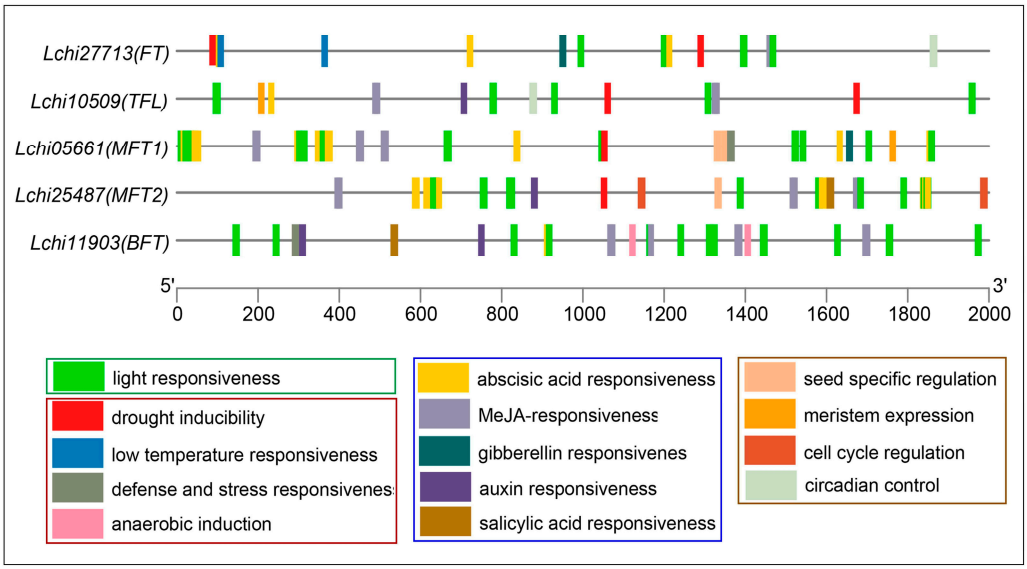

Figure 2. Cis-acting elements within the 2000 bp upstream region of PEBP Genes in *Liriodendron chinense*. Within Figure 2, a spectrum of cis-acting elements is denoted by diverse, color-coded boxes. Notably, elements associated with light responsiveness, hormone responses, plant development, and stress responses are delineated by green, blue, brown, and red rectangles, respectively.

3.4. Phylogenetic Analysis

To explore the phylogenetic relationships of the PEBP gene family in *Liriodendron*, we investigated the four characterized PEBP members from *Liriodendron* hybrids and 33 PEBP genes from *A. thaliana*, *Populus tomentosa*, *Cinnamomum micranthum*, etc. (Figure 3). The phylogenetic tree divided the 37 proteins into three subgroups: the *FT*-like subgroup, the *TFL1*-like subgroup, and the *MFT*-like subgroup. *LhFT*, *LhTFL1*, and *LhMFTs* belonged well to the eudicot group of the relative clades. *LhFT* had the closest relationship with *CmFT* genes from *C. micranthum*. *LhTFL1* and *LtTFL1* from *L. tulipifera* were clustered together and then clustered with *ZmTFL1* of *Zea mays*, forming a subgroup of *TFL1*. *MFT*-like genes were divided into three subgroups. *LhMFT1* and *LhMFT2* were clustered into two different groups, with *LhMFT2* clustering with *CmMFT* from *C. micranthum*, while *LhMFT1* was clustered with most herbs and gymnosperms and seemed to have a more ancient origin than *LhMFT2*.

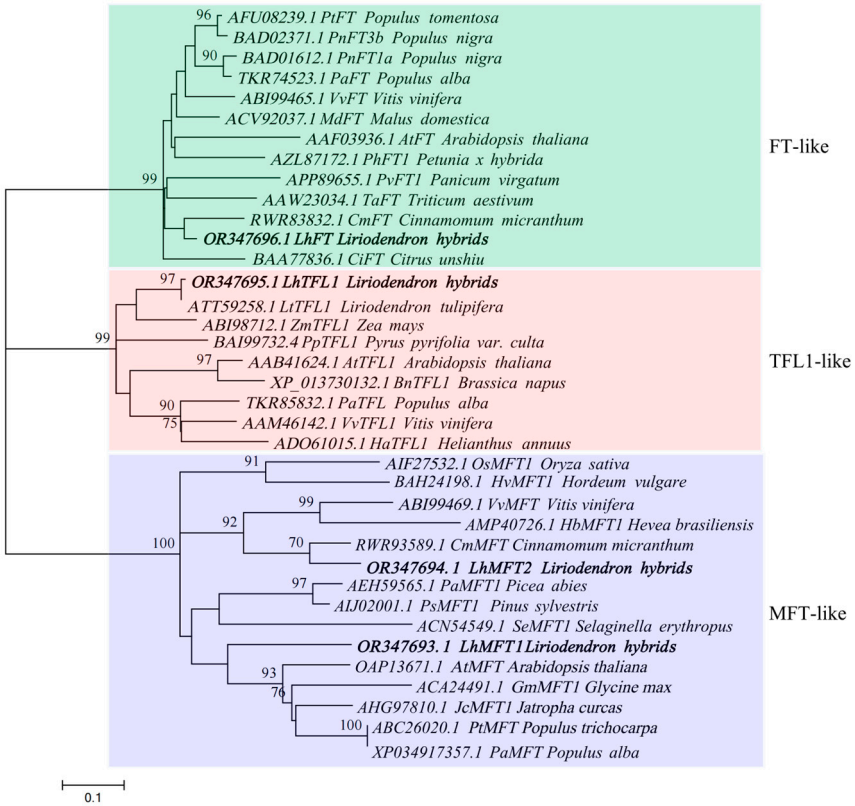

Figure 3. The phylogenetic tree of PEPB family proteins from *Liriodendron* hybrids and other plants. The tree was constructed using the maximum likelihood method in MEGA 6.0. The number at the nodes represents the bootstrap values based on 1000 replications (%). The *FT*-like, *TFL1*-like, and *MFT*-like subfamilies are marked by green, red, and blue, respectively. *LhPEBPs* are marked in bold font.

3.5. Flowering Analysis of LhPEBPs in Transgenic Arabidopsis Plants

To examine the function of *LhPEBP* genes, the coding sequences of four *LhPEBPs* were introduced into *A. thaliana* (Col-0). We successfully obtained several transgenic lines for the four *LhPEBPs*. To explore the function of the four *LhPEBPs* in flower regulation, we compared the flowering phenotype of *LhPEBPs* in overexpressing lines and other control lines. Through phenotypic observation, we found that the overexpression of *LhPEBPs* did not result in obvious floral morphology changes in the stem height, the number of lateral branches, the shape of leaves, and the feature of flowers and siliques, but resulted in different flowering times and rosette leaves in transgenic Arabidopsis under long-day conditions.

As shown in Figure 4, the *LhFT* lines flowered earlier than the wild type, approximately six days earlier (18.70 ± 2.21 DAS vs. 24.72 ± 3.66 DAS), which was significantly earlier than the other phenotypes. The function of promoting flowering was further verified by the result that the *LhFT* transgenic lines had the fewest rosette leaves. For the *LhTFL1* lines, the flowering time was the most flexible (24.29 ± 3.95 DAS; Figure 4 and Table S3) but showed no significant differences from that of the wild type and other transgenic types. However, the average number of rosette leaves of the *LhTFL1* transgenic lines was significantly greater than that of Col-0.

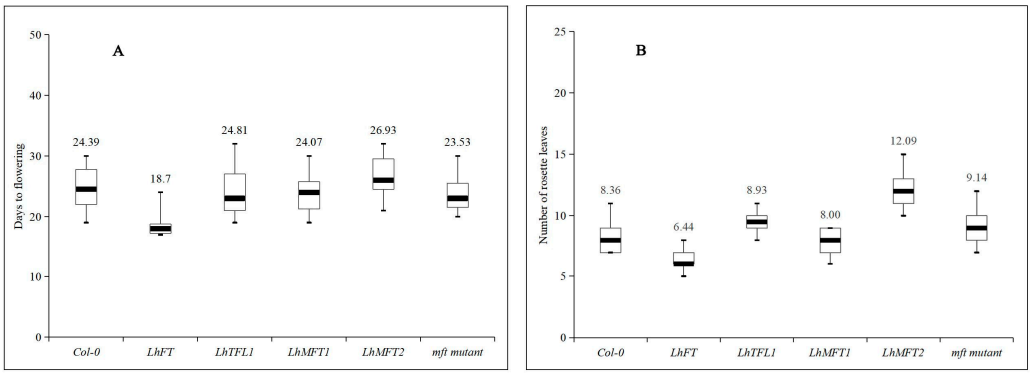

Figure 4. Phenotype pattern of *LhPEBP* genes affecting flowering in *Arabidopsis* in a phytotron under LD conditions. (**A**) Flowering time of Col-0, four *LhPEBP* transgenic lines and the *mft* mutant. (**B**) Rosette leaf numbers of Col-0, four *LhPEBP* transgenic lines and the *mft* mutant.

The *mft* mutants showed no significant differences in either bolting time or rosette leaves compared with the wild type in the present study, proving that the *AtMFT* gene in *A. thaliana* may have a weak function in flowering. Although weak, the function of the two *LhMFTs* in flowering showed two contrasting directions, with the *LhMFT1* lines flowering slightly earlier than the wild type and the *LhMFT2* plants flowering later than the wild type, but the flowering time of *LhMFT2* was significantly later than that of *LhMFT1*. This was further confirmed by the rosette leaves (Figure 4). The number of rosette leaves of *LhMFT2* was not only significantly greater than that of *LhMFT1* but also greater than that of the Col-0 and *mft* mutants. On the other hand, the *LhMFT1* lines have relatively fewer rosette leaves than the Col-0 and other transgenic lines except for *LhFT* (Figure 4).

3.6. Germination Analysis of LhPEBPs in Transgenic Arabidopsis Plants

In view of the likely function of PEBP genes in seed germination, we analyzed the seed germination process of *LhPEBPs* in overexpressing Arabidopsis lines. The average final seed germination ranged from 0.872 to 0.972 under 1/2 MS (Figure 5 and Table S4). All the *LhPEBP* transgenic lines increased the germination of seeds, although it was not significant in *LhFT* lines. When treated with abscisic acid (ABA) and gibberellic acid (GA), the transgenic *LhPEBP* lines largely had the same trend, with seed germination delayed one to two days under a 10 μM ABA treatment and largely promoted in the early germination stage under a 1 μM GA3 addition. Although not significant, the 1 μM GA3 treatment increased the seed germination except for the *LhMFT2* transgenic lines. The 10 μM ABA treatment significantly decreased the final seed germination percentage of the *LhFT* and *LhTFL1* lines and Col-0, while it had a relatively weak influence on the *LhMFT* lines and *mft* mutants (Figure 5).

The seed germination rate of the *LhMFT1* lines and *mft* mutants was significantly higher than that of the other lines in the early stage of seed germination (Figure 5 and Table S4). When treated with 1 μM GA3, the seed germination rate of *LhFT* and *LhTFL1* in the early stage increased significantly compared with that under 1/2 MS; in particular, the *LhFT* lines showed a sudden outbreak of seed germination on the second day under the hormone action of GA3. When treated with 10 μM ABA, in accordance with Col-0 lines, the seed germination of the *LhFT* and *LhTFL1* was rare on the third day, and their germination was suppressed during the whole process, but the degree of decrease was reduced in the later germination stage for the *LhMFTs* transgenic seeds and *mft* mutants.

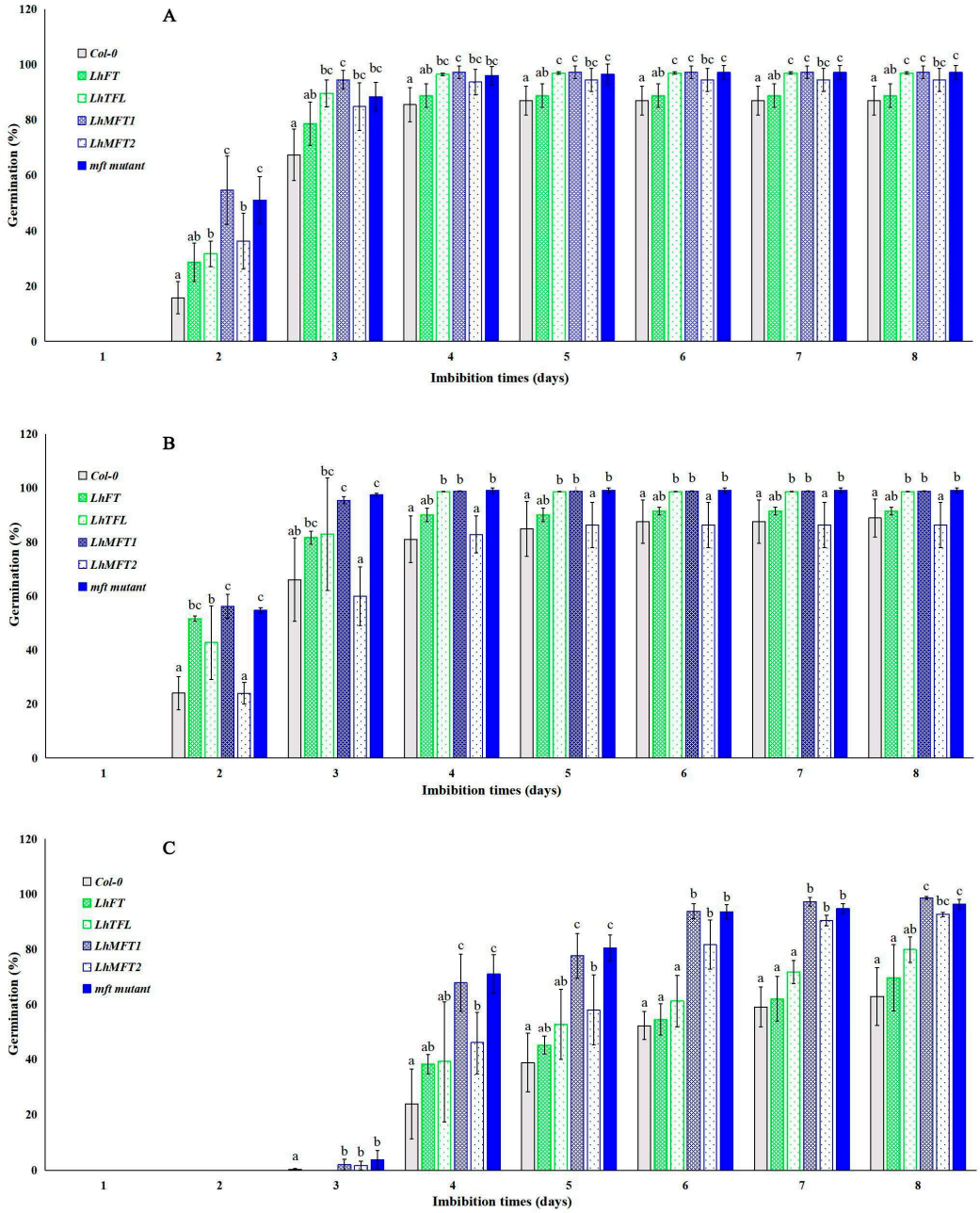

Figure 5. Time course of seed germination for Col-0, overexpressed *LhPEBP* transgenic lines, and *mft* mutants in Arabidopsis. Three situations, (**A**) under 1/2 MS, (**B**) 1 μM GA3, and (**C**) 10 μM ABA, are presented; a–c indicate the results based on Duncan's test at a significance value of 0.05.

4. Discussion

4.1. The Characteristics and Evolution of PEBP Genes in Liriodendron Hybrids

Collectively, we identified five members of the *LcPEBP* genes from the reported genome [42]. Unlike the traditional identification of gene family members from the reported genome, we characterized four potentially functional *LhPEBP* members from the expressed genes in *Liriodendron* hybrids. As the PEBP genes were mainly formed by gene duplication from their ancient ancestor, and functions diverged from each other after duplication [26], the number of PEBP genes varies in different species. The number of *LhPEBPs* is less than that in *Populus nigra* [43], *Perilla frutescens* [44], sugarcane [45], and soybean [46], and it is largely the same as that in moso bamboo [47], *Pyrus communis* [48], and *Picea abies* [3].

Our phylogenetic analysis showed that the four PEBP genes fall into three typical clades: one each into the *FT*-like clade and *TFL*1-like clade, and two into the *MFT*-like clades (Figure 3). This result was consistent with the common ancestor duplication and diverged orders of PEBP genes [26,49]. *LhFT* and *LhTFL* share more sequence similarity with *Populus* tree species than with the herb Arabidopsis. Nevertheless, *LhMFTs* shared largely the same sequence similarity with the two species (Table 1). Furthermore, *LhMFT1* showed more sequence consistency with *Populus* and Arabidopsis than *LhMFT2*. This was further proven by the ML tree of 37 PEBP proteins (Figure 3). *LhMFT1* was located in the largest subclade of the *MFT*-like clades, including most herbs and gymnosperms, such as *A. thaliana*, *Populus alba*, and *Picea abies*. However, *LhMFT2* was separated into a relatively small clade. Evolutionary analyses of the PEBP family have also provided us with valuable information to better understand functional diversification. From this perspective, the observed divergent functions of *LhMFTs* in flowering, seed germination, and hormone response (Figures 4 and 5) may largely indicate a different adaptive evolution after ancient gene duplication. Such functional divergence of PEBP genes within the same clade was also found in other species. For example, in *Populus tremula*, *FT2* paralogs control summer growth, and the *FT1* paralog controls the release of winter dormancy in response to winter temperatures [17,50,51]; *OsMFT2* delays germination [32], whereas *OsMFT1* also maintains flowering regulation in a manner similar to *FT*-like or *TFL1*-like genes [33].

In the present study, although we successfully amplified the gDNA sequences of *BFT* in the genomes of all *Liriodendron* species, we failed to obtain the expressed mRNA of *BFT* genes in *L. chinense* or *Liriodendron* hybrids. The tissue of flowers and the mixture of young and old leaves were all screened in the present study. In addition, Sheng [52] also searched for the PEBP family gene in the transcriptome dataset of *L. chinense*, covering five developmental time points, including autumn bud, spring bud, and flower buds at three successive developmental phases, and no *BFT* transcripts were found. We argued that except for a special case not captured, for example, peaking in the early evening [53], this gene was more likely functionally redundant in *Liriodendron*. Loss of the *BFT* gene occurs in many species, such as grapevine [54], *Picea abies* [3], moso bamboo [47], and sugarcane [45]. In the process of evolution, gene functions are repeated and redundant, sometimes leading to the formation or selection of pseudogenes. For example, *AcBFT2* from kiwifruit has the potential to reduce plant dormancy with no adverse effect on flowering [55]. Wang [26] reported that *BFT*, *TFL1*, and *ATC* were three daughter lineages created by two separate duplication events of the common *TFL1* ancestor. From this aspect, *BFT* may function like *TFL1*; for example, *BFT* of Arabidopsis possesses *TFL1*-like activity and functions redundantly with *TFL1* in inflorescence meristem development [53].

4.2. The Function of LhFT/LhTFL1 Was Conserved, and LhMFTs Functioned Differently

We identified one *FT* and one *TFL* gene in *Liriodendron* hybrids. This enabled them to perform the basic functions of promoting and inhibiting flowering [5,9,56]. *LhFT* has a significant flowering promotion effect (Figure 4). Similar early-flowering phenotypes of transgenic Arabidopsis appeared upon the ectopic overexpression of *FT* genes from heterologous plants. For example, *GbFT* in *Ginkgo biloba* [57], *PbFT* in *Pyrus betuleafolia* [48], and *PfFT1* in *Perilla frutescens* [44] all promote flowering in transgenic Arabidopsis. Furthermore, the function of *FT* splicing functioned contrarily, prolonging the blooming period [52] or negatively inducing flowering [58]. In *Liriodendron* hybrids, it showed a wide range of flowering timing in *LhTFL1* transgenic lines, although the average time was similar with Col-0. The function of *TFL* was usually to delay the flowing time, such as in *MiTFL1* genes in mangos [25] and *ScFT1* genes in sugarcanes [59], but it was also found to function oppositely in alpine snow tussocks [60]. The machinery determining flower bud initiation in Arabidopsis may be disturbed by the activity of *LhTFL1.* On the other hand, the number of rosette leaves was increased in *LhTFL1* overexpressing plants, partially showing the strengthening of its early vegetative growth, and indicating a certain regulation of the transition from vegetative growth to reproductive growth conducted by *LhTFL1*. In addition to

the original flowering time control, *FT*/*TFL1*-like genes have been found to be involved in more diverse functions. For example, the balance between vegetative and reproductive growth is regulated in pears [48] and tomatoes [11]. A cis-element analysis of the promoter of the PEBP genes in *L. chinese* also showed circadian control elements, indicating the possibility of their function in the growth cycle. For example, the *FT* paralogs in *Populus tremula* regulate different aspects of the tree's yearly growth cycle [17,51]. In *Picea abies*, *PaFT4* correlates with both the photoperiod-controlled bud set and temperature-mediated bud burst [3]. In Arabidopsis, *FT* interacts with the transcription factor *BRANCHED1* (*BRC1*) to regulate branching [61].

As the sister clade of the common ancestor of the *FT* and *TFL1* subfamilies [26], *MFT*-like genes have maintained their potential to function like *FT* and *TFL1*. A few *MFT*-like genes regulate flowering in a manner similar to *FT*-like or *TFL1*-like genes [27,28]. Hence, previous studies have proven that *MFTs* from different species have diverse functions in flowering time. For example, in *Arabidopsis*, the overexpression of *AtMFT* caused slightly early flowering under long-day conditions [27]. In contrast, the overexpression of the orchid *DnMFT* [62] and rubber tree *HbMFT1* also delayed floral initiation [63]. However, many studies also found that *MFT* had no effect on flowering; for example, *GmMFT* [30], *PaMFT1*, and *PaMFT2* [3], *GhMFT1*, *GhMFT2* [31], and *MiMFT* [28] did not affect the flowering time of transgenic plants. Consistent with the analysis results of ginkgophytes, cycadophytes, and pinophytes [49], we also identified two distinct clades of *MFT* proteins (*LhMFT1*/*LhMFT2*) in *Liriodendron* hybrids. Regarding their functions in flowering, the functions of *LhMFT*s seem to be contradictory. The overexpression of *LhMFT1* caused slightly early flowering under long-day conditions, similar to the functions of *MFT* in Arabidopsis, which closely clustered together in the phylogenetic tree (Figure 3), while *LhMFT2* delayed flowering, in accordance with the opposite examples, including rubber tree *HbMFT1* [63], which is located in the same subclade of *MFT*-like genes in our constructed phylogenetic tree.

In addition to being involved in flowering regulation, the function of *MFT*-like genes in seed dormancy and germination was prominent. In the present study, we found seed specific cis-acting elements in the promoter of the two *MFT* genes in *L. chinense* (Figure 2). Furthermore, the function of *MFTs* in seed germination is different. *AtMFT* was reported to promote seed germination in Arabidopsis [29]. Yang also reported that the overexpression of *PhFT5* (*MFT*-like clade) from moso bamboo promoted the seed germination rate in Arabidopsis [47]. However, more studies have proven that the overexpression of *MFTs* inhibits seed germination in transgenic Arabidopsis, such as cotton *GhMFT1* and *GhMFT2* [31], mango *MiMFT* [28], and wheat *TaMFT* [64]. In the present study, we found that *LhMFTs* promote seed germination, and the effect of *LhMFT1* is higher than that of *LhMFT2* (Table S4). The functional trend of *LhMFT1* in determining the flowering time and seed germination in transgenic Arabidopsis was roughly the same. This consistent functional trend in flowering and seed germination was also found in other species, such as *MFTs* in cotton [31], mangos [28] and rubber trees [63].

Abscisic acid (ABA) and gibberellic acid (GA) are two antagonistic phytohormones that regulate plant growth and seed germination in response to biotic and abiotic environmental stresses [28,29,64,65]. We found that *LhPEBPs* were hormone-responsive genes. The seed germination of the *LhMFT* lines was relatively less influenced by ABA, and the transgenic *LhFT* and *LhTFL1* lines were sensitive to both ABA and GA3 (Figure 5 and Table S4). The involvement of *MFTs* and seed germination in ABA signaling was actually controversial [29,32,66]. In *Liriodendron* hybrids, the application of exogenous ABA significantly inhibited the germination of PEBP transgenic lines, but the germination inhibition of overexpressed *LhMFT* lines was less than that for others (Figure 5). In mangos, the overexpression of *MiMFT* also increased the tolerance to ABA stresses [28]. The function of *LhFT* in seed germination was strongly promoted by GA3 in the early stage, and we also found GA responsive cis-acting elements in the promoter region of the *FT* and *MFT1* gene in *L. chinense*, which may promote flowering and seed germination. We argue that the sensitivity of *LhFT* to GA and the relative insensitivity of *LhMFTs* to ABA may contribute to

heterosis in *Liriodendron* hybrids. Nevertheless, further experiments are needed to elucidate their actual functions in *Liriodendron* hybrids.

5. Conclusions

In summary, we isolated four members of the PEBP family from *Liriodendron* hybrids, including one *LhFT*, one *LhTFL1*, and two *LhMFTs*, and identified the functions of their products. Our results provide the first relatively comprehensive description of the PEBP family genes in *Liriodendron* species, and to our knowledge, this is the first report to demonstrate the function of PEBP genes in *Liriodendron* hybrids. This study provides insight into the characteristics and function of four PEBP family genes from *Liriodendron* hybrids in the regulation of flowering and seed germination in *A. thaliana*.

In accordance with the reported functions of most PEBP genes, *LhFT* promoted floral induction and exhibited a rapid response to GA3 in the seed germination of transgenic Arabidopsis. Conversely, the *LhTFL1* gene induced a wide dispersion of the flowering timing. Both *LhMFT* genes promoted seed germination and they were less influenced by ABA. However, they functioned differently, with *LhMFT2* inhibiting flowering and *LhMFT1* slightly promoting it. This suggested that amino acid divergence following duplication drove the adaptive functional differentiation of PEBP family genes. However, further studies are needed to characterize the *LhPEBP* family genes by genetic approaches to understand their actual roles in flowering, germination, and other related functions related to heterosis in *Liriodendron* species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14102103/s1, Figure S1: Phylogenetic analysis of *BFT* genes in *Liriodendron*; Table S1: Primers and original accession information for *LhPEBP* genes in *Liriodendron* hybrids; Table S2: Information for *BFT* genes in *Liriondendron*. Table S3: Flowering traits of *Col-0, LhPEBP* transgenic lines and *mft* mutants. Table S4: Time course of the seed germination for *Col-0*, over expressed *LhPEBPs* transgenic lines and *mft* mutations in *Arabidopsis* under 1/2 MS, with or without 1 μM GA3 or 10 μM ABA treatment. Different letters in the right corner indicate significant differences of ANOVA tests under the Duncan method for all the gene lines with different treatment in the same day ($p < 0.05$)

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