

Article

Comprehensive Characterization of the PEBP Gene Family in *Osmanthus fragrans* Reveals Genetic Diversity and Potential Roles in Floral Development

Hui Xia ^{1,2} , Yuanhang Wu ¹, Jie Yang ¹, Hongguo Chen ¹ , Jingjing Zou ¹ and Yingting Zhang ^{1,*}

¹ Key Laboratory of National Forestry and Grassland Administration on *Osmanthus fragrans*, Hubei University of Science and Technology, Xianning 437100, China; xiahui625649@163.com (H.X.); 15257932995@163.com (Y.W.); yj_anna@hbust.edu.cn (J.Y.); chen_hongguo1969@163.com (H.C.); silence@hbust.edu.cn (J.Z.)

² State Key Laboratory of Woody Oil Resources Utilization, College of Forestry, Central South University of Forestry and Technology, Changsha 410018, China

* Correspondence: ytzhang0308@163.com; Tel.: +86-139-1598-1398

Abstract

The phosphatidylethanolamine-binding protein (PEBP) gene family plays essential roles in flowering regulation, plant development, and stress responses. However, its characteristics in *Osmanthus fragrans* remains largely unexplored. Here, a genome-wide analysis was conducted to comprehensively investigate the *OfPEBP* gene family. A total of 11 *OfPEBP* genes were identified and classified into three subgroups: FT-, TFL1-, and MFT-like. Genes within the same subgroup exhibited similar motif compositions and exon–intron structures. Promoter analysis revealed abundant light- and hormone-responsive *cis*-elements. Population genetic analyses identified significant differentiation in *OfPEBP3*, *OfPEBP7*, and *OfPEBP8*, with their single nucleotide polymorphisms (SNPs) and haplotypes closely associated with seasonal flowering phenotypes. DNA methylation profiling indicated that CpG and CHG methylation predominated across the *OfPEBP* family and remained relatively stable during flower development, whereas *OfPEBP6*, *OfPEBP9*, and *OfPEBP10* displayed higher methylation levels. Segmental duplication was identified as the primary driver of family expansion, and duplicated gene pairs experienced strong purifying selection. Expression analyses revealed tissue-specific expression patterns and differential responses to temperature, ethylene, and DNA methylation inhibitor (Aza) treatments. These findings provide useful insights into the potential involvement of *OfPEBP* genes in seasonal flowering and offer candidate genetic resources for future functional studies and molecular breeding in *O. fragrans*.

Keywords: *Osmanthus fragrans*; *OfPEBP* gene family; gene structure; epigenetic modification; SNP variation; expression analysis



Academic Editor: Haiying Liang

Received: 30 May 2026

Revised: 7 July 2026

Accepted: 13 July 2026

Published: 15 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and

conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Phosphatidylethanolamine-binding proteins (PEBPs) are a highly conserved protein family widely distributed in eukaryotes. They were first isolated from bovine brain tissue and named for their specific affinity for phosphatidylethanolamine [1,2]. Structural studies have shown that PEBPs contain a conserved core domain, which consists of a large central β -sheet, a smaller β -sheet, and several α -helices [3,4]. In plants, PEBP family members are involved in many biological processes, including floral induction, plant growth and development, seed germination, and morphogenesis [5–7].

To date, PEBP family members have been identified in many plant species, including rice (*Oryza sativa*) [7], maize (*Zea mays*) [8], and tomato (*Solanum lycopersicum*) [9]. In the model plant *Arabidopsis thaliana*, six *AtPEBP* genes have been reported: FLOWERING LOCUS T (*FT*), TWIN SISTER OF FT (*TSF*), TERMINAL FLOWER1 (*TFL1*), BROTHER OF FT AND TFL1 (*BFT*), ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG (*ATC*), and MOTHER OF FT AND TFL1 (*MFT*) [10,11]. Based on phylogenetic relationships and functional divergence, plant *PEBP* genes are generally classified into three major subfamilies: FT- [12,13], TFL1- [14], and MFT-like [15,16].

The FT- and TFL1-like subfamilies are major regulators of flowering time in plants. The FT-like subfamily mainly includes *FT* and *TSF*. Among them, *FT* is a key regulator of floral transition and promotes the shift from vegetative to reproductive growth [17]. Under long-day conditions, *FT* expression is induced in leaves. The FT protein is then transported to the shoot apical meristem, where it interacts with the b-ZIP transcription factor (TF) FD to form the FT–FD complex. This complex subsequently activates flowering-related genes, such as *APETALA1* (*AP1*) and *LEAFY* (*LFY*), thereby promoting floral initiation [18,19]. In *O. sativa*, the *FT* homologs HEADING DATE 3a (*Hd3a*) and RICE FLOWERING LOCUS T1 (*RFT1*) promote heading and flowering through activation of the *AP1* orthologs *OsMADS14* and *OsMADS15* [20]. In contrast, the TFL1-like subfamily, including *TFL1*, *BFT*, *ATC*, and *CENTRORADIALIS* (*CEN*), generally acts as a floral repressor. Previous studies have shown that *TFL1* suppresses flowering by competing with FT for shared interacting proteins [21,22]. In cucumber (*Cucumis sativus*), *CsTFL1* inhibits terminal flower development by competing with *CsFT* for association with the CsNOT2a–CsFDP complex [23]. Similarly, in rice, the TFL1-like protein RCN delays flowering by competing with 14-3-3 and FD proteins, thereby preventing formation of the florigen activation complex [24]. However, the MFT-like subfamily is considered the most ancient lineage within the PEBP family. It is mainly involved in seed germination and hormone signaling. In *A. thaliana*, *MFT* has been shown to regulate seed germination through the coordinated action of abscisic acid (ABA) and gibberellin (GA) signaling pathways [25]. Phylogenetic evidence suggests that MFT-like genes may represent the ancestral form of the PEBP family in land plants. Later gene duplication and functional divergence likely gave rise to the FT- and TFL1-like subfamilies, contributing to the evolution of flowering regulatory mechanisms in plants.

Sweet osmanthus (*Osmanthus fragrans*) is an important ornamental species in the Oleaceae family. It has considerable ornamental, edible, and medicinal value. Because of its distinctive fragrance and diverse flower colors, *O. fragrans* is widely used in landscape greening, fragrance processing, and functional product development. However, its flowering period is relatively short. The optimal ornamental and harvesting stage generally lasts only 2–3 days, which limits both ornamental application and industrial utilization [26]. Therefore, clarifying the molecular basis of flowering regulation is important for germplasm improvement and the sustainable development of the *O. fragrans* industry. As a perennial woody plant, *O. fragrans* has a long growth cycle. These features have limited research on its flowering regulatory mechanisms. Current studies on flower development and flowering regulation in *O. fragrans* have mainly focused on transcriptome analyses and the functional characterization of several flowering-related genes. In contrast, the PEBP gene family has not been systematically characterized in this species. In this study, genome-wide identification and comprehensive analyses of the PEBP gene family were conducted based on the *O. fragrans* genome. This work aims to clarify the composition, evolutionary features, and expression profiles of the *PEBP* gene family in *O. fragrans*. The results provide a basis for future studies on flowering regulation, flowering-time control, molecular breeding, and elite germplasm improvement in *O. fragrans*.

2. Materials and Methods

2.1. Identification of the OfPEBP Gene Family

The genome sequence data of the *O. fragrans* cultivar “Liuye Jingui” was downloaded from the OfIR database (<https://yanglab.hzau.edu.cn/OfIR/home/>, accessed on 10 May 2026) [27], based on the published genome assembly [28]. The AtPEBP protein sequences of *A. thaliana* (Supplementary Table S1) were obtained from TAIR (<http://www.arabidopsis.org/>) and used as queries to perform BLASTP v2.14.0 searches against the *O. fragrans* protein database to identify candidate OfPEBP family members. In addition, the conserved PEBP domain (PF01161) was retrieved from Pfam (<http://pfam.xfam.org/>), and HMMER3.0 (<http://hmmmer.org/>) were used to search the *O. fragrans* protein dataset with the corresponding hidden Markov model profile. After redundant sequences were removed, all candidate OfPEBP proteins were verified using the CDD (<https://www.ncbi.nlm.nih.gov/cdd>, accessed on 1 May 2026) and SMART database (<http://smart.embl.de/>, accessed on 7 May 2026) to confirm the presence of the conserved PEBP domain. Chromosomal length and gene location information were extracted from the *O. fragrans* genome using TBtools [29], and the identified *OfPEBP* genes were then renamed according to their chromosomal distribution order.

2.2. Analysis of Physicochemical Properties, Subcellular Localization, and Structure Prediction of OfPEBP Proteins

The physicochemical properties of OfPEBP proteins, including molecular weight (MW), theoretical isoelectric point (pI), instability index, and grand average of hydropathicity (GRAVY), were predicted using the ExPASy ProtParam tool (<http://web.expasy.org/protparam/>, accessed on 8 May 2026). Subcellular localization was predicted using PSORT (<https://www.psort.org/>, accessed on 8 May 2026). Secondary structure prediction was performed using the SOPMA server (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html, accessed on 9 May 2026). The three-dimensional (3D) structures were predicted using the SWISS-MODEL (<https://swissmodel.expasy.org/>). Homology models were built using templates with relatively high sequence coverage and sequence identity greater than 30%. The best models were selected based on Global Model Quality Estimation (GMQE) and Qualitative Model Energy Analysis (QMEAN) scores. Model reliability and stereochemical quality were further evaluated using Ramachandran plot analysis [30].

2.3. Phylogenetic Tree Construction, Conserved Motif, and Gene Structure Analysis

The PEBP sequences of *A. thaliana* were obtained from the TAIR database, whereas the PEBP protein sequences of *O. sativa* and *P. trichocarpa* were downloaded from the Phytozome database [31]. Relevant gene information was collected according to Liu et al. [32] (Supplementary Table S1). The OfPEBP protein sequences were then combined with those from the above species for comparative analysis. Multiple sequence alignment was performed using the ClustalW algorithm in MEGA11 [33]. A phylogenetic tree was subsequently constructed using the Neighbor-Joining (NJ) method with 1000 bootstrap replications, the Poisson substitution model, and complete deletion for missing sites.

Conserved motifs of OfPEBP proteins were predicted using the MEME Suite online platform (<https://meme-suite.org>). The parameters followed She et al. [34], with the maximum number of motifs set to 10. All other parameters were kept at their default settings. Gene structure information, including exon–intron organization, was extracted from the genome annotation files of each species. Conserved motifs and gene structures were then visualized using CFVisual v2.1 [35].

2.4. Cis-Acting Element Prediction and Synteny Analysis

The 2000 bp genomic sequences upstream of the translation initiation codon (ATG) of each *OfPEBP* gene were extracted as promoter regions. These sequences were submitted to the PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 12 May 2026) to predict *cis*-acting regulatory elements.

Synteny analysis was performed using MCScanX [36]. This analysis identified collinearity relationships among *PEBP* genes within the *O. fragrans* genome and between *O. fragrans* and *A. thaliana*, *O. sativa*, or *P. trichocarpa*. MCScanX detects syntenic relationships by scanning collinear blocks across genomes. Each alignment represents a collinear block containing corresponding homologous genes. Tandem duplication events were defined as gene pairs located within 100 kb on the same chromosome and sharing $\geq 70\%$ sequence similarity. Synonymous substitution rates (Ks), nonsynonymous substitution rates (Ka), and Ka/Ks ratios of duplicated *PEBP* gene pairs were calculated using KaKs_Calculator 3.0 [37].

2.5. DNA Methylation Analysis

To investigate DNA methylation patterns of the *OfPEBP* gene family, the genome annotation file and corresponding methylation datasets of *O. fragrans* were obtained from the OfIR database (<https://yanglab.hzau.edu.cn/OfIR/home/>, accessed on 10 May 2026). Methylation information within *OfPEBP* genomic regions was extracted, and methylation levels in three sequence contexts, CpG, CHG, and CHH, were analyzed, where H represents A, T, or C. Combined with gene structure information, the distribution of methylation sites across different regions of *OfPEBP* genes were further examined. Hierarchical clustering heatmaps were generated to visualize methylation patterns and to provide preliminary insight into the possible association between DNA methylation and *OfPEBP* gene regulation.

2.6. Population Differentiation Index (*Fst*), Nucleotide Diversity (*Pi*), and Haplotype Analysis

Fst, *Pi*, and haplotype analyses were conducted to evaluate population genetic structure, genetic differentiation, and potential selection signals. Based on the high-quality single nucleotide polymorphisms (SNP) dataset generated from whole-genome resequencing data of 126 *O. fragrans* accessions in our previous study (PRJNA679852) [28], SNPs located within the genomic regions of *OfPEBP* genes were extracted for downstream analysis. VCFtools [38] was used to calculate the *Fst* and *Pi* for each variant site between the two populations: Autumn, representing autumn-flowering accessions, and Season, representing continuous-flowering accessions. The parameters `--weir-fst-pop` and `--site-pi` were used for *Fst* and *Pi* calculations, respectively. *Fst* was used to estimate the degree of genetic differentiation between populations, whereas *Pi* represented the level of nucleotide diversity within populations. By integrating *Pi* and *Fst* analyses, potential selective regions within the *PEBP* gene family were further explored. The results were visualized using the ggplot2 package in R v4.3.0.

For SNP loci located within *OfPEBP* genes, haplotype analysis was performed using genotype data obtained from the OfIR database (<https://yanglab.hzau.edu.cn/OfIR/home/>, accessed on 10 May 2026). SNPs within the target regions were extracted, haplotypes were defined based on SNP combinations, and the frequency of each haplotype was calculated across different populations. In addition, local association analysis was performed using logistic regression to identify potential variants associated with the continuous-flowering trait in *O. fragrans*. To reduce false positives caused by multiple testing, raw *p* values were adjusted using the Benjamini–Hochberg method, and variants with adjusted *p* < 0.05 were considered significantly associated. For significant SNPs, chi-square tests were further used to compare genotype frequency differences at each locus

between continuous-flowering and non-continuous-flowering accessions. Statistical tests and visualization were performed using the R package ggstatsplot.

2.7. TFs Regulatory Network and Enrichment Analysis of OfPEBP Genes

To explore the potential functions and regulatory relationships of *OfPEBP* genes, co-expression analysis between *OfPEBP* family members and TFs was performed based on transcriptome expression data. Expression profiles of *OfPEBP* genes and TFs under different tissues and treatment conditions were extracted and normalized prior to analysis. Pearson correlation analysis was then performed to evaluate expression associations between *OfPEBP* genes and TFs. Gene pairs with a correlation coefficient $|r| > 0.8$ were considered strongly coexpressed. The significantly coexpressed pairs were then imported into Cytoscape v3.10 for the construction and visualization of the coexpression regulatory network.

Subsequently, functional annotation and Gene Ontology (GO) enrichment analyses of *OfPEBP* proteins and their coexpressed TFs were performed using the GO tools in OfIR database platform (<https://yanglab.hzau.edu.cn/OfIR/tools/go/>, accessed on 10 May 2026) to identify enriched terms associated with Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). GO enrichment significance was determined using a false discovery rate threshold of $FDR < 0.05$. The enriched GO terms were further visualized using ggplot2 package with R v4.3.0 software.

2.8. RNA-Seq Expression Analysis

The expression profiles of *OfPEBP* genes across different tissues, petal opening and senescence stages, and under low-temperature, DNA methylation inhibitor 5-azacytidine (Aza), and ethephon (ETH) treatments were obtained from the OfIR database and publicly available RNA-seq datasets (PRJNA679852, PRJNA564449, and PRJNA642321; Supplementary Table S2). For the petal opening and senescence dataset, petal samples from six developmental stages (S1–S6) were analyzed, with three biological replicates per stage. For low-temperature treatment, S1-stage floral buds of the cultivar ‘Yanhong Gui’ were treated at 23 °C for 2, 4, and 6 days or at 19 °C for 2, 4, and 6 days, with three biological replicates per treatment. For Aza and ETH treatments, S1-stage flowers (day 0) on intact plants were treated with Aza, ETH, or ddH₂O as the control (CK), and samples were collected at day 1 (S1 stage, 0 d), day 5 (S3 stage, 5 d), and day 8 (S5 stage, 8 d), with three biological replicates for each treatment and control. RNA-seq reads were aligned to the ‘Liuye Jingui’ reference genome [28] using HISAT2 v2.1.0 (<https://daehwankimlab.github.io/hisat2/download/>, accessed on 29 May 2026), converted and sorted with Samtools v1.6 (<https://github.com/samtools/samtools>, accessed on 29 May 2026), and quantified using featureCounts v2.0.6 (<http://subread.sourceforge.net>). Transcript abundance of each *OfPEBP* gene was calculated as fragments per kilobase of exon per million mapped reads (FPKM), and expression profiles were visualized as heatmaps using TBtools v2.475 software. The qRT-PCR was performed to validate the expression patterns of selected *OfPEBP* genes during flower development using the $2^{-\Delta\Delta C_t}$ method referring to Livak and Schmittgen [39], and the primers are listed in Supplementary Table S3.

3. Results

3.1. Identification and Characterization of the *OfPEBP* Gene Family

A total of 11 *OfPEBP* genes were identified in *O. fragrans* and designated as *OfPEBP*1–11 according to their chromosomal positions. The physicochemical properties of the encoded proteins are summarized in Figure 1a. Protein lengths ranged from 145 (*OfPEBP*5) to 217 aa (*OfPEBP*10), while the molecular weights varied from 16.26 (*OfPEBP*5) to 23.99 kDa (*OfPEBP*10). The predicted theoretical isoelectric points (pI) ranged from 5.68 (*OfPEBP*8) to 9.99 (*OfPEBP*5), and the aliphatic index values ranged from 73.85 (*OfPEBP*7) to

89.54 (OfPEBP6). Except for OfPEBP5–7, all OfPEBP proteins has instability index values above 40, suggesting that most OfPEBP proteins may be unstable. The GRAVY values ranged from −0.42 (OfPEBP8) to −0.03 (OfPEBP6), indicating that all OfPEBP proteins are hydrophilic in nature.

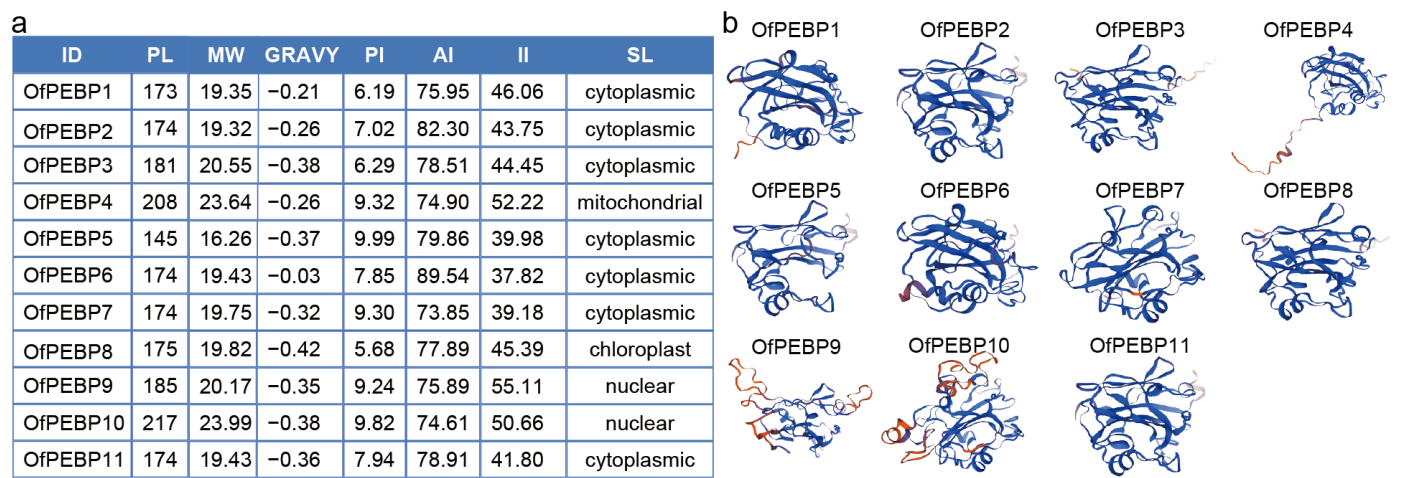


Figure 1. Physicochemical properties and predicted tertiary structures of OfPEBP proteins. **(a)** Physico-chemical characteristics of OfPEBP proteins. PL, protein length; MW, molecular weight; GRAVY, grand average of hydropathicity; pI, theoretical isoelectric point; AI, aliphatic index; II, instability index. **(b)** Predicted tertiary structures of OfPEBP proteins.

Subcellular localization prediction showed that OfPEBP9 and OfPEBP10 proteins were localized to the nucleus. OfPEBP4 was predicted to localize to the mitochondria, and OfPEBP8 to the chloroplasts. The remaining OfPEBP proteins were predicted to localize in the cytoplasm (Figure 1a). In addition, secondary structure prediction showed that OfPEBP proteins were mainly composed of random coils (56.67–66.30%), extended strands, and α -helices (Figure 1b; Supplementary Table S4).

3.2. Gene Structure, Conserved Motif, and Phylogenetic Analyses of the OfPEBP Gene Family

To examine the evolutionary relationships between the *OfPEBP* genes and PEBP members from other plant species, we constructed a phylogenetic tree using PEBP sequences from *A. thaliana*, *O. sativa*, *P. trichocarpa*, and *O. fragrans*. In total, 6 *AtPEBP*s, 19 *OsPEBP* proteins, 6 *PtPEBP* proteins, and 11 *OfPEBP* proteins were included in multiple sequence alignment and phylogenetic analysis (Figure 2a). The phylogenetic results showed that all PEBP proteins could be classified into three major subgroups: FT-, MFT-, and TFL1-like. OfPEBP3 and OfPEBP8 were clustered within the FT-like subgroup, while OfPEBP6 belonged to the MFT-like subgroup. The remaining OfPEBP members were assigned to the TFL1-like subgroup. These results suggest that the PEBP family in *O. fragrans* has undergone substantial functional divergence, with different subgroups potentially carrying out distinct biological roles.

Conserved motif analysis revealed that all *OfPEBP* proteins contained motifs 2, 3, and 5 (Figure 2b). Different subgroups exhibited distinct subgroup-specific motif compositions. FT-like members additionally contained motifs 1, 4, and 6, whereas the MFT-like member possessed motifs 1 and 8 (Figure 2b). In contrast, TFL1-like members exhibited different motif combinations, including motifs 4, 1, and 6 or motifs 7, 1, and 6 (Figure 2b), suggesting that subgroup-specific structural characteristics may have arisen during functional divergence. Gene structure analysis showed that members of the FT- and MFT-like subgroups each contained four coding sequence (CDS) regions. Within the TFL1-like subgroup, most members also possessed four CDS regions, except for OfPEBP5 and OfPEBP9, which con-

tained two and five CDS regions, respectively (Figure 2c). Overall, members within the same subgroup displayed highly similar motif compositions and gene structural characteristics, further supporting the phylogenetic classification and suggesting that proteins with similar structural features may share related biological functions.

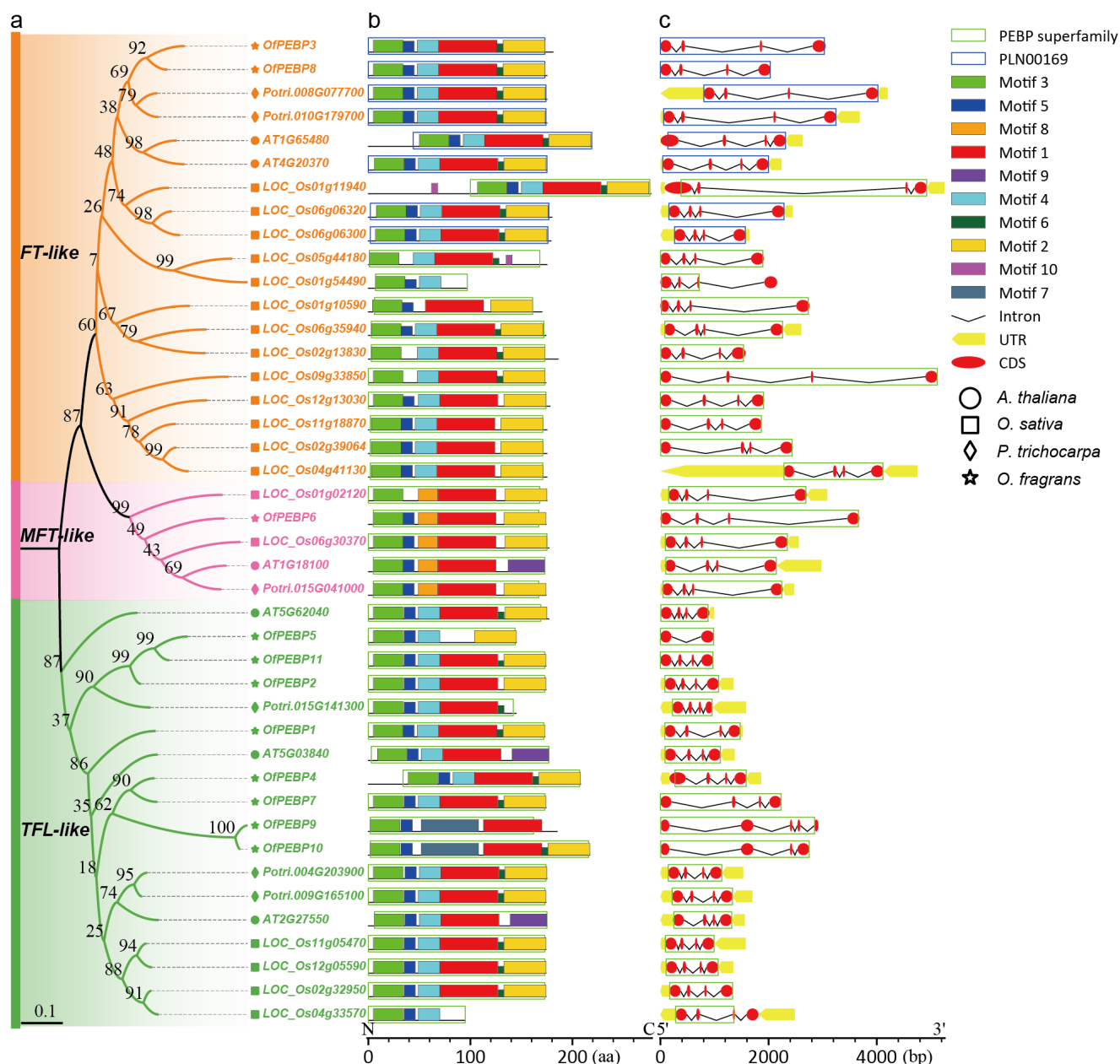


Figure 2. Phylogenetic relationships, conserved motifs, and gene structures of the OfPEBP family. (a) Phylogenetic tree of OfPEBP proteins constructed using the NJ method with 1000 bootstrap replicates. Numbers on branches indicate bootstrap support values, and different colors and shapes represent proteins from different species. (b) Conserved motif composition of OfPEBP proteins. Motifs with different conserved features are shown in different colors. (c) Exon–intron structures of OfPEBP genes. Red ovals indicate coding sequences (CDS), yellow pentagons indicate untranslated regions (UTRs), and black lines indicate introns.

3.3. Prediction of Cis-Acting Regulatory Elements

Cis-acting regulatory elements play essential roles in gene expression regulation and responses to environmental signals. To investigate the potential transcriptional regulatory mechanisms of OfPEBP genes, cis-acting element analysis was performed on the upstream

promoter regions of each gene. A total of 34 different types of *cis*-acting elements were identified and classified into six functional categories: growth- and development-related (4 types), hormone (9 types), light (16 types), metabolic (1 type), stress (3 types), and temperature responsiveness (1 type) (Figure 3a–c). A total of 275 *cis*-acting elements were identified among the 11 *OfPEBP* genes. Among these, light-responsive elements were the most abundant, with 123 elements accounting for 44.73% of the total, followed by hormone-responsive elements, which comprised 101 elements and represented 36.73% of all identified elements (Figure 3a,b). These results suggest that light signals and plant hormones may serve as major regulatory factors controlling *OfPEBP* gene expression, indicating their potential involvement in photoperiodic regulation, plant growth, and hormone signal transduction.

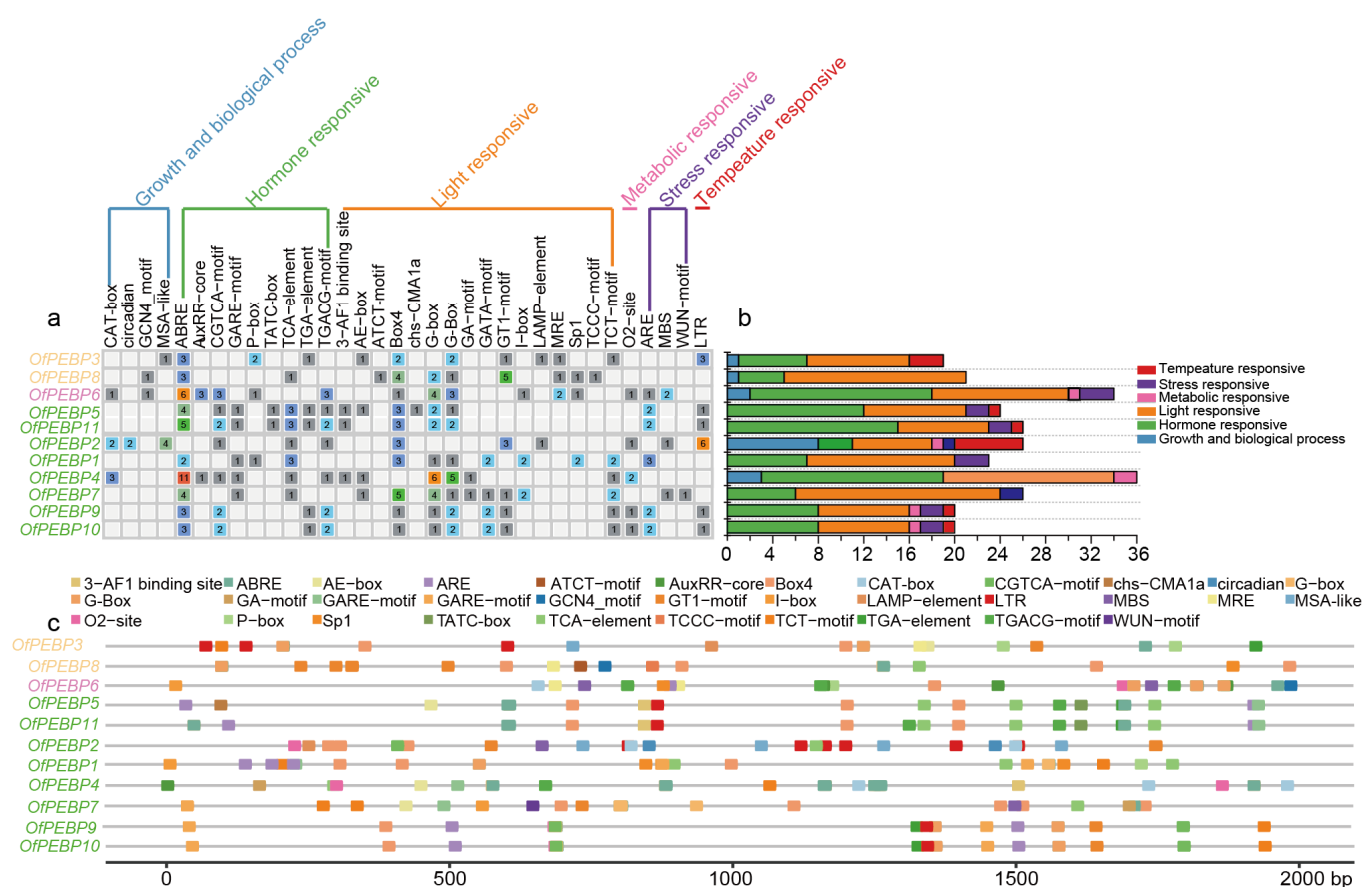


Figure 3. Analysis of *cis*-acting elements in the promoter regions of *OfPEBP* genes. (a) Classification and abundance of *cis*-acting elements in *OfPEBP* promoters. Numbers in different colors indicate the abundance of each type of *cis*-acting element. (b) Composition and number of *cis*-acting elements in individual *OfPEBP* genes, shown as a stacked bar chart. Different fill colors represent the five major categories of *cis*-acting elements. (c) Distribution of *cis*-acting elements within the promoter regions of *OfPEBP* genes. Different colors indicate different *cis*-acting element categories.

Further analysis revealed that all *OfPEBP* genes contained both light- and hormone-responsive elements (Figure 3b). In addition, substantial differences in *cis*-element composition were observed among different family members. Besides light- and hormone-responsive elements, *OfPEBP3* and *OfPEBP8* also contained growth- and development-related responsive elements (Figure 3b). *OfPEBP6* uniquely possessed metabolic- and stress-responsive elements in addition to growth- and development-related elements. The remaining *OfPEBP* members contained an additional 1–4 categories of responsive elements (Figure 3b). These findings indicate that different *OfPEBP* genes may participate in diverse

biological processes through distinct *cis*-regulatory patterns and may play differential roles in developmental regulation and environmental adaptation.

3.4. Genomic Variation and Haplotype Analysis of the *OfPEBP* Gene Family

For the seven *OfPEBP* genes containing SNP variants within their genomic regions, combined haplotype frequency distributions were analyzed. These genes exhibited substantial differences in haplotype diversity, reflecting different levels of genetic variation between the Season and Autumn populations (Figure 4a). *OfPEBP1* and *OfPEBP2* showed relatively simple haplotype compositions in both populations, dominated by a few shared major haplotypes, particularly Hap1 and Hap2. In contrast, *OfPEBP3*, *OfPEBP4*, and *OfPEBP6–8* displayed more complex haplotype patterns, including both shared haplotypes and multiple “other” haplotype types, indicating greater divergence between the two populations. Notably, *OfPEBP3*, *OfPEBP6*, and *OfPEBP8* showed relatively high proportions of “other” haplotypes. Further comparison of shared haplotype frequencies between the Season and Autumn groups revealed clear differences in haplotype composition, especially for *OfPEBP3*, *OfPEBP4*, and *OfPEBP6*, suggesting potential population differentiation and selection associated with flowering phenotype.

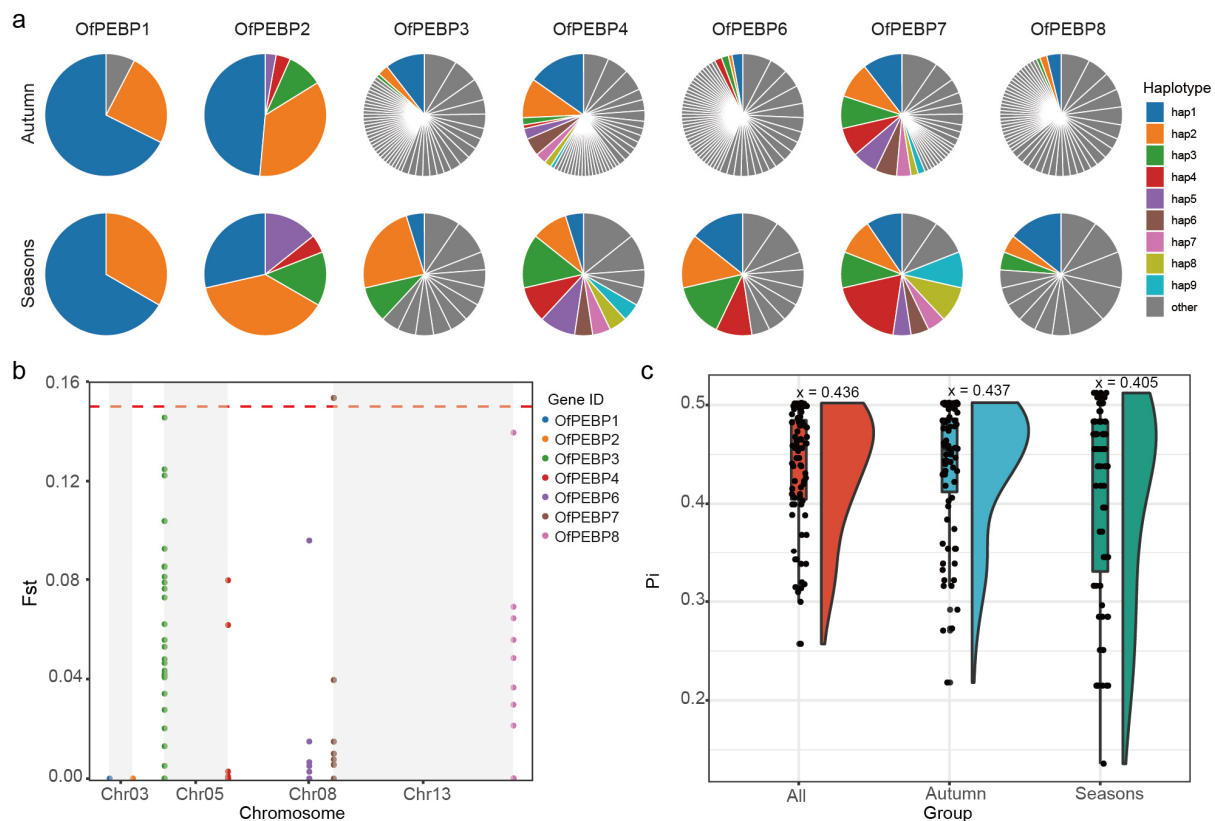


Figure 4. Population genetic variation analysis of *OfPEBP* genes in seasonal- and autumn-flowering *O. fragrans* populations. (a) Frequency distribution of different SNP-based haplotypes of *OfPEBP* genes in seasonal-flowering and autumn-flowering populations. For each gene, pie chart sectors with the same color in the Season and Autumn groups represent the same haplotype, whereas distinct low-frequency haplotypes are grouped as “Other” in gray. Sector size indicates haplotype frequency. (b) Population differentiation analysis of SNP loci within *OfPEBP* genes. The red dashed line at $F_{st} = 0.15$ indicates moderate to relatively high genetic differentiation. Dots in different colors represent SNP loci located in different *OfPEBP* genes. (c) Comparison of nucleotide diversity between seasonal- and autumn-flowering populations for *OfPEBP* genes, shown using boxplots and density distributions. The y-axis represents π values, colors indicate population groups, and “x” marks the group mean.

Population differentiation analysis showed that *OfPEBP7* contained one locus on Chr08 with an *Fst* value exceeding 0.15 (Figure 4b), representing the highest level of genetic differentiation among all examined genes. *OfPEBP3* possessed multiple loci with moderate to high differentiation on Chr05, indicating an overall elevated level of genetic differentiation, whereas loci within *OfPEBP8* on Chr13 exhibited a gradient distribution ranging from low to moderate differentiation (Figure 4b). By contrast, loci within *OfPEBP1*, *OfPEBP2*, *OfPEBP4*, and *OfPEBP6* generally exhibited relatively low *Fst* values (Figure 4b). These results indicate that *OfPEBP7* and *OfPEBP3* displayed the strongest differentiation signals, implying that these genes may have experienced selective pressures associated with population divergence.

Pi analysis further revealed differences in genetic polymorphism among the examined groups (Figure 4c). The average Pi values for all samples and the Autumn population were 0.436 and 0.437, respectively, showing highly similar and concentrated distributions, with most loci falling within the 0.4–0.5 range, indicative of relatively high and stable genetic diversity (Figure 4c). In comparison, the Seasons population exhibited a lower average Pi value of 0.405 and a more dispersed distribution, with a greater proportion of low-Pi loci, suggesting reduced overall genetic polymorphism (Figure 4c). These findings indicate that the Seasonal population may have undergone stronger directional selection or population structure differentiation, resulting in reduced nucleotide diversity, whereas the Autumn population maintained a relatively stable level of genetic diversity comparable to that of the overall population.

3.5. SNP–Phenotype Association Analysis

Association signals from multiple SNP loci were detected across different chromosomes, and detailed information for each SNP is provided in Supplementary Table S5. Among these loci, the SNP locus within *OfPEBP7* on Chr13 exhibited the highest $-\log_{10}(P)$ value, exceeding the significance threshold and displaying the strongest association signal. In addition, SNP loci within *OfPEBP3* (Chr05) and *OfPEBP8* (Chr13) also showed relatively strong association signals (Figure 5a). For *OfPEBP7*, the T/T genotype were mainly associated with the Autumn flowering phenotype, accounting for 94% of individuals, while only 6% exhibited the Seasons phenotype. Among individuals with the T/C genotype, 75% showed the Autumn phenotype and 25% showed the Seasonal phenotype. In contrast, individuals with the C/C genotype showed equal proportions of Autumn and Seasons phenotype (50% each), with no significant difference (ns) (Figure 5b). For *OfPEBP3*, the Autumn phenotype accounted for 87% and 88% of individuals with the A/A and A/G genotypes, respectively. These proportions were much higher than those of the Seasons phenotype. By contrast, among individuals with the G/G genotype, the Seasons phenotype accounted for 67%, while the Autumn phenotype accounted for 33%. However, this difference was not statistically significant (ns) (Figure 5c). For *OfPEBP8*, all individuals with the G/G genotype showed the Autumn phenotype. Among individuals with the G/C genotype, 83% displayed the Autumn phenotype and 17% displayed the Seasons phenotype. Individuals with the C/C genotype included 68% Autumn and 32% Seasons phenotypes (Figure 5d). Overall, distinct genotype-dependent distributions of Autumn and Seasons flowering phenotypes were observed for *OfPEBP3*, *OfPEBP7*, and *OfPEBP8*. These results suggest that polymorphisms within these genes are associated with flowering-type variation and may contribute to the genetic differentiation of flowering phenotypes in *O. fragrans*.

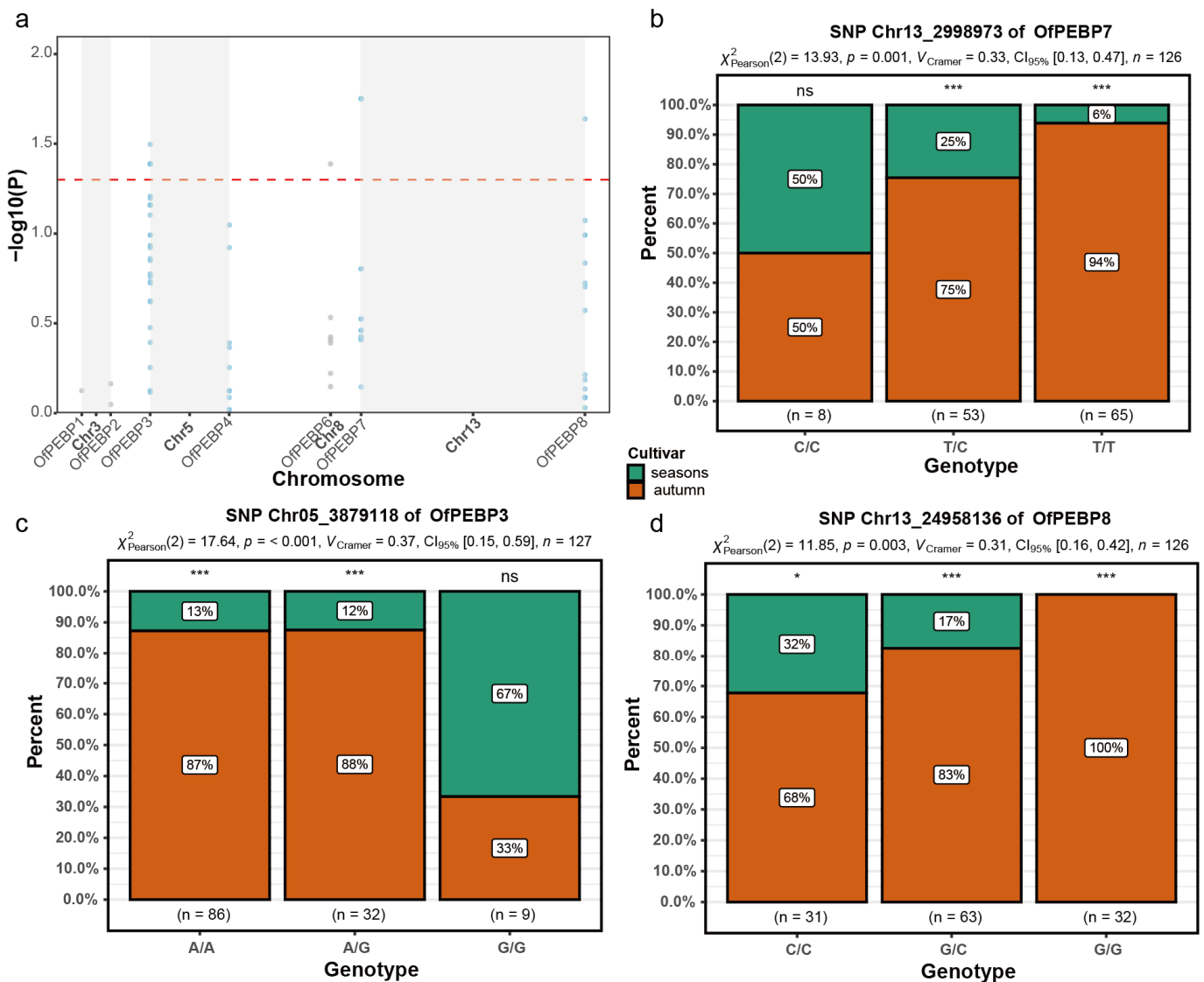


Figure 5. Association analysis between SNP loci in *OfPEBP* genes and flowering phenotypes in *O. fragrans*. **(a)** Manhattan plot showing the local association analysis of SNP loci located within *OfPEBP* genes across different chromosomes. The x-axis represents the genomic positions of SNP loci within *OfPEBP* genes, and the y-axis represents the $-\log_{10}(p)$ values obtained from the logistic regression after Benjamin–Hochberg adjust. “Seasons” and “Autumn” denote continuous-flowering and non-continuous-flowering accessions, shown in green and orange, respectively. The red dashed line indicates the significance threshold of adjusted $p < 0.05$, above which the genotype frequency of a SNP locus differs significantly between the two flowering types. **(b–d)** Genotype frequency distributions of three significantly associated SNP loci, Chr13_2998973 in *OfPEBP7*, Chr05_3879118 in *OfPEBP3*, and Chr13_24958136 in *OfPEBP8*, respectively, between the two flowering types. Significant associations were further detected using chi-square tests. The overall p value indicates the significance of association at each SNP locus. Pairwise differences in genotype frequency distributions are indicated as *** $p < 0.001$ and * $p < 0.1$; ns, not significant.

3.6. Methylation Pattern Analysis During Flowering Development Stages

CpG, CHG, and total methylation levels exhibited highly consistent variation patterns among the *OfPEBP* family members (Figure 6a–c). Hierarchical clustering analysis revealed that *OfPEBP6*, *OfPEBP9*, and *OfPEBP10*, which displayed relatively high methylation levels, were clustered into the same branch (Cluster I), with methylation levels markedly higher than those of the other family members. In contrast, *OfPEBP1–5*, *OfPEBP7*, *OfPEBP8*, and *OfPEBP11* were grouped into Cluster II and generally exhibited low to moderate

methylation levels. In addition, methylation levels of individual genes showed only minor fluctuations across the S1–S6 developmental stages and remained relatively stable overall (Figure 6a–c). Compared with the other three methylation types, CHH methylation levels were substantially lower (Figure 6d). Only *OfPEBP2* and *OfPEBP8* displayed relatively elevated CHH methylation signals (> 0.1), whereas the remaining members maintained extremely low CHH methylation levels with little variation among developmental stages (Figure 6d). Overall, CpG and CHG methylation represented the predominant methylation forms within the *OfPEBP* gene family. Although considerable differences in methylation levels were observed among different family members, methylation patterns remained relatively stable throughout the S1–S6 developmental stages. These findings suggest that the methylation profiles of *OfPEBP* genes are highly conserved and may contribute to maintaining stable gene expression states and developmental regulation during flower development.

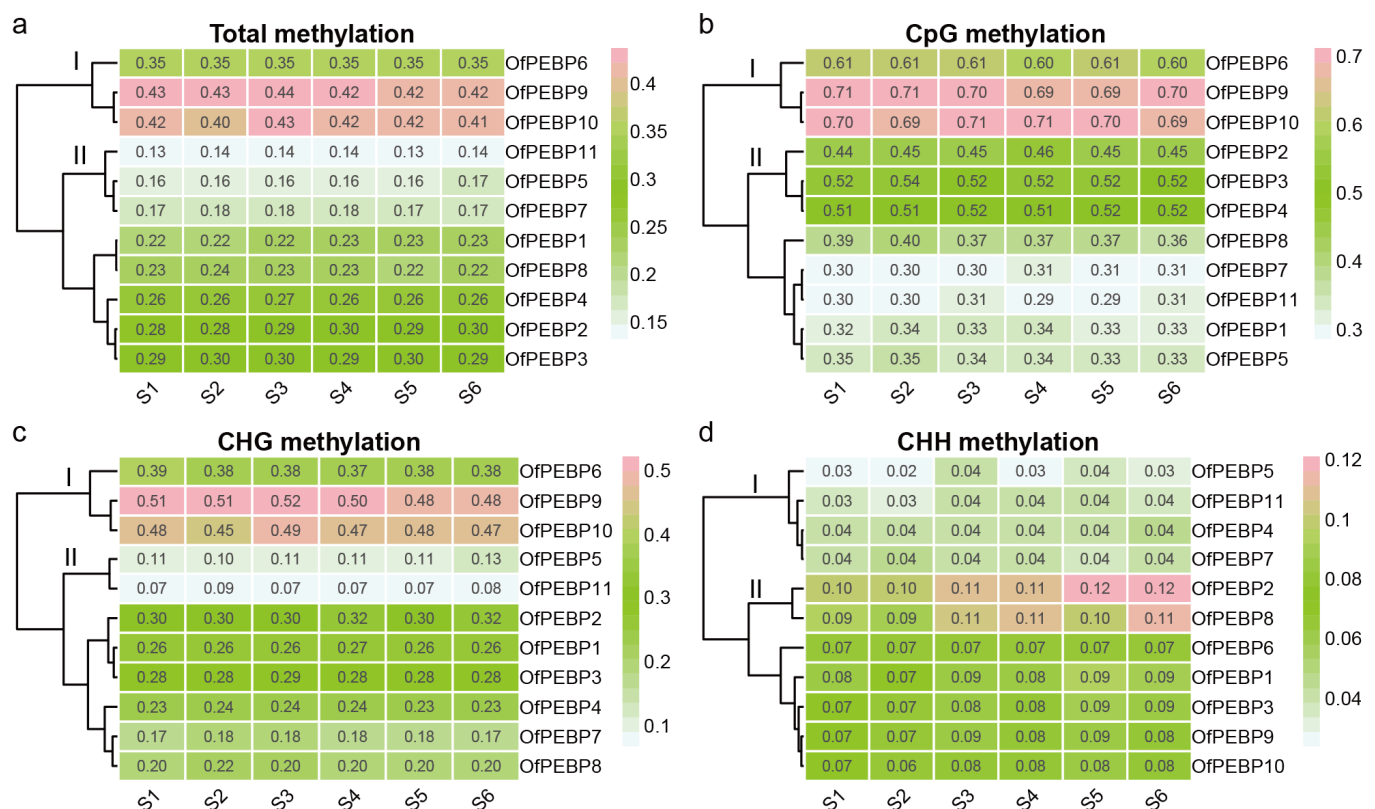


Figure 6. DNA methylation patterns of *OfPEBP* genes during petal development and senescence in *O. fragrans*. (a) Total methylation level, calculated as the sum of CpG, CHG, and CHH methylation levels. (b) CpG methylation level. (c) CHG methylation level. (d) CHH methylation level. For each gene, methylation levels were calculated as the summed methylation values across different positions within the corresponding genomic region. Raw methylation values are displayed as heatmaps, with deeper red indicating higher methylation levels. Genes were grouped by hierarchical clustering.

3.7. Chromosomal Distribution and Intra-/Interspecies Synteny Analysis

Except for one gene located on the contig, a total of 10 *OfPEBP* genes were unevenly distributed across six chromosomes of *O. fragrans* (Figure 7a). Among them, Chr3, Chr5, Chr13, and Chr23 each contained two genes, whereas Chr6 and Chr8 each harbored one gene (Figure 7a). Within the *O. fragrans* genome, six duplicated gene pairs were identified, all of which were derived from segmental duplication events (Figure 7a). Comparative synteny analysis identified one collinear gene pair between *O. sativa* and *O. fragrans*, whereas five collinear gene pairs were detected between *O. fragrans* and *P. trichocarpa*, as well as between

O. fragrans and *A. thaliana* (Figure 7b). Furthermore, all duplicated gene pairs exhibited K_a/K_s ratios lower than 1 (Supplementary Table S6), indicating that these duplicated genes have mainly undergone purifying selection during evolution.

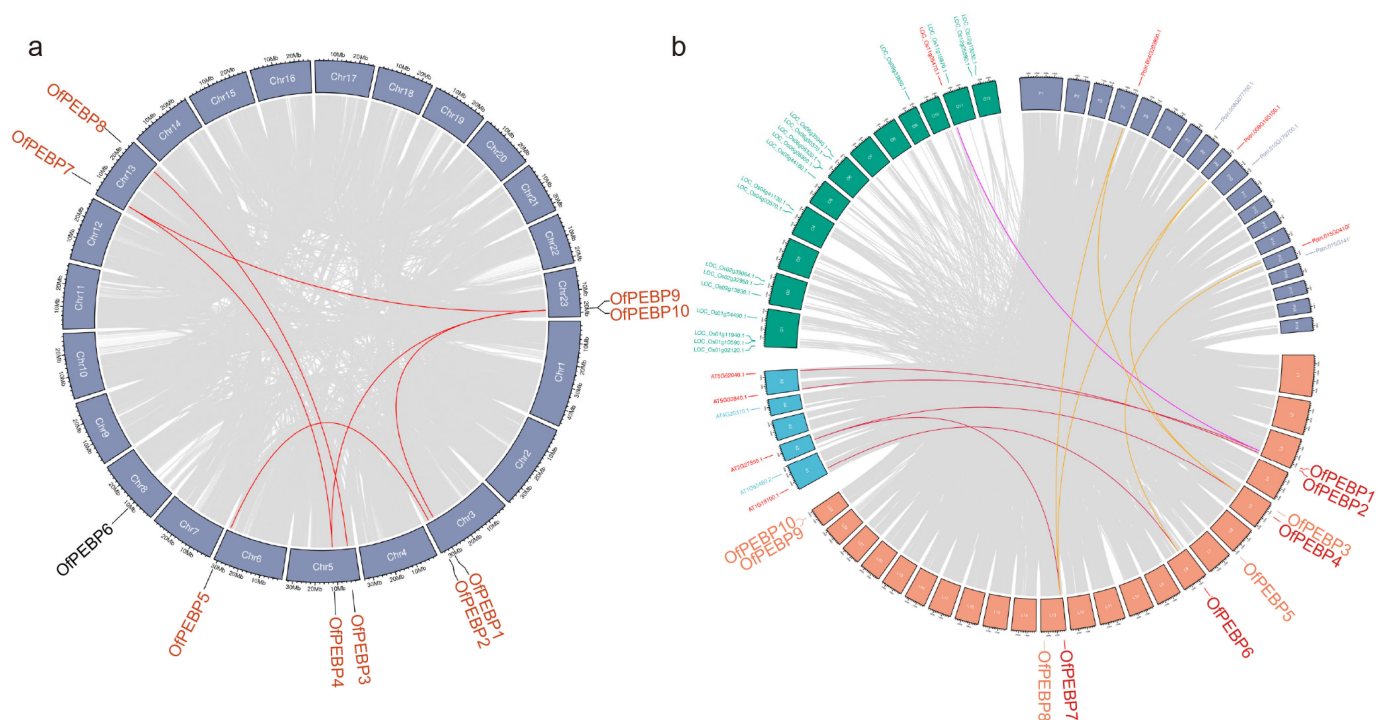


Figure 7. Synteny relationships of *PEBP* genes within *O. fragrans* and between *O. fragrans* and representative plant species. (a) Intraspecies synteny analysis of *OfPEBP* genes in *O. fragrans*. (b) Interspecies synteny analysis of *PEBP* genes between *O. fragrans* and other plant species. Gray lines indicate collinear blocks, and red lines indicate syntenic *PEBP* gene pairs.

3.8. TF Network and GO Enrichment Analysis

To investigate the regulatory relationships and potential functions of the *OfPEBP* gene family, TF regulatory network analysis was performed for the 11 *OfPEBP* genes. The results showed that six *OfPEBP* genes were coexpressed with 122 TFs and eight downstream genes, including *LYG035628*, *LYG022132*, *LYG021780*, *LYG009683*, *LYG006219*, *LYG034319*, and *LYG000730* (Figure 8a). Based on phylogenetic relationships and functional characteristics, the *OfPEBP* family was classified into three subgroups: FT- (*OfPEBP3/8*), TFL1- (*OfPEBP1/5/11*), and MFT-like (*OfPEBP6*), each forming relatively independent yet interconnected functional modules within the regulatory network (Figure 8a). Specifically, *OfPEBP3/8* were mainly co-expressed with members of the *OfMIKC_MADS* (14), *OfERF* (7), *OfB3* (7), and *OfBBR-BPC* (5) TF families. *OfPEBP1/5/11* were primarily associated with *OfHD-ZIP* (17), *OfDof* (9), *OfBBR-BPC* (9), and *OfMIKC_MADS* (8) TFs, whereas *OfPEBP6* showed strong coexpression associations with *OfERF* (24), *OfBBR-BPC* (9), and *OfMIKC_MADS* (6) TFs. In addition, extensive co-expression links were observed among the three subgroups, with *OfBBR-BPC* and *OfMIKC_MADS* TFs commonly associated with all three subfamilies (Figure 8a). Overall, the *OfPEBP* family exhibited clear functional divergence while retaining a certain degree of conservation, providing an important basis for further elucidation of their molecular functions and regulatory mechanisms.

GO enrichment analysis of the 130 coexpressed genes demonstrated that these genes were significantly enriched in biological processes associated with plant growth and development, hormone signal transduction, and stress responses (Figure 8b). In particular, hormone-related pathways, including “response to ethylene (ETH)”, “response to jasmonic

acid”, “response to gibberellin”, “ETH-activated signaling pathway”, and “jasmonic acid-mediated signaling pathway”, were significantly enriched (Figure 8b). Moreover, several developmental processes, such as “shoot system morphogenesis”, “leaf morphogenesis”, “meristem maintenance”, and “regulation of flower development”, also exhibited high enrichment levels (Figure 8b). These enrichment results suggest that the OfPEBP family not only participates in plant morphogenesis, meristem maintenance, and flowering regulation, but may also coordinately regulate plant adaptive processes through hormone signaling and stress-response pathways.

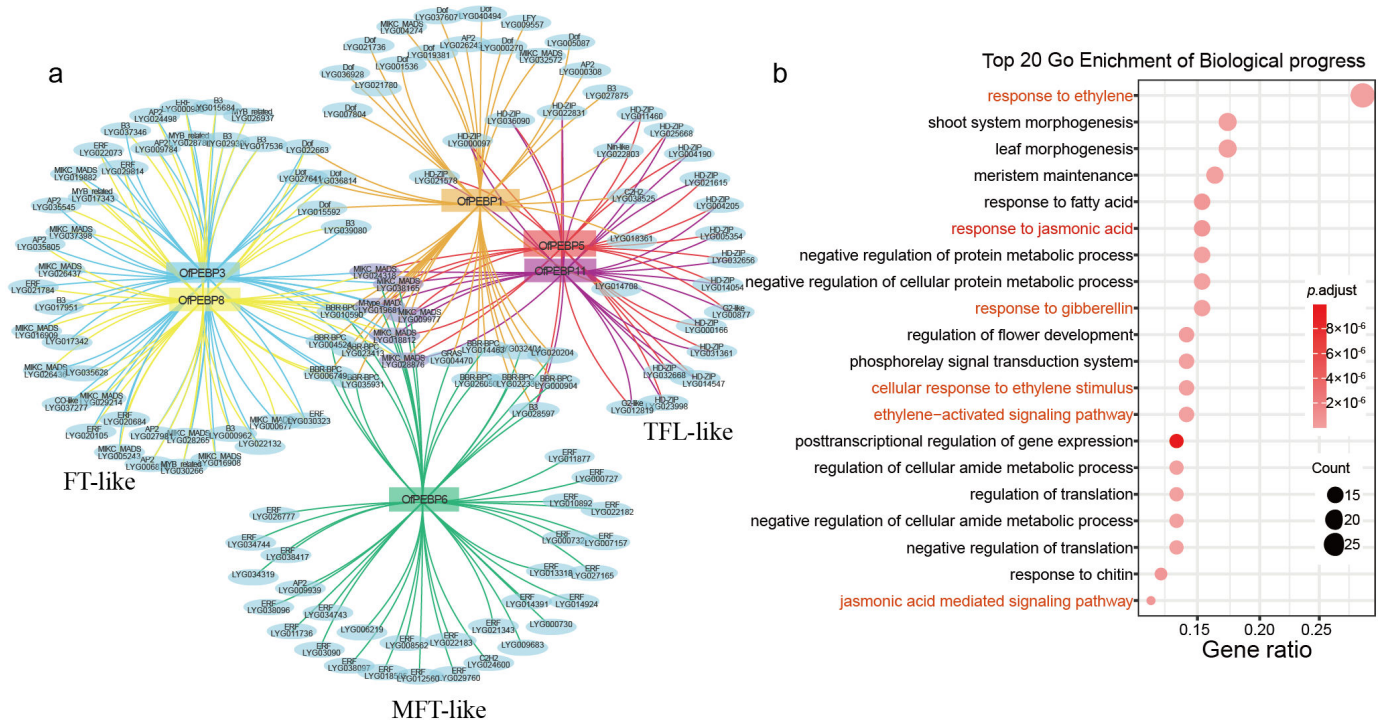


Figure 8. Coexpression regulatory network and functional enrichment analysis of *OfPEBP* genes. (a) Coexpression regulatory network between *OfPEBP* genes and their coexpressed transcription factors (TFs). Edges indicate coexpression relationships, and only correlations with absolute values greater than 0.8 are shown. Rectangles in different colors represent *OfPEBP* genes, whereas light-blue ellipses represent coexpressed genes or TFs. (b) Gene Ontology (GO) enrichment analysis of genes coexpressed with *OfPEBP* genes. The x-axis represents the gene ratio, dot size indicates the number of enriched genes, and color intensity represents the adjusted significance level.

3.9. Gene Expression Patterns Under Different Tissues, Flowering Stages, and Hormone Treatments

Members of the *OfPEBP* family exhibited clear tissue-specific expression patterns (Figure 9a). Among them, *OfPEBP8* showed specifically high expression in stems (FPKM = 120.09), whereas *OfPEBP1/2* were highly expressed in buds, and *OfPEBP3* exhibited relatively high expression in leaves (Figure 9a). Most other members had very low or undetectable expression across tissues (Figure 9a). *OfPEBP1* and *OfPEBP2*, which may be related to flowering regulation, showed opposite expression trends during flower development (Figure 9b). *OfPEBP1* maintained relatively high expression from S1 to S3 and then gradually decreased, whereas *OfPEBP2* was strongly upregulated at the late flowering stage (S6), where it reached its highest expression level. (Figure 9b).

OfPEBP1 and *OfPEBP8* responded markedly to temperature changes (Figure 9c). Under low-temperature treatment (19 °C), *OfPEBP1* expression reached the highest level at 6 d, while high temperature (23 °C) strongly induced the expression of *OfPEBP8* (Figure 9c). ETH and Aza treatments also affected the expression of *OfPEBP1/2/8* (Figure 9d). Specifically, ETH and Aza treatments suppressed *OfPEBP1* expression, with the

highest expression observed at the early stage of the control, ETH, and Aza treatments. In contrast, *OfPEBP2* expression was upregulated at 5 d in the control and at day 8 under ETH and Aza treatments. *OfPEBP8* expression peaked at 1 d in the control and at 5 d following ETH and Aza treatments (Figure 9d). Overall, members of the *OfPEBP* family exhibited highly specific expression patterns across tissues and developmental stages, and different members showed distinct responses to temperature and hormone treatments. These results suggest that *OfPEBP* genes may have diverse roles in flowering-time regulation and environmental responses in *O. fragrans*.

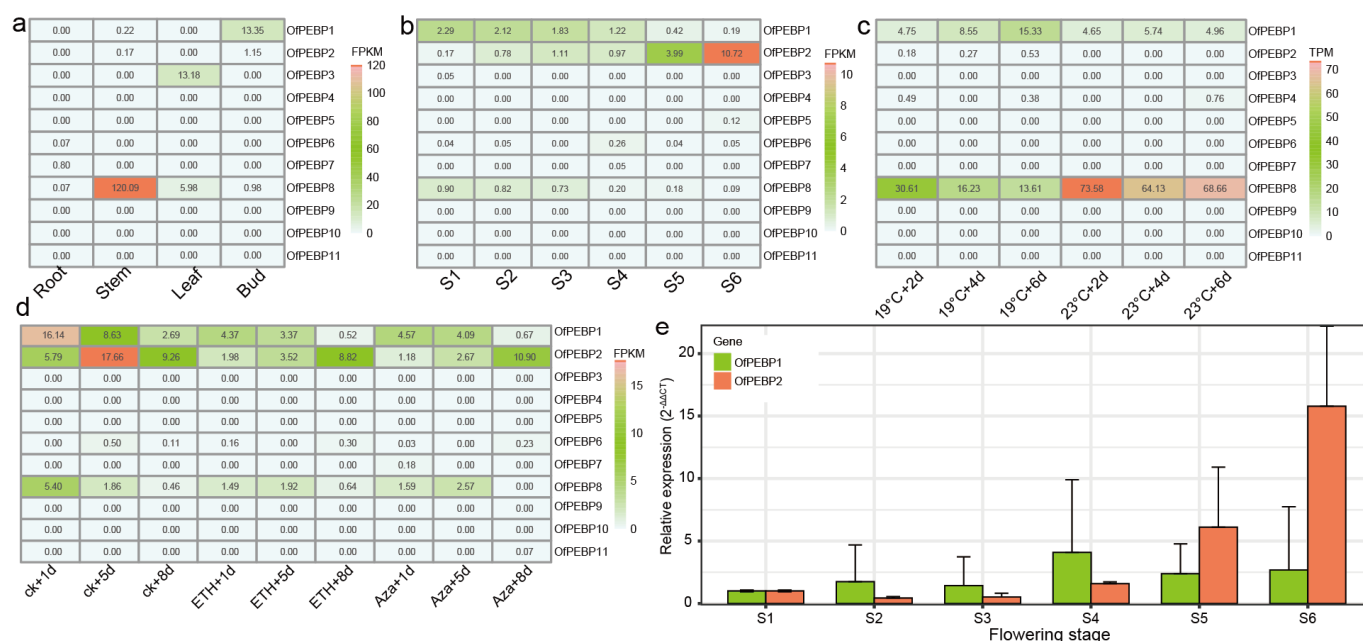


Figure 9. Expression patterns of *OfPEBP* genes across different tissues, flower developmental stages, and under various treatments. (a) Expression profiles of *OfPEBP* genes in different tissues. (b) Expression patterns of *OfPEBP* genes during flower development from S1–S6. (c) Expression profiles of *OfPEBP* genes under 19 °C and 23 °C treatments. (d) Expression responses of *OfPEBP* genes to ethylene and Aza treatments. (e) qRT-PCR validation of selected *OfPEBP* genes at different flower developmental stages (S1–S6), short horizontal bars indicate standard errors. All heatmaps were generated using raw FPKM or TPM values without normalization, with deeper red indicating higher expression levels.

4. Discussion

4.1. Characterization and Evolution of the *OfPEBP* Gene Family

A total of 11 *OfPEBP* family members were identified in *O. fragrans* (Figure 1a), which is fewer than the numbers reported in *O. sativa* (19) and maize (25), but greater than those identified in *A. thaliana* (6) and poplar (6). Variations in the PEBP gene family size among plant species suggest distinct evolutionary trajectories, indicating that the PEBP family has undergone different degrees of expansion and contraction during plant evolution [9]. Previous studies have shown that monocot species generally contain more PEBP members than dicot species, likely due to whole-genome duplication (WGD), tandem duplication, and segmental duplication events, whereas some duplicated genes may subsequently undergo pseudogenization or gene loss over the course of long-term evolution [40]. Rice and maize experienced multiple rounds of genome duplication, resulting in the retention of a greater number of PEBP copies. In contrast, woody dicotyledonous species generally exhibit relatively limited expansion of this gene family. Although *O. fragrans* is a woody dicot, it possesses more *PEBP* members than *A. thaliana* and poplar, suggesting that additional

expansion events may have occurred during the evolution of the *O. fragrans* lineage. In this study, six pairs of segmentally duplicated genes were identified (Figure 7a), and all Ka/Ks ratios were lower than 1 (Supplementary Table S6), indicating that segmental duplication may have played a major role in the expansion of the OfPEBP family [41,42]. Furthermore, these duplicated gene pairs appear to have undergone strong purifying selection during evolution, thereby preserving their essential biological functions.

Phylogenetic analysis classified the *OfPEBP* genes into three subfamilies, including one MFT-, eight TFL1-, and two FT-like genes (Figure 2a), consistent with observations reported in other plant species [9,43,44]. These three subfamilies generally exhibit distinct functional divergence. FT-like members are widely recognized as key promoters of flowering [18,19], whereas TFL1-like proteins primarily function in maintaining meristem identity and delaying flowering [21,22]. In contrast, MFT-like genes are considered an ancestral branch associated with seed germination, hormone signaling, and stress responses [25]. Therefore, the substantially higher number of TFL1-like genes relative to FT-like genes in *O. fragrans* suggests that this species may possess a more complex regulatory network governing growth and flowering time.

Further analyses revealed subgroup-specific motif compositions and gene structural characteristics (Figure 2b,c). All *OfPEBP* members contained the conserved motifs 2, 3, and 5, suggesting that these motifs are essential for maintaining the conserved core structure and biological functions of PEBPs. Nevertheless, distinct motif combinations were observed among different subgroups (Figure 2b,c), a pattern commonly reported in other plant species [9,43,44]. For example, although FT and TFL1 proteins share high sequence similarity, substitutions at only a few critical amino acid residues can shift their biological functions between flowering promotion and flowering repression. Specifically, FT proteins contain the conserved xGxGGR motif associated with flowering promotion, whereas TFL1 proteins possess the TAARRR motif related to flowering repression, and these motif differences underlie their antagonistic regulatory roles in flowering control [45,46].

Gene structure analysis further demonstrated that members within the same subgroup displayed highly conserved CDS numbers and arrangements, whereas certain variations were observed among different subgroups (Figure 2b,c). Both FT- and MFT-like members contained four CDS regions. In contrast, within the TFL1-like subgroup, *OfPEBP5* and *OfPEBP9* possessed two and five CDSs, respectively, whereas the remaining members retained four CDSs (Figure 2c). Similar structural patterns have been reported in PEBP gene families from other plant species [9,47]. Overall, the number of exons in *OfPEBP* genes ranged from two to five, with most genes containing four exons. Such structural variation may contribute to functional divergence among subgroups, as differences in CDS number and arrangement could influence alternative splicing diversity or fine-tune protein conformation [48,49], thereby affecting distinct biological functions. Collectively, the conserved motifs likely ensure the fundamental functions of the PEBP family, whereas subgroup-specific motif compositions and CDS variations may provide the molecular basis for the functional diversification of *OfPEBP* genes in flowering regulation, meristem maintenance, and stress responses.

4.2. Differential Expression and Methylation Patterns of *OfPEBP* Genes Are Associated with Flowering Regulation and Responses to Low Temperature, and Hormonal Treatments

Promoter *cis*-acting elements serve as specific binding sites for regulatory proteins and play crucial roles in transcriptional regulation [50,51]. In this study, light-responsive elements accounted for the largest proportion of *cis*-elements identified in *OfPEBP* promoters, followed by hormone-responsive elements (Figure 3a,b). The enrichment of light- and hormone-responsive elements suggests that most *OfPEBP* promoters may function as inducible promoters regulated by light and hormone signaling pathways. In addition,

OfPEBP3 and *OfPEBP8* contained growth- and development-related elements besides light- and hormone-responsive elements, whereas *OfPEBP6* harbored metabolism- and stress-responsive elements. The remaining *OfPEBP* members contained an additional 1–4 types of *cis*-elements (Figure 3a–c), suggesting potential functional divergence among family members. For example, the *cis*-element composition of *OfPEBP3* and *OfPEBP8* implies possible involvement in growth and developmental processes, such as floral bud differentiation and flowering regulation (Figure 9b) [6,52], whereas *OfPEBP6* may be associated with metabolic regulation and stress responses [53,54]. However, these functional inferences are based on promoter element prediction and supporting expression patterns, and have not yet been validated by transgenic, knockout, or other targeted functional experiments. Therefore, the *cis*-element analyses should be regarded as providing candidate regulatory clues for future functional validation of *OfPEBP* genes.

DNA methylation analysis revealed that CpG and CHG methylation were the dominant methylation types within the *OfPEBP* family. *OfPEBP6*, *OfPEBP9*, and *OfPEBP10* exhibited relatively high methylation levels that remained generally stable throughout the S1–S6 flowering stages (Figure 6a–c). In contrast, *OfPEBP2* and *OfPEBP8* displayed relatively higher CHH methylation levels (Figure 6d), suggesting their potential involvement in stage-specific or environmentally responsive regulation. Combined with temperature and hormone treatment experiments, *OfPEBP1* and *OfPEBP2* exhibited differential expression patterns during petal opening and senescence stages (Figure 9b). Notably, *OfPEBP1* showed a strong expression response under low temperature (19 °C), whereas *OfPEBP8* responded more markedly under 23 °C treatment (Figure 9c). In addition, ETH and Aza treatments affected the expression of *OfPEBP1/2/8* (Figure 9d), suggesting a possible association between *PEBP* gene expression and hormone- or DNA methylation-related responses. These observations are consistent with previous reports that FT-like genes participate in low-temperature-mediated flowering regulation [55,56] and ETH-related flowering pathways [57]. However, because this study did not quantitatively examine the relationship between DNA methylation levels and transcript abundance, the involvement of methylation in *OfPEBP* regulation remains speculative; and direct functional and methylation-expression validation was not performed in this study, the potential involvement of *OfPEBP* genes in environmentally responsive flowering regulation through epigenetic mechanisms should be regarded as a hypothesis requiring further experimental confirmation.

TF coexpression network analysis further suggested that different *OfPEBP* subgroups were associated with distinct TF families. FT-like members were mainly coexpressed with *OfMIKC_MADS* and *OfERF* TFs, whereas TFL1-like members were primarily associated with *OfHD-ZIP* and *OfDof* TFs. The MFT-like subgroup showed strong co-expression associations with *OfERF* factors, and partial cross-connections among different subgroups were also observed (Figure 8a). These findings indicate that the *OfPEBP* family exhibits both functional divergence and conserved regulatory characteristics within the transcriptional network, thereby enabling coordinated regulation of flowering development, hormone signaling, and stress responses. Furthermore, GO enrichment analysis revealed that downstream target genes of *PEBP* members are mainly involved in plant morphogenesis, meristem maintenance, and flowering regulation (Figure 8b). These functions are highly consistent with those reported for *PEBP* family members in model plants and other woody species, thereby providing a theoretical basis for future studies on their molecular functions and regulatory mechanisms.

4.3. SNPs and Haplotype Variations in *OfPEBP* Genes May Contribute to Seasonal Flowering in *O. fragrans*

Haplotype analysis revealed distinct patterns of genetic variation among different *OfPEBP* genes within the population. *OfPEBP1* and *OfPEBP2* exhibited relatively simple

haplotype compositions, whereas *OfPEBP3*, *OfPEBP4*, and *OfPEBP6–8* displayed more diverse haplotype structures, with a relatively high proportion of unique or other rare haplotypes (Figure 4a). Further comparison between the Autumn and Seasons populations revealed substantial changes in the proportions of shared haplotypes for several genes, suggesting that a certain degree of genetic differentiation has occurred between different flowering types. Fst analysis demonstrated that *OfPEBP7* on Chr08 exhibited the strongest differentiation signal, while multiple moderately to highly differentiated loci were identified in *OfPEBP3* on Chr05 (Figure 4b; Figure 5), indicating that these genes may have experienced stronger selective pressure during evolution. In contrast, although *OfPEBP8* showed relatively low overall differentiation, it still exhibited a continuous differentiation gradient across loci (Figure 4b; Figure 5). Meanwhile, the Pi of the Seasons population was lower than that of both the Autumn population and the total population (Figure 4c), suggesting that the continuous-flowering population may have undergone stronger directional selection, leading to reduced genetic diversity. Previous studies have shown that reduced nucleotide diversity is commonly associated with domestication or long-term artificial selection. Therefore, advantageous allelic variations may have been retained during the formation and improvement of the continuous-flowering trait.

SNP–phenotype association analysis further supported this hypothesis. Extensive genetic variations were identified in FT-like genes (*OfPEBP3* and *OfPEBP8*) and the TFL1-like gene (*OfPEBP7*) (Figure 5a). The distribution frequencies of different genotypes varied substantially between the Autumn and Seasons populations. For example, the T/T genotype of *OfPEBP7* was mainly enriched in the Autumn population, whereas the G/G genotype of *OfPEBP3* occurred at a significantly higher frequency in the Seasons population (Figure 5b–d). Notably, *OfPEBP3* and *OfPEBP8* belong to the FT-like subgroup, whose members are generally recognized as flowering promoters involved in floral transition. Therefore, genetic variations in these genes may influence flowering time and recurrent flowering ability by affecting gene expression, protein activity, or regulatory networks. Similar phenomena have been reported in multiple plant species, where natural variations in PEBP family genes are closely associated with flowering regulation and seasonal adaptation [58,59]. Taken together, these results suggest that specific SNPs and haplotype variations in *OfPEBP3*, *OfPEBP7*, and *OfPEBP8* may contribute to the formation of the continuous-flowering trait in *O. fragrans* and may represent the combined outcome of long-term natural selection and artificial domestication.

5. Conclusions

In this study, 11 *OfPEBP* genes were systematically identified and classified into three subgroups: FT-, TFL1-, and MFT-like. Phylogenetic, conserved motif, and gene structure analyses revealed a high degree of conservation and potential functional divergence among these subgroups. Population genetic analyses demonstrated that SNPs and haplotype variations in *OfPEBP3*, *OfPEBP7*, and *OfPEBP8* were closely associated with seasonal flowering phenotypes, although further functional validation is needed to confirm their biological roles. DNA methylation analysis showed that the *OfPEBP* family was predominantly characterized by CpG and CHG methylation patterns, which remained relatively stable throughout flowering development. Expression profiling further revealed distinct tissue-specific expression patterns and differential responses of *OfPEBP* genes to temperature, ETH, and Aza treatments. These findings provide useful insights into the potential involvement of the *OfPEBP* family in flowering regulation, environmental responses, and continuous flowering traits, while also offering candidate genes and a theoretical foundation for future functional studies and molecular breeding in *O. fragrans*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070857/s1>, Table S1: PEBP protein sequences of *Arabidopsis thaliana*, *Oryza sativa*, and *Populus trichocarpa* used in this study; Table S2: Expression data of *OfPEBP* genes obtained from the *O. fragrans* Information Resource (OfIR) database; Table S3: Primers used in this study; Table S4: Predicted secondary structures of *OfPEBP* proteins; Table S5: Detailed information of SNP loci identified in *OfPEBP* genes; Table S6: KaKs analysis.

Author Contributions: Data curation, Formal analysis, Software, Visualization, and Writing—original draft preparation: H.X.; Validation: Y.W.; Formal analysis: J.Y.; Project administration: J.Z.; Supervision, Resources, and Investigation: H.C.; Conceptualization, Funding acquisition, Methodology, and Writing—review and editing: Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (32401647), the Hubei Youth Sci-tech Talent Training Special Project (2025DJ A045), the Hubei Young Sci-tech Talent Funding Project (E Kexieban 2025[67]), and PhD grant from Hubei University of Science and Technology (BK202503).

Data Availability Statement: The original contribution presented in this study are added in the article or Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Bernier, I.; Jollès, P. Purification and characterization of a basic 23 kDa cytosolic protein from bovine brain. *Biochim. Biophys. Acta Protein Struct. Mol. Enzym.* **1984**, *790*, 174–181. [\[CrossRef\]](#) [\[PubMed\]](#)
- Bernier, I.; Tresca, J.; Jollès, P. Ligand-binding studies with a 23 kDa protein purified from bovine brain cytosol. *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1986**, *871*, 19–23. [\[CrossRef\]](#) [\[PubMed\]](#)
- Banfield, M.J.; Barker, J.J.; Perry, A.C.; Brady, R.L. Function from structure? The crystal structure of human phosphatidylethanolamine-binding protein suggests a role in membrane signal transduction. *Structure* **1998**, *6*, 1245–1254. [\[CrossRef\]](#) [\[PubMed\]](#)
- Serre, L.; Vallée, B.; Bureaud, N.; Schoentgen, F.; Zelwer, C. Crystal structure of the phosphatidylethanolamine-binding protein from bovine brain: A novel structural class of phospholipid-binding proteins. *Structure* **1998**, *6*, 1255–1265. [\[CrossRef\]](#) [\[PubMed\]](#)
- Nadal Bigas, J.; Fiers, M.; van der Wal, F.; Willems, L.A.J.; Willemsen, V.; Nijveen, H.; Angenent, G.C.; Immink, R.G.H. The *PEBP* genes *FLOWERING LOCUS T* and *TERMINAL FLOWER 1* modulate seed dormancy and size. *J. Exp. Bot.* **2025**, *76*, 1049–1067. [\[CrossRef\]](#) [\[PubMed\]](#)
- Song, C.; Zhang, W.; Manzoor, M.A.; Sabir, I.A.; Pan, H.; Zhang, L.; Zhang, Y. Differential involvement of *PEBP* genes in early flowering of *Peucedanum praeruptorum* Dunn. *Postharvest Biol. Technol.* **2024**, *212*, 112860. [\[CrossRef\]](#)
- Zhao, C.; Zhu, M.; Guo, Y.; Sun, J.; Ma, W.; Wang, X. Genomic Survey of *PEBP* gene family in rice: Identification, phylogenetic analysis, and expression profiles in organs and under abiotic stresses. *Plants* **2022**, *11*, 1576. [\[CrossRef\]](#) [\[PubMed\]](#)
- Danilevskaya, O.N.; Meng, X.; Hou, Z.; Ananiev, E.V.; Simmons, C.R. A genomic and expression compendium of the expanded *PEBP* gene family from maize. *Plant Physiol.* **2008**, *146*, 250–264. [\[CrossRef\]](#) [\[PubMed\]](#)
- Sun, Y.; Jia, X.; Yang, Z.; Fu, Q.; Yang, H.; Xu, X. Genome-wide identification of *PEBP* gene family in *Solanum lycopersicum*. *Int. J. Mol. Sci.* **2023**, *24*, 9185. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kardailsky, I.; Shukla, V.K.; Ahn, J.H.; Dagenais, N.; Christensen, S.K.; Nguyen, J.T.; Chory, J.; Harrison, M.J.; Weigel, D. Activation tagging of the floral inducer *FT*. *Science* **1999**, *286*, 1962–1965. [\[CrossRef\]](#) [\[PubMed\]](#)
- Fornara, F.; de Montaigu, A.; Coupland, G. SnapShot: Control of flowering in *Arabidopsis*. *Cell* **2010**, *141*, 550. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ruonala, R.; Rinne, P.L.H.; Kangasjärvi, J.; van der Schoot, C. CENL1 expression in the rib meristem affects stem elongation and the transition to dormancy in *Populus*. *Plant Cell* **2008**, *20*, 59–74. [\[CrossRef\]](#) [\[PubMed\]](#)
- Shalit, A.; Rozman, A.; Goldshmidt, A.; Alvarez, J.P.; Bowman, J.L.; Eshed, Y.; Lifschitz, E. The flowering hormone florigen functions as a general systemic regulator of growth and termination. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8392–8397. [\[CrossRef\]](#) [\[PubMed\]](#)
- Argiriou, A.; Michailidis, G.; Tsafaris, A.S. Characterization and expression analysis of terminal flower1 homologs from cultivated allotetraploid cotton (*Gossypium hirsutum*) and its diploid progenitors. *J. Plant Physiol.* **2008**, *165*, 1636–1646. [\[CrossRef\]](#) [\[PubMed\]](#)
- Xi, W.; Liu, C.; Hou, X.; Yu, H. Mother of FT and TFL1 regulates seed germination through a negative feedback loop modulating ABA signaling in *Arabidopsis*. *Plant Cell* **2010**, *22*, 1733–1748. [\[CrossRef\]](#) [\[PubMed\]](#)

16. Hedman, H.; Källman, T.; Lagercrantz, U. Early evolution of the MFT-like gene family in plants. *Plant Mol. Biol.* **2009**, *70*, 359–369. [[CrossRef](#)] [[PubMed](#)]
17. Adeyemo, O.S.; Hyde, P.T.; Setter, T.L. Identification of FT family genes that respond to photoperiod, temperature and genotype in relation to flowering in cassava (*Manihot esculenta*, Crantz). *Plant Reprod.* **2019**, *32*, 181–191. [[CrossRef](#)] [[PubMed](#)]
18. Abe, M.; Kobayashi, Y.; Yamamoto, S.; Daimon, Y.; Yamaguchi, A.; Ikeda, Y.; Ichinoki, H.; Notaguchi, M.; Goto, K.; Araki, T. FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **2005**, *309*, 1052–1056. [[CrossRef](#)] [[PubMed](#)]
19. Wigge, P.A.; Kim, M.C.; Jaeger, K.E.; Busch, W.; Schmid, M.; Lohmann, J.U.; Weigel, D. Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* **2005**, *309*, 1056–1059. [[CrossRef](#)] [[PubMed](#)]
20. Komiya, R.; Ikegami, A.; Tamaki, S.; Yokoi, S.; Shimamoto, K. Hd3a and RFT1 are essential for flowering in rice. *Development* **2008**, *135*, 767–774. [[CrossRef](#)] [[PubMed](#)]
21. Lee, C.; Kim, S.J.; Jin, S.; Susila, H.; Youn, G.; Nasim, Z.; Alavilli, H.; Chung, K.S.; Yoo, S.J.; Ahn, J.H. Genetic interactions reveal the antagonistic roles of FT/TSF and TFL1 in the determination of inflorescence meristem identity in *Arabidopsis*. *Plant J.* **2019**, *99*, 452–464. [[CrossRef](#)] [[PubMed](#)]
22. Ahn, J.H.; Miller, D.; Winter, V.J.; Banfield, M.J.; Lee, J.H.; Yoo, S.Y.; Henz, S.R.; Brady, R.L.; Weigel, D. A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. *Embo J.* **2006**, *25*, 605–614. [[CrossRef](#)] [[PubMed](#)]
23. Wen, C.; Zhao, W.; Liu, W.; Yang, L.; Wang, Y.; Liu, X.; Xu, Y.; Ren, H.; Guo, Y.; Li, C.; et al. CsTFL1 inhibits determinate growth and terminal flower formation through interaction with CsNOT2a in cucumber. *Development* **2019**, *146*, dev180166. [[CrossRef](#)] [[PubMed](#)]
24. Kaneko-Suzuki, M.; Kurihara-Ishikawa, R.; Okushita-Terakawa, C.; Kojima, C.; Nagano-Fujiwara, M.; Ohki, I.; Tsuji, H.; Shimamoto, K.; Taoka, K. TFL1-Like proteins in rice antagonize rice FT-Like protein in inflorescence development by competition for complex formation with 14-3-3 and FD. *Plant Cell Physiol.* **2018**, *59*, 458–468. [[CrossRef](#)] [[PubMed](#)]
25. Xi, W.; Yu, H. Mother of FT and TFL1 regulates seed germination and fertility relevant to the brassinosteroid signaling pathway. *Plant Signal. Behav.* **2010**, *5*, 1315–1317. [[CrossRef](#)] [[PubMed](#)]
26. Yan, Q.; Xia, H.; Huang, W.; Li, Z.; Zhang, T.; Wu, Y.; Chen, H.; Zhang, Y.; Zou, J. The OfJAZ gene family in *Osmanthus fragrans*: IV-OfJAZs regulate petal senescence via hormonal crosstalk. *Ind. Crop. Prod.* **2026**, *242*, 122966. [[CrossRef](#)]
27. Zou, J.; Liu, D.; Chen, X.; Yang, J.; Luo, C.; Zeng, X.; Cai, X.; Zhang, Q.; Zeng, J.; Li, Z.; et al. A multi-omics database for the biological study of *Osmanthus fragrans*. *Hortic. Plant J.* **2024**, *11*, 2237–2249. [[CrossRef](#)]
28. Chen, H.; Zeng, X.; Yang, J.; Cai, X.; Shi, Y.; Zheng, R.; Wang, Z.; Liu, J.; Yi, X.; Xiao, S.; et al. Whole-genome resequencing of *Osmanthus fragrans* provides insights into flower color evolution. *Hortic. Res.* **2021**, *8*, 98. [[CrossRef](#)] [[PubMed](#)]
29. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* **2020**, *13*, 1194–1202. [[CrossRef](#)] [[PubMed](#)]
30. Park, S.W.; Lee, B.H.; Song, S.H.; Kim, M.K. Revisiting the Ramachandran plot based on statistical analysis of static and dynamic characteristics of protein structures. *J. Struct. Biol.* **2023**, *215*, 107939. [[CrossRef](#)] [[PubMed](#)]
31. Goodstein, D.M.; Shu, S.; Howson, R.; Neupane, R.; Hayes, R.D.; Fazo, J.; Mitros, T.; Dirks, W.; Hellsten, U.; Putnam, N.; et al. Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Res.* **2012**, *40*, D1178–D1186. [[CrossRef](#)] [[PubMed](#)]
32. Liu, Y.Y.; Yang, K.Z.; Wei, X.X.; Wang, X.Q. Revisiting the phosphatidylethanolamine-binding protein (PEBP) gene family reveals cryptic flowering locus T gene homologs in gymnosperms and sheds new light on functional evolution. *New Phytol.* **2016**, *212*, 730–744. [[CrossRef](#)] [[PubMed](#)]
33. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [[CrossRef](#)] [[PubMed](#)]
34. She, K.; Pan, W.; Yan, Y.; Shi, T.; Chu, Y.; Cheng, Y.; Ma, B.; Song, W. Genome-wide identification, evolution and expressional analysis of OSCA gene family in barley (*Hordeum vulgare* L.). *Int. J. Mol. Sci.* **2022**, *23*, 13027. [[CrossRef](#)] [[PubMed](#)]
35. Chen, H.; Song, X.; Shang, Q.; Feng, S.; Ge, W. CFVisual: An interactive desktop platform for drawing gene structure and protein architecture. *BMC Bioinform.* **2022**, *23*, 178. [[CrossRef](#)] [[PubMed](#)]
36. Wang, Y.; Tang, H.; Wang, X.; Sun, Y.; Joseph, P.V.; Paterson, A.H. Detection of colinear blocks and syntenic and evolutionary analyses based on utilization of MCScanX. *Nat. Protoc.* **2024**, *19*, 2206–2229. [[CrossRef](#)] [[PubMed](#)]
37. Zhang, Z. KaKs_Calculator 3.0: Calculating selective pressure on coding and non-coding sequences. *Genom. Proteom. Bioinf.* **2022**, *20*, 536–540. [[CrossRef](#)] [[PubMed](#)]
38. Danecek, P.; Auton, A.; Abecasis, G.; Albers, C.A.; Banks, E.; DePristo, M.A.; Handsaker, R.E.; Lunter, G.; Marth, G.T.; Sherry, S.T.; et al. The variant call format and VCFtools. *Bioinformatics* **2011**, *27*, 2156–2158. [[CrossRef](#)] [[PubMed](#)]
39. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
40. Yang, Z.; Chen, L.; Kohnen, M.V.; Xiong, B.; Zhen, X.; Liao, J.; Oka, Y.; Zhu, Q.; Gu, L.; Lin, C. Identification and characterization of the PEBP family genes in moso bamboo (*Phyllostachys heterocycla*). *Sci. Rep.* **2019**, *9*, 14998. [[CrossRef](#)] [[PubMed](#)]

41. Innan, H.; Kondrashov, F. The evolution of gene duplications: Classifying and distinguishing between models. *Nat. Rev. Genet.* **2010**, *11*, 97–108. [[CrossRef](#)] [[PubMed](#)]
42. Qiao, X.; Li, Q.; Yin, H.; Qi, K.; Li, L.; Wang, R.; Zhang, S.; Paterson, A.H. Gene duplication and evolution in recurring polyploidization–diploidization cycles in plants. *Genome Biol.* **2019**, *20*, 38. [[CrossRef](#)] [[PubMed](#)]
43. Wang, Q.; Zhou, Y.; Wang, F.; Li, X.; Yu, Y.; Yu, R.; Fan, Y. Genome-wide identification of *PEBP* gene family in *Hedychium coronarium*. *Front. Plant Sci.* **2025**, *16*, 1482764. [[CrossRef](#)] [[PubMed](#)]
44. Tian, Y.; Wang, J.; Wang, X.; Wang, D.; Wang, X.; Liu, J.; Zhang, H.; Zhang, J.; Yu, L. Genome-wide identification, phylogeny, and expression analysis of *PEBP* gene family in *Castanea mollissima*. *Front. Genet.* **2025**, *16*, 1530910. [[CrossRef](#)] [[PubMed](#)]
45. Hanzawa, Y.; Money, T.; Bradley, D. A single amino acid converts a repressor to an activator of flowering. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7748–7753. [[CrossRef](#)] [[PubMed](#)]
46. Wickland, D.P.; Hanzawa, Y. The *FLOWERING LOCUS T*/*TERMINAL FLOWER 1* gene family: Functional evolution and molecular mechanisms. *Mol. Plant* **2015**, *8*, 983–997. [[CrossRef](#)] [[PubMed](#)]
47. Wang, M.; Tan, Y.; Cai, C.; Zhang, B. Identification and expression analysis of phosphatidyl ethanolamine-binding protein (*PEBP*) gene family in cotton. *Genomics* **2019**, *111*, 1373–1380. [[CrossRef](#)] [[PubMed](#)]
48. Pham, M.T.; Milevskiy, M.J.G.; Visvader, J.E.; Chen, Y. Incorporating exon–exon junction reads enhances differential splicing detection. *BMC Bioinform.* **2025**, *26*, 193. [[CrossRef](#)] [[PubMed](#)]
49. Kang, H.; Vázquez, F.X.; Zhang, L.; Das, P.; Toledo-Sherman, L.; Luan, B.; Levitt, M.; Zhou, R. Emerging β -sheet rich conformations in supercompact huntingtin exon-1 mutant structures. *J. Am. Chem. Soc.* **2017**, *139*, 8820–8827. [[CrossRef](#)] [[PubMed](#)]
50. Zou, C.; Sun, K.; Mackaluso, J.D.; Seddon, A.E.; Jin, R.; Thomashow, M.F.; Shiu, S. Cis-regulatory code of stress-responsive transcription in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 14992–14997. [[CrossRef](#)] [[PubMed](#)]
51. Molina, C.; Grotewold, E. Genome wide analysis of *Arabidopsis* core promoters. *BMC Genom.* **2005**, *6*, 25. [[CrossRef](#)] [[PubMed](#)]
52. Zhang, X.; Ouyang, Y.; Zhao, L.; Li, Z.; Zhang, H.; Wei, Y. Genome-wide identification of *PEBP* gene family in pineapple reveal its potential functions in flowering. *Front. Plant Sci.* **2023**, *14*, 1277436. [[CrossRef](#)] [[PubMed](#)]
53. Wang, J.; Liu, X.; Wang, Q.; Shi, M.; Li, C.; Hou, H.; Lim, K.; Wang, Z.; Yang, Z. Characterization of pecan *PEBP* family genes and the potential regulation role of CiPEBP-like1 in fatty acid synthesis. *Plant Sci.* **2024**, *351*, 112326. [[CrossRef](#)] [[PubMed](#)]
54. Zheng, X.M.; Wu, F.Q.; Zhang, X.; Lin, Q.B.; Wang, J.; Guo, X.P.; Lei, C.L.; Cheng, Z.J.; Zou, C.; Wan, J.M. Evolution of the *PEBP* gene family and selective signature on FT-like clade. *J. Syst. Evol.* **2016**, *54*, 502–510. [[CrossRef](#)]
55. Zhou, H.; Zeng, R.F.; Liu, T.J.; Ai, X.Y.; Ren, M.K.; Zhou, J.J.; Hu, C.G.; Zhang, J.Z. Drought and low temperature-induced NF-YA1 activates FT expression to promote citrus flowering. *Plant Cell Environ.* **2022**, *45*, 3505–3522. [[CrossRef](#)] [[PubMed](#)]
56. Lyu, J.; Cai, Z.; Li, Y.; Suo, H.; Yi, R.; Zhang, S.; Nian, H. The floral repressor GmFLC-like is involved in regulating flowering time mediated by low temperature in soybean. *Int. J. Mol. Sci.* **2020**, *21*, 1322. [[CrossRef](#)] [[PubMed](#)]
57. Liu, C.; Fan, C. De novo transcriptome assembly of floral buds of pineapple and identification of differentially expressed genes in response to ethephon induction. *Front. Plant Sci.* **2016**, *7*, 00203. [[CrossRef](#)] [[PubMed](#)]
58. Song, C.; Li, G.; Dai, J.; Deng, H. Genome-wide analysis of *PEBP* genes in *Dendrobium huoshanense* unveiling the antagonistic functions of FT/TFL1 in flowering time. *Front. Genet.* **2021**, *12*, 687689. [[CrossRef](#)] [[PubMed](#)]
59. Song, H.; Sun, H.; Xin, J.; Yang, D.; Deng, X.; Liu, J.; Li, J.; Zhang, M.; Wang, Y.; Yang, M. *FLOWERING LOCUS T* genes control floral induction in lotus. *Plant Physiol. Biochem.* **2024**, *207*, 108339. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.