Genome-wide characterization of PEBP family genes in nine Rosaceae tree species and their expression analysis in P. mume

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Abstract

Background

Phosphatidylethanolamine-binding proteins (PEBPs) constitute a common gene family found among animals, plants and microbes. Plant PEBP proteins play an important role in regulating flowering time, plant architecture as well as seed dormancy. Though PEBP family genes have been well studied in Arabidopsis and other model species, less is known about these genes in perennial trees.

Results

To understand the evolution of PEBP genes and their functional role in flowering control, we identified 56 PEBP members belonging to three gene clades (MFT-like, FT-like, and TFL1-like) and five lineages (FT, BFT, CEN, TFL1, and MFT) across nine Rosaceae perennial species. Structural analysis revealed highly conserved gene structure and protein motifs among Rosaceae PEBP proteins. Codon usage analysis showed slightly biased codon usage across five gene lineages. With selection pressure analysis, we detected strong purifying selection constraining divergence within most lineages, while positive selection driving the divergence of FT-like and TFL1-like genes from the MFT-like gene clade. Spatial and temporal expression analyses revealed the essential role of FT in regulating floral bud breaking and blooming in P. mume. By employing a weighted gene co-expression network approach, we inferred a putative FT regulatory module required for dormancy release and blooming in P. mume.

Conclusions

We have characterized the PEBP family genes in nine Rosaceae species and examined their phylogeny, genomic synteny relationship, duplication pattern, and expression profiles during flowering process. These results revealed the evolutionary history of PEBP genes and their functions in regulating floral bud development and blooming among Rosaceae tree species.

Background

Proper timing of flowering is a key adaptive strategy in plant species, especially temperate woody perennials [1-3]. The flowering time in annual or biennials is largely determined by the timing of the transition from vegetative growth to reproductive growth [4, 5]. However, in temperate tree species, flower buds initiate and develop during summer, undergo a short period of dormancy, exit dormancy after exposure to chilling temperatures and finally bloom in suitable environments [6]. Therefore, the blooming time of temperate woody perennials is mainly determined by intrinsic state of flower buds and external environments [7, 8]. Within the context of global climate change, warm winters and irregular occurrences of extreme weather have disrupted the timing of spring phenological events in tree species, increased the risk of frost damage, and caused abnormal fertility and poor fruit setting due to insufficient winter chill [9-12]. Therefore, it is important to study the flowering time control in perennial species and understand their adaptation mechanisms in synchronizing the timing of floral bud breaking and reproduction with local climate [10, 13, 14].

Phosphatidylethanolamine-binding proteins (PEBPs) form a superfamily of genes containing a PEBP domain, which is highly conserved across taxa, from bacteria and insects to mammals and plants [15-17]. Mammalian PEBPs are globular proteins composed of a functional binding site for acetate, phosphate groups and phosphorylethanolamine [18, 19]. Plant PEBP homologs share similar conserved motifs, except their C-terminal part is deleted [20, 21]. Animal PEBP proteins were reported to function as serine proteases or Raf kinase inhibitors, controlling cell growth and differentiation [22-25]. In plants, PEBP genes are central regulators in determining the flowering time, plant architecture and seed germination [26-30]. In angiosperms, members of the PEBP family fall into three clades of genes: FLOWERING LOCUS T (FT), TERMINAL FLOWER 1 (TFL1) and MOTHER OF FT AND TFL1 (MFT) [31, 32]. It was reported that MFT-like genes exist in both basal land plants and seed plants, while FT-like and TFL1-like genes were only found in gymnosperms and angiosperms, indicating that the MFT clade might be the evolutionary ancestor to FT-like and TFL1-like genes [32, 33]. Despite extensive sequence similarity among PEBP members, their functions have diverged from each other [34].

FT and TFL1 are two major PEBP proteins that are well studied in Arabidopsis and in many other plant species [35-38]. In Arabidopsis, FT acts as a floral signal transducer, moving from leaves to the shoot apical meristem to promote flowering, while TFL1 maintains inflorescence meristem identity in shoot apex by antagonizing FT function [39-41]. The balance of FT and TFL1 modulates floral transition and inflorescence architecture by affecting determinacy of meristem identity [30, 42]. FT and TFL1 share ~60% of their amino acid sequence identity, but only a few amino acid changes can convert FT from a floral promoter to a TFL1-like floral repressor [37, 43]. In addition to FT and TFL1, the Arabidopsis PEBP gene family includes MOTHER OF FT AND TFL1 (MFT), TWIN SISTER OF FT (TSF), BROTHER OF FT AND TFL1 (BFT), and CENTRORADIALIS (CEN) [27]. MFT integrates abscisic acid (ABA) and gibberellic acid (GA) signaling pathways and acts in a PIF1-dependent manner to repress seed germination under far-red light [28, 44]. TSF encodes the closest homolog of FT and resembles FT as a floral inducer under non-inductive SD conditions [45]. BFT and CEN are two floral repressors in Arabidopsis, and the overexpression of either one resulted in a late flowering phenotype similar to plants overexpressing TFL1 [46-48].

Although the PEBP gene family has been recognized as key floral regulators in model species, their molecular evolution and function remains less clear in woody perennials. The Rosaceae family consists of over 2500 species from approximately 90 genera, most of which are native to temperate zones around the world [49-51]. Prunus is a large genus belonging to the tribe Amygdalae and contains about 430 species, many of which are important fruit crops, such as plums, cherries, apricots and peaches [52]. Additionally, Prunus includes a large number of spring-blooming trees with high ornamental and economic value. Prunus mume is one of the earliest flowering species, which blooms in late winter or early spring, followed by apricots, peaches, cherries and plums that flower during March to April. Apple and pear trees from the tribe Malae bloom much later, around April to May in Northern China [53]. With the divergent flowering times among Rosaceae tree species, it is of great interest to investigate the evolution of PEBP family genes and their functional roles in governing flowering time among Rosaceae tree species.
Here, we provide a systematic study on the molecular evolution and function of the PEBP gene family in Rosaceae tree species. We identified 56 PEBP family genes across nine Rosaceae species and analyzed the sequence conservation, protein motifs, gene structures, and codon usage patterns of these genes. We then performed genome synteny and duplication analysis, along with nonsynonymous/synonymous substitution (dN/dS) ratio tests, to determine the evolutionary trajectory of PEBP family genes. We also analyzed the spatial and temporal expression patterns of PEBPs across tissues and in floral buds from floral initiation to bud blooming. Furthermore, we performed weighted gene co-expression network analysis (WGCNA) to determine the FT coexpressed genes in P. mume. In summary, our study provides insight into the molecular evolution of PEBP genes among Rosaceae tree species and adds information regarding their function in regulating floral bud development and blooming in woody perennials.

Results

Characterization of PEBP genes in Rosaceae species

By combining HMM and BLAST searches, we identified 56 PEBP-like proteins across nine Rosaceae tree species (Table 1). Each putative gene was validated by blasting against SMART, Pfam and NCBI CDD to ensure that they contained complete PEBP domain. We then assigned all Rosaceae PEBPs to their closest Arabidopsis homologs (Figure 1; Table 1). In total, these Rosaceae PEBPs included 12 FT/TSF-like, 11 TFL-like, 11 CEN-like, 10 MFT-like and 12 BFT-like genes (Table 1). TFL1 and CEN-like genes showed the highest identities of 72.25-80.0% with their Arabidopsis orthologs, while BFT-like proteins showed the lowest identities of 62.07 to 67.82% compared with AtBFT. Five to six PEBPs were detected among Prunus species, while the average number of PEBPs almost doubled in M. domestica and Pyrus communis (Table 1). The duplicated paralogous gene pairs, such as MdTFL1 and MdTFL2, PcTFL1 and PcTFL2, were retained in the genomes of M. domestica and Pyrus communis, while only one copy of MFT was present in both species (Table 1).

Phylogenetic analyses

Phylogenetic trees were constructed based on protein sequence alignment of Arabidopsis and Rosaceae PEBPs using three approaches: the neighbor-joining, maximum likelihood, and Bayesian inference methods (Figure 2; Figure S1). All three phylogenetic trees shared similar topologies (Figure 2; Figure S1). The phylogenetic trees showed that the total of 62 PEBP proteins can be clustered into three major clades, which are the FT-clade, TFL1-clade, and MFT-clade (Figure 2). The FT-clade could be further split into FT/TSF-like genes and BFT-like genes, and the TFL1-clade can be split into TFL1-like and CEN-like subfamily genes (Figure 2). Within each subfamily, the genes of Prunus species first group closely together, then group with the genes of Malus species including M. domestica and Pyrus communis, and finally group with genes of R. occidentalis and Arabidopsis genes (Figure 2). Among Prunus PEBPs, proteins within the same subgenus tend to group together, for example, P. dulcis and P. persica from the Amygdalus subgenus, P. armeniaca and P. mume from the Prunus subgenus, and proteins of P. yedoensis and P. avium from the Cerasus subgenus (Figure 2). The duplicated paralogous gene pairs from M. domestica and Pyrus communis within the TFL1, CEN and BFT subfamilies were grouped separately, for example, PcTFL1-MdTFL1 and PcTFL2-MdTFL2 form separate clusters, rather than genes of the same species grouping together (Figure 2).

Structural analysis of PEBP family genes

Rosaceae PEBP family genes displayed conserved gene structures and high amino acid sequence similarity (Figure 3; Figure S2). The length of the coding regions of PEBPs ranged from 507 to 576 bps, with FT-like genes falling between 522 to 543 bps, MFT-like genes between 507 to 576 bps, BFT-like genes between 519 to 525 bps, TFL1-like genes between 516 to 519 bps, and CEN-like genes between 519 to 522 bps. All PEBP genes have a rather loose gene structure consisting of four exons and three introns (Figure S2). For example, BFT-like genes harbor the shortest intron total lengths, ranging from 522 to 534 bp (Figure S2).

Sequence alignment revealed a high degree of conservation across the entire protein and within the PEBP domains present in all 65 genes (Figure 1; Figure 3). The phylogeny structure inferred from the alignment of PEBP domains was generally in accordance with that of the whole protein sequence alignment, suggesting the PEBP domain as the major factor driving the evolution of Rosaceae PEBPs (Figure 3a). Five motifs covering 160 amino acids were identified by the MEME program among Rosaceae PEBP proteins (Figure 3b). Among these, Motifs 1, 2, 4, and 5 together spread over the whole PEBP domain (Figure 3b). Motifs DPDXP (Asp-Pro-Asp-X-Pro) and GIHR (Gly-Ile-His-Arg), which are essential for anion-binding activity, were present in the fourth exon of the PEBPs (Figure 1). We also found that residues distinguishing FT-like genes from TFL1-like proteins were conserved among the two gene lineages (Figure S3). Previously reported key residues conferring the flowering-promoting role of FT including V76, Y91, E115, L134, Y140, G144, W145, Q147, and N159 were present in all Rosaceae FT-like proteins (Figure S3) [20, 43, 54]. The corresponding residues I(1)/T76, H91, E115, (K/N/T)134, (F/N)140, (P/S)144, S145, D147, and D159 were found in all TFL1-like proteins (Figure S3). Residues determining the 14-3-3 receptor binding interface (R68, F107, R137) were shared by both protein types (Figure S3).

Microsynteny and duplication analysis of PEBP genes

To understand the evolution origin of PEBP family genes, we performed inter- and intra-genomic synteny analysis with MCScanX for Arabidopsis and seven Rosaceae species with chromosome-level genome assemblies. We observed large interspecies collinear blocks between four Prunus species, P. avium, P. persica, P. armeniaca, and P. mume, which indicates high level of microsynteny among Prunus species (Figure S4). The genome comparisons between R. occidentalis and M. domestica and between M. domestica and P. avium revealed large-scale chromosomal rearrangements including translocation and fusion-fission events that possibly occurred during the genome evolution of Rubus, Malus, and Prunus genera (Figure S4). Based on intra-genomic comparisons, we classified the duplication origin of orthologous gene pairs for Arabidopsis and other Rosaceae species (Table S1). Among all duplication types, whole-genome duplication (WGD)/segmental duplication was the major type for M. domestica, tandem duplicated genes were mostly found in A. thaliana, P. armeniaca, and P. persica, and dispersed duplication events were enriched in the genomes of R. occidentalis, P. mume, P. avium, and P. dulcis (Table S1).
Furthermore, we characterized the duplication modes of *PEBP* family genes across species (Table S2; Figure 4; Figure S5). In *Arabidopsis*, *R. occidentalis*, and *P. armeniaca*, all *PEBP* gene members were predicted to be originated from dispersed duplications (Figure S5; Table S2). In four *Prunus* species, FTs, MFTs, and BFTs were classified as having dispersed duplication, while TFL1-like and CEN-like genes were classified as exhibiting WGD/segmental duplication (Figure S5; Table S2). The inter-genomic comparison of *Prunus* species confirmed that TFL1 and CEN genes were within shared syntenic blocks between species, indicating a shared duplication origin of TFL1-like and CEN-like genes in *Prunus* species (Figure 4). Within the genome of *M. domestica*, we detected seven syntenic blocks consisting of three WGD/segmental duplication gene pairs, including MdTFL1-MdTFL2, MdCEN1-MdCEN2, and MdBFT1-MdBFT2, and two dispersed duplication events related to MdFT and MdMFT (Figure S5; Table S2). The inter-genomic comparisons between *M. domestica* and *R. occidentalis* and between *M. domestica* and *P. avium* also confirmed that the duplicated gene pairs MdCEN1-MdCEN2, MdTFL1-MdTFL2, and MdBFT1-MdBFT2 are likely resulted from an independent WGD event unique to the *Malus* tribe (Figure 4).

Codon usage bias and other gene parameters

We observed differentially preferred codons and different gene features across five *Rosaceae* *PEBP* gene lineages (Figure 5; Figure S6; Table 2). For arginine, codons AGA and AGG were most frequently used by all lineages compared with other codons (Figure S6). Codon UCU encoding serine was mostly used in CEN-like and MFT-like proteins, while codon UCU was mostly employed by the TFL1 lineages (Figure S6). We also observed significant differences in other gene parameters among different *PEBP* lineages (all Kruskal-Wallis tests *p*val<0.01) (Figure 5; Table 2). The codon adaptive index (CAI) of MFT and TFL1 genes is significantly larger than those of the other gene groups (Kruskal-Wallis test *p*val= 2.75e⁻²) (Figure 5; Table 2). In contrast, the effective number of codons (ENC) estimated for MFT and TFL1 genes is much lower than those of the other groups (Kruskal-Wallis test *p*val= 0.005) (Figure 5; Table 2). The average ENC values ranging from 51.24 to 54.95 indicated weak codon bias among *PEBP* genes. Analysis of the GC content revealed that MFT lineage genes had much higher GC content indices compared to other genes (Figure 5; Table 2). In contrast, TFL1 and BFT lineage genes appear to have lower GC1% and GC3% but relatively higher GC2% compared to other groups (Figure 5; Table 2). All gene parameters showed no variation among species (Figure 5; Table 2). Strong pairwise correlations between gene parameters were observed (Table S3). For example, the CAI was positively correlated with the total GC% and GC3% (both correlation coefficient ≥0.56) but was negatively correlated with the ENC (*r*=-0.62) (Table S3). On the other hand, the ENC displayed a negative correlation with the total GC content and GC3% (Table S3).

Molecular evolution of different *PEBP* lineages

To investigate the evolution of *PEBP* genes in *Rosaceae* species, we performed selection scans on coding sequences of all *PEBP* genes using the branch model, site model, and branch-site model in the CODEML program of PAML (Table 3; Table S4; Table S5). Branch models with different *ω* parameters specified for foreground lineages (i.e., FT-like, TFL1-like, CEN-like, and BFT-like lineages and FT/TFL1 clades) were compared with the fixed ratio model (Table S4). The likelihood ratio tests (LRT) on models specifying individual lineages of FT, TFL1, CEN and BFT genes as the foreground branch showed no significant difference in *ω* between the foreground and background branch (*p*>0.05) (Table S4). However, the LRT test on the branch model specifying the FT and TFL1 clades as the foreground branch suggested significant divergence among FT/TFL1 and MFT clade genes (*p*<0.001) (Table S4). We then applied the site model LRT test and detected signs of positive selection among sites of *PEBP* proteins (Table S5). The branch-site LRT tests further revealed strong positive selection within TFL1 lineage and slight positive selection within FT lineages at specific protein sites (Table 3). The Empirical Bayes model suggested modest selection at positions 19 and 106 when FT lineage was set as the foreground branch and at positions 11 and 18 when TFL1 lineage was set as the foreground branch (Table 3). We further validated the results by performing selective pressure analysis on five gene lineages separately with the software Selecton. Only the FT and TFL1 lineages showed the signature of positive selection, in which residues 40N, 56N, 128S, and 181L in the FT lineage (with *RoFT1* as the reference gene) and 4T, 73V, 134P, 141S, 157L, and 161S in the TFL1 lineage (with PyTFL as the reference gene) were mostly selected (Figure 6). In contrast, the genes of the other three lineages all showed signs of purifying selection across most sites (Figure S8).

Cis-acting element analysis of the FT promoter

We extracted the 2000 bp region of FT genes and scanned for putative cis-elements by searching against the PlanPan and the PlantCARE databases (Table 4). We compared the type and copy number of cis-elements for 11 FT genes from *A. thaliana*, *P. trichocarpa*, *M. domestica*, *Pyrus communis*, *R. occidentalis*, *P. armeniaca*, *P. avium*, *P. mume*, and *P. persica* (Table 4). Within the promoter region of the investigated FTs, three to ten CCACA boxes (binding site for CO) were identified across nine species, while none were found for *PpFT* (Table 4). CAG boxes, the binding site for the MADS transcription factor, were present in all FT promoters, among which the *AtFT* promoter contained the most (Table 4). Light-response elements including the G-box, AE-box, GATA-motif, GT1-motif, and CCT-motif were present within all FT genes but in different types (Table 4). In addition, binding sites for MYB, MYC transcription factor, ethylene-responsive transcription factor, and abscisic-acid responsive element (ABRE) were present in all FT promoters (Table 4). Gibberellin-responsive elements of different types were detected in FT promoters, with GARE-motif in the promoters of *MdFT*, *PtFT*, *RoFT*, *PvFT* and P-box in the promoters of *AtFT*, *PpFT*, *PaFT*, *PvFT*, *PmFT*, and *PpFT*. We also observed some cis-elements with species-specific distribution patterns. For example, the low-temperature responsiveness (LTR) element was only detected within the promoters of *AiFT*, *PcFT*, *RoFT*, *PvFT* and *PmFT* (Table 4). The W-box, which is the binding site for WRKY transcription factor, was detected exclusively in *RoFT*, *PpFT*, and *Prunus* FT promoter regions (Table 4).

Tissue-specific expression patterns of *PEBPs*

To explore the functional roles of *PEBP* genes, we examined their expression patterns in different tissues of four *Rosaceae* species, *P. persica*, *P. mume*, *P. yedoensis*, and *R. occidentalis* (Figure 7a-d). In general, we observed a differentiated expression preference of *PEBP* genes across different tissues (Figure 7). Among the five *PEBP* subfamilies, FT-like and TFL1-like genes were expressed in both vegetative tissues such as leaf and stem, and reproductive organs such as flower bud and fruit (Figure 7). The transcription of CENs, as the closest paralogs of TFL1, was barely detected in any organs, except in the root tissues of *P. mume* (Figure 7). MFT was only detected in seed embryos of *P. persica* and fruit tissues of *P. yedoensis* and *R. occidentalis* (Figure 7). BFT was detected in the fruit tissues of all species but was relatively highly expressed in leaf and stem tissues in *P. yedoensis* and *R. occidentalis*, respectively (Figure 7). We validated
the tissue-specific expressions of five PEBP genes by real-time quantitative PCR (qRT-PCR) in *P. mume* (Figure S9). *PmFT* is highly expressed in floral buds compared with its expression in leaf and stem, which is consistent with result of the above tissue transcriptome sequencing in *P. mume* (Figure 7; Figure S9). *PmTFL* and *PmCEN* were relatively highly expressed in root tissues (Figure S9). *PmBFT* and *PmMFT* was barely detected in the four examined tissue types (Figure S9). The somewhat inconsistent tissue-specific expression patterns of PEBP orthologs across examined species are likely a result of non-uniformity in the sampling time, plant physiological state, and tissue specificity across four independent studies. Despite the inconsistency, the divergent expression of PEBP members across different tissue types indicates significant functional differentiation of PEBP gene lineages.

**Expression analysis of PEBP genes during floral bud development in *P. mume***

We analyzed the expression of *PEBP* genes in flower buds of different developmental stages from July 10th, 2019 to January 12th, 2020 by qRT-PCR analysis. The expression of *PmFT* first decreased as the bud initiated the floral meristem from July to August, increased as floral organ initiated and developed (from August to October), slightly decreased during bud dormancy, and then significantly increased as the floral bud exited dormancy and bloomed (Figure 8). *PmBFT* maintained a low expression level throughout the whole process, with only a minor increase during floral bud development in August and September (Figure 8). The other PEBP members retained barely detected expression levels in floral buds of all developmental stages (Figure 8). These results imply that *PmFT* is possibly the primary *PEBP* member participating in regulating floral bud development and bud flushing in *P. mume*.

**Co-expression network analysis of FT during the blooming process in *P. mume***

To explore the regulatory network of *FT* in flowering regulation in trees, we reanalyzed the transcriptome changes of *P. mume* during dormancy release and the floral bud opening process [55] and performed a weighted co-expression network analysis (WGCNA). We identified 23 modules with distinct expression patterns (Figure S10a). Module-association analysis revealed four modules, 'brown', 'turquoise', 'dark green', and 'salmon', associated with the progression of bud flushing (R² > 0.8). Among them, module 'brown' showed the strongest correlation with the FPKM of *PmFT* (Figure S10b). The 'brown' module genes were significantly enriched in biological processes including cell cycle (GO:0007049), flower development (GO:0009908), glucon metabolic process (GO:0009251), auxin transport (GO:0060918), and responses to abiotic stimulus (GO:0009628). We further identified the top 50 genes most associated with *PmFT* and 15 known flowering-related genes such as *PmLFY*, *PmAP1*, and *PmCOL* (Table S6) [56, 57]. Among genes in the 'brown' module, *SVP* (SHORT VEGETATIVE PHASE), *SOC1* (SUPPRESSOR OF OVEREXPRESSION OF CO 1), *GI* (GIGANTEA), and *CIB1* (CRYPTOCROME-INTERACTING BASIC-HELIX-LOOP-HELIX) were previously identified as key players in the FT-dependent floral regulation in *Arabidopsis* [58, 59] (Figure 9a). Four tandem-duplicated *PmDAMs* (*PmDAM1, PmDAM4, PmDAM5, PmDAM6*) from the 'brown' module also exhibited expression patterns negatively correlated with that of *PmFT* (Figure 9a-b). The expression patterns of other known floral regulators such as *COL* (CONSTANS-LIKE) from the 'turquoise', *LHY1* (LATE ELONGATED HYOCOTYL 1) and *AP1* (APETALA1) from 'dark green' module were not highly correlated with *PmFT* (R² < 0.62) (Figure 9b). *PmFT* showed a relatively weak transcription level in endodormant floral buds (Figure 9b). As the floral bud continued accumulating chilling units and exiting dormancy, *PmFT* expression significantly increased and showed the highest expression in flushing buds (Figure 9b). *PmCIB1* and 37 other genes showed similar expression patterns to that of *PmFT*, while *PmPHYB* (*Pm008367*), *PmGL, PmLHY, PmCOL, PmSVP, PmSOC1*, and four *PmDAMs* displayed contrasting expression patterns, with their expression decreasing as the floral buds exited endodormancy (Figure 9b). The expression patterns of *FT* and its coexpressed genes were further verified by qRT-PCR analysis (Figure 9c).

**Discussion**

**Evolution trajectory of PEBP family genes in Rosaceae genomes**

PEBP family genes are ancient family central to many plant developmental processes, including floral transition, plant architecture, and seed germination [30, 32, 60]. In *Arabidopsis*, the PEBP family constitutes six genes grouped into three distinct clades, *FT*-like (*FT* and *TSF*), *TFL1*-like (*TFL1* and *CEN*), and *MFT*-like genes [31]. Though previous studies have characterized the functions of PEBP family genes in model plants, none have focused on a comparative analysis of the PEBP family in tree species. Our study conducted a systematic search across nine Rosaceae genomes and identified 56 PEBP family genes orthologous to six *Arabidopsis* genes, *FT, TSF, TFL1, CEN, BFT*, and *MFT*. The number of PEBP family members in *Prunus* species (chromosome 2n = 2x = 16) was approximately the same as that in *Arabidopsis* (five to six copies), while PEBP members were expanded in *M. domestica* and *Pyrus communis* (chromosome 2n = 2x = 34). Genome synteny and duplication analyses together supported that duplicated ortholog pairs *MdTFL1-MdTFL2*, *MdCEN1-MdCEN2*, and *MdBFT1-MdBFT2* are likely originated from a recent whole-genome duplication (WGD) event that occurred in the *Maleae* clade after splitting from *Prunus* [61]. However, only one copy of *MdFT, MdMFT*, and *PcMFT* was retained in apples and pears, indicating that the duplicated copy may have been lost during species evolution after the WGD [62]. The duplication mode analysis also suggested a shared origin of *TFL1* and *CEN* from segmental or WGD duplication in *Prunus* species (Table S2). Previous studies reported that the angiosperm *TFL1*-like gene experienced duplication after splitting from basal angiosperms, followed by functional divergence, resulting in *TFL1* and *CEN* gene lineages in eudicots [63]. Given the conserved sequence alignment of *Prunus TFL1/CEN* orthologs with other Rosaceae species, it is unlikely that *Prunus TFL1/CEN* arose from a recent segmental duplication or WGD unique to *Prunus* species. Therefore, the syntenic relationship may have been caused by the preservation of genomic segments containing *TFL1, CEN*, and their neighboring genes through rounds of chromosome rearrangements during *Prunus* species evolution. In *Arabidopsis*, the *TSF* gene, which is a homolog of *FT*, highly resembles *FT* in its coding sequence and flowering promoting role [64]. The absence of *TSF* in the Rosaceae genome suggests that the gene duplication of *FT/TSF* possibly occurred in *Brassicaceae* after splitting from their common ancestors [65].

The PEBP gene family experienced two ancient duplications, giving rise to three types: *FT*-like genes promoting flowering, *TFL1*-like genes repressing flowering and maintaining indeterminate state of meristems, and *MFT*-like genes controlling seed germination [17, 27, 32]. The phylogenetic analysis suggests that Rosaceae PEBPs can be clustered into three distinct clades (*FT, TFL1, and MFT*), which is consistent with other species [17, 27, 32]. The *FT*-like clade can be further divided into *FT* and *BFT* lineages, and the *TFL1*-like clade can be divided into *TFL1* and *CEN* lineages. Based on maximum-likelihood test on branch
models specifying different gene lineages (FT, TFL1, CEN, MFT, and BFT) as the foreground branch, we detected no evidence of positive selection acting on any of them. However, we observed significant selection acting on FT/TFL1 clade genes with the MFT clade specified as the background branch, which supports the theory that functional divergence of the FT/TFL1 clade occurred after splitting from the MFT clade [33]. Through likelihood ratio tests on branch-site models, we detected a few slightly selected codons within the FT lineage and a few strongly selected codons in the TFL1 lineage, which is consistent with results of Selecton analysis on individual lineages. In summary, these results indicate that adaptive evolution is driving the divergence of the FT and TFL1 clades from the MFT clade, as well as the diversification among FTs and TFL1s in Rosaceae species. These result are consistent with a previous study reporting that positive selection on FT-like genes especially within the fourth exon is driving their divergence from MFT and TFL1 clade [17]. We also observed strong purifying selection constraining protein evolution within the MFT, CEN, and BFT lineages in Rosaceae species. However, this does not rule out the possibility of positive selection acting on a few codons masked by strong purifying selection in preserving the other sites [17].

Additionally, we examined the codon usage patterns of PEBP genes across Rosaceae species. Codon usage bias refers to the nonrandom choice of synonymous codons in specific genes or species and can affect the translation efficiency and accuracy, protein folding, and biological functions [66, 67]. The codon usage pattern usually reflects the balanced effect of mutation pressure and selection constraints during long-term evolution [68, 69]. Several codons for amino acids were differentially preferred across five PEBP lineages. Among all codons, the most frequently used codon for arginine was AGG for FT, CEN, and MFT and AGA for the BFT and TFL1 lineages (Figure S6). Several other codons, including TCC, TCA, TCT for serine and CCT for proline, were preferred by specific PEBP gene lineages, indicating differentially selected codons by different PEBP gene lineages. To further understand the factors influencing codon usage patterns, we compared the GC content, gene length, CAI, and ENC of different PEBP lineages and species. The CAI measures the optimal codon usage for a gene and is commonly used as an index for the expression level [70]. The ENC has been widely used to determine the level of codon bias for individual genes [71]. We observed significant differences in these gene features estimated for different gene lineages but not for species. Despite the differences, all genes had a relatively high CAI (range 0.81-0.87) and moderate ENC (above 47), indicating high translational efficiency and slightly biased codon usage among PEBP genes. Furthermore, the strong pairwise correlations between ENC and GC content, ENC and CAI indicate that the nucleotide composition and gene expression level are two factors possibly contributing to the differentiated codon preference among different PEBP gene lineages [69].

Functional role of FT/TFL1 genes in Rosaceae tree species

Structural analysis of Rosaceae PEBP proteins revealed a highly conserved gene structure and amino acid sequence, especially within the PEBP functional domain (Figure 1; Figure 3; Figure S2). All PEBP family genes shared a common gene structure with exactly four exons of similar sizes. Among the conserved protein motifs, the anion-binding D-P-D-x-P and G-x-H-R motifs are important for the conformation of the ligand binding site in PEBP proteins [72]. Mutations close to this region may affect the binding of FT protein with phosphate ions and thus alter its interaction with FD (FLOWERING LOCUS D) [73]. Segment B on exon 4 encodes an external loop, and together with its adjacent segment C, determines the opposite functions of FT and TFL1 [35]. Another key protein motif is the 14-3-3 binding domain that is essential for FT/TFL1 interaction with 14-3-3 receptors to modulate flowering [20]. Key residues within these motifs are critical in determining FT/TFL1 functions. For example, the substitution of an amino acid (replacing His-88 in TFL1 with Tyr) can convert TFL1 into FT protein motif is the 14-3-3 binding domain that is essential for FT/TFL1 interaction with 14-3-3 receptors to modulate flowering [20]. Key residues within these motifs are critical in determining FT/TFL1 functions. For example, the substitution of an amino acid (replacing His-88 in TFL1 with Tyr) can convert TFL1 into FT-like repressor [43]. The amino acids at each of these critical positions were highly conserved and specific to FT-like and TFL1-like proteins, which suggests that the floral promoting and repressing role of FT/TFL1 genes in Rosaceae species is possibly conserved.

Recent molecular studies have characterized the function of Rosaceae FT/TFL1-like genes in several Rosaceae perennials [33]. The overexpression of MdFT in both Arabidopsis and apple lead to precocious flowering [74]. The ectopic expression of PmFT and RoFT in tobacco leads to extremely advanced flowering [75]. Similarly, the late-flowering phenotype of Arabidopsis ft mutant can be rescued by overexpressing PpFT, indicating the conserved floral promoting role of FT in examined Rosaceae species [76]. On the other hand, prolonged vegetative growth and a late-flowering phenotype were observed for transgenic Arabidopsis/tobacco overexpressing PtTFL1, PmTFL1, RoTFL1, MdTFL1-1/2, suggesting that the Rosaceae TFL1-like genes can complement the TFL1 function in Arabidopsis [77-79].

Despite the conservative function of Rosaceae FT/TFL1-like genes in herbaceous plant systems, their regulatory roles in perennial trees may differ. For example, two homologs of PcFTs showed differed annual expression patterns in the apical buds of Pyrus communis [80]. The ectopic expression of PcFT2 caused early flowering in tobacco but delayed dormancy and leaf senescence in M. domestica [80]. Another study in pears reported that the expression of FTs was not induced in the reproductive meristem prior to floral initiation, while the transcripts of TFL1s rapidly decreased and maintained a very low level, indicating the essential role of TFL1 in floral induction in Pyrus pyrifolia [36]. In our study, the minimal level of TFL1 throughout all floral bud stages may indicate that the repression of TFL1 is necessary for determinate floral meristem identity and terminal flower formation during floral bud development in P. mume. The multifaceted role of FT/TFL1-like genes was also observed in other tree species [33]. In poplar, PFT1 functions as a floral promoter activated by chilling temperatures, while vegetative growth and dormancy breaking are promoted by PFT2 [81]. Plum trees transformed with PpFT1 displayed a shrub-like growth habit, a reduced chilling requirement, and insensitivity to short-day signals [82]. In gymnosperms, FT-like genes exhibited contrasting roles in regulating growth cycling and bud setting [83]. For example, expression of FT/TFL1-like genes in Norway spruce (PaTFL2) and Scots pine (PsTFL2) increase during bud setting in autumn and decrease during bud bursting in the next spring [84-86]. Thus, FT/TFL1-like genes may undertake some novel functions concerning floral transition, plant architecture, and growth-dormancy cycling during the evolution of tree species.

Regulatory role of FT in promoting bud break and blooming in perennial trees

Flowering is a major developmental process that is key to the fitness and reproduction of higher plants [87]. Plants have synchronized their seasonal timing of flowering with favorable environmental conditions to ensure sexual reproduction success and seed production [87, 88]. The regulation of flowering times requires an intricate network of signaling pathways, which has been studied in many plant species but is best characterized in Arabidopsis [57, 87, 89]. FT functions as a gene hub integrating five major floral induction pathways, including the photoperiodic pathway, vernalization pathway, autonomous pathway,
gibberellin pathway, and age pathway [56, 59]. In *Arabidopsis*, the transcription of *FT* is activated by the transcription factor CONSTANS (CO), which is affected by the circadian regulatory GI [90, 91]. The GI-CO-FT module not only is used to regulate photoperiod-dependent flowering in *Arabidopsis* and temperate cereals [92, 93] but also showed a conserved function in regulating short-day induced bud dormancy in poplar [94]. In addition to CO, SVP FLC (FLOWERING LOCUS C), and PI4 (PHYTOCHROME INTERACTING FACTOR 4) from the vernalization pathway can also regulate *FT* transcription through directly binding the *FT* promoter or intronic regions [90, 95-97]. Upon induction by long-photoperiod signals, FT, together with other floral pathway integrators SOC1 and LFY (LEAFY), activates floral meristem identity genes such as AP1, APETALA2 (AP2), FRUITFULL (FUL), CAULIFLOWER (CAL), and LFY, which convert the vegetative meristem to the floral meristem in *Arabidopsis* [59, 98, 99].

Though flowering regulation is well understood in model species, it is still unclear in temperature tree species. Unlike annual or biennials, many trees in temperate environments initiate floral buds in the preceding summer, cease growth in autumn, with floral buds remaining dormant during winter, and then bloom early in spring after exposure to chilling temperatures [6, 10]. Therefore, perennial flowering marks the event of the floral bud exiting dormancy and flushing instead of the time of floral meristem initiation in annual species [6]. So far, many studies on floral bud breaking regulation have been reported; however, the molecular mechanism is still far from complete. Apart from regulating floral initiation, *FT* has been suggested to participate in regulating bud dormancy in temperate trees [100]. Poplar exhibited constitutive expression of *FT*1 initiated flower-like structures directly from tissue culture and showed delayed growth cessation in short-days [81, 94], while *FT*2 was predominantly expressed during vegetative growth and is likely responsible for growth cessation and vegetative bud set [81]. The ectopic expression of poplar *FT*1 in plum causes precious flowering and reduces the chilling requirement for dormancy release [82]. Moreover, Rinne et al. (2011) reported that *FT* is hyper-induced during bud breaking in poplar, indicating that *FT* may also participate in regulating dormancy release in poplar [101]. In pear, chilling reduces the expression of *DAM* genes, which are well-known floral repressors, releasing the repression of *FT* and promoting floral bud breaking [102, 103]. Our expression analysis confirmed that *FT* is significantly induced during chilling-mediated floral bud breaking in *P. mume*.

To further understand the regulatory module of *FT* during floral bud breaking, we used WGCNA and identified a number of candidate genes whose expression patterns strongly correlated with *FT* in *P. mume*. Among these candidates, *PmDAM1*, *PmDAM4*, *PmDAM5*, and *PmDAM6* were found to be downregulated during the progression of bud breaking. Another MADS-box gene *PmSVP* displaying a similar expression pattern to that of *PmDAMs* was reported to maintain bud dormancy in apples [104]. Thus, *PmDAMs* and *PmSVP* may function as FT repressors in the same manner as in *Arabidopsis* by binding to the CArG box in the promoter region of *PmFT* [105]. A number of genes previously identified upstream *FT*, including *PmCOL*, *PmGL* and *PmCIB1*, were found to be induced by chilling in endodormant buds before the activation of *PmFT*. These genes may act directly or indirectly to activate *FT* expression during dormancy release in *P. mume*. We also observed that some known *FT* regulated genes, namely, *AP1*, *SOC1*, and *LFY*, peaked before the induction of *FT*, indicating their functional role during flower bud development prior to bud breaking [6, 106]. Additionally, a number of *FT* co-expressed genes were annotated to pathways that did not show relatedness to bud breaking or flowering in previous studies. Future functional studies are required to characterize the regulatory mechanisms of *FT* in floral induction and bud breaking in *Rosaceae* tree species.

**Conclusion**

In this study, we systematically characterized the *PEBP* gene family in nine *Rosaceae* species and examined their gene structure, protein features, evolutionary trajectories, and expression profiles. The 56 *PEBP* genes can be divided into three major clades, namely, *FT*-like, *TFL1*-like, and *MFT*-like genes. We observed highly conserved protein motifs and gene structure among *PEBP* genes. Selection scans showed that positive selection is driving the divergence of the *FT* and *TFL1* clades, while strong purifying selection is restraining diversification within most lineages. Expression analysis of *PEBP* genes suggested the essential role of *FT* in floral bud development and blooming. Furthermore, we identified a number of *FT* co-expressed genes, revealing a *FT*-related regulatory model in *Prunus* species different from those in annual or biennial plants. In summary, the comprehensive analysis of the *PEBP* family in our study provided evidence of structural and functional conservation of *PEBP* genes among *Rosaceae* woody perennials and provided insight into the adaptive evolution of the *PEBP* gene family over the evolutionary history of perennial trees.

**Methods**

**Identification of the *PEBP* gene family**

We obtained the most recent versions of genomes for *P. persica* [107], *P. mume* [108], *P. yedoensis* [109], *P. avium* [110], *P. dulcis*, *P. ameniaca* [111], *M. domestica* [112], *Pyrus communis* [113], and *Rubus occidentalis* [114] from GDR (Genome Database For Rosaceae) [115]. To identify the *PEBP* genes of each species, we retrieved the HMM model PF01161 of PBP domain from the Pfam database (https://pfam.xfam.org) and searched the genome protein databases with an e-value cutoff of 1.0×e-5 using HMMER 3.1 software [116]. In addition, we used protein sequences atFt (At1g65480.1), AtTSF (At4g20370.1), AtFT1 (At5g03840.1), AtBFT (At5g62040.1), and AtMFT (At1g18100.1) downloaded from TAIR (The Arabidopsis Information Resource) (www.arabidopsis.org) as query sequences to blast against the local protein databases of nine species, and we only retained putative PEBP proteins with identities > 40% and e-values ≤ 1.0×e-10. The genes identified by both methods were considered candidate PEBP family genes and were then verified with SMART [117], Pfam [118], and the CDD database [119] to ensure the completeness of the PBP domain. Redundant sequences or sequences with incomplete PEBP domains were excluded from the following analyses.

**Phylogenetic analysis**

Multiple sequence alignment was performed using the protein sequences with software MUSCLE v3.8 [120] and was visualized with GeneDoc v2.6 [121]. Phylogenetic trees were constructed using neighbor-joining (NJ) method with MEGAG7 [122], maximum likelihood (ML) analysis with RAxML v8.1 [123], and Bayesian inference (BI) with MrBayes 3.1 [124]. The BI method was performed with 100,000 generations of MCMC processes. Bayesian inference was
performed with 100,000 generations of Markov-chain Monte Carlo (MCMC) simulations, discarding the first 2500 trees as 'burn-in'. With consistent tree topologies inferred by these three approaches, the neighbor-joining tree was chosen to display the phylogeny of Rosaceae full PEBP protein sequences. Furthermore, amino acids within the regions of predicted PEBP domains were extracted and used to construct a PEBP domain tree by the NJ method.

Gene structure and protein motif detection

The exon and intron locations of PEBP genes were analyzed by comparing the coding sequences with their genome sequences. The MEME (Multiple Expectation Maximization for Motif) online tool (http://meme-suite.org/tools/meme) was used to predict protein motifs [125]. The protein motifs were further annotated with the Pfam [118], SMART [117] and CDD [119] online tools. The chromosome distributions of PEBP genes were obtained based on genome GFF3 files. Finally, the gene structures, protein motifs, and chromosome locations were visualized with the software TBtools [126].

Microsynteny analysis and codon usage evaluation

To identify the synteny of PEBP family genes among species, we performed all-to-all BLASTP between the genomes of A. thaliana, Rubus occidentalis, M. domestica, P. avium, P. persica, P. armeniaca, and P. mume. We also performed self-blast by comparing protein-coding genes against their own genome using BLASTP. All BLASTP hits with e-values <1e-10 were used as input for software MCScanX (Multiple Collinearity Scan toolkit) [127] to identify possible collinear blocks within and between genomes of different species. Based on the self-blast results, we classified the duplication origin of orthologous genes pairs including PEBP family genes with the ‘duplicate_gene_classifier’ toolkit built in MCScanX for each species. All intra/inter-genomic synteny relationships were visualized with TBtools [126].

Gene parameters including the GC content (total GC%, GC1%, GC2%, and GC3%), CAI, and ENC were computed using CAICal (http://genomes.urv.cat/CAICal/) [128, 129]. CAI provides an estimate of directional translational selection in optimizing the codon usage patterns of genes and is used to predict highly expressed genes [70]. ENC is a number between 20 to 61 that measures the degree of codon usage bias (where ENC=20 refers to the preference of only one codon per amino acid, while ENC=61 refers to complete unbiased codon usage) [130]. We compared these gene parameters for FT, TFL1, CEN, BFT, and MFT gene lineages and across species using the Kruskal Wallis Test with the ‘kruskal.test’ function in R. The relative synonymous codon usage (RSCU) is defined as the ratio of the observed codon frequency to the expected frequency of all synonymous codons per amino acid and is calculated using software MEGA7 [131].

Molecular evolution of PEBP genes

To investigate the signatures of positive selection on Rosaceae PEBP genes, we extracted the coding sequences of PEBP genes and aligned them with MUSCLE v3.8 [120]. The sequence alignment was then trimmed with Gblocks [132] in 'codon' mode, and the resulting alignments were used to infer phylogenetic relationships with RAxML [123]. The ratios (ω) of nonsynonymous substitution sites (dN) and synonymous substitution sites (dS) were computed for each PEBP lineage using the branch model, site model and branch-site model with the codeML package in PAML 4.0 [133]. To test the hypothesis of adaptive evolution in specific PEBP lineages and across sites, we performed likelihood ratio tests to evaluate the fit of branch models (FT, TFL1, CEN, BFT and (FT, TFL1, CEN, BFT) set as foreground branch), sites models, and branch site models. The positively selected sites were detected by Bayes Empirical Bayes analysis in PAML 4.0 [133]. To better visualize the site-specific selection on amino acids within each PEBP lineage, we performed a selection pressure test with site model M8 and visualized the results with Selecton Server [134].

Cis-element analysis of the FT promoter region

To investigate the conservation of the cis-regulatory model of FT genes across different species, we extracted the 2 kb upstream region of the start codon (ATG) and submitted the sequences to the PlantCARE [135] and PlantPan 2.0 databases [136]. The cis-acting elements predicted by both methods were integrated and considered as putative cis-acting elements.

Tissue-specific expression profiles of PEBP genes

The tissue transcriptome sequencing data of P. mume, P. yedoensis, P. persica, and Rubus occidentalis was retrieved from four independent studies: GSE4760162 from the GEO database [108] and SRP136962, SRA053230, and SRP149938 from the NCBI SRA database [109, 137]. The raw SRA files were first dumped to FASTQ format using SRA toolkit and preprocessed with Trimmomatic v0.38 [138] to trim off poor-quality reads. Clean paired reads were aligned with the reference genomes of P. mume, P. yedoensis, P. persica, and Rubus occidentalis, respectively, with software HISAT2 [139]. The genic count was computed with HTSeq [140] and normalized to RPKM with R package 'edgeR' [141]. The RPKM value of each PEBP gene across different tissues of P. mume, P. persica, P. yedoensis, and Rubus occidentalis was extracted and visualized using the 'pheatmap' package in R. The relative expression of PEBP genes in leaf, stem, root, and floral bud tissues was tested in P. mume using real-time PCR analysis with detailed procedure described below.

Expression analysis of PEBP genes during the flower bud development process

To further understand the functional role of PEBP genes in floral bud initiation and the bud flushing process, we performed real-time quantitative PCR analysis to examine the temporal expression patterns of PEBP genes. Lateral floral bud samples were collected from P. mume ‘Fei Lv’ tree grown in the Jiufeng sunlight greenhouse approximately every four weeks from July 10th, 2019 to January 12th, 2020. The total RNA was extracted from mixed bud samples using the E.Z.N.A.® Plant RNA Kit following the manufacturer's instructions (Omega Bio-tek, Norcross) and was reverse-transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan). We performed real-time PCR experiments with at least three technical replicates on the PikoReal real-time PCR platform (Thermo Fisher Scientific, Germany). The temperature was set as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s; 60 °C for 30 s;
and ending 20 °C. We used protein phosphatase 2A (PP2A) as an internal reference and calculated the relative transcription levels of target genes using the 2−ΔΔCt method [142]. The primers used for qRT-PCR experiments are listed (Table S7).

Co-expression network of FT during the blooming process in P. mume

To investigate the functional role of FT during oral bud flushing, we obtained the transcriptome data of four successive stages during dormancy release and blooming in P. mume from a previous study reported by Zhang et al. (2018). The procedure of sample collection, RNA extraction, sequencing library construction, quality control, and gene expression quantification was described in detail [55]. We normalized the gene expression and performed weighted gene co-expression network analysis with WGCNA v1.67 package in R [143]. The Dynamic Tree Cut algorithm was applied to detect gene modules (power β of 4; height cutoff of 0.3; minimal module size of 30). To identify the key modules coexpressed with FT, we calculated the module-trait association and ranked genes by their correlation with the FPKM value of PmFT. Finally, the top 50 candidate genes (R² > 0.6) coexpressed with PmFT and 15 FT interacting factors identified in Arabidopsis flowering pathways [56, 57] were selected to construct the coexpression network of FT. The FT regulatory network was visualized with Cytoscape 3.1 [144]. The expression levels of FT and putative co-expressed genes were further validated by qRT-PCR analysis. The primers are described in the supplementary data (Table S8).

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and materials
The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that no competing interests exist.

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Authors' contributions
Zhang M designed and conducted the study; Li P and Yan XL performed the blast analysis and sequence curation; Wang J and Cheng T provided help with transcriptome analysis; Zhang Q supervised the project and revised the manuscript.

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Reference

1. Ågren J, Oakley CG, Lundemo S, Schemske DW: Adaptive divergence in flowering time among natural populations of Arabidopsis thaliana: Estimates of selection and QTL mapping. Evolution 2017, 71(3):550-564.
2. Gaudinier A, Blackman BK: Evolutionary processes from the perspective of flowering time diversity. New Phytologist 2019, 225(5):1883-1898.
3. Townsend T, Albani M, Wilkinson M, Coupland G, Battey N: The Diversity and Significance of Flowering in Perennials. 2018:181-197.
4. Khan MRG, Ai XY, Zhang JZ: Genetic regulation of flowering time in annual and perennial plants. Wiley Interdisciplinary Reviews: RNA 2014, 5(3):347-359.
5. Huijser P, Schmid M: The control of developmental phase transitions in plants. Development 2011, 138(19):4117-4129.
6. Wilkie JD, Sedgley M, Olesen T: Regulation of floral initiation in horticultural trees. Journal of Experimental Botany 2008, 59(12):3215-3228.
7. Basler D, Komer C: Photoperiod and temperature responses of bud swelling and bud burst in four temperate forest tree species. Tree Physiology 2014, 34(4):377-388.
8. Rohde A, Bastien C, Boerjan W: Temperature signals contribute to the timing of photoperiodic growth cessation and bud set in poplar. Tree Physiology 2011, 31(5):472-482.
9. Rafferty NE, Scaven VL: Physiological effects of climate warming on flowering plants and insect pollinators and potential consequences for their interactions. Current Zoology 2013, 59(3):418-426.
10. Abbott AG, Zhebentyayeva T, Barakat A, Liu Z: Chapter Six - The Genetic Control of Bud-Break in Trees. In: Advances in Botanical Research. Edited by Plomion C, Adam-Blondon A-F, vol. 74: Academic Press; 2015: 201-228.
11. Dirlewanger E, Quero-Garcia J, Le Dantec L, Lambert P, Ruiz D, Dondini L, Illa E, Quilot-Turion B, Audergon JM, Tartarini S et al. Comparison of the genetic determination of two key phenological traits, flowering and maturity dates, in three Prunus species: peach, apricot and sweet cherry. Heredity 2012, 109(5):280-292.

12. Vitasse Y, Lenz A, Körner C: The interaction between freezing tolerance and phenology in temperate deciduous trees. Frontiers in Plant Science 2014, 5.

13. Ladwig LM, Chandler JL, Guiden PW, Henn JJ: Extreme winter warm event causes exceptionally early bud break for many woody species. Ecosphere 2019, 10(1).

14. Cattani AM, Sartor T, da Silveira Falavigna V, Porto DD, Silveira CP, Dias da Oliveira PR, Revers LF: The Control of Bud Break and Flowering Time in Plants. 2018, 88:277-325.

15. Chautard H, Jacquet M, Schoentgen F, Bureaud N, Bénédetti H: Tfs1p, a member of the PEBP family, inhibits the Ira2p but not the Ira1p Ras GTPase-activating protein in Saccharomyces cerevisiae. Eukaryotic Cell 2004, 3(2):459-470.

16. Yeung K, Seitz T, Li S, Janosch P, McC Ferran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H et al. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature 1999, 401(6749):173-177.

17. 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. Journal of Systematics and Evolution 2016, 54(5):502-510.

18. Serre L, Vallaé 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(10):1255-1265.

19. Banfield MJ, Barker JJ, Perry ACF, Brady RL: Function from structure? The crystal structure of human phosphatidylsphingolipid-binding protein suggests a role in membrane signal transduction. Structure 1998, 6(10):1245-1254.

20. Tsuji H, Taoka K-t: Chapter Five - Florigen Signaling. In: The Enzymes. Edited by Machida Y, Lin C, Tamanoi F, vol. 35: Academic Press; 2014: 113-144.

21. Vallaé’e BS, Coadou G, Labbeé H, Sy D, Vovelle F, Schoentgen F: Peptides corresponding to the N- and C-terminal parts of PEBP are well-structured in solution: new insights into their possible interaction with partners in vivo. The Journal of Peptide Research 2008, 61(2):47-57.

22. Hengst U, Albrecht H, Hess D, Monard D: The Phosphatidylethanolamine-binding Protein Is the Prototype of a Novel Family of Serine Protease Inhibitors. Journal of Biological Chemistry 2001, 276(1):535-540.

23. Keller ET, Fu Z, Brennan M: The role of Raf kinase inhibitor protein (RKIP) in health and disease. Biochemical Pharmacology 2004, 68(6):1049-1053.

24. Lorenz K, Lohse MJ, Quitterer U: Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. Nature 2003, 426(6966):574-579.

25. Odabaie G, Chatterjee D, Jazirehi AR, Goodglick L, Yeung K, Bonavida B: Raf-1 kinase inhibitor protein: structure, function, regulation of cell signaling, and pivotal role in apoptosis. Advances in cancer research 2004, 91:169-200.

26. Bradley D, Carpenter R, Copsey L, Vincent C, Rothstein S, Coen E: Control of inflorescence architecture in Antirrhinum. Nature 1996, 379(6568):791-797.

27. Karlgren A, Gyllenstrand N, Kallman T, Sundstrom JF, Moore D, Lascoux M, Lagercrantz U: Evolution of the PEBP Gene Family in Plants: Functional Diversification in Seed Plant Evolution. Plant Physiology 2011, 156(4):1967-1977.

28. Vaistij FE, Barros-Galvão T, Cole AF, Gilday AD, He Z, Li Y, Harvey D, Larson TR, Graham IA: MOTHER-OF-FT-AND-TFL1 represses seed germination under far-red light by modulating phytohormone responses in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 2018, 115(33):8442-8447.

29. Mackenzie KK, Coelho LL, Lütken H, Müller R: Phylogenetic Analysis of the PEBP Gene Family from Kalanchoë. Agronomy 2019, 9(4):171.

30. Jin S, Nasim Z, Susila H, Ahn JH: Evolution and functional diversification of FLOWERING LOCUS T/TERM NAL FLOWER 1 family genes in plants. Seminars in Cell & Developmental Biology 2020.

31. Hedman H, Källman T, Lagercrantz U: Early evolution of the MFT-like gene family in plants. Plant Molecular Biology 2009, 70(4):359-369.

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 Phytologist 2016, 212(3):730-744.

33. Wickland Daniel P, Hanzawa Y: The FLOWERING LOCUS T/TER MINAL FLOWER 1 Gene Family: Functional Evolution and Molecular Mechanisms. Molecular Plant 2015, 8(7):983-997.

34. Wang Z, Zhou Z, Liu Y, Liu T, Li Q, Ji Y, Li C, Fang C, Wang M, Wu M et al. Functional Evolution of Phosphatidylethanolamine Binding Proteins in Soybean and Arabidopsis. The Plant Cell Online 2015, 27(2):323-336.

35. Ahn JH, Miller D, Winter VJ, Banfield MJ, Lee JH, Yoo SY, Henz SR, Brady RL, Weigel D: A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. The EMBO Journal 2006, 25(3):605-614.

36. Bai S, Tuan PA, Saito T, Ito A, Ubi BE, Ban Y, Moriguchi T: Repression of TERMINAL FLOWER1 primarily mediates floral induction in pear (Pyrus pyrifolia Nakai) concomitant with change in gene expression of plant hormone-related genes and transcription factors. Journal of Experimental Botany 2017, 68(17):4899-4914.

37. Hanzawa Y, Money T, Bradley D: A single amino acid converts a repressor to an activator of flowering. Proceedings of the National Academy of Sciences 2005, 102(21):7748-7753.

38. Koskela EA, Säntstebay A, Flachowsky H, Heide OM, Hanke M-V, Elomaa P, Hytönen T: TERMINAL FLOWER1 is a breeding target for a novel everbearing trait and tailored flowering responses in cultivated strawberry (Fragaria × ananassa Duch.). Plant Biotechnology Journal 2016, 14(9):1852-1861.

39. Baumann K, Venail J, Berbel A, Domenich MJ, Money T, Conti L, Hanzawa Y, Madueno F, Bradley D: Changing the spatial pattern of TFL1 expression reveals its key role in the shoot meristem in controlling Arabidopsis flowering architecture. Journal of Experimental Botany 2015, 66(15):4769-4780.
40. Chen Q, Payyavula RS, Chen L, Zhang J, Zhang C, Turgeon R: **FLOWERING LOCUS T mRNA is synthesized in specialized companion cells in Arabidopsis and Maryland Mammoth tobacco leaf veins.** *Proceedings of the National Academy of Sciences* 2018, 115(11):2830-2835.

41. Luccioni L, Krzymuski M, Sánchez-Lamas M, Karayevok E, Cerdán PD, Casal JJ: **CONSTANS delays Arabidopsis flowering under short days.** *The Plant Journal* 2019, 97(5):923-932.

42. Lee C, Kim S-J, Jin S, Susila H, Youn G, Nasim Z, Alavilli H, Chung K-S, Yoo S-J, Ahn JH: **Genetic interactions reveal the antagonistic roles of FT/TSF and TFL1 in the determination of inflorescence meristem identity in Arabidopsis.** *The Plant Journal* 2019, 99(3):452-464.

43. Ho WWH, Weigel D: **Structural Features Determining Flower-Promoting Activity of Arabidopsis FLOWERING LOCUS T.** *The Plant Cell* 2014, 26(2):552-564.

44. Foottit S, Öljær-Footitt H, Hambidge AJ, Finch-Savage WE: **A laboratory simulation of Arabidopsis seed dormancy cycling provides new insight into its regulation by clock genes and the dormancy-related genes DOG1, MFT, CIPK23 and PHYA.** *Plant, Cell & Environment* 2017, 40(8):1474-1486.

45. Jin S, Jung HS, Chung KS, Lee JH, Ahn JH: **FLOWERING LOCUS T has higher protein mobility than TWIN SISTER OF FT.** *Journal of Experimental Botany* 2015, 66(20):6109-6117.

46. Huang N-C, Jane W-N, Chen J, Yu T-S: **Arabidopsis thaliana CENTRORADIALIS homologue (ATC) acts systemically to inhibit floral initiation in Arabidopsis.** *The Plant Journal* 2012, 72(2):175-184.

47. Liu X, Zhang J, Abuhamad A, Franks RG, Xie D-Y, Xiang Q-Y: **Analysis of two TFL1 homologs of dogwood species (Cornus L.) indicates functional conservation in control of transition to flowering.** *Planta* 2016, 243(5):1129-1141.

48. Ryu JY, Lee H-J, Seo PJ, Jung J-H, Ahn JH, Park C-M: **The Arabidopsis Floral Repressor BFT Delays Flowering by Competing with FT for FD Binding under High Salinity.** *Molecular Plant* 2014, 7(2):377-387.

49. Shulaev V, Korban SS, Sosinski B, Abbott AG, Aldwinckle HS, Fitma K, Iezzoni A, Main D, Arus P, Dandekar AM et al: **Multiple Models for Rosaceae Genomics.** *Plant Physiology* 2008, 147(3):985-1003.

50. Soundararajan P, Won SY, Kim JS: **Insight on Rosaceae Family with Genome Sequencing and Functional Genomics Perspective.** *BioMed Research International* 2019, 2019:1-12.

51. Yamamoto T, Terakami S: **Genomics of pear and other Rosaceae fruit trees.** *Breeding Science* 2016, 66(1):148-159.

52. Xiang Y, Huang C-H, Hu Y, Wen J, Li S, Yi T, Chen H, Xiang J, Ma H: **Evolution of Rosaceae Fruit Types Based on Nuclear Phylogeny in the Context of Geological Times and Genome Duplication.** *Molecular Biology and Evolution* 2016:msw242.

53. Gottschalk C, van Nocker S: **Diversity in Seasonal Bloom Time and Floral Development among Apple Species and Hybrids.** *Journal of the American Society for Horticultural Science* 2013, 138(5):367-374.

54. Pin PA, Benlloch R, Bonnet D, Wremer-Weich E, Kraft T, Gielan J, NLlsson O: **An Antagonistic Pair of FT Homologs Mediates the Control of Flowering Time in Sugar Beet.** *Science* 2010, 330(6009):1397-1400.

55. Zhang Z, Zhuo X, Zhao K, Zheng T, Han Y, Yuan C, Zhang Q: **Transcriptome Profiles Reveal the Crucial Roles of Hormone and Sugar in the Bud Dormancy of Prunus mume.** *Scientific Reports* 2018, 8(1).

56. Amasino R: **Seasonal and developmental timing of flowering.** *The Plant Journal* 2010, 61(6):1001-1013.

57. Song YH, Ito S, Imaizumi T: **Flowering time regulation: photoperiod- and temperature-sensing in leaves.** *Trends in Plant Science* 2013, 18(10):575-583.

58. Komeda Y: **Genetic regulation of time to Flower in Arabidopsis thaliana.** *Annual Review of Plant Biology* 2004, 55(1):521-535.

59. Teotia S, Tang G: **To Bloom or Not to Bloom: Role of MicroRNAs in Plant Flowering.** *Molecular Plant* 2015, 8(3):359-377.

60. Kaikiewicz M, Rychel S, Nelson MW, Wynra K, Naganaswa B, Wolko B: **Expansion of the phosphatidylethanolamine binding protein family in legumes: a case study of Lupinus angustifolius L. FLOWERING LOCUS T homologs, LanFTc1 and LanFTc2.** *BMC Genomics* 2016, 17(1).

61. Wu J, Wang Z, Shi Z, Zhang S, Ming R, Zhu S, Khan MA, Tao S, Korban SS, Wang H et al: **The genome of the pear (Pyrus bretschneideri Rehdc.).** *Genome Research* 2012, 23(2):396-408.

62. Xu G, Ma H, Nei M, Kong H: **Evolution of F-box genes in plants: Different modes of sequence divergence and their relationships with functional diversification.** *Proceedings of the National Academy of Sciences* 2009, 106(3):835-840.

63. Gao J, Huang B-H, Wan Y-T, Chang J, Li J-Q, Liao P-C: **Functional divergence and intron variability during evolution of angiosperm TERMINAL FLOWER1 (TFL1) genes.** *Scientific Reports* 2017, 7(1).

64. Yamaguchi A, Kobayashi Y, Goto K, Abe M, Araki T: **TWIN SISTER OF FT (TSP) Acts as a Floral Pathway Integrator Redundantly with FT.** *Plant and Cell Physiology* 2005, 46(8):1175-1189.

65. Hu Y, Liu B, Sun C, Liu J, Wang X, Cheng F, Liang J, Wang X, Wu J: **Evolution of TWIN SISTER OF FT (TSP) Genes in Brassicaceae.** *Horticultural Plant Journal* 2016, 2(1):16-25.

66. Komar AA: **The Yin and Yang of codon usage.** *Human Molecular Genetics* 2016, 25(R2):R77-R85.

67. Yu C-H, Deng Y, Zhou Z, Wu C, Zhao F, Sachs Matthew S, Liu Y: **Codon Usage Influences the Local Rate of Translation Elongation to Regulate Cotranslational Protein Folding.** *Molecular Cell* 2015, 59(5):744-754.

68. Eyre-Walker AC: **An analysis of codon usage in mammals: Selection or mutation bias?** *Journal of Molecular Evolution* 1991, 33(5):442-449.

69. Guan D-L, Ma L-B, Khan MS, Zhang X-X, Xu S-Q, Xie J-Y: **Analysis of codon usage patterns in Hmutunaria manilensis reveals a preference for GC-ending codons caused by dominant selection constraints.** *BMC Genomics* 2018, 19(1).

70. Sharp PM, Li W-H: **The codon adaptation index—a measure of directional synonymous codon usage bias, and its potential applications.** *Nucleic Acids Research* 1987, 15(3):1281-1295.

71. Wright F: **The effective number of codons used in a gene.** *Gene* 1990, 87(1):23-29.
72. Banfield MJ, Brady RL: The structure of Antirhinum centrotoradialis protein (CEN) suggests a role as a kinase regulator. Journal of Molecular Biology 2000, 297(5):1159-1170.

73. Si Z, Liu H, Zhu J, Chen J, Wang Q, Fang L, Gao F, Tian Y, Chen Y, Chang L et al. Mutation of SELF-PRUNING homologs in cotton promotes short-branching plant architecture. Journal of Experimental Botany 2018, 69(10):2543-2553.

74. Kotoda N, Hayashi H, Suzuki M, Igarashi M, Hatsuysama Y, Kidou S-i, Iigasaki T, Nishiguchi M, Yano K, Shimizu T et al. Molecular Characterization of FLOWERING LOCUS T-like Genes of Apple (Malus × domestica Borkh.). Plant and Cell Physiology 2010, 51(4):561-575.

75. Wang Z, Yang R, Devisetty UK, Maloof JN, Zuo Y, Li J, Shen Y, Zhao J, Bao M, Ning G: The Divergence of Flowering Time Modulated by FT/TFL1 Is Independent to Their Interaction and Binding Activities. Frontiers in Plant Science 2017, 8.

76. Zhang X, An L, Nguyen TH, Liang H, Wang R, Liu X, Li T, Qi Y, Yu F: The Cloning and Functional Characterization of Peach CONSTANS and FLOWERING LOCUS T Homologous Genes PpCO and PpFT. Plas One 2015, 10(4):e0124108.

77. Mimida N, Kotoda N, Ueda T, Igarashi M, Hatsuysama Y, Iwanami H, Moriya S, Abe K: Four TFL1/CEN-Like Genes on Distinct Linkage Groups Show Different Expression Patterns to Regulate Vegetative and Reproductive Development in Apple (Malus × domestica Borkh.). Plant and Cell Physiology 2009, 50(2):394-412.

78. Esumi T, Kitamura Y, Hagihara C, Yamane H, Tao R: Identification of a TFL1 ortholog in Japanese apricot (Prunus mume Sieb. et Zucc.). Scientia Horticulturae 2010, 125(4):608-616.

79. Hanano S,goto K: Arabidopsis TERMINAL FLOWER1 is involved in the regulation of flowering time and in flower development through transcriptional regulation. The Plant Cell 2011, 23(9):3172-3184.

80. Freiman A, Golovitch S, Yablotsky V, Belavoev S, Dahan Y, Peer R, Avraham L, Freiman Z, Evenor D, Reuveni M et al. Expression of flowering locus T2 transgene from Pyrus communis L. delays dormancy and leaf senescence in Malus × domestica Borkh., and causes early flowering in tobacco. Plant Science 2015, 241:164-176.

81. Hsu CY, Adams JP, Kim H, No K, Ma C, Strauss SH, Drnevich J, Vandervelde L, Ellis JD, Rice BM et al. FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. Proceedings of the National Academy of Sciences 2011, 108(26):10756-10761.

82. Park S, Srinivasan C, Dardick C, Callahan A, Scorza R: Plum (Prunus domestica) Trees Transformed with Poplar FTL1 Result in Altered Architecture, Dormancy Requirement, and Continuous Flowering. PLoS ONE 2012, 7(7):e40715.

83. Kliétnán S, Pin PA, Benlloch R, Ingvarsen PK, Nilsson O: Analysis of conifer FLOWERING LOCUS T/TERMINAL FLOWER1-like genes provides evidence for dramatic biochemical evolution in the angiosperm FT lineage. New Phytologist 2012, 196(4):1260-1273.

84. Avis K, Kärkkäinen K, Lagercrantz U, Savolainen O: Association of FLOWERING LOCUS T/TERMINAL FLOWER1-like gene FTL2 expression with growth rhythm in Scots pine (Pinus sylvestris). New Phytologist 2014, 204(1):159-170.

85. Gyllenstrand N, Clapham D, Kallman T, Lagercrantz U: A Norway Spruce FLOWERING LOCUS T Homolog Is Involved in Control of Growth Rhythm in Conifers. Plant Physiology 2007, 144(1):248-257.

86. Karlsgren A, Gyllenstrand N, Clapham D, Lagercrantz U: FLOWERING LOCUS T/TERRNIAL FLOWER1-Like Genes Affect Growth Rhythm and Bud Set in Norway Spruce. Plant Physiology 2013, 163(2):792-803.

87. Mouradov A, Cremer F, Coupland G: Control of Flowering Time. The Plant Cell 2002, 14(suppl 1):S111-S130.

88. Putterill J, Laurie R, Macknight R: It's time to flower: the genetic control of flowering time. BioEssays 2004, 26(4):363-373.

89. Song YH, Shim JS, Kimnmonth-Schultz HA, Imaizumi T: Photoperiodic Flowering: Time Measurement Mechanisms in Leaves. Annual Review of Plant Biology 2015, 66(1):441-464.

90. Samach A, Gover A: Photoperiodism: The consistent use of CONSTANS. Current Biology 2001, 11(16):R651-R654.

91. Yang S, Weers BD, Morishige DT, Mullet JE: CONSTANS is a photoperiod regulated activator of flowering in sorghum. BMC Plant Biology 2014, 14(1):148.

92. Hayama R, Coupland G: The Molecular Basis of Diversity in the Photoperiodic Flowering Responses of Arabidopsis and Rice. Plant Physiology 2004, 135(2):677-684.

93. Distelfeld A, Li C, Dubcovsky J: Regulation of flowering in temperate cereals. Current Opinion in Plant Biology 2009, 12(2):178-184.

94. Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O: CCFT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science 2006, 312(5776):1040-1043.

95. Deng W, Ying H, Hellwell CA, Taylor JM, Peacock WJ, Dennis ES: FLOWERING LOCUS C (FLC) regulates development pathways throughout the life cycle of Arabidopsis. Proceedings of the National Academy of Sciences 2011, 108(16):6680-6685.

96. Hartmann U, Hohmann S, Nettesheim K, Wisman E, Saedler H, Huijser P: Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. The Plant Journal 2000, 21(4):351-360.

97. Kumar SV, Lucyshyn D, Jaeger KE, Aloe E, Alvey E, Harberd NP, Wigge PA: Transcription factor PIF4 controls the thermosensory activation of flowering. Nature 2012, 484(7393):242-245.

98. Lee J, Lee I: Regulation and function of SOC1, a flowering pathway integrator. Journal of Experimental Botany 2010, 61(9):2247-2254.

99. Teper-Bammolker P: The Flowering Integrator FT Regulates SEPALATA3 and FRUITFULL Accumulation in Arabidopsis Leaves. The Plant Cell Online 2005, 17(10):2661-2675.

100. Pin PA, Nilsson O: The multifaceted roles of FLOWERING LOCUS T in plant development. Plant, Cell & Environment 2012, 35(10):1742-1755.

101. Rinne PLH, Welling A, Vahala J, Ripel L, Ruonala R, Kangasjärvi J, van der Schoot C: Chilling of Dormant Buds Hyperinduces FLOWERING LOCUS T and Recruits GA-Inducible 1,3-β-Gluocanases to Reopen Signal Conduits and Release Dormancy in Populus. The Plant Cell 2011, 23(1):130-146.
102. Niu Q, Li J, Cai D, Qian M, Jia H, Bai S, Hussain S, Liu G, Teng Y, Zheng X. Dormancy-associated MADS-box genes and microRNAs jointly control dormancy transition in pear (Pyrus pyrifolia white pear group) flower bud. *Journal of Experimental Botany* 2016, 67(1):239-257.

103. Ito A, Saito T, Sakamoto D, Sugiuira T, Bai S, Moriguchi T, Nåsholm T: Physiological differences between bud breaking and flowering after dormancy completion revealed by DAM and FT/TFL1 expression in Japanese pear (Pyrus pyrifolia). *Tree Physiology* 2016, 36(1):109-120.

104. Rongmei W, Sumathí T, Sakuntala K, Tustin SD, Hellens RP, Allan AC, Macknight RC, Erika VG: *SVPlike MADS Box Genes Control Dormancy and Budbreak in Apple*. *Frontiers in Plant Science* 2018, 07(55).

105. Mateos JL, Madrigal P, Tsuda K, Rawat V, Richter R, Romera-Branchat M, Formara F, Schneebberger K, Krajewski P, Coupland G: Combinatorial activities of SHORT VEGETATIVE PHASE and FLOWERING LOCUS C define distinct modes of flowering regulation in Arabidopsis. *Genome Biology* 2015, 16(1).

106. Kurokura T, Mimida N, Battey NH, Hytönen T: The regulation of seasonal flowering in the Rosaceae. *Journal of Experimental Botany* 2013, 64(14):4131-4141.

107. Verde I, Jenkins J, Dondini L, Micalli M, Pagliarani G, Vendramin E, Paris R, Aramini V, Gazza L, Rossini L et al. The Peach v2.0 release: high-resolution linkage mapping and deep resequencing improve chromosome-scale assembly and contiguity. *BMC Genomics* 2017, 18(1).

108. Zhang Q, Chen W, Sun L, Zhao F, Huang B, Yang W, Tao Y, Wang J, Yuan Z, Fan G et al. The genome of Prunus mume. *Nature Communications* 2012, 3(1).

109. Baek S, Choi S, Kim G-B, Yu H-J, Cho A, Jang H, Kim C, Kim H-J, Chang KS, Kim J-H et al. Draft genome sequence of wild Prunus yedoensis reveals massive inter-specific hybridization between sympatric flowering cherries. *Genome Biology* 2018, 19(1).

110. Shirasawa K, Isuzugawa K, Ikenaga M, Saito Y, Yamamoto T, Hirakawa H, Isobe S: The genome sequence of sweet cherry (Prunus avium) for use in genomics-assisted breeding. *DNA Research* 2017, 24(5):499-508.

111. Jiang F, Zhang J, Wang S, Yang L, Luo Y, Gao S, Zhang M, Wu S, Hu S, Sun H et al. The apricot (Prunus armeniaca L.) genome elucidates Rosaceae evolution and beta-carotene biosynthesis. *Horticulture Research* 2019, 6(1).

112. Daccord N, Celton J-M, Linsmith G, Becker C, Choisnne N, Schijlen E, van de Geest H, Bianco L, Micheletti D, Velasco R et al. High-quality de novo assembly of the apple genome and methylome dynamics of early fruit development. *Nature Genetics* 2017, 49(7):1099-1106.

113. Tinker NA, Chagné D, Crowhurst RN, Pindo M, Thrimawithana A, Deng C, Ireland H, Fiers M, Dzierzon H, Cestaro A et al. The Draft Genome Sequence of European Pear (Pyrus communis L. ‘Bartlett’). *PLoS ONE* 2014, 9(4):e92644.

114. VanBuren R, Wai CM, Colle M, Wang J, Sullivan S, Bushakra JM, Liachko I, Vining KJ, Dossett M, Finn CE et al. A near complete, chromosome-scale assembly of the black raspberry (Rubus occidentalis) genome. *GigaScience* 2018, 7(8).

115. Jung S, Jesudurai C, Staton M, Du Z, Ficklin S, Cho I, Abbott A, Tomkins J, Main D: GDR (Genome Database for Rosaceae): integrated web resources for genomics and genetics research. *BMC Bioinformatics* 2004, 5(1):130.

116. Potter SC, Luciani A, Eddy SR, Park Y, Lopez R, Finn RD: HMmer web server: 2018 update. *Nucleic Acids Research* 2018, 46(W1):W200-W204.

117. Letunic I, Doerks T, Bork P: SMART: recent updates, new developments and status in 2015. *Nucleic Acids Research* 2015, 43(D1):D257-D260.

118. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J et al. Pfam: the protein families database. *Nucleic Acids Research* 2014, 42(D1):D222-D230.

119. Marchler-Bauer A, Bo Y, Han L, He J, Lanczyczi CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Research* 2017, 45(D1):D200-D203.

120. Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 2004, 32(5):1792-1797.

121. Nicholas KB: GeneDoc: Analysis and visualization of genetic variation, EMBNEW. *Emnbew News* 1997, 4.

122. Kumar S, Stecher G, Tamura K: MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* 2016, 33(7):1870-1874.

123. Stamatakis A: *RAxML version 8*: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014, 30(9):1312-1313.

124. Ronquist F, Huelsenbeck JP: MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003, 19(12):1572-1574.

125. Bailey TL, Johnson J, Grant CE, Noble WS: The MEME Suite. *Nucleic Acids Research* 2015, 43(W1):W39-W49.

126. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R: *TBtools*: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Molecular Plant* 2020, 13(8):1194-1202.

127. Wang Y, Tang H, DeBarry JD, Tan X, Li J, Wang X, Lee Th, Jin H, Marler B, Guo H et al. *MCScanX*: a toolkit for detection and evolutionary analysis of gene synteny and collinearly. *Nucleic Acids Research* 2012, 40(7):e49-e49.

128. Garcia-Gil MR, Lecoy J: Evolutionary analysis of LP3 gene family in conifers: an ASR homolog. *bioRxiv* 2020.

129. Puigbó P, Bravo IG, Garcia-Vallve S: CaCal: A combined set of tools to assess codon usage adaptation. *Biology Direct* 2008, 3(1):38.

130. Fuglsang A: The ‘effective number of codons’ revisited. *Biochemical and Biophysical Research Communications* 2004, 317(3):957-964.

131. Xu X-z, Liu Q-p, Fan L-j, Cui X-f, Zhou X-p: Analysis of synonymous codon usage and evolution of begomoviruses. *Journal of Zhejiang University SCIENCE B* 2008, 9(9):667-674.

132. Castresana J: Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis. *Molecular Biology and Evolution* 2000, 17(4):540-552.

133. Yang Z: *PAML 4: Phylogenetic Analysis by Maximum Likelihood*. *Molecular Biology and Evolution* 2007, 24(8):1586-1591.

134. Stern A, Doron-Faigenboim A, Erez E, Marz E, Bacharach E, Pupko T: Selection 2007: advanced models for detecting positive and purifying selection using a Bayesian inference approach. *Nucleic Acids Research* 2007, 35(Web Server):W506-W511.
135. Lescot M: PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research* 2002, 30(1):325-327.

136. Chow C-N, Zheng H-Q, Wu N-Y, Chien C-H, Huang H-D, Lee T-Y, Chiang-Hsieh Y-F, Hou P-F, Yang T-Y, Chang W-C: PlantPAN 2.0: an update of plant promoter analysis navigator for reconstructing transcriptional regulatory networks in plants. *Nucleic Acids Research* 2016, 44(D1):D1154-D1160.

137. Lazzari B, Caprera A, Vecchietti A, Stella A, Milanesi L, Pozzi C: ESTree db: a Tool for Peach Functional Genomics. *BMC Bioinformatics* 2005, 6(Suppl 4):S16.

138. Bolger AM, Lohse M, Usadel B: Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014, 30(15):2114-2120.

139. Kim D, Langmead B, Salzberg SL: HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* 2015, 12(4):357-360.

140. Anders S, Pyl PT, Huber W: HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* 2014, 31(2):166-169.

141. Robinson MD, McCarthy DJ, Smyth GK: edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2009, 26(1):139-140.

142. Livak KJ, Schmittgen TD: Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* 2001, 25(4):402-408.

143. Langfelder P, Horvath S: WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 2008, 9(1).

144. Saito R, Smoot ME, Ono K, Ruscheinski J, Wang P-L, Lotia S, Pico AR, Bader GD, Ideker T: A travel guide to Cytoscape plugins. *Nature Methods* 2012, 9(11):1069-1076.

**Tables**

**Table 1.** Detailed information of PEBP genes from *A. thaliana* and nine Rosaceae species
| Gene lineage | Species | Gene accession number | NCBI accession | Notation |
|--------------|---------|-----------------------|----------------|----------|
| FT           | Arabidopsis thaliana | AT1G65480.1; AT4G20370.1 | | AFT; ATSF |
| Malus domestica | MD12G1262000 | NM_001293862.1 | | MdFT |
| Pyrus communis | PCP004421.1; PCP023373.1 | | PcFT1; PcFT2 |
| Rubus occidentalis | Ro04_G00016; Ro06_G09261 | | RoFT1; RoFT2 |
| Prunus persica | Prupe.6G364900.1 | XM_007205940.2 | PpFT |
| Prunus mume | Pm003733 | NM_001293253.1 | PmFT |
| Prunus armeniaca | PARG03266m01 | | PaFT |
| Pyrus yedoensis | PQ005805.1; PQ009349.1 | | PyFT1; PyFT2 |
| Prunus avium | Cps0077204G3m0 | XM_021948448.1 | PvFT |
| Prunus dulcis | Prudul26A015211P1 | XM_034364192.1 | PdFT |
| TFLI         | Arabidopsis thaliana | AT5G03840.1 | | ATFL1 |
| Malus domestica | MD14G1021100; MD12G1023900 | NM_001293865.1; NM_001293958.1 | MdTFL1; MdTFL2 |
| Pyrus communis | PCP003730.1; PCP025869.1 | | PcTFL1; PcTFL2 |
| Rubus occidentalis | Ro06_G14897 | | RoTFL |
| Prunus persica | Prupe.7G112600.1 | XM_007202602.2 | PpTFL |
| Prunus mume | Pm026188 | XM_008243028.1 | PmTFL |
| Prunus armeniaca | PARG26714m01 | | PaTFL |
| Prunus yedoensis | PQ096161.1 | | PyTFL |
| Prunus avium | Cps0034G256m0 | XM_021954469.1 | PvTFL |
| Prunus dulcis | Prudul26A021958P1 | XM_034369048.1 | PdTFL |
| CEN          | Arabidopsis thaliana | AT2G27550.1 | | ATCEN |
| Malus domestica | MD11G1163500; MD03G1143000 | NM_001294011.1; NM_001293884.1 | MdCEN1; MdCEN2 |
| Pyrus communis | PCP019918.1; PCP022206.1 | | PcCEN1; PcCEN2 |
| Rubus occidentalis | Ro03_G20412 | | RoCEN |
| Prunus persica | Prupe.6G128400.1 | XM_007205944.2 | PpCEN |
| Prunus mume | Pm001309 | XM_008230265.2 | PmCEN |
| Prunus armeniaca | PARG01261m01 | | PaCEN |
| Prunus yedoensis | PQ012971.1 | | PyCEN |
| Prunus avium | Cps00116G158m0 | XM_021966077.1 | PvCEN |
| Prunus dulcis | Prudul26A027558P1 | XM_034364411.1 | PdCEN |
| MFT          | Arabidopsis thaliana | AT1G18100.1 | | AMFT |
| Malus domestica | MD06G1229900 | XM_008376608.2 | MdMFT |
| Pyrus communis | PCP033759.1 | | PcMFT |
| Rubus occidentalis | Ro03_G03590 | | RoMFT |
| Prunus persica | Prupe.5G230900.1 | XM_007209625.2 | PpMFT |
| Prunus mume | Pm025099 | XM_008241952.1 | PmMFT |
| Prunus armeniaca | PARG25179m01 | | PaMFT |
| Prunus yedoensis | PQ05244.1; PQ015508.1 | | PyMFT1; PyMFT2 |
| Prunus avium | Cps0021G127m0 | XM_021947485.1 | PvMFT |
| Prunus dulcis | Prudul26A015523P1 | XM_034360958.1 | PdMFT |
| BFT          | Arabidopsis thaliana | AT5G62040.1 | | ABFT |
| Malus domestica | MD01G1198400; MD07G1265900 | NM_001293841.1; XM_008378317.3 | MdBFT1; MdBFT2 |
| Pyrus communis | PCP030682.1; PCP007692.1 | | PcBFT1; PcBFT2 |
| Rubus occidentalis | Ro07_G09463 | | RoBFT |
| Prunus persica | Prupe.2G291900.1 | XM_007221111.2 | PpBFT |
| Prunus mume | Pm019359 | XM_008236052.2 | PmBFT |
| Prunus armeniaca | PARG19444m01 | | PaBFT |
| Prunus yedoensis | PQ001551.1; PQ034355.1 | | PyBFT1; PyBFT2 |
| Prunus avium | Cps0033G388m0 | XM_021971342.1 | PvBFT |
| Prunus dulcis | Prudul26A027512P1 | XM_034348832.1 | PdBFT |

Rosaceae species include M. domestica, Pyrus communis, R. occidentalis, P. persica, P. mume, P. armeniaca, P. yedoensis, P. avium and P. dulcis. Gene notations were assigned to Rosaceae PEBPs based on their Arabidopsis ortholog.

Table 2. Average gene parameters estimated for FT, TFL1, CEN, BFT, and MFT gene lineages.

| Name | Length | CAI | total GC% | GC1% | GC2% | GC3% | ENC |
|------|--------|-----|-----------|------|------|------|-----|
| BFT  | 524.590| 0.842| 47.142 | 52.158| 44.133| 45.142| 54.133|
| CEN  | 523.364| 0.843| 45.327 | 52.118| 38.427| 45.491| 54.264|
| FT   | 526.750| 0.826| 48.583 | 56.058| 44.533| 45.108| 54.950|
| MFT  | 533.700| 0.866| 55.500 | 58.660| 44.600| 63.240| 51.240|
| TFL1 | 519.273| 0.851| 47.982 | 51.627| 45.891| 46.427| 52.291|
Table 3. Parameter estimates and likelihood values for branch-site models among sites and lineages of PEBP.

| Branch-site model | Foreground branch | Estimate of parameters | Model comparison | 2*|lnL₁−lnL₂| | df | pLRT | Selected sites |
|------------------|-------------------|------------------------|------------------|-----------------|------|-------|----------------|
| Model A          | FT                | proportion p=0.54412, 0.44507, 0.00595, 0.00486 | Model A vs Model Aa | 3.816 | 1 | 0.05 * | 19,106 |
|                  |                   | background ω=0.13622, 1.00000, 0.13622, 1.00000 |                  |                 |      |       |                |
|                  |                   | foreground ω₁=0.13622, 1.00000, 26.81509, 26.81509 |                  |                 |      |       |                |
|                  | TFL1              | proportion p=0.16400, 0.49980, 0.08306, 0.25314 | Model A vs Model Aa | 10.381 | 1 | 0.0013 ** | 11,18 |
|                  |                   | background ω=0.14566, 1.00000, 0.14566, 1.00000 |                  |                 |      |       |                |
|                  |                   | foreground ω=0.14566, 1.00000, 999.00000, 999.00000 |                  |                 |      |       |                |
|                  | CEN               | proportion p=0.23775, 0.76225, 0.00000, 0.00000 | Model A vs Model Aa | 2E-06 | 1 | P>0.05 | - |
|                  |                   | background ω=0.14634, 1.00000, 0.14634, 1.00000 |                  |                 |      |       |                |
|                  |                   | foreground ω=0.14634, 1.00000, 1.00000, 1.00000 |                  |                 |      |       |                |
|                  | BFT               | proportion p=0.20425, 0.66599, 0.03046, 0.09930 | Model A vs Model Aa | 2.878 | 1 | P>0.05 | - |
|                  |                   | background ω=0.12885, 1.00000, 0.12885, 1.00000 |                  |                 |      |       |                |
|                  |                   | foreground ω=0.12885, 1.00000, 7.38132, 7.38132 |                  |                 |      |       |                |
|                  | (FT, TFL1, CEN, BFT) | proportion p=0.23291, 0.72041, 0.01140, 0.03528 | Model A vs Model Aa | 0.521 | 1 | P>0.05 | - |
|                  |                   | background ω=0.15263, 1.00000, 0.15263, 1.00000 |                  |                 |      |       |                |
|                  |                   | foreground ω=0.15263, 1.00000, 10.76576, 10.76576 |                  |                 |      |       |                |

Significant chi-squared comparisons are indicated with * (pLRT<0.05), ** (pLRT<0.01), and *** (pLRT<0.001). Positively selected sites in the foreground lineages were detected by Bayes Empirical Bayes analysis with a probability ≥0.7.

Table 4. Summary statistics of putative cis-elements present in the 2 kb upstream promoter region of FTs across nine species.
| TF Family | Organism | Motif | Description | A. thaliana | Populus trichocarpa | M. domestica | Pyrus communis | R. occidentalis | P. armeniac |
|-----------|----------|-------|-------------|-------------|---------------------|-------------|--------------|----------------|-------------|
| CCACA box | A. thaliana | CCACA | Binding site for CO | 5 | 4 | 0 | 6 | 5 | 3 | 10 | 6 |
| CArG box | A. thaliana | CC[A/T]6GG | Binding site for MADS-domain transcription factor | 10 | 1 | 3 | 2 | 2 | 1 | 2 | 2 |
| MYB box | A. thaliana | CAACAG | Binding site for MYB transcription factor | 5 | 3 | 1 | 2 | 3 | 4 | 5 | 3 |
| hHLH motif | A. thaliana | ATGTG/AGGTG | Binding site for MYC | 7 | 3 | 3 | 5 | 16 | 8 | 6 | 7 |
| TCT motif | A. thaliana | TCTTAC | Part of a light-responsive element | 2 | 2 | 0 | 2 | 1 | 2 | 0 | 4 |
| GATA motif | A. thaliana | AAGATAAGATT | Part of a light-responsive element | 0 | 1 | 0 | 1 | 1 | 2 | 2 | 0 |
| AE-box | A. thaliana | AGAAACAA | Part of a light-responsive element | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| G-box | A. thaliana | TACGTG | Cis-acting regulatory element involved in light responsiveness | 2 | 1 | 1 | 1 | 2 | 2 | 2 | 3 |
| GT1 motif | A. thaliana | GGTTAA | Light-responsive element | 2 | 1 | 2 | 0 | 0 | 0 | 2 | 3 |
| MSA-like | A. thaliana | (T/C)C(T/C)AACGG(T/C)A | Cis-regulatory element involved in cell cycle regulation | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| GARE motif | Brassica oleracea | TCTGTTG | Gibberellin-responsive element | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| P-box | Oryza sativa | CCTTTTG | Gibberellin-responsive element | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| AP2: ERF | A. thaliana | CCGAC | Ethylene-responsive transcription factor | 7 | 3 | 5 | 8 | 6 | 8 | 6 | 2 |
| ABRE | A. thaliana | ACGTG | Cis-acting element involved in the abscisic acid responsiveness | 1 | 3 | 2 | 1 | 4 | 4 | 5 | 2 |
| LTR | Hordeum vulgare | CCGAAA | Cis-acting element involved in low-temperature responsiveness | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| W box | A. thaliana | CCGAAA | Binding site for WRKY transcription factor | 0 | 0 | 2 | 0 | 0 | 1 | 1 | 1 |

The organism column indicates in which organism the motif was characterized.

**Figures**

![Sequence alignment of 62 PEBP family proteins from nine Rosaceae species and A. thaliana. The sequences were aligned using Muscle. The conserved protein motif 14-3-3 interaction interface and anion-binding site are underlined in pink and purple, respectively (Mackenzie KK et al., 2019). A, B, C, and D represent four segments in exon 4 (Ahn et al., 2006), which are underlined in orange, blue, green and brown.](image-url)
Figure 1

Sequence alignment of 62 PEBP family proteins from nine Rosaceae species and A. thaliana. The sequences were aligned using Muscle. The conserved protein motif 14-3-3 interaction interface and anion-binding site are underlined in pink and purple, respectively (Mackenzie KK et al., 2019). A, B, C, and D represent four segments in exon 4 (Ahn et al., 2006), which are underlined in orange, blue, green and brown.

Figure 2

Phylogenetic tree of PEBPs from Rosaceae species and A. thaliana constructed by the neighbor-joining method. All PEBP proteins can be clustered into three clades and five subfamilies.
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Phylogenetic tree of PEBPs from Rosaceae species and A. thaliana constructed by the neighbor-joining method. All PEBP proteins can be clustered into three clades and five subfamilies.

Figure 3

Sequence conservation of the PEBP protein domains within 62 PEBP genes. (a) Phylogenetic analysis of sequences within the PEBP domain; (b) Five major motifs were predicted with MEME and visualized with TBtools.
Arabidopsis thaliana
Rubus occidentalis
Malus domestica
Prunus avium
Prunus persica
Prunus armeniaca
Prunus mume

Figure 4

Inter-genomic synteny blocks related to PEBP family genes in A. thaliana, R. occidentalis, M. domestica, P. avium, P. persica, P. armeniaca and P. mume. Chromosomes of Rosaceae species are labeled as Ro, Md, Pv, Pp, Pa, and Pm and are colored differently. We used purple, red, orange, green, and blue lines to connect collinear blocks containing MFTs, FTs, CENs, TFL1s, and BFTs, respectively.

Arabidopsis thaliana
Rubus occidentalis
Malus domestica
Prunus avium
Prunus persica
Prunus armeniaca
Prunus mume

Figure 5

Comparison of gene parameters including the (a) CAI, (b) total GC%, (c) ENC, (d) GC1%, (e) GC2%, and (f) GC3% estimated for BFT, CEN, FT, MFT, and TFL1 genes.
Figure 5
Comparison of gene parameters including the (a) CAI, (b) total GC%, (c) ENC, (d) GC1%, (e) GC2%, and (f) GC3% estimated for BFT, CEN, FT, MFT, and TFL1 genes.

Figure 6
Selective pressure analysis of FT and TFL1 lineage genes identified positively selected sites. Amino acids colored with yellow/purple indicate sites of positive/purifying selection, respectively.
Figure 7

Tissue-specific expression of PEBP genes in four Rosaceae species including (a) P. persica, (b) P. yedoensis, (c) P. mume and (d) Rubus occidentalis.

Figure 8

Relative expression levels of PEBP genes during oral bud development and bud blooming in P. mume.
Relative expression levels of PEBP genes during oral bud development and bud blooming in P. mume.

Figure 9

Co-expression network of FT during floral bud blooming in P. mume. (a) Cytoscape visualization of candidate genes co-expressed with PmFT during dormancy release. Candidate genes from the ‘brown’, ‘dark green’, ‘green-yellow’, ‘turquoise’, and ‘cyan’ modules are colored in brown, green, green-yellow, turquoise, and cyan, respectively. The circle size represents the significance of gene expression correlation with PmFT. (b) Expression patterns of PmFT and putative co-expressed genes during oral bud blooming. (c) Relative expression of PmFT and putative co-expressed genes verified by qRT-PCR analysis.
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Co-expression network of FT during floral bud blooming in P. mume. (a) Cytoscape visualization of candidate genes co-expressed with PmFT during dormancy release. Candidate genes from the ‘brown’, ‘dark green, ‘green-yellow, ‘turquoise’, and ‘cyan’ modules are colored in brown, green, green-yellow, turquoise, and cyan, respectively. The circle size represents the significance of gene expression correlation with PmFT. (b) Expression patterns of PmFT and putative co-expressed genes during oral bud blooming. (c) Relative expression of PmFT and putative co-expressed genes verified by qRT-PCR analysis.

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