Genome-wide identification of the 14-3-3 gene family and its participation in response to floral transition by interacting with TFL1/FT in Apple

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Abstract

Background: Apple (Malus domestica Borkh.) is one of the most popular cultivated fruit crops in China. Apple floral transition is an important process but liable to be affected by various environmental factors. The 14-3-3 proteins are involved in regulating diverse biological processes in plants, and some 14-3-3 members have been identified to play vital roles in flowering. However, little information was available about the 14-3-3 members in apple.

Results: In the current study, we identified eighteen 14-3-3 gene family members from apple genome database, designated MdGF14a to MdGF14r. The isoforms possess a conserved core region composed of nine antiparallel α-helices and divergent N and C termini. According to their structural and phylogenetic features, Md14-3-3s classified into two major evolutionary branches, the epsilon (ε) group and the non-epsilon (non-ε) group. Moreover, expression profiles derived from transcriptome data and quantitative reverse-transcription PCR (qRT-PCR) analysis exhibited diverse expression patterns of Md14-3-3 genes in various tissues and in response to different sugars and hormones treatments during floral transition phase. Four Md14-3-3 isoforms (MdGF14a, MdGF14d, MdGF14i, and MdGF14j) exhibiting prominent transcriptional responses to sugars and hormones were selected for further investigation. Furthermore, yeast two-hybrid and bimolecular fluorescence complementation (BiFC) experiments showed that the four Md14-3-3 proteins interact with key floral integrators, MdTFL1 (TERMINAL FLOWER1) and MdFT (FLOWERING LOCUS T). Subcellular localization of four selected Md14-3-3 proteins demonstrated their localization in both the cytoplasm and nucleus.

Conclusion: We comprehensively identified Md14-3-3s family in apple. Some Md14-3-3 genes are predominantly expressed during apple floral transition stage, and may participate in regulation of flowering through association with flower control genes. Our results provide a preliminary framework for further investigation into the roles of Md14-3-3s for floral transition.

Background

The 14-3-3 family consists of multiple genes and protein isoforms, which is present in all eukaryotic organisms, such as yeast [1], human [2] and Arabidopsis [3]. At the very beginning it was first identified as essential component of protein/G box complex in Arabidopsis thus named as “G box factor 14-3-3,” in a word “GF14” [4]. 14-3-3 proteins belong to a highly conserved protein family and regulate a variety of multiple cellular processes through interaction with other proteins. In plants, 14-3-3s typically function as homo- or hetero-dimers [5], each monomer is capable of binding a separate phosphorylated target protein by the recognized binding motifs, namely mode I-RXX(pS/pT)XP, mode II-RX(F/Y)X(pS)XP [6]. A modified mode I LX(R/K)SX(pS/pT)XP motif widely exists in plants [7]. More recently, a mode III-SW(pT)X-COOH has also been defined [8]. However, a few of 14-3-3 binding proteins do not match these phosphorylated consensus motifs, and in some cases the binding does not depend on phosphorylation of target proteins [9].
At the cellular level, 14-3-3 proteins generally serve as a molecular escort and regulate the function of targets through physical obstruction, scaffolding, or distorted conformational changes. The effects caused by 14-3-3 proteins can alter protein stability, enzymatic activity and subcellular localization of their binding partners, allowing them to respond quickly and accurately to altered signals [8, 10, 11]. For instance, in Arabidopsis, the activity of the plasma membrane H+ATPase was positively mediated through a direct association with 14-3-3 proteins [10]. Additionally, recent finding has reported that the phosphorylated transcription factor PHYTOCHROME-INTERACTING FACTOR 7 (PIF7) can be sequestered by 14-3-3 proteins in the cytoplasm [12].

Plant growth and development sense constantly environment signals, leading to changes of biological processes in vivo, such as signal pathways and metabolic regulations. In particular, plant 14-3-3s have been found to be important regulators of primary metabolism and membrane transport. For example, nitrate reductase and sucrose-phosphate synthase are key enzymes in nitrogen and carbon metabolism, respectively, and both are inhibited by 14-3-3 binding [13]. In plant, 14-3-3 complexes were highlighted to be involved in cell signals, stress responses and transcription regulations [11, 14-17]. Several studies have also established a role for 14-3-3s in the hormonal signaling, such as gibberellins (GA), abscisic acid (ABA), brassinosteroids (BR), cytokinins and auxin [18]. In tobacco, 14-3-3 proteins can bind to a bZIP transcriptional activator RSG (REPRESSION OF SHOOT GROWTH) to regulate gibberellin biosynthesis [8, 19]. Interaction between 14-3-3 proteins and members of the ABA responsive-element binding factor (ABF) family is involved in the regulation of GA and ABA signaling [20]. Moreover, 14-3-3s can regulate the localization and activity of the transcription factor brassinazole resistant 1 (BZR1) in the BR signaling pathway [21]. All of the above studies have shown that 14-3-3 proteins play a role in the cross-talk between these pathways.

Recent studies indicated that 14-3-3 proteins can affect the transition between vegetative growth and reproductive growth, which is a tightly regulated process controlled by both environmental and endogenous conditions. In Arabidopsis, research in photoperiodic flowering control indicated that 14-3-3 ν and μ proteins physically interact with CONSTANS (CO) [22], which is a major regulator of the photoperiodic pathway by directly activating FT expression for flowering. Mutant plants of T-DNA insertion for 14-3-3 ν and μ flower late slightly [22]. In rice, compared to wild-type plants, transgenic plants overexpressing GF14c (a rice 14-3-3 protein) exhibited delayed flowering while the knockout mutants displayed early flowering [11]. Moreover, in plants, 14-3-3 proteins have been shown to interact with floral integrators, FT and TFL1 [23, 24].

FT and TFL1, both of which belong to the same phosphatidylethanolamine binding protein (PEBP) family, share similar amino acid sequence, however, they have antagonistic role in flower induction. Overexpression of FT in apple results in an early-flowering phenotype [25]. FT protein is generated in leaves and then transported to the shoot apical meristem (SAM) to promote floral transition [26-28]. Loss of TFL1 function lead to early flowering and a terminal flower phenotype in apple [29, 30]. Overexpression of TFL1 in Arabidopsis leads to a strong late flowering phenotype [31]. TFL1 is weakly expressed in the center of the SAM during the vegetative phase and strongly upregulated at the floral transition, thereby
inducing the regulation of flowering time [31, 32]. In previous reports, TFL1 and FT interact with the bZIP transcription factors FD [33, 34], which regulate the expression transcription of floral identity gene \textit{APETALA1 (AP1)} leading to flowering [33, 35]. FT forms a ternary ‘florigen activation complex’ (FAC) with 14-3-3 protein and FD. In contrast, TFL1 also makes a ‘florigen repression complex’ (FRC) to repress FT [35-37]. Hence, the interactions of TFL1/FT with FD are mediated by 14-3-3 proteins.

Apple is a widely cultivated profitable fruit tree in the world. Apple floral transition is a serious problem and some apple varieties like Fuji are susceptible to alternate bearing, which directly causes production fluctuations. The flowering process is highly affected by nutrient conditions and hormonal signals [38-40]. 14-3-3 proteins are known to influence flowering by integrating multiple signals [22, 35, 36]. Studies uncovered many details of 14-3-3 proteins in \textit{Arabidopsis} [41], Rice [42], Soybean [43], Tomato [44], and \textit{Populus} [45]. However, the diversity of 14-3-3 proteins in apple and their potential roles in apple floral transition are still a question mark. In the present study, we identified 18 apple 14-3-3 genes and their chromosomal locations, gene structures, and evolutionary relationships were also analyzed in detail. Global expression profiles were performed to determine their responses to sugars and plant hormones treatments. Their subcellular localizations in tobacco leaves were also detected. Furthermore, we confirmed MdTFL1 (MdTFL1-1 and MdTFL1-2) and MdFT as 14-3-3s binding partners using yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. Their subcellular localizations in tobacco leaves were also detected. Identification of apple 14-3-3 family members and interaction with target proteins laid the foundation for further understanding of the 14-3-3 gene family in apple.

**Results**

**Genome-wide identification and chromosomes location of 14-3-3 genes family in the apple genome**

To identify 14-3-3 family members in apple, previously published 15 \textit{Arabidopsis} 14-3-3 protein sequences were served as queries against the Apple Genome Database using BLASTp program (E-value <1e-5). After manually removing sequences containing the incomplete 14-3-3 domain, 18 putative Md14-3-3 genes were identified, named \textit{MdGF14a-MdGF14r} based on their chromosomal positions (Table 1; Additional file 1: Figure S1). The 18 Md14-3-3 genes identified were located on 9 of the 17 chromosomes of apple, and 2 genes (\textit{MdGF14a} and \textit{MdGF14b}) were still mapped on unanchored scaffolds. The basic information of these Md14-3-3 genes is provided in Table 1. The putative Md14-3-3 proteins contained 252 (MdGF14f and MdGF14h) to 302 (MdGF14q) amino acid residues.

**Gene structure and multiple sequence alignment of 14-3-3 genes**

To determine the gene structures of Md14-3-3 family members, we investigated the divergence of Md14-3-3s exon-intron structures (Fig. 1), revealing the evolutionary relationships. The full-length amino acid
sequences of Md14-3-3s was used to construct the phylogenetic tree by using maximum likelihood method in MEGA. As shown in Fig. 1, the family members of Md14-3-3s grouped into two major evolutionary branches, the group and the non- group. The group is itself split into the isoforms MdGF14k, MdGF14o, MdGF14d, MdGF14j, MdGF14b, MdGF14r, MdGF14f and MdGF14m. The non- group is made up of the isoforms MdGF14a, MdGF14i, MdGF14g, MdGF14n, MdGF14e, MdGF14p, MdGF14q, MdGF14h, MdGF14c and MdGF14l (Fig. 1). Moreover, the group breaks into four subbranches. The non-group breaks down into three very distinct subbranches. The group and non- groups are well supported by intron-exon structure. The members have six exons and six introns (including an additional C-terminal intron). Different from the group, most non- members contain four exons and three introns, except for MdGF14c with three exons and MdGF14e, MdGF14p and MdGF14p containing an extra in the 5’ leader (Fig. 1). Besides, to detect the sequence conservation of 14-3-3 family members, we performed multiple sequence alignment of the 18 full-length Md14-3-3 protein sequences (Additional file 2: Figure S2). It was worth noting that the amino acid sequences of the N-terminal and C-terminal regions are significantly different (little amino acid conservation), while other central regions composed of nine antiparallel α-helices (α1-α9) are relatively conserved (Additional file 2: Figure S2), especially α1, α3, α5, α7, and α9 domains which possibly play a very conservative function during the evolution.

Phylogenetic conduction and synteny analysis

To gain further insights into the evolutionary relationships of 14-3-3 proteins in different species, we constructed a phylogenetic tree by maximum likelihood method using the 14-3-3 protein sequence alignments of six plant species: Arabidopsis thaliana, Malus domestica, Oryza sativa, Medicago truncatula, Glycine max and Populus trichocarpa (Fig. 2). The detailed information of all 14-3-3 genes identified in this study was provided in Additional file 3: Table S1. As shown in the phylogenetic analysis (Fig. 2), the 14-3-3 family members from the six plant species were divided into two major classes (group and non- group), the same as described previously [3]. Most 14-3-3 members from apple and Populus clustered together (Fig. 2), exhibiting a closer phylogenetic relationship.

The evolution and expansion of gene families are closely related with the occurrence of tandem duplication and segmental duplication events. Tandem duplication usually was characterized as multiple family members forming gene clusters. Segmental duplication, which occurs most frequently in plants, might cause scattered family members on different chromosomes [46]. To understand the expansion patterns of the Md14-3-3 genes in apple genome, we performed the tandem and segmental duplicated analysis. As shown in Fig. 3A, four Md14-3-3 genes (MdGF14m/MdGF14n and MdGF14g/MdGF14f) were clustered into two tandem duplication regions on apple 08 and 15 linkage groups. Besides, the MdGF14l/MdGF14c, MdGF14k/MdGF14o and MdGF14j/MdGF14d gene pairs may be generated by segmental duplications because they are located on different and non-homologous chromosomes (Fig. 3A). Additionally, a syntenic map of 14-3-3s in apple and Arabidopsis were also created. A total of four pairs of orthologous genes (MdGF14o-AtGRF10, MdGF14f/MdGF14g-AtGRF12/AtGRF13, MdGF14c-AtGRF6, MdGF14c-AtGRF8) were found (Fig. 3B). These results indicated that some Md14-3-3 genes were
possibly generated by gene duplication which plays a major driving force for Md14-3-3 evolution. In a word, synteny analysis and phylogenetic comparison of Md14-3-3 genes provided deep insight into the evolutionary characteristics of apple 14-3-3 genes.

Cis-elements in the promoter of Md14-3-3 genes

To further explore the function and regulatory patterns of Md14-3-3 genes, the 2,000bp upstream intergenic regions from start codon of the 18 Md14-3-3 genes were scanned for putative cis-regulatory elements using the PlantCARE database. A series of cis-acting elements involved in hormonal responses, light and abiotic stress responses were found in the promoter region of these Md14-3-3s (Additional file 4: Table S2). Among the cis-acting regulatory elements involved in hormone responses, abscisic acid responsive element (ABRE) was present almost all members of Md14-3-3 family except MdGF14i. In addition, the numbers of hormone-related cis-regulatory elements showed great variance among different Md14-3-3 genes. For example, four of the gibberellin response elements (P-box) were present in MdGF14r promoter, but none in the promoters of MdGF14g, MdGF14h, MdGF14k and MdGF14n genes. MeJA-related element (CGTCA-motif and TGACG-motif), auxin-responsive element (AuxRR-core and TGA-element) and salicylic acid-related element (TCA-element) were also observed in the promoters of 14, 11 and 12 Md14-3-3 genes, respectively. Also, the numbers of light-responsive cis-elements were also found to be the most abundant among all 14-3-3 genes, including G-box, Box 4, AE-box, TCCC-motif, GATA-motif, I-box, TCT-motif and AT1-motif, which may reflect the response of the 14-3-3 involving light signals to regulate plant growth. Circadian-responsive element existed in the upstream flanking regions of MdGF14d, MdGF14m, MdGF14p and MdGF14q. Meanwhile, stress response (e.g., drought and low temperature) were also identified in promoter sequences of a portion of the Md14-3-3 genes (Additional file 4: Table S2). The presence of abundant elements in the promoters suggested that 14-3-3s are involved in multiple biological processes.

Expression profiles of Md14-3-3 genes in RNA-seq datasets

Some reports claimed that 14-3-3 genes were involved in plant hormonal responses, such as cytokinins, GA, and ABA [16, 18, 21] as well as sugar metabolism [44, 47]. To further determine the potential role of Md14-3-3s genes in the context of apple flower induction, we performed a preliminary analysis of the transcript expressions of 18 Md14-3-3 genes in response to 6-benzylaminopurine (6-BA), glucose, and sucrose treatment based on the transcriptomic sequence databases. For 6-BA and glucose treatment, RNA-seq datasets were retrieved from NCBI Sequence Read Archive (SRA) datasets (SRR6510620 [48] and SRP226830, respectively). The fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) values of Md14-3-3 genes were listed in Additional file 5: Table S3, and a heat map was generated to display the expression profiles of the Md14-3-3 genes (Fig. 4). In general, most genes with closer relationships of 14-3-3s exhibited similar expression patterns (Fig. 4). For example, the expression of MdGF14d was induced or inhibited by 6-BA and sugar at one or more time points, which is
consistent with the expression pattern of \textit{MdGF14j}. This indicated that they may have similar functions. \textit{MdGF14o} showed no or very low expression levels (less than 1) during different flower bud developmental stages in all treatments, indicating that it did not function to a large extent in flower development. Besides, the expression levels of five genes (\textit{MdGF14c, MdGF14f, MdGF14m, MdGF14k} and \textit{MdGF14q}) were also significantly lower. On the contrary, the expression levels of genes, such as \textit{MdGF14a, MdGF14b, MdGF14d, MdGF14e, MdGF14g, MdGF14h, MdGF14i, MdGF14j}, and \textit{MdGF14n}, were significantly higher, indicating that they may play a major role in the flower induction phase. It is remarkable that \textit{MdGF14a, MdGF14i} were down-regulated while \textit{MdGF14d, MdGF14j} were up-regulated in response to 6-BA and glucose treatments at the early stage, which is a key stage for flower induction (Fig. 4). Overall, Md14-3-3s showed different and multiple expression patterns in transcriptome data, implying functional diversity.

\section*{Expression patterns of Md14-3-3 genes in various tissues and their responses to \textit{GA}_3\textit{ treatment by qRT-PCR}}

To investigate the possible roles of the Md14-3-3 genes, tissue-specific (leaves, stems, leaf buds, flower buds, flowers and fruits) gene expressions were determined by qRT-PCR (Fig. 5, Additional file 6: Table S4). As shown in Fig. 5, some Md14-3-3 genes exhibited similar expression patterns in different tissues, while other Md14-3-3s showed tissue-specific transcript accumulation patterns, potentially suggesting the functional divergence of Md14-3-3 genes during apple growth and development. For example, four Md14-3-3 (\textit{MdGF14b, MdGF14h, MdGF14q} and \textit{MdGF14r}) genes were ubiquitously high expressed in nearly all tested tissues, whereas \textit{MdGF14p} genes were almost undetectable in leaf buds. It is noteworthy that the transcription level of \textit{MdGF14o} alone cannot be detected in any selected tissues by qRT-PCR, due to its very low abundance of \textit{MdGF14o}. Similarly, in soybean, both \textit{SGF14q} and \textit{SGF14r}, the closest homolog of \textit{MdGF14o}, were not detected in the EST database [43]. Some Md14-3-3 genes showed a very high level in specific tissues (Fig. 5). For example, \textit{MdGF14e}, and \textit{MdGF14k} exhibited strong preferential expression in flowers, signifying their putative role in the regulation of flower development. Besides, genes with closer relationships (\textit{MdGF14a} and \textit{MdGF14i}) showed similar expressions, and both were expressed at a higher level in the tested tissues (Fig. 5), demonstrating that they play similar roles in tissue development. Furthermore, two pair Md14-3-3s in the segmental duplications also showed similar expression patterns (Fig. 5). For example, \textit{MdGF14d} and \textit{MdGF14j} with similar gene structure were mainly expressed in stems and flowers. \textit{MdGF14c} and \textit{MdGF14l} showed relatively high expression level in the stem. However, some genes in tandem duplicated regions displayed different expression patterns (Fig. 5). \textit{MdGF14m} were expressed to very higher level in stem compared to other tissues, while \textit{MdGF14f} were highly expressed in flowers. \textit{MdGF14g} and \textit{MdGF14n} displayed higher expression level in flowers and fruits, respectively. These results indicated that some Md14-3-3 genes maybe play multiple important roles in apple growth and development.
Gibberellin promotes vegetative growth, but inhibit floral transition, resulting a significant reduction of fruit load the following year in apple [40]. To gain insight into the potential roles of the Md14-3-3s in response to GA signal, qRT-PCR was used to analyze the expression of the Md14-3-3 genes under GA treatment (Fig. 6). It is noteworthy that *MdGF14o* was not expressed at various stages of flower bud development in GA treatment. In the early stages of GA induction, the expressions of 11 Md14-3-3 genes, including *MdGF14b, MdGF14c, MdGF14d, MdGF14e, MdGF14f, MdGF14g, MdGF14h, MdGF14j, MdGF14k, MdGF14m and MdGF14p* were extremely reduced and remained at a lower level. Rather, significant up-regulation of *MdGF14a* and *MdGF14i* was observed at 30 DAFB (Fig. 6). The transcription level of *MdGF14n* did not differ significantly at first, but subsequently increased by 4-fold at the second sampling point after the GA treatment (Fig. 6). Interestingly, *MdGF14l, MdGF14q* and *MdGF14r* showed similar expression patterns during flower induction, which were up- or down-regulated at different time points after treatment (Fig. 6), indicating that they might have similar roles in hormonal stress responses or apple development.

**Md14-3-3s can interact with MdTFL1, and MdFT**

To address how Md14-3-3s participate in floral transition, we focused on the floral pathway integrators, TFL1 and FT. In fact, we previously used full-length MdTFL1 protein as a bait protein to conduct yeast two-hybrid screening in apple flower bud cDNA library, and screened the MdGF14a and MdGF14j protein. In addition, MdGF14i and MdGF14d are closely related to MdGF14a and MdGF14j, respectively (Fig. 1), and they exhibited prominent transcriptional responses to sugars and hormones. Therefore, we chose these four genes for further analysis. Previous studies reported that 14-3-3 protein can interact with TFL1 and FT [11, 35, 36]. In apple, there are two MdTFL1 encoding genes, MdTFL1-1 and MdTFL1-2 [50]. An experiment further confirmed both MdTFL1 (MdTFL1-1 and MdTFL1-2) proteins can interact with four 14-3-3 isoforms (MdGF14a, MdGF14d, MdGF14i and MdGF14j) using the yeast system (Fig. 7A). Moreover, the 14-3-3 isoforms preference of MdTFL1 can also be comparable with that of MdFT, the four 14-3-3 isoforms also interact with MdFT in yeast two-hybrid assays (Fig. 7A).

Also, we used a BiFC assay to determine the interactions between Md14-3-3 proteins and MdTFL1 or MdFT in vivo (Fig. 7, Additional file 7: Figure S3). Different fusion protein combinations containing the expression vectors for MdTFL1-1, MdTFL1-2 and MdFT proteins fused to the pSPYNE and the four Md14-3-3 proteins fused to the pSPYCE were transiently introduced into the *Nicotiana benthamiana* leaves, yellow fluorescent protein (YFP) fluorescence signals from MdTFL1-1-Md14-3-3s (Fig. 7B), MdTFL1-2-Md14-3-3s (Fig. 7C) and MdFT-Md14-3-3s interactions (Fig. 7D) were detected both in the cytoplasm and nuclear, but mainly in the cytoplasm. Thus, these results clearly showed that Md14-3-3s can interact with MdTFL1 and MdFT in yeast and in plant cells.

**Subcellular localization of 14-3-3 proteins**
To determine the subcellular localization of Md14-3-3 proteins, we made a fusion construct green fluorescent protein (GFP)-linked Md14-3-3s driven by the cauliflower mosaic virus (CaMV) 35S promoter and analyzed the intracellular localization of the four Md14-3-3s. These constructs were introduced into Nicotiana benthamiana leaves and fluorescent signals were observed in the cytoplasm and nucleus (Fig. 8), consistent with previous studies [11].

Discussion

Plants required a series of regulators to sense and respond to complex environmental changes, a situation that seems relevant to the existence of a large number of diverse 14-3-3 family. All eukaryotes possess multiple 14-3-3 gene paralogs, increasing functional complexity of this regulatory protein family. Arabidopsis and rice genome consist of 15 and 8 14-3-3 genes, respectively [3, 41, 51]. In this study, we identified a total of 18 Md14-3-3 genes in apple. The isoforms range in length from 252 to 302 amino acids (Table 1). In plants, the 14-3-3 proteins form homo- and hetero-dimeric proteins. Each monomer in dimer contains a conserved core region composed of nine antiparallel α-helices (α1-α9) forming an amphiphilic groove. It is capable of binding a separate phosphorylated target protein [6, 41], which enables 14-3-3s participation in diverse functions. The primary diversity among Md14-3-3 isoforms occurs at the N and C termini (Additional file 2: Figure S2), which are related to dimerization and client binding, respectively [52, 53]. These subtle changes in the internal loops and the highly divergent termini are thought to contribute to the 14-3-3 isoform specificity by regulating differential affinity between individual 14-3-3 isoforms towards possible targets [41, 47, 54]. Recent studies in Arabidopsis suggested 14-3-3 target specificity and further confirmed that the extreme C termini of 14-3-3s play an important role in ligand interaction [55-56], although the precise mechanism is not fully understood.

The phylogenetic analysis showed that Md14-3-3s family members were classified into two major evolutionary branches, the non group and the non- group (Fig. 1; Fig. 2), consistent with the identified 14-3-3 family members in other species [41-43, 45]. Exon-intron structural divergence analysis help to better insight into the evolutionary relationships of the Md14-3-3 genes. As show in Fig. 1, the members all possess an intron-exon structure distinct from the non- group. The highly similar gene structure was identified in the same group of the Md14-3-3 genes. For example, all ten Md14-3-3s in group contained six exons, and most members within non- group had four exons except for MdGF14c (Fig. 1).

Additionally, most Md14-3-3 gene members in each group also exhibited nearly identical exon lengths. The intron arrangements and intron lengths are distinct between subfamilies. Similar results can also be found in other species [43, 45]. These results suggested that exon-intron structure reveal the evolutionary relationship of Md14-3-3 gene family. The presence of highly similar 14-3-3s in these plant species could have resulted from their whole genome duplication event. Tandem and segmental duplication events have an important contribution to the expansion of gene family members during plant evolution [46]. Our results further elucidate the expansion mechanism of the 14-3-3 gene family in apple. Two pairs of tandem duplication Md14-3-3 genes and three pairs of segmental duplication Md14-3-3 genes were detected (Fig. 3). These results suggested that both segmental and tandem duplications play a crucial
role in the expansion of the Md14-3-3 gene family during their evolution. Taken together, the evolution and phylogeny of the 14-3-3 family exhibit diversity and complexity, reflecting functional divergence.

The 14-3-3 proteins are known as a component associated with several different proteins in signal transduction pathways [58]. Schoonheim et al. [18] identified a large number of target proteins of 14-3-3 isoforms using yeast two-hybrid screens. A number of proteins have a well-known function in plant hormone signaling pathways, such as the auxin transport protein PIN1 [59] and NPH3 [60], the major BR signaling related proteins [61], and the ABF transcription factor family members [62]. Interestingly, we found numerous hormone-related elements in the promoter of Md14-3-3 family genes (Additional file 4: Table S2), which may explain that plant 14-3-3 proteins play an important role in the regulation of many hormones signaling pathways. Again, this conclusion has also been confirmed through transcriptome data and qRT-PCR analysis in our study (Fig. 4, Fig. 6). Additionally, 14-3-3 proteins also have mechanistic roles in the perception of light signaling through interacting with light-related proteins, such as PIF and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) [63]. These results are also supported by the presence of multiple light-responsive and circadian-responsive elements in the promoter regions of the 14-3-3 isoforms (Additional file 4: Table S2).

A recent study reported the mechanisms of plant 14-3-3 proteins in regulating the development of multiple organs and the promoter-driven GUS reporter assays initially demonstrated expression of 14-3-3s in seed, flower, leaf, and root [58]. Our data demonstrated that most Md14-3-3s are expressed in all detected tissues and are particularly higher in stem and flower (Fig. 5), suggesting that the expression of 14-3-3s are essential and important to maintain or respond to plant growth requirements. It is worth noting that several Md14-3-3 isoforms with closer evolutionary relationships (e.g. *MdGF14a* and *MdGF14i*) showed similar expression patterns in different tissues, while other Md14-3-3s showed tissue-specific transcript accumulation patterns. For example, *MdGF14e* and *MdGF14k* displayed higher expression level in flowers, which suggested that they might be involved in regulation of flower development. *MdGF14m* were ubiquitously high expressed in stems, indicating involvement in stem growth. Furthermore, *MdGF14d* and *MdGF14j* in the segmental duplications, were mainly expressed in stems and flowers, signifying their putative role in the regulation of stem and flower development. Taken together, these results potentially suggested functional diversity (overlapping or specific function) of Md14-3-3s genes during apple growth and development, which is also supported by 14-3-3 sequence conservation and diversity that exist across isoforms of many species [42, 43, 45].

Flower induction in apple is regulated by complex gene networks that involve multiple signal pathways to ensure flower bud formation next year. Phytohormones and sugars participated in the growth and floral transition in apple [64]. Our previous study showed that 6-BA treatment contributes to increase the proportion of short branches and promote floral transition [48]. Sugar as an energy substance is involved in the flowering regulation [49]. Several studies showed that 14-3-3 genes are directly involved in floral development. In *Arabidopsis*, 14-3-3v and μ knockout lines display late flowering [22]. In tomato, overexpression of 14-3-3 genes compensates for the loss of function of SELF-PRUNING (SP), which is a homologous gene of TFL1, by increasing the indeterminacy of the shoot apical meristem, and by
increasing the vegetative properties of the inflorescence [23]. In rice, functional analysis of GF14c (a 14-3-3 protein) indicated that GF14c-overexpressing plants exhibited a delay in flowering and the knockout mutants displayed early flowering relative to the wild-type plants under short-day conditions [11]. Therefore, it is of interest to understand the role of Md14-3-3s in the transition from vegetative to reproductive growth in apple. In this study, RNA-based sequencing data in response to 6-BA and sugar, combined with qRT-PCR analysis using GA treatment, enabled us to screen Md14-3-3 genes that respond to hormones or sugar. Our present study showed that the expression trends of 14-3-3s are diverse and change depending on developmental stages under sugars and hormones treatments (Fig. 5; Fig. 6). It is well known that 6-BA and GA have antagonistic action on apple flowering, and act as a positive promoter and a negative regulatory factor, respectively [40, 48]. This antagonistic effect is also consistent with the expression patterns of most Md14-3-3 genes. For example, at 30 DAFB (a key point for floral induction), the expressions of \textit{MdGF14a} and \textit{MdGF14i} were significantly increased by GA$_3$ treatment (Fig. 6). However, under 6-BA treatment, \textit{MdGF14a} and \textit{MdGF14i} showed opposite expressions patterns (Fig. 5). Moreover, most Md14-3-3 genes (e.g. \textit{MdGF14b}, \textit{MdGF14c}, \textit{MdGF14d}, \textit{MdGF14e} and \textit{MdGF14j}) were upregulated at early stage after 6-BA treatment, and downregulated after GA$_3$ treatment (Fig. 5, Fig. 6). Collectively, we preliminary judgment these Md14-3-3 genes may have significant roles in the regulation of floral transition, and their functions merit further investigation.

In apple, MdFT is an ortholog of MdTFL1 but has a converse function in flower development [65]. Overexpression of MdFT-encoding gene in apple results in precocious flowering. Recent research showed that \textit{MdFT1} transcripts are expressed to appreciable levels in the apical bud, but are not significantly affected by 6-BA treatment [66] and GA treatment [67] during the floral transition. Interestingly, the expression of \textit{MdTFL1} was decreased by 6-BA treatment [66], while was significantly increased by GA treatment (or fruit load) during flower induction [67, 68]. In other words, GA inhibition of apple flowering appears to be mediated by inducing a significant increase in \textit{MdTFL1} levels. In plants, 14-3-3 proteins have been shown to interact with TFL1 and FT [24, 36]. In our study, identification of Md14-3-3s (MdGF14a, MdGF14d, MdGF14i, and MdGF14j) as MdTFL1 and MdFT partners have been confirmed by yeast two-hybrid assays and BiFC assays (Fig. 7). These indicated that 14-3-3 proteins are involved in the regulation of flowering through direct association with floral genes. The subcellular distribution of the four Md14-3-3 isoforms showed their localization in the cytoplasm and nucleus (Fig. 8). Several evidences indicated that the subcellular distribution of 14-3-3 proteins seems to be highly dependent on interaction with targets [8, 11, 12, 19]. Previous reports also found that the binding of 14-3-3 proteins regulates their partner proteins through a variety of mechanisms, such as altering their catalytic activity, subcellular localization, stability or their interaction with targets [69]. To understand whether the protein interactions would affect the subcellular localization of MdTFL1 and MdFT, we performed a BiFC experiment. Notably, the MdTFL1-Md14-3-3s or MdFT-Md14-3-3s BiFC signal were mainly detected in the cytoplasm and weakly in the nucleus (Fig. 7B, C, D), consistent with previous reports [11, 36]. These results potentially suggeste that MdTFL1/MdFT and Md14-3-3s interactions increase MdTFL1 or MdFT cytoplasmic retention and inhibite shutting of them from the cytoplasm into the nucleus.

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Much is known about the antagonistic roles of TFL1 and FT in the regulation of flowering time. Therefore, interactions of MdTFL1/MdFT with Md14-3-3s led to a hypothesis that MdTFL1 antagonizes MdFT through competition with Md14-3-3 binding. Current and previous studies have provided several evidences to strongly support this hypothesis. For example, in *Arabidopsis*, both TFL1 and FT interact with FD and 14-3-3 and act as transcription repressors (FRC) or transcription activators (FAC), respectively, functionally antagonistically to regulate the downstream floral meristem identity genes, including *LFY* and *AP1* [70]. In tomato, there also are associations between SP (TFL1 homolog) and SFT (FT homolog) and 14-3-3 isoforms [71]. In rice, RCN (a rice TFL1 homolog) as well as Hd3a (a rice FT homolog) can interact with OsFD1 through 14-3-3 binding and increasing the amount of RCN can antagonize Hd3a in a 14-3-3-dependent manner. [36]. These results suggested that TFL1 acts against FT for 14-3-3 binding. The balance between FAC and FRC for regulation of reproductive development is thought to be a general mechanism in plants [35]. A high FAC/FRC ratio in the meristem promotes determinate growth and induces transition to the reproductive phase, eventually converting the SAM into a terminal flower, while a low FAC/FRC balance maintains indeterminate plant growth. The resulting balance serves an important role in accurate modulating of plant’s response to flower-induced signals (Fig. 9). However, how MdTFL1 antagonizes MdFT for 14-3-3 binding needs further research in apple.

**Conclusion**

This study presents a comprehensive classification of the Md14-3-3 gene family in apple and provides evidence for their possible roles in apple flowering. In total, there are 18 Md14-3-3 genes in apple genome, and they can be grouped into ε and non-ε groups. The diverse expression patterns of the Md14-3-3s in different tissues and in response to sugars and hormones treatments suggested that 14-3-3 family members serve as positive or negative regulators mediating floral transition. Further clues indicated that MdTFL1 (MdTFL1-1 and MdTFL1-2) and MdFT, which are important floral integrators, act as 14-3-3s binding partners. Nevertheless, the studies on the functional mechanism of apple 14-3-3s during floral transition should be strengthened.

**Methods**

**Identification and chromosomal location of 14-3-3 family in apple**

To identify potential 14-3-3s gene family members in the apple genome, we retrieved previously published 15 *Arabidopsis* 14-3-3 protein sequences from *Arabidopsis* genome database (http://www.arabidopsis.org/), they were used as queries in BLASTp (E-value <1e-5) searches against the apple genome (GDDH13V1.1; https://www.rosaceae.org/). Furthermore, candidate 14-3-3s family members were confirmed their highly conserved domain using Pfam (http://pfam.xfam.org/) and the Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Only those sequences having a full-length 14-3-3 domain were selected as Md14-3-3 proteins and used for the
subsequent analyses. The candidate 14-3-3 genes annotations and their chromosomal locations were obtained from the apple genome.

**Sequence alignment, gene structure, *cis*-element analysis and phylogenetic tree construction**

Multiple alignments of Md14-3-3 proteins sequences were performed in DNAMAN software (V 6.0). The Md14-3-3 exon-intron structures were generated using the online tool of Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) [72]. The 2,000bp upstream intergenic regions of Md14-3-3 genes were derived from the start codon based on the apple genome. Then they were searched against the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify the putative *cis*-elements. Muscle program was used to create multiple sequence alignment of 14-3-3 proteins. Based on the alignment results, a phylogenetic tree was constructed using MEGA7.0 program maximum likelihood method with a bootstrap analysis (1000 replicates) based on poission model and partial deletion.

**Tandem duplication and synteny analysis**

Tandem duplication and synteny relationships were carried out using Circos version 0.63 (http://circos.ca/) [73]. Tandem duplications of Md14-3-3 genes were identified according to their physical locations on individual chromosomes in the apple genome. Details regarding the syntenic blocks within the apple genome and between the apple and *Arabidopsis* genomes were downloaded from the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/).

**Expression profiles of Md14-3-3 Genes in RNA-seq Datasets**

The expression profiles of putative Md14-3-3 genes was determined using the RNA-seq datasets, including transcriptional response to 6-BA treatment, glucose and sucrose treatment during key period of floral transition. RNA-seq data for expression profiles in response to 6-BA were retrieved from NCBI Short Read Archive (SRA) under the accession number SRR6510620 (https://www.ncbi.nlm.nih.gov/sra/?term=SRR6510620) [48]. RNA-seq data in response to glucose treatment were retrieved from NCBI Sequence Read Archive (SRP226830) (https://www.ncbi.nlm.nih.gov/sra/?term=SRP226830). The six-year-old ‘Nagafu No.2’ trees were used for glucose treatment. 15,000 and 30,000 mg L⁻¹ glucose was spray at 25 and 30 days after full bloom (DAFB), respectively. Control plants were also treated with an equivalent amount of water. In addition, RNA-seq datasets under sucrose treatment (data not shared online) are available to analyze the expression profiles of Md14-3-3 genes. The specific operations were as follows, sucrose treatment liquid (15,000 mg L⁻¹ and 20,000 mg L⁻¹ sucrose for 29 and 36 DAFB, respectively) were sprayed on apple leaves with a handheld sprayer until run-off. At the same time, the water was sprayed as a blank control. Three biological replicates were performed for each treatment. The
terminal buds of the short shoots (< 5cm) were collected for further RNA-seq. Library construction and sequencing were performed using Illumina HiSeq platform. The analysis of RNA-seq data was based on the previous method [48]. The FPKM values were used to estimate the gene expression level. FPKM values of all types of treatment involved three sampling time points: 30, 50 and 70 DAFB. The heatmap of Md14-3-3 genes was revealed using HemiL 1.0 software.

**Plant growth condition and GA$_3$ treatment**

Plant samples were collected from six-year-old 'Nagafu No.2'/‘M26’/M. robusta Rehd trees which had not exhibited alternate bearing. They are planted at the Apple Demonstration Nursery of Yangling Modern Agriculture Technology Park, Shaanxi Province of China (108°70′ E, 34°52′ N).

The six tissue samples including leaves, stems, leaf buds, flower buds, flowers and fruits were collected for organ-specific expression analysis. Mature leaves were collected from the adjacent terminal buds of spur. Stems were collected from fresh shoot pulled out in the spring. Additionally, leaf buds were collected from bourse shoots apices with adjacent developing fruits, which are difficult to form flowers next year. Flower buds were collected from plump terminal buds of the short shoot. Moreover, fruits with diameters of 3-4 cm were also collected after 40 DAFB. All samples were immediately frozen in liquid nitrogen and stored at -80 °C for qRT-PCR analysis.

For GA$_3$ treatment, 500 mg L$^{-1}$ GA$_3$ was sprayed in a clear morning at 25 and 30 DAFB. At the same time, water was sprayed as a control. GA$_3$ and control solutions were applied with a handheld sprayer until run-off. The spur terminal buds were collected at 30, 50 and 70 DAFB and stored for further qRT-PCR analysis.

**RNA extraction, cDNA synthesis, and Gene expression analysis RT-PCR**

Next we extract total RNA using a polysaccharide polyphenol plant total RNA Extraction Kit (Foregene, China) by following the manufacturer's instructions. First-strand cDNA was synthesized using a PrimeScript RT Reagent kit (Takara Bio, Shiga, Japan). Expression levels of Md14-3-3 genes in different tissues and different developmental stages of flowering induction were analyzed by qRT-PCR using an iCycler iQ Real-Time PCR Detection System (Bio-Rad, USA). The qRT-PCR reaction mix (20µL) consisted of 2µL cDNA samples (diluted 1:8), 10µL TB Green Premix Ex Taq II (Takara Bio), 1µL of each primer (10 µM), and 6bµL distilled deionized H$_2$O. The housekeeping histone H3-encoding gene (LOC103406086, XM_008345103) was used as a reference gene to calculate the relative expression of selected genes [74]. For each time point, three independent biological repeats with three technical repeats were performed. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [75]. Specific primer pairs were
designed using Primer-BLAST online tool in NCBI database (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the primers are listed in Additional file 6: Table S4.

**Yeast two-hybrid assay**

To confirm the interaction between MdTFL1/MdFT and Md14-3-3 proteins, the full-length coding sequences of MdTFL1-1, MdTFL1-2 and MdFT were cloned into the bait vector pGBK7, and Md14-3-3s (MdGF14a, MdGF14d, MdGF14i and MdGF14j) were cloned into the prey vector pGADT7. The recombinant bait vector was then transformed into the yeast strain Y2H gold for a self-activation and self-toxicity check. Next, the bait-prey interactions were tested on SD medium without Leu, Trp, His and Ade according to the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech). The primers used in this assay are listed in Additional file 6: Table S4.

**Bimolecular fluorescence complementation**

Full-length coding sequences of MdTFL1/MdFT and candidate Md14-3-3 interacting proteins were respectively cloned into the pSPYNE and pSPYCE vectors for protein-protein interaction assays [76]. The resulting plasmids were transformed into Agrobacterium tumefaciens (strain GV3101), incubated, harvested and resuspended in infiltration buffer (10mM MES, 10mM MgCl₂ and 0.2mM acetosyringone, pH 5.6). Then, the two candidate Agrobacterium cultures were mixed in equal volumes and co-transformed into the *Nicotiana benthamiana* leaves. Infected tissues were analyzed 48h after infiltration. Yellow fluorescent protein (YFP) signals were detected using confocal laser microscopy (LSM 510, Zeiss, Oberkochen, Germany). The primers used for BiFC are listed in Additional file 6: Table S4.

**Subcellular location**

The full-length Md14-3-3s without the stop codon were cloned into pCAMBIA2300 vector fused with a green fluorescent protein (GFP). Agrobacterium strains (GV3101) transformed with the target vectors were suspended in infiltration buffer (10mM MES, 10mM MgCl₂ and 0.2mM acetosyringone, pH 5.6) to final concentrations (A₆₀₀=0.6). Next, the injection of *Nicotiana benthamiana* leaves was performed. The infected plants were incubated at 25 °C for 48h before observation using confocal laser microscopy (LSM 510, Zeiss, Oberkochen, Germany). Relevant primer information is listed in Additional file 6: Table S4.

**Additional Files**

Additional file 1: Figure S1. Chromosome map of Md14-3-3 genes in apple.
Additional file 2: Figure S2. Sequence alignment of Md14-3-3 proteins in apple. Identical residues are shown in blue and similar residues are in red. Nine antiparallel α-helices were marked as α1-α9.

Additional file 3: Table S1. The gene name and gene ID of 14-3-3s in apple and other plant species.

Additional file 4: Table S2. Analysis of the cis-elements in the Md14-3-3 promoter. The 2,000bp upstream from the start codon of Md14-3-3 genes were analyzed using the PlantCARE database.

Additional file 5: Table S3. FPKM values of Md14-3-3s from RNA-seq statistics during floral transition in 6-BA, glucose and sucrose treatment.

Additional file 6: Table S4. Primers used in the present study.

Additional file 7: Figure S3. Negative control of BiFC assay. MdGF14a, MdGF14d, MdGF14i and MdGF14j were independently cloned into the pSPYCE vector. The empty pSPYNE vector served as the control. Bar =25um.

Abbreviations
GA: gibberellin; ABA: abscisic acid; BR: brassinosteroids; 6-BA: 6-benzylaminopurine; DAFB: Days after full bloom; FPKM: The fragments per kilobase of transcript sequence per million base pairs sequenced; TFL1: TERMINAL FLOWER1; FT: FLOWERING LOCUS T; CO: CONSTANS; PEBP: phosphatidylethanolamine binding protein; SAM: shoot apical meristem; PIF: PHYTOCHROME-INTERACTING FACTOR; AP1; APETALA1; COP1: CONSTITUTIVE PHOTOMORPHOGENIC1; SP: SELF-PRUNING; qRT-PCR: quantitative reverse-transcription PCR; GFP: green fluorescent protein; BiFC: bimolecular fluorescence complementation.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
All relevant data analyzed during this study are included in this article and in Additional files. RNA-seq data in response to 6-BA and glucose treatment were acquired from NCBI Short Read Archive (SRA) (the accession number: SRR6510620 and SRP226830, respectively) (https://www.ncbi.nlm.nih.gov/sra/?
term=SRR6510620 and https://www.ncbi.nlm.nih.gov/sra/?term=SRP226830). The apple RNA-seq data for sucrose treatment used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

ZD, ZCP, HMY, and ZXY designed the study and revised the manuscript. ZXY, WSX, XW performed the experiments. YHR, ZSG, TM and AN analyzed the data. ZXY wrote the manuscript. All authors have read and approved the final manuscript.

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Table

Table 1 Apple 14-3-3 genes information.

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Figures

Figure 1

Analysis of Md14-3-3 gene structures. (A) The unrooted phylogenetic tree of Md14-3-3 protein sequences was constructed by the maximum likelihood method with 1,000 bootstrap replicates. The two major
groups are marked with pink or light green. (B) Exon-intron composition of Md14-3-3 genes. The blue, yellow boxes and black lines represent UTR, exon and intron positions, respectively.

Figure 2

Phylogenetic tree showing evolutionary relationships of Md14-3-3 proteins in apple and other plant species. The maximum likelihood method with 1,000 bootstrap replicates was used to build the phylogenetic tree with the MEGA7.0 program. Protein designations consist of the prefixes A. thaliana, (At, yellow circles), M. domestica, (Md, red triangles), O. sativa, (Os, green rhombus), M. truncatula, (Mt, blue circles), G. max (Gm, pink rhombus) and P. trichocarpa (Pt, blue triangles). Detail information of 14-3-3s from those plant species were listed in Additional file 3: Table S1.
Figure 3

Syntenic relationships of apple and Arabidopsis 14-3-3 genes. (A) Chromosomal distribution and duplication relationships of Md14-3-3 genes. (B) Syntenic relationship between apple and Arabidopsis. The colored curves represent apple and Arabidopsis syntenic gene regions.

Figure 4

[Table and heat map data]
Expression profiles of Md14-3-3s during floral transition phase in 6-BA treatment, glucose and sucrose treatment. Water was used as a blank control. FPKM values were used to generate their expression profiles. Samples were collected at three time points (30, 50, 70 days after full bloom (DAFB)) during floral transition. The diagram was drawn using Heml 1.0 software. For other details, see Additional file 5: Table S3.

Figure 5

Quantitative real-time PCR analysis of Md14-3-3 gene expressions in different tissues. Each value represents the standard error of three replicates.
Figure 6

Quantitative real-time PCR analysis of Md14-3-3 gene expressions in apple buds treated with gibberellin (GA3). Samples were collected at 30, 50, and 70 days after full bloom (DAFB). Each value represents the standard error of three replicates. The asterisks (*) indicate a significant difference between control and treatment (p<0.05).
Figure 7

Yeast two-hybrid and BiFC assays of interactions between candidate Md14-3-3 proteins and MdTFL1/MdFT. (A), Yeast two-hybrid assays. MdTFL1-1, MdTFL1-2, MdFT, and Md14-3-3 proteins were fused to the GAL4 activation domain (AD) or DNA-binding domain (BD) to generate the bait constructs or prey constructs. The empty pGADT7 vector was used as control. -LT, yeast medium lacking leucine and tryptophan. -LTAH, yeast medium lacking leucine, tryptophan, adenine and histidine. (B, C, D) BiFC assays. Interactions between Md14-3-3s and MdTFL1-1 (B), MdTFL1-2 (C) and MdFT (D), respectively. MdTFL1-1, MdTFL1-2, and MdFT were cloned into pSPYNE respectively, and MdGF14a, MdGF14d, MdGF14i and MdGF14j were independently cloned into the pSPYCE vector. The empty pSPYCE vector served as the control. The negative control of empty pSPYNE vector was shown in Additional file 7: Figure S3. YFP, yellow fluorescence protein; CHL, Chlorophyll. Bar =25um.
Figure 8

Subcellular localization of the four Md14-3-3s proteins (MdGF14a, MdGF14d, MdGF14i and MdGF14j) in Nicotiana Benthamiana leaves. All candidate genes were independently cloned into pCAMBIA2300 vector fused with green fluorescent protein (GFP). Free GFP was used as the control. Bar =25μm.
Figure 9

A model for interaction of 14-3-3 with TFL1 and FT. The interaction complexes TFL1-14-3-3s or FT-14-3-3s mainly occurs in the cytoplasm and forms a larger ternary complex with FD in the nucleus, thereby regulating the expression of downstream floral meristem identity gene AP1 for flower transition. TFL1 interacts with the 14-3-3-FD to form florigen repression complex (FRC) (A), while FT interacts with the 14-3-3-FD to form florigen activation complex (FAC) (B). The balance between FRC and FAC controls the vegetative and reproductive growth in apical meristem, modulating plant architecture and optimizing crop productivity.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile3TableS1.xlsx
- Additionalfile5TableS3.xls
- Additionalfile4TableS2.xlsx
- Additionalfile2FigureS2.tif
- Additionalfile1FigureS1.tif
- Additionalfile7FigureS3.tif
- Additionalfile6TableS4.xlsx