

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, 17 of them are transcribed. 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. Subcellular localization of four selected Md14-3-3 proteins demonstrated their localization in both the cytoplasm and nucleus. 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).

Conclusion: We comprehensively identified Md14-3-3s family in apple. Some Md14-3-3 genes are predominantly expressed during apple flowering 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 flower 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 client's function 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 transcriptional 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 to regulate gibberellin biosynthesis [8, 19]. Interaction between 14-3-3 proteins and the ABF transcription factor family members involves in the regulation of GA and ABA signaling [20]. Moreover, 14-3-3s can regulate the localization and activity of 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.

Most recently, 14-3-3 proteins were thought to 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]. Conversely, loss of TFL1 function lead to early flowering and a terminal flower phenotype in apple [29, 30], while 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 *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 flower 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 *Arabidopsis* [41], Rice [42], Soybean [43], Tomato [44], and *Populus* [45], however the diversity of 14-3-3 proteins in apple and their potential roles involving in apple floral transition are still a question mark. In the present study, we identified 18 apple 14-3-3 genes and their chromosomes locations, gene structures, evolutionary relationship 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. 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 genes in apple, *Arabidopsis* 14-3-3 protein sequences were served as queries against the Apple Genome Database using BLASTp. After manually removing sequences containing the incomplete 14-3-3 domain, 18 putative Md14-3-3 genes were identified, named *MdGF14a-MdGF14r* based on their chromosomal positions (Table 1; Additional file 2: Figure S1). The 18 Md14-3-3 genes identified were located on 9 of the 17 chromosomes of apple, and 2 genes (MdGF14a and 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 family members of Md14-3-3s grouped into two major evolutionary branches, the Ψ group and the non- Ψ group. The Ψ group is

itself split into the isoforms *MdGF14k*, *MdGF14o*, *MdGF14d*, *MdGF14j*, *MdGF14b*, *MdGF14r*, *MdGF14f* and *MdGF14m*. The non- β group is made up of the isoforms *MdGF14a*, *MdGF14i*, *MdGF14g*, *MdGF14n*, *MdGF14e*, *MdGF14p*, *MdGF14q*, *MdGF14h*, *MdGF14c* and *MdGF14l* (Fig. 1). Moreover, the β group breaks into two subbranches. The non- β group breaks down into three very distinct subbranches. The β and non- β groups are well supported by intron-exon structure. The β members have six exons and six introns (including an additional C-terminal intron). Different from the β group, the non- β members contain four exons and three introns, except for *MdGF14e*, *MdGF14p* and *MdGF14q* 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 3: 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 (α 1- α 9) are relatively conserved (Additional file 3: Figure S2), especially α 1, α 3, α 5, α 7, and α 9 domains which possibly play a very conservative function during the evolution.

Phylogenetic construction and synteny analysis

To gain further insights into the evolutionary relationships of 14-3-3 proteins in different species, we constructed a phylogenetic tree using the 14-3-3 protein sequence alignments of five 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 4: Table S2. Phylogenetic analyses of 14-3-3s provide a robust tree and the 14-3-3 family members from the five plant species were divided into two major classes (β class and non- β class), the same as described previously [3].

To understand the expansion patterns of the Md14-3-3 genes in apple genome, we performed the tandem duplicated analysis. As shown in Fig. 3A, four Md14-3-3 genes (*MdGF14m*/*MdGF14n* and *MdGF14g*/*MdGF14f*) were clustered into two tandem duplication event 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, a 2,000bp promoter region of the 18 Md14-3-3 genes was scanned for putative cis-regulatory elements using the PlantCARE database.

Notably, various cis-acting elements involved in hormonal responses such as ABA, GA, MeJA, auxin and salicylic acid were found in the promoter region of those Md14-3-3s (Additional file 5: Figure S3). Also, the numbers of light-responsive cis-elements were also found to be the most abundant among all 14-3-3 genes (Additional file 5: Figure S3). This 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 5: Figure S3). The presence of abundant elements in the promoters suggested that 14-3-3s are involved in multiple biological processes.

Expression pattern of Md14-3-3 genes in different tissues

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. 4). Noticeably, our results showed that the transcription level of MdGF14o alone cannot be detected in all selected tissues by qRT-PCR, due to its very low abundance of MdGF14o. Therefore, we conclude that despite the presence of 18 14-3-3 genes in apple genome, 17 isoforms are transcribed. Similar results were also found in other species [43, 45]. Moreover, *MdGF14d*, *MdGF14e*, *MdGF14f*, *MdGF14g*, *MdGF14j* and *MdGF14k* expression patterns were consistent and exhibited strong preferential expression in flowers, while *MdGF14m* and *MdGF14p* were expressed to higher level in stem compared to other tissues (Fig. 4). However, *MdGF14m* has no transcript in leaf buds and flower buds. Besides, genes with closer relationships (*MdGF14a* and *MdGF14i*, *MdGF14h* and *MdGF14l*) showed similar expressions in all tissues (Fig. 4), suggesting that they act largely redundantly in the control of apple growth and development.

Expression of Md14-3-3 genes after hormone and sugar treatments

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, 46]. Moreover, 14-3-3 protein family also plays an important role in the regulation of flowering time. These suggested that 14-3-3 protein may act as a crosstalk point in signal transduction networks to regulate floral transition. Our previous study showed that 6-BA treatment contributes to the increase the proportion of short branches and promotes floral transition [47]. Sugar as an energy substance involved in the flowering regulation [48]. Consequently, to further determine the potential role of Md14-3-3s genes in the context of apple flower induction, research on their expressions in response to sugars and hormones is very necessary. We first performed a preliminary analysis of Md14-3-3s expression after 6-BA [47], sucrose treatment [48] and glucose treatment (data not published) by analyzing transcriptome data (Fig. 5, Additional file 6: Table S3). In general, most orthologous genes of 14-3-3s exhibited similar expression patterns. *MdGF14o* showed no or very low expression levels (less than 1), indicating that it did not function to a large extent in flower

development. Besides, the expression levels of *MdGF14c*, *MdGF14f*, *MdGF14m*, *MdGF14k* and *MdGF14q* were also significantly lower. On the contrary, the expression levels of genes such as *MdGF14a*, *MdGF14b*, *MdGF14d*, *MdGF14e*, *MdGF14g*, *MdGF14h*, *MdGF14i*, *MdGF14j*, and *MdGF14n*, were significantly higher, indicating that they may play a major role in the flower induction phase. Noticeably, at the early stage, which is a key stage for flower induction, *MdGF14a*, *MdGF14i* were down-regulated while *MdGF14d*, *MdGF14j* were up-regulated in response to 6-BA and glucose treatments. Overall, Md14-3-3s showed different and multiple expression patterns in transcriptome data, implying functional diversity.

Furthermore, to gain insight into the response of Md14-3-3s to GA signal, we examined their expression patterns using qRT-PCR under GA3 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 GA3 treatment (Fig. 6). Interestingly, *MdGF14l*, *MdGF14q* and *MdGF14r* showed similar expression patterns during flowering induction, which were up- or down-regulated at different time points after treatment (Fig. 6), indicating that they might have roles in hormonal stress responses or apple development.

Subcellular localization of 14-3-3 proteins

Based on gene expression data, we selected four candidate Md14-3-3 isoforms (*MdGF14a*, *MdGF14d*, *MdGF14i* and *MdGF14j*) for further analysis, which may be related to flower induction in apple. To gain insight into the molecular function 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. 7).

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. Previously, TFL1 and FT were reported to interact with 14-3-3 proteins [11, 35, 36]. In apple, there are two MdTFL1 encoding genes, MdTFL1-1 and MdTFL1-2 [49]. We performed yeast two-hybrid assays and confirmed that both MdTFL1 (MdTFL1-1 and MdTFL1-2) proteins can interact with four 14-3-3 isoforms (*MdGF14a*, *MdGF14d*, *MdGF14i* and *MdGF14j*) (Fig. 8A). 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 system (Fig. 8A).

Also, we used a BiFC assay to determine the interactions between Md14-3-3 proteins and MdTFL1 or MdFT in vivo (Fig. 8B, 8C, 8D). 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. 8B), MdTFL1-2-Md14-3-3s (Fig. 8C) and MdFT-Md14-3-3s interactions (Fig. 8D) 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.

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, 50]. In this study, we identified a total of 18 Md14-3-3 genes in apple, 17 isoforms are transcribed. 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 containing a conserved core region composed of nine antiparallel α -helices (α 1- α 9) forming an amphiphilic groove 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 3: Figure S2), which are related to dimerization and client binding, respectively [51, 52]. 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 differentially affinity between individual 14-3-3 isoforms towards possible targets [41, 46, 53]. 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 [54-56], although the precise mechanism is not fully understood.

The Md14-3-3 family members break into two major evolutionary branches, the Ψ group and the non- Ψ group (Fig. 1; Fig. 2), consistent with the identified 14-3-3 family members in other species [41-43, 45]. Surprisingly, the Ψ members all possess an intron-exon structure distinct from the non- Ψ group. The evolution and phylogeny of the 14-3-3 family exhibit diversity and complexity, reflecting functional divergence.

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 [57]. Our data demonstrates that most Md14-3-3s are expressed in all detected tissues and are particularly higher in stem and flower (Fig. 4), 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, such as *MdGF14a/MdGF14i* and *MdGF14d/MdGF14j*, show similar expression patterns in different tissues.

These imply that 14-3-3 isoforms overlap and specific functions coexist in regulating plant growth, which is also supported by 14-3-3 sequence conservation and diversity that exist across isoforms of many species [42, 43, 45].

The 14-3-3 proteins are known as a component associated with several different proteins in signal transduction pathways [57]. 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 [58] and NPH3 [59], the major BR signaling related proteins [60], and members of the ABA responsive-element binding factor (ABF) family [61]. Interestingly, we found numerous hormone-related elements in the promoter of Md14-3-3 family genes (Additional file 5: Figure S3), 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. 5; 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) [62]. 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 5: Figure S3).

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 [63]. 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. 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). Notably, at 30 DAFB (a key point for floral induction) in GA₃ treatment (a negative factor of apple flower formation), the expressions of *MdGF14a* and *MdGF14i* were significantly up-regulated, while most Md14-3-3 genes (such as *MdGF14d* and *MdGF14j*) were sharply down-regulated (Fig. 6). In contrast, in 6-BA treatment (a positive promoter of flowering), *MdGF14a* and *MdGF14i* showed opposite expressions patterns (Fig. 5) at 30 DAFB. Together, these results suggested that 14-3-3 family members negatively or positively mediate the regulation of flowering induction.

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. 8). 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. 7). Previous reports indicated that 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 [64]. 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. 8), consistent with previous reports [11, 36]. These results strongly suggest that MdTFL1/MdFT and Md14-3-3s interactions increase MdTFL1 or MdFT cytoplasmic retention and inhibit shuttling of them from the cytoplasm into the nucleus.

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 or transcription activators, respectively, functionally antagonistically to regulate the downstream floral meristem identity genes, including *LFY* and *AP1* [65]. 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]. In tomato, there also are associations between SP (TFL1 homolog) and SFT (FT homolog) and 14-3-3 isoforms [66]. These results suggested that TFL1 acts against FT for 14-3-3 binding. The balance between FAC and FRC 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, of which 17 were found to be transcribed. The diverse expression patterns of the Md14-3-3 s 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 14-3-3 s binding partners. Biochemical analyses indicated that MdTFL1/MdFT and Md14-3-3 s interactions increase MdTFL1 or MdFT cytoplasmic retention and inhibit shuttling of them from the cytoplasm into the nucleus. Nevertheless, the studies on the functional mechanism of apple 14-3-3 s 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 published *A. thaliana* 14-3-3 protein sequences from *A. thaliana* genome database (<http://www.arabidopsis.org/>), they were used as queries in BLASTp 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/>). 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>) [67]. The 2,000bp upstream regions of Md14-3-3 genes were derived from the transcription start site 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. A phylogenetic tree was constructed using the maximum likelihood method in MEGA7.0 software with 1,000 bootstrap replicates and partial deletion option.

Tandem duplication and synteny analysis

Tandem duplication and synteny relationships were carried out using Circos version 0.63 (<http://circos.ca/>) [68]. 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 *A. thaliana* genomes were downloaded from the Plant Genome Duplication Database (<http://chibba.agtec.uga.edu/duplication/>).

Plant materials and treatment

The study was conducted at the Apple Demonstration Nursery of Yangling Modern Agriculture Technology Park, Shaanxi Province of China (108°70' E, 34°52' N). Plant materials were collected from randomly selected healthy, uniform six-year-old 'Nagafu No.2' / 'M26' / *M. robusta* Rehd trees and grouped into five groups (each group with nine trees divided into three blocks), where each group was sprayed with sucrose, glucose, 6-BA, GA3 and water (control). 15,000 mg L⁻¹ and 20,000 mg L⁻¹ sucrose was treated at 29 and 36 day after full bloom (DAFB), respectively [48]. 15,000 and 30,000 mg L⁻¹ glucose was spray at 25 and 30 DAFB, respectively. Meanwhile, 300 mg L⁻¹ 6-BA treatment was applied at 27 and

30 DAFB, as described in the previous report [47]. GA₃ treatment with a concentration of 500 mg L⁻¹ at 25 and 30 DAFB was sprayed in a clear morning. Besides, similar nine trees were sprayed with water as a control. Next we collect the terminal buds of the short shoots (< 5cm) at 30, 50 and 70 DAFB and stored for further use.

At the same time, different tissue samples were collected from six-year-old 'Nagafu No.2' /'M26'/ *M. robusta* Rehd trees. 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. While 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 further 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 6μL distilled deionized H₂O. The housekeeping histone H3-encoding gene (LOC103406086, XM_008345103) was used as a reference gene to calculate the relative expression of selected genes [69]. For each time point, three independent biological repeats with three technical repeats were performed. Relative gene expression levels were calculated using the 2^{-ΔΔCt} method [70]. Specific primer pairs were designed using Primer-BLAST online tool in National Center for Biotechnology Information (NCBI) database and are listed in Additional file 1: Table S1.

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 1: Table S1.

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 pGBKT7, 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 1: Table S1.

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 [71]. The resulting plasmids were transformed into *Agrobacterium tumefaciens* (strain GV3101), incubated, harvested and resuspended in infiltration buffer. 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 1: Table S1.

Additional Files

Additional file 1: Table S1. Primers used in the present study.

Additional file 2: Figure S1. Chromosome map of Md14-3-3 genes in apple.

Additional file 3: 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 $\alpha 1$ - $\alpha 9$.

Additional file 4: Table S2. The gene name and gene ID of 14-3-3s in apple and other plant species.

Additional file 5: Figure S3. Analysis of the *cis*-elements in the Md14-3-3 promoter. The 2,000bp sequences upstream of the start codon of Md14-3-3 genes were analyzed using the PlantCARE database.

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

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

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Availability of data and materials

All relevant data analyzed during this study are included in this article and its Additional files. The transcriptomic sequence data from glucose treatment used during the current study are available from the corresponding author on reasonable request.

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.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Table

Table 1 Apple 14-3-3 genes information.

Name	Gene identifier	CDS (bp)	Peptide (aa)	Gene locationa	Strand
MdGF14a	MD00G1018600	786	261	3143713-3146570	-
MdGF14b	MD00G1176300	798	265	40806633-40810797	+
MdGF14c	MD01G1037700	762	253	13038612-13047589	-
MdGF14d	MD05G1301400	786	261	43423732-43426889	-
MdGF14e	MD07G1096000	789	262	10468901-10472640	+
MdGF14f	MD08G1184000	759	252	22921142-22936861	-
MdGF14g	MD08G1187400	795	264	23593019-23594916	+
MdGF14h	MD08G1193600	759	252	24908573-24911343	-
MdGF14i	MD10G1084500	786	261	12408819-12411711	+
MdGF14j	MD10G1280300	786	261	37120031-37122984	-
MdGF14k	MD13G1063600	792	263	4396805-4399529	-
MdGF14l	MD15G1315300	765	254	32152582-32157628	+
MdGF14m	MD15G1370500	768	255	45033182-45036653	-
MdGF14n	MD15G1373400	792	263	45588403-45590135	+
MdGF14o	MD16G1062100	849	282	4419295-4422061	-
MdGF14p	MD17G1074900	786	261	6068710-6072300	-
MdGF14q	MD17G1105100	909	302	8914153-8917345	+
MdGF14r	MD17G1231000	783	260	27941075-27945515	+

Figures

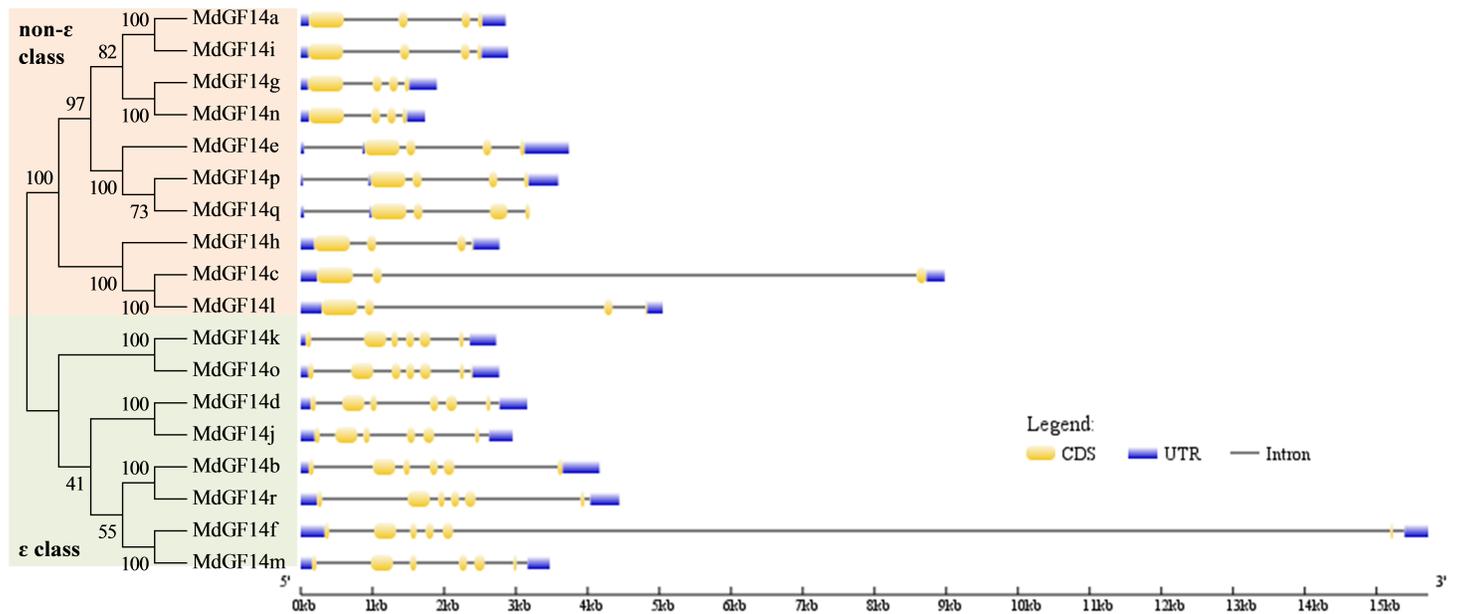


Figure 1

Analysis of Md14-3-3 gene structures. (A) The unrooted phylogenetic tree of Md14-3-3 protein sequences. (B) Exon-intron composition of Md14-3-3 genes. The blue, yellow boxes and black lines represent UTR, exon and intron positions, respectively.

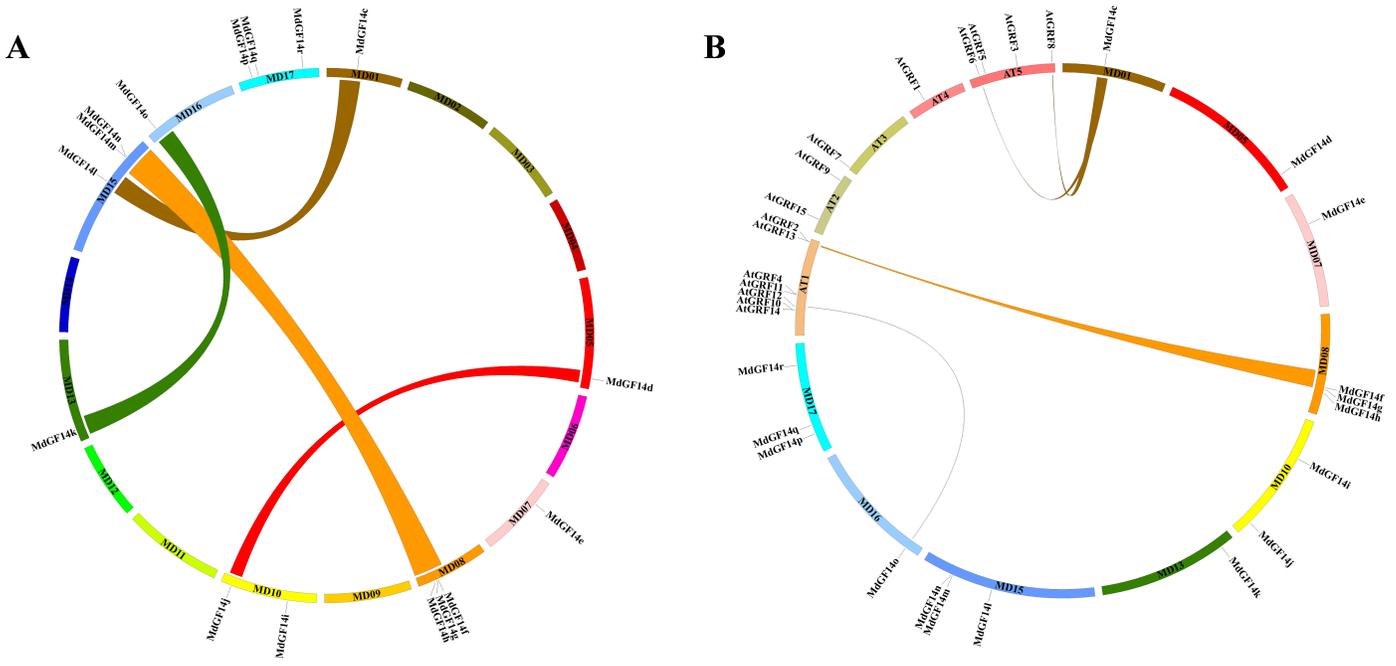


Figure 3

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.

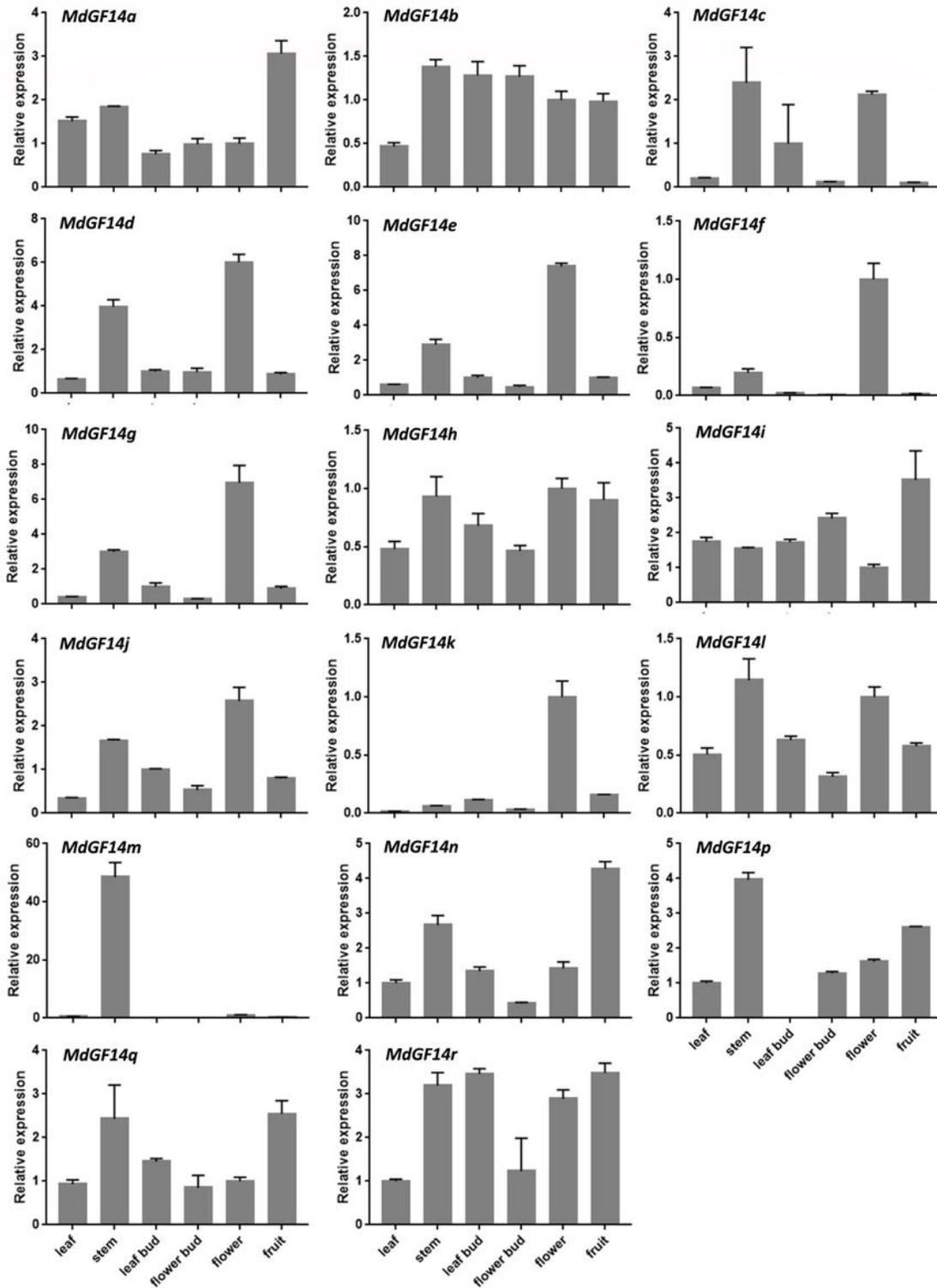


Figure 4

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

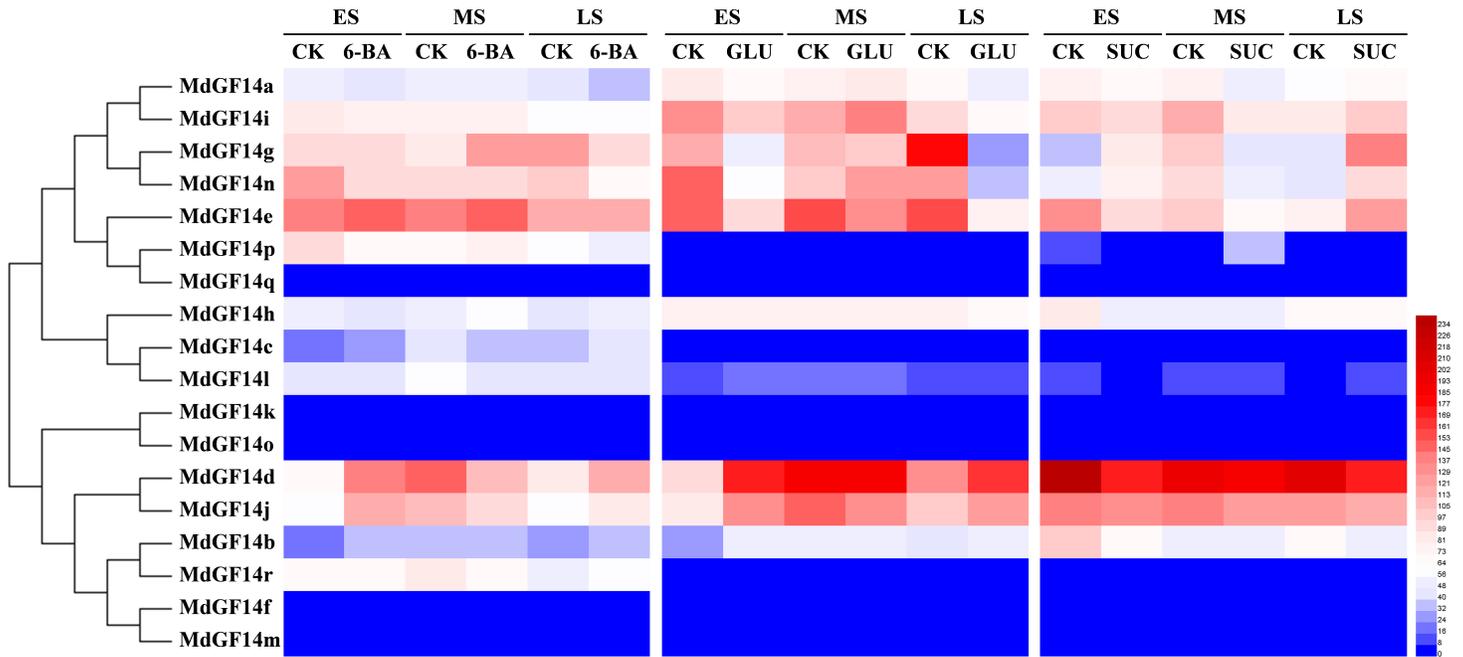


Figure 5

Heat map representation of response patterns of Md14-3-3s during floral transition phase in 6-BA treatment, glucose and sucrose treatment. FPKM values were used to generate their expression profiles. ES, MS, and LS represent early (30 DAFB), middle (50 DAFB) and late stages (70 DAFB) of flower formation, respectively. The diagram was drawn using Heml 1.0 software. For other details, see Additional file 6: Table S3.

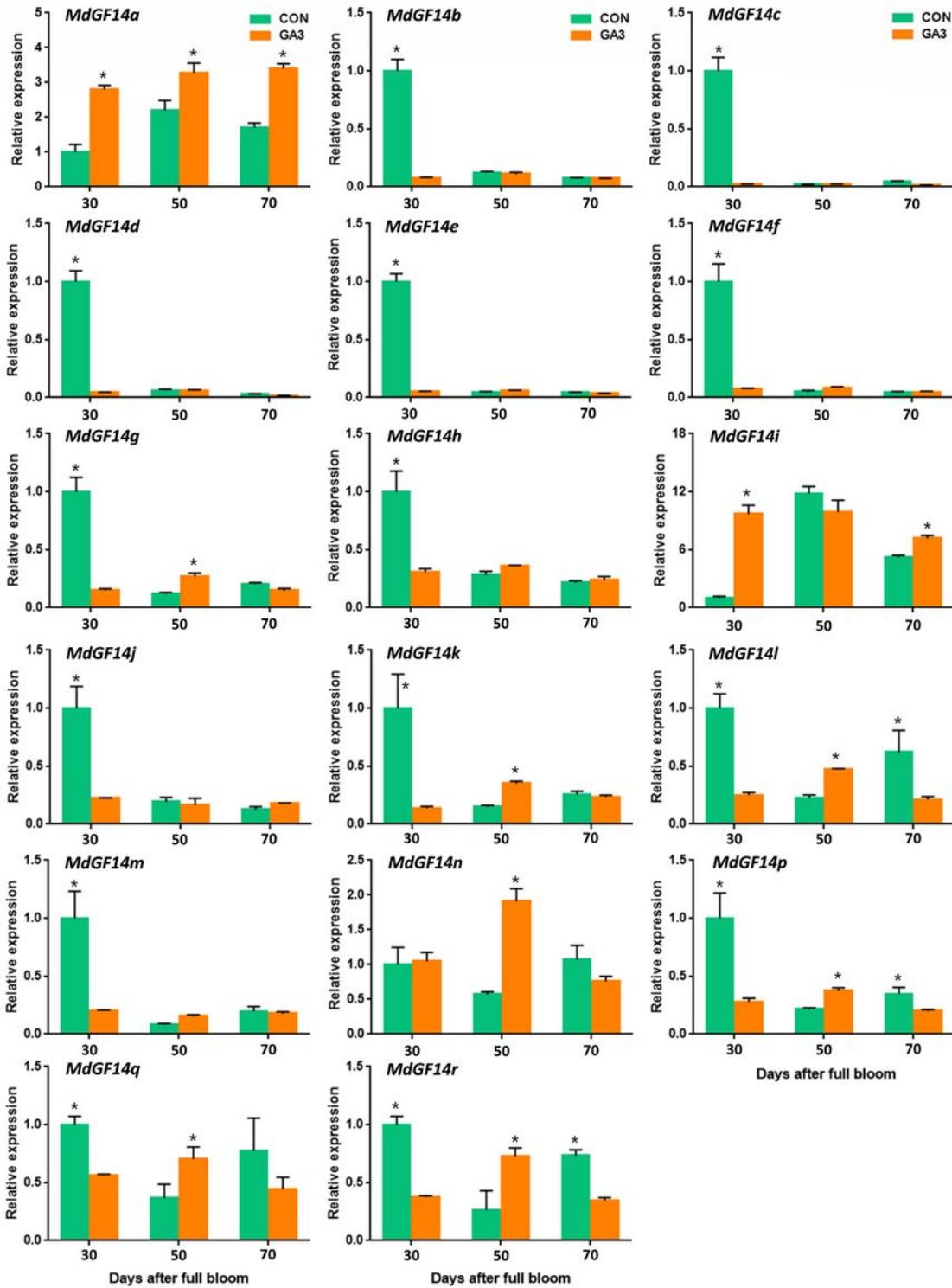


Figure 6

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.

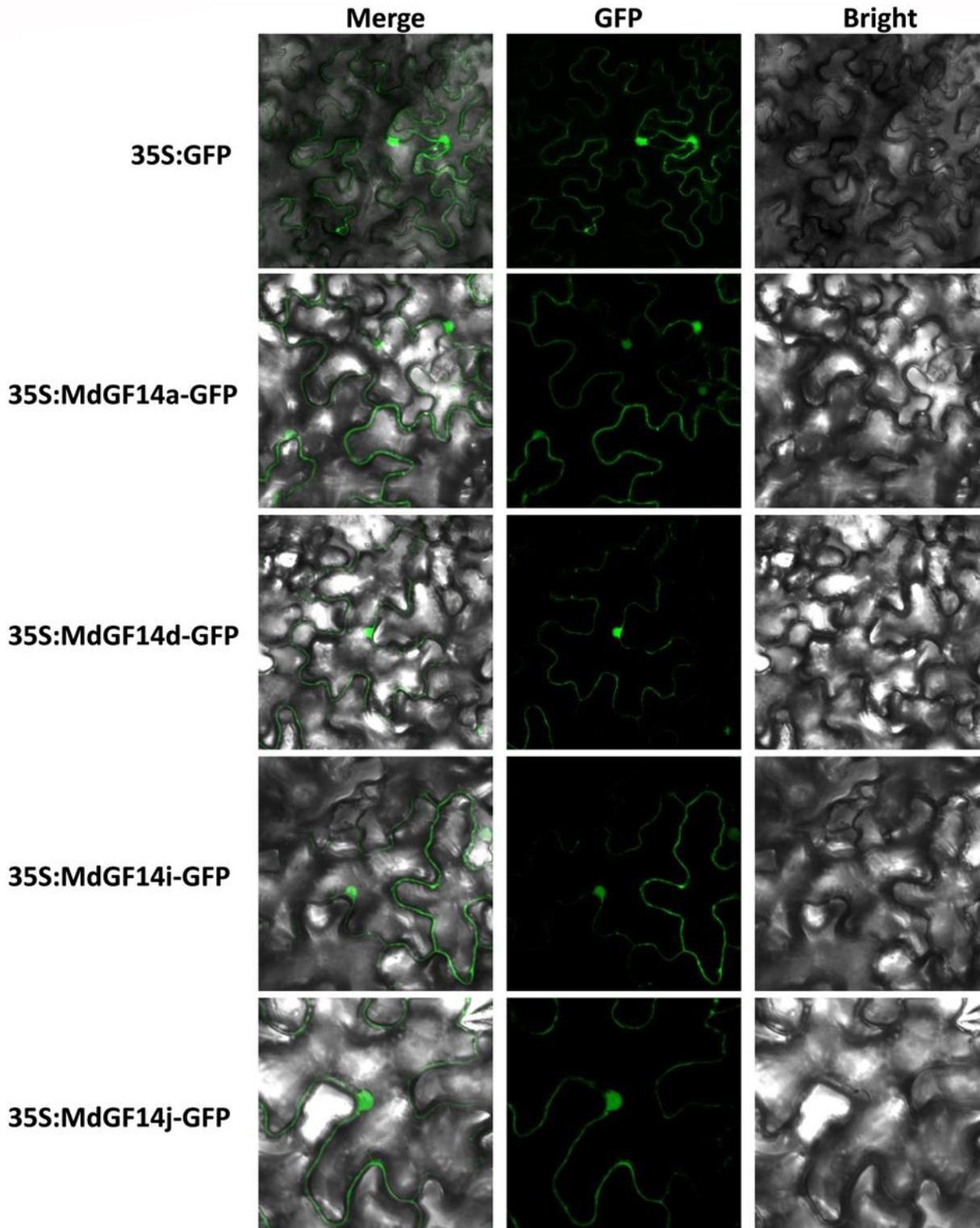


Figure 7

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.

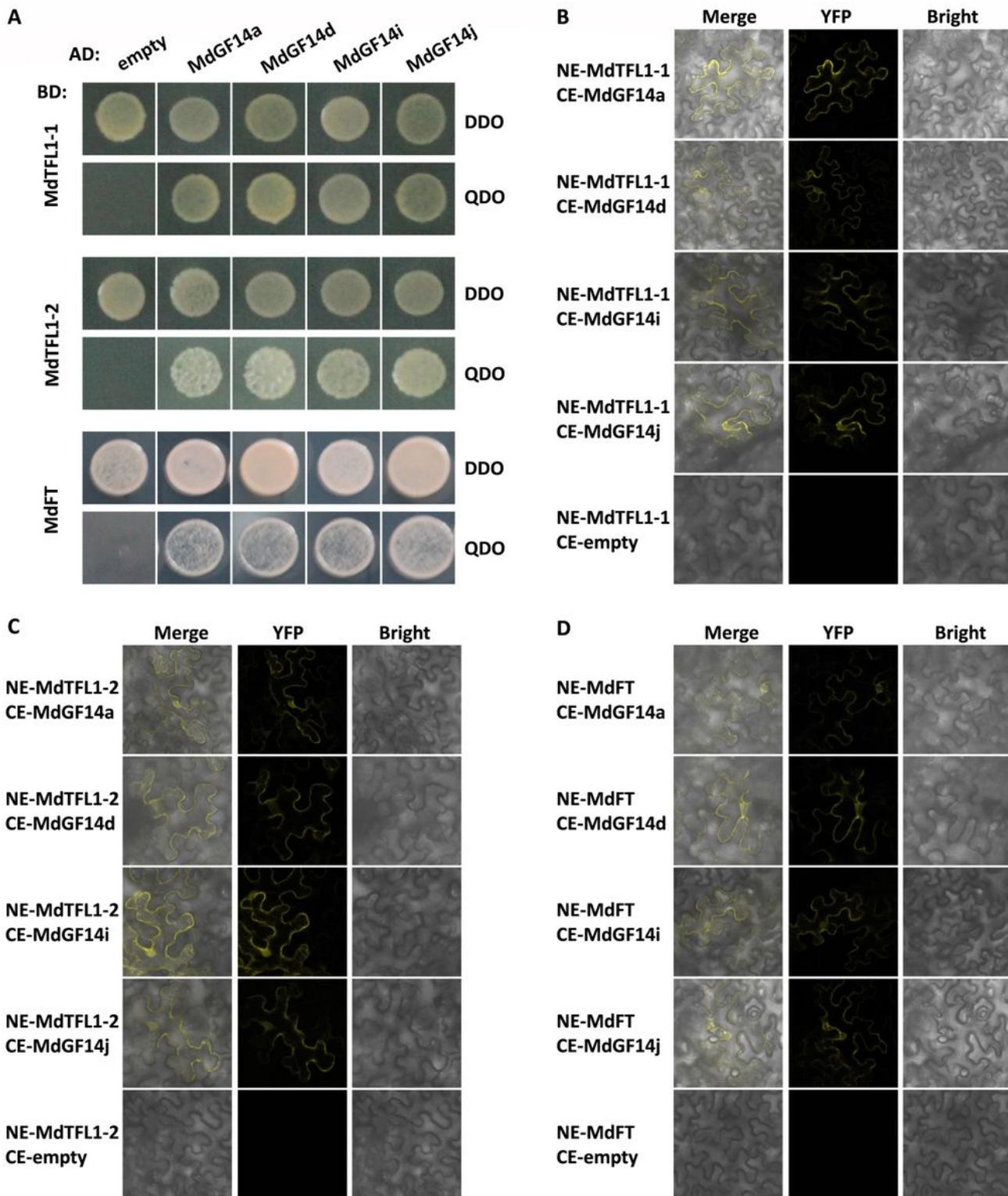


Figure 8

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 Cs14-3-3 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. (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.

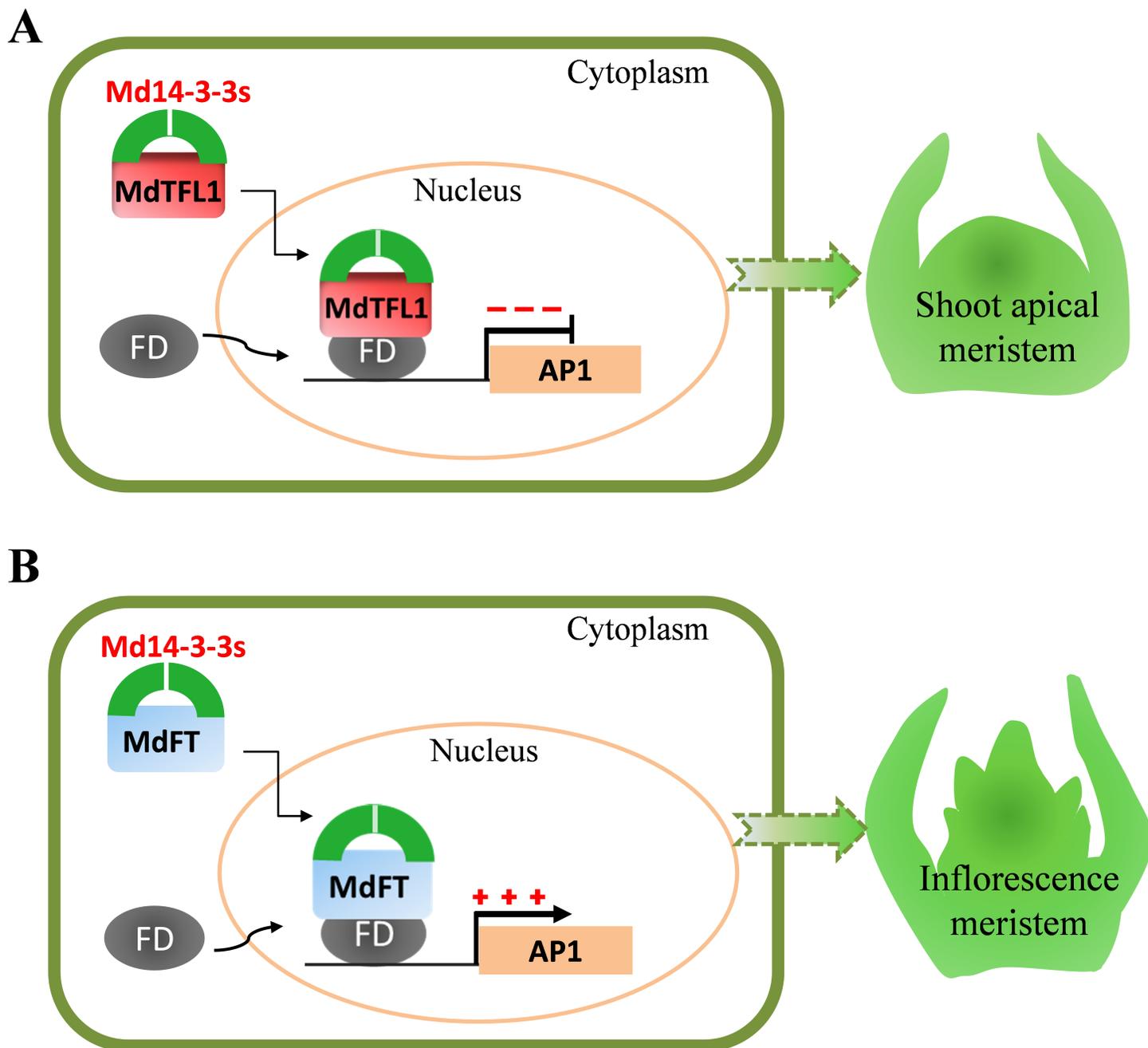


Figure 9

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 of crop productivity.

Supplementary Files

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

- [Additionalfile4TableS2.xlsx](#)
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- [Additionalfile2FigureS1.tif](#)
- [Additionalfile1TableS1.xlsx](#)
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