Functional conservation and divergence of five *AP1/FUL*-like genes in Marigold (*Tagetes erecta*)

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Abstract

Background: Members of *AP1/FUL* subfamily genes play an essential role in the regulation of floral meristem transition, floral organ identity, and fruit rippling. At present, there have been insufficient studies to explain the function of the *AP1/FUL*-like subfamily genes in Asteraceae.

Results: Here, we cloned two *euAP1* clade genes *TeAP1-1* and *TeAP1-2*, and three *euFUL* clade genes *TeFUL1*, *TeFUL2*, and *TeFUL3* from marigold (*Tagetes erecta*). Expression profile analysis demonstrated that *TeAP1-1* and *TeAP1-2* were mainly expressed in receptacles, sepals, petals, and ovules. *TeFUL1* and *TeFUL3* were expressed in floral buds, stems and leaves as well as in productive tissues, while *TeFUL2* was mainly expressed in floral buds and vegetative tissues. Transgenic Arabidopsis lines showed that overexpression *TeAP1-2* or *TeFUL2* resulted in early flowering, implying that these two genes might regulate the floral transition. Yeast two-hybrid analysis indicated that TeAP1/FUL proteins only interacted with TeSEP proteins to form heterodimers, and that TeFUL2 could also form a homodimer.

Conclusion: In general, TeAP1-1 and TeAP1-2 might play a conserved role in regulating sepal and petal identity, just like the role of MADS-box class A genes, while *TeFUL* genes might display divergent functions. This study provides an insight into molecular mechanism of *AP1/FUL*-like genes in Asteraceae species.

Background

Although flowers show great diversity in morphology, structure, composition, color and function, they are usually composed of four distinct concentric whole floral organs: sepals in the outermost outer, petals in the second whorl, stamens (male reproductive organs) in the third whorl, and carpels (female reproductive organs) in the innermost whorl [1]. The exploration of the mechanism of distinct floral organ formation has undergone a long-term challenge in plant developmental genetics [1–4]. Based on the study of the floral formation in model plants, such as *Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia hybrida*, the fate of the different floral organs was determined by a complex regulatory network composed of MADS-box proteins [5].

The MADS-box protein family is one of the most widely studied transcriptional factor family in angiosperm, which plays a key role in regulating the floral meristem development, floral organ identity, fruit and seed development, vegetable tissues development as well as flowering time [6–8]. MADS box genes in higher plants are reported to have undergone several duplication events which play an important role in the evolution of morphological complexity of flower [9], allowing MADS-box genes to cluster into several major subfamilies [10–12]. One subfamily of MADS-box genes forms the angiosperm-specific *APETALA1/FRUITFUL* (*AP1/FUL*) lineage via gene duplication. Phylogenetic analyses reveal that *AP1/FUL* lineage have undergone numerous duplication events throughout angiosperm diversification [13, 14]. *AP1* genes are diverged into two types of *AP1* lineage genes within core eudicot, namely, *euAP1* (Arabidopsis *AP1* and Antirrhinum *SQUA*) and *euFUL*, which duplication is likely part of the whole genome duplication that occurs before the diversification of the core eudicots, which is often referred to as the gamma event [15, 16]. Contrary to core eudicots, non-core eudicots have only *FUL*-like clade genes [13, 14]. Within model plant Arabidopsis, *euAP1* clade is subdivided into *AP1* and *CAULIFLOWE* (*CAL*) genes [17, 18] whose amino acid sequences possess an acidic domain and a farnesylation motif (CaaX) in their 3' end of coding sequence [13, 19]. *AP1* and *CAL* are accumulated in floral meristems, sepals, and petals primordia [20, 21]. In Arabidopsis, *ap1* mutation results in the absence of petals, the transformation of sepals into bract-like structure, and secondary flowers produce from the axils of these first whorl organs. [20, 22]. The overexpression of *AP1* leads to remarkably early flowering and transformation of inflorescence shoot apical meristem into floral meristem [23]. In single Arabidopsis *cal* mutants, there are no remarkable changes in floral organs, but the *cal* mutation enhances the repetitive branching pattern in floral meristem of *ap1* mutants [22, 24]. In other core eudicots, the *ap1* mutation only changes sepal structure, but it does not affect petal structure [27, 28]. Furthermore, ectopic expression of *Fortunella crassifolia* *FcAP1* in Arabidopsis [25] and heterologous overexpression of *AP1*-like gene *Betula pendula* *BpMADS3* [26] and *Pisum sativum* *PEAM4* [27] in *Nicotiana tabacum* cause early flowering without affecting floral meristem development. In general, the *euAP1* clade genes exhibit a conserved function in specifying the floral meristem and sepal identity in core eudicot plants.

The *euFUL* genes show a conserved function in promoting the transition from vegetative meristems to reproductive meristems and in regulating fruit development in core eudicots. The *euFUL* proteins are characterized by a conserved six-hydrophobic-amino-acid motif (*FUL*-like motif) in C domain, and this motif is considered to be conserved in the entire the ancestral gene lineage, and its occurrence is prior to the *euFUL/euAP1* duplication, but its function remains unclear [13, 14]. In Arabidopsis, duplication event of *euFUL* clade allows *euFUL* to divide into *FUL* and *AGAMOUS*-like 79 (*AGL79*) [28–30]. Different from *euAP1*, *FUL* is mainly expressed in growing leaves, inflorescence meristems, carpel primordia, and young siliques [23, 31]. Arabidopsis *FUL* is redundant with *AP1* and *CAL* in regulating the floral meristem identity, and it also regulates the flowering time, axillary meristem activation, meristem determinacy, and plant longevity [21, 32]. In *ful* mutants, the cauline leaf development was defected and the floral development was disrupted [31, 33]. The transcript of *AGL79* is detected in roots, but its function remains unclear [28, 29]. Only limited functional analysis of *euFUL* genes in other core eudicots is available. Similar to *FUL* gene function in Arabidopsis, ectopic expression of *DEFICIENS-homolog28* (*DEFH28*, a *euFUL* gene, Antirrhinum) [34] in Arabidopsis causes early flowering, transformation of inflorescence into terminal flower, and silique indehiscence. Moreover, the overexpression of *NtFUL* in *Nicotiana tabacum* also results in early flowering and failure in capsule dehiscence [35]. In *Petunia hybrida*, silencing *PETUNIA FLOWERING GENE* (*PFG*, a *euFUL* gene) leads to the interruption of inflorescence formation and the maintenance of vegetative growth [36].

Asteraceae is one of most abundant and widespread family of flowering plants, and has specific capitulum consisting of two flower types: the outer are the sterile ray florets and the inner are the fertile disk florets. The specific inflorescence makes Asteraceae a suitable material in studying the evolution and function of MADS-box genes related to floral organ development. Nevertheless, the available functional information on *AP1/FUL* genes in Asteraceae is restricted to *Gerbera hybrida* [37], *Chrysanthemum lavandulifolium* [38], and *Chrysanthemum morifolium* [39, 40]. The expression patterns of *AP1/FUL*-like genes are different among various Asteraceae species. The *euAP1*-like genes *CDM111* (*C. morifolium*) and *GASQUA3* (*G. hybrida*) are highly expressed in sepals and petals, while their homologous gene *GASQUA1* (*G. hybrida*) was not expressed in floral meristem or in perianth primordia [37, 39, 41]. The *FUL*-like genes *GSQUA2* and *GSQUA5* are only expressed in florescence and floral organs, while *GSUA4* and *CDM41* are also expressed in leaves [37, 39]. The function of *AP1/FUL*-like genes in Asteraceae remains unclear. Overexpression of *AP1*-like gene *CDM111* or *FUL*-like gene *CIM8* in Arabidopsis results in the altered

flowering time and inflorescence structure [38, 39]. However, overexpression of *GSQUA2* (a *FUL*-like gene, homology to *DEFH28*) results in dwarf plant, early flowering, and vegetative abnormality, but it does not affect inflorescence structure [37].

Marigold (*Tagetes erecta*) is a popular ornamental plant and industrial crop, in whose flower lutein is rich. As a member of Asteraceae, marigold also has a typical capitulum. Compared to that of Chrysanthemum, the whole life cycle of marigold lasts only 2–3 months from sowing to flowering. In addition, in the evolutionary history of Asteraceae family, marigold undergoes a long evolution process and it is located in a derived Calenduleae clade [42]. These characteristics make marigold a valuable material in the study of function of *AP1/FUL* genes. In this study, we cloned and characterized five *AP1/FUL*-like genes in marigold, whose distinct expression patterns, protein interaction manner and different phenotypes of Arabidopsis transgenic lines might imply their divergent functions in regulating the floral meristem development, floral organ identity, and flowering time.

Results

Isolation and phylogenetic analysis of TeAP1/FUL-like genes

The full-length sequences of five *AP1/FUL*-like genes were amplified by using gene-specific primers with cDNA generated from buds as a template. In order to further identify the putative homologues of *AP1* and *FUL* genes, we blasted nucleotide sequences of these five genes against NCBI. The blast search results indicated that two different *AP1*-like genes and three different *FUL*-like genes were detected with the former designated as *TeAP1-1* (Acc. No. MT394170), *TeAP1-2* (Acc. No. MT394171), and the latter designated as *TeFUL1* (Acc. No. MT394172), *TeFUL2* (Acc. No. MT394173), and *TeFUL3* (Acc. No. MT394174), respectively. Sequence analysis revealed that the putative proteins based on these five genes coding sequences were composed of 246, 247, 235, 235, and 242 amino acids, respectively. The two putative AP1 proteins shared over 89% amino-acid identity, while the three putative FUL homologous proteins shared relatively low identity (Supplementary Table S3). Multiple sequence alignment and conserved analysis of AP1/FUL proteins indicated that all TeAP1/FUL proteins contained a conserved MADS domain, a I domain, a less conserved K domain, and a variable C-terminal domain (Fig. 1). The putative proteins of TeAP1-1 and TeAP1-2 possessed a typical euAP1-motif (CFPS) containing both an acidic domain and a farnesylation motif (CaaX, shown at their C termini). In addition, characteristic FUL motif was shared by the three TeFUL proteins (Fig. 1).

To investigate the relationship between *TeAP1/FUL* genes and other members of *AP1* and *FUL* clades, a phylogenetic analysis was carried out by using amino acid sequences of AP1/FUL clade from other plant species and *O. sativa* OsMADS1 belonging to SEP subfamily as an outgroup (Fig. 2). TeAP1-1 and TeAP1-2 were co-orthologous to Arabidopsis AP1 and Antirrhinum SQUA. TeFUL1 and TeFUL3 were phylogenetically close to euFUL, and TeFUL2 was co-orthologs of the Antirrhinum protein DEFH28. Remarkably, TeFUL1 and HaFUL (*Helianthus annuus*) shared 78.39% amino-acid identity, and TeAP1-1 and HAM75 (*H. annuus*) were more closely related to each other with over 97.45% amino-acid identity. The high homology might indicate the functional similarity between them.

Expression analysis of TeAP1/FUL-like genes in marigold

The expression patterns for the five *AP1/FUL* genes in different vegetable tissues, floral organs, and different development phases of floral buds were examined by qRT-PCR. *TeAP1-1* was mainly expressed in leaves, receptacles, bracts, sepals of ray florets, petals of disk florets, and ovules, but not expressed in floral buds and roots (Fig. 3, S1). Compared to *TeAP1-1*, *TeAP1-2* was weakly expressed in different development phases of floral buds, but was highly expressed in receptacles, sepals of ray and disk florets, petals of disk florets, and ovules (Fig. 3, S1). *TeFUL1* and *TeFUL3* shared a similar expression pattern, and they were widely expressed in vegetative and productive tissues (Fig. 3, S1). The difference in expression was detected between *TeFUL1* and *TeFUL3*. For example, *TeFUL1* was expressed mainly in petals of disk florets, stamens, ovules and sepals of ray florets, while *TeFUL3* was highly expressed in all floral organs of two-type florets and receptacles (Fig. 3, S1). Contrary to *TeFUL1* and *TeFUL3*, *TeFUL2* was highly expressed in vegetative tissues, flower buds, receptacles, and bracts, and weakly expressed in floral organs (Fig. 3, S1).

Interactions between TeAP1/FUL proteins and other MADS-box proteins in marigold

In order to investigate whether difference in expression pattern of these five *TeAP1/FUL* genes caused their difference in protein interactions, a yeast two-hybrid analysis was performed. The ability of pairwise interaction between TeAP1/FUL and other MADS-box proteins in marigold was determined by yeast two hybrid method. The marigold proteins were individually fused to the binding domain and the activation domain, and were combined in both directions. No autoactivation was observed among these five proteins (Supplementary Fig. S2a). As shown in Table 1 and Supplementary Fig. S1b-e, TeAP1/FUL proteins only interacted with SEPATELLA (SEP) proteins, but they did not interact with class B (TePI, TeAP3-1, TeAP3-2, TeTM6-1 and TeTM6-2), class C (TeAG1 and TeAG2), and class D (TeAGL11-1 and TeAGL11-2) proteins. TeAP1-1 and TeAP1-2 exhibited a same protein interaction manner, both of which interacted with class E proteins TeSEP3-2 and TeSEP3-3 to form heterodimers (Table 1, Supplementary Fig. S2b, e). Contrary to TeAP1 proteins, TeFUL proteins displayed a different protein interaction manner. TeFUL1 only interacted with TeAGL6. TeFUL2 interacted with TeSEP3-2, TeAGL6, and itself. TeFUL3 exhibited a variety of protein interaction manners, which could form heterodimers with TeSEP1, TeSEP3-1, TeSEP3-2, TeSEP-3, and TeSEP4 (Table 1, Supplementary Fig. S2b, e).

Table 1
Interactions of Marigold AP1/FUL proteins detected by yeast two-hybrid assays.

	BD- TeAP1- 1	BD- TeAP1- 2	BD- TeFUL1	BD- TeFUL2	BD- TeFUL3	BD- TePI	BD- TeAP3- 1	BD- TeAP3- 2	BD- TeTM6- 1	BD- TeTM6- 2	BD- TeAG1	BD- TeAG2	BD- TeAGL11- 1	BD- TeAGL11- 2
AD- TeAP1-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AD- TeAP1-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AD- TeFUL1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AD- TeFUL2	-	-	-	+	-	-	-	-	-	-	-	-	-	-
AD- TeFUL3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AD-TePI	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeAP3-1	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeAP3-2	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeTM6- 1	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeTM6-2	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeAG1	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeAG2	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeAGL11- 1	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeAGL11- 2	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeSEP1	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeSEP3-1	-	-	-	-	-	/	/	/	/	/	/	/	/	/
AD- TeSEP3-2	+	+	-	+	+	/	/	/	/	/	/	/	/	/
AD- TeSEP3-3	-	-	-	-	+	/	/	/	/	/	/	/	/	/
AD- TeSEP4	-	-	-	-	++	/	/	/	/	/	/	/	/	/
AD- TeAGL6	-	-	-	-	-	/	/	/	/	/	/	/	/	/
Note: ++, strong interaction; +, weak interaction; -, no interaction; /, no done.														

Early flowering caused by ectopic expression of TeAP1-2 and TeFUL2 genes in Arabidopsis

To explore the potential function of *TeAP1-1*, *TeAP1-2*, *TeFUL1*, *TeFUL2*, and *TeFUL3* genes, functional analyses were performed by overexpressing these five genes into Arabidopsis with the cauliflower mosaic virus 35S promoter. After selection through kanamycin and confirmation through PCR, a total of 63, 31, 42, 26 and 45 independent T1 transgenic lines were obtained for *35S:TeAP1-1*, *35S:TeAP1-2*, *35S:TeFUL1*, *35S:TeFUL2*, and *35S:TeFUL3*, respectively. The transcript level analysis revealed that five *AP1-1/FUL*-like genes *TeAP1-1*, *TeAP1-2*, *TeFUL1*, *TeFUL2*, and *TeFUL3* were successfully expressed in Arabidopsis plants (Fig. 4a, b, Supplementary Fig. S3a-c). According to the phenotypic alterations, 3–6 T2 transgenic lines of *35S:TeAP1-1*, *35S:TeAP1-2*, *35S:TeFUL1*, *35S:TeFUL2*, and *35S:TeFUL3* were selected for further experiment. Compared with wild-type plants, the transgenic lines containing *35S:TeAP1-1*, *35S:TeFUL1*, and *35S:TeFUL3* exhibited no visual phenotypical changes. However, overexpression of *TeAP1-2* and *TeFUL2* into Arabidopsis caused obvious early flowering (Fig. 4d, f, h). In addition, ectopic expression of *TeAP1-2* also led to the collapse of the last two rosette leaves and the curl of cauline leaves (Fig. 4e, g).

Expression Analysis Of Endogenous Genes In Transgenic Plants

To reveal the mechanism underlying phenotypic changes of transgenic lines *35S:TeAP1-2* and *35S:TeFUL2*, the expression levels of endogenous genes regulated by *AP1* were analyzed when the T3 transgenic and wild-type seedlings were 10 days old. As shown in Fig. 5, *TeAP1-2* and *TeFUL2* displayed a similar function in regulating the expression level of *AP1* downstream genes. For example, the expressions of *AP1*, *FT*, *LFY*, *SOC1*, *SPE3*, and *TFL* in *35S:TeAP1-2* and *35S:TeFUL2* transgenic lines were obviously higher than those in wild-type plants. The expression level of *AGL24* showed no remarkable changes in both *35S:AP1-2* and *35S:FUL2* transgenic lines. However, it should be noted that genes *TeAP1-2* and *TeFUL2* specifically regulated some downstream genes. For instance, *SPL9* was significantly upregulated in *35S:TeAP1-2* transgenic lines, but it exhibited no change in *35S:FUL2* transgenic lines. In contrast to *SPL9*, *SVP* was significantly highly expressed in *35S:FUL2* transgenic lines, but no significant change in *35S:AP1-2* transgenic lines.

Discussion

The study of many *AP1/FUL*-like genes from various species has demonstrated that *AP1/FUL* genes play key roles in flowering time, flower and fruit development. Like genes *APETALLA3* (*AP3*, B class gene) and *AGAMOUS* (*AG*, C class gene), the *AP1/FUL* genes undergo several duplication events, resulting in the occurrence of *euAP1* and *euFUL* clade in core eudicots [13, 14]. In this study, five *AP1/FUL*-like genes were obtained from marigold. Sequence alignment analysis indicated that all these 5 *AP1/FUL*-like proteins are typical MIKC proteins, and they contained conserved motif at their C terminal domain (Fig. 1). *TeAP1-1* and *TeAP1-2* were clustered into *euAP1* clade proteins harboring an acidic domain and a farnesylation motif (Fig. 1), and the *TeFUL1*, *TeFUL2* and *TeFUL3* possessed a conserved *FUL* motif (Fig. 1) that was demonstrated to be all members of *FUL* clade proteins [13, 14]. Such changes in amino acid sequence have been explained by a frameshift mutation in an ancestral *AP1/FUL*-like gene [13, 43] and are responsible for gene-specific functions.

Our phylogenetic analysis indicated that *TeAP1-1* and *TeAP1-2* were members of *AP1* clade, and seemed to be homologous to antirrhinum *SQUA* which was previously reported to be involved in regulating the floral meristem development and specifying the sepal and petal identity [44]. *TeFUL1*, *TeFUL2* and *TeFUL3* were clustered into the *FUL* clade, and *TeFUL1* and *TeFUL3* proteins were closer to the *euFUL* group. *TeFUL2* belonged to the *euFULII* group (Fig. 2). The *TeFUL2* was orthologous to the antirrhinum gene *DEFH28* which was also clustered into *euFULII* group, and this gene participated in regulation of floral meristem development, fruit development, and flowering time [34]. Based on the expression pattern analysis, the *TeFUL2* was mainly expressed at the early stage of inflorescence development (Fig. 3, S1), which was similar to the expression pattern of the early function genes represented by Arabidopsis *FUL* [21] and petunia *PFG* [36], implying a role of *TeFUL2* in meristem identity. However, *TeFUL1* and *TeFUL3* were expressed in vegetative tissues, different stages of floral buds, and floral organs (Fig. 3, S1). Based on these findings, it could be speculated that *TeFUL2* and *TeFUL1*(or *TeFUL3*) might arise due to gene duplication, and that this duplication event might cause the change in their expression patterns. Many previous studies reveal that functional divergence is caused by gene duplication which further drives evolution [10, 45]. Therefore, we speculated that the duplication events and the modification of transcript pattern of *TeFUL* genes might imply the diversification of their functions in marigold.

Conserved function of *AP1/FUL* genes in flowering performance

Function analysis of the *AP/FUL*-like genes in core eudicots and non-core eudicots revealed that *AP1/FUL*-like genes displayed conserved roles in regulating the flowering time. For example, overexpression of *AP1* or *FUL* in Arabidopsis both leads to early flowering [25, 46]. Furthermore, the similar phenomena were also observed in the case of ectopic overexpression of *AP1*-like or *FUL*-like genes from Asteraceae species, such as *C. morifolium* (*CDM111*) [39], *C. lavandulifolium* (*CIM8*) [38] and *G. hybrida* (*GSUQA2*) [37]. In this study, heterologous expression of *TeAP1-2* and *TeFUL2* into Arabidopsis resulted in early flowering without affecting floral organ identity (Fig. 4d, f, h). Additionally, ectopic expression of *TeAP1-2* also led to the curl of rosette leaf and cauline leaf (Fig. 4e, g), which was similar to the function of the *AP1/FUL*-like gene *MBP20* [47]. The MADS-box transcription factors possess a DNA-binding domain to regulate their downstream gene expression [45]. Therefore, we speculated that the early flowering phenotypes observed in *35S:TeAP1-2* and *35S:TeFUL2* transgenic lines might be related to the change in endogenous gene expression level. In this study, *AP1*, *FT*, *LFY*, *SOC1*, *SPE3*, and *TFL* were significantly up-regulated in 10-day-old seedlings of transgenic lines containing *35S:TeAP1-2* and *35S:TeFUL* fusion vectors (Fig. 5), suggesting *TeAP1-2* and *TeFUL2* might share overlapping regulation network of a series of downstream genes in Arabidopsis. In Arabidopsis, *AP1* directly represses *SVP*, *AGL24*, and *SOC1* to partially specify floral meristem identities [48]. However, in our study, no remarkable change in the expression level of *AGL24* was observed in both *35S:TeAP1-2* and *35S:TeFUL2* transgenic lines (Fig. 5). Additionally, the expression level of the flowering repressor gene *SVP* was significantly activated in *35S:TeFUL2* transgenic lines, but not in *35S:AP1-2* transgenic lines (Fig. 5). In contrast to *SVP*, *SPL9* was significantly upregulated in *35S:TeAP1-2* transgenic lines, but not in *35S:FUL2* transgenic lines (Fig. 5). These results revealed that *TeAP1-2* and *TeFUL2* had a divergent function in regulating downstream genes, which was further supported by their difference in protein interaction manners (Table 1, Supplementary Fig. S2b-e).

Potential redundant function of *TeAP1-1* and *TeAP1-2* as class A genes

In Arabidopsis, *AP1* is an early-acting gene and functions as an class A gene to specify sepal and petal identity [20, 49]. *AP1* is expressed in floral meristems and developing sepal and petal primordia [20, 21, 24, 50]. However, in other core eudicots, the *AP1*-like genes can be also expressed in bracts and reproductive organs [27, 39, 51]. Similarly, *TeAP1-1* and *TeAP1-2* were both highly expressed in sepals of two-type florets and petals of disk florets as well as in bracts, receptacles, and ovules (Fig. 3, S1). In Arabidopsis, *AP1* only interacted with *SEP3* to form heterodimer. Furthermore, in Asteraceae species, the *AP1*-like proteins *C. morifolium* CDM111 [39, 40], *G. hybrida* GSQUA1, and GSQUA3 [37] also had a limited protein interaction manner. In other words, they only formed heterodimers with *SEP3* proteins. In this study, *TeAP1-1* and *TeAP1-2* shared a similar protein interaction pattern to form heterodimers with *TeSEP3-2* and *TeSEP3-3* (Table 1, Supplementary Fig. S2b, e), suggesting that *euAP1*-like proteins shared a conserved protein interaction manner. Taken together, *TeAP1-1* and *TeAP1-2* may play a redundant role as class A genes.

Divergent functions among *TeFULs* genes

The functions of *FUL*-like genes in the transition from vegetative meristems to reproductive meristems and in fruit development were well-known in many core eudicots and non-core eudicots. In model plant Arabidopsis, *FUL* regulates the cell differentiation during fruit development [31, 33, 52] and participates in

specifying floral meristem identity with *AP1* and *CAL* [21]. In basal eudicots, the *Aquilegia coerulea* *FUL*-like genes regulate leaf morphogenesis and inflorescence development [8]. Additionally, in monocots, the *Oryza sativa* homologues genes *OsMADS14* and *OsMADS15* are involved in specifying the meristem identity, palea and lodicule identity [7]. In contrast to the *AP1*-like genes, the *FUL*-like genes are widely expressed in vegetative and productive tissues [6, 31, 46].

In our study, *TeFUL1* and *TeFUL3* were expressed in stems and leaves as well as in productive tissues (Fig. 3, S1), which was consistent with the typical *FUL*-like expression pattern [6, 31, 46], implying that *TeFUL1* and *TeFUL3* might play a role as *FUL* genes. Furthermore, ectopic expression of *TeFUL1* or *TeFUL3* into *Arabidopsis* led to no visible phenotype changes. In *Arabidopsis*, *FUL* functions redundantly with *CAL* and *AP1* to specify the floral meristem identity, and single *ful* mutation has no ability to affect floral organ identity [21]. In general, we speculated that *TeFUL1* and *TeFUL3* might function redundantly in regulating the floral meristem identity, or that *TeFUL1* and *TeFUL3* need to work together with *AP1*-like genes to regulate the floral meristem development. However, the striking difference in protein interaction manner was observed between *TeFUL1* and *TeFUL3* (Table 1, Supplementary Fig. S2b-e). *TeFUL1* only interacted with *TeAGL6*, while *TeFUL3* interacted with *TeSEP1*, *TeSEP3-1*, *TeSEP3-2*, *TeSEP3-3*, and *TeSEP4* to form heterodimers (Table 1, Fig. S2b-e). Different protein interaction patterns might be related to their different conserved regions at C domains (Fig. 1). The above results suggested that *TeFUL1* and *TeFUL3* might be partially functionally redundant, but they might have their own specific functions in regulating floral organ identity.

In contrast to *TeFUL1* and *TeFUL3*, *TeFUL2* was highly expressed in floral buds and vegetative tissues, and weakly expressed or unexpressed in floral organs and ovules (Fig. 3, S1). Additionally, *TeFUL2* could form homodimer, and heterodimers with *TeAGL6* and *TeSEP3-2* (Table 1, Supplementary Fig. S2b, e). Ectopic expression of *TeFUL2* into *Arabidopsis* also led to early flowering with less number rosette leaves (Fig. 4h), which was consistent with phenotype of the overexpressed *euFULII* (*DEFH28*) clade genes from core eudicots and non-core eudicots [28, 37]. The above results suggested that *FUL1* and *FUL3* might lose some functions, but these functions might have been retained in *FUL2*. Overexpression of Antirrhinum *DEFH28* (*euFULII* clade genes) into *Arabidopsis* resulted in early flowering, two to four carpel formation, and failure to silique dehiscence [28]. However, ectopic expression of *TeFUL2* into *Arabidopsis* did not affect floral organ identity and silique dehiscence (Fig. 4), which was in line with the study results of *Gerbera GSQUA2*. In general, *TeFUL2* might retain a conserved role in regulating the meristem transition rather than fruit ripping.

Conclusion

In conclusion, marigold has five *AP1/FUL*-like genes, two of which are clustered into *euAP1* clade, and three of which *FUL*-like clade. Based on the results of expression pattern and protein interaction manner, *TeAP1-1* and *TeAP1-2* are likely to play a redundant role as class A genes in regulating sepal and petal identity. Additionally, ectopic expression of *TeAP1-2* resulted in early flowering, implying that *TeAP1-2* might be involved in the regulation of meristem transition. However, three *FUL*-like genes display divergent functions. *TeFUL1* and *TeFUL3* are more functionally close to *euFUL* genes, whereas *TeFUL2* is more functionally close to antirrhinum *DEFH28* belonging to *euFULII* gene. Our results will provide a theoretical basis for the study of class A genes in Asteraceae. Considering the great difference in florescence structure between marigold and *Arabidopsis*, the function analysis of these five *AP1/FUL*-like genes has been insufficient. Therefore, we need to further explore the potential functions of these five genes by transforming them into marigold.

Methods

Plant materials and growth conditions

Marigold (*T. erecta*, M525B-1) is an inbred line with more than 10 generations of continuous self-crossing, and this marigold has only one whorl of ray florets outside capitulum [53]. The plants were grown in the experimental field of Huazhong Agricultural University (lat. 30°28'36.5" N, long. 114°21'59.4" E) under natural conditions. To investigate *AP1/FUL*-like genes expression patterns, the samples of vegetative tissues, different sizes of flower buds, and floral organs in the blooming period were collected as described by Ai et al [54], and were frozen immediately in liquid nitrogen and stored at -80 °C.

For functional analysis of *AP1/FUL*-like genes of marigold, *Arabidopsis* ecotype Columbia (Col-0) plants were used in this study. Plants were grown in chamber at 22 °C under long-day conditions (16 h light, 8 h dark) with 70% relative humidity.

Total RNA extraction, isolation, and bioinformatics analysis of *AP1/FUL*-like genes from marigold

The total RNA of each sample was isolated with PLANTpure kit (Aidlab, Beijing, China) according to the manufacturer's protocol. The quantity and the quality of RNA samples were analyzed by Nano-Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and by running test gels with ethidium bromide staining. The first-strand cDNA was synthesized by the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) with the Oligo-dT primers. Five *AP1/FUL*-like genes selected from the transcriptome sequence (accession number SRP066084) [54] were named *TeAP1-1*, *TeAP1-2*, *TeFUL1*, *TeFUL2*, and *TeFUL3*, respectively. To verify the accuracy of these five gene sequences, the specific primers of *TeAP1-1*-full-F/R, *TeAP1-2*-full-F/R, *TeFUL1*-full-F/R, *TeFUL2*-full-F/R, and *TeFUL3*-full-F/R were designed in the 3' and 5' terminal region by Primer Premier 5 (Supplementary Table S1) and used to clone full length of gene sequences. The PCR amplified fragments were purified and then cloned into *pMD18-T* vector (Takara, Dalian, China). Positive clones were verified by PCR using *M13*-F/R universal primers, and 3-5 of them were selected and sequenced in the Sangon company in Shanghai.

The Open Reading Frame (ORF) of these five *AP1/FUL*-like genes were predicted on line (<https://www.ncbi.nlm.nih.gov/orffinder/>) and were blasted against the NCBI to search for homologous sequences. To identify the conserved motifs of *AP1/FUL* amino acids, the multiple sequence alignment was performed by using the DNAMAN (v.6.0) software (<https://www.lynnon.com>) and BoxShade (https://embnet.vital-it.ch/software/BOX_form.html). A total of 38 *AP1/FUL*-like genes and a *SEP*-like gene *OsMADS1* derived from *Oryza sativa* were downloaded from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) for phylogenetic analysis. Gene accession number was listed in supplementary Table S2. The *O. sativa* *OsMADS1* belonging to *SEP* subfamily was used as outgroup. The construction of phylogenetic tree was based on amino acid alignment using the default settings of MUSCLE in

MEGA (v. 7.0). A phylogenetic tree was constructed by the neighbor-joining (NJ) method with a bootstrap confidence values of 1000 replicates, and distances were calculated with Poisson corrections for multiple substitutions.

Gene expression analysis

The analysis of expression patterns of *AP1/FUL*-like genes in different tissues and different floral bud development phases was performed by quantitative real-time PCR (qRT-PCR). The specific primers were designed within the non-conservative C-terminal region by the Primer Premier 5.0 and were listed in Supplementary Table S1. The specific and unique PCR products for each primer pair were confirmed by 1.2% agarose gel electrophoresis. QRT-PCR was carried out in an optical 384-well plate in the QuantStudio 6 Flex real-time PCR system (Applied Biosystems) with SYBR Primix Ex Taq kit (TaKaRa, Dalian, China) according to manufacturer's instructions. QRT-PCR data were analyzed in the ABI 7500 Detection System (Applied Biosystems, USA). The qRT-PCR products were amplified in 10 µl reaction solution containing 1 µl template of the reaction mixture, 5 µl 2 × SYBR Green Master Mix, 0.2 µl forward primer and reverse primer (10 µmol/µl for primers), and double-distilled water to supply final volume of 10 µl. The PCR was performed as follows: 95°C for 2 min and 40 cycles of 95°C for 10 s and 60°C for 20 s. The expression level of each gene was summarized from three replicates for each sample. The house-keeping gene *beta-actin* was used as an internal control for qRT-PCR and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Yeast two-hybrid assay

The full-length coding sequences of *TeAP1-1*, *TeAP1-2*, *TeFUL1*, *TeFUL2*, and *TeFUL3* were amplified using primers with specific restriction sites and cloned into the activation domain plasmid pGBKT7 (Clontech, Palo Alto, CA, USA) and binding domain plasmid pGADT7 (Clontech, Palo Alto, CA, USA), respectively. All constructs were confirmed by sequencing analyses. The primers were supplied in Supplementary Table S1. The bait and prey constructs of five class B genes (*TeAP3-1*, *TeAP3-2*, *TePI*, *TeTM6-1*, and *TeTM6-2*) and class C+D genes (C: *TeAG1* and *TeAG2*, D: *TeAGL11-1* and *TeAGL11-2*) were previously described by Ai et al [54] and Zhang et al [55], respectively. The full-length sequences, six class E genes (*TeSEP1*, *TeSEP3-1*, *TeSEP3-2*, *TeSEP3-3*, *TeSEP4* and *TeAGL6*) were downloaded from NCBI, and bait and prey recombinants of their proteins were also constructed, respectively. Both bait and prey constructs were transformed into yeast cell strain *AH109* using LiAc method (Clontech) following the Frozen-EZ Yeast Transformation II Kit protocols (Zymo Research Corp, Irvine, CA, USA). Interaction results between bait proteins and empty AD, prey proteins and empty BD, and empty BD and empty AD were used as negative controls. The interaction products between pGBKT7-53 and pGADT7-T vectors were used as positive control. Yeast double transformants were plated on SD medium without tryptophane (Trp) and leucine (Leu) (Sigma, St. Louis, MO, USA, A8056), and medium was incubated at 30 °C for 3-5 days. Positive clones were confirmed by PCR with general primers AD-R/F or BD-F/R (Listed in supplementary Table S1). Three randomly selected positive yeast cells were spotted on the X-α-gal-supplemented selection medium without Leu, Trp, histidine (His), and adenine (Ade). The interaction between the tested proteins was analyzed after incubating the positive yeast cell for 3-5-day at 30 °C.

Vector construction and plant transformation

The full-length coding sequences of *TeAP1-1*, *TeAP1-2*, *TeFUL1*, *TeFUL2*, and *TeFUL3* were amplified by using primer pairs with specific restriction sites (Supplementary Table S1), and the amplification products were ligated to the plasmid under the control of *CaMV35S* promoter. The recombinant plasmids were named *35S:TeAP1-1*, *35S:TeAP1-2*, *35S:TeFUL1*, *35S:TeFUL2*, and *35S:TeFUL3*, respectively. All the recombinant plasmids were introduced into *Escherichia coli* DH5α and tested by sequencing. These plasmids were separately transformed into chemically competent *Agrobacterium tumefaciens* strain GV3101. Wild-type Arabidopsis ecotype Columbia plants were transformed by floral dip method [56]. Transgenic plants of T1 and T2 generations were selected in *kanamycin* and verified by PCR with general forward primer of *35S-F* and gene-specific reverse primers *35S-TeAP1-1-R*, *35S-TeAP1-2-R*, *35S-TeFUL1-R*, *35S-TeFUL2-R*, and *35S-TeFUL3-R* (listed Supplementary Table S1), respectively. The genomic DNA was isolated from the transgenic plants and wild-type Arabidopsis, respectively. The transcript levels of *TeAP1-1*, *TeAP1-2*, *TeFUL1*, *TeFUL2*, and *TeFUL3* were analyzed by semi-quantitative PCR (Semi-PCR). The total RNA of blooming flowers from T1 transgenic lines and wild type plants was isolated and reverse-transcribed with the above-mentioned reagent kit. The Arabidopsis house-keeping gene *EF1α* (*AtEF1α*, AT5G60390) was used as a control. Phenotype changes of transgenic plants of both T1 and T2 generations were analyzed. The main morphological traits of the control and transgenic plants of T2 generations were recorded. The transcript levels of some endogenous genes of the T3 generations were analyzed.

Expression Analysis of Endogenous Genes in transgenic plants

In order to investigate the functional conservation of *AP1/FUL*-like genes in marigold and to reveal the mechanism underlying phenotypic changes of *35S:TeAP1-2* and *35S:TeFUL2* transgenic lines, the transcript levels of some endogenous genes regulated by *AP1* (including *LFY*, *FT*, *SEP3*, *SOC1*, *SVP*, *TFL1*, *AGL24*, and *SPL9*) were analyzed by qRT-PCR [57]. Total RNA was isolated from *35S:TeAP1-2*, *35S:TeFUL2* transgenic (T3) and wild-type Arabidopsis seedlings 10 days old. Reverse transcription and qRT-PCR were performed in the same way as described above. The gene-specific primers were listed in Supplementary table S1.

Abbreviations

AP1, *APETALA1*

FUL, *FRUITFULL*

AGL79, *AGAMOUS-like 79*

DEFH28, *DEFICIENS-homolog28*

qRT-PCR, real-time quantitative RT-PCR

Semi-PCR, semi-quantitative PCR

Declarations

Author Contributions

Conceived and designed the experiments: YHH, CLZ. Performed the experiments: CLZ. Analyzed the data: CLZ, YLS. Contributed reagents/materials/analysis tools: CLZ, LDW, WJW. Wrote the paper: CLZ, YHH. Plant cultivation: CLZ, LDW, WJW, HL. Revised the paper: CLZ, YHH, MZB.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The sequences information of *TeAP1/FUL*-like genes was uploaded to the NCBI (TeAP1-1: Acc. No. MT394170, TeAP1-2: Acc. No. MT394171, TeFUL1: Acc. No. MT394172, TeFUL2: Acc. No. MT394173, and TeFUL3: Acc. No. MT394174).

Declaration of competing interests

The authors declare that no competing commercial or financial interests exist.

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Figures

Figure 1

Alignment of *Tagetes erecta* AP1/FUL-like amino acid sequence from model plants (*Arabidopsis* and *Antirrhinum*) and Asteraceae species. The MADS domain is marked with a bold red line. The I domain is marked with a bold black line. The K domain is marked with a bold yellow line. The C domain is marked with a bold blue line. The FUL protein motif is marked with red box. EuAP1-like proteins contain both an acidic domain (shown in yellow dotted box), and a farnesylation motif (shown in blue dotted box) at their C termini. The whole domain marked red dotted line represents the euAP1 motif.

Figure 2

Phylogenetic tree based on the amino-acid alignment of TeAP1/FUL proteins. The tree was generated with the MEGA v7.0 software, using the neighbor-joining (NJ) method and 1000 bootstrap replicates. The TeAP1-1, TeAP12, TeFUL1, TeFUL2 and TeFUL3 are marked with black stars.

Figure 3

Expression levels of TeAP1/FUL genes in different tissues and organs of marigold. (a) Heatmap of relative expression of TeAP1/FUL genes by qRT-PCR in different tissues and organs. Rt: root; Sm: stems; Le: leaves; FB1-FB4: flower buds were 0-1mm, 2-3mm, 4-5 mm and 6-7mm in diameter, respectively; Re: receptacle; Br: bract; RS: sepal of ray floret; RP: petal of ray floret; RPi: pistil of ray floret; Se: sepal of disk floret; Pe: petal of disk floret; St: stamen of disk floret; Pi: pistil of disk floret; Ov: ovary. (b) Heatmap of TeAP1/FUL genes in the inflorescence of marigold based on the relative expression by qRT-PCR. Blank control: structural model of capitulum in marigold, different colors represent different floral organs.

Figure 4

Abnormal morphology of transgenic *Arabidopsis* plants of constitutively expressed *TeAP1-2* and *TeFUL2* genes. (a) Expression of *TeAP1-2* in seedlings of T1 transgenic lines by semi-RT-PCR; (b) Expression of *TeFUL2* in seedlings of T1 transgenic lines by semi-RT-PCR; (c) The wild-type seedling; (d) Wild-type (left) and early flowering transgenic plant (right) of 35S:*TeFUL2* transgenic lines; (e) The transgenic seedlings with severe curled rosette leaves in 35S:*TeAP1-2* transgenic lines; (f) Wild-type (left) and early flowering transgenic plant (right) of 35S:*TeAP1-2* transgenic lines; (g) The curled cauline leaves of 35S:*TeAP1-2* transgenic lines; WT1: wild-type line 1; WT2: wild-type line 2; L1: 35S:*TeAP1-2* or 35S:*TeFUL2* line 1; WL2: 35S:*TeAP1-2* or 35S:*TeFUL2* line 2; L3: 35S:*TeAP1-2* or 5S:*TeFUL2* line3. a-e, bar=5mm. (h) Statistics for main morphological traits of the control and transgenic plants., * significant difference at $P < 0.05$.

Figure 5

qRT-PCR analysis of endogenous flowering related genes in 10-D-old seedlings of Arabidopsis wild-type and 35S:TeAP1-2 and 35S:TeFUL2 transgenic lines. WT1: wild-type line 1; WT2: wild-type line 2; * expression level of endogenous genes in transgenic plants was 2 times higher or 1/2 lower than that in wild-type plants.

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