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 ripening. 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: sepal 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, vegetative 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 higher plants 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 inferenceshoot 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 BpMADS2 [26] and Pism 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 avene 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 AGAMOUSlike 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 cauleine 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 siliqua indehiscence. Moreover, the overexpression of NFUL 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 inflorescence 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 TeAP1, TeSEP3-1, TeSEP3-2, TeSEP3-3, and TeSEP4 (Table 1, Supplementary Fig. S2b, e).
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, 35S:TeAP1-1, 35S:TeAP1-2, 35S:TeFUL1, 35S:TeFUL2, and 35S:TeFUL3, respectively. The transcript level analysis revealed that five AP1/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**

\[ \text{Table 1} \]

| Interactions of Marigold AP1/FUL proteins detected by yeast two-hybrid assays |
|---------------------------------------------------------------|
| **TeAP1-1** | **TeAP1-2** | **TeFUL1** | **TeFUL2** | **TeFUL3** | **TeAP3-1** | **TeAP3-2** | **TeTM6-1** | **TeTM6-2** |
|---------------------------------------------------------------|
| **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.
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 DEFH29 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 AP1/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 (CLM9) [38] and G. hybrida (GSSQ42) [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 upregulated in 10-day-old seedlings of transgenic lines containing 35S:TeAP1-2 and 35S:TeFUL2 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 sepal 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, palaе 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 euFUL1 (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 (euFULI 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 euFULI 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 NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and by running test gels with ethidium bromide staining. The first-strand cDNA was synthesized by the PrimeScriptTM 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/](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](https://www.lynnon.com)) and BoxShade ([https://embnet.vital-it.ch/software/BOX_form.html](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](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^(-ΔΔCt) 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 pGBK T7 (Clontech, Palo Alto, CA, USA) and binding domain plasmid pGAD T7 (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 pGBK T7-53 and pGAD T7-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-a-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: and gene-specific reverse primers 35S:TeAP1-1-R, 35S:TeAP1-2-R, 35S:TeFUL1-R, 35S:TeFUL2-R, and 35S:TeFUL3-R (Listed in 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 EF1a (ATEF1a, 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 (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
PFG, PETUNIA FLOWERING GENE

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 date 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 of interests

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

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**Figures**

![Figure 1](image)

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, TeAP1-2, 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; 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 line 3. 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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