Identification, characterization and functional analysis of AGAMOUS subfamily genes associated with floral organs and seed development in Marigold (Tagetes erecta)

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Abstract

Background: AGAMOUS (AG) subfamily genes regulate the floral organs initiation and development, fruit and seed development. At present, there has been insufficient study of the function of AG subfamily genes in Asteraceae. Marigold (Tagetes erecta) belongs to Asteraceae family whose unique inflorescence structure makes it an important research target for understanding floral organ development in plants.

Results: Four AG subfamily genes of marigold were isolated and phylogenetically grouped into class C (TeAG1 and TeAG2) and class D (TeAGL11–1 and TeAGL11–2) genes. Expression profile analysis demonstrated that these four genes were highly expressed in reproductive organs of marigold. Subcellular localization analysis suggested that all these four proteins were located in the nucleus. Protein-protein interactions analysis indicated that class C proteins had a wider interaction manner than class D proteins. Function analysis of ectopic expression in Arabidopsis thaliana revealed that TeAG1 displayed a C function specifying the stamen identity and carpel identity, and that TeAGL11–1 exhibited a D function regulating seed development and petal development. In addition, overexpression of both TeAG1 and TeAGL11–1 led to curling rosette leaf and early flowering in Arabidopsis thaliana.

Conclusions: This study provides an insight into molecular mechanism of AG subfamily genes in Asteraceae species and technical support for improvement of several floral traits.

Keywords: Marigold, Floral organs, MADS-box genes, AGAMOUS subfamily genes, Functional analysis

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Background

Flowers are the reproductive organs of a plant, which are regarded as an important morphological innovation in plant evolution. The MADS-box transcription factors are key elements in floral organ identity [1, 2], fruit and seed development [3, 4], and leaf and root development as well [5, 6]. Based on the genetic studies, a well-known ABCDE model is introduced to explain genetic regulation in floral organ determination. In this model, class A and E genes determine the sepals; class A, B, and E genes specify the petals; class B, C, and E genes regulate the stamens fate; class C and E genes control the carpel formation; and class D and E genes direct the ovule development [7, 8]. The AGAMOUS (AG) subfamily genes belonging to MADS-box classes C/D are involved in the regulation of floral organ, floral meristem, and fruit development. Previous reports demonstrated that AG subfamily genes are most likely to be arose from several paraphyletic lineages with multiple whole-genome duplication events (WGDs) in flowering plants, leading to possible subfunctionalization [9–12]. The first WGDs probably result in the generation of AG (class C) lineage and AGAMOUS-LIKE11 (AG11, class D) lineage [10, 11]. The AG lineage then undergoes the second WGDs in core eudicots, resulting in two sub-clades of euAG and PLENA (PLE) [9, 10].

The genes in AG lineage determining the floral meristem development and reproductive organ (stamen and carpel) identity [13] were firstly identified in Arabidopsis (Arabidopsis thaliana, AG) [13] and Antirrhinum (Antirrhinum majus, PLE) [10]. In Arabidopsis, the class C genes prevent the class A genes from functioning in inner whorl floral organs, which is clearly supported by reducing the AG expression in Arabidopsis, resulting in homeotic mutation of reproductive organs such as petal-like stamens and sepal- or petal-like carpels [14–16]. The function of AG lineage genes has been previously characterized in other eudicot species including Chrysanthemum (Chrysanthemum morifolium) [17], Petunia (Petunia hybrida) [18], Populus (Populus trichocarpa) [2], and also in monocot species such as Rice (Oryza sativa) [19].

Then, the FLORAL BINDING PROTEIN7 (FBP7) and FLORAL BINDING PROTEIN11 (FBP11) regulating ovule identity in petunia were determined as class D genes, and these two genes belong to the AG11 lineage [20, 21]. In Arabidopsis, there are three D class genes, named SEEDSTICK (STK; formerly known as AG11), SHATTERPROOT1 (SHP1), and SHATTERPROOT2 (SHP2), which redundantly overlap in their function in regulating ovule development [22–24]. This is verified by the phenotypes of single gene and triple gene mutants. In triple mutant, integuments are transformed into carpelloid structures, and female gametophyte development is interrupted just after megasporogenesis. However, in stk single mutant, only the ovule funiculus is larger than that of the wild type, and the mature seeds are not detached from the silique [24–27]. In addition, the similar phenotype changes have been reported in cultivated Grapevine (Vitis vinifera) [28] and Populus [2], indicating that STK/AG11 has a conservative function in regulating the ovule development.

Asteraceae is one of the largest plant families of flowering plants, which bears a unique head-like inflorescence consisting of hundreds of ray florets and disk florets. For head-like inflorescence, the outer are the sterile ray florets without stamen, and the inner are the fertile disk florets with complete four whorl organs. There are multiple floret morphological traits existing in a single inflorescence, such as fertility, symmetry, and organ fusion. Therefore, Asteraceae provides an unparalleled opportunity to study the genetic regulation of the above-mentioned phenomena. Previous studies have revealed that the auxin and genes LFY and UFI0 are involved in pattern formation of the head flower, and that CYC-like genes regulate the flower symmetry [29–31]. The MADS-box family genes play an important role in the regulation of floral meristem and floral organ development, and their functions in regulating the formation of head-like inflorescence in Asteraceae have been a research hotspot. Until now, the functions of class B genes have been reported in many Asteraceae species [32–34], but there have been few reports on AGAMOUS subfamily genes in Asteraceae species. Up to now, the AG gene functions in Gerbera (Gerbera hybrid) [35], Chrysanthemum [36], and Sunflower (Helianthus annuus) [37] have been reported, and the results reveal that AG function in Asteraceae is conservative in specifying the stamen and carpel identity. Unlike AG lineage genes, the functions of AG11 lineage genes have not been reported in Asteraceae.

Marigold (Tagetes erecta) is a popular ornamental plant. As a member of Asteraceae family, marigold has typical head flower consisting of two morphologically distinct types with ray (sterile) florets in the periphery and disk (fertile) florets in the center. The growth period of marigold is 2–3 months from sowing to flowering. In the evolutionary history of Asteraceae family, marigold undergoes a long evolutionary process, and it is located in a derived Calenduleae clade [38]. These characteristics make marigold an important plant in the study of floral organ development. Here, two AG lineage genes (TeAG1 and TeAG2) and two AG11 lineage genes (TeAG11–1 and TeAG11–2) of marigold were cloned, their expression patterns were investigated, and their subcellular localization was determined. Yeast two-hybrid assay and ectopic transformation were conducted to predict the gene functions in the formation of floret organs and the development of seeds.
Fig. 1  Multiple alignments of the predicted amino acid sequence of TeAG1, TeAG2, TeAGL1–1 and TeAGL1–2. The MADS domain is marked with black line. The I domain is marked with blue line. The C domain is marked with green line. The K domain is marked with red line. The red box in K-domain indicates the conservative amino acid residues. The black boxes in the C-domain indicate the AG motif I and motif II, respectively.
Results

Isolation and molecular characterization of marigold AGAMOUS subfamily genes

To study the functions of AG subfamily genes in marigold, we amplified TeAG1 (991 bp), TeAG2 (837 bp), TeAGL11–1 (735 bp), and TeAGL11–2 (831 bp). Their sequences included the open reading fragment, partial 5′ untranslated region and partial 3′ untranslated region. Multiple alignment with other typical C/D proteins from model plants and Asteraceae species showed that these four proteins were typical MADS-box proteins containing MADS-domain, I-domain, K-domain with conservative amino acid residues, and AG motif I and AG motif II in C-terminal end (Fig. 1). Phylogenetic analysis using

![Phylogenetic tree based on the amino-acid alignment of AG and AGL11 proteins. The tree was generated with the MEGA v6.0 software, using the neighbor-joining (NJ) method and 1000 bootstrap replicates. The TeAG1, TeAG2, TeAGL11–1 and TeAGL11–2 are marked with black stars](image-url)
neighbor-joining (NJ) method showed that these proteins were divided into two main branches of AG and AGL11 lineages corresponding to the MADS-box class C and class D genes, respectively (Fig. 2). The first WGDs was found to have occurred during evolutionary history of marigold. Two C class proteins TeAG1 and TeAG2 were clustered to core eudicot euAG lineage, and two D class proteins TeAGL11–1 and TeAGL11–2 were clustered into AGL11 lineage (Fig. 2). TeAG1 and TeAG2 proteins are putative orthologs of Sunflower HAM45 and HAM59 proteins, respectively, both of which shared amino acid identity as high as over 85% (Table S2). TeAGL11–1 and TeAGL11–2 proteins shared high similarity with their orthologs Sunflowers HaAGL11–1 and HaAGL11–2 proteins with amino acid identity of 70.76 and 65.38%, respectively (Table S3).

Expression patterns of TeAG and TeAGL11 genes

Here, qRT-PCR was conducted to investigate the expression patterns of the four genes in marigold. In order to determine whether genes’ transcripts were stage-dependent or not, we preliminarily detected their expression levels at four stages of floral buds (FB1–FB4). The qRT-PCR analysis showed that the transcript levels of TeAG1, TeAG2, and TeAGL11–2 showed an increase tendency during floral bud development, while the expression level of TeAGL11–1 was very weak and exhibited no significant changes in four floral bud development stages (Fig. 3a, Fig. S1, Table S6).

We further analyzed the expression levels of the four genes in vegetative tissues, and anthesis stage of flower organs (Fig. 3a, b, Fig. S1, Table S6). The results showed that these genes were highly expressed in floral organs.

Fig. 3 Expression levels of TeAG and TeAGL11 genes in different tissues and organs of marigold. (a) Heatmap of relative expression of TeAG1, TeAG2, TeAGL11–1 and TeAGL11–2 genes by qRT-PCR in different tissues and organs. Rt: root; Sm: stems; Le: leaves; FB1–FB4: flower buds were 0–1 mm, 2–3 mm, 4–5 mm and 6–7 mm 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 TeAG1, TeAG2, TeAGL11–1 and TeAGL11–2 genes in the inflorescence of marigold based on the relative expression by qRT-PCR. Blank control: structural model of capitulum in T. erecta, different colors represent different floral organs.
The TeAG1 and TeAG2 were more preferentially expressed in reproductive organs (stamens, pistils and ovaries) than in sepals and petals. Interestingly, the transcript level of TeAG1 was significantly higher in stamens than in pistils and ovaries, while that of TeAG2 was higher in stamens and pistils than in ovaries. The expression patterns of the two AGL11 genes varied in floral organs. TeAGL11–1 had a wide expression region in disk florets, including sepals, petals, stamens, and pistils, whereas this gene was detected only in sepals and pistils of ray flowers, as well as in ovaries. Remarkably, the high expression level of TeAGL11–1 was detected in stamens. In contrast, the TeAGL11–2 was higher expressed in pistils, and ovaries than in stamens, sepals, and petals.

Subcellular localization of TeAG and TeAGL11 proteins
To gain an insight to the subcellular localization of these four genes, four fusion vectors 35S:YFP-TeAG1, 35S:YFP-TeAG2, 35S:YFP-TeAGL11–1, and 35S:YFP-TeAGL11–2 were transiently co-transformed with 35S:RFP-N7 vector into the leaf of tobacco, respectively. The fluorescence signals of these four fusion vectors were mainly observed in the nucleus outside the nucleolus (Fig. 4).

Protein interactions of TeAG and TeAGL11
To confirm the interaction among the four proteins, the yeast two-hybrid experiment was performed. Self-activation of BD constructs was assessed. The results indicated that no autoactivation was observed (Fig. S2a). Although TeAG1 and TeAG2 proteins shared a high similarity in sequences, their interaction manner with other AGAMOUS subfamily proteins were different. As shown in Table 1 and Fig. S2b, the TeAG2 formed heterodimers with TeAG1, TeAGL11–1, and TeAGL11–2, and formed homodimer with itself. However, the TeAG1 only interacted with TeAG2 and TeAGL11–1, but it formed no homodimer. The TeAGL11–1 and TeAGL11–2 showed a limited interactive ability with other AGAMOUS subfamily proteins. Neither homodimer nor heterodimer were formed through the

**Table 1** Interactions of marigold TeAG and TeAGL11 proteins detected by yeast two-hybrid assays

|          | AD-TeAG1 | AD-TeAG2 | AD-TeAGL11–1 | AD-TeAGL11–2 | AD-empty | AD-T7 |
|----------|----------|----------|--------------|--------------|----------|-------|
| BD-TeAG1 | –        | ++       | +            | –            | –        | /     |
| BD-TeAG2 | ++       | ++       | +            | ++           | –        | /     |
| BD-TeAGL11–1 | ++ | –        | –            | –            | –        | /     |
| BD-TeAGL11–2 | –       | –        | –            | –            | –        | /     |
| BD-empty | –        | –        | –            | –            | –        | /     |
| BD-S3   | /        | /        | /            | /            | /        | /     |

Note: ++, strong interaction; +, weak interaction; –, no interaction; / not determined
Fig. 5 (See legend on next page.)
interaction between AGL11–1 and AGL11–2. TeAGL11–1 and TeAGL11–2 interacted with TeAG2 unidirectionally. In addition, TeAGL11–1 strongly interacted with TeAG1, while TeAGL11–2 had no ability to interact with TeAG1.

**Dramatic effect of overexpression of TeAG1 in Arabidopsis on sepal and petal identity**

To further study the functions of TeAG1 and TeAG2, functional analyses were performed using ectopic expression in Arabidopsis. Eighteen 35S:TeAG1 transgenic lines and twenty-three 35S:TeAG2 transgenic lines were obtained. Transcript levels of TeAG1 and TeAG2 were further analyzed by semi-quantitative RT-PCR with the flower cDNA as templates (Fig. S3a, b). The 35S:TeAG2 transgenic lines did not show any evident morphological changes, compared with the wild type. However, five of the 35S:TeAG1 transgenic lines displayed severe phenotypes (named Sl-TeAG1), seven showed weak phenotypes (named Wl-TeAG1), and six had no remarkable phenotypic changes. Compared with the wild type, Sl-TeAG1 and Wl-TeAG1 transgenic lines displayed early flowering, rosette leaf curling, and small plant size (Fig. 5a, b, c, h, l, Table 2). Furthermore, only in Sl-TeAG1 transgenic lines, normal sepal and petal formations were disrupted (Fig. 5d, e, f, g, Table 2). Homeotic conversion of sepal to pistil-like structure was detected at the top margin of sepal. Similar conversion of petal to stamenoid structure was observed (Fig. 5e, g, Table 2). The sepals, petals, and stamens retained at base of silique (Fig. 5, k). The silique were more bumpy and smaller, and seed setting rate was lower than those of normal siliques in wild-type lines (Fig. 5, Table 2, S4).

The four whorls of floral organs from Sl-TeAG1 lines and wild-type lines were observed by SEM (i, j, k, l, 6). Compared with the sepals structure of wild type (Fig. 6a, b), a cluster of papilla-like cells occurred at the top of carpelloid sepals in transgenic lines (Fig. 6a), and the rough cells with stomata in normal sepalst (Fig. 6a) were replaced by the smooth rectangle epidermis cells in adaxial surface of carpelloid sepals (Fig. 6d). In addition, the abaxial epidermis cells were converted from the normal rough types with stomata (Fig. 6b) into irregular smooth convex structure (Fig. 6e), which was similar to the epidermal cell structure of style (Fig. 6p). The second whorl of floral organs in the transgenic plants were converted from stamen-like petals with an anther-like structure (Fig. 6f) consisting of squamous cells (Fig. 6i). Furthermore, the epidermal cells in the lower region of the stamen-like petals were changed from a rough spindle structure (Fig. 6g) to a smooth filament-like structure (Fig. 6k, n). No obvious change was found in stamens and carpels in transgenic lines (Fig. 6u). In general, the overexpression of TeAG1 in Arabidopsis resulted in homeotic mutation of flower organs such as carpelloid sepals and stamen-like petals.

Since the phenotypes of ectopic expression of TeAG1 were visually focused on sepal and petal identity, the AP1, AP3, PI, AG, and STK genes in Arabidopsis were selected to detect whether their transcriptional levels were changed, based on the ABCDE model. The results (Fig. 6v, Table S7) showed that the transcript levels of PI, AG and STK were significantly up-regulated in transgenic line Sl-TeAG1, while that of AP1 was remarkably down-regulated, suggesting that ectopic expression of TeAG1 (class C gene) might suppress the expression levels of AP1 (class A gene) in Arabidopsis. No

### Table 2 Mutant morphological traits of the transgenic plants via overexpression TeAG1 and TeAGL11–1

| Rosette leaf             | Flowering time | Sepal                  | Petal                  | Stamen                  | Pistil                  | Silique                  |
|-------------------------|----------------|------------------------|------------------------|-------------------------|-------------------------|--------------------------|
| Sl-TeAG1                | Less and curled rosette leaves | Early flowering | Carpelloid sepals | Stamen-like petals | –            | –                         | Bumpy and small, low seed setting rate |
| Wl-TeAG1                | Less and curled rosette leaves | Early flowering | –                      | –                      | –                       | –                         |
| Sl-TeAGL11–1           | Less and curled rosette leaves | Early flowering | Curled petal          | –                      | –                       | Bumpy and small, almost seedless |
| Wl-TeAGL11–1           | Less and curled rosette leaves | Early flowering | Curled petal          | –                      | –                       | Bumpy and small, low seed setting rate |

Note: –, no morphological change compared with wild-type lines
Fig. 6 Scanning electron micrograph of floral organs and expression levels of genes related to floral organ development and seed formation between TeAG1 transgenic Arabidopsis and wild-type lines. (a, u) Scanning electron micrograph of floral organs between SI-TeAG1 transgenic Arabidopsis and wild-type lines; (a, b) Adaxial (a) and abaxial (b) epidermis cells of sepals of wild-type; (c) The papilla-like cells at the top of the carpelloid sepals of transgenic plant; (d, e) Adaxial (d) and abaxial (e) epidermis cells of carpelloid sepals of transgenic plant; (f, g) The epidermal cells at the upper (f) and bottom portion (g) of the petals of wild-type; (h) The petals transformed into anther-like structure in transgenic lines; (i, k) The epidermal cells at the top (i) and bottom (k) part of anther-like structure; (l) The anther structure of wild-type lines; (m, n) The epidermal cells of anther (m) and filament (n) in wild-type lines; (o) The papilla cells of stigma in wild-type lines; (p) the epidermal cells of style in wild-type lines; (q) The anther structure of transgenic plant; (r, s) The epidermal cells of anther (r) and filament (s) in transgenic plant; (t) The papilla cells of stigma in transgenic plant; (u) The epidermal cells of style in transgenic plant. (v) Expression levels of genes related to floral organ development and seed formation in control and transgenic Arabidopsis flowers by qRT-PCR analysis. WT1: wild-type line 1; WT2: wild-type line 2; WL1: Wl-TeAG1 line 1; WL2: Wl-TeAG1 line 2; SL1: SI-TeAG1 line 1; SL2: SI-TeAG1 line 2. * expression level of endogenous genes in transgenic plants was 2 times higher or 1/2 lower than that in wild-type plants.

Fig. 7 Abnormal morphology of transgenic Arabidopsis plants containing 35S:TeAGL11–1 and expression levels of genes related to floral organ development and seed formation. (a) The wild-type seedling. (b, c, e, f, h, i, j) Phenotype of SI-TeAGL11–1 lines. (b) The transgenic seedlings with severely curled rosette leaves. (c) Wild-type and 35S:TeAGL11–1 transgenic plant with early flowering. (d, g) Wild-type flowers. (e, f, h) Transgenic flowers. (i) Wild-type (left) and transgenic plant (right) with smaller plants. (j, k) The siliques are almost seedless and short with unabsceded sepals. (j) compared to those from wild-type. (k) Phenotype of WI-TeAGL11–1 lines. (l) The transgenic seedlings with severely curled rosette leaves. (m) Wild-type (left) and transgenic plant (right) with smaller plants. (n) Transgenic flowers. (o) The smaller siliques (right). se: sepal; pe: petal; st: stamen; pi: pistil, a-c, i, l, m, o, bar = 5 mm; d-h, j, k, n bar = 1 mm. (p) Statistics for main morphological traits of the control and transgenic plants, * significant difference at P < 0.05. (q) Expression levels of genes related to floral organ development and seed formation in control and transgenic Arabidopsis flowers by qRT-PCR analysis. WT1: wild-type line 1; WT2: wild-type line 2; WL1: Wl-TeAGL11–1 line 1; WL2: Wl-TeAGL11–1 line 2; SL1: SI-TeAGL11–1 line 1; SL2: SI-TeAGL11–1 line 2. * expression level of endogenous genes in transgenic plants was 2 times higher or 1/2 lower than that in wild-type plants.
significant difference in the transcript level of AP3 (B class gene) was observed between wild type and SI-TeAG1 lines (Fig. 6v, Table S7). Compared with the results observed in SI-AG1 line, similar change tendency and mild expression level changes of AP1, PI, AP3, AG and STK were detected in WI-AG1 lines (Fig. 6v, Table S7).

Effect of ectopic expression of TeAGL11–1 in Arabidopsis on petals and seed development
In order to investigate the function of TeAGL11–1 and TeAGL11–2, the two genes were also ectopically expressed in Arabidopsis. We obtained twenty-one 35S:TeAGL11–1 transgenic lines with seven severe phenotype lines (SI-TeAGL11–1), ten weak phenotype lines (WI-TeAGL11–1), and four lines without phenotypic changes. We obtained forty-six 35S:TeAGL11–2 transgenic lines without any evident phenotypic alteration, compared with the wild-type lines. Transcript levels of TeAGL11–1 and TeAGL11–2 were further analyzed by semi-quantitative RT-PCR with flower cDNA as templates (Fig. S3c, d). The overexpression of TeAGL11–1 in Arabidopsis resulted in upward and inward curling of rosette leaves, obvious petal curling, early flowering, and small plant size (Fig. 7a, b, c, d, e, f, g, h, i, l, m, n, p, Table 2). In SI-TeAGL11–1 lines, the siliques were almost seedless and smaller than those in wild-type lines, and the sepals were not detached from siliques (Fig. 7), k, p, Table 2, S5). However, in WI-TeAGL11–1 lines, only bumpy and small siliques were observed (Fig. 7), k, o, p, Table 2).

To explore whether the phenotype was affected by the expression of the endogenous gene API, PI, AP3, AG, and STK regulating the floral organs and ovule development, the qRT-PCR analysis was performed in the two severe phenotype lines, two weak phenotype lines, and two wild-type lines. As shown in Fig. 7q and Table S8, the transcript levels of API and AP3 exhibited no significant difference among the six samples. The expression level of PI was obviously down-regulated in both SI-TeAGL11–1 and WI-TeAGL11–1 lines, but the expression level of STK was lower in SI-TeAGL11–1 lines than in WI-TeAGL11–1 lines, suggesting that the seedless phenotype in SI-TeAGL11–1 lines might be related to the downregulation of STK.

Expression profile analysis of endogenous genes related to early flowering and curled leaves
We also detected the expression level of endogenous genes related to flowering time (API, FT, LFY, SOC1, AG and SEP3) and curled leaves (GRF1, GRF2, GRF5, TCP3, TCP18, TCP20, and ARF2), when the transgenic and wild-type seedlings were 10 days old. As shown in Fig. 8, the expression levels of API, FT, SOC1, AG and SEP3 were significantly higher in all the 35S:TeAG1 transgenic seedlings than in wild-type seedlings. However, the expression level of the LFY was remarkably increased in SI-AG1 lines, and slightly increased in WI-AG1 lines (Fig. 8a, Table S9). Transcripts analysis of leaf development-related genes in 35S:TeAG1 transgenic seedlings indicated that expression levels of ARF2, GRF1, GRF5, TCP20 and TCP3 had no significant difference among the six samples (Fig. 8a, Table S9), whereas the expression levels of GRF2 and TCP18 were obviously higher than those in wild-type lines, suggesting that high expression of GRF2 and TCP18 might have caused the leaf curling.

In WI-TeAGL11–1 lines and SI-TeAGL11–1 lines, API, AG, FT and SEP3 were strongly up-regulated. The expression levels of SOC1 were increased in SI-TeAGL11–1 lines, and no significant difference in the expression level of SOC1 was observed between WI-TeAGL11–1 lines and wild-type lines (Fig. 8b, Table S10). The results suggested that API, AG, FT and SEP3 might contribute

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**Fig. 8** qRT-PCR analysis of endogenous flowering and leaf development-related genes in 10-day-old seedlings of the wild-type, 35S:TeAG1 and 35S:TeAGL11–1 transgenic lines of Arabidopsis. (a) qRT-PCR analysis of endogenous flowering and leaf development-related genes in 35S:TeAG1 transgenic lines. (b) qRT-PCR analysis of endogenous flowering and leaf development-related genes in 35S:TeAGL11–1 transgenic lines. * expression level of endogenous genes in transgenic plants was 2 times higher or 1/2 lower than that in wild-type plants.
to the early flowering in 35S:TeAG11–1 transgenic lines. Expression levels of leaf development-related genes showed a complex expression pattern in 35S:TeAGL11–1 transgenic lines. The transcript level of TCP18 in Sl–TeAGL11–1 lines was up-regulated and obviously higher than that in wild-type lines, and slightly increased in WI–AG11–1 lines (Fig. 8b). ARF2 and TCP3 were down-regulated in Sl–TeAGL11–1 lines, and TCP3 was significantly decreased in WI–TeAGL11–1 lines. Expression levels of GRF1, GRF2, GRF5 and TCP20 had no significant changes, compared with those in wild type (Fig. 8b, Table S10).

Discussion
Evolutionary conservation and diversity of marigold AGAMOUS-like genes
In flowering plants, numerous MADS-box genes are important regulators for plant growth and development. The AG subfamily MADS-box genes are involved in regulating floral meristem and fruit development, and specifying reproductive organ identity in many species. Previous studies demonstrated that AG subfamily genes originated from several paraphyletic lineages in flowering plants, most of which probably arose from multiple whole-genome duplication events (WGDs) in flowering plants during long-term evolutionary process [9–12]. AG and AGL11 lineages might arise from the first WGDs [10, 11]. The AG lineage underwent second WGDs in lower eudicots, resulting in the generation of two subclades, namely, euAG and PLE [9, 10]. Recently, duplication events of PLE lineages observed in Arabidopsis [23] and tomato [39] made their function different from that of euAG. In marigold, TeAG1, TeAG2, TeAGL11–1, and TeAGL11–2 were grouped into AG subfamily proteins. These four proteins contained highly conservative AG motif I and AG motif II in the C-terminal regions (Fig. 1) [10, 40]. According to our phylogenetic analysis, TeAG1 and TeAG2 belonged to euAG lineages, TeAGL11–1 and TeAGL11–2 were classified into AGL11 lineages, whereas no PLE lineage proteins were found in marigold transcriptome data (Fig. 2). Dreni and Kater’s proposal suggests that PLE lineage proteins might lost in Asteraceae family, which might be related to dry, indehiscent seed in Asteraceae [9].

Specific expressions of marigold AGAMOUS-like genes in inner two whorls of floral organs and ovules
In marigold, we found that the expressions of four AG subfamily genes were highly tissue-specific. The expression patterns of TeAG1 and TeAG2 (C class genes) in floral organs were consistent with those reported in Gerbera [35]. These two genes were preferentially expressed in reproductive organ and ovules (Fig. 3a, b, Fig. S1a, b), indicating that TeAG1 and TeAG2 might play an important role in regulating the reproductive organ development and specifying ovary identity. Furthermore, TeAG1 and TeAG2 were slightly expressed in sepals and petals, suggesting TeAG1 might be involved in regulating four-whorl floral organ developments. Unlike TeAG2, TeAG1 was highly expressed in pistils and ovaries, indicating their functional differentiation. Similar to TeAG1 and TeAG2 expression pattern, TeAGL11–1 and TeAGL11–2 (D class genes) were highly expressed in pistils and ovaries (Fig. 3a, b, S1a–d), suggesting that both C class and D class genes in marigold might be involved in regulating pistil and ovary development in the evolutionary process. The expression patterns of TeAGL11–1 and TeAGL11–2 were clearly different from each other. TeAGL11–1 was highly expressed in disk flower organs including stamens, sepals, and ovaries, while TeAGL11–2 was highly expressed in pistils and ovules (Fig. 3a, b, S1c, d). Combining the above finding with the fact of low amino acid sequence identity between TeAGL11–1 and TeAGL11–2 proteins, we speculated that these two genes might have various functions in regulating flower development. The expression patterns of TeAGL11–1 and TeAGL11–2 differed from those of FBP7, FBP11, or STK whose expressions were limited to seed and ovule [41, 42]. Thus, the specific expression of D class genes in marigold suggested some extra functions of TeAGL11–1 or TeAGL11–2 in addition to regulating seed development.

Conservative role of TeAG1 in specifying stamen and carpel identities
Compared to previous studies in other plants, the similar phenotypic changes of flower organs and seed development were observed in 35S:TeAG1 transgenic lines. The ectopic expression of TeAG1 in Arabidopsis resulted in homeotic conversion of sepals into pistillolid structures and that of petals into stamenoid structures (Figs. 5e, g, 6e, f, h, i, Table 2), which was consistent with the results of ectopic expression of C class genes in Chrysanthemum [43] and Carnation (Dianthus caryophyllus) [44]. For example, ectopic expression of CDM37 (C class gene) in Chrysanthemum resulted in the conversion of petals into antheroid structure, and that of sepals into pistillloid tissues [43]. Based on ABCDE model, ectopic expression of functional class C orthologue would suppress the A class homeotic genes expression in the first and second whorls, leading to the transformation of sepals and petals into carpels and stamens, respectively. The analysis of the expression pattern of endogenous genes AP1, AP3, PI, AG, and STK revealed that TeAG1 developed activities to repress A class genes (Fig. 6v). Based on the specific expression of TeAG1 and the noticeable phenotypic alteration of transgenic plants, we
speculated that TeAG1 might regulate the stamen and carpel developments.

**Function of TeAGL11–1 in seed and petal development**

The phenotypical changes caused by TeAGL11–1 overexpression in Arabidopsis suggested that D class genes might have conservative and divergent function. The almost seedless phenotype in 35S:TeAGL11–1 lines was similar to that in other plant species in which D class gene expression were suppressed, suggesting that the function of class D genes in transgenic Arabidopsis is partially inhibited. In Arabidopsis, the triple stk/shp1/shp2 abolished the development of ovule and seed [24]. In Petunia, simultaneous down-regulation of FBP7 and BP11 formed the seedless phenotype [18, 20, 41]. In addition to model plants, similar phenotypes resulting from down-regulation the D class genes were also observed in other species [2, 3]. The down-regulation expression of endogenous gene STK also supported this phenotypic change (Fig. 7q), suggesting that ectopic expression TeAGL11–1 in Arabidopsis led to co-suppression phenotype. Co-suppression phenomenon was observed in other ectopic expression lines of MADS-box genes. For example, overexpression HAM5as and HAM59 (C class) genes from Sunflower in tobacco downregulated the expression level of endogenous C class genes, further resulting in the conversion of stamen into petals [45]. In addition, the curled petals in 35S:TeAGL11–1 lines were observed, but this phenotype was different from those resulting from over-expression of D class genes in Arabidopsis and tomato where the sepals were transformed into carpeloid organs bearing ovules [42] or a fleshy organ [3]. On one hand, the different phenotypes might be attributed to the functional difference between TeAGL11–1 and STK (Arabidopsis) or SI-AGL11 (tomato, Solanum lycopersicum). On the other hand, different gene regulatory networks in marigold and Arabidopsis might lead to their difference in heterologous transformation phenotype. The decrease in PI transcript level might explain the petal curling (Fig. 7q).

**Functional conservation and diversity of TeAG1 and TeAGL11–1 genes**

The early flowering phenotype was observed in transgenic plants containing 35S:TeAG1 and 35S:TeAGL11–1 constructs (Figs. 5c, l, 7c, p, Table 2), which was consistent to the phenotype of transgenic Arabidopsis with the overexpression of AG or STK [42, 46]. In addition, overexpression TeAG1 and TeAGL11–1 in Arabidopsis led to rosette leaf curling (Figs. 5b, 7b, l, Table 2). Similar phenotype was also observed in transgenic plants in which C or D class genes were ectopically expressed [47, 48]. Remarkably, in Arabidopsis, ectopic expressions of TeAG1 and TeAGL11–1 resulted in short siliques, low seed setting rate, and sepal retainment at the bottom of mature siliques. This result was consistent with the finding reported in Cymbidium [49]. The expression analysis of endogenous gene STK suggested that short siliques with low seed setting rate might be correlated with the increased STK expression level in 35S:TeAG1 lines, while the short siliques with low seed setting rate might be associated with the decreased STK transcript level in TeAGL11–1 transgenic lines. In general, these results supported our prediction that ectopic expression of C and D class genes might have similar functions in regulating the floral development in Arabidopsis [50]. In addition, the divergent function between TeAG1 and TeAGL11–1 were also observed. Compared with phenotypic changes of 35S:TeAG1 transgenic plants, the overexpression of TeAGL11–1 caused the petals to curl inwards, but it could not induce homologous alteration of floral organs (Fig. 7e, f, h, n, Table 2).

Previous studies indicated that the early flowering and curled leaves caused by overexpression of MADS-box genes might be usually associated with expression changes of upstream or downstream genes [51–54]. In this study, the transcript levels of endogenous genes regulating flowering time and curled leaves in 35S:TeAG1, 35S:TeAGL11–1 lines and wild-type plants were analyzed. The results indicated that the transcript levels of API, FT, AG, and SEP3 were up-regulated in 35S:TeAG1 and 35S:TeAGL11–1 lines. Previous report on soybean (Glycine max) indicated that the early flowering was related to the up-regulated transcript levels of API, FT, and SEP3 [55]. Taken together, the increased expression levels of API, FT, AG, and SEP3 might promote the formation of flowers and early flowering in transgenic plants (Fig. 8a, b). The analysis of gene expression and phenotypic changes revealed that the leaf curling in 35S:TeAG1 transgenic lines might be correlated with the transcript changes of GRF2 and TCP18 (Fig. 8a), which was consistent with results that TCP [56] and GRF [57] could regulate leaf development. In addition, the down-regulation of ARF2 and TCP3 and up-regulation of TCP18 might result in the curled leaves in 35S:TeAGL11–1 lines (Fig. 8b). The expression of LFY was evidently increased in transgenic lines with higher expression level of TeAG1, suggesting the expression levels of LFY might be related to the transgene expression.

**Conclusions**

This study reveals that TeAG1 and TeAGL11–1 regulate the development of floral organs, seeds and vegetative tissues, and that TeAG2 and TeAGL11–2 might lose their ability to regulate the floral organ and ovules developments, or they need bind with other MADS-box genes to regulate the flower development and ovules.
formation. Our results expand the understanding of the development of stamens, pistils and ovules in Asteraceae family, and provide technical support for the subsequent creation of horticultural traits. However, further situ hybridization analysis and homologous transformation phenotyping experiments are still needed to explore the accurate expression regions and potential functional mechanism of these four genes.

Methods

Plant materials and growth conditions

An inbred line M525B-1 of marigold was derived from 10 generations of continuous self-crossing of M525B which is a male fertile type plant from the two-type (male sterile/male fertile) line M525AB isolated by He [58] in our lab. M525B-1 had one whorl of ray florets in the periphery of the capitulum. They were grown in the natural conditions in fall 2016 at Huazhong Agricultural University, Wuhan, Hubei Province, China (lat. 30°28′ 36.5″ N, long, 114°21′59.4″ E).

The seeds from Arabidopsis thaliana accession Columbia (Col-0) plants used for the functional analysis were first sterilized and cultured on the agar containing Skoog (MS) and 1/2× Murashige at 4°C for 2 days. After 10 days, the seedlings were transplanted to growth chamber under long-day conditions (16 h light/8 h dark) at 22/20°C day/night temperature with 60% relative humidity until genetic transformation.

Total RNA extraction, isolation and bioinformatics analysis of AG and AGL11 genes from marigold

Various vegetative tissues, floral buds in four developmental stages, and floral tissues from marigold were collected and quickly frozen in liquid nitrogen as described by Ai et al. [33]. There were four different sizes of flower buds: FB1: 0–1 mm in diameter, the ray floret primordium and the outermost disk floret primordium were formed; FB2: 2–3 mm in diameter, the sepals and petals of the ray florets and the outmost disk florets were developed; FB3: 4–5 mm in diameter, the stamens of the outmost disk florets were developed; and FB4: 6–7 mm in diameter, the pistils of the ray florets and the outmost disk florets were developed. Total RNA of each sample was isolated by PLANTpure kit (Aidlab, Beijing, China) according to the manufacturer’s protocol. The quality and quantity of RNA were tested by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The first-strand cDNA was synthesized by using PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Based on the transcriptome sequence (accession number SRP066084) [33], four AG subfamily genes were selected and named TeAG1 (comp38613_c0, comp38613_c1), TeAG2 (comp68705_c0), TeAGL11–1 (comp199520_c0), and AGL11–2 (comp50841_c0). The full length of TeAG1, TeAG2, TeAGL11–1, TeAGL11–2 were cloned with the specific primers TeAG1-Full-F/R, TeAG2-Full-F/R, TeAGL11–1-Full-F/R, and TeAGL11–2-Full-F/R, respectively (Supplementary Table S1). The PCR amplified products were purified and cloned into pMD18-T vector (Takara, Dalian, China). Positive clones (3–5 replicates) were confirmed by sequencing in the Sangon company in Shanghai.

Multiple sequence alignment of TeAG1, TeAG2, TeAGL11–1, TeAGL11–2 proteins with other known C/D class genes was performed by using the DNAman (v.6.0) software (https://www.lynnnon.com). To analyze the phylogenetic relationships of C/D class genes, a number of AG and AGL11 genes were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Full-length amino-acid sequences were first aligned using the default settings in MUSCLE implemented in MEGA (v.6.0), and then adjusted manually with the reference alignment provided by Zahn et al. [59]. Phylogenetic tree was constructed by MEGA (v.6.0) software by using the neighbor-joining (NJ) method under 1000 bootstrap replicates.

Quantitative real-time PCR for expression analysis

Expression levels of the C/D class genes in different tissues (roots, tender stems, and fresh leaves), floral tissues (sepals, petals, and pistils of ray and disk florets, stamens of disk florets, receptacles, bracts, and ovaries), and different sizes of flower buds in marigold were analyzed by quantitative real-time PCR (qRT-PCR). Gene-specific primers (Supplementary Table S1) for qRT-PCR were designed within the non-conservative C-terminal region by the Primer Premier 5.0. QRT-PCR experiments were performed, as described in previous study [33]. The expression levels of these four genes per sample were repeated three times. In these experiments, the reference gene beta-actin was used for normalization and the relative expression levels were calculated using the 2^ΔΔCt method. The data were analyzed using the TBtools software and normalized using row scale.

Subcellular localization of AG and AGL11 proteins from marigold

The coding sequences of TeAG1, TeAG2, TeAGL11–1, TeAGL11–2 with removed stop codon were amplified and introduced into a pYellow vector under the control of the 3SS CaMV promoter to generate fusion vectors 3SS:YFP-TeAG1, 3SS:YFP-TeAG2, 3SS:YFP-TeAGL11–1, 3SS:YFP-TeAGL11–2. The primers were listed in supplementary Table S1. The YFP signal could be observed in both cytoplasm and nucleus when transfected with 3SS:YFP vector. The 3SS:YFP empty vector was used as a
negative control, and the 35S:RFP-N7 vector including the N7 nuclear targeting signal was used as a nucleus control. The control vectors and recombinant vectors were transformed into agrobacterium tumefaciens strain GV3101, respectively. Then, the recombinant vectors and the nucleus control 35S:RFP-N7 or the pYellow-YFP empty and nucleus control 35S:RFP-N7 in tumefaciens strain GV3101 were simultaneously injected into tobacco (Nicotiana benthamiana) leaves, separately [60]. After incubation at 25 °C for 48 h, the YFP fluorescence signal and RED fluorescence signal in tobacco leaves were detected by confocal microscopy (Leica, TCS SP2, Wetzlar, Germany).

Yeast two-hybrid assay
The cDNA of TeAG1, TeAG2, TeAGL11–1, and TeAGL11–2 were amplified using primers with specific restriction sites (Table S1). The PCR fragments were recombined with the plasmid pGBK7 and pGADT7 (Clontech, Palo Alto, CA, USA), respectively. The PGAD T7 and PGBK7T7 recombinant vectors were co-transformed into strain AH109 by the LiAc/DNA/PEG method following the the Frozen-EZ Yeast Transformation II Kit protocols (Zymo Research Corp, Irvine, CA, USA). The transformants were selected on selection medium lacking leucine (Leu) and tryptophan (Trp). The autoactivation and toxicity of BD clones were tested by co-transforming with empty AD plasmid. Simultaneous transfer of empty AD and empty BD vectors or pGBK7–53 and pGADT7-T vectors into AH109 was set as the negative control or positive control. The positive yeast cells were verified by PCR with AD-F/–R and BD-F/–R (Supplementary Table S1). Then positive yeast cells were further tested by spotting assays on X-a-gal supplemented medium without Leu, Trp, histidine (His), and adenine (Ade). The result was observed after incubation of plates at 30 °C for 3–5 days. In this study, the positive signal of interaction ability was scored if any direct interaction caused yeast to grow on the selection plate.

Vectors construction and ectopic expression in Arabidopsis
The coding regions of TeAG1, TeAG2, TeAGL11–1 and TeAGL11–2 were amplified using specific primers with restriction sites (Supplementary Table S1), and then were cloned into p2300 vector under the control of the CaMV 35S promoter. The obtained fusion vectors were named 35S:TeAG1, 35S:TeAG2, 35S:TeAGL11–1, and 35S:TeAGL11–2. These fusion vectors were introduced into Escherichia coli DH5a, and tested by PCR. These fusion vectors were then transformed into Agrobacterium tumefaciens strain GV3101. These resultant constructs were transformed into wild-type Arabidopsis ecotype Columbia plants by using the floral dip method [61]. Transformants were selected on a medium containing 50 μg ml⁻¹ kanamycin, and were further verified by PCR and semi-quantitative RT-PCR (semi-RT-PCR) analysis with the primers 35S-F, 35S:TeAG1–R, 35S:TeAG2–R, 35S:TeAGL11–1–R, 35S:TeAGL11–2–R, qRT-TeAG1–F/ R, qRT-TeAG2–F/R, RT-TeAGL11–1–F/R, RT-TeAGL11–2–F/R, and AtEF1α–F/R (Supplementary Table S1). Phenotype changes of T1 and T2 transgenic lines were analyzed.

Scanning electron microscopy
The blooming flowers of 35S:TeAG1 and wild-type plants were collected and fixed overnight in 2.5% (v/v) glutaraldehyde at 4 °C. Then the flowers were dehydrated every 15 min with an ethanol series (30–100%). Ethanol was replaced with isoamyl acetate/ethanol (1/1) and isoamyl acetate for every 10 min. The dried samples were critical point dried (CPD 020; Balzers Union, http://www.bal-tec. com/), sputter-coated with gold (NanotechSEMPrep II sputtercoater, Nanotech Ltd., Prestwick, UK), and fixed on the specimen stubs by double-sided tape. The samples were examined and photographed using a LEO 435VP scanning electron microscope (LEO Electron Microscopy Ltd., http://www. smt.zeiss.com/).

Expression analysis of endogenous genes in transgenic plants
To further analyze the mechanism of floral organs and seed phenotype changes in 35S:TeAG1 and 35S:TeAGL11–1 transgenic plants, the expression levels of AP1, AP3, PI, AG and AGL11 of Arabidopsis were detected by qRT-PCR. Total RNA of blooming flowers from 35S:TeAG1, 35S:TeAGL11–1 transgenic plants and wild-type plants was isolated by using PLANTpure (Aidlab, Beijing, China), and then reverse-transcribed by using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Arabidopsis EF1α (AtEF1α, AT5G60390) was used as housekeeping gene. In addition, the transcript levels of some endogenous genes related to flowering time (FT, SOC1, LFY, AP1, SEP3, AG) [54] and leaf development (GRF1, GRF2, GRF5, TCP3, TCP18, TCP20, and ARF2) were analyzed [62], when the transgenic and wild-type seedlings were 10 days old. All the samples of 10-day-old seedlings were collected at the same time under light conditions. The primers were listed in Supplementary Table S1.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-020-02644-5.

Additional file 1: Table S1. Sequence of primers.
Additional file 2: Table S2. Amino acid sequence alignment of C class proteins.
Additional file 3: Table S3. Amino acid sequence alignment of D class proteins.

Additional file 4: Fig. S1. Expression levels of TeAG1, TeAG2, TeAGL11−1 and TeAGL11−2 in different tissues and organs. Rf: root; Ss: stems; Le: leaf; Fb1−Fb4: flower buds 0−1 mm, 2−3 mm, 4−5 mm and 6−7 mm in diameter, respectively; Re: receptacle; Br: bract; Rs: sepals of ray floret; Rp: petals of ray floret; Pp: pistil of ray floret; Se: sepals of disc floret; Pd: petals of disc floret; St: stamens of disc floret; Pl: pistil of disc floret; Ov: ovary.

Additional file 5: Fig. S2. Interactions of TeAG and TeAGL11 proteins of marigold by yeast two−hybrid assays. (a) Assay of self−activation and auto−activation of AD and BD constructs. (b) Ten−fold serial dilutions from 10−1 to 10−6 of each culture were spotted on the selected 3D−Leu−Try−His−Ade plates with X−a−gal.

Additional file 6: Fig. S3. Expression of TeAG1, TeAG2, TeAGL11−1 and TeAGL11−2 in seedlings of T1 transgenic lines by semi−quantitative RT−PCR. (a−1) 35S:TeAG1 transgenic lines. (b−1) 35S:TeAG2 transgenic lines. (c−1) 35S:TeAG1−1 transgenic lines. (d−1) 35S:TeAG1−2 transgenic lines. WT: wild type line; Sl: strong phenotypic line; Wk: weak phenotypic line; L: transgenic line. (a−2, b−2, c−2, d−2) the constitutive gene is Arabidopsis keeping−house gene Sl−TeAG11−1. (DOCK 654 kb).

Additional file 7: Table S4. Statistics for seed setting rate between control and Sl−TeAG11 transgenic lines.

Additional file 8: Table S5. Statistics for seed setting rate between control and Sl−TeAG11−1 transgenic lines.

Additional file 9: Table S6. Raw data of Ct value in qRT−PCR for expression levels of TeAG1, TeAG2, TeAGL11−1, and TeAGL11−2 in different tissues and organs of marigold.

Additional file 10: Table S7. Raw data of Ct value in qRT−PCR for expression levels of AP1, AP3, PI, AG, and STK in flowers from 35S:TeAG1 transgenic lines and wild−type Arabidopsis.

Additional file 11: Table S8. Raw data of Ct value in qRT−PCR for expression levels of AP1, AP3, PI, AG, and STK in flowers from 35S:TeAG1−1 transgenic lines and wild−type Arabidopsis.

Additional file 12: Table S9. Raw data of Ct value in qRT−PCR for expression levels of AP1, AP3, PI, AG, and STK in seedlings of 35S:TeAG1 transgenic lines and wild−type Arabidopsis.

Additional file 13: Table S10. Raw data of Ct value in qRT−PCR for expression levels of AP1, AP3, PI, AG, and STK in seedlings of 35S:TeAG1−1 transgenic lines and wild−type Arabidopsis.

Abbreviations
AP1: APETALA1; AP3: APETALA3; SEP3: SEPELLATA3; P: PISTILLATA; TCP: TEOSINTEBRANCHED 1, CYCLOIDEA, PROLIFERATING FACTOR; GRF: GROWTH−REGULATING FACTOR; ARF2: AUXIN RESPONSE REGULATOR 2; FT: FLOWERING LOCUS T; SOC1: SUPPRESSOR OF OVEREXPRESSING CO 1; LFY: LEAFY; AG: AGAMOUS; WGDs: whole−genome duplication events; AGAMOUS−LIKE1: PLE: PLENA; FBP7: FLORAL BINDING PROTEIN 7; FBP11: FLORAL BINDING PROTEIN 11; STK: SEEDSTICK; SHP1: SHATTER ROOT1; SHP2: SHATTERPROOT1; YFP: yellow fluorescent protein; REP: red fluorescent protein; Leu: leucine; Trp: tryptophan; His: histidine; Ade: adenine; RT−qPCR: quantitative real−time PCR; Semi−PCR: semi−quantitative−PCR.

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Authors’ contributions
Conceived and designed the experiments: YHH, CLZ. Performed the experiments: CLZ. Analyzed the data: CLZ, YQW. Contributed reagents/materials/analysis tools: CLZ, LDW, WJW, WQQ, HL, ZC. Wrote the paper: CLZ, YHH, HL, ZC. All authors read and approved the final manuscript.

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Availability of data and materials
The sequences information of AGAMOUS−like proteins was uploaded to the NCBI (TeAG1: MT452648, TeAG2: MT452649, TeAGL11−1: MT394168, and TeAGL11−2: MT394169).

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interests.

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