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The *AGL6*-like Gene *CpAGL6*, a Potential Regulator of Floral Time and Organ Identity in Wintersweet (*Chimonanthus praecox*)

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Abstract Wintersweet (*Chimonanthus praecox*), a deciduous aromatic shrub endemic to China, has high ornamental value for developing beautiful flowers with strong fragrance. The transition from the vegetative to the reproductive phase in wintersweet takes 4-5 years. The molecular mechanism regulating flower development in this basal angiosperm is largely unknown. Here we characterized the molecular features and expression patterns of the *C. praecox AGL6*-like gene *CpAGL6* and investigated its potential role in regulating floral time and organ development via ectopic expression in *Arabidopsis thaliana*. The expression of *CpAGL6* is highly tissue-specific, with the highest level in the middle tepals, moderate levels in inner tepals and carpels, and weak levels in stamen and young leaf tissues. Its dynamic expression in the flower is coincident with tepal opening. Ectopic expression of *CpAGL6* in *Arabidopsis* retarded the vegetative growth and led to precocious flowering, mainly correlated with the inhibition of the floral repressor *FLC* and promotion of the floral promoters *AP1* and *FT*. Although no ectopic floral organs have been observed, transgenic plants exhibited abnormal stamen and carpel development in later-developing flowers, with fertility reduced to varying degrees. These results suggest that *CpAGL6*, the *AGL6*-like gene from the basal angiosperm *C. praecox*, is a potential E-function regulator involved in specifying floral time and organ identity, functionally homologous to those *AGL6*-like genes from higher eudicots and monocots.

Keywords *Chimonanthus praecox* L. · *AGL6*-like gene · Expression pattern · Ectopic expression · Floral time · Floral organ identity

The nucleotide sequence reported in this article has been submitted to GenBank under the accession number FJ807387 (*CpAGL6*).

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Introduction

Floral transition and further development is crucial for sessile flowering plants to reproduce successfully. One family of genes with a key role in flower initiation and development is the MADS-box family. These genes obtained the name from the initials of the first four members: yeast *MCM1*, *Arabidopsis AGAMOUS*, snapdragon *DEFICIENS A*, and human *SRF*, sharing a highly conserved region encoding the MADS domain (Schwarz-Sommer and others 1990). Members of the MADS-box gene family play fundamental roles in developmental control and signal transduction in probably all eukaryotes (Becker and Theissen 2003). During flower development, some MADS-box genes such as *SEP3* and *AP1* function in both floral transition and organ formation (Liljegren and

others 1999; Ferrario and others 2003). Others may act only as floral time genes or organ identity genes. The floral repressor *FLC* and the floral transition pathway integrator *SOC1* (*AGL20*) are also MADS-box genes, without directly participating in floral organ formation (Onouchi and others 2000; Sheldon and others 2000).

The "ABCDE" model is now widely accepted for flower development, with A and E functions specifying the identity of sepals, A and B together with E specifying petals, B and C as well as E specifying stamens, C and E specifying carpels, and D and E specifying ovules (Theissen and Saedler 2001). Most of A-, B-, C-, D-, and E-function genes are MIKC-type MADS-box genes, and their homologs have been identified in almost all major lineages of angiosperms (Theissen 2001; Irish 2003). *SEPALLATA1* (*SEP1*)-like genes, the so-called "class E genes", are critical for specifying the identities of all four whorls of floral organs. *SEP1*-like genes are prominently involved in floral organ specification already in the most recent common ancestor of extant flowering plants. Recently, it has been shown that these genes also play an important role in specifying floral meristem identity (Liu and others 2009).

AGL6-like genes form a superclade together with *SEP1* and *SQUA*-like genes in most phylogenetic reconstructions. It has been proposed that proteins from the AGL6/SEP1/SQUA-clade are in the center of the floral developmental kernel and constitute the postulated morphogen, when concerning the evolutionary origin of the flower (Melzer and others 2010). Recent studies on *AGL6*-like genes in petunia, maize, rice, and *Arabidopsis* led to the hypothesis that a common function of *SEP1*- and *AGL6*-like genes is ancient and was conserved during evolution (Ohmori and others 2009; Rijpkema and others 2009; Thompson and others 2009; Koo and others 2010). However, what is still lacking is experimental evidence from basal angiosperms and other plants at lower evolutionary positions.

Wintersweet (*Chimonanthus praecox*), also known as Wax shrub, is an important deciduous aromatic shrub endemic to China. It is one of the most precious epibiotic species of the Tertiary period and classified as a second-grade protected wild plant in China (Zhang and Liu 1998). Used in potted landscaping, cut-flower, and Chinese traditional medicinal materials, this plant has a long history of cultivation owing to the high ornamental value of its yellow flowers with strong fragrance. As the name in Latin indicates, it blooms from December to February, which is almost the whole winter, in areas as different as eastern China and southwestern China. The closest relatives include *Calycanthus* which is in North America, *Sinocalycanthus* is in China, and *Idiospermum* is in Australia (Ming and Ming 2004; Zhou and others 2006). All four genera are diploid ($2n = 22$), belonging to the Calycanthaceae, Laurales, Magnoliidae category, which is characterized by opposite leaves and numerous spirally arranged tepals and stamens, is dichogamous and cross-pollinated predominantly by bees or beetles, and achenes are enclosed in a concave receptacle that is usually capsule-like at maturity (Nicely 1965; Zhang and Liu 1998).

Four or five years is often needed for wintersweet flowering after seed germination. Wintersweet (*C. praecox*) flower buds differentiate from buds in the axil of the spring shoot when shoot elongation gradually slows down in April. The blooming process is normally divided into six consecutive stages: I, sprout stage; II, flower-bud formation stage; III, petal-emerging stage; IV, early-blown stage; V, full-blown stage; and VI, wither stage (Wu and Hu 1995). However, the molecular mechanism of this process are largely enigmatic. So far no research on transcriptional regulation of wintersweet flowering has been reported. In this report we present the molecular characterization and ectopic expression analysis of an *AGL6*-like MADS-box gene from the wintersweet flower. Our results suggest that the *AGL6*-like gene, in basal angiosperms such as the *Laurales* species, functions as a potential E class gene, regulating both floral time and floral organ identity.

Materials and Methods

Plant Materials and Growth Conditions

Plants of wintersweet (*Ch. praecox* var. *grandiflora*) used in this study were grown in the research garden of Shanghai Academy of Agricultural Sciences (SAAS), Shanghai, China. The developmental stages of bud and flower were identified using the morphologic index described previously (Wu and Hu 1995). To obtain tissues representing each floral organ, flowers of the early-blown stage (stage IV) were dissected. All tissue samples were snap-frozen in liquid nitrogen and stored at −72°C pending further analysis. For ectopic expression analysis, seeds of *Arabidopsis* (Col-0) were surface-sterilized (with 8% commercial bleach for 5 min) and placed on half MS medium at 4°C for 3 days. The seedlings were then grown in a growth chamber (SANYO, MLR-351H, Japan) with a 16-h light/8-h dark cycle, 120 μmol m^{-2} s^{-1} at 22 ± 2°C for 10 days before transplanting to soil.

Cloning of Full-length cDNA for *CpAGL6*

A cDNA library was constructed with a SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA; K1051-1) using the mRNA from wintersweet (*Ch. praecox* var. *grandiflora*) flowers of all six developmental stages (Sui and others 2007). A random expressed

sequencing tag (EST) sequencing project combined with the RACE (rapid amplification of cDNA ends) technique led to the isolation of a 959-bp-long cDNA. This cDNA harbors a 726-bp open reading frame (ORF) encoding a 241-amino-acid peptide. The deduced amino acid sequence shows a greater than 90% similarity to an AGL6-like MADS-box protein from avocado (*Persea americana*). This gene was named *CpAGL6* and submitted to GenBank (accession No. FJ807387).

RNA Extraction and Reverse Transcription PCR (RT-PCR)

Total RNA was isolated from wintersweet or *Arabidopsis* tissues using the TRIzol reagent (Sangon, Shanghai, SK1321) following the instructions in the User's Manual. The crude RNA extracts were treated with RNase-free DNase I (TaKaRa, Dalian, Cat. D2215) for 30 min to remove genomic DNA contamination. For quality and quantity control, RNA extracts were examined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA) and visualized by 1% agarose gel electrophoresis under denaturing conditions.

First-strand cDNA was synthesized from total RNA (3 μg) using M-MLV reverse transcriptase (Promega, Madison, WI, USA, Cat. M1705). Semiquantitative RT-PCR (in a volume of 25 μl) was performed on a Heijingang amplifier (Eastwin, China, EDC-810), and PCR products were visualized by 2% gel electrophoresis. Quantitative RT-PCR (in a volume of 25 μl) was performed on an ABI 7300 Real-Time Cycler (Applied Biosystems, Foster City, CA, USA) using a SYBR® Green-I Real-time PCR Master Mix kit (Toyobo, Japan, QPK-201). Fluorescence data collected during the 72°C step were analyzed using ABI 7300 Analysis Software ver. 1.4.0. The amounts of cDNA template in each sample were normalized using the amplification of *Arabidopsis Actin* with the primer pair (5′-3′) TCTCTATGCCAGTGGTCGTA and CCTCAGGACAACGGAATC, or wintersweet *Ubiquitin* with the primers AGGCTAAGATTCAAGACAAGG and TTGGTCGCAGCTGATTGCTGTG. Gene-specific primers for expression level analysis are listed in Supplementary Appendix 1.

Plasmid Construction and *Agrobacterium*-mediated *Arabidopsis* Transformation

An 873-bp fragment ranging from the 5′ untranslated region (UTR) to the 3′ UTR of *CpAGL6* was amplified from wintersweet cDNAs with the primer pair AAAACAGAAGGAAGAAGA and CAGTCAAAGAGCCTATTA. The product was cloned into the pUCm-T vector (Sangon, Shanghai, SK2212) and sequenced for confirmation. A site-directed cloning strategy was later used to transfer *CpAGL6* cDNA from pUCm-T to the vector pCambia2301 between the *Xba*I and *BamH*I sites downstream of the cauliflower mosaic virus (CaMV) 35S promoter. The resultant expression vector was transformed into Col-0 via *Agrobacterium tumefaciens* EHA105 using the floral-dip method (Clough and Bent 1998).

Transgenic Plant Analysis

The obtained transgenic seeds were sterilized and plated on half MS medium containing 90 μg ml^{-1} kanamycin. Selected resistant *Arabidopsis* plants were cultivated in a growth chamber under the same conditions as described above. After molecular confirmation of the ectopic expression of *CpAGL6* via RT-PCR, T2 and T3 progeny of three independent transgenic lines that showed a 3:1 segregation ratio in a resistance test were used for phenotypic analysis. Whole plants were photographed with a SONY digital camera (SONY G1, Japan). Flowers of transgenic plants were viewed and photographed under dark-field illumination with an Olympus dissecting microscope (Olympus SZ61TR, Japan).

Results

Molecular Homology of *CpAGL6*

To examine the homology of *CpAGL6*, we performed sequence alignment and generated a phylogenetic tree using deduced AGL6-like MADS box amino acid sequences from wintersweet and other plants. Phylogenetic analysis assigns CpAGL6 highly close to Pe.am.AGL6.2 from avocado in the dicotyledon subclade (Fig. 1a). The amino acid sequence of CpAGL6 shows 85% identity and 91% similarity to Pe.am.AGL6.2. CpAGL6 also shows high sequence identity (73%) and similarity (82%) to other AGL6 orthologs such as MFO1/OsMADS6 from rice.

The *CpAGL6* cDNA sequence encodes a 241-amino-acid protein, with a MADS-box (amino acids 1-59) at the N terminus and a K-box (amino acids 74-172) in the middle region (Fig. 1b). The MADS-box domain of CpAGL6 is 100% identical to that of rice MFO1 and maize BDE/ZAG3, and 98% identical to that of *Hyacinthus orientalis* L. HoAGL6. The putative protein dimerization domain of the K-box in CpAGL6 also shares 88, 77, and 74% identities to those in Pe.am.AGL6.2, MFO1, and BDE, respectively.

In the C-terminal region of AGL6-like proteins there exist two well-conserved sequences, named the AGL6-I motif and the AGL6-II motif. It has been suggested that the C-terminally conserved motifs of AGL6-like proteins, with high similarity to the SEP I/II motifs, have potential

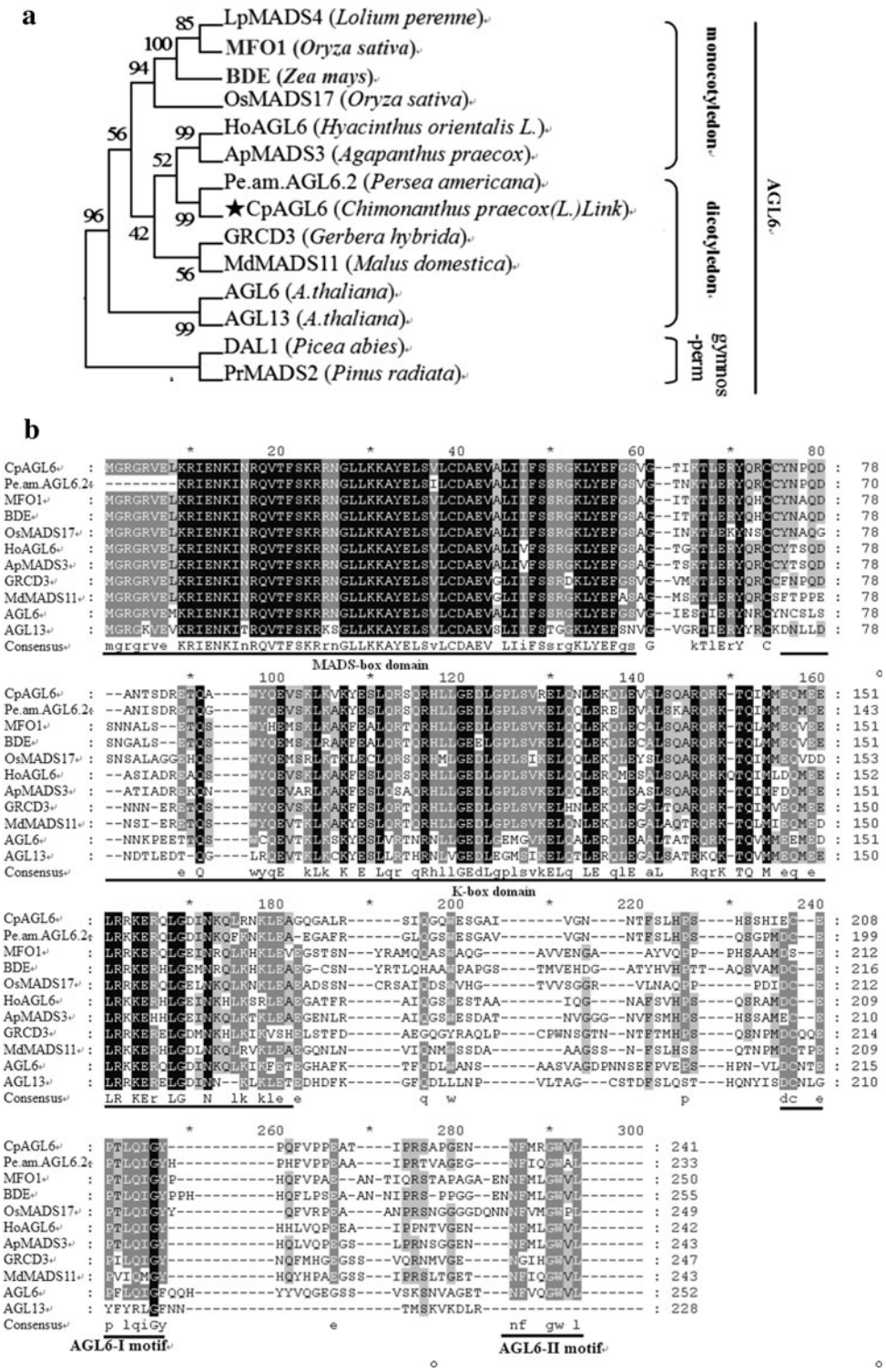

Fig. 1 Phylogenetic analysis of CpAGL6 and related proteins. **a** Phylogenetic tree of plant AGL6-like MADS-box proteins. Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar and others 2004). The tree was constructed by the neighbor-joining method. The bootstrap values (%) of 100 replicates are shown at the branching points. **b** Sequence alignment of CpAGL6 and related AGL6-like MADS box proteins and analyzed by ClustalW program. The positions of conserved regions are *underlined*. Identical and conservative amino acids are shown in *black* and *gray* shading, respectively. Except for CpAGL6, the other 13 AGL6-like proteins involved are PrMADS2 (AAD09207) from *Pinus radiata*, DAL1 (CAA56864) from *Picea abies*, AGL6 (AAA79328) and AGL13 (AAC49081) from *A. thaliana*, GRCD3 (CAH04879) from *Gerbera hybrida*, MdMADS11 (CAA04325) from *Malus domestica*, Pe.am.AGL6.2 (ABG49494) from *P. americana*, HoAGL6 (Fan and others 2007) from *H. orientalis* L., ApMADS3 (BAC66964) from *Agapanthus praecox*, MFO1 (AAB64250) and OsMADS17 (AAF21900) from *Oryza sativa*, BDE (AAB00078) from *Zea mays*, and LpMADS4 (AAO45878) from *Lolium perenne*

transcriptional activation activity (Ohmori and others 2009). The AGL6-I motif is located in the middle part of the C-terminal region, with the 10-amino-acid sequence DCEPTLQIGY forming a consensus. In CpAGL6, this motif is well conserved, although the first Asp is substituted with Glu, similar to ApMADS3 from *Agapanthus praecox* (Fig. 1b). The AGL6-II motif is at the end of the C-terminal region, with the 10-amino-acid residue sequence ENNFMLGWVL as a consensus. CpAGL6 has one amino acid substitution in the middle of this motif (Leu to Arg).

Preferential Expression of *CpAGL6* in the Middle Tepals at Early-blown Stage

The expression pattern of *CpAGL6* in different tissues of wintersweet was first analyzed by reverse-transcription PCR. Because the squama-shaped russety sepal (outer tepal) could not be homogenized, even with liquid nitrogen, no RNA could be extracted from this floral organ for expression analysis. The wintersweet petal was divided into the middle tepal and inner tepal for analysis. As shown in Fig. 2a–c expression of *CpAGL6* is highly tissue-specific, mainly confined to floral organs. Its transcripts are the highest in middle-tepal tissue but are undetectable in vegetative tissues such as the stem and shoot apical meristem (SAM) from spring shoots. The inner tepal and pistil (carpel) from flowers also accumulates *CpAGL6* transcripts. In addition, this gene is weakly expressed in stamen and young leaf tissue.

The blooming process in wintersweet has been divided into the six stages previously described (Wu and Hu 1995). Accumulation of *CpAGL6* transcripts changes dynamically and is associated with tepal opening (Fig. 2b and d). From the sprout stage (I) to the petal-emerging stage (III), *CpAGL6* transcript accumulation is not significantly changed. However, this accumulation is sharply elevated to the highest level at the early-blown stage (IV). Later, a gradual but obvious decrease in *CpAGL6* expression occurs. It is unclear why the transcript level of *CpAGL6* in withered flowers is three times as high as that in the flower bud (stage II) and petal-emerging bud (stage III).

Retarded Vegetative Growth and Early Flowering Resulted from Ectopic Expression of *CpAGL6*

The ectopic expression of *CpAGL6* was studied in *Arabidopsis*. Fifteen independent lines of *Pro35S:CpAGL6* transgenic *Arabidopsis* showing an early flowering phenotype were obtained. Three lines with approximate 3:1 segregation in a kanamycin resistance test were propagated for further analysis. The ectopic expression of *CpAGL6* in these lines was confirmed via RT-PCR, as shown in Supplementary Appendix 2.

Days to flowering (DTF), the time from seed germination until the first appearance of flower buds, were recorded daily in both transgenic and wild-type *Arabidopsis*. Under the previously described long-day growth conditions, the first flower bud occurred in some plants of the *OX11* line at 23 days after germination (DAG), just 6 days before that in wild type (see Supplementary Appendix 3). The first flower bud of most wild-type plants was visible at 32-34 DAG, whereas the first flower bud of most transgenic plants was visible at 27-30 DAG. The early flowering was concomitant with the repressed growth of vegetative organs in transgenic plants. As compared to wild type, transgenic plants developed relatively smaller and darker green leaves. In addition, the average number of rosette leaves in flowering transgenic plants was less than that of flowering wild type (data not shown). Typical whole-plant morphology of wild type and transgenic lines *OX1* and *OX11* is shown in Fig. 3a-f. Promotion of flowering in *Pro35S:CpAGL6* plants was stronger in line *OX11* than in line *OX1*.

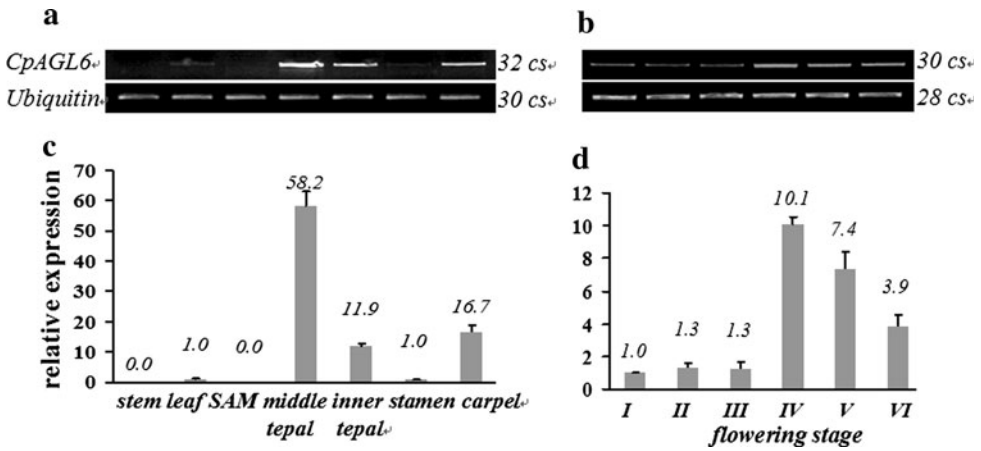

Fig. 2 Expression pattern of *CpAGL6* in *C. praecox*. **a** Semiquantitative RT-PCR analysis in different tissues. Repeat cycle for *CpAGL6* is 32 and for *Ubiquitin* 30. Tissue names are the same as shown in **c**. **b** Semiquantitative RT-PCR analysis during flowering. Repeat cycle for *CpAGL6* is 30 and for *Ubiquitin* 28. Identities of plant materials are the same as shown in **d**. **c** Quantitative real-time PCR analysis of *CpAGL6* transcript level in different tissues relative to that in stamen. **d** Quantitative RT-PCR analysis during flowering. The amounts of cDNA template in each sample were normalized by the amplification of *Ubiquitin*. Stem, leaf, and SAM (shoot apical meristem) were sampled from young shoots in mid-April. All other tissues were from stage IV flowers in mid-December. Whole flowers (buds) were used for dynamic pattern analysis. The experiment was independently conducted three times. Error bars represent the standard deviations within the triplicate

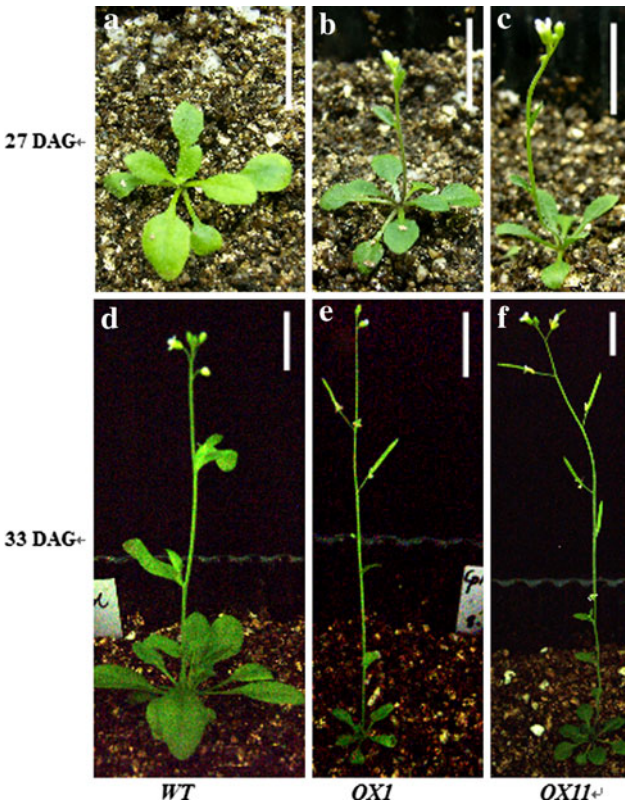

Fig. 3 The growth and flowering phenotype of transgenic *Arabidopsis* plants ectopically expressing *CpAGL6*. **a-c** Wild type (*Col*) and two independent transgenic lines *OX1* and *OX11* (T2 progeny) at 27 days after germination (DAG). **d-f** *Col*, *OX1*, and *OX11* at 33 DAG. Plants were grown in a growth chamber with a 16-h light/8-h dark cycle, 120 μmol m^{-2} s^{-1} at 22 ± 2°C. Scale bar = 1 cm

Numerous previous experiments via molecular genetic approaches have already revealed the regulatory network controlling floral time in *Arabidopsis* (Corbesier and Coupland 2006). These floral time genes were tested in this study to investigate potential mechanisms for flowering promotion via *CpAGL6* ectopic expression. Compared to that in wild type, the level of *FT* transcripts is obviously elevated in transgenic plants at 24 DAG (Fig. 4b). Upregulation of the *AP1* gene in *Pro35S:CpAGL6* plants was detected later than that observed for *FT* (27 and 30 DAG when 3- and 5-fold increases of *AP1* transcripts in transgenic plants were observed, respectively; Fig. 4c). Conversely, expression of *FLC*, the negative regulator for flowering, is notably downregulated in *Pro35S:CpAGL6* plants at all three stages tested (Fig. 4f). In addition, a moderate increase of *LFY* and *SOC1* expression was found at 30 DAG in transgenic line *OX11* (Fig. 4d and e). It is quite puzzling that the level of *CO* transcripts significantly decreased in transgenic plants at 24 DAG (Fig. 4a). The above results support the suggestion that early flowering in *Pro35S:CpAGL6* transgenic *Arabidopsis* plants might be an integrated result of complex changes in the flowering regulatory network, correlated with the downregulation of repressor *FLC* and the upregulation of promoters *AP1* and *FT*.

Partial Sterility Resulting from *CpAGL6* Ectopic Expression in *Arabidopsis*

No ectopic development of any whorl floral organ has been observed in *Pro35S:CpAGL6* transgenic plants. The sepal, petal, stamen, and carpel in most flowers from transgenic plants are normal. However, aberrant flowers randomly occurred in later-developed flowers of each transgenic plant. The phenotypic alterations in these aberrant flowers could be divided into four types: stamen pattern altered, stamen number reduced, carpel length elongated, and the production of partially to nonfunctional stamens (staminodes) (Fig. 5).

Wild-type flowers produce six stamens: four long stamens of identical length closely around the carpel, and two short symmetrical stamens as the second stamen whorl (Fig. 5a). In *Pro35S:CpAGL6 Arabidopsis* plants, stamens in some flowers lose symmetry, and the model of four long with two short could be changed to five long with one short (Fig. 5b) or two long with four short (Fig. 5c). The six stamens could be reduced to five (Fig. 5d) or four (Fig. 5e) of identical length in some transgenic flowers. Furthermore, the carpel was markedly elongated in some flowers (Fig. 5f). Contrary to pollinating stamen that produce functional pollen, staminode that normally has a different shape and loses the capability of producing viable pollen has been observed in all transgenic lines examined (Fig. 5, as arrows indicated). This type of homeotic conversion was often more pronounced in line *OX11*: five of the six stamens could be changed to staminodes (Fig. 5g). In some cases, the same flower could present combined morphologic alterations, with an elongated carpel and one stamen altered, as shown in Fig. 5i.

It has been suggested that the identity of each floral organ in *Arabidopsis* is determined by different tetramers of MADS-box proteins. The predicted composition of tetramers in the inner two whorls is AG–SEP–AP3–PI in whorl 3 to specify stamens, and AG–AG–SEP–SEP in whorl 4 to specify carpels (Krizek and Fletcher 2005). Expression of these regulators was examined in both wild-type and *Pro35S:CpAGL6* plants to seek probable causes for the above phenotypes. The ectopic expression of *CpAGL6* in *Arabidopsis* results in marked elevation of *SEP3* expression compared to that in wild type. An obvious increase of transcript level is also observed for *SEP2*, *AP3*, and *PI*. The expression of *AG* is undetectable in the wild-type material used in this study but is detected in transgenic plants, though at quite low levels (Fig. 6). These results clearly indicate that ectopic expression of *CpAGL6* could

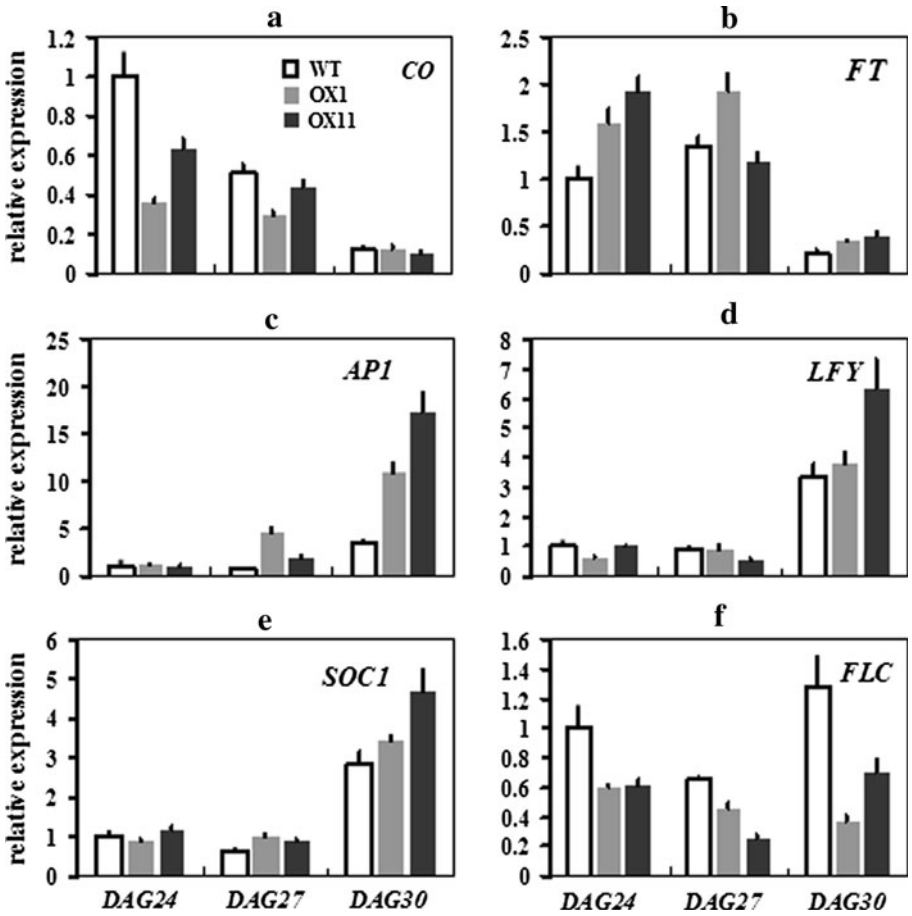

Fig. 4 Quantitative real-time PCR analysis of flowering time regulators in wild type (*Col*) and the *Pro35S:CpAGL6* transgenic *Arabidopsis*. *WT* wild type; *OX1* and *OX11* two independent transgenic lines (T3 progeny). Icons representing different plant genotypes are shown in **a** and are similar for the other panels (**b-f**). *Y* axes in all panels indicate gene expression level relative to that in wild type at 24 days after germination (DAG). *X* axes in all panels show developmental stages, as shown in **e** and **f**. The whole upper parts of 5-10 seedlings at certain development stages were used for RNA extraction and expression analysis. The experiment was independently conducted three times. Error bars reflect the standard deviations within the triplicate

regulate the transcription level of several MADS-box genes crucial for stamen and carpel specification.

Discussion

AGAMOUS-like6 (*AGL6*) genes belong to typical MIKC-type MADS-box genes and are closely related to *SEP*- and *SQUA*-like genes. A number of *AGL6*-like genes have been isolated from diverse gymnosperms and angiosperms. It is clear that homologous genes could be subject to different control of their spatial and temporal expression. *AGL6* mRNA is expressed in all four floral organs and ovules in *Arabidopsis*, and in maize the *AGL6*-like gene *BDE* shows a very similar pattern to *AGL6* (Mena and others 1995). The orchid *AGL6*-like gene *OMADS1* is expressed only in the lip and carpel of flowers, whereas *OMADS7* is located in sepal, petal, lip, and carpel, and barely detected in the stamen (Hsu and others 2003; Chang and others 2009). The expression of *CpAGL6* is confined mainly in whorls 2 and 4 of the wintersweet flower at the early-blown stage. Similarly, the expression of the rice *AGL6*-like gene *MFO1* is preferentially confined to whorls 2 and 4 (Ohmori and others 2009). It is conceivable that conservation in *cis*-regulatory elements and corresponding transcriptional factors may result in similar tissue-specific expression of *CpAGL6* and rice *MFO1*, at least in part.

Attenuated vegetative growth, early flowering, terminal flower production, and homeotic conversion are often observed in transgenic *Arabidopsis* when ectopically expressing *AGL6*-like genes from other species such as orchid, hyacinth, *Dendrocalamus latiflorus*, and Norway spruce (Hsu and others 2003; Carlsbecker and others 2004; Tian and others 2005; Fan and others 2007; Chang and others 2009). Here the ectopic expression of *CpAGL6* caused similar changes in vegetative growth and flowering time in transgenic *Arabidopsis*. However, no terminal flower has been detected after *CpAGL6* ectopic expression. Inhibition of the flower repressor *FLC* and promotion of the flower promoters such as *AP1* and *FT* may account for this early flowering phenotype. A recent study on *Arabidopsis AGL6* suggests that AGL6 functions in the regulation of flowering time by suppressing the function of floral repressors rather than by activating a floral activator (Koo and others 2010). The overexpression of both *AGL6* and its transcriptional repressor form *AGL6::EAR* causes a similar early flowering phenotype. However, the transcript level of the critical floral repressor *FLC* was significantly elevated

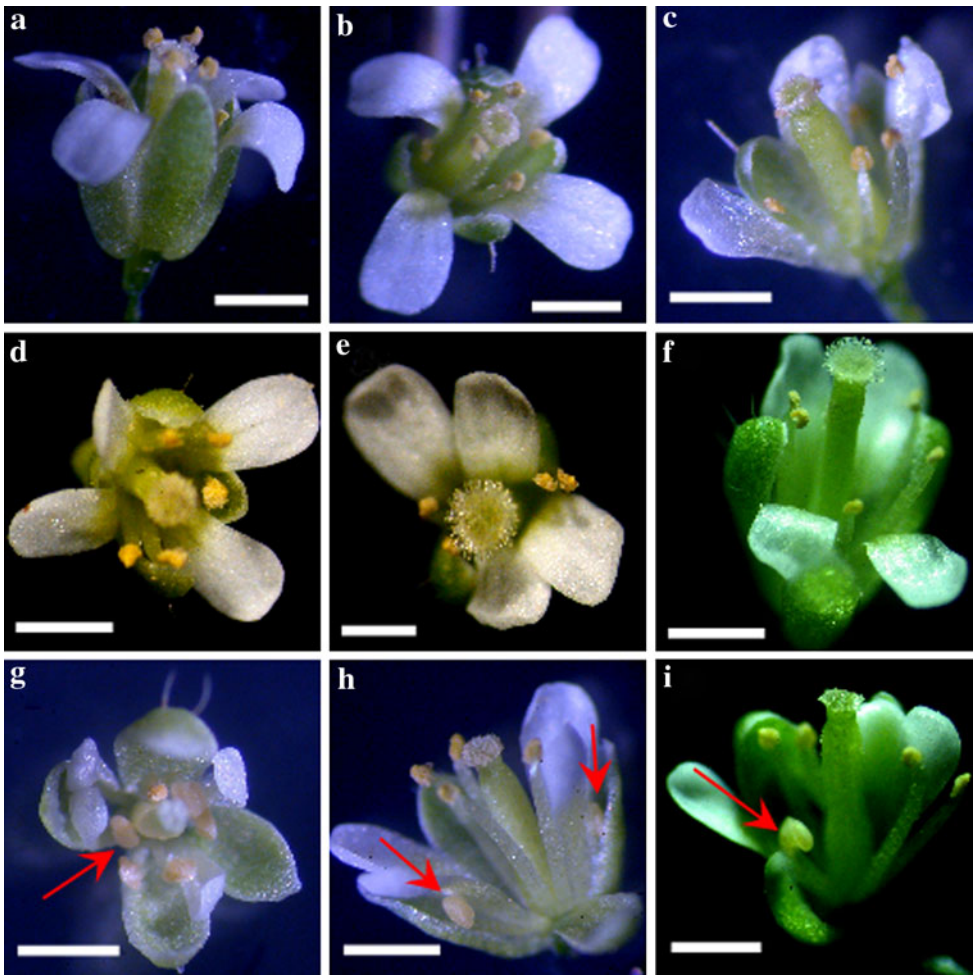

Fig. 5 The ectopic expression of *CpAGL6* in *Arabidopsis* resulted in partial sterility due to various changes in floral organs. **a** A flower of wild type containing six stamens with four long (as long as the carpel) and two short. **b-i** Flowers from transgenic plants. **b** Six stamens with five long and one short. **c** Six stamens with two long and four short. **d** Five stamens. **e** Four stamens. **f** Five stamens with the carpel elongated. **g** Six stamens of identical length and 5 being sterile. **h** Two of six stamens being sterile. **i** One of five stamens being sterile with the carpel elongated. The patterns from **g** and **h** were specifically presented in the *OX11* line, whereas patterns from **e** and **i** were only in the *OX1* line. Other patterns (**b**, **c**, **d**, **f**) were observed in both lines. Arrows indicate sterile stamens. Scale bar = 500 μm

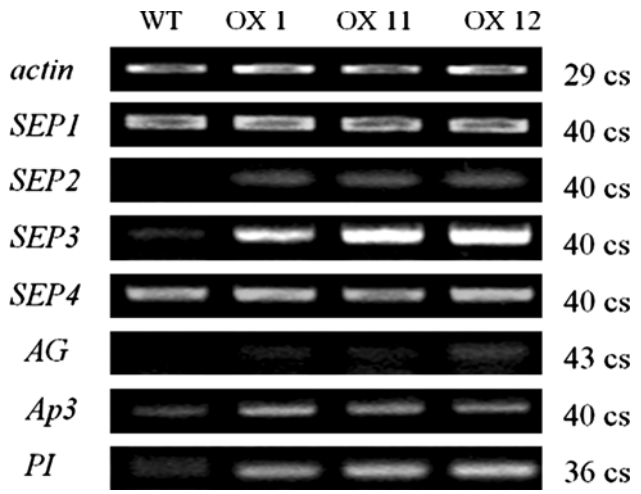

Fig. 6 Semiquantitative RT-PCR analysis of MADS box genes specifying the identity of stamen and carpel in *Arabidopsis*. *WT* wild type; *OX1*, *OX11*, and *OX12* three independent *Pro35S:CpAGL6* transgenic lines (T3 progeny). The amplification of *Actin* (At5g09810) was used as an internal control. The upper parts of shoots, including cauline leaves and flowers from 5-10 seedlings at 30 DAG were used for RNA extraction and expression analysis. The experiment was independently conducted three times

in both cases (Koo and others 2010). Which floral repressor is the target of AGL6 remains a mystery. Probably, the molecular mechanism underlying the early flowering phenotype may vary with the *AGL6*-like gene from different species being ectopically expressed.

Ectopic expression of *CpAGL6* also disturbed the expression of several MADS-box genes, including *SEP*-like genes, *AG*, *AP3*, and *PI*, resulting in various aberrations in stamen and carpel with fertility reduced. Because MADS homeotic proteins recognize the DNA of target genes in an organ-specific way as multimeric protein complexes (Melzer and others 2010), altered expression of the above-mentioned floral time regulators and floral organ identity regulators directly or indirectly resulted from the formation of ectopic MADS-box protein complexes in transgenic *Arabidopsis*. Especially, *AGL6*-like genes are sisters to the *SEP1*-like genes (E-function). They share conserved hydrophobic motifs in the C-terminal domain and might interact, either in the endogenous plants or when ectopically expressed in heterologous plants (Rijpkema and others 2009).

Recent reports on loss-of-function mutants for *AGL6*-like genes in higher eudicots and monocots uniformly suggest that *AGL6*-like genes share common functions with *SEP1*-like genes in specifying floral meristem identity and floral organ identity, although the definite function of one particular *AGL6*-like gene may vary in certain plant species (Ohmori and others 2009; Rijpkema and others 2009; Thompson and others 2009; Koo and others 2010; Li and others 2010).

This study on *CpAGL6* has produced new evidence from a basal angiosperm supporting the above hypothesis. Our results also provide useful information about the role of MADS-box genes in wintersweet flower development and the interaction among MADS-box regulators in flower transition and flower organ specification. Undoubtedly, the present study does not exclude the possibility that the wintersweet genome harbors novel *AGL6*-like gene(s), and there exists subfunctionalization or redundancy between *CpAGL6* and hitherto unknown homolog(s). Further isolation of *AGL6*-like genes from wintersweet and studies on their homology, expression patterns, and ectopic expression effects will contribute significantly to a better understanding of the relationship between the evolution of *AGL6*-like MADS lineage and flower development.

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