Science in China Series C: Life Sciences

Ectopic expression of a hyacinth *AGL6* homolog caused earlier flowering and homeotic conversion in *Arabidopsis*

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MADS-box genes are involved in floral organ development. Here we report that an *AGL6*(*Agamous-like 6*)*-like* MADS-box gene, *HoAGL6*, was isolated from *Hyacinthus orientalis* L. Expression pattern analysis demonstrated that *HoAGL6* transcript was detected in inflorescence buds, tepals, carpels and ovules, but not in stamina, leaves or scales. Transgenic *Arabidopsis* plants ectopically expressing *HoAGL6* exhibited novel phenotypes of significantly reduced plant size, extremely early flowering, and losing inflorescence indeterminacy. In addition, wide homeotic conversion of sepals, petals, and leaves into carpel-like or ovary structures, and disappearance or number reduction of stamens in 35S::*HoAGL6 Arabidopsis* plants were also observed. RT-PCR analysis indicated that the expressions of flowering time gene *SOC1* and flower meristem identity gene *LFY* were significantly up-regulated in 35S::*HoAGL6* transgenic *Arabidopsis* plants, and the expression levels of floral organ identity genes *AG* and *SEP1* in leaves were also elevated. These results indicated that *HoAGL6* was involved in the regulation of flower transition and flower organ formation.

AGL6 homologue, flower development, flowering time, MADS-box genes, *Hyacinthus orientalis*

Studies of floral homeotic mutants in *Arabidopsis thaliana*, *Antirrhinum majus* and *Petunia hybrida* have shown that the underlying mechanism of flower organ identity determination is largely conserved[1,2]. One achievement of such studies is the establishment of ABC model[3]. For its simplicity and wide applicability in plant kingdom, the model has been accepted as a general principle of floral organ development. In this model, most of the genes encode MADS-box proteins, each containing a conserved MADS domain at the N-terminus and a K-box characterized by a coiled-coil structure in the middle region, from which the molecular basis for the conservation of ABC model in flower development is thus revealed[4, 5].

Further studies brought about a revision of the model by introducing new classes of D- and E-function genes. In *Arabidopsis*, it was recently shown that *SHP1* and *SHP2* acted redundantly with *STK* in promoting determination of ovule identity, and in the *stk shp1 shp2* triple mutants, normal ovule and seed development was completely disrupted, with some of the ovules being converted to leaf-like or carpel-like structures[6]. Previously, the homeotic transformation of ovules into carpeloid structures was found in petunia as a result of the cosuppression of two MADS-box genes, *FBP7* and *FBP11* which were homologues of *STK*[7]. *Arabidopsis SHP1*, *SHP2* and *STK*, and *Petunia FBP7* and *FBP11* together were referred to as D-function genes which were required for ovule development[8]. Other genetic studies showed that three closely related MADS-box genes *SEP1*, *SEP2* and *SEP3* in *Arabidopsis* (previously de-

Received March 16, 2007; accepted May 25, 2007
doi: 10.1007/s11427-007-0083-4
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scribed as *AGL2*, *AGL4* and *AGL9*, respectively), being homologues of *FBP2, FBP5* and *FBP9* in petunia correspondingly, were necessary for determining the identity of petals, stamina, and carpels[9]. Single *sep* mutants displayed subtle phenotypical changes, whereas the triple mutant produced indeterminate flowers composed of only sepals. A similar phenotype was also obtained by cosuppression of the MADS-box gene *FBP2* in petunia[8]. These morphological changes suggest that the *SEP* genes and their homologues were in some way required for the activity of class B and class C organ identity genes, and functioned redundantly. In yeast, the SEP3 had protein-protein interaction with AP1, AP3/PI or AG. Furthermore, quadruple transgenic *Arabidopsis* plants overexpressing *AP1-PI-AP3-SEP3* or *PI-AP3-SEP3-AG* showed that vegetative leaves were respectively converted to petal-like or stamen-like organs, while combination of ectopic expression of *SEP3* and *AG* transformed normal leaves into carpel-like structures[10,11]. These data provided confirmative evidence that *SEP*s acting as E-function genes determine the floral organ fate at least during the period of petal, stamen and carpel development.

MADS-box genes involved in floral organ development have been classified into five major functional types. Analyses of their relationship in floral organ determination led to the revision of the original ABC model to an ABCDE model. According to the revised model, the expression of A-function genes alone determines sepal development; the combination of the A-, B- and E-function genes specifies petal; the B-, C- and E-function genes control stamen development; the C- and E-function genes work together to regulate carpel formation; and the D-function genes are involved in ovule initiation[4,5,8,12−17]. Up to now, the molecular mechanism of floral organ determination has seemed to be completely revealed. However, a specific combination of the genes in ABCDE model is never competent for induction of a kind of perfect floral organ identical to that in the wild type, but a metamorphic and nonfunctional structure, such as the carpeloid structures arising due to the ectopic co-expression of *AG* and *SEP3*[11]. This indicates that there is something else unknown in floral organogenesis.

The gene family encoding MADS domain transcription factors comprises 107 members in the *Arabidopsis* genome, but a large percentage (84%) of the members are function-unknown[18,19]. These MADS-box genes have been divided into five subfamilies (named Mα, Mβ, Mγ, Mδ, and MIKC respectively) by phylogenetic analyses[2,18−20]. The MADS-box genes involved in flower development are almost restricted to the MIKC subfamily. Although being originated from different plant species and sharing similar expression patterns and the related developmental functions, these MADS-box genes, having different A, B, and C (D, E) functions, fall into separate phylogenetic clades. *AGL6* and its homologues were classified into the *AP1*/*AGL9* group of MIKC subfamily. A protein-protein interaction map in a yeast two-hybrid system showed that every MADS-box member in MIKC subfamily had a specific protein-protein interaction pattern in *Arabidopsis*, in which the interaction pattern of AGL6 was very similar to that of AP1 and SEPs[20], suggesting that AGL6 may function in a similar manner like AP1 and SEPs.

Despite their involvement in floral organ formation, some of MADS-box genes in the MIKC subfamily are also thought to play a role in the regulation of floral transition in *Arabidopsis*. For example, *AGL24* and *SVP*, two similar MADS-box genes, have opposite functions in the controlling of floral transition in *Arabidopsis*, with the former functioning as a promoter and the latter as a repressor[21]. A floral repressor *FLC* and a floral transition pathway integrator *SOC1* (known as *AGL20*) are also MADS-box genes, without directly participating in flower organ formation[22−24]. The other two MADS-box genes, *SEP3* and *AP1*, which are most similar to *AGL6* in sequence, not only regulate floral transition, but also play a certain role in flower organ formation[25−27].

AGL6-like genes have been cloned from different plant species[28−38]. However, their function has not been well characterized. In *Arabidopsis*, *AGL6* is expressed in all of 4 flower whorls and ovules[28], and *ZAG3*, an *AGL6* orthologue in maize (*Zea mays*), also shows flower-specific expressing pattern[33]. Until very recently, it was revealed that *OMADS1* of orchid (*Oncidium* Gower Ramsey), an *AGL6*-like gene, with its expression detectable in apical meristem, lip and carpel of flower, participated in flower transition by acting as an activator for *FT* and *SOC1* and flower organ formation deduced from its ectopic expression in *Arabidopsis*[39].

Here, we present the molecular evidence for the function of *HoAGL6* from *Hyacinthus orientalis* L., based on the analysis of its transgenic plants in *Arabidopsis*. Al-

though it promoted flowering, similarly to the function of *OMADS1* in orchid[39], *HoAGL6* was noticeably different in transforming leaves, sepals, and petals into carpel structures or perfect ovaries when ectopically expressed in *Arabidopsis*. It was found that the expression level of two genes, *LFY* and *SOC1* which act in the floral transtion pathway to activate most of flower organ identity genes, was obviously increased in the *HoAGL6* transgenic *Arabidopsis*. Hence, the result has given a cue for the early flowering in the transgenic *Arabidopsis*. Furthermore, expressions of both *AG* and *SEP* were activated in leaves of the transgenic *Arabidopsis* plants, indicating a novel mechanism by which *AGL6*-like genes regulate floral organ development.

1 Materials and methods

1.1 Plant materials

H. orientalis L. cv. Deft Blue used in this study was originated from Holland. Plants were grown in test field. The hyacinth, an ornamental flower of monocot, was in a mature bulb, where scores of succulent scales always enclosed an inflorescence consisting of many flowers and bearing a few cauline leaves at its base (Figure 1(a)). A flower of the hyacinth always possessed three whorls of floral organs: a gamophyllous tepal known as a kind of petal-like (blue-violet in the cultivar), stamina, and an ovary in the center (Figure1(b)).

A. thaliana (ecotype Columbia) was used to investigate the function of the hyacinth MADS-box gene *HoAGL6*. Seeds were sterilized, and then placed on agar plates containing 0.5 B5 medium (0.8% agarose) at 4℃ for 2 d. Seedlings were transferred into pots, growing in a growth chamber under long-day conditions (16 h light/8 h dark, 120 μmol·m^{-2}·s^{-1}) at 22℃.

1.2 Isolation of total RNA

Total RNA was isolated from various organs of *H. orientalis* L., wild-type and transgenic *Arabidopsis* using Trizol Kit (GIBCO BRL, USA) according to the manufacturer's protocol.

1.3 Isolation and sequence analysis of *HoAGL6*

After being digested with RNase-free DNase I (Promega), 2 μg of total RNA isolated from flower buds of hyacinth, oligo-d(T) primer B26 and M-MLV reverse transcriptase (TaKaRa) was used for synthesizing the first strand cDNA. Two degenerate primers, a forward primer AG5-1 and a reverse primer AG3-1 which were designed based on the amino acid sequences conserved in plant *AGL6*-like genes, were used in the PCR for amplification of the internal conservative fragment of *HoAGL6*. One gene-specific primer AG3-R and B26 primer were used in 3′-RACE, and another gene-specific primer AG5-P and abridged universal amplification primers (AAP and AUAP) were used in the 5′-RACE, with a System for Rapid Amplification of cDNA 5′ ends v2 kit (Invitrogen).

To verify the integrity of the cDNA sequences of *HoAGL6*, RT-PCR was carried out to amplify nearly full-length cDNA, using a forward primer AG-P and a reverse primer AG-R added with *Bam*H I and *Sac* I restriction sites respectively.

The amplified products were then separated on a 1% agarose gel and the bands visualized by ethidium bromide staining to estimate the amplifications. The PCR products were inserted into pGEM-T Easy vector (Promega) according to the manufacturer's instructions, and then followed by sequencing.

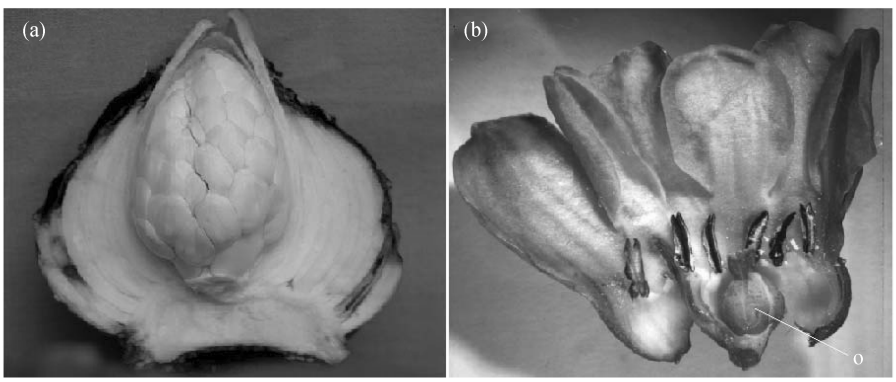

Figure 1 Bulb and flower structures of *H. orientalis* L. (a) A section of a bulb; (b) a dissected flower. The hyacinth flower is composed of a six-lobe tepal, six stamina attached to the tepal tube, and an ovary (o) fused with three carpels marked by 3 stigmas.

Escherichia coli DH5α was used as the recipients for routine cloning experiments. Primers for isolation of *HoAGL6* are listed as follows:

B26: 5′-GACTCTAGACGACATCGATTTTTTTTT-TTTTTTTT-3′;

AUAP: 5′-GGCCACGCGTCGACTAGTAC-3′;

AAP: 5′-GGCCACGCGTCGACTAGTACGGGIIGG-GIIGGGIIG-3′;

AG3-1: 5′-AA(A/G)AA(A/G)GC(A/T/C/G)TA(T/C)-GA(A/G)(T/C)T-3′;

AG5-1: 5′-GA(A/G)(A/C)G(C/G)TA(T/C)CA(A/G)(A/C)G(A/T/C/G)TG-3′;

AG5-P: 5′-CTGCCGAACTCGTAGAGCT-3′;

AG3-R: 5′-AGGTCGCCCTCATCGTCTTC-3′;

AG-P: 5′-AAGGATCCAGCGCGGGATGTTTCCCA-G-3′ (*Bam*H I);

AG-R: 5′-AAGAGCTCTAGCACAGATAAGTCTAG-G-3′ (*Sac* I).

Sequence analysis of *HoAGL6* was performed using DNAman 4.0 and BLAST search. Other plant MADS-box gene sequences in the analysis were obtained from the GenBank database at the National Center for Biotechnology Information at the web site (http://www.ncbi.nlm.nih.gov).

1.4 Plasmid construction and *Agrobacterium*-mediated *Arabidopsis* transformation

The nearly full-length cDNA of *HoAGL6* containing the open reading frame (ORF) in pGEM-T Easy vector was digested with *Bam*H I and *Sac* I, and then subcloned into pBI121 vector downstream of Cauliflower Mosaic Virus (CaMV) 35S promoter, verified by sequencing.

The 35S::*HoAGL6* construct was introduced into *Agrobacterium tumefaciens* (strain GV3101) and subsequently transferred into *Arabidopsis* by a vacuum-infiltration method[40]. Seeds collected from the vacuum-infiltrated plants were sterilized and plated onto selection plates containing B5 medium (0.8% agarose) and 50 μg/mL kanamycin. After being transferred into pots, the selected *Arabidopsis* plants grew in the growth chamber under the same conditions as described above. Phenotype analyses were performed based on the original transformants (T_0); after being confirmed by RT-PCR, the death-lines which could not survive until flowering were excluded.

1.5 RNA gel blot analysis

A fragment of *HoAGL6* cDNA (494－892 nt) amplified with a primer pair HA5-P (5′-AGCACAGAAGTCTA-GG-3′) and HA3-R (5′-AGCGTCATCTCGGAGAGA-3′) was ligated into pGEM-T Easy vector. The plasmid was cleaved at both ends of the insert by *Eco*R I. After the resultant fragment was recovered by Agarose Gel DNA Fragment Recovery Kit (TaKaRa), the cDNA was labeled with ^{32}P-dCTP using a Primer-a-Gene Labeling System (Promega), serving as the probe for Northern blotting.

Total RNA (10 μg), isolated from various organs of hyacinth and a typical 35S::*HoAGL6* transgenic *Arabidopsis* plant, was loaded to each lane, and fractionated by electrophoresis on a 1.0% agarose gel after denaturation with glyoxal at 50℃ for 1 h. The fractionated RNA bands were transferred to a piece of Hybond N+ nylon membrane (Amersham Biosciences, Buckinghamshire, UK) and fixed by baking at 80℃ for 2 h after UV cross-linking. The membrane was prehybridized for 2 h, and then hybridized with the ^{32}P-labeled DNA probe overnight at 42℃ in the same solution (0.25 mol/L Na_2HPO_4, pH 7.2, 7% (*w/v*) SDS). After hybridization, the membrane was washed twice in solution 1 (20 mmol/L Na_2HPO_4, pH 7.2, 5% (*w/v*) SDS) and once in solution 2 (20 mmol/L Na_2HPO_4, pH 7.2, 1% (*w/v*) SDS)), each for 30 min. After being dried, the blot was covered with plastic wrap, and then autoradiographed.

1.6 RT-PCR Southern analyses

For cDNA synthesis, 1 μg of total RNA which was isolated from wild type or 35S::*HoAGL6* transgenic *Arabidopsis* plants was reverse-transcribed in a 20-μL reaction mixture using the BcaBEST RNA PCR system (TaKaRa). 5 mL of each cDNA sample of the reverse-transcribed products was used for a 25-cycle PCR reaction. The PCR reactions were carried out as: after a denaturation step (94℃ for 3 min), then 94℃ (1 min), 58℃ (1 min), 72℃ (2 min) for cycles, finally 72℃ for 10 min as extension. The PCR product (10 μL) in each reaction was analyzed by electrophoresis in a 1.5% (*w/v*) agarose gel.

Primers specific for *SOC1*, *LFY*, *SEP1* and *AG* used in RT-PCR are listed below: *SOC1*, SOC1-3 (5′-GTTT-CTGAAGAAAATATGCAGCATT-3′) and SOC1-5 (5′-GAACAAGGTAACCCAATGAACAA-3′); *LFY*, LFY-3 (5′-TCATTTGCTACTCTCCGCCGCT-3′) and LFY-5 (5′-CATTTTTCGCCACGGTCTTTAG-3′); *SEP1*, SEP 1-3 (5′-ACGCGCATCATCAAGCTCAG-3′) and SEP1-

5 (5′-AGGATTTGCCTTTGGCGCAG-3′); *AG*, AG-3 (5′-GAGGATCTAACTACGAGCAG-3′) and AG-5 (5′-GCAGGAATTGGTAATTAA-3′). A fragment of *ACT* gene was amplified as an internal control. Primers specific for *ACT* were ACT-1 (5′-ATGAAGATTAAGGTCGTGGCA-3′) and ACT-2 (5′-TCCGAGTTTGAAGAGGCTAC-3′).

The hybridizations were carried out under a highly stringent condition at 65℃, and the preparation of the probes was similar to that for Northern blotting.

2 Results

2.1 *HoAGL6* is a homolog of *Arabidopsis AGL6*

HoAGL6 (GenBank accession No. AY591333) is 913 bp in length with an ORF that encodes a putative protein of 242 amino acids, bearing a MADS-box (amino acids 1 to 60) at the N terminus and a K-box (amino acids 90 to 165) in the middle region. Comparison of HoAGL6 with other members within MIKC subfamily using full-length amino acid sequences was carried out (Figure 2). The result indicates that HoAGL6 protein has the highest similarity to AGL6 clade members in the AP1/AGL9 group of MIKC subfamily, such as 75% identity with maize ZAG3, 62% with *Arabidopsis* AGL6, and 53% with orchid OMADS1. In the MADS-box domain, 98%, 97% and 94% of the amino acids of HoAGL6 are identical to that of ZAG3, OMADS1 and AGL6 respectively. In addition to the MADS-box domain, the K-box of HoAGL6 also shares high homology to that of ZAG3, OMADS1 and AGL6, with 79%, 84% and 71% identities respectively.

The phylogenetic tree analysis revealed that AP1/AGL9 group was split into SEP, AGL6 and AP1 clades and AGL6 clade was the sister of SEP clade. AGL6 clade was further divided into three groups which coincided with gymnosperm, dicotyledon and monocotyledon taxon. HoAGL6 was inserted in the monocotyledon group within AGL6 clade (Figure 3). The highest sequence similarity between *HoAGL6* and other *AGL6*-like genes demonstrates that *HoAGL6* is a homolog of *Arabidopsis AGL6*, and is most similar to maize *ZAG3*.

2.2 Gene expression of *HoAGL6*

RNA gel blot analysis was carried out to examine the expression for *HoAGL6* in hyacinth organs. As shown in Figure 4, the inflorescence meristem of *HoAGL6* transcripts was detected at two developmental stages: before and just after emergence of flower meristems. The expression level of *HoAGL6* obviously increased with further development of the flower buds, suggesting that *HoAGL6* had an action in flower bud development. When floral organs were examined, the expression of *HoAGL6* was detected in petal-like tepal, carpels and ovules, but absent in vegetative leaves, scales and stamens (Figure 4). The floral-whorl specific expression pattern strongly indicates that *HoAGL6* has a function in regulating flower organ development.

A high expression level of *HoAGL6* was also detectable in a transgenic *Arabidopsis* plant (Figure 4). The extensive accumulation of *HoAGL6* mRNA in the transgenic plant suggests that transgenic phenotypes should originate from the genome integration and transcription of *HoAGL6* in *Arabidopsis*.

2.3 Up-regulated expression of *HoAGL6* caused early flowering by enhancing the expressions of *SOC1* and *LFY*

To investigate the function of *HoAGL6*, we conducted transgenic analysis of *HoAGL6* in *Arabidopsis*. The near full-length cDNA of *HoAGL6* driven by cauliflower mosaic virus 35S promoter was therefore introduced into *Arabidopsis* plants by *A. tumefaciens*-mediated transformation.

Twenty-two independent lines of transgenic *Arabidopsis* plants were obtained. Nine of them were phenotypically indistinguishable from wild-type plants, whereas other 13 plants showed novel phenotypes. A clear reduction in the time to flowering was observed in the 13 transgenic lines. All of them exhibited extreme reduction in plant size (less than 10 cm tall when flowering), production of only 2—5 small curled filamentous rosette leaves, and loss of inflorescence indeterminacy, earlier production of a terminal flower or floral organ-like structures without any inflorescence branch (Figure 5(a), (c), (d) and (f)). In contrast, wild-type plants of the same age produced only a few nearly round rosette leaves and remained in vegetative development (Figure 5(e)). According to their defect degree in flower production, the 13 plants were classified into three different groups. The first group containing two plants showed the weak phenotype. They produced a cluster of less than 6 flowers, instead of a branched inflorescence (Figure 5(a)), in which the terminal flower displayed the most severe defects, completely losing the petal whorl (Figure 5(b)). The second group of the 8 plants with the

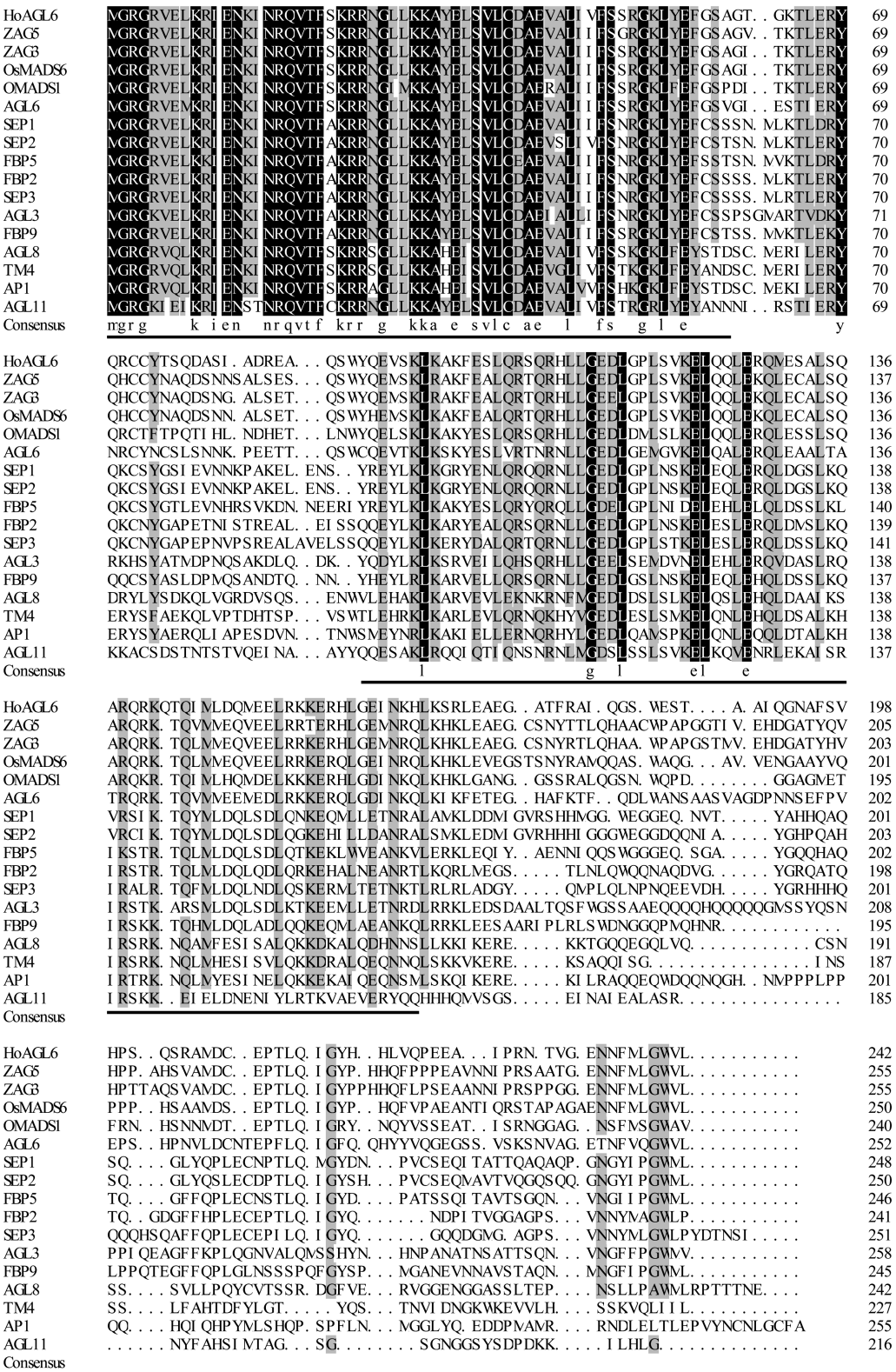

Figure 2 Alignment of the HoAGL6 amino acid sequence with the selected AP1/AGL9 group proteins: HoAGL6, AGL3, AGL6, AGL8, AGL11 (Q38836), SEP1, SEP2, SEP3, AP1, TM4, FBP2, FBP5, FBP9, ZAG3, ZAG5, OsMADS6, and OMADS1. The MADS-box domain at the N terminus and the K-box domain in the middle region are underlined respectively. Besides AGL11, their accession numbers are stated in Figure 3.

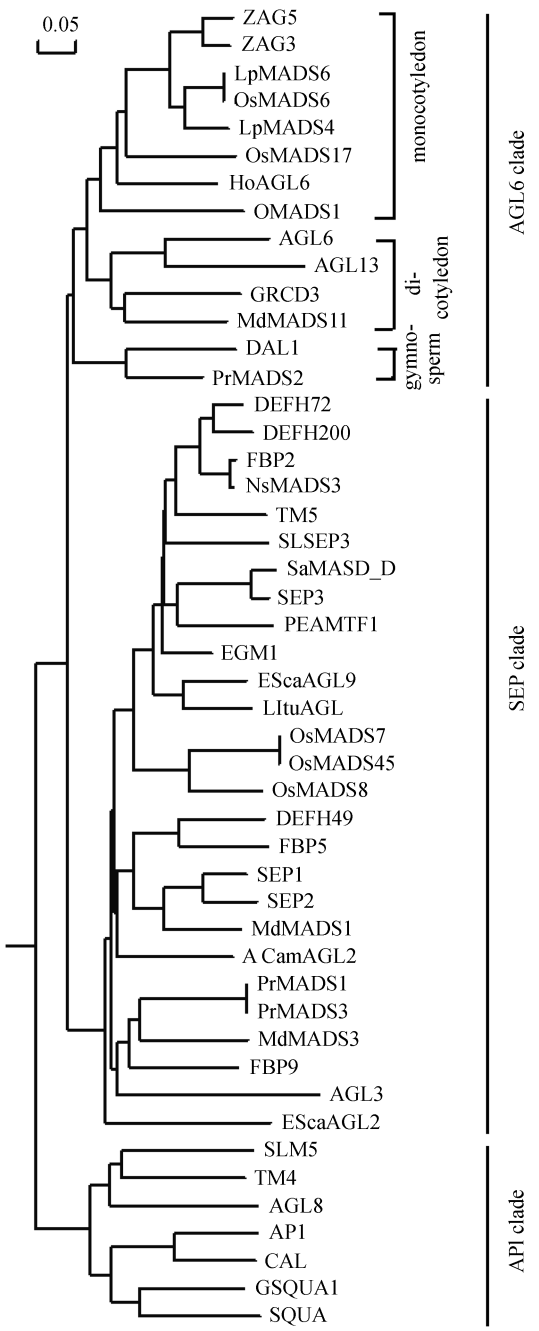

Figure 3 Phylogenetic tree of HoAGL6 and other AP1/AGL9 group proteins. The gene members of AP1/AGL9 group listed in the tree are as follows: *SEP1* (M55551), *SEP2* (M55552), *SEP3* (AY850180), *AGL3* (U81369), *AGL6* (M55554), *AGL8* (U33473), *AGL13* (U20183), *CAL* (NM_102395) and *AP1* (Z16421) from *A. thaliana*; *DEFH49* (X95467), *DEFH72* (X95468), *DEFH200* (X95469) and *SQUA* (X63701) from *A. majus*; *EGM1* (AF029975) from *Eucalyptus grandis*; *GRCD3* (AJ784157), *GSQUA1* (AJ009727) from *Gerbera hybrida*; *MdMADS1* (U78947), *MdMADS3* (U78949) and *MdMADS11* (AJ000763) from *Malus domestica*; *TM4* (X60757) and *TM5* (X60480) from *Lycopersicon esculentum*; *NsMADS3* (AF068722) from *Nicotiana sylvestris*; *OsMADS6* (U78782), *OsMADS7* (Q6Q9I1), *OsMADS8* (U78892), *OsMADS17* (Q7XUN2) and *OsMADS45* (U31994) from *Oryza sativa*; *FBP2* (M91666), *FBP5* (AAK21248) and *FBP9* (AAK21249) from *P. hybrida*; *DAL1* (X80902) from *Picea abies*; *PrMADS1* (U42399), *PrMADS2* (U42400) and *PrMADS3* （U42399） from *Pinus radiata*; *PEAMTF1* (AJ223318) from *Pisum sativum*; *SLM5* (X80492) and *SlSEP3* (AB162020) from *Silene latifolia*; *SaMADS_D* (Y08626) from *Sinapsis alba*; *ZAG3* (L46397) and *ZAG5* (L46398) from *Zea mays*; *EScaAGL9* (AY850180) and *EScaAGL2* (AY850181) from *Eschscholzia californica*; *LItuAGL9* (AY850182) from *Liriodendron tulipifera*; *LpMADS6* (AY198329), *LpMADS4* (AY198331) from *Lolium perenne*; *ACamAGL2* (AY850184) from *Acorus americanus*. *OMADS1* of *Oncidium* Gower Ramsey was obtained from a published article [38]. The scale, observed divergency.

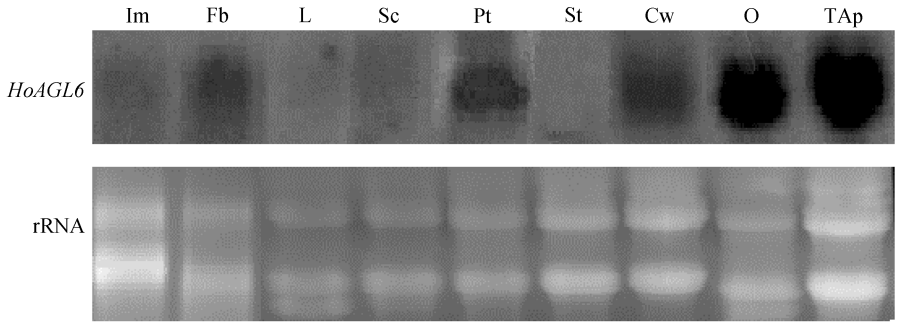

Figure 4 Northern analysis for *HoAGL6*. Total RNAs were isolated from inflorescence meristem (Im) before emergence of flower meristem and from young floral buds (Fb) just after emergence of flower meristem in mid June; also from vegetative leaves (L), scales (S); from petal-like tepals (Pt), stamens (St), carpel wall (Cw, with ovules removed) and ovules (O) of mature flowers. To detect the expression of *HoAGL6*, total RNA was extracted from transgenic *Arabidopsis* plants (TAp). Ethidium bromide-stained rRNA before hybridization is shown as control.

moderate phenotype produced a solitary terminal flower at the top of stem (Figure 5(c), (d)). Three transgenic plants with the most severe defects were classified as the last group. The 3 plants grew 3—5 small rosette leaves, a shortened inflorescence stem where 2—3 thickened and flower organ-like (carpeloid) cauline leaves and a stagnant terminal bud were bearing (Figure 5(f)). Though they could not produce any typical flower, their flower organ-like cauline leaves displayed the quality for the entrance of flowering stage. The precocious presence of flower or flower organ-like cauline leaves in the three groups of transgenic plants suggests that *HoAGL6* is involved in flowering time regulation in *Arabidopsis* plants.

Flower time regulation pathways which have been revealed in *Arabidopsis* and other species suggest that if a gene has conferred earlier flowering on plant, it should have activated flower meristem gene *LFY*. In the pathways, *SOC1* just functions as an activator upstream of *LFY*. To illustrate how *HoAGL6* promotes flowering, we investigated the expression of *SOC1* and *LFY* in transgenic *Arabidopsis* plants with RT-PCR Southern analysis. In the investigation, it was found that both *SOC1* and *LFY* mRNA levels were distinctly elevated in the transgenic seedlings contrasting to the control in the wild type (Figure 6). Hence, *HoAGL6* could have a function in flowering promotion by targeting the *SOC1-LFY* activating pathway.

2.4 Ectopic expression of *HoAGL6* caused the homeotic conversion among floral and vegetative organs probably by activation of *SEP1* and *AG*

Phenotypic alterations of the 35S::*HoAGL6* transgenic plants were also observed in flower organs, and it was found that homeotic conversion always happened among floral organs and cauline leaves. Stigmatic structures were often produced from cauline leaves of the transgenic plants, whatever the transgenic phenotypes were weak, moderate or severe (Figure 5(a), (c), (d), (f) and (i)), while stigmatic papillae were also found on inflorescence stems in the severe phenotype lines (Figure 5(k)). Cauline leaves in the severe phenotype lines changed into carpeloid structures characterized by abnormal thickening, morphological incurvature, transparent texture, and the attachment with stigmatic structures. These carpeloid leaves were much resembling the carpel in the wild type. Carpel-like cauline leaves also were observed in plants of ectopic co-expression of *AG* and *SEP3*[8]. The phenotypic exhibition indicated that *HoAGL6* alone was sufficient to transform cauline leaves into carpeloid structures, and that it preferably promoted determination of carpel identity.

When flowers of the transgenic plants in the weak and moderate phenotype groups were analyzed, it was clearly found that sepals and petals homeotically converted into carpel-like organs or morphologically perfect ovaries. In the weak phenotype lines, the first-whorl sepals were converted into carpel-like organs which usually produced stigmatic papillae on the tips of these structures, or/and presented brilliant yellow and transparency (Figure 5(a), (b) and (h)). In the moderate phenotype lines, 4 sepals became 2 quite perfect ovaries rather than carpel-like sepal organs present in the weak phenotype lines or in the *AG* and *SEP3* co-overexpressed transgenic *Arabidopsis* plants[41, 42]. The petals in the second whorl were always converted into carpel-like structures, producing bundles of stigmatic papillae in the moderate phenotype lines (Figure 5(a) and (b)) or small

Figure 5 Phenotypes of transgenic *Arabidopsis* plants ectopically expressing *HoAGL6*. (a) A twenty-day-old 35S::*HoAGL6 Arabidopsis* plant with the weak phenotype flowered earlier than wild-type plant, and produced only 5 small curled rosette leaves, and three cauline leaves, and a cluster of 6 flowers with a terminal flower (tf) imperfect. A carpel-like cauline leaf (cc) characterized by attachment with stigmatic papillae (sp) was shown in a close-up. Bar = 1.0 cm. (b) A cluster of flowers of a 35S::*HoAGL6* transgenic plant. The sepals exhibited carpel-like (cs), light yellow, transparent or/and attached with stigmatic papillae. The number of stamens (st) reduced. (c) A twenty-day-old 35S::*HoAGL6 Arabidopsis* plant with the moderate phenotype also flowered significantly earlier, and produced three rosette leaves, two cauline leaves and one solitary flower. (d) Carpel-like cauline leaves characterized by stigmatic papillae, and a solitary terminal flower with two morphologically perfect ovary structures (o) in the place of sepals presented in a 35S::*HoAGL6 Arabidopsis* plant with the moderate phenotype. (e) A 20-day-old wild-type *Arabidopsis* plant did not enter the flowering stage, producing a few nearly-round rosette leaves only. (f) A 20-day-old 35S::*HoAGL6 Arabidopsis* plant with the severe phenotypes, small in size, produced 3 curled rosette leaves and two carpel-like cauline leaves (cc) characterized by stigmatic papillae, but no typical flowers. Its terminal bud (tb) was stagnant. (g) A close-up of a solitary terminal flower in a 35S::*HoAGL6* plant showed its ovary-like structures (o) in the place of sepals with regular-form stigma, and carpeloid petal (cp) with stigmatic papillae attached. (h) A carpel-like sepal (cs) attached with stigmatic papillae at the tip. (i) A close-up of carpel-like cauline leaves showed their stigmatic papillae. (j) A flower of 35S::*HoAGL6 Arabidopsis* plants had carpel-like petals and an under-developmental ovary (ov), but lost the stamen whorl. (k) Stigmatic papillae structures emerged from an inflorescence stem. (l) A flower of wild type *Arabidopsis*.

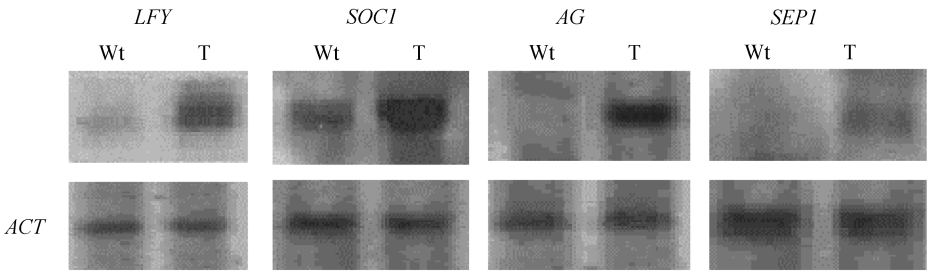

Figure 6 RT-PCR Southern analysis on the expressions of the genes related with flowering time and floral organ determination in 35S::*HoAGl6* transgenic *Arabidopsis* plants. The total RNA for *LFY* and *SOC1* detection was isolated from the 7 d wild-type seedlings on B5 medium and the 7 d resistance-selected seedlings (the RNA sample was also confirmed by RT-PCR) grown on B5 medium after 35S::*HoAGL6* transformation, and the total RNA for *SEP1* and *AG* detection was isolated from the leaves of wild-type seedlings and 35S::*HoAGL6* plants. A fragment of *ACT* gene was amplified as an internal control. Wt, wild-type plants; T, 35S::*HoAGl6* transgenic plants.

outgrowth dots in the weak phenotype lines (data not shown). In the third whorl, the stamina showed normal morphology but the number reduced by 1—2 in the weak phenotype lines, and they completely disappeared in the moderate phenotype lines (Figure 5(a), (b) and (j)). An ovary was usually under-developmental, occasionally regular in the weak phenotype lines, presenting in the center of a flower (Figure 5(a) and (b)). These phenotypes in 35S::*HoAGL6* transgenic *Arabidopsis* suggested that *HoAGL6* participated in the determination of flower organs, influencing the development of four-whorl floral organs.

According to the ABCDE model, carpel identity is specifically controlled by combination of *SEP* and *AG*[8]. Presence of stigmatic papillae in various organs means their carpel properties. In other words, *SEP* or *AG* alone cannot fully determine carpel. These phenotypes of 35S::*HoAGL6* transgenic plants were so similar to those observed in *Arabidopsis* plants with both *AG* and *SEP3* overexpressed, that we speculated that *SEP* and *AG* would be activated by *HoAGL6*. As we expected, *SEP1* and *AG* mRNAs had an obvious accumulation in leaves of 35S::*HoAGL6* transgenic plants (Figure 6), determined by RT-PCR Southern analyses. This result not only gave a reasonable interpretation of the homeotic conversions of vegetative organs (leaves), sepals and petals into carpel-liked structures or ovaries in the transgenic plants, but also indicated a novel mechanism how *AGL6*-like genes took part in floral organ development.

3 Discussion

To investigate the role of MADS-box genes in regulating flower development, we cloned a hyacinth MADS-box gene *HoAGL6* via means of PCR amplification and compared the *HoAGL6* with many other MADS-box genes identified from other plant species. Sequence analysis indicated that *HoAGL6* showed the highest identity to *AGL6*-like genes from other plant species (Figure 2). Phylogenetic analysis indicated that *HoAGL6* sequence was closely related to other *AGL6*-like genes, demonstrating that *HoAGL6* was potentially an *AGL6* homologue in hyacinth. The sequence similarity among *AGL6*-like genes indicated that they might share similar functions.

In addition to sequence similarity, *HoAGL6* showed a similar expression pattern as one of other *AGL6*-like genes in spermatophyte, where their expression was detectable in flower buds and floral organs[29, 32, 33, 36, 38, 39, 43]. The flower-specific expression pattern supported that the primary role of *AGL6*-like genes was to control flowering time and floral organ formation. Although specific for flower, expression of *AGL6*-like genes in floral organ whorls showed a similar pattern within a taxon, but somewhat divergence among different taxa. In monocots, similarly to *HoAGL6* (Figure 4), orchid *OMADS1*[38] and rice *OsMADS6* [39] were also expressed in petals (petal-like perianth) and carpels, but not in sepals and stamina, given that the paleas and lemmas are modifications of sepals, and lodicules are modifications of petals[44—46]. In dicots, *Arabidopsis AGL6*/*AGL13*[28,47] and *Petunia pMADS4*[31] were whorl-specifically expressed in all of the four whorls which were very same domains as the SEP-like genes. In gymnosperm, transcripts of *AGL6*-like genes were found in juvenile-to-adult transition buds, ovuliferous scales and microsporophylls, such as *GGM9* and *GGM11* in *Gnetum parvifolium*[37], *PrMADS2* and *PrMADS3* in *Pinus radiata*[29] and *DAL1* in *Picea abies*[48], while expression of *PrMADS3* was

also detectable in a group of cells giving rise to needle primordia in differentiated vegetative buds[29]. From these expression data, *AGL6*-like genes could be expressed in all of floral organs or their analogs. It is reasonable to believe that the obvious difference in expression patterns of *AGL6*-like genes may serve the development of different reproductive structures in different plant species.

In addition to the similarity of their expression patterns, the ectopic phenotypes of *AGL6*-like genes in *Arabidopsis* consistently supported that they have similar functions: promoting floral induction and determining floral organ identities. We have shown that ectopic expression of *HoAGL6* in *Arabidopsis* caused earlier flowering and homeotic conversion (Figure 5). Similar phenotypical changes were also observed in the plants of ectopically expressed orchid *OMADS1*[38] and *Picea abies DAL1*[43] in *Arabidopsis*. For example, transgenic *Arabidopsis* plants ectopically expressing *OMADS1* showed extremely reduced plant size, significantly early flowering, lost inflorescence indeterminacy, and homeotical conversion of sepals into carpel-like structures and petals into staminoid structures. Moreover, the presence of morphologically perfect ovaries and carpel-like leaves in the 35S::*HoAGL6* transgenic plants indicated the importance of *AGL6*-like genes in ovary determination.

The common mechanism of *AGL6* members to promote flowering was primarily revealed. The result of RT-PCR Southern analyses for 35S::*HoAGL6* transgenic plants gave two probable pathways for promoting floral transition. Pathway 1 was that *HoAGL6* activated *SOC1* in *Arabidopsis*, then *SOC1* activated *LFY* to promote flowering. This assumption was supported by two lines of evidence in our experiment. The first one was the detection of *HoAGL6* expression in the inflorescence meristem of the hyacinth before formation of flower meristem (Figure 4). The second evidence came from the fact that *HoAGL6* up-regulated the expression of *SOC1* and *LFY* in leaves (Figure 6). So the early flowering phenotype of 35S::*HoAGL6* transgenic *Arabidopsis* plants was caused by promoting expression of *SOC1* and *LFY*. We can also cite for illustration that the expression levels of *SOC1* and *LFY* were also significantly up-regulated in 35S::*OMADS1* transgenic *Arabidopsis*, resulting in precocious flowering[38]. As a crucial factor to control flowering time, *SOC1* integrates signals from the photoperiod, vernalization, gibberellin response and autonomous pathways during floral induction to activate floral meristem genes *LFY* and *AP1* in *Arabidopsis*[49−51]. AGL6 has protein dimerization with SOC1 in yeast hybrid system[19], so *HoAGL6* probably collaborates with *SOC1* to promote expression of *LFY*. These results support the promoting pathway *HoAGL6*/*SOC1*-*LFY*-*AG*. Pathway 2 also may exist independently, in which *SEP1* and *AG* are activated by *HoAGL6* to promote flowering. The hypothesis was supported by the fact that *Arabidopsis* plants that overexpressed *SEP3* or *AG* also exhibited the precociously flowering phenotype[41, 52].

One significant event for *AGL6*-like genes is their involvement in floral organ morphogenesis. The conversion of leaves, sepals and petals into carpel-like structures or ovaries in 35S::*HoAGL6* transgenic *Arabidopsis* was very similar to that observed in the *Arabidopsis* plants ectopically expressing both *AG* and *SEP3*[8] (Figure 5). Naturally, activation of *SEP1* and *AG* was revealed in the 35S::*HoAGL6* transgenic plants (Figure 6). In *Arabidopsis*, as a key factor for activation of floral organ identity genes, *LFY* not only directly activated *AG* and *AP1*[51, 53, 54], but also was required for the activation of *AP3/PI*[55]. Hence *HoAGL6* positively regulated the floral organ identity gene *AG*, probably following the flowering pathway: *HoAGL6*/*SOC1*-*LFY*-*AG*. Expression of *SEP1* which was exclusive from vegetative organs could be detected in leaves of ectopically expressing *HoAGL6* plants (Figure 6). The pathway of activating *SEP1* was never observed before. The activation of *SEP1* and *AG* might be a common mechanism for *AGL6*-like genes to regulate floral organ formation.

In order to explain how *AGL6*-like genes regulate floral organ formation, we provided the evidence that both *AG* and *SEP1* could be activated by hyacinth *HoAGL6*. Previous yeast two-hybrid assays revealed that the MADS-box proteins in ABCDE model could form multimeric complexes, putative tetrameric complexes，to control various transcriptional programs in specific organ types[4, 10, 11]. Further, it was found that AGL6 has protein-protein interaction with SEP1, AP1, SHP1, SHP2 or FUL in *Arabidopsis*[19], OMADS1 with OMADS3 (an AP3-like protein) in orchid[38, 56], and OsMADS6 with some of other members of AP1/AGL9 group in rice[39]. AGL6-like proteins should act as their partners in determining the four whorls of flower organs. This hypothesis was also strengthened by the functional analyses of *OMADS1*. We can infer that *AGL6*-like genes as a new class of factors are involved in the mechanism of floral organ development revealed in the

ABCDE model, serving as an activator and partner.

In the ABCDE model, AP1-AP1-SEP-SEP, AP1-AP3-PI-SEP, AP3-PI-AG-SEP, and AG-AG-SEP-SEP complexes are required for the formation of sepals, petals, stamens, and carpels, respectively. A resurvey is required for the model, because of the involvement of *AGL6*-like genes and other MADS-box genes. Here, we tried to propose a dynamical polymerization model, in which the MADS-box proteins dynamically would polymerize as groups of quartets in a dosage-dependent manner to specify groups of cells to form functionally and morphologically perfect floral organs. In the model *AGL6*-like genes would not only be an activator of other floral organ identity genes but also a partner during floral organ determination. The first sepal whorl is sufficiently specified by concomitance of AP1, SEP, SVP and AGL6. The four factors orderly compose a group of polymers, one of which is the AP1-AP1-SEP-SEP polymer. The presence in the quartets of SVP and AGL6-like genes is strengthened by pieces of new evidence: AP1-SVP dimer interacts with the LUG-SEU corepressor complex to inhibit expression of *AG*[57, 58], while *AP1-SEP3* interacts with *SEU* to release *AG* from being inhibited[59]. Furthermore, overexpression of two *SVP*-like MADS-box genes, barley (*Hordeum vulgare*) *BM10* and petunia *UNSHAVEN* causes conversion of floral organs into sepaloid organs with leaf-like features in *Arabidopsis*[60, 61]. These results display that *SVP*-like genes inhibit expression of *AG* and participate in the polymerization in determination of sepal. *HoAGL6* activates *AG* and *SEP1*, and AGL6 has protein-protein interaction with SEP and SVP[19], suggesting that AGL6-like protein is a component in the polymers for sepal. Furthermore, *SVP* and *AGL6* have opposite actions, with the former promoting sepal features and the latter facilitating petal and carpel. In monocots, the expression of *AGL6*-like genes is generally absent in the sepal whorl, resulting in that the perianth whorls are well undistinguished between sepal and petal, exhibiting much sepaloid. In addition，an invasion of other factors into this whorl could change its morphology. For example, in a petaloid-sepal mutant of *Habenaria radiate*, *HrGLO1*, *HrGLO2* and *HrDEF* (*AP3* homologue) present in the sepal whorl[62], suggesting that *AP3*-like genes also influence differentiation between sepal and petal. Similarly to the sepal whorl, the petal whorl could be fully specified by concomitance of AP1, AP3/PI, SEP and AGL6. The four types of factors also dynamically compose a group of tetramers, such as AP1-AP3-PI-SEP. All of *AGL6*-like genes transcript in petal and *HoAGL6* alone can transform leaves into petals in petunia *in vitro* (unpublished data). These results suggested that *AGL6*-like genes were essential factors in the determination of petal fate. The third stamen whorl would be determined by a group of polymers basically composed of AP3/PI, SEP, AG and AGL6. Ectopic expression of *AGL6*-like genes severely influences stamen development, predicting that AGL6 dose changes stamen regulation program. *AGL6*-like genes are generally expressed in stamen primordia at a very early stage, such as *PrMADS2* and *PrMADS2* in *P. radiata*[29]. Transcripts of *AGL6*-like genes are so strictly limited to stamen primordia at the very early stage that they cannot be well detected by Northern blot, thus the gene expression data are unavailable, or the detected signals are very weak, such as *pMADS4*, *OMADS1* and *HoAGL6*. Therefore a more accurate detection such as RNA hybridization *in situ* is required. AGL6-like protein may be involved in the polymerization in stamen primordia at a very early stage. The fourth carpel whorl could be controlled by a tetramer array of STK/SHP, AG, SEP and AGL6. This proposal is strongly supported by the general production of carploid structures and morphologically perfect ovaries in 35S::*HoAGL6* transgenic *Arabidopsis*. All of *AGL6*-like genes are extensively expressed in carpels and ovules, suggesting that they have a function in determination of ovule and carpel identity. Because the ectopic co-expression of SEP3 and SHP1 and/or STK is not sufficient to homeotically transform vegetative organs into carpels with ovules[62,63], the putative AG-AG-SEP-SEP and AG-SHP-SEP-STK tetramers do not fully determine carpel identity. These data suggest AGL6 is a potential partner involved in the polymerization for the formation of ovaries. In addition, *STK* promotes carpel development in the absence of *AG* activity[8], so it should be a component of the quartets. We think that the ectopic co-expression of *AG*, *SHP*, *SEP* and *AGL6* would homeotically transform vegetative organs into ovaries with ovules.

Based on the mechanistic analysis of hyacinth *HoAGL6* in floral organ determination, we propose that the dynamic behavior of the four groups of MADS-box proteins is perfectly responsible for morphogenesis of the four floral organ types. The model can meet various morphologies of floral organs of various floral structures in plant kingdom.

We thank Mr. Zou Qi for valuable suggestions and critical reading of the manuscript.

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