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Characterization of B- and C-class MADS-box genes in medicinal plant *Epimedium sagittatum*

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Abstract

The basal eudicot Epimedium (Barrenwort) exhibits innovative floral morphology in the form of petal spurs filled with nectar and petaloid sepals. The B-class MADS-box genes *APETALAS3* (*AP3*) and *PISTILLA* (*PI*) determine sepal and petal identity while the C-class gene *AGAMOUS* (*AG*) determines carpel and stamen identity in *Arabidopsis*. Complex histories of gene duplication resulted in subsequent subfunctionalization or neofunctionalization of paralogs. Here, a total of four B- and two C-class genes were successfully isolated from *E. sagittatum*. Phylogenetic analysis showed that *EsAP3-1*, *EsAP3-2*, and *EsAP3-3* are part of the AP3 clade; *EsPI* is part of the PI group; and *EsAG* and *EsAG11* clustered into the AG and AG11 groups, respectively. Quantitative real-time PCR was utilized to detect the expression patterns of these genes, and all B-class genes except for *EsAP3-3* were found to be universally expressed. The transcribed *EsAG* and *EsAG11* genes were confined to reproductive organs. In addition, yeast three and two hybrid assays were used to explore the status of protein complexes. EsAP3-1 was found to have broadly interactive partners, and EsPI can form a heterodimer with EsAP3-1 and EsAP3-2. Transgenic EsAG overexpression in wild-type Arabidopsis confirmed the conserved function determining carpel development.

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INTRODUCTION

MADS-box transcription factors (TFs), which incorporate an evolutionarily conserved domain (MADs), can be found across a diverse array of eukaryotes[1]. Land plants contain a higher proportion of MIKC*- and MIKC^C-type MADS-box TFs than any other group of organisms[2,3]. Theoretically, the accumulation of MADS-box genes in angiosperms is likely to have facilitated the morphological diversity exhibited by this group of plants. The 'ABC' model of floral organogenesis was proposed following genetic studies in *Antirrhinum majus* and *Arabidopsis thaliana*[4]. The model was later revised to the 'ABC(D)E' model, after the addition of D and E class and MIKC^C genes[5,6]. In general, it is believed that MADS-box proteins act synergistically during the process of primordial floral organogenesis: C + D + E class genes specify ovules; C + E class genes specify carpels; B + C + E class genes specify stamens; A + B + E class genes specify petals; and A + E class genes specify sepals[7,8]. Several genes related to floral organogenesis have been characterized in Arabidopsis, including the E class genes *SEPALLATA1-4* (*SEP1-4*), the C class gene *AGAMOUS* (*AG*), the B class genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the A class genes *APETALA1* (*AP1*) and *APETALA1* (*AP2*). Each of these genes is considered a MIKC^C MADs-box genes, except for *AP2*.

Two paralogous MADS-box gene lineages, *PISTILLATA* (*PI*) and *APETALA3* (*AP3*), are likely to have arisen before the rise of the angiosperms during a duplication event. In both Arabidopsis and snapdragon, in the third whorl, these genes determine stamen identity and in the second whorl they determine petal identity[9,10]. Across core eudicots and monocots, the expression of B class genes is relatively conserved in stamens, petals, or lodicules. However, divergent expression of B class genes outside of the core eudicots is associated with poorly differentiated perianth. Novel floral morphologies are often associated with B class gene diversification in both function and expression. In *Petunia* for example, stamen filament and petal tube fusion is regulated by *GLO1*[11]. Staminodia, novel floral organs in *Aquilegia*, appear to be specified by B class genes[12]. In Magnoliids, Ranunculales, and basal angiosperms and monocots, homeotic petaloid organogenesis is affected by B class MADS box gene ectopic expression. The expression patterns of B homologous genes from basal eudicots *Akebia trifoliata*, *Platanus acerifolia* and *Trochodendron aralioides* have been investigated deeply in the developing tissues, indicating that divergent function probably involved floral organ identity[13–15].

As with the duplication event resulting in the establishment of the *PI* and *AP3* genes, a whole genome duplication (WGD) event occurring before the rise of the angiosperms likely

contributed to the *AGL11* and *AG* gene lineages, previously defined as C and D lineages[16,17]. In Arabidopsis, the C class gene *AGAMOUS* (*AG*) is responsible for carpel and stamen organogenesis, determination of floral meristem fate, and prevention of A class gene misexpression in floral tissues[18]. In addition to C class gene function, the *AGL11*-like gene *STK* exhibits a particular function in ovular funiculus development[19,20]. In the basal eudicot *Papaver somniferum*, stamen and carpel features are determined by an *AG* orthologue, with *AG-1* involved in stamen and carpels *AG-2* involved only in carpels[21].

Because *Epimedium* plants have evolved unique floral morphologies, including petaloid sepals and spur or sac-like petals with nectariferous tissue on their inner faces[22], these plants are ideal investigation models. Previous studies primarily characterized the function of the *Crabs claw* gene in the development of nectaries and the *AP1/SEP/AGL6* MADS-box superclade in flower development and flavonoid synthesis[19,23–25]. Here, we have functionally characterized the *AP3*, *PI*, *AG*, and *AG11* homologs of the basal eudicot *Epimedium sagittatum* (Berberidaceae, Ranunculales). The results of this study will provide a clearer understanding of how C and B class genes are regulated in *E. sagittatum*.

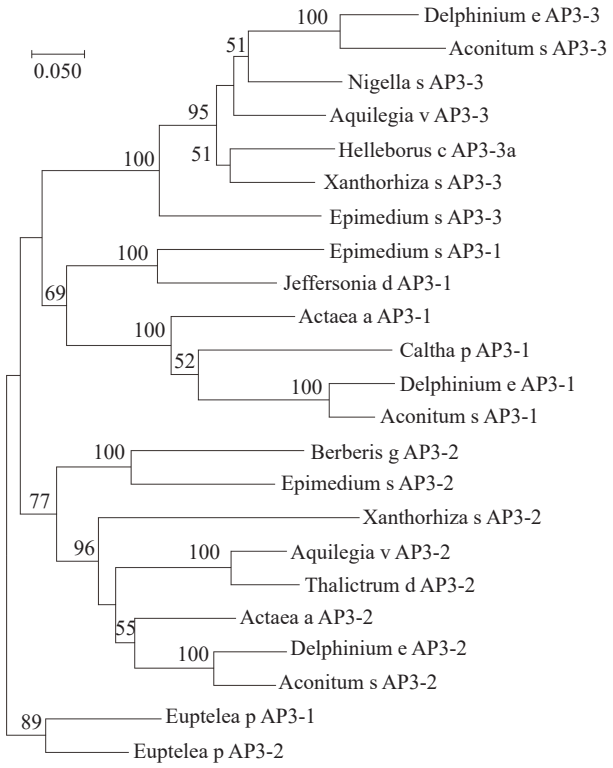

Fig. 1 Phylogeny of *AP3* lineage genes. The maximum likelihood tree was produced using MEGA5 from multiple nucleotide alignments and reveals that the phylogenetic position of *EsAP3-1*, *EsAP3-2* and *EsAP3-3*. Bootstrap values over 50 was shown with 1000 replications. Caltha p AP3-1, EU481813.1; Actaea a AP3-1, HQ647375.1; Delphinium e AP3-1, EU481804.1; Aconitum s AP3-1, EU481818.1; Jeffersonia d AP3-1, EU481791.1; Xanthorhiza s AP3-2, EU481797.1; Delphinium e AP3-3, EU481802.1; Aconitum s AP3-3, EU481816; Nigella s AP3-3, HQ694794.1; Helleborus c AP3-3a AY162876.1; Aquilegia v AP3-3, EF489476.1; Xanthorhiza s AP3-3, EU481796.1; Berberis g AP3-2 AY162859.1; Euptelea p AP3-1, GU357449.1; Euptelea p AP3-2, GU357450.1; Aquilegia v AP3-2, EF489477.1; Thalictrum d AP3-2, AY867876.1; Actaea a AP3-2 HQ647376.1; Delphinium e AP3-2, EU481803.1; Aconitum s AP3-2, EU481817.1.

RESULTS

Phylogenetic analysis of *E. sagittatum* C and B class genes

Five MADS-box genes were discovered in the floral tissues of *E. sagittatum*. Through a BLAST search against the NCBI database, these genes were originally assigned to the C and B class MADS-box genes. MEGA5 was utilized to perform a phylogenetic characterization of the *AG11/AG* and PI/AP3 genes from *E. sagittatum* and other plants using the maximum likelihood method with MEGA5 software[26]. *EsAP3-1*, *EsAP3-2*, and *EsAP3-3* were found to belong to the previously characterized and evolutionarily conserved *AP3-III*, *AP3-II*, and *AP3-I* paralogous lineages (Fig. 1). In contrast, no duplication events were found for the *PI-like* gene (Fig. 2). Based on their topology, *EsAG* and *EsAG11* were assigned to the *AG* and *AG11* lineages (Fig. 3).

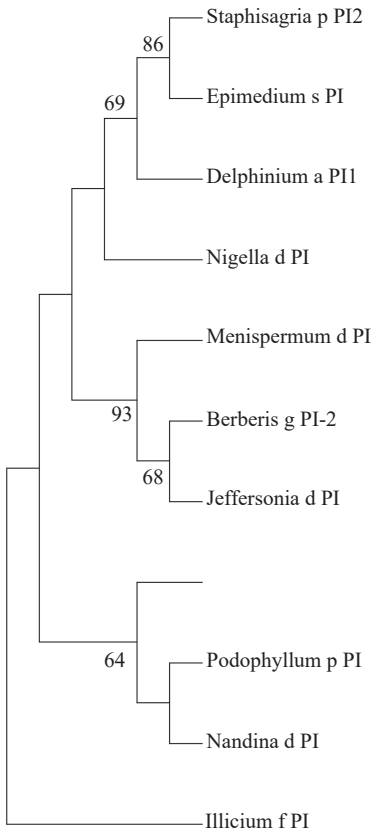

Fig. 2 Phylogeny of *PI* lineage genes. The ML phylogenetic tree from multiple nucleotide alignments of PI homologs from *E. saggitatum* and other species was conducted by MEGA software (version 5). The PI sequence from *Illicium floridanum* was used as an outgroup. Bootstrap test was estimated with 1000 replicate analysis, above 50 support was shown. Genbank Accession Numbers: Podophyllum p PI, HQ694792; Sinofranchetia c PI JQ806397; Staphisagria p PI2 OL469304; Berberis g PI-2 AY162861; Jeffersonia d PI EU481792; Nigella d PI KT984414;Delphinium a PI1 OL469301; Nandina d PI HQ694793; Menispermum d PI EU481787; Illicium f PI AY936224.

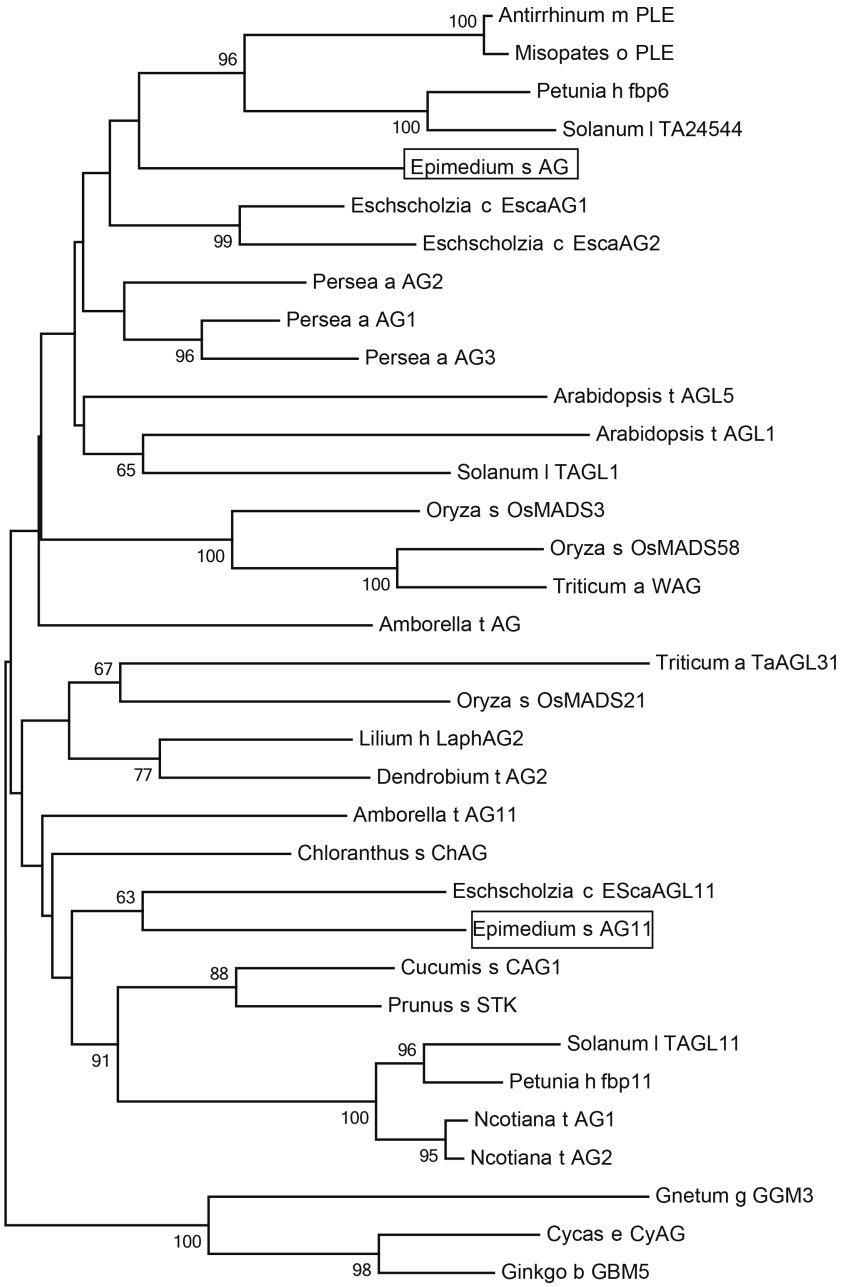

Fig. 3 Phylogeny of AG and AG11 lineage genes from homologs from *E. sagittatum* and other angiosperm species. The C class genes from *Gnetum gnemon*, *Cycas edentata* and *Ginkgo biloba* sequences were used as an outgroup. The ML phylogenetics tree was constructed by MEGA (version 5). Bootstrap test was estimated with 1000 replicate analysis, above 50 support was shown. Genbank Accession Numbers: Arabidopsis t AGL5, M55553.1; Triticum a TaAGL31, DQ512349.1; Oryza s OsMADS21, FJ750944.1; Arabidopsis t AGL1 NM_118013.2; Eschscholzia c EScaAGL11, DQ088998.1; Lilium h LaphAG2, AB359184.1; Dendrobium t AG2, DQ017703.1; Amborella t AG11, XM_006858527.2; Chloranthus s ChAG, AY464099.1; Solanum l TAGL11, AY098736.2; Nicotiana t AG1, KM205819.1; Nicotiana t AG2, NM_001325570.1; Petunia h fbp11, X81852.1; Cucumis s CAG1, NM_001280577.1; Prunus s STK, GU332504.1; Gnetum g GGM3, AJ132209.1; Oryza s OsMADS3, L37528.1; Oryza s OsMADS58, AB232157.1; Triticum a WAG, AB084577.1; Cycas e CyAG, AF492455.1; Ginkgo b GBM5, GU563899.1; Antirrhinum m PLE, AB516404.1; Misopates o PLE, AM162212.1; Petunia h fbp6, X68675.1; Solanum l TA24544, AY098735.2; Amborella t AG, NM_00 1305835.1; Eschscholzia c EscaAG1, DQ088996.1; Eschscholzia c EscaAG2, DQ088997.1; Persea a AG2, DQ398011.1; Persea a AG1, DQ398021.1; Persea a AG3, DQ398023.1.

Patterns of expression of *E. sagittatum* floral MADS-box genes

Gene expression of both C and B class MADS-box genes in mature floral tissue of *E. sagittatum* was evaluated using qRT-PCR (Fig. 4). We combined stamens and carpels due to the difficulty of separating them. Consistent with previous studies on *Epimedium*, *EsAP3-3* expression was restricted to floral nectar spurs. Both *EsAP3-1* and *EsAP3-2* were expressed in leaves, roots, and flowers. *EsPI* exhibited high expression in petals, but displayed minimal expression in stamens/carpels and sepals. *EsAG* was expressed in stamens/carpels, petals, and sepals, while *EsAG11* exhibited stamen/carpel-specific expression.

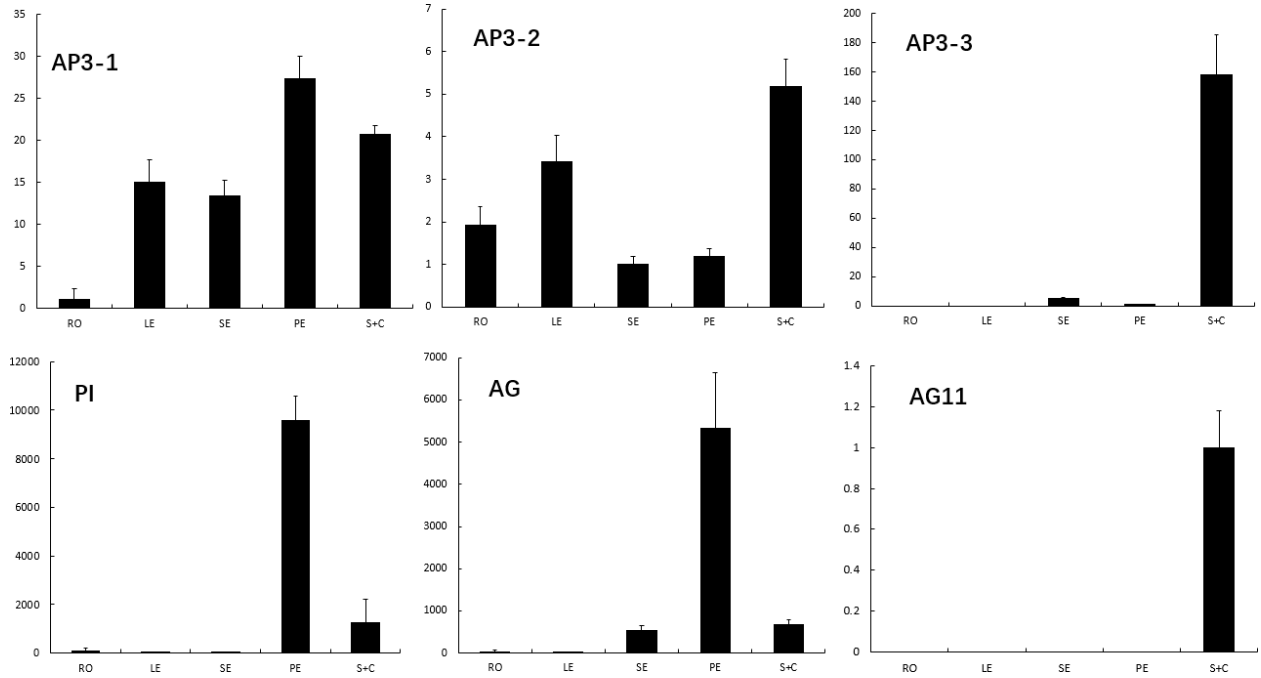

Fig. 4 The expression of *E. sagittatum* B and C class genes in root, leaf, seqal, petal and combination of stamen and carpel. Error bars represent SE for three technical replicates. The expression of each gene was normalized against the *Epimedium actin* gene.

Interaction analysis of the *E. sagittatum* C and B class proteins

Interactions between C and B class proteins were probed using a yeast-two-hybrid assay. Self-activation was not observed for any of the tested proteins after fusion to the GAL4 domain. Additionally, B class proteins exhibited no interactions with C class proteins, and vice versa. Compared with other B class proteins, EsAP3-1 has broadly interactive partners. Furthermore, EsPI may be able to form a heterodimer with EsAP3-1 and EsAP3-2. Additionally, homodimer and semihomodimer interactions were observed between EsAP3-1, EsAP3-2, and EsAP3-3 (Table 1).

Multimeric complex analysis C and B class proteins

The ability of *E. sagittatum* B and C class MADs-box proteins to organize into synergistic complexes was explored in yeast. As previously reported, neither EsAG nor EsAG11 were observed to organize into a heterodimer with the B class proteins. However, AP3-2 (AP3-2-IKC) appears to be able to bring either EsAG or EsAG11 and EsPI together to form a complex (Table 2). In addition, EsAG11 and EsPI interacted strongly when co-expressed together with either AP3-3 (AP3-3-IKC) or PI (PI-IKC) sans MADS-box.

Functional analysis of *EsAG* through Arabidopsis transformation

Agrobacterium-mediated transformation of wild-type Arabidopsis was used for functional characterization of *EsAG*. Specifically, the *EsAG* gene was cloned into ectopic expression vectors containing the CaMV 35S promoter, and four transgenic *35S::EsAG* Arabidopsis lines were obtained. The transformed lines were subsequently confirmed by qRT-PCR (Fig. 5a). A few of the transgenic plants exhibited distinct phenotypes (Fig. 5b), and sepals were homeotically transformed into stigmatic papillae-containing carpelloid structures in other lines (Fig. 5c).

DISCUSSION

To study their roles in the regulation of floral organogenesis, we identified and characterized four B class MADS-box genes (*EsAP3-3*, *EsAP3-1*, *EsAP3-2*, and *EsPI*) through motif and phylogenetic analyses. We found that the three *AP3*-like genes belonged to three phylogenetic lineages, consistent with previous reports suggesting that, prior to the divergence of Ranunculaceae and Berberidaceae, two WGD events occurred[27]. Furthermore, gene duplication events resulting in

Table 1. Data from yeast two-hybrid assay. EsAP3-1, EsAP3-2, EsAP3-3, and EsPI were added to both an activation and GAL4 binding domains. Interactions were analyzed using SD/-Ade/-His/-Trp/-Leu plates containing 10 mM of 3-amino-1,2,4-triazole. Three replicates were analyzed for each experiment.

	BD-EsAP3-1	BD-EsAP3-2	BD-EsAP3-3	BD-EsPI	BD-EsAG	BD-EsAG11
AD-EsAP3-1	+	+	+	+	–	–
AD-EsAP3-2	+	–	+	–	–	–
AD-EsAP3-3	–	–	–	–	–	–
AD-EsPI	+	–	+/–	–	–	–
AD-EsAG	–	–	–	–	–	–
AD-EsAG11	–	–	–	–	–	–

Table 2. Data from yeast three-hybrid assay examining the formation of higher order protein complexes.

Ternary complex	-LTAH + 10 mM 3AT, 28C and LacZi
AD-EsPI + pTFT-AP3-1ΔM + BD	–
AD-EsPI + pTFT-AP3-2ΔM + BD	–
AD-EsPI + pTFT-AP3-3ΔM + BD	–
AD-EsPI + pTFT-PIΔM + BD	–
AD-EsPI + pTFT-AP3-1ΔM + BD-EsAG	–
AD-EsPI + pTFT-AP3-1ΔM + BD-EsAG11	–
AD-EsPI + pTFT-AP3-2ΔM + BD-EsAG	+
AD-EsPI + pTFT-AP3-2ΔM + BD-EsAG11	+
AD-EsPI + pTFT-AP3-3ΔM + BD-EsAG	–
AD-EsPI + pTFT-AP3-3ΔM + BD-EsAG11	+
AD-EsPI + pTFT-PIΔM + BD-EsAG	–
AD-EsPI + pTFT-PIΔM + BD-EsAG11	+
AD + pTFT-AP3-1ΔM + BD-EsAG	–
AD + pTFT-AP3-1ΔM + BD-EsAG11	–
AD + pTFT-AP3-2ΔM + BD-EsAG	–
AD + pTFT-AP3-2ΔM + BD-EsAG11	–
AD + pTFT-AP3-3ΔM + BD-EsAG	–
AD + pTFT-AP3-3ΔM + BD-EsAG11	–
AD + pTFT-PIΔM + BD-EsAG11	–
AD + pTFT-PIΔM + BD-EsAG11	–

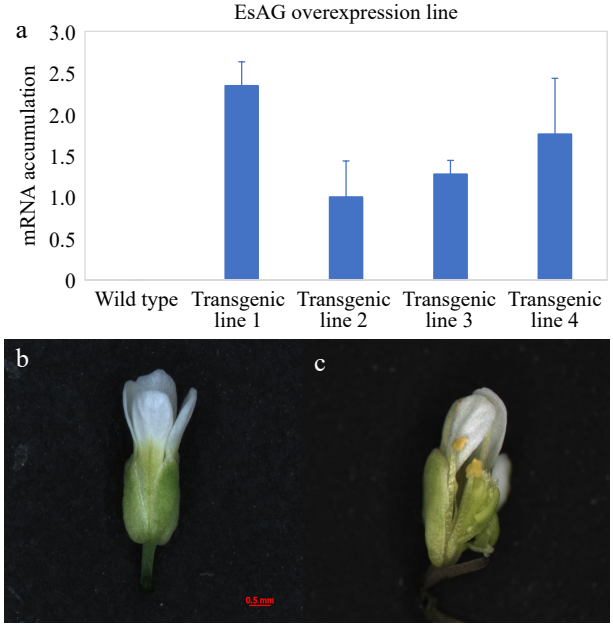

Fig. 5 Functional analysis of *EsAG* in wild-type Arabidopsis. (a) Analysis of EsAG overexpression lines in *35S::EsAG* transgenic Arabidopsis plants. A fragment of *Tubulin* gene was amplified as an internal control. Error bars represent SE for three technical replicate reactions. (b) The flower of the wild-type Arabidopsis. (c) Phenotype of *35S::EsAG* transgenic Arabidopsis in the wild-type background.

alterations in gene expression or coding regions occurred at different scales after plant polyploidy.

EsAP3-1 and *EsAP3-2* exhibited broad expression across floral tissues, although *EsAP3-3* exhibited petal-specific expression, which is consistent with data suggesting that the expression patterns *AP3-3* orthologs among Ranunculales is conserved[27–30]. The absence of *AP3-3* paralog expression in *Aquilegia vulgia* and *Nigella damascene* is associated with the loss of petal initiation[29]. Functional validation will be required to fully characterize the role of *AP3-3* paralogs in petal formation in *Epimedium*. Furthermore, differential heterodimerization patterns among *AP3-3* paralogs is implicated in the diverse roles these genes play in floral organ formation. Our study of the AP3-PI and C protein complex suggests that all three *E. sagittatum AP3*-like genes are likely to have been subfunctionalized after WGD, similarly to *EsAP3-1* and *EsAP3-2*.

We found two novel conserved AG motifs (motifs II and I) in the EsAG and EsAG11 C-terminal regions, which is consistent with previous studies[16,31]. These conserved motifs are likely related to the core eudicot duplication event which produced the AG and AG11 lineages. As with other D and C class genes, both *EsAG* and *EsAG11* exhibit floral tissue-specific expression patterns, where petal and stamen formation are controlled by these orthologs. In Arabidopsis, AG orthologs regulate carpel and stamen organogenesis dictate ovule identity though synergistic interactions with E and D class genes, limit the determinacy of floral meristematic tissue, and act as repressors of A class genes[4] . The ability of both EsAG and EsAG11 to differentially interact with the B protein dimer complex in *E. sagittatum* indicates the possibility that they work to regulate floral organogenesis. Transgenic overexpression of *AG* in *Arabidopsis* caused sepals to homeotically transform into carpelloid structures, and petals to transform into stamen-like structures[32]. Here, we also observed phenotypic alterations in the *EsAG* transgenic *Arabidopsis* line, suggesting that *EsAG* regulates carpel and stamen organogenesis across plant lineages.

MATERIALS AND METHODS

Plants and cultivation

Field-grown *E. sagittatum* transplants were cultivated at the Wuhan Botanical Garden at the Chinese Academy of Sciences (Wuhan, China). Columbia ecotype *Arabidopsis thaliana* plants were grown using 8 h of dark and 16 h of light.

Cloning of C and B class genes from *E. sagittatum*

Leaves, roots, and inflorescences of *E. sagittatum* were used to extract total RNA with the Trizol reagent (Invitrogen, CA, USA), and subsequently digested using RQ1 RNase-free DNase (Promega, WI, USA). First-strand cDNA was transcribed with and the poly-T primer 3'-CDS and Superscript III reverse transcriptase (Invitrogen, CA, USA). 5'- and 3'-RACE PCR were used to isolate the *EsAP3-I*, *II*, *III*, *EsPI*, *EsAG*, and *EsAG11* genes. 3'-RACE was performed using the p5B and SMARTII primers for B class genes, and the B2 primer for *EsAG* and *EsAG11*. First strand synthesis was carried out with the 5'-CDS and SMART II primers. Two 5' RACE reactions were carried out using the 5' specific primer 1 and UPM for the first strand, and 5' specific primer 2 and NUP for the second strand. Both the 3' and 5' UTRs were utilized to generate full-length sequences. The primers used for this experiment can be found in Supplemental Table S1.

qRT-PCR

The expression of *EsAP3-I*, *EsAP3-II*, *EsAP3-III*, *EsPI*, *EsAG*, and *EsAG11* were investigated in leaves and floral organs, including carpels, stamens, petals, and sepals, at both preanthesis and anthesis. Prior to qRT-PCR, a PrimeScript RT kit (Takara, Japan) was utilized to perform first strand synthesis and DNA decontamination. All assays were performed using a 20 μl reaction volume, including 10 ng of cDNA, and 10 μM SYBR Premix Ex

Taq II and primers (Takara, Japan). qRT-PCR was performed with an ABI7500 Real-Time PCR system (ABI, USA), using the following protocol: denaturation stage (30 s at 95 °C, then 40 cycles of 3 s at 94 °C and 30 s at 60 °C) and dissociation stage (15 s at 95 °C, 1 min at 60 °C, 30 s at 60 °C). Gene expression was quantified using the two-delta CT relative calculation method. *EsActin* was used as the internal reference. Each experiment consisted of three technical replicates for each of three biological replicates. Data was handled using software provided in ABI7500.

Phylogenetic analysis

Phylogenetic analyses of specific C and B class genes were performed on full-length nucleotide alignments. The maximum likelihood (ML) method was employed in MEGA5 with the GTR model and four categories of rate substitution. ML analyses were carried out using 500 bootstrap replications and the default settings. Data were used to estimate the Gamma distribution. The tree topology was obtained through the nearest-neighbor interchange strategy.

Yeast three- and two-hybrid assays

EsAG and *EsAG11* full-length genes together with the IKC parts from full-length *EsAP3-I*, *EsAP3-II*, *EsAP3-III*, and *EsPI* were amplified from pMD19-T using primers that introduced 3′ and 5′ restriction sites. The resultant PCR products were subsequently digested using the corresponding restriction enzymes and introduced into the pGADT7 and pGBKT7 vectors (Clontech, CA, USA). For the yeast three-hybrid assay, the third protein, containing both the ADE selectable marker gene and the full-length ADH1 promoter, was expressed from the pTFT1 vector. M-domain lacking EsAP3-I, EsAP3-II, EsAP3-III, and EsPI were cloned, in frame with the SV40 coding region, into the pTFT1 vector. Negative controls consisted of co-transformed AD or BD empty and insertion vectors. For the yeast two-hybrid assay, the constructed empty pGADT7 and pGBKT7 were introduced into AH109 yeast (*Saccharomyces cerevisiae*) according to the LiAc transformation procedure to detect autoactivation. Yeast three- and two-hybrid assays were tested by using SD medium lacking tryptophan, histidine, adenine, and leucine, and containing 10 mM 3-Amino-1, 2, 4-triazole.

Transgenic analysis

In order to construct the p35Spro EsAG vector, KpnI and SalI were utilized to cut the full-length versions of each gene from the PMD19-T vector (Takara, Japan). The genes were then inserted into the pMV binary vector (derived from pBI121) after previous digestion with KpnI and XhoI. *Agrobacterium tumefaciens* GV3101 was used to introduce the construct into Arabidopsis through floral dipping[33]. Murashige and Skoog medium at half strength containing 50 ug/mL Kanacymin was used to select transgenic seedlings. Transgenic leaf-level gene expression was determined using qRT-PCR, with *Tublin* as the internal reference gene.

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Conflict of interest

Wei Sun is the Editorial Board member of journal *Medicinal Plant Biology*. He was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and his research group.

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