Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.083737

ARTICLE

PsSEP3L1 Positively Regulates Petal Number in Tree Peony (*Paeonia suffruticosa*) by Interacting with MADS-Box Family Proteins

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Received: 09 April 2026; Accepted: 15 May 2026; Published: 29 June 2026

ABSTRACT: *SEP3*, one of the SEPALLATA (SEP) genes, plays a crucial role in the regulation of floral organ morphogenesis in plants. However, its specific function and molecular regulatory mechanisms remain largely unclear in tree peony (*Paeonia suffruticosa* Andr.). In this study, the *PsSEP3L1* sequence of the tree peony cultivar 'Luoyang Hong' was obtained by homologous cloning. The open reading frame of *PsSEP3L1* is 738 bp and encodes 245 amino acids. Phylogenetic analysis indicated that PsSEP3L1 was most closely related to the SEP3 homolog from *cassava*. The expression level of *PsSEP3L1* in petals, petaloid petals, and stamens was significantly higher than that in sepals. Specifically, in the petaloid petals of the tree peony cultivar 'Luoyang Hong', the expression level was 23.73-fold higher than that in sepals. Functional assays demonstrated that overexpression of *PsSEP3L1* significantly increased petal number in tree peony, whereas silencing of this gene resulted in a significant reduction in petal number, confirming its positive regulatory role in petal number determination. The Yeast two-hybrid and Bimolecular fluorescence complementation assays revealed that PsSEP3L1 protein interacts with MADS-box family proteins, including PsSEP1L, PsAP3, and PsAG. Collectively, *PsSEP3L1* may participate in regulating petal number in tree peony by forming protein complexes with other MADS-box proteins, thereby contributing to floral organ morphogenesis. This study provides a basis for further mechanistic studies on floral organ morphogenesis in tree peony.

KEYWORDS: Tree peony; *PsSEP3L1*; MADS-box; floral organ

1 Introduction

Flower type is a key trait that determines the ornamental value of horticultural plants and represents an important focus in horticultural plant breeding. In flowering plants, floral morphology arises from the differentiation and development of floral organs, a process governed by a complex genetic regulatory network [1–5]. The ABCDE model was developed based on earlier studies elucidating the genetic basis of floral organ identity [6–9]. According to this model, class C genes are responsible for pistil development, class D genes are involved in ovule development, class A and B genes cooperatively regulate petal development, and class B and C genes jointly control stamen identity. Notably, class E genes serve as key cofactors, forming multi-protein complexes with other MADS-box gene classes to participate in floral organ identity and developmental regulation.

The class E gene SEP plays a key role in the regulation of flower development. They are involved in floral organ differentiation and contribute to a complex regulatory network during this process. In Arabidopsis thaliana, loss of function of SEP1, SEP2, and SEP3 (which are normally expressed in petals,

stamens, and carpels) causes all floral organs to develop into sepal-like structures [10], while *SEP4* is involved in the development of sepals, petals, and stamens [11]. SEP genes are key components of the floral regulatory network, and the proteins they encode can form complexes with MADS-box proteins and participate in the regulation of floral organ identity and floral meristem regulation [12,13]. In *Rosa* × *hybrida*, SEP proteins interact with AP3, PI, and AG and contribute to the regulation of floral organ formation [14]. Similarly, in orchids, SEP proteins form complexes with class B proteins and affect floral organ structure [15].

Tree peony is a traditional ornamental flower widely known in China and holds an important place in horticulture and the flower industry. Long periods of cultivation and repeated artificial selection have produced many cultivars with diverse flower forms [16–18]. Among these cultivars, clear variation appears in petal number, the level of stamen petalization, and the spatial arrangement of floral organs. These traits not only contribute to the ornamental value of tree peony but also provide significant resources for examining the development and variation of floral organs [19–21]. The development of floral organs in tree peony is mainly regulated by the MADS-box gene family, and several functional genes have been identified, including *PsAP1*, *PsAP2*, *PsPI*, *PsMADS1*, *PsMADS5*, *PsMADS9*, and *PsAG* [22–25].

Previous studies have characterized the expression patterns of MADS-box genes in different floral organs of tree peony. For instance, Wang [26] reported that *PsAP1* and *PsAP2* are mainly expressed in bracts and sepals; *PsAP3* and *PsPI* are highly expressed in petals and stamens; *PsAG* shows high expression in stamens; *PsSEP3* is expressed in all four floral organ whorls; and *PsSEP4* is mainly expressed in sepals and stamens. Related studies have cloned *PsuSEP3L* and *PsuSEP3S* from tree peony, and transient expression analysis revealed that PsuSEP3L and PsuSEP3S have distinct functions. Through interactions with PsuAP1 and PsuAG, they exert different effects on floral organ formation in tree peony [27].

In this study, *PsSEP3L1* in tree peony was used as the research subject, and its molecular characteristics were analyzed through gene cloning and bioinformatics analysis. Expression pattern analysis was performed to examine the relative expression levels of this gene in different floral organs across different tree peony cultivars during flower development, and to analyze its potential function. The function of *PsSEP3L1* in organ development was further assessed using homologous transient transformation in tree peony. Additionally, a yeast two-hybrid assay was used to investigate the interaction between PsSEP3L1 and other MADS-box proteins. Collectively, this study on *PsSEP3L1* in tree peony aims to explore the role of class E genes in floral organ development and to provide a theoretical basis for understanding the molecular mechanisms of floral organ morphogenesis in tree peony.

2 Materials and Methods

2.1 Plant Materials

The experimental materials were collected from the experimental field (34.60404° N, 112.42325° E) of Jingyuan Farm, Henan University of Science and Technology. Four tree peony cultivars were selected: the single-petal cultivar ‘Ziban’, the semi-double-petal cultivar ‘Baqiandaichun’, the double-petal cultivar ‘Doulv’, and the multi-flowered cultivar ‘Luoyang Hong’. The sepals, outer petals, inner petals, and stamens (Se, OP, IP, St) of the single-petal cultivar ‘Ziban’ and the sepals, petals, petaloid petals, and stamens (Se, Pe, Pet, St) of the semi-double-petal, double-petal, and multi-flowered cultivars ‘Baqiandaichun’, ‘Doulv’, and ‘Luoyang Hong’ were quickly frozen in liquid nitrogen and stored at −80°C.

2.2 Total RNA Extraction, cDNA Synthesis and Gene Cloning of Tree Peony

Total RNA was extracted from each tissue of tree peony cultivars ‘Luoyang Hong’, ‘Ziban’, ‘Baqiandaichun’ and ‘Doulv’ using an RNA extraction kit (TianGen, Beijing). The resulting RNA was

reverse transcribed into cDNA using a reverse transcription kit (Accurate, Hunan). The tree peony cultivar 'Luoyang Hong' cDNA was used as a template to amplify *PsSEP3L1* using a high-fidelity enzyme (TransGen Biotech, Beijing). The target DNA fragment was excised from the gel at the expected size and recovered using a gel recovery kit equipped with a microcolumn concentration system (Zoman, Beijing). The primers used in this study are listed in Table S1.

2.3 Bioinformatics Analysis

The amino acid sequence, physicochemical properties, transmembrane domain, phosphorylation sites, secondary structure, tertiary structure, conserved domain, signal peptide, hydrophilicity, hydrophobicity, subcellular localization and phylogenetic tree of PsSEP3L1 were predicted and analyzed using software and online platforms (Table S2).

2.4 Expression Analysis of PsSEP3L1 in Tree Peony

Four varieties of tree peonies were selected for qRT-PCR expression analysis of *PsSEP3L1*: the single-petal cultivar 'Ziban', the semi-double-petal cultivar 'Baqiandaichun', the double-petal cultivar 'Doulv', and the multi-flowered cultivar 'Luoyang Hong'. Sepals, outer petals, inner petals, and stamens (Se, OP, IP, and St) from the single-petal cultivar, and sepals, petals, petaloid petals, and stamens (Se, Pe, Pet, and St) from the semi-double-petal and double-petal cultivars at full bloom were selected. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and statistically analyzed with SPSS software. The primers used in this study are listed in Table S1.

2.5 Experimental Methods for the Subcellular Localization of PsSEP3L1

The CDS of *PsSEP3L1*, excluding the stop codon, was amplified to generate the 35S::*PsSEP3L1*-GFP fusion construct using gene-specific primers listed in Table S1. Agrobacterium cultures harboring 35S::GFP and 35S::*PsSEP3L1*-GFP were infiltrated into leaves of *Nicotiana benthamiana* respectively. To induce protein expression, the infiltrated Nicotiana benthamiana leaves were incubated at 25°C in the dark for 48 hours. The subcellular localization of PsSEP3L1 in tobacco leaf cells was then examined using a laser scanning confocal microscope.

2.6 PsSEP3L1 Homologous Transient Transformation in Tree Peony

According to the *PsSEP3L1* sequence, the *PsSEP3L1*-1300 overexpression vector and the *PsSEP3L1*-TRV2 silencing vector were successfully constructed (Table S1). The tree peony cultivar 'Luoyang Hong' was used as the experimental material. The study found that during the flower organ development process of tree peony, the big-bell stage represents a critical developmental period of flower type formation [28,29]. Therefore, we selected the big-bell stage for experimental investigation. TRV1 was mixed in equal volumes with the bacterial solutions containing PsSEP3L1-TRV2 or TRV2. The bacterial solutions containing the empty 1300 vector and PsSEP3L1-1300 were resuspended in infection solution and incubated in the dark for 3–4 h. The bacterial suspension was then injected into the pedicels using a 1 mL sterile syringe every 5 to 7 days until the color emergence stage of flower buds and pedicels. When the flowers reached the full-bloom stage, phenotypic observations were recorded, the number of petals was counted for analysis, and samples were immediately taken for qRT-PCR to detect the changes in gene expression. Six independent biological replicates were conducted for the transient transformation experiment. The *t*-test was performed using SPSS 16 to verify statistical differences and the results were visualized using Prism 5.0.

2.7 Yeast Two-Hybrid Assay (Y2H)

Based on the database previously established by our group, the genes *PsSEP1L*, *PsAP1*, *PsAP3*, and *PsAG*, which may interact with *PsSEP3L1*, were screened. Restriction sites EcoRI and BamHI were selected according to the pGBKT7 and pGADT7 vector sequences, and cloning primers were designed according to the gene sequence and the vector restriction site (Table S1). The pGBKT7-*PsSEP3L1*, pGADT7-*PsSEP3L1*, pGADT7-*PsSEP1L*, pGADT7-*PsAP1*, pGADT7-*PsAP3*, and pGADT7-*PsAG* fusion vectors were constructed. Yeastmaker™ Yeast Transformation System 2 was used to prepare Y2H Gold yeast competent cells and perform yeast transformation. The recombinant plasmids pGADT7 and pGBKT7 were co-transfected into yeast cells. The transformed yeast strains were coated with SD/-Trp solid medium and SD/-Leu/-Trp/x-α-gal solid medium to verify whether gene autoactivation existed. After the single colony grew on SD/-Trp solid medium, it was picked and transferred into SD/-Trp liquid medium. After 10 h, the OD value of the bacterial solution was detected to verify whether the bait gene was toxic. After co-transformation, the bacterial solution was coated on SD/-Leu/-Trp solid medium, serially diluted in four gradients, and then dot-coated on SD/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp/X-α-Gal solid media, respectively. After culturing at 30°C for 3 to 5 days, blue and white spots were observed.

2.8 Bimolecular Fluorescence Complementation Assay (BiFC)

The CDS of *PsSEP3L1* was inserted into the cYFP vector, while the CDSs of *PsSEP1L*, *PsAP1*, *PsAP3*, and *PsAG* were individually inserted into the nYFP vector, generating the fusion constructs cYFP-*PsSEP3L1*, nYFP-*PsSEP1L*, nYFP-*PsAP1*, nYFP-*PsAP3*, and nYFP-*PsAG*. cYFP-*PsSEP3L1* was co-infiltrated with nYFP-*PsSEP1L*, nYFP-*PsAP1*, nYFP-*PsAP3*, or nYFP-*PsAG* into tobacco leaves respectively. Conduct both positive and negative controls simultaneously. After 48–72 h of cultivation, yellow fluorescent protein (YFP) signals were observed using a confocal laser scanning microscope. The primers used in this study are listed in Table S1.

3 Experimental Results

3.1 Cloning and Bioinformatic Analysis of the PsSEP3L1 in Tree Peony

Using cDNA from the tree peony cultivar 'Luoyang Hong' as a template, *PsSEP3L1* was obtained by homologous cloning, and its full-length sequence was 752 bp (Fig. 1A). Bioinformatic analysis showed that the open reading frame (ORF) of PsSEP3L1 was 738 bp, encoding 245 amino acids. Prediction of the physicochemical properties of PsSEP3L1 protein indicated that its molecular formula was $C_{1247}H_{1972}N_{354}O_{377}S_8$, with a total of 3958 atoms. The theoretical isoelectric point (pI) of PsSEP3L1 is 8.64, and its relative molecular mass is 28,212.0 Da. The protein contains 34 positively charged residues (Arg + Lys) and 31 negatively charged residues (Asp + Glu). With an instability index of 41.53, the protein is predicted to be unstable. Hydrophilicity and hydrophobicity analyses indicate that PsSEP3L1 is a hydrophilic protein (Fig. 1D). Transmembrane structure prediction using TMHMM showed that the protein does not contain a transmembrane domain (Fig. 1E). Phosphorylation site prediction showed that PsSEP3L1 contained 45 putative phosphorylation sites on threonine residues (Fig. 1F). Protein structure prediction showed that the secondary structure of PsSEP3L1 consisted mainly of α-helix (56.33%), random coil (30.61%), extended strand (8.98%), and β-corner (4.08%) (Fig. 1B). Swiss-model analysis revealed a predicted tertiary protein structure (Fig. 1C). Conserved domain analysis revealed that PsSEP3L1 contained a MADS-box and a K-box conserved domain (Fig. 1H). Subcellular localization studies revealed that the PsSEP3L1 protein is localized in the nucleus (Fig. 1G).

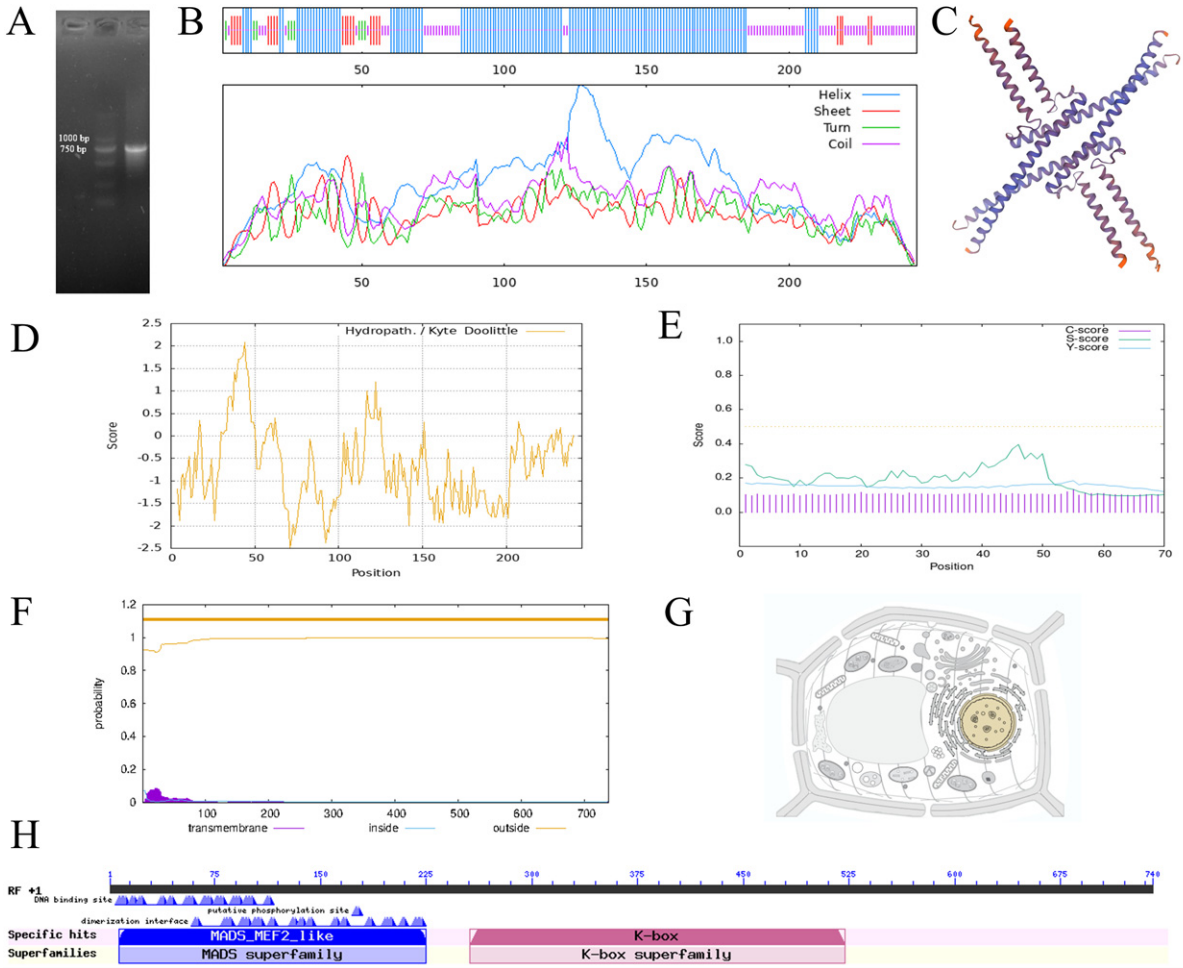

Figure 1: Cloning and bioinformatics analysis of *PsSEP3L1*. (**A**): Gel electrophoresis of PsSEP3L1, (**B**): Secondary structure of the PsSEP3L1 protein, (**C**): Tertiary structure protein of the PsSEP3L1 protein, (**D**): Hydrophilic-hydrophobic analysis of the PsSEP3L1 protein, (**E**): Signal peptide of the PsSEP3L1 protein, (**F**): Transmembrane domain analysis of the PsSEP3L1 protein, (**G**): Subcellular localization prediction of the PsSEP3L1 protein, (**H**): Conserved domain analysis map of the PsSEP3L1 protein.

3.2 Amino Acid Sequence Alignment and Phylogenetic Tree Analysis of PsSEP3L1 in Tree Peony

The predicted amino acid sequence of PsSEP3L1 was analyzed using BLAST (National Center for Biotechnology Information, NCBI). The results showed that PsSEP3L1 shared 62.10% sequence identity with the SEP3 protein of *Malania oleifera* and 60.47% similarity with that of *Ipomoea batatas*. Amino acid sequences of SEP3 proteins from the nine plant species most closely related to PsSEP3L1 were retrieved from the NCBI database and aligned using DNAMAN (Fig. 2A). The resulting phylogenetic analysis showed that the protein encoded by PsSEP3L1 is closely related to that of *Manihot esculenta*, but more distantly related to those of *Vitis riparia* and *Herrania umbratica* (Fig. 2B).

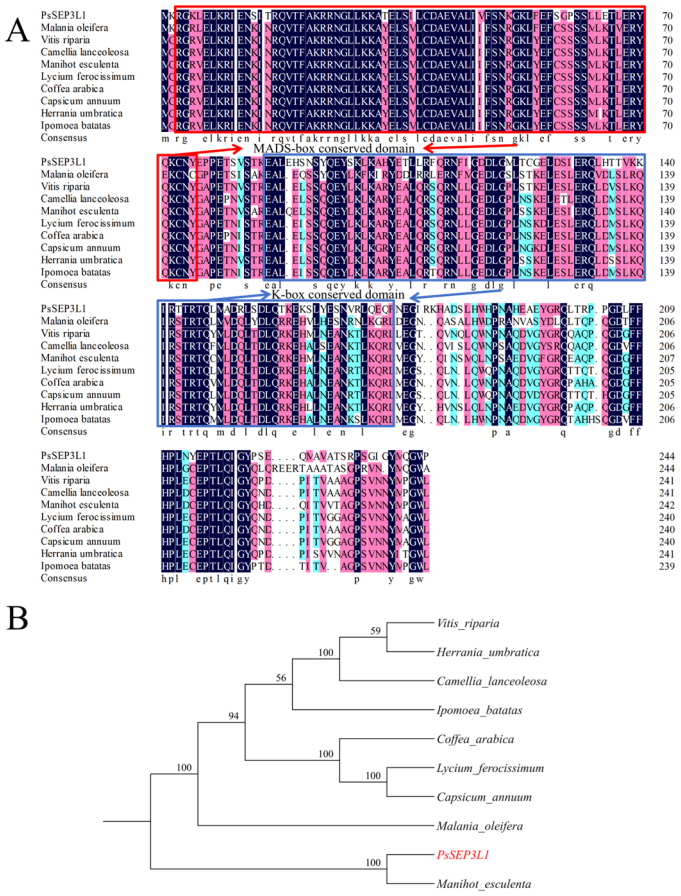

Figure 2: Comparison of amino acids sequences encoded by the tree peony *PsSEP3L1* and phylogenetic tree analysis of PsSEP3L1. (**A**): Amino acid sequence alignment of *PsSEP3L1* with SEP3 proteins from other species; The red box contains the MADS-box domain, while the blue box contains the K-box domain; (**B**): Phylogenetic analysis of PsSEP3L1.

3.3 Analysis of Expression Pattern of PsSEP3L1 in Tree Peony

To explore the expression pattern of *PsSEP3L1* in tree peony, its expression levels in different cultivars and floral organs were analyzed using qRT-PCR. The results showed that the relative expression level of *PsSEP3L1* differed significantly among different cultivars and tissues (Fig. 3). In the single-petal cultivar 'Ziban', the relative expression of *PsSEP3L1* in the outer and inner petals was 2.41- and 3.00-fold higher than that in the sepals, respectively. In the semi-double-petal cultivar 'Baqiandaichun', its expression in petals, petaloid petals, and stamens was 5.04, 6.88, and 5.38 times higher than that in sepals. In the double-petal cultivar 'Doulv', the relative expression of *PsSEP3L1* in petals, petaloid petals, and stamens was 6.41, 5.52, and 3.98 times higher than that in sepals. In the multi-flowered cultivar 'Luoyang Hong', the relative expression level of *PsSEP3L1* was 13.76-fold higher in petals, 23.73-fold higher in petaloid petals, and 10.61-fold higher in stamens compared with that in sepals (Fig. 3). Collectively, these analyses showed that *PsSEP3L1* exhibited the highest expression levels in petals and petaloid petals.

The above analysis revealed that *PsSEP3L1* was expressed in all floral organs with significant differences in the single-petal varieties, with higher relative expression in the outer and inner petals. In cultivars with semi-double, double, and multi-petal flowers, the relative expression levels of *PsSEP3L1* in petals, petaloid stamens, and stamens were significantly higher than those in sepals. Therefore, these findings indicated that

PsSEP3L1 is mainly expressed in petals, petaloid petals, and stamens, and may play a role in the formation of tree peony floral organs.

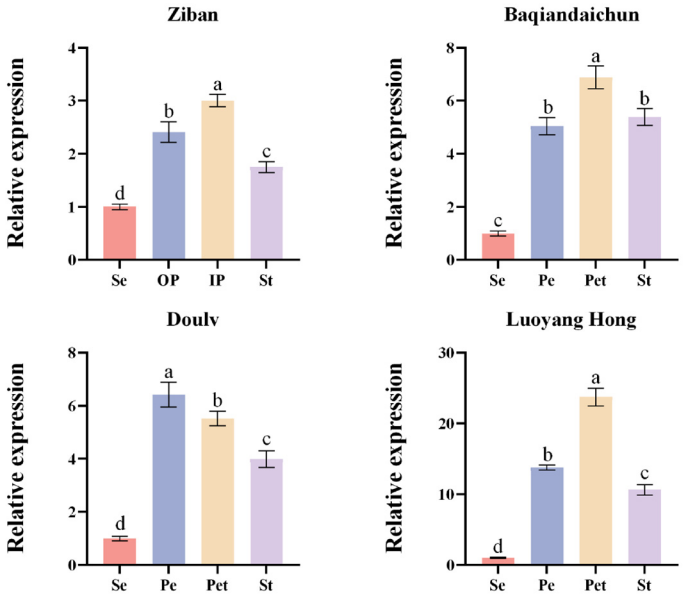

Figure 3: Relative expression levels of PsSEP3L1 in different floral organs among four tree peony cultivars. Se: Sepal, OP: Outer petal, IP: Inner petal, Pe: Petal, Pet: Petaloid organ, St: Stamen; Different letters indicate significant differences at $p < 0.05$ (one-way ANOVA).

3.4 Subcellular Localization of PsSEP3L1

PsSEP3L1 was transiently expressed in *N. benthamiana* leaves, and its subcellular localization was examined using laser scanning confocal microscopy. Fluorescence signals observed in leaves infiltrated with 35S::*PsSEP3L1*-GFP indicated that PsSEP3L1 is localized in the nucleus (Fig. 4). This finding is consistent with the prediction.

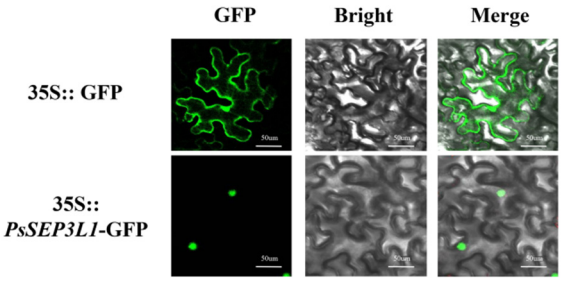

Figure 4: Subcellular localization of PsSEP3L1.

3.5 PsSEP3L1 Increases the Number of Petals

To further investigate the function of *PsSEP3L1*, transient overexpression and virus-induced gene silencing (VIGS) assays were used on the flowers of the tree peony cultivar 'Luoyang Hong' at the big-bell stage. Observation and statistical analysis of flower organs in the flowering stage of transient plants (Fig. 5A) showed that overexpression of *PsSEP3L1* significantly increased the number of tree peony petals (Fig. 5B),

while silencing this gene resulted in a significant reduction in the number of petals (Fig. 5C), indicating that *PsSEP3L1* positively regulates the number of tree peony petals.

RNA was extracted from petals, petaloid petals, and stamens (Pe, Pet, and St) of different transient plants, and cDNA was synthesized for quantitative real-time PCR analysis. The relative expression of *PsSEP3L1* in overexpressed plants was significantly higher than that in the empty vector. The relative expression levels in petals, petaloid petals, and stamens of overexpressed plants were 2.19-fold, 3.60-fold, and 1.83-fold higher than those in the empty vector, respectively, and the highest relative expression level was found in petaloid petals (Fig. 5D). Conversely, in PsSEP3L1-silenced plants, the relative expression levels of *PsSEP3L1* in petals, petaloid petals, and stamens were decreased by 33.23%, 42.32%, and 40.48%, respectively, compared with the TRV2 empty vector, and the decrease was the largest in petaloid petals (Fig. 5E). Collectively, these results demonstrate that PsSEP3L1 plays a role in regulating petal number in tree peony.

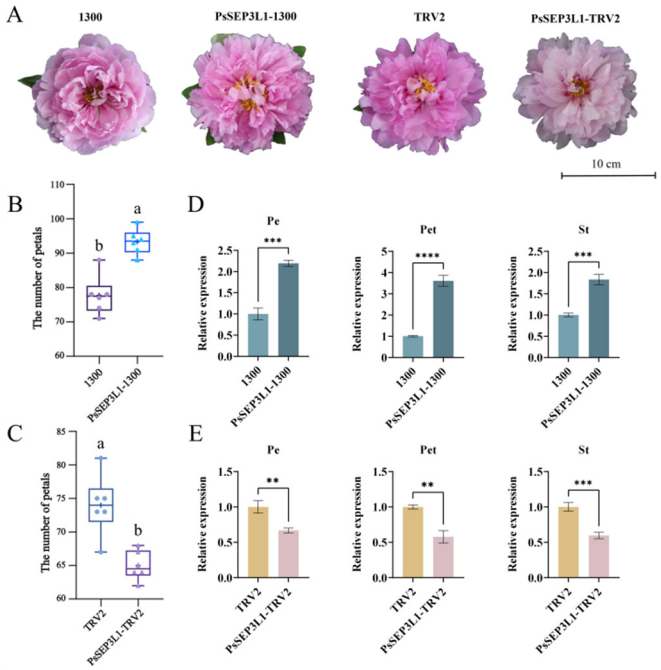

Figure 5: Transient transformation panel of *PsSEP3L1* in tree peony. (**A**): Morphological observation of transient transformation, (**B**): Petal count changes after overexpression, (**C**): Petal count changes after silencing, (**D**): Relative expression levels of *PsSEP3L1* after overexpression, (**E**): Relative expression levels of *PsSEP3L1* after silencing. Pe: Petals, Pet: Petaloid organ, St: Stamen. Asterisks denote significant differences: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Different letters indicate significant differences ($p < 0.05$), as determined by one-way ANOVA.

3.6 PsSEP3L1 Interacts with PsSEP1L, PsAP3, and PsAG

To explore the regulatory mechanism of PsSEP3L1 in flower organ number in tree peony, yeast two-hybrid experiments were performed. Using cDNA from the tree peony cultivar 'Luoyang Hong' petals as a template, the cDNA was cloned into pGBKT7 and pGADT7 vectors via homologous recombination. The pGBKT7-*PsSEP3L1*, pGADT7-*PsSEP1L*, pGADT7-*PsAP1*, pGADT7-*PsAP3*, and pGADT7-*PsAG* fusion vectors were successfully constructed. The following combinations were co-transformed into yeast cells: pGADT7-T with pGBKT7-LAM (negative control), pGADT7-T with pGBKT7-p53 (positive control), pGADT7-*PsSEP1L* with pGBKT7-*PsSEP3L1*, pGADT7-*PsAP1* with pGBKT7-*PsSEP3L1*, pGADT7-*PsAP3* with

pGBKT7-*PsSEP3L1*, and pGADT7-*PsAG* with pGBKT7-*PsSEP3L1*. The co-transformed Y2HGold yeast strain grew normally on SD/-Leu/-Trp medium. Among them, the combinations pGADT7-T with pGBKT7-p53, pGADT7-*PsSEP1L* with pGBKT7-*PsSEP3L1*, pGADT7-*PsAP3* with pGBKT7-*PsSEP3L1*, and pGADT7-*PsAG* with pGBKT7-*PsSEP3L1* were able to grow on SD/-Leu/-Trp/-His/-Ade medium and produced blue colonies (Fig. 6A), indicating that PsSEP1L, PsAP3, and PsAG interact with PsSEP3L1. BiFC further assay indicated that PsSEP3L1L with PsSEP1L, PsAP3 and PsAG inter-acted in the nucleus of *N. benthamiana* (Fig. 6B). Given that PsSEP3L1 is a member of the MADS-box family, we speculate that it may function in combination with other MADS-box proteins to form multi-protein complexes, thereby co-regulating floral organ development in tree peony.

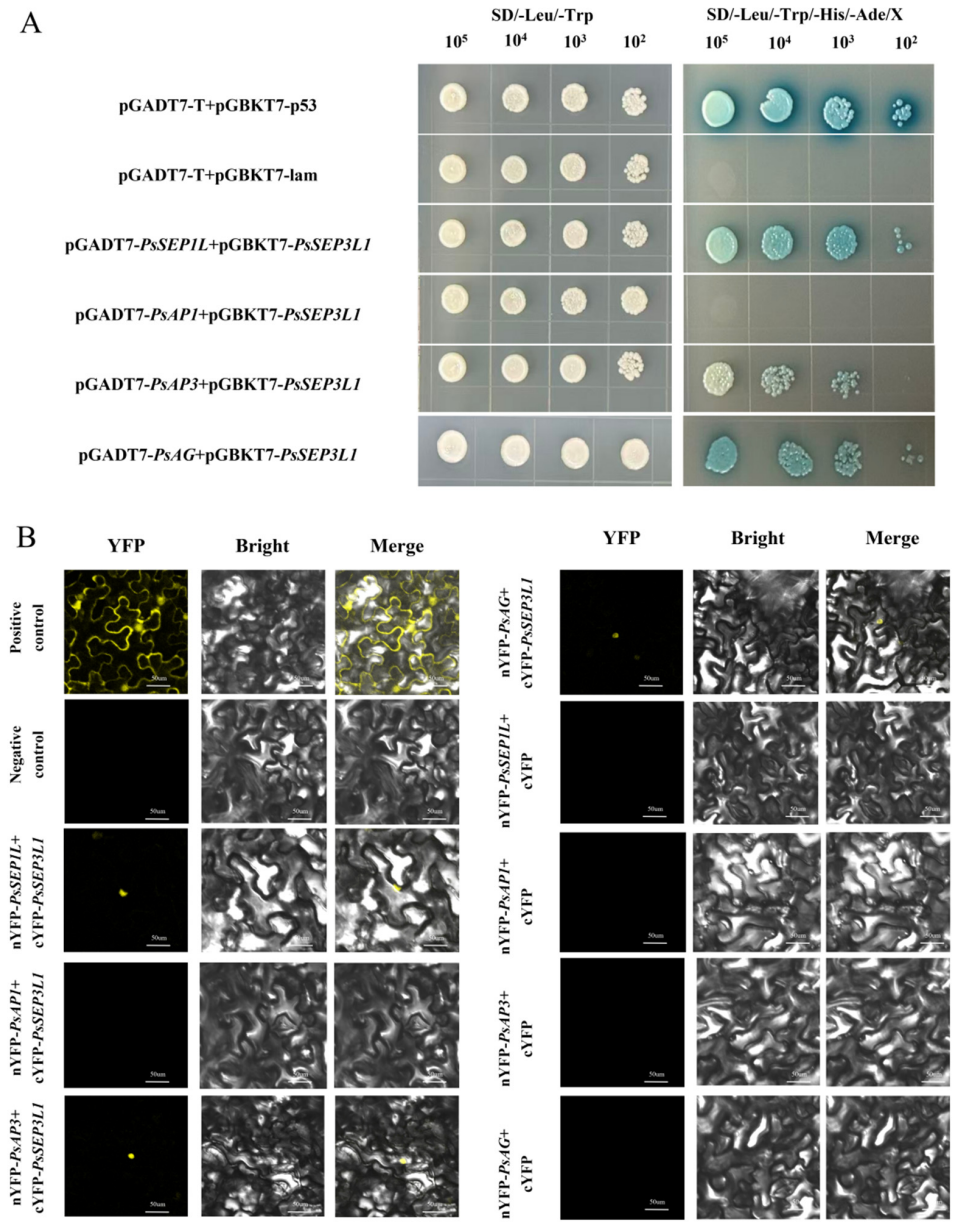

Figure 6: PsSEP3L1 interacts with PsSEP1L, PsAP3, and PsAG. (**A**): Yeast two-hybrid assay of PsSEP3L1, (**B**): Bimolecular fluorescence complementation assay of PsSEP3L1.

4 Discussions

Flower type is an important trait affecting the ornamental value of plants, and variation in floral organ morphology forms the basis of floral diversity [30]. Studies have shown that numerous transcription factors regulate floral organ development. Among these, the MADS-box gene family is crucial for multiple floral tissues and developmental stages, playing a particularly vital role in floral organ formation [31]. MADS-box proteins form a complex regulatory network that determines floral organ identity [32–34]. Among them, SEPALLATA (SEP) genes, as important members of class E functional genes, play a key regulatory role in floral organ development, mainly functioning by forming tetrameric complexes through interactions with other proteins and jointly participating in floral organ formation [35–39].

In *Arabidopsis*, loss of or mutations in class E genes lead to defects in floral organ development. In *SEP1/2/3/4* quadruple mutants, all floral organs change into leaf-like structures [10]. Similarly, in *Oryza sativa*, simultaneous knockdown of *OsMADS1/5/7/8* (four *SEP-like* genes) also leads to the transformation of floral organs into leaf-like structures, while knockdown of *OsMADS7* and *OsMADS8* results in the conversion of stamens and carpels into grass-specific structures [40]. These findings show that SEP genes play key roles in the control of flower development. Studies in different plants show that the function of *SEP3* genes in floral organ development is largely conserved [41]. SEP3 homologs have been cloned in several horticultural plants, including *A. thaliana*, *Iris germanica*, and *Prunus* [42–44]. These genes are mainly expressed in floral parts, while they show low expression in non-floral organs. *SEP3* genes are highly expressed in petals and stamens [2,10].

In this study, the tree peony cultivar 'Luoyang Hong' was used as the experimental material to investigate the function of *PsSEP3L1* in floral organ development. Expression analysis showed that *PsSEP3L1* is expressed in different tree peony varieties. Its expression is mainly observed in petals, petaloid petals, and stamens. This expression pattern differs from previous reports of *SEP3* homologs in other plants. In *Petunia* × *hybrida* and *Solanum lycopersicum*, *SEP3-like* genes are mainly expressed in inner floral organs, and show little or no expression in sepals [45,46]. In contrast, in *Orchidaceae* and *Lilium*, *SEP3-like* genes are expressed in both petals and sepals [47,48]. This difference in expression pattern probably reflects the differences in floral organ structure among plants. In plants such as *Arabidopsis* and *Petunia*, sepals and petals are highly differentiated in both morphology and function. In certain monocotyledonous plants, the morphological characteristics of sepals and petals often exhibit similarity; consequently, the genes governing petal development might also sustain specific expression levels within sepals [49]. These findings indicate that *SEP3* genes could assume distinct functions in the regulation of floral organ differentiation across various plant species, with the expression patterns potentially attributable to species-specific factors.

To further investigate the function of *PsSEP3L1* in floral organ development of tree peony, overexpression and silencing vectors were generated in this study, and Agrobacterium-mediated transient transformation was performed in the tree peony cultivar 'Luoyang Hong' at the balloon stage for functional validation. The experimental results showed that overexpression of *PsSEP3L1* led to an increase in the number of petals compared with the empty vector control. This suggests that the gene may influence petal number by regulating floral organ development. This finding is similar to previous research [50], which showed that overexpression of *SnMADS37* caused more petals in *Arabidopsis*. Conversely, the number of petals decreased in the gene silencing treatment, which may be related to the decrease in the expression of endogenous *SEP3* in tree peony. This phenomenon was consistent with the result that the antisense expression of *GmMADS28* in *Nicotiana benthamiana* caused the reduction in the number of petals [51]. The opposite phenotypic changes produced by overexpression and silencing treatment further suggested that

PsSEP3L1 plays a regulatory role in tree peony petal formation. Collectively, these results indicate that *PsSEP3L1* promotes petal formation and regulates petal number in tree peony.

SEP3 functions as a core component of MADS-box transcription factor complexes, coordinating with multiple floral development regulators to participate in floral organ formation [52]. Our research group previously identified PsSEP1L, PsAP1, PsAP3, and PsAG as candidate interacting partners of PsSEP3L1. Y2H and BiFC assays further in this study confirmed that PsSEP3L1 interacts with PsSEP1L, PsAP3, and PsAG, but does not interact with PsAP1. According to the floral quartet model, *SEP* acts as an E-class MADS-box gene, forming complexes with *AP1*, *AP3*, and *PI* to regulate petal development, and with *AG*, *AP3*, and *PI* to regulate stamen development [53,54]. The observed interactions of PsSEP3L1 with PsAP3 and PsAG in tree peony are consistent with findings in Arabidopsis [35], indicating functional conservation of *SEP3* across plant species. The lack of interaction between PsSEP3L1 and PsAP1 may reflect species-specific divergence in MADS-box family protein interactions, possibly due to relatively weak direct interaction signals between *PsAP1* and PsSEP3L1 that are difficult to detect [27]. By contrast, *HrSEP1* interacts with *HrAP1* in sea buckthorn [39], suggesting that the evolutionary divergence of the *AP1* gene family across different species may account for this discrepancy. Interestingly, the observed interaction between PsSEP3L1 and PsSEP1L in tree peony suggests either functional synergy or partial redundancy among E-class gene members, consistent with the notion that E-class proteins exhibit a certain degree of functional substitutability [55]. In summary, this study demonstrates that PsSEP3L1 interacts with PsSEP1L, PsAP3, and PsAG to coordinately regulate petal number and influence floral morphology in tree peony. These findings provide a theoretical basis for elucidating the molecular mechanisms underlying floral organ morphogenesis in this species.

5 Conclusions

Using homologous cloning, we obtained the *PsSEP3L1* sequence from the tree peony cultivar ‘Luoyang Hong’ for this study. Bioinformatics analysis showed that the open reading frame of *PsSEP3L1* was 738 bp, encoding 245 amino acids. The molecular formula is $C_{1247}H_{1972}N_{354}O_{377}S_8$, the total number of atoms is 3958, the theoretical isoelectric point (pI) is 8.64, and the relative molecular mass is 28,212 Da. The protein encoded by *PsSEP3L1* is a hydrophilic unstable protein, and there is no transmembrane structure. The protein contains a MADS-box domain and a K-box domain. Phylogenetic analysis showed that *PsSEP3L1* had the closest evolutionary relationship with cassava. Quantitative results showed that *PsSEP3L1* was expressed in various organs of flowers of different cultivars, and the relative expression levels were significantly different between different organs. The relative expression levels were higher in the outer and inner petals of single-petal cultivars. The relative expression levels in petals, petaloid petals, and stamens were significantly higher than those in sepals in both semi-double and double cultivars. The expression level in the petals of the tree peony cultivar ‘Luoyang Hong’ was 23.73-fold higher than that in sepals. Functional validation revealed that overexpression of *PsSEP3L1* significantly increased petal number in tree peony. Conversely, knockdown of the gene resulted in fewer petals. This confirms that *PsSEP3L1* positively affects the number of petals in tree peony. Y2H and BiFC assays further in this study confirmed that the PsSEP3L1 protein can interact with other MADS-box family proteins, including PsSEP1L, PsAP3, and PsAG, to co-regulate flower morphology in tree peony.

Acknowledgement: Thanks to all researchers for their contribution to this work.

Funding Statement: This work was financially supported by the National Natural Science Foundation of China (U23A20211) and the Henan Province Traditional Chinese Medicine Industry Technology System (HARS-22-11-S).

Author Contributions: Renjie Li and Ruiya Li designed and executed the study; analyzed the data and wrote the first draft of the paper; Yuying Li, Lili Guo and Qi Guo Xiangnan He designed and analyzed the experimental results; Xiangnan He and Yunxin Zhou wrote and revised the manuscript of the thesis; Xiaogai Hou initiated and led the project, guiding the design, analysis, and writing and revision of the paper. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: All sequencing data will be made available on request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The supplementary material is available online at https://www.techscience.com/doi/10.32604/phyton.2026.083737/s1.

References

1. Kaufmann K, Wellmer F, Muiño JM, Ferrier T, Wuest SE, Kumar V, et al. Orchestration of floral initiation by APETALA1. Science. 2010;328(5974):85–9. [CrossRef].
2. Smaczniak C, Immink RGH, Angenent GC, Kaufmann K. Developmental and evolutionary diversity of plant MADS-domain factors: Insights from recent studies. Development. 2012;139(17):3081–98. [CrossRef].
3. Chanderbali AS, Berger BA, Howarth DG, Soltis PS, Soltis DE. Evolving ideas on the origin and evolution of flowers: New perspectives in the genomic era. Genetics. 2016;202(4):1255–65. [CrossRef].
4. Stewart D, Graciet E, Wellmer F. Molecular and regulatory mechanisms controlling floral organ development. FEBS J. 2016;283(10):1823–30. [CrossRef].
5. Chen D, Yan W, Fu LY, Kaufmann K. Architecture of gene regulatory networks controlling flower development in *Arabidopsis thaliana*. Nat Commun. 2018;9:4534. [CrossRef].
6. Theißen G, Saedler H. Plant biology: Floral quartets. Nature. 2001;409(6819):469–71. [CrossRef].
7. Theißen G. Development of floral organ identity: Stories from the MADS house. Curr Opin Plant Biol. 2001;4(1):75–85. [CrossRef].
8. Rijpkema AS, Vandenbussche M, Koes R, Heijmans K, Gerats T. Variations on a theme: Changes in the floral ABCs in angiosperms. Semin Cell Dev Biol. 2010;21(1):100–7. [CrossRef].
9. Bowman JL, Smyth DR, Meyerowitz EM. The ABC model of flower development: Then and now. Development. 2012;139(22):4095–8. [CrossRef].
10. Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF. B and C floral organ identity functions require *SEPALLATA* MADS-box genes. Nature. 2000;405(6783):200–3. [CrossRef].
11. Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky MF. The SEP4 gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. Curr Biol. 2004;14(21):1935–40. [CrossRef].
12. Dreni L, Ferrándiz C. Tracing the evolution of the SEPALLATA subfamily across angiosperms associated with neo- and sub-functionalization for reproductive and agronomically relevant traits. Plants. 2022;11(21):2934. [CrossRef].
13. Hugouvieux V, Blanc-Mathieu R, Janeau A, Paul M, Lucas J, Xu X, et al. SEPALLATA-driven MADS transcription factor tetramerization is required for inner whorl floral organ development. Plant Cell. 2024;36(9):3435–50. [CrossRef].
14. Yan H, Zhang H, Wang Q, Jian H, Qiu X, Baudino S, et al. The *Rosa chinensis* cv. viridiflora phyllody phenotype is associated with misexpression of flower organ identity genes. Front Plant Sci. 2016;7:996. [CrossRef].
15. Lin ZY, Zhu GF, Lu CQ, Gao J, Li J, Xie Q, et al. Functional conservation and divergence of *SEPALLATA*-like genes in floral development in *Cymbidium sinense*. Front Plant Sci. 2023;14:1209834. [CrossRef].
16. Li P, Shen J, Wang Z, Liu S, Liu Q, Li Y, et al. Genus *Paeonia*: A comprehensive review on traditional uses, phytochemistry, pharmacological activities, clinical application, and toxicology. J Ethnopharmacol. 2021;269:113708. [CrossRef].

17. Yan Z, Xie L, Li M, Yuan M, Tian Y, Sun D, et al. Phytochemical components and bioactivities of novel medicinal food–Peony roots. Food Res Int. 2021;140:109902. [CrossRef].
18. Li Y, Guo L, Wang Z, Zhao D, Guo D, Carlson JE, et al. Genome-wide association study of 23 flowering phenology traits and 4 floral agronomic traits in tree peony (*Paeonia* section *Moutan* DC.) reveals five genes known to regulate flowering time. Hortic Res. 2023;10(2):uhac263. [CrossRef].
19. Zhao D, Tao J, Han C, Ge J. Flower color diversity revealed by differential expression of flavonoid biosynthetic genes and flavonoid accumulation in herbaceous peony (*Paeonia lactiflora* Pall.). Mol Biol Rep. 2012;39(12):11263–75. [CrossRef].
20. Du H, Wu J, Ji KX, Zeng QY, Bhuiya MW, Su S, et al. Methylation mediated by an anthocyanin, *O*-methyltransferase, is involved in purple flower coloration in *Paeonia*. J Exp Bot. 2015;66(21):6563–77. [CrossRef].
21. Luan Y, Chen Z, Tang Y, Sun J, Meng J, Tao J, et al. Tree peony PsMYB44 negatively regulates petal blotch distribution by inhibiting dihydroflavonol-4-reductase gene expression. Ann Bot. 2023;131(2):323–34. [CrossRef].
22. Ge J, Zhao D, Han C, Wang J, Hao Z, Tao J. Cloning and expression of floral organ development-related genes in herbaceous peony (*Paeonia lactiflora* Pall.). Mol Biol Rep. 2014;41(10):6493–503. [CrossRef].
23. Li Y, Lu J, Chang Y, Tang W, Yang Q. Comparative analysis of tree peony petal development by transcriptome sequencing. Acta Physiol Plant. 2017;39(10):216. [CrossRef].
24. Wu Y, Tang Y, Jiang Y, Zhao D, Shang J, Tao J. Combination of transcriptome sequencing and iTRAQ proteome reveals the molecular mechanisms determining petal shape in herbaceous peony (*Paeonia lactiflora* Pall.). Biosci Rep. 2018;38(6):BSR20181485. [CrossRef].
25. Yang Y, Sun M, Li S, Chen Q, Teixeira da Silva JA, Wang A, et al. Germplasm resources and genetic breeding of *Paeonia*: A systematic review. Hortic Res. 2020;7:107. [CrossRef].
26. Wang S, Gao J, Xue J, Xue Y, Li D, Guan Y, et al. De novo sequencing of tree peony (*Paeonia suffruticosa*) transcriptome to identify critical genes involved in flowering and floral organ development. BMC Genom. 2019;20(1):572. [CrossRef].
27. Li R, Zuo D, Wang D, Song C, Zhou Y, Li R, et al. Global landscape of alternative splicing in tree peony floral organs and the mechanism underlying *PsuSEP3L*- and *PsuSEP3S*-mediated flower type diversification. Plant Cell Environ. 2026;49(1):447–9. [CrossRef].
28. Yuan J, Jiang S, Jian J, Liu M, Yue Z, Xu J, et al. Genomic basis of the giga-chromosomes and giga-genome of tree peony *Paeonia ostii*. Nat Commun. 2022;13:7328. [CrossRef].
29. Chang Y, Hu T, Zhang W, Zhou L, Wang Y, Jiang Z. Transcriptome profiling for floral development in reblooming cultivar 'High Noon' of *Paeonia suffruticosa*. Sci Data. 2019;6:217. [CrossRef].
30. Wils CR, Kaufmann K. Gene-regulatory networks controlling inflorescence and flower development in *Arabidopsis thaliana*. Biochim Biophys Acta BBA Gene Regul Mech. 2017;1860(1):95–105. [CrossRef].
31. Zhang Z, Zou W, Lin P, Wang Z, Chen Y, Yang X, et al. Evolution and function of MADS-box transcription factors in plants. Int J Mol Sci. 2024;25(24):13278. [CrossRef].
32. Hu J, Chen Q, Idrees A, Bi W, Lai Z, Sun Y. Structural and functional analysis of the *MADS*-box genes reveals their functions in cold stress responses and flower development in tea plant (*Camellia sinensis*). Plants. 2023;12(16):2929. [CrossRef].
33. Zhang A, He H, Li Y, Wang L, Liu Y, Luan X, et al. MADS-box subfamily gene GmAP3 from *Glycine* max regulates early flowering and flower development. Int J Mol Sci. 2023;24(3):2751. [CrossRef].
34. Liu C, Wang F, Li R, Zhu Y, Zhang C, He Y. Marigold (*Tagetes erecta*) MADS-box genes: A systematic analysis and their implications for floral organ development. Agronomy. 2024;14(9):1889. [CrossRef].
35. Honma T, Goto K. Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature. 2001;409(6819):525–9. [CrossRef].
36. Becker A, Saedler H, Theissen G. Distinct MADS-box gene expression patterns in the reproductive cones of the gymnosperm *Gnetum gnemon*. Dev Genes Evol. 2003;213(11):567–72. [CrossRef].
37. Ruelens P, Zhang Z, van Mourik H, Maere S, Kaufmann K, Geuten K. The origin of floral organ identity quartets. Plant Cell. 2017;29(2):229–42. [CrossRef].
38. Wang Q, Dan N, Zhang X, Lin S, Bao M, Fu X. Identification, characterization and functional analysis of C-class genes associated with double flower trait in carnation (*Dianthus caryphyllus* L.). Plants. 2020;9(1):87. [CrossRef].

39. Cong D, Zhao X, Ni C, Li M, Han L, Cheng J, et al. The SEPALLATA-like gene *HrSEP1* in *Hippophae rhamnoides* regulates flower development by interacting with other MADS-box subfamily genes. Front Plant Sci. 2025;15:1503346. [CrossRef].
40. Cui R, Han J, Zhao S, Su K, Wu F, Du X, et al. Functional conservation and diversification of class E floral homeotic genes in rice (*Oryza sativa*). Plant J. 2010;61(5):767–81. [CrossRef].
41. Feng M, Xi X, Zhang X, Qiu F, Song J, Li L. Saffron *CsSEP3* regulates flowering time and flower organ morphogenesis. Gene. 2025;972:149818. [CrossRef].
42. Gao W, Zhang L, Wang J, Liu Z, Zhang Y, Xue C, et al. ZjSEP3 modulates flowering time by regulating the *LHY* promoter. BMC Plant Biol. 2021;21(1):527. [CrossRef].
43. Liu G, Li F, Shi G, Wang L, Wang L, Fan L. Identification of MADS-box transcription factors in *iris laevigata* and functional assessment of *IlSEP3* and *IlSVP* during flowering. Int J Mol Sci. 2022;23(17):9950. [CrossRef].
44. Zhou Y, Xu Z, Yong X, Ahmad S, Yang W, Cheng T, et al. SEP-class genes in *Prunus mume* and their likely role in floral organ development. BMC Plant Biol. 2017;17(1):10. [CrossRef].
45. Angenent GC, Franken J, Busscher M, van Dijken A, van Went JL, Dons HJ, et al. A novel class of MADS box genes is involved in ovule development in *Petunia*. Plant Cell. 1995;7(10):1569–82. [CrossRef].
46. Pnueli L, Hareven D, Broday L, Hurwitz C, Lifschitz E. The TM5 MADS box gene mediates organ differentiation in the three inner whorls of tomato flowers. Plant Cell. 1994;6(2):175–86. [CrossRef].
47. Chang YY, Chiu YF, Wu JW, Yang CH. Four orchid (*Oncidium* Gower Ramsey) *AP1*/*AGL9*-like *MADS* box genes show novel expression patterns and cause different effects on floral transition and formation in *Arabidopsis thaliana*. Plant Cell Physiol. 2009;50(8):1425–38. [CrossRef].
48. Tzeng TY, Hsiao CC, Chi PJ, Yang CH. Two lily *SEPALLATA*-like genes cause different effects on floral formation and floral transition in Arabidopsis. Plant Physiol. 2003;133(3):1091–101. [CrossRef].
49. Kanno A. Molecular mechanism regulating floral architecture in monocotyledonous ornamental plants. Hortic J. 2016;85(1):8–22. [CrossRef].
50. Yuan S, Piao CL, Zhang X, Cui ML. Overexpression of *SEPALLATA3*-like gene *SnMADS37* generates green petal-tip flowers in *Solanum nigrum*. Plants. 2025;14(13):1891. [CrossRef].
51. Huang F, Xu G, Chi Y, Liu H, Xue Q, Zhao T, et al. A soybean MADS-box protein modulates floral organ numbers, petal identity and sterility. BMC Plant Biol. 2014;14(1):89. [CrossRef].
52. Immink RG, Tonaco IA, de Folter S, Shchennikova A, van Dijk AD, Busscher-Lange J, et al. SEPALLATA3: The 'glue' for MADS box transcription factor complex formation. Genome Biol. 2009;10(2):R24. [CrossRef].
53. Theißen G, Melzer R, Rümpler F. MADS-domain transcription factors and the floral quartet model of flower development: Linking plant development and evolution. Development. 2016;143(18):3259–71. [CrossRef].
54. Fatima M, Ma X, Zhang J, Ming R. Genome-wide analysis of MADS-box genes and their expression patterns in unisexual flower development in dioecious spinach. Sci Rep. 2024;14:18635. [CrossRef].
55. Espinosa-Soto C, Immink RG, Angenent GC, Alvarez-Buylla ER, de Folter S. Tetramer formation in *Arabidopsis* MADS domain proteins: Analysis of a protein-protein interaction network. BMC Syst Biol. 2014;8(1):9. [CrossRef].