CLONING AND EXPRESSION OF FLORAL ORGAN IDENTITY GENES IN PAEONIA OSTII 'FENGDAN'

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Abstract

Tree peony is the most popular and important ornamental plant in China and has many different flower types. Although many studies about tree peony cultivation have been published, the regulatory mechanism of floral organ identity has not been explored. *Paeonia ostii* 'Fengdan' is a typical single-flower variety, and many important tree peony cultivars in China originated from homoploid hybridization between 'Fengdan' and other Paeonia species. Peony tea made from 'Fengdan' has already been introduced to the market, and the quality and price of peony tea are closely related to the flower type of 'Fengdan'. This research cloned six floral organ identity genes in 'Fengdan', namely, *PsAP1fd*, *PsAP2fd*, *PsAP3fd*, *PsPIfd*, *PsACfd*, and *PsSEP1fd*. The bioinformatics analysis was performed, and results showed that all these genes encoded MADS-box proteins, except *PsAP2fd*, and *PsAP2fd* encoded an AP2 protein. Five MADS-box proteins encoded by these genes contained two conserved motifs: MADS-MEF2 and K-box domain. PsSEP1fd was hydrophilic and stable, whereas the other five proteins were hydrophilic and unstable. The results of qRT-PCR displayed that *PsAP1fd* was mostly observed in the petals and sepals, and *PsAP2fd* was observed in the petals, followed by that in the stamens. The highest expression level of *PsAGfd* was observed in the petals, followed by that in the stamens. The highest expression level of *PsAGfd* was observed in the flower type formation of 'Fengdan'. Our work would help reveal the molecular mechanism underlying flower type formation in 'Fengdan' and promote quality control for peony tea products.

Key words: Cloning, Floral organ identity, Gene expression, MADS-box gene, Paeonia ostii 'Fengdan'.

Introduction

Flower development is strictly regulated. In 1991, the ABC model was proposed according to researches on Arabidopsis and Antirrhinum (Coen & Meyerowitz, 1991). The ABC model indicated that the flowers of Arabidopsis and Antirrhinum have four whorls, referred to as sepals, petals, stamens, and carpels, and flower organs are determined by A-, B-, and C-class genes (Coen & Meyerowitz, 1991). Sepal formation is controlled by A-class genes, while petal formation is regulated by A- and B-class genes. Stamen formation is controlled by B- and C-class genes, whereas the formation of carpel is determined by Cclass genes (Coen & Meyerowitz, 1991). Furthermore, Aand C-class genes are antagonistic (Bowman et al., 1991). The mutations of A-class gene resulted in sepals to change into pistils, as well as petals to change into stamens. B-class gene mutations resulted in petals and stamens to change into sepals and pistils, respectively. The mutations of C-class gene promote the transition of stamens into petals and transition of pistils into sepals (Bowman et al., 1991; Coen & Meyerowitz, 1991; Weigel & Meyerowitz, 1994). APETALA1 (AP1) and AP2 in Arabidopsis are A-class genes, whereas AP3 and PISTILLATA (PI) in Arabidopsis, GLOBOSA (GLO) and DEFICIENS (DEF) in Antirrhinum, pMADS1, pMADS2/FBP3 (FLORAL BINDING PROTEIN3) and FBP1 in petunia are B-class genes (Riechmann & Meyerowitz, 1998; Eckardt, 2003; Pařenicová et al., 2003). PLENA in snapdragon, AGAMOUS (AG) in Arabidopsis, and pMADS3 in petunia are C-class genes (Eckardt, 2003).

ABC model had been comprehensively studied and extended to ABCDE model. *FBP7* and *FBP11* in petunia (Angenent *et al.*, 1995), *SHATTERPROOF1* (*SHP1*), *SHP2*, *SEEDSTICK* (*STK*) in *Arabidopsis* (Favaro *et al.*, 2003) are all D-class genes, which determine ovule development and have redundant effects similar to C-class genes (Colombo *et al.*, 1995; Jack, 2004).

The discovery of E-class genes is a significant improvement for the ABC model. These genes were first found in tomato MADS box gene no.5 (TM5) (Pnueli et al., 1994), as well as petunia FBP2 (Angenent et al., 1994; Ferrario et al., 2003). Some researchers attempted to change the expression levels of ABC genes to induce the transition of leaves into floral organs, but they were unsuccessful (Mizukami & Ma, 1992; Krizek & Meyerowitz, 1996; Pelaz et al., 2000). ABC genes are significant to floral organs formation, and another class of floral organ identity genes are also essential to the transition of vegetative organs into floral organs. In Arabidopsis, the formation of the complexes of ABC proteins and SEP proteins is sufficient for converting vegetative organs into floral organs (Pelaz et al., 2000; Honma & Goto, 2001; Pelaz et al., 2001b). Therefore, the ABCE model was updated according to the ABC model. The ABCE model indicated that A+E regulates sepal formation (Pelaz et al., 2001a), A+B+E regulates petal formation, B+C+E regulates stamen formation (Honma & Goto, 2001; Pelaz et al., 2001b; Ferrario et al., 2003), C+E regulates pistil formation (Fan et al., 1997; Pelaz et al., 2000).

Tree peony (*Paeonia suffruticosa* Andrews) has a long history of cultivation in China, which belongs to section *Moutan* DC of the genus *Paeonia* and family Paeoniaceae. It is called "king of flowers" for its beautiful and bright colors and large and diverse flowers. *Paeonia ostii* 'Fengdan' is a popular ornamental, medicinal and oil-seed tree peony variety in China (Liu *et al.*, 2019). In 2011, the seeds of 'Fengdan' were identified as novel sources of edible plant oil in China. Seed oil extracted from 'Fengdan' was found to be rich in unsaturated fatty acids, especially the proportion of α -linolenic acid in peony seed oil is extremely high (Li et al., 2015). In 2013, the flowers of 'Fengdan' were identified as a new food resource in China and found to be rich in flavonoids (Zhang et al., 2017). 'Fengdan' was used in genetic map construction and OTL analysis (Guo et al., 2017; Zhang et al., 2019). The callus, direct somatic embryogenesis, and shoot organogenesis were induced in 'Fengdan' (Du et al., 2020; Ren et al., 2020). Some peony MADS-box genes were identified, and the expression patterns of these genes were analyzed, which are involved in flower organ formation (Wang et al., 2019). However, the regulation mechanism of flower organ formation in tree peony has not been fully clarified. The flower of P. ostii 'Fengdan' has typical four whorls: sepals, petals, stamens, and pistils, and many important peony cultivars in China originated from homoploid hybridization between P. ostii and other Paeonia species. Consequently, studies on 'Fengdan' will increase the understanding of floral organ identity in tree peony and provide basic knowledge of cultivar breeding.

This research used RT-PCR to clone *PsAP1fd* and *PsAP2fd* (A-class genes); *PsAP3fd* and *PsP1fd* (B-class genes); *PsAGfd* (C-class gene); and *PsSEP1fd* (E-class gene) in 'Fengdan'. These genes are involved in flower type formation. Bioinformatics analysis and expression patterns of these genes in different flower organs were carried out. This research will serve as a foundation for the study of the mechanism of flower type formation in tree peony.

Materials and methods

Plant materials: All the plants were grown in Henan University of Science and Technology, Luoyang, Henan Province, China. 'Fengdan' flowers at the bloom stage were collected in April 2017. The flowers were divided into sepals, petals, stamens, and pistils for further analysis.

RNA extraction and reverse transcription: A MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan) was used for RNA Extraction of 'Fengdan'. Then, the samples were measured using a Multiskan Go microplate spectrophotometer (Thermo Scientific, USA). The A_{260}/A_{280} values ranged from 1.8 to 2.0. From each sample, 1000 ng of RNA of each sample was used as the template and a PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Japan) was used for reverse transcription.

Isolation of genes: According to transcriptome sequencing performed in our laboratory (unpublished), Primer Premier 5.0 (Premier Biosoft, Palo Alto, USA) was used for primer pair design (Table 1). PCR reactions were carried out using *TaKaRa Ex Taq*® (TaKaRa, Japan). PCR reactions were performed using three-step cycling conditions: *PsAP1fd*, *PsAP3fd*, *PsP1fd*, *PsAGfd*, and *PsSEP1fd*: 94°C for 5 min, then 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min; *PsAP2fd*: 94°C for 5 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 10 min. Then, 1.5% agarose gel electrophoresis was used for the detection of PCR products. A gel extraction kit (CoWin Biosciences, China)

was used in purifying the incised gels. PMD18-T vector, as well as *E.coli* DH5 α Competent Cells (TaKaRa, Japan) were used in cloning the extracted products. Recombinant plasmids were selected, then sequenced by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China).

Sequence analysis: DNAMAN6.0 software was used in analyzing *PsAP1fd*, *PsAP2fd*, *PsAP3fd*, *PsPIfd*, *PsAGfd*, and *PsSEP1fd* sequences. ORF search was carried out according to the NCBI ORF Finder (<u>https://www.ncbi.nlm.nih.gov/orffinder/</u>), as well as conserved domain analysis performed using NCBI Conserved Domains Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?). The properties of these proteins were analyzed on ExPASy (http://us.expasy.org/tools/protparam.html). MEME (http://meme-suite.org/tools/meme) was used in identifying the conserved protein motifs of the proteins. Homology search was investigated according to NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene expression analysis: The expression patterns of PsAP1fd, PsAP2fd, PsAP3fd, PsPIfd, PsAGfd, and PsSEP1fd were detected through qRT-PCR. LightCycler 96 (Roche, Germany) was used. P. suffruticosa GAPDH (according to transcriptome sequencing performed by our laboratory) was used as the internal control. Primers of qRT-PCR were designed as above (Table 2). The TB GreenTMPremix Ex TaqTMII (Tli RNaseH Plus) (TaKaRa, Japan) was used for qRT-PCR. The amplification was performed as this conditions: 94°C for 30 s, 40 cycles of 94°C for 5 s, and 60°C for 30 s. The relative expression levels of the detected genes were computed according to the methods described by Schmittgen and Livak (Schmittgen & Livak, 2008). The expression level in the petals was used as the control. Triplicate reactions were analyzed. Statistical analysis was carried out using Microsoft Excel, and data were analyzed using one-way ANOVA test.

Results

Morphological description of flower in 'Fengdan': *P. ostii* 'Fengdan' is a typical single-flower variety, with only two whorls of petals, which are broad and flat (Fig. 1a). The sepals, petals, stamens, and pistils of 'Fengdan' developed normally (Fig. 1b) and were thus good materials for studying the mechanism of flower type formation in tree peony.

Isolation of floral organ identity genes in 'Fengdan': The full-length cDNAs of *PsAP1fd*, *PsAP2fd*, *PsAP3fd*, *PsP1fd*, *PsAGfd* and *PsSEP1fd* were successfully identified from 'Fengdan' through RT-PCR (Table 3). The cDNAs of *PsAP1fd*, *PsAP2fd*, *PsAP3fd*, *PsP1fd*, *PsAGfd* and *PsSEP1fd* were 799, 1697, 815, 791, 925, and 869 bp, respectively, containing ORFs of 729, 1533, 666, 639, 777, and 735 bp, respectively, and encoding proteins of 242, 510, 221, 212, 258, and 244 aa, respectively.

The isolated genes were deposited in the GenBank, and the accession numbers were MT822685 (*PsAP1fd*), MT822686 (*PsAP2fd*), MT822687 (*PsAP3fd*), MT822688 ((*PsP1fd*), MT822689 (*PsAGfd*), and MT822690 (*PsSEP1fd*; Table 3).

| I able 1. | Table 1. Primer sequences used for floral organ identity genes isolation in "Fenguan". | | | | |
|-----------|--|-------------------------------|--|--|--|
| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | | | |
| PsAP1fd | TTGTCTGTTTGGGTGGTGGGA | CATAACAGTCCGAAGGAGTGC | | | |
| PsAP2fd | GAGTCTCATAGAGTAATCAGC | GAAGAAAGAATCTCACAAGC | | | |
| PsAP3fd | CCATTGGAGGTGATTGCTA | ATTGGACCATGGGTTGAGTTG | | | |
| PsPIfd | TTGTGGCTAGACTTGAAGAGA | TCACACAAACCAAGTTCAT | | | |
| PsAGfd | CCTGCTCAGATTTTGTGGGA | CCGCAGAATTTGATGACAG | | | |
| PsSEP1fd | AGATCAGCTGGTTCCCAAGAG | GTTACAAATTCCAAGCAAGC | | | |
| | | | | | |

Table 1. Primer sequences used for floral organ identity genes isolation in 'Fengdan'.

Table 2. Gene-specific primer sequences for detection by qRT-PCR.

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|----------|------------------------|-------------------------------|
| GAPDH | TGTTCACTCCATCACTGCTAC | ACATCCACAGTAGGAACACGA |
| PsAP1fd | GGAGAACCAACAGAAATGAG | ATACACCAAAGCACCCAAG |
| PsAP2fd | TATACAAGTGAGGCAAACG | GAGATGGAACAATGTGAAG |
| PsAP3fd | GGAGAATGAGGGAGACTATG | CATGACTCAAGAGAGGTGC |
| PsPIfd | ATGGAATTTCCCAAGAGGC | GGAAGGCGTAAGGAATCAG |
| PsAGfd | CAAATGAACTTGATGCCAG | ATTGAAGAGCGATTTGGTC |
| PsSEP1fd | GTTCAGACCAAATGACGGC | ACTCAGAGCATCCATCCAGG |

| Table 3. Gene seq | uences of floral | organ identity | genes in | 'Fengdan'. |
|-------------------|------------------|----------------|----------|------------|
| | | | | |

| | | ODE | | | | |
|----------|---------------|---------------|---------------|---------------|-------|-----------|
| Gene | Full length | ORF | 5'-UTR | 3'-UTR | Amimo | Accession |
| Gelle | (bp) | (bp) | (bp) | (bp) | acid | number |
| PsAP1fd | 799 | 729 | 35 | 35 | 242 | MT822685 |
| PsAP2fd | 1697 | 1533 | 51 | 113 | 510 | MT822686 |
| PsAP3fd | 815 | 666 | 36 | 113 | 221 | MT822687 |
| PsPIfd | 791 | 639 | 28 | 124 | 212 | MT822688 |
| PsAGfd | 925 | 777 | 113 | 35 | 258 | MT822689 |
| PsSEP1fd | 869 | 735 | 79 | 55 | 244 | MT822690 |

Table 4. Physical and chemical parameters of proteins related to floral organ identity in 'Fengdan'.

| Protein | Formula | Molecular weight | Theoretical | Instability index | GRAVY value |
|----------|--|---------------------|-------------|----------------------|----------------|
| | ~ ~ ~ ~ ~ ~ ~ | 0 | <u>pl</u> | | |
| PsAP1fd | $C_{1217}H_{1976}N_{360}O_{375}S_{11}$ | 28003.92 | 9.01 | 50.47 | -0.788 |
| PsAP2fd | $C_{2461}H_{3794}N_{740}O_{793}S_{18}$ | 57012.76 | 6.66 | 51.93 | -0.911 |
| PsAP3fd | $C_{1122}H_{1803}N_{325}O_{341}S_{12}$ | 25686.35 | 9.30 | 43.17 | -0.822 |
| PsPIfd | $C_{1068}H_{1745}N_{319}O_{334}S_{10}$ | 24719.14 | 8.65 | 48.67 | -0.850 |
| PsAGfd | $C_{1268}H_{2057}N_{391}O_{403}S_{10}$ | 29548.26 | 9.47 | 52.98 | -0.914 |
| PsSEP1fd | $C_{1220}H_{1960}N_{354}O_{375}S_{10}$ | 27907.73 | 8.78 | 29.76 | -0.654 |

Sequence analysis of floral organ identity genes in 'Fengdan': Conserved domain analysis confirmed that all proteins encoded by the genes contained MADS-MEF2like and K-box domain, except PsAP2fd (Fig. 2). PsAP2fd contained two typical AP2 domains, which belong to the AP2 family (Fig. 2b). The Molecular weight varied from 24.72 KDa to 57.01 KDa, and the theoretical pI varied from 6.66 to 9.47. Only the instability index of PsSEP1fd was less than 40, and it was stable. The other five proteins detected were considered unstable. The GRAVY values of the proteins were all less than 0, and thus they were all predicted to be hydrophilic (Table 4).

BLAST analysis showed that PsAP1fd shared 75.20-99.59% identity with AP1 from *Paeonia suffruticosa*, *Paeonia lactiflora*, *Vitis riparia*, *Herrania umbratica*, *Durio zibethinus*, and *Rhamnella rubrinervis*. PsAP2fd shared 66.67-100.00% identity with AP2 from *Paeonia suffruticosa*, *Paeonia lactiflora*, *Vitis vinifera*, *Vitis riparia*, *Theobroma cacao*, *Nyssa sinensis*, and *Durio zibethinus*. PsAP3fd shared 70.97-100.00% identity with AP3 from *Paeonia suffruticosa*, *Paeonia lactiflora*, *Vitis vinifera*, *Cephalotus follicularis*, *Nyssa sinensis*, and *Mercurialis annua*. PsPIfd shared 71.70-99.06% identity with PI from Paeonia suffruticosa, Paeonia lactiflora, Mercurialis annua, Vitis vinifera, Vitis riparia, Manihot esculenta, and Jatropha curcas. PsAGfd shared 78.93-97.29% identity with AG from Paeonia suffruticosa, Cercidiphyllum japonicum, Vitis riparia, Prunus serotina, Manihot esculenta, and Tripterygium wilfordii. PsSEP1fd shared 77.46-97.13% identity with SEP1 from Paeonia lactiflora, Carica papaya, Vitis riparia, Vitis vinifera, Theobroma cacao, and Durio zibethinus (Table 5).

Conserved domain analysis showed that the proteins detected were MADS-box proteins, except PsAP2fd, which belongs to the AP2 family (Fig. 2). To examine the common feature of 'Fengdan' MADS-box proteins, the MEME suite was used in identifying their conserved motifs and sequence logos. Five conserved motifs (called Motif 1-5) were identified, and only three motifs were usable (the E-values of Motif 4 and Motif 5 were larger than 0.05; Fig. 3b). The motifs were then matched to two different domains. Motif 1 and 3 at the N-terminus were in the MADS domain, and Motif 2 was in the K-box domain. A less-well-conserved I (intervening) domain, as well as a variable C-terminal region were found in these proteins (Fig. 3a).

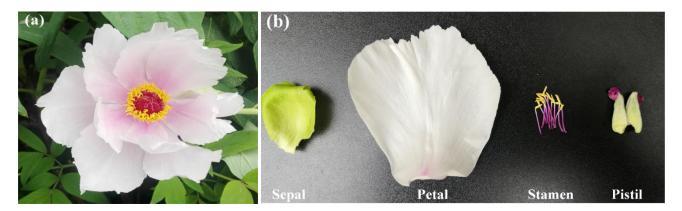


Fig. 1. Phenotype of *P. ostii* 'Fengdan'. (a) The flower of 'Fengdan'. (b) Different floral organs of 'Fengdan'.

| Protein | Species | GenBank accession no. | Indentity (%) |
|----------|--------------------------|-----------------------|---------------|
| PsAP1fd | Paeonia suffruticosa | AJO68022.1 | 99.59 |
| | Paeonia lactiflora | AGH61290.1 | 97.11 |
| | Vitis riparia | XP_034699499.1 | 81.74 |
| | Herrania umbratica | XP_021292999.1 | 78.19 |
| | Durio zibethinus | XP_022776709.1 | 77.78 |
| | Rhamnella rubrinervis | KAF3431618.1 | 75.20 |
| PsAP2fd | Paeonia suffruticosa | AEK33829.1 | 100.00 |
| | Paeonia lactiflora | AGI61068.1 | 96.75 |
| | Vitis vinifera | NP_001267881.1 | 71.80 |
| | Vitis riparia | XP_034691428.1 | 71.61 |
| | Theobroma cacao | XP_007047337.2 | 68.33 |
| | Nyssa sinensis | KAA8547120.1 | 68.27 |
| | Durio zibethinus | XP_022740198.1 | 66.67 |
| PsAP3fd | Paeonia suffruticosa | AEK33828.1 | 100.00 |
| | Paeonia lactiflora | AGH61291.1 | 91.86 |
| | Vitis vinifera | NP_001267937.1 | 73.27 |
| | Cephalotus follicularis | GAV72187.1 | 72.35 |
| | Nyssa sinensis | KAA8531940.1 | 71.69 |
| | Mercurialis annua | QER90709.1 | 70.97 |
| PsPIfd | Paeonia suffruticosa | QCQ84555.1 | 99.06 |
| | Paeonia lactiflora | AGH61293.1 | 96.23 |
| | Mercurialis annua | ALK01328.2 | 76.89 |
| | Vitis vinifera | NP_001267875.1 | 76.53 |
| | Vitis riparia | XP_034676852.1 | 76.06 |
| | Manihot esculenta | XP_021601944.1 | 73.71 |
| | Jatropha curcas | XP_012078322.1 | 71.70 |
| PsAGfd | Paeonia suffruticosa | | 97.29 |
| | Cercidiphyllum japonicum | ASY97759.1 | 83.56 |
| | <i>Vitis riparia</i> | XP_034696943.1 | 81.70 |
| | Prunus serotina | | 80.69 |
| | Manihot esculenta | XP_021599035.1 | 79.57 |
| | Tripterygium wilfordii | KAF5736281.1 | 78.93 |
| PsSEP1fd | Paeonia lactiflora | AQM56645.1 | 97.13 |
| | Carica papaya | ACD39982.1 | 79.67 |
| | <i>Vitis riparia</i> | XP_034705766.1 | 79.51 |
| | Vitis vinifera | | 79.18 |
| | Theobroma cacao | XP_007032865.1 | 78.28 |
| | Durio zibethinus | XP_022727577.1 | 77.46 |

Table 5. Comparisons of deduced floral organ identity proteins in 'Fengdan' with other plants.

(b)

1

61 21

121 41

181 61

241 81

130 GAAGATEGAI E D G

70 80 90 100 110 AGGGETAAAGGTETTEAATGACTATEGAATGAGAATTCAAGTTETTEGETG

190 200 210 220 2 AGCAAGATATTIGOCITITICTOTGACDOCAAATGAAGAAGAGAGACITG S K I F G F S S T P

250 260 270 280 290 GACCETETETAACCEGECACTETETETECGEECGAGGETETAGAAATGG

| (a) | |
|------------|---|
| (4) | , 10 20 30 40 50 60 |
| 1 1 | AYSGEAAGAGEMEGETYEMACYGAAGEXTYYGGAAADAADAADAYCAYCXGCAADTGAC M <mark>G R G R V Q L K R I E N K I N R Q V T</mark> |
| 61 21 | 70 50 00 100 110 120 тестеммерозизоватоствалейместем тектом теттотой Р S K R R G G L L K K A H E I S V L C D |
| | 130 140 150 160 170 180 |
| 121 41 | GCTGAGGTTGCTTTGCTTGTCTCTCTCTACAGGGASGCTGTTTGAGTACTACAGAT <mark>A B V A L I V F S T K G K L F B Y S T D</mark> |
| 181 61 | 190 200 210 220 230 240 TCTASCATEGASAGATACTOSACCETTADIAGCSATATTCTATTECDAGEGACAACTS <mark>S S M E K T L D R Y E R Y S</mark> T A E R Q L |
| 241 51 | 250 260 270 280 280 300 GTREGGAACEREATCACAGEAACTRETOCTAGATACTICAACTAGEGCIAAS V G E P G S <mark>Q C N W S L E Y S K L R A X</mark> |
| 301 101 | 310 320 330 340 360 360 Atagagetettacaangaaaccaangegetettatorgagaagatettgategetege <mark>I K L L Q R N Q R X F Q G E D L D S L S</mark> |
| 361 121 | 370 380 290 400 410 420 ССТАМАЛТСТСТАЛАЛТАТОБАССИЛСКАГТГОНСТИАЛАЛЛАТАОЛТКА РКДІЧХИ ВООСІЛУ SLXNIR <mark></mark> |
| 421 | 430 440 450 465 470 480 4684A4AAATCAACTAATHIATGAGTCAATTIC5G8027TCAG4AGAAG665A4G663ATC |
| 481 | <u>ККЛЦЦЛУК SISКЦЦККЕ КА</u> 490 - 500 - 510 - 520 - 530 - 540 Сморарскаласкаяттостарскаларскал торскала |
| 481 | <mark>o e o n n l l a ko i k</mark> e ke kit ha o |
| 541 181 | 550 550 570 580 500 580 500 CAGGEDEGATUGGGEGGGAATTCATCATCATCATCATCATCA Q A Q W B Q Q I H H G P N A S A Y L L S |
| 601 201 | 610 620 630 640 650 660 CCTCATGAACTTACTACTCTAAACAT65GT66CAATTAOCAA65A6AACGAAACGAAAT5 P B E L T T L N V G 6 N Y Q G B P T B M |
| 661 221 | 670 680 800 700 710 720 АббАбдалозастскаюстскостобалскалтятятастотокостобобтостт R R X E L D L T L E P I Y T C H L G C P |
| 721 241 | GETERATER G & * |
| (d) | |
| 1 | 10 20 30 40 50 60 АГСССАНДСТАНСКІСТАЛСКІСТІ ЛАЛІСТАЛАТАЛСЯССКІСТСКО М <mark>С R C K I E I K R I E S S N N R Q V T</mark> |
| 61 21 | 70 80 90 100 110 120 ТАСТСАА БАЕБАСТОВБАТСТВА ЕФА ОКОЛОВИЕТТА МАЙЕТТСТВСТВАТ У S K R R T G I L K K A T E I T V L C D |
| 121 | 130 140 150 160 170 180 67154167111010115115010010064545441650354130001165 |
| 41 | A H V S L V I F A T S F K N H E Y C S P 190 200 210 220 230 240 TOLKCHOGOTIGATATOTIGATACITICAAGOATCTACAAGOATGG |
| 61 | STTVIDICOCK AND AND A CONTRACT AND |
| 241 81 | GATICITAASCATGAGAATCITCHECAATGANTGGANTGGANTGAAGAAAGAAAAGAAAAGAAAA |
| 301 101 | 310 320 330 340 350 360 Atgenerations for the state of th |
| 361 121 | 370 380 390 400 410 420 CTCATAGOCTAGAGGAAGCCTTCAGAATOGTCTTCGGAGAGCAGCAGCTC LIALEETLENGLASVRDX000000000000000000000000000000000000 |
| 421 141 | 430 440 450 460 47D 48D GAATITCCCMARAGETTAARAAAGCTTGAMATTGGAMATGAGAACAGCMCCC <mark>K F P K R L K K R V D K L E D K N X Q L</mark> |
| 481 161 | 490 500 510 520 530 540 ΑCTOCAT DOGASTCANTA DOGASTCANDECASCICATOC T C I A S Q Y E X D M E D N V R E N C T |
| 541 181 | 350 500 570 580 590 000 664747CATCAGA6666C0TTAG66666ACTACAATIOCCA6AATCCTTAG6C0CTTAG6CCTTAG6 G Y III Q R A Y R G D Y N S Q T P Y A F R |

610 620 530 GTGCAGCCTATCCAGCCASATTTACAGCACAGCATATAG VQPIQPNLQDRI*

 $\frac{601}{201}$

| 101 301 | 310 320 340 350 380 GETGETEGENERGENE DESETTECEMBERCIENCESCETENAMETTERENE A B G K F P R H W G K F C Q |
|-------------|---|
| 361 121 | 370 380 390 400 410 420 ТОБАЛОСТПИТАТОБАЛАКСКАЛОБТКАЛСПТИТАКСИТТКАЛАЛ SEPLYPGNGKSVEVSQPLKK |
| 421 141 | 400 440 450 460 470 480 Accodigade Techara technic technic SRRGPRSRS <mark>0 TRG TRG TPTRR</mark> |
| 481 161 | 490 500 510 520 530 540 Altigicologiacticical Altificada Alacticaticaticatica C G R N B S III V D C G K Q V V L G G R |
| 541 181 | 550 560 570 580 590 600 сасадарсасаторастороссалорараарсарс сосратсала тор сосората <mark>р т л н л л л г л г л г л л г к р г с л</mark> |
| 601 201 | 1610 E20 630 640 650 680 Gaggenanattickfretigagataogagataagagataagagataa <mark>B A D T N F S L E D Y E E</mark> D L K Q H T X |
| 061 221 | 670 680 690 700 710 720 ТГАНХААЫБААБАНТІБТІХАТСТАЛТІССКОВАСААБТАЛТІБССТІССААБАХБА І.ТКЕЕГУНКL RRQSTGLPRG |
| 721 241 | 730 740 750 760 770 780 Agendealathangedetean tegangedegaan tegangetegangege S S <mark>K Y R G Y T L II K C G R V C A R M G</mark> |
| 781 261 | 750 806 810 826 830 840 CAATTTINGCCAAAATINITITINIGCCITTITINIGCCAATTGAACTULA D F L G K K Y Y L G L F D T K L K A 4 |
| 841 281 | 850 860 870 880 890 900 Aggegetangagaale dixeathastgegatgegetagegetetereadaactiteget <mark>8 a y d k a a t k c n 6 k d a v t n f b</mark> |
| 901 301 | 910 920 930 940 950 980 Сосноститерала Голастска с боласт с 450 980 <mark>Р S T Y R N</mark> R L N S T D G S R K S G D II |
| 961 321 | 970 980 990 1000 1010 1020 Алестисантискостилаютаетискосантисколителерали N L D L S L G N S T S E S S S B L G D |
| 1021 341 | НОЗО НОНО НОТО НОВО НОГО НОВО Антибносотитетскоснатвантабосалтости советскато своетскате антика NSPYYTMN6FISLQPTPAEAD |
| 1081 361 | 1090 J100 JJ10 L120 J130 L140 TEGREGARGARGTRAGECEARCTRAGECEATATAGARGTRAG WR X X S G F R P K L N L H Q G P X T S E |
| 1141 381 | НБО НБО Н7О НВО Н190 1200 GCMARCGMANCTATGCASCTTTTGGGCCMACCACTATGAMCTOCTAATGAMCTAA ANETMQ1. I. GQTHYQTPNEMY |
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| 1261 421 | 1270 1280 1290 1300 1310 1320 ТТСАЛСТСКИСКАЛСКАТСКАТСКАТСКАССКАССКАССКАЛОБСКАВССКАЛБ РNSSNVQMNQPPSXSNGSRN |
| 1321 441 | 1330 1340 1330 1360 1360 1380 A TOBERETERATIONERGENERGENERGENETETTATACTRONOMICARCHARCH M 6 V N 6 G R 6 G D P C L V T R P Q Q Q |
| 1381 461 | 1190 1400 1410 1420 1430 1440 Тереласкарскартостостоастаттерала поста ТЕР Q P G P P Q L F A T A A A S S G F P |
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| 1501 501 | 1510 1520 1530 ТПССИСТИЛСИТСКАТЬКАВАССКИТИСКАТАА РПУSLM КРSР∗ |
| (f) | 10 20 30 40 50 60 |
| 1 | ATGGGGAGGGGAGGGTGGAGGAGGAGGAGGAGGAGGAGGGAGGGAGGGAGGGAGGGAGGGAGGGTGGAGG M <mark>6 R G R V E L K R I E Y K I N R Q V T</mark> 70 50 90 100 110 120 |
| 61 21 | TTTGCAAAGAAAGGAATGCACTOCTCAAGAAAGCTTATGAGCTCTCTCTCTGTGA FAKRRNGLLKKAYBLSVLCO |
| 121 41 | 130 140 150 160 170 180 Gengatergesterlateatetterteataagenetateagenttergegac <mark>& D V & L I I F S N R G K L V B F C S N</mark> |
| 181 61 | 190 200 210 220 238 240 Техарсатоварскаасаюттоватальство алагосаюттоссяттерсатова <mark>S N M V K T L J K Y Q K C S Y G</mark> A V B V |
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| 301 101 | 310 329 350 340 359 360 TTIGAGGTTIMIAMAATTICKIGAGAAGATTIGGGGAGGTTIAGAT <mark>F B A L Q Q S Q R H L L G B D L G P L D</mark> |
| 361 121 | 370 380 390 400 410 420 Texaggagetagagetagagetagetagetagetagetageta |
| 421 141 | 430 440 450 460 470 480 Асалабастолагаталостося телестрополостиса акадамскостата <mark>р к т о у м l d q l a d l o x k b h v l</mark> |
| 481 161 | 490 500 510 520 530 540 ATGGAATGTAACAATGCTTTAGCAAGAAAGCTGGATGGAAGTTAATGCTAAGAAAGCAGCTC W E S N N A L A R K L D E L N A K N H L |
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| (c) | | |
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| | 10 20 30 40 50 | e |
| 1 | ATGGEACGTEGGAAGATCGAGATTAAGAAGATAGAGAATCCCCACAAACAGGCA | |
| 1 | M <mark>G R G K I E I K K I E N P T N R Q</mark> | 1 1 1 |
| | 70 80 90 100 110 | 12 |
| 61 | TACTCAAAGOGTOGCAATGGCATCTTCAAGAAGGCTCAAGAACTCACTGTTCT | |
| 21 | Y S K R R N G I F K K A Q E I T V L | . с р |
| | 130 140 150 160 170 | 18 |
| 121 | GCTAAGGTTTCTCTCATCATGATTTCTAATADGEGGRAARTCCATGAATACAT | |
| 41 | A KYSLINISNTGKIHEYI | - 5-1 |
| | 190 200 210 220 230 | 24 |
| 181 | ACCACTACAACGAAAAAGATATATGATCAATATCAAAAGGTTATGAAGACOGA | |
| 61 | ΤΤΤΤΚΚΙΥΟΨΥΘΚΥΜΚΤΟ | |
| | 250 260 270 280 290 | 34 |
| 241 | AAATCTCACTACEAGAAAATECTAGATADCTTGAAAAGACACAAEGAGGTCAA | |
| 81 | K S H Y K K N L D T L K R H K E V N | נאו |
| | 310 320 330 34D 350 | 36 |
| 301 | CTGAGAAGABAGATCAAGEAAAGAATGGETGAAGATTTAAAECATCTGAGTTA | |
| 101 | L R R E I K Q R M B E D L N H L S Y | 9 H |
| | 370 380 390 400 410 | 42 |
| 361 | TISCICAFI CITIFAGLAAAATATSGASGELTR/IFTOBCCATAATACHXGAACS | |
| 121 | I. R. S. I. E. Q. N. H. E. S. V. A. I. I. R. E. | : К 1 |
| | 430 440 450 460 470 | 48 |
| 421 | CAUMAGETCAAAACCUMGACTGATACUTACAUXAGAAAGETGAATGGTGTAGA | |
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| | 490 500 510 520 530 | 51 |
| 481 | ATTGAAAATCICATGETAGGCITTGAGGEGAAATGTBGGGATCCACATTATGE | |
| 161 | <mark>I E N L N L</mark> G F E A K C G D P H Y A | |
| | 550 560 570 580 590 | 60 |
| 541 | GAGAATGAGGGAGACTATGAATCTGCAGCTGCATTTBCAAATGGGGCCTCTAA | |
| 181 | ENEGDYESAAAFANGASN | |
| | 510 620 630 640 550 | 68 |
| 601 | GCTTTCCSCCTGDATTCGAGDCADCATGGAGAAGGTTACATGATGTGCACCTC | |
| 201 | A F R L H S S H H B E G Y N N C T S | 5 L I |
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| e) | | |
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| | ATGAAAACTTEGGATCTTEGCACAGGAAAGCCAACAACCAGTTTEGCATCCATGG | |
| | Ν ΚΤ Ψ D L A T G K P T T Q F A S M | B L |
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| 1 | Ν ΚΤ Ψ D L A T G K P T | TQFASMEL |
|------------|---|------------------------------|
| | 70 80 90 | 100 110 120 |
| 61 | AGGAATGATCUTTUAABGGAGGAATUTUUAGAGAB | |
| 21 | TNDPSREESPQR | K N <mark>G R G K I E</mark> |
| | | |
| | 130 140 150 | 160 170 160 |
| 121 | ATCAAGEOGATCEAGAACACAAATAATEGCEAAGT | |
| 11 | IKRIENTNNRQV | TFCKRRNG |
| | 190 200 210 | 220 230 240 |
| 181 | TTACTCAAAAAGGIZTATGAATIGTUTGTUTTIG | |
| 61 | LLKKAYELSVLC | |
| 01 | | 0 8 6 7 8 6 1 7 |
| | 250 260 270 | 250 230 300 |
| 241 | TECTCAACCECTGCAEGCETTTTTGAGTATGCTAA | CARCAGEGETTAGAGGAAGAATTGAG |
| 81 | FSTRGRLFEYAN | NSVRATIE |
| | | |
| | 310 320 330 | 340 350 360 |
| 301 | AGGUATAAAAAAGGUAAGIGUAGATTIDZUUDGGUAG | TG6GTETGFFTETGAGGDCAATGET |
| 101 | <mark>R Y K K A S A D S</mark> S G T | G S V S <mark>E A N A</mark> |
| | | |
| | 370 380 390 CAGTATTACCACCAGAAGCCTCAAAACTGCGTGC | 400 410 420 |
| 361 121 | 0 Y Y O O E A S K L R A | |
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| 481 | GAGTETAAAATAGAGAAAGGAATTAGAAATATCEG | |
| 161 | ESKIEKCIRNIR | SKKNELLF |
| | 550 560 570 | 580 590 600 |
| 541 | TECERGATEGAAGACATECAAAAGAEGGAAATEGA | |
| 181 | SEIED NOKREID | |
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| | 610 620 630 | 640 650 660 |
| 601 | CHAGEAANAATTGETGAGAACGAAAGAGECCAGCA | AATGAACTEGATGCEAGGTGGAM:A |
| 201 | RARIA ENERAQQ | инсирост. |
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| | 670 680 690 | 700 710 720 |
| 661 | AACTATGAGCTCTTACCATCTCAACCATTCGACTC | |
| 221 | NYELLPSQPFDS | кирраура |
| | 730 740 750 | 760 770 |
| 721 | TISCAGECEGA ICATAACTATICICECCAAGACCA | |
| 241 | L Q P N H N Y S R Q D Q | |
| | | |

Fig. 2. ORF sequences of *PsAP1fd* (a), *PsAP2fd* (b), *PsAP3fd* (c), *PsP1fd* (d), *PsAGfd* (e), *PsSEP1fd* (f) and their deduced amino acid sequences. Yellow: MADS-MEF2-like domain; Blue: K-box domain; Green: AP2 domain.

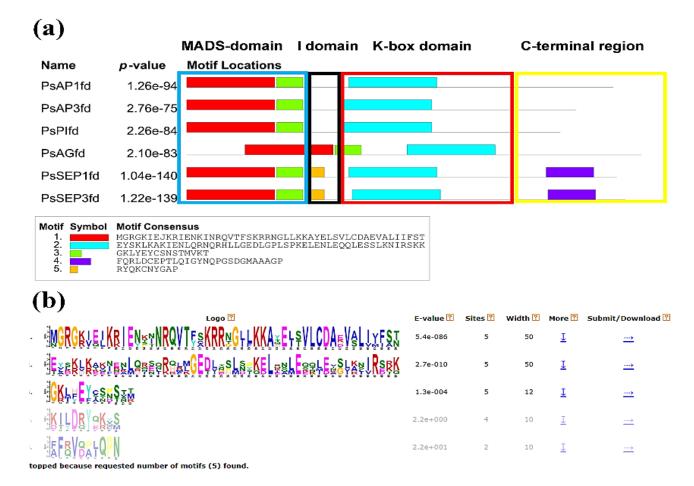


Fig. 3. Distribution of conserved motifs in MADS-box proteins in 'Fengdan'. (a) Motif distribution in each MADS-box protein in 'Fengdan'. Motif 1 and 3 were in MADS domain at N-terminus, followed by Motif 2 in K-box domain. A less-well-conserved I domain and a variable C-terminal region were also found. (b) Only 3 motifs were usable because the E-values of Motif 4 and Motif 5 were larger than 0.05.

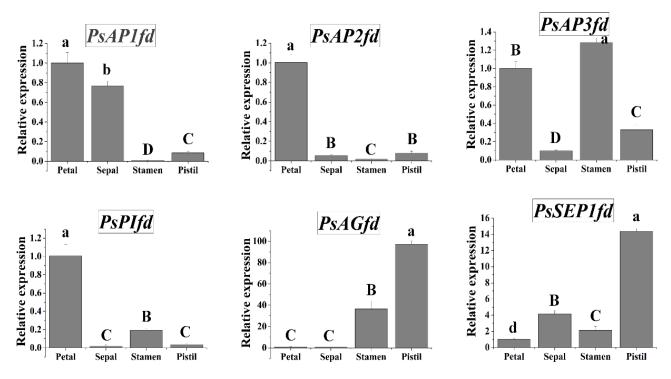


Fig. 4. Expression analysis of floral organ identity genes in the different floral organs of 'Fengdan'. The values are mean \pm SD and the bars with different letters indicate significant differences at p<0.05 (lower case letters) or p<0.01 (capital letters), respectively (based on the one-way ANOVA test).

Expression patterns of floral organ identity genes in 'Fengdan': The expression patterns of these genes were investigated through qRT-RCR. The expression levels of the genes considerably varied among different floral organs. PsAP1fd was predominantly observed in the sepals and petals, as well as the highest expression level of PsAP2fd was found in the petals. By contrast, PsAP3fd had strong expressions in the petals and stamens, but had low expressions in the sepals and pistils. The highest expression level of *PsPIfd* was observed in the petals, followed by the stamens, but the gene was hardly detected in the sepals and pistils. PsAGfd was predominantly expressed in the stamens and pistils, and its highest expression level was found in the pistils. The expressions of *PsAGfd* were hardly detected in the sepals and petals. PsSEP1fd was observed in all the whorls of the flower organs, and the highest expression level of PsSEP1fd was found in the pistils, followed by the sepals (Fig. 4).

Discussion

MADS-box genes encoded transcriptional regulators that are active in diverse plant development processes, such as floral transition, flowering time regulation, and floral organ identify (Becker & Theißen, 2003; Dornelas et al., 2011). Our results showed that in 'Fengdan', the floral organ identity genes examined were all MADS-box family, except *PsAP2fd*, which belongs to the AP2 family. This result is consistent with previous research (Pařenicová et al., 2003; Wang et al., 2019). The proteins encoded by these MADS-box genes had two conserved domains: which were MADS-box and K-box domain, respectively. A less-well-conserved I domain, as well as a variable C-terminal region were also found. Therefore, the proteins were MIKC-type proteins (type II MADSdomain proteins). The MADS-box domain is important to many functions, such as DNA binding, nuclear localization, accessory factor binding and dimerization (Theissen et al., 2000; Ng & Yanofsky, 2001; Immink et al., 2002). The K-box domain is important for dimerization, while the I domain is involved in regulatory determinant for the selective formation of DNA-binding dimers, as well as the C-terminal region is associated with functional specificity, formation of ternary or quaternary complexes, and transcriptional activation protein (Riechmann & Meyerowitz, 1997; Egea-Cortines et al., 1999; Honma & Goto, 2001; Lamb & Irish, 2003). The genomic DNA sequence and coding sequence of the Bclass genes PsTM6 from 23 different tree peony cultivars were obtained and analyzed, and the results showed that the electronic charge and polarity of PsTM6 paralogs varied because of amino acid substitution leading to functional differentiation, which significantly affected stamen petalody and caused variations in flower shapes in tree peony (Shu et al., 2012). Different selection forces generated the different regions of PsTM6, especially in the K-box domain (Shu et al., 2012).

The ABCE model is closely related to flower type formation, and this relationship has been confirmed in many species (Wagner *et al.*, 1999; Lenhard *et al.*, 2001; Lohmann *et al.*, 2001; Krizek & Fletcher, 2005). Previous research showed that the MADS-box proteins related to

flower organ identity often function as complexes: A+E regulates sepal development (Pelaz et al., 2001a), A+B+E regulates petal formation, B+C+E regulates stamen development (Honma & Goto, 2001; Pelaz et al., 2001b; Ferrario et al., 2003), C+E regulates pistil development (Fan et al., 1997; Pelaz et al., 2000). Furthermore, protein complexes comprising AG, SEP, STK or AG, SEP, SHP both control ovule development in Arabidopsis (Favaro et al., 2003). Flower type is a valuable ornamental characteristic, and tree peony has 10 flower types, including lotus, crown, chrysanthemum, rose, globular, and crown-proliferation (Wang & Yuan, 2003). Increase in petals, stamen petalody, pistil petalody, and flower overlapping generate different flower types in tree peony (Wang & Yuan, 2003). Diverse flower types are one of the most significant characteristics for cultivar classification (Wang & Yuan, 2003; Shu et al., 2012). Nevertheless, the molecular mechanism underlying floral organ identity in tree peony remains unclear. Some ABCE genes in tree peony and herbaceous peony have been studied (Shu et al., 2012; Ge et al., 2014; Gong et al., 2017; Wang et al., 2019), but further functional research is needed. Ge et al. isolated ABE genes in herbaceous peony and detected their expression patterns in the three cultivars, which had different flower types. The results suggested that the expression levels of A- and E-class genes increased, while these of B-class genes reduced with the depth of stamen petaloidy. This study focused on stamen petaloidy rather than on floral organ identity (Ge et al., 2014). According to our study in 'Fengdan', PsAP1fd (A-class gene) and PsSEP1fd (E-class gene) had strong expressions in the sepals. PsAP1fd, PsAP2fd (A-class genes), as well as PsAP3fd, PsPIfd (B-class genes) had high expressions in the petals. PsAP3fd, PsPIfd (B-class genes), as well as PsAGfd (C-class gene) had strong expressions in the stamens. PsSEP1fd (E-class gene) had normal expression. The highest expression levels of PsAGfd (C-class gene) and PsSEP1fd (E-class gene) were detected in the pistils. These results were consistent with those of the ABCE model and previous studies (Wang et al., 2019).

In Arabidopsis, there are 4 E-class genes named SEP1, SEP2, SEP3 and SEP4, respectively, which have specific expression levels in diverse flower organs (Pelaz et al., 2000: Honma & Goto, 2001: Pelaz et al., 2001a. 2001b; Ditta et al., 2004). In tree peony 'Ziluo Lan', SEP1 is primarily expressed in the sepals, stamens, and pistils; SEP3 is detected in all the whorls of the flower organs; SEP4 has high expression levels in the sepals, as well as stamens (Wang et al., 2019). PISEP3 had extremely high expression in the sepals of P. lactiflora 'Hangshao' (Ge et al., 2014). In our study, the highest expression level of *PsSEP1fd* was found in the pistils, followed by the sepals. These results indicated that ABC genes play the same role in flower organ formation in 'Fengdan' and the expression patterns of E-class genes varied among cultivars, exhibiting a significant role in flower type formation.

In 2013, the flowers of 'Fengdan' were identified as new food resource in China and were found to contain abundant flavonoids (Zhang *et al.*, 2017). Peony tea made by 'Fengdan' has been introduced to the market, and the quality and price of peony tea are closely related to the flower type of 'Fengdan'. This research will serve as a foundation for the mechanism of the flower type formation in tree peony, as well as promotion of quality control for peony tea products.

Conclusion

Genes in the four floral organs of *P. ostii* 'Fengdan' were isolated through RT-PCR and identified: *PsAP1fd* and *PsAP2fd* (A-class genes); *PsAP3fd* and *PsP1fd* (B-class genes); *PsAGfd* (C-class gene); and *PsSEP1fd* (E-class genes). They are all MADS-box family except *PsAP2fd*, which belongs to the AP2 family. The six genes played different roles during floral organ development. *PsAP1fd* was primarily observed in the sepals and petals. *PsAP2fd* was primarily found in the petals. *PsAP3fd* was significantly expressed in the petals, as well as stamens. The highest expression level of *PsP1fd* was observed in the space of *PsAGfd* was predominantly detected in the stamens and pistils, and its highest expression levels were found in the pistils. *PsSEP1fd* was mainly expressed in the pistils.

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