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# Expression of floral MADS-box genes in *Sinofranchetia chinensis* (Lardizabalaceae): implications for the nature of the nectar leaves

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• *Background and Aims* The perianths of the Lardizabalaceae are diverse. The second-whorl floral organs of *Sinofranchetia chinensis* (Lardizabalaceae) are nectar leaves. The aim of this study was to explore the nature of this type of floral organ, and to determine its relationship to nectar leaves in other Ranunculales species, and to other floral organs in *Sinofranchetia chinensis*.
• *Methods* Approaches of evolutionary developmental biology were used, including 3′ RACE (rapid amplification of cDNA ends) for isolating floral MADS-box genes, phylogenetic analysis for reconstructing gene evolutionary history, *in situ* hybridization and tissue-specific RT-PCR for identifying gene expression patterns and SEM (scanning electron microscopy) for observing the epidermal cell morphology of floral organs.
• *Key Results* Fourteen new floral MADS-box genes were isolated from *Sinofranchetia chinensis* and from two other species of Lardizabalaceae, *Holboellia grandiflora* and *Decaisnea insignis*. The phylogenetic analysis of *AP3*-like genes in Ranunculales showed that three *AP3* paralogues from *Sinofranchetia chinensis* belong to the *AP3-I*, *-II* and *-III* lineages. *In situ* hybridization results showed that *SIchAP3-3* is significantly expressed only in nectar leaves at the late stages of floral development, and *SIchAG*, a C-class MADS-box gene, is expressed not only in stamens and carpels, but also in nectar leaves. SEM observation revealed that the adaxial surface of nectar leaves is covered with conical epidermal cells, a hallmark of petaloidy.
• *Conclusions* The gene expression data imply that the nectar leaves in *S. chinensis* might share a similar genetic regulatory code with other nectar leaves in Ranunculales species. Based on gene expression and morphological evidence, it is considered that the nectar leaves in *S. chinensis* could be referred to as petals. Furthermore, the study supports the hypothesis that the nectar leaves in some Ranunculales species might be derived from stamens.

**Key words:** Nectar leaves, perianth, petals, Ranunculales, Lardizabalaceae, *Sinofranchetia chinensis*, MADS-box, expression pattern, evolutionary developmental biology.

## INTRODUCTION

The reproductive organs of most angiosperms are enclosed by a sterile outer structure, which is usually called the perianth. The perianth, as a remarkable novelty in angiosperms, generally contains two whorls and shows great morphological diversity. The perianth shape ranges in different lineages from undifferentiated to bipartite (i.e. differentiated into sepals and petals; Cronquist, 1988; Takhtajan, 1997; Zanis *et al.*, 2003; Endress and Matthews, 2006; Ronse De Craene, 2008). The various morphologies of perianths in different angiosperm lineages have led to the suggestion that perianths could have different origins (Endress, 1994, 2006). Particularly, petals (the inner part of the perianth) have been thought to have evolved several times independently from sterile stamens (andropetals) or bracts (bracteopetals) during angiosperm evolution (e.g. Eames, 1961; Weberling, 1989; Friis and Endress, 1990; Takhtajan, 1991; Endress, 1994, 2001). However, the molecular evolutionary mechanisms responsible for the different origins of petals remain unclear.

Ranunculales, the earliest-diverging lineage in eudicots, displays extreme diversity in perianth morphology and has received increasing attention from evolutionary-developmental biologists (e.g. Albert *et al.*, 1998; Kramer *et al.*, 1998, 2003, 2007; Kramer and Irish, 1999, 2000; Theissen *et al.*, 2002; Rasmussen *et al.*, 2009; Kramer and Hodges, 2010; Sharma *et al.*, 2011). This order is composed of seven families according to recent molecular phylogenetic studies: Ranunculaceae, Berberidaceae, Menispermaceae, Lardizabalaceae, Circaeasteraceae, Papaveraceae and Eupteleaceae (Angiosperm Phylogeny Group III, 2009; Wang *et al.*, 2009). The majority of genera in the Ranunculaceae, Berberidaceae, Menispermaceae, Lardizabalaceae and Papaveraceae have bipartite perianths, whereas there is no clear differentiation of the perianth in Circaeasteraceae, and the flowers lack perianths in Eupteleaceae (Qin, 1997; Damerval and Nadot, 2007; Rasmussen *et al.*, 2009; Takhtajan, 2009; Wang *et al.*, 2009; Zhang and Ren, 2011). Diverse perianth architectures are present in Ranunculales, including petaloid sepals, floral nectariferous organs and spurs (Qin, 1997; Damerval and Nadot,

2007; Rasmussen *et al.*, 2009; Takhtajan, 2009; Wang *et al.*, 2009; Zhang *et al.*, 2009; Zhang and Ren, 2011). The floral nectariferous organs are also called nectar leaves, which are nectar-bearing and formed between the perianth and androecium (Janchen, 1949). In Ranunculales, nectar leaves are found in some species of Ranunculaceae, Berberidaceae, Menispermaceae and Lardizabalaceae (Erbar *et al.*, 1998; Ronse De Craene, 2010). Furthermore, nectar leaves in different species of Ranunculales display different morphologies: some are small and greenish, such as in *Sinofranchetia* (Lardizabalaceae); some are large and petaloid, such as in *Ranunculus* (Ranunculaceae); some are converted to long spurs, such as in *Aquilegia* (Ranunculaceae) (Leppik, 1988; Weberling, 1989; Zhang *et al.*, 2009). The diversification of nectar leaves in different species of Ranunculales is also reflected in floral morphogenesis: some types of nectar leaves share common primordia with stamens, such as in Berberidaceae and *Holboellia* (Lardizabalaceae); some develop from individual primordia distinguished from that of stamens, such as in *Sinofranchetia* (Lardizabalaceae) (Zhang *et al.*, 2009; Ronse De Craene, 2010; Zhang and Ren, 2011). In addition, the nectar leaves have ever been referred to as 'petals', especially in some species of Ranunculaceae and Berberidaceae, because these nectar leaves are colourful, sterile and positioned in the second whorl of the bipartite perianth, fitting the broader definition of petals (e.g. Cronquist, 1981; Kramer *et al.*, 2007; Kramer and Hodges, 2010). However, some other studies considered that the term 'petals' for these organs is mainly related to the function of display rather than the morphological concept (Leppik, 1988; Weberling, 1989). Furthermore, some previous studies of the species of Ranunculaceae and Berberidaceae suggested that the nectar leaves might be derived from the sterilization of stamens (Endress, 1995; Ronse De Craene, 2010).

In the past two decades, great advances have been made in evolutionary developmental biology studies of angiosperm flowers. New models and theories have been proposed based on the evolution, expression and functional analyses of genes involved in floral development, besides floral morphology and morphogenesis (Albert *et al.*, 1998; Baum and Whitlock, 1999; Kramer and Irish, 1999, 2000; Irish, 2003; Kramer *et al.*, 2003; Soltis *et al.*, 2004). These models and theories promote our understanding of the molecular mechanisms for the origin and evolution of angiosperm flowers. Among them, the best-known one is the floral ABCE model, which was proposed from genetic analyses of two core eudicot species, *Arabidopsis thaliana* and *Antirrhinum majus*. In this model, the identity of floral organs is determined by the combinations of four classes of genes: A + E class genes are responsible for the specification of sepals, A + B + E for petals, B + C + E for stamens and C + E for carpels (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Colombo *et al.*, 1995; Ma and dePamphilis, 2000; Pelaz *et al.*, 2000; Theissen, 2001*b*). Most of the A-, B-, C- and E-class genes are MIKC$^{C}$-type MADS-box genes and they belong to the *AP1*/*FUL* (A-class), *AP3*/*PI* (B-class), *AG* (C-class) and *SEP* (E-class) MADS-box gene subfamilies, respectively (Litt and Irish, 2003; Kramer *et al.*, 2004; Irish and Litt, 2005; Zahn *et al.*, 2005*a*, *b*, 2006; Kramer and Zimmer, 2006; Shan *et al.*, 2007, 2009). The ABCE model works relatively well for most core eudicot flowers with well-differentiated sepals and petals, but not for most species of basal eudicots and basal angiosperms with less derived perianth architecture (Soltis *et al.*, 2007). Accordingly, some modified ABCE models have been put forward, such as the 'sliding boundary' model and 'fading borders' model (Soltis *et al.*, 2007). The 'sliding boundary' model suggests that the boundary of B-class gene expression can slide across the developing flower from its pre-existing location to the outer perianth whorl (e.g. Kanno *et al.*, 2003; Kramer *et al.*, 2003; Ochiai *et al.*, 2004; Kramer and Jaramillo, 2005; Hintz *et al.*, 2006; Kramer and Zimmer, 2006; Ronse De Craene, 2007). In addition, the labile petal/stamen boundary in the flower has been suggested to correspond to the sliding of the A–C boundary (Goto *et al.*, 2001; Theissen, 2001*a*; Kim *et al.*, 2005; Chanderbali *et al.*, 2006, 2009; Xu *et al.*, 2006; Dubois *et al.*, 2010). The 'fading borders' model suggests that the gradual transitions in floral organ morphology are due to the gradient in the expression levels of floral organ identity genes (Buzgo *et al.*, 2004; Kim *et al.*, 2005; Soltis *et al.*, 2006; Soltis *et al.*, 2007).

Both the ABCE model and its derived models emphasize the importance of B-class MADS-box genes for petal identity specification in core eudicots and for the development of petal-like structures in basal eudicots and basal angiosperms (Weigel and Meyerowitz, 1994; Albert *et al.*, 1998; Kramer *et al.*, 1998, 2003, 2007; Kramer and Irish, 2000; Theissen *et al.*, 2002; Lamb and Irish, 2003; Aoki *et al.*, 2004; Kim *et al.*, 2004; Kramer and Jaramillo, 2005; Zahn *et al.*, 2005*b*; Rasmussen *et al.*, 2009; Sharma *et al.*, 2011). Phylogenetic studies have indicated that two major gene duplication events occurred during the evolution of B-class genes, one before the origin of angiosperms giving rise to the *AP3* and *PI* lineages, and the other before the divergence of core eudicots leading to the eu*AP3* and *TM6* lineages (Kramer *et al.*, 1998, 2006; Kramer and Irish, 2000; Aoki *et al.*, 2004; Kim *et al.*, 2004; Stellari *et al.*, 2004). These gene duplication events and subsequent functional diversification have resulted in modifications of floral organ identity programmes in different plant groups (Kramer *et al.*, 1998, 2003; Rasmussen *et al.*, 2009; Specht and Bartlett, 2009). It was also found that two recent gene duplication events had occurred during the evolution of *AP3*-like genes in Ranunculales, giving rise to three *AP3* lineages (*AP3-I*, *-II* and *-III*), which in turn enable gene subfunctionalization (Kramer *et al.*, 2003; Rasmussen *et al.*, 2009; Sharma *et al.*, 2011). The recent studies in Ranunculales have suggested that the diversification of *AP3*-like genes is responsible for petaloidy diversity (e.g. Rasmussen *et al.*, 2009; Specht and Bartlett, 2009). In particular, the genes from the *AP3-III* lineage are found to be petal-specific in the Ranunculaceae and Berberidaceae (Kramer *et al.*, 2003; Rasmussen *et al.*, 2009; Sharma *et al.*, 2011). Therefore, *AP3*-like genes are good candidates for studying the molecular mechanisms regulating petal or petal-like structure specification across different species.

In this study, as an initial step towards understanding the development of nectar leaves in Lardizabalaceae at the molecular level, we identified the floral MADS-box genes in *Sinofranchetia chinensis* and especially studied the evolution

of the B-class MADS-box genes in Ranunculales. Furthermore, we investigated the expression patterns of the floral MADS-box genes and observed the epidermal cell morphology of floral organs in *S. chinensis*. By integrating molecular, developmental and morphological data, we hope to explore the nature of the nectar leaves in *S. chinensis*, and reveal its relationship to nectar leaves in other Ranunculales species, and to other floral organs in *S. chinensis*.

## MATERIALS AND METHODS

### *Plant materials*

*Sinofranchetia chinensis* is a liana with unisexual flowers. The functionally unisexual flowers are bisexual in organization at the floral bud stages, and the unisexuality is found in mature flowers (Zhang *et al.*, 2009). A *Sinofranchetia* flower has petaloid sepals with a purple margin in the first whorl, greenish nectar leaves in the second whorl, stamens or sterile staminodes in the third whorl, and rudimentary carpels or carpels in the forth whorl (Figs 1 and 6A). Floral buds and mature flowers of *S. chinensis* at different developmental stages were collected from Taibai Mountain (1200–1500 m a.s.l.), Meixian County, Shaanxi Province, China. They were treated in one of three ways: fixed in FAA (formalin to acetic acid to 50% alcohol in the ratio of 5 : 6 : 89) for morphological observation; stored in liquid nitrogen for RNA isolation; or fixed in PFA (4 % paraformaldehyde) and embedded in Paraplast (Sigma, St Louis, MO, USA) for *in situ* hybridization. Plant materials of *Holboellia grandiflora* and *Decaisnea insignis* were also collected for RNA extraction.

### *Gene cloning*

Total RNA of floral buds and young flowers was extracted using Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). Poly(A) mRNA was purified from total RNA using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen). We performed 3′ RACE (rapid amplification of cDNA ends) PCR with the degenerate primers and the adapter primer AP (5′-CCGGATCCTCTACA GCGGCCGC-3′). To clone B-class MADS-box genes, hemi-nested PCR assay was carried out with the B-class gene-specific degenerate primer B1 and the adapter primer AP.

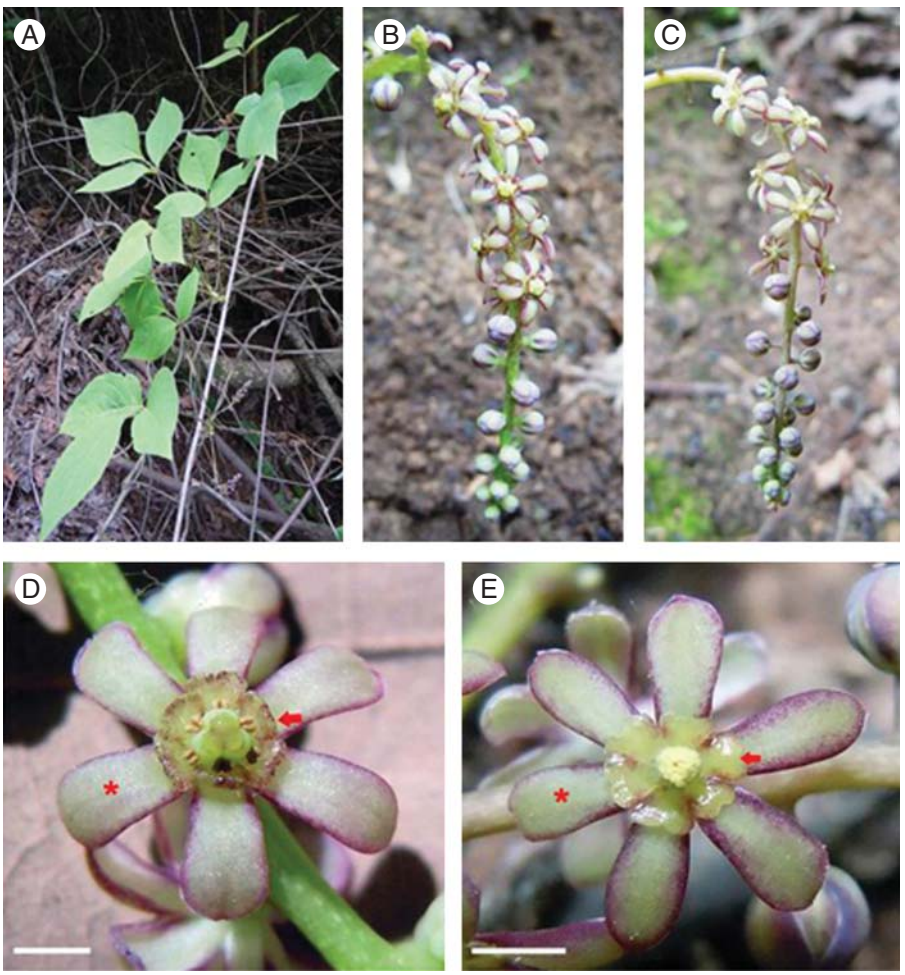


FIG. 1. Morphology of *Sinofranchetia chinensis* flowers: (A) plant with inflorescences; (B) female inflorescence; (C) male inflorescence; (D) female flower; (E) male flower. Red asterisks indicate sepals; red arrows indicate nectar leaves. Scale bars = 2 mm.

The PCR reaction was heated at 94 °C for 4 min, followed by ten cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, and then 25 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and finally extended at 72 °C for 10 min. A second B-class gene-specific degenerate primer B2 and AP were then used to amplify the PCR products obtained in the first step. The PCR was performed with 35 cycles, and the annealing temperature was set at 52 °C. The amplified fragments over 800 bp were purified and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). The plasmid DNA was isolated by alkaline lysis precipitation (Sambrook *et al.*, 1989). The positive clones were identified by restriction enzyme analysis of the plasmids, and at least three independent clones were sequenced for each identified locus using forward primer T7 (5′-TAATACGACTCACTATAGGG-3′) and reverse primer SP6 (5′-ATTTAGGTGACACTATAG-3′). In a similar way, other floral MADS-box genes were cloned. Only nucleotide sequences with Phred quality scores >20 were used for further analysis. The degenerate primers for B-, C- and E-class gene amplification and the number of sequenced clones for each gene are listed in Supplementary Data Table S1.

### *Sequence retrieval and alignment*

Aside from all the floral MADS-box genes cloned from *S. chinensis*, *H. grandiflora* and *D. insignis*, we also obtained homologous sequences from other species using BLAST against the publicly available databases, including NCBI (http://www.ncbi.nlm.nih.gov) and the TIGR plant transcript assembly database (http://plantta.jcvi.org/). The full-length amino acid sequences for phylogenetic analyses of A-, B-, C- and E-class MADS-box genes (referred to as global analysis hereafter), *AP3*-like genes in Ranunculales (referred to as *AP3* analysis hereafter) and *PI*-like genes in Ranunculales (referred to as *PI* analysis hereafter) were aligned with ClustalX 1.83 using the default parameters (Thompson *et al.*, 1997). Alignments were adjusted manually using GeneDoc (Nicholas and Nicholas, 1997). The corresponding DNA matrices were generated by aa2dna (https://homes.bio.psu.edu/people/faculty/nei/software.htm) using the well-aligned amino acid matrices. In addition, we used ClustalX 1.83 to estimate the column scores of the amino acid matrices for the global analysis, *AP3* analysis and *PI* analysis, respectively, and the residues with higher-than-12 quality scores were kept in the alignment (Thompson *et al.*, 1997; Zahn *et al.*, 2005*a*; Shan *et al.*, 2007). Based on these residues, we generated the corresponding nucleotide matrices for further phylogenetic analyses, and the length of the dataset for the global analysis was 588 bp, 606 bp for the *AP3* analysis and 612 bp for the *PI* analysis.

### *Phylogenetic analysis*

Phylogenetic analyses were performed for each DNA matrix using the maximum likelihood (ML) method in PhyML version 2.4.4 (Guindon and Gascuel, 2003). The best fit model of nucleotide evolution for the DNA matrices was GTR + I + Γ, which was chosen by running MODELTEST version 3.06 (Posada and Crandall, 1998). The proportion of invariable sites and gamma distribution parameter values for the GTR + I + Γ model were optimized by using MODELTEST. Each ML analysis used a BIONJ tree as a starting point (Gascuel, 1997). The support value for each tree branch was based on the best ML tree filtered through 1000 bootstrap replicates in PhyML (Felsenstein, 1985).

### In situ *hybridization*

The floral buds of *S. chinensis* at various developmental stages were used for *in situ* hybridization. The gene-specific primers were designed for amplifying the partial CDS (coding sequences) and 3′ UTR (untranslated regions) of *Sinofranchetia* floral MADS-box genes to make RNA probes (Supplementary Data Table S2). The sense and antisense digoxigenin-labelled RNA probes for *in situ* hybridization were synthesized using a DIG northern starter kit (Roche Diagnostics, Mannheim, Germany). Embedding of plant materials, pretreatment, hybridization and washing of the sections were performed as previously described (Li *et al.*, 2005; Shan *et al.*, 2006) with slight modifications.

### *Tissue-specific RT-PCR*

The expression patterns of *AP3*, *PI* and *AG* homologues in the mature flowers of *S. chinensis* were investigated by using tissue-specific RT-PCR. The RNA used in RT-PCR was extracted from sepals, petals, staminodes and carpels of mature female flowers, and sepals, petals and stamens of mature male flowers and from young leaves. The rudimentary carpels in the male flowers are too small to be collected, so they were not included in the analysis. The extraction of total RNA, purification of poly (A) mRNA, and synthesis of the first-strand cDNA were performed according to the methods described above. The amounts of templates were normalized using the control gene *ACTIN*. Gene-specific forward and reverse primers were used to detect gene expression (Supplementary Data Table S2). The PCR thermocycling conditions used were: initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55–60 °C (depending on the melting temperature of primer pairs) for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were fractionated in 1 % agarose gels and digitally photographed. We repeated the RT-PCR experiments three times independently.

### *Scanning electron microscopy*

The characteristics of the epidermal cells are an important criterion for identifying morphological equivalents or homologues among different floral organs (Endress, 1994; Krizek *et al.*, 2000; Pelaz *et al.*, 2000; Jaramillo and Kramer, 2004; Geuten *et al.*, 2006). We therefore performed SEM (scanning electron microscopy) analysis of epidermal cell shapes for the sepal, nectar leaf, sterile staminode/stamen and carpel/rudimentary carpel in the female and male flowers of *S. chinensis*. Young flowers were collected at 7-d intervals and immediately fixed with FAA. Subsequently, the materials were dehydrated in an alcohol–isoamyl acetate series, treated by critical point drying in $CO_2$, mounted, and then coated with

gold. Observations of the epidermal cell morphology of different floral organs were performed using a Hitachi S-800 scanning electron microscope.

## RESULTS

### *A-, B-, C- and E-class MADS-box genes in* S. chinensis

Six floral MADS-box genes were isolated from *S. chinensis*, four from *H. grandiflora* and four from *D. insignis*. Through BLAST against NCBI databases, these genes were preliminarily grouped into B-, C- and E-class MADS-box genes, and these classifications were further supported by ML analysis (Fig. 2). The studied species, gene name and accession number for 14 newly isolated genes in this study, two previously published genes from *S. chinensis* (Shan *et al.*, 2007) and 92 representative floral MADS-box genes from other species downloaded from databases are listed in Supplementary Data Table S3. All floral MADS-box genes analysed here formed four distinct well-supported clades in the ML tree, corresponding to the *AP1*/*FUL* (A-class), *AP3*/*PI* (B-class), *AG* (C-class) and *SEP* (E-class) subfamilies of MADS-box genes (Fig. 2). Furthermore, the relationships among these genes are largely consistent with the species phylogeny (Fig. 2). Accordingly, the eight floral MADS-box genes from *S. chinensis* were classified as two *FUL*-like genes (*SIchFL1* and *SIchFL2*), three *AP3*-like genes (*SIchAP3-1*, *SIchAP3-2* and *SIchAP3-3*), one *PI*-like gene (*SIchPI*), one *AG*-like gene (*SIchAG*) and one *SEP3*-like gene (*SIchSEP3*); the four floral MADS-box genes from *H. grandiflora* were one *AP3*-like gene (*HOgrAP3*), one *PI*-like gene (*HOgrPI*) and two *AG*-like genes (*HOgrAG1* and *HOgrAG2*); and the four floral MADS-box genes from *D. insignis* were two *AP3*-like genes (*DEinAP3-1* and *DEinAP3-2*), one *PI*-like gene (*DEinPI*) and one *AG*-like gene (*DEinAG*) (Fig. 2). The phylogenetic analysis indicated that the following paralogous gene pairs might be the result of gene duplication events that took place before the divergence of the Lardizabalaceae (Fig. 2): *SIchFL1* and *SIchFL2*; *SIchAP3-1*, *SIchAP3-2* and *SIchAP3-3*; *HOgrAG1* and *HOgrAG2*; and *DEinAP3-1* and *DEinAP3-2*.

### *Sequence and phylogenetic analysis of B-class MADS-box genes in Ranunculales*

Our phylogenetic analysis of *AP3*-like genes in Ranunculales showed that the three *AP3*-like genes from *S. chinensis* were grouped into three paralogous *AP3* lineages of Ranunculales (Fig. 3 and Supplementary Data Table S4). *SIchAP3-1* belongs to the *AP3-I* lineage, *SIchAP3-2* to the *AP3-II* lineage, and *SIchAP3-3* to the *AP3-III* lineage. Furthermore, SIchAP3-1 and SIchAP3-2 protein sequences show 67 % identity; SIchAP3-2 and SIchAP3-3 share 62 % identity; and SIchAP3-1 and SIchAP3-3 share 56 % identity. The highly conserved MADS domain and K domain, as well as PI-derived motif and paleoAP3 motif, were found in the three SIchAP3 proteins from multiple sequence alignment with other AP3-like proteins of Ranunculales; however, the SIchAP3-2 protein lacks 29 amino acids in the K domain (Supplementary Data Fig. S1). At least four families (Ranunculaceae,

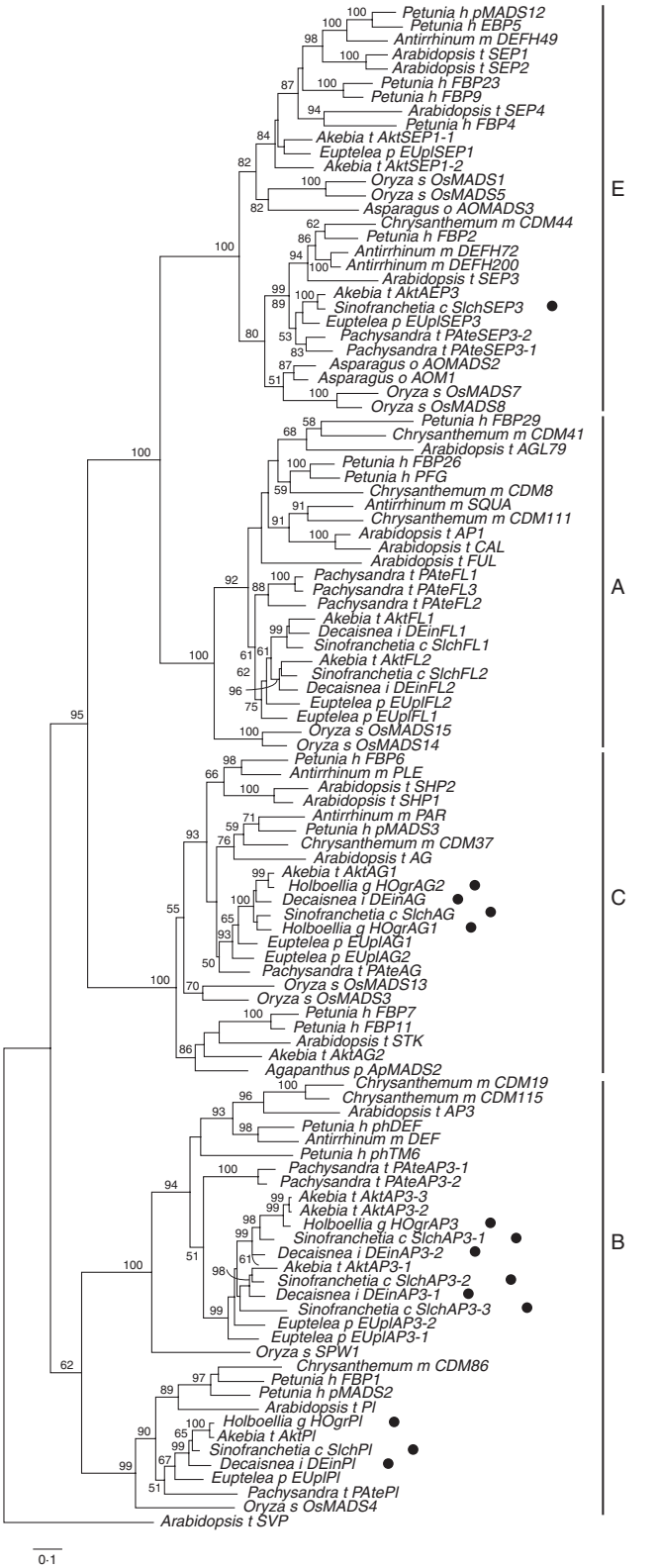


FIG. 2. Phylogenetic relationships of 108 representative floral MADS-box genes. Bootstrap values (>50 %) are shown above the branches. Genes obtained in this study are indicated with dots.

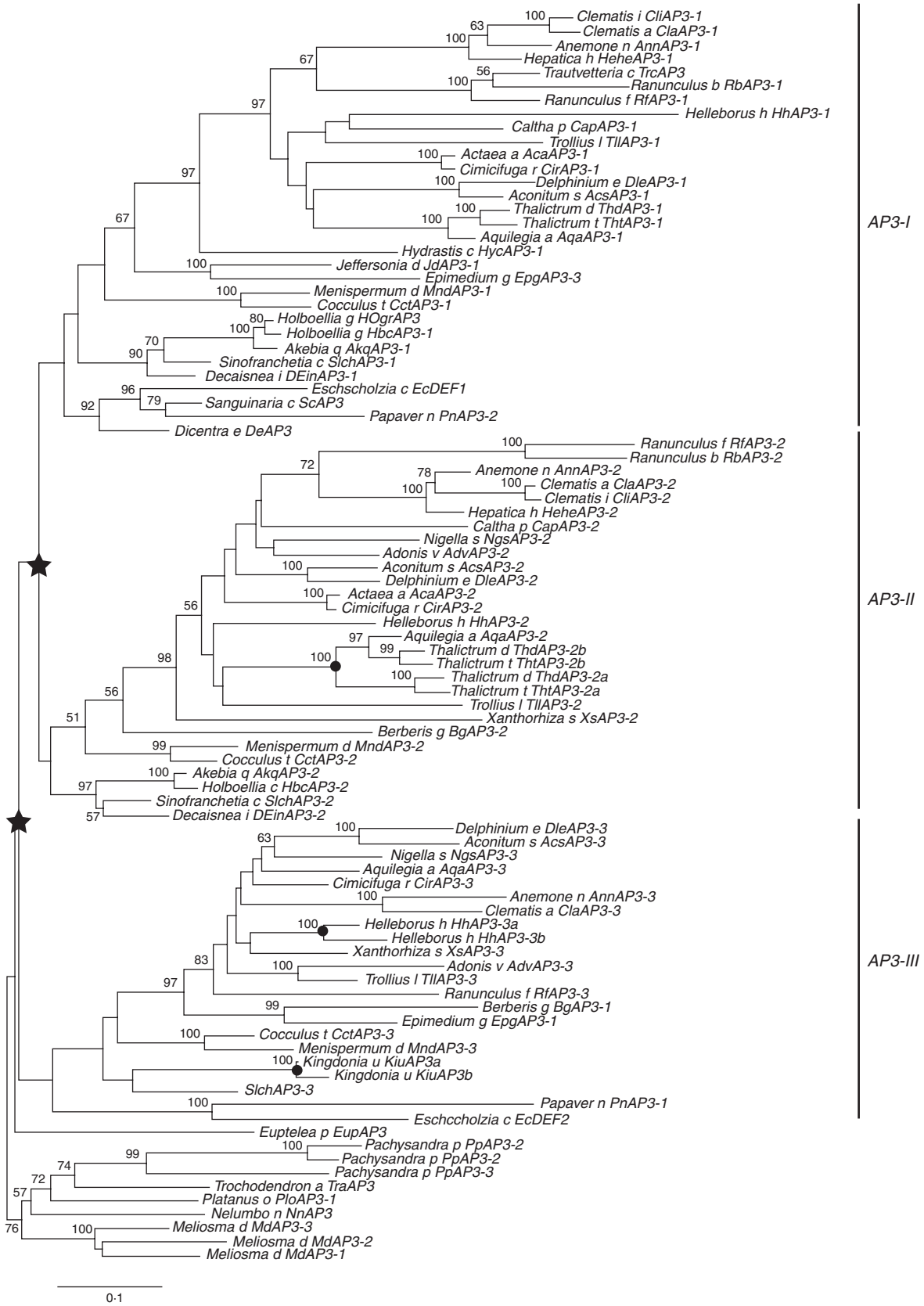


FIG. 3. Maximum-likelihood tree of *AP3*-like genes in Ranunculales. Bootstrap values (>50 %) are shown above the branches. In Ranunculales, the inferred two major, successive gene duplication events are highlighted by stars, and the inferred small-scale gene duplication events are indicated with dots.

Berberidaceae, Menispermaceae and Lardizabalaceae) of Ranunculales have all three paralogues of *AP3*-like genes, but the relationships among these three *AP3* lineages in Ranunculales are still uncertain (Fig. 3).

In this study, we have added three new *PI* homologues from Lardizabalaceae to the phylogenetic analysis of *PI*-like genes of Ranunculales (Supplementary Data Table S5 and Fig. S2). These were from three different species of Lardizabalaceae, only one copy in each species (Supplementary Data Fig. S2).

### *Spatiotemporal expression patterns of floral MADS-box genes in* S. chinensis

To detect the spatiotemporal expression patterns of floral MADS-box genes in *S. chinensis* at the floral bud stages, we performed *in situ* hybridization experiments. The results for B-class MADS-box genes are presented in Fig. 4. At the early stages, *SIchAP3-1* is highly expressed in the primordia of the nectar leaves, as well as in the androecial and gynoecial primordia (Fig. 4A). Later on, the expression signals of *SIchAP3-1* were mainly detected in the nectar leaves and developing stamens, but the expression level in the carpels reduces gradually during floral development (Fig. 4B). At the late stages, *SIchAP3-1* expression is mainly restricted to the nectar leaves and stamens (Fig. 4C). The expression pattern of *SIchAP3-2* is mostly like that of *SIchAP3-1*, but weak expression of this gene was also detected in the sepal primordia, and its expression level in the carpels is constant during the late stages (Fig. 4D–F). *SIchAP3-3* expression is ubiquitously in the whole flower (Fig. 4G) and gradually narrows to the inner three whorls (Fig. 4H). At the late stages, *SIchAP3-3* is expressed strongly in the nectar leaves, but very weakly in the stamens and carpels (Fig. 4I). *SIchPI* is expressed in the nectar leaves and stamens but not in the carpels (Fig. 4J, K).

The expression patterns of A-, C- and E-class MADS-box genes in *S. chinensis* are shown in Fig. 5. *SIchFL1* has very strong expression signals throughout the floral organ primordia at the early stages (Fig. 5A), and high expression levels are maintained in the nectar leaves, stamens and carpels at the late stages (Fig. 5B, C). The expression level of *SIchFL2* is lower than that of *SIchFL1*, and its transcripts were found in

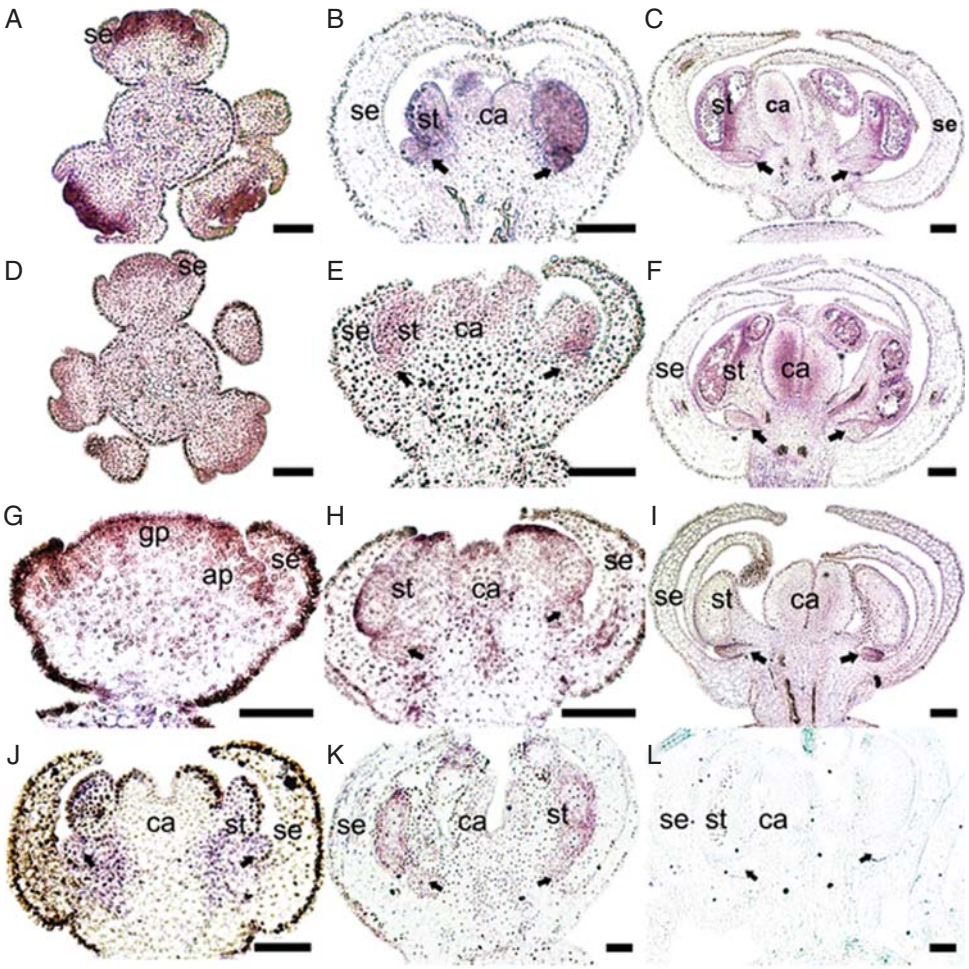


FIG. 4. Expression patterns of B-class MADS-box genes of *Sinofranchetia chinensis* as revealed by *in situ* hybridization analyses: (A–C) *SIchAP3-1*; (D–F) *SIchAP3-2*; (G–I) *SIchAP3-3*; (J, K) *SIchPI*; (L) negative control with sense probe for *SIchPI*. (A) and (D) show a young inflorescence with multiple flowers, whereas all other panels show only one flower. Abbreviations: gp, gynoecial primordium; ap, androecial primordium; se, sepal; st, stamen; ca, carpel. Arrows indicate nectar leaves. Scale bars = 100 μm.

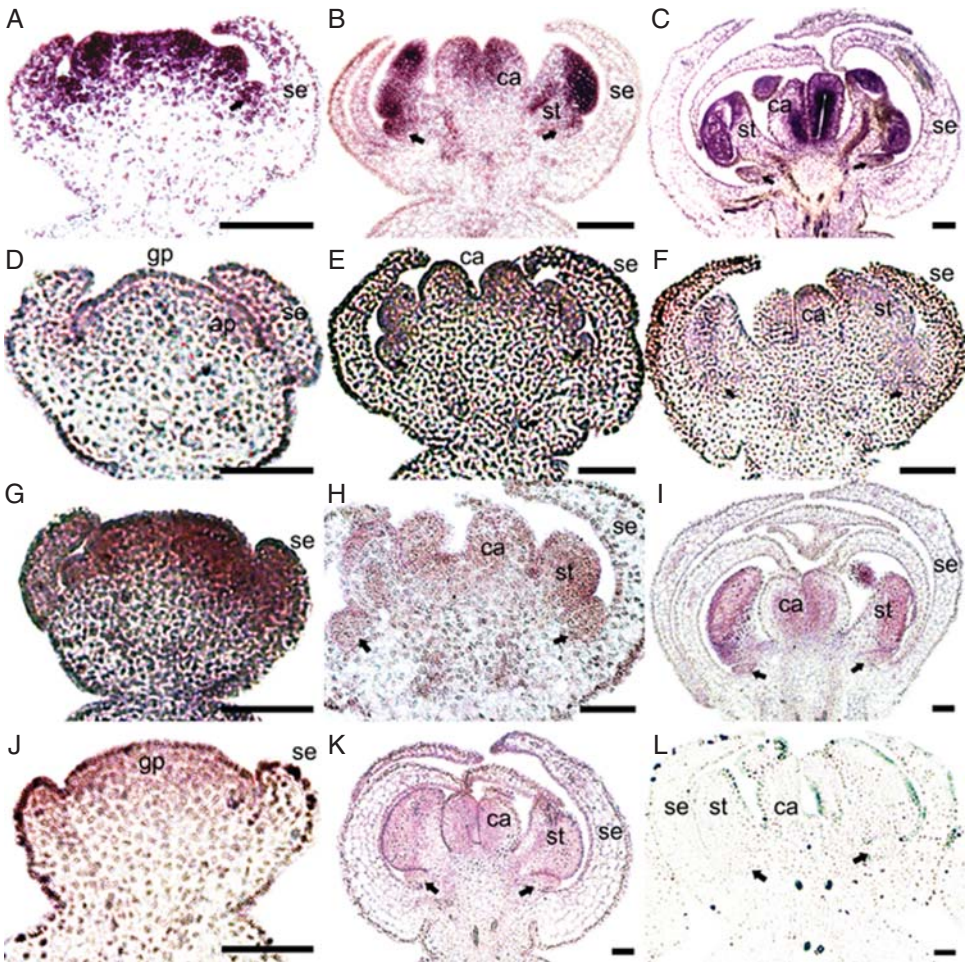


FIG. 5. Expression patterns of A-, C- and E-class MADS-box genes of *Sinofranchetia chinensis* as revealed by *in situ* hybridization analyses; (A–C) *SIchFL1*; (D–F) *SIchFL2*; (G–I) *SIchAG*; (J, K) *SIchSEP3*; (L) negative control with sense probe for *SIchAG*. Abbreviations: gp, gynoecial primordium; ap, androecial primordium; se, sepal; st, stamen; ca, carpel. Arrowheads indicate nectar leaves. Scale bars = 100 μm.

the floral meristems and all the floral organs (Fig. 5D–F). Initially, *SIchAG* is highly expressed throughout all the floral organ primordia (Fig. 5G). During the later stages of development, *SIchAG* is expressed in the nectar leaves, stamens and carpels (Fig. 5H, I). *SIchSEP3* is ubiquitously expressed in the whole flower, at the early and late stages (Fig. 5J, K).

Furthermore, we investigated the expression patterns of B- and C-class MADS-box genes of *S. chinensis* in mature flowers by tissue-specific RT-PCR (Fig. 6). The mature male and female flowers can be distinguished from each other, and B- and C-class MADS-box genes are differentially expressed in the male and female flowers (Fig. 6B). *SIchAP3-1* is highly expressed in the nectar leaves and stamens/staminodes in both male and female flowers, whereas there is very low expression of *SIchAP3-1* in the carpels of the female flowers. Its expression was also found in the sepals, and the expression level is higher in the female flowers than in the male flowers. In addition, a high expression signal of *SIchAP3-1* was also observed in the leaves. *SIchAP3-2* shows higher expression in the nectar leaves and stamens in the male flowers, and lower expression in the sepals, nectar leaves and staminodes in the female flowers. No expression signal of *SIchAP3-2* was found in the carpels or leaves. In comparison, the expression of *SIchAP3-3* is mainly restricted in the nectar leaves in both male and female flowers, and the expression level is higher in the male flowers. Very low expression of *SIchAP3-3* was also detected in the stamens of the male flowers, but not at a significant level and not in the staminodes of the female flowers. *SIchPI* is expressed at high level in the sepals, nectar leaves and stamens/staminodes in both male and female flowers and at low levels in the carpels of the female flowers. In the female flowers, the transcripts of *SIchAG* were found in the staminodes and carpels. In comparison, *SIchAG* is expressed at relatively low levels in the nectar leaves and stamens in the male flowers (Fig. 6B).

### *Epidermal cell morphology of different floral organs in* S. chinensis

There is no significant difference in morphology of the epidermal cells of different floral organs between female flowers and male flowers (Fig. 7). The adaxial epidermal sepal cells are asymmetrically conical-papillate (Fig. 7A, I); the abaxial

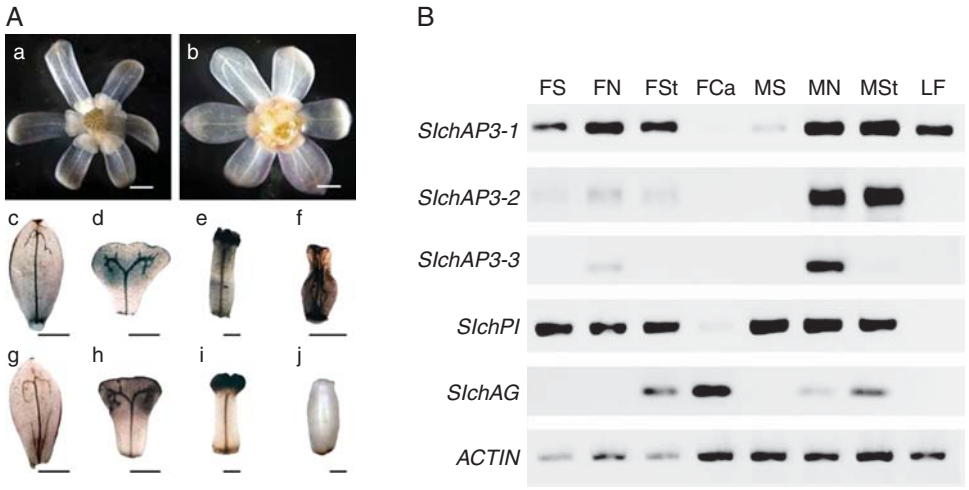


FIG. 6. Flowers and floral organs with corresponding expression analyses in *Sinofranchetia chinensis*: (A) FAA-fixed flowers and floral organs of *S. chinensis*: (a) male flower; (b) female flower; (c–f) floral organs in male flower; (g–j) floral organs in female flower; (c) and (g) sepal; (d) and (h) nectar leaf; (e) stamen; (f) rudimentary carpel; (i) staminode; (j) carpel. Scale bars = 2 mm. (B) Tissue-specific RT-PCR results for *SIchAP3-1*, *SIchAP3-2*, *SIchAP3-3*, *SIchPI*, *SIchAG* and *ACTIN* (control gene). Abbreviations: FS, female flower sepal; FN, female flower nectar leaf; FSt, female flower staminode; FCa, female flower carpel; MS, male flower sepal; MN, male flower nectar leaf; MSt, male flower stamen; LF, leaf; male flower rudimentary carpel is not included in RT-PCR.

ones are flat, irregular and with slightly sunken stomata (Fig. 7E, M). The conical epidermal cells were found on the adaxial surface of the nectar leaf (Fig. 7B, J). The abaxial surface of the nectar leaf consists of flat and irregular cells (Fig. 7F, N). The epidermal cells of the anther are irregular in shape (Fig. 7C, K), and those of the filament are flat and rectangular (Fig. 7G, O). The epidermis of the carpel/rudimentary carpel is covered with irregularly shaped cells (Fig. 7D, H, L, P).

## DISCUSSION

In this study, the A-, B-, C- and E-class MADS-box genes were found to be expressed in the nectar leaves of *S. chinensis* at different levels during different developmental stages (Figs 4–6). It implies that these floral MADS-box genes might contribute to the developmental regulation of the nectar leaves in *S. chinensis*. In addition, the A- and E-class genes display relatively broad expression patterns in most of the floral organs in *S. chinensis*, while the B- and C-class genes have major expression regions, and some even show relatively specific expression in the nectar leaves at mature stages. Therefore, the B- and C-class genes might be preferential candidates to explore the nature of the nectar leaves in *S. chinensis* at the molecular level.

Three *AP3*-like genes were identified in *S. chinensis*, and, importantly, *SIchAP3-3* is the first representative gene of the *AP3-III* lineage from Lardizabalaceae, based on the updated phylogenetic tree of *AP3*-like genes in Ranunculales (Fig. 3). Meanwhile, our phylogenetic analysis suggested that the two major gene duplication events involved in the evolution of *AP3*-like genes in Ranunculales occurred at least before the divergence of the Ranunculaceae, Berberidaceae, Menispermaceae and Lardizabalaceae (Fig. 3; Rasmussen *et al.*, 2009; Sharma *et al.*, 2011). Furthermore, the three *AP3*-like genes of *S. chinensis* show distinct and complex expression patterns (Figs 4A–I and 6B). They were all detected in the floral meristems as the sepals initiated, and their expression domains diverged gradually during floral development, especially at mature stages (Figs 4A–I and 6B). These results suggest that the three copies of *AP3*-like genes in *S. chinensis* might have undergone subfunctionalization after gene duplication; similar cases were also reported in other Ranunculales species, such as *Aquilegia vulgaris* (Ranunculaceae) and *Papaver somniferum* (Papaveraceae) (Force *et al.*, 1999; Lynch and Force, 2000; Kramer *et al.*, 2003, 2007; Moore *et al.*, 2005; Drea *et al.*, 2007).

In addition, we compared the expression pattern of *AP3*-like genes in *S. chinensis* with that in *A. vulgaris*, a well-studied Ranunculaceae species. Both of these two species have three copies of *AP3*-like genes and nectar leaves, except that the nectar leaves in *A. vulgaris* have become spurs and are larger (Kramer *et al.*, 2007). At early stages, the expression of *SIchAP3-2* and *SIchAP3-3* is broader than that of corresponding *Aquilegia* genes, *AqvAP3-2* and *AqvAP3-3* (Fig. 4D, G; Kramer *et al.*, 2007), and *SIchAP3-1* shares the same expression pattern with *AqvAP3-1* (Fig. 4A; Kramer *et al.*, 2007). At mature stages, the expression domains of *SIchAP3-1*, *SIchAP3-2* and *SIchAP3-3* generally resemble those of *AqvAP3-1*, *AqvAP3-2* and *AqvAP3-3*, respectively, except for the expression of *SIchAP3-1* in leaves (Fig. 6B; Kramer *et al.*, 2007). *SIchAP3-3* and *AqvAP3-3* are all mainly expressed in nectar leaves and weakly expressed in stamens but not in staminodes (Fig. 6B; Kramer *et al.*, 2007). And another B-class gene, *SIchPI*, which is a *PI*-like gene, is strongly expressed in the nectar leaves and stamens/staminodes at late stages, like *AqvPI* (Figs 4K and 6B; Kramer *et al.*, 2007). In general, the expression pattern of B-class genes in *S. chinensis* and *A. vulgaris* is very similar, especially at mature stages. More interestingly, the expression pattern of B-class genes mentioned above is also found in other Ranunculales species with nectar leaves, such as *Trollius laxus* and *Xanthorhiza simplicissima* (Ranunculaceae), *Berberis gilgiana* and *Epimedium grandiflora*

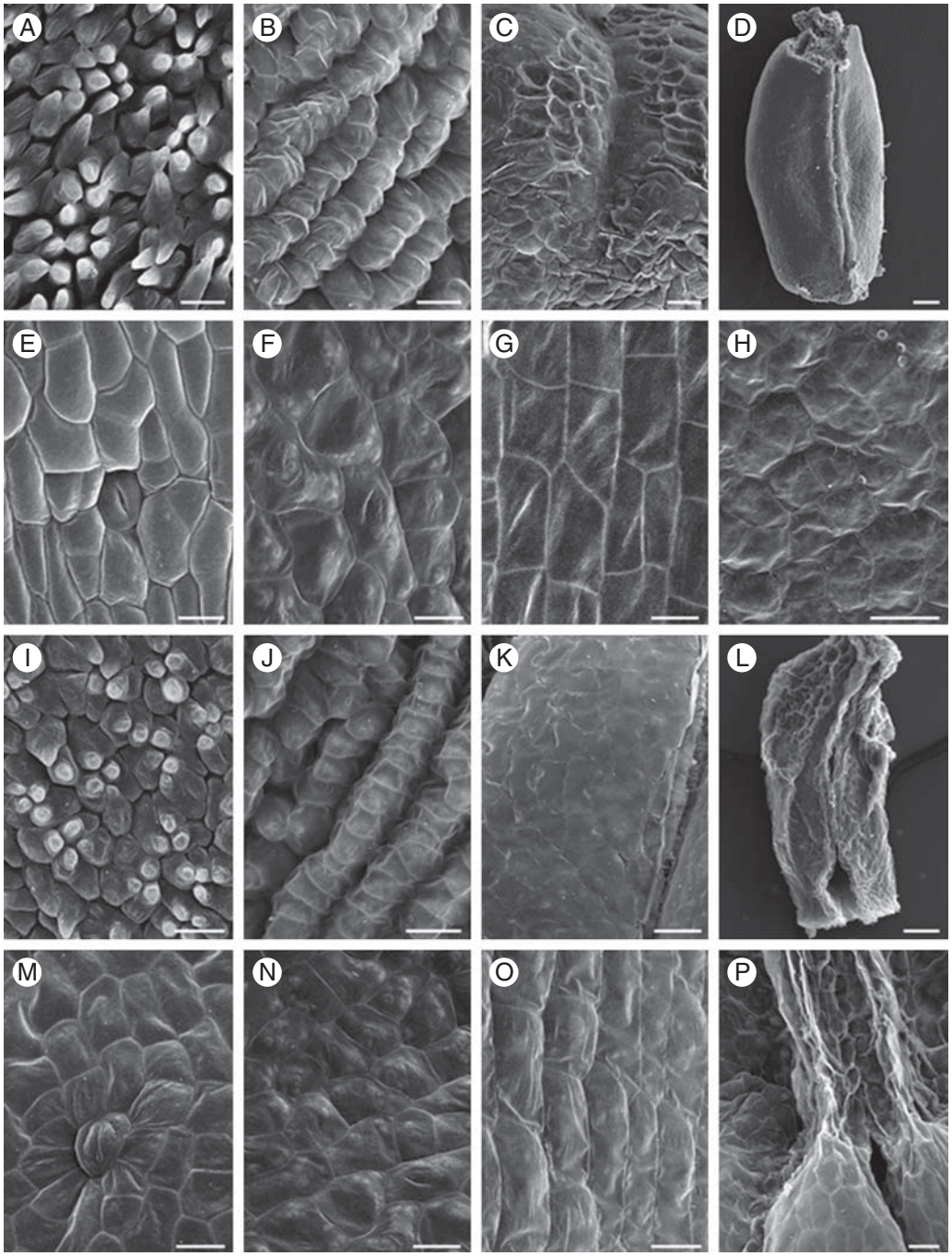


FIG. 7. Epidermal cell morphology of floral organs of female and male flowers in *Sinofranchetia chinensis* under SEM: (A–H) female floral organs; (I–P) male floral organs. (A) adaxial and (E) abaxial surface of the sepal; (B) adaxial and (F) abaxial surface of the nectar leaf; (C) anther and (G) filament of the staminode; (D) carpel of female flower; (H) surface of the carpel; (I) adaxial and (M) abaxial surface of the sepal; (J) adaxial and (N) abaxial surface of the nectar leaf; (K) anther and (O) filament of the stamen; (L) rudimentary carpel of male flower; (P) surface of the carpel. Scale bars: (A–C, E–K, M–O) = 20 μm; (P) = 50 μm; (L) = 100 μm; (D) = 250 μm.

(Berberidaceae) (Rasmussen *et al.*, 2009). These gene-expression data imply that the development of the nectar leaves in *S. chinensis* might be under a similar gene regulatory programme as other nectar leaves in Ranunculales species, although the shapes of these nectar leaves are variable in different species. Taking account of the expression pattern for B-class genes in other eudicots, the expression in nectar leaves and stamens in some Ranunculales species seems more or less corresponding to the expression in petals and stamens in core eudicots (e.g. Goto and Meyerowitz, 1994; Kramer *et al.*, 1998; Kramer and Irish, 1999, 2000; Lamb and Irish, 2003). It might support the reference to 'nectar leaves' as 'petals' (e.g. Cronquist, 1981; Kramer *et al.*, 2007; Kramer and Hodges, 2010).

What is more, the expression for the C-class gene *SIchAG* of *S. chinensis* was observed not only in the stamens/staminodes and carpels, but also in the nectar leaves (Figs 5G–I and 6B). Compared with the relatively conserved and concentrated expression in stamens and carpels of *AG*-like genes in most angiosperms, it seems that the *SIchAG* gene shifts the expression domain outwards, which just fits the 'shifting boundary' model (Kramer *et al.*, 2003, 2004). Furthermore, it has been suggested that the shifts in the expression domain of genes could lead to the genetic shifts in floral architecture and result in changes in floral structure, or even homeotic transformations (Bowman, 1997; Albert *et al.*, 1998; Kramer *et al.*, 2003). For example, the ectopic expression of C-class genes in the second whorl of the flower has resulted in staminoid petals or even true stamens instead of petals in *Antirrhinum* (Bradley *et al.*, 1993). Therefore, the morphological similarity between nectar leaves and stamens/staminodes in *S. chinensis* (Figs 1 and 6A) might have some relationships with the expression pattern of *SIchAG*, which needs to be investigated by gene-function analysis in the future. More importantly, the nectar leaves share more common expressed floral MADS-box genes with the stamens/staminodes than other floral organs in *S. chinensis*, which might reflect the close genetic relationship between nectar leaves and stamens (Figs 4–6). To some degree, it suggests that the nectar leaves in *S. chinensis* might be derived from stamens, which is consistent with the hypothesis reported for species of Ranunculaceae and Berberidaceae (Endress, 1995; Ronse De Craene, 2010).

Based on the morphological data, there are sepals, nectar leaves, stamens/staminodes and carpels from outer whorl to inner whorl in *S. chinensis*. Since a colourful perianth could enhance the attractiveness to potential pollinators, the showy petaloid sepals of *S. chinensis* might take more responsibility for attracting potential pollinators, compared with the small greenish nectar leaves (Glover and Martin, 1998). Moreover, the nectar leaves could secrete nectar, which could be a 'reward' to the pollinators, and there may be a trade-off between size/attractiveness and nectar production for the nectar leaves (Nepi *et al.*, 2009). In addition, the nectar leaves and sepals are all covered with cone-shaped cells on the adaxial surface, which can be easily distinguished from the morphology of the stamen/staminode epidermis (Fig. 7). The conical epidermal cell has been suggested to be a hallmark for petaloidy, possibly aiding pollinator orientation on the flowers in previous studies (Glover and Martin, 1998; Geuten *et al.*, 2006; Kramer *et al.*, 2007; Kramer and Hodges, 2010; Whitney *et al.*, 2011). It seems that the nectar leaves more or less play the role, just like petals. Our analysis also showed that the major cells on the abaxial surface of the sepals, nectar leaves and stamens in *S. chinensis* are generally similar, except that the sepals have stomata in their epidermis (Fig. 7).

In this study, *SIchAP3-3*, the first representative gene of the *AP3-III* lineage from Lardizabalaceae, was observed to be specifically expressed in the nectar leaves at mature stages, which is very similar to the expression pattern of *AP3-III* members in other Ranunculales species with nectar leaves (Fig. 6B; Kramer *et al.*, 2007; Rasmussen *et al.*, 2009). It suggests that the development of nectar leaves in *S. chinensis* might share a similar genetic regulatory code with other nectar leaves in Ranunculales species. Our study suggests that the nectar leaves in *S. chinensis* could be referred to as petals, and they might preserve the genetic footprint of the stamen ancestor. However, all of these need to be explored in further and more comprehensive studies.

## SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used for gene cloning in this study. Table S2: primers used for *in situ* hybridization and RT-PCR in this study. Table S3: representative floral MADS-box genes used in the phylogenetic analysis. Table S4: genes used in the phylogenetic analysis of *AP3*-like genes in Ranunculales. Table S5: genes used in the phylogenetic analysis of *PI*-like genes in Ranunculales. Figure S1: multiple sequence alignment for AP3-like proteins of representative species from Ranunculales. Figure S2: maximum-likelihood tree of *PI*-like genes in Ranunculales.

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