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

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**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,
The floral nectariferous organs are also called nectar leaves, which are nectar-bearing and formed between the perianth and androecium (Janchen, 1949). In Ranunculaceae, 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 Ranunculaceae 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 Ranunculaceae 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; Solitis 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 de Pamphilis, 2000; Pelaz et al., 2000; Theissen, 2001b). Most of the A-, B-, C- and E-class genes are MIKC-type MADS-box genes and they belong to the AP1/FUL (A-class), AP3/IP (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., 2005a, 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 (Solitis et al., 2007). Accordingly, some modified ABCE models have been put forward, such as the ‘sliding boundary’ model and ‘fading borders’ model (Solitis 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, 2001a; Kim et al., 2005; Chandlerbali 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; Solitis et al., 2006; Solitis 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., 2005b; 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 euAP3 and TM6 lineages (Kramer et al., 1998, 2006; Kramer and Irish, 2000; Aoki et al., 2004; Kim et al., 2004; Stellarri 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 Ranunculaceae, 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 Ranunculaceae 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′-CCGGATCCTCTACGGCGGCCGC-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
identified by restriction enzyme analysis of the
clones were selected for each identified locus using forward primer T7
(5′-TAATACGACTCACTATAGGG-3′) and reverse primer
SP6 (5′-TTTAGTTGACACATAAG-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. insigne, 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 ana-
lysis 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., 2005a; 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

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 sepal, petals, staminodes and carpels of mature female
flowers, and sepal, 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 (de-
pending 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 homolo-
gues among different floral organs (Endress, 1994; Krizak
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 CO2, 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 (*SlchFL1* and *SlchFL2*), three AP3-like genes (*SlchAP3-1, SlchAP3-2* and *SlchAP3-3*), one PI-like gene (*SlchPI*), one AG-like gene (*SlchAG*) and one SEPI-like gene (*SlchSEPI*); 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 Lizardzabaleaceae (Fig. 2): *SlchFL1* and *SlchFL2*; *SlchAP3-1, SlchAP3-2* and *SlchAP3-3, HOgrAG1 and HOgrAG2; and DEinAP3-1 and DEinAP3-2.

Sequence and phylogenetic analysis of floral 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). *SlchAP3-1* belongs to the AP3-I lineage, *SlchAP3-2* to the AP3-II lineage, and *SlchAP3-3* to the AP3-III lineage. Furthermore, *SlchAP3-1* and *SlchAP3-2* protein sequences show 67% identity; *SlchAP3-2* and *SlchAP3-3* share 62% identity; and *SlchAP3-1* and *SlchAP3-3* 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 *SlchAP3* proteins from multiple sequence alignment with other AP3-like proteins of Ranunculales; however, the *SlchAP3-2* protein lacks 29 amino acids in the K domain (Supplementary Data Fig. S1). At least four families (Ranunculaceae,
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, SlchAP3-1 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 SlchAP3-1 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, SlchAP3-1 expression is mainly restricted to the nectar leaves and stamens (Fig. 4C). The expression pattern of SlchAP3-2 is mostly like that of SlchAP3-1, 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). SlchAP3-3 expression is ubiquitously in the whole flower (Fig. 4G) and gradually narrows to the inner three whorls (Fig. 4H). At the late stages, SlchAP3-3 is expressed strongly in the nectar leaves, but very weakly in the stamens and carpels (Fig. 4I). SlchPI 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. SlchFL1 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 SlchFL2 is lower than that of SlchFL1, 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) SlchAP3-1; (D–F) SlchAP3-2; (G–I) SlchAP3-3; (J, K) SlchPI; (L) negative control with sense probe for SlchPI. (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.
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 sepal, and the expression level is higher in the female flowers than in the male flowers. In addition, a high expression signal of SIchAP3-2 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 sepal, 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 sepal, nectar leaves and stamens/staminodes in both male and female flowers and at low levels in the carpels of the female flowers. In the male 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

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**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.
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 (Fig. 7F, N), 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, *SlcAP3-3* 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 *SlcAP3-2* and *SlcAP3-3* is broader than that of corresponding *Aquilegia* genes, *AqvAP3-2* and *AqvAP3-3* (Fig. 4D, G; Kramer et al., 2007), and *SlcAP3-1* shares the same expression pattern with *AqvAP3-1* (Fig. 4A; Kramer et al., 2007). At mature stages, the expression domains of *SlcAP3-1*, *SlcAP3-2* and *SlcAP3-3* generally resemble those of *AqvAP3-1*, *AqvAP3-2* and *AqvAP3-3*, respectively, except for the expression of *SlcAP3-1* in leaves (Fig. 6B; Kramer et al., 2007). *SlcAP3-3* 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, *SlcPI*, 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*.
(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).

**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.
What is more, the expression for the C-class gene SlchAG 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 SlchAG 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 SlchAG, 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, SlchAP3-3, 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 alignment 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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