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Analysis of the *APETALA3*- and *PISTILLATA*-like genes in *Hedyosmum orientale* (Chloranthaceae) provides insight into the evolution of the floral homeotic B-function in angiosperms

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• *Background and Aims* According to the floral ABC model, B-function genes appear to play a key role in the origin and diversification of the perianth during the evolution of angiosperms. The basal angiosperm *Hedyosmum orientale* (Chloranthaceae) has unisexual inflorescences associated with a seemingly primitive reproductive morphology and a reduced perianth structure in female flowers. The aim of this study was to investigate the nature of the perianth and the evolutionary state of the B-function programme in this species.
• *Methods* A series of experiments were conducted to characterize B-gene homologues isolated from *H. orientale*, including scanning electron microscopy to observe the development of floral organs, phylogenetic analysis to reconstruct gene evolutionary history, reverse transcription–PCR, quantitative real-time PCR and *in situ* hybridization to identify gene expression patterns, the yeast two-hybrid assay to explore protein dimerization affinities, and transgenic analyses in *Arabidopsis thaliana* to determine activities of the encoded proteins.
• *Key Results* The expression of *HoAP3* genes was restricted to stamens, whereas *HoPI* genes were broadly expressed in all floral organs. *HoAP3* was able to partially restore the stamen but not petal identity in *Arabidopsis ap3-3* mutants. In contrast, *HoPI* could rescue aspects of both stamen and petal development in *Arabidopsis pi-1* mutants. When the complete C-terminal sequence of *HoPI* was deleted, however, no or weak transgenic phenotypes were observed and homodimerization capability was completely abolished.
• *Conclusions* The results suggest that *Hedyosmum AP3*-like genes have an ancestral function in specifying male reproductive organs, and that the activity of the encoded PI-like proteins is highly conserved between *Hedyosmum* and *Arabidopsis*. Moreover, there is evidence that the C-terminal region is important for the function of HoPI. Our findings indicate that the development of the proposed perianth in *Hedyosmum* does not rely on the B homeotic function.

Key words: Floral homeotic B function, *Hedyosmum orientale*, Chloranthaceae, C-terminal region, *HoAP3*, *HoPI*, homodimerization, *APETALA3*, *PISTILLATA*.

INTRODUCTION

Flowers of core eudicots typically show a standardized architecture with a fixed number of floral organs in often four distinct whorls and differentiated sepals and petals (Endress, 1994). However, in basal angiosperms floral organization is much more diverse, with some taxa exhibiting an undifferentiated perianth of spirally arranged tepals (e.g. *Amborella*), others having a well-differentiated perianth of distinct sepals and petals (e.g. *Asimina* and *Saruma*), and still others apparently lacking a perianth altogether (e.g. *Eupomatia* and *Chloranthus*) (Doyle *et al.*, 2003; Kim *et al.*, 2005). The ABC model, which was proposed to explain the determination of floral organ identities, is considered essential for understanding the developmental mechanisms that underlie the ontogenetic development of reproductive structures in angiosperms (Coen and Meyerowitz, 1991; Colombo *et al.*, 1995; Theißen and Saedler, 2001; Alvarez-Buylla *et al.*, 2010). According to this model, the B-function genes determine petal and stamen identities in floral development. It thus appears likely that they also played a key role in the origin and diversification of the perianth during the evolution of the angiosperms (Kramer and Irish, 1999; Theissen *et al.*, 2000; D. Soltis *et al.*, 2006).

There are two main lineages of class-B genes in angiosperms, the *APETALA3/DEFICIENS* lineage (for simplicity, henceforth termed the *AP3/DEF* lineage) and the *PISTILLATA/GLOBOSA* lineage (henceforth the *PI/GLO* lineage) (Sommer *et al.*, 1990; Jack *et al.*, 1992; Tröbner *et al.*, 1992; Goto and Meyerowitz, 1994). Phylogenetic analysis has shown that class-B genes underwent two major duplication events from an ancestral B gene possessing both a paleoAP3 motif and a PI motif in the C-terminus of the protein (Kramer *et al.*, 1998). When the lineage that led to angiosperms branched off from the gymnosperm lineage, the ancestral B gene underwent a duplication event, giving rise to the paleo*AP3* and *PI* lineages (Kramer *et al.*, 1998; Theissen *et al.*, 2000; Hernández-Hernández *et al.*, 2007). The paleo*AP3* lineage genes have preserved both the paleoAP3 motif and the PI-derived motif, whereas the *PI* lineage members have lost the paleoAP3 motif. Subsequently, the paleo*AP3* lineage underwent another duplication event at the base of the core eudicots, resulting in two sublineages, the *TM6* sublineage and the eu*AP3* sublineage (Kramer *et al.*,

1998; Kramer and Irish, 1999, 2000). While *TM6* has preserved the PI-derived motif as well as the paleoAP3 motif, the eu*AP3* lineage has replaced the paleoAP3 motif with a new euAP3 motif, presumably by a frameshift mutation in the paleoAP3 motif (Vandenbussche *et al.*, 2003; Kramer *et al.*, 2006).

In the model species *Arabidopsis thaliana* and *Antirrhinum majus*, the *AP3/DEF* and *PI/GLO* gene products function as obligate AP3–PI (in *Arabidopsis*) or DEF–GLO (in *Antirrhinum*) heterodimers to establish the identity of petals and stamens in the organ primordia developing in the second and third whorls, and the strong continuous expression of these genes in petals and stamens is positively autoregulated by the functional AP3–PI or DEF–GLO heterodimers (Carpenter and Coen, 1990; Sommer *et al.*, 1990; Jack *et al.*, 1992, 1994; Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992; Goto and Meyerowitz, 1994; Riechmann *et al.*, 1996). The genetic programme of the B function appears to be highly conserved in core eudicot taxa such as *Petunia hybrida* (Angenent *et al.*, 1993; Tsuchimoto *et al.*, 2000; Vandenbussche *et al.*, 2004) and *Nicotiana tabacum* (Hansen *et al.*, 1993; Kempin *et al.*, 1993), as well as in the derived monocots rice and maize (Kang *et al.*, 1998; Moon *et al.*, 1999; Ambrose *et al.*, 2000; Ma and dePamphilis, 2000; Nagasawa *et al.*, 2003; Whipple *et al.*, 2004). However, in basal angiosperms, even though homologues of the floral organ identity genes do exist, their expression patterns are often broader or more variable than those observed in core eudicots (Kramer and Irish, 2000; Kim *et al.*, 2005; D. Soltis *et al.*, 2006, 2007; Chanderbali *et al.*, 2009; P. Soltis *et al.*, 2009). For example, in the basalmost taxa *Amborella* and *Nuphar*, *AP3* and *PI* homologues are not only expressed in tepals and stamens, but also in carpels (Kim *et al.*, 2005). The expression of B-function homologues observed in the petaloid perianth and stamens in basal angiosperms suggests that these organs share the developmental genetic programme of eudicot petals and stamens (D. Soltis *et al.*, 2007). However, the broader expression patterns of class-B genes observed throughout the undifferentiated perianth and carpels may represent the ancestral condition for angiosperms and explain the lack of a clear morphological distinction between outer and inner tepals in these taxa (Kim *et al.*, 2005; D. Soltis *et al.*, 2007).

Besides the broader expression patterns, equally complex interaction patterns of AP3- and PI-like proteins have also been uncovered in plant species outside the core eudicots. For instance, the AP3-like proteins CsAP3 from the basal angiosperm *Chloranthus spicatus* (Su *et al.*, 2008), LMADS1 from the lily *Lilium longiflorum* (Tzeng and Yang, 2001), OMADS3, OMADS5 and OMADS9 from the orchid *Oncidium* 'Gower Ramsey' (Hsu and Yang, 2002; Chang *et al.*, 2009) and the PI-like proteins CsPI from *C. spicatus* (Su *et al.*, 2008) and LRGLOA and LRGLOB from the lily *Lilium regale* (Winter *et al.*, 2002*b*) are able not only to form heterodimers, but also to form homodimers, even though it is not clear whether these homodimers function *in vivo* (Winter *et al.*, 2002*b*). Together, these findings of expression and interaction analyses suggest that the B-function programme had already been constituted in the most recent common ancestor of extant angiosperms, but some aspects of the ancestral B-function programme displayed a high degree of plasticity and may not have become fixed until later in angiosperm evolution (Kramer and Irish, 2000). Thus, the question of which evolutionary forces led to the fixation of the B-function programme arises. To answer this question requires more insight into the phylogeny and function of class-B genes in the basal angiosperm groups in general and in taxa with special floral morphology in particular.

Chloranthaceae may provide hints in understanding the origin and early diversification of angiosperms because of this family's near-basal position in the angiosperm tree, seemingly primitive floral morphology, and extensive and deep fossil record extending back to the Early Cretaceous (Eklund *et al.*, 2004; Antonelli and Sanmartin, 2011). This family consists of the four genera *Chloranthus*, *Sarcandra*, *Ascarina* and *Hedyosmum*. Molecular analyses have already indicated that the genus *Hedyosmum* is sister to the other three genera and that *Ascarina* is sister to *Sarcandra* and *Chloranthus* (Qiu *et al.*, 1999; Zanis *et al.*, 2002; Zhang and Renner, 2003; Antonelli and Sanmartin, 2011). Flowers of living species of the derived *Chloranthus*, *Sarcandra* and *Ascarina* do not develop a perianth, whereas the ancestral *Hedyosmum*, the most diverse and species-rich genus in the family Chloranthaceae, possesses a soft tissue interpreted as a perianth in female flowers (Edwards, 1920; Leroy, 1983; Endress, 1987; Doyle *et al.*, 2003). All *Hedyosmum* species are confined to the neotropics except for one Asian species, *Hedyosmum orientale* (Todzia, 1988). *H. orientale* of the southeastern coast of tropical China is dioecious. Male inflorescences are compound spikes, often two opposite lateral and a terminal, in the axil of foliage leaves or bracts (see Fig. 3D), while female inflorescences are globose green spadices, terminating leafy shoots or found in the axil of foliage leaves (Fig. 3T). It remains to be determined whether the male catkins of *Hedyosmum* are real polystaminate flowers or rather inflorescences with 150–200 unistaminate, ebracteate flowers spirally arranged along an axis (Edwards, 1920; Leroy, 1983; Endress, 1987; Doyle *et al.*, 2003). The slightly more complex female flower (Fig. 3X), which is unicarpellate and enveloped by a large hood-shaped bract, is enclosed in a soft structure that has generally been believed to represent a perianth, but is sometimes interpreted as androecial in origin or ovarian tissue (Burger, 1977; Leroy, 1983; Endress, 1987; Doyle *et al.*, 2003). This perianth terminates at the apex in three minute lobes. It is greenish and slightly yellow, and never conspicuous either in size or colour. The characteristics of *Hedyosmum* flowers noted above raise intriguing questions about aspects such as the true nature of the proposed perianth in these plants and whether the class-B gene homologues are involved in the development of this tissue.

In this study, two *AP3*-like and five *PI*-like mRNAs, of which two are the results of alternative splicing, were isolated from *H. orientale*. We performed detailed sequence and expression analyses of the corresponding genes and explored their functional characteristics in heterologous systems. Specifically, the phenotypes of overexpression of these cDNAs under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter and their capacity to complement *ap3* and *pi* mutants was analysed in *A. thaliana*. Finally, we determined the protein interaction patterns of the corresponding gene products by yeast two-hybrid analyses. The genetic analysis of *Hedyosmum* B-gene homologues in this study advances our understanding of the proposed perianth organ in *Hedyosmum* and provides novel information about the evolution of the floral homeotic B function programme in angiosperms.

MATERIALS AND METHODS

Plant materials

Hedyosmum orientale plants were collected from Diaoluo mountain in Hainan province, China (18°41′N–18°44′N, 109°50′E–109°55′E) (Cui *et al.*, 2011). Entire inflorescences and floral buds at different stages from early development to anthesis were harvested and stored in liquid nitrogen for RNA isolation or embedded in paraplast for *in situ* hybridization.

Isolation of cDNAs of putative class-B genes from H. orientale

Total RNA was prepared from male and female inflorescences of *H. orientale* using TRIzol reagent (Invitrogen, USA). After RNA extraction, we treated RNA samples with RNase-free recombinant DNase I (Takara, Dalian, China) to avoid potential contamination by genomic DNA. Complementary DNA (cDNA) was synthesized using Superscript III Reverse Transcriptase (Invitrogen). cDNA synthesis was followed by 3′ and 5′ rapid amplification of cDNA ends (3′RACE and 5′RACE) experiments. Primer sequences are presented in Supplementary Data Table S1. PCR products of about 1·0 kb were cloned into pGEM-T Easy vectors (Promega) and sequenced.

Alignment and phylogenetic analyses

The cDNA sequences of the two *AP3*-like and three *PI*-like genes isolated from *H. orientale* and sequences of their homologues in eudicots, monocots, other basal angiosperms and gymnosperms, retrieved from the NCBI database, were used for phylogenetic analyses. Accession numbers, species names and plant families for each gene are presented in Supplementary Data Table S2. Multiple alignment was performed as described previously (Shan *et al.*, 2006). We eliminated the poorly aligned positions and the divergent C-terminal regions. The MADS, I and K domains were used to construct the phylogenetic tree. For the DNA matrices, PHYML version 2.4.3 (Guindon and Gascuel, 2003) was used to construct a maximum-likelihood tree under default settings. The general time-reversible substitution model was used, with optimization of the proportion of invariable sites, nucleotide frequencies and the gamma shape parameter. One thousand bootstrap replicates were performed.

Reverse transcription–PCR (RT–PCR) and quantitative real-time PCR (qRT–PCR) analyses

Total RNA was isolated from different reproductive tissues of *H. orientale* and from inflorescences of transgenic *Arabidopsis* plants. Extraction of total RNAs and reverse transcription were performed according to the methods described above. The *ACTIN1* gene was used as an internal control. In RT–PCR analyses, the forward primer PI-1F and gene-specific reverse primers PI-1R, PI-1BinserR, PI-2R and PI-3R were used to detect the expression profiles of different *HoPI* transcripts in *H. orientale*.

To investigate the expression of transgenes in transgenic *Arabidopsis* plants, triplicate qRT–PCR assays were performed using SYBR Premix Ex Taq (Takara, Dalian, China) using a Rotor-Gene 3000 (Corbett Research, Qiagen, Hilden, Germany) detection system and software. Primers used are presented in Supplementary Data Table S1.

In situ *hybridization*

Male and female inflorescences and flowers of *H. orientale* collected from Hainan province were fixed and embedded in paraffin (Sigma). The specimens were cut into 10-μm-thick sections and hybridized with gene-specific probes according to a method described previously (Li *et al.*, 2005). The partial C-terminal and 3′ untranslated region-specific sequences of *HoAP3_1* (459–677), *HoPI_1* (532–779) and *HoPI_3* (543–891) were amplified as templates and then transcribed *in vitro* to produce sense and antisense probes using the Digoxigenin RNA Labelling Kit (Roche, Mannheim, Germany). Primer sequences are presented in Supplementary Data Table S1.

Plasmid construction

A series of cDNAs, including several *HoAP3* genes and several *HoPI* genes, were obtained by PCR amplification and then cloned into binary vector SN1301 (possessing CaMV *35S* promoter) to drive nearly ubiquitous expression of the transgenes on a wild-type *A. thaliana* background. In complementation experiments, the *Arabidopsis AP3* promoter (*pAtAP3*, from position −1767 to −1 relative to the translation start codon ATG of the *Arabidopsis AP3* gene) and *Arabidopsis PI* promoter (*pAtPI*, from position −1573 to −3 relative to the translation start codon ATG of the *Arabidopsis PI* gene) were amplified and replaced the 35S promoter of SN1301 to drive expression of the transgenes on *Arabidopsis ap3-3* and *pi-1* mutant backgrounds, respectively. The final constructs were verified by sequencing and restriction enzyme analyses. Primers are presented in Supplementary Data Table S1.

Arabidopsis thaliana *transformation and genotyping*

The plasmid constructs described above were transformed into wild-type *A. thaliana* (Landsberg erecta), and heterozygous *ap3-3* or *pi-1* mutant plants, respectively, by the floral dip method (Clough and Bent, 1998). The seeds of transgenic plants were selected on solid 0·6× MS medium containing 25 mg L^{-1} hygromycin B and 1 g L^{-1} carbenicillin disodium salt and were genotyped by PCR with transgene-specific primers. Homozygous *ap3-3* or *pi-1* transformants were identified using the dCAPS finder programme as described previously (Lamb and Irish, 2003). The homozygous mutant plants containing transgenic cDNAs were then analysed.

Scanning electron microscopy

Hedyosmum orientale material collected from Diaoluo mountain in Hainan province (China) and flowers from transgenic and wild-type *A. thaliana* and *pi-1* and *ap3-3* mutants were fixed, dried and coated as described (Shan *et al.*, 2006), and then photographed with a Hitachi S-800 scanning electron microscope (SEM).

Yeast two-hybrid analysis

Yeast two-hybrid assays were performed using the GAL4-based MATCHMAKER Two-Hybrid System (Clontech, Mountain View, CA, USA). *Saccharomyces cerevisiae* strain AH109, GAL4 activation domain expression vector pGADT7 and GAL4 DNA-binding domain expression vector pGBKT7 were used. The coding cDNA sequences of *HoAP3* and *HoPI* were amplified and fused into the pGADT7 and pGBKT7 vectors, respectively. All constructs were verified by restriction enzyme analysis and sequencing. Primers used are listed in Supplementary Data Table S1. For an autoactivation test, the single transformants were checked as described (Shan *et al.*, 2006). No colony grew on the corresponding selective medium, indicating that these transformants could be used for further two-hybrid assays. Confirmation of the transformants and interaction analyses were performed as previously described (Shan *et al.*, 2006).

RESULTS

Identification and phylogenetic analyses of putative class-B genes in H. orientale

Seven full-length cDNA sequences of putative class-B genes were isolated from young inflorescences of *H. orientale*. Multiple sequence alignments indicated that two of them represented *AP3*-like genes, designated *HoAP3_1* (GenBank accession no. JX069759) and *HoAP3_2* (JX069760), whereas the remaining five represented *PI*-like transcripts, designated *HoPI_1* (JX069754), *HoPI_1A* (JX069755), *HoPI_1B* (JX069756), *HoPI_2* (JX069757) and *HoPI_3* (JX069758). *HoPI_1A* and *HoPI_1B* were alternative splicing isoforms of *HoPI_1*. Both *AP3*-like genes encoded proteins with a length of 224 amino acids, including the diagnostic paleoAP3 and PI-derived motifs in the C-terminal region, which characterized them as members of the paleo*AP3* lineage (Fig. 1A). The putative HoPI_1 and HoPI_2 proteins contained the diagnostic PI motif in the C-terminal region, while the putative HoPI_1A and HoPI_3 proteins contained an atypical PI motif. The putative HoPI_1B protein lacked the complete C-terminal region (Fig. 1B).

Nucleotide sequence alignment showed that the only difference between the two alternative splicing isoforms *HoPI_1A* and *HoPI_1B* and the gene *HoPI_1* was in the C-terminal region (Supplementary Data Fig. S1). Compared with *HoPI_1*, *HoPI_1A* had a 92-nucleotide deletion at position +597 (relative to ATG; Supplementary Data Fig. S1), which resulted in a frameshift mutation and altered the last 15-amino-acid sequence of the C-terminus (Fig. 1B). However, *HoPI_1B* had a 105-nucleotide insertion at position +493 that introduced a premature stop codon at position +505 (Supplementary Data Fig. S1) and produced a 168-amino-acid premature protein of HoPI_1B that lacked the complete C-terminal region (Fig. 1B). Genomic sequence analysis showed that the 105-bp insertion sequence of *HoPI_1B* and the 92-bp deletion sequence of *HoPI_1A* were present in intron 6 and exon 7 of the *HoPI_1* DNA sequence, respectively (Fig. 1C), providing strong evidence that *HoPI_1A* and *HoPI_1B* are produced by exon skipping and intron retention splicing of the *HoPI_1* locus, respectively.

Phylogenetic analyses were performed using the predicted amino acid sequences of the MIK domains. The results confirmed the placement of the three *HoPI* genes in the *PI* clade and the two *HoAP3* genes in the *AP3* clade (Supplementary Data Fig. S2). They furthermore indicated that the three *PI*-like and the two *AP3*-like genes of *H. orientale* originated from *Hedyosmum*-specific duplication events.

Expression patterns of AP3 *and* PI *homologues in* H. orientale *flowers*

To determine the expression patterns of the *Hedyosmum AP3*- and *PI*-like genes, RT–PCR and qRT–PCR analyses were performed using RNA from dissected reproductive tissues. We found that the expression of *HoAP3_1* and *HoAP3_2* was restricted to male organs, and that the mRNA level of *HoAP3_1* was up to 25 times higher than that of *HoAP3_2* (Fig. 2A, C). In contrast to the stamen-specific expression of *HoAP3*, the *HoPI* transcripts turned out to be broadly expressed in all floral organs, including stamens, perianth organs and carpels, but at different levels, with high levels in stamens and carpels and low levels in perianth organs (Fig. 2B). Moreover, the expression levels of *HoPI_1* and *HoPI_3* were found to be higher than those of *HoPI_1A*, *HoPI_1B* and *HoPI_2* (Fig. 2B, C).

To further investigate the spatial and temporal expression patterns of these genes, *in situ* hybridizations were performed at a series of developmental stages of male and female reproductive tissues. RT–PCR analysis showed that paleo*AP3* paralogue *HoAP3_2* and *PI* paralogue *HoPI_2* are expressed at very low levels and were therefore excluded from the *in situ* hybridization analysis.

In male reproductive tissues, as soon as the male reproductive primordia emerged, signals of *HoAP3* and *HoPI* genes could be detected in the entire male reproductive meristems (black arrows in Fig. 3E, I, M). Subsequently, the expression signals became confined to the basal part of the adaxial surface area of the male reproductive structure where the stamen primordia develop (red arrows in Fig. 3E, I, M). As stamen primordia initiated and differentiated on its surface from the base to the top (Fig. 3B), the expression signals were maintained throughout the succeeding developmental stages of stamens, from nascent stamen primordia to nearly mature stamens (Fig. 3F, J, N). At very late stages (Fig. 3C), *HoPI_3* was detected throughout the stamen (Fig. 3O), but *HoAP3_1* and *HoPI_1* were restricted to the microsporangium region, which gives rise to fertile pollen (Fig. 3G, K).

In female reproductive tissues, no *HoAP3* transcripts could be detected at any developmental stages of female organs (data not shown), consistent with the results of RT–PCR analyses. The *in situ* hybridization results were identical for each set of *HoPI* paralogues in female reproductive tissues (data not shown), so results are shown only for *HoPI_3* and the genes are simply referred to as *HoPI*. When the female floral primordia emerged (Fig. 3Q), the expression signals of *HoPI* genes were first detectable in the female floral primordia, where the perianth and carpel primordia emerge a short time later (blue arrow in Fig. 3U). As the perianth and gynoecium primordia initiated and differentiated on the flanks and middle of the female flower primordium (Fig. 3R), *HoPI* remained strongly expressed in these organs (green and yellow arrows in Fig. 3V). Subsequently, perianth tissue developed gradually in the base, top and angle regions, resulting in

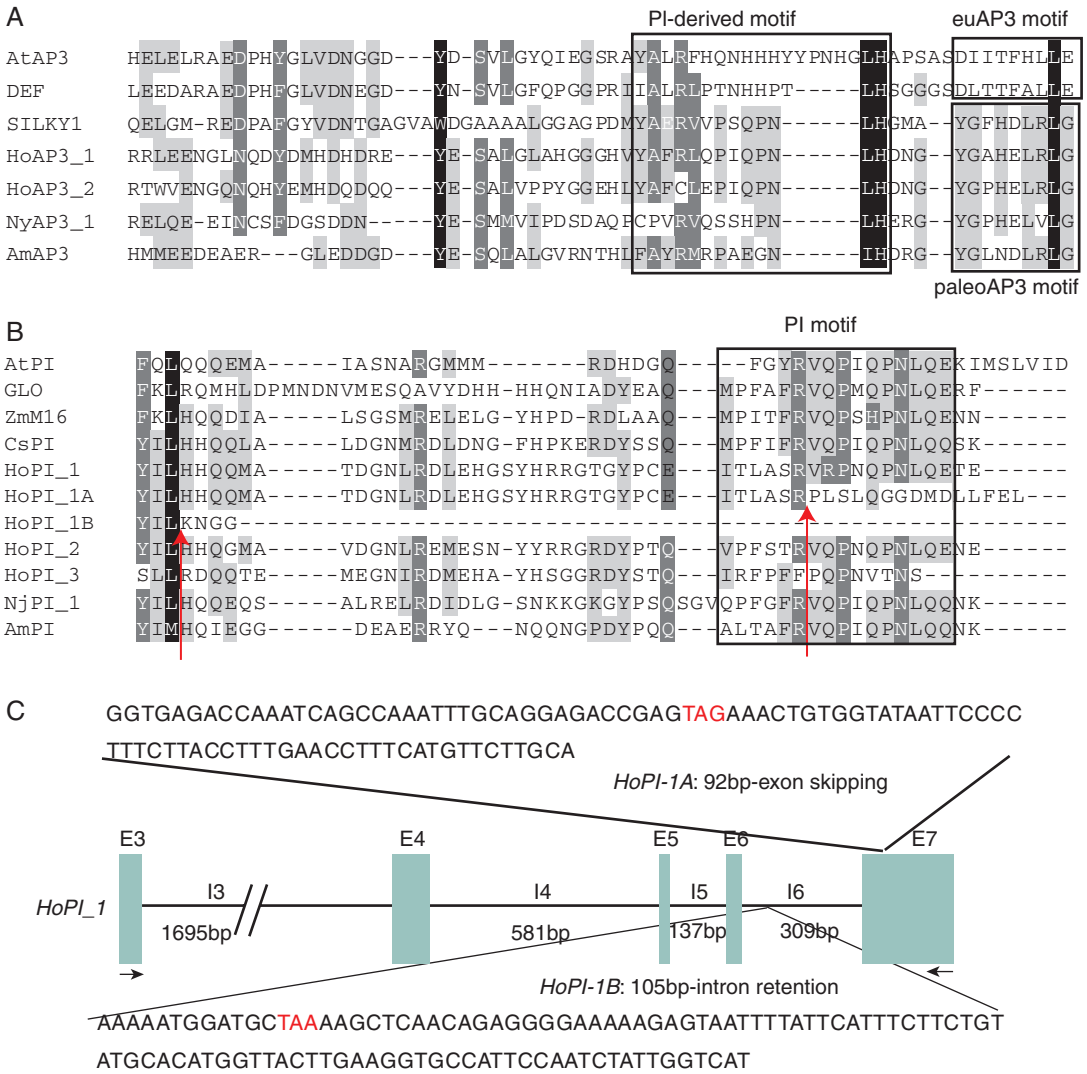


FIG. 1. Sequence analyses of AP3/PI homologues. (A, B) Alignment of the C-terminal region of the predicted AP3-like (A) and PI-like (B) protein sequences. Conserved PI and AP3 motifs are indicated by boxes. Arrows in (B) indicate the positions of intron retention in *HoPI_1B* and exon skipping in *HoPI_1A* that mediate the frameshift. (C) Exon–intron structure of *HoPI_1* and alternative splicing of *HoPI_1A* and *HoPI_1B*. Arrows indicate primers used in amplifying the DNA sequence of *HoPI_1*.

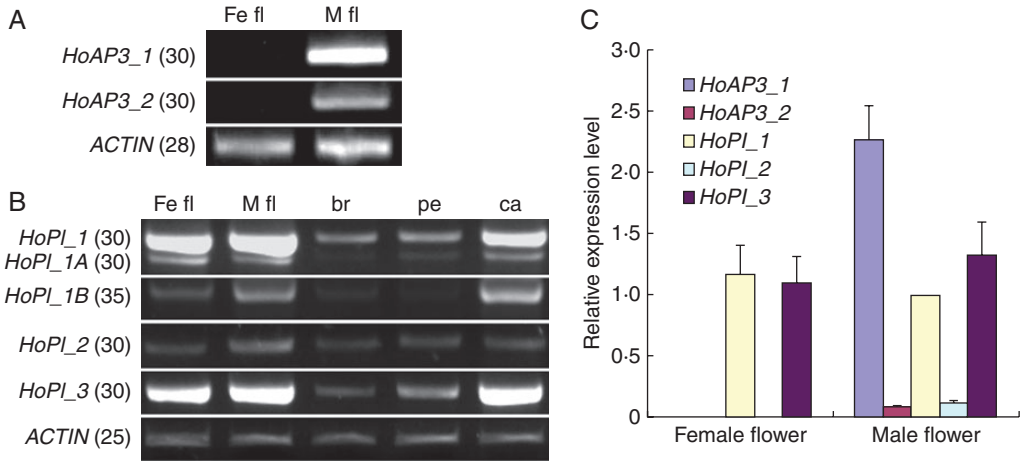


FIG. 2. Expression patterns of *HoAP3* and *HoPI* transcripts in *Hedyosmum orientale* reproductive tissues. (A, B) RT–PCR analysis of *HoAP3* (A) and *HoPI* (B) transcripts in male flowers (M fl), female flowers (Fe fl), dissected bracts (br), perianth organs (pe) and carpels (ca). Figures in parentheses are numbers of PCR cycles. (C) qRT–PCR analysis of *HoAP3* and *HoPI* genes in male and female flowers. The fold expression level is relative to *HoPI_1* expression in female flowers.

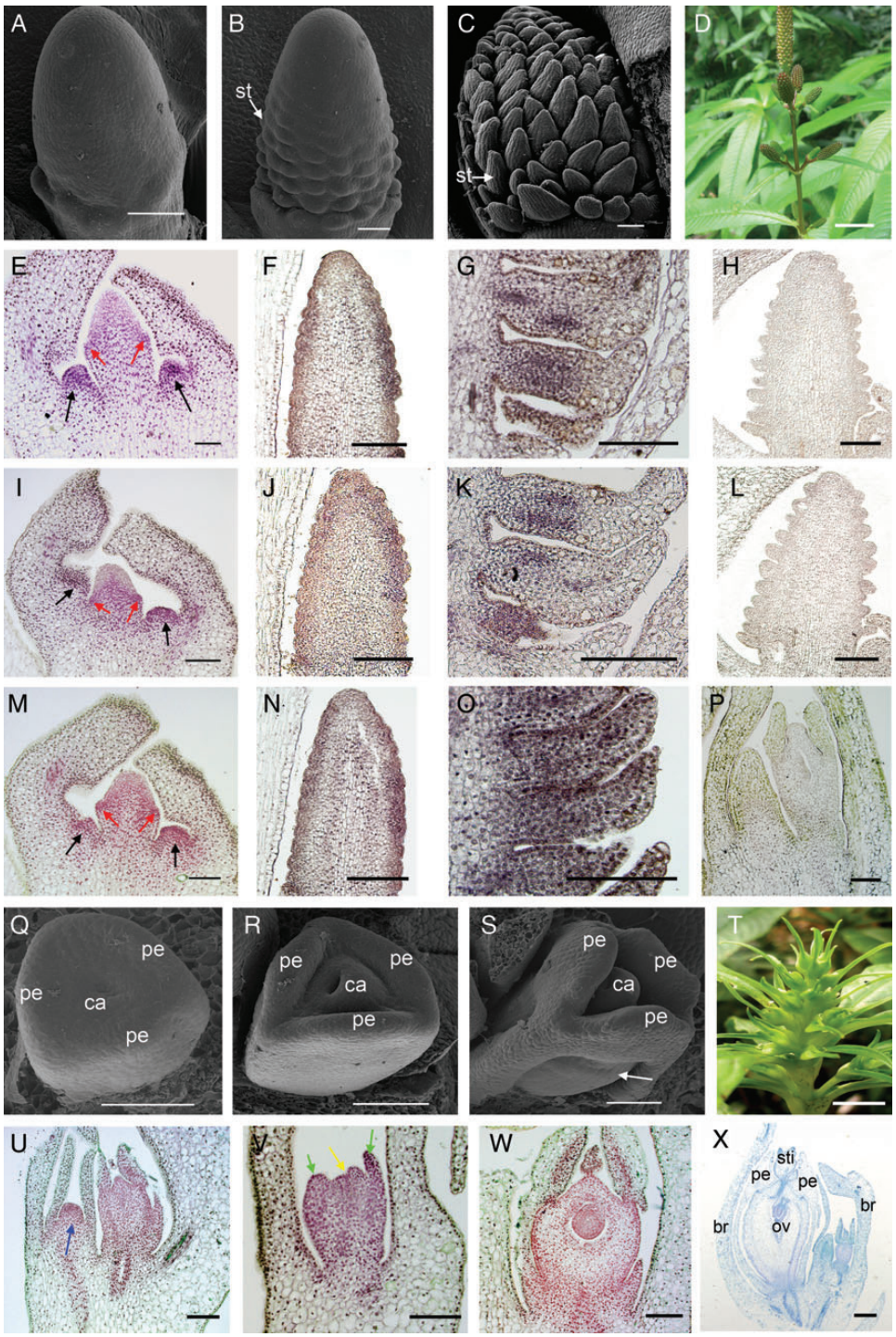


FIG. 3. *In situ* hybridization of *HoAP3_1*, *HoPI_1* and *HoPI_3* in *Hedyosmum orientale* floral organs. (A–C) SEM of the development of the male reproductive tissue. (A) Male flower before initiation of stamen primordia; (B) development of stamen primordia from the base part to the top of male flower; (C) late developmental stages of male flower with almost mature stamens. (D) Male inflorescence. (E–G) *In situ* hybridization of *HoAP3_1* in male floral meristems. (I–K) *HoPI_1*. (M–O) *HoPI_3*. (H, L, P) *In situ* hybridization with *HoAP3_1*, *HoPI_1* and *HoPI_3* sense probes, respectively. (Q–S) SEM of development of the female flower. (Q) Early developmental stages of a female flower before initiation of perianth and carpel promordia; (R) female flower with initiation of a single perianth whorl and inferior ovary tissue; (S) almost mature female flower with three large pores (white arrow) on the sides of the fruit. (T) Female inflorescence. (U–W) *In situ* hybridization of *HoPI_3* in female floral meristems. (X) Longitudinal section of female flower. Photographs arranged vertically [(A, E, I, M), (B, F, J, N), (C, G, K, O), (Q, U), (R, V) and (S, W)] each relate to a single developmental stage. ov, ovule; sti, stigma; br, bract (primordia); pe, perianth (primordia); ca, carpel (primordia); st, stamen (primordia). Scale bars: (A–C, E–S, U–X) = 100 μm; (D, T) = 3 cm. Black arrows, male floral primordia; red arrows, stamen primordia; blue arrows, female flower primordia; green arrows, perianth lobes; yellow arrows, developing carpels.

three large pores on the sides of the fruit (arrow in Fig. 3S). At maturity, the developing perianth tissue extended over the entire flat sides of the fruit, the full-grown perianth surrounded the carpel to form a perigynous whorl (Fig. 3X), and the expression signal of *HoPI* appeared to be restricted to the adaxial domain of the perianth and throughout the carpel (Fig. 3W).

Ectopic expression of AP3 *and* PI *homologues of* H. orientale *in* Arabidopsis

Hedyosmum orientale has not yet been developed as a molecular genetic model. This makes it unfeasible to conduct genetic experiments or induce mutations to study gene function directly in this species. Therefore, we generated ectopic expression and complementation lines for *HoAP3* and *HoPI* transcripts in transgenic *A. thaliana* plants with wild-type and *ap3-3* and *pi-1* mutant backgrounds, taking advantage of the many details known about *A. thaliana AP3* (*AtAP3*) and *PI* (*AtPI*) genes in this dicotyledonous species.

A number of independent transgenic lines were generated for each of the constructs. *Arabidopsis* plants that ectopically expressed *AtAP3* throughout the flower showed a homeotic conversion of carpels into stamens in the fourth floral whorl (Jack *et al.*, 1994). In our study, neither *35S::HoAP3_1* nor *35S::HoAP3_2* lines showed a homeotic conversion of carpels into stamens in the fourth whorl (Table 1, Fig. 4F, G), despite the high expression levels of *HoAP3_1* and *HoAP3_2* detected in these lines (Supplementary Data Fig. S4A, B). However, flowers of the *pAtAP3::HoAP3_1;ap3-3* and *pAtAP3::HoAP3_2;ap3-3* complementation lines showed weak rescue of stamen development to mosaic (combining the characters of both stamens and carpels) or filament-like organs in the third whorl (Table 2, Fig. 4M, N), whereas no complementation of petal development was observed in the second whorl (Fig. 4O). It has been demonstrated that *AP3*-like genes appear to be sensitive to gene expression level, so the failure of *AP3*-like genes to rescue *ap3-3* phenotypes is most likely due to suboptimal expression levels of genes (Piwarzyk *et al.*, 2007). However, when *HoAP3_1* and *HoAP3_2* were expressed under the control of the 35S promoter, the phenotypes of *35S::HoAP3_1;ap3-3* and *35S::HoAP3_2;ap3-3* transgenic plants resembled that of *pAtAP3::HoAP3_1;ap3-3* and *pAtAP3::HoAP3_2;ap3-3* complementation plants (data not shown), which makes it very unlikely that suboptimal expression is the only reason for the prevention of complementation.

Most of the transgenic lines bearing *35S::HoPI_1* (74%), *35S::HoPI_1A* (84%) or *35S::HoPI_3* (66%) displayed similar phenotypes to those of *35S::AtPI* transgenic *A. thaliana* (Krizek and Meyerowitz, 1996), i.e. a partial conversion of the first-whorl sepals into petaloid organs (Table 1, Fig. 4H). SEM analyses confirmed that the adaxial and abaxial surfaces of the petaloid organs showed the mosaic feature of wild-type sepals and petals (Fig. 4I, J). By contrast, no homeotic conversion was observed in flowers of the *35S::HoPI_1B* and *35S::HoPI_2* transgenic lines (Table 1).

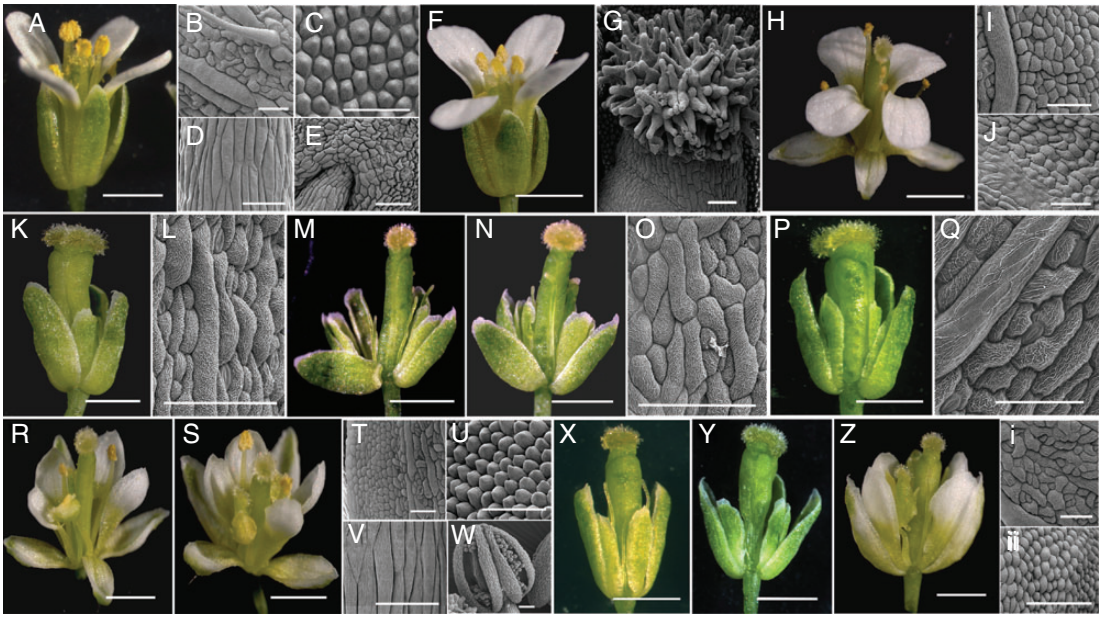
For all *HoPI* complementation constructs, a number of independent transformants homozygous for *pi-1* were identified, showing a range of phenotypes. To facilitate the description of the observed phenotypes, the degree of rescue is categorized as 'no rescue', 'weak rescue', 'medium rescue' and 'strong rescue', as shown in Supplementary Data Fig. S3. Flowers of plants in the no rescue category were indistinguishable from *pi-1* mutant flowers. Flowers of weak rescue lines had mosaic (combining the characters of both stamens and carpels) or filament-like organs in the third whorl, but no rescue of petal identity in the second whorl (Supplementary Data Fig. S3A–D). In flowers of medium rescue plants, the third-whorl organs were generally mosaic organs (possessing both stamen and carpel characters), and the second whorl organs were white and petal-like in appearance, but were smaller than the wild-type petals (Supplementary Data Fig. S3E–H). In plants in the strong rescue category, the second-whorl petals were indistinguishable from wild-type petals, whereas the third-whorl organs were usually stamenoid or very similar to wild-type stamens but not fully elongated (Supplementary Data Fig. S3I, J). In some lines with strong rescue, the restored stamens could extend fully and produced fertile pollen grains (Supplementary Data Fig. S3K, L), and all progenies had an identical phenotype (data not shown).

All *pAtPI::HoPI_2;pi-1* lines (38/38, 100%) showed no rescue, with phenotypes indistinguishable from *pi-1* mutants (Table 2, Fig. 4Y). Most of the *pAtPI::HoPI_3;pi-1* lines (36/58, 62%) showed medium rescue (Table 2, Fig. 4Z), while most of the *pAtPI::HoPI_1;pi-1* (11/19, 58%) and *pAtPI::HoPI_1A;pi-1* lines (43/85, 51%) could be put in the strong rescue category (Table 2); in these cases, not only the *pi-1* phenotype in whorls 2 and 3 was rescued, but also sepal-to-petal transformations in whorl 1 occurred (Fig. 4R, S). In contrast, all *pAtPI::HoPI_1B;pi-1* lines (53/53, 100%) showed only weak rescue (Table 2), with filament-like structures in the third whorl, but no occurrence of petal identity in the second whorl (Fig. 4X). Sequence analyses had shown that the only difference between HoPI_1B and HoPI_1 (or HoPI_1A) was the absence of the C-terminal sequence (Fig. 1B), so it was reasonable to hypothesize that the different degrees of phenotypes observed in transgenic analyses were due to the presence or absence of the C-terminal sequence. To confirm this result, we generated a truncated *HoPI_3ΔC* construct that lacked the complete C domain of HoPI_3 from amino acid 164 downwards. Flowers of the *pAtPI::HoPI_3ΔC;pi-1* lines exhibited a phenotype similar to that of the *pAtPI::HoPI_1B;pi-1* lines, i.e. they showed partial restoration of stamens but not of petals. These data suggest that the C-terminal region is required for the activity of HoPI proteins.

To explore whether transgene expression level and the severity of the transgenic phenotype are correlated, qRT–PCR analyses were performed. As shown in Supplementary Data Fig. S4, there was a strong correlation between the level of transgene

TABLE 1. *Summary of phenotypes of all overexpressing lines*

Phenotype	No. (%) of plants with petaloid sepals	No. of plants with stamenoid carpels	No. of plants
35S::HoPI_1	29 (74%)	–	39
35S::HoPI_1A	82 (84%)	–	98
35S::HoPI_1B	0	–	28
35S::HoPI_2	0	–	51
35S::HoPI_3	78 (66%)	–	118
35S::HoAP3_1	–	0	52
35S::HoAP3_2	–	0	82



Gene	Ectopic	Rescue	
		Whorl 2	Whorl 3
HoAP3_1	–	–	+
HoAP2_2	–	–	+
HoPI_1	+++	+++	+++
HoPI_1A	+++	+++	+++
HoPI_1B	–	–	+
HoPI_2	–	–	–
HoPI_3	++	++	++

FIG. 4. Phenotypes of *HoAP3* and *HoPI* transgenic *Arabidopsis* lines. (A) Wild-type flower. (B–E) SEMs of (B) sepals, (C) petals, (D) filaments and (E) anthers of the flower shown in (A). (F) Flower of *35S::HoAP3_1*. (G) Carpels of the flower shown in (F) do not show conversion to stamens. (H) Flower of *35S::HoPI_1*. (I, J) SEMs of adaxial (I) and abaxial (J) petaloid sepals of the flower shown in (H). (K) *ap3-3* homozygous flower. (L) Abaxial epidermis of sepaloid petal in (K). (M) *pAtAP3::HoAP3_1;ap3-3*. (N) *pAtAP3::HoAP3_2;ap3-3*. (O) Abaxial epidermis of sepaloid petal in (M) and (N). (P) *pi-1* homozygous flower. (Q) Abaxial epidermis of sepaloid petals in (P). (R) *pAtPI::HoPI_1;pi-1*. (S) *pAtPI::HoPI_1A;pi-1*. (T–W) Epidermal cells of (T) petaloid sepals, (U) second whorl petals, (V) filament, and (W) anther from *pAtPI::HoPI_1;pi-1* (R) and *pAtPI::HoPI_1A;pi-1* (S). (X) *pAtPI::HoPI_1B;pi-1*. (Y) *pAtPI::HoPI_2;pi-1*. (Z) *pAtPI::HoPI_3; pi-1*; (i) abaxial epidermis of first-whorl organs in (Z); (ii) abaxial epidermis of second-whorl organs in (Z). In (P, X, Y, Z), one sepal has been removed to reveal the inner whorls of the flower. Scale bars: (A, F, H, K, M, N, P, R, S, X–Z) = 1 mm; (B–E, G, I, J, L, O, Q, T, W, i, ii) = 50 μm. The table summarizes floral organ phenotypes observed in transgenic analyses. The degree of ectopic phenotype or rescue is indicated. –, no ability to generate an ectopic phenotype or complement the mutant phenotype; +, some ability to generate an ectopic phenotype or complementation; ++, moderate ectopic phenotype or rescue; +++, strong ectopic phenotype or full complementation.

TABLE 2. *Summary of rescue phenotypes*

Phenotype	No rescue	Weak rescue	Medium rescue	Strong rescue	No. of plants
pAtPI::HoPI_1;pi-1		4 (21%)	4 (21%)	11 (58%)	19
pAtPI::HoPI_1A;pi-1		18 (21%)	24 (28%)	43 (51%)	85
pAtPI::HoPI_1B;pi-1		53 (100%)	0	0	53
pAtPI::HoPI_2;pi-1	38 (100%)	0	0	0	38
pAtPI::HoPI_3;pi-1		12 (21%)	36 (62%)	10 (17%)	58
pAtAP3::HoAP3_1;ap3-3		52 (100%)	0	0	52
pAtAP3::HoAP3_2;ap3-3		24 (100%)	0	0	24

expression and the degree of phenotypes for different transformants that contained the same construct. This result supports the view that the gain-of-function and complementation phenotypes observed in the transgenic *Arabidopsis* lines were due to the heterologous expression of the *Hedyosmum* genes.

Yeast two-hybrid interactions among HoAP3 and HoPI proteins

In order to determine the dimerization affinities of *Hedyosmum AP3*- and *PI*-like gene products, we analysed their interaction patterns by the yeast two-hybrid system. No interactions were observed when the full-length sequences (comprising the complete MIKC regions) were used (data not shown). Therefore, we truncated the MADS domain of all the class-B proteins in this experiment, as had been done in a previous study (Yang *et al.*, 2003), generating 'IKC' constructs (Table 3).

As shown in Table 3, both MADS-deleted HoAP3_1 and HoAP3_2 proteins could interact with the MADS-deleted HoPI proteins, although the HoAP3_1-HoPI dimerization was particularly strong. All the MADS-deleted HoAP3 and HoPI proteins were able to form HoAP3-AtPI or HoPI-AtAP3 heterodimers with their distant *Arabidopsis* homologues. HoAP3_1, HoAP3_2, HoPI_1A and HoPI_3 also displayed medium to strong homodimerization capabilities. In addition, we also detected a strong interaction between the two HoAP3 proteins and medium to weak heterodimerization between HoPI_1A and HoPI_3. However, no homodimerization interactions were observed for HoPI_1, HoPI_1B and HoPI_2.

It is interesting that, in contrast to HoPI_1A, HoPI_1B revealed no homodimerization interactions (Table 3). We thus hypothesized that the C-terminal region of HoPI proteins may be required for homodimerization. To confirm this result, we truncated the C-terminal sequence of HoPI_3 and examined the dimerization affinity of the resulting derivative HoPI_3 (IK). As shown in Fig. 5, the homodimerization capability of the truncated HoPI_3 (IK) was greatly diminished while its heterodimerization capability was not affected, reflecting a similar dimerization affinity to that of HoPI_1B. These results indicate that the C-terminal region of HoPI proteins is required for homodimerization, but not essential for heterodimerization.

Given that the ancestral B protein GGM2 from the gymnosperm *Gnetum gnemon* binds to DNA as a homodimer form (Winter *et al.*, 2002*b*; Wang *et al.*, 2010), we tested whether the *in vitro* HoPI_1A or HoPI_3 homodimer had an actual function *in vivo* by ectopically expressing *HoPI_1A* or *HoPI_3* in *Arabidopsis ap3-3* mutants. However, we observed no rescue

TABLE 3. *Interactions between* Arabidopsis *AP3/PI and* Hydyosmum *AP3/PI in the Y2H systems*

Interaction	HoPI_1 (IKC)	HoPI_1A (IKC)	HoPI_1B(IKC)	HoPI_2 (IKC)	HoPI_3 (IKC)	HoAP3_1 (IKC)	HoAP3_2 (IKC)
HoPI_1(IKC)	–	+ /–	–	–	+ /–	+ + +	+
HoPI_1A(IKC)	+ /–	+ +	–	+ /–	+	+ +	+
HoPI_1B(IKC)	–	–	–	–	–	+ + +	+
HoPI_2(IKC)	–	+ /–	–	+ /–	–	+ +	+ +
HoPI_3(IKC)	+ /–	+	–	–	+ +	+ + +	+
HoAP3_1(IKC)	+ + +	+ +	+ + +	+ +	+ + +	+ + +	+ + +
HoAP3_2(IKC)	+	+	+	+ +	+	+ + +	+ + +
AtAP3(IKC)	+ + +	+ + +	+ + +	+ + +	+ + +	+	+
AtPI(IKC)	–	–	–	+ +	–	+ + +	+ +

–, no interaction; + /–, marginal interaction; +, weak interaction; + +, moderate interaction; + + +, strong interaction.

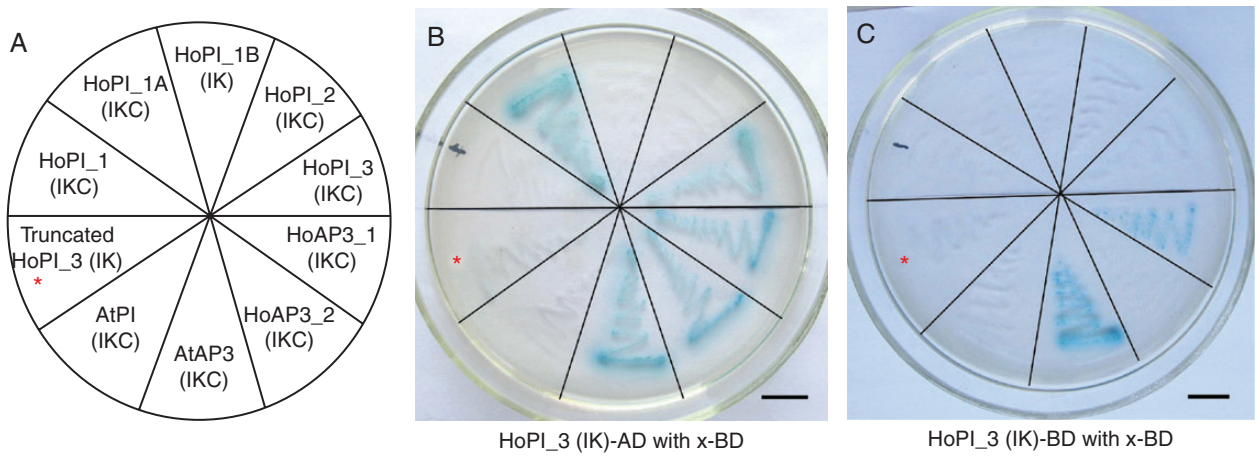


FIG. 5. Interaction analyses of the truncated HoPI_3 (IK) form (indicated by red asterisks). (A) Schematic summarizing yeast genotypes streaked on the plates in (B) and (C). (B) Truncated HoPI_3 (IK) activation domain with AP3/PI binding domain; (C) Truncated HoPI_3 (IK) binding domain with AP3/PI activation domain. Scale bars = 1 cm.

phenotype in *35S::HoPI_1A;ap3-3* and *35S::HoPI_3;ap3-3* transgenic plants (data not shown). This indicates that the HoPI_1A or HoPI_3 homodimer has no practical function in the context of *Arabidopsis*, and that HoPI rescues *Arabidopsis pi-1* mutants by forming functional heterodimers with the endogenous AtAP3 (Table 3).

DISCUSSION

The evolutionary states of class-B gene homologues in Hedyosmum

Gene duplication events are often associated with shifts in expression patterns and/or changes in coding sequence, giving rise to the diversification of gene function (Moore and Purugganan, 2005; Sharma *et al.*, 2011; Sharma and Kramer, 2013). In this study, the different expression levels observed may indicate that the relatively highly expressed *HoPI_1*, *HoPI_1A* and *HoPI_3* could have maintained a predominant role in *Hedyosmum* floral development, whereas *HoPI_1B* and *HoPI_2*, with low expression levels, might have partially lost their roles in this process (Fig. 2B). This hypothesis is supported by the strong ectopic and rescue phenotypes observed in transgenic *Arabidopsis* plants bearing *HoPI_1*, *HoPI_1A* and *HoPI_3* (Fig. 4H, R, S, Z) and the weak or no homeotic phenotype observed in *HoPI_1B* and *HoPI_2* transgenic *Arabidopsis* plants (Fig. 4X, Y). Moreover, *in situ* hybridization analysis further showed that, though the expression patterns of the two highly expressed *PI* paralogues *HoPI_1* and *HoPI_3* were identical in the early developmental stages of stamen (Fig. 3I, J, M, N), they differentiated in late stages. Specifically, *HoPI_3* was expressed throughout the nearly mature stamen (Fig. 3O), whereas *HoPI_1* was only expressed in the microsporangium (Fig. 3K). It is likely that the two *PI* loci both act to promote stamen identity, but experience differentiation at very late stages, with *HoPI_3* being the crucial player and *HoPI_1* being essential only for pollen development.

Two *HoAP3* paralogues were almost completely restricted to stamens, and the expression level of *HoAP3_1* was much higher than that of *HoAP3_2* (Fig. 2A, C). In the phylogenetic tree, the length of the branch leading to *HoAP3_1* is much shorter than that leading to *HoAP3_2* (Supplementary Data Fig. S2). In addition, the yeast two-hybrid assays demonstrated that the dimerization affinity of HoAP3_1 was much stronger than that of HoAP3_2 (Table 3). Overall, there were distinct expression levels, molecular evolution patterns and dimerization affinities for the two *HoAP3* paralogues, which may indicate a diversification between the two loci after gene duplication. However, both *HoAP3* coding sequences displayed similar activity in transgenic analyses. They did not function as well as *Arabidopsis AP3*, but substituted only some functional aspects of *AP3*, namely specifying stamen identity to some extent in the *Arabidopsis ap3-3* mutant (Fig. 4M, N). These results are consistent with previous studies of *CsAP3*, the paleo*AP3* orthologue in *C. spicatus* (Chloranthaceae), which is also exclusively expressed in stamens and only partially able to rescue stamen identity, but is biochemically incapable of promoting petal identity in the *Arabidopsis ap3-3* mutant (Li *et al.*, 2005; Su *et al.*, 2008). These observations strongly support the view that the paleo*AP3*-like genes in the family Chloranthaceae are different from the eu*AP3*-like genes in core eudicots. They may have an ancestral function in specifying male reproductive organs and in distinguishing male and female reproductive organs like that of the putative B genes in gymnosperms (Mouradov *et al.*, 1999; Sundström *et al.*, 1999; Winter *et al.*, 1999, 2002*a*; Fukui *et al.*, 2001).

As shown in Fig. 6, the *Hedyosmum AP3* orthologues are restricted to male organs and there is no *AP3* homologue in the perianth; thus the B function, which requires the concerted expression of *AP3* and *PI* homologues, may not play a role in perianth identity and development in *Hedyosmum*. The distinctive stamen-specific characteristic of paleo*AP3* genes revealed in the Chloranthaceae differs sharply from the broad expression of paleo*AP3* homologues that has been observed in *Amborella* of the ANITA grade (Kim *et al.*, 2005). Similarly, it is also different from paleo*AP3*-like genes in the large magnoliid clade, for which expression signals have been detected in petaloid tepals and in stamens (Kramer and Irish, 2000; Kim *et al.*, 2005). Chloranthaceae is one of the basal angiosperm lines above the basalmost ANITA grade (Qiu *et al.*, 1999; Zanis *et al.*, 2002). Since most of the magnoliids and ANITA members have flowers with a petaloid perianth and paleo*AP3* genes with broad expression, the extremely simple flower and the stamen-specific state of paleo*AP3* genes in Chloranthaceae are likely derived rather than primitive.

In general, coexpression of the *AP3* and *PI* genes is correlated with the production of petaloid organs, regardless of their position (van Tunen *et al.*, 1993; Kramer and Irish, 2000; Kanno

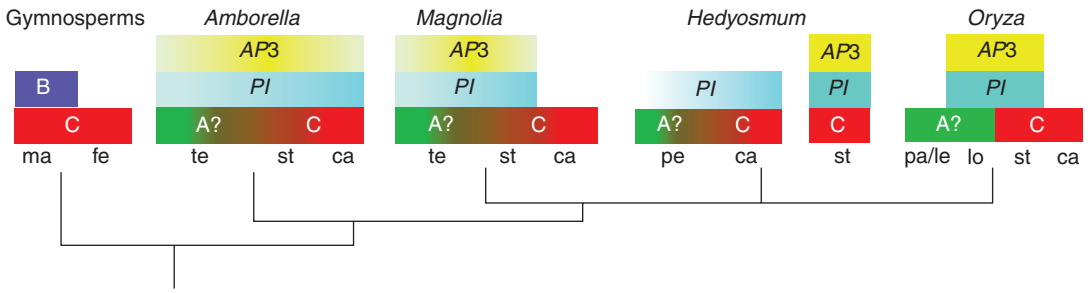


FIG. 6. Evolution of ABC genes in basal angiosperms. The simplified angiosperm phylogeny is based on Fig. 3 of Kim *et al.* (2005). The expression patterns of B genes in gymnosperms are summarized from Fukui *et al.* (2001), Mouradov *et al.* (1999), Sundström *et al.* (1999); Winter *et al.* (1999) and Winter *et al.* (2002*a*). The modified ABC model for *Hydyosmum* is based on expression data reported here. The ABC models for *Amborella*, *Magnolia* and *Oryza* are modified from Fig. 6 of Álvarez-Buylla *et al.* (2010) (data summarized from Kim *et al.*, 2005; D. Soltis *et al.*, 2007; Whipple *et al.*, 2007). Abbreviations: ma, male cone; fe, female cone; te, tepals or petaloid tepals; st, stamens; ca, carpels; pe, perianth organs; pa/le, palea/lemma; lo, lodicules.

et al., 2003; Kramer *et al.*, 2003, 2007; Jaramillo and Kramer, 2004; Tsai *et al.*, 2004; Kim *et al.*, 2005; Mondragón-Palomino and Theissen, 2008, 2009; Feng *et al.*, 2012; Vekemans *et al.*, 2012). It is likely that overlap of *AP3* and *PI* homologue expression is limited to the stamens in *Hedyosmum* because it lacks a petaloid perianth organ. However, we still cannot rule out the possibility that changes in floral homeotic genes (such as *HoAP3*) are causally linked to the greatly reduced perianth in *Hedyosmum*. Important changes in *cis*-regulatory elements or *trans*-regulatory factors that regulate paleo*AP3*-like genes may have taken place during or after the emergence of the family Chloranthaceae, resulting in the stamen-specific expression of these genes in this family (Li *et al.*, 2005). A deeper understanding of the molecular mechanisms involved in the origin of stamen-specific expression of paleo*AP3* homologues in the Chloranthaceae family would be quite informative.

The C-terminal region is essential for HoPI proteins

Previous studies suggested that the C-terminal region is essential for protein function despite its rapid rate of evolutionary change and its obvious variation in length and sequence (Kempin *et al.*, 1995; Theissen *et al.*, 2000). It has been hypothesized that this variable region of class-B proteins and the highly conserved motifs in the C-terminus of these proteins may have played a crucial role in the evolution of B-function proteins, especially in the acquisition of new functions in petal identity specification (Lamb and Irish, 2003; Vandenbussche *et al.*, 2003; Chen *et al.*, 2012). However, our analysis of HoPI_1A and previous studies of the truncated PI-like proteins have shown that, though these protein forms have an atypical PI motif or lack the PI motif in the C-terminus, they are functional in specifying petal and stamen identities in transgenic analysis (Fig. 4S in this study; Berbel *et al.*, 2005; Piwarzyk *et al.*, 2007; Benlloch *et al.*, 2009), which suggests that the C-terminus PI motif is dispensable for PI activity. Moreover, study of a PI-like protein in a lily has shown that the PI-like protein LMADS8 can completely rescue the second-whorl petal of the *Arabidopsis pi* mutant, while the other PI homologue, LMADS9, lacking most of the C domain (29 C-terminal amino acids of the 45 amino acids of the C region), only partially rescues the second-whorl organs into small petals (Chen *et al.*, 2012). This suggests that, even though the PI motif is not required for PI activity, the rescue ability of PI-like proteins in petal development is decreased when more amino acids are deleted. In the present study, data on the truncated HoPI_3ΔC and HoPI_1B further demonstrated that, when the entire C-terminal region was truncated, the petal identity function of a PI-like protein was completely abolished. This result provides strong evidence that the amino acid sequence at the start of the C region is valuable for petal identity specification and that the complete C-terminal region of PI-like proteins plays a crucial role in petal development.

Besides its great importance for biochemical activity concerned with petal identity, the C-terminal region of PI proteins is also essential for the homodimerization capability of some of these proteins. The HoPI_1A and HoPI_3 proteins are able not only to form heterodimers with AP3-like proteins, but also to form homodimers (Table 3). Given the inability of HoPI_1A or HoPI_3 homodimers to rescue *Arabidopsis ap3-3* mutants, it is likely that the only functional form of the B-protein complex is based on heterodimerization, and the homodimerization of *Hedyosmum* PI-like proteins may be a vestige of the functional homodimerization seen in gymnosperms. However, we also cannot rule out the possibility that, *in vivo*, HoPI_1A and HoPI_3 homodimers can activate unique targets or interact with novel protein partners in *H. orientale*. It is interesting to note that when the C-terminal region was truncated, the heterodimerization ability of the truncated HoPI_3 (IK) and HoPI_1B was not altered, whereas the homodimerization capability was diminished or lost (Table 3 and Fig. 5). This indicates that the C-terminal region of HoPI proteins is essential for homodimerization. This may provide some clues concerning the evolution of the dimerization of B-function proteins.

It has been proposed that the obligate heterodimerization of class B proteins in the higher eudicots evolved from ancestral homodimerization, with the facultative homo- and heterodimerization in some species being a transitory state leading to obligate heterodimerization (Winter *et al.*, 2002*b*; D. Soltis *et al.*, 2004). Consequently, there are two key events that are crucial for the transformation from homo- to hetero- dimerization, one being the acquisition of heterodimerization and the other being the elimination of homodimerization. Concerning the acquisition of heterodimerization, previous studies have shown that a dramatic diversification in the K domain is to a great extent responsible for the heterodimerization of AP3- and PI-like proteins (Yang *et al.*, 2003; Yang and Jack, 2004). On the other hand, some studies have also shown that the C-terminal region is essential for homodimer formation by AP3 lineage proteins (Hsu and Yang, 2002; Tzeng *et al.*, 2004). Taking these results together, it is quite likely that, while the K domain contributes much to the heterodimerization of AP3 and PI lineage proteins, the C-terminal region is important for homodimerization capability. Revisiting the question posed in the Introduction—the question of which evolutionary forces led to the fixation of the B-function programme—we conclude that the complete disappearance of homodimerization resulted from sequence diversification in the C-terminal region; this finally eliminated the interaction flexibility of *AP3*- and *PI*-like gene products, which had persisted for a long period of time during evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used in this study. Table S2: accession information for all loci used in the phylogenetic analyses. Figure S1: alignment of the coding sequence of *HoPI_1*, *HoPI_1A* and *HoPI_1B*. Fig S2: maximum likelihood phylogeny of *AP3*/*PI*-like genes in seed plants. Figure S3: calibration of the degree of rescue of transgenic flowers. Figure S4: expression of *HoAP3* and *HoPI* in transgenic *Arabidopsis* plants.

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