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Altered expression patterns of TCP and MYB genes relating to the floral developmental transition from initial zygomorphy to actinomorphy in *Bournea* (Gesneriaceae)

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Summary

• The shift from zygomorphy to actinomorphy has been intensively studied in molecular genetic model organisms. However, it is still a key challenge to explain the great morphological diversity of derived actinomorphy in angiosperms, since different underlying mechanisms may be responsible for similar external morphologies. *Bournea* (Gesneriaceae) is of particular interest in addressing this question, as it is a representative of primarily derived actinomorphy characteristic of a unique developmental transition from zygomorphy to actinomorphic flowers at anthesis.

• Using RNA *in situ* hybridization, the expression patterns were investigated of three different *Bournea* orthologues of TCP and MYB genes that have been shown to control floral symmetry in model species.

• Here, it is shown that the initial zygomorphic pattern in *Bournea* is likely a residual zygomorphy resulting from conserved expression of the adaxial (dorsal) identity gene *BlCYC1*. As a key novel event, the late downregulation of *BlCYC1* and *BlRAD* and the correlative changes in the late specific expression of the abaxial (ventral) identity gene *BlDIV* should be responsible for the origin of the derived actinomorphy in *Bournea*.

• These results further indicate that there might be diverse pathways in the origin and evolution of derived actinomorphy through modifications of pre-existing zygomorphic developmental programs under dynamics of regulatory networks.

Key words: altered expression pattern, *Bournea* (Gesneriaceae), *CYCLOIDEA*, derived actinomorphy, *DIVARICATA*, evolution of floral symmetry, *RADIALIS*.

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Introduction

One important event during the evolution of angiosperms is the secondary shift from zygomorphy (bilateral symmetry along the dorsoventral axis) to actinomorphy (radial symmetry) of their flowers. The shift in floral symmetry has attracted considerable interest for over 200 yr since Carl Linnaeus first described a natural peloric mutant of toadflax (*Linaria vulgaris*) (Gustafsson, 1979). In the past decade, significant progress has been made in the area of flower symmetry regulation especially in model genetic systems including *Antirrhinum majus* (Luo *et al.*, 1996, 1999; Cubas *et al.*, 1999a; Endress, 1999; Citerne *et al.*, 2006; Feng *et al.*, 2006; Howarth & Donoghue, 2006; Damerval *et al.*, 2007). However, the evolutionary shift from zygomorphy to actinomorphy is still a vast unexplored field at the molecular developmental level in naturally occurring nonmodel organisms. In Lamiales *sensu lato*, a major clade of predominantly zygomorphically flowered

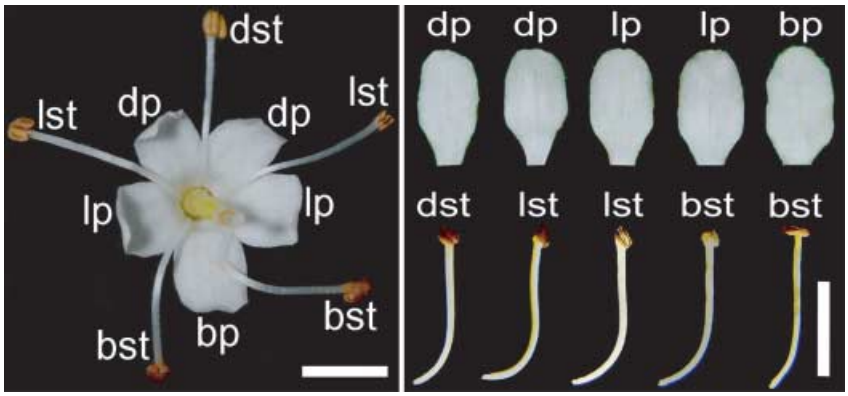

Fig. 1 Mature whole and dissected flowers of *Bournea leiophylla*. Actinomorphy (radial symmetry) was observed in mature *Bournea leiophylla* flowers with nearly identical adaxial, lateral and abaxial petals and stamens. bp, abaxial (ventral) petal; bst, abaxial (ventral) stamen; dp, adaxial (dorsal) petal; dst, adaxial (dorsal) stamen; lp, lateral petal; lst, lateral stamen. Bar, 5 mm.

angiosperms, derived actinomorphy frequently occurs (Donoghue *et al.*, 1998; Endress, 1998).

Note that actinomorphy is considered the ancestral state for angiosperms, and zygomorphy has evolved several times independently from actinomorphic ancestors (Crepet, 1996; Donoghue *et al.*, 1998). However, zygomorphy is frequently lost in the Asteridae, especially in Lamiales *sensu lato* (Donoghue *et al.*, 1998; Endress, 1998). The secondary shift from zygomorphy to actinomorphy may be a reversal to an earlier ancestral form, but may also be an innovative process or innovative homoeotic transformation (Citerne *et al.*, 2006). Since it is not exactly known to date which genes or gene interactions are involved in controlling the ancestral actinomorphy, the term 'derived actinomorphy' as used here is not concerned whether it is a reversal or innovative process, but only indicates an actinomorphy derived from an otherwise zygomorphic clade.

In *A. majus* with zygomorphic flowers, the floral dorsoventral (adaxial/abaxial) asymmetry is established through transcriptional and post-transcriptional interactions among three key genes encoding transcription factors of the TCP and MYB families. *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*), encoding two related transcription factors of the TCP (Teosinte Branched 1, CYCLOIDEA and PCF) family (Cubas *et al.*, 1999b), function redundantly in specifying the adaxial (dorsal) identity (Luo *et al.*, 1996, 1999). Conversely, *DIVARICATA* (*DIV*) encodes a MYB transcription factor and determines the abaxial (ventral) petal identity (Galego & Almeida, 2002; Perez-Rodriguez *et al.*, 2005). The function of *CYC* and *DICH* is mediated through activating the downstream target *RADIALIS* (*RAD*), another MYB gene, whose encoded protein antagonizes DIV protein function in the adaxial region and therefore limits *DIV* to the abaxial domain (Corley *et al.*, 2005; Costa *et al.*, 2005). In *Antirrhinum*, the *cyc/dich* double mutant has a fully abaxialized (ventralized) peloric flower because *RAD* is not activated (Corley *et al.*, 2005) to exert an antagonistic effect on *DIV* in the adaxial region. The abaxialized peloric mutants of both *A. majus* and *L. vulgaris* are caused by complete silencing of *CYC* and *LCYC* (*Linaria CYC*) through transposon insertion and extensive DNA methylation, respectively (Luo *et al.*, 1996; Cubas *et al.*, 1999a). In legumes, distantly related to *Antirrhinum*, *CYC* homologues *legCYC* genes are involved in the establishment of zygomorphy (Feng *et al.*, 2006). Actinomorphic flowers in legumes come from *legCYC* expression in all five petals, similar to the adaxialized (dorsalized) mutant in *Antirrhinum* as a result of ectopic expression of *CYC* (Citerne *et al.*, 2006). However, the formation of the great diversity of actinomorphy derived from different clades of zygomorphy in nature seems not to involve simple loss-of-function or gain-of-function mutations and the same mechanism that underlies the genetic control of floral symmetry in the model genetic system (Donoghue *et al.*, 1998; Reeves & Olmstead, 2003; Smith *et al.*, 2004). Using the mechanisms that underlie mutant phenotypes in model species as a starting point, detailed explorations of natural types of derived actinomorphy may reveal new evolutionary pathways from zygomorphy to actinomorphy, involving different suites of genes and acting at different times during development.

The family Gesneriaceae, sister to the remainder of Lamiales *s. l.* (Wortley *et al.*, 2005), with weak floral zygomorphy (especially in subfamily Cyrtandroideae), has the most diverse forms of derived actinomorphy and possesses the largest proportion of genera with actinomorphic flowers in Lamiales *s. l.* (Endress, 1998, 1999). In Gesneriaceae, *Bournea leiophylla* exhibits actinomorphic flowers at anthesis (Fig. 1). However, its very short corolla tube with five filaments adnate to the corolla and the adaxial petals somewhat smaller than others hints at their evolution from a zygomorphic ancestor (Fig. 1). Phylogenetic analyses confirm that the ancestor to *Bournea* was likely a species with zygomorphic flowers (Li & Wang, 2004; Du & Wang, 2008). The floral development further shows that the flowers in *B. leiophylla* undergo a morphological transition from a zygomorphic pattern during organ initiation and early development to actinomorphy at anthesis (Figs 1 and 2). This developmental pattern implies that *CYC*-like genes should be functional in controlling the floral dorsoventral asymmetry in *B. leiophylla*, at least in early floral development. Given that Gesneriaceae and Veronicaceae (family of *Antirrhinum*) are relatively closely related (Wortley *et al.*, 2005) and *CYC*-like genes function in arresting stamen development

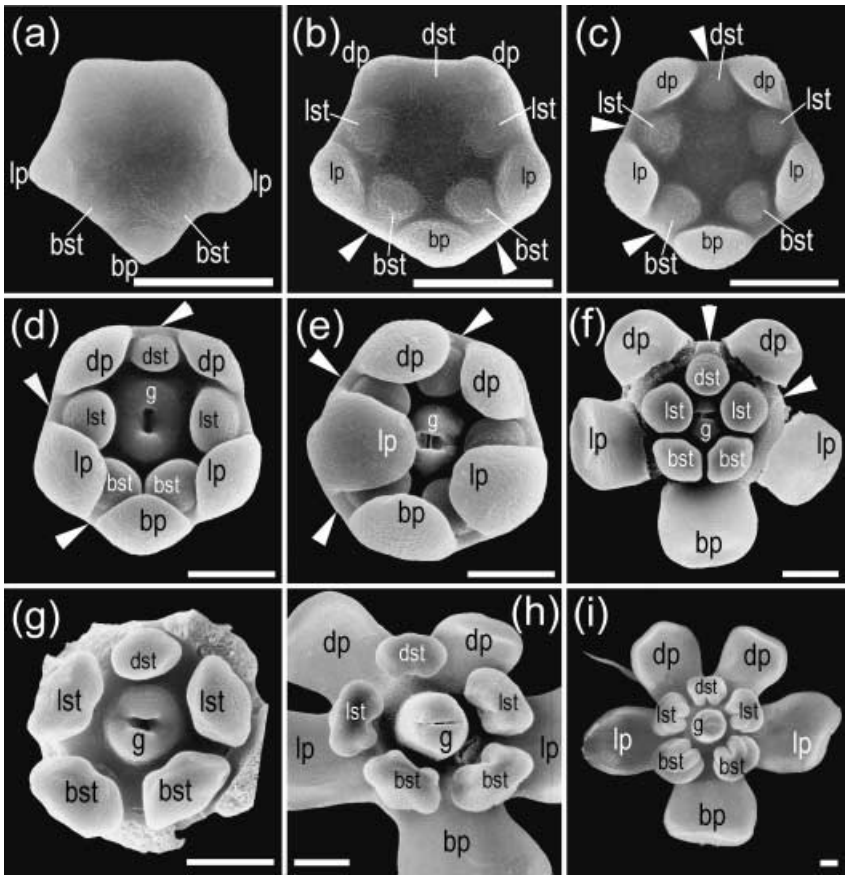

Fig. 2 Morphological developments of *Bournea leiophylla* flowers. Zygomorphy was observed at early stages of floral development in *B. leiophylla*. After sepal initiation (not shown), the petals and stamens are unidirectionally initiated from the abaxial side and subsequently extended to the adaxial side of developing flowers at end of stages 4 and 5, and the adaxial stamen was much delayed in initiation (a–c). After petal and stamen initiation, lateral growth between adjacent petals and stamens results in the formation of the corolla tube (arrowheads) (c–d). Lateral growth only lasts a short duration during early development (arrowheads) (d–f). As two carpels emerge and grow at stages 6, adaxial petals and stamen are significantly smaller than others in each whorl (d). Then, the growth of the adaxial stamen gradually catches up with that of the abaxial and lateral ones as anthers are swollen (e–h). When anther sacs are defined and two carpels are closed into the ovary from stage 9 onward, five stamens become almost equal in size (h,i). Up to stage 10, the adaxial petals are only slightly smaller than other petals but all petals have a similar shape (i). bp, abaxial petal; bst, abaxial stamen; dp, adaxial petal; dst, adaxial stamen; lp, lateral petal; lst, lateral stamen; g, gynoecium. Bar, 150 μm.

(Luo *et al*., 1996, 1999; Hileman *et al*., 2003), it is unlikely that the actinomorphic flower in *B. leiophylla* results from either loss-of-function or gain-of-function of *CYC*-like genes. Instead, the developmental transition from the initial zygomorphy to actinomorphy at anthesis may involve an alteration in the regulatory interaction among TCP and MYB family genes during floral development.

Recent molecular genetic studies have begun to reveal the dynamics of gene-regulatory interaction, and the complex combinatorial mechanisms that create a distinct final floral architecture and form (Krizek & Fletcher, 2005). *Bournea* represents an ideal candidate for exploring a potentially novel evolutionary pathway for derived actinomorphy in angiosperms, especially in Lamiales *s. l.* To address this, we carried out a comprehensive investigation on the expression patterns of all three kinds of floral symmetry genes known in *Antirrhinum* (i.e. *CYC*, *RAD* and *DIV* homologues; *DICH* only exists in Antirrhineae) (Hileman & Baum, 2003; Cubas, 2004). The first goal of this study was: to identify whether the early expression patterns of the three kinds of floral symmetry genes are correlated with the initial floral zygomorphy in *B. leiophylla* as in those in *A. majus*; to identify whether their expression is altered when the floral development shifts to an actinomorphic pattern; to understand the functional and developmental homology of the floral symmetry genes between *B. leiophylla* and *A. majus*. Second, we focused on how the expressional alteration and evolution of floral symmetry genes relate to the developmental transition from zygomorphy to actinomorphy in *B. leiophylla*, which would provide novel evolutionary pathway for derived actinomorphy in angiosperms.

Materials and Methods

Molecular cloning and sequence analyses

We isolated *CYC*, *RAD* and *DIV* homologues from *Bournea leiophylla* using degenerate oligonucleotide primers in 3′- and/or 5′-RACE (rapid amplification of cDNA ends) according to described methods (Sambrook & Russell, 2001) and the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), respectively. Total RNA was extracted from the young floral buds of *B. leiophylla* using the Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were synthesized from total RNA for 3′-RACE with the Supertranscript III RNase H$^-$ Reverse Transcriptase (Invitrogen). To examine the intron/exon structures we isolated and sequenced the corresponding genomic DNA of *BlCYC*, *BlRAD* and *BlDIV* from leaves. The oligonucleotide sequences for primers are included in the Supplementary Material Text S1.

To evaluate homologues, we analysed BlDIV1, BlDIV2, DIV, DVL1, LeMYBI, PHAn2 and known or predicted DIV-like proteins from *Arabidopsis thaliana* (Quattrocchio *et al*., 1993; Rose *et al*., 1999; Galego & Almeida, 2002; Guo *et al*., 2005); BlRAD, RAD, BlDIV1, BlDIV2 and DIV were also aligned (Galego & Almeida, 2002; Corley *et al*., 2005). Similarly, alignment of BlCYC1, BlCYC2, ObCYC1, ObCYC2, CYC and DICH was carried out (Luo *et al*., 1996, 1999; Du & Wang, 2008).

According to the known and putative amino acid information, phylogenetic analyses of DIV-like and RAD-like proteins were conducted, respectively (Rose *et al*., 1999; Galego & Almeida, 2002; Barg *et al*., 2004; Guo *et al*., 2005; Baxter *et al*., 2007). Protein sequences to be analysed were aligned using CLUSTALX 1.81 (Thompson *et al*., 1994) or DNAMAN version 5.2.2 (Lynnon BioSoft, Pointe-Claire, Quebec, Canada) and manually refined. Amino acid sequences for phylogenetic analysis were aligned and all gaps were removed manually. The neighbor-joining method with p-distance was carried out using MEGA 3.1 (Kumar *et al*., 2004). Bootstraps (1000 replicates) were conducted to assess the statistical reliability of the inferred topology with MEGA 3.1 (Kumar *et al*., 2004). The sequences reported in the paper for *B. leiophylla BlCYC1*, *BlCYC2*, *BlRAD*, *BlDIV1* and *BlDIV2* have been deposited in the GenBank database (Accession Nos. EF211118, EF211119, EF211120, EF211121, EF207557, EF211122, EF486282, EF486283 and EF486284).

RNA *in situ* hybridizations

Materials for *in situ* hybridization were fixed, sectioned and hybridized to digoxygenin-labeled probes of *BlCYC1*, *BlRAD*, *BlDIV1* and *BlDIV2* with reference to described methods (Bradley *et al*., 1993). The templates for transcriptions of probes specific to *BlCYC1*, *BlRAD*, *BlDIV1* and *BlDIV2* include translated and nontranslated regions. The fragments were amplified from single-strand cDNA of young floral buds from *Bournea* by using primer pairs specific for *BlDIV1*, *BlDIV2*, *BlRAD*, and *BlCYC1*, and then purified and cloned into pGEM-T easy vectors. Linearized templates were amplified by using primers YT7 and YSP6, and then single-strand digoxygenin-labeled antisense and sense probes were prepared from the linearized templates (Divjak *et al*., 2002). The oligonucleotide sequences for primers are included in the Supplementary Material Text S1.

Locus-specific reverse transcriptase polymerase chain reaction (RT-PCR)

Tissues used for RT-PCR including petals and sepals were dissected from middle-late *B. leiophylla* flowers whose organs had undergone a degree of differentiation. Sepals were dissected from the flowers. The petals were partitioned into abaxial, lateral and adaxial regions. Total RNA was extracted from the three types of petal regions and from the sepal tissues respectively. cDNA was prepared from 4 μg of different total RNA with the Supertranscript™ III RNase H$^-$ Reverse Transcriptase (Invitrogen). To make sure each pair of primers was suitable, we first used them to amplify genomic DNA of *B. leiophylla.* The PCR products were then cloned. At least 20 clones of each PCR product were sequenced and only the primers that could specifically amplify *BlDIV1*, *BlDIV2*, *BlRAD*, and *BlCYC1*, *BlCYC2* genes were used further. The oligonucleotide sequences for primers are in the Supplementary Material Text S1. The PCR reaction mixes included 0.75 units of Ex-taq (Takara, Tokyo, Japan), 2 mM MgCl, 200 μM of each dNTP, 40 mM of corresponding primers and 1.5 μl corresponding first-strand cDNA. The amplification program was: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52°C or 54°C for 30 s, and 72°C for 40 s and a final 8-min extension at 72°C. Amplification of *ACTIN* was used as a positive control (Jin *et al*., 2000). The primers for *ACTIN* are actinF and actinR. All RT-PCR products were cloned into pGEM-T easy vectors, and 20 clones from each RT-PCR were sequenced to confirm locus-specificity.

Scanning electron microscopy

Floral buds were collected at different developmental stages and were examined under a Hitachi S-800 scanning electron microscope in Institute of Botany, CAS. The electron microscopy was performed according to previously described protocol (Matthews & Endress, 2004).

Results

Floral development in *Bournea leiophylla*

We first examined the floral developmental process in *Bournea leiophylla*, using scanning electron microscopy, and compared it with the previously described floral developmental stages in *A. majus* (Galego & Almeida, 2002; Vincent & Coen, 2004) (also see 'Definition and additional description of the floral developmental stages in *Bournea leiophylla*' in the Supplementary material Text S2). It was found that the petals and stamens were unidirectionally initiated from the abaxial side and subsequently extended to the adaxial side of developing flowers, and the adaxial stamen was much delayed in initiation (Fig. 2a–c), exactly matching stages 4 and 5 of floral development in *A. majus* and zygomorphic taxa in Gesneriaceae (Wang *et al*., 1997, 2002; Vincent & Coen, 2004). However, rather than stop growth after carpel initiation (stage 6) as in *Antirrhinum* and zygomorphic taxa in Gesneriaceae, the adaxial stamen of *B. leiophylla* continued to elongate and expand in size (Fig. 2d). In *A. majus* and zygomorphic taxa in Gesneriaceae (Wang *et al*., 1997, 2002; Vincent & Coen, 2004), Stage 7 is characteristic of the abaxial (ventral) and lateral stamens beginning to widen (or swell) (microsporangia) with corolla

almost covering the stamens and Stage 8 shows an important feature of basic external structure of the anther becoming visible and the rudimentary filament seen. During stages 7 and 8 in *B. leiophylla*, the development of the corolla and stamens shows a common feature with those of *A. majus* and zygomorphic taxa in Gesneriaceae except for the adaxial corolla lobes (data not shown) and the adaxial stamen gradually catching up with the abaxial and lateral ones in size (Fig. 2e–g). When anther sacs were clearly defined and two carpels were closed into the ovary, which is defined as stage 9 and 10 in *A. majus* (Vincent & Coen, 2004), the adaxial stamen became almost equal in size with other stamens, and two adaxial petals were only slightly smaller than others but similar in shape (Fig. 2 h–i). Lateral growth between adjacent petals and stamens only lasted a short duration, forming a short corolla tube (Fig. 2b–f). At anthesis, all five petals were equally expanded and similar in shape albeit the two adaxial petals were slightly smaller than others. All five stamens were almost equal in length and were extending radially and horizontally (Fig. 1).

Sequence analyses of BlCYC, BlRAD and BlDIV

There are two *CYC* homologous genes in *B. leiophylla* (see the Materials and Methods section). They encode two related proteins of 333 and 331 amino acids, respectively. We designated them as *BlCYC1* and *BlCYC2*. Sequence analysis shows that BlCYC1 and BlCYC2 are, respectively, 48% and 45% identical to CYC over the entire amino acid sequence. When comparing the TCP and R functional domains, BlCYC1 and BlCYC2 share 94.8% and 90.9% amino acid sequence identity with CYC, respectively, suggesting they are functionally related (Supplementary Material Fig. S1a). When compared with *CYC*-like genes from other species in Gesneriaceae, BlCYC1 and BlCYC2 are 91% and 86% identical to ObCYC1 and ObCYC2 from *Oreocharis*, respectively (Supplementary material Fig. S1a). *Oreocharis*, a genus with weak zygomorphy, is closely allied with *Bournea* both in morphology and *GCYC* phylogeny in Gesneriaceae (Li & Wang, 2004; Du & Wang, 2008). Proteins encoded by these *CYC*-like genes are very similar in the amino acid sequences in the three motifs, TCP domain, R domain and end box (see the Supplementary Material Fig. S1a). The sequence differentiation among these *CYC*-like genes are mainly located within the nonconserved regions (Fig. S1a). The close phylogenetic relationship between *CYC*-like genes in Gesneriaceae and *CYC* in *Antirrhinum* was shown in previous phylogenetic analyses (Möller *et al.*, 1999; Citerne *et al.*, 2000; Smith *et al.*, 2004; Du & Wang, 2008) (The sequences of BlCYC1 and BlCYC2 are included in the phylogenetic tree in Du & Wang (2008) and alignment in this paper).

Two *DIV* homologues were isolated from *B. leiophylla*, which encode two proteins of 295 and 291 amino acids, respectively. We designated these two genes as *BlDIV1* and *BlDIV2*. They are 91% identical at the nucleotide sequence level. At the amino acid level, BlDIV1 and BlDIV2 are 71% and 73% identical to DIV, respectively. All three proteins share a conserved R3 domain (Fig. S1b).

DIV, BlDIV1 and BlDIV2 belong to the atypical plant R2R3-MYB class of transcription factors (i.e. the DIV-like class) (Galego & Almeida, 2002). Distinctively different from the typical plant R2R3MYB protein, DIV-like proteins have different substitutions for the conserved tryptophan residue, longer linkers between R2 and R3 MYB-domains and a specific 'VASHAQKYF' motif (Fig. S1b). Sequence alignments revealed that the *Arabidopsis* At5g58900 has a higher sequence similarity to BlDIV1, BlDIV2, DIV and DVL1 than to any other known R2R3-MYB protein from *A. thaliana*. These five genes also share the same intron insertion positions and phases (Fig. S1b). In their *C*-terminal regions, there were some conserved motifs (Fig. S1c). Phylogenetic analyses based on neighbor-joining showed that BlDIV1, BlDIV2, DIV, DVL1 and At5g58900 formed a monophyletic clade with a high bootstrap support (Fig. 3a). Within the clade, BlDIV1, BlDIV2, DIV and DVL1 were further clustered together, sister to At5g58900 with a bootstrap value of 100% (Fig. 3a).

The *RAD* homologue was isolated from *B. leiophylla* and it was designated as *BlRAD*. It shares 69% amino acid identity with RAD from *Antirrhinum* (Fig. S1d). BlRAD has a single 84aa-MYB-domain conserved among RAD and DIV-like proteins, including DIV and BlDIV proteins (Fig. S1d). Phylogenetic analyses based on neighbor-joining of proteins encoded by *RAD*-like genes showed that BlRAD was sister to RAD from *Antirrhinum* with high support and they were further clustered with AtRL1 and AtRL2 proteins from *Arabidopsis* in a monophyletic clade (Fig. 3b). Other *RAD*-like genes from *Antirrhinum* and *Arabidopsis* that do not function in controlling floral dorsoventral asymmetry were shown to not be closely related to BlRAD (Fig. 3b).

Tissue-specific expressions of *BlCYC*, *BlRAD and BlDIV*

Full-length *BlCYC*, *BlRAD* and *BlDIV* cDNA was isolated from developing floral tissues. To elucidate their role in floral development, their temporal and spatial expression patterns were examined during floral development in *B. leiophylla* using RNA *in situ* hybridization. Transcripts of *BlCYC1* were first detected at the adaxial side of the floral meristem (Fig. 4a). When petals and stamens were just becoming visible, the mRNA of *BlCYC1* was specifically detected at the adaxial side of the floral apex inside the adaxial sepal (Fig. 4a). After initiation of petals and stamens at stage 5 in floral development, *BlCYC1* was highly expressed in the two adaxial petals and one adaxial stamen up to stage 6 after carpel initiation (Fig. 4b). The *BlCYC1* transcripts were then sharply reduced in the adaxial petals and stamen when stamens began to enlarge laterally and differentiate into anthers and filaments at stage 7 or at early stage 8 in floral development, and eventually became

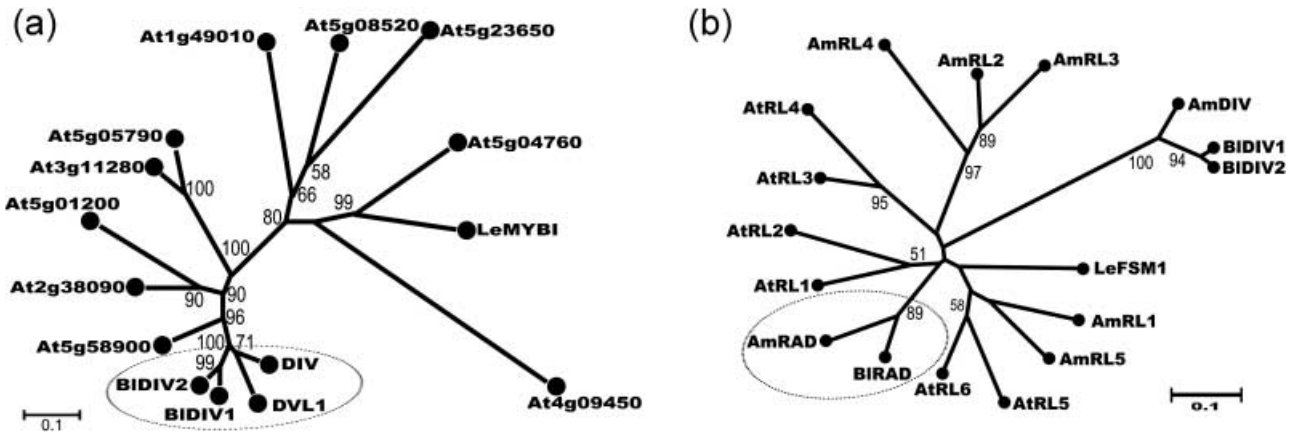

Fig. 3 Neighbor-joining trees among proteins encoded by the *DIV*-like and *RAD*-like genes using MEGA 3.1. (a) Neighbor-joining tree among proteins encoded by *DIV*-like genes. DIV and DVL1 proteins are from *Antirrhinum majus*. LeMYBI is from *Lycopersicon esculentum*. Others are from *Arabidopsis thaliana*, which include all the known or predicted R2R3-MYB proteins with 'SHAQKY' motif. Neighbor-joining analysis shows that BlDIV1, BlDIV2, DIV, DVL1 and At5g58900 form a clade, and BlDIV1, BlDIV2, DIV and DVL1 are clustered together, sister to At5g58900. (b) Neighbor-joining tree among proteins encoded by *RAD*-like genes. AmRAD and AmRL1–AmRL5 are from *Antirrhinum majus*, LeFSM1 is from *Lycopersicon esculentum* and six RAD-like proteins (AtRL1–AtRL6) are from *Arabidopsis thaliana*. Neighbor-joining tree shows that BlRAD and RAD form a clade that is further clustered with AtRL1 and AtRL2 proteins from *Arabidopsis*. Other *RAD*-like genes from *Antirrhinum* and *Arabidopsis* that do not function in controlling floral dorsoventral asymmetry are shown not be closely related to BlRAD. Bootstrap values with support ≥ 50% are shown.

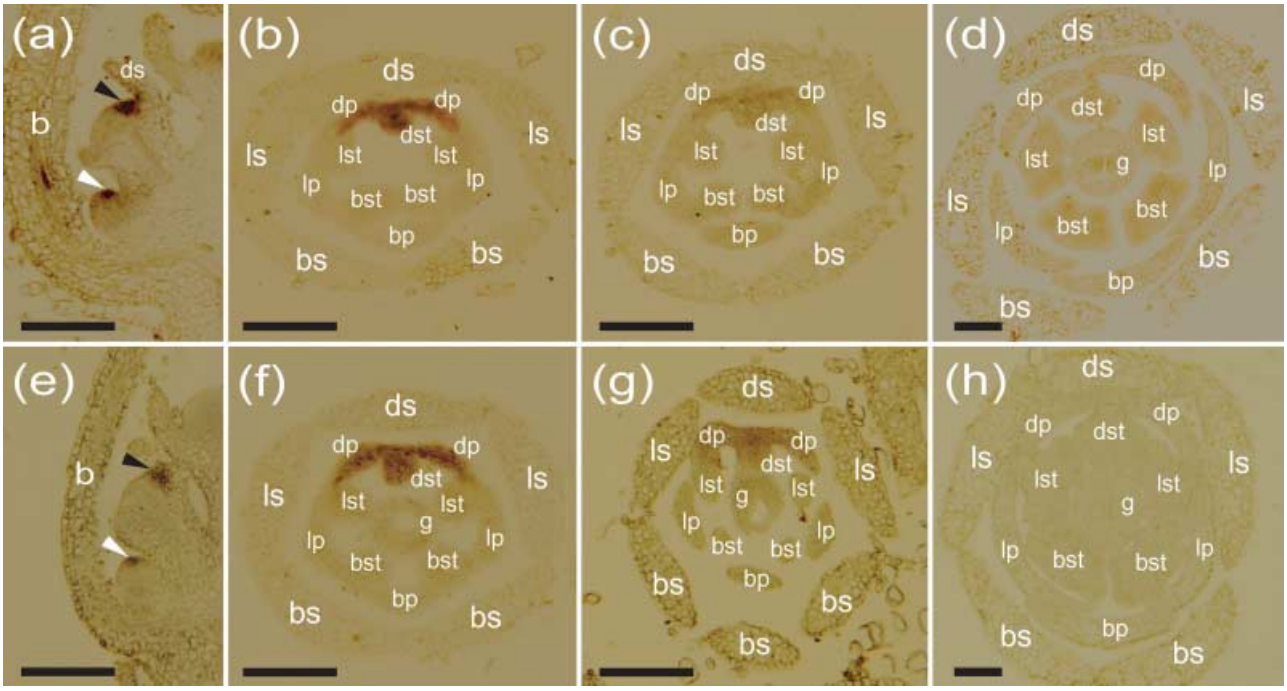

Fig. 4 Tissue-specific expression of *BlCYC1* and *BlRAD* during floral development in *Bournea leiophylla*. RNA *in situ* hybridizations was used to detect spatial and temporal expression of *BlCYC1* and *BlRAD* genes in longitudinal (a,e) and cross-sections (b–d, f–h) of flowers at different developmental stages. Transcripts of *BlCYC1* are first detected at the adaxial side of a floral apex (white arrowhead) (a). When petals and stamens just become visible, the mRNA of *BlCYC1* is specifically detected at the adaxial side of the floral apex inside the adaxial sepal (black arrowhead) (a). *BlCYC1* transcripts still densely accumulate in the two adaxial petals and one adaxial stamen at stage 6 or early stage 7 after carpel initiation (b). Then, *BlCYC1* transcripts are quickly reduced at stage 7 or early stage 8 and become barely detected in the developing flower from stage 8 onward (c,d). The mRNA of *BlRAD* is also first detected at the adaxial side of the floral meristem (white arrowhead) (e). Then, *BlRAD* is expressed in the floral adaxial region inside the adaxial sepal where adaxial petal or/and stamen primordia were becoming visible before early stage 5 (black arrowhead) (e). Transcripts of *BlRAD* are still accumulated to a high level in the adaxial regions including two adaxial petals and one adaxial stamen at stage 6 or early stage 7 (f). Then, the signal of *BlRAD* is rapidly reduced and barely detected in the adaxial petals and stamen (g,h). Contrast and color balance were adjusted by using PHOTOSHOP 7.0 (Adobe). b, Bract; bp, abaxial petal; bs, abaxial sepal; bst, abaxial stamen; dp, adaxial petal; ds, adaxial sepal; dst, adaxial stamen; lp, lateral petal; ls; lateral sepal; lst, lateral stamen; g, gynoecium. Bar, 200 μm.

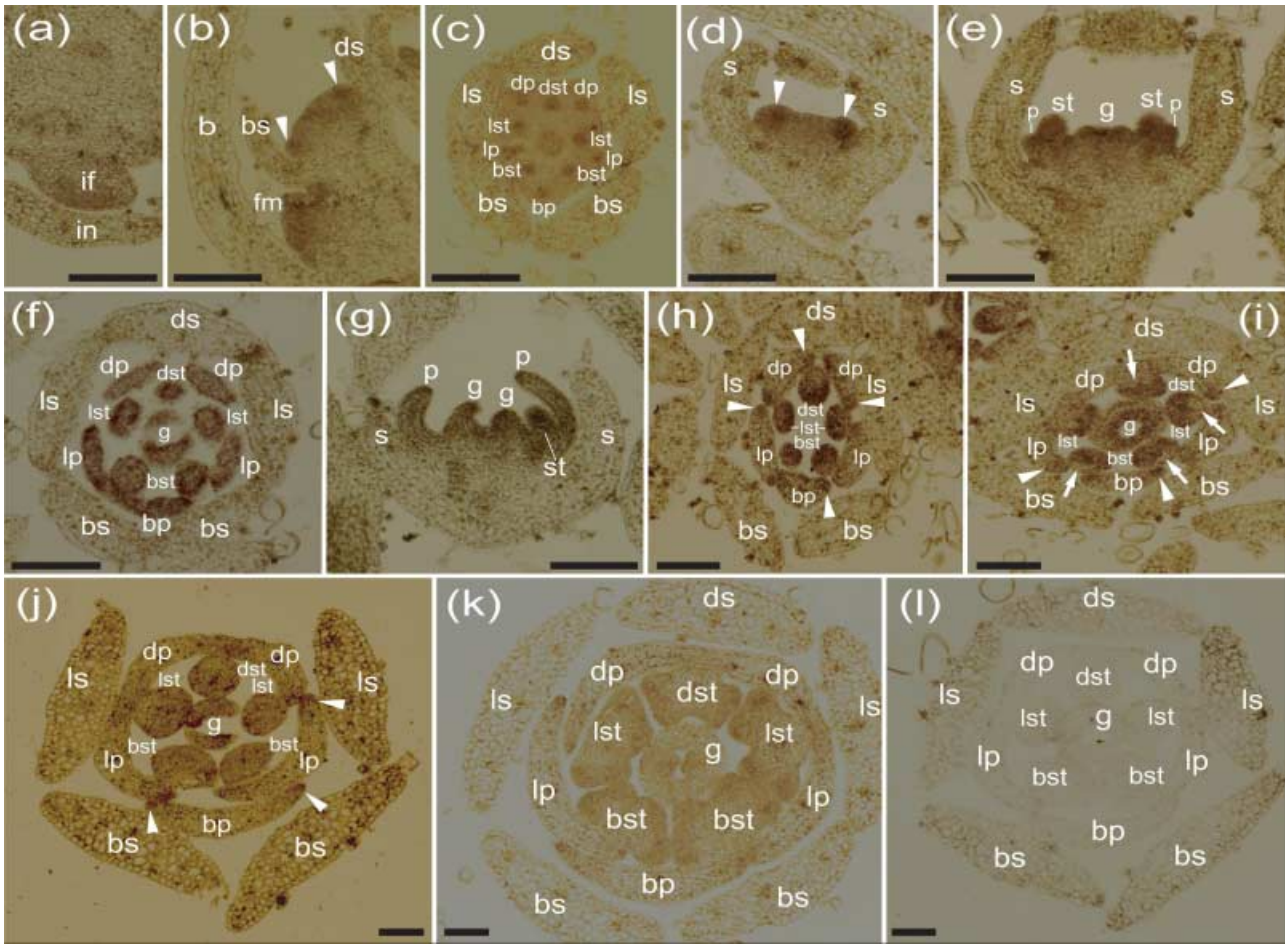

Fig. 5 Tissue-specific expression of *BlDIV1* during floral development in *Bournea leiophylla*. RNA *in situ* hybridizations was used to detect spatial and temporal expression of *BlDIV1* gene in longitudinal (b,d,e,g) and cross (a,c,f,h–l) sections of flowers at different developmental stages. *BlDIV1* transcripts are detected in inflorescence and floral meristem before initiation of floral organs (a,b). The mRNA of *BlDIV1* is specifically detected in the regions where petal or stamen primordia become visible at end of stage 4 (arrowheads) (b–d). From stage 5 to stage 6, transcripts of *BlDIV1* intensively accumulate in all petal, stamen and carpel primordia (c–f). After stage 6, the signal of *BlDIV1* sharply weakens in all petals except for the lateral edges (arrowheads) (g–j). During stages 7 and 8, the signal is still accumulated to a high level in all five stamens but is absent in small patches on the abaxial side (arrows in i) (h,i). From stage 9 onward, the expression of *BlDIV1* is gradually reduced in petals and stamens (j,k). Negative control using *BlDIV1* sense riboprobe is shown (l). Contrast and color balance were adjusted by using PHOTOSHOP 7.0 (Adobe). b, Bract; bp, abaxial petal; bs, abaxial sepal; bst, abaxial stamen; dp, adaxial petal; ds, adaxial sepal; dst, adaxial stamen; lp, lateral petal; ls, lateral sepal; lst, lateral stamen; g, gynoecium; if, inflorescence; fm, floral meristem; in, involucrum; p, petal; s, sepal; st, stamen. Bar, 200 μm.

barely detectable in the developing flower from stage 8 onward (Fig. 4 c,d).

Similar to *BlCYC1*, *BlRAD* transcripts were first detected at the adaxial side of the floral meristem (Fig. 4e). Then, *BlRAD* was highly expressed in the two adaxial petals and one adaxial stamen up to stage 6 or early stage 7 after carpel initiation (Fig. 4e–f). Like *BlCYC1*, *BlRAD* transcripts were then rapidly reduced and barely detectable from stage 8 onward (Fig. 4g,h).

Our results showed that the expression patterns of *BlDIV1* and *BlDIV2* were indistinguishable during all stages of flower development (data not shown). During stage 1 to stage 3, *BlDIV1* and *BlDIV2* mRNA accumulated in both inflorescence and floral meristems (Fig. 5a,b). Between stages 4 and 6, when floral organs were initiated, *BlDIV1* and *BlDIV2* were highly expressed in the primordia of petals, stamens and carpels (Fig. 5b–f). After stage 6, the level of *BlDIV1* and *BlDIV2* transcripts were sharply reduced in petals and their mRNA accumulation became restricted to the lateral edges of each petal (Fig. 5g–i). Transcripts of *BlDIV1* and *BlDIV2* were only detected at the lateral edges of each of the five petals at stage 8 (Fig. 5j). While greatly reduced in petals, the transcripts of *BlDIV1* and *BlDIV2* were still accumulated to a high level in all five stamens after stage 6 but were absent in small patches on the abaxial side (Fig. 5h–i). From stage 9 onward, transcript signal of *BlDIV1* and *BlDIV2* gradually became weak in petals and stamens (Fig. 5j,k).

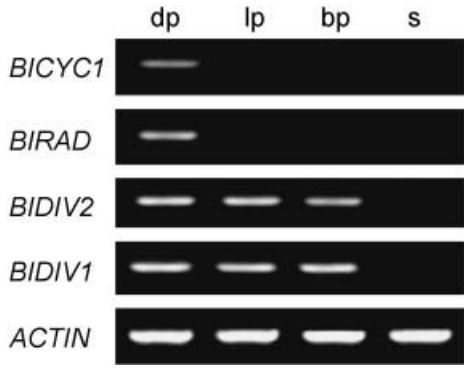

Fig. 6 Gene-specific reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *BlCYC*, *BlRAD* and *BlDIV* from dissected *Bournea leiophylla* flowers buds. Adaxial, lateral and abaxial petals were dissected from mid-to-late stage flowers as described in experimental procedures. *BlCYC1* indicates expression of *BlCYC1*. *BlRAD* indicates expression of *BlRAD*. *BlDIV1* and *BlDIV2* indicate expression of *BlDIV1* and *BlDIV2*, respectively. *ACTIN* is included as a positive control. *BlDIV1* and *BlDIV2* are detected in all petals, whereas *BlRAD* and *BlCYC1* are found only in adaxial petals. dp, Adaxial petals; lp, lateral petals; bp, abaxial petals; s, sepals.

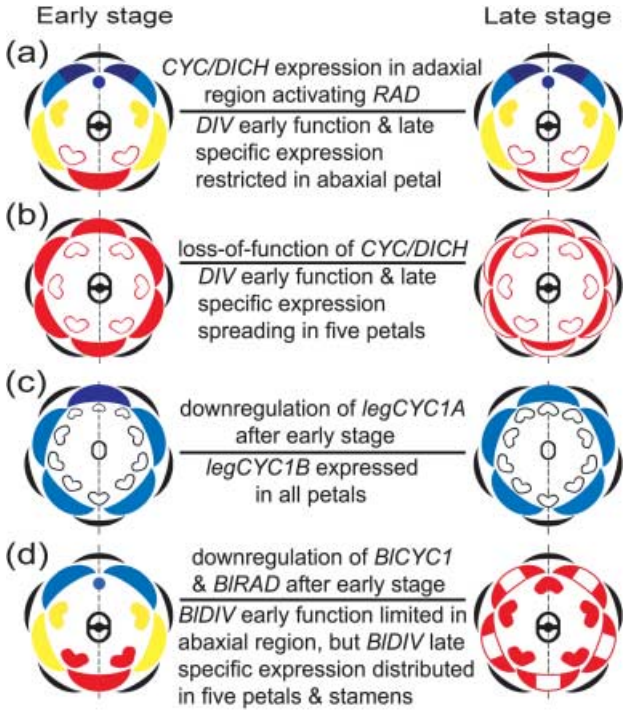

Fig. 7 Diagrammatic comparisons of expression patterns and functional interactions of related floral symmetry regulatory genes during early and late floral development in *Antirrhinum*, *Cadia* and *Bournea*. (a,b) *Antirrhinum*: (a) wild type of flower, (b) *cyc/dich* double mutant, showing interaction among *CYC*, *DICH*, *RAD* and *DIV* genes in wild and mutant flowers. (c) *Cadia*, showing interaction between *legCYC1A* and *legCYC1B* from early to late stages. (d) *Bournea*, showing expression pattern of *BlCYC*, *BlRAD* and *BlDIV* genes changed from early to late stages. Note: blue indicates adaxial petal and stamen expressed by *CYC* and *CYC*-like genes; deep blue shows overlapped expression of *CYC/DICH* or two *CYC*-like genes; blue circle indicates the adaxial stamen suppressed in growth in early stage controlled under *CYC/DICH* in *Antirrhinum* or *BlCYC* in *Bournea*, which becomes a staminode in late stage in *Antirrhinum*; yellow indicates lateral petals and stamens; red shows abaxial petal and *DIV* expression or both abaxial petals and stamens and *BlDIV* expression, in which the locally filled red color indicates intrapetal differentiation between *DIV* and *BlDIV*; stamens without filled color indicate no expression in stamens of *DIV* in *Antirrhinum* and no expression (or unknown in expression) in stamens of *legCYC1A* and *legCYC1B* in *Cadia* (It is not clear whether or not they are expressed in stamens in early floral development because the information is only shown in the reverse transcriptase polymerase chain reaction (RT-PCR) at more advanced developmental stages. See also Citerne *et al*., 2006).

To confirm our RNA *in-situ* hybridization data, we performed RT-PCR analysis of the expression of *BlCYC*, *BlRAD* and *BlDIV* genes using gene-specific primers and dissected petals from mid-to-late stages of developing flowers. The expression of *BlDIV1* and *BlDIV2* was detected in all petals (Fig. 6), while the expression of *BlCYC1* and *BlRAD* was only observed in the adaxial petals (Fig. 6). The RT-PCR results confirmed our RNA *in situ* hybridization data. Interestingly, no *BlCYC2* transcripts were detected in sepals, petals and stamens of all stages of flowers that we tested (data not shown), suggesting that *BlCYC2* may not play a significant role in floral development in *B. leiophylla*. This is not surprising because we failed to isolate *BlCYC2* from cDNA pools prepared from total RNA isolated from flower buds by RACE-PCR.

Discussion

Early zygomorphy and expression patterns of *BlCYC1*, *BlRAD* and *BlDIV*

The early pattern of *BlCYC1*, *BlRAD* and *BlDIV* expression in *B. leiophylla* is quite similar to the pattern of *CYC*, *RAD* and *DIV* expression in *Antirrhinum* (Luo *et al*., 1996, 1999; Galego & Almeida, 2002; Corley *et al*., 2005; Costa *et al*., 2005) (Fig. 7a,d). Similarly, both plants have flowers showing dorsoventral asymmetry at this early stage. In *Antirrhinum*, even though *DIV* is transcribed in all petals during early floral development, its early effect on growth is restricted to the abaxial petal and its adjacent region of the lateral petals. This is because *DIV* is inhibited post-transcriptionally by *CYC/DICH* through an antagonistic effect of *RAD* on *DIV* in the adaxial petals (Galego & Almeida, 2002; Corley *et al*., 2005) (Fig. 7a). Because of a high degree of sequence homologies and similar patterns of expression during early flower development, it is likely that a similar antagonistic interaction between BlRAD and BlDIV is involved in the growth control of the abaxial petal at the early developmental stages (Fig. 7d). By contrast, the delay in the development of two adaxial petal and one adaxial stamen primordia correlates well with the expression of *BlCYC1* and *BlRAD* being restricted to these organs. Together, our data suggest that *BlCYC1*, *BlDIV* and

BlRAD, similar to *CYC*, *DIV* and *RAD* in *Antirrhinum*, play a role in the establishment of the early zygomorphic floral development in *Bournea*.

Accumulating evidence suggests that *CYC*-like genes are widely conserved in controlling the adaxial floral organs in eudicots (Donoghue *et al.*, 1998; Citerne *et al.*, 2000, 2003, 2006; Clark & Coen, 2002; Hileman *et al.*, 2003; Hileman & Baum, 2003; Reeves & Olmstead, 2003; Smith *et al.*, 2004; Howarth & Donoghue, 2005, 2006; Feng *et al.*, 2006; Damerval *et al.*, 2007). However, the expression data of *DIV*-like and *RAD*-like genes have not been reported to date outside *Antirrhinum*, except for *RAD*-like genes recently investigated in *Arabidopsis* (Baxter *et al.*, 2007). In *A. thaliana*, *RAD*-like genes are not activated in the adaxial regions of the flower meristem where they express the *Arabidopsis CYC*-like gene *TCP1* (Costa *et al.*, 2005; Baxter *et al.*, 2007). Therefore, it is still not clear whether the basic function of *RAD* and *DIV* relating to floral symmetry exists in homologues out of *Antirrhinum*. These early expression patterns suggest that *BlCYC1*, *BlRAD* and *BlDIV* in *B. leiophylla* are homologous with *CYC*, *RAD* and *DIV* in the basic function of controlling floral dorsoventral asymmetry at the earliest stages of floral development (Fig. 7a,d).

Late actinomorphy and *BlDIV* late specific expression

The later stages of floral development as well as corresponding gene expression differ between *Antirrhinum*, which results in a zygomorphic flower at anthesis, and *Bournea*, which is nearly actinomorphic at anthesis. In *Antirrhinum*, a *cyc/dich* double mutation does not express *RAD* in the adaxial region, thus, the abaxial pattern of the late specific expression of *DIV* in *Antirrhinum* spreads to all five petals, resulting in an abaxialized peloric flower (Luo *et al.*, 1996, 1999; Galego & Almeida, 2002) (Fig. 7b). In *Bournea*, both *BlCYC1* and *BlRAD* expressions in the adaxial petals are downregulated after stage 7. Coincidentally, the pattern of *BlDIV* expression is changed in late floral development in *Bournea*. We found that the expression of *BlDIV* genes is restricted to the lateral edges of all five petals from stage 7 onward in *Bournea* (Fig. 7d). This pattern of expression is similar to the late specific expression of *DIV* in *cyc/dich* double mutants in that *DIV* is no longer restricted abaxially (Galego & Almeida, 2002), but unique in that it is only found in the lateral edges of the petals (Fig. 7d). *DIV* is mainly expressed in the inner epidermis of the furrow corresponding to the boundary between corolla tube and lobe. Therefore, there are additional differences in the intrapetal spatial patterns with respect to the late specific expression between *BlDIV* in *Bournea* and *DIV* in snapdragon (Fig. 7b,d). The downregulation of *BlCYC1* accompanied by *BlRAD* in the adaxial petals after early floral development is most likely responsible for the late *BlDIV* specific expression distributed in all five petals, which are correlated with the developmental change from initial zygomorphy to actinomorphy at anthesis in *Bournea*. Our RNA *in-situ* hybridization data further indicate that *BlDIV* transcripts were accumulated in stamen primordia during early development and were continuously accumulated in all five stamens after stage 6 except in small patches on the abaxial side. This pattern of *BlDIV* expression has not been observed for the corresponding *DIV* gene in snapdragon. However, it correlates well with the observation that all five stamens are almost equal in size at anthesis (Fig. 7d), suggesting that *BlDIV* may also play a role in stamens as in petal development in *Bournea* (Fig. 7d).

Significance of the changed pattern from zygomorphy to actinomorphy in *Bournea*

As the sister group to the remainder of Lamiales *s. l.* (Wortley *et al.*, 2005), Gesneriaceae is characteristic of predominantly weak zygomorphy with four fertile stamens plus an adaxial staminode (Endress, 1999; Cubas, 2004; Li & Wang, 2004). In the derived actinomorphic taxa in Gesneriaceae, some groups have noticeable zygomorphic vestiges, such as *Thamnocharis*, *Niphaea*, *Phinaea*, and *Bournea*, while some genera have completely actinomorphic flowers, resembling 'natural pelories', such as *Tengia*, *Bellonia* and *Marssonia* (Donoghue *et al.*, 1998; Endress, 1998; Smith *et al.*, 2004; Li & Wang, 2004). For example, the adaxial petals and stamen are usually somewhat smaller or shorter than others in *Thamnocharis* and *Bournea*, while no residual zygomorphy can be easily observed in the flowers of *Tengia* (Li & Wang, 2004). Between them, there are some taxa with almost completely actinomorphic flowers, such as *Ramonda* and *Protocyrtandra* (Donoghue *et al.*, 1998; Endress, 1998; Citerne *et al.*, 2000; Cubas, 2004). According to Citerne *et al.* (2000), *Ramonda* shows no sign of residual zygomorphy when petals and stamens are initiated. The lack of zygomorphy suggests that the *CYC* homologues in *Ramonda* may be expressed before floral organ initiation (Citerne *et al.*, 2000). In *Oreocharis*, which is closely related to *Bournea*, both in terms of morphology and *CYC* sequence homologies in Gesneriaceae (Li & Wang, 2004; Du & Wang, 2008), *ObCYC1* is expressed in the adaxial petals and stamen (staminode) throughout floral development (Du & Wang, 2008). This is consistent with mature flowers of *Oreocharis* being characteristic of a weakly zygomorphic (slightly bilabiate) corolla with four didynamous stamens plus a staminode at the adaxial position (Li & Wang, 2004; Du & Wang, 2008). Several other studies have also implicated *CYC* homologues functioning in the development of floral symmetry in Gesneriaceae. The genus *Chirita* develops zygomorphic flowers with bilabiate corolla, two fertile abaxial stamens and three staminodes at the adaxial and lateral positions. In *Chirita heterotricha*, a *CYC* homologue *ChCYC1D* is expressed in the adaxial floral region, while the expression of another copy *ChCYC1C* is expanded from the adaxial to the lateral region, correlating with the abortion of both the adaxial and lateral stamens (Gao Qiu, unpublished). This expression

pattern is similar to *McCYC/McDICH* expression in the flower of *Mohavea*, which aborts both the adaxial and lateral stamens (Hileman *et al*., 2003). In *Sinningia speciosa*, a zygomorphic species in Gesneriaceae, its actinomorphic cultivars carry a frame-shift mutation in the single *Sinningia CYC* homologue (*GCYC*) that yields a truncated protein, suggesting that the actinomorphic flowers may be caused by the mutation of *GCYC* (Citerne *et al*., 2000; Cubas, 2004). In *Arabidopsis thaliana*, a model eudicot species with ancestral actinomorphic flowers, a *CYC* homologue *TCP1* is transiently expressed at the adaxial region of the floral meristem that prepatterns the dorsoventral asymmetry (Cubas *et al*., 2001). In the basal eudicot family Papaveraceae *sensu lato*, it has been shown that the duplication and diversification of *CYC*-like TCP genes is accompanied by alterations in expression patterns, some of which play a role in floral symmetry as the adaxial identity genes (Damerval *et al*. 2007). Given that *CYC*-like genes are widely conserved in the adaxial identity function in eudicots and the expression patterns and loss-of-function of *CYC*-like genes are correlated with both natural and mutant phenotypes in Gesneriaceae, we come to the conclusion that *CYC* homologues in Gesneriaceae also function as adaxial identity genes in establishing the floral dorsoventral asymmetry. Therefore, the initial zygomorphic pattern of floral development in *Bournea* may be interpreted as a residual zygomorphy that results from the conserved pattern of early *BlCYC1* and *BlRAD* expression. The key novel event seen in *Bournea* is the downregulation of *BlCYC1* accompanied by *BlRAD* after early floral development and the correlative changes in the late specific expression of the abaxial identity gene *BlDIV* that is distributed in lateral edges of all five petals and stamens, which should be responsible for the origin of the derived actinomorphy in *Bournea*.

The epimutant flowers of *L. vulgaris* caused by heavy DNA methylation of *LCYC* provide an example that the abaxialized actinomorphic flower in nature can come about via the same mechanism that underlies the *cyc/dich* double mutant phenotype in *Antirrhinum* (i.e. loss-of-function of adaxial identity TCP genes) (Cubas *et al*., 1999a). The study in *Cadia* indicates that an adaxialized mutant resulting from ectopic expression of *CYC* homologues can also occur in natural species (i.e. the adaxial identity gene, *legCYC1B* expression distributed in all five petals) (Citerne *et al*., 2006) (Fig. 7c). However, the floral actinomorphy in *Cadia* is established in consequence of the late downregulation rather than complete loss-of-function of another copy, *legCYC1A* (Citerne *et al*., 2006). Our findings provide another example that the down-regulation of a *CYC* homologue triggers the developmental change from initial zygomorphy to the actinomorphic flower in natural species. The difference between *Bournea* and *Cadia* lies in the late specific expression of the abaxial identity gene (*BlDIV*) (Fig. 7d), rather than the expression of the adaxial identity gene (*legCYC1B*) (Fig. 7c), distributed in all five petals and stamens, which has been previously observed only in the model species snapdragon upon complete loss-of-function of *CYC*-like genes (Fig. 7b). The expression data in *Bournea*, along with previous reports, suggest that there might be diverse pathways in the origin of derived actinomorphy through modifications of a pre-existing zygomorphic developmental program. A special organ type usually involves dynamics of the gene-regulatory interaction in elaborate networks of positive and negative factors that intersect at various levels to regulate floral morphogenesis, finally creating a distinct floral architecture and form (Krizek & Fletcher, 2005). Our results further shed light on the evolution of actinomorphy from zygomorphy dependent upon the spatial and temporal changes in gene-regulatory interactions between *RAD* and *DIV* homologues promoted by *CYC*-like genes in floral development. Different shifts from zygomorphy to actinomorphy may depend on the timing of when the expression of *CYC*-like genes is downregulated in floral development, attributed to the degree of conserved early expression pattern in different lineages switching from zygomorphy to actinomorphy in Lamiales *s. l.*, especially in Gesneriaceae. Further investigation of the expression patterns and functional analyses of the key regulatory genes in additional taxa with derived actinomorphy, as well as identification of upstream primary genes responsible for the spatiotemporal switches of expression of *CYC* genes promises to shed new light on mechanisms that underlie the vast morphological diversification of derived actinomorphy in Lamiales *s. l.*

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Sequence alignment of BlCYC, BlRAD and BlDIV with other related proteins.

Text S1 Oligonucleotide sequences for primers used in this study.

Text S2 Definition and additional description of floral developmental stages in *Bournea leiophylla*.

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