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Acquisition and diversication of tendrilled leaves in Bignonieae (Bignoniaceae) involved changes in expression patterns of SHOOTMERISTEMLESS (STM), LEAFY/FLORICAULA(LFY/ FLO), and PHANTASTICA(PHAN)

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Research

Acquisition and diversification of tendrilled leaves in Bignonieae (Bignoniaceae) involved changes in expression patterns of *SHOOTMERISTEMLESS* (*STM*), *LEAFY/FLORICAULA* (*LFY/FLO*), and *PHANTASTICA* (*PHAN*)

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Summary

- Leaves have undergone structural modifications over evolutionary time, and presently exist in many forms. For instance, in Fabaceae and Bignoniaceae, leaf parts can be modified into tendrils. Currently, no data are available on genic control of tendrilled leaf development outside Fabaceae.
- Here, we conducted a detailed study of three representatives of Bignonieae: *Amphilophium buccinatorium*, *Dolichandra unguis-cati*, and *Bignonia callistegioides*, bearing multifid, trifid, and simple-tendrilled leaves, respectively. We investigated the structure of their petioles, petiolules, leaflets, and tendrils through histological analyses. Additionally, the expression of *SHOOTMERISTEMLESS* (*STM*), *PHANTASTICA* (*PHAN*), and *LEAFY/FLORICAULA* (*LFY/FLO*) during leaf development was analyzed by *in situ* hybridizations.
- Tendrils share some anatomical similarities with leaflets, but not with other leaf parts. Transcripts of both *STM* and *LFY/FLO* were detected in leaf primordia, associated with regions from which leaflets and tendril branches originate. *PHAN* expression was found to be polarized in branched tendrils, but not in simple tendrils.
- In Bignonieae, tendrils are modified leaflets that, as a result of premature completion of development, become bladeless organs. Bignonieae leaves develop differently from those of peas, as both *LFY/FLO* and *STM* are expressed in developing leaves of Bignonieae. Moreover, *PHAN* is probably involved in tendril diversification in Bignonieae, as it has distinct expression patterns in different leaf types.

Introduction

Leaves have undergone major functional and structural modifications over evolutionary time, and currently exist in a wide diversity of forms, sizes, and arrangements (Sinha, 1999; Piazza *et al.*, 2005). For example, leaf parts can turn into tendrils, which are filiform structures that twine around nearby objects in search of support for climbing (Putz & Holbrook, 1991). Diverse families, such as Fabaceae, Polemoniacae, and Bignoniaceae, have developed this strategy, and use tendrils to reach the canopy and obtain the light necessary for development (Darwin, 1875; Fischer *et al.*, 2004; Wilken, 2004).

Bignonieae is a large monophyletic tribe within Bignoniaceae characterized by the presence of two-foliolate leaves with terminal leaflets modified into tendrils, among other traits (Lohmann, 2006). In Bignonieae, leaves develop acropetally (Sousa-Baena *et al.*, 2014), and tendrils may be simple or divided in different ways varying from bifid to trifid or multifid (Lohmann, 2006; Figs 1, 2a–c). Ancestral character state reconstructions allied to developmental analyses suggest that trifid tendrils arose early in Bignonieae and that the evolution of different tendril types involved heterochrony (Sousa-Baena *et al.*, 2014).

Angiosperm compound leaf development is generally controlled by *class I Knotted-like* (*KNOXI*) genes (Bharathan *et al.*, 2002) which are responsible for maintaining the meristematic state of leaf margins. These genes are down-regulated in leaf founder cells but reactivated later in leaf development during leaflet formation. This mechanism seems to be the most common for the maintenance of the undifferentiated state in the leaf margins of angiosperms during secondary morphogenesis, allowing for greater elaboration of leaf blades (Bharathan *et al.*, 2002).

In contrast, in pea (*Pisum sativum*) and other species belonging to the inverted-repeat-lacking clade (IRLC; Wojciechowski *et al.*, 2000; Hofer *et al.*, 2009) of legumes, *LEAFY/FLORICAULA* (*LFY/FLO*) is employed in the maintenance of the meristematic state in primordium marginal tissue, replacing the function of

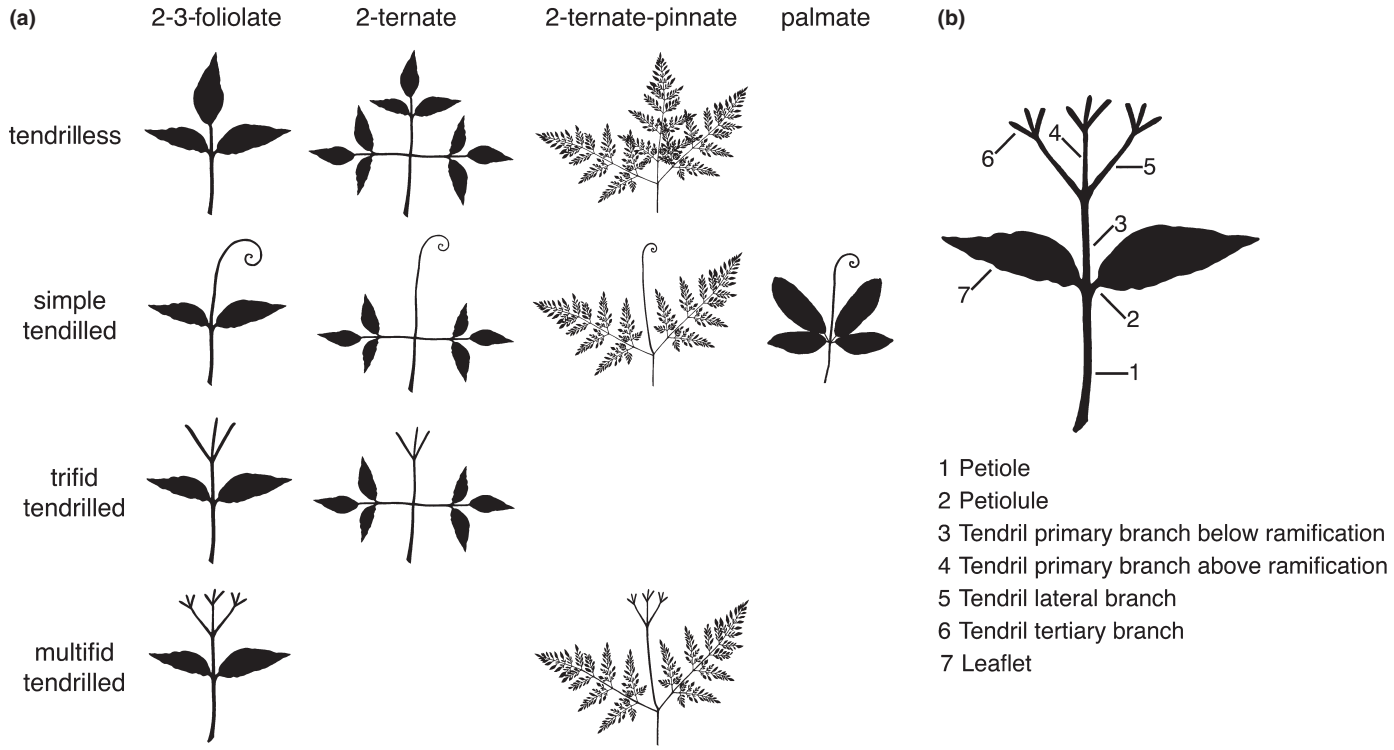

Fig. 1 Schematic diagrams showing leaf structure and diversity in Bignonieae. (a) Schematic drawings illustrating types of leaf and tendrils in Bignonieae species. (b) Parts of the Bignonieae adult leaf.

KNOX1 completely (Hofer *et al.*, 1997; Gourlay *et al.*, 2000). *LFY/FLO* is expressed in leaf primordia of many simple and compound-leaved species besides peas (Busch & Gleissberg, 2003); however, functional studies have shown that *LFY/FLO* has only a minor role during leaf development in those species. For instance, it was observed that in soybean (*Glycine max*; a legume outside the IRCL clade), the silencing of *LFY/FLO* only led to a reduction in the number of leaflets on the second node (Champagne *et al.*, 2007).

Recently, *PRESSED FLOWER/WUSCHEL*-related Homeobox 3 (*PRS/WOX3*) and *WOX1* were shown to have an important role, downstream of polarity genes, in Arabidopsis leaf margin development (Nakata *et al.*, 2012). Furthermore, in maize (*Zea mays*), *NARROWSHEATH1* (*NS1*) and *NS2* encode proteins similar to the Arabidopsis *WUSCHEL*-related homeodomain protein *PRESSED FLOWER* (Matsumoto & Okada, 2001); more specifically, the *NS1/2* double mutant in maize shows that *WOX3* is essential for marginal meristem activity in this species (Nardmann *et al.*, 2004).

KNOX1 genes play important roles in leaf development through interaction with other genes. In tomato (*Lycopersicon esculentum*) and Arabidopsis, *asymmetric leaves/rough sheath/phantastica* (*ARP*) genes (*Asymmetric leaves* in Arabidopsis, *Rough sheath 2* in maize, and *Phantastica* in *Antirrhinum majus*) repress the expression of *KNOX1*, inducing cells to enter the differentiation pathway (Byrne *et al.*, 2000; Koltai & Bird, 2000). In simple-leaved plants, *KNOX1* and *ARP* are expressed in complementary and nonoverlapping patterns (Tsiantis *et al.*, 1999; Byrne *et al.*, 2000). However, their expression is temporally and spatially coincident during compound leaf development; this concerted expression is thought to have enabled the rise of compound leaves in angiosperms (Koltai & Bird, 2000; Kim *et al.*, 2003b).

ARP genes are also involved in the establishment of the abadaxial polarity of leaves in Euasterids (Kidner & Timmermans, 2007). In *A. majus*, tomato, and *Nicotiana*, the suppression of *ARP* results in the development of needle-like leaves that are abaxialized (Waites & Hudson, 1995; Kim *et al.*, 2003a; McHale & Koning, 2004). In pea, *ARP* was shown to be involved in the adaxial fate acquisition of leaflets, but not in tendril formation (Tattersall *et al.*, 2005). Thus, *ARP* genes probably play distinct roles during the development of compound leaves controlled by *LFY/FLO* and *KNOX1*.

Considerable amounts of information are available on the development of leaves of model organisms (Tsukaya, 2010). However, the only tendrilled species in which leaf development has been studied in detail is pea. Thus, little is still known about the molecular regulation of the development of foliar tendrils outside the Fabaceae. Here, we studied the structure and evolution of leaves in Bignonieae, and compared them to the general organization of leaves in pea in order to trace parallels in the anatomical organization of leaf parts among these species and investigate the origins of tendrils in Bignonieae. To investigate whether the development of leaves in Bignonieae was controlled by *LFY/FLO* and not by *SHOOTMERISTEMLESS* (*STM*), we isolated orthologs of both genes in representatives of Bignonieae with different tendril types, and analyzed their expression patterns through *in situ* hybridizations during leaf development. In

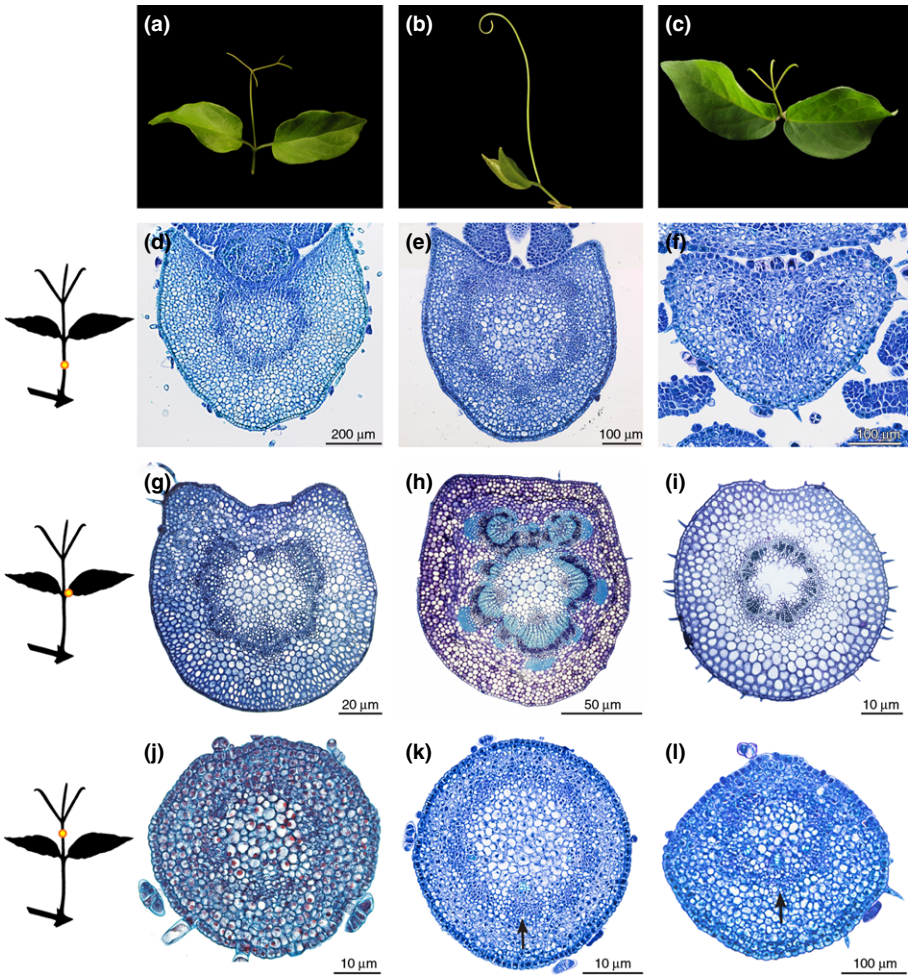

Fig. 2 Leaf morphology and anatomy of Bignonieae species. (a, d, j) *Amphilophium buccinatorium*. (b, e, k) *Bignonia callistegioides*. (c, f, l) *Dolichandra unguis-cati*. (a–c) Morphology of adult leaves. (d–f) Anatomical sections of young petioles. (g–i) Anatomical sections of young petiolules. (j–l) Anatomical sections of young tendrils. The leaf cartoon in the right column indicates the placement of the individual sections presented in (d)–(l).

addition, we cloned *PHANTASTICA* (*PHAN*), and analyzed its expression pattern through RNA *in situ* hybridizations to investigate the establishment of polarity in tendrils. We relate patterns of expression of *PHAN*, *STM*, and *LFY/FLO* to leaflet and tendril development, and discuss possible roles of these genes during leaf development in Bignonieae, as well as their involvement in the diversification of leaf morphology.

Materials and Methods

Taxon sampling

For this study, we selected three species of Bignonieae with different tendril types: *Bignonia callistegioides* Cham. (simple-tendrilled), *Dolichandra unguis-cati* (L.) L.G. Lohmann (trifid-tendrilled), and *Amphilophium buccinatorium* (DC.) L.G. Lohmann (multifid-tendrilled). Samples were collected from adult individuals growing in different localities at Davis, CA (USA). For details, see Supporting Information Table S1.

Anatomical study

Histological analyses were conducted in young and mature leaflets, petioles, petiolules, and tendrils. Leaves were collected from two individuals and four leaves were analyzed per species. Fresh samples and embedded material were used in these analyses, as follows.

Embedded material Shoot apices were fixed for 16 h in 4% paraformaldehyde. Apices were subsequently dehydrated in a graded ascending series of ethanol, and gradually embedded in paraplast (Garcês & Sinha, 2009a). Embedded specimens were sectioned using a rotary microtome, mounted on slides, and stained with toluidine blue (O'Brien *et al.*, 1964). Sections were permanently mounted with 'permount'.

Fresh material Hand-sectioned material was cut in water, and then clarified in 50% bleach for 5 min. The material was subsequently rinsed in water five times and then stained with toluidine blue for 30 min. Sections were then rinsed in water until excess dye had been removed, and were mounted on 50% glycerol.

Gene cloning

Total RNA was extracted from shoot apices from *B. callistegioides*, *D. unguis-cati*, and *A. buccinatorium* using the RNeasy Mini Kit (Qiagen). For each sample, cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen).

Gene fragments were cloned through RT-PCR, which was run for 40 cycles.

Degenerate primers described by Uchida *et al.* (2007) were used for *STM* amplification, while specific primers were designed for *ARP* and *LFY/FLO* based on *A. majus* sequences (AJ005586 and M55525, respectively; Table S2). The expected size fragments were cloned into the pCR®2.1-TOPO® vector (Invitrogen) and sequenced using the T7 promoter primer. Bignonieae sequences were compared against GenBank using the BLASTN algorithm at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) to confirm identity.

Phylogenetic analysis

A phylogenetic analysis was performed based on the deduced amino acid alignment, along with the sequences from *A. majus*, sequences from other species belonging to Lamiales, and sequences from model species (see Figs S1–S3, and species selected for the phylogenetic analysis in Tables S3–S5). The alignment was performed using CLUSTAL W1.4 within BIOEDIT 7.0.5.3 and edited using the same version of BIOEDIT (Hall, 1999). Residues 1–154 of *STM*, 87–266 of *ARP*, and 184–278 of *LFY/FLO* were selected for the phylogenetic analysis. Gene trees were estimated using maximum likelihood criteria and the software MEGA 5.05 (Tamura *et al.*, 2011). The Jones–Taylor–Thornton model of amino acid change was used for the phylogeny reconstruction. Rate variation among sites was considered uniformly distributed. Gaps and missing data were eliminated only in pairwise sequence comparisons. Tree support was estimated through maximum likelihood (ML) bootstrap, using 1000 replications.

Gene expression

We investigated expression patterns of *STM*, *PHAN*, and *LFY/FLO* in the three representatives of Bignonieae through *in situ* hybridizations. Apices were collected from two individuals for each species, and a minimum of ten shoot apices per species were used for the analysis of each gene. Shoot apical meristems (SAMs) were processed following the protocol of Garcês & Sinha (2009a). Riboprobes were generated as described in Garcês & Sinha (2009b). Probes of 682, 509, and 286 bp were used for the *STM*, *ARP*, and *LFY/FLO* hybridizations, respectively. Probe hybridization, washing, and immunolocalization followed Garcês & Sinha (2009b). Hybridization was performed at 53°C for *STM* and *ARP*, and at 54°C for *LFY/FLO*. For details of the probe concentrations used for each gene, see Table S6. Nomenclaturally, we followed leaf developmental stages previously established for the Bignonieae by Sousa-Baena *et al.* (2014). Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: JN182849 (*AbSTM*), JN182846 (*AbPHAN*), JN182843 (*AbFLO*), JN182851 (*DuSTM*), JN182848 (*DuPHAN*), JN182845 (*DuFLO*), JN182850 (*BcSTM*), JN182847 (*BcPHAN*), and JN182844 (*BcFLO*).

Ancestral leaf reconstruction

The single tree that resulted from the ML analysis of a combined molecular data set (*Pep*C + *ndh*F; Lohmann, 2006) was used to reconstruct the ancestral states of the character 'leaf type.' Coding of the character 'leaf type' for all 104 species was extracted from the morphological matrix of Lohmann (2003), and its respective states were coded as discrete, nonoverlapping, and multi-state. Leaf type reconstruction was conducted under ML assumptions (Maddison & Maddison, 2009), with the character treated as unordered and unweighted.

In Bignonieae, leaf morphological characteristics were employed to code two distinct phylogenetic characters: leaf type, which takes into account general patterns of ramification of the leaf main axis, as well as that of the leaflets; its states are two–three-foliolate, palmate, two-ternate, and two-ternate-pinate (Fig. 1a); and tendril type, which relates specifically to the terminal portion of leaves, which are often replaced by tendrils of different forms; its states are simple, bifid, trifid, and multifid (Fig. 1). In order to understand the evolution of leaves as a whole entity in Bignonieae, we reconstructed the ancestral states of the character leaf type, and indicated the evolutionary shifts that occurred in the character tendril type on the resulting tree. Data on the evolution of tendril type were retrieved from ancestral character state reconstructions conducted under ML assumptions (Sousa-Baena *et al.*, 2014).

Results

Anatomical analyses

Petiole symmetry Petioles from all species studied are bilaterally symmetrical; this arrangement can be identified by the shape of the petioles in cross-section, and by the arrangement of their vasculature, which is generally an open arc. In cross-section, young petioles of *A. buccinatorium* and *B. callistegioides* are semicircular, with vascular tissues also arranged as such (Fig. 2d,e). However, young petioles of *D. unguis-cati* are triangular, with vascular tissues arranged in an open V-shaped arc (Fig. 2f).

Petiolule symmetry In cross-section, the shape of the petiolules is similar to that of the petioles in *A. buccinatorium* and *B. callistegioides* (Fig. 2g,h). In *B. callistegioides*, the organization of petiolules is similar to that of the petioles. The major difference in the anatomy between the two structures is the presence of two accessory bundles that develop facing the adaxial side in the petiolules (Fig. 2h). In *D. unguis-cati*, the petiolule is nearly round in cross-section, with the vasculature arranged in a ring (Fig. 2i).

Tendril symmetry All three species have tendrils that are bilaterally symmetrical, such characteristic is evidenced by a larger vascular bundle that is formed on the abaxial side (Fig. 2j–l). *Bignonia callistegioides* presents the most radialized shape of the three species, bearing tendrils that are circular in cross-section (Fig. 2k). In *D. unguis-cati*, the tendril is widely ovate and the

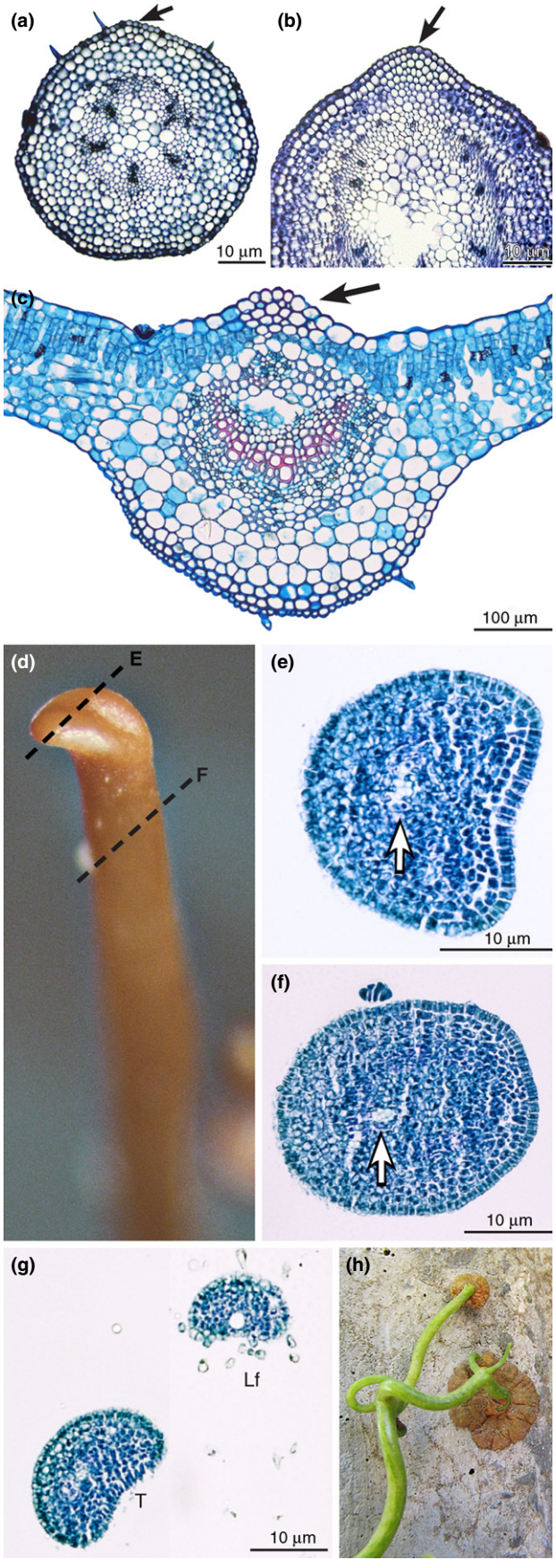

Fig. 3 Anatomical structure of tendrils and leaflets of *Dolichandra unguis-cati* (a–c) and *Amphilophium buccinatorium* (d–h). (a, b) Tissue projection (arrows) on the adaxial side of tendrils. (c) The arrow indicates the tissue projection on the adaxial side of the leaflet midvein region. (d) Leaf-like expansion at the tendril tip. The dashed lines 'E' and 'F' indicate the regions where sections presented in (e) and (f) were taken. (e, f) Serial transversal sections of a young tendril, showing leaf-like expansion at the tip (e), and the round shape of the tendril in the region below the tip (f). Arrows indicate vasculature in the initial development stage. (g) Section illustrating the similar shape of the tendril tip (T) and leaflet (Lf) in young leaves. (h) Adhesive disks at the tip of a mature tendril.

vascular system is open, with a gap facing the adaxial surface (Fig. 2l). Furthermore, tendrils and leaflets of *D. unguis-cati* share a similar morphological feature, that is, the same ribbed projection is seen in the adaxial side of both structures (Fig. 3). In *A. buccinatorium*, a flattened leaf-like expansion develops in tendril tips (Fig. 3d,e); however, the tendril is cylindrical immediately below this structure (Fig. 3f,g). In particular, the tendril tip has a semicircular shape in cross-section similar to that of the leaflet primodium tip (Fig. 3g). In *A. buccinatorium* in particular, the leaf-like expansion undergoes a massive proliferation and originates an adhesive disk (Fig. 3h) whenever the tendril is fully developed and finds a support to which to attach.

Gene cloning and orthology

Partial sequences of *STM*, *ARP*, and *LFY/FLO* orthologs for *A. buccinatorium*, *D. unguis-cati*, and *B. callistegioides* were cloned. The 682-bp *STM* fragment comprises the 5′ untranslated region (UTR) and part of the coding sequence (CDS) that includes part of the KNOX domain (Fig. S4a). The *ARP* ortholog fragment comprises *c.* 509 bp of the CDS, including the end of the second MYB repeat (Fig. S5a). For *LFY/FLO*, the cloned fragment spans 286 bp corresponding to a highly conserved portion of the third exon (Fig. S6a). The fragments isolated from *A. buccinatorium* were named *AbSTM*, *AbPHAN*, and *AbFLO*; fragments isolated from *D. unguis-cati* were named *DuSTM*, *DuPHAN*, and *DuFLO*; and fragments isolated from *B. callistegioides* were named *BcSTM*, *BcPHAN*, and *BcFLO*.

The deduced amino acid sequences of *STM*, *PHAN*, and *FLO* cloned from Bignonieae species were aligned with orthologs from several angiosperm species to assess the degree of similarity among them, and investigate whether the obtained sequences were genuinely orthologous to the corresponding reference genes through phylogenetic analyses. Sequence analyses and the gene trees reconstructed in this study confirmed that *BigSTM*, *BigPHAN*, and *BigFLO* (Figs S4–S6, respectively) are indeed the Bignonieae orthologs of the well functionally characterized genes *STM*, *PHAN*, and *LFY/FLO* involved in leaf development control in model species.

Gene expression

SHOOTMERISTEMLESS Expression of *STM* was detected in the SAM, leaf primordia, and axillary buds of the three

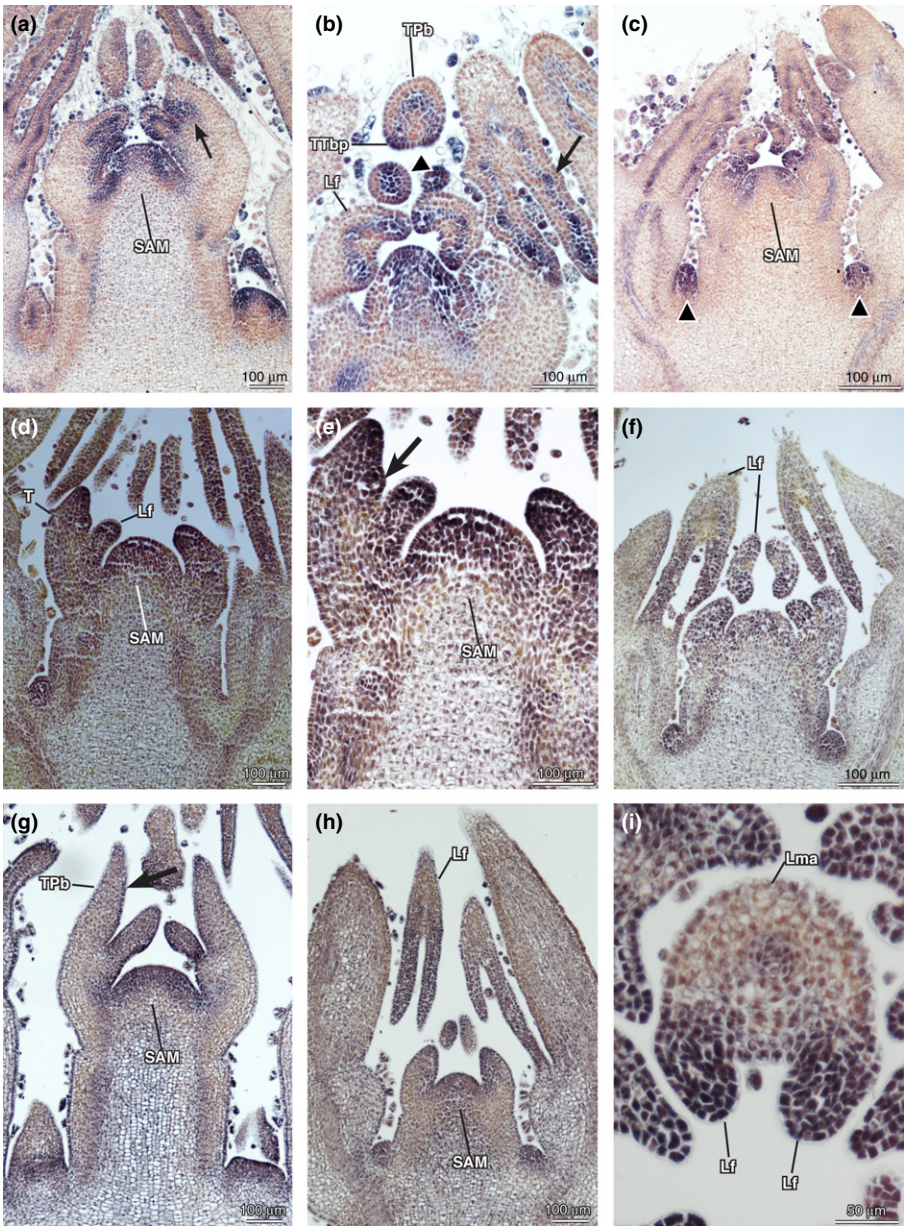

Fig. 4 *In situ* hybridizations of *SHOOTMERISTEMLESS* (*STM*) in leaf shoot apices of Bignonieae species. (a–c) *Amphilophium buccinatorium*. (d–f) *Dolichandra unguis-cati*. (g–i) *Bignonia callistegioides*. (a) *STM* expression is confined to the adaxial region of the tendril primordium (arrow). (b) Transverse section illustrating *AbSTM* expression in the adaxial side of lateral branches of the tendril (arrowhead), in the region of the primary branch (TPb) where the tertiary branches (TTpb) are being formed, in the boundary between the abaxial and adaxial domains on leaflet primordia, and in older primodium lateral veins (arrow). (c) Strong expression of *STM* in axillary buds (arrowheads), and lack of expression in the center of the shoot apical meristem (SAM). (d, e) *DuSTM* is strongly expressed in tendril and leaflet primordium tips, including the region where lateral branches are developing (arrow) in (e). (f) Expression of *DuSTM* in the developing lamina of leaflet primordia. (g) Tendril primary branch showing a lack of expression of *BcSTM* (arrow) in P2. (h) Older leaf primordium showing expression of *BcSTM* throughout the developing lamina. (i) Transverse section of leaf primordium at the P2 stage showing strong expression of *BcSTM* during leaflet development. Lf, leaflet primordium; Lma, leaf main axis; TPb, tendril primary branch; T, tendril primordium; TTbp, tendril tertiary branch primordium.

species analyzed (Fig. 4). All species have opposite leaves, which allowed us to identify a lack of expression of *STM* in the middle of the SAM (P0), in longitudinal sections that passed through the peripheral portions of the stem (Fig. 4c). However, sections passing through the stem midplane showed expression of *STM* in all other regions of the SAM (Fig. 4d,e,g). High levels of expression of *STM* were detected in the leaflet primordia of all species (Fig. 4a,d,g), with *STM* expressed through the whole primordia during the earlier stages of leaf development (Fig. 4i). However, *STM* transcripts were detected in the boundary between adaxial and abaxial leaflet surfaces (middle domain) and in the midrib vasculature in later stages of development (P3 and P4; Fig. 4b,f).

In the two species with branched tendrils, *A. buccinatorium* and *D. unguis-cati*, high levels of *STM* expression were detected in primary branch tips, in the region where tendril lateral branches were developing (Fig. 4b,e). In *A. buccinatorium*, *STM* mRNA was also detected in the middle domain of the tendril primary branch at late P3 (Fig. 4b), which seems to mimic the expression pattern found in leaflet primordia. In addition, *STM* expression was detected in the adaxial domain of lateral branches of tendrils (Fig. 4b). In *B. callistegioides* (simple-tendrilled), *STM* was expressed strongly in leaflet primordia, and weakly expressed in the adaxial domain of tendril primordia (Fig. 4g,i). Moreover, *STM* mRNA was detected in the developing blade of young leaves, where its expression was uniform through the lamina in *D. unguis-cati* and *B. callistegioides* (Fig. 4f,h), but stronger in lateral veins of *A. buccinatorium* (Fig. 4b).

PHANTASTICA *PHAN* transcripts were detected in the SAM, leaf primordia, and axillary buds of all species studied (Fig. 5);

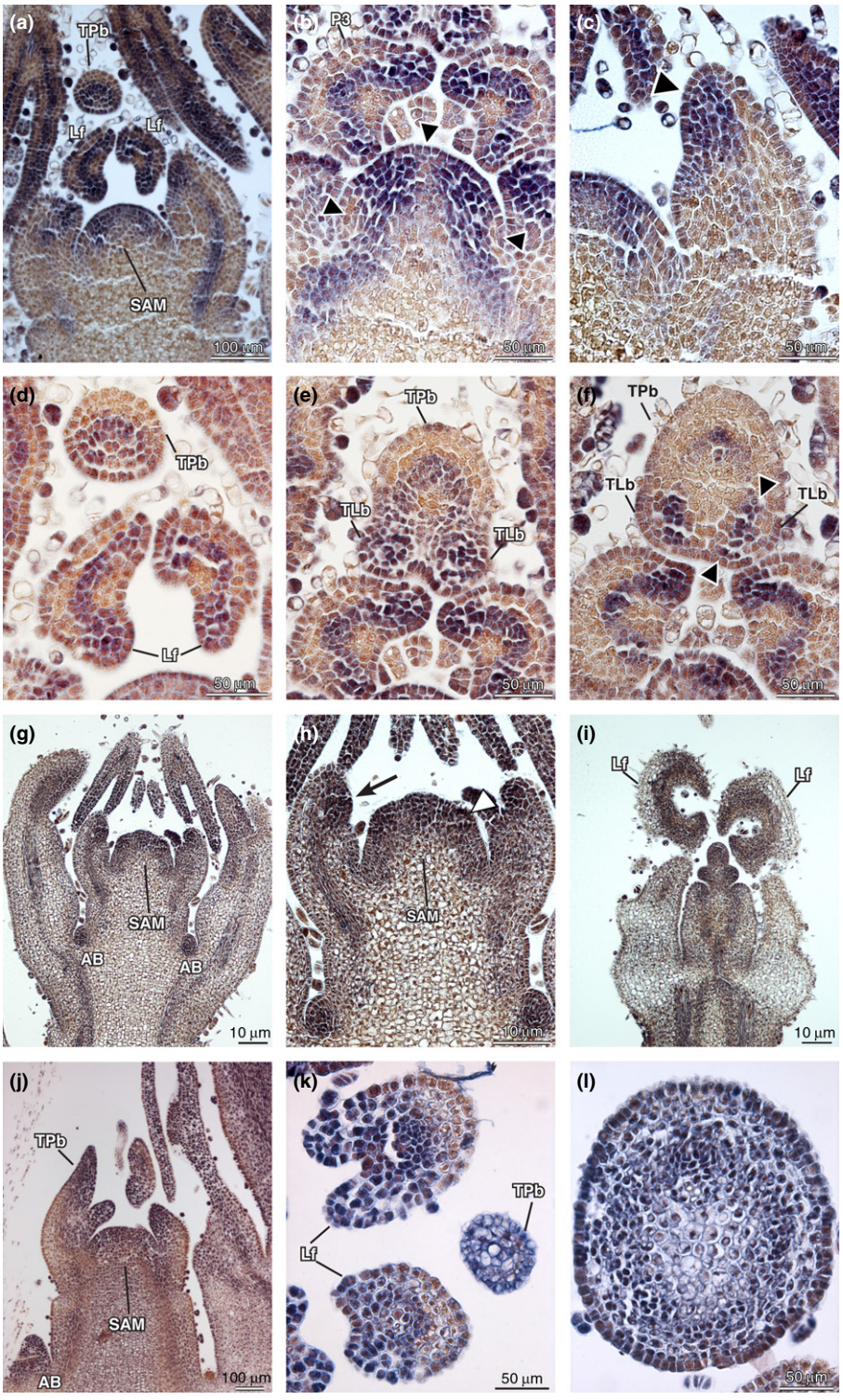

Fig. 5 *In situ* hybridizations of *PHANTASTICA* (*PHAN*) in shoot apices of Bignonieae species. (a–f) *Amphilophium buccinatorium*. (g–i) *Dolichandra unguis-cati*. (j–l) *Bignonia callistegioides*. (a, g, j) All species show accumulation of *PHAN* transcripts in the shoot apical meristem (SAM), leaflet primordia, tendril primordia, and leaf primordium vasculature. (b) *AbPHAN* transcripts were not detected in the flanks or center of the SAM (arrows). *AbPHAN* transcripts accumulated in the leaflet midrib vasculature and margins of P3. (c) Expression of *AbPHAN* concentrated in the adaxial region of the tendril primordium tip (arrowhead). (d) *AbPHAN* expression was detected in the adaxial side of the tendril primary branch (TPb) and in leaflet primordia. (e) Strong expression of *AbPHAN* in the region of the tendril where lateral branches (TLb) are developing from the primary branch (TPb). (f) Accumulation of *AbPHAN* transcripts in the adaxial domain of the tendril lateral branch primordia (arrowheads) in a section passing immediately below the section presented in (e). (h) Expression of *DuPHAN* in the flanks of the SAM (white arrowhead) where new leaves are beginning to develop and during formation of lateral branches of tendrils (arrow). (i) Expression of *DuPHAN* in the adaxial region of older leaflet primordia. (k) Expression of *BcPHAN* in the adaxial side of the leaflet primordium, and throughout the tendril tip. (l) The tendril median region showing the expression of *BcPHAN* throughout the organ. AB, axillary bud; TLb, tendril lateral branch; Lf, leaflet primordium; TPb, tendril primary branch. The developmental stage of leaf primordia is indicated by P#, where '#' gives the stage of development of the primordium in question.

however, expression patterns varied among species. In *A. buccinatorium* (multifid-tendrilled), no *AbPHAN* mRNA was detected in the center and flanks of the SAM at the more peripheral sections of the shoot apex (Fig. 5b). However, *AbPHAN* transcripts were detected in the whole SAM in sections passing through the midplane of the stem (Fig. 5a). *AbPHAN* was expressed strongly in tips of tendrils confined to the adaxial region, at P1, and in the vasculature of the leaf primordia (Fig. 5a). Serial sections of P3 showed that *AbPHAN* is also expressed in the midrib vasculature of the leaflet primordia, in the middle domain (Fig. 5d), and in the adaxial region of tendril tips (Fig. 5c). Sections in the region where tendril lateral branches were developing showed that *AbPHAN* is expressed strongly in lateral branch primordia (Fig. 5e). However, expression of

AbPHAN was weaker in the tendril region below the insertion of the lateral branches, and was confined to a smaller domain of the adaxial region (Fig. 5f). In older primordia, *AbPHAN* was detected in developing lamina (Fig. 5a).

In *D. unguis-cati* (trifid-tendrilled), strong *DuPHAN* expression was detected in the SAM, including the flanks where new leaves were developing (Fig. 5g,h). Additionally, *DuPHAN* transcripts were detected in the leaf primordia, where lateral branches were being formed and in leaflet primordia (Fig. 5h). In P4, *DuPHAN* transcripts accumulated in the adaxial side of leaflets (Fig. 5i).

In *B. callistegioides* (simple-tendrilled), *BcPHAN* expression was detected throughout leaflet primordia, as well as in tendril tips at the P2 stage (Fig. 5j). At later stages, *BcPHAN* expression was concentrated in the adaxial region of leaflet primordia and in their midrib vasculature (Fig. 5k). In addition, *BcPHAN* expression was detected throughout the tendril tip and median region (Fig. 5k,l).

FLORICAULA *FLO* transcripts were detected in the SAM, in the leaf primordia, and in the axillary buds of all three species (Figs 6a–c, 7a,e). In *A. buccinatorium*, *AbFLO* mRNA accumulated at the tip of the leaf primodia, especially on the adaxial side of the primordia (Fig. 6b). Transverse serial sections from the shoot apex of *A. buccinatorium,* including P1 to P3 primordia, revealed that *AbFLO* expression in the developing lateral branch of the tendril (Fig. 6e–g) is a reiteration of the pattern found in primary branches and leaflets, that is, a broader expression domain comprising the whole adaxial domain in their tips (Fig. 6d,h,k relating to P3, P2, and P1, respectively), and a more restricted domain, becoming confined to the middle domain, in their median region (Fig. 6g,i,l relating to P3, P2, and P1, respectively). At P3, *AbFLO* expression was clearly associated with the emergence of tendril lateral branches (Fig. 6e,f), and at P2 and P1 with the rising of leaflets (Fig. 6j,l, respectively). *AbFLO* expression was restricted to vascular traces in the basal region of leaf primordia at P3 (Fig. 6j,k) and in petioles at P2 and P3 (Fig. 6n,o). Furthermore, *AbFLO* was expressed in the tip of the SAM (Fig. 6l), with sections below this region showing that *AbFLO* expression was excluded from the flanks of the SAM (Fig. 6m,n).

The expression domain of *FLO* was broader in *D. unguis-cati* and *B. callistegioides*. In the SAM of both species, *FLO* was expressed in the flanks, in several layers below the protodermis, and in the center of the meristem (Fig. 7a,e). However, in *B. callistegioides*, the expression domain was broader, with transcripts found in the young stem (Fig. 7e,f). *FLO* was expressed strongly in the developing leaves in the flanks of the meristem (Fig. 7b) and in the adaxial side of the leaflet and tendril primordia (Fig. 7c,g). In *D. unguis-cati*, *DuFLO* was highly expressed in the tendril primordium region where lateral branches were being formed (Fig. 7c,d). By contrast, *FLO* expression was excluded from the region marking the boundary between emerging leaves and meristem flanks in both species (Fig. 7a,f). More specifically, in *D. unguis-cati*, a few cells from the ground meristem, subjacent to P1, did not express *DuFLO*, as well as a constricted region of the protodermis, comprising approximately five cells (Fig. 7b). Moreover, in both species expression of *FLO* was also lacking from a band of cells subtending the axillary portion of leaflet primordia, at the boundary between P3 and the meristem (Fig. 7c,f).

Ancestral leaf reconstructions

The ancestral leaf type of the tribe Bignonieae was reconstructed as two-ternate (58%; Fig. 8), and the 'Core Bignonieae' node as bearing two–three-foliolate leaves with trifid tendrils (84%; Fig. 8). There have been five evolutionary shifts to simple-tendrilled leaves from the node 'Core Bignonieae': in *Adenocalymma*, in internal nodes of *Bignonia* and *Lundia*, in *Tanaecium*, and in an internal node of the clade '*Fridericia* and allies' (Sousa-Baena *et al.*, 2014; Fig. 8). Two reversals to two–three-foliolate trifid-tendrilled leaves occurred: in *Tanaecium*, and in the ancestor node of *Tynanthus* (Fig. 8).

In contrast, some leaf forms have arisen by changes in the lateral leaflets only, as in *Xylophragma* and *Cuspidaria* (Fig. 8). Tendrils were lost in ten species, generating three-foliolate leaves. Two of these losses were from trifid-tendrilled ancestors, while eight losses were from ancestors bearing two–three-foliolate simple-tendrilled leaves (Fig. 8). In *Adenocalymma*, a tendril-less leaf evolved from an ancestor that presented two-ternate-pinate leaves with a terminal simple tendril, through replacement of the simple tendril by a pinnate leaflet (Fig. 8).

Two lineages showed higher levels of diversification in leaf morphology: *Adenocalymma*/*Neojobertia* and *Pleonotoma*/*Manaosella*. The ancestor node of the *Adenocalymma*/*Neojobertia* clade presented two–three-foliolate trifid-tendrilled leaves. From this node derived the ancestor of *Neojobertia*, which was probably two-ternate trifid-tendrilled, and the ancestor of *Adenocalymma*, which was probably two–three-foliolate simple-tendrilled. In *Neojobertia*, a more complex leaf arose from the genus most recent common ancestor; this leaf presented pinnate leaflets and multifid tendrils (Fig. 8). Within *Adenocalymma*, three different leaf forms evolved: a three-foliolate leaf; a two-ternate-pinate and simple-tendrilled leaf; and a two-ternate-pinate tendrilless leaf (Fig. 8).

Three evolutionary shifts from two- to three-foliolate trifid-tendrilled ancestors were observed within *Pleonotoma*/*Manaosella*: one in which tendrils became multifid; one in which the tendril type was maintained and leaflets became ternate; and one in which tendrils became simple, and leaflets pinnate (Fig. 8).

Discussion

Symmetry of tendrils and other leaf parts in Bignonieae

In order to further understand the origin of an apparently radial structure (tendrils) from an organ with dorsoventral polarity (leaves), we analyzed and compared histological sections of leaflets, petioles, petiolules, and tendrils in an attempt to establish a connection between the plane of symmetry of these structures

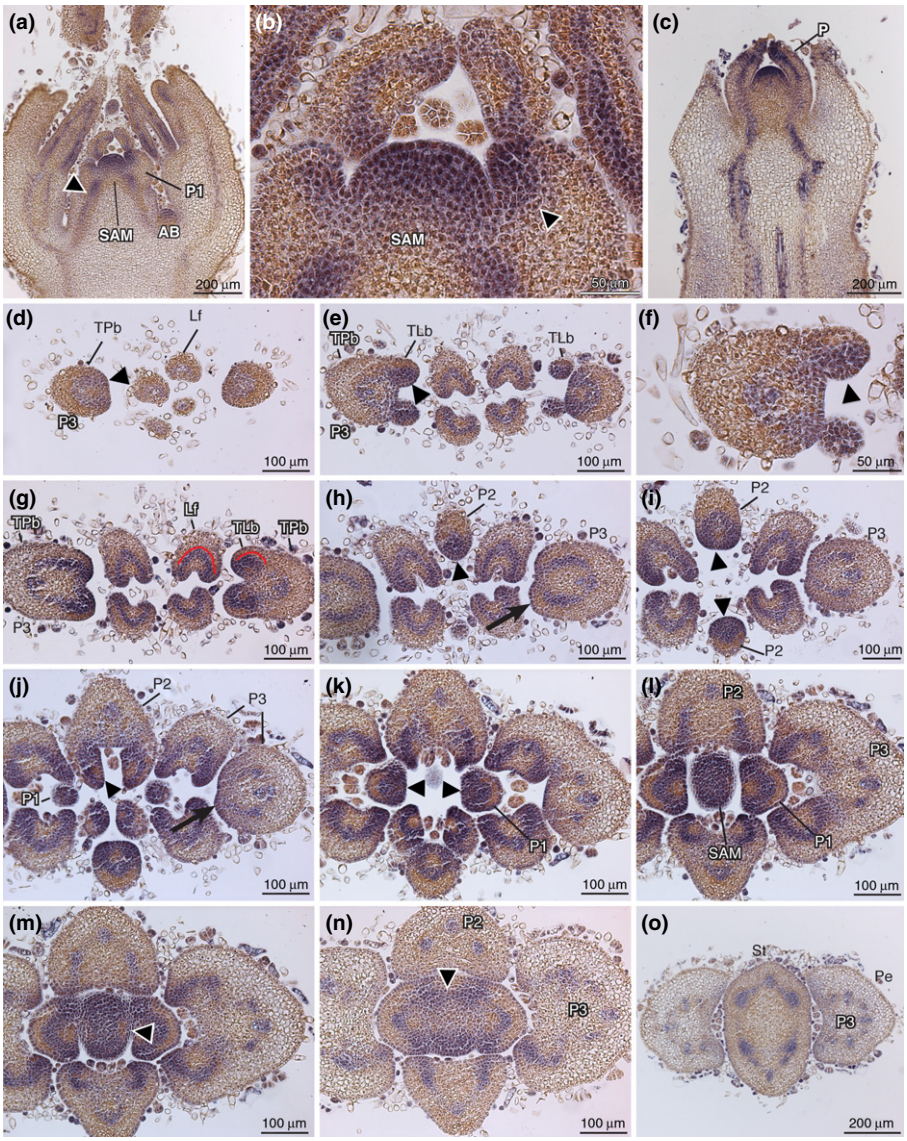

Fig. 6 *In situ* hybridization of *FLORICAULA* (*AbFLO*) in shoot apices of *Amphilophium buccinatorium*. (a) Expression of *AbFLO* in the shoot apical meristem (SAM), leaf primordia, and stem vascular traces (arrowhead). (b) Strong expression of *AbFLO* in the adaxial region of the leaf primordium tip (arrowhead). (c) *AbFLO* transcripts detected in axillary buds and in the vasculature of the prophylls. (d–f) Transcripts of *AbFLO* concentrated where tendril lateral branches are developing at P3 (arrowheads). (g) Expression of *AbFLO* in the vasculature and in the boundary between the abaxial and adaxial domains of the tendril lateral branches (TLb). Note coincident patterns of expression between leaflets and tendril lateral branches (red arcs). (h, i) Section below the region where the tendril lateral branches are being formed at P3, showing lack of expression of *AbFLO* in the adaxial region of the tendril (arrow) and strong expression of *AbFLO* in the adaxial region of the tendril in P2 (arrowhead). (j) Expression of *AbFLO* throughout the tendril tip at P1 and in the leaflet primordia at P2 (arrowhead), and lack of expression in the adaxial side of the tendril at P3 (arrow). (k) Expression of *AbFLO* in the adaxial region at P1 (arrowheads). (l) Expression of *AbFLO* throughout the SAM and P1 midrib vasculature, and in the boundary between the abaxial and adaxial domains. (m) Lack of expression of *AbFLO* in a small area in the flanks of the SAM (arrowhead). (n) *AbFLO* is not expressed in the flank of the meristem, marking the boundary between the next leaf primordium and the meristem (arrowhead). (o) Weak expression of *AbFLO* in the vasculature of petioles at P3 and in the young stem. (a–c) Shoot apex longitudinal sections. (d–l) Shoot apex transverse sections. AB, axillary bud; TLb, tendril lateral branch; Lf, leaflet primordium; P, prophyll; TPb, tendril primary branch; Pe, petiole; St, stem. The developmental stage of leaf primordia is indicated by P#, where '#' gives the stage of development of the primordium in question.

and the rise of tendrils on leaves. All leaf parts analyzed from *A. buccinatorium*, *B. callistegioides*, and *D. unguis-cati* showed bilateral symmetry. Petioles from all species were clearly bilaterally symmetrical, as their shape in cross-section is not radial, and petiole vasculature does not assume a ring form as seen in peltate compound leaves (Kim *et al.*, 2003a). Petiolules are generally similar in structure to petioles, and neither petioles nor petiolules presented a structural organization that was similar to that of tendrils. Therefore, tendrils could not be interpreted as a structure originating from those foliar parts.

The pea tendril has been thought to represent a determined rachis because of the anatomical similarity between rachis and

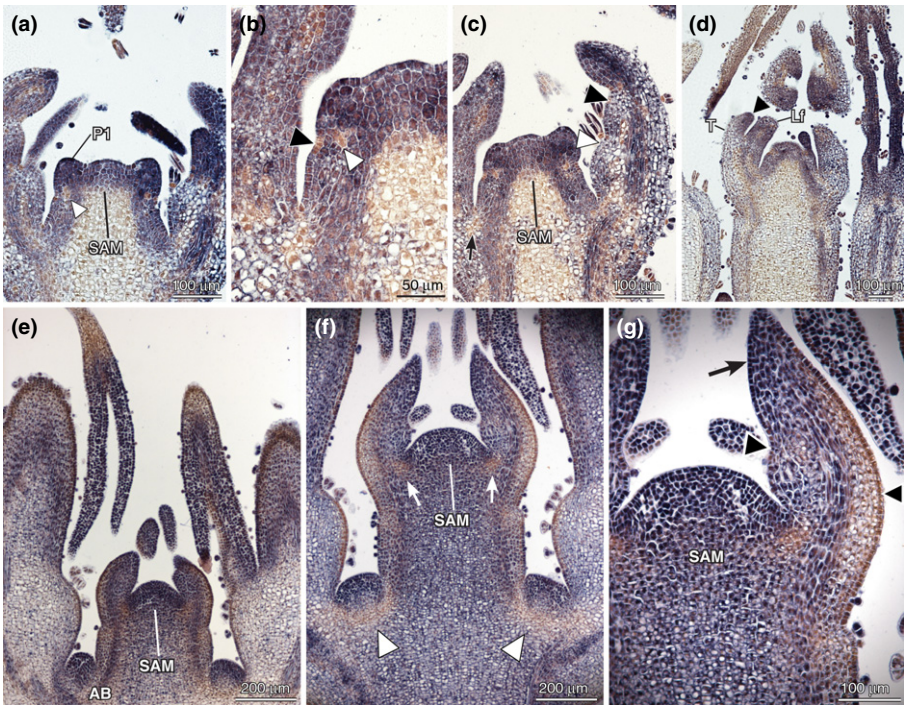

Fig. 7 *In situ* hybridizations of *FLORICAULA* (*FLO*) in shoot apices of *Dolichandra unguis-cati* (a–d) and *Bignonia callistegioides* (e–g). (a) Strong expression of *DuFLO* at P1 and lack of expression in the boundary between the new emerging leaves and the meristem flanks (arrowhead). (b) Lack of *DuFLO* expression in some cells of the protodermis (black arrowhead) and in the flanks of the shoot apical meristem (SAM) (white arrowhead). (c) Lack of *DuFLO* transcripts in the boundary between the leaflet primordium and tendril lateral branches at P3 (white arrowhead), between the tendril lateral and primary branches (black arrowhead), and in the boundary between P3 and the meristem (arrow). (d) Expression of *DuFLO* in the leaflet primordium and tendril primordium tip (arrowhead) at P2. (e) Transcripts of *BcFLO* in the SAM, leaf primordia, and axillary buds. (f) Lack of *BcFLO* in the boundary between the meristem and the axillary buds (arrowheads), as well as between the meristem and the leaf primordium (arrows). (g) Expression of *BcFLO* in the tendril tips (black arrow), with a lack of expression of *BcFLO* in the leaf primordium indicated by arrowheads. AB, axillary bud; Lf, leaflet; T, tendril. The developmental stage of leaf primordia is indicated by P#, where '#' gives the stage of development of the primordium in question.

tendril (Lu *et al.*, 1996; Tattersall *et al.*, 2005), although more recent data have demonstrated that pea tendrils are modified leaflets (Hofer *et al.*, 2009). In Bignonieae, leaves have only two leaflets and terminal tendrils, and no rachis is evident. A change in structure along the tendril length, that is, the basal region of the tendrils being anatomically different from the apical region, could denote the presence of a rachis in the tendril basal portion. However, branched tendrils have a similar anatomical structure in regions above and below the ramification, and simple tendrils have similar tissue organization all along their length. This, allied to the absence of a rachis in two–three-foliolate leaves, indicates that it is unlikely that tendrils originate from the rachis in Bignonieae.

Our histological analyses indicated that Bignonieae tendrils seem to represent modified leaflets. A morphological ribbed projection in the adaxial side of the leaflet midrib of *D. unguis-cati* that is similar to that formed in tendrils supports this hypothesis (Fig. 3a–c). In both leaf parts, this projection is caused by increased numbers of cell layers below the epidermis, in the cortex or mesophyll. Moreover, tendril tips of *A. buccinatorium* presented blade-like tissue expansions that are similar in shape to leaflet tips (Fig. 3d–g). In addition, the lack of major anatomical dissimilarities between the tendrils and leaflets of *A. buccinatorium* and *B. callistegioides*, along with the similar gene expression patterns observed in these structures, also supports the homologous nature of tendrils and leaflets in these taxa.

Individuals of Bignonieae can bear tendrilless and tendrilled leaves (Lohmann, 2003), suggesting that the conversion of leaflets into tendrils and vice versa results from relatively simple processes. Moreover, some species that do not develop adhesive disks develop a small leaf on the tendril tip (Fig. S7), indicating that the blade developmental program can be switched on/off easily, as well as that the establishment of the tendril/leaflet domain is quite plastic. These characteristics further support the hypothesis that tendrils of Bignonieae are derived from leaflets. Alternatively, the primordium distal tip may never go through a leaf-like ground state, having a 'tendril' fate determined in early stages of development; these different fates could be established as early as the proximo-distal polarity.

STM, *PHAN* and *LFY/FLO* are expressed during tendril development in Bignonieae

SHOOTMERISTEMLESS In contrast to simple leaf primordia, compound leaf primordia retain an organogenetic zone in their margins, the blastozone, where leaflets are formed (Hagemann & Gleissberg, 1996). In compound leaves, *KNOX1* genes are expressed in the blastozone, extending the meristematic capacity

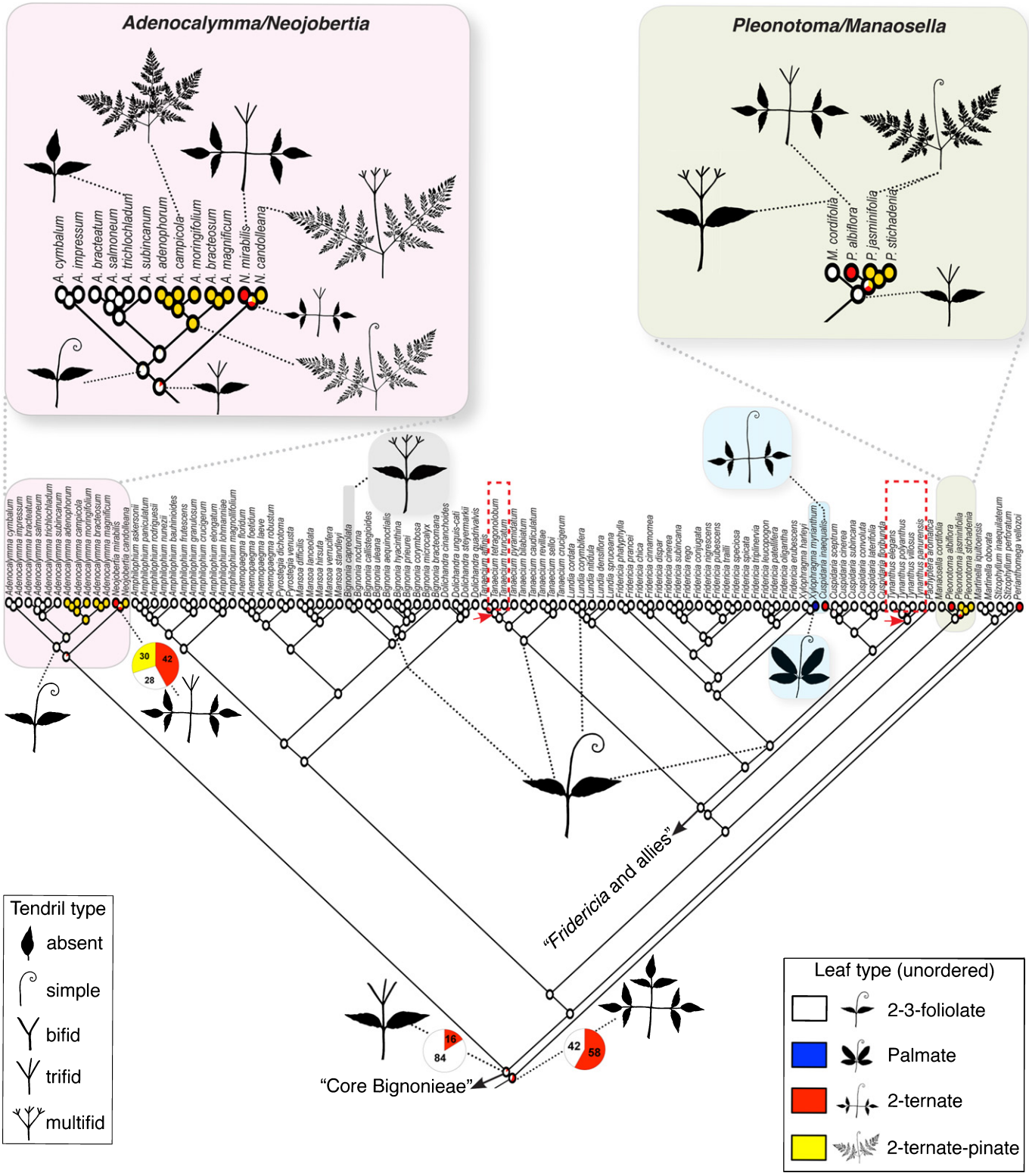

Fig. 8 Maximum likelihood ancestral state reconstruction of the character 'leaf type' plotted on the single optimum maximum likelihood tree derived from the analysis of combined *ndh*F and *Pep*C sequences of 104 species of Bignonieae (from Lohmann, 2006) plus *Perianthomega vellozoi*. Pie charts represent the likelihoods of the individual reconstructions. The color-coded key summarizes the four character states of 'leaf type' in Bignonieae (extracted from Lohmann, 2003). Leaf drawings placed on the tree indicate evolutionary shifts of the character 'tendril type' (retrieved from Sousa-Baena *et al.*, 2014), allied to shifts in lateral leaflets. Pink and green rectangles show in detail points of the tree at which many evolutionary shifts have occurred. The gray rectangle indicates a case in which 'leaf type' was maintained, but 'tendril type' changed; blue rectangles indicate species in which the opposite situation has occurred. Red dashed rectangles indicate reversals from two–three-foliolate, simple-tendrilled to two–three-foliolate, trifid-tendrilled leaves; red arrows indicate the nodes in which these reversals occurred.

of this tissue, delaying the transition from primary to secondary morphogenesis, and allowing the formation of leaflets (Bharathan *et al.*, 2002).

In Bignonieae, *STM* mRNA was detected in the SAM and developing leaves, indicating that blastozone activity of Bignonieae leaves is also regulated by *KNOX1*. This pattern of reactivated *KNOX* expression has also been documented in other compound-leaved species, such as tomato, Papaveraceae species, and *Cardamine hirsuta* (Janssen *et al.*, 1998; Kim *et al.*, 2003b; Groot *et al.*, 2005; Hay & Tsiantis, 2006).

In branched-tendrilled leaves of Bignonieae, *STM* expression is strongly associated with the initiation of leaflets, as well as with the initiation of tendril lateral and tertiary branches. In *B. callistegioides* (simple-tendrilled species), *STM* was associated with leaflet development, with a weak expression in tendrils. Thus, the expression of *STM* in tendrilled leaves of Bignonieae was clearly associated with regions undergoing extensive cell division, which is consistent with the role of *STM* in the prevention of cell differentiation.

PHANTASTICA In Euasterids (a clade that encompasses Bignoniaceae), *ARP* mutants develop clear adaxial–abaxial polarity phenotypes (Kidner & Timmermans, 2007). In contrast, the *AS1* mutant of Arabidopsis (Eurosid) does not present polarity defects, but symmetry disruption, which shows that the *PHAN* ortholog has been subject to differential recruitment in these species.

In Bignonieae, *PHAN* expression was detected in the SAM and developing leaves, similar to what was reported for tomato (Kim *et al.*, 2003a,b). However, *PHAN* transcripts were confined to the adaxial side of tips of young leaf primordia in Bignonieae, but were distributed throughout the young leaf primordia in tomato, in which such transcripts become restricted to the adaxial side of leaflets only at later stages of development (Kim *et al.*, 2003a,b). Furthermore, tomato *LePHAN* is expressed in the region of leaflet primordium initiation in older leaves, suggesting that *LePHAN* might be involved in leaflet formation and in the establishment of leaflet ab-adaxiality (Kim *et al.*, 2003b). The same pattern as described for *LePHAN* was observed in the developing leaves of Bignonieae.

In Bignonieae, *PHAN* is expressed strongly during initiation and development of leaflets, tendril lateral branches, and tendril tertiary branches. In *A. buccinatorium*, for instance, *PHAN* expression was high in regions where tendrils were branching, suggesting that *PHAN* plays an important role in ramification development. At later stages of leaflet development (e.g. P3), *PHAN* expression is restricted to the midrib vasculature, and to the primordium middle domain, similar to the pattern observed in the compound leaves of *C. hirsuta* and *Vitex cannabifolia* (Kim *et al.*, 2003a; Hay & Tsiantis, 2006).

Molecular studies of *CRISPA*, the *PHAN* ortholog in pea, showed that tendril development was not altered in *CRISPA* mutants (Tattersall *et al.*, 2005). In pea, *CRISPA* transcripts are restricted to the middle mesophyll layers of leaflet primodia, and are not polarized in tendril primordia (Tattersall *et al.*, 2005). This pattern differs from what we encountered in *D. unguis-cati* and *A. buccinatorium* (branched-tendrilled species), but is similar to that described for *B. callistegioides* (simple-tendrilled species). In the pea *P. sativum*, the disruption of adaxial domain establishment does not prevent tendril ramification. However, given the epistatic relationship between *PHAN* and *KNOX1* and the fact that *PHAN* is expressed in leaves of Bignonieae, it is likely that *PHAN* may play a greater role in the establishment of polarity in Bignonieae than in the pea *P. sativum*, affecting blastozone functioning and consequently tendril development and ramification.

Moreover, in tomato, *LeT6* is strongly expressed in the center of the SAM and excluded from P0, with *LePHAN* being expressed in the flanks of the SAM and in P0 (Kim *et al.*, 2003a, b). In Bignonieae, in contrast, both transcripts are excluded from P0. As leaf primordia pairs emerge from the SAM at *c.* 90° from each other, in more superficial longitudinal sections P0 is identified, in the central part of the apical region, by the lack of expression of *PHAN* and *STM* in this region (Figs 4c, 5b, S8). However, in longitudinal sections passing through the middle of the stem, both transcripts are observed in the flanks and center of the SAM.

Studies on many angiosperm species indicate that the function of ARP proteins in *KNOX* regulation has been conserved; however, its role in adaxial–abaxial polarity is variable (Kidner & Timmermans, 2007). The *ARP* expression pattern does not always correlate with its function; for example, *ARP* is expressed uniformly throughout the leaf primordium in Arabidopsis and *A. majus* (Kidner & Timmermans, 2007); however, *ARP* is correlated with adaxial–abaxial polarity establishment in *A. majus* (Waites & Hudson, 1995), but not in Arabidopsis (Byrne *et al.*, 2000). In a broad survey of angiosperm compound leaves, *ARP* expression was found to be adaxially localized and correlated with the pattern of leaflet production (Kim *et al.*, 2003a). Together, these data suggest that *ARP* genes are generally involved in polarity establishment in Euasterids, and thus possibly have a significant role in the development of Bignonieae leaves.

LEAFY/FLORICAULA Expression of *LFY/FLO* has been detected in the flanks of the SAM and in emerging leaf primordia in a wide range of angiosperms, including several compound-leaved taxa (Busch & Gleissberg, 2003). In the species of Bignonieae analyzed here, transcripts of *LFY/FLO* were detected in the SAM, leaf primordia, and axillary buds. Moreover, *LFY/FLO* was strongly expressed in the distal portions of the leaf primordium, similar to what was detected for pea (Hofer *et al.*, 1997). In pea, the expression of *LFY/FLO* was observed immediately above the insertion of the lateral tendril pair (DeMason & Schmidt, 2001), in a pattern similar to that observed in the leaves of *D. unguis-cati* and *A. buccinatorium* (Fig. 6j). However, differently from what was observed in Bignonieae, *LFY/FLO* is not expressed in the SAM of the pea *P. sativum* (Hofer *et al.*, 1997).

Leaves of Bignonieae present concomitant expression of *LFY/FLO* and *STM* during development, which is similar to the expression patterns found in *Eschscholzia californica* (Groot *et al.*, 2005). *FLO* expression in Bignonieae is similar to that in tomato, in which *FLO* is expressed in the SAM, stem vascular traces,

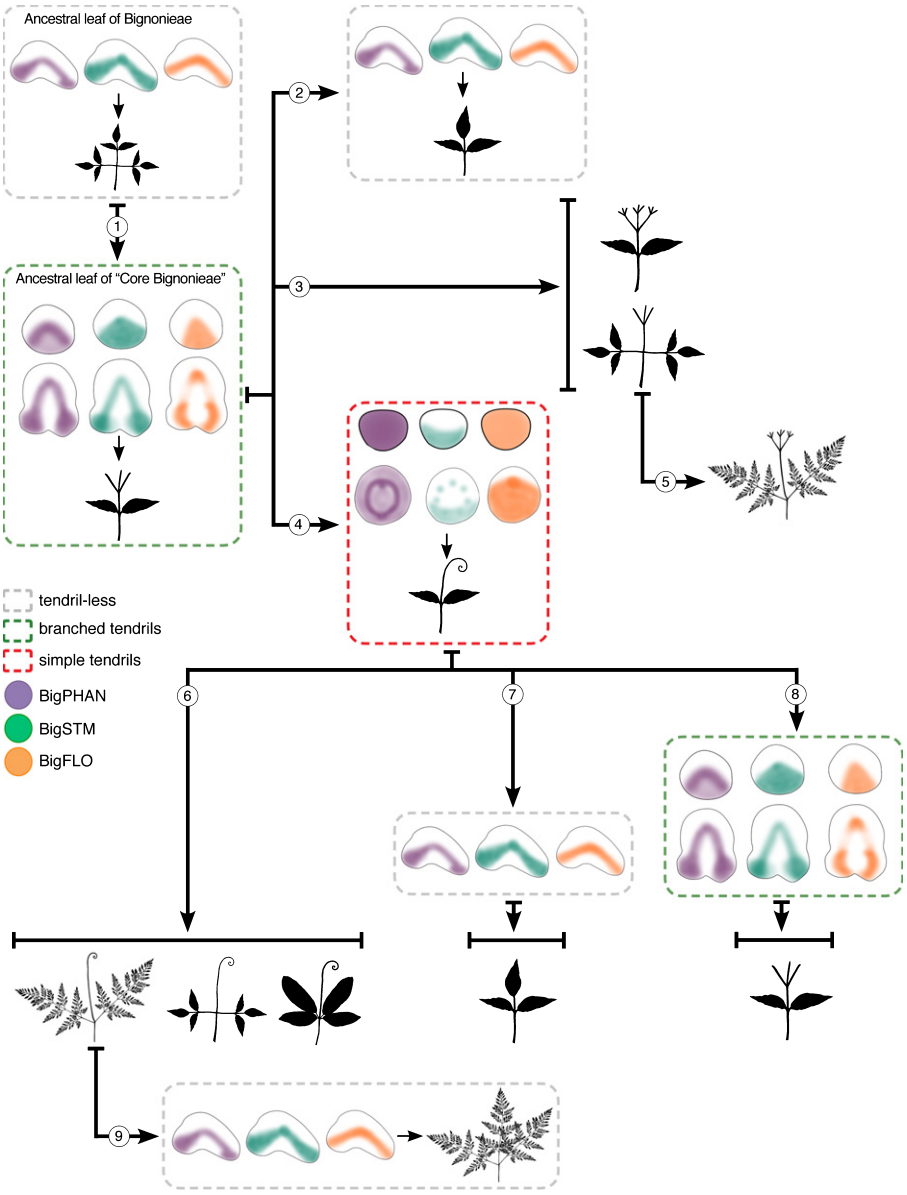

Fig. 9 Diagram summarizing leaf evolutionary shifts that occurred in the tribe Bignonieae, with schematic representations of the associated changes in patterns of expression of *PHANTASTICA* (*BigPHAN*), *SHOOTMERISTEMLESS* (*BigSTM*) and *FLORICAULA* (*BigFLO*). Expression pattern schemes are represented in the tendril and leaflet in cross-section. Inside green and red rectangles, the first row represents the tendril tip, and the second row shows the region of the tendril from which lateral branches emerge in branched tendrils, or the correspondent region in simple tendrils. Inside gray rectangles, the gene expression pattern is represented on leaflets in cross-section. Information on 'tendril type' evolutionary shifts was retrieved from Sousa-Baena *et al.* (2014).

axillary buds, and developing leaves, with higher expression in the leaf adaxial side and leaf margins (Molinero-Rosales *et al.*, 1999). A recent study showed that *LFY/FLO* regulates adaxial establishment in Arabidopsis pedicels (Yamaguchi *et al.*, 2012); hence, *LFY/FLO* may also be involved in adaxial domain establishment in Bignonieae.

Leaf evolutionary shifts in Bignonieae: changes in meristematic competence and gene expression generating diversity of tendril forms

Temporal and spatial changes in meristematic competence, associated with the pattern of partition of the leaf primordium tip, generated the current patterns of leaf morphology encountered in Bignonieae. According to the hypothesis of leaf and tendril type evolution based on our reconstructions (data from this work and from Sousa-Baena *et al.*, 2014), the first shift that occurred in Bignonieae was from a tendrilless, two-ternate leaf to a trifid-tendrilled, two-foliolate leaf in the 'Core Bignonieae' (Fig. 9). Our anatomical, morphological and evolutionary data indicate that each branch of a trifid tendril probably represents a reduction of one foliolule of the terminal leaflet, which comprises three foliolules.

This first evolutionary shift involved a drastic reduction in leaf primordium meristematic capacity. The three-foliolate lateral and terminal leaflets were reduced to a single leaflet and to a trifid tendril, respectively. This reduction probably resulted from a shortened duration of *KNOX1* expression in leaf primordia, but might also have involved changes in *WOX1/3* activity (Nakata *et al.*, 2012). Furthermore, the onset of expression of genes antagonistic to *KNOX1* (e.g. *ARP*, Class III homeodomain-leucine zipper genes, *YABBY* and *KANADI*; Husbands *et al.*, 2009) must have happened earlier in leaf development, promoting the precocious activation of the differentiation pathway.

BigPHAN expression in leaflets and branched tendrils presented the patterns expected for compound leaves, with expression in the adaxial domain. Hence, other genes that promote tissue differentiation and interact with the *KNOX1* and *ARP* genes, such as *TCP* (*TB1/CYC/PCF*) genes, may also be involved in the shift from three-foliolulate leaflets to branched tendrils (Efroni *et al.*, 2008; Li *et al.*, 2012). Furthermore, *Trifoliate* might also be involved in the precocious termination of leaf development in Bignonieae (Naz *et al.*, 2013). In simple-tendrilled species of Bignonieae, the marginal blastozone is narrower, and trichomes cover nearly all the extension of the primordium by the P2 stage, resulting in earlier differentiation of simple-tendrilled primordia compared with trifid-tendrilled primordia (Sousa-Baena *et al.*, 2014). This is consistent with the changes observed in tomato *trifoliate* mutants, in which early maturation of the marginal blastozone, which is narrower than in its wild-type counterpart, results in accelerated maturation of leaf primordia (Naz *et al.*, 2013). Indeed, trifid and multifid tendril morphologies in Bignonieae seem to result from mechanisms such as the reiteration of the ancestral pattern of division of the leaf primordium, and premature completion of leaf development, resulting in organs without expanded lamina.

Recently, Nakata *et al.* (2012) demonstrated that *WOX3* and *WOX1* are expressed in the boundary between the adaxial and abaxial sides of the leaf primordia and are essential for lamina expansion in Arabidopsis. This middle domain is established through the mutual interaction among *WOX3*/*WOX1*, adaxial, and abaxial polarity genes. Furthermore, *WOX3*/*WOX1* maintain the expression of *ASYMMETRIC LEAVES2* (*AS2*) restricted to the adaxial epidermal cells. In Arabidopsis, *AS2* and *AS1* act in concert during blade development (Xu *et al.*, 2003). In Bignonieae, it is possible that the interaction among *AS2*, *PHAN*, and *WOX3*/*WOX1* enables lamina expansion in the basal domain of the leaf primordia, generating leaflets, whereas this genetic program is suppressed or altered in the apical domain.

Tendrilless, multifid, and simple-tendrilled leaves (Fig. 9, arrows 2, 3, and 4, respectively) were hypothesized to have evolved from trifid-tendrilled ancestors. In tendrilless leaves, the tendril was replaced by a single leaflet, indicating that the ability to develop lamina in this position was regained, which could be interpreted as a partial reversal to the ancestral two-ternate form. Furthermore, our results showed that *BigPHAN* expression is not polarized in simple tendrils (Fig. 5l); this may influence *KNOX1*, as well as the expression of *PIN-FORMED* (*PIN*) genes, as *PHAN* represses *KNOX1* and up-regulates *PIN* through the interaction with *AS2* and *JAGGED LATERAL ORGANS* (Rast & Simon, 2012).

Furthermore, according to the leaf and tendril reconstructions, other leaf forms evolved from the two–three-foliolate simple-tendrilled leaf. Some forms have arisen via changes in the lateral leaflets only, keeping tendrils simple (Fig. 9, arrow 6), although a tendrilless, three-foliolate form also evolved from this ancestor (Fig. 9, arrow 7), probably through regaining of the ancestral expression pattern of *BigPHAN*, *BigFLO* and *BigSTM* present in ancestral two-ternate leaves. Reversals to trifid tendrils have also occurred (Fig. 9, arrow 8), but in this context, ancestors with simple-tendrilled leaves can never originate species with multifid-tendrilled leaves, which can only evolve from trifid-tendrilled ancestors (Fig. 9, arrows 3 and 5). Apparently, some key components of the genetic pathway for the generation of more complex tendril forms were irreversibly lost or changed in the shift from trifid- to simple-tendrilled leaves (Fig. 9, arrow 4). The expression of *BigPHAN* is disrupted in simple tendrils, which may be correlated with the inability of simple-tendrilled ancestors to evolve to multifid-tendrilled species. Alternatively, it is possible that the ancestor of Bignonieae bore two-foliolate leaves (42% likelihood; Fig. 8) with trifid tendrils (37% likelihood; Sousa-Baena *et al.*, 2014). That would probably not cause significant changes in the reconstruction of leaf and tendril type at deeper nodes of the tree, as leaves with two leaflets and a terminal tendril were also, in this scenario, hypothesized to have arisen early in Bignonieae, in the node 'Core Bignonieae.'

Together, our data suggest that a successive shut-down of components of the polarity establishment pathway has occurred in the evolutionary history of Bignonieae. The evolutionary sequence of events starts with leaflets in all leaf domains (two-ternate ancestor) being replaced by trifid tendrils, which still present polarized *PHAN* expression (but probably disruption in other polarity genes), culminating in the rise of simple tendrils, in which *PHAN* expression is disrupted. This reduction of tendril complexity happened early in the tribe, and was maintained in many species (Sousa-Baena *et al.*, 2014).

Results from this study advance our understanding of the roles of *STM*, *PHAN*, and *FLO* during development of leaf tendrils considerably. Tendrilled leaves of Bignonieae species are not exclusively controlled by *LFY/FLO* as occurs in the pea *P. sativum*. Hence, although the tendrils of Bignoniaceae and Fabaceae species are similar, the similarity results from an evolutionary convergence, with tendrils presenting different subjacent developmental pathways in these families. In addition, the differences in *PHAN* expression pattern between simple and branched tendrils strongly suggest that this gene is involved in the establishment of the adaxial domain and ramification of tendrils in Bignonieae, although this is not the case in pea.

This study also provided clues to the evolutionary and developmental origins of different types of leaves in Bignonieae. However, functional studies are still needed in order to achieve a complete understanding of the exact mechanisms that are responsible for such high leaf diversity in Bignonieae. Indeed, Bignonieae represents an excellent model for studies on the origin of the changes in gene expression leading to diversity in leaf morphology. Apart from the great leaf diversity encountered in the group, a well-supported phylogeny (Lohmann, 2006) and a time-calibrated phylogeny (Lohmann *et al.*, 2013) are available, allowing detailed evolutionary studies to be conducted, within a temporal framework.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Deduced amino acid sequence alignment of *STM* orthologs.

Fig. S2 Deduced amino acid sequence alignment of *ARP* orthologs.

Fig. S3 Deduced amino acid sequence alignment of *LFY/FLO* orthologs.

Fig. S4 Schematic drawing showing the portion of *STM* that was cloned for Bignonieae, amino acid alignment of *STM* orthologs, and *STM* phylogeny.

Fig. S5 Schematic drawing showing the portion of *ARP* that was cloned for Bignonieae, amino acid alignment of *ARP* orthologs, and *ARP* phylogeny.

Fig. S6 Schematic drawing showing the portion of *LFY/FLO* that was cloned for Bignonieae, amino acid alignment of *LFY/FLO* orthologs, and *LFY/FLO* phylogeny.

Fig. S7 Variation in leaflet number and tendril morphology in Bignonieae.

Fig. S8 Expression patterns of *STM* and *PHAN* in shoot apices of *B. callistegioides* and *D. unguis-cati*.

Table S1 Taxa sampled for this study, followed by voucher information

Table S2 Primers used in this study

Table S3 *ARP* orthologs used in phylogeny reconstruction

Table S4 *LFY/FLO* orthologs used in phylogeny reconstruction

Table S5 *STM* orthologs used in phylogeny reconstruction

Table S6 Amount of RNA probe used for each gene

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