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Floral symmetry genes and the origin and maintenance of zygomorphy in a plant-pollinator mutualism

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The evolution of floral zygomorphy is an important innovation in flowering plants and is thought to arise principally from specialization on various insect pollinators. Floral morphology of neotropical Malpighiaceae is distinctive and highly conserved, especially with regard to symmetry, and is thought to be caused by selection by its oil-bee pollinators. We sought to characterize the genetic basis of floral zygomorphy in Malpighiaceae by investigating *CYCLOIDEA2*-like (*CYC2*-like) genes, which are required for establishing symmetry in diverse core eudicots. We identified two copies of *CYC2*-like genes in Malpighiaceae, which resulted from a gene duplication in the common ancestor of the family. A likely role for these loci in the development of floral zygomorphy in Malpighiaceae is demonstrated by the conserved pattern of dorsal gene expression in two distantly related neotropical species, *Byrsonima crassifolia* and *Janusia guaranitica*. Further evidence for this function is observed in a Malpighiaceae species that has moved to the paleotropics and experienced coincident shifts in pollinators, floral symmetry, and *CYC2*-like gene expression. The dorsal expression pattern observed in Malpighiaceae contrasts dramatically with their actinomorphic-flowered relatives, Centroplacaceae (*Bhesa paniculata)* and Elatinaceae (*Bergia texana*). In particular, *B. texana* exhibits a previously undescribed pattern of uniform *CYC2* expression, suggesting that *CYC2* expression among the actinomorphic ancestors of zygomorphic lineages may be much more complex than previously thought. We consider three evolutionary models that may have given rise to this patterning, including the hypothesis that floral zygomorphy in Malpighiaceae arose earlier than standard morphology-based character reconstructions suggest.

CYCLOIDEA | development | gene duplication | Malpighiaceae | phylogeny

Most flowers are either bilaterally symmetrical (i.e., zygomorphic) and have a single plane of symmetry or radially symmetrical (i.e., actinomorphic) and have several planes of symmetry (1). Floral zygomorphy has evolved independently at least 38 times (2–4) and is a hallmark feature of the most diverse angiosperm clades, including Asteraceae (23,600 spp.), Orchidaceae (21,950 spp.), Fabaceae (19,400 spp.), and Lamiales (23,275 spp.) (5). Plant evolutionary biologists therefore propose that the origin of floral zygomorphy may have been a key innovation for promoting speciation throughout the course of angiosperm evolution (6). The driving force behind the origin of floral zygomorphy has long been thought to be a consequence of selection by specialization on certain insect pollinators (1, 7), which has recently gained experimental support (8).

The tropical plant family Malpighiaceae exhibits a strong association between floral zygomorphy and insect pollinator attraction. The floral morphology of the more than 1,000 New World species of this clade is very distinctive and highly conserved, especially with regard to symmetry and pollinator reward. The single upright/dorsal banner petal is strongly differentiated from other petals in the corolla whorl, and appears to help orient and attract an extremely limited suite of pollinators, principally female bees of the tribes Centridini and Tapinotaspidini (Fig. 1) (9, 10–12). Furthermore, the very narrowed base of the petals provides the bees access to oil glands, which are borne in pairs on the abaxial surface of the sepals. The stereotypical floral morphology of New World Malpighiaceae, despite tremendous variation in vegetative and fruit morphology, led Anderson (9) to hypothesize that floral uniformity in the group results from their specialization on these oil-bee pollinators.

Interpreting the origin and maintenance of this unique floral morphology in a comparative evolutionary framework, however, has remained elusive, in large part because of our lack of understanding of the closest phylogenetic relatives of Malpighiaceae. Fortunately, this problem has recently been resolved: the family is successively sister to two species-poor clades that possess actinomorphic flowers, Elatinaceae and Centroplacaceae [Fig. 1; Malpighiaceae (1,300 spp.), Elatinaceae (35 spp.), Centroplacaceae (6 spp.)] (13–16). These findings, together with morphology-based character state reconstructions of floral symmetry in the group, demonstrate that Malpighiaceae evolved from actinomorphic-flowered ancestors (Fig. S1). Moreover, these results suggest that the origin of Malpighiaceae and their unique flowers appear to correspond with a dramatic shift in speciation rates (14, 15). During the course of this radiation, there appears to have been seven subsequent dispersal events from the New World that gave rise to Old World Malpighiaceae (17–19). The Old World tropics lack the oil-bee pollinators that visit New World Malpighiaceae (ref. 20, p. 913), and these geographic transitions have resulted in alterations in both floral symmetry and petal morphology, as well as the loss of the oil gland morphology among Old World members of the family (17, 18, 21). Thus, the majority of Old World species possess flowers that are either truly actinomorphic or zygomorphic in a manner that is very divergent from the pattern exhibited by New World species. These zygomorphic Old World species possess two dorsal petals, two lateral petals, and one ventral petal. Malpighiaceae therefore provide a rare opportunity to elucidate the ways in which important morphologies originate and are alternatively maintained or remodeled following changes in a selective regime, such as a shift in pollination system.

One way to approach this problem is to investigate the floral developmental genetic basis for these shifts, both in terms of the origin and maintenance of zygomorphy in New World Malpighiaceae, and the secondary loss of this conserved morphology among Old World species. Fortunately, the developmental genetics of floral zygomorphy is being elucidated at a rapid pace.

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Fig. 1. Comparative floral morphology of Malpighiaceae and their closest actinomorphic flowered relatives, Elatinaceae and Centroplacaceae. *Banisteriopsis argyrophylla, Bergia texana,* and *Bhesa paniculata* represent Malpighiaceae, Elatinaceae, and Centroplacaceae, respectively. Dotted lines indicate planes of symmetry. Note: the flower of *B. texana* was forced opened for illustration. The exact floral orientation of *B. paniculata* is unclear given the congested nature of their inflorescences.

Genes belonging to the recently defined *CYC2* clade (22) of the TCP [*Teosinte Branched 1*, *CYCLOIDEA* (*CYC*), and PCF] transcription factor family have been shown to play a critical role in the development of zygomorphy. In *Antirrhinum*, the two closely related *CYC2*-like genes, *CYC* and *DICHOTOMA* (*DICH*), demarcate the dorsal region of the flower by differentially regulating the rate of cell growth in the developing floral organs (23, 24). *CYC*/*DICH* expression is continuous throughout floral development in the dorsal region of this species. In *cyc*/*dich* double mutants, however, zygomorphy is lost—the dorsal petals become ventralized, resulting in actinomorphic corollas. The emerging paradigm from this and numerous subsequent studies is that zygomorphy has evolved independently through the repeated recruitment of *CYC2*-like genes in several phylogenetically diverse clades (reviewed in refs. 25, 26).

The present study builds on this strong developmental framework and suggests that *CYC2*-like genes are similarly associated with the origin and maintenance of zygomorphy in Malpighiaceae. We identified two forms of *CYC2*-like genes in Malpighiaceae, which resulted from a gene duplication in the common ancestor of the family. Both gene copies, *CYC2A* and *CYC2B*, are expressed in the dorsal region of the flowers in distantly related New World species with the stereotypical floral morphology (i.e., *Byrsonima crassifolia* Kunth and *Janusia guaranitica* A. Juss.). Further evidence of the function of *CYC2* in establishing floral symmetry in Malpighiaceae derives from expression patterns in an Old World species that has lost its association with the New World oil-bee pollinators and its stereotypical morphology. In the zygomorphic-flowered species *Tristellateia australasiae* A. Rich., *CYC2A* expression mirrors a dramatic shift in the plane of floral symmetry, whereas *CYC2B* has been lost. The dorsal patterning of gene expression we observed in Malpighiaceae contrasts dramatically with their actinomorphic-flowered relatives, Centroplacaceae (*Bhesa paniculata* Arn.) and Elatinaceae (*Bergia texana* Seub. ex Walp.). Results from the latter species suggest unique insights on *CYC2* patterning in actinomorphic-flowered species, and highlight the importance of investigating *CYC2*-like genes in other actinomorphic core eudicot clades beyond the model species *Arabidopsis thaliana*.

Results and Discussion

Gene Duplication and Loss. Thirty-three species from 29 genera of Malpighiaceae representing all major clades of the family, two species from both genera of Elatinaceae, and one species from Centroplacaceae, were used for assembling the *CYC2* genealogy (Table S1). *Oxalis herrerae* R. Knuth was used as an outgroup (16). One to six copies of the *CYC2*-like genes were isolated from each taxon. As with previous studies (e.g., ref. 22), the TCP and R domains were highly conserved, but the intervening coding region was variable across all taxa. Partial TCP and R domains (i.e., 26 of 60 and two of 18 total amino acid sequences were obtained for each region, respectively) and the entire intervening coding region were included in our analyses. The aligned *CYC2* matrix included 78 sequences and was 384 bp in length; 78 of these bps were constant and 278 were parsimony-informative.

The phylogenetic relationships inferred from the *CYC2* homologues mirror our understanding of accepted species tree relationships (14, 15, 17, 19) (Fig. 2 and Fig. S2). Centroplacaceae and Elatinaceae are successive sisters to Malpighiaceae with 100% maximum likelihood (ML) bootstrap support (BP) and 100% Bayesian posterior probability (PP; reported for simplicity here as percentages) and 70% BP/75% PP, respectively. A comparison of the *CYC2*-like gene tree with accepted species tree relationships indicates that the origin of Malpighiaceae coincided with a duplication in the *CYC2* clade, which yielded two major copies, *CYC2A* and *CYC2B*. Both copies receive moderate to high support (*CYC2A*, 57% BP/65% PP; *CYC2B*, 80% BP/99% PP). We inferred additional gene duplications in *CYC2A* in *Galphimia, Hiptage*, *Tristellateia*, and *Verrucularia*, and in *CYC2B* in *Banisteriopsis, Flabellariopsis, Hiptage*, *Janusia*, *Ptilochaeta*, *Ryssopterys,* and *Spachea*. *CYC2* duplications were also inferred in the outgroup taxa *Bergia texana* and *Elatine minima* (Elatinaceae). These results are consistent with previous analyses that have uncovered *CYC2* duplications across diverse phylogenetic groups (22). Furthermore, they demonstrate that *CYC2* gene duplications have occurred independently in Malpighiaceae and Elatinaceae, with the former duplication corresponding to the origin of Malpighiaceae and their unique floral zygomorphy (Fig. S1). Importantly, this duplication is not likely to be the result of polyploidization, as there is no evidence of genome doubling associated with the origin of Malpighiaceae (27, 28).

Similarly, we uncovered evidence for the loss of *CYC2A* or *B* in various Malpighiaceae clades, which has also been observed in other groups (22). Nearly all species of New World Malpighiaceae possess *CYC2A* and *CYC2B*, but some species, especially Old World Malpighiaceae, appear to retain only one copy (Table S1). We further verified the copy number of *CYC2*-like genes by Southern hybridization in a subset of taxa (Fig. S3 and S4). *Bergia texana*, *Byrsonima crassifolia, J. guaranitica,* and *T. australasiae* have six, two, four, and one copy of *CYC2*-like genes respectively, which is consistent with our initial assessment of gene copy number ascertained from PCR and clone screening. These results suggest that our PCR/clone screens provide a reliable estimate of copy number in taxa for which Southern hybridizations were not conducted.

Recruitment of *CYC2* and the Maintenance of Floral Zygomorphy in Malpighiaceae. To determine if the evolution of floral zygomorphy in Malpighiaceae is likely a result of changes in the regulation of *CYC2* homologues, we investigated the pattern of *CYC2* expression using locus-specific RT-PCR. RT-PCR was conducted on *J. guaranitica* and *B. crassifolia* (New World Malpighiaceae), *T. australasiae* (Old World Malpighiaceae), *B. texana* (Elatinaceae), and *B. paniculata* (Centroplacaceae; Fig. 3). Each of these taxa was carefully selected based on its phylogenetic affinities to elucidate changes in gene expression related to (*i*) the origin of zygomorphy in Malpighiaceae, (*ii*) the conservation of floral morphology of New World Malpighiaceae, and (*iii*) the loss of the stereotypical New World floral morphology in Old World Malpighiaceae.

Several studies from phylogenetically diverse species indicate that zygomorphy has evolved independently via the repeated recruitment of *CYC2*-like genes (reviewed in ref. 25). *CYC2* expression in Malpighiaceae similarly suggests that these genes are responsible for the unique floral symmetry of New World Malpighiaceae (Fig. 3). During the later stages of floral development, *CYC2A* and *CYC2B* are differentially expressed along the dorsoventral plane of symmetry in *Byrsonima crassifolia* and *J. guaranitica*, which mirrors their floral morphology. *B. crassi-*

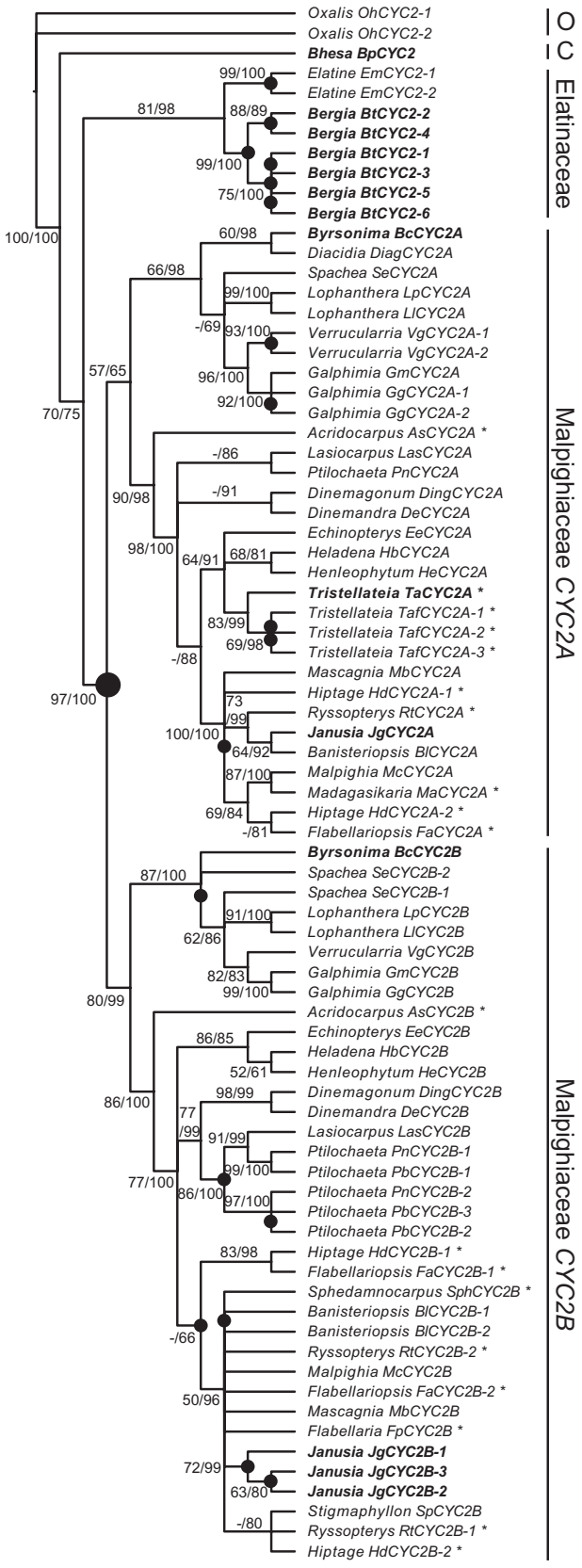

Fig. 2. Phylogeny of *CYC2*-like genes inferred using maximum likelihood (ML) and Bayesian analyses. Majority rule consensus shown depicting clades with >50% bootstrap support and >60% Bayesian posterior probability depicted above lines, respectively. ML bootstrap support <50% indicated with a hyphen. Black circles indicate inferred gene duplication events. Inferred gene tree is reflective of accepted species tree relationships (13-16, 19) (Fig. S2). Accessions shown in boldface were additionally analyzed for gene expression using RT-PCR. Asterisks indicate Old World clades. Species identities and voucher information can be found in Table S1. (O, Oxalidaceae; C, Centroplacaceae.)

folia has two *CYC2* genes: *CYC2A* (*BcCYC2A*) and *CYC2B* (*BcCYC2B*; Fig. 2). *J. guaranitica* has four *CYC2* genes: *CYC2A* (*JgCYC2A*) and three copies of *CYC2B* (*JgCYC2B-1, -2*, and *-3*). *BcCYC2A* of *B. crassifolia* and *JgCYC2A* and *JgCYC2B-3* of *J. guaranitica* are expressed broadly in the dorsal region of the flower, i.e., in the dorsal and lateral petals and sepals, and weakly in the gynoecium (Fig. 3). In contrast, *BcCYC2B* of *B. crassifolia* and *JgCYC2B-1* and *JgCYC2B-2* of *J. guaranitica* are expressed only in the single dorsal banner petal, and weakly in the gynoecium. Whether differential expression of these *CYC2*-like genes affects the symmetry of the calyx is unclear. In *J. guaranitica*, *CYC2*-like genes are differentially expressed in the dorsal and lateral sepals with oil glands, but not in the eglandular ventral sepal. This raises the possibility that *CYC2* might be involved in the development of the dorsoventral asymmetry of the calyx. However, all of the sepals of *B. crassifolia* are identical and bear oil glands, despite the fact that *CYC2* expression was detected only in the dorsal and lateral sepals.

Overlapping expression patterns of duplicated *CYC2* homologues, as observed here in Malpighiaceae, are common in numerous zygomorphic-flowered groups (23–25, 29, 30). Without functional assays, however, it is difficult to discern if the *CYC2A* and *2B* paralogues have significant functional redundancy, as in *Antirrhinum* (23, 24), or have more differentiated functions, as in Fabaceae, where one copy is more broadly expressed and the other is specific to the dorsal flag petal (29, 30). Regardless, duplication and functional divergence of *CYC2*-like genes may have contributed to the evolution of the unique zygomorphy in New World Malpighiaceae. The two species examined here, *B. crassifolia* and *J. guaranitica*, span the basal node of crown group Malpighiaceae, and diverged from one another at least 64 Mya (17), yet their pattern of gene expression is identical. This highly conserved pattern provides a likely molecular explanation for the stereotypical floral morphology in New World Malpighiaceae, which is in turn the basis of a well documented plant-pollinator mutualism.

An important test of our hypothesis that *CYC2* controls floral zygomorphy in Malpighiaceae is to examine gene expression in those clades that have lost the stereotypical New World morphology. *T. australasiae* is an Old World species with zygomorphic flowers that has lost its association with the oil-bee pollinators, and the characteristic New World morphology (Fig. 3). Instead, *T. australasiae* possesses two dorsal, two lateral, and one ventral petal. Although this species superficially resembles a resupinate New World flower, its altered symmetry does not result from resupination. If this were the case, the innermost petal of *T. australasiae*, which is homologous with the dorsal flag/banner petal of all New World species (31), would initiate in the dorsal position and terminate in the ventral position during development. This is not observed: *T. australasiae* produces an innermost petal that begins and ends in the dorsal position. Hence, the shift of floral zygomorphy in *T. australasiae* is best explained as a 36° rotation in the plane of floral symmetry, rather than a 180° resupination (cf. ref. 32; Figs. S5 and S6). This is likely a developmental feature that is established very early in floral development. How this happens remains an open question that we will seek to address in future studies.

We have shown that *T. australasiae* has lost the *CYC2B* gene and that *CYC2A* (*TaCYC2A*) expression closely mirrors its dramatic shift in the dorsoventral plane of floral symmetry (Fig. 3).

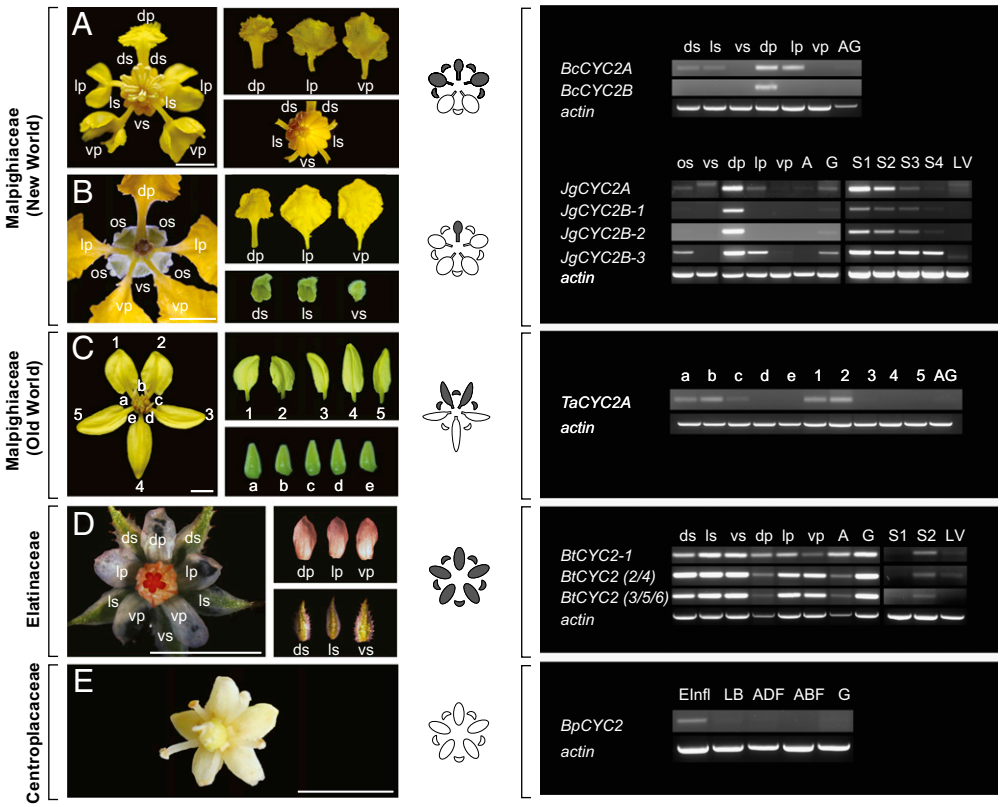

Fig. 3. *CYC2*-like gene expression in Malpighiaceae, Elatinaceae, and Centroplacaceae. RT-PCR was examined in whole flower buds at early developmental stages, dissected floral organs at later stages, and leaves. The stereotypical flowers of New World Malpighiaceae are represented here by *Byrsonima crassifolia* (*A*) and *J. guaranitica* (*B*). *T. australasiae* (*C*) is an Old World species of Malpighiaceae that has lost the stereotypical New World morphology and instead has two dorsal petals and eglandular sepals. Flowers of *Bergia texana* (Elatinaceae, *D*) and *Bhesa paniculata* (Centroplacaceae, *E*) are actinomorphic and eglandular. Gray highlighting on flower diagrams indicates the spatial pattern of *CYC2* expression in the corolla and calyx. *Actin*-specific primers were used as a positive control. ds, dorsal sepal; ls, lateral sepal; vs, ventral sepal; dp, dorsal petal; lp, lateral petal; vp, ventral petal; AG, androecium and gynoecium; os, sepal with oil glands; A, androecium; G, gynoecium; S, developmental stages of floral buds (S1, <1 mm in diameter; S2, 1–2 mm; S3, 2–3 mm; S4, 4–5 mm); LV, leaves; b, dorsal sepal; a, c, lateral sepal; d, e, ventral sepal; 1, 2, dorsal petal; 3, 5, lateral petal; 4, ventral petal; EInfl, early inflorescence; LB, large buds (2–3 mm); ADF, adaxial flower (including sepals, petals, and stamens); ABF, abaxial flower. Note: our sequence analysis confirmed that the larger fragment amplified from the ventral sepal of *J. guaranitica* is not a TCP family gene. (Scale bars, 5 mm.)

TaCYC2A is still expressed in the dorsal region of the flower, but now the domain encompasses the two dorsal petals and one dorsal and two lateral sepals. We hypothesize that changes in the regulation of *TaCYC2A* expression contributed to the shift in flower morphology of *Tristellateia*, and may reflect adaptations to a different pollination strategy. *Tristellateia* is visited by xylocopine bees whose reward for visiting the flowers appears to be pollen, rather than oil (14, 15, 21).

Additional expression studies of the *CYC2* homologues across the six remaining Old World Malpighiaceae clades, plus two small New World clades that have lost the stereotypical New World floral morphology, will provide further insights into the genetic and developmental basis of how floral zygomorphy has been altered or lost in this group. This will enable us to begin to determine whether convergent floral morphologies have evolved via convergent genetic changes in what appears to be a broadly conserved developmental program.

***CYC2* and the Actinomorphic-Flowered Relatives of Malpighiaceae, Centroplacaceae, and Elatinaceae.** Our results demonstrate that the two actinomorphic sister clades of Malpighiaceae, represented by *B. paniculata* (Centroplacaceae) and *B. texana* (Elatinaceae), have different patterns of *CYC2*-like gene expression, suggesting that different processes may be involved in controlling similar floral symmetries. The single *CYC2*-like gene of *B. paniculata* (*BpCYC2*) is expressed only during early stages of flower development (Fig. 3), which is similar to the well studied actinomorphic-flowered rosid *A. thaliana* (L.) Heynh. For the *Arabidopsis TCP1* gene, dorsal gene expression is detected in the young floral meristem but greatly declines before the floral organ primordia are initiated (33). It remains to be determined whether early *BpCYC2* expression is similarly localized in the dorsal region of the floral meristem in *Bhesa*.

In contrast, a unique temporal and spatial pattern of *CYC2*-like gene expression was discovered in the actinomorphic flowers of *Bergia texana* (Fig. 3). The six *CYC2*-like genes in *B. texana* exhibit a high degree of sequence similarity (i.e., 78–95%), suggesting that they are most likely recently evolved paralogues (Fig. 2). As sequence variation among some of these paralogues was especially minor (i.e., ≤5%), we distinguished three groups for RT-PCR analysis: (*i*) *BtCYC2-1*, (*ii*) *BtCYC2-2* and *BtCYC2-4*, and (*iii*) *BtCYC2-3*, *BtCYC2-5*, and *BtCYC2-6*. All six *BtCYC2* genes exhibit no sign of early expression, but are expressed uniformly across the floral organs during later stages of development. Given the apparent uniformity of gene expression in these paralogues, it is most likely that the expression pattern we observed in *B. texana* evolved before gene duplication. In terms of the temporal pattern of *CYC2* expression, our result most closely resembles that of the zygomorphic-flowered species *Iberis amara* L. (Brassicaceae), in which early expression of *IaTCP1* has been lost but late-stage expression is present. Spatial expression in *I. amara* is differential, however, which is consistent with the

fact that its flowers are zygomorphic (34). In this respect, the uniform spatial expression of *BtCYC2*s throughout the corolla of *B. texana* is similar to the pattern in *Cadia purpurea* Forssk., an actinomorphic-flowered member of the otherwise zygomorphic-flowered Fabaceae (35). The uniform expression of *LgCYC1B* in *Cadia* is thought to be the result of a homeotic transformation, which accounts for the apparent morphological reversal to actinomorphy, i.e., gene expression in the lateral and ventral petals has become dorsalized. The main difference between *CYC2*-like gene expression in *Cadia* and *Bergia* is that *LgCYC1B* expression appears to be corolla-specific whereas expression of *BtCYC2*s in *Bergia* is not restricted to the corolla, and is instead expressed throughout all of the floral organs examined (Fig. 3).

Interpreting the Origin of Floral Zygomorphy in Malpighiaceae. Differential expression of the *CYC2*-like genes during the late stage of floral development is required for establishing zygomorphic flowers in several phylogenetically diverse rosid [*Iberis, Lotus, Lupinus,* and *Pisum* (29, 30, 34, 35)] and asterid [*Antirrhinum, Gerbera, Linaria*, and *Senecio* (23, 24, 36–38)] clades, suggesting that the recruitment of *CYC2*-like function at later developmental stages is necessary for evolving zygomorphic flowers. To better understand the evolution of gene expression that likely contributed to the origin of zygomorphy in Malpighiaceae, we reconstructed the evolutionary pattern of late stage *CYC2* expression in New World Malpighiaceae [i.e., which represents the ancestral floral morphology for the family (9, 19, 27)] and their closest actinomorphic-flowered relatives (Fig. S7).

Our results indicate that the most recent common ancestor of Centroplacaceae–Elatinaceae–Malpighiaceae likely exhibited the pattern of gene expression observed in Centroplacaceae (*B. paniculata*), which is also similar to the actinomorphic-flowered rosid, *A. thaliana* (Fig. 4 and Fig. S7). *CYC2* expression in the most recent common ancestor of Malpighiaceae–Elatinaceae, however, is equivocal.

In view of the uncertain pattern of *CYC2*-like gene expression in the most recent common ancestor of Malpighiaceae–Elatinaceae, we propose three alternative, and equally parsimonious, scenarios to explain the origin of floral zygomorphy in Malpighiaceae (Fig. 4). In the first scenario, the most recent common ancestor of Malpighiaceae–Elatinaceae had actinomorphic flowers with no late-stage *CYC2* expression. Under this scenario, floral zygomorphy and differential *CYC2* expression evolved in the common ancestor of Malpighiaceae and the broad expression in Elatinaceae must have evolved independently. The second scenario proposes that the most recent common ancestor of Malpighiaceae–Elatinaceae was actinomorphic and exhibited uniform *CYC2* expression across the floral meristem. Under this scenario, floral zygomorphy in Malpighiaceae originated through the loss of ventral gene expression. The third scenario proposes that the most recent common ancestor of Malpighiaceae–Elatinaceae possessed zygomorphic flowers with differential *CYC2* expression. Under this scenario, floral zygomorphy and differential *CYC2* expression evolved in the common ancestor of Malpighiaceae–Elatinaceae. Actinomorphic flowers in Elatinaceae would therefore have been secondarily derived from a zygomorphic ancestor. Support for this model derives from the *Cadia* example discussed earlier, which is what we hypothesize here to explain the gene expression pattern observed in Elatinaceae. This scenario is especially intriguing because it is one that we would not have previously predicted given our character state reconstructions of floral evolution based on standard morphological characters (Fig. S1). Those analyses clearly indicate that zygomorphy arose in the most recent common ancestor of Malpighiaceae and that actinomorphy in Elatinaceae was plesiomorphic. The precedent established by *Cadia*, combined with these analyses, however, raises the distinct possibility that

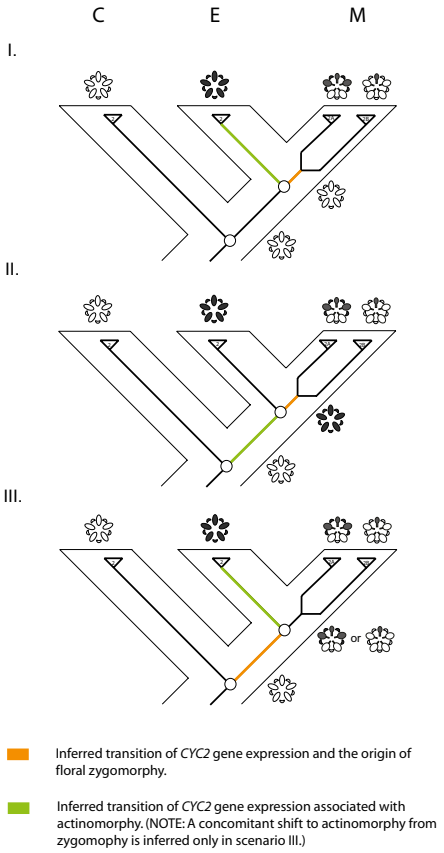

Fig. 4. Hypothesized scenarios for the origin of floral zygomorphy in Malpighiaceae. Gray highlighting in the flower diagram indicates the spatial pattern of *CYC2* expression. Orange coloring on the tree indicates the inferred transition to differential *CYC2* expression and floral zygomorphy; green indicates the transition to actinomorphic *CYC2* expression in actinomorphic-flowered species. In scenario (I), floral zygomorphy evolved in stem lineage Malpighiaceae from an actinomorphic-flowered ancestor with no late stage *CYC2* expression. In scenario (II), floral zygomorphy evolved in stem lineage Malpighiaceae from an actinomorphic-flowered ancestor with actinomorphic late stage *CYC2* expression. In scenario (III), floral zygomorphy and differential *CYC2* expression evolved from an actinomorphic-flowered ancestor with no *CYC2* expression along the stem lineage leading to the most recent common ancestor of Malpighiaceae and Elatinaceae. (C, Centroplacaceae; E, Elatinaceae; M, Malpighiaceae.)

actinomorphy in Elatinaceae may instead be better explained as a result of a homeotic transformation from a zygomorphic-flowered ancestor, which ultimately gave rise to Malpighiaceae.

Overall, this complex set of alternatives demonstrates that analyzing molecular data, and gene expression data in particular, in an explicit phylogenetic framework enhances comparative studies and can add a level of richness to our understanding of morphological evolution. Determining which of these models is most likely, however, will require additional comparative expression data, particularly from other actinomorphic rosids, as well as functional studies of *CYC2*-like genes in Elatinaceae and Malpighiaceae. It is important to note here that most studies of *CYC2* homologues have focused exclusively on zygomorphic-flowered groups (e.g., Asteraceae, Dipsacales, Fabaceae, Lamiales), meaning that we actually know very little about comparative patterns of *CYC2*-like gene expression in actinomorphic-flowered groups. This is especially relevant given the diverse actinomorphic-flowered ancestors that gave rise to these zygomorphic clades. Our results, as well as those from Busch and Zachgo (34), indicate that further expression studies are needed to determine if early dorsal expression followed by gene repression is the ancestral condition

for many actinomorphic angiosperm clades as is commonly assumed (cf. *Arabidopsis*), or if the ancestral condition is, in fact, highly variable across the angiosperms.

Materials and Methods

Sequence Alignments and Phylogenetic Analyses. *CYC2*-like gene sequences we obtained from Malpighiaceae–Elatinaceae–Centroplaceae–Oxalidaceae (*SI Materials and Methods*) were assembled with Sequencher 4.7 (Gene Codes) and aligned by eye with reference to the translated amino acid sequences using MacClade 4.06 (39). We applied the WAG + G model of amino acid evolution to the aligned *CYC2* data set as determined by the Akaike Information Criterion in ProtTEST (40). One hundred ML bootstrap replicates were conducted using RAxML-VI-HPC (41). Bayesian analyses were implemented in MrBayes version 3.1.2 (42) under the same model using default priors for the rate matrix, branch lengths, and γ-shape parameter. A Dirichlet distribution was used for the base frequency parameters and an uninformative prior was used for the starting tree topology. Four chains were initiated with a random starting tree and run for two million generations sampled every 1,000 generations. Following a burn-in of 1,000 trees as determined by Tracer version 1.4.1 (*http://tree.bio.ed.ac.uk/software/tracer/*), trees were sampled from the posterior distribution to calculate clade posterior probabilities.

RT-PCR. Floral organs were dissected directly in the field from flower buds ranging in size from 1 mm in diameter to open flowers, and preserved immediately in cryodewars or RNAlater (Ambion). For the samples prepared in RNAlater, the floral organs were finely chopped on sterile Petri dishes to facilitate penetration of the preservative into the tissues. These materials were processed in our laboratory using the RNAqueous kit (Ambion). RT-PCR was performed as described (43) using locus-specific primers to examine the expression of each *CYC2*-like gene copy (Table S2). The sequence identity of RT-PCR fragments was confirmed by sequencing.

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Supporting Information

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SI Materials and Methods

Morphology-Based Character State Reconstruction of Floral Symmetry. We used maximum likelihood (ML) character state reconstruction as implemented in Mesquite version 2.6 (1) to infer the evolution of floral symmetry in Malpighiaceae and its closest relatives, Elatinaceae and Centroplacaceae. Floral morphology for 353 species was scored as either zygomorphic or actinomorphic and treated as unordered and optimized onto the rate-smoothed topology for the family (2, 3). Absolute divergence time estimates were ascertained using the methods described by Davis et al. (4, 5) and included nearly one quarter of all species of Malpighiaceae, plus numerous outgroup species of Elantinaceace and Centroplacaceae. These data were analyzed using the Mk1 model (6) with rate parameters estimated directly from the data.

Isolation of CYCLOIDEA Homologues in Malpighiales–Oxalidaceae–Celastrales. We used 3′ RACE to obtain all *CYC* homologues, including *CYC1*, *CYC2*, and *CYC3*, across the closely related clades Malpighiales, Oxalidales, and Celastrales (7). Fresh floral tissue was collected over a broad range of developmental stages (i.e., buds from multiple developmental stages from <1 mm to mature size, and open flowers) from four species of Malpighiaceae (*Byrsonima lucida* DC., *Janusia guaranitica* A. Juss., *Malpighia coccigera* L., and *Tristellateia australasiae* A. Rich.), two additional species of Malpighiales [*Hypericum perforatum* L. (Hypericaceae) and *Euphorbia milii* Des Moul. (Euphorbiaceae)], one species of Oxalidales [*Oxalis herrerae* R. Knuth (Oxalidaceae)], and one species of Celastrales [*Euonymus alatus* (Thunb.) Siebold (Celastraceae)].

Total RNAs were purified using the Concert Plant RNA Reagent (Invitrogen). Single-stranded cDNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) by priming with the oligonucleotide 5′-CCGGATC CTCTAGAGCGGCCGC$(T)_{17}$. This poly-T primer was then used with primer 5′-AARGAYMGICAYAGYAARAT (modified from ref. 8) for PCR. PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) and used as the template in a secondary PCR with the degenerate forward primer, 5′-GCIAGRAARTTYTTYGAYYTICARGAYATG, and the poly-T primer described earlier. PCRs were performed in 100 μL of buffer [60 mM Tris-SO_4, pH 8.9; 18 mM $(NH_4)_2SO_4$; 2.5 mM $MgSO_4$] containing 50 pmol 5′ primer, 10 pmol poly-T primer, 25 pmol of dNTPs, and 3.75 units of PlatinumTaq polymerase (Invitrogen). The PCR amplification began with a 12-min activation step at 95 °C, followed by 37 cycles of a 1-min incubation step at 95 °C, a 30-s annealing step at temperatures ranging from 40 °C to 60 °C, and a 1-min extension at 72 °C. The reaction was terminated with a 10-min incubation at 72 °C. Gel-purified secondary PCR products were cloned using the pGEM-T easy vector system (Promega) and XL1-Blue competent cells (Stratagene). Fifty to 200 colonies for each taxon were screened by restriction site analysis and sequencing using the protocols established at Functional Biosciences. The identity of *CYC*-like genes was determined by the presence of the highly conserved TCP domain. Phylogenetic analyses including numerous TCP genes from previously published studies confirmed the identity of *CYC* homologues.

Isolation of CYC2-Like Genes from Malpighiaceae–Elatinaceae–Centroplacaceae–Oxalidaceae. Based on the *CYC*-like gene alignment, we designed degenerate primers to amplify the *CYC2*-like genes from genomic DNA. Our broad taxonomic sampling ensured that these degenerate primers covered a broad range of sequence variation across a diverse set of taxa. One set of nested degenerate primers (i.e., the forward primer located in the TCP domain, 5′-GCIMGIAARTTYTTYGAYYTKCAA, and the reverse primer in the R domain, 5′-GCYCKYGCYCTIGCYYTHKCYCTWGA), was selected to amplify a 350- to 400-bp fragment of the *CYC2*-like genes from Malpighiaceae and its sister clades. Gel-purified PCR products were cleaned and cloned. Fifty to 200 colonies were initially screened by restriction site analysis, and at least five clones were sequenced for each variant identified using this initial screen. Our criteria to further distinguish these variants included the degree of nucleotide variability and the presence of unique indels. Sequence variants containing no indels and differing by less than 5% sequence similarity were treated as alleles. The coding regions of *CYC2A* and *CYC2B* that fall between the TCP and R domains are 176 ± 14 and 184 ± 5 nucleotides long, respectively. *CYC1*- and *CYC3*-like genes, which were rarely amplified with *CYC2*, were distinguished by using phylogenetic analysis and excluded from subsequent consideration.

Nucleotide Sequence Analyses. Our analyses in the main text focused on amino acid sequence analyses. Analyses using nucleotide sequence data with third codon positions excluded, yielded a topology nearly identical to the amino acid sequence data. The parameters of the best-fit model for our nucleotide data were estimated using MODELTEST 3.06 (9). The Akaike Information Criterion (10) recommended a general time reversible model with added parameters for invariable sites and a Γ distribution ("GTR + I + Γ"). One hundred ML bootstrap replicates were conducted with the optimal model of sequence evolution. Bayesian analyses were also conducted using this model.

Southern Hybridization. Ten micrograms of genomic DNA was digested from *Bergia texana*, *Byrsonima crassifolia, J. guaranitica,* and *T. australasiae* with restriction enzymes (i.e., HindIII, EcoRI, and HindIII plus EcoRI), fractionated on 0.8% agarose gels, and blotted onto a positively charged nylon membrane (GE Healthcare BioSciences). In addition, for *Janusia*, we ran lanes containing *CYC1*, *CYC2*, and *CYC3* plasmid DNA as controls to test probe efficiency and specificity.

A fragment containing the 3′ end of the TCP domain and the variable region between the TCP and R domains was used as a template to synthesize probes for detecting *CYC2*-like genes (Fig. S3*B*). For *J. guaranitica*, a mixture of *JgCYC2A* (*CYC2A*) and *JgCYC2B-3* (*CYC2B*) sequences in equal molar concentration was used as a template to synthesize probes with ^{32}P-dCTP (Perkin-Elmer) using the Prime-It II kit (Stratagene). This gene region exhibits no more than 31% pair-wise sequence similarity among the *CYC1/2/3* paralogues, which ensures probe specificity. The hybridization was carried out in hybridization solution [900 mM NaCl; 60 mM $NaH_2PO_4 \bullet H_2O$; 6 mM EDTA; 5× Denhart solution (from 50×; Amresco); 1% SDS; 10 μg/mL sheared salmon sperm DNA; pH 7.4] at 65 °C for 18 h. The membranes were then washed at low stringency (900 mM NaCl; 60 mM $NaH_2PO_4 \bullet H_2O$; 6 mM EDTA; 1% SDS; pH 7.4) at 65 °C and exposed for 90 h to phosphor imaging (GE Healthcare Bio-Sciences) to detect for the presence of all *CYC2* homologues.

There are four bands for *J. guaranitica* in the EcoRI digest of the *CYC2* probe (Fig. S3*A*). This result is identical to our cloning experiments, which also identified four copies of *CYC2*. The *CYC1* and *CYC3* plasmid controls gave very faint signals experimentally, demonstrating that the *CYC2* probes are lineage specific. The number of bands in the EcoRI digest therefore

reflects the approximate *CYC2* copy number. In the HindIII and double digests, we expected more than four bands as a result of the presence of a restriction site within the probed region.

Given the ability of our probe to detect lineage specific *CYC2* gene copies, we conducted Southern hybridizations only for *CYC2* on the remaining taxa (i.e., *B. texana*, *B. crassifolia,* and *T. australasiae*). *BtCYC2-1* and *BtCYC2-2* of *B. texana*, *BcCYC2A* and *BcCYC2B* from *B. crassifolia*, and *TaCYC2A* of *T. australasiae* were mixed in equal molar concentrations and used as a template to synthesize our ^{32}P-labeled probes. Our *CYC2*-specific probes revealed two bands in *B. crassifolia*, one band in *T. australasiae*, and six bands in *B. texana* (see EcoRI digest in Fig. S4), which was identical to the *CYC2* copy number inferred by PCR and cloning.

Floral Organ Arrangement in New and Old World Malpighiaceae. New World Malpighiaceae, e.g., *B. crassifolia* and *J. guaranitica*, possess a dorsal flag/banner petal that is always innermost in bud. The remaining four petals can be arranged in one of two ways that form mirror images of each other (Fig. S5*A*). These enantiomorphic flowers can be found on the same inflorescence within a single species. The Old World species *T. australasiae* has a similar petal aestivation (Fig. S5*B*; also see main text). The innermost petal of *T. australasiae* is homologous to the New World dorsal flag/banner petal and can also initiate on the left or right side of the dorsoventral plane of symmetry (Fig. S5*B*). We used the relative positions of these floral organs to sample homologous tissue types from these New and Old World species, and took great care to sample only those *Tristellateia* flowers for RT-PCR in which the innermost petal was located on the left side of the dorsoventral plane of symmetry.

Notes on the Floral Orientation of *Bergia texana* and *Bhesa paniculata*. *B. texana* has a single dorsal petal with respect to the stem, which is uncommon relative to most angiosperms, which have one ventral petal (11, 12). Two dorsal and one ventral sepal are glandular, and two lateral sepals are eglandular (Fig. 3 in the main text). Moreover, the two dorsal sepals tightly clasp the stem, so it is relatively easy to determine that there is a single dorsal petal in *B. texana*. In Centroplacaceae, the orientation is more difficult to interpret. The flowers of *B. paniculata* are sessile, and three tightly congested flowers are commonly borne in an inflorescence. More than one kind of floral orientation was observed, in which case these flowers may twist during development.

Character State Reconstruction of CYC2-Like Gene Expression. We used ML character state optimization as implemented in Mesquite 2.6 (1) to reconstruct the evolution of *CYC2*-like gene expression. We used the single ML topology inferred from *CYC2* nucleotide sequences to reconstruct the pattern of *CYC2* gene expression under the general Mk1 model (6) with the rate parameter estimated from the data. The character states of gene expression were coded as follows: none, uniform, broad differential, and narrow differential. Ancestral character states were reconstructed assuming that all transition states are unordered. Although we used genetic distance as an approximate measure of the "opportunity for selection" (13), we also conducted our analysis with the topology calibrated for absolute divergence time estimates (4, 5, 14). Those results are very similar to those presented here and do not affect our conclusions.

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Fig. S1. Ancestral character state reconstruction of floral symmetry. ML analysis indicates the relative likelihood of floral symmetry at the nodes of Centroplacaceae–Elatinaceae–Malpighiaceae (actinomorphy, 0.77; zygomorphy, 0.23), Elatinaceae–Malpighaceae (actinomorphy, 0.75; zygomorphy, 0.25), and crown group Malpighiaceae (actinomorphy, 0.0; zygomorphy, 1.0). These results indicate that the common ancestor of Centroplacaceae–Elatinaceae–Malpighiaceae and Elatinaceae–Malpighiaceae are likely actinomorphic, and that zygomorphy evolved in the common ancestor of all Malpighiaceae.

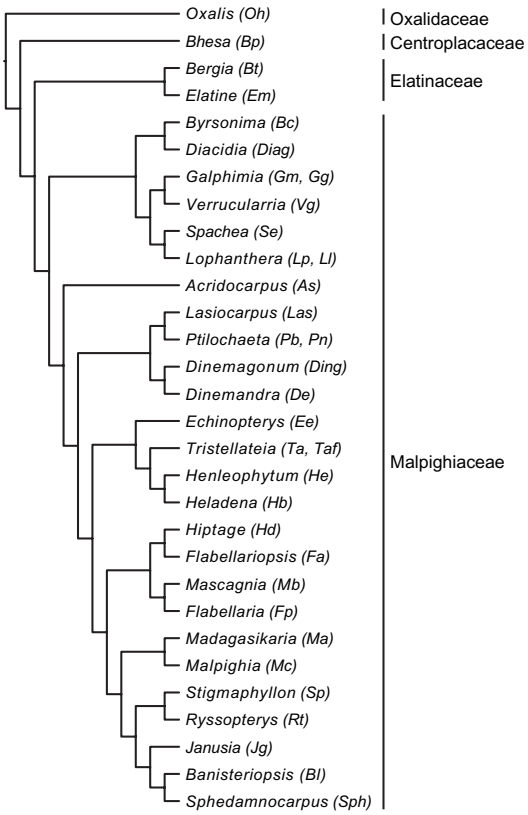
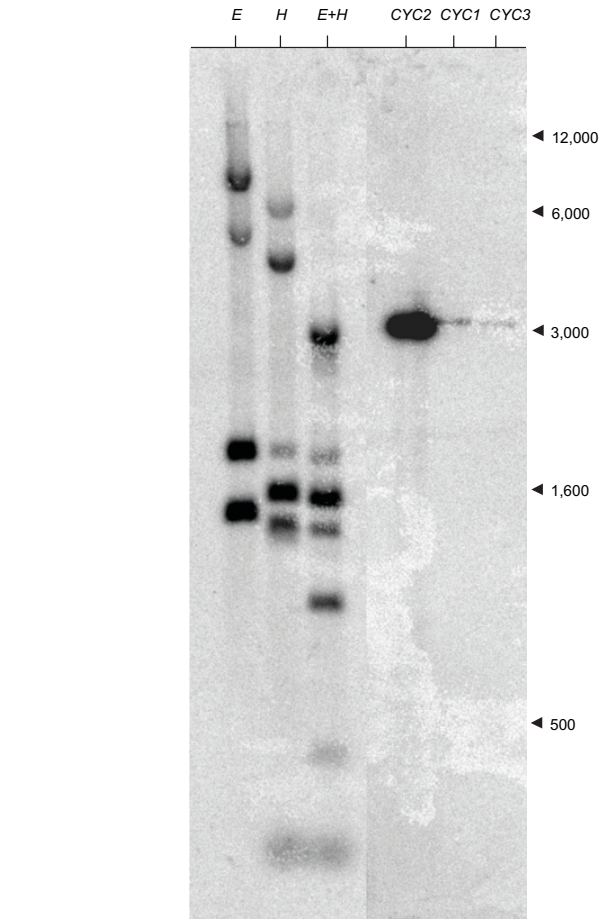
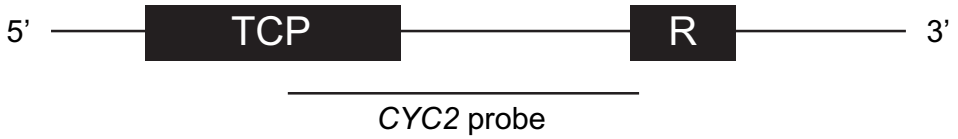


Fig. S2. Phylogeny showing accepted species relationships (derived from refs. 2, 3, 15–17).

A.



B.



C.

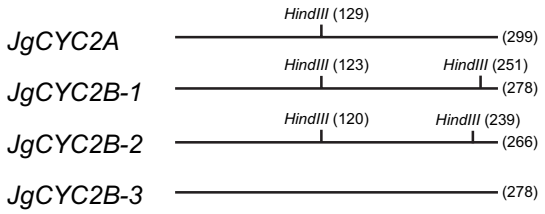


Fig. S3. *CYC2* Southern hybridization results for *J. guaranitica*. (*A*) To assess gene copy number, we developed a *CYC2*-specific probe and tested it against *J. guaranitica*. That probe preferentially identified only *CYC2* and not other *CYCLOIDEA* homologues in *Janusia*, i.e., *CYC1* and *CYC3*. The result of this test of the probe's specificity is shown on the right side of the *Janusia* blot. Restriction digests using EcoRI (E), HindIII (H), and EcoRI + HindIII (E+H) are shown for *Janusia*. (*B*) The position of the probe region within *CYC*-like genes. (*C*) Restriction cut sites were inferred from sequence analysis and are indicated on the *CYC2* gene copies shown at bottom. Arrows and numbers indicate molecular size markers (in base pairs).

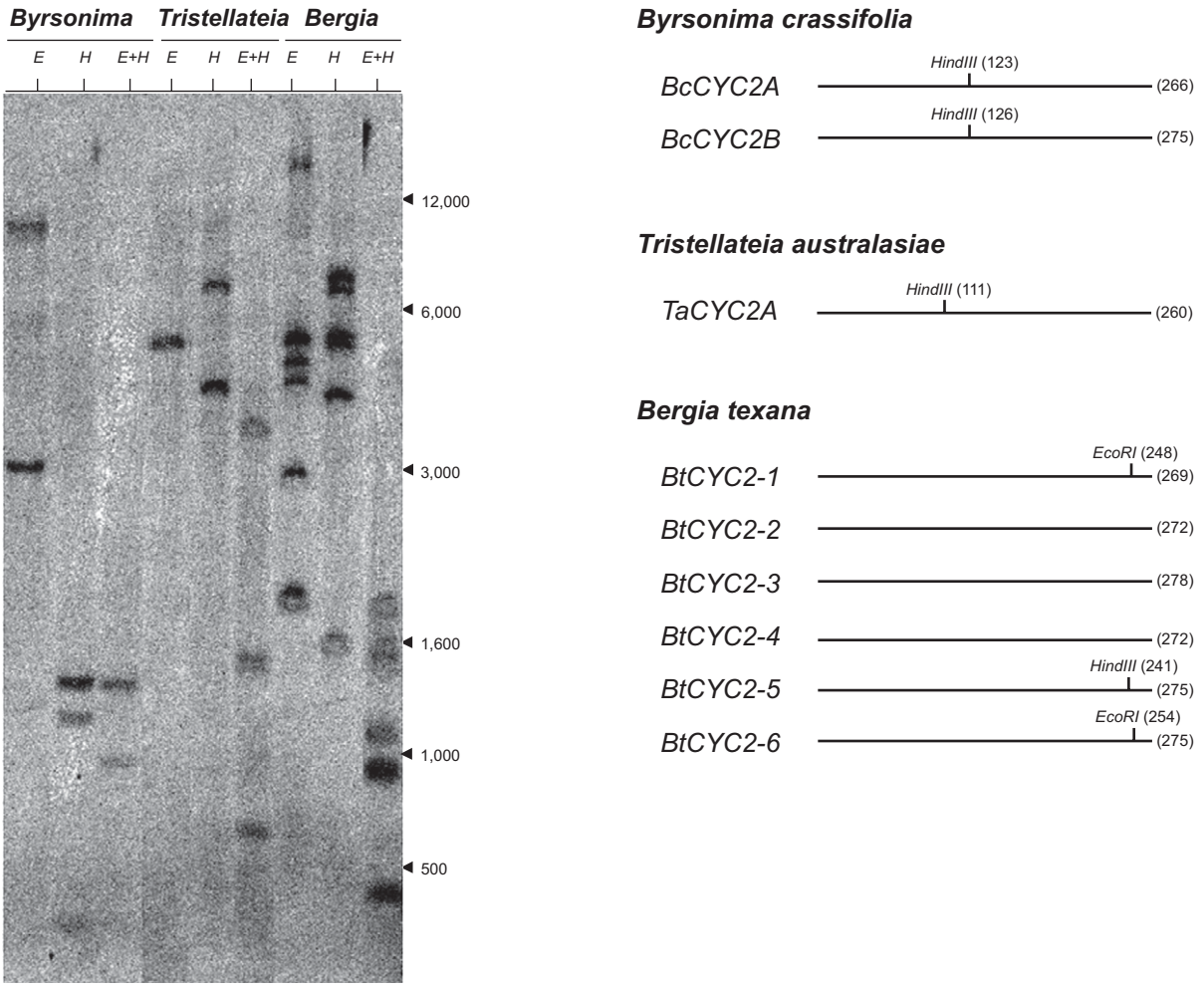


Fig. S4. *CYC2*-like gene Southern hybridization results for *Byrsonima crassifolia*, *T. australasiae*, and *Bergia texana*. (*A*) Restriction digests using EcoRI (E), HindIII (H), and EcoRI + HindIII (E+H) are shown for *B. crassifolia*, *T. australasiae*, and *B. texana*. (*B*) Restriction cut sites were determined from sequence analysis and are indicated on the *CYC2* gene copies shown at bottom. Arrows and numbers indicate molecular size markers (in base pairs).

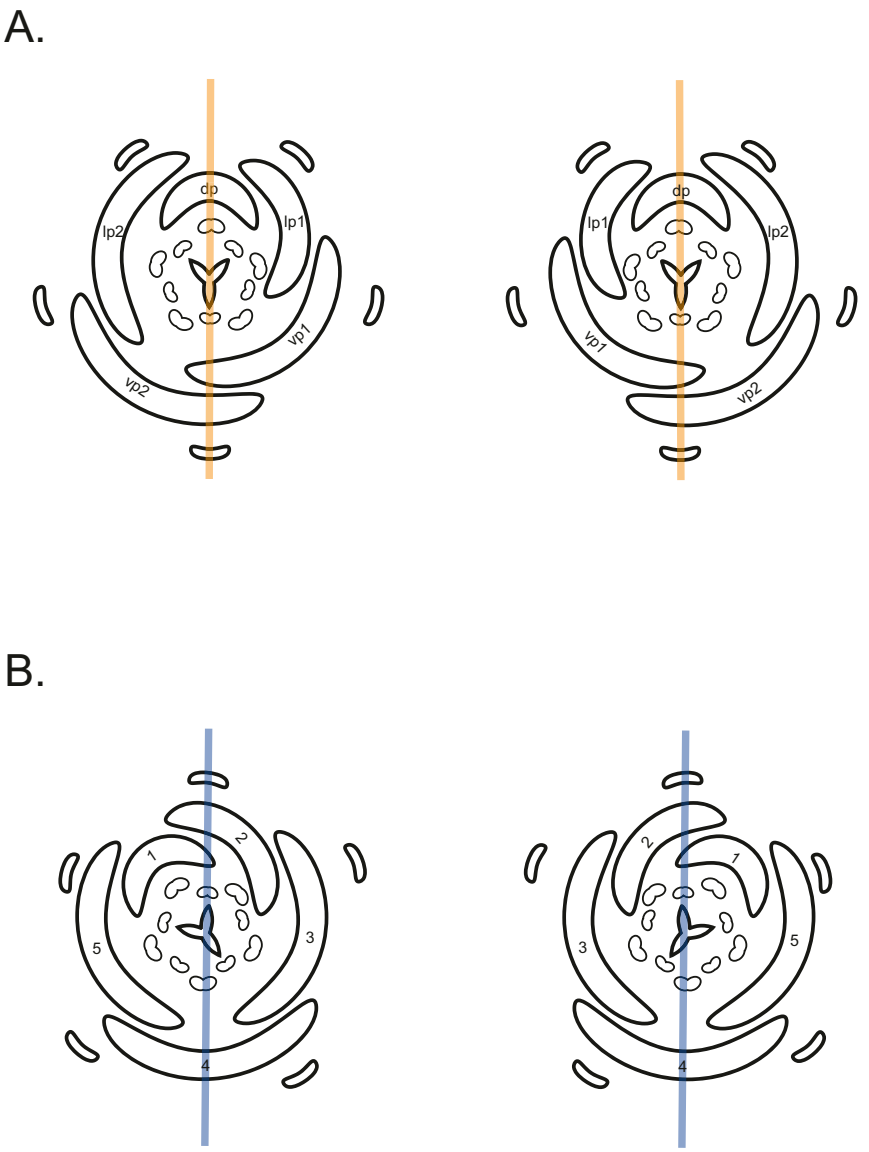


Fig. S5. Floral aestivation and enantiomorphy of Malpighiaceae. (*A*) New World Malpighiaceae. Petal identities are indicated as follows: dp, dorsal petal; lp1-2, lateral petals; vp1-2, ventral petals. (*B*) *T. australasiae*, an Old World Malpighiaceae species. The dorsoventral planes of floral symmetry are indicated with a colored vertical line. Petal identities are indicated as follows: 1 and 2, dorsal petals; 3 and 5, lateral petals; 4, ventral petal. The petals are not intended to be drawn proportional to scale in *Tristellateia*.

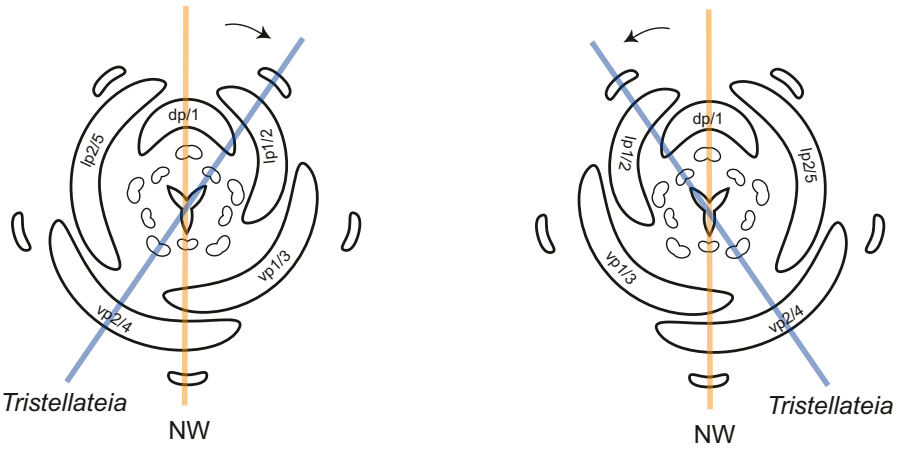


Fig. S6. Hypothesized shift in the dorsoventral plane of symmetry in the Old World species *T. australasiae*. Floral arrangement of New World Malpighiaceae shown for comparison. The dorsoventral planes of floral symmetry of New World Malpighiaceae and *Tristellateia* are indicated in orange and blue, respectively. The arrow illustrates the hypothesized 36° rotation in *Tristellateia* relative to their New World ancestors. For the New World arrangement abbreviations are as follows: dp, dorsal petal; lp1-2, lateral petals; vp1-2, ventral petals. For the Old World arrangement, abbreviations are as follows: 1 and 2, dorsal petals; 3 and 5, lateral petals; 4, ventral petal.

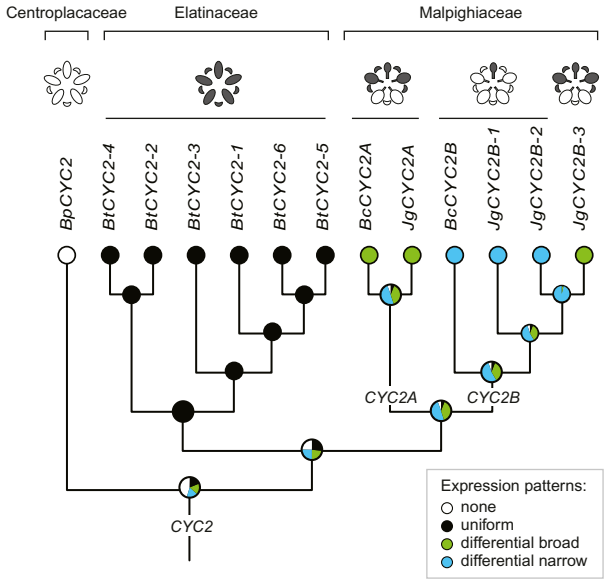


Fig. S7. Character state reconstruction of *CYC2* gene expression. Expression patterns are treated as character states shown in different colors. Areas of pies indicate the relative degree of support for alternative ancestral character states. Gray highlighting in flower diagram indicates the spatial pattern of *CYC2* expression. The most recent common ancestor of Centroplacaceae–Elatinaceae–Malpighiaceae likely exhibited the pattern of gene expression observed in Centroplacaceae (*Bhesa paniculata*). *CYC2* expression in the most recent common ancestor of Malpighiaceae–Elatinaceae is equivocal. The most recent common ancestor of Malpighiaceae likely exhibited differential expression of *CYC2* before the *CYC2A/B* duplication. In addition, our results support the independent evolution of a broader pattern of gene expression of *JgCYC2B-3*, one of the *CYC2B* copies in *J. guaranitica*, from a narrow patterned *CYC2B* ancestor.

Table S1. Species sampled, with collection locations, voucher information, and *CYC2* identities

Species	Location	Voucher	Identity of obtained *CYC2* copies		
			2	2A	2B
Acridocarpus smeathmanni Guill. and Perr.	Ghana	Davis 99–13 (A)	–	*AsCYC2A*	*AsCYC2B*
Banisteriopsis latifolia (A.Juss.) B. Gates	Distrito Federal, Brazil	Azeuedo 698 (MICH)	–	*BlCYC2A*	*BlCYC2B-1*, *BlCYC2B-2*
Bergia texana Seub. ex Walp.	Butte County, California, US	Zhang, Ahart, and Bartholomew 84 (A)	*BtCYC2-1* ~ *BtCYC2-6*	–	–
Bhesa paniculata Arn.	Negeri Sembilan, Malaysia	Zhang and Boufford 160 (A)	*BpCYC2*	–	–
Byrsonima crassifolia Kunth	Cult. OEB, Harvard U.	Matamoros and Cerda 301	–	*BcCYC2A*	*BcCYC2B*
Diacidia galphimioides Griseb.	Amazonas, Venezuela	Berry et al., 5275 (MICH)	–	*DiagCYC2A*	-
Dinemagonum gayanum A.Juss.	Chile	Simpson 83–10-23–2c (MICH)	–	*DingCYC2A*	*DingCYC2B*
Dinemandra ericoides A.Juss.	Chile	Dillon and Teillier 5103 (MICH)	–	*DeCYC2A*	*DeCYC2B*
Echinopterys eglandulosa (A. Juss.) Small	Sonora, Mexico	Van Devender 98–178 (MICH)	–	*EeCYC2A*	*EeCYC2B*
Elatine minima (Nutt.) Fisch. & C. A. Meyer	Gemini lake, North Michigan, US	Voss 11739 (MICH)	*EmCYC2-1*, *EmCYC2-2*	–	–
Flabellaria paniculata Cav.	Tanzania	Congdon 414 (K)	–	–	*FpCYC2B*
Flabellariopsis acuminata (Engl.) R. Wilczek	Tanzania	Faulkner 783 (K)	–	*FaCYC2A*	*FaCYC2B-1*, *FaCYC2B-2*
Galphimia gracilis Bartl.	Fairchild T.G., Florida, US	FTG 79–235 (FTG)	–	*GgCYC2A-1*, *GgCYC2A-2*	*GgCYC2B*
Galphimia mexiae C.E. Anderson	Jalisco, Mexico	Anderson and Anderson 6122 (MICH)	–	*GmCYC2A*	*GmCYC2B*
Heladena bunchosioides A. Juss.	Espírito Santo, Brazil	Folli 4653 (MICH)	–	*HbCYC2A*	*HbCYC2B*
Henleophytum echinatum (Griseb.) Small	nr. Havana, Cuba	Curtiss 688 (K, NY)	–	*HeCYC2A*	*HeCYC2B*
Hiptage detergens Craib	Thailand	Middleton et al., 2095 (A, MICH)	–	*HdCYC2A-1*, *HdCYC2A-2*	*HdCYC2B-1*, *HdCYC2B-2*
J. guaranitica A. Juss.	Cult. OEB, Harvard U.	Zhang 165 (A)	–	*JgCYC2A*	*JgCYC2B-1*, *JgCYC2B-2*, *JgCYC2B-3*
Lasiocarpus sp.	Mexico	Anderson 13828 (MICH)	–	*LasCYC2A*	*LasCYC2B*
Lophanthera longifolia Griseb.	Amazonas, Venezuela	Zimmerman 27 (MICH)	–	*LlCYC2A*	*LlCYC2B*
Lophanthera pendula Ducke	Amazonas, Brazil	Lima and Lima 3185 (MICH)	–	*LpCYC2A*	*LpCYC2B*
Madagasikaria andersonii C. Davis	Madagascar	Davis 20–01 (A)	–	*MaCYC2A*	–
Malpighia coccigera L.	UMBG	UMBG 20626 (MICH)	–	*McCYC2A*	*McCYC2B*
Mascagnia bracteosa Griseb.	Manaus, Brazil	Anderson 13777 (MICH)	–	*MbCYC2A*	*MbCYC2B*
Oxalis herrerae R.Knuth	Cult. OEB, Harvard U.	Zhang 20 (A)	*OhCYC2-1*, *OhCYC2-2*	–	–
Ptilochaeta bahiensis Turcz.	Bahia, Brazil	Anderson 13725 (MICH)	–	–	*PbCYC2B-1*, *PbCYC2B-2*, *PbCYC2B-3*
Ptilochaeta nudipes Griseb.	Jujuy, Argentina	Anderson 13588 (MICH)	–	*PnCYC2A*	*PnCYC2B-1*, *PnCYC2B-2*
Ryssopterys timoriensis (DC.) Blume ex A. Juss.	Cult. Bogor	XVIII.F.172 (BO)	–	*RtCYC2A*	*RtCYC2B-1*, *RtCYC2B-2*
Spachea elegans A. Juss.	Guyana	Janson-Jacobs et al., 3907 (MICH)	–	*SeCYC2A*	*SeCYC2B-1*, *SeCYC2B-2*
Sphedamnocarpus sp.	Madagascar	Phillipson et al., 4104 (MICH, MO, P)	–	–	*SphCYC2B*
Stigmaphyllon paralias A. Juss.	Bahia, Brazil	Anderson 13693 (MICH)	–	–	*SpCYC2B*
Tristellateia africana S. Moore	Dar es Salaam, Tanzania	Davis 99–25 (A)	–	*TafCYC2A-1*, *TafCYC2A-2*, *TafCYC2A-3*	–
T. australasiae A. Rich.	Cult. OEB, Harvard U.	Zhang 163 (A)	–	*TaCYC2A*	–
Verrucularia glaucophylla A. Juss.	Bahia, Brazil	Amorim 3662 (CEPEC, MICH)	–	*VgCYC2A-1*, *VgCYC2A-2*	*VgCYC2B*

Arnold Arboretum (Arn. Arb.) is in Jamaica Plain, MA. A, Arnold Herbarium, Harvard University Herbaria; BO, Herbarium Bogoriense, Bogor, West Java, Indonesia; CEPEC, Herbário Centro de Pesquisas do Cacau, Bahia, Brazil; FTG, Fairchild Tropical Botanic Garden; K, Royal Botanic Gardens, Kew, England; MICH, University of Michigan Herbarium; MO, Missouri Botanical Garden, St. Louis, Missouri; NY, New York Botanical Garden, Bronx, New York; *P*, Muséum National d'Histoire Naturelle, Paris. GenBank numbers are given for each copy found. The GenBank numbers are GU982187–GU982264.

Table S2. RT-PCR primer sequences used in this study

Name	Taxa	Forward (5′ to 3′)	Reverse (5′ to 3′)
BtCYC2-1	*Bergia texana*	GGTCTTACAATTCTACTAGTGAATTACTTG	GAATTCTGGAAGCAAACTTTTGTAAT
BtCYC2 (-2, -4)	*Bergia texana*	CAAGAWACTYTAGGGTTTGATAAAGCAA	CAAATGACTCCATYTTTGAAACTGTCC
BtCYC2 (-3, -5, -6)	*Bergia texana*	CAAGAWACTYTAGGGTTTGATAAAGCAA	GAATCCAMCTTTGAAACTSTTGTCC
BpCYC2	*Bhesa paniculata*	GATCTTCAAGACATTCTAGGGTTTGAC	GACTCCTTTGCAAGAAGTGTACTG
BcCYC2A	*Byrsonima crassifolia*	AAGATTTGTTAGGGTTTGATAGGG	AGGTCTCATTTCACTATAATCAACACA
BcCYC2B	*Byrsonima crassifolia*	AAGACCTTCTAGGGTTTGATAGGG	TCTCCCCTCACTAGAATCAACAGT
JgCYC2A	*J. guaranitica*	ACAATCTCTGGAGCTGAAAAGG	ACTCACATCCTGCCTGAACC
JgCYC2B-1	*J. guaranitica*	TTCCATAYTCAAGATCCGATTTA	CTCAATTGTTCTGATGATGACCT
JgCYC2B-2	*J. guaranitica*	TTCCATAYTCAAGATCCGATTTA	CTTCCCATGATTTGCAGTATACTTATT
JgCYC2B-3	*J. guaranitica*	CTAACAAGCGATCAAATCGAA	GATCTCAATTGTTCTGGTGATCTT
TaCYC2A	*T. australasiae*	TTAGGGTTTGACAGGGCAAG	GCTTAGCAAGAAGTGGGATTT