DIFFERENTIAL EXPRESSION OF CYC2 GENES AND THE ELABORATION OF FLORAL MORPHOLOGIES IN HIPTAGE, AN OLD WORLD GENUS OF MALPIGHIACEAE

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Editor: Gerhard Prenner

Premise of research. The primarily Neotropical Malpighiaceae exhibit an elegant suite of floral morphological characteristics associated with a specialization mutualism with oil bee pollinators, including bilaterally symmetrical flowers and paired oil glands on the calyx. One clade within the family, Hiptage Gaertn., has migrated to the paleotropics and lost its association with oil bees. Corresponding to this transition, some members of Hiptage have evolved a highly elaborate zygomorphic corolla with strongly reflexed petals and striking dorsoventral heteranthery. Previously, we demonstrated that expression of CYCLOIDEA2-like (CYC2-like) genes is correlated with the evolution of floral symmetry in Malpighiaceae. Here, we examine CYC2 expression in relation to the evolution of elaborate floral zygomorphy in Hiptage benghalensis.

Methodology. CYC2-like genes were cloned from H. benghalensis. The spatial pattern of CYC2 expression was examined with quantitative reverse-transcription PCR on the dissected floral organs.

Pivotal results. While most Neotropical Malpighiaceae express two CYC2-like genes, CYC2A and CYC2B, we demonstrate that H. benghalensis has experienced further duplications yielding four copies, which are expressed in all four whorls of the flower. As in Neotropical Malpighiaceae, CYC2A homologs HbCYC2A-1 and HbCYC2A-2 are expressed broadly in the dorsal region of the flower, but unlike that in other Neotropical species, expression also extends to the dorsal stamens. The CYC2B copies HbCYC2B-1 and HbCYC2B-2 are intensely expressed in the single dorsal petal (as in Neotropical Malpighiaceae), but their expression is further detected in the other floral whorls, especially in the stamens of the dorsal region.

Conclusions. The relaxation of the conserved expression of CYC2-like genes in Neotropical Malpighiaceae and the expansion to broader floral regions, including the dorsal androecium, correlate with the development of dorsoventral heteranthery in H. benghalensis. We propose that changes in the pattern of CYC2 expression may have contributed to the elaborated androecium of H. benghalensis, which was crucial for its adaptation to a novel pollination strategy.

Keywords: CYC2-like genes, development, floral evolution, floral symmetry, gene duplication, Hiptage benghalensis.

Online enhancements: appendix table and figure.

Introduction

The remarkable diversity of floral morphology results from genetic modifications in the regulators that control organ development, but it remains elusive how such alterations have occurred during the course of evolution in angiosperms (Glover et al. 2015; Specht and Howarth 2015). The plant family Malpighiaceae originated in the New World (NW; Anderson 1990; Cameron et al. 2001; Davis et al. 2001, 2002, 2004, 2014; Davis and Anderson 2010), where most species produce flowers with a typical morphology, including bilateral symmetry (zygomorphy) and oil-producing glands on the sepal (fig. 1A). This conserved floral morphology is thought to be maintained by plant-pollinator mutualisms involving NW Malpighiaceae and specialized female bees from several genera of Tapinotaspi-dini and Centridini (Apidae; Vogel 1990; Sigrist and Sazima 2004; Davis et al. 2014). Female bees secure themselves to the flower by grasping the narrow claw of the banner petal, i.e., the single dorsal petal, with their mandibles and then use their middle and hind legs to collect oil secretions from the paired glands on the abaxial surface of the sepal. Floral conservation in Malpighiaceae has been relaxed independently seven times, however, when members of the family migrated from the NW to the Old World (OW), where the obligate oil bee pollinators...
are absent (Anderson 1979, 1990; Davis 2002; Davis et al. 2002, 2004, 2014). In contrast to the conserved floral morphology of NW Malpighiaceae, the floral morphology of OW Malpighiaceae is highly diverse (Davis 2002; Davis and Anderson 2010; Zhang et al. 2010, 2012, 2013; Davis et al. 2014). Most of the OW species have lost the distinctive banner-petal morphology, resulting in radially symmetrical (actinomorphic) flowers. Furthermore, the oil glands have become completely lost, or the glands have become modified to produce sugar-based rewards (Lobreau-Callen 1989). The OW Malpighiaceae primarily provide pollen and, less commonly, nectar to their OW pollinators (Vogel 1990; Davis 2002).

**Hiptage** Gaertn., one of the seven OW clades, has evolved elaborate floral morphology, presumably as an adaptation to OW pollinators. *Hiptage* comprises ~25 species of woody lianas, all endemic in tropical forests ranging from Pakistan and India to Taiwan, the Philippines, and Indonesia (Chen and Chen 1997; Anderson et al. 2006–). The flowers of *Hiptage* are zygomorphic in all four whorls (fig. 1B; Niedenzu 1928; Anderson et al. 2006–). A single large calyx gland is diagnostic for the genus when present (Niedenzu 1928; Anderson et al. 2006–). While the sepal glands of two species of *Hiptage* still produce oil (Arumugasamy et al. 1989; Subramanian et al. 1990), the secretions of the single sepal gland in *Hiptage benghalensis* contain only sugars and amino acids instead of lipid rewards (Arumuganathan et al. 1994).

Additional floral features have also diverged from the ancestral morphology. The single dorsal petal in *Hiptage*, which is homologous to the banner petal of its NW relatives, is morphologically distinct in size and color and possesses a highly thickened claw (Anderson et al. 2006–). Importantly, strong doroventral heteranthery is obvious and synapomorphic in...
**Material and Methods**

**Specimen Collection**

Specimens of *Hiptage benghalensis* Kuntze were collected from naturalized plants in the Secret Woods Nature Center, Fort Lauderdale, Broward County, Florida.

**Isolation of CYC2-Like Genes and Phylogenetic Analysis**

We followed the methods described in Zhang et al. (2010, 2012, 2013) to clone the CYC2-like genes from *H. benghalensis*. DNA sequences of the four newly acquired CYC2-like genes have been deposited in GenBank, under accession numbers KX021861–KX021864. The newly obtained sequences were aligned with our previous CYC2-like gene matrix and followed the previously outlined approaches for phylogenetic reconstruction (Zhang et al. 2010).

**RNA Sample Preparations**

Floral buds from two stages representing late stages of floral development, open flowers, and leaves were prepared in liquid nitrogen in the field. The buds were grouped into two categories: medium (<40%–60% of full-size buds) and large (<70%–90% of full-size buds). All materials were preserved in cryogenic containers and processed in the lab with the RNAqueous kit (Ambion, Austin, TX). We examined two developmental stages for organ-specific CYC2 expression. First, we examined floral organs pooled from multiple flower buds, representing the large buds (<70%–90% of full-size buds), prepared in the field from fresh plants. Second, we examined floral organs from a single medium-sized bud (<50% of full-
size buds). The medium buds were too small to be dissected with accuracy in the field without a stereomicroscope. The medium buds were thus dissected in the lab, after being thawed from −80°C, with the RNAlater-ICE kit (Ambion-Applied Biosystems, Austin, TX). Two buds were examined as biological replicates. The microdissected samples were processed with the RNAqueous Micro kit (Ambion). DNA contamination was removed with a DNA-free kit (Ambion). RNA quality was assessed with an Agilent 2100 Bioanalyzer equipped with the RNA 6000 Nano Labchip kit for our pooled samples and the RNA 6000 Pico Labchip kit for each organ dissected from a single bud (Agilent Technologies, Palo Alto, CA). Details of this method were described in Zhang et al. (2012).

**Quantitative Reverse-Transcription PCR**

First-strand complementary DNA synthesis was performed with 0.5 μg total RNA for the pooled samples in a 20-μL reaction using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). For the four target CYC2 paralogs, primer pairs that hybridize to the gene copy–specific sequences were designed and evaluated by Primer3 (ver. 2.2.3) software (Untergasser et al. 2012) and were synthesized by Integrated DNA Technologies (Coralville, IA; table A1, available online). Class I β-tubulin was used as a control to normalize the quantitative reverse-transcription PCR (qRT-PCR), as previously described (Zhang et al. 2012). The qRT-PCR reactions were conducted with PerfeCTa SYBR GreenFastMix, Low ROX (Quanta BioSciences, Gaithersburg, MD), using the Stratagene MX3005P QPCR System (Stratagene, San Diego, CA) running a program with an initialization step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min, and melting-curve analyses were used to verify primer specificity. The qRT-PCR samples were also run on a 2% agarose gel to further confirm specificity. Absence of genomic DNA was further confirmed with our β-tubulin control, which spanned a 90-bp intron region. No β-tubulin amplicons were observed for the higher-molecular-weight intron-bearing copy. Thus, our RNA preparations were free of genomic DNA contamination. The identity of all amplicons was confirmed by sequencing. The amplification efficiency was determined for all genes (table A1; Pfaffl 2001). One biological replicate (i.e., one extraction from >20 flower buds from an individual plant) was analyzed for the latest stages; two biological replicates (i.e., two extractions from two flower buds from an individual plant) were analyzed for the medium-sized bud stages. Three technical replicates (i.e., three separate qRT-PCRs from a single extracted sample) were analyzed for each biological replicate. Standard errors were calculated from all technical replicates. CYC2 expression levels were calculated relative to β-tubulin with the 2−△△Ct method (Livak and Schmittgen 2001). Detailed methodology and statistical analyses are also described in Zhang et al. (2012).

**Reverse-Transcription PCR**

Reverse-transcription PCR (RT-PCR) was performed with locus-specific primers (table A1) for 30 cycles to examine the expression of CYC2-like genes in floral buds, open flowers, and leaves to determine whether the expression of CYC2-like genes was flower specific. The sequence identity of the RT-PCR fragments was further confirmed by sequencing.

**Usage of “Dorsal”/“Ventral” versus “Adaxial”/“Abaxial”**

To describe meristems, we use “dorsal” and “ventral” to refer, respectively, to the upper and lower regions of the zygomorphic floral meristem; “adaxial” and “abaxial” refer, respectively, to the upper and lower surfaces of individual lateral determinate organs.

**Results**

**Floral Morphology of Hiptage benghalensis**

The petals of *H. benghalensis* are pinkish white and have fringed margins and a short claw (fig. 1B). The single dorsal petal bears a bright-yellow central spot, and its claw is thickened. The base of the blade of the dorsal petal is pouched and bent (fig. 1B). When the dorsal petal is flattened, it is larger than the lateral and ventral petals. During anthesis, all five petals are strongly reflexed to cover the sepals, including the sugary secretion produced by the single dorsal gland located on the abaxial surface between the two dorsal sepals. *Hiptage* has 10 stamens, like its NW counterparts. The filament of the single ventral stamen, however, is twice as long and is thickened when compared to that of the remaining nine stamens (fig. 1B). Like that in all Malpighiaceae, the gynoecium of *H. benghalensis* comprises three superior uniiovulate carpels. The upwardly curved style, which is as long as the elongated ventral stamen, is presented well outside the flower (fig. 1B). The style is deflected to either the left or the right of the main floral axis and the single ventral stamen, creating left and right mirror-image flowers on the same inflorescence. In addition, the flowers of *H. benghalensis* are strongly fragrant, which is rare in Malpighiaceae.

**Evolution of CYC2-Like Genes in Hiptage**

We cloned four CYC2-like sequences from *H. benghalensis*. Phylogenetic analysis confirmed that two of the obtained sequences, *HbCYC2A-1* and *HbCYC2A-2*, belong to the CYC2A lineage, while the other two sequences, *HbCYC2B-1* and *HbCYC2B-2*, belong to the CYC2B lineage (fig. 2). These results are consistent with our previous findings, where the corresponding CYC2 paralogs were found in another species of *Hiptage*, *H. detergens* Craib (Zhang et al. 2010; fig. 2). The paralogs of *HbCYC2As* and *HbCYC2Bs* share 86.2% and 93.1% nucleotide identity, respectively. On the basis of gene genealogy, the duplications that gave rise to *CYC2A-1/CYC2A-2* and *CYC2B-1/CYC2B-2* in *Hiptage* took place at least in the common ancestor of the hiptagoid clade, which includes *Hiptage* and the African genus *Flabellariopsis*. R. Wilczek, which has actinomorphic flowers (Davis and Anderson 2010). It is unclear whether the absence of *CYC2A-1* in *Flabellariopsis* is due to gene loss.

**Expression of CYC2-Like Genes in H. benghalensis**

To test our hypothesis that evolution of dorsoventral heterancihy in *Hiptage* is the result of changes in expression of the
CYC2 homologs, the expression of the four CYC2-like genes from *H. benghalensis* was examined. The expression of CYC2 genes was detected in late stages of flower development and in open flowers in *H. benghalensis* (fig. A1, available online). The qRT-PCR results indicate that the CYC2 paralogs in *H. benghalensis* are differentially expressed along the dorso-ventral plane of floral symmetry (fig. 3). The CYC2A copies, *HbCYC2A-1* and *HbCYC2A-2*, are expressed in all four whorls of the flowers, with a higher concentration restricted to a broad dorsal region, including the dorsal and lateral positions in the calyx and corolla. *HbCYC2B-1* and *HbCYC2B-2* are also expressed in all four whorls of the flowers, with extremely high expression in the single dorsal petal of the flowers of *Hiptage* (fig. 3C, 3D). Expression levels of *HbCYC2B* in the dorsal petal are 40–80-fold greater than those in other floral organs. The relative expression levels of *HbCYC2B* in the dorsal sepal, lateral petals, and dorsal stamens appear to be comparable to those observed for *HbCYC2As*, suggesting that the dorsal petal expression levels are unusually high. Patterns of gene expression among the CYC2 paralogs are differentially expressed, especially between *HbCYC2B-1* and *HbCYC2B-2*.

**Discussion**

**Gene Duplication of CYC2 in the Common Ancestor of Hiptage and Flabellariopsis of the OW**

Hiptage is the sister group of Flabellariopsis, and together they form the hiptageoid clade, one of seven OW lineages (Davis and Anderson 2010). Interestingly, although *Hiptage* and *Flabellariopsis* are closely related genera, they have very different floral symmetries. *Hiptage* has elaborate floral zygomorphy in all four floral whorls, while *Flabellariopsis* produces radially symmetrical flowers and an eglandular calyx. The NW sister of the hiptageoid clade is among *Carolus*, *Dicella*, and *Tricommia* (Davis and Anderson 2010), all of which retain the typical NW floral morphology, including a banner petal and glandular sepals. This implies that the ancestral NW floral zygomorphy was possibly modified independently in *Hiptage* and *Flabellariopsis* when the two groups diverged in the OW. It is clear that the duplications that gave rise to the paralogs of the *CYC2A* and *CYC2B* lineages predate the ancestor of *Hiptage* + *Flabellariopsis*, but their exact timing relative to one another remains uncertain. Given that the base chromosome number in the subfamily Malpighioideae is *n*= 10 (Anderson 1993; Cameron et al. 2001), *Hiptage benghalensis*, with *n*= 28, 29, or 30 chromosomes (Devar and Borai 1981; Bir et al. 1982; Sandhu and Mann 1988; Gill et al. 1990), may implicate a hexaploid ancestry for the clade, but determining whether this is related to the origin of the CYC2 paralogs will require further investigation.

**Differential Expression of CYC2 and Elaborated Floral Zygornorph in Hiptage**

CYC2-like genes have been shown to control aspects of dorsal organ morphology in the context of floral zygomorphy in multiple lineages of core eudicots and often exhibit shifts in expression in association with changes in floral symmetry (reviewed in Hileman 2014). In the typical flowers of NW Malpighiaceae...
Fig. 3  Quantitative reverse-transcription PCR for the four CYC2-like genes in *Hiptage benghalensis*. The two CYC2A genes, *HbCYC2A-1* and *HbCYC2A-2*, are expressed differentially in the calyx, corolla, and androecium in the dorsal region of the flower (A, B); the two CYC2B genes, *HbCYC2B-1* and *HbCYC2B-2*, are expressed at very high levels in the single dorsal petal but also at detectable levels in other floral organs (C, D). Two different sampling strategies were included in the analysis of developing floral buds sized 0.6 cm × 0.9 cm and the pooled samples from multiple late stages of floral development. ds = dorsal sepals; ls = lateral sepals; vs = ventral sepals; dp = dorsal petal; lp = lateral petal; vp = ventral petal; 9ust = 9 upper stamens; 1vst = single ventral stamen; ca = carpels.
examined to date, CYC2-like genes are differentially expressed along the dorsoventral axis of floral symmetry, but expression is absent or present at low levels in the androecium, as in the NW Byrsonima crassifolia and Janussia guarantica (Zhang et al. 2010). Examination of NW Malpighiaceae representing phylogenetically diverse clades supports the conservation of the expression of CYC2-like genes correlated with the conservation of the NW floral zygomorphy (Zhang et al. 2010, 2012, 2013). The expression of the four CYC2 paralogs in H. benghalensis diverges from the NW pattern in a number of key ways. Although the expression of HbCYC2As is similar to that found in NW CYC2A homologs (fig. 1A), their expression also extends to the androecium, with the highest levels in the dorsal region. The CYC2B orthologs are also expanded relative to their NW counterparts to varying degrees. In addition to their unusually high expression in the dorsal petal, HbCYC2Bs are detected in the lateral petals, dorsal androecium, and carpels. Further examination of these four CYC2 paralogs in other species of Hiptage and comparison with their closest NW counterparts will help us to better understand the evolution of dorsoventral heteranthery, a synapomorphic trait of Hiptage. When viewed collectively, these individual expression patterns create a dramatic dorsoventral gradient in CYC2 expression that is highest in the dorsal banner petal and nine dorsal stamens and then declines to weakest expression in the exerted ventral stamen and petals. Other expression studies have observed similar dorsoventral gradients, suggesting that CYC2-like genes function in a dosage-sensitive manner influencing morphology (Zhang et al. 2010; Howarth et al. 2011). Moreover, functional data from both Antirrhinum and legumes has uncovered additive effects among CYC2 paralogs (Luo et al. 1996, 1999, Wang et al. 2008). This would be consistent with a model in which higher expression of CYC2-like genes in the nine shortened dorsal stamens is responsible for their differentiated morphology relative to the elongated ventral stamen. Furthermore, the nine dorsal stamens in Hiptage are functionally different from the single ventral stamen (Ren et al. 2013). The single exerted ventral stamen is reported to produce significantly more viable pollen than the smaller stamens, such that the anthers of the nine smaller stamens are thought to be “fodder” anthers, providing pollen to pollinators as food, while the single large anther is the “pollinating” anther. This pattern is reminiscent of the role of CYC and its paralog DICH (DICHTOMA) in Antirrhinum, in which they promote abortion of the dorsal stamen (Luo et al. 1996, 1999). At the same time, it is important to note that CYC2-like genes often play variable roles in controlling cell proliferation, in some cases repressing it (e.g., the Antirrhinum dorsal stamen [Luo et al. 1996, 1999] or Iberis dorsal petals [Busch and Zachgo 2007]) and in other cases promoting it (the Antirrhinum dorsal petals; Luo et al. 1996, 1999). Therefore, the extraordinarily high expression levels of HbCYC2Bs observed in the dorsal petal may be more consistent with a role in sculpting this organ’s dramatic three-dimensional morphology than with simply suppressing cell proliferation. This finding, however, does not exclude the possibility of the involvement of other genes acting downstream of the CYC2 homologs, or parallel genetic pathways, in the modification of floral zygomorphy in Hiptage. Regardless, it appears that a broadly consistent pattern extends across Malpighiaceae, such that transitions to the OW are always associated with changes in both floral symmetry and patterns of CYC2-like gene expression, which in the case of Hiptage may include dosage-dependent contributions to the evolution of dramatic heteranthery.

Acknowledgments

We thank P. Howell for help with fieldwork. This work is supported by National Science Foundation grants DEB-1355109, DEB-0544039, and AToL EF 04-31242.

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