Differential regulation of symmetry genes and the evolution of floral morphologies

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Shifts in flower symmetry have occurred frequently during the diversification of angiosperms, and it is thought that such shifts play important roles in plant–pollinator interactions. In the model developmental system Antirrhinum majus (snapdragon), the closely related genes CYCLOIDEA (CYC) and DICHTOMA (DICH) are needed for the development of zygomorphic flowers and the determination of adaxial (dorsal) identity of floral organs, including adaxial stamen abortion and asymmetry of adaxial petals. However, it is not known whether these genes played a role in the divergence of species differing in flower morphology and pollination mode. We compared A. majus with a close relative, Mohavea confertiflora (desert ghost flower), which differs from Antirrhinum in corolla (petal) symmetry and pollination mode. In addition, Mohavea has undergone a homeotic-like transformation in stamen number relative to Antirrhinum, aborting the lateral and adaxial stamens during flower development. Here we show that the patterns of expression of CYC and DICH orthologs have shifted in concert with changes in floral morphology. Specifically, lateral stamen abortion in Mohavea is correlated with an expansion of CYC and DICH expression, and internal symmetry of Mohavea adaxial petals is correlated with a reduction in DICH expression during petal differentiation. We propose that changes in the pattern of CYC and DICH expression have contributed to the derived flower morphology of Mohavea and may reflect adaptations to a pollination strategy resulting from a mimetic relationship, linking the genetic basis for morphological evolution to the ecological context in which the morphology arose.

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gush Mohavea from Antirrhinum flowers. We hypothesized that lateral stamen abortion during Mohavea flower development is due to an expansion of CYC and/or DICH homologs into the lateral staminodes and that the higher degree of internal petal symmetry in Mohavea adaxial petals is due to expansion or contraction of DICH expression, leading to uniform expression across these petals (Fig. 3).

Materials and Methods
Cloning of CYC and DICH Orthologs and Phylogenetic Analysis. PCR was used to amplify, clone, and sequence CYC and DICH orthologs from genomic DNA of Antirrhinum coronatum, Antirrhinum coulterianum, Antirrhinum leptaleum, Antirrhinum multiflorum, Antirrhinum nuttalianum, Antirrhinum ova-tum, Antirrhinum subcordatum, Antirrhinum vexillo-calculatum, Antirrhinum virga, and Mohavea confertiflora according to published methods (12). An additional forward primer, 5'-CACATACCTACATCTCCTCAGG-3', was used. The sequences reported here have been deposited in the GenBank database (accession nos.: A. cornutum, AF512687, AF512697, AF512716, and AF512706; A. coulterianum, AF512688, AF512698, AF512717, and AF512707; A. leptaleum, AF512689 and AF512708; A. multiflorum, AF512690, AF512699, AF512718, and AF512709; A. nuttalianum, AF512691, AF512700, AF512719, and AF512710; A. ovatum, AF512692, AF512701, AF512720, and AF512711; A. subcordatum, AF512693, AF512702, AF512721, and AF512712; A. vexillo-calculatum, AF512695, AF512704, AF512722, and AF512714; A. virga, AF512694, AF512703, and AF512713; and M. confertiflora, AF512696, AF512705, AF512723, and AF512715). CYC and DICH sequences were aligned manually with reference to both nucleotide and hypothetical amino acid information.

To evaluate gene orthology, we conducted phylogenetic analysis of the isolated genes and published CYC and DICH sequences from A. majus (Y16313 and AF199465, respectively), Chaenorrhinum villosum (AF512601 and AF512591), and Misopates orontium (AF512600 and AF512594). CYC and DICH sequences were combined into a single matrix and analyzed together. Phylogenetic analyses were conducted by using PAUP*4.0b1 (16). We estimated the maximum likelihood tree by using a random taxon addition sequence, tree bisection reconnection heuristic search under the general time-reversible model of evolution with a discrete gamma model, allowing for four
RNA in Situ Hybridization. RNA in situ hybridization was performed according to described methods (17) with the following modifications: tissue fixation in FAA (50% EtOH/10% formalin/5% acetic acid/0.1% DMSO), probes were alkaline hydrolyzed to 400 bp, and, after signal development, tissues were counterstained with Calcofluor (0.002%). Digoxigenin-labeled probes of McCYC1, McCYC2, McDICH1, and McDICH2 were prepared from linearized templates cloned into pCR4 plasmid (Invitrogen). RNA probes were gene-specific and included the coding region sequenced for each locus; this region corresponded to ~94% and 95% of the coding sequence for CYC and DICH loci, respectively.

RT-PCR. Tissues used for RT-PCR consisted of floral organs dissected from relatively late-stage *M. confertiflora* flowers in which petal and stamen primordia had undergone a high degree of differentiation (Fig. 1e). Sepals were removed from the outer whorl. The corolla tube and petal lobes, including the attached abaxial stamens and lateral and adaxial staminodes of 110 flowers, were dissected into abaxial, lateral, and adaxial regions. Total RNA was extracted (18) from the tissues of the three corolla plus stamen/staminode regions and from the sepal tissues for RT-PCR experiments. RT-PCR was performed as described (18) by using locus-specific primers: McCYC1 (forward 5’-GCTGCTACTTTGGTGTCT-3’; reverse 5’-AATGCCT- CACGAGTACCC-3’), McCYC2 (forward 5’-GCCGCTACGTCGTTGTTT-3’; reverse 5’-AAGCCCTCGGATTACCT-3’), McDICH1 (forward 5’-CACGACGTGGATTCCGAG-3’; reverse 5’-GGACACGCGGTTGACAG-3’) and McDICH2 (forward 5’-CATGACGTTGATTTCTGGC-3’; reverse 5’-CCTCATATAATTGTTGAGGAC-3’). Primers that amplify actin were used as a positive control (19). RT-PCR products were cloned, and between 5 and 12 clones from each RT-PCR were sequenced to confirm locus-specificity.

Results

To test our hypothesis that lateral stamen abortion and internal petal symmetry in *Mohavea* are due to changes in the regulation of CYC and/or DICH homologs during flower development (Fig. 3), the CYC and DICH orthologs of *M. confertiflora* were cloned and sequenced. As with other CYC and DICH homologs, these sequences lacked introns in the coding regions. Phylogenetic analysis of the resultant sequences confirmed that two CYC loci (McCYC1 and McCYC2) and two DICH loci (McDICH1 and McDICH2) were isolated (data not shown). This result is expected because *Mohavea* is tetraploid relative to *A. majus* (ref. 20; Fig. 2). Apart from six to eight triplet in-frame indels, McCYC and McDICH loci share ~94.4% and 92.0% nucleotide identity with *AmCYC* and *AmDICH*, respectively. RNA in situ hybridization and locus-specific RT-PCR were used to determine the spatial and temporal patterns of McCYC and McDICH gene expression in *M. confertiflora*.

RNA in situ hybridization in *M. confertiflora* revealed that McCYC1 and McCYC2 expression patterns are indistinguishable across all observed stages of flower development (Fig. 4a–d and data not shown). Expression is first detected before the initiation of organ primordia, with RNA concentrated in the adaxial region of early floral meristems (Fig. 4a). Once sepal, petal, and stamen primordia have initiated, McCYC1 and McCYC2 expression differ markedly from that of *AmCYC*, *McCYC1* and *McCYC2* expression becomes concentrated in regions of the developing lateral and adaxial stamen primordia (Fig. 4b and c). The expression in adaxial petals of *M. confertiflora* (Fig. 4b and c) is similar to that observed for *AmCYC* (4). Therefore, it is specifically McCYC expression in lateral stamen primordia that differs from CYC expression in *A. majus*, correlating perfectly with the additional stamen abortion seen during *Mohavea* flower development.

McDICH1 and McDICH2 differ in the timing of their expression. Transcripts of McDICH1 first accumulate in the adaxial region of early floral meristems (Fig. 4c) in a similar pattern to McCYC, *AmCYC*, and *McDICH*. In contrast, multiple hybridizations of McDICH2 probed to similar stage flowers did not detect expression (Fig. 4i). After the initiation of sepal, petal, and stamen primordia, McDICH1 and McDICH2 are expressed in adaxial and lateral stamen primordia (Figs. 4f, g, and j), with expression declining in later stages (Fig. 4h; data for McDICH2 not shown). McDICH1 and McDICH2 expression differs markedly from *AmDICH* expression (4), and correlates with additional stamen abortion during *Mohavea* flower development.

In the corolla, McDICH1 (Fig. 4f), but not McDICH2 (Fig. 4j), is expressed in initiating petal primordia. However, neither McDICH1 nor McDICH2 expression is detected in petals during mid (Fig. 4g and k) and later stages (Fig. 4h; data not shown for McDICH2, but similar to McDICH1). *AmDICH* expression during flower development when petals undergo differentiation. This finding is significantly different from *A. majus* developing petals, where *AmDICH* is expressed in the inner region of each adaxial petal through petal differentiation, resulting in internal petal asymmetry (4). The lack of McDICH expression in *M. confertiflora* petals by using *in situ* hybridization correlates with the higher degree of internal petal symmetry observed in *Mohavea* flowers.

Locus-specific RT-PCR results using dissected petal plus stamen/staminode tissue from relatively late-stage flowers that have undergone a high degree of differentiation (Fig. 1e) are in line with the observed *in situ* expression patterns. Expression of McCYC1, McCYC2, McDICH1, and McDICH2 is observed in adaxial and lateral regions of dissected corolla plus staminate tissue (Fig. 5). By using *in situ* hybridization, expression of McDICH1 and McDICH2 was not detected during later stages of flower development. This discrepancy between the RT-PCR and *in situ* hybridization results likely reflects a higher sensitivity of RT-PCR to low levels of gene expression in later-stage flowers. The similarity in RT-PCR for McCYC1 relative to McCYC2, and McDICH1 relative to McDICH2, suggests that the similar patterns observed with *in situ* hybridization for the paralogous McCYC and McDICH loci are not entirely due to probe cross-reactivity.

Discussion

McCYC and McDICH expression in *M. confertiflora* fits the hypothesis that changes in the regulation of these flower-symmetry genes played a causal role in morphological evolution. Most notably, expression of McCYC and McDICH in lateral stamen primordia is correlated with their abortion during *Mohavea* flower development. Changes in DICH expression may correlate with differences in corolla morphology between *A. majus* and *M. confertiflora*. Unlike in *A. majus*, where medially restricted *AmDICH* expression in the adaxial petals results in petal-lobe asymmetry (4), *in situ* hybridization did not detect McDICH expression in *Mohavea* adaxial corollas at stages when petals undergo differentiation. However, the RT-PCR results show that McDICH1 and McDICH2 are expressed in the adaxial and lateral regions of later-stage flowers at levels that are apparently too low to be detected by *in situ* hybridization. Given that *in situ* hybridization shows McDICH1 and McDICH2 expression in staminodes but not petals at midstages of development (Fig. 4g and k), the RT-PCR results likely reflect low levels of McDICH expression in the adaxial and lateral staminodes in late-stage flowers. If McDICH is expressed in the medial region...
of adaxial petals at low levels, then the higher degree of internal adaxial petal symmetry in *Mohavea* may be due to the decrease in **McDICH** expression or to changes in downstream genes involved in cell division and expansion. In any case, alterations to the **DICH** pathway affecting adaxial petal morphology in *Mohavea* were likely only a single component of multiple evolutionary modifications to gene function and/or expression that resulted in the superficially actinomorphic appearance of *Mohavea* corollas.

Although the data do not allow identification of the specific mutations responsible for the derived flower morphology of *Mohavea*, they suggest that the effects of these mutations were partially mediated through the developmental control of adaxial flower identity, specifically, changes in the expression of the adaxial identity genes **CYC** and **DICH**. In the petal whorl, potential reduction in **McDICH** expression across adaxial petals may have evolved through cis- or trans-regulatory modifications. Because both **McCYC** and **McDICH** genes are expressed in *Mohavea* lateral stamen primordia, it is possible that changes in the cis-regulatory sequences of **McCYC1**, **McCYC2**, **McDICH1**, and **McDICH2** have resulted in their expanded expression. However, this explanation would require four separate cis-regulatory changes. More parsimoniously, changes in the expression domain of an upstream regulator in the **CYC/DICH** pathway may be responsible for alterations in **McCYC/McDICH** expression in the stamen whorl of the *Mohavea* lineage.

The **cyc** mutant phenotype in *A. majus* was described by Carpenter and Coen (26) as homeotic in nature, in that the

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**Fig. 4.** Observed patterns of mRNA in situ hybridization in developing *M. confertiflora* flower meristems. (a–c) **McCYC1** antisense probe hybridized to *M. confertiflora* early through later stage flowers. (a) Transverse section through inflorescence; **McCYC1** expression is detected in the adaxial region of early floral meristems. (b and c) Transverse sections through mid-stage (b) and later stage (c) flowers; **McCYC1** expression is detected in adaxial and lateral staminodes and across the adaxial corolla. **McCYC2** expression patterns are identical to those of **McCYC1** across all observed stages of flower development (data not shown). (d) **McCYC1** sense probe hybridized to *M. confertiflora* flowers. (e–h) **McDICH1** antisense probe hybridized to *M. confertiflora* early through later stage flowers. (e) Transverse section through inflorescence; **McDICH1** expression is detected in the adaxial region of early floral meristems. (f) Oblique section through early stage flower; **McDICH1** expression is detected in the adaxial staminode and the adaxial petal primordia. (g) Oblique section through mid-stage flower; **McDICH1** expression is detected in the lateral staminodes but not in the adaxial petals. (h) Transverse section through later stage flower; **McDICH1** expression is not detected. (i–k) **McDICCH2** antisense probe hybridized to *M. confertiflora* early through mid-stage flowers. (i) Transverse section through inflorescence; **McDICCH2** expression is not detected in early floral meristems. (j) Oblique section through early stage flower; **McDICCH2** expression is detected in the adaxial staminode but not in the adaxial petal primordia. (k) Oblique section through mid-stage flower; **McDICCH2** expression is detected in the lateral staminodes but not in the adaxial petals. (l and m) **McDICHI1** and **McDICCH2** sense probes, respectively, hybridized to *M. confertiflora* flowers. (Scale bars, 100 μm.) Arrowheads indicate position of adaxial and lateral staminodes; asterisks indicate position of adaxial petal primordia; B, bract; la, inflorescence axis; efm, early floral meristem; G, gynoecium; Ads, adaxial sepal; AdC, adaxial corolla.
This group. Therefore, the expression of such phenotypes contribute to interspecific differences in floral symmetry. No evidence exists for homeotic-like transformations, and thus, whether natural selection might have played a role in the observed evolutionary changes in CYC and DICH regulation. Whereas most Antirrhinum are specialized for pollination by nectar-foraging bees (21), Mohavea is unusual in being pollinated exclusively by pollen-collecting bees (22, 23). Furthermore, it appears that M. confertiflora flowers even though they provide minimal pollen reward (22, 23). Selection in Mohavea for mimetic similarity to M. involucrata likely favored mutations that enhance the actinomorphic appearance of the corolla. A component of the genetic changes leading to enhanced actinomorphy likely included the reduction of MCDICH expression in the medial part of Mohavea adaxial petals. Likewise, the loss of lateral stamens may also be associated with the shift to pollen-collecting bees. Reduction in Mohavea stamen number is correlated with a change in abaxial anther position and pollen consistency. Together, these changes in stamen number and morphology are likely to reduce pollen loss to grooming after Mohavea flowers are visited by the pollen-collecting bee specialist (25). Although further ecological work is clearly needed (e.g., studies of how pollen-collecting bees respond to actinomorphic vs. zygomorphic flowers and further studies of pollen loss to grooming), it is clear that an integrative approach that bridges ecology, genetics, and development (34) has the potential to greatly improve our understanding of the mechanisms for adaptive evolution.

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Mohavea represents the first clear correlation between changes in gene expression and homeotic-like evolutionary transformations in angiosperms.

Our observations provide direct evidence that major changes in floral morphology between species, including a homeotic-like transformation, are associated with changes in the regulation of floral symmetry gene expression. One critical aspect of this study is that the genetic basis for evolutionary changes in flower form can be linked to the ecological context in which the novel flower morphology arose. It is therefore important to ask whether adaptive significance can be attached to the derived features of Mohavea flowers, and, thus, whether natural selection might have played a role in the observed evolutionary changes in CYC and DICH regulation. Where sympatric Antirrhinum are specialized for pollination by nectar-foraging bees (21), Mohavea is unusual in being pollinated exclusively by pollen-collecting bees (22, 23).

The expression of CYC and DICH in adaxial regions of flowers took on abaxial identity. Although homeotic mutations are generally studied within individual species, it has become widely accepted that homeotic-like transformations may play an important role in establishing morphological diversity (27–29). Shifts in gene expression correlated with such homeotic-like morphological transformations have been well documented in arthropods (30–33). Lateral stamen abortion in Mohavea can similarly be considered a homeotic-like transformation whereby lateral stamens have acquired adaxial identity, which in this case leads to abortion. We have established a strong correlation between expansion in the expression of the CYC and DICH genes and the ultimate abortion of these organs. Cubas et al. (9) have elegantly demonstrated that epigenetic mutations at the CYC locus are responsible for radially symmetrical mutants in populations of Linaria vulgaris. Although this phenotype can be considered a homeotic-like transformation, no evidence exists that such phenotypes contribute to interspecific differences in this group. Therefore, the expression of CYC and DICH in adaxial, lateral, and abaxial corolla and connate (attached) stamens was dissected from later stage buds (Fig. 1e). Sepal tissue was used as a negative control of CYC and DICH expression. Actin primers were used as a positive control. Locus specificity was confirmed by sequence analysis of RT-PCR products.