Origin of floral asymmetry in *Antirrhinum*

Da Luo, Rosemary Carpenter, Coral Vincent, Lucy Copsey & Enrico Coen

Genetics Department, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Dorsoventral asymmetry in flowers is thought to have evolved many times from a radially symmetrical ancestral condition. The first gene controlling floral asymmetry, *cycloidea* in *Antirrhinum*, has been isolated. The *cycloidea* gene is expressed at a very early stage in dorsal regions of floral meristems, where it affects growth rate and primordium initiation. Expression continues through to later stages in dorsal primordia to affect the asymmetry, size and cell types of petals and stamens.

FLOWERS can be classified into two basic types according to their symmetry: irregular flowers have a single plane of symmetry, whereas regular flowers have two or more planes of symmetry[1]. Both flower types have a radial axis that establishes concentric whorls of different types of organs, typically sepals, petals, stamens and carpels[2,3]. Irregular flowers have an additional axis of asymmetry such that organs from the same whorl have distinct identities according to their dorsoventral position[4]. The irregular condition is thought to have evolved many times from the regular one as a specialized adaptation to animal pollinators. One approach to understanding the origin and mechanisms underlying dorsoventral asymmetry is through the analysis of mutants in which asymmetry is reduced or lost. Such mutations have been known since the classic study on peloric forms of *Linaria* described by Linnaeus in 1744 (ref. 5), but so far they have not been the subject of detailed developmental or molecular analysis. We have chosen to address this problem in *Antirrhinum majus*, a species with irregular flowers that is amenable to molecular genetics.

The wild-type *Antirrhinum* flower has an axis of dorsoventral asymmetry which is orientated such that the dorsal (upper or adaxial) part is nearer the stem, whereas the ventral (lower or abaxial) part is nearer to the bract, a small leaf-like organ that subtends each flower. Dorsoventral asymmetry is most pronounced in the petals and stamens, each of which can be divided into three types: dorsal, lateral and ventral. Mutants with a peloric phenotype have radially symmetrical flowers in which all organs have a ventral identity, indicating that they lack genetic functions that are normally active in the dorsal and lateral regions of the flower[4,6]. The semipeloric phenotype is intermediate between peloric and wild type. Two groups of semipeloric mutants were identified, mapping to two linked loci[6]. To avoid possible confusion between the two loci, here we call one locus *cycloidea (cyc)* and the other *radialis* (*rad*), names given to some of the original mutants identified in the classical genetic studies[15].

We describe the isolation of *cyc* by transposon tagging and show that it is expressed in a dorsal domain of wild-type floral meristems, before any morphological dorsoventral asymmetry in the meristem is visible. The phenotypic consequences of its expression can be divided into early effects on primordium initiation and later effects on organ morphology. Early expression of *cyc* retards growth rate and reduces organ number in the dorsal region of the floral meristem, perhaps helping to ensure that the domain of *cyc* expression is aligned precisely with the arrangement of primordia in the flower. At later stages, *cyc* continues to be strongly expressed in dorsal organ primordia, where it interacts with the radial organ identity genes to affect the asymmetry, size and cell-types of petals and stamens. No expression of *cyc* is detected in lateral organs, suggesting that it may act non-autonomously to influence lateral identity. Peloric mutants derived from *cyc* result from mutations in a second gene, *dichotoma* (*dich*), indicating that both *cyc* and *dich* are needed to establish full dorsoventral asymmetry.

Phenotype and development

Each mature wild-type flower has five sepals, five petals, four stamens and two united carpels. The petals are united for part of their length to form a corolla tube ending in five separate lobes. The petals can be classified according to size, shape and epidermal cell characteristics into three types: two dorsal, two lateral and one ventral. The dorsal petals have large lobes and are relatively free of hairs whereas the lateral and ventral petals have smaller lobes with yellow areas and hairs in the tube (Fig. 1). The ventral petal has bilateral symmetry whereas both the dorsal and lateral petals are individually asymmetric along the dorsoventral axis (this is more easily seen in the flattened petal lobe diagrams in Fig. 1). Alternating with the petals, three types of stamen primordia are initiated in whorl 3: one dorsal, two lateral and two ventral. The dorsal stamen primordium arrests at an early stage of development to form a small structure called a staminode; the two lateral stamens can be distinguished from the ventral pair by their shorter length and lack of hairs at their base. The filaments of the lateral and ventral stamens twist in a consistent way such that all anthers eventually face ventrally (floral diagram, Fig. 1). Looking down on the flower, filaments on the left twist clockwise whereas those on the right twist anticlockwise, implying that the lateral and ventral stamen filaments have individual asymmetry along the dorsoventral axis.

In *Antirrhinum* flowers with a peloric phenotype, there are typically six sepals, six petals, six stamens and two united carpels. All of the petals are bilaterally symmetrical and resemble the ventral petals of wild type, except that the lobes are slightly smaller (Fig. 1). The stamens resemble the wild-type ventral stamens and the filaments do not twist significantly so that all anthers tend to face towards the centre of flower (floral diagram, Fig. 1). The flowers therefore have a ventralized phenotype in which the number, type and internal symmetry of organs are affected.

The flowers of *cyc*-608 mutants usually have six sepals, six petals, six stamens and two united carpels. Most commonly, three petals have a ventral identity and the remaining petals have a combination of dorsal and lateral characteristics (Fig. 1). In some cases, however, the axis of asymmetry is differently aligned with respect to the organs, giving four petals with ventral identity and two with dorsal/lateral identity (Fig. 1, floral diagrams). Four or five stamens have a ventral identity, depending on the alignment of the dorsoventral axis, whereas the remaining stamens resemble the lateral stamens of wild type (Fig. 1). The twisting of stamen filaments is variable and the anthers end up facing in various directions (Fig. 1).

Development of wild type and mutant flowers was investigated by scanning electron microscopy (SEM). Floral meristems initiate sequentially at a rate of about one every 10 hours on the periphery of the inflorescence apex in the axils of bract primordia[7]. A sequence of developmental stages can be observed along the nodes of the inflorescence, starting with the earliest stage near the top (node 0), and progressively later stages below[8]. In wild type, the lateral and ventral sepal primordia first become visible at about node 10–11. The dorsal sepal primordium appears about a node later and its centre often seems to be offset to one side of the flower meristem (early stage 4, Fig. 2*a*). The dorsal primordium remains smaller than the other primordia through stages 4 and 5 (Fig. 2*d*, *g*). When petal and stamen primordia become clearly visible, their dorsal primordia are smaller than the others (stage 6, Fig. 2*j*). Eventually, development of the dorsal stamen arrests, whereas the growth of dorsal petals and sepals appears to catch up with the other organs in each whorl.

Differences between wild-type, semipeloric and peloric meristems were first detected at the time that sepal primordia started to appear. In peloric and semipeloric mutants, six sepal primordia usually emerge, although the two upper primordia are smaller, particularly in semipeloric meristems (Fig. 2*a*–*f*). By stages 5 and 6, six petal and six stamen primordia are visible, the upper petal and stamen primordia being noticeably smaller than the others in semipeloric meristems (stage 6, Fig. 2*k*, *l*). Some peloric and semipeloric flowers develop with five instead of six organs in a whorl. The numbers of petals, sepals and stamens are often correlated with each other but there are some flowers with more sepals than petals or vice versa.

The *cyc* locus and mutant alleles

The *cyc*-608 allele arose from a transposon-mutagenesis experiment and is unstable, reverting to wild type at a rate of 0.34% (ref. 6). To determine whether *cyc*-608 was caused by a known transposon, genomic DNA from *cyc*-608 mutant and revertant plants was digested with various restriction enzymes and probed with different transposons. This showed that *cyc*-608 was caused by the insertion of a transposon belonging to the CACTA family[9–12], allowing the *cyc* locus to be cloned (Fig. 3). The sequence of the locus revealed an uninterrupted open reading frame (ORF) encoding a putative protein of 286 amino acids (Figs 3*c*, 4). RNA blots using a probe containing the ORF detected a transcript of 1.3 kb in the wild type but not in *cyc*-25 or *cyc*-608 mutants, and the transcript was found in young inflorescences but not in leaves (data not shown). A complementary DNA library was screened with a *cyc* probe and three cDNA clones containing sequence identical to the *cyc* genomic ORF were obtained. 5′ and 3′ RACE PCR (see Methods) confirmed the full length of the *cyc* transcript.

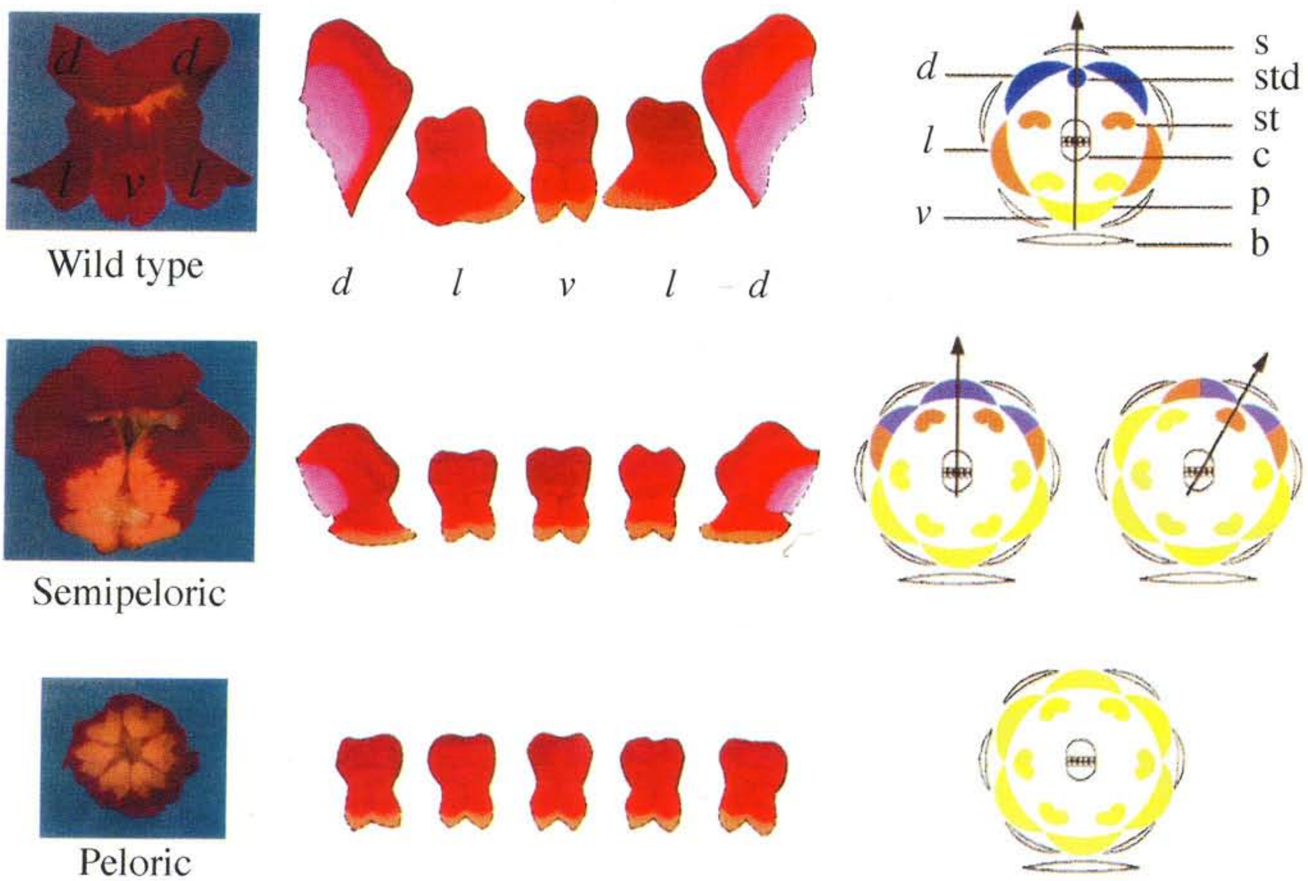

FIG. 1 Wild-type, semipeloric and peloric flowers. The wild-type *Antirrhinum* flower has an axis of dorsoventral asymmetry which is orientated such that the dorsal (upper or adaxial) part is nearer the stem whereas the ventral (lower or abaxial) part is nearer to the bract. On the left, flowers are photographed in face view with the dorsal (d), lateral (l) and ventral (v) petal lobes indicated for wild type. The characteristics of the different petal lobes are shown on the right of each flower, after they have been cut off the flower and flattened out (dotted lines indicate the sites of cutting). In the case of peloric and semipeloric flowers, only five petal lobes are shown for simplicity. The floral diagrams on the right show the relative position of different organs, with identities indicated by colours: dark blue (dorsal, top diagram); brown (lateral); yellow (ventral). Semipeloric flowers lack petals with full dorsal identity but have petals regions with some dorsal characteristics (light blue, middle diagrams). The axis of asymmetry, as defined by the different types of organs is indicated by an arrow. In wild type, the axis of floral asymmetry is always aligned with the floral organs in a fixed way. For the semipeloric phenotype, the two floral diagrams indicate that the identity of floral organs varies depending on how the residual axis of asymmetry is aligned with respect to the organs. In peloric flowers there is no dorsoventral axis of asymmetry based on organ identity. Symbols: b, bract; c, carpel; p, petal; d, dorsal petal; l, lateral petal; v, ventral petal; s, sepal; st, stamen; std, staminode.

Based on restriction enzyme mapping and PCR, five *cyc* alleles were shown to have been generated by transposon insertions (Fig. 3*c*). Two of the alleles, *cyc*-650 and *cyc*^{neo}, carried insertions 5′ to the ORF and had a slightly weaker phenotype than *cyc*-608 and *cyc*-25. The allele with the weakest phenotype, *cyc*^{abnor}, had an insertion in the second intron.

Analysis of the longest ORF of *cyc* shows that there is a bipartite motif, similar to a consensus nuclear localization signal (Fig. 4). This motif has been defined as two basic amino acids, followed by a spacer region, and five amino acids containing three basic residues[13]. No significant homology was found between *cyc* and other sequences in the gene banks, except for several expressed sequence tags (ESTs) from *Arabidopsis* with no known function.

The expression pattern of cyc

The expression pattern of *cyc* was analysed by RNA *in situ* hybridization. Digoxigenin-labelled antisense *cyc* RNA was used as a probe against inflorescence apices of wild type and *cyc* mutants. Serial sections through individual inflorescences were prepared to include different stages of floral development. In both longitudinal and transverse sections of wild type, *cyc* expression could only be detected in a dorsal region of developing floral meristems. Expression could be detected as early as at node 4, corresponding to stage 1 of floral development[8]. Expression of *cyc* was initiated near the junction between inflorescence meristem and the floral meristem within a domain 2–4 cells wide, that gradually expanded as floral meristems grew larger (Fig. 5*a*, *d*). When the sepal primordia became visible, *cyc* expression was detectable in the dorsal sepal and in the dorsal part of the floral dome (Fig. 5*b*). In some wild-type genetic backgrounds, *cyc* expression was also detected in the dorsal part of the two lateral sepals (Fig. 5*e*). In late stage 4 (node 14), *cyc* expression was more concentrated in regions where the primordia of dorsal petals and the staminode were forming (Fig. 5*e*). When petal, stamen and carpel primordia were clearly visible (stage 6), *cyc* expression could only be detected within the two dorsal petals and the retarded staminode (Fig. 5*c*, *f*). In transverse sections, *cyc* expression in dorsal petals appeared to end abruptly a few cells away from the junction with the lateral petals (Fig. 5*f*). In more mature flowers, expression was weakly detected only in the staminode and the two dorsal petals (data not shown). Therefore, the asymmetric expression pattern of *cyc* was maintained during flower development. The inflorescences of different *cyc* mutants, that is, *cyc*-25 and *cyc*-608, did not show any expression of *cyc* (data not shown).

Analysis of peloric mutants

One explanation for the semipeloric phenotype of *cyc*-608 mutants was that other factors present in the genetic background were responsible for the residual dorsoventral asymmetry in the

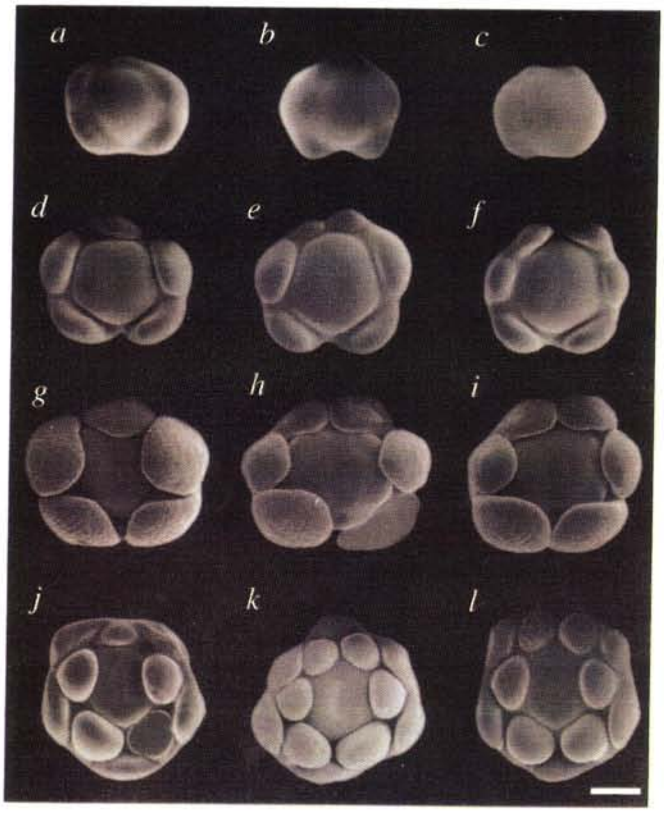

FIG. 2 Development of wild-type, semipeloric and peloric floral meristems as revealed by SEM. Four stages are shown: early stage 4 (sepal primordia initiate; top row, *a*–*c*), late stage 4 (sepal primordia are clearly visible; second row, *d*–*f*), stage 5 (petal primordia visible; third row, *g*–*i*) and stage 6 (sepals are removed to reveal all floral organ primordia; bottom row, *j*–*l*). In wild type, the dorsal sepal primordium appears about one node later than the lateral and ventral sepals (*a*), and is retarded (*d*) before catching up to be about the same size as the others by stage 5 (*g*). Similarly, the dorsal petal and dorsal stamen primordia appear later than the others, and are retarded (*j*). In semipeloric meristems, the dorsal sepal, petal and stamen primordia appear slightly later and are more retarded than those in other positions (*b*, *e*, *h* and *k*). In the peloric mutant derived from *cyc*-608, all the primordia of sepals, petals and stamens appear at about the same time, although a little retardation in the dorsal primordia is normally observed at initiation.

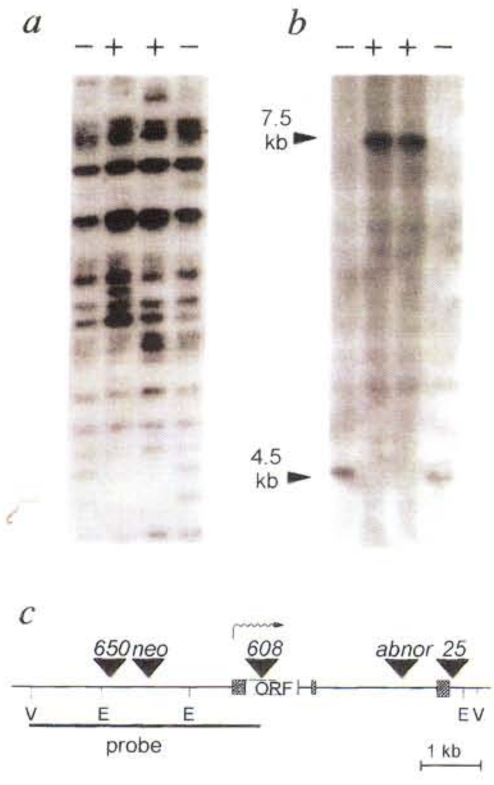

FIG. 3 Isolation and structure of the *cyc* gene. *a*, Genomic DNA from two *cyc*-608 mutant plants (−) and two independent revertants (+) were digested with *Eco*RV and blotted. The blot was probed with a 600-base pair (bp) fragment from the conserved end of Tam4 (refs 9–12), and a novel band of 4.5 kb was observed in mutants but not in revertants. *b*, The 4.5-kb fragment was cloned and the region flanking the transposon (*c*), was used to probe the same blot as in *a*, revealing the different banding patterns between mutants (4.5 kb) and revertants (7.5 kb). Different banding patterns between mutants and revertants were also observed in DNA from two other alleles *cyc*-25 and *cyc*-650, confirming that a fragment of the *cyc* locus had been cloned (not shown). *c*, Map of the *cyc* locus. The exons and predicted ORF are indicated in rectangles and the arrow indicates the direction of transcription. Black triangles represent the sites of transposon insertion for different *cyc* alleles. Restriction enzyme digestion sites within transposons are not shown. *E*, *Eco*RI; V, *Eco*RV.

flowers. This could be tested by analysing two peloric plants that had been derived from transposon-mutagenized *cyc*-608 plants. Neither of the peloric derivatives showed a detectable alteration within the 15-kilobase (kb) region of the *cyc* locus compared with their *cyc*-608 progenitor, indicating that the peloric phenotype might have resulted from a mutation in a second gene. To confirm this, one of the peloric mutants was backcrossed to wild type, and the phenotypes of F_2 plants were scored. In addition to peloric, semipeloric and wild type, there was a novel phenotype in which the dorsal petal lobes were separated from each other by a deeper divide. This was similar to the phenotype of *dichotoma* (*dich*) mutations[14,15], suggesting that the peloric mutants derived from *cyc*-608 had arisen by a mutation in the *dich* gene.

Analysis of an independently derived peloric line (stock-25) has shown that this also carries mutations in both *cyc* and *dich* (J. Almeida, M. Rocheta and L. Galego, personal communication). The *cyc*-608-derived peloric mutant was therefore crossed to stock-25 to test for complementation. All the plants in the F_1 and F_2 generations had peloric phenotypes, except for a few plants that displayed single mutant phenotypes, presumably as a result of either *cyc* or *dich* having reverted. To confirm further that the peloric phenotype resulted from the combination of *cyc* and *dich* mutations, the *cyc*-608 line was crossed to a line carrying *dich*. F_1 plants had wild-type flowers and the F_2 gave some plants with a peloric or semipeloric phenotype, as expected from segregation of two mutant alleles. However, the frequency of pelorics (13%) was higher than the expected 1/16, indicating that *dich* might not be behaving in a fully recessive manner. Further genetic analysis showed that *dich* had a variable phenotype and confirmed that it was not fully recessive in a *cyc* background (our unpublished results). This would account for the recovery of peloric plants in the direct progeny of *cyc*-608[6], because only one copy of the *dich* gene needs to be inactivated to give peloric flowers in this background.

Discussion

The *cyc* and *dich* genes are both needed to set up full dorsoventral asymmetry in *Antirrhinum* flowers. Mutants that lack both *cyc* and *dich* activity have radially symmetrical peloric flowers, mutants for *cyc* alone have semipeloric flowers and *dich* single mutants have flowers with altered dorsal petals. This implies that the *cyc* and *dich* genes can substitute for each other to some extent, allowing for residual asymmetry in each single mutant. One possibility is that *cyc* and *dich* encode related products that function in a similar way. We have recently confirmed this by isolating *dich* and showing that its sequence and expression pattern are indeed very similar to those of *cyc* (our unpublished results). However, the role of *cyc* appears to be more critical than *dich* for establishing dorsoventrality as its mutant phenotype is more extreme.

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  1 ATGTTTGGGAAGAACACATACCTACATCTCCCTCAGGTTTCATCCTCCCTTCACTCTCGCGCCGCTACT  69
  1  M  F  G  K  N  T  Y  L  H  L  P  Q  V  S  S  S  L  H  S  R  A  A  T  23

 70 TCTGTTGTCGATCTCAACGGCAATGAGATCCAGCTCCACGACATGCTCTCCGGTCATTACTTAACAACC 138
 24 S  V  V  D  L  N  G  N  E  I  Q  L  H  D  M  L  S  G  H  Y  L  T  T   46

139 GCGAATGCTCCGGTTCTTGAGTCCACCGCTTTGTTCAACAACAACAACAACTTCAATCACGACGTCGTT 207
 47 A  N  A  P  V  L  E  S  T  A  L  F  N  N  N  N  N  F  N  H  D  V  V   69

208 AATGGCCTAAACAGAGATCCTTCTCCCACATTTCCGACAAAACAGGCCGTGAAAAAAGATCGCCACAGC 276
 70 N  G  L  N  R  D  P  S  P  T  F  P  T  K  Q  A  V  K  K  D  R  H  S   92

277 AAAATATACACATCACAAGGGCCAAGGGACAGGAGAGTCCGTTTATCCATCGGCATTGCGAGAAAGTTC 345
 93 K  I  Y  T  S  Q  G  P  R  D  R  R  V  R  L  S  I  G  I  A  R  K  F  115

346 TTTGATCTACAAGAGATGCTAGGTTTCGACAAGCCGAGCAAAACCCTTGATTGGCTGCTCACTAAGTCG 414
116 F  D  L  Q  E  M  L  G  F  D  K  P  S  K  T  L  D  W  L  L  T  K  S  138

415 AAAACCGCTATCAAAGAGCTTGTGCAGTCTAAAAGTACTAAGAGCAACTCTTCCTCCCCTTGTGATGAT 483
139 K  T  A  I  K  E  L  V  Q  S  K  S  T  K  S  N  S  S  S  P  C  D  D  161

484 TGTGAGGAAGTTGTGTCTGTAGATTCTGAGAATGTGACAGATCATTCAAAGGGGAAATCACTGAAGGCT 552
162 C  E  E  V  V  S  V  D  S  E  N  V  T  D  H  S  K  G  K  S  L  K  A  184

553 AACAACAAATGTAAAGAAGCAATGGATTCACATCAAGCAGCAGCCAAAGAGTCGAGGGCGAAGGCGAGA 621
185 N  N  K  C  K  E  A  M  D  S  H  Q  A  A  A  K  E  S  R  A  K  A  R  207

622 GCGAGAGCTAGGGAAAGAACTAAGGAGAAGATGTGTATCAAACAACTGAATGAAGCTATAGTACTGAGG 690
208 A  R  A  R  E  R  T  K  E  K  M  C  I  K  Q  L  N  E  A  I  V  L  R  230

691 AACCATCAATTTGAAGTTTCGGGTACCCGTGAGGCGTTTGTTCATCCAGTTTTCGGGTTTCATCAGCAA 759
231 N  H  Q  F  E  V  S  G  T  R  E  A  F  V  H  P  V  F  G  F  H  Q  Q  253

760 AACTATGGCAACGCATCTCATGAGAACTGGGATCAGAGTAACCTTTCTTCGCAGTCGAACCAGCTATGC 828
254 N  Y  G  N  A  S  H  E  N  W  D  Q  S  N  L  S  S  Q  S  N  Q  L  C  276

829 GCCATTTTGAATCAGCACAAGTTCATCAATTAG                                     861
277 A  I  L  N  Q  H  K  F  I  N  *                                      286
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FIG. 4 Sequence of the *cyc* ORF and the deduced protein sequence. The underlined amino acids indicate the putative bipartite motif similar to a consensus nuclear localization signal[13]. The triangle indicates the insertion site of the transposon in *cyc*-608. A few ESTs (accession numbers: T45419, R29994 and R30409) from *Arabidopsis* have been found to share some homology with *cyc*.

The *cyc* gene encodes a protein with no significant homology to other known proteins, although it does contain a putative nuclear localisation signal, indicating that it may play a role in transcriptional regulation. Transcripts of *cyc* accumulate specifically in the dorsal region of wild type floral meristems, consistent with its role in setting up dorsoventral asymmetry. Its expression is first detected at a very early stage, before any morphological dorsoventral asymmetry in the meristem is visible by SEM, and continues through to later stages in the dorsal petal and stamen primordia (Fig. 6*a*–*e*). The phenotypic consequences of this expression pattern can be conveniently divided into early effects on primordium initiation and later effects on organ morphology.

Early effects of *cyc* on primordium initiation. During early stages, Cyc^+ activity retards growth and reduces the number of primordia in dorsal regions of the flower meristem. In meristems of semipeloric flowers, two dorsal sepal primordia are typically formed and their development is almost in synchrony with the other primordia (Fig. 6*f*–*j*). In contrast, wild-type floral meristems form a single dorsal sepal primordium which is retarded in growth relative to the other sepal primordia. The region of retarded growth corresponds to the domain where *cyc* transcripts accumulated in wild type, indicating that localized Cyc^+ activity represses growth and/or primordium initiation (Fig. 6). It is possible that two dorsal primordia are initiated in wild type but that only one continues to develop and become visible. This might account for the observation that the dorsal sepal primordium often appears to be displaced to one side of wild-type meristems in early stages of growth (Fig. 6*d*, *e*). Increased sepal number in *perianthia* mutants of *Arabidopsis* also correlates with more synchronous sepal initiation, although in this case the wild-type gene appears to advance growth in ventral regions rather than retard it in dorsal regions[16]. Expression of *cyc* in emerging dorsal petal and stamen primordia correlates with their reduced growth and number, indicating that *cyc* may affect all dorsal organ primordia in a similar way at early stages (Fig. 6*e*). Unlike *cyc*, the *dich* gene does not appear to have a major effect on organ number.

The early effects of *cyc* may help to bring the sites of organ initiation into register with the axis of asymmetry. By reducing growth, Cyc^+ activity could ensure that the sites of dorsal organ primordia become centred within the *cyc* expression domain. In this way, asymmetry could be coupled more consistently with the position of primordia in the flower. A prediction is that in *cyc* mutants, the residual asymmetry conferred by $Dich^+$ activity should be more variably aligned with respect to organ primordia. This appears to be the case, because some *cyc* mutant flowers have two dorsal/lateral and four ventral petals rather than the more usual three dorsal/lateral and three ventral petals (see floral diagrams in Fig. 1).

Later effects of *cyc* on organ morphology. Transcripts of *cyc* are detected in dorsal petal and staminode primordia, from their inception through to later stages of development. The domain of *cyc* expression ends at a boundary lying a few cells away from the junction between dorsal and lateral petals in whorl 2, and around the staminode in whorl 3 (Fig. 5*f*). The effects of *cyc* expression on the developmental fate of dorsal primordia vary between whorls. In whorl 2, the dorsal petal lobes of wild type are larger in area than in *cyc* mutants, indicating that a role of Cyc^+ activity is to promote petal lobe growth. In contrast, in whorl 3, the dorsal stamen of wild type becomes increasingly retarded relative to the other stamens, indicating that *cyc* activity in the dorsal stamen primordium acts to slow down and eventually arrest growth. The later effects of *cyc* on organ development therefore vary according to the whorl, in contrast to the early effects on growth rate and organ number which are more similar between whorls. This may be because early *cyc* expression occurs before that of organ identity genes (that is, before stage 4; refs 17, 18), whereas at later stages, *cyc* can interact in a combinatorial fashion with organ identity genes.

Even though *cyc* transcripts cannot be detected outside the dorsal domain, *cyc* has clear effects on the development of lateral organs, altering petal shape and stamen length. We cannot rule out the possibility that *cyc* transcripts are present in lateral organs below the level of detection. However, it seems more likely that *cyc* expression in the dorsal domain acts non-autonomously, through cell–cell signalling, to influence the behaviour of lateral organ primordia. Perhaps the flower meristem is first divided into two main regions: a dorsal domain expressing *cyc* and the remaining domain with no *cyc* expression. This early partition could then be further elaborated by cell–cell interactions to generate three distinct domains: dorsal, lateral and ventral. Candidate genes involved in this further elaboration are the *radialis* gene[6], which gives a phenotype similar to *cyc* and *divaricata*, which confers a lateralized phenotype (ref. 15; J. Almeida, M. Rocheta and L. Galego, personal communication).

In addition to establishing distinctions between dorsal, lateral and ventral organs, the *cyc* and *dich* genes are also required to set up dorsoventral asymmetry within individual organs. Unlike the ventral petal, which is bilaterally symmetrical, dorsal and lateral petals are asymmetric along the dorsoventral axis of wild-type flowers. Similarly, the left- or right-handed twists of lateral and ventral stamen filaments indicates that they may have different growth rates along the dorsoventral axis, ensuring that the anthers are presented in specific orientations. These aspects of internal petal and stamen asymmetry are lost in *cyc:dich* double mutants, suggesting that in addition to setting up differences between organs, *cyc* and *dich* are also needed to establish subdomains within organs along the dorsoventral axis.

The *cyc* expression pattern, together with its phenotypic effects, raise the question of how its role has evolved. It is possible that the early effects of *cyc* on retarding growth rate and primordium initiation are more highly conserved than the later effects on organ morphology. For example, in many species belonging to the same family as *Antirrhinum* or to closely related families, the dorsal petal lobes are smaller than the lateral or ventral lobes, indicating that in these cases *cyc* homologues may act to reduce final lobe size rather than increase it as in *Antirrhinum*. It has also been proposed that early aspects of floral asymmetry in the Leguminosae are more conserved than later characteristics[19], although it is not clear if *cyc* homologues are involved in this case because dorsoventral asymmetry in this family is thought to have evolved independently from that in *Antirrhinum*. It is also unclear whether any of the early or late roles of *cyc* might also be

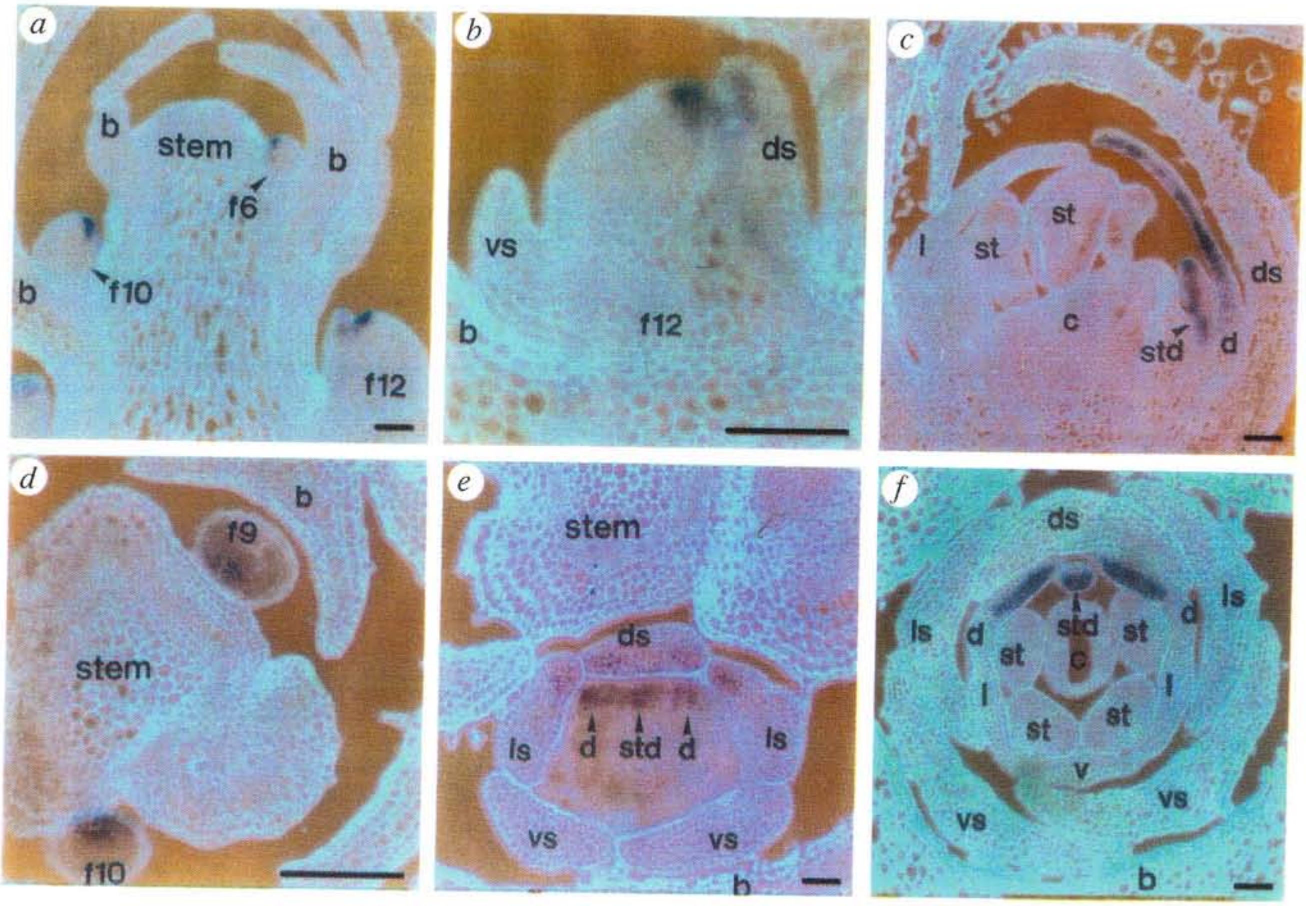

FIG. 5 RNA *in situ* hybridization of wild-type sections probed with *cyc*. The top row (*a–c*) shows longitudinal sections and the bottom row (*d–f*) shows transverse sections. The sections were probed with digoxigenin-labelled *cyc* antisense RNA and viewed under bright field, which gives a dark blue signal on turquoise background tissue. *a*, Section through an inflorescence apex; the node of each floral primordium is indicated. The signal can be seen in the dorsal (upper) regions of each floral meristem. *b*, Sections through a node-12 floral meristem, showing a signal in the dorsal region of the floral dome and in the dorsal sepal primordium. *c*, Floral meristem at a late stage, showing the signal in the dorsal petal and staminode. *d*, Floral primordia at stage 2, showing a signal in the dorsal regions near the junction with the inflorescence stem. *e*, Stage-5 meristem, showing the signal in the primordia of the dorsal sepal, part of the lateral sepals, dorsal petals and staminode. *f*, Floral meristem at a late stage, showing the signal in the staminode and dorsal petals. Symbols: b, bract; c, carpel; p, petal; d, dorsal petal; l, lateral petal; v, ventral petal; s, sepal; st, stamen; std, staminode; f, floral meristem (node number indicated); ds, dorsal sepal; ls, lateral sepal; vs, ventral sepal. Scale bar, 100 μm.

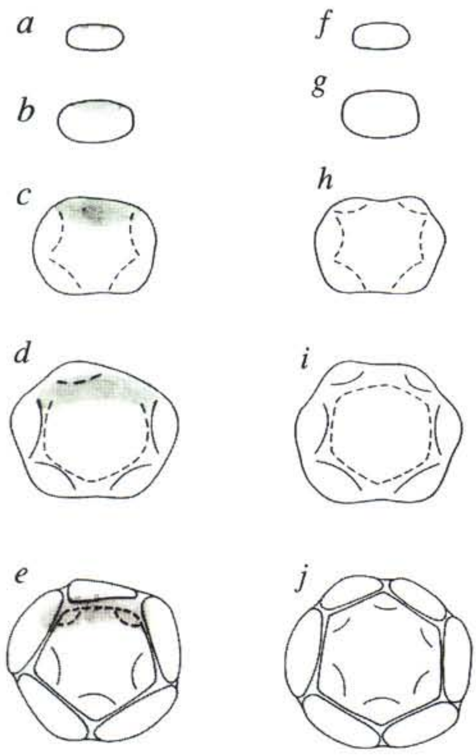

FIG. 6 Summary of the *cyc* expression pattern and its early effects on floral development in wild type as compared to the *cyc*-608 mutant. *a–e*, Wild type, showing domain of *cyc* expression (grey area) at various developmental stages based on reconstructions from serial sections. *f–j*, Semipeloric *cyc*-608 mutant, showing more growth and primordium initiation in the region where *cyc* is normally expressed in wild type. Solid lines indicate outlines of clearly visible primordia; dotted lines indicate regions of primordium initiation.

present under the guise of a different expression pattern in species with radially symmetrical flowers. The isolation and analysis of *cyc* homologues from species with different patterns of floral symmetry should allow some of these possibilities to be investigated. □

Methods

Plant Material. Plants of wild type (JI-75), semipeloric (JI-608) and a peloric mutant derived from *cyc*-608 (JI-659), were grown either in the greenhouse or the field as described previously[20]. Lobes were dissected from flowers and then flattened between two glass slides before drawing. For floral diagrams, five flowers from six plants of each phenotype grown in the greenhouse were examined. For SEMs, plants were grown in growth rooms at 20 °C. SEMs were made on plastic replicas as described[8].

DNA and RNA analysis. DNA extraction and blot analysis were done as described previously[12]. A 600-bp DNA from the 3′ end of Tam4, conserved among the CACTA transposon family in *Antirrhinum majus*[9–12], was used as a probe to reveal a 4.5-kb *Eco*RV band which was only present in the mutants. The 4.5-kb *Eco*RV fragment was cloned as pJAM608, using the λgt10 vector (Amersham PRN1713, N334L). The DNA flanking the transposon in pJAM608 was used to screen a genomic library (kindly provided by H. Sommer) to obtain λJAM151 containing the *cyc* locus. Most of the 15-kb insertion in λJAM151 was sequenced and the ORF identified. Restriction enzyme site mapping was used to identify the approximate insertion positions of the transposons in different *cyc* alleles. Four alleles, *cyc*-25 and *cyc*-608 from the John Innes collection; $cyc^{abnormis}$ (cyc^{abnor}) and $cyc^{neohemiradialis}$ (cyc^{neo}) from the Gatersleben collection, have been described[6,14,15,21]. The *cyc*-650 allele was obtained in a transposon-mutagenesis experiment (R.C. and E.C., unpublished results). PCR on genomic DNA of each allele was done to determine the exact transposon insertion site, using oligonucleotides to the conserved end of the CACTA transposon family and to the *cyc* sequence. DNA spanning the ORF was used as a probe to screen a cDNA library made from young inflorescence of wild type[22], and from about 2×10^6 recombinants, three independent cDNA clones (pJAM167, pJAM168 and pJAM169) were obtained and sequenced. Two of them had the same structure, indicating that the *cyc* gene had three exons, the ORF being contained within the first exon. The third cDNA was a 2.1-kb variant, containing an unspliced second intron. The transcription start and full-length cDNA were confirmed by 3′ and 5′ RACE PCR (5′ RACE system: GibcoBRL).

The methods for digoxigenin labelling of RNA probes, tissue preparation and *in situ* hybridization were done as described[23]. The poly(A)$^+$ tail of a *cyc* cDNA subclone, pJAM167, was deleted to obtain a plasmid pJAM193, which was used to generate antisense and sense probes using either T3 or T7 polymerases.

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CORRESPONDENCE and requests for materials should be addressed to E.C.