was a high degree of band-sharing between individuals, probably due to past inbreeding in this population. The large number of monomorphic and common bands provided a reference ladder that allowed 28 apparently homologous polymorphic minisatellite fragments in the size range 4.0–23.0 kilobases (kb) to be identified between fingerprints, thus enabling the ready comparison of all individuals. The order of the samples was randomized within and between gels, and the band patterns were scored by an assistant who had no knowledge of the lek sites of the individual birds.

Eight free-ranging full-trained displaying lek males whose mating success varied were removed from Whipsnade Park during February 1991 and transferred to a peacock farm in Norfolk, UK. The peacocks were housed in separate pens and four naive adult peahens, known to be at least 2 years old, provided by the farm, were measured and randomly assigned to each pen on 14 March. Pens were checked daily for eggs (it was not possible to know which of the four hens laid which egg unless egg laying was observed) and any eggs found were labelled and removed. Groups of eggs originating from several different pens over several dates were mixed and placed under broody domestic chickens for incubation. Egg weights were removed from the hens after 26 days and placed in a hatcher in batches, where each egg was hatched in a hatcher in batches, consisting of one young of the same sex from each pen. The fate of the offspring was recorded by a field assistant who had care to release the offspring in batches of eight, consisting of one young of the same sex from each pen. The sex of the offspring was monitored in January and February 1992, 12 offspring (7 males and 5 females) from each of the 8 males (3 from each of the 4 females) were introduced into Whipsnade Park. A matched sample of young was chosen from each pen so that there were no overall significant differences in hatching dates or weights of the offspring between fathers (at day 84, F1, 28 = 0.838, P = 0.393; at hatching, F1, 28 = 0.338, P = 0.562).

Care was taken to ensure that the offspring were reared together. Females produced 519 eggs and the growth of the surviving 349 offspring was monitored. In January and February 1992, 12 offspring (7 males and 5 females) from each of the 8 males (3 from each of the 4 females) were introduced into Whipsnade Park. A matched sample of young was chosen from each pen so that there were no overall significant differences in hatching dates or weights of the offspring between fathers (at day 84, F1, 28 = 0.838, P = 0.393; at hatching, F1, 28 = 0.338, P = 0.562). Care was taken to ensure that the offspring were reared together. Females produced 519 eggs and the growth of the surviving 349 offspring was monitored. In January and February 1992, 12 offspring (7 males and 5 females) from each of the 8 males (3 from each of the 4 females) were introduced into Whipsnade Park. A matched sample of young was chosen from each pen so that there were no overall significant differences in hatching dates or weights of the offspring between fathers (at day 84, F1, 28 = 0.838, P = 0.393; at hatching, F1, 28 = 0.338, P = 0.562).

Mantel tests that randomized the pairwise physical distances or band-sharing values, respectively, were performed on square-root transformed distances using the program RT v2.1 (ref. 21). We analysed nearest-neighbour associations using a program that randomized (100,000 times) the positions of relatives and non-relatives, as appropriate, and counted the number of occasions on which nearest neighbours were relatives. Received 11 March, accepted 2 July 1999.


Although there have been many molecular studies of morphological mutants generated in the laboratory, it is unclear how these are related to mutants in natural populations, where the constraints of natural selection and breeding structure are quite different. Here we characterize a naturally occurring mutant of Linaria vulgaris, originally described more than 250 years ago by Linnaeus3–5, in which the fundamental symmetry of the flower is changed from bilateral to radial. We show that the mutant carries a defect in Lecy, a homologue of the cycloidea gene which controls dorsoventral asymmetry in Antirrhinum4. The Lecy gene is extensively methylated and transcriptionally silent in the mutant. This modification is heritable and co-segregates with the mutant phenotype. Occasionally the mutant reverts phenotypically during somatic development, correlating with demethylation of Lecy and restoration of gene expression. It is surprising that the first natural morphological mutant to be characterized should trace to methylation, given the rarity of this mutational mechanism in the laboratory. This indicates that epigenetic mutations may play a more significant role in evolution than has hitherto been suspected.

Mature wild-type flowers of Linaria vulgaris (toadflax) have five petals that are part of their length to form a corolla tube ending in five separate lobes (Fig. 1c, d). Dorsoventral asymmetry is clearly evident in the shape and colour of the petals. The two dorsal (adaxial) petals have relatively long strap-shaped lobes; the two lateral petals have wider lobes with a partially orange lip; and the ventral (abaxial) petal has a small lobe with an orange lip, and a spur-shaped nectary at its base. Dorsoventral asymmetry is also evident in the stamens: the dorsal stamen is arrested early in development to give a sterile staminode (Fig. 1d), and the two lateral stamens are shorter and less hairy than the two ventral stamens.

Flowers of naturally occurring peloric mutants in Linaria are radially symmetrical (Fig. 1a–d). All five petals resemble the ventral petal of wild type, each having a small lobe with an orange lip, and a spur at their base. Similarly, there are five stamens, all of which closely resemble the ventral stamens of wild type in length and hairiness. In being fully ventilized, these mutant Linaria flowers resemble peloric mutants of Antirrhinum which lack the activity of two related genes, cycloidea (cyc) and dichotomy4–6. The peloric mutation in Linaria is recessive, as crosses to wild type yielded essentially wild-type F1 progeny. Only one of the F1 individuals occasionally gave one or two extra spurs.

We compared the development of wild-type and peloric flowers of Linaria by scanning electron microscopy. No differences were
seen early on, when sepal primordia were being initiated on the periphery of the floral meristem (Fig. 2a–d). However, differences were evident shortly after this, when stamen and petal primordia had emerged. The dorsal stamen primordium was retarded in wild type but not in peloric mutants (Fig. 2e, f). Similarly, the dorsal petal primordia had a distinctive shape in wild type but were similar to the other petals in peloric mutants (Fig. 2g–j). These effects on early stamen and petal development are comparable to those seen for peloric mutants of Antirrhinum, although in the case of Antirrhinum there are also earlier effects on organ number.

To investigate whether the peloric mutation might be caused by an alteration in a homologue of the cyc gene of Antirrhinum, a genomic study was performed. RNA in situ hybridizations of wild-type floral meristems probed with Lcyc. All panels show longitudinal sections; the inflorescence stem is to the left. The signal is seen as dark blue. k–n, later stages when all organ primordia are visible. Scale bars, 100 μm. Stages are defined according to ref. 23. br, Bract primordium; ca, carpel primordium; dp, dorsal petal primordium; ds, dorsal sepal primordium; ls, lateral sepal primordium; lst, lateral stamen primordium; stm, staminode primordium; vp, ventral petal primordium; vs, ventral sepal primordium; vst, ventral stamen primordium.
clone of a Linaria cyc-like gene, Lcyc, was isolated (Fig. 3) and used to probe DNA blots of an F2 population segregating for peloria. Digestion with several restriction enzymes revealed restriction-fragment length polymorphisms (RFLPs) linked to the peloric phenotype, suggesting that the mutation was either within, or closely linked to, the Lcyc gene (Fig. 4a, b). In Antirrhinum, a second gene, related to cycloidea-like gene, was isolated (Fig. 3) and used to probe genomic sequences of the mutant and wild type. Apart from a partial digestion with an insensitive enzyme (Fig. 3), no sequence polymorphisms specific to the peloric mutant were found within the Lcyc coding region or in the 930 base pairs downstream of the coding region (Fig. 3, boxed nucleotides).

The effect of the peloric mutation on Lcyc expression was determined by RNA in situ hybridization. In wild type, Lcyc expression was detected in floral meristems from peloric individuals. Similar expression was found at later stages, indicating that in Linaria there may be less redundancy with respect to the role of these genes in the control of floral asymmetry.

To determine the nature of this mutation, we compared the genomic sequences of Lcyc in mutant and wild type. Apart from a nucleotide polymorphism in the 3′ region (Fig. 3, boxed nucleotides), no sequence polymorphisms specific to the peloric mutant were found within the Lcyc coding region or in the 930 base pairs (bp) of upstream sequence. Surprisingly, we observed no difference at restriction sites previously shown to be polymorphic by RFLP analysis (Fig. 3). Because the enzymes that detected RFLPs were sensitive to cytosine methylation, one explanation was that the mutation was either within, or closely linked to, the Lcyc promoter region. The effect of the peloric mutation on Lcyc expression was probably responsible for the peloric phenotype.

Figure 4 RFLP analysis and methylation of Lcyc. a, Restriction map of the Lcyc genomic region showing location of sites that are methylated in peloric plants. Lollipops indicate complete (filled), partial (half-filled) or no methylation (empty) at the sites. Numbers indicate the size of each fragment in kilobases. b, Genomic DNA from young leaves of wild-type (+) and peloric (P) individuals digested with HindIII and partial or complete digestion with PstI due to methylation are shown. H, HindIII; PstI; D, DdeI. Numbers indicate the size of each fragment in kilobases. c, Genomic DNA from wild-type (+) and peloric (P) plants digested with Mbol and Sau3A, blotted and hybridized with the Lcyc probe A (see a). The first two lanes show DNA from the parents; other lanes show DNA from siblings with a wild-type or mutant phenotype taken from four different F2 families (1–4). Based on a DNA-sequence polymorphism at the 3′ end of Lcyc, the wild-type F2 segregants shown were heterozygous for the peloric allele. c, Genomic DNA of wild-type (+) and peloric (P) plants digested with Mbol or Sau3A, blotted and hybridized with probe A. In addition to the 0.4-kb fragment, three bands of 139 bp, 144 bp and 148 bp were expected but were too small to be resolved on the gel. Lower panel, same blot after stripping and hybridizing with a homologue of the centroradialis gene from Linaria (control): no other bands were visible on the blot. The 0.7-kb band hybridizing to centroradialis in peloric plants corresponds to an RFLP fragment (Fig. 4a, b). In Antirrhinum, a second gene, related to cycloidea-like gene, was isolated (Fig. 3) and used to probe genomic sequences of the mutant and wild type. Apart from a partial digestion with an insensitive enzyme (Fig. 3), no sequence polymorphisms specific to the peloric mutant were found within the Lcyc coding region or in the 930 base pairs (bp) of upstream sequence. Surprisingly, we observed no difference at restriction sites previously shown to be polymorphic by RFLP analysis (Fig. 3). Because the enzymes that detected RFLPs were sensitive to cytosine methylation, one explanation was that the mutation was either within, or closely linked to, the Lcyc promoter region. The effect of the peloric mutation on Lcyc expression was probably responsible for the peloric phenotype.

To determine the nature of this mutation, we compared the genomic sequences of Lcyc in mutant and wild type. Apart from a nucleotide polymorphism in the 3′ region (Fig. 3, boxed nucleotide), no sequence polymorphisms specific to the peloric mutant were found within the Lcyc coding region or in the 930 base pairs (bp) of upstream sequence. Surprisingly, we observed no difference at restriction sites previously shown to be polymorphic by RFLP analysis (Fig. 3). Because the enzymes that detected RFLPs were sensitive to cytosine methylation, one explanation was that the mutant allele was methylated. We checked this by digesting wild-type and peloric genomic DNA with a pair of isoschizomers (Mbol and Sau3A) that differed in sensitivity to methylation. Probing with Lcyc revealed no difference in digests with the insensitive enzyme.
there is an association between plants carrying heavily methylated DNA and the semi-peloric phenotype. However, even DNA from wild type plants showed evidence of some methylation (see bands at 2.2 and 2.4 kilobases (kb) in Figs 4b, 5b), perhaps reflecting somatic modifications of Lcyc in a proportion of wild-type cells.

These results might be explained in two ways: either a methylated Lcyc allele from the semi-peloric parent was being transmitted through the germ line to the F2, or Lcyc was being methylated de novo in a proportion of the F2 progeny. These possibilities could be distinguished by following the segregation of a DNA-sequence polymorphism between the parents in the 3′ region of Lcyc. Sequencing of this region in several peloric and wild-type F2 segregates showed that all peloric individuals analysed were homozygous for the Lcyc allele from the semi-peloric parent, whereas the wild types were either homozygous or heterozygous for the allele from the wild-type parent. Thus, the phenotype in the F2 appeared to reflect transmission of a modified allele of Lcyc.

Although these results indicated that the peloric mutation was linked to a methylated Lcyc allele, they did not prove that methylation affected Lcyc activity. For example, the methylated allele might have carried a DNA sequence change in an upstream region that was primarily responsible for reduced Lcyc expression. This could be tested by analysing peloric plants that showed somatic instability. Several F2 peloric plants produced some branches with almost wild-type flowers, or flowers that were intermediate between peloric and wild type (semipeloric; Fig. 5a). Cuttings were taken from branches with different flower phenotypes, and the methylation of Lcyc was analysed in the propagated plants (Fig. 5b). The phenotype correlated with the state of Lcyc methylation: the nearly wild-type plants were partially demethylated, whereas the peloric and semipeloric plants were heavily methylated. Probing blots of DNA digested with methylation-insensitive 6-bp cutters (for example, HincII, Xbal) revealed no other alterations, such as excision of a transposon, around 10 kb of the Lcyc locus, although excisions of less than 200 bp could not be ruled out. RNA in situ hybridization showed that demethylation of Lcyc correlated with recovery of a wild-type Lcyc transcription pattern in all three layers of floral meristems (data not shown). Thus, the lack of Lcyc expression in the peloric plants was not due to a defect in the Lcyc DNA sequence, but to a heritable epimutation involving DNA methylation. This epimutation was not completely stable during somatic development, occasionally reverting through demethylation.

It is surprising that the first natural morphological mutant to be characterized should trace to an epimutation because most mutations recovered from studies of laboratory stocks are due to DNA-sequence alterations or transpositions. In plants, epimutations of endogenous genes have occasionally been described in laboratory strains, as with the alterations at the P locus of maize and at the SUPERMAN locus of Arabidopsis. Studies on animals have not so far revealed heritable mutations of this kind, although epimutations have been recovered as somatic events in some cancers. One possible explanation is that, unlike the situation in animals, there is no early separation between germ line and soma in plants. Thus, an epimutation arising in a plant meristem can be transmitted to subsequent generations either by sexual means or through vegetative propagation. The mechanism for generating epimutations in plants is not known, but it may reflect aberrant activation within meristems of a process that can operate to silence genes in some non-meristematic cells. In the case of Linaria, which is an outbreeding perennial, such epimutations may be more likely to underlie a natural mutant phenotype than DNA-sequence alterations which require two mutant alleles to come together to form a homozygote. This contrasts with laboratory populations where homozygoty is continually promoted by inbreeding and where the diminished role of vegetative propagation means there is less opportunity for somatic mutations to accumulate progressively in meristems.

Epimutations appear to be less stable than DNA-sequence alterations, as illustrated by the various degrees of somatic reversion in Linaria. Nevertheless, they may have longer-term consequences, depending on how the variation they cause interacts with variation generated by DNA-sequence changes. Furthermore, methylated DNA is more prone to mutation and may influence the local frequency of recombination. Epimutations may therefore have both a short- and long-term significance for plant evolution.

Methods

Plant material and crosses

Wild-type Linaria vulgaris plants were grown from seed (Unwins). Seeds were germinated on plates containing MS growth medium, grown in cabinets (at 20°C for 16 h in light). Seedlings were then transplanted to pots and grown in a cool greenhouse. The peloric mutant was maintained by cuttings. For floral diagrams, more than ten flowers from different plants were examined for each phenotype. SEMs were carried out on plastic replicas as described.

Five F1 individuals were intercrossed (Linaria is self-incompatible) to generate eight F2 segregating families. These generally contained few individuals owing to poor seed set and poor germination. In total, 39 F2 individuals were obtained, of which 5 were fully peloric and the rest were wild-type. The segregations were (first number, wild type; second number, peloric): family 1: 16, 1; family 2: 1, 1; family 3: 1, 1; family 4: 5, 1; family 5: 0, 1; family 6: 6, 0; family 7: 4, 0; family 8: 1, 0. One of the peloric plants showed somatic instability during the first year, and three other peloric plants showed somatic instability after several years growth in the greenhouse. Seed capsules obtained from self-pollination of F1 individuals showed a degree of self-compatibility; 18 F2 individuals, all of which were phenotypically wild type. This was possible due to linkage of Lcyc to the self-incompatibility locus, as described in Antirrhinum.

DNA and RNA analysis

Genomic DNA was obtained from young leaves. DNA extraction and blot analysis was done as described. The 3′ end of the Lcyc transcript was isolated by RACE PCR: cDNA was synthesized from wild-type Linaria mRNA as described and amplified by PCR using an Antirrhinum cyclamen-specific oligonucleotide primer (GAGAGGATCCGTACCGA-CAAGAGCCAGACAAACCCTTGATTGGG-3′) together with B25 using similar conditions apart from annealing temperature, which was 50°C instead of 45°C. PCR products were gel-purified with Qiaquick (Qiagen), cloned in pGEM-T (Promega) and sequenced automatically (ABI system, Perkin Elmer). The genomic region flanking Lcyc was isolated by inverse PCR: 2–4 μg of wild-type and peloric genomic DNA was digested with HindIII, cloned with a Wizard Clean-up kit (Promega), deluted fourfold, self-ligated with T4 ligase (Gibco) and used as a template for PCR, using oligonucleotides of Lcyc directed outwards from the gene (5′-ATGGAGTTGATCTCGTGGCGG-3′) and

![Figure 5](image-url)
A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer

Sandrine Millet†1, Kenneth Campbell†1,‡, Douglas J. Epstein†1,‡, Kasia Lossoi§, Esther Harris‡ & Alexandra L. Joyner‡§

†Developmental Genetics Program and Howard Hughes Medical Institute, Skirball Institute of Biomolecular Medicine, §Department of Cell Biology and Physiology and Neuroscience, NYU School of Medicine, 540 First Avenue, New York, New York 10016, USA

These authors contributed equally to this work.

The mid/hindbrain (MHB) junction can act as an organizer to direct the development of the midbrain and anterior hindbrain1,2. In mice, Otx2 is expressed in the forebrain and midbrain and Gbx2 is expressed in the anterior hindbrain, with a shared border at the level of the MHB organizer. Here we show that, in Gbx2−/− mutants, the earliest phenotype is a posterior expansion of the Otx2 domain during early somite stages. Furthermore, organizer genes are expressed at the shifted Otx2 border, but not in a normal spatial relationship. To test whether Gbx2 is sufficient to position the MHB organizer, we transiently expressed Gbx2 in the caudal Otx2 domain and found that the Otx2 caudal border was indeed shifted rostrally and a normal appearing organizer formed at this new Otx2 border. Transgenic embryos then showed an expanded hindbrain and a reduced midbrain at embryonic day 9.5–10. We propose that formation of a normal MHB organizer depends on a sharp Otx2 caudal border and that Gbx2 is required to position and sharpen this border.

Otx2 null mutants have a deletion of the brain rostral to hindbrain homobmere 3 (r3), due to a failure of induction of the anterior neural plate during gastrulation1–3. Otx1 mutants have only subtle defects4. In double Otx1/Otx2 mutants5,6 that have only one Otx2 wild-type allele, the Otx2 caudal limit and the MHB organizer are shifted anteriorly at early somite stages (ESS). Subsequently, no mesencephalon (midbrain) and caudal forebrain form and the cerebellum (normally arising from the anterior hindbrain or metencephalon) is expanded rostrally. In contrast, Gbx2 null mutants lack the rostral hindbrain and have a caudal expansion of the midbrain at E12.5, and have abnormalities in the MHB organizer at E9.5 (ref. 9).

To investigate the specific role of Gbx2 in formation of the MHB organizer, we reanalysed MHB gene expression in Gbx2 mutants at ESS. At 4–6 somites in these mutants, the Otx2 domain was clearly expanded and the posterior limit shifted caudally from the middle of the MHB region to the r3/r4 border (Fig. 1a–d). Furthermore, the Otx2 limit did not sharpen in the mutant embryos (Fig. 1c, d). At ESS, the domain of expression of the organizer gene Foxf1 was expanded and shifted caudally (Fig. 1e–h) from r2 to r4 in Gbx2 mutants, and the gradient of expression was inverted in the strongest expression r4 (Fig. 1h). The normal expression pattern of the organizer gene Wnt1 at ESS (Fig. 1i, k) can be subdivided into a domain of expression along the lateral edges (future dorsal midline) between the diencephalon and the MHB junction and caudally from r4 into the spinal cord, and a domain of expression in the mesencephalon. In Gbx2 mutants, the expression along the lateral edges was continuous without a negative gap in the mesencephalon (Fig. 1j, l), indicating that Gbx2 could be required to repress Wnt1 in this region. The Wnt1 domain in the mesencephalon was slightly expanded and clearly shifted caudally, so that it was

References


Acknowledgements

We thank the Linnean Society of London for permission to photograph the specimen of peloric Linaria kept in Linnaeus’ herbarium and thank C. Jarvis from the Natural History Museum in London for providing the photograph; we also thank M. Cragg-Barber for providing a living peloric specimen from the UK; N. Hartley for sequencing the genomic Lyc sequence; and R. Carpenter, D. Bradley, O. Ratcliffe, I. Amaya and U. Nath for comments on the manuscript. This work was supported by the Gatesy Charitable Foundation. P.C. was an EMBO postdoctoral fellow and a EU postdoctoral fellow.

Correspondence and requests for materials should be addressed to E.C. (e-mail: coeren@bbsrc.ac.uk). The Genbank accession number for the Lyc sequence is Bank AF 161252.

NATURE | VOL 401 | 9 SEPTEMBER 1999 | www.nature.com

© 1999 Macmillan Magazines Ltd