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. Eggs were removed from the hens after 26 days and placed in a hatcher in batches, where each egg had its own compartment; each of the hatched chicks was given an individual colour ring combination. Each batch of eggs was incubated and hatched separately at approximately weekly intervals from May to August. Each batch of chicks was provided with a heat lamp and food and water *ad libitum*; batches were subsequently pooled and 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 per male) 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, $F_{7,95} = 0.838$, $P = 0.559$; at introduction, $F_{7,95} = 0.358$, $P = 0.924$). Care was taken to release the offspring in batches of eight, consisting of one young of the same sex from each pen. The fate of the offspring was recorded by a field assistant who had no knowledge of the relatedness of any of the individuals, and the birds were observed every spring until they established permanent display sites in 1995 (aged 4). Of the introduced males, 19 were observed to have established permanent display sites in 1995 (Fig. 3).

Mantel tests[7] 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.

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An epigenetic mutation responsible for natural variation in floral symmetry

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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 Linnaeus[1–3], in which the fundamental symmetry of the flower is changed from bilateral to radial. We show that the mutant carries a defect in *Lcyc*, a homologue of the *cycloidea* gene which controls dorsoventral asymmetry in *Antirrhinum*[4]. The *Lcyc* 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 *Lcyc* 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 united for 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 ventralized, these mutant *Linaria* flowers resemble peloric mutants of *Antirrhinum* which lack the activity of two related genes, *cycloidea* (*cyc*) and *dichotoma*[4,5]. The peloric mutation in *Linaria* is recessive, as crosses to wild type yielded essentially wild-type F_1 progeny. Only one of the F_1 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

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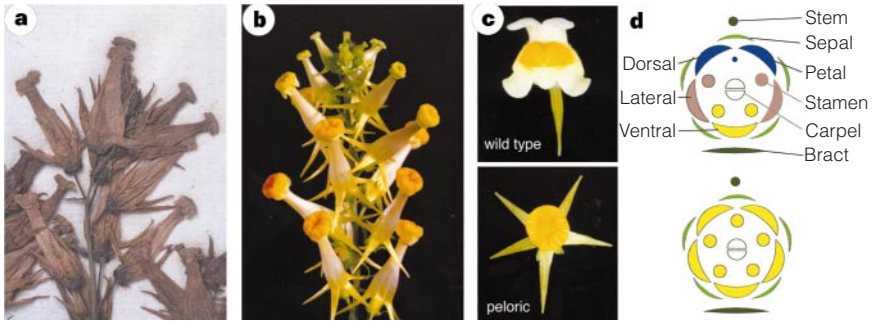

Figure 1 Wild-type and peloric *Linaria vulgaris* flowers. **a**, Original herbarium specimen of peloric *Linaria* inflorescence collected by Linneaus and currently housed in the Linnean Society, London. **b**, Peloric *Linaria* inflorescence from a living specimen. **c**, Face view of a wild-type *Linaria* flower compared to a peloric mutant. **d**, Floral diagrams of wild-type (top) and peloric (bottom) flowers showing the relative positions of different organs, with identities indicated by colours: blue (dorsal) brown (lateral) yellow (ventral). The wild-type flower has an axis of dorsoventral asymmetry orientated such that the dorsal (upper or adaxial) part is nearer the stem whereas the ventral (lower or abaxial) part is nearer to the subtending leaf. The peloric flower is radially symmetrical, with all petals resembling the ventral petal of the wild type.

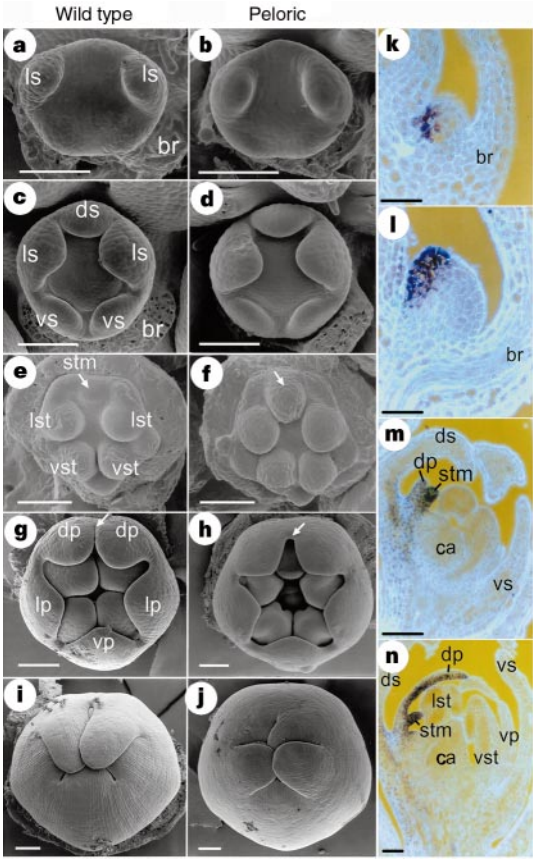

Figure 2 Development of wild-type and peloric flowers as revealed by scanning electron microscopy (SEM) and expression of *Lcyc* during floral development. **a**–**j**, Five stages studied by SEM are shown. No difference between mutant and wild type are observed at early stage 4 (**a**, **b**; sepal primordia initiated) or late stage 4 (**c**, **d**; all five sepal primordia are clearly visible). By early stage 6 (**e**, **f**; sepal primordia have been removed to show stamen primordia), the dorsal stamen (stm) of wild type was relatively small and retarded in growth (**e**); whereas in the peloric mutant, the dorsal stamen was indistinguishable from the other stamen primordia (**f**, arrow). Differences in petal development became evident at a slightly later stage, when the corolla tube and lobes started to form (**g**, **h**; stage 7). In wild type the dorsal petal lobes had a distinctive shape and were separated by a relatively narrow gap (**g**, arrow), whereas in the peloric mutant, all the petal lobes were identical (**h**). At later stages, when petals enclose the stamens and carpel (**i**, **j**), the wild-type dorsal petal lobes consistently cover the other petals, whereas the petals of the mutant are more equivalent. **k**–**n**, 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**, Stage 2; **l**, stage 3; **m**, **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; lp, lateral petal primordium; ls, lateral sepal primordium; lst, lateral stamen primordium; stm, staminode primordium; vp, ventral petal primordium; vs, ventral sepal primordium; vst, ventral stamen primordium.

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[4].

To investigate whether the peloric mutation might be caused by an alteration in a homologue of the *cyc* gene of *Antirrhinum*, a genomic

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   1  GATAAAGCTTGCGGCCCCTCCTTACACACACACTCCACACACTGGTCTAATTGAAGTAAA   60
  61  GACAATCAATATATGAATGACTGTAAGAAAAAACGATATGATTAACCCTAGTGGTTTGAC  120
 121  CTGTATTGGATTAAAACATTGCATTGCATCAATCCTGATTGATAATAGTGTGATACCCGC  180
 181  TGTTGCTCTATTTTGTATGTCTAAAATGTTTTCTTTATGATCGTCAGGGGTTGATAGGGC  240
 241  CCCCCCCCTTCACTCCATCAGATTGGTCATTTCTCATTTACTACTTCTTTAGTAGTACTA  300
 301  TAATAAAATCCTTTTTATTTCAATGGACATAAAAGTACAACCAAATGAATCATTATATAT  360
 361  TTGTGCTGAAGTGGACGAAACTGTTGTTAGGTTACACCAATTGATTGTACGTACCAAAAG  420
 421  GTTTTAGTAAACTAGGTTTAGTCACCAATTAATTAATTATACCCATATATCTGAGCCAGA  480
 481  TTTTGACGTTGTTGGAGTTCTAAAACTTTTAGAAGCAGAGAAAGTACAAAATAATACTTA  540
 541  GTTATAATGCATGCACCTGAAAGAAACCCCAGCCTTCCTTATTTCATCATTCATTATGTA  600
 601  TTGATATTCATTCATCCTTCCTTTTCTCCCCTCCAGCTATATAGCCCTCCAGTACTCTTG  660
 661  CACAGGATTAGTCCTTTTCCATGTAAACAAAAGAAATTGACAGCTTTATTTCTTTCTTGC  720
 721  CTTCAATAAAATAAAACATGTTAAATAGAGTCATATATTAATTCACCTCACTAAGAAAGG  780
 781  TAAACAAAAGCCCCTATTTTCCATGGCAAAGATCATAAAACTCTTTCAAGGATTTCTTCT  840
 841  GAAACAGCTTCATGTGAGCTCAAAATAATAGGGTTTCTTTGTACCCTTCTTTCAATCATT  900
 901  CACTCCCAACACAACAAAGGAAATTAGAAAATGTTTGGGAAGAACACATACCTACACCTT  960
 301                          M  F  G  K  N  T  Y  L  H  L           320
 961  CCTCATCAGGTTTCAACCTCTCTTAACTCTCGTGCCGTTGTTGACCTAAACGGCAACGAG 1020
 321  P  H  Q  V  S  T  S  L  N  S  R  A  V  V  D  L  N  G  N  E   340
1021  ATCCAACTCCATCTCCAGCATCACCACCACGACATACTCTCCGGTCATTACTTGACCAAC 1080
 341  I  Q  L  H  L  Q  H  H  H  H  D  I  L  S  G  H  Y  L  T  N   360
1081  GGTCCTGTTCTTGAGTCCACAGCCTTGTTCAACAACTTCAACAATCAACATGTTGTTAGT 1140
 361  G  P  V  L  E  S  T  A  L  F  N  N  F  N  N  Q  H  V  V  S   380
1141  CACGGGATGAACTGCCATGATCCTGATCCTGCCAATATGGCAGAAACATTCCAAACCAAA 1200
 381  H  G  M  N  C  H  D  P  D  P  A  N  M  A  E  T  F  Q  T  K   400
1201  CAAAGTACTGTGAAAAAGGACCGGCACAGCAAGATATACACTGCTCAAGGGCCGAGAGAC 1260
 401  Q  S  T  V  K  K  D  R  H  S  K  I  Y  T  A  Q  G  P  R  D   420
1261  AGGAGAGTTCGTCTGTCCATCGGCATTGCAAGGAAGTTCTTTGATCTACAAGAGATGCTA 1320
 421  R  R  V  R  L  S  I  G  I  A  R  K  F  F  D  L  Q  E  M  L   440
1321  GGTTTCGACAAGCCAAGCAAGACCCTCGACTGGCTCCTTACTAAGTCGAAAACAGCCATA 1380
 441  G  F  D  K  P  S  K  T  L  D  W  L  L  T  K  S  K  T  A  I   460
1381  AAAGAGCTAGTGCAGTCCAAAAGTACGAAGAGCAATTCTTCTAATTCCCATTCTGATGAT 1440
 461  K  E  L  V  Q  S  K  S  T  K  S  N  S  S  N  S  H  S  D  D   480
1441  GATTGTGATGATGAGGTTGTGTCTGCCGAAGGAGATTCTGAGAACGCTGCAGATTCGAAG 1500
 481  D  C  D  D  E  V  V  S  A  E  G  D  S  E  N  A  A  D  S  K   500
1501  GGGAAGTCGGTGCTTATAAAAGGTAACTACAAATGTAAAGAAGCTGCTTCTGCAATGGAT 1560
 501  G  K  S  V  L  I  K  G  N  Y  K  C  K  E  A  A  S  A  M  D   520
1561  TCTCAACAAGCAGCACTCAATCTCGTGAAAGAGTCGAGGGCAAAGGCGAGAGCAAGAGCT 1620
 521  S  Q  Q  A  A  L  N  L  V  K  E  S  R  A  K  A  R  A  R  A   540
1621  AGGGAAAGAACCAAGGAGAAAATGTGTATCAAACAACAACTGAATGAAGCTAGGAACAAC 1680
 541  R  E  R  T  K  E  K  M  C  I  K  Q  Q  L  N  E  A  R  N  N   560
1681  AACAAGGGTGGTGACTGGATCAATAATCCCTTCAACAATGTAATCCAGTCTAACAGGAAC 1740
 561  N  K  G  G  D  W  I  N  N  P  F  N  N  V  I  Q  S  N  R  N   580
1741  CATCAACAGTTCGAAAGTCGCGAGGCGGCATTTGTTCATCATCCGGTTTTTGGGTTTCAT 1800
 581  H  Q  Q  F  E  S  R  E  A  A  F  V  H  H  P  V  F  G  F  H   600
1801  CAGCAAAACTACAGCAACGCATCTGCCTCCTCTCATGAGAACTGGGATCAGTCGAACCAG 1860
 601  Q  Q  N  Y  S  N  A  S  A  S  S  H  E  N  W  D  Q  S  N  Q   620
1861  CTATGCGCCATTTTGAATCAGCACAAGTTCATCAATTAGGTAAAAGAAGAAGAAATTAAA 1920
 621  L  C  A  I  L  N  Q  H  K  F  I  N  *                       640
1921  CTATTTTATAAAGTTGTTTTCCAATTTTGTGCCATACCATGTCAGGTCAAATGTGATTTA 1980
1981  TGGTTTGCTTTTGTCTAACAGCAAGTTGAACTATAGGAAACCTTTTGCATTCCTCAAAGA 2040
2041  TCTCAAGGTCAGAAAGGTTCTTTTAGCTTGAAGAATTTTATGTTTTGTTACCTTCTTTT 2100
2101  TTCAGCAGGGTACATGAATCTTATCAATCTAATCTTAGTATTAAAAAGGTATTATGAATA 2160
2161  GTATATTTAGTATTTGGATCTAATTATATCCATGTTTCTGCTAGATTATACTTAAAACAA 2220
2221  CTCCTGGACATTATCTAAATACTGCAGTCAAAGCTAACTCATGTGACTGATTTTAGTGGT 2280
2281  CTTTTGTAATTGTCTGTTTCCAATATTATGCAAGGTTATTTGCTTATGTGACCACTAAGC 2340
2341  ATGGAAATTTTTTTAGTTTCAATCGAGGTATGAAAGCAGAGACAAGCTACTATCATGTTT 2400
2401  TAACATCAAAATCATGAGATAAAGCTTG  2428
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Figure 3 Sequence of *Lcyc*. Wild-type genomic sequence containing the *Lcyc* ORF. The similarity between *Lcyc* and *cycloidea* is 87% identity at the DNA level. The deduced protein sequence is indicated underneath in the standard one-letter notation. Restriction sites that are methylated in peloric plants are underlined. *Hae*III GGCC; *Msp*I CCGG; *Pst*I, CTGCAG; *Sau*3AI, GATC; *Mae*II, ACGT, *Dde*I, CTNAG (not all the *Dde*I sites were analysed). Site of nucleotide polymorphism in the 3′ region is boxed.

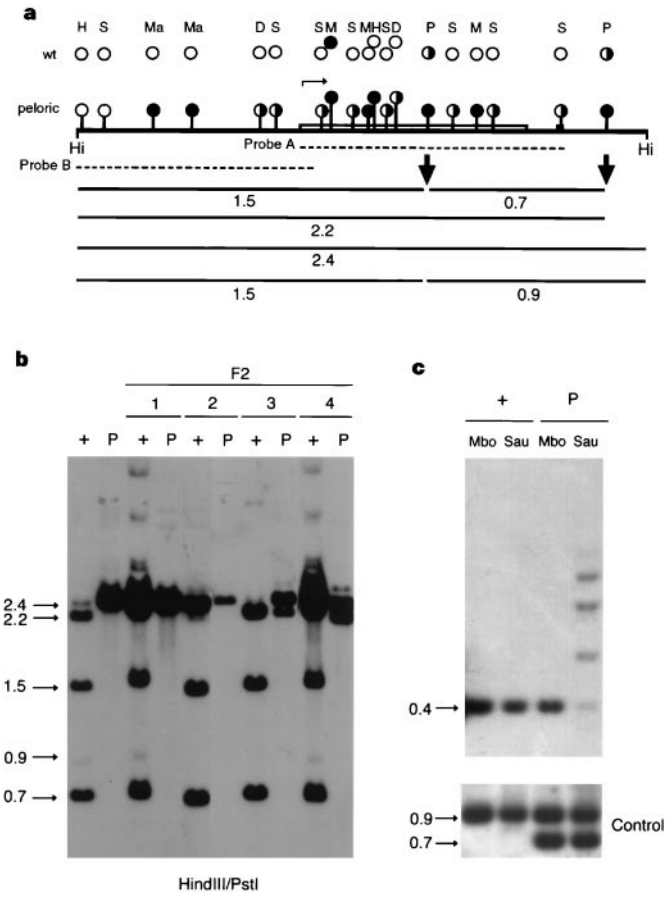

Figure 4 RFLP analysis and methylation of *Lcyc*. **a**, Restriction map of the *Lcyc* genomic region showing location of sites that are methylated in peloric plants. Lollipops indicate complete (filled), partial (half-filled) or no methylation (empty) at the sites. The ORF region is indicated by an open box. Probe A was used in **b** and **c** and in Fig. 5b; probe B was used to analyse the methylation at the promoter region of *Lcyc*. Fragments resulting from complete digestion with *Hin*dIII and partial or complete digestion with *Pst*I due to methylation are shown. H, *Hae*III; Hi, *Hin*dIII; M, *Msp*I; S, *Sau*3AI, Ma, *Mae*II, D, *Dde*I. Numbers indicate the size of each fragment in kilobases. **b**, Genomic DNA from young leaves of wild-type (+) and peloric (P) individuals digested with *Hin*dIII/*Pst*I, 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 F_2 families (1–4). Based on a DNA-sequence polymorphism at the 3′ end of *Lcyc*, the wild-type F_2 segregants shown were heterozygous for the peloric allele. **c**, Genomic DNA of wild-type (+) and peloric (P) plants digested with *Mbo*I or *Sau*3A, 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[24] from *Linaria* ('control': no other bands were visible on the blot). The 0.7-kb band hybridizing to *Lcentroradialis* in peloric plants corresponds to an RFLP present in this line. Similar blots were stripped and hybridized with a second *Lcyc*-like gene and with a ubiquitin cDNA probe, which revealed no differences between wild-type and peloric restriction patterns (not shown).

clone of a *Linaria cyc*-like gene, *Lcyc*, was isolated (Fig. 3) and used to probe DNA blots of an F_2 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 *Lyc* gene (Fig. 4a, b). In *Antirrhinum*, a second gene, related to *cyc*, also needs to be inactivated to give a fully peloric phenotype[4,5], indicating that in *Linaria* there may be less redundancy with respect to the role of these genes in the control of floral asymmetry.

The effect of the peloric mutation on *Lcyc* expression was determined by RNA *in situ* hybridization. In wild type, *Lcyc* was expressed in the dorsal region of floral meristems from very early stages of development (Fig. 2k, l). At later stages, expression became restricted to the dorsal staminode and dorsal petal primordia (Fig. 2m, n). The overall expression pattern of *Lcyc* was therefore similar to that of *cyc* in *Antirrhinum*[4], consistent with a role for *Lcyc* in establishing the asymmetry of the *Linaria* flower. No expression of *Lcyc* was detected in floral meristems from peloric individuals (data not shown). Taken together with our RFLP analysis, this suggested that a mutation blocking *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 (*Mbo*I and *Sau*3A) that differed in sensitivity to methylation. Probing with *Lcyc* revealed no difference in digests with the insensitive enzyme

(*Mbo*I), whereas the methylation-sensitive enzyme *Sau*3A gave incomplete digestion of *Lcyc* specifically in the peloric mutant (Fig. 4c). This differential methylation did not extend to all genes, as similar blots probed with other *Linaria* genes revealed no difference between wild-type and mutant DNA (Fig. 4c). Thus, there is an association between plants carrying heavily methylated *Lcyc* and the peloric phenotype. However, even DNA from wild type showed evidence of some methylation (see bands at 2.2 and 2.4 kilobases (kb) in Figs 4b, 5b), perhaps reflecting somatic modification of *Lcyc* in a proportion of wild-type cells.

These results might be explained in two ways: either a methylated *Lcyc* allele from the peloric parent was being transmitted through the germ line to the F_2, or *Lcyc* was being methylated *de novo* in a proportion of the F_2 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 F_2 segregants showed that all peloric individuals analysed were homozygous for the *Lcyc* allele from the peloric parent, whereas the wild types were either homozygous or heterozygous for the allele from the wild-type parent. Thus, the phenotypes in the F_2 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 F_2 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, *Hin*cII, *Xba*I) 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.

Figure 5 Somatic instability of the peloric phenotype correlates with demethylation of *Lcyc*. **a**, Flowers from a peloric plant showing somatic instability. Arrows point to extra spurs on the lateral petals of the semipeloric flowers. The almost wild-type flower on the left has an extra dorsal stamen within the flower. **b**, DNA from young leaves of wild-type (+) and peloric (P) individuals digested and blotted as for Fig. 4b. The first two lanes corresponds to DNA from parental individuals; lane 3, DNA from an F_2 unstable mutant (U); lanes 4–6, DNA from cuttings from the unstable plant with peloric (P), semipeloric (SP) and near wild-type (+) phenotypes. Of the cuttings, only those with near wild-type phenotype show extensive demethylation of *Lcyc*. Based on the relative intensity of the 2.2-kb and 2.4-kb bands, semipelorics appeared to less heavily methylated than pelorics, consistent with their less extreme phenotype.

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[6] and at the *SUPERMAN* locus of *Arabidopsis*[7]. Studies on animals have not so far revealed heritable mutations of this kind, although epimutations have been recovered as somatic events in some cancers[8,9]. 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[10], 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 homozygosity 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[11] and may influence the local frequency of recombination[12]. Epimutations may therefore have both a short- and long-term significance for plant evolution[13,14]. □

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[15].

Five F_1 individuals were intercrossed (*Linaria* is self-incompatible) to generate eight F_2 segregating families. These generally contained few individuals owing to poor seed set and poor germination. In total, 39 F_2 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 F_1 individuals showing a degree of self-compatibility gave 58 F_2 individuals, all of which were phenotypically wild type. This was possibly due to linkage of *Lcyc* to the self-incompatibility locus, as described in *Antirrhinum*[16,17].

DNA and RNA analysis

Genomic DNA was obtained from young leaves. DNA extraction and blot analysis was done as described[18]. The 3′ end of the *Lcyc* transcript was isolated by RACE PCR: cDNAs were synthesized from wild-type *Linaria* mRNA as described[19] and amplified by PCR[20] using an *Antirrhinum cycloidea*-specific oligonucleotide primer (5′-GAAAGTTCTTT-GATCTACA-3′) from a very conserved region of the gene, the TCP domain[21], together with oligonucleotide B25, which is complementary to the 3′ end of the cDNA[20] (5′-GAC TCG AGT CGA CAT CGA-3′). PCR conditions were: 1 cycle at 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 45 °C for 2 min and 72 °C for 3 min, and a final step at 72 °C for 10 min. Seminested PCR was carried out on 1 μl of the previous PCR reaction with a further *cyc*-specific oligonucleotide nearer the 3′ end (5′-GATGCTAGGTTTCGA-CAAGCCGAGCAAAACCCTTG ATTGG-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 *Hin*dIII, cleaned with a Wizard Clean-up kit (Promega), diluted fourfold, self-ligated with T4 ligase (Gibco) and used as a template for PCR, using oligonucleotides of *Lcyc* directed outwards from the gene (5′-ATGGAG TTGGATCTCGTTGCCG-3′) and

(5′-GTTCGAAAGTCGCGAGGCGGC-3′). 1 μl from the reaction was used in a second PCR in which nested oligonucleotides further out (5′-ACAACGGCACGAGAGTTAA-GAGAGG-3′ and 5′-AACT ACAGCAACGCATCTGCCTCC-3′) were used. PCR was carried out with an Expand Long Template PCR system (Boehringer Mannheim) under the conditions recommended by the manufacturer. Wild-type and peloric PCR products were cloned as pJAM2167 and pJAM2169, respectively, in pGEM-T (Promega) and sequenced. The 1.1-kb fragment used as probe A in the blots shown in Figs 3c, d and 4d was obtained by PCR carried out on wild-type genomic DNA and using oligonucleotides of *Lcyc* pointing towards the gene (5′-TTTGGGAAGAACACATACC-3′ and 5′-AGATCTTTGAGGAATGCAAAA GGTTTCC-3′). The fragment was cloned as pJAM2166 in pGEM-T as described. Probe B (Fig. 4a) is a *Hin*dIII/*Sac*II fragment obtained from pJAM2167. RFLP analysis of the F_2 families was carried out by digesting genomic DNA of the 39 individuals with *Pst*I. The peloric phenotype showed complete linkage with absence of a 0.7-kb band. To analyse the segregation of a DNA polymorphism located 3′ of the *Lcyc* ORF, PCR was carried out on genomic DNA from four F_2 peloric plants and 16 F_2 wild types using oligonucleotides pointing towards the polymorphic region (5′-AAAGGTATT ATGAATAGTATATTTAGTATTTGG-3′ and 5′-ATCTCATGAATTTTGATGTTAAAA-CATGATAGTAGC-3′). The resulting 290-bp fragments were purified with a Wizard clean-up kit (Promega) and automatically sequenced (ABI system, Perking Elmer). The four peloric plants were homozygous for a G at position 2,228 (Fig. 3), like the peloric parental plant, whereas their wild-type siblings were either homozygous (A/A, 3 plants) like the wild-type parental plant or heterozygous (A/G, 13 plants). The four F_1 plants analysed were heterozygous (A/G). In the heterozygotes, two peaks, corresponding to two different nucleotides, were visualized at the polymorphic position.

The methods for digoxigenin labelling of RNA probes, tissue preparation and *in situ* hybridization have been described[22].

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A role for *Gbx2* in repression of *Otx2* and positioning the mid/hindbrain organizer

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The mid/hindbrain (MHB) junction can act as an organizer to direct the development of the midbrain and anterior hindbrain[1,2]. 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 rhombomere 3 (r3), due to a failure of induction of the anterior neural plate during gastrulation[3–5]. *Otx1* mutants have only subtle defects[6]. In double *Otx1/Otx2* mutants[7,8] 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 r3/4 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 *Fgf8* was expanded and shifted caudally (Fig. 1e–h) from r2 to r4 in *Gbx2* mutants, and the gradient of expression was inverted with the strongest expression in 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 metencephalon (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

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