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Genetic control of flower shape in *Antirrhinum majus*

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SUMMARY

The asymmetric shape of the *Antirrhinum* corolla is determined by genes acting differentially along the dorsoventral axis of the flower. Genes so far identified determine asymmetry by acting in dorsal regions. We describe a gene, *divaricata*, which in contrast to previously identified genes acts in ventral regions of the flower. We show by the analysis of mutant combinations that the *divaricata* gene is negatively regulated by the dorsal genes *cycloidea* and *dichotoma*. In addition, we show by the analysis of chromosomal duplications that the *divaricata* gene acts in a dosage-dependent manner, suggesting that the level of its product is critical for determining ventral identities.

Key words: flower development, genetic interactions, flower shape, *Antirrhinum*, *divaricata*, *cycloidea*, *dichotoma*

INTRODUCTION

In *Antirrhinum*, development along a radial axis of floral meristems results in flowers with four types of organs, sepals, petals, stamens and carpels, arranged in concentric whorls. Within each whorl there are several organs which differ in shape according to their positions relative to a dorsoventral axis of the flower (e.g., 5 petals in whorl 2, see Fig. 1). The pattern of these differences is such that the flower has only one plane of symmetry, coinciding with the dorsoventral axis (Coen, 1991; Coen and Nugent, 1994; Luo et al., 1996).

Two systems of genes characterized in *Antirrhinum* determine either the radial or the dorsoventral patterns (for reviews, see Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1990). When inactivated, genes determining the radial pattern typically cause the differences between whorls to be reduced whilst dorsoventral differences may be retained. Mutations in genes controlling the dorsoventral pattern reduce differences between organs within whorls, without altering the radial pattern. In extreme cases, this may result in all organs within the same whorl being identical, giving radially symmetric flowers. Mechanisms controlling the radial and dorsoventral patterns may therefore be separate, at least to some extent, perhaps providing flexibility in the evolution of flower structure and shape. These mechanisms may also differ in that they generate differences in cell and regional identities which are sharply defined along the radial axis but more subtle along the dorsoventral axis. Here, we further address the problem of how genes control the dorsoventral pattern.

The best characterized dorsoventral mutants analysed in *Antirrhinum* give rise to ventralized phenotypes (Carpenter and Coen, 1990; Luo et al., 1996). For example, whereas wild-type flowers have five petals of three identities, one ventral, two laterals and two dorsals, in mutants with radially symmetric flowers all petals have ventral identity. Ventralization in these mutants indicates that the genes affected act in dorsal regions, consistent with the finding that one of these genes, *cycloidea* (*cyc*), is expressed only in dorsal parts of the flower (Luo et al., 1996). In addition, ventralization suggests that one role of dorsal-acting genes may be to restrict other determinants of the dorsoventral pattern to ventral domains. So far, however, no such determinants have been identified.

We describe a semidominant mutation, *divaricata* (*div*), which, in contrast to previously described mutations, affects ventral regions of the flower and confers a lateralized phenotype. We show by the analysis of mutant combinations that the dorsal genes *cyc* and *dichotoma* (*dich*) negatively regulate the *div* gene. In addition, by analysing the effects of chromosomal duplications we show that the *div* gene acts in a dosage-dependent manner, suggesting that the level of its product may be critical for determining subtle differences in regional identities in ventral regions of the corolla.

MATERIALS AND METHODS

Construction of mutant combinations with *Div*, *cyc* and *dich*

The *cyc* allele used in this work was contained in line JI25 which has radially symmetric (peloric) flowers (Carpenter and Coen, 1990). This line carries the *cyc*-25 allele and is also mutant at the unlinked *dich* locus, as shown by test-crosses to a single *dich* mutant (obtained from Drs U. Wobus and K. Hammer, Gatersleben, Germany). In a $Dich^+$ background, the *cyc*-25 allele gives a semipeloric phenotype, similar to that of *cyc*-608 (Carpenter and Coen, 1990). The three possible double mutants with *cyc*, *dich* and *div* were obtained in 150 F_2 progeny derived from crossing JI25 to a single *div* mutant homozygote. The genotypes of the double mutants were determined by test-crosses to single mutants. However, the triple mutant could not be obtained in this F_2. The *cyc;dich* double mutants (peloric) occurred only in combination with $Div^+;Div^+$ or to $Div^+;div$. This was as

expected because *dich* is linked to *div* (about 25 cM in the sequence *dich-pal-div*; see Stubbe, 1996). The triple mutant *div;cyc;dich* was therefore obtained in F_3 families derived from *cyc;dich* homozygotes that were heterozygous for *div*. The genotype of the triple mutant was then confirmed by test-crosses to single and double mutants. Further genetic tests involving the *cyc*, *dich*, *pal* and *div* loci gave results consistent with *dich* being responsible for the peloric/semipeloric difference in a *cyc* mutant background. For example, in progenies from crosses between double heterozygotes for *dich* and *div* which were homozygous at *cyc* (semipeloric) and the *div;dich;cyc* homozygote (peloric), the peloric/semipeloric difference segregated linked to *div*, giving 20 recombinat gametes out of 94 tested, in close agrreement with the known distance between *div* and *dich*.

Analysis of *div*-dosages

To select for changes at the *div* locus, progenies from crosses between*Div*$^+$ and *div* homozygotes were screened for deviations from the expected phenotype of heterozygotes (see Fig. 5). The *Div*$^+$ progenitors in these crosses were derived from line JI2 (Martin et al., 1985) which carries an insertion of the transposon Tam3 at *pallida* (*pal*), a gene controlling flower colour which is linked to *div*. The *pal* allele carrying Tam3 (*pal*-2) gives ivory flowers with clones of red cells unlike *Pal*$^+$ which gives red flowers. 200 *Pal*$^+$ revertants, most of which were heterozygous *Pal*$^+$/*pal*-2, were grown at 15°C, a temperature at which Tam3 transposition is stimulated (Carpenter et al., 1987), and crossed to a *div* line carrying *pal*-35, an allele giving pale colored flowers (Almeida et al., 1989).

In 4000 progeny from these crosses, we selected four plants with wild-type flower shape and colour, all of which subsequently gave mostly normal F_2 progeny segregating for *div* and *pal*. Two of these four F_2 families segregated for the three different *pal* alleles, indicating that their F_1 progenitors carried a duplication of the *pal* locus. This was confirmed by probing Southern blots of DNAs from the F_1 wild-types digested with *Eco*RI and *Nru*I with a 1.6 kb *Eco*RI-*Bam*HI fragment of the *pal* clone pJAM501 (Coen et al., 1986; Almeida et al., 1989). To show that the *div* locus was contained in the duplicated region, the same blots were probed with a 3.4 kb *Eco*RI-*Hin*dIII fragment of the *rcp* clone pJAM5A (Robbins et al., 1989) and a cDNA clone of the *flo* gene (pJAM101, Coen et al., 1990).

In progenies from the F_1 wild-types we identified 2 *Div*$^+$/*Div*$^+$/*div*, 4 *Div*$^+$/*div*/*div* and 2 *Div*$^+$/*Div*$^+$/*Div*$^+$ genotypes by Southern analysis as above. A *Div*$^+$/*Div*$^+$/*Div*$^+$ genotype carrying three doses of *rcp*^{D} (see Fig. 6), the three different *pal* alleles and two doses of *flo*^{D} and one of *flo*^{d} (hence containing *pal-35-Div*$^+$ and *Div*$^+$-*flo*^{d} recombinant segments) was crossed to line JI25 which contains *cyc*-25 and is *Div*$^+$ but carries *rcp*^{d}. Thus, in the progeny from this cross the dosages of *pal*, *rcp* and *flo* could be readily determined by Southern analysis. Probing this progeny with a *cyc* clone showed that all plants carried one copy of *Cyc*$^+$ and one copy of *cyc*-25 irrespective of *Div*$^+$ dosage.

RESULTS

Wild-type dorsoventral pattern

Dorsoventral differences are particularly striking in the corolla of the wild-type *Antirrhinum* flower, which comprises five petals of three identities: two dorsals, two laterals and one ventral (Fig. 1). Each of the petals in lateral or dorsal positions is asymmetric whilst the ventral petal is bilaterally symmetrical. The five petals are united for part of their length forming a tube which ends in a sharp border with the petal lobes. This border can be represented as a wavy line with peaks or troughs at petal boundaries, reflecting a continuous variation in tube length along the dorsoventral axis (Fig. 2). In addition to tube length, a conspicuous marker for dorsoventral differences in the tube is provided by two stripes of yellow hairs on the internal surface of the tube, located to either side of the boundaries between ventral and lateral petals.

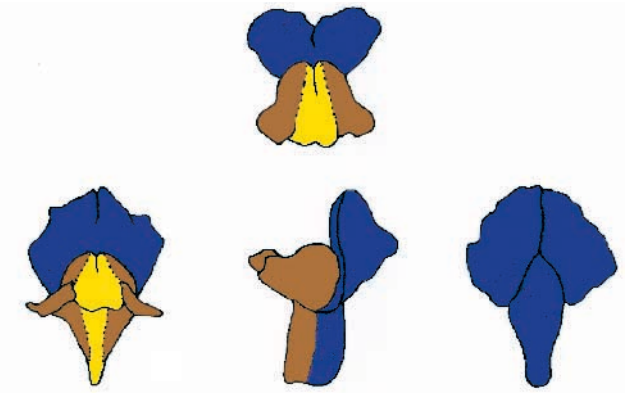

Fig. 1. Wild-type corolla. Top: dorsoventral view; botom, left to right: ventral, lateral and dorsal views. The ventral petal is in yellow, the laterals in brown and the dorsals in blue. Dorsal is towards the inflorescence apex.

Phenotypic effect of *div*

The wild-type dorsoventral pattern is altered by a semidominant mutation, *div* (see Stubbe, 1966), which causes ventral regions of the corolla to adopt a lateral identity whilst the dorsal petals remain unaltered (Fig. 2). In *div* mutant flowers, each half of the ventral petal becomes nearly a mirror-image of its adjacent part of the lateral petal. In mutant homozygotes, the domain affected includes the ventral petal and adjacent parts of the lateral petals, resulting in the loss or drastic reduction in the width of the stripes of hairs that mark the junctions between ventral and lateral petals. The altered domain is narrower in heterozygotes in which only the ventral petal is clearly affected. The boundaries of the region altered by *div* in the lateral petals cannot be precisely defined as the effect of *div* is strongest at the most ventral position decreasing in a graded manner towards lateral positions.

Interactions between *div*, *cyc* and *dich*

In contrast to the *div* gene which is needed for ventral identity, the *cyc* and *dich* genes together are needed for dorsal and lateral identities. In flowers from single *cyc* mutants the petals at ventral and lateral positions have ventral identity and those in dorsal positions have laterodorsal identity (Luo et al., 1996). The *dich* mutation only affects the shape of the dorsal petals which have reduced asymmetry relative to wild-type (see Stubbe, 1966; Luo et al., 1996). The effect of *dich* becomes clearer in *cyc;dich* double mutants which have radially symmetric flowers in which all petals have ventral identity (Luo et al., 1996; see also materials and methods and Fig. 3). Thus, in a *cyc* background, the *dich* mutation causes the normally asymmetric petals at dorsal positions to adopt the symmetric ventral identity.

To investigate how the *div* gene might interact with the *cyc* and *dich* genes, we constructed all possible combinations of the three mutants. *cyc;dich;div* triple mutants have radially symmetric flowers in which all petals have lateral identities, showing that the domain affected by the *div* gene extends all round the flower in a *cyc;dich* mutant background (Fig. 3). This

Fig. 2. Phenotypic effects of *div*. (A) Top: ventral views of the corollas. Middle: internal surfaces of tubes. The corollas were dissected at the borders between tubes and lobes. The tubes were then dissected along the junction between the dorsal petals. Hence, for each genotype ventral is in the middle and dorsal to the sides. Bottom: symmetry patterns between halves of ventral petal lobes (to the left) dissected along the planes of bilateral symmetry and the lateral lobes. (B) Schematic representation of the corolla tubes of the various *Div* genotypes. For wild-type, colours indicate petal identities as in Fig. 1. Letters along the base line indicate regional identities. In the lateral petal of wild-type these are b, c and d from dorsal to ventral. The region of the lateral petal close to its junction with the dorsal petal is unaffected by the *div* mutation, retaining the b identity.

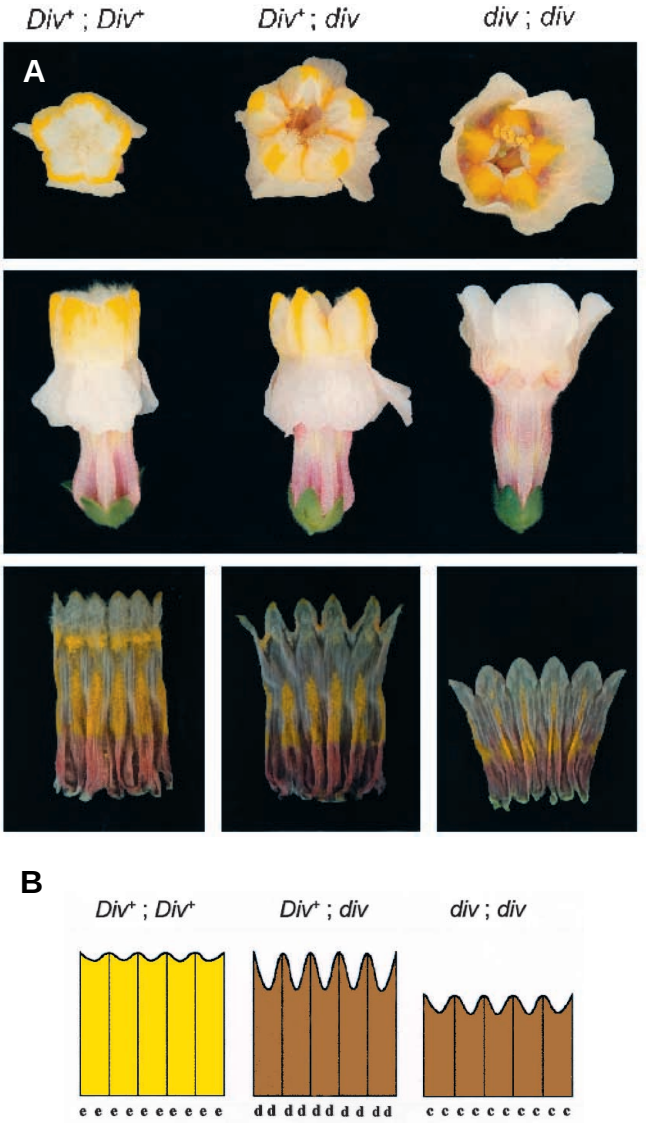

Fig. 3. (A) Phenotypes of the 3 *div* genotypes in a *cyc;dich* background with (top) dorsoventral, (middle) ventral views and (bottom) a view of the internal surfaces of tubes, obtained as described in Fig. 2A. (B) Schematic representation of the phenotypes shown in A (for details see legend to Fig. 2B). In addition to its effect on petal identities, the *cyc* mutation causes an increase in organ numbers, with the corolla bearing six rather than the usual five petals. However, as observed by Luo et al. (1996) some flowers from *cyc* mutants have only five petals. Triple *cyc;dich;div* mutants can also have five or six petals, all with lateral identity, showing that the effect of *cyc* on petal number is independent of *div*. Here and in Fig. 4, we show mutant flowers with five petals to facilitate comparisons with wild-type.

indicates that the dorsal genes normally act negatively on the *div* gene.

The phenotypes of double mutants allow the effects of *cyc* and *dich* on the *div* gene to be distinguished. In *div;cyc* double mutants the ventral and lateral petals are symmetrical and have lateral identity, as in the triple mutant, but the dorsal petals have laterodorsal identity (Fig. 4). The dorsal petals can be divided in two domains. One, close to their junction with the lateral petals is altered in *div;cyc* double mutants relative to single *cyc* mutants, with an identity similar to that of of the triple mutant in the same region. Thus, the domain affected by the *div* gene extends towards lateral and dorsal positions in a *cyc* background. In the other domain, close to the most dorsal position, the *div;cyc* double mutant is similar to the single *cyc* mutant, indicating that the effect of *div* does not reach the most dorsal position (Fig. 4). This reflects a negative action of *Dich*$^{+}$ on the *div* gene, because in the triple mutant the domain affected by *div* extends all round the flower. However, *Dich*$^{+}$ only acts

Fig. 4. Phenotypes of homozygous *Div*$^+$ or *div* genotypes in a *cyc* mutant background, with ventral (top) and dorsal (bottom) views. Note the similarity in dorsal identities irrespective of *div* genotypes.

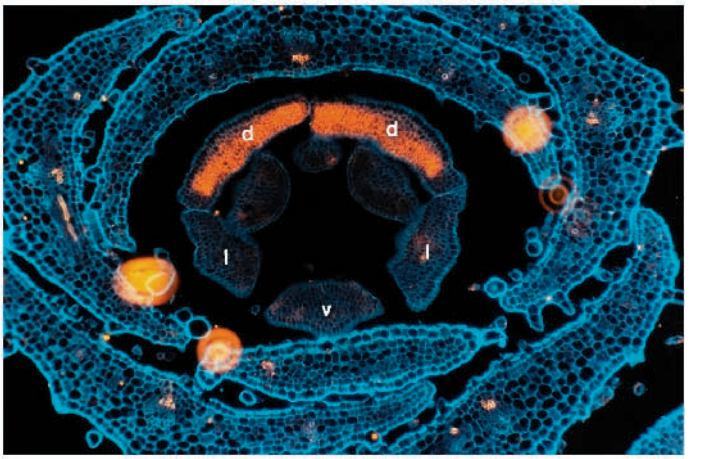

Fig. 5. Spatial distribution of *cyc* mRNA in a transverse section through a *div* bud at a stage when the five petals have become distinct (for details of in situ hybridization, see Luo et al., 1996). *cyc* mRNA is detected as the orange signal in blue cells. The petals are marked d (dorsal), l (lateral) and v (ventral).

on the *div* gene in a *cyc* mutant background, because in *div;dich* double mutants the dorsal petals have the same identity as in the single *dich* mutant and the ventral and lateral petals have the same identity as in the single *Div* mutant (not shown). Therefore, both the *cyc* and *dich* genes can act negatively on the *div* gene. However, *cyc* acts on *div* in lateral and dorsal petals irrespective of *dich* whereas *dich* only acts on *div* in dorsal petals in a *cyc* mutant background.

The domain affected by *cyc* includes the entire lateral petal in a *Div*$^+$ background (petals in lateral positions are converted from a bcd to an ee identity; see Figs 2B and 3B). However, in a *div* mutant background, the domain affected by *cyc* includes only the region of the lateral petals close to their junctions with the dorsal petals. In this case, petals in lateral positions are converted from a bc to a cc identity (Figs 2B and 3B). Thus, the domain affected by *cyc* appears to be narrower in a *div* mutant background. One possible explanation is that the mRNA expression domain of the *cyc* gene could become more restricted in a *div* mutant background. To test this possibility, we determined the pattern of *cyc* mRNA expression in the *div* mutant by in situ hybridization. The pattern of *cyc* expression in *div* flowers could not be distinguished from that of wild-type. This was particularly clear in transverse sections of flower buds at stages when all organs have been initiated and each of the five petals can be easily defined. As in wild-type, at such stages *cyc* mRNA was found only in dorsal petals of the *div* mutant ending abruptly three to five cells away from the junction with the lateral petals (Fig. 5). Therefore, the reduced effect of *cyc* on the lateral petals in *div* mutants is not due to an altered pattern of *cyc* mRNA expression.

div-dosage effects

The semidominance of *div* suggests that *Div*$^+$ may act in a dosage-dependent manner in ventral regions. Alternatively, *div* might be a neomorphic or gain-of-function allele. To distinguish between these possibilities, we determined the effects of different *div* allele dosages in plants carrying three copies of the *div* locus. If *Div*$^+$ acts in a dosage-dependent manner and *div* is a loss-of-function allele, *Div*$^+$/*Div*$^+$/*div* genotypes should have the wild-type phenotype and *Div*$^+$/*div*/*div* genotypes should have the same phenotype as normal heterozygotes. In addition, three doses of *Div*$^+$ might have a ventralizing effect. However, if *div* is a neomorphic or gain-of-function allele, increases in the dosage of the *div* mutant allele should have a lateralizing effect on ventral regions. To obtain such genotypes, we carried out a mutagenesis experiment in which progenies of crosses between *Div*$^+$ and *div* homozygotes were screened for deviations from the expected phenotype of heterozygotes (Fig. 6A).

Out of about 4000 F_1 progeny from these crosses, most plants had the expected phenotype of *div* heterozygotes and none had the phenotype of *div* homozygotes. However, four exceptional plants had the wild-type flower shape, suggesting that they might carry an extra dose of *Div*$^+$ giving a *Div*$^+$/*Div*$^+$/*div* genotype. To test this, we took advantage of differences between their progenitors in genetic and molecular markers flanking the *Div*$^+$ and *div* alleles (Fig. 6A). Southern analysis using probes for three flanking markers (*pal*, *rcp* and *flo*, Fig. 6B) showed that the exceptional F_1 progeny with a wild-type phenotype had inherited one copy of the chromosome segment carrying the *div* mutant allele and two copies of the segment containing the *Div*$^+$ allele.

This was particularly clear in the case of *pal*, a gene affecting flower colour, because the F_1 wild-types carried three different *pal* alleles, each of which gives a distinctive restricion pattern (Fig. 6B). In addition, genetic evidence for the duplication at *pal* could be obtained in progenies derived from the F_1 wild-types, because each of the three *pal* alleles confers an unique flower colour phenotype. Differences in the restriction patterns at each of the other two loci flanking *div*, provided a means of determining their dosages from changes in the relative intensities of the bands that they originated.

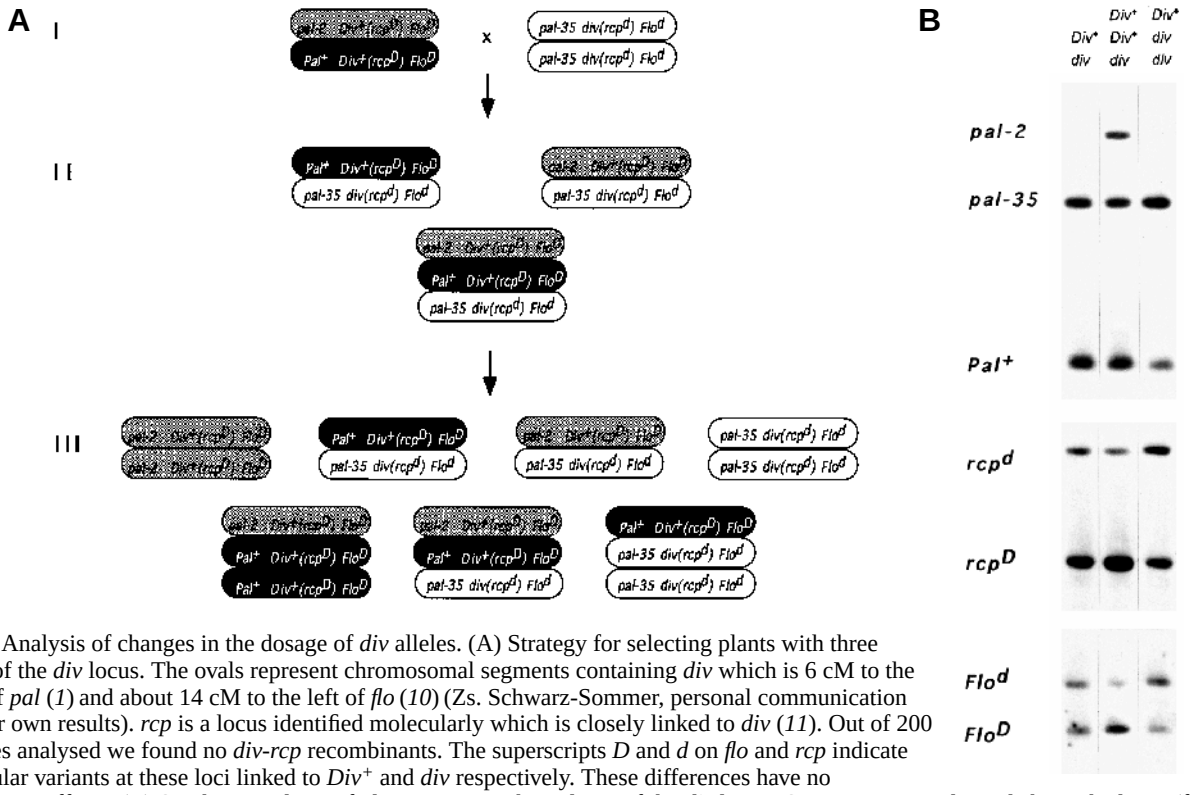

Fig. 6. Analysis of changes in the dosage of *div* alleles. (A) Strategy for selecting plants with three doses of the *div* locus. The ovals represent chromosomal segments containing *div* which is 6 cM to the right of *pal* (*1*) and about 14 cM to the left of *flo* (*10*) (Zs. Schwarz-Sommer, personal communication and our own results). *rcp* is a locus identified molecularly which is closely linked to *div* (*11*). Out of 200 gametes analysed we found no *div-rcp* recombinants. The superscripts *D* and *d* on *flo* and *rcp* indicate molecular variants at these loci linked to Div^+ and *div* respectively. These differences have no phenotypic effects. (B) Southern analysis of plants carrying three doses of the *div* locus. Genotypes are indicated above the lanes (for $Div^+/Div^+/Div^+$ see Materials and methods). Bands corresponding to the different variants at *pal*, *rcp* and *flo* are indicated along the lanes and designated as in A. For the Pal^+;*pal*-2;*pal*-35 genotype each of the alleles gives a different band. For *rcp*, *flo* and other *pal* genotypes, the duplications lead to changes in the ratio of the intensities of the bands produced by two variants, relative to normal heterozygotes. As determined by densitometry the rcp^d/rcp^D intensities were about 1:2 for Div^+/div, 1:4 for $Div^+/Div^+/div$ and 1:1 for $Div^+/div/div$. Probing similar blots with a clone of the unlinked *cyc* locus revealed no differences between these genotypes (not shown).

Determining the dosages of the markers above in progenies derived from the F_1 wild-types allowed four plants with $Div^+/div/div$ genotypes to be identified (Fig. 6B), all of which gave the same phenotype as normal Div^+/div heterozygotes, consistent with Div^+ acting in a dosage-dependent manner and *div* being a loss-of-function allele. In addition, we obtained two plants with three copies of the wild-type Div^+ allele conferring a novel phenotype in which the lateral petals appeared to be slightly ventralized. To obtain a clear phenotypic marker for ventralization conferred by three doses of Div^+, we crossed a $Div^+/Div^+/Div^+$ genotype to a *cyc* mutant. The heterozygous Cyc^+/cyc progeny, were either wild-type or had flowers with a notch in the lateral petals close to their junctions with the dorsal petals (Fig. 7). This phenotype is typical of a weak ventralization as it is also observed in plants doubly heterozygous for *cyc* and *rad*, mutations that both have ventralizing effects (Carpenter and Coen, 1990). Analysis of the genotypes of six plants with this phenotype showed that they all carried three doses of Div^+ whereas six of their sibs with the wild-type phenotype carried the normal two doses.

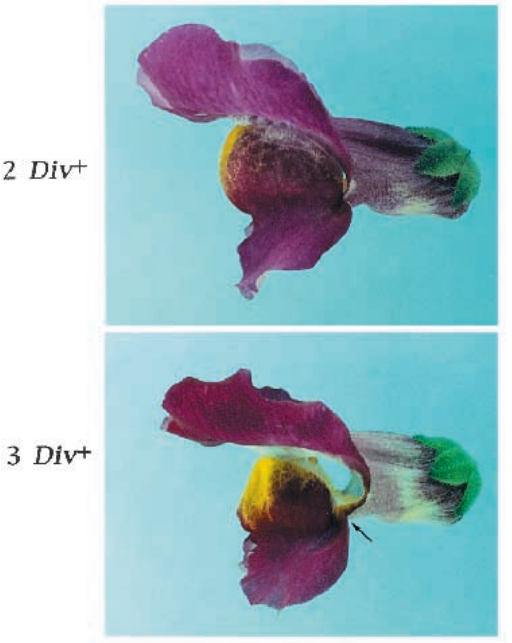

Fig. 7. Lateral views of flowers from *cyc* heterozygotes carrying either two or three doses of Div^+. The arrow in the $Div^+/Div^+/Div^+$ flower shows the notch in the lateral petal close to the junction with the dorsal petal.

DISCUSSION

We have analysed mechanisms by which genes acting differentially along the dorsoventral axis of the *Antirrhinum* corolla control its shape. The wild-type corolla has five petals of three

identities, two dorsals, two laterals and one ventral. This pattern is altered by a semidominant mutation, *div*, which causes a ventral region of the corolla to adopt a lateral identity. As a result, each half of the ventral petal becomes nearly a mirror-image of the adjacent part of the lateral petal, an effect reminiscent of that of segment-polarity mutations in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980).

In contrast to the *div* gene which is needed for ventral identity, the *cyc* and *dich* genes together are required for lateral and dorsal identities. Plants mutant for both *cyc* and *dich* have radially symmetric flowers in which all petals have ventral identity. The expression of ventral identity all round the flower in these mutants, suggests that one role of Cyc^+ and $Dich^+$ might be to restrict Div^+ to ventral regions. To test this possibility we introduced the *div* mutation in a *cyc;dich* mutant background. Consistent with our prediction, *cyc;dich;div* triple mutants have radially symmetric flowers in which all petals have lateral identities, showing that the domain altered by *div* extends all round the flower in a *cyc;dich* background.

The two dorsal genes, *cyc* and *dich*, differ in their domains of action and in the contexts in which they negatively regulate *div*. Whereas the *cyc* gene can by itself affect the action of Div^+ in dorsal and lateral petals, the *dich* gene affects a dorsalmost region of the corolla and only acts on Div^+ in a *cyc* mutant background. This is consistent with the view that the *cyc* and *dich* genes can substitute for each other to some extent (Luo et al., 1996).

Interestingly, the region affected by *cyc* appears to become more restricted in a *div* mutant background. The petals in lateral positions adopt ventral identities in *cyc* mutants but retain lateral identities in *cyc;Div* double mutants. This is not due to an altered pattern of *cyc* mRNA expression, because in *div* mutant flowers, as in wild-type, *cyc* mRNA was found only in dorsal petals ending abruptly three to five cells away from the junction with the lateral petals. A more likely explanation is that Div^+ mediates a non-cell-autonomous process in which Cyc^+ activity spreads from dorsal to lateral regions. However, the action of *Div* is not necessary for all the effects of *cyc* on lateral petals because their symmetry is still altered by *cyc* in a *div* mutant background.

The semidominance of *div* reflects the action of Div^+ in a dosage-dependent manner, as shown here by determining the effects of different *div* allele dosages in plants carrying three copies of the *div* locus. Consistent with Div^+ acting in a dosage-dependent manner, we found that $Div^+/Div^+/div$ genotypes have the wild-type phenotype and $Div^+/div/div$ genotypes have the same phenotype as normal heterozygotes. In addition, we found that plants carrying three doses of Div^+ have flowers in which the lateral petals are slightly ventralized. This may reflect a shift in the balance between the activities of the *div* gene and of genes acting dorsally because in a background heterozygous for *cyc*, $Div^+/Div^+/Div^+$ genotypes had the same weakly ventralized phenotype as that of plants doubly heterozygous for *cyc* and *rad*, mutations that both have ventralizing effects (Carpenter and Coen, 1990).

Action of Div^+ in a dosage-dependent manner indicates that the level of its product may be critical for determining petal identities in ventral and lateral regions. One possibility is that the level of Div^+ product peaks at the most ventral position and declines gradually along the dorsoventral axis to a low point as a result of the negative action of Cyc^+ and $Dich^+$ in dorsal regions. Regional identities at different positions would in this case reflect directly their levels of Div^+ product. This would be in line with the dosage-dependent action of genes expressed as gradients (Driever and Nüsslein-Volhard, 1988). Graded effects could alternatively reflect Div^+ acting in a relay system of signals which would interact with a Div^+ product in a concentration-dependent manner to determine petal identities. Semidominance would then reflect a fine balance between the concentrations of the Div^+ product and of the other signals. In this case, Cyc^+ might act through these other signals to determine asymmetry in lateral petals. Isolation of the *div* gene might allow these possibilities to be distinguished.

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