Quantitative Control of Organ Shape by Combinatorial Gene Activity

Min-Long Cui¹ᵃ, Lucy Copsey¹, Amelia A. Green¹ᵇ, J. Andrew Bangham², Enrico Coen¹*¹
¹ Department of Cell and Developmental Biology, John Innes Centre, Norwich, United Kingdom, ² University of East Anglia, School of Computing Sciences, Norwich, United Kingdom

Abstract

The development of organs with particular shapes, like wings or flowers, depends on regional activity of transcription factors and signalling molecules. However, the mechanisms that link these molecular activities to the morphogenetic events underlying shape are poorly understood. Here we describe a combination of experimental and computational approaches that address this problem, applying them to a group of genes controlling flower shape in the Snapdragon (Antirrhinum). Four transcription factors are known to play a key role in the control of floral shape and asymmetry in Snapdragon. We use quantitative shape analysis of mutants for these factors to define principal components underlying flower shape variation. We show that each transcription factor has a specific effect on the shape and size of regions within the flower, shifting the position of the flower in shape space. These shifts are further analysed by generating double mutants and lines that express some of the genes ectopically. By integrating these observations with known gene expression patterns and interactions, we arrive at a combinatorial scheme for how regional effects on shape are genetically controlled. We evaluate our scheme by incorporating the proposed interactions into a generative model, where the developing flower is treated as a material sheet that grows according to how genes modify local polarities and growth rates. The petal shapes generated by the model show a good quantitative match with those observed experimentally for each petal in numerous genotypes, thus validating the hypothesised scheme. This article therefore shows how complex shapes can be accounted for by combinatorial effects of transcription factors on regional growth properties. This finding has implications not only for how shapes develop but also for how they may have evolved through tinkering with transcription factors and their targets.

Introduction

Although major progress has been made in the genetic dissection of organ and appendage development, the process whereby gene activities lead to particular tissue shapes is still poorly understood. For example, wing morphogenesis in Drosophila is one of the best defined developmental systems [1], yet little is known about how regional activities in the imaginal disc are translated into final wing shape [2]. Addressing this problem has not been easy for several reasons. First, genes that modify shape are normally identified through their overall phenotypic effects, making it difficult to establish how particular regions of the tissue are affected. Second, shape is often described in qualitative terms like “rounder” or “more elongated,” making it difficult to quantify and compare the effects of different gene combinations. Third, we lack modelling frameworks that allow hypotheses for how genes control morphogenesis to be evaluated quantitatively.

Here we combine molecular genetic and morphometric approaches to address these issues, using the Snapdragon (Antirrhinum majus) flower as a model system. A key advantage of choosing a plant system is that the lack of cell movement means that morphogenesis arises mainly through differential growth. Shape changes can therefore be described in terms of genes modifying rates of growth in particular orientations [3]. So far, this approach has been applied to studying the effects of genes on overall growth rates of an organ [4]. However, it should be possible to extend this principle to the subregions within an organ, thus allowing final shape to be dissected into genetically determined modulations in the local rates and orientations of growth.

The Antirrhinum flower is particularly suitable for this approach as specific shapes can be generated through inactivation or over-expression of key transcription factors. Each flower comprises two upper petals (dorsals) and three lower petals (laterals and ventral) that together form the corolla (Figure 1A–D). The petals are united proximally to form a tube while the distal regions form five lobes. The shapes of the upper and lower petals are precisely matched at the boundary between tube and lobe, termed the rim, so that the overall structure forms a closed mouth hinged at its edges.

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Abbreviations: D, dorsal (petal); DV, dorsoventral; GPT-framework, Growing Polarised Tissue framework; L, lateral (petal); PC, principal component; PCA, principal component analysis; V, ventral (petal). * E-mail: enrico.coen@bbsrc.ac.uk

ᵃ Current address: Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China
ᵇ Current address: Department of Systems Biology, Harvard Medical School, Boston, Massachusetts, United States of America
The distinctive shapes of the upper and lower petals depend on the activities of four dorsoventral genes: CYCLOIDEA (CYC), DICHOTOMA (DICH), RADIALIS (RAD), and DIVARICATA (DIV) [5–9]. CYC and DICH encode TCP transcription factors that are expressed from an early stage in the dorsal domain of the flower bud. Mutants lacking both CYC and DICH have flowers with all petals resembling the ventral petal of wild type. RAD and DIV encode Myb-like transcription factors. RAD is switched on by CYC and DICH and promotes dorsal identity, while DIV is active in lower petals and promotes ventral identity. DIV is initially expressed throughout the corolla, but RAD is thought to antagonise its activity, preventing DIV from acting in dorsal petals. At later developmental stages, DIV expression becomes restricted to lateral and ventral petals through the action of the dorsally expressed genes. A cis-acting dominant mutant of CYC (backpetals) has been characterised in which CYC is ectopically expressed, leading to lower petals acquiring dorsal identity [9]. However, it is unclear whether the phenotype is a result of ectopic expression of CYC and/or its target gene RAD.

The changes in shape resulting from inactivation or overexpression of genes may be quantified using morphometric methods. Such methods have been applied to genetically controlled shape variations, such as mandible shape in vertebrates, wing shape in Drosophila, and leaf shape in plants [10–13]. This approach involves placing landmarks at key positions on the organ, aligning the resulting points, and then using multivariate methods to extract major trends in variation. The advantages of taking a quantitative approach are that average shapes for each genotype can be extracted and the main features under genetic control can be highlighted. Additionally, this approach potentially allows quantitative comparisons to be made between experimentally generated shapes and shapes generated by computational modelling, enabling hypotheses about morphogenesis to be evaluated.

Here we show that the genetic control of flower shape can be accounted for by a combination of region-specific effects. We quantify these effects through shape analysis of previously described mutants and of lines in which RAD is over-expressed in a range of genetic backgrounds. The shapes observed for multiple genotypes can be summarised with a scheme in which dorsoventral transcription factors act in combination with gene activities along the proximodistal and mediolateral axes to modulate the length or breadth of each petal region. Morphogenetic hypotheses for how these phenotypic effects might arise were evaluated using a modelling framework in which genes modify local polarities and specified growth rates [14,15]. The petal shapes generated by the resulting model show a good quantitative match with those observed experimentally for each petal from 10 different genotypes, thus validating the underlying hypothesis. Our results suggest that evolution of shape involves a process of “tinkering”, through which size and shape of regions is adjusted by piecemeal modification of local growth properties under the control of transcription factors.

Results

Quantifying the Morphology of Wild-Type and Mutant Petals

As a first step towards evaluating the effects of different genes on organ shape, the corolla was subdivided into several regions along its proximodistal axis. Most proximal is a continuous cylinder of tissue, the proximal tube. Beyond this region, the tube tissue extends to form the upper and lower palate (Figure 1E–H). The palate ends distally with a boundary called the rim, which acts as a line of transition between the tube and the lobes. The proximal region of the lobes comprises the lip, over which the lobes of adjacent petals are united (yellow dotted lines in Figure 1E–H). The lip is greatly reduced at the junction between the dorsal and lateral lobes, creating a hinge that allows the corolla to be opened by pollinators. The lobes are separate over the remaining distal region of the lobes.

To quantify the effects of dorsoventral genes on shape, the outline and size of the various regions of the corolla were captured. First, the 3-D structure of the flower was converted into a series of 2-D shapes. To achieve this conversion, the upper and lower sections of the corolla were separated by making cuts along the junction between lateral and ventral petals. The resulting petal sections were then flattened (Figure 1I–L). Second, the outlines of the regions for each petal were captured using a series of landmarks. Eight primary landmarks (green dots in Figure 1J,L) were located at recognisable morphological features, such as where the lobes become separate or where the tube rim and petal junctions intersect. Cell type patterns, which vary along the proximodistal axis of the tube, were also used to define primary landmarks for internal boundaries such as those between ventral and lateral petals. In cases where there were no discernable palate or lip regions, the landmarks bounding these regions were overlaid. The remaining 47 secondary landmarks (yellow dots in Figure 1J,L) were spaced evenly along the outlines of each region between the primary landmarks.

Taken together, the coordinates for the 55 landmarks summarise the shape and size of the regions for each petal. These coordinate values will vary in a correlated manner between petals depending on how the shapes and sizes of the regions are influenced by genotype and petal identity. The main trends or correlations can be captured using Principal Component Analysis (PCA) [16]. To implement this procedure, 110 coordinate values (from 55 landmarks) were determined for dorsal, lateral, and ventral petals from wild type as well as the various genotypes described below. Dorsal, lateral, and ventral petals were sampled from five different flowers for each genotype. Petal shapes were...
aligned by translation and rotation (Procrustes alignment). The average position for each landmark gave the mean petal shape and region outlines for the population. The major trends of variation about this mean were then determined by PCA on the covariance. This analysis showed that 94% of the variance in coordinate positions could be captured with four principal components (PCs).

PC1 accounts for 56% of the variance and captures variation in palate and lip size (Figure 2A). Increasing the value of PC1 gives longer petals with extended lip and palate regions, while reducing PC1 gives shorter petals with a reduced lip and palate. PC2 accounts for 23% of the variance and captures petal asymmetry (Figure 2A). Increasing the value of PC2 gives asymmetric petals with shorter lip and palate regions and a longer distal lobe on one side, while reducing PC2 gives bilaterally symmetrical petals. PC3 accounts for 11% of the variance and captures variation in distal lobe size: increasing the value of PC3 gives a smaller distal lobe, while reducing PC3 gives a larger distal lobe. PC4 accounts for 4% of the variance, with an increase in the PC4 value giving a petal that twists in one direction and a decrease giving a petal twisting the opposite way.

To determine the contribution of each PC to the specification of petal shapes, average PC values for wild-type dorsal, lateral, and ventral petals were determined and then used to reconstruct the petal shapes (Table S1, Figure 2B). If all four PC values were used for reconstruction, the resulting shapes closely resemble the observed shapes (compare top row of Figure 2B with Figure 1J, L). This result is expected because these four PCs capture 94% of the variance in petal shape. A good match was also obtained using just PC1 and PC2, showing that these two PCs are sufficient to capture the main features of the regional shapes. This finding allowed the main shape variations to be represented within a 2-D space that has PC1 and PC2 as its axes. This space will be referred to as the DorsoVentral (DV) space (Figure 2C). Each petal sample corresponds to a point in DV space. The origin of DV space, where all PC values are set to 0, corresponds to the mean petal shape. Samples of the same petal type (e.g., dorsal) form a cloud of points clustered around the mean for that petal type (Figure 2C). The dorsal and lateral clouds are near each other but well separated from the ventral cloud. This clustering reflects the similarity in overall shape and asymmetry of the dorsal and lateral petals and the difference in shape and symmetry of the ventral petals.

**Control of Ventral Petal Development**

To determine the effect of the four dorsoventral genes on the ventrally positioned petal, we analysed its shape in several mutant...
The only dorsoventral gene expressed in the wild-type ventral petal is \textit{DIV}. The ventral petal of the \textit{div} mutant therefore expresses no dorsoventral genes and can be considered to represent a \textit{ground state}. Relative to the wild-type ventral petal, that of \textit{div} has a reduced palate, is wider, and is not bent back at the rim (Figure 3B). The reduced palate corresponds to a lower value of PC1 (PC1 < 0). The \textit{div} mutant is therefore shifted to the left in DV space relative to the wild-type ventral petal (Figure 3K, arrowed). The position of the \textit{div} ventral ground state will be shown in all further DV spaces as a common point of reference. In wild type, expression of \textit{DIV} in the ventral petal throughout development leads to a longer palate and narrower petal than the ground state. Additionally, the wild-type ventral petal bends back at the rim. These observations indicate that \textit{DIV} acts to increase palate length, reduce petal width, and promote bending back at the rim.

\textit{CYC}, \textit{DIC}, and \textit{RAD} are not expressed in the lower corolla section, so we would not expect these genes to have much effect on ventral petal shape. Consistent with this expectation, the shapes of the \textit{cyc dich} and \textit{rad} mutant ventral petals are similar to wild type.
Figure 4. Effect of ectopic RAD expression in Antirrhinum majus. Comparison between wild-type (A,B) and 35S::RAD (C,D) flowers. Face views on left (A,C), side views on right (B,D). Scale bar = 1 cm.

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(Figure 3C,D) and map to similar positions in DV space (Figure 3K). In contrast, the ventral petal of backpetals is markedly different from wild type, showing a reduced lip (Figure 3E). The reduced lip size correlates with a leftward shift in DV space (Figure 3K). Additionally, the distal lobe region of backpetals is larger than wild type, particularly along its lateral edges (giving a low value of PC3; Table S1). Also, similar to the ground state, the ventral lobe does not bend back at the rim in backpetals. Backpetals is a semidominant CYC allele that expresses CYC and its downstream target RAD ectopically in the ventral and lateral petals [9]. The effect of backpetals on ventral petal shape may therefore reflect the action of CYC or RAD or the combined action of both genes.

To separate the contributions of CYC and RAD, we generated plants that expressed RAD ectopically, by introducing RAD under the control of the 35S promoter. The ventral petals from these transgenic plants should express RAD but not CYC. Three transgenics were obtained, two of which showed strong petal phenotypes (Figure 4). No phenotypic effects were observed in leaves, even though RAD expression was detected by RT-PCR of the transgenics but not in wild type (unpublished data).

The most noticeable effect of ectopically expressing RAD in the ventral petal was reduction of both the lip and palate regions (Figures 4 and 3F). This reduction resulted in the 35S::RAD point cloud mapping to a similar position to backpetals in DV space (with a low value of PC1) (Figure 3K). Also, like backpetals, the 35S::RAD ventral petal lobe does not bend back. Thus, RAD can exert an autonomous effect on petal shape in the absence of CYC. However, the phenotype of 35S::RAD is not identical to that of backpetals. Unlike 35S::RAD, backpetals has a slightly enlarged medial palate and a large distal lobe (compare Figure 3E,F), indicating that CYC acts partly independently of RAD to increase the length of these regions.

To explore interactions between the dorsoventral genes further, 35S::RAD was introduced into several mutant backgrounds (Figure 3G,J). Analysis of the ventral petals showed that the tube of 35S::RAD div resembled that of the div ground state, having a reduced palate (compare Figure 3B with Figure 3G). This result is consistent with previous proposals that a major effect of RAD is to antagonise DIV [7,8]. Additionally, the 35S::RAD ventral lip is greatly reduced compared to div, and the palate is also further reduced (the PC1 value for 35S::RAD is much less than for div). This finding indicates that RAD acts independently of DIV to reduce lip and palate length. The phenotype of 35S::RAD in ventral petals resembles that of 35S::RAD rad and 35S::RAD cyc dich. This result is expected because RAD, CYC, and DICH are not normally expressed in ventral petals. In a backpetals mutant background, 35S::RAD had little effect on ventral petal shape, also expected as RAD is already expressed ectopically in the backpetals mutant.

Control of Dorsal Petal Development

We next analysed the effect of dorsoventral genes on dorsally positioned petals (Figure 5). Wild-type dorsal petals express CYC, DICH, and RAD and also DIV at early stages. The main difference between wild-type dorsal petals and the ground state is the increased value of PC2, reflecting a marked asymmetry in petal shape. This asymmetry involves a reduced lip on one (lateral) side of the petal and an extended palate on the other (dorsal) side (Figure 5A). Extension of the palate on the dorsal side of the petal probably reflects DICH activity, as palate asymmetry is not observed in the ventral petal of backpetals (Figure 3E), which only differs from wild-type dorsal petals in not expressing DICH. Reduction of length on the lateral side of the wild-type dorsal petal depends on RAD activity. In the rad mutant, lip length is restored to this side, reducing the degree of petal asymmetry (Figure 5D). The rad dorsal petals remain asymmetric because DICH activity increases palate length on the more dorsal side.

In cyc dich mutants the dorsally positioned petals are fully ventralised (Figure 5C). The petals are bilaterally symmetric because they lack both DICH and RAD expression (activation of RAD depends on CYC and DICH). The absence of RAD also leads to ectopic DIV activity in cyc dich dorsal petals (RAD normally antagonises DIV), accounting for the extended palate and higher value of PC1 relative to the ground state (Figure 5K). If RAD is ectopically expressed in cyc dich dorsal petals (35S::RAD cyc dich), the PC1 value drops below that of the ground state, as lip and palate regions both become reduced (Figure 5H). This result is consistent with RAD reducing lip and palate length and also further reducing palate length by antagonising DIV.

The div mutation does not affect dorsal petal development (Figure 5B, presumably because DIV activity is normally blocked in dorsal petals by expression of RAD. Dorsal petal development is also not affected by the backpetals mutation (Figure 5E), as expected because backpetals does not modify gene expression in the dorsal domain. 35S::RAD also had little or no effect on dorsal petals in wild-type, div, or backpetals backgrounds (Figure 5G,J). Again this result was expected because the endogenous RAD gene is expressed in dorsal petals. 35S::RAD rad dorsal petals have a wild-type phenotype, showing that the transgene complements rad in dorsal regions. This result demonstrates that the shape of the wild-type dorsal petal does not depend on spatial regulation of RAD expression within the dorsal petal.
Figure 5. Analysis of dorsal petals for various genotypes. (A–J) Flattened upper corolla sections with mean dorsal petal shape to the right for wild type (wt) and a series of other genotypes. Petal regions colour-coded as in Figure 1. (K) Positions of dorsal petals of various genotypes (colour-coded red or pink) projected onto DV space. Arrow points to ground state (div ventral petal, pale yellow). Positions of wild-type dorsal (D, red), lateral (L, orange), and ventral (V, dull yellow) shown for reference. Diagrams show mean shapes for each petal type reconstructed using PC1 and PC2 values. Units for the axes are in standard deviations from the mean. bp, backpetals.

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Control of Lateral Petal Development

We next analysed laterally positioned petals in various genetic backgrounds. Similar to the wild-type dorsal petal, each wild-type lateral petal is asymmetric with a reduced lip and palate on one (lateral) side and extended lip and palate on its other (ventral) side (Figure 6A). This morphology places lateral petals in a similar position to dorsal petals in DV space. However, in lateral petals asymmetry of the palate depends on DIV rather than DICH. In the div mutant, the palate is shortened on its ventral side, leading to a more symmetric shape (lower PC2 value, Figure 6B,K). The div lateral petals are still asymmetric because lip and palate length is reduced on the more lateral side of the petal. This reduction involves RAD. In rad mutants, the lateral petal becomes bilaterally symmetrical, with extended lip and palate regions (Figure 6D). The extended palate mainly reflects ectopic DIV activity (DIV is no longer antagonised by RAD), while the extended lip reflects lack of RAD activity. As RAD is not normally expressed in the lateral domain, the reduction of lateral lip growth in wild-type lateral petals involves a non-autonomous effect of RAD expression from the adjacent dorsal domain. If RAD is expressed ectopically in the lateral petal, as in 35S::RAD genotypes, the length of the lip and palate regions becomes negligible and the petal bilaterally...
symmetrical, with a low PC2 value, similar to that of the ground state (Figure 6F). The value of PC1 value drops below the ground state, reflecting $\text{RAD}$ antagonising $\text{DIV}$ and also reducing lip length (Figure 6K). Lateral petals of $35\text{S}::\text{RAD}$ backpetals are bilaterally symmetrical, like $35\text{S}::\text{RAD}$, but have a partially extended medial palate (Figure 6J). This suggests that expressing $\text{CYC}$ counteracts the effect of $\text{RAD}$ on reducing palate length in medial regions.

**Discussion**

Analysis of petal phenotypes in wild-type, mutant, and transgenic backgrounds reveals that the dorsoventral genes have several region-specific effects on shape. These effects on local shape can be accounted for by a scheme in which the dorsoventral genes interact combinatorially with a pattern of gene activities along the proximodistal and mediolateral axes (Figure 7). Candidate genes for the proximodistal gene activities are the $\text{LIP1}$ and $\text{LIP2}$ genes, which encode AP2-like transcription factors that increase palate and lip length [17], and $\text{CIN}$, which encodes a TCP transcription factor that increases lip length [18]. These genes may play an equivalent role to proximodistal systems involved in animal limb development [19]. Less is known about mediolateral systems in plants [20], although a notable feature in our scheme is that it involves graded changes, allowing lengths to be increased or decreased smoothly. This pattern may be similar to the way graded mediolateral information is provided by Dpp during *Drosophila* wing development [21,22]. The scheme also involves graded effects for $\text{RAD}$ activity, which spreads non-autonomously from the dorsal into the lateral domain to restrict $\text{DIV}$ function. This spread may reflect direct movement of the $\text{RAD}$ protein, as described for other small plant Myb proteins [23], or more indirect spreading mediated by signalling molecules.

Although the scheme in Figure 7 can account for the observed phenotypes through combinatorial effects on the shape and size of regions, it does not define the morphogenetic processes through which shapes are generated. To generate phenotypic outcomes, such as an increase or decrease in length of a petal region, genes presumably modify rates of growth along particular orientations within the region as it develops. However, predicting the consequences of particular hypotheses for growth control can be difficult for several reasons. One is that local orientations may become deformed through differential growth, dynamically modifying the principal orientations in which a region grows. Secondly, the extent to which a region grows may be mechanically constrained by neighbouring regions; so specified growth need not be the same as resultant growth. To address these issues, a computational modelling approach for growing tissues, called the GPT-framework (Growing Polarised Tissue framework), was used to determine the consequences of particular hypotheses [24]. The petal was modelled as a growing material sheet of tissue that can deform in 3-D, incorporating the combinatorial interactions described in Figure 7 [14]. Dorsoventral genes such as $\text{CYC}$ and $\text{DICH}$ were assumed to be expressed uniformly throughout development within their domains. According to the GPT-framework, genes influence shape by modifying tissue polarity and specified rates of growth (rates of extension along axes defined by the local polarity). For example, the combination $\text{DIV} \cdot \text{PAL}$ increases palate length by promoting specified growth parallel to

![Figure 7. Combinatorial effects of dorsoventral genes.](image)

In the following summaries of gene interactions, a dot (.) indicates “in combination with” while a tilde (∼) indicates “in the absence of.” (A) Ground state (div ventral petal). The basic petal shape is determined by gene activities that vary along the proximodistal (PTB, PLT, LIP, and DTL) and mediolateral (MED and LAT) axes. (B) Wild-type ventral petal. $\text{DIV}$ is expressed throughout the petal. $\text{DIV}$ reduces petal width while $\text{DIV} \cdot \text{PLT}$ increases palate length. $\text{DIV} \cdot \text{RIM}$ promotes bending back of the lobe (dotted line). (C) Wild-type lateral petal. Non-autonomous $\text{RAD}$ activity from the dorsal side restricts $\text{DIV}$ activity towards the ventral side at later stages of development. $\text{RAD} \cdot \text{LIP}$ and $\text{RAD} \cdot \text{PLT}$ reduce palate and lip length on one side while $\text{DIV} \cdot \text{PLT}$ increases palate length on the other. The lobe is bent back by $\text{DIV} \cdot \text{RIM}$ (at early stages, when $\text{DIV}$ is expressed throughout the petal). (D) Wild-type dorsal petal. $\text{CYC}$ and $\text{RAD}$ are expressed throughout while $\text{DICH}$ is expressed in the most dorsal half. $\text{CYC}$ increases petal width. $\text{CYC} \cdot \text{PLT}$ and $\text{DICH} \cdot \text{PLT}$ increase palate length, while reduction in length by $\text{RAD} \cdot \text{PLT}$ is antagonised by $\text{DICH}$ and $\text{CYC} \sim \text{LAT}$. Reduction in lip length by $\text{RAD} \cdot \text{LIP}$ is antagonised by $\text{DICH} \cdot \text{LAT}$, leading to a visible lip on the dorsal side. $\text{CYC} \cdot \text{DTL}$ increases length of the distal lobe, which is antagonised by $\text{DICH}$ on the dorsal side.

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Figure 8. Comparison of observed corolla shapes with growth model corolla shapes. (A) Model corolla at initial developmental stage. (B) Initial model stage at same scale as (C). (C) Side view of wild-type corolla generated by growth model. (D) Ventral view of wild-type flower generated by growth model. (E) Ventral view of real flower. (F) Computationally flattened dorsal (d), lateral (l), and ventral (v) petals from the growth model. Petal regions colour-coded as in Figure 1. (G–O) Ventral view of mutants described in this article, with real flower on left and result from growth model on right.
the local polarity. Tissue polarity is established through three
organisers (proximal, central, and distal), from which polarity
signals propagate through the tissue. The activity of these
organisers is also influenced by dorsoventral genes [14]. Figure 8
shows the output from the growth model for wild type, from
the starting shape of a small lobed cylinder of tissue (Figure 8A,B)
through to the final shape (Figure 8C,D).

To test the hypotheses underlying the computer model, the
various genotypes described in this article were generated by
setting the relevant gene activity in the model to 0 (null mutants)
or to 1 everywhere (over-expression lines). The resulting corollas
showed a good qualitative match to observed flowers (Figure 8G–
O). To give a more quantitative comparison, petals from each
model corolla were computationally flattened (e.g., Figure 8F) and
their outlines processed in the same way as the observed petal
data. The PC values from the model were then compared to the
PC values observed experimentally for the corresponding
genotype and petal (Table S1; Figure 8P–S). As can be seen in
Figure 8P,Q, there is a strong correlation between model output
and observational data for PC1 ($R^2 = 0.87$, $p < 0.0001$) and PC2
($R^2 = 0.91$, $p < 0.0001$). This result shows that the model captures
the main relationships between genes and shape for each petal and
thus provides quantitative validation of the proposed combinatorial
interactions between the dorsoventral genes proposed in Figure 7.
Values for PC3 also show a significant correlation between observed and modelled ($R^2 = 0.56$, $p < 0.0001$; Figure 8R),
suggesting that the model also captures this aspect of petal shape
variation. However, PC4 showed little correlation ($R^2 = 0.04$,
$p = 0.28$; Figure 8S), which is not surprising because this PC
captures only minor shape variations.

In the growth model, each dorsoventral gene has several
region-specific effects on rates or orientations of growth. This
hypothesis is consistent with these genes encoding transcription factors that act in combination with other factors to influence a
variety of target genes. These interactions may have been
elaborated during the evolution of the Antirrhinum lineage, leading
to the formation of a corolla with a closed mouth, hinged at its
dorsal or ventral palate growth, by $\text{DIC}H$ and $\text{DIV}$, respectively, repression of lip growth at the lateral petal
boundaries by $\text{RAD}$ to create a hinge, and promotion of tissue
polarity organisers at particular locations. Thus, the close match
between upper and lower petals depends on a history of multiple
regional modifications. Similar principles may underlie the close
match between the upper and lower jaws of vertebrates,
illustrated by mutants in which the lower jaw protrudes or
recedes [26–28]. The evolution of matched tissue shapes can be
compared to the way protein domains may evolve to match each
other [29]. In both cases shape-matching arises through
tinkering, involving either a sequence of adjustments in regional
growth properties and polarities as described here or a series of
modifications to protein shape through piecemeal amino acid
changes.

**Materials and Methods**

**Antirrhinum majus Stocks**

Plants of JI 7 (wild type), JI 98 (wild type), JI 726 (rad-726), JI
609 (rad-609), JI 721 (cyt-721), JI 608 (cyt-608), JI 705 (backpetals-
705), JI 13 (div-35 [5]), and JI 718 (cyt-608 dich-719) were grown in
the greenhouse as described previously [30] and recurrently
crossed with 35S::RAD transgenic *Antirrhinum majus* lines. Stocks JI
7 and JI 98 were used as the standard wild type for comparison
with the mutants.

**Antirrhinum majus Transformation**

The 35S::RAD construct was cut from a pGREEN0029 [31]
vector and transformed into a binary vector pBIN 19 [32]. This
expression vector was transformed into *Agrobacterium* strain
GV3101 and used to transform *Antirrhinum majus* as described by
[33]. Three kanamycin resistant shoots were obtained and
analysed by PCR using a set of primers for the kanamycin
resistance gene (Neomycin phosphotransferase II), 5'-GATG
GATTGCACGCAGGTTG-3' and 5'-GTGGTGCGATG GGC
AGGTAG-3'. A strong phenotype 35S::RAD transgenic line and a
weak phenotype 35S::RAD transgenic line were crossed with each of the mutants listed above.

**Genotyping**

The back-crossed plants were screened on MS medium
containing 50 mg/l kanamycin and genotyped using the primer
sets described below. Genotyping of mutant alleles was performed
by PCR using combinations of gene-specific or transposon-specific
primers. Primers were 5'-agtttcatgcaagatgtgg-3' and 5'-agtttt
tatgcaagatgtgg-3' for rad-726; 5'-atgatttgggaagacaca-3' and 5'-
taatgattgagactgtgt-3' for cyt-721; 5'-agtttgcaatctcgc-3' and 5'-
tgtcagctccctgtaagg-3' for backpetals-705; 5'-agttttgcaatctggtgg-
tc-3' and 5'-taagggtatcctgtaacgcg-3' for rad-609; 5'-atggtagga-
gacaca-3' and 5'-tgaggcaactcactgg-3' for cyt-606; and 5'-
-ggggtagcttgcagag-3' and 5'-tacatgcttgcagagag-3' for div-33.
The *div* mutant allele was detected by sequencing PCR
products.

**Analysis of Expression**

To detect RAD and transgene expression, total RNA was
extracted from young leaves using an RNeasy Plant Mini Kit
(Qiagen, UK). First-strand cDNA was synthesised using the
SuperScript III First-Strand Synthesis System for RT-PCR
(Invitrogen) on 5 μg of total RNA treated with a TURBO
RNase-free kit (Ambion). RT-PCR was carried out using specific
primer sets: 5'-atgcttgcagctccctgtaagg-3' and 5'-agttttgcaagatgtgg-
tc-3' for RAD expression; 5'-agtttcatgcaagatgtgg-3' and 5'-
tgtcagctccctgtaagg-3' for NPTII expression; and 5'-agttttgcaatctggtgg-
tc-3' and 5'-taagggtatcctgtaacgcg-3' for ubiquitin expression.
PCR was performed for 4 min at 94°C and then 30 cycles
consisting of 40 s at 94°C, 40 s at 61°C and 60 s at 72°C,
followed by 10 min at 72°C.

**Shape Model Analysis**

Flower samples were collected from eight individual plants each
from mutant and transgenic lines, when flowers were fully opened.
Each flower was dissected by cutting in a proximodistal direction
along the tube conjunction of dorsal and lateral petals, using a
razor. The upper petals (including two dorsal petals) and lower
petsals (including two lateral petals and a ventral petal) were
flattened by gluing onto paper and photographed using a Nikon
Coolpix 995 digital camera. All images were normalised to 4000
pixels/cm² using an ImagePrep tool written in Matlab. Fifty-five
landmarks (eight primary landmarks and 47 secondary landmarks)
were fitted to each of the dorsal, lateral, and ventral petals (Figure 1J,L) to build the shape model using the AAMToolbox (http://fizz.cmp.uca.ac.uk/wiki/DArT_Toolshed/index.php/Main_Page) in Matlab (version: 7.2), as described in [12]. A statistical PCA model of flower petal shape and size was generated from the petal point models of the mutant and transgenic plant dataset, projected to a morphospace defined by PC1 and PC2.

Supporting Information

Table S1 Principal Component values for petals of various genotypes compared to values obtained from petals generated with the growth model. DP, dorsal petal; LP, lateral petal; VP, ventral petal.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: MLC EC. Executed the experiments: MLC LC. Analyzed the data: MLC AAG JAB EC. Wrote the paper: MLC AAG EC.

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