Formation and Shaping of the *Antirrhinum* Flower through Modulation of the *CUP* Boundary Gene

**Highlights**
- Boundary genes play a key role in the evolution of diverse 3D plant shapes
- *CUP* boundary gene needed for formation of the complex lower Snapdragon corolla
- *CUP* can either promote or inhibit growth depending on tissue context
- Petal fusion involves clearing of boundary gene activity at petal junctions

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**In Brief**
Rebocho et al. shows how the *CUP* boundary gene plays a key role in the shaping of the ornate Snapdragon flower by controlling differential growth. Clearing of *CUP* expression from petal junctions allows the flower tube to form, while activation of *CUP* in the lower petal promotes growth and formation of the convoluted lower palate and lip.
Formation and Shaping of the Antirrhinum Flower through Modulation of the CUP Boundary Gene

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SUMMARY

Boundary domain genes, expressed within or around organ primordia, play a key role in the formation, shaping, and subdivision of planar plant organs, such as leaves. However, the role of boundary genes in formation of more elaborate 3D structures, which also derive from organ primordia, remains unclear. Here we analyze the role of the boundary domain gene CUPULIFORMIS (CUP) in formation of the ornate Antirrhinum flower shape. We show that CUP expression becomes cleared from boundary subdomains between petal primordia, most likely contributing to formation of congenitally fused petals (sympetally) and modulation of growth at sinuses. At later stages, CUP is activated by dorsoventral genes in an intermediary region of the corolla. In contrast to its role at organ boundaries, intermediary CUP activity leads to growth promotion rather than repression and formation of the palate, lip, and characteristic folds of the closed Antirrhinum flower. Intermediary expression of CUP homologs is also observed in related sympetalous species, Linaria and Mimulus, suggesting that changes in boundary gene activity have played a key role in the development and evolution of diverse 3D plant shapes.

INTRODUCTION

The formation and shaping of plant outgrowths, such as leaves or petals, depends on differential growth at boundary domains. These boundaries are evident at the base of organ primordia or within primordia where serrations form. In cases where organ primordia are organized in whorls, we may distinguish between two types of boundary: intra-whorl boundaries, which delimit adjacent organs belonging to the same whorl, and inter-whorl boundaries, which delimit adjacent organs belonging to different whorls. Defects in the intra-whorl boundaries can lead to formation of cup-shaped structures, such as congenitally fused cotyledons or sepals (calyx). Defects in inter-whorl boundaries can lead to hybrid structures such as congenitally fused petal-stamens, or stamen-carpels. Loss of boundaries within organs can also lead to reduced dissection (e.g., loss of leaf serrations). All of these phenotypes have been observed for mutants in NAC-domain boundary genes, which include NO APICAL MERISTEM (NAM, Petunia), CUP-SHAPED COTYLEDON (CUC, Arabidopsis), GOBLET (tomato), and CUPULIFORMIS (CUP, Antirrhinum) [1–8]. These genes are expressed at the boundaries of organ primordia or serrations, where they are thought to repress growth, though they may also promote growth outside these domains by influencing auxin distribution [9, 10]. Changes in NAC gene expression are thought to contribute to the evolution of planar leaf shape complexity [8, 11]. These findings raise the question of whether NAC boundary genes might also play a role in formation and shaping of non-planar structures, such as the corolla of sympetalous flowers, as these also derive from primordia.

Sympetalous (gamopetalous) flowers, have petals that are congenitally fused (united) or postgenitally fused for all or part of their length. This arrangement is thought to be evolutionary derived from the polypetalous (choripetalous) condition, in which petals grow separately [12, 13]. Sympetalous species might be expected to show diminished activity of genes that promote intra-whorl boundary formation in the second whorl. If reduced NAC gene activity contributes to enhanced petal fusion, mutations that diminish NAC function in sympetalous species should either further increase the degree of petal fusion or have little effect. Contrary to this expectation, inactivation of NAM in Petunia by virus-induced silencing leads to increased petal separation [14]. This observation suggests that NAM activity promotes petal fusion rather than intra-whorl boundary formation. However, this interpretation is called into question by the observation that occasional flowers generated by nam mutants have normally united corollas, even though inter-whorl fusions between stamens and petals are observed [1]. This result indicates that removing NAM activity has no impact on sympetally. A further difficulty in interpreting these results is that, although NAM expression is detected in corollas by qPCR [14], the detailed expression pattern in developing Petunia corollas has not been determined by in situ hybridization. Thus, the role of NAC boundary domain genes in the evolution of sympetally remains unclear.

To further explore the role of NAC domain genes in corolla development, we analyze the role of the NAM ortholog, CUP, in...
Antirrhinum (Snapdragon). Like Petunia, Antirrhinum flowers are sympetalous. However, instead of an open trumpet shape, Antirrhinum corollas have a closed mouth and exhibit strong dorsoventral asymmetry (zygomorphy). The wild-type Antirrhinum corolla comprises five petals: two dorsal (D), two lateral (L), and one ventral (V). The proximal regions of the petals are united (congenitally fused) to form a tube, the distal part of which extends to form the upper and lower palate (Figure 1C). The tube is bounded distally by a fold (rim), beyond which are the petals lobes. The dorsal and lateral lobes are separated by a sinus, forming a hinge, which allows the flower to be opened by pollinators. Growth occurs proximal to the other sinuses allowing two subregions of the lobe to be defined—a proximal united region termed the lip, and a distal lobe region separated by sinuses. The palate and lip of the lower corolla form a wedge-shaped fold, which fits tightly, like a closed lid, over the triangular shaped upper palate [15, 16].

Mutations in CUP affect both vegetative and floral development in Antirrhinum. The cotyledons of cup mutants are united, similar to the situation observed for nam mutants of Petunia and cuc1 cuc2 mutants of Arabidopsis [1, 2, 4]. Adventitious shoots develop from the hypocotyl of cup mutant seedlings and usually terminate with similar cup-shaped leaves (Figure 1D [4]). Eventually, secondary shoots may form with a functional apical meristem, similar to what happens with nam mutants in combination with other transcription factors [13]. However, the role of CUP in corolla development is unclear.

Computational modeling of Antirrhinum corolla development shows that the growth patterns and final shape can be accounted for by combinatorial interactions between genes expressed along three axes: dorsoventral, proximodistal, and mediolateral [15, 16]. These genes act in combination to control specified growth rates parallel and perpendicular to local tissue polarity. Tissue polarity is initially oriented proximodistally to form a polarity field, which becomes deformed, together with gene expression patterns, as the tissue grows. CYCLOIDEA (CYC), DICHOTOMA (DICH), RADIALIS (RAD), and DIVARICATA (DIV) establish different identities along the dorsoventral axis of the corolla [17–19]. CYC and DICH are dorsally expressed and encode TCP transcription factors that activate expression of the MYB gene RAD. RAD acts antagonistically to the DIV MYB gene, restricting its activity to ventral and lateral regions [19, 20]. The dorsoventral genes are thought to interact with genes expressed in domains along the proximodistal and mediolateral axes to produce the characteristic Antirrhinum corolla shape [16]. For example, the wedge-shape form of the lower corolla depends on interactions between DIV and inferred gene activities in the primordial palate and lip regions (proximodistal axis), together with genes in the midline and edges of each petal (mediolateral axis) [15]. However, unlike the dorsoventral axis,
genes underlying the proximodistal and mediolateral domains have yet to be identified. Moreover, the initial starting tissue shape for these computational models is a cylinder with five lobes and thus already exhibits sympetally, raising the question of how this initial form is generated.

Here, we analyze CUP expression in *Antirrhinum*, from floral initiation to later stages of development and relate this expression pattern to mutant and wild-type morphogenesis. We reveal a gap in CUP expression between proximal and distal regions at boundaries between adjacent petal primordia. Through computational modeling, we show that this gap most likely reflects reduced growth repression needed for formation of the sympetalous corolla. At later stages, distal CUP expression is cleared from the lateral-ventral sinuses, where the lip forms, suggesting a role for CUP in modulating growth at sinuses.

We further show that CUP is activated by dorsoventral genes at later developmental stages in an intermediary domain of the lower and upper corolla. This domain corresponds to the presumptive palate and lip regions, and CUP is required for their development. Unlike the role of CUP at boundaries, expression in intermediary regions leads to promotion rather than inhibition of growth, most likely reflecting interactions with other region-specific factors. Intermediary expression of CUP is found in other species with dorsoventrally asymmetric corollas, such as *Mimulus* and *Linaria*. Our findings thus suggest that changes in CUP expression have played a key role in the formation and shaping of the sympetalous corolla.

**RESULTS**

**Early Role of CUP in Antirrhinum Corolla Development**

The role of NAC genes in the formation of the *Antirrhinum* corolla was investigated through analysis of flowers on escape shoots generated by the cup mutant (white arrow in Figure 1D, red box in Figure 1E). Mutant corollas have an open mouth and narrow tube and lack the complex folds and shaping characteristic of the palate and lip regions of wild-type. Dorsal, lateral, and ventral petals are more similar to each other in shape than those of wild-type (Figure 1F). Sinuses are still present between petal lobes, and the stamen filaments are often fused with the corolla tube (Figures 1G–1J).

To determine the pattern of regional identities in cup mutant corollas, we visualized the cell types in wild-type and cup petals by scanning electron microscopy. In wild-type dorsal and ventral petals, cell types could be identified that correlated with the proximal tube, palate, lip, and distal lobe regions (Figures 2A and 2B). By contrast, only the proximal tube and distal lobe cell types could be clearly identified in cup mutants (Figures 2C and 2D).
To understand how CUP influences corolla development, we analyzed CUP expression by RNA in situ hybridization. We first considered expression at early stages (5–10 DAI, days after floral meristem initiation). In early wild-type floral meristems (5–6 DAI), CUP was expressed in a meristematic ring internal to whorl 1 sepal primordia (Figures 3A–3D). By 7 DAI, regions lacking CUP expression, most likely corresponding to petal primordia, could be detected (Figures 3F and 3G, arrowed in blue inset of Figure 3G). By 8 DAI, CUP expression around petal junctions became separated into a proximal and distal domain, with a gap in-between on the abaxial side. The gap was evident for both the ventral-lateral junctions (Figure 3J, V-L SIN, red bracket) and lateral-dorsal junctions (Figure 3J, L-D SIN, red bracket). By 10 DAI, the gap had enlarged and was also evident on the adaxial side (Figure 3K, V-L SIN and L-D SIN, red brackets). Thus, CUP is initially expressed in a continuous domain, both between whorls and at petal junctions (Figure 3L). The junction regions later

2C and 2D), showing that regions displaying palate and lip identities were not evident.

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Figure 4. Modeling Sympetalous Corolla Formation

(A) Initial cylindrical canvas representing primordial corolla ring at 6.5 DAI, with domains of expression used to establish growth patterns and polarity. BASE and DISTAL define regions at the proximal and distal ends of the cylinder, respectively. The proximal half of the cylinder expresses TUBE and the distal half LOBE. JUN is expressed at the petal junctions.

(B) Uniform isotropic specified growth. Expression domains of TUBE, LOBE, and inter-whorl CUP (red) are shown at 10 DAI.

(C) Repression of isotropic specified growth rate by CUP at petal junctions leads to formation of deep sinuses between petal primordia. (D) As in (C) except CUP expression inhibited by TUBE, leading to formation of shallow sinuses between petal primordia and a united tube. Cylinder slopes inward because of reduced circumferential growth caused by CUP.

(legend continued on next page)
become separated into a more distal domain, corresponding to the petal sinus (e.g., green domain within red box in Figure 3M), and a proximal domain corresponding to the boundaries of whorl 2 with whorls 1 and 3 (green line at bottom of Figure 3M).

The subdivision of the CUP domain at junctions into proximal and distal subdomains may be related to the formation of sympetalous corollas. To explore this possibility, we modeled formation of the corolla lobes and sinuses, using the growing polarized tissue (GPT) framework [21] (see STAR Methods for further details). In this framework, tissue is treated as a connected continuous material, termed the canvas. We used a shallow cylinder to represent the corolla meristematic ring around the floral meristem at 6.5 DAI and subdivided it into four domains along the proximodistal axis: BASE, TUBE, LOBE, and DISTAL (Figure 4A). We also subdivided each petal primordium along its mediolateral axis with JUN, which is expressed at the lateral edges or junctions of each primordium.

We first considered the effects of isotropic specified growth in the plane of the canvas. If areal specified growth rate was uniform (Figure 4B, 6.5 DAI), the cylindrical canvas simply became enlarged (Figure 4B, 10 DAI). Inter-whorl CUP expression was indicated at the corolla base (shown in red, Figure 4B, 10 DAI). To account for the development of separate petals, we introduced repression of growth at the petal junctions, mediated by the action of CUP (Figure 4C, 6.5 DAI). The depth of the divisions was reduced if junctional CUP was repressed in the tube region, corresponding to a gap in the CUP junctional domain (Figure 4D, 6.5 DAI). This led to the formation of an inwardly sloping corolla tube with sinuses between the lobes (Figure 4D, 10 DAI). The inward sloping (a passive effect of growth repression) could be reduced by inhibiting growth of the canvas by BASE (Figure 4E). Thus, if CUP acts by repressing growth, the generation of a sympetalous flower may reflect inhibition of CUP in a petal junction zone, allowing the non-expressing region to grow and form the tube.

Clonal analysis indicates that early petal growth is anisotropic, being higher parallel to the proximodistal axis than perpendicular to it [16]. To determine how this feature influences shape, we introduced a polarity field, with specified growth rate higher parallel to the polarity (Figure 4F, 6.5 DAI). Running this model gave a taller and narrower cylinder than for the isotropic model (Figure 4F, 10 DAI). Repression of growth at the petal junctions through CUP (Figure 4G, 6.5 DAI) gave deep sinuses between the petals and an inward sloping corolla (Figure 4G, 10 DAI). Inhibiting CUP expression in the tube (Figure 4H, 6.5 DAI) gave shallower sinuses (Figure 4H, 10 DAI). The inward sloping of the corolla could be reduced by inhibiting growth by BASE (Figure 4I). This shape resembled the starting shape used for modeling corolla morphogenesis (compare Figure 4I with Figure 4J, see also [16]). Although specified growth was only repressed in inter-whorl domains in these models, the deformation of tissue extended to nearby regions, illustrated by the deformation of an initially square grid near the sinus (magnified region in Figure 4I). The grid becomes curved and shows modified growth outside the CUP domain. Thus, a simple model for early corolla growth is that CUP represses anisotropic growth at petal junctions, except for the tube junctional region where CUP is cleared.

Sinuses still form in cup mutant corollas, showing that CUP is not essential for sinus formation. It is possible that boundary genes other than CUP can repress growth at the sinus. Another possibility is that sinus formation depends on not only growth repression at boundaries, but also growth promotion within the body of the primordium. To model this hypothesis, specified growth parallel to the polarity was promoted in and around the midline of each petal primordium, using a mediolateral factor MED (Figure 4J). Inward sloping of the corolla was also reduced by inhibiting growth by BASE as in previous models. Running this model gave five primordia separated by shallow sinuses (Figure 4K). Expression of CUP throughout the petal junction, led to further sharpening and deepening of the sinuses (Figure 4L). Repression of CUP in the tube region, gave a sympetalous corolla with five lobes (Figure 4M). If CUP was maintained in only three junctions, by making its expression depend on factor RIGHT (Figure 4N), an asymmetric corolla was generated with a lip region at sinuses where CUP was lacking (blue arrow). Thus, shaping and growth around sinuses may reflect a combination of growth repression at different boundaries and promotion within primordia.

Later Role of CUP in Palate and Lip Growth
At 11 DAI, a new CUP expression domain was observed. In addition, to the proximal and sinus domains, CUP expression was seen in an intermediary region of the ventral and adjoining lateral petals, around the junction of the tube and lobe (Figure 5A, V MID and V-L SIN, blue bracket). Serial sections indicated that this domain formed a crescent shape (Figure 5D, large green domain). There was also a clearing or reduction of CUP expression at the lateral-ventral sinuses (Figure 5A, V-L SIN, white arrow) creating two pockets of low expression (Figure 5D, in green within red box). The gap between distal and proximal domains of CUP was also evident at the dorsal-dorsal junction at this stage (Figure 5A, D-D SIN, red bracket).

The crescent-shaped domain of CUP expression in the lower corolla enlarged along the proximodistal axis as the petals

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(E) As in (D) but with growth rates repressed by BASE, preventing inward sloping of cylinder (only 10 DAI shown, as same scale as in (D)).
(F) Uniform anisotropic specified growth oriented by a proximodistal polarity field (6.5 DAI, arrows).
(G) As in (F) but with growth repressed by CUP at petal junctions, leading to formation of deep sinuses.
(H) As in (G) except CUP expression is inhibited by TUBE. A cylindrical tube forms with five lobes.
(I) As in (H) except that growth inhibited by BASE (only 10 DAI shown, at same scale as for H). Magnified region shows how an initially square grid is deformed by growth repression at the sinus.
(J) Expression domain of MED.
(K) Anisotropic growth enhanced by MED, giving sinuses where MED is low.
(L) As in (K) with growth also repressed by CUP at petal junctions, leading to deeper sinuses.
(M) As in (L) except CUP is inhibited by TUBE.
(N) As in (M) except distal CUP only expressed in the presence of factor RIGHT, allowing growth of a lip region (blue arrow) where CUP is not expressed. Color coding of growth rates rescaled in (K), (L), and (M) for clarity.
Figure 5. *CUP* Expression Pattern and Growth Analysis at Later Stages of Development

(A–C) Expression of *CUP* from 11–14 DAI. 11 DAI (A) shows intermediary region of *CUP* (blue bracket). A gap in junctional *CUP* expression domain is visible at the dorsal-dorsal petal junctions (red bracket). At 13 DAI (B), the ventral domain has expanded and expression is lost at the distal end of ventral-lateral junctions (green bracket, V-L SIN). By 14 DAI (C), the palate (pink line) and lip (red) regions can be identified by the furrow that lies between them (yellow line). Extended expression around the dorsal-dorsal junction is visible (D-D SIN). Planes of sectioning are mid-ventral (V MID, light blue), ventral-lateral sinus (V-L SIN, dark blue), mid-dorsal (D MID, light green), dorsal-dorsal sinus (D-D SIN, dark green), mid-lateral (L MID, pink), and lateral-dorsal sinus (L-D SIN, purple).

(D–F) Schematic of *CUP* expression (green shading) on flattened petal diagrams from 11–14 DAI. Blue triangle indicates where virtual cut was made to flatten the petal. At 11 DAI (D), a crescent-shaped domain of *CUP* expression is established in the lower corolla with a gap in expression around the ventral-lateral sinus (red (legend continued on next page)
grew (Figures 5B and 5C and 5E and 5F). In addition, expanded expression was observed near the dorsal-dorsal junction, forming a semi-circular domain (Figure 5C, D-D SIN, Figures 5E and 5F). From 14 DAI, morphological landmarks could be used to map the domains of CUP expression. For the lower corolla, the palate-proximal tube boundary (red dashed line, Figure 5C, V MID and V-L SIN) could be identified by a change in trichome densities, the rim (yellow line) by the bend in the petal, and the distal limit of the lip (white dashed line) by tracing a line between petal sinuses (see also white dashed lines in Figures 5D–5F). These assignments showed that the CUP crescent domain lies over the palate and lip regions.

As the crescent of CUP expression enlarged during growth, so did the pockets of low expression at the lateral-ventral junctions (green bracket in Figure 5B V-L SIN). The pockets of CUP low expression were positioned at the lip region of ventral-lateral junctions (Figures 5D–5F). The clearing of CUP expression in these regions from 11 DAI onward coincides with growth of the lip at these junctions. By contrast, CUP expression was maintained at the sinuses of other junctions, which show little or no lip growth. Thus, growth of the lip at palatal junctions correlates with clearing of CUP from the sinus domain (Figures 5E and 5F).

Expression of CUP in the ventral and dorsal intermediary regions was maintained until 15 DAI (Figure 5G). By 17 DAI, downregulation of CUP was observed in the region of palate trichome development (white arrow in enlarged panel of Figure 5H). Taken together, these results suggest that the intermediary CUP domain broadly corresponds to the palate and lip regions of the corolla, consistent with these regions not growing in cup mutants.

The increase in length of the CUP crescent domain along the proximodistal axis during development might be explained by growth of the CUP-expressing region and/or cell-to-cell spread of gene expression. To determine whether growth alone might be sufficient to account for the data, we compared the rate of increase in CUP domain length with growth rates estimated from a previously published model of corolla morphogenesis [16]. In this model, the lower palate and lip regions derive from a hypothetical strip of ventral tissue (Figure 5I), which could correspond to the CUP crescent domain. The rates of growth of the hypothetical palatal- and CUP crescent domains would not be expected to be exactly the same, as the hypothetical domain was assigned a somewhat arbitrary initial size, and intermediate markers were not available to tune its growth rate. Nevertheless, we would expect these domains to grow similarly in relation to other regions. In particular, the ventral palatal-lip domain was proposed to grow faster than regions proximal or distal to it (2.9%/hr compared to 1.3%/hr and 1.7%/hr Figure 5L), leading to formation of the extended ventral palate and lip (Figures 5J and 5K).

To see whether this enhanced growth was also observed for the CUP crescent, the growth rates of the CUP crescent and domains distal and proximal to it were estimated by measuring the length of these regions from in situ hybridizations on longitudinal sections through midline (passing through the middle of the ventral petal) of flower buds at different developmental stages. The CUP crescent was initially about 100 μm along the proximodistal axis (11 DAI) and grew to a length of 800 μm by 14 DAI. As in the model, the CUP crescent grew faster in the proximodistal orientation than domains proximal or distal to it (2.0% ± 0.11%/hr, compared to 1.4% ± 0.1%/hr and 1.45% ± 0.09%/hr, Figure 5M). This comparison, taken together with the cup mutant not forming a palate or lip, suggests that the CUP intermediary expression domain most likely maps to the hypothetical palate-lip domain and expands largely through growth, though a further contribution from cell-cell spreading cannot be ruled out.

The CUP crescent is a feature of the lower corolla and might thus be expected to be influenced by genes conferring ventral identity. We therefore analyzed expression of CUP in the dorso-ventral mutant div which lacks ventral identity. In contrast to wild-type, the CUP crescent domain was not observed in div mutants. CUP expression was still seen at the sinuses and proximal inter-whorl domain, showing that these domains are not under DIV control (Figures 6A–6E; and compare Figures 6P–6R with Figures 6S–6U). Expression at the ventral-lateral sinuses was cleared at later stages (Figure 6R), consistent with growth of the lip in div mutants. Mutants with ectopic ventral identity such as cyc dich, showed an uninterrupted ring of intermediary CUP expression corresponding to ventralization of the flower (Figures 6F–6J). This ectopic activity depended on DIV, as in the cyc dich div mutant CUP expression was again reduced to the petal sinuses and proximal domains (Figures 6K–6O). The extended semicircular domain characteristic of the dorsal petals was also absent in this triple mutant. Thus, DIV upregulates CUP expression, leading to the crescent of expression in the wild-type lower corolla.

Inter-whorl fusions were not observed in div mutants, which had greatly reduced intermediary CUP expression. Moreover, inter-whorl fusions were observed at all petal junctions in cup mutants, including dorsal-lateral junctions. These observations suggest that organ fusion is most likely due to reduced CUP activity in the inter-whorl boundary domain rather than in the intermediary domain.

Both boundary and intermediary CUP activity could be integrated within previous models of corolla, in which morphogenesis depends on combinatorial interactions between factors expressed along three axes: dorsoventral, proximodistal, and dorso-ventral. In this regard, the observations suggest that organ fusion is most likely due to reduced CUP activity in the inter-whorl boundary domain rather than in the intermediary domain.
Figure 6. CUP Expression in Dorsoventral Mutants
(A–E) The div mutant (flower shown in A) lacks a ventral palate region and lacks the crescent-shaped domain of CUP expression in the ventral corolla at ~13 DAI (B and C) and ~14 DAI (D and E). Expression of CUP still visible around the sinus (red arrows) with a distal gap corresponding to the lip (yellow arrows).
(F–J) The cyc dich mutant (flower shown in F) is fully ventralized and expresses CUP in an intermediary domain (white arrows) in all petals at ~12 DAI and (G and H) and ~15 DAI (I and J).
(K–O) The cyc dich div mutant (flower shown in K) lacks intermediary CUP but retains expression at the sinuses (red arrow).

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mediolateral [15, 16]. CUP expression at early stages would contribute to shaping the early sympetalous corolla (illustrated in models of Figure 4), which is taken as a starting shape for the previously published model. This would be achieved by CUP expression at petal junctions being activated by mediolateral factors, which would act in combination with CUP to inhibit growth. Junctional CUP expression would be inhibited by a proximodistal factor (TUBE) to allow growth of the corolla tube (united tube). Expression at the distal junction would be inhibited at later stages by mediolateral factors (LIP), modulated by dorsoventral factors (RAD), to allow growth of the lateral-ventral lip junction (united lip). Intermediary CUP activity would be incorporated by activating CUP with a combination of proximodistal (PALATE, LIP) and dorsoventral (DIV, CYC, and DICH) genes. CUP in combination with these factors would then promote growth of the palate and lip regions. These interactions would replace roles previously assigned directly to the combined dorsoventral, proximodistal, and mediolateral factors.

Possible downstream targets of CUP are YUCCA auxin biosynthetic genes. In Arabidopsis, YUCCA1 is expressed in a similar domain to CUC and can depend on CUC activity [22]. In situ hybridizations with the Antirrhinum ortholog of YUCCA1, AmYUCCA1, showed that it was expressed in a very similar pattern to CUP, at the base of primordia, sinuses, and intermediary domain (Figure S1). Moreover, AmYUCCA1 was cleared at later stages from around the lateral-ventral sinus, similar to CUP (Figures S1L and S1M). Expression of AmYUCCA1 in these domains was not observed in cup mutants (Figure S2), suggesting that they are under CUP control.

As the CUP crescent is involved in formation of the ventral palate and lip, we tested whether a similar intermediary region of expression was present in other sympetalous flowers of the order Lamiales. Linaria has a corolla with a closed mouth similar to that of Antirrhinum and belongs to the same tribe (Antirrhineae) of the family Plantaginaceae (Figure 7A). Early expression of Linaria CUP was in boundary domains, similar to that in Antirrhinum (Figures 7C–7E). At later stages, intermediary region of CUP expression was observed in the lower petals, similar to that seen in Antirrhinum, suggesting that it shares the crescent domain (Figure 7F, blue bracket). Mimulus is a more distant genus from Antirrhinum, belonging to a different plant family (Phrymaceae). The corolla is not fully hinged like Antirrhinum but nevertheless has a bilaterally symmetric shape with palate and lip regions (Figure 7B). Early expression of Mimulus CUP was in boundary domains, similar to those in Antirrhinum (Figures 7G and 7H). At later stages, expression of Mimulus CUP was also detected in an intermediary region of the lower petals (blue bracket Figure 7I). Expression then diminished (Figure 7J). Thus, the intermediary region of CUP expression was found in other species with sympetalous corollas exhibiting strong zygomorphy.

**DISCUSSION**

CUP expression in the developing Antirrhinum corolla shows two novel features. First, shortly after petal initiation, expression at the junction between adjacent petal primordia becomes subdivided into a proximal and distal domain, separated by a gap. The proximal domain remains at the inter-whorl boundary, the gap grows to become the junctional region of the corolla tube, and the distal domain forms the sinus. Second, at later stages of development, CUP is expressed in an intermediary region, in a crescent-shaped domain in the lower corolla, and in a semi-circular domain in the upper corolla. These intermediary domains correspond to the palate and lip regions that grow to form the characteristic wedge shape of the lower corolla and apposing triangular region of the upper corolla.

Both of these novel features of CUP expression may be connected with the formation and shaping of the corolla. A major role of CUP and its homologs is to repress growth at the base of primordia, ensuring their separate development [2, 3, 23–25]. This repression may operate at both intra-whorl and inter-whorl boundaries. We show, through computational modeling, that repression of specified growth rates at inter-whorl boundaries leads to relative shortening of the boundary domain and deformation of nearby tissue to generate a sinus. If growth repression is excluded from the observed gap in the CUP expression domain, a sympetalous corolla with a united tube is generated.

Thus, the sympetalous corolla can be accounted for by repression of CUP at petal junctions, creating a gap that allows united growth to occur. Clearing of CUP also occurs at later stages from the lateral-ventral sinuses of the corolla, correlated with growth of the lip in these regions. Such clearing is not observed at the dorsal-lateral sinuses where lip growth is greatly reduced, corresponding to the hinge of the corolla. Thus, selective inhibition of CUP may play a key role in sympetally and modulating growth proximal to the sinus by allowing subregions of boundary domains to grow. Sinuses still form in cup mutants and at lateral-ventral petal junctions, showing that CUP-independent mechanisms are involved in sinus formation. These mechanisms may involve action of other boundary genes and/or growth promotion within primordia through the action of auxin or other growth regulators [9, 10].

Similar to orthologous mutants in other sympetalous species, nam in Petunia [1] and gob in tomato [7], flowers of cup mutants show inter-whorl fusion (e.g., petal-stamen fusions), while intra-whorl fusion of petals is not markedly affected. These findings contrast with results of virus-induced silencing of NAM in Petunia, which leads to reduced intra-whorl fusion and petal separation [14]. A possible explanation for this discrepancy is that rather than eliminating NAC activity, virus-induced silencing modifies NAC gene regulation, leading to ectopic boundary gene activity.

---

P–R) CUP expression at the ventral-lateral sinus in div mutant. Expression is retained at the sinus (dashed black line) at ~10 DAI (P) and ~11 DAI (Q). By ~13.5 DAI, a gap in expression is seen at the sinus (yellow arrow) corresponding to formation of the lip region (R).

(S–U) CUP expression near the ventral-lateral sinus in wild-type. Expression is observed at the sinus at ~10 DAI (S, red arrow), with more extended expression by ~11 DAI (T, white arrow) and gap near the sinus where the lip is forming (yellow arrow). By 13.5 DAI, the ventral crescent (white arrows) is extensive (U).

Planes of sectioning are indicated by white lines in the top right of each in situ image. Only half of the section plane is shown in close-ups (C, H, M, and P–U). “V,” mutant ventral petal; “V–L,” mutant ventral and lateral petal junction; D, dorsal; ca, carpel; st, stamen. Scale bars represent 1 cm for (A), (F), and (K) and 100 μm for the other panels.

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In Antirrhinum, CUP has acquired a further role, in establishing lip and palate domains. This role of CUP involves expression in an intermediary domain along the proximodistal axis, at the tube-lobe boundary. For the lower corolla, the intermediary domain grows to form a crescent shape, while for the upper corolla it grows to form a semi-circular shape. These intermediary CUP domains depend on activity of the dorsoventral genes. In cup mutant flowers, the corolla has a greatly simplified shape without an observable palate or lip. Thus, the evolution of Antirrhinum corolla shape most likely involved the CUP gene being co-opted and brought under the control of dorsoventral genes to modulate palate and lip growth. This co-option must have occurred prior to the divergence of Antirrhinum, Linaria, and Mimulus, as all three species exhibit intermediary domains of CUP. Linaria is the closer relative of Antirrhinum and has a similar closed mouth with palate and lip. Mimulus is a more distant member of the Lamiales and has a bilaterally symmetric sympetalous corolla, with upper and lower palates and lips. However, the Mimulus flower does not have a closed mouth, suggesting that the specific shaping of the corolla depends on targets of CUP and dorsoventral genes.

NAC-domain genes typically repress growth within their expression domains (e.g., boundaries), though they may promote growth outside these domains, by influencing auxin distribution [9, 10]. By contrast, the zone of intermediary CUP expression shows enhanced growth. Growth enhancement by CUP may involve generation of auxin as the auxin biosynthetic gene, AmYUCCA1, is expressed in the intermediary domain, and this expression is absent in cup mutants. YUCCA expression is also activated in boundary domains of CUP and CUC [22], though unlike the intermediary zone, growth is believed to be repressed within these domains [5]. Thus, rather than necessarily being a repressor of growth, the CUP transcription factor may acquire different roles, presumably reflecting its combinatorial interactions with other genes. These interactions would then lead to modulation of growth-modulating target genes, such as AINTEGUMENTA [26–28], known to be expressed in the ventral palate and lip of Snapdragon petals [29].

Shaping of the Snapdragon flower, with its united corolla, hinged palate, and extended lower lip, depends on the interactions between boundary genes, such as CUP, and factors expressed along the different axes of the flower. Similar interactions may underlie the shaping of other structures, such as the grass leaf, in which the ligule and auricle provide a hinge at the sheath-blade boundary, where NAC genes are expressed [30]. Thus, our analysis provides further support for...
the notion that modulations in boundary genes and their interactors may play an important role in the evolution of diverse plant morphologies.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.07.064.

AUTHOR CONTRIBUTIONS

A.B.R. conceived and designed the study, acquired the data, performed computational modeling, analyzed and interpreted the data, and revised the manuscript. J.R.K. analyzed and interpreted the data and provided GFPbox modeling support. J.A.B. conceived and designed the study, E.C. conceived and designed the study, analyzed and interpreted the data, performed computational modeling, and drafted and revised the manuscript.

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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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Software and Algorithms


CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Enrico Coen (enrico.coen@jic.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Antirrhinum majus* wild-type (JIC stock 7), *div-13* [18], *cyc dich* and *cyc dich div* [16] and *cup*<sup>sem</sup> [4], were grown in the greenhouse at the John Innes Centre. Each *cup* mutant generates several escape shoots, with 2-3 flowers per escape shoots. We have grown and observed more than 30 *cup* mutants. Seed of *Linaria maroccanna* was obtained from Jorge Almieda [31], and that of *Mimulus guttatus* from John Willis. Staging of flowers in Days After Initiation (DAI) was as described in [32]. Mutants were staged by: 1) measuring dorsal petal width in case of *div-13* as this mutant does not affect the development of the dorsal petals; 2) measuring carpel and stamen width in case of *cyc dich* and *cyc dich div*, as a proxy of the wild-type carpel and stamen width values.

METHOD DETAILS

**In situ Hybridization**

In situ hybridizations were carried out as described previously [15, 33]. To generate specific digoxigenin-labeled riboprobes probes, the ORF of *CUP*, the ORF of *Linaria CUP* ortholog and the ORF of *Mimulus CUP* ortholog were amplified using TAQ DNA polymerase PCR (201205, QIAEN) and cloned into pCR4-TOPO TA vector (K4575-01, Life Technologies) following manufacture instructions. The cloned fragments were amplified using a forward specific primers and the M13F primer in the case of CUP and the M13R primer in the case of the other two probes (pCR4-TOPO kit), column purified (28104, Qiaquick PCR purification) followed...
by a phenol/chlorophorm extraction. Antisense probe was obtained by RNA transcription using the T7 promoter (10881767001, Roche through Sigma-Aldrich) in the case of CUP, and the T3 promoter (11031163001, Roche through Sigma-Aldrich) in the case of the other probes, and DIG-UTP (11209256910, Roche through Sigma-Aldrich), according to manufacture instructions. The probe was hydrolysed at 60°C using a 200 mM carbonate buffer pH 10.2 solution, for 65 min in the case of CUP and 35 min in the case of the other two probes.

To identify the YUCCA orthologs expressed during Snapdragon flower development, a draft genome sequence of Snapdragon was used to isolate nine snapdragon YUCCA genes. AtYUCCA1 and AtYUCCA4, in Arabidopsis, and FLOOZY, in petunia, were shown to be expressed during petal emergence [34, 35]. Focusing on the snapdragon YUCCA1/FLOOZY ortholog, primers were used to amplify the open reading frame from JIC stock2 cDNA.

Microscopy

Scanning electron microscopy was carried out as described in [32]. In situ hybridized material was imaged using a Leica DM 6000 and Nomarski settings, x10 and x20 dry lens and a DFC420 digital camera were used to photographed the in situ sections. All measurements made from in situ images were calculated using the ImageJ software (http://imagej.nih.gov/ij/).

Computational Modeling

Because of tissue connectivity, repression of growth in one region may cause passive deformations in adjacent regions. Computational modeling is therefore needed to determine the shapes that would be generated by particular patterns of growth repression. Models are based on the Growing Polarized Tissue framework (GPT-framework) [21]. This model, tissue is treated as a connected continuous material, termed the canvas. The extent to which a tissue region grows depends not only on its intrinsic growth specification but also on mechanical constraints from neighboring regions. We distinguish between two types of growth: specified and resultant. Specified growth is how a region of tissue would deform if it was free from the mechanical constraints of its neighboring regions. Resultant growth is how a region deforms in the context of neighboring mechanical constraints, and includes anisotropies, rotations, and curvature that emerge passively from such constraints [15].

As the main focus of the modeling was to understand corolla formation, we considered growth of whorl 2 alone. The initial canvas comprised a shallow cylinder to represent a corolla meristematic ring around the floral meristem at 6.5 DAI. The base of the cylinder, which corresponds to the petal inter-whorl boundary, was anchored in the z direction. The canvas has two surfaces (A and B). Identity and signaling factors can be specified throughout the canvas. The distribution of factors is shown in Figures 4A and 4J. A growth regulatory network (KRN) controls the specified growth parallel ($K_{par}$) and perpendicular ($K_{per}$) to the local polarity, established by taking the gradient of a diffusible factor POLARIZER (POL). For anisotropic growth models, the polarity field is established by producing POL at the bottom of the canvas (through factor BASE) and fixing it to a low concentration at the top (through factor DISTAL).

**Figure 4B: Uniform isotropic growth**

To model uniform specified isotropic growth the KRN equations are:

$$K_{par} = 0.0165$$
$$K_{per} = 0.0165$$
$$K_{nor} = 0.005$$

where $K_{nor}$ is specified growth rate in canvas thickness.

**Figure 4C: Isotropic growth inhibited by CUP at junction**

The KRN equations are:

$$K_{par} = 0.0165 \cdot \text{inh}(100, i_{\text{cup}})$$
$$K_{per} = 0.0165 \cdot \text{inh}(100, i_{\text{cup}})$$
$$K_{nor} = 0.005$$

where $i_{\text{cup}}$ is the CUP expressed at the petal junctions (intra-whorl boundary), and inh (x, i) denotes inhibition by factor i by an amount x [16].

**Figure 4D: Isotropic growth inhibited by CUP when excluded from TUBE**

As for Figure 4C except that the expression of $i_{\text{cup}}$ is inhibited by TUBE.

**Figure 4E: Isotropic growth as in D with growth inhibited at base**

The KRN equations are:

$$K_{par} = 0.0165 \cdot \text{inh}(100, i_{\text{cup}}) \cdot \text{inh}(0.5, i_{\text{base}})$$
$$K_{per} = 0.0165 \cdot \text{inh}(100, i_{\text{cup}}) \cdot \text{inh}(0.5, i_{\text{base}})$$
$$K_{nor} = 0.005$$

where $i_{\text{base}}$ is the BASE identity factor.
**Figure 4F: Uniform anisotropic growth**
The KRN equations are:

\[
\begin{align*}
K_{\text{par}} &= 0.022 \\
K_{\text{per}} &= 0.011 \\
K_{\text{nor}} &= 0.005
\end{align*}
\]

**Figure 4G: Anisotropic growth inhibited by CUP at junction**
The KRN equations are:

\[
\begin{align*}
K_{\text{par}} &= 0.022 \cdot \text{inh}(100, i_{\text{cup}}) \\
K_{\text{per}} &= 0.011 \cdot \text{inh}(100, i_{\text{cup}}) \\
K_{\text{nor}} &= 0.005
\end{align*}
\]

**Figure 4H: Anisotropic growth inhibited by CUP when excluded from TUBE**
As for Figure 4G except that the expression of \(i_{\text{cup}}\) is inhibited by TUBE.

**Figure 4I: Anisotropic growth as in H with growth inhibited at base**
The KRN equations are:

\[
\begin{align*}
K_{\text{par}} &= 0.022 \cdot \text{inh}(100, i_{\text{cup}}) \cdot \text{inh}(0.5, i_{\text{base}}) \\
K_{\text{per}} &= 0.011 \cdot \text{inh}(100, i_{\text{cup}}) \cdot \text{inh}(0.5, i_{\text{base}}) \\
K_{\text{nor}} &= 0.005
\end{align*}
\]

**Figure 4K: Anisotropic growth promoted within primordia**
The KRN equations are:

\[
\begin{align*}
K_{\text{par}} &= 0.022 \cdot \text{pro}(0.3, s_{\text{med}}) \cdot \text{inh}(0.5, i_{\text{base}}) \\
K_{\text{per}} &= 0.011 \cdot \text{inh}(0.5, i_{\text{base}}) \\
K_{\text{nor}} &= 0.005
\end{align*}
\]

where \(s_{\text{med}}\) is the MED factor expressed within primordia (Figure 4J), and pro \((x, s_z)\) denotes promotion by factor \(s_z\) by an amount \(x\) \[16\].

**Figure 4L: Anisotropic growth promoted within primordia combined with CUP repressing growth at junctions**
The KRN equations are:

\[
\begin{align*}
K_{\text{par}} &= 0.022 \cdot \text{pro}(0.3, s_{\text{med}}) \cdot \text{inh}(100, i_{\text{cup}}) \cdot \text{inh}(0.5, i_{\text{base}}) \\
K_{\text{per}} &= 0.011 \cdot \text{inh}(100, i_{\text{cup}}) \cdot \text{inh}(0.5, i_{\text{base}}) \\
K_{\text{nor}} &= 0.005
\end{align*}
\]

**Figure 4M: Anisotropic growth promoted within primordia combined with CUP excluded from tube**
As for Figure 4L except that the expression of \(i_{\text{cup}}\) is inhibited by TUBE.

**Figure 4N: CUP expression at a subset of junctions**
As for Figure 4M except that \(i_{\text{cup}}\) expression depends on factor RIGHT.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Growth rates for the CUP domain, and domains distal and proximal to it, were determined by measuring of these domains from staged in situ. Data from 27 buds at various stages were obtained and log of length of the CUP domain in medial sections through the ventral petal, plotted against estimated age (in DAI). The slope of linear fits gave the estimated relative growth rate, and the standard deviation of the slopes was obtained using the LINEST function in excel. As the measurements came from different buds, the standard deviations relate to both technical variation in length/staging estimates and biological variation between samples. In developing the model used for comparison, growth parameters were adjusted to generate approximately the correct final dimensions and were not tuned to domain lengths at intermediate stages. The parameters chosen, and thus the growth rates, were therefore influenced by the initial size of the domain and simplifying assumptions about intermediate stages.

**DATA AND SOFTWARE AVAILABILITY**

GFT-box software and programs used for modeling can be downloaded from http://cmpdartsvr1.cmp.uea.ac.uk/downloads/software/OpenSourceDownload_CurrentBiology_Rebocho_2017/GPT_Antirrhinum_CUP.zip.