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Evolution of spur-length diversity in *Aquilegia* petals is achieved solely through cell-shape anisotropy

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The role of petal spurs and specialized pollinator interactions has been studied since Darwin. *Aquilegia* petal spurs exhibit striking size and shape diversity, correlated with specialized pollinators ranging from bees to hawkmoths in a textbook example of adaptive radiation. Despite the evolutionary significance of spur length, remarkably little is known about *Aquilegia* spur morphogenesis and its evolution. Using experimental measurements, both at tissue and cellular levels, combined with numerical modelling, we have investigated the relative roles of cell divisions and cell shape in determining the morphology of the *Aquilegia* petal spur. Contrary to decades-old hypotheses implicating a discrete meristematic zone as the driver of spur growth, we find that *Aquilegia* petal spurs develop via anisotropic cell expansion. Furthermore, changes in cell anisotropy account for 99 per cent of the spur-length variation in the genus, suggesting that the true evolutionary innovation underlying the rapid radiation of *Aquilegia* was the mechanism of tuning cell shape.

Keywords: petal shape; cell shape; evolution; pollination syndrome; morphogenesis; nectar spur

1. INTRODUCTION

Floral spurs are tubular pockets that grow out from developing floral organs (figure 1), typically with nectar-producing glands at their distal tip. Nectar spurs have evolved multiple times across the angiosperms, often in association with dramatic speciation events, such as in the families Tropaeolaceae (nasturtium), Fumariaceae (bleeding-heart) and Lentibulariaceae (bladderwort) [1]. A particularly striking example of morphological diversity is seen in the genus *Aquilegia*, commonly known as columbine. Species of *Aquilegia* vary dramatically in spur length over a 16-fold range, matching the tongue lengths of their major pollinators (i.e. bees, hummingbirds and hawkmoths) [2] (figure 1; electronic supplementary material, figures S1 and S2). The fit between the pollinator's tongue length and a species' spur length is apparently driven by selection acting to maximize pollen removal and receipt [2,3], resulting in very rapid evolution of spur length at the time of speciation, and thereby contributing to the rapid radiation of the genus [2]. Despite their critical role in the ecology and diversification of *Aquilegia*, remarkably little is understood about spur morphogenesis and its evolution. Here, we have used molecular, developmental and morphometric approaches to understand spur morphogenesis and the developmental basis of spur diversity in *Aquilegia*.

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2. SPUR DEVELOPMENT: CONNECTING TISSUE MORPHOGENESIS WITH CELL SHAPE

Since Darwin [4], botanists have appreciated the evolutionary significance of petal spurs, yet spur development remains largely uncharacterized. In *Aquilegia*, traditional botanical hypotheses based on early histological studies hold that spur development is driven by meristematic knobs flanking the attachment point in the developing petal [5,6]. In this scenario, continued cell divisions combined with cell expansion is the primary driver of spur growth. Since Tepfer [5], the idea that spur growth occurs by essentially adding one cell at a time has been widely accepted [6,7], but has never been verified.

We experimentally tested this meristem hypothesis in *Aquilegia* by marking cell divisions with *in situ* hybridization [8] of *AqHistone4* (*AqHIS4*), which marks DNA-replicating cells, in developing petal spurs (figure 2; electronic supplementary material, §M1). This analysis revealed that while cell divisions are initially diffuse throughout the petal primordium, they cease early during development in a wave that begins at the distal petal tip and progresses towards the site of the initiating spur (figure 2*a*–*d*). Cell divisions are no longer visible anywhere in the young spur once it achieves a cup-like shape of approximately 5 mm length (figure 2*d*). Furthermore, by directly counting the number of cells in a single cell file extending along the entire spur length, we determined that cell divisions completely cease early in development once the spur reaches a length of approximately 5–9 mm (figure 2*e*; electronic supplementary material, §M2). Together, these results unequivocally demonstrate that spur growth is not driven by a

Figure 1. *Aquilegia* flowers exhibit considerable spur-length diversity. (a) *A. vulgaris*, (b) *A. canadensis*, (c) *A. coerulea* and (d) *A. longissima*. Scale bars, 1 cm.

meristematic zone. Thus, cell expansion, not cell division, must be the primary driver of spur outgrowth once the pre-pattern is established by localized cell division. However, isotropic cell expansion alone would simply result in a scaled-up version of the initial cup-like spur; clearly, an additional mechanism is needed to achieve the observed slender, elongated morphology (figure 1; electronic supplementary material, figures S1 and S2).

To investigate if and how cellular mechanisms are responsible for spur sculpting, we measured cell size and shape along a continuous transect of the outer (abaxial) epidermis in developing *Aquilegia coerulea* 'Origami' red/white spurs (hereafter referred to as *A. coerulea*) at 11 developmental stages following the cessation of cell division and until spur maturity (figure 3a,b). Since cells are consistently oriented along the long axis of the spur, we defined and measured cell length $l(s)$ and cell width $w(s)$ at a distance s (in millimetres) from the nectary tip, for a total of approximately 7000 cell measurements (figure 3b; electronic supplementary material, figure S4 and §§M3–M4). Given that petal lamina thickness is virtually uniform throughout the spur (electronic supplementary material, figure S4), cell size can be characterized by cell area $A(s) = lw$, while cell shape is characterized by the anisotropy defined as $\epsilon(s) = l/w$ along the spur. We see that although cell area increases uniformly along the entire spur during development (electronic supplementary material, figure S5), cell anisotropy varies along the length of the spur (figure 3b,c). To characterize the temporal development of the spur, we scaled the distance s by the instantaneous length of the spur L, a measure of developmental time, so that the scaled distance $z = s/L$ varies from $z = 0$ at the nectary tip to $z = 1$ at the attachment point (figure 3a) at each developmental stage. This allowed us to compare cell anisotropy $\epsilon(z)$ through development (figure 3c) and shows that although young spurs start out with $\epsilon(z) \approx 1$ (cells approximately isotropic), as development progresses, $\epsilon(z)$ increases non-uniformly along the length of the spur, reaching a maximum value just above the nectary. In figure 3d, the maximum cell anisotropy ϵ_{max} is plotted against the spur length L, demonstrating that spur development is associated with increasing cell anisotropy.

In addition to cell morphology measurements during development, we also recorded the shape of the entire spur at each stage. While cell columns along the length of the spur twist slightly during growth (electronic supplementary material, figure S6), spur shape remains cylindrically symmetric throughout development, but becomes increasingly slender and elongated. Thus, spur shape can be quantified by measuring its radial profile $r(s)$ (figure 3; electronic supplementary material, figures S7–S9 and §M5). To correlate cell morphology changes during development with the observed shape of the spur, we started with an 'initial' spur shape obtained by averaging radial profiles of two young (approx. 8 mm) *A. coerulea* spurs. This model spur profile was then numerically 'grown' using experimental measurements of cell area $A(s)$ and cell anisotropy $\epsilon(s)$ to achieve spur profiles at the same developmental stages shown in figure 3a. The profiles were then rotated about the long axis of the spur to generate spur shapes at each developmental stage. The good agreement between the numerical and experimental spur profiles and shapes (figure 3e,f), with no adjustable parameters, demonstrates the critical role of cell shape in spur morphogenesis, and directly connects measured cellular level data with organ level morphology. This is further confirmed by comparing the profiles calculated using only cell area changes while ignoring cell anisotropy, which result in deformed, short, wide spurs (electronic supplementary material, figure S10 and §M6).

Having linked changes in cell anisotropy to the sculpting of spur morphology, we sought to experimentally perturb cell shape. In plant cells, the cytoskeleton constrains the direction of cell elongation by orienting cellulose deposition [9]. Since disruption of the cytoskeleton should perturb cell anisotropy and therefore spur morphosis, we treated developing *Aquilegia chrysantha* spurs with oryzalin, a microtubule depolymerization agent [10,11] (details in electronic supplementary material, figure S11 and §M7). As shown in figure 4, the treated spur is much shorter and wider than untreated spurs from the same flower. Examination of cells in the treated tissue verified that changes in cell area A are unaffected, while cell anisotropy remains at $\epsilon \approx 1$ (figure 4b,c) for all time points. These findings further confirm that anisotropic cell expansion, and not extended meristematic growth, determines spur morphogenesis.
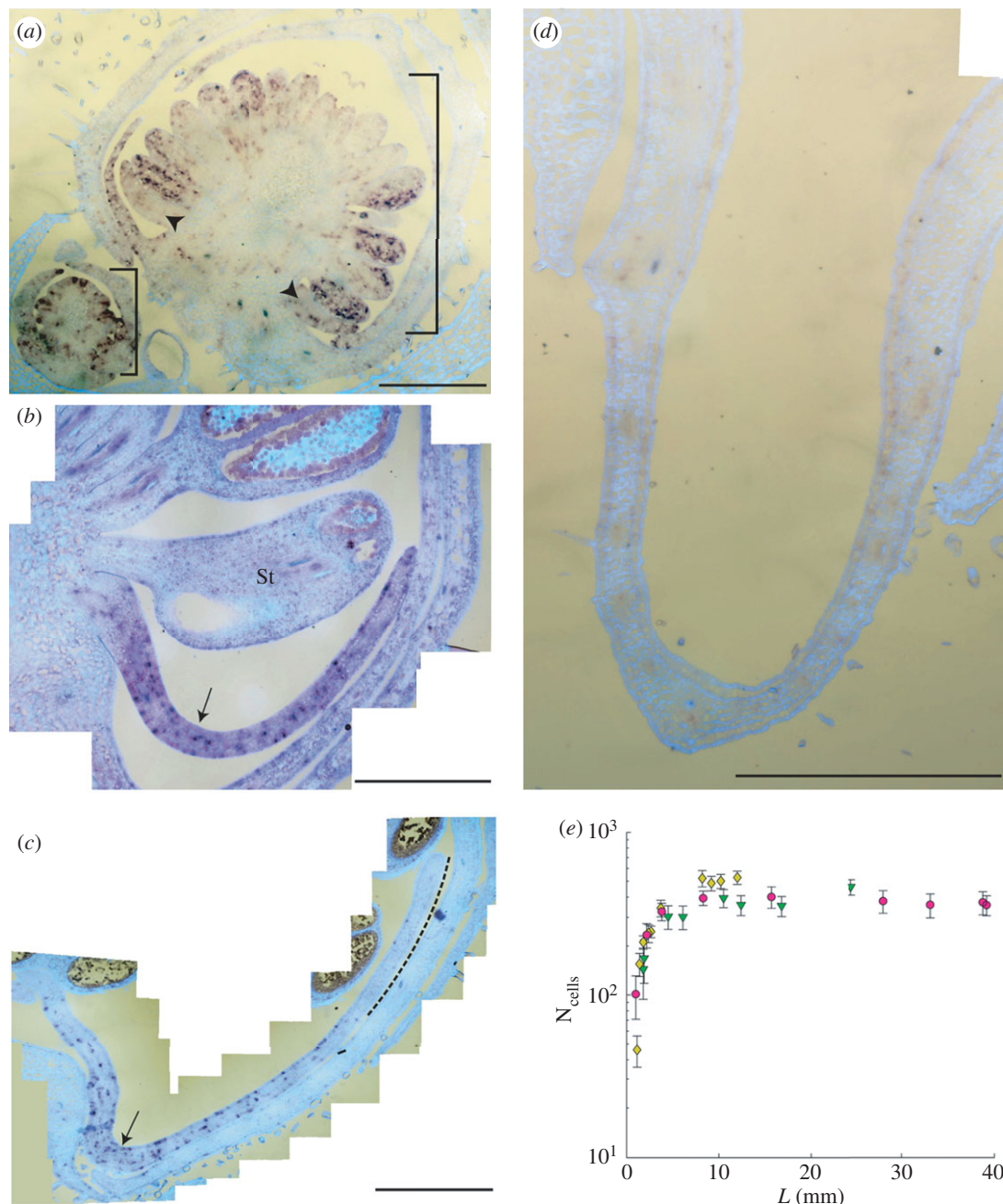
Figure 2. Cell divisions cease very early in spur development. (a–d) *In situ* localization of *AqHIS4* in developing *A. vulgaris* flowers was used to determine the pattern and extent of cell divisions in early petal development. *AqHIS4* expression, visualized by purple staining, marks cell divisions. Arrowheads, petals; arrow, initiating spur. (a) Two young *A. vulgaris* flower buds (in brackets) showing ubiquitous *AqHIS4* expression indicating diffuse cell divisions. (b) Older flower showing ubiquitous cell divisions in the petal while cell divisions have ceased in the stamens (St). (c) *A. vulgaris* petal with initiating spur. Cell divisions are most concentrated at the initiating spur and have ceased in the tip of the developing petal, as indicated by a dotted line. (d) *A. vulgaris* spur of length $L \approx 5$ mm with no *AqHIS4* expression evident, indicating that all cell divisions have ceased. (e) The number of cells in a single cell file extending the entire length of developing *A. canadensis* (green inverted triangles), *A. coerulea* (pink circles) and *A. longissima* (yellow diamonds) spurs, counted from the attachment point to the nectary. The number of cells plateaus to a constant value early in development when the spur is approximately 5–9 mm long. Error bars indicate counting errors. Scale bars, (a,b) 0.5 mm and (c,d) 1 mm.

3. CELL ANISOTROPY AND SPUR-LENGTH DIVERSITY

The essential role of cell anisotropy in *A. coerulea* spur morphogenesis raised the question of how variations in this parameter contribute to evolutionarily significant diversification of spur shape and length. Since mature petal spurs in *Aquilegia* range in length from $L \approx 1 - 15$ cm, with the majority in the 2–6 cm range [2,12], four *Aquilegia* species were studied to sample this entire range: *A. vulgaris* (final spur length $L_f \approx 2.4$ cm), *A. canadensis* ($L_f \approx 2.6$ cm), *A. coerulea* ($L_f \approx 5.1$ cm) and *A. longissima* ($L_f \approx 15.9$ cm; figures 1 and 5a). These species also represent a breadth of associated pollinators from bees (short, curled spurs in *A. vulgaris*) to hummingbird (short, straight spurs in *A. canadensis*) to hawkmoth (long, slender spurs in *A. coerulea* and *A. longissima*). For each species, cellular measurements from two to four biological replicates were imaged at
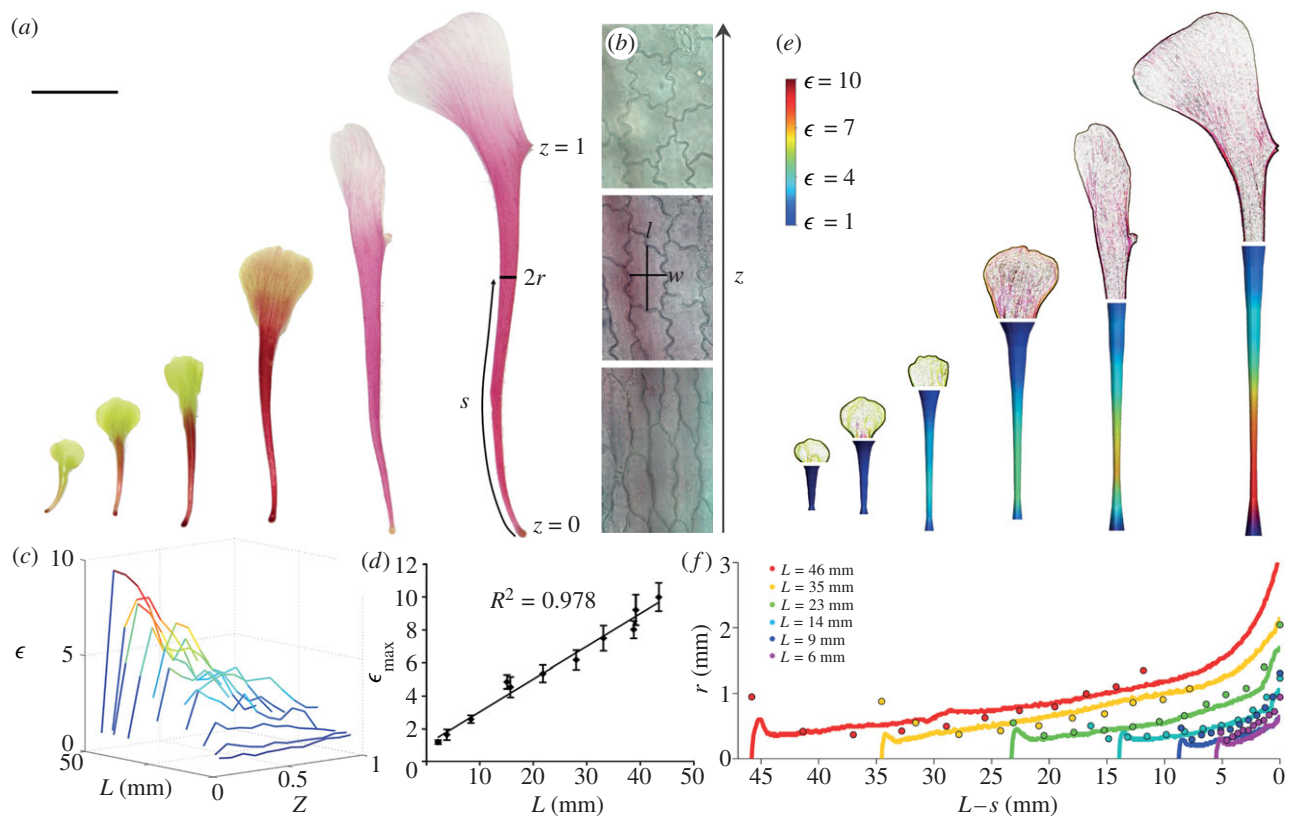
Figure 3. Cell anisotropy drives *A. coerulea* petal spur development. (*a*) Developmental series of *A. coerulea* petals. Both cellular measurements and spur radius r are recorded at the position s as measured from the nectary tip along the length of the spur. To compare between developmental stages, the position along the spur is also measured by z, which increases from 0 at the nectary to 1 at the attachment point. (*b*) Light microscope images are analysed to determine cell anisotropy $\epsilon = l/w$ and cell area $A = kw$ at the position z along the spur. (*c*) Waterfall plot of ϵ versus z at different developmental stages measured by the spur length L. (*d*) The maximum cell anisotropy $\epsilon_{\max}$ is highly correlated with spur length L. (*e*) Using measurements of cell anisotropy and cell area, in concert with an initial spur determined by averaging experimental spur profiles, numerically calculated spur shapes are generated without any free parameters at the same developmental stages shown in panel (*a*). Numerical spurs are shaded according to local cell anisotropy. (*f*) Numerically calculated spur profiles (circles) are overlaid on experimentally measured spur profiles (solid curves). Scale bars, 1 cm.

multiple developmental stages, using environmental scanning electron microscopy at three equally spaced locations along the axis of the spur and one point on the petal blade, for a total of approximately 6500 independent cellular measurements (electronic supplementary material, figures S12–S13 and §M8).

There are three possible contributors to the diversity in *Aquilegia* spur length: variation in cell number, cell size or cell anisotropy. We have addressed the issue of cell number in two independent ways. First, as described above, we have demonstrated that all cell divisions cease in *A. vulgaris* petals at approximately 5 mm. At this stage, spurs from the other study species are indistinguishable, as are their cell size and shape, implying that cell number should not vary considerably between species. To verify this, we have also directly counted the number of cells in mature spurs from *A. canadensis*, *A. coerulea* and *A. longissima* flowers (figure 2*e*). We find that the number of cells in each species varies by less than 30 ± 21 per cent, whereas spur length varies by up to 600 per cent (electronic supplementary material, §M2).

Having eliminated cell number as the primary contributor to spur-length diversity, we expect to find that changes in cell size and/or cell anisotropy will be correlated with relative increase in spur length for each species. In figure 5*b*, we show that the relative increase

in cell area, A_f/A_i (final cell area at spur maturity/cell area at the initial stage) is uncorrelated with the ratio of final to initial spur length, L_f/L_i. Here, the initial spur length L_i is the length of the spur once cell divisions have ceased (about 7 ± 2 mm; figure 2*e*). However, the relative increase in cell anisotropy, ϵ_f/ϵ_i, is strongly correlated with the ratio of final to initial spur length (figure 5*c*). The R^2 value of 0.99 indicates that variations in cell anisotropy account for 99 per cent of the observed variation in mature spur length. Furthermore, each of the species follows the same growth curve (figure 5*d*), where total petal length, L_p, including the blade, is reported because spur-length measurements in attached young petals are obstructed by sepals. Thus, length differences between these species are achieved through variations in the duration of cell elongation. For example, the developmental duration of the shortest spur studied, *A. vulgaris*, is approximately 10 days, while in the longest spur studied, *A. longissima*, this duration is approximately 16 days, so that longer periods of cell elongation lead to higher cell anisotropy, and consequently longer petal spurs.

4. DISCUSSION
We have shown that the *Aquilegia* petal spur is initially formed by a short period of localized cell divisions
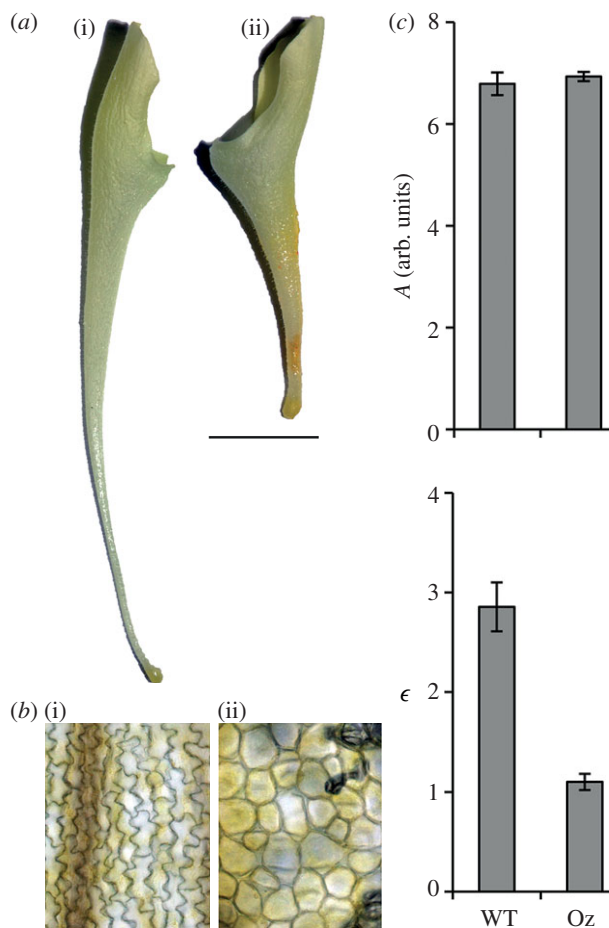
Figure 4. Cytoskeleton perturbations decouple isotropic cell expansion from cell anisotropy. (*a*) Oryzalin (Oz), a microtubule depolymerization agent, was applied to the entire surface of single *Aquilegia* spurs after they had achieved a short tubular shape of length $L \approx 1$ cm (ii). Untreated petal from the same flower is shown as a control (i). Photos of petals were taken approximately 6 days after initial application of oryzalin. (*b*)(i) Anisotropically shaped cells from untreated spur. (ii) Image of oryzalin-treated spur showing isotropically shaped cells. (*c*) Comparison of cell area A and anisotropy ϵ between cells from oryzalin-treated spurs ($n = 270$) and from untreated samples ($n = 127$). Scale bar, 1 cm.

followed by an extended process of collectively oriented cell elongation. Furthermore, diversity in spur length is mediated by variation in the degree of anisotropic cell elongation rather than the number or size of cells. The tight correlation of cell anisotropy with spur length suggests that even the extreme outlier *A. longissima* can reach its extraordinary spur length simply by increasing a single developmental parameter. Thus, minimal elaboration of an existing developmental mechanism can rapidly generate spur-length variation in the genus in concert with a specific ecological pressure, the presence of a pollinator with a dramatically longer tongue. Interestingly, there are taxa within the genera *Semiaquilegia* and *Urophysa*, which are very closely related to *Aquilegia*, that lack elongated spurs but produce small nectary cups or extremely short spurs [6,13,14], similar to very early developmental stages in *Aquilegia*. This implies that the evolutionary innovation underlying spur formation and the rapid radiation of *Aquilegia* may have been the mechanism of tuning cell anisotropy, which led to the elaboration of the nectary cup.
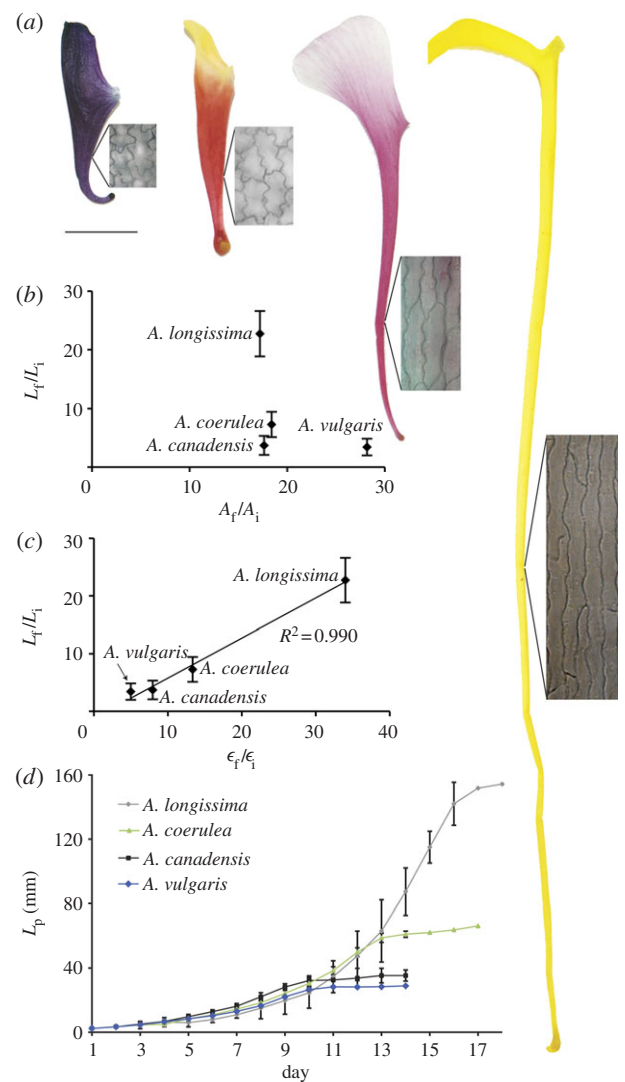
Figure 5. Cell anisotropy plays an essential role in spur-length diversity. (*a*) Petals from four different *Aquilegia* species. From left to right: *A. vulgaris*, *A. canadensis*, *A. coerulea* and *A. longissima*. Insets for each species show a cellular region of identical width of approximately 30 μm. (*b*) The ratio of final to initial spur length L_f/L_i versus the fractional increase in cell area A_f/A_i is plotted to show that changes in spur length are not correlated with changes in cell area ($R^2 = 0.233$, Pearson's $r = -0.482$). (*c*) L_f/L_i is plotted versus the fractional increase in cell anisotropy ϵ_f/ϵ_i, measured at $z \approx 1/3$, indicating that spur-length diversity is characterized by cell anisotropy ($R^2 = 0.990$, Pearson's $r = 0.995$). (*d*) Total petal length L_p is plotted versus time, demonstrating that all species follow the same growth curve but differ in developmental duration. Vertical error bars indicate range in initial spur length L_i and horizontal error bars in (*b*,*c*) are comparable with marker size. Scale bar, 1 cm.

It is useful to consider the sculpting observed in *Aquilegia* spurs in a broader context of tissue elongation, which is at the heart of organ morphogenesis. Tissue elongation without cell division can occur via a combination of two mechanisms: convergent extension driven by cell migration in animals [15], or changes in cell shape anisotropy in instances where cells are immobile, such as in plants [16]. In tissues with active cell division, oriented divisions followed by isotropic cell expansion can also result in tissue elongation. Since any of these

microscopic reorganizations would lead to indistinguishable macroscopic deformations, all of these possibilities must be considered in phenotypic analysis of tissue morphogenesis [16,17]. In the context of plant morphodynamics [18], our study has emphasized that in addition to differential cell division and isotropic cell expansion, differential cell anisotropy can also play a dominant role in evolutionarily significant shape change. Petal spur sculpting and spur-length diversity across the genus *Aquilegia*, even in its most extreme expressions, can be explained solely through variation in cell anisotropy. Developmental perturbations using oryzalin have further demonstrated that changes in cell anisotropy are dependent on cytoskeletal arrangement. We know from work done in model plants that several major hormone pathways, as well as perturbations of the cytoskeleton itself, can influence oriented cell elongation [10,19,20]. Contrary to what has been suggested in Lamiales [21], our developmental measurements imply that the duration of cell elongation plays a critical role in determining spur length. Genes underlying both hormone pathways that influence cell anisotropy and developmental duration should be explored as candidates for the control of spur development in *Aquilegia*, as well as for the genetic basis of new pollinator syndromes that are associated with speciation of the genus. Diversification in association with pollinators is often associated with correlated shape variation in floral organs such as stamens, styles, corolla tubes, petals and sepals [1,22,23], and raises the question of whether tuning cell anisotropy is exploited in other systems that exhibit evolutionarily significant morphological diversity.

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