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Understanding the development and evolution of novel floral form in *Aquilegia*

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Flowers of the lower eudicot *Aquilegia* (columbine) possess morphological innovations, namely elaborate petal spurs and a fifth distinct organ identity, the staminodium, that are well suited to the investigation of key questions in developmental evolution. The recent evolution of these characteristics combined with a growing set of genetic and genomic resources has provided insight into how the traits arose and diversified. The petal spur appears to represent a key innovation that diversified largely via modification of specific aspects of cell expansion. In the case of the staminodium, gene duplication has played a role in allowing a novel organ identity to be carved out of the traditional ABC program.

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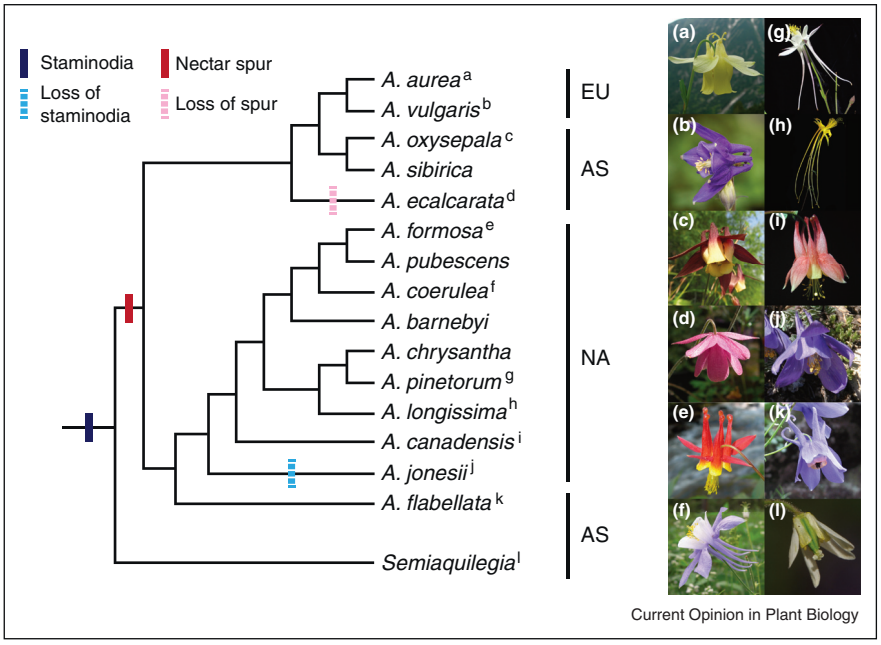
Introduction

The established plant genetic models have provided us with a detailed understanding of the genetic programs controlling the development of structures such as lateral organs, including the floral organs (reviewed [1,2]). However, we must recognize that these models capture only a very limited component of the enormous diversity in floral morphology. Commonly known as columbine, *Aquilegia* is a member of the basal eudicot family Ranunculaceae whose morphology and evolutionary history make it a powerful tool for studying the evolution of novel morphology [3,4]. The genus encompasses 70 recently diversified species, which although morphologically varied (Figure 1), remain largely interfertile [5]. In addition to this interesting recent history, the phylogenetic position of *Aquilegia*, roughly intermediate between the core eudicots and grasses, provides an attractive data point for deeper comparisons across the flowering plants. *Aquilegia* possesses five distinct types of floral organs: petaloid sepals in the first whorl; nectiferous, spurred petals in the second whorl; four to seven whorls of 10 stamens each; a whorl of 10 sterile organs termed the staminodia; and an innermost whorl of four to seven carpels (Figure 2a,d). Although the floral bauplans of the diverse *Aquilegia* species are very similar, the flowers differ in color, many aspects of spur morphology, and, in one case, the presence of staminodia. Several groups have been using a growing set of genetic and genomic tools to investigate the genetic basis of the novel floral features of *Aquilegia*. These tools include a high-quality Sanger-sequenced genome for *A.* × *coerulea* 'Goldsmith' [6] with detailed gene annotation utilizing multiple deeply sequenced expressed sequence tag (EST) and RNAseq libraries (e.g., http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=aquilegia), resequencing of 13 additional species as well as the sister genus *Semiaquilegia* (Figure 1), two bacterial artificial chromosome (BAC) libraries [7], and a well-established virus induced gene silencing (VIGS) protocol [8].

There are a number of immediate questions suggested by *Aquilegia*'s floral morphology in relation to the otherwise conserved ABC model of floral organ identity. The ABC model suggests that floral organ identity is established by the interaction of three classes of gene activity: A class alone determines sepals; A + B, petals; B + C, stamens; and C alone, carpels [9]. This model fails to explain, however, how a flower could produce two whorls of morphologically distinct petaloid organs, as in the sepals and petals of *Aquilegia*, or even more dramatically, an entirely novel fifth organ such as the staminodium. Expression and functional studies of A, B and C gene homologs in *Aquilegia* have shed light on these questions. In regard to A function, it has previously been demonstrated that this role is not well conserved, even within the core eudicots [10,11]. Consistent with this, the *Aquilegia* homologs of the Arabidopsis A gene *APETALA1* (*AP1*), *AqFL1a* and *AqFL1b*, control leaf morphogenesis and inflorescence structure but do not contribute significantly to flower development [12••]. In contrast, C class function, represented by homologs of *AGAMOUS* (*AG*), is generally well-conserved across the angiosperms in terms of promoting stamen and carpel identity as well as floral meristem determinacy. Ongoing functional studies of the *Aquilegia AG* paralogs *AqAG1* and *AqAG2* suggest that this is equally true for the loci, although a role in novel staminodium identity requires further investigation (B Sharma *et al.*, unpublished data).

This leaves us to focus on the role of the B class genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in Arabidopsis.

Figure 1



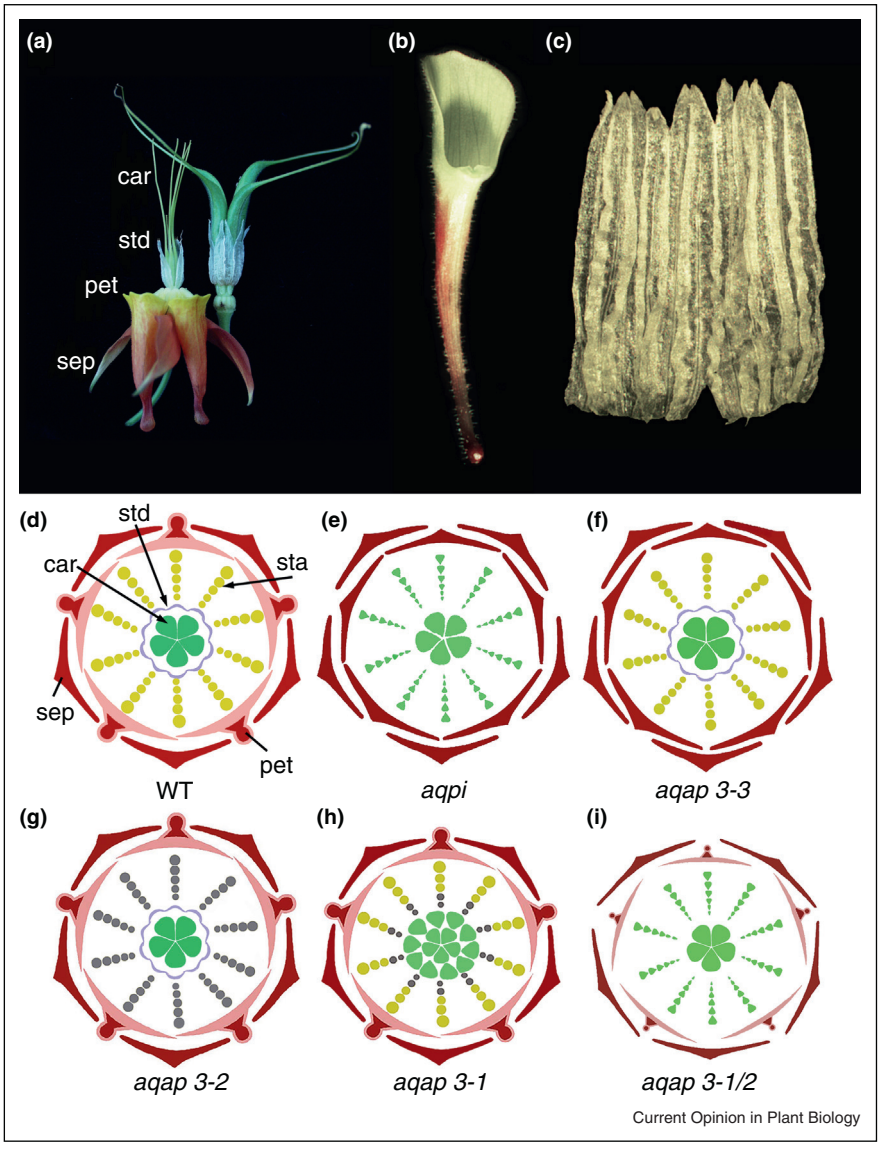
Simplified phylogeny of *Aquilegia* species relative to the sister genus *Semiaquilegia* (based on [22,30]). All species represented have been the subject of genome resequencing or, in the case of *A. jonesii*, deep RNA-seq. The evolution of several features is represented by colored bars, specifically the initial gain and subsequent loss of both petal nectar spurs and staminodia. The geographic distribution of the species is also indicated to the right of the phylogeny. EU = European, AS = Asian, and NA = North American. Panels (a)–(l) are photographs of the species, as indicated by the corresponding superscript letters. **(a)** *A. aurea*. **(b)** *A. vulgaris*. **(c)** *A. oxysepala*. **(d)** *A. ecalcarata*. **(e)** *A. formosa*. **(f)** *A. coerulea*. **(g)** *A. pinetorum*. **(h)** *A. longissima*. **(i)** *A. canadensis*. **(j)** *A. jonesii*. **(k)** *A. flabellata*. **(l)** *Semiaquilegia*. All photographs by SA Hodges except (c) and (k) by Hong-Xing Xiao.

They offer an obvious starting point since the novel features of *Aquilegia* involve both different forms of petaloidy and derivatives of the stamens. Early studies found that there are three major paralogous lineages of *AP3* in the Ranunculid order and, in *Aquilegia*, due to a more recent duplication, the genus actually possesses four *AP3*-like genes: *AqAP3-1*, *AqAP3-2*, *AqAP3-3* and *AqAP3-3b* [13,14]. These proteins all appear to function as heterodimers with the protein of the single *PI* homolog, AqPI, but each have distinct expression patterns [14]. *AqAP3-1* expression is initially broad but rapidly becomes restricted to the staminodia, *AqAP3-2* is also broad at early stages but is only consistently expressed in stamens, and both *AqAP3-3* and *AqAP3-3b* are entirely restricted to the petals, with *AqAP3-3b* expressed at much lower levels. Consistent with the dependence of each AP3 protein on AqPI, the latter is broadly expressed across the petals, stamens and staminodia. Furthermore, transient RNAi targeting *AqPI* demonstrates that the B functional domain in *Aquilegia* is expanded to include the staminodia as well as the canonical petals and stamens (Figure 2e) [14]. Following from these data, the questions that have received recent attention are: Do the *AP3* paralogs, in fact, show subfunctionalization and neofunctionalization as suggested by their expression patterns? and How has both petal and staminodium morphology diversified down-stream of organ identity?

Petal identity and diversification

Work in *Aquilegia* has also informed questions about the evolution of petal identity itself. The diversity of Ranunculid petal form, combined with a variability even of their presence within the family, prompted botanists to conclude that they evolved from stamens many times independently [15,16]. With the advent of gene expression studies, however, Rasmussen *et al.* [17] suggested that this diversity was instead controlled by a commonly inherited identity program, which was lost in some cases rather than gained multiple times. More recent work in *Aquilegia* has supported this hypothesis: the MADS-domain *AqAP3-3/3b* paralogs have been shown to specifically promote petal identity with no contribution to stamen or staminodium development (Figure 2f) [18]. Furthermore, similar studies in the distantly related Ranunculaceae genus *Nigella* have recovered the same result [19••,20•]. While this evidence does seem to argue for a conserved petal identity controlled by *AqAP3-3* orthologs, the question remained: What happens to these genes when petals are naturally lost? This point has been addressed by a study that examined multiple pairs of closely related genera in which one taxon had petals while the other lacked them [19••]. In every apetalous genus, expression of the *AqAP3-3* ortholog is dramatically reduced, if not completely eliminated, and the genomic loci show a variety of evidence of pseudogenization. All of these data

Figure 2



Novel floral organs of *Aquilegia* and homeotic RNAi phenotypes that affect their identity. **(a)** Flowers of *A. formosa*. On the left, a pre-anthesis flower with all stamens removed. The petaloid sepals (sep) and spurred petals (pet) are visible along with the inner staminodia (std) surrounding the carpels (car). Note that the staminodia are free from each other. On the right, a post-anthesis flower in which the sepals, petals and stamens have naturally abscised while the persistent staminodia are fused to form a continuous sheath that surrounds the carpels. Photograph by SA Hodges. **(b)** The spurred petal of *A.* × *coerulea* 'Goldsmith'. **(c)** The staminodial sheath of *A.* × *coerulea* 'Goldsmith'. Photographs in (b) and (c) by B Sharma. **(d)** Floral diagram for a wildtype (WT) flower showing five distinct organ identities: sepals (sep), petals (pet), stamens (sta), staminodia (std), and carpels (car). **(e)** Phenotype of *AqPI*-silenced flower (*aqpi*) in which petals are replaced by sepals and both stamens and staminodia by carpels. **(f)** In *AqAP3-3*-silenced flowers (*aqap3-3*), the petals are replaced by sepals with no effect on the other floral organs [18]. **(g)** *AqAP3-2*-silenced flowers (*aqap3-2*) show stunted, sterilized stamens, indicated in grey [32]. **(h)** *AqAP3-1*-silenced flowers (*aqap3-1*) display strong transformation of staminodia into carpels and some perturbation of inner stamen development [32]. **(i)** Double silencing of *AqAP3-1* and *AqAP3-2* (*aqap3-1/2*) results in complete transformation of all stamens and staminodia into carpels with no effect on petal identity but some reduction in size [32].

are, therefore, consistent with a model in which petal identity was commonly inherited but turned off in many separate instances. Of course, one may also ask why the loss of petals is so commonly tolerated in the family. One likely explanation is the presence of petaloid sepals in almost all of the taxa. This transference of primary attractive function from the second to the first whorl could have released constraint on the petals, allowing them to diversify in morphology or, on occasion, be lost [17,19••].

Having established the genetic basis of petal identity, we can now turn our attention to the elaboration of these organs. *Aquilegia* flowers feature a diverse array of petal nectar spurs, the shape of which is tightly associated with specialized pollinators that range from bees to hummingbirds to hawkmoths (Figure 1; [21,22]). Tight correspondence between a pollinator's tongue and the spur length in any particular species is considered a product of selection for pollen removal and receipt, driving spur length over a 16-fold range and reproductive isolation between species [22]. Combined with the explosive worldwide radiation of *Aquilegia* species after the evolution of the nectar spur, this novelty is thought to be the key innovation that enabled *Aquilegia* to become a textbook example of adaptive radiation [23]. Despite the clear ecological and evolutionary significance of spur length, however, almost nothing was known about *Aquilegia* spur morphogenesis until very recently.

Early hypotheses posited that spur development is caused primarily by 'meristematic knobs' thought to flank the spur attachment point, adding one cell at a time until the final spur shape is achieved [24]. Recently, however, spur growth has been shown to be an extreme example of organ curvature, progressing in two dynamic phases. During phase I, diffuse cell divisions occur throughout petal primordium but when the nascent spur cup reaches ~1 mm in length, the division domain contracts in a wave that begins at both the blade and base, progressing eventually toward the future nectary [25••]. The result is that cell divisions persist in the spur region for longer than in the blade and base, which promotes the initial outpocketing of the spur. This localized domain of cell division continues to contract until the spur reaches ~5 mm, when it enters phase II. This consists of highly anisotropic cell elongation that drives the bulk of spur growth and continues until the spur reaches its final length. The inference that spur shape ontogeny is governed by the these two simple phases was confirmed by modeling: spurs were computationally 'grown' using thousands of experimental measurements of cell area and anisotropy to achieve spur profiles that were in agreement with natural profiles and shapes [25••]. Another critical finding of this study is that the considerable variation in spur length across various *Aquilegia* species appears to be entirely controlled by variation in anisotopic cell elongation, rather than cell number. The degree of elongation is, in turn, controlled by the duration of the elongation period, which suggests that a macromorphological feature — spur length — is controlled by a cell level parameter, which is itself mediated by heterochronic shifts. A clear target of future work is to understand the genetic basis of this morphological novelty, which will take advantage of genome sequence resources that cover a wide range of species with various spur shapes and lengths, as well as the secondarily spurless *A. ecalcarata* (Figure 1d). This natural variation is a powerful tool because the species are interfertile, which allows genetic dissection through segregation studies [26] and there are even natural hybrid zones where association mapping is promising [27].

Aquilegia staminodia, more than sterilized stamens

Unlike the staminodia of many other angiosperms, which may simply be aborted stamens, the staminodia of *Aquilegia* are a continuous whorl of sterile, laterally expanded organs that surround the carpel whorl (Figure 2a,c). After anthesis, when all of the outer floral organs abscise, the staminodia stay attached to the receptacle and undergo late congenital fusion to form a cylindrical sheath [14]. The current hypothesis is that this reflects an herbivory deterrence mechanism protecting the early developing fruits [28], but this has yet to be tested in the field. In *Semiaquilegia*, the sister genus to *Aquilegia* (Figure 1l), similar organs are present in the same position, but they are more variable in number and morphology [29]. No other genus in the Ranunculaceae has similar staminodia in this position, suggesting that they evolved recently in a stepwise fashion, becoming sterilized in the last common ancestor of *Aquilegia* + *Semiaquilegia* ~8 mya and further elaborated in the lineage leading to *Aquilegia* ~6 mya [30•].

As described above, expression studies implicated the *AP3* paralogs *AqAP3-1* and *AqAP3-2* in the differential control of staminodium and stamen identity, respectively. Using VIGS, a transient RNAi approach, each of these paralogs has been silenced individually and in combination [32••]. In *AqAP3-2* silenced plants, stamens show broad anther necrosis and, in the most severe phenotypes, anthers were highly reduced to yield naked sterile filaments (Figure 2g), reminiscent of the underdeveloped staminodia seen in *Semiaquilegia*. There was no effect on the actual staminodia or other floral organs in *AqAP3-2*-silenced flowers, however. In the *AqAP3-1* knockdowns, the primary phenotype was homeotic conversion of the staminodia toward carpel identity with some weak necrosis or transformation to carpel identity in the inner stamens (Figure 2h). These findings would appear to suggest that *AqAP3-2* is essential to the proper development and fertility of the stamens while the main role of *AqAP3-1* is staminodium identity with some gradient of influence on the innermost stamens. These conclusions are supported by the double silencing phenotype, in which all of the stamens and staminodia were strongly transformed to carpels (Figure 2i). There was also some reduction in petal size but no obvious effect on identity. These findings reveal a complex picture of a modified ABC model following gene duplication. While *AqAP3-3* experienced a relatively ancient subfunctionalization to control only petal identity, more recent evolutionary changes to the *AqAP3-1* and *AqAP3-2* paralogs have allowed the definition of a new organ identity derived from the innermost

whorl of stamens. Both *AqAP3-1* and *AqAP3-2* are broadly expressed throughout the stamen/staminodium domain at very early stages and it appears that this early *AqAP3-1* expression is sufficient to promote some degree of stamen identity. Because of the later establishment of differential expression, we see that proper stamen development requires *AqAP3-2* while staminodium identity depends on *AqAP3-1*. Again, the available genomic resources for *Aquilegia* as well as its sister genus *Semiaquilegia*, will hopefully facilitate a better understanding of how this differential expression pattern evolved, as well as how the entire staminodium identity program was derived from the ancestral stamen program.

Conclusion

Aquilegia's recent evolutionary history makes it a particularly useful model for studying traits related to the evolution of floral novelty. That being said, there are many other developmental features of interest where *Aquilegia*, along with its genomic resources, will represent an important model, including compound leaves, cymose inflorescence structure, flower color, perennial life-form and the requirement for vernalization in controlling flowering time. For instance, the genus has been used to demonstrate that the role of *CUC* homologs in the dissection of leaf margins is deeply conserved across eudicots [31]. As our tools continue to improve, these comparisons will grow even more powerful.

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Functional demonstration the subfunctionalization and neofunctionalization of the *AqAP3-1* and *AqAP3-2* paralogs. Data also suggest potential auto-regulatory or cross-regulatory interactions among the *AP3* paralogs.