Evolving Ideas on the Origin and Evolution of Flowers: New Perspectives in the Genomic Era

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ABSTRACT The origin of the flower was a key innovation in the history of complex organisms, dramatically altering Earth's biota. Advances in phylogenetics, developmental genetics, and genomics during the past 25 years have substantially advanced our understanding of the evolution of flowers, yet crucial aspects of floral evolution remain, such as the series of genetic and morphological changes that gave rise to the first flowers; the factors enabling the origin of the pentamerous eudicot flower, which characterizes ~70% of all extant angiosperm species; and the role of gene and genome duplications in facilitating floral innovations. A key early concept was the ABC model of floral organ specification, developed by Elliott Meyerowitz and Enrico Coen and based on two model systems, *Arabidopsis thaliana* and *Antirrhinum majus*. Yet it is now clear that these model systems are highly derived species, whose molecular genetic-developmental organization must be very different from that of ancestral, as well as early, angiosperms. In this article, we will discuss how new research approaches are illuminating the early events in floral evolution and the prospects for further progress. In particular, advancing the next generation of research in floral evolution will require the development of one or more functional model systems from among the basal angiosperms and basal eudicots. More broadly, we urge the development of "model clades" for genomic and evolutionary-developmental analyses, instead of the primary use of single "model organisms." We predict that new evolutionary models will soon emerge as genetic/genomic models, providing unprecedented new insights into floral evolution.

KEYWORDS ABC model; basal angiosperms; evo-devo; fading borders model: floral diversity; flower evolution; Pentapetalae

THE origin of the flower during the late Jurassic to early Cretaceous eras (most recent estimates are between 150 and 190 MYA; Magallón *et al.* 2015) was a key evolutionary innovation that profoundly altered the Earth's biota. Flowering plants (angiosperms), with reproductive security and speed conferred by the flower, replaced other seed plants in most ecosystems. Diversification of flowers and the resulting fruit spurred coevolutionary change in pollinators and dispersers, with subsequent wide-ranging effects on herbivores, mycorrhizae, and other interacting organisms. The development of human civilization during the past 10 millenia was likewise closely linked to the flower, as seeds and fruit—especially grains—are the basis of agriculture in both agrarian and modern society. Thus, elucidation of the genetic basis of the origin and evolution of the flower has fundamental implications for both our understanding of organismal evolution and our ability to increase food production through bioengineering of key angiosperm crops.

A broad spectrum of multidisciplinary research involving phylogenetics, developmental genetics, and genomics spurs this work and facilitates revised views of floral evolution. One key element has been the development of a robust phylogenetic framework for the angiosperm branch of the Tree of Life (*e.g.*, Soltis *et al.* 2011) to place the developmental genetics of the flower in the appropriate evolutionary context (evolutionary-developmental biology, evo-devo). Accordingly, the original genetic models used to unravel flower developmental genetics, *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz 1991), are highly derived rosid and asterid species, respectively, embedded within the core eudicot clade of angiosperms (Figure 1). Key aspects of the

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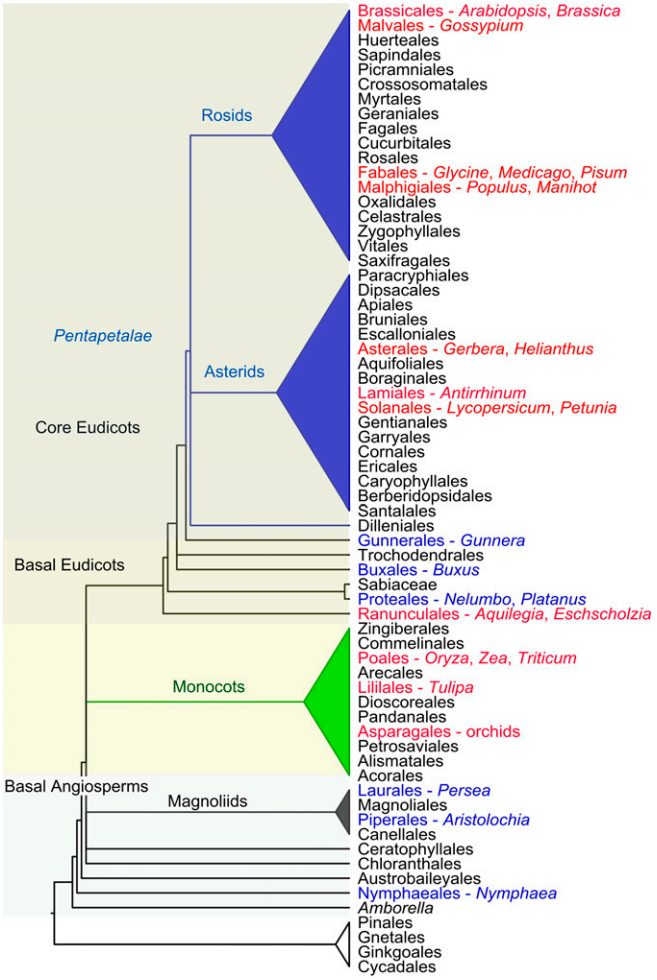

Figure 1 Summary tree of seed plant phylogeny showing the main lineages of flowering plants and the sister group, the extant gymnosperms. Species with established resources for flower developmental genetics, indicated in red, are distributed predominantly among the asterid and rosid clades of the *Pentapetalae*. Additional "evolutionary models," shown in blue, are needed to address questions regarding the genetic basis of major transitions in floral evolution.

genetics of *Arabidopsis* and *Antirrhinum* flower development also operate in genetic models of the highly derived grass family (Poaceae), including *Oryza* and *Zea* (Mena *et al.* 1996; Ambrose *et al.* 2000), which are nested within the monocot clade. Despite this apparent conservation across much of angiosperm diversity, a synthesis of comparative molecular studies suggests that the floral genetic programs of *Arabidopsis* and *Antirrhinum* are evolutionarily derived, and a new paradigm (described below) is necessary to describe the early evolution of flowers (Soltis *et al.* 2002, 2006a,b, 2007, 2009a; Kanno *et al.* 2003; Albert *et al.* 2005; Kim *et al.* 2005; Chanderbali *et al.* 2006, 2009, 2010; Shan *et al.* 2006; Kramer *et al.* 2007; Theissen and Melzer 2007; Broholm *et al.* 2008; Specht and Bartlett 2009; Rasmussen *et al.* 2009; Yoo *et al.* 2010a,b; Brockington *et al.* 2013; Ronse de Craene and Brockington 2013; Hileman 2014a,b; Specht and Howarth 2015; Glover *et al.* 2015; Galimba and Di Stilio 2015).

In this paper, we will review central tenets of floral developmental evolution, linking specific floral innovations to known genetic programs and propose new directions for understanding the genetic bases for evolutionary diversification of flowers. We focus on three scales of evolutionary innovation: the origin of the first flowers; the origin of flowers of *Pentapetalae*, a major subclade representing ~70% of living angiosperm species; and the origin of specific floral innovations. See Table 1 for a glossary of terms related to this article.

Conservation and Divergence in Floral Morphology and Developmental Genetics

Origin and evolution of floral developmental genetics

Twenty-five years ago, a combinatorial genetic model for the specification of floral organ identity, the so-called ABC model, was proposed by Enrico Coen and Elliot Meyerowitz (Coen and Meyerowitz 1991), based on studies of two of the major plant model systems of the time, *Arabidopsis thaliana* and *Antirrhinum majus*. Per this model, floral organ identities are specified through the action of three key gene functions (Figure 2) such that A function alone specifies sepals, A and B functions together determine petals, combined B and C functions specify stamens, and C function alone determines carpels (*e.g.*, Bowman *et al.* 1989; Irish and Sussex 1990; Schwarz-Sommer *et al.* 1990; Coen and Meyerowitz 1991; Ma 1994). Subsequently, D and E functions were described and added to the model, with D controlling aspects of ovule development and E interacting with A, B, and C functions to specify organ identity (*e.g.*, Colombo *et al.* 1995; Pelaz *et al.* 2000; Honma and Goto 2001; Ma 2005). Given the requirement of E-class genes for floral organ specification, the ABC model is now often referred to as the ABCE model. In *Arabidopsis*, the A-function genes are *APETALA1* (*API*) and *APETALA2* (*AP2*), B function is provided by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), C function by *AGAMOUS* (*AG*), and E function by multiple *SEPALLATA* genes (*SEP1–4*). All but one (*AP2*) of the ABCE genes are members of the MADS-box gene family (Jofuku *et al.* 1994; Ma and dePamphilis 2000; Becker and Theissen 2003).

The ABCE model was developed through observations of homeotic conversion of floral organs in genetic mutants. For example, sepals replace petals and carpels replace stamens in B-function mutants, while C-function mutants exhibit homeotic conversion of stamens into petals (Bowman *et al.* 1989; Coen and Meyerowitz 1991). Recognition that such dramatic changes in floral structure can be rapidly obtained by disrupting individual ABCE functions soon led to the suggestion that evolutionary changes in floral form might involve shifts of ABCE functions across spatial domains of the flower, or the "shifting boundary" hypothesis (van Tunen *et al.* 1993; Bowman 1997; Albert *et al.* 1998; Kramer *et al.* 2003). For example, instead of a dimorphic perianth that is differentiated into sepals and petals, an entirely petaloid perianth could develop through the activation of B function in an organ that would be positionally homologous to a sepal. This genetic

Table 1 Glossary

Glossary of Terms
Amborella: a genus that contains a single species, *Amborella trichopoda*, and widely regarded as the sister group of all other extant angiosperms.
ANA grade: the three successively basal-most branches (grade) of angiosperms; the acronym ANA derives from the names of its three constituent lineages, *Amborella*, Nymphaeales, and Austrobaileyales.
Asterids: one of the two major subclades of core eudicots, the other being the rosids. The asterids are characterized by fused petals (sympetaly). Examples include potato, tomato, and sunflowers.
Austrobaileyales: one of the three ANA-grade lineages. Includes the spice "star anise."
Basal angiosperms: an informal name for the flowering plants outside of the large, and derived, eudicot and monocot clades. They include the ANA grade and magnoliids.
Basal eudicots: an informal name for a paraphyletic group comprising the eudicot lineages outside of the core eudicot clade.
Carpel: the female reproductive organ of a flower.
Core eudicots: a monophyletic group comprising all eudicots apart from the basal eudicots, ~70% of all angiosperm species.
Eudicots: the eudicots are the largest clade of flowering plants, characterized by pollen grains that exhibit three colpi or grooves paralleling the polar axis (tricolpate pollen).
Gymnosperm cones: the reproductive structure in gymnosperms composed of a central stalk densely covered with leaf-like organs (sporophylls); female cones bear ovules on the surface of their sporophylls; the sporophylls of male cones bear pollen sacs.
Nymphaeales: an order with three families of aquatic plants, Hydatellaceae, Cabombaceae, and Nymphaeaceae (water lilies). It is one of the three early-diverging basal angiosperm lineages that constitute the ANA grade.
Magnoliids: the largest clade of basal angiosperms. Familiar species include avocado, bay laurel, black pepper, cinnamon, magnolias, nutmeg, and tulip tree.
Monocots: the second largest clade of flowering plants, and one of the major groups into which the flowering plants have traditionally been divided. They are characterized by seeds with a single cotyledon (embryonic leaf) and many other synapomorphies.
Pentapetalae: all core eudicots except Gunnerales.
Perianth: a collective term for all parts of the flower external to the stamens and carpels.
Petals: the whorl of floral organs, usually colored, that surrounds the stamens.
Rosids: one of the two major subclades of core eudicots, the other being the asterids. In contrast to the fused petals of asterids, the petals of rosids are free. Examples include many familiar plants, such as roses, peaches, and the legumes (*e.g.*, peanuts).
Sepals: the outer, often leaf-like, floral organs that surround the petals, stamens, and carpels.
Stamen: the male reproductive organ of a flower.
Synorganization: the close and precise interrelationship of floral organs of the same or different kinds during development, usually involving fusion of the parts involved.
Whole-genome duplication: the duplication of a complete genome, for example, of a diploid genome (with two copies of each chromosome) to form a tetraploid (with four copies of each chromosome); this term is sometimes used to refer to the process of duplication (*i.e.*, polyploidization) and sometimes in reference to the state of having multiple, duplicate genomes (*i.e.*, polyploidy).

mechanism appears to operate in some basal eudicots, such as *Aquilegia* (columbines, Ranunculaceae; Kramer *et al.* 2003), and perhaps many monocots, including *Tulipa* (tulips, Liliaceae; van Tunen *et al.* 1993; Kanno *et al.* 2003), in which sepals and petals differ only (or primarily) in position rather than in morphology.

The basal angiosperm lineages (the ANA grade of *Amborella*, Nymphaeales, and Austrobaileyales; Chloranthales; magnoliids) accommodate only a few percent of angiosperms (Drinnan *et al.* 1994) but exhibit tremendous floral diversity, particularly in the number and arrangement of floral organs (Figure 3), and are pivotal to questions about floral development in the earliest angiosperms. Developmental genetic studies conducted for basal angiosperms indicate that they have a broader pattern of expression of B- (and to a lesser extent, C- and E-) function homologs than do eudicots (Kim *et al.* 2005; Soltis *et al.* 2009a) and prompted the formulation of a new model, the "fading borders" model (*e.g.*, Buzgo *et al.* 2004, 2005; Soltis *et al.* 2006b, 2007; Chanderbali *et al.* 2010). The fading borders model interprets the gradual transition in floral morphology observed in basal angiosperms, magnoliids, and basal eudicots as reflecting a gradient in expression levels of floral organ identity genes across the developing floral meristem, where weak expression at the margin of the range of activity of a given gene overlaps with the expression of another regulator in adjacent organs. Gradually fading influence toward the periphery of broadly expressed organ identity functions, of B function in particular, imparts some features of one set of organs onto adjacent floral organs. Subsequent restriction of expression (and function) to specific regions of the floral meristem resulted in discrete whorls of morphologically distinct floral organs that together characterize the core eudicots. Genetic models are currently lacking among basal angiosperms, but petals in an *Arabidopsis* mutant that exhibits reduced B function are morphologically intermediate between petals and sepals (Bowman *et al.* 1989), offering genetic support for the fading borders concept.

Recognition that the B and C components of the ABCE system are conserved in the specification of reproductive organ development in gymnosperms (Winter *et al.* 1999; Theissen and Becker 2004) led to hypotheses of how genetic changes in the gymnosperm system could have produced the first flowers (Frohlich 2003; Baum and Hileman 2006; Theissen and Melzer 2007). Given that gymnosperm cones are unisexual, these evolutionary scenarios involve the modification of separate genetic programs for male (BC) and/or female (C) gymnosperm reproductive organs that would transform a unisexual cone into a bisexual structure with male organs (stamens) below female organs (carpels). Hypothesized genetic mechanisms include shifting gradients of

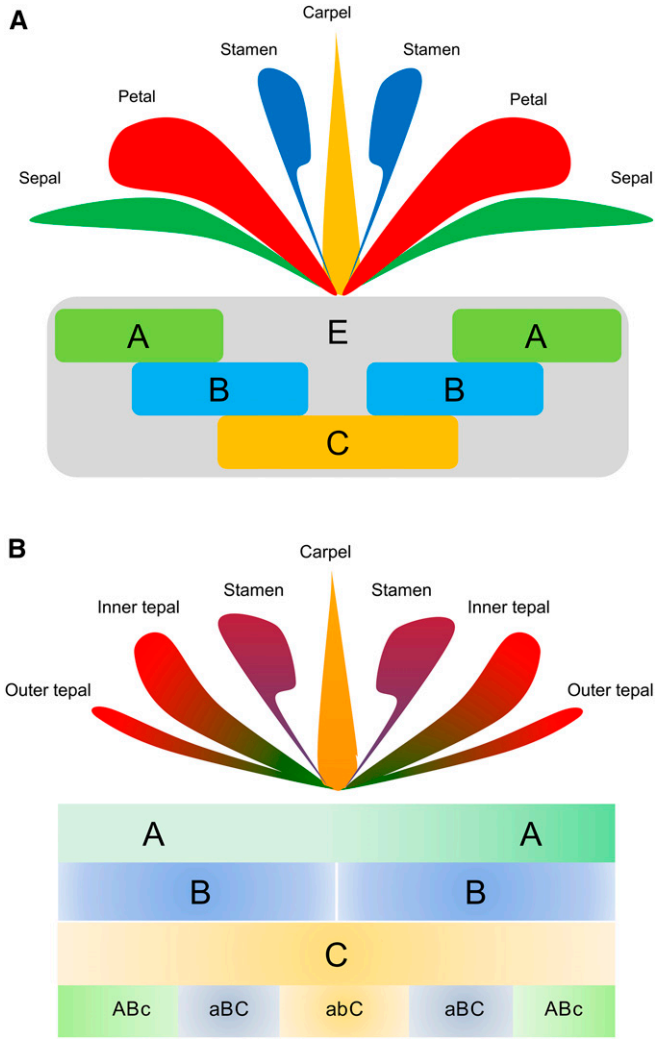

Figure 2 Classic ABCE model of floral organ identity *vs.* fading borders model. (A) The classic ABCE model specifies four morphologically discrete floral organs: sepals are produced where only A function acts, petals are produced where A and B functions overlap, stamens occur where B and C functions overlap, and carpels are produced where C function acts alone. (B) In contrast, in the fading borders model, the borders between A, B, and C functions are blurred to produce a gradual transition of organ identity programs across the floral meristem. Hence, floral organs are influenced by "ABc," "aBC," and "abC" activities, where lowercase font indicates lower functional influence. These three combinations of gene activities promote the development of morphologically intergrading petaloid organs (tepals), stamens, and carpels, respectively. Modified from Chanderbali *et al.* (2010).

organ identity functions in male ("out of male") or female ("out of female") gymnosperm cones (Baum and Hileman 2006; Theissen and Melzer 2007), or ectopic imposition of established female organ identity programs onto male cones in the "mostly male" scenario (Frohlich 2003). Further genetic transformations are also needed to produce a recognizable flower. These changes include condensation of the initial cone-like axis such that stamens appear to surround carpels, the origin of an external envelope of sterile perianth appendages surrounding the sexual organs (producing a structure similar to basal angiosperm flowers), and perianth dimorphism into sepals and petals to produce the typical flower specified by the ABCE model (Baum and Hileman 2006).

Clearly, the genetic regulators of floral organ specification are shared across angiosperms and were likely inherited from gymnosperm ancestors. Modifications to the activities of these key floral regulators may underlie the origin of the flower; can alter floral morphology, in some cases dramatically; and drive divergence in floral form. Given current evidence, the ancestral flower likely had a fading borders type of floral developmental program with broadly overlapping expression domains that produced morphologically intergrading floral organs, similar to those seen in a number of extant basal angiosperms. Over evolutionary time, restriction of ABCE function to specific regions of the floral meristem resulted in discrete whorls of the four morphologically distinct floral organs (sepals, petals, stamens, carpels) that characterize the *Pentapetalae* (Figure 2). Positive autoregulatory control and obligate heterodimerization are possible molecular mechanisms through which the ABCE model was refined via subsequent evolution (Theissen and Becker 2004; Theissen and Melzer 2007). Thus, although the ABCE model, given its priority and influence, has been considered the default floral developmental program, with variants viewed as derivatives of this program, comparative studies conducted in a phylogenetic context demonstrate instead that the model is evolutionarily derived.

Origin of the Pentapetalae flower

Sometime after the origin of the flower, a novel floral ground plan was established in the most species-rich major clade of angiosperms—the *Pentapetalae* (as defined in Cantino *et al.* 2007) (see Figure 1). The changes to floral organization that occurred with the origin of *Pentapetalae* include concentric whorls of floral organs, with parts typically in fives or multiples thereof (pentamery), and morphologically distinct perianth organs (sepals and petals). The genetic basis for the origin of this canonical floral ground plan represents one of the major unresolved mysteries of flowering plant evolution. Independent studies have identified three evolutionary events that correspond closely with the origin of *Pentapetalae*, but their precise roles are unclear. First, two whole-genome duplication (WGD) events are believed to have occurred in close succession prior to the origin of the *Pentapetalae*, but their exact positions are uncertain (Jiao *et al.* 2012). Second, it is well documented that duplicate copies of most flower development genes, which may have originated through these WGDs, are maintained in the genomes of *Pentapetalae* species (*e.g.*, Kramer and Irish 2000; Soltis and Soltis 2004; Kim *et al.* 2004; Zahn *et al.* 2005; Howarth and Donoghue 2006, 2009; Soltis *et al.* 2009a; Boyden *et al.* 2012; Pabón-Mora *et al.* 2014). Together, these genes pattern the development of morphological traits such as organ identity, symmetry, fusion, polarity, elongation, and growth, and thus have functions that could have contributed to the origin of a whorled pentamerous flower. Third, there appears to have been a shift from the fading borders model of

Figure 3 Floral variation in ANA grade, magnoliid, and basal eudicot angiosperms. Although comprising only a few percent of extant angiosperm species, these lineages exhibit enormous floral variation compared to the more canalized flowers of core eudicots and monocots. (A) *Nymphaea caerulea* (Nymphaeaceae; basal angiosperm). (B) *Austrobaileya scandens* (Austrobaileyaceae; basal angiosperm). (C) *Persea americana* (Lauraceae; magnoliid). (D) *Piper neesiasnum* (Piperaceae; magnoliid). (E) *Aristolochia veraguensis* (Aristolociaceae; magnoliid). (F) *Asimina incana* (Annonaceae; magnoliid). (G) *Magnolia champaca* (Magnoliaceae; magnoliid). (H) *Argemone albiflora* (Papaveraceae; basal eudicot). (I) *Anemone canadensis* (Ranunculaceae; basal eudicot). (J) *Ranunculus ficaria* (Ranunculaceae; basal eudicot). A is courtesy of Deborah Chanderbali; B is courtesy of Douglas Soltis; C is courtesy of Andre Chanderbali; and D–J are courtesy of Walter Judd.

floral developmental gene expression of basal angiosperms and basal eudicots to the canalized ABCE model in *Pentapetalae* (Chanderbali *et al.* 2009, 2010; Voelckel *et al.* 2010; Yoo *et al.* 2010b), but the precise phylogenetic location of this transition is uncertain.

Although flowers of *Pentapetalae* are considerably canalized (*i.e.*, fixed in the arrangement, merosity, and morphology of its organs) compared to early-diverging lineages of angiosperms (Endress 1996, 2006; Soltis *et al.* 2002), they often exhibit extensive modifications to their floral organs. Although it is not clear whether such modifications originated directly via natural selection or as side effects of developmental changes (the spandrels of San Marco phenomenon; Gould and Lewontin 1979), the canalization of the *Pentapetalae* flower may have facilitated some such alterations through "synorganization"—a close association, fused or otherwise, among floral organs. Synorganization is hypothesized to have led to the evolution of novel morphologies and functions and is dependent on a whorled phyllotaxis with a fixed number of floral parts, as was established in *Pentapetalae* (Endress 1990, 1996, 2006). A notable example of synorganization is the fusion of petals (sympetaly) into the tubular corolla—recognized as a morphological innovation for centuries (de Jussieu 1789; Reichenbach 1827)—that characterizes the large asterid clade of *Pentapetalae* and has itself been further modified multiple times during asterid evolution (Stuurman *et al.* 2004; Wu *et al.* 2007).

The flowers of monocots are also very stable in number and arrangement of floral organs and are typically trimerous. Although monocots do not exhibit the extent of synorganization present in *Pentapetalae*, the orchids provide a parallel example of this phenomenon (Endress 2015). Studies of floral modifications offer a wealth of next-generation research possibilities that promise new insights into angiosperm floral

innovations. Much progress has been made, for example, in understanding the genetic basis of sympetaly (Zhong and Preston 2015), petal differentiation (Huang and Irish 2015), and floral symmetry (Hileman 2014a,b). The available genetic data suggest that these innovations are regulated by genes that operate in parallel with, or downstream from, the ABCE organ identity program.

Origins of floral development genes

Phylogenomic analyses of the evolutionary history of functionally validated genetic regulators of flower development (232 *Arabidopsis* genes) suggest that ~70% belong to orthogroups (sets of homologous genes representing narrowly defined gene lineages) that originated in nonflowering plants, ~10% originated in the most recent common ancestor of angiosperms, and the remaining 20% evolved during angiosperm diversification (*Amborella* Genome Project 2013). Importantly, an ancient WGD event that occurred in the common ancestor of all angiosperms (Jiao *et al.* 2011) would have been the source of many new genes that contributed to the origin of the flower and other important angiosperm innovations (Buzgo *et al.* 2005; Zahn *et al.* 2005; De Bodt *et al.* 2005; *Amborella* Genome Project 2013). For example, many of the floral genes exist as paralogous gene lineages, likely due to this WGD event, in extant angiosperms. Gene Ontology (GO) annotations related to reproduction (flower development, reproductive developmental process, pollination, and similar terms) and several MADS-box gene lineages are overrepresented in this set of new genes (*Amborella* Genome Project 2013). They include the B, C, and E components of the ABCE program (*i.e.*, *AP3/PI*, *AG/STK*, *SEP1/SEP3*). On the other hand, many novel gene lineages arose through multiple rounds of WGD during angiosperm diversification (Soltis *et al.* 2009b, 2010), and some have acquired new functions in specific floral organs within evolutionarily derived angiosperm lineages (Irish 2006; Soltis *et al.* 2006b; Zahn *et al.* 2006).

Thus, it seems reasonable to conclude that: (1) orthologs of most floral genes existed long before their specific roles in flowering were established; (2) novel gene lineages first appeared with the origin of the angiosperms and probably contributed to the origin of the flower; and (3) after a functional flower evolved, genetic innovations continued as new genes originated and/or were recruited into floral genetic programs.

Moving Forward

Next-generation tools for next-generation evo-devo studies

Advances in next-generation sequencing (NGS) have revolutionized much of plant evolutionary biology (*e.g.*, Egan *et al.* 2012; Godden *et al.* 2012; Soltis *et al.* 2013), as well as biological research in general. It is now possible to obtain enormous amounts of genomic and transcriptomic sequence data for virtually any plant system that poses intriguing evolutionary questions and to do so at low cost. For example, a wealth of new data has been generated through projects such as the 1KP project (Matasci *et al.* 2014), the Floral Genome Project (Albert *et al.* 2005), and many clade-focused phylogenetic projects from the past decade. These technological advances provide unprecedented research opportunities to characterize and compare floral genetic programs to elucidate the genetic basis of novel floral ground plans. Toward this end, investigations of developmental gene regulatory networks (GRNs) that underlie floral diversity will be equally as valuable. Similar to GRN evolution in animal development (Levine and Davidson 2005; Peter and Davidson 2011), in plants, there is increasing evidence that: (1) most changes in floral morphology result from altered timing, location, and/or level (s) of GRN activity; and (2) similar GRNs are repeatedly coopted as similar adaptive traits are gained and lost (Specht and Howarth 2015). The GRNs and molecular mechanisms underlying the formation of *Arabidopsis* flowers have been studied in some detail, and technological advances such as translating ribosome affinity purification (TRAP) (Jiao and Meyerowitz 2010) promise even greater resolution in the future (Ó'Maoiléidigh *et al.* 2014). Improved knowledge of GRNs involved in floral organ identity, symmetry, cell type, floral color, and synorganization has become more attainable with technological advances in recent years, and further clarification of GRNs should be a goal in the study of flower developmental genetics.

Growing numbers of tools are now available to study gene function in nonmodel plants. Virus-induced gene silencing (VIGS) uses the plant's innate defense response to invading viruses to silence specific genes inserted in modified viral genomes (Dinesh-Kumar *et al.* 2003). This technique overcomes the time-consuming steps of genetic transformation (specific genes can be silenced in a few weeks) and is limited primarily by the susceptibility of plants to infection by VIGS vectors (Senthil-Kumar and Mysore 2014). VIGS has been applied successfully in a diverse range of eudicot and monocot species (Becker and Lange 2010), including functional investigations of genes involved in floral organ identity and symmetry in basal eudicots and core eudicots (*e.g.*, Gould and Kramer 2007; Sharma *et al.* 2011; Hidalgo *et al.* 2012; Gonçalves *et al.* 2013; Preston *et al.* 2014).

Another recently developed avenue of research is genome editing via the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas (CRISPR/associated) system (Sorek *et al.* 2013). Whereas VIGS reduces post-transcriptional gene activity, the CRISPR/Cas system facilitates genome editing by taking advantage of the Cas9 nuclease and a single guide RNA (sgRNA) to target a specific sequence in the genome. In addition to genome editing, which facilitates efficient and precise reverse genetics, genome engineering, and targeted transgene integration experiments, the CRISPR/Cas technology is suitable for studies of gene regulation through both transcriptional activation and repression. Moreover, the type III-B CRISPR/Cas system facilitates post-transcriptional silencing of gene expression in a manner that may be more specific than VIGS-mediated RNA interference (Bortesi and

Fischer 2015). The CRISPR/Cas system can be delivered through *Agrobacterium*-mediated transformation of tissue culture preparations and may be applied in any angiosperm species amenable to these techniques. Thus far, CRISPR/Cas has been successfully applied in several eudicot and monocot species, including *A. thaliana*, *Citrus sinensis*, *Nicotiana benthamiana*, *Oryza sativa*, *Solanum lycopersicum*, *Sorghum bicolor*, *Triticum aestivum*, and *Zea mays* (Bortesi and Fischer 2015).

From model species to model clades

As next-generation techniques for assaying gene expression and function become more accessible, it may be increasingly feasible to apply these methods to multiple species within a clade of interest. Using a "model-clade" approach (*i.e.*, not just a single species but a group of species within a defined clade targeted for gene expression, functional, and/or classical genetics studies), research questions can focus on related taxa that often encompass multiple trait gains, losses, or modifications (see Howarth and Dunn 2016). For instance, a model clade could include a single shift in a trait of interest as well as multiple independent iterations of similar shifts. Moreover, a model clade can include transitional forms of the trait of interest, facilitating investigations of the stepwise evolution of evolved gene function and phenotypic change. A model clade would therefore possess a number of features that would make it useful for floral evo-devo studies: (1) a well-supported species phylogeny; (2) high diversity of floral traits; and (3) multiple species, spread throughout the clade, that are easy to obtain and amenable to gene expression and/or functional techniques. These species need not be sister taxa, but are chosen to represent phenotypic variation across the clade. Studies of model clades therefore could rapidly provide clues about the genetic basis of evolutionary change that would not be achievable via the analysis of a single species from that clade. This model clade approach is well exemplified by evo-devo studies of orchid flower development, derived from comparisons of wild-type and peloric terata (Mondragón-Palomino 2013). Other examples include the evolution of zygomorphy in Zingiberales (Bartlett and Specht 2010, 2011), Dipsacales (Howarth *et al.* 2011) and Malpighiaceae (Zhang *et al.* 2010; Zhang *et al.* 2013a), petal evolution in Ranunculaceae (Zhang *et al.* 2013b) and Aizoaceae (Caryophyllales; Brockington *et al.* 2012, 2013), and the origins of petaloid bracts in *Cornus* (Cornaceae; Feng *et al.* 2012).

NGS technology can thus be applied to generate the data required to characterize floral GRNs; these GRNs, in turn, can be compared to identify candidate regulatory changes underlying floral evolutionary shifts. These "candidate genes" may then be functionally investigated *in planta* via CRISPER/Cas and related systems. Efforts to develop such integrative research programs are needed. Here, we advocate for avenues of research where these tools can be relatively easily developed and applied toward a more complete understanding of the genetic programs that underlie evolutionary changes in flowers.

Evolution of floral developmental genetic programs

Evolutionary genetic scenarios for the origin of the flower involve shifting gradients of gene expression/function in gymnosperm cones (out of male or out of female), reminiscent of the fading borders program of basal angiosperm flowers, or ectopic imposition of established female identity programs on male organs (mostly male) similar to the shifting boundary concept (see Theissen and Melzer 2007). Elucidation of the genetic programs of flowers that appear to represent intermediate steps in the transition from bisexual cone to flower, as seen in basal angiosperms and basal eudicots (Figure 2) is pivotal to understanding the origin and evolution of flowers, and floral diversification generally. For example, what is the evolutionary origin of the ancestral floral perianth? How are the spatial distribution and relative strengths of organ identity functions in basal angiosperm flowers determined? How are these determinants of organ identity functions manifested in monocots and basal eudicots *vs.* core eudicots such as *Arabidopsis*? To address such questions, new evolutionary model systems among the basal angiosperms and basal eudicots are necessary.

Among basal angiosperms, there are a few phylogenetically pivotal species with good potential to be tractable genetic models. Several members of *Nymphaea* (water lilies; Nymphaeaceae) have small genomes (*e.g.*, *Nymphaea thermarum*, *Nymphaea caerulea*, and *Nymphaea capensis*; Pellicer *et al.* 2013) and are easily cultivated in aquaria or tubs. Collections of mutant water lilies could be maintained in greenhouse research facilities to facilitate forward genetic screens. *N. thermarum* has already been proposed as a potential model (Povilus *et al.* 2014), on account of its rapid life cycle (5–6 months), small size, an apparently selfing breeding system, and relatively small genome- its haploid genome size (1C value) is 0.51 pg (~500 Mb), which is approximately three times the size of that of *Arabidopsis*. Likewise, a small genome (1C = 0.58), ease of culture, and ongoing genome sequencing as part of the *Amborella* Genome Project make *N. caerulea* another attractive target among the water lilies. *Cabomba caroliniana* has also been proposed as a possible genetic model for basal angiosperms (Vialette-Guiraud *et al.* 2011). It is also easily cultivated, but has a large genome with a 1C value of 3.55 pg (Pellicer *et al.* 2013), which is considerably larger than either of the above-mentioned species of *Nymphaea*. Importantly, *Nymphaea* species have been interbred by horticulturalists for >100 years to obtain hybrids with desirable floral characteristics (Les *et al.* 2004), indicating that this group could be used as a model clade in classical genetic studies.

Among magnoliids, *Aristolochia fimbriata* (pipevine; Aristolochiaceae) has been proposed as a potential experimental system and has numerous features that facilitate genetic studies (Bliss *et al.* 2013). Whereas most magnoliids are woody, this species is herbaceous, easily cultured with a rapid life cycle (3 months), transformable, and can be regenerated via tissue culture. Notably, flowers of *Aristolochia* exhibit synorganization in the perianth and are zygomorphic (bilaterally

symmetrical), representing a derived floral state among the basal angiosperms (Soltis *et al.* 2005; Judd *et al.* 2007). The genome size is also small (1C = 0.45; Bliss *et al.* 2013), but no genome sequencing is underway. *Persea americana* (the avocado; Lauraceae) is also a potential candidate among magnoliids. This commercially valuable crop species is amenable to genetic transformation, tissue culture, *in vitro* mutagenesis, and related technologies such as cryopreservation, as well as *in vitro* and *ex vitro* micrografting to circumvent the long juvenile period (reviewed in Chanderbali *et al.* 2008). A draft nuclear genome sequence (1C = 0.92; Arumuganathan and Earle 1991) is also close at hand (L. Herrera-Estrella, V. A. Albert, A. Herrera-Estrella, M. Rendon, and E. Ibarra-Laclette, personal communication). As tissue culture protocols are already in place for both of these magnoliid species, it should be possible to conduct CRISPR/Cas-mediated genetic manipulation of the ABCE genes and other candidates that emerge from RNA-sequencing (RNA-Seq) and GRN analyses.

Among basal eudicots, genome sequences are currently available for members of Ranunculales (*Aquilegia coerulea*; Kramer 2009) and Proteales (*Nelumbo nucifera*; Ming *et al.* 2013), but similar efforts are needed for representatives of other basal eudicot lineages. Exemplars from economically important groups (*e.g.*, poppies, Papaveraceae; plane tree, Platanaceae; boxwood, Buxaceae) could be targeted for complete genome sequencing. A genome sequence for a basal core eudicot would also be an important evolutionary reference, with *Gunnera* (Gunneraceae), the sister lineage of *Pentapetalae*, as a logical candidate. Indeed, efforts are underway to obtain a genome sequence for *Gunnera manicata* (L. Fay-Wei, personal communication). All of these resources would be invaluable to investigations of the evolutionary transition from basal eudicots to *Pentapetalae*. Promising target species for CRISPR/Cas-mediated genome editing and VIGS are members of Ranunculales, in which the application of reverse genetics is relatively well advanced, as in *Aquilegia* (Gould and Kramer 2007; Rasmussen *et al.* 2009; Kramer and Hodges 2010; Galimba and Di Stilio 2015) and *Eschscholzia* (Tekleyohans *et al.* 2013).

Summary: New Evo-Devo Approaches for Understanding Flower Developmental Genetics

Studies of genetic model plants, primarily *Arabidopsis*, have identified a limited number of transcription factors with crucial roles in floral development and led to the formulation of the ABCE model. This elegantly simple model appears to have its evolutionary roots in a gymnosperm "BC" system that has been modified and elaborated during flowering plant evolution. At the inception of the ancestral floral development program, specification of floral organ identity was likely deployed through a fading borders program, which still appears to specify the morphologically intergrading floral organs of basal angiosperms such as *Amborella* and water lilies. Sharpened borders of organ identity functions likely underlie the origin of the canalized *Pentapetalae* flower, as seen in *Arabidopsis*. Moreover, shifting borders of petal identity functions appear to promote the development of identical petaloid organs in the perianth of some flowers (*e.g.*, tulips). Imposed on these fundamental changes in floral form, still further modifications are evident in individual lineages of flowering plants, including multiple origins of bilateral symmetry and synorganization with its attendant novelties.

Comparative studies of orthologs and/or homologs of known floral regulators across angiosperms often suggest conserved roles in specific floral traits; however, they also highlight ample opportunities for neo- or subfunctionalization of duplicated genes as a consequence of multiple WGDs during the diversification of angiosperms. These findings underscore the likelihood that the regulation of flower development in distantly related angiosperms might involve genes that are not orthologous to known candidate genes, and/or the regulatory networks may be substantially different. To advance from a comparative approach based on candidate genes to a more mechanistic account of floral diversity, the establishment of collections of mutant phenotypes in phylogenetically relevant nonmodel plant species would be especially valuable. Although these resources are currently not available, forward genetic approaches and/or high-throughput transcriptome sequencing combined with reverse genetic screening may increasingly be feasible in select basal angiosperm and basal eudicot species. The development of these systems will herald a new generation of multidisciplinary evo-devo research during which many new plant systems can be the focus of study—species that afford the opportunity to address questions of floral evolution and organization that cannot be addressed with the current set of model systems. The development of these approaches would rapidly elucidate evolutionary changes in the regulatory networks underlying floral development.

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