Tansley review

Adaptation in flower form: a comparative evodevo approach

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Summary

Evolutionary developmental biology (evodevo) attempts to explain how the process of organismal development evolves, utilizing a comparative approach to investigate changes in developmental pathways and processes that occur during the evolution of a given lineage. Evolutionary genetics uses a population approach to understand how organismal changes in form or function are linked to underlying genetics, focusing on changes in gene and genotype frequencies within populations and the fixation of genotypic variation into traits that define species or evoke speciation events. Microevolutionary processes, including mutation, genetic drift, natural selection and gene flow, can provide the foundation for macroevolutionary patterns observed as morphological evolution and adaptation. The temporal element linking microevolutionary processes to macroevolutionary patterns is development: an organism's genotype is converted to phenotype by ontogenetic processes. Because selection acts upon the phenotype, the connection between evolutionary genetics and developmental evolution becomes essential to understanding adaptive evolution in organismal form and function. Here, we discuss how developmental genetic studies focused on key developmental processes could be linked within a comparative framework to study the developmental genetics of adaptive evolution, providing examples from research on two key processes of plant evodevo – floral symmetry and organ fusion – and their role in the adaptation of floral form.

I. Introduction

For decades, developmental geneticists have been elucidating the genes and genetic pathways involved in floral development in model species in laboratory environments. Simultaneously, evolutionary biologists have investigated genetic variation in natural populations as a means of understanding the genetic architecture underlying the development and evolution of adaptive (= heritable

beneficial phenotype) traits (Mackay *et al.*, 2009). Our next step is to determine which naturally occurring genetic changes affect ecologically relevant phenotypes (Colautti *et al.*, 2012) and how natural selection acts upon those phenotypes to confer adaptive evolution. While the underlying genetic networks are often conserved across plant lineages, genes identified to be important in the regulation of phenotypic changes for wildtype and mutant plants in model species may not translate into the types of genetic and phenotypic changes that are selected upon and affect survival and reproductive fitness under natural environmental conditions. Additionally, rampant polyploidy in natural populations results in duplicate gene copies that can undergo differential selection and allow for temporal and positional alternations in genetic networks to emerge, conferring novel phenotypes upon which selection may act.

Large numbers of comparative studies have demonstrated that changes in expression patterns of developmental genes are correlated with the evolution of morphological phenotypes (Remington & Purugganan, 2003; Zhang *et al.*, 2008; Bartlett & Specht, 2011; Howarth *et al.*, 2011; Mondragón-Palomino & Theissen, 2011). These data underlie the 'candidate gene' approach, in which genes that are functionally determined to cause particular phenotypes when mutated are hypothesized to be responsible for similar phenotypic changes during evolution. This approach requires two assumptions: one is that genes and gene regulatory networks (GRNs) responsible for developmental processes are conserved over large evolutionary distances. The second is that changes in the regulation of these genes, resulting in altered temporal and spatial expression patterns, affect the phenotype and thus are responsible for fixed differences in organismal morphology.

For many studied developmental processes, these two assumptions appear to hold true. For example, changes in the expression of the TCP gene lineage during floral development of *Antirrhinum* were shown to cause alterations in petal morphology in this model species, resulting in a shift from bilaterally symmetrical to radially symmetrical flowers (Luo *et al.*, 1996). Subsequent studies demonstrated that this same class of genes was responsible for the evolution of symmetry across many lineages of flowering plants, with similar yet independently evolved genetic mechanisms underlying differential gene expression patterns (Cubas *et al.*, 2001; Citerne *et al.*, 2003; Howarth & Donoghue, 2005, 2006; Feng *et al.*, 2006; Zhang *et al.*, 2013). Thus, a relationship was drawn between the differential expression of the TCP developmental gene(s) and phenotypic variation associated with fixed morphological diversification across angiosperm flowers. This fixed diversity is demonstrated across large evolutionary distances and across floral organ types, demonstrating the conserved nature of the TCP gene lineage and its role in floral symmetry patterns.

What is not clear from these studies, however, is the diversity of mechanisms by which the TCP genes may underlie changes in floral symmetry across these diverse lineages, or the selection process by which the genetic modifications underlying shifts in patterns of symmetry occurred. For instance, while the expression of TCP genes is spatially restricted in similar ways in groups in which bilateral symmetry has evolved independently, the expression patterns are not identical for each evolutionary event (Hileman, 2014a). Additionally, TCP gene expression is acting to regulate a cascade of genes that vary in their copy number and expression patterns (Hileman, 2014b). Studies in both model systems and in diverse plant lineages have demonstrated that floral morphology is not controlled by a single candidate symmetry gene or even a single set of genes, but rather by a complex network of genes that regulate symmetry patterns and lead to the diversity in shape within lineages (Hileman, 2014b). Differential expression and interactions of the network genes can underlie symmetry differences among flowers by influencing characters such as petal shape, stamen growth and cell type differentiation within flowers (Luo *et al.*, 1996; Almeida *et al.*, 1997; Galego & Almeida, 2002; Perez-Rodriguez *et al.*, 2005), and lineage-specific changes in copy numbers of these genes, each copy having the potential to acquire mutations in regulatory or coding regions, provide opportunities for novel developmental and morphological consequences.

Thus, in order to study the genetics of adaptation in floral form, the full developmental networks underlying organ morphology must be determined for the lineage under study. Using a comparative approach among related species or within polymorphic populations, mutation(s) causing an observed change in gene expression or alterations in protein interactions can be identified. In many cases, differential copy numbers of the genes involved in the networks may contribute to a shift in timing or placement of gene expression, resulting in novelty in form (Sharma *et al.*, 2014). Thus, both regulatory changes as well as novel or altered DNA binding and/or protein interactions (i.e. coding changes) must be investigated (see Box 1 for definitions). Here, we discuss how regulatory hypotheses of developmental evolution can be used to investigate the phenotypic changes associated with adaptive diversification, providing an evolutionary developmental biology (evodevo) approach for the study of adaptive evolution. Beginning with a review of the scientific discussion concerning the types of regulatory changes important for morphological evolution, we discuss how genetic changes can effect changes in organismal development that influence the evolution of developmental processes. We bring together a diverse set of literature in an attempt to find common ground between population-level approaches focused on adaptive evolution and the model system-driven studies of molecular mechanisms of development. We argue that only by understanding the regulatory networks that underlie organismal form and function in a comparative context can we determine how developmental evolution can lead to adaptive changes in morphology. Finally, we discuss two developmental processes (floral symmetry and organ fusion) and outline how understanding the GRNs underlying these processes will allow us to investigate how these processes have enabled the adaptive evolution of floral form.

II. Evodevo and the regulatory hypothesis

The regulatory hypothesis predicts that phenotypic evolution is, in most cases, associated with changes in gene expression (Hoekstra & Coyne, 2007; Prud'homme *et al.*, 2007; Carroll, 2008). Unlike the more stringent *cis*-regulatory hypothesis championed by Carroll (2008), the more general regulatory hypothesis makes no clear prediction about the molecular nature of the genetic changes

Box 1 Definitions of terms used in studying developmental evolution of adaptive phenotypes

Adaptive trait: a feature that has become prevalent in a population because of a selective advantage conveyed by that feature in the improvement of function; a heritable beneficial phenotype.

Adnation: fusion of different plant organs into a single structure, that is, stamens fused to petals.

Compitum: in a flower, this is the structure formed by the fusion of independent styles from separate carpels, providing a single pathway for pollen tube growth and pollination.

Connation: fusion of similar plant organs together, that is, petals fused to form a tube.

***Cis*-regulatory element:** a sequence of noncoding DNA that is located nearby a gene coding region and which is responsible for its regulation of transcription.

Developmental constraint: any restriction during the process of development causing a particular phenotype to be more predictably expressed and reproducible under a precise scenario of conditions.

Developmental degeneracy: resulting in part from genetic redundancy, a developmental process characterized as being composed of different elements performing a similar or overlapping function. Promotes stability in a self-organizing system and allows elements to functionally diverge by an evolutionary process without loss of coherence to the original system, thus serving as structural variation underpinning developmental plasticity (Maleszka *et al.*, 2013).

Developmental plasticity: the ability of an organism to change or react to the environment by changes in development that alter its phenotype; a subset of phenotypic plasticity.

Functional divergence: the process by which genes, after gene duplication, shift in function from an ancestral function. Subfunctionalization and neofunctionalization are the two possible outcomes of functional divergence.

Fusion: the process by which two or more organs become joined during development.

Postgenital fusion: the process that occurs following organogenesis such that separate organs merge together.

Congenital fusion: the process that occurs before or during organogenesis such that the organs are never separate from one another. This is not a developmental process, *per se*, but rather the lack of separation of organs that in other plants may be separate.

Gene cascade: a cascade or network of regulating genes, where genes regulate each other in a chain.

Genetic redundancy: when two or more genes perform the same function such that inactivation of one of these genes has little or no effect on the biological phenotype.

Gene regulatory network (GRN): a collection of genes that interact with each other indirectly (through their RNA and protein expression products) and with other substances in a cell to govern the expression levels of mRNA and proteins.

Merism: number of similar parts, used to describe the number of organs in each floral whorl.

Neofunctionalization: the process by which a gene acquires a new, derived function after a gene duplication event.

Regulatory evolution: changes in noncoding sequences controlling timing, position or levels of gene expression.

Shoot apical meristem (SAM): a pool of meristematic (dividing and undifferentiated) cells at the tip of a growing shoot. The SAM gives rise to leaves as lateral organs and, upon flowering, will become the floral meristem to produce the flower organs (sepals, petals, stamens, carpels).

Subfunctionalization: the process by which two duplicate paralogues undergo a division of labour by retaining different parts (subfunctions) of their ancestral function. Neither is able to complete the full function of the ancestral gene.

***Trans*-regulatory element (TRE):** genes that modify or regulate the expression of genes at a distance. Typically TREs encode transcription factors that can regulate gene expression at a distance.

underlying regulatory evolution (i.e. whether they are *cis* or *trans*), emphasizing only that regulation is key to developmental evolution (Hoekstra & Coyne, 2007). As changes in the coding region, especially of transcription factors, can cause downstream changes within a GRN that result in spatial and/or temporal alterations in gene expression, a mutation does not have to be in a regulatory region to cause a heritable regulatory change. Thus, while regulatory changes in gene expression are a major factor in evodevo (Stern & Orgogozo, 2008), these changes may be caused by different types of functional mutations (Box 1).

Cis-regulatory changes can evolve *de novo* by accumulation of mutations in *cis*-regulatory elements, or can be altered by increases in number and function of transcription factor (TF) binding sites following gene or whole-genome duplication events. When a gene acquires a new TF binding site, it can alter its expression domain either by adding new expression or by repressing expression in certain locations, times or under certain conditions (de Bruijn *et al.*, 2012). While studies in animal systems show that a gain of new expression patterns is rare relative to changes in the timing or level of gene expression, the expansion or restriction of spatial expression

domains, or the loss of expression features (Prud'homme *et al.*, 2007), gene duplication and rampant polyploidy that are inherent to plants enables the possibility for a gain of new expression to likewise provide a considerable source of developmental variation.

Across the diversity of life there is, by and large, considerable conservation of protein coding sequences; still, the number of coding changes that have the capacity to impact gene regulation, protein interactions and DNA-binding properties can be significant (Eyre-Walker, 2006). Expression can be influenced by changes to variable upstream TF binding regions (*cis*-regulatory elements), by changes to coding sequences that alter protein–protein interactions essential for transcriptional regulation, by changes in heterochromatin location that silence or unsilence expression, or by changes in copy number that result in additional genes that can be coexpressed or subfunctionalized to effect new expression patterns. As was recently demonstrated for the regulation of the floral homeotic gene *AGAMOUS* by orthologues of *LEAFY* in different flowering plants (Moyroud *et al.*, 2010, 2011), variation in TF binding sites can simultaneously form the basis for conserved as well as divergent regulatory interactions, leading to novel phenotypes: observed changes in expression patterns that appear to be similar may not be caused by homologous genetic processes. In plants, where gene duplication followed by differential selection on resulting copies enables novel regulatory networks to form, in part or in whole, regulatory evolution must be investigated in the context of these networks (Rosin & Kramer, 2009). In an effort to investigate adaptive evolution, it is essential to determine the genetic causes underlying the regulatory changes, and to determine how selection acted upon these mutations to influence regulatory change within a developmental gene network.

III. Adaptive evolution and regulatory changes

Accelerated rates of regulatory gene evolution have been shown to accompany rapid morphological diversification in adaptive radiations (Barrier *et al.*, 2001), demonstrating a link between morphological and genetic diversification in regulatory regions of developmental genes. Within a population, morphological change can occur via divergence in regulatory genes, leading to variation in gene expression (Garfield *et al.*, 2013), and thus the extrapolation to morphological evolution proceeding via diversification in regulatory loci provides a logical foundation for investigating the genetics underlying developmental evolution.

Novel phenotypes arise as a result of a particular mutation or combination of mutations becoming fixed in a population, but, in order for this to occur, the genetic change must first be tolerated by the developing organism and be inherited by its offspring. Unlike biochemical or physiological traits, morphological traits are typically specified by genes that act upstream in major developmental pathways (Wessinger & Rausher, 2012). As such, mutations in coding regions ('functional' mutations) were considered to incur sufficiently deleterious pleiotropy to be untenable for effecting morphological evolution (but see Wessinger & Rausher, 2012). Mutations in regulatory regions were thus thought to be the main force for morphological evolution, as changes in gene regulation impacting timing and/or position of gene expression were likely to be better tolerated as a result of their more subtle effects in a restricted set of tissues during development (Hoekstra & Coyne, 2007; Stern & Orgogozo, 2008; Wessinger & Rausher, 2012). Unlike with functional mutations, regulatory mutations would not impact the physical interactions and function of the encoded protein, enabling essential metabolic or cellular functions to proceed.

Because of modularity in plant development and redundancy in GRNs underlying developmental processes, however, a change in a protein sequence may not have the massive negative pleotropic effects that were once considered to eliminate such changes from playing a role in developmental evolution (Hoekstra & Coyne, 2007; Prud'homme *et al.*, 2007). The same benefits of modularity that were evoked to hypothesize why *cis*-regulatory changes would be less pleiotropic than functional changes (Carroll, 2006) apply as well to the modularity of GRNs as effectors of gene regulation and expression, with the GRN acting as a buffer against any widespread negative effects of a coding mutation in a developmental gene (Garfield *et al.*, 2013). Mutations in the coding regions of genes involved in a regulatory network can cause changes in protein–protein interactions, but these interactions could occur in a restricted, tissue-specific manner and impact a subset of developmental functions. The presence of duplicated genes means that mutations and subsequent drift or natural selection affecting genes or their targets can occur without necessarily changing the essential function of an existing GRN. As we move forward to study adaptive phenotypes and the dynamics of morphological evolution, it is important to consider changes to whole GRNs, regardless of whether the mutations are in regulatory or protein-coding regions. This involves a comparative approach that focuses on regulatory genomics of developmental processes (de Bruijn *et al.*, 2012).

IV. Gene duplications, GRNs and diversification of form

It is estimated that between 47 and 70% of angiosperms are polyploids (Ramsey & Schemske, 1998) and that all flowering plants have undergone multiple lineage-specific whole-genome duplication events throughout their evolutionary history. More recent phylogenomic and synteny-based examinations of plant genomic data indicate that there have been multiple whole-genome duplications during flowering plant evolution, including duplication events that correlate with large shifts in morphology such as duplication in the ancestral lineage prior to the diversification of angiosperms and independently in the lineage leading to the core eudicots (Jaillon *et al.*, 2007; Ming *et al.*, 2008; Jiao *et al.*, 2011; Vekemans *et al.*, 2012). As such, most plant lineages harbour duplicated genes and have the potential to differentially retain certain gene families that can lead to the acquisition of novel traits.

The propensity for gene and whole-genome duplication in plants provides raw genetic material for the evolution of novel floral features and for shifts in the timing and location of developmental processes to occur, altering floral morphology (Soltis & Soltis, 2014). Shifts in timing and location of expression, effected by changes in regulatory elements, as well as nucleotide changes impacting protein function can result in the neofunctionalization and subfunctionalization of duplicate copies, both situations

leading to the development and evolution of novel morphologies. Clear cases have been characterized in which novel morphological features arise as a result of divergent gene expression of paralogous gene lineages (Kramer *et al.*, 2004, 2007).

Gene duplications provide robustness to networks enabling evolutionary innovations (Wagner, 2008). In addition to redundancy, many duplicate gene functions have diversified within and among species. Diversification post-duplication is expected if robustness caused by gene duplications provides a substrate for morphological evolution (Wagner, 2008). The SEP gene family provides an example: while redundant in *Arabidopsis*, the SEP homologs have evolved different functions and expression profiles in other plant lineages, including the potential for inflorescence and floral organ morphological diversification in monocot lineages (Malcomber & Kellogg, 2004; Yockteng *et al.*, 2013). Thus robustness originally caused by duplication can facilitate diversification at the molecular level, which is a prerequisite for morphological evolution (Wagner, 2008).

While most duplicated genes are lost or degraded relatively quickly, it has been shown that in model plant systems, paralogues involved in signalling and transcriptional regulation are shown to be preferentially retained, leading to the hypothesis that polyploidy events can drive the evolution of novelties in form and function (Blanc & Wolfe, 2004; Jiao & Paterson, 2014; Rensing, 2014). Functional bias of retained genes is more dependent on the lineage of plants than on the mode of gene duplication (Carretero-Paulet & Fares, 2012). This implies that selection pressures, either biotic or abiotic, could play a role in determining gene content via paralogue retention post-duplication and that these selection pressures are likely to act in a lineage-dependent fashion: this is in addition to responding to known genomic features such as molecular/cellular selection pressures, gene-specific domain numbers, chromosome location and GC content, which also affect which genes are most likely to be retained for dosage, neofunctionalization and subfunctionalization (Jiang *et al.*, 2013). Preferentially retained genes provide candidates for studies of adaptive evolution: genes that are differentially expressed between tissues are prone to positive selection, and this pattern of differentiated expression is found most commonly among recent paralogues, indicating that their positive selection and differentiation may facilitate retention while playing a role in adaptive diversification (Jiang *et al.*, 2013).

Evolutionary gain in morphological complexity has been correlated with an expansion of genes encoding transcriptional regulators in plants (Lang *et al.*, 2010). In plants, unique protein domains are often shuffled or recombined to create novel gene functions (Kersting *et al.*, 2012); as duplicated genes are subject to domain shuffling, rapid changes in the regulation of gene expression post-duplication can lead to morphological novelty. Families of TFs tend to expand in number following whole- or segmental genome duplication, providing a mechanism for increasing number and complexity of GRNs and providing the raw material for neofunctionalization and subfunctionalization of networks, increasing morphological complexity. Genes encoding proteins involved in transcriptional regulation include TFs binding to DNA regulatory elements as well as transcriptional proteins that act via chromatin or protein–protein interactions, all of which increase in number following whole-genome duplication (Rensing, 2014).

Recent data have indicated that gene involvement in networks and network connections can play a large role in determining fate post-duplication (Zhu *et al.*, 2013). Genes involved in networks tend to be preferentially retained, however only a very small portion of whole-genome duplication pairs maintain exactly the same set of network neighbours, indicating that pure subfunctionalization and neofunctionalization are rare (Zhu *et al.*, 2013). In fact, a large fraction of duplicates undergo rapid subfunctionalization followed by a long period of neofunctionalization. As divergence of whole-genome duplication pairs correlates strongly with gene expression and fitness, whole-genome duplication combined with the development of novel regulatory network interactions has the potential to play a major role in morphological adaptive evolution.

The developmental redundancy or 'degeneracy' that networks provide, particularly following whole-genome duplication, has been considered a link among evolvability, robustness and complexity in biological systems (Box 1; Whitacre, 2010, 2011). Degeneracy supports robustness in developmental processes while enabling evolvability of organ or organismal form or function. Investigating degenerate functional elements within a GRN provides an opportunity to study the developmental mechanisms that enable adaptation and evolution within particular structures (Wellmer *et al.*, 2014) or between structures of closely related organisms. Changes in the expression of several genes, or alterations in the interactions among several genes, may be needed to allow the creation of a new steady state of a network in order to effect a robust change in morphology (de Bruijn *et al.*, 2012).

V. Ontogeny, homology and the study of plant adaptation

Genetic studies of developmental processes and pathways in model systems provide some insight into the conservation of developmental processes; for example, the same genetic mechanisms are involved in making lateral organs within a plant, including leaves of varying shapes and sizes (Moon & Hake, 2011; Byrne, 2012) as well as floral organs (Wellmer *et al.*, 2014). Thus, all lateral organs have conserved genetic pathways that underlie their growth and development within the organism. These ontogenetically conserved pathways may be those that are most conserved phylogenetically, with similar pathways underlying similar processes across large evolutionary distances.

In order to advance our understanding of the GRNs underlying novel developmental consequences, studies must advance to characterizing the fundamental developmental processes involved in morphological differentiation and the patterns of selection on the genes, both functional and regulatory, that form the GRNs underlying these processes. Processes such as polarity, patterning, cell differentiation, growth and expansion are essential to creating the diversity of features during ontogeny, and the differential realization of each process is likely to function in the evolution of form as well. It's these developmental programs and their

underlying genetic networks that evolve and give rise to structural diversity on which adaptive forces can act. Using these principles, studying traits that have evolved multiple times independently provides statistical power to investigate the potential for developmental evolution to explain adaptive trait diversification, taking into consideration the role of developmental constraints leading to phenotypic convergence.

This is a different approach from identifying homology in identity, or homology of pattern, and it removes challenges associated with making homology statements about mature structures as though they were static objects rather than the result of developmental programmes. Instead, it is critical to characterize which developmental processes are involved in the evolution of a feature that can be acted on by selection, and then determine how the GRNs that affect those processes have been altered to give rise to the potentially adaptive phenotype. Were gene duplications involved? Does evolution involve novel expression patterns or novel protein interactions? How were GRNs altered to give rise to changes in the developmental processes that enable the evolution of novelty? Finally, as we develop the tools that enable us to study the mutational changes that result in adaptive evolution, we can start to answer key evodevo questions, such as the following. What is the role of single large-effect mutations vs the accumulation of many small-effect mutations in the evolution of developmental processes? How does homoplasy involve both ontogenetic and phylogenetic convergence of genetic and developmental traits? Which types of genetic changes typically occur during the evolution of novel structures? (Nadeau & Jiggins, 2010).

The following case studies in floral symmetry and fusion of floral organs demonstrate how studying the processes of developmental diversification within the context of a GRN that are at least partially understood can provide evodevo researchers with the power to test for adaptive evolution of organismal form.

VI. Case study 1: Symmetry and adaptation of floral form

During diversification of flowering plants, similar adaptive traits have been gained and even lost repeatedly, often utilizing or coopting similar GRNs. This trend is highlighted by the recurrent shifts in floral symmetry, in which there have been a hypothesized 70 shifts from radial to bilaterally symmetrical flowers (Citerne *et al.*, 2010), and countless reversals back to radial symmetry. While flower symmetry is generally categorized as radially symmetrical or bilaterally symmetrical, there are clearly many variable traits that make each separate species unique. For example, a shift from radial to bilateral symmetry involving the petal whorl involves a transition from a single petal form within the flower to the individualization of petals within the whorl and the resulting possibility of having multiple petal forms. In core eudicots this ultimately results in three petal types: a medially positioned abaxial (ventral) petal, two lateral petals and two adaxial (dorsal) petals (Donoghue *et al.*, 1998). Each of these separate groups (two dorsal petals, two lateral petals, and single ventral petal) can vary in multiple morphological characteristics to result in a floral form with a single dorsoventral plane of symmetry. These characteristics often include petal position, with the most frequent being the 2 + 3 form in which two petals are arranged dorsally and three are arranged ventrally. However, other forms (4 + 1, 0 + 5) have also evolved to give rise to bilaterally symmetrical flowers. In addition to position, changes in morphological characteristics such as symmetry in petal shape (Luo *et al.*, 1996), presence or absence of trichomes on petals (Perez-Rodriguez *et al.*, 2005), colour differences among petals, and changes in cell types on petal surfaces (Perez-Rodriguez *et al.*, 2005) can also lead to shifts in floral symmetry. Thus any GRN governing flower symmetry must be utilized to set up general patterns of bilateral symmetry while still providing for genetic variation that underlies different organ-specific developmental traits within the flower. Here we examine the complexity of GRNs by examining one thread of the floral symmetry pathway: TCP and MYB genes and the petal whorl.

1. TCP genes

An increasing number of studies from across angiosperms indicate that morphological shifts between radial symmetry and bilateral symmetry are correlated with independent transitions in asymmetric expression of members of the ECE clade of TCP TFs (Howarth & Donoghue, 2006; Preston & Hileman, 2009), of which *CYCLOIDEA* (CYC) was the first characterized member (Luo *et al.*, 1996). Specifically, in core eudicots, members of the CYC2 clade of ECE genes (Howarth & Donoghue, 2006) independently shift from equivalent dorsoventral expression across the corolla (either ubiquitously expressed or not expressed) in radially symmetric flowers to dorsally restricted expression in bilaterally symmetrical flowers (Fig. 1). This sets up the hypothesis that species with bilaterally symmetrical flowers are homologous in their capacity to undergo a shift to dorsal expression, even though the location of gene expression itself is independently derived across independently evolved bilaterally symmetrical groups.

Nearly all characterized bilaterally symmetrical lineages contain two or more duplicates of CYC2 genes (see Howarth *et al.*, 2011). Plants with floral heads (compact inflorescences) that contain both radial and bilaterally symmetric flowers, such as those in Asteraceae and Dipsaceae, appear to have the greatest numbers of CYC2 genes (Chapman *et al.*, 2008; Carlson *et al.*, 2011) with differential expression tied to changes in floral symmetry across the inflorescence axis (Broholm *et al.*, 2008). In Dipsacales, the first shift from radial to bilateral symmetry is correlated with a duplication event, resulting in two gene copies, and the partial restriction of both copies to exclude the ventral petal lobe (Howarth *et al.*, 2011). Subsequent shifts to more strongly bilaterally symmetrical flowers involved further restriction of the duplicate copies and a decoupling of expression, such that one copy is more dorsally restricted than the other (Howarth *et al.*, 2011). Similar patterns in which one duplicate is more dorsally restricted than the other exist across the eudicots (*Antirrhinum majus* (Luo *et al.*, 1996), Malphigiaceae (Zhang *et al.*, 2008), Dipsacales (Howarth *et al.*, 2011) and *Pisum sativum* (Wang *et al.*, 2008)). This indicates that a similar pattern of differentially restricted expression occurs independently in the evolution of bilateral symmetry from radially symmetric ancestors. Therefore, we find independent cooption of not just the same

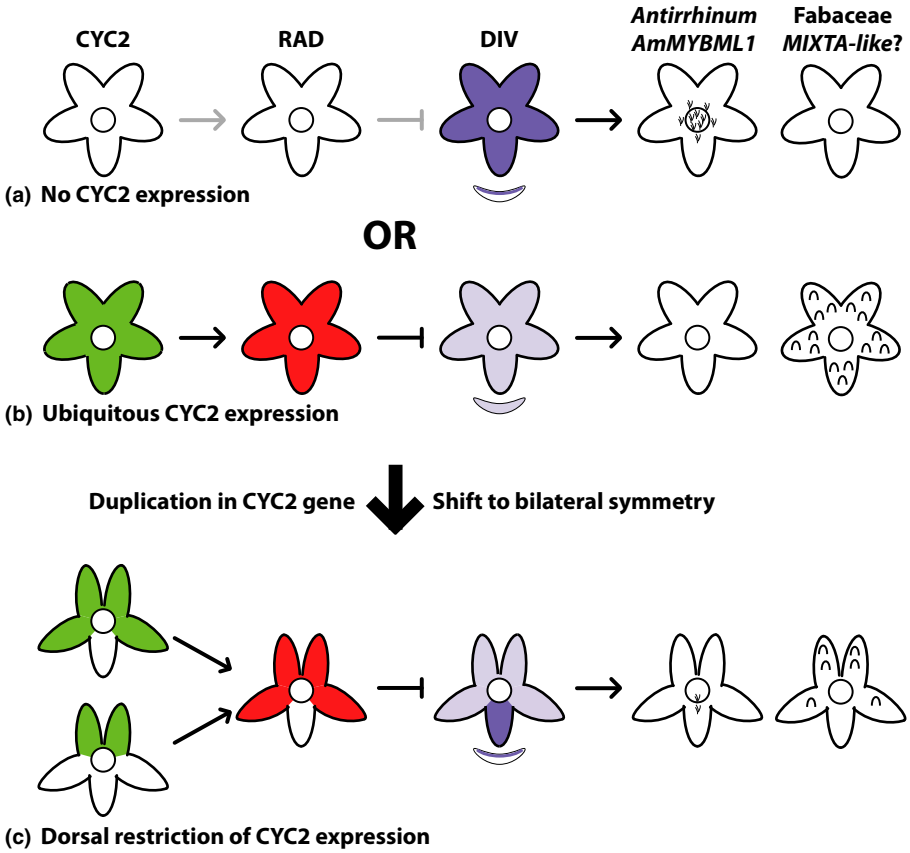

Fig. 1 General gene regulatory network (GRN) governing floral symmetry. Head-on images of generalized flowers showing expression of each gene group. In radially symmetrical flowers, expression of CYCLOIDEA2 (CYC2) genes have been shown to be either not expressed (a) or ubiquitously expressed (b). (a) When *CYC2* is not expressed, *RADIALIS* (*RAD*) is no longer activated. In the absence of *RAD*, DIVARICATA (DIV) localizes to the surface epidermal cells where it may up-regulate *AmMYBL1* (a MIXTA-like gene), resulting (at least in *Antirrhinum majus*) in trichome formation throughout the corolla tube. (b) When *CYC2* is ubiquitously expressed, then *RAD* is activated throughout the corolla, which inhibits *DIV*. DIV remains diffusely expressed throughout the corolla, perhaps not sufficiently to result in the up-regulation of *AmMYBL1*, leading to the loss of trichomes. (c) A shift to a bilaterally symmetrical flower generally entails two correlated events: the duplication into two *CYC2* paralogues and the dorsal restriction of both paralogues. Additionally, in many groups one paralogue is more dorsally restricted than the other paralogue. In *A. majus*, both paralogues are expressed in the dorsal petals only; however, one copy is more restricted than the other. In other groups, however, the *CYC2* expression pattern shown in (c) has been found. *CYC2* genes then activate *RAD* in the broadest zone of expression. This results in differential expression of *DIV*, with diffuse expression in dorsal regions and localized epidermal expression in ventral regions. This could result in trichomes developing only in the ventral region. In *Pisum* and *Lotus* (Fabaceae), this gene cascade has not yet been examined; however, loss of function of CYC2 paralogues ultimately leads to loss of dorsally restricted conical cells. This effect on conical cell formation from changes in CYC expression has not been supported in *Antirrhinum*, suggesting different outcomes farther down the cascade. The crescent under the DIV flower shows a cross-section of the ventral petal.

genes, but also of the dynamic molecular process involving restricted gene expression across the floral apex.

2. MYB genes

A proposed molecular model of floral symmetry development in *A. majus* hypothesizes that the two TCP genes *CYC* (*CYCLOIDEA*) and *DICH* (*DICHOTOMA*), formed from a duplication event specific to the Antirrhineae (Hileman & Baum, 2003), control the activation of the MYB TF *RADIALIS* (*RAD*). *RAD* is expressed in the dorsal regions where *CYC2* genes are also expressed. *RAD* in turn inhibits another MYB TF, *DIVARICATA* (*DIV*), restricting *DIV* to function only in the ventral portion of the corolla (Corley *et al.*, 2005). Thus mutants of *cyc/dich* and *rad* result in radially symmetrical, ventralized flowers (Luo *et al.*, 1996; Corley *et al.*, 2005), while mutants of *div* (in a *cyc/dich* background) result in radially symmetrical, lateralized flowers in *A. majus* (Almeida *et al.*, 1997). *DIV* likely up-regulates the expression of yet another MYB gene, *AmMYBML1*. *AmMYBML1*, a MIXTA paralogue, is involved in the formation of conical cells and trichomes as well as modification of cell expansion. Increased *AmMYBML1* expression, probably as a result of increased *DIV* expression in the ventral petal, results in trichomes only in the ventral region of the corolla (Perez-Rodriguez *et al.*, 2005).

Outside of *A. majus*, little is known about the expression or function of the MYB TFs (*DIV* and *RAD*). Expression data from other Veronicaceae and *Bournea* (Gesneriaceae) are, however, consistent with a model of *CYC-like*, *DIV-like* and *RAD-like* gene interaction being conserved at least across Lamiales (Zhou *et al.*, 2008; Preston & Hileman, 2009). Additionally, previous data on *DIV* expression in *Heptacodium* (Dipsacales) indicated that there is a dorsoventral expression pattern of a DIV orthologue in the

corolla, supporting the possibility that the pathway is similarly coopted across asterids (Howarth & Donoghue, 2009). An orthologue of *RAD* in *Lonicera* (Dipsacales) is expressed in a similar pattern to *CYC2*, indicating that the potential for an interaction between *CYC* and *RAD* spans the asterids (Boyden *et al.*, 2012).

3. Floral symmetry GRN

Studies in model systems can start to piece together a modular GRN that underlies floral symmetry and to investigate how the dynamics of this network can lead to different symmetry patterns. It remains unknown, however, how many of the interactions and gene functions are similarly utilized for morphological shifts in independent groups, that is, how much of the genetic process is homologous across lineages. The current model, mostly derived from *A. majus*, involves sequential steps leading to a shift from radial to bilateral symmetry in the petal whorl: (1) *CYC2*-like genes are dorsally restricted (Luo *et al.*, 1999); (2) *CYC2*-like genes up-regulate *RADIALIS* (*RAD*) in the dorsal region (Corley *et al.*, 2005); (3) RAD negatively regulates weakly ubiquitous *DIVARICATA* (*DIV*), leaving DIV (Raimundo *et al.*, 2013) to localize in the outer epidermis of only the ventral region (Galego & Almeida, 2002); and (4) DIV possibly up-regulates *AmMYBML1*, which functions in forming trichomes, conical cells, and the hinge of the ventral petal (Perez-Rodriguez *et al.*, 2005). This sequential process provides an example of how a conserved GRN could be dynamically altered in divergent groups to evolve a bilaterally symmetrical flower from an actinomorphic ancestor (Fig. 1). The molecular and genetic changes leading to a bilateral petal whorl have been shown to affect pollinator preference, with developmental changes in form and the locations of trichomes and conical cells leading to fewer successful bee visitations (Martin & Glover, 2007), which could potentially lead to restricted gene flow and subsequent speciation.

In radially symmetrical groups, CYC2 clade members either are not expressed in corolla tissue or are ubiquitously expressed (Howarth *et al.*, 2011; Zhang *et al.*, 2013). By contrast, the dorsal restriction of usually two CYC2 paralogues has been found in all bilaterally symmetrical core eudicots to date (Hileman, 2014a). This suggests that the capability for *CYC2* to be dorsally restricted, ultimately leading to a bilaterally symmetrical flower, is homologous even if the asymmetrical *CYC2* expression itself is not directly ancestrally derived. It could be argued that the interactions of orthologues of *CYC2*, *RAD*, *DIV* and *AmMYBML1* genes form a phenotypic cascade that is independently triggered in different groups. At the top of the cascade, the function of CYC2 is highly conserved across multiple groups (see Hileman, 2014a). The dorsal expression of *RAD*-like genes appears to be shared at least across asterids (Boyden *et al.*, 2012) and that of *DIV* at least across Lamiids (Zhou *et al.*, 2008), although possibly across asterids as well (Howarth & Donoghue, 2009). The MIXTA gene family appears to regulate epidermal projections such as conical cell growth and trichome development across angiosperms (Brockington *et al.*, 2013), although conservation of their expression patterns and regulation by *DIV*-like genes is unknown. While the expression patterns of these individual genes have been found to be similar across groups with independent derivations of bilateral symmetry, it is not yet known whether this GRN is also shared. Clues from legumes suggest the use of a similar GRN, given that dorsal petal-specific conical cells are lost in CYC2 mutants, suggesting that loss of CYC2 expression ultimately leads to loss of expression of a MIXTA-like paralogue (Feng *et al.*, 2006; Wang *et al.*, 2008). This implies that these TFs could be interacting in the same GRN at least across core eudicots but that their mechanisms for interactions may be independently evolved.

These data indicate that the transitions near the start of the cascade may be more conserved, triggering the cascade of events that develop into the specific phenotype. However, the differences among the phenotypes in different groups may indicate more lability in downstream members of the network. For instance, MIXTA-related genes function in different tissues in the plant to regulate epidermal projections. Different plant groups have differing numbers of MIXTA and MIXTA-like genes, utilizing different paralogues to regulate different projections in the different plant groups (Brockington *et al.*, 2013). Conversely, at least one CYC2 member is always dorsally restricted in the corolla in bilaterally symmetrical groups, coopted in a very similar way in divergent groups. Additionally, nearly all transitions to bilaterally symmetrical groups are correlated with having at least two paralogues of CYC-like genes. This provides a hypothesis that the duplication of CYC2 genes has the potential to be the trigger that leads to a similar pattern of dorsal restriction in expression, thereby triggering a shift to bilateral symmetry. The key will be locating precise regulatory mechanisms that could be changed as a result of having duplicate gene copies, and identifying the downstream developmental and morphological consequences.

Changes in gene expression can be modified through gene duplication or through modifications to regulatory regions. As we investigate the role of developmental changes in morphological diversification, it becomes increasingly clear that changes in gene expression, not only the location and timing but also subtle changes in concentration, play a major role in developmental evolution. For instance, *DipsCYC2A* and *DipsCYC2B* both shift ventrally in *Lonicera sempervirens*, a species with a flower that is more radially symmetrical in form than the 'typical' *Lonicera* flower (Howarth *et al.*, 2011). Likewise, differential expression levels of *MIXTA* across *Antirrhinum* species correlate with changes in morphology that influence pollinator preferences (Baumann *et al.*, 2007). Finally, different modules of regulatory networks can interact differently across the tree of life. For example, in *Pisum sativum* conical cells occur only on petals with *CYC2* expression and are lost when *CYC2* expression is lost (Wang *et al.*, 2008), implying a developmental constraint within this network that could be relaxed in other taxa.

Moving forward, studies are needed that examine expression of all genes within a proposed GRN to investigate how changes in copy number and changes in molecular interactions impacted by sequence evolution and timing/position of expression can result in developmental differentiation across a given lineage or among lineages that share a particular derived morphology. Examining the evolution of such networks in groups that have independently

derived bilaterally symmetry would help to determine how much similarity there is in the gene network underlying pattern similar morphological traits. Once the consequences of regulatory evolution on developmental diversification are better understood, researchers can begin to investigate the fitness of variable traits across a clade. Changes in petal symmetry, petal reflection and cell shape can change the size of the visual floral display and colour saturation, creating differences in likely visitation by pollinators, thus effecting speciation via reproductive isolation among individuals with differing patterns of gene expression. In the context of this case study, the link to adaptive evolution would be to examine effective pollination in flowers that have shifts in developmental processes that are correlated with shifts in expression of this GRN.

VII. Case study 2: Organ fusion and adaptation of floral form

Fusion of floral organs occurs in a myriad ways across the angiosperm bauplan. Even among closely related lineages, fusion of different organs and organ whorls can yield an astounding diversity of floral form. For example, flowers of the palms (Arecaceae) can have a fused outer perianth whorl (e.g. *Pseudophoenix*), a fused inner perianth whorl (e.g. *Roystonea*), or both whorls can be fused into a single tube (e.g. *Coccothrinax*) (Endress, 2011). Shifts in timing and position of fusion within a lineage have traditionally been used taxonomically to differentiate among genera and tribes of different plant lineages; however, the developmental mechanisms underlying such phenotypic differences have not been investigated in a systematic context. It is not clear if the shift in fusion among organs reflects the novel acquisition of a process ('fusion') in a different spatial and temporal context, or if different mechanisms altogether are involved when considering the fusion of different organs during different developmental stages. Furthermore, fusion as a process may more accurately be defined as a lack of organ separation, and thus understanding the diversity of mechanisms that underlie boundary formation followed by primordia growth and organogenesis could be essential to investigating the mechanisms of organ fusion. However, the fusion (or lack of separation) of floral organs appears multiple times across the angiosperm tree of life and during different developmental phases of floral organogenesis, and is consistently linked with key innovations in floral shape, size and pollination interactions (Box 1). It is thus an ideal developmental process to investigate from an evolutionary perspective (Wake *et al.*, 2011) and to test for adaptive significance in the various lineages in which it occurs.

Floral fusion provides enormous potential for the diversification of the flower (Endress, 2011). Fusion within (connation) or between (adnation) organ whorls (Fig. 2a) provides the flower with a diversity of architectures that can be explored for adaptive fit to a variety of specialized pollination associations and to structural stability under diverse environmental conditions. By creating a floral tube that restricts access to a reward, fusion also provides a means of directing specialized and efficient pollination, thereby potentially increasing individual fitness. The fusion of various floral organ whorls enables coordinated development among the fused parts, providing mechanisms for genetic isolation and differential selection via highly specialized coordination of pollen and stigma placement in the elaboration of pollination syndromes (Fenster *et al.*, 2004).

Fusion as a developmental process can occur early or late during the development of an organ series (congenital fusion), or can occur at the margins of fully formed organs (postgenital fusion). Within the congenital fusion spectrum, fusion of the developing primordia can occur early or late during organogenesis. Late fusion occurs when the primordia appear as distinct early on but the individual organs eventually fuse during development, often at the base, leaving the tips of the primordia free (usually found in asterids). By contrast, early fusion occurs when the fused organs appear first as a ring meristem or fascicle on which individual organ primordia appear later (usually found in rosids) (Endress, 2001a,b); thus, the base of the organs are fused from inception.

1. Perianth

Fusion of the petals (*sympetaly*) characterizes the asterids and especially the euasterids, but also occurs independently in some monocots, early diverging eudicots (e.g. some Ranunculaceae) and core eudicots (e.g. Malvaceae, Rosaceae) (Endress, 2011). Sympetaly itself involves the combination of two developmental processes: meristem fusion of petal primordia; and the establishment of an intercalary meristem that produces a floral tube via intercalary elongation. Perianth synorganization can include fusion of petals alone, or can unite the petals with the stamens into a floral tube, resulting in concerted evolution of floral form (Endress & Matthews, 2006).

Functionally, the fusion of perianth organs can offer protection in bud as well as promoting specific associations to enhance pollination (Endress, 2011). Fusion of stamens and petals may also present opportunities for the emergence of elaborations on petals and staminodes that play a major role in nectar production and canalization for purposes of effective pollination (Endress & Matthews, 2006). Finally, fusion events within the canonical perianth whorls may result in the formation of novel boundary regions, resulting in additional floral whorls; the corona, a novel organ that forms between the perianth and the androecium, is prominent in several monocot and core eudicot lineages as a result of novel boundary generation between the petal and stamen whorls.

2. Stamens and carpels

Unlike perianth fusion, which occurs early in angiosperm evolution and defines large clades, stamen fusion occurs multiple times independently and does not tend to define major angiosperm clades, although it is locally important for species-level diversification and is likely to have adaptive significance given the impact on mating patterns in plant populations (Ren, 2008). Stamen fusion, including fusion of filaments, fusion of anthers and fusion to the style, can be found in over 70 families of flowering plants occurring independently with differing mechanisms of fusion (Ren, 2008). Interestingly, fusion of filaments alone is typically found in the early diverging angiosperms in flowers with many petals, while fusion events involving both anthers (connective) and filament are

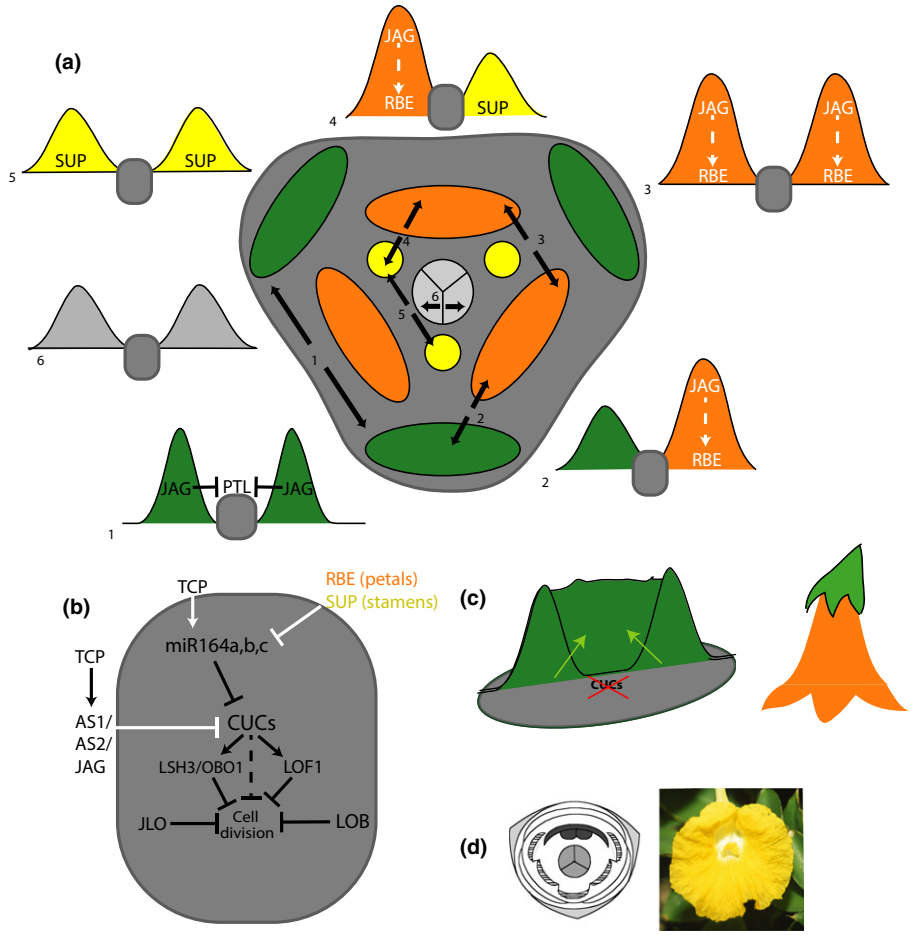

Fig. 2 Boundary formation gene regulatory network (GRN) and fusion of floral organs. (a) A floral diagram with four concentric whorls showing different planes along which fusion (or lack of separation) of floral organs typically occurs (numbered 1–6): (1) connation of sepals, resulting in a calyx tube; (2) adnation of sepals and petals; (3) connation of petals; (4) adnation of petals and stamen; (5) connation of stamens to form a staminodial tube; (6) connation of carpels leading to a syncarpous gynoecium. Fusion of stamen, petals and carpels into a single floral tube (not pictured) also appears throughout angiosperm evolution and appears to be mediated by different mechanisms that either stamen–stamen or carpel–carpel fusion. Organ colours: green, sepal; orange, petal; yellow, stamen; light grey, carpel. Grey shading indicates the localized expression of the organ boundary GRNm either between floral organs (1–6) or underlying the entire floral meristem. Intersepal *PETAL LOSS* (*PTL*) expression, resulting in *JAGGED*-regulated boundary formation parallel to the organ boundary network, is indicated in (1); organ-specific gene expression of *SUPERMAN* (*SUP*) and *RABBIT EARS* (*RBE*) is indicated (2–5) (see text for details). (b) The organ boundary GRN, resulting in repressed cell division and separation of organ primordia, with known gene network interactions indicated as black arrows (promotion) or lines (repression). In addition to genes expressed within the actual boundary region, genes expressed in the primordia defining the boundary region are indicated by white lines of influence. (c) Cartoon of fusion between primordia as a result of repression of *CUP-SHAPED COTYLEDON* (*CUC*) gene expression and a hypothetical flower resulting from processes A1 (sepal fusion) and A3 (petal fusion). (d) Floral diagram (left) and photograph (right) of a flower of Costaceae (*Monocostus uniflorus*) with five petaloid staminodes fusing together to form the labellum. The single fertile stamen is not involved in the fusion. Formation of boundaries on either side of the fertile stamen may be mediated by *TCP*-based regulation of the boundary formation GRN, leading to suppression of the expression of *CUC* genes abaxially in the floral meristem during stamen whorl development. *TCP* genes can suppress *CUC* gene expression by activating either *miR164* expression or *ASSYMETRIC LEAVES1* (*AS1*) expression in the primordia surrounding the area where the boundary should form.

typically correlated with petal fusion, indicating that stamen fusion may have adaptive significance in flowers with tubular petals, increasing fitness through improved mechanisms of pollen reception or dispersal (Ren, 2008). Anther fusion has been demonstrated to enhance pollen removal in the flower of *Campsis grandiflora*, one of many flowers with didynamous (two pairs of unequal length) stamens (Ren & Tang, 2010).

During the evolution of the angiosperm flower, the ovule-bearing carpels become enrolled and sealed with the ovules inside, with carpel fusion being a derived state for the angiosperm flower (Endress & Doyle, 2009). Once the carpel is formed, syncarpy, or the congenital fusion of multiple carpels to form a multicarpellate ovary, dominates angiosperm evolution, with an estimated 80% of all angiosperms bearing syncarpous ovaries (Endress, 2011). Syncarpy is considered to be a key innovation, estimated to have evolved independently at least 17 times (Armbruster *et al.*, 2002) and correlated with increased rates of fertilization and enhanced offspring fitness.

Congenitally united carpels benefit from the development of the compitum, a single pollen tube transmitting tract resulting from the fusion in the stigmatic region (see Endress, 2011). This enables flowers with multiple ovaries to maintain centralized pollen tube selection, which has an adaptive advantage for targeted fertilization (Endress, 1982; Armbruster *et al.*, 2002). The compitum is

considered a key innovation that evolved independently in monocots (Igersheim *et al.*, 2001) and in eudicots (Williams *et al.*, 2010) with various degrees of fusion involved in the formation of the structure. Both syncarpy and the additional fusion of the stigmatic area to form a functional compitum are cases in which fusion provides an adaptive advantage (Armbruster *et al.*, 2002).

3. Evolution of fusion

Considering the ancestral angiosperm (Endress & Doyle, 2009), it is likely that lack of fusion among and between most organs is the ancestral condition for the angiosperm flower, with the potential exception of basal fusion of the outermost perianth. For example, fusion of the carpel margins to form an enclosed carpel arose multiple times and is not considered homologous owing to the diversity of carpel types degrees of completeness of fusion (Endress & Doyle, 2009; p.41 and their fig. 9A). The fusion of individual carpels into a gynoecium occurred several times independently among angiosperms and proceeded through separate developmental mechanisms; either via fusion at the centre of the gynoecium resulting in axile placentation or fusion into a unilocular ovary with parietal placentation. Both types may have evolved independently from unfused carpels (Endress & Doyle, 2009; their fig. 10B).

Fusion of parts does not become a major element in angiosperm evolution until the establishment of whorled floral phyllotaxy and the establishment of a fixed merism, or the arrangement of the flower into defined whorls comprising regular multiples of organs (Fig. 2a; Endress & Doyle, 2009; Soltis *et al.*, 2009). However, the capacity for fusion of plant parts was clearly present in early angiosperms, as basal fusion of the outermost perianth can even be found in the early diverging *Amborella*, in which a short fusion zone among the tepals occurs before the fusion with the stamen whorl (Endress & Doyle, 2009), as well as in the early diverging lineages, including *Cabomba* (Endress, 2008), Canellales, Aristolochiaceae, Myristicaceae and *Degeneria* (Magnolialaes) (Endress & Doyle, 2009). These authors note that fusion tends to be more labile in the outermost perianth than in petals, a phenomenon that may predict different genetic mechanisms underlying organ separation. In some cases, developmental characters within a flower are highly correlated with fusion of particular tissues; for example, early fusion of petals is predominantly found in flowers with inferior ovaries and reduced sepal whorl(s) (Endress, 2001b). This indicates that fusion of organs is a multifaceted process that contributes in predictable ways to the diversity of floral form, providing opportunities for comparative analyses of adaptive floral development across angiosperms.

4. Developmental genetics of fusion in plants

Through recent studies in developmental genetics, the processes involved in discrete organ definition during development have begun to emerge. Because the model genetic systems largely maintain unfused organs (with the exception of carpels), they provide a practical system to investigate the genetic mechanisms involved in organ separation based on genetic mutants developing fused organ phenotypes.

In order for lateral primordia to distinguish themselves as a population of cells separate from the shoot apical meristem (SAM), a meristem-to-organ boundary must be created (Žádníková & Simon, 2014). Mutants lacking appropriate genetic mechanisms for separation of organ primordia into distinct lateral organs are considered to be mutants in the process of 'boundary formation' (Huang *et al.*, 2012). The boundary zone itself expresses a specific set of TFs that locally repress cell division (Fig. 2b). Mutations in these regulators or their downstream targets cause organ fusion as well as alterations in organ development and phyllotactic patterning (Žádníková & Simon, 2014) and such developmental defects can occur during embryogenesis, lateral organ formation and even leaf margin serration (see Huang *et al.*, 2012 for review). Because of the diversity of developmental stages influenced by what appears to be a conserved process, we hypothesize that a conserved 'boundary formation GRN' plays a central role in boundary formation at different hierarchies during plant development (Fig. 2b). This boundary formation network is a robust developmental network: redundant yet specialized. In addition to maintaining redundancy in its conserved core genes, spatially and temporally regulated network regulators, such as the miRNA164 genes, provide the network with developmental robustness (Sieber *et al.*, 2007), enabling it to function in the face of various developmental perturbations.

The first indication that boundary formation was a fundamental process of plant development came with the characterization of *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2*, genes found to be essential for proper cotyledon separation as well as separation of sepal and stamen primordia during flower development (Aida *et al.*, 1997). These were the first molecular data to indicate that a common mechanism may be involved in the separation of lateral organs within the same whorl, whether during embryonic/vegetative (cotyledons) or reproductive (sepals, stamens) development. These findings also point to the SAM as the site of action of the boundary network, while indicating that not all whorls to emerge laterally from the SAM would share the same mechanism for organ separation; for example, no mutants with fused petals were recovered from the early *cuc1cuc2* screens (Aida *et al.*, 1997). In some *CUC* mutants, stamens were fused to carpels, indicating a potential role of the *CUC*s in connation as well as adnation. Organ fusion occurred early in development, with the basal part of very young primordia fused in sepal and stamen whorls (Aida *et al.*, 1997), indicating early congenital fusion as the basis for both connation and adnation in *CUC* mutants.

Subsequent studies implicated microRNA (miRNA) function in the proper separation of adjacent primordia during floral organ development (Mallory *et al.*, 2004), including the petal whorl (Baker *et al.*, 2005). Specifically miR164 a, b and c are responsible for regulating the expression of the boundary-forming *CUC1* and *CUC2* transcriptional regulators, both in regulating the spatial domain of their transcription and in regulating transcript accumulation (Fig. 2b). As such, the miR164 miRNAs increase the precision of the developmental processes associated with organ separation. How they do so may differ slightly in the context of the

floral meristem: while petal and stamen primordia are fused (fail to separate) with loss of *CUC* gene expression, extra petals form when *MIR164* fails to properly repress *CUC1* and *CUC2* in the second whorl of *Arabidopsis*, causing a proliferation of petals (Baker *et al.*, 2005). This points to the possibility of whorl or organ-specific upstream regulation while demonstrating the importance of the positional and spatial (physical) interplay with genetic networks during floral development (Barrio *et al.*, 2010).

Upstream of miR164, *RABBIT EARS* (*RBE*) (Krizek *et al.*, 2006) functions as a transcriptional repressor that regulates all three miR164 genes and directly binds to the promoter of mir164c to repress transcription (Huang *et al.*, 2012). *RBE* is expressed in petal primordia (Fig. 2a) while its function appears to be restricted to first and second whorl boundary formation (Takeda *et al.*, 2004; Krizek *et al.*, 2006). Petals tend to be lost or aberrant in *rbe* mutants while sepals are fused, again indicating a potentially different mechanism for petal vs calyx fusion and highlighting the physical impact of one whorl's development on neighbouring primordia formation. While *rbe* petals were never found to be fused into a floral tube, they were often lost or reduced in size and/or in number. Reduction of stamen number was also prevalent, and was likely the result of lack of stamen primordia definition or reduced cell division with the loss of *CUC* expression.

In mutants of the *PETAL LOSS* (*PTL*) boundary gene, fusion of adjacent sepals resulted in the development of fewer petals, probably as a result of interference of margin definition or loss of petal initiation signal (Lampugnani *et al.*, 2012). *PTL* is a trihelix TF expressed in boundaries between developing sepal primordia, functioning within the sepal whorl to repress proliferation between sepal primordia (Fig. 2a). *PTL* is reported to act downstream of *ASSYMETRIC LEAVES1* and *2* (*AS1*/*AS2*) (Xu *et al.*, 2008), which are expressed in surrounding primordia and activate *PTL* expression. PTL also seems to act upstream of RBE (Takeda *et al.*, 2004); yet despite these common connections, *PTL* appears to act independently of the *CUC* genes and their role in intersepal boundary definition. Its expression limited to the sepal whorl of the flower provides the potential for independence of the mechanism for sepal whorl fusion from the canonical organ boundary GRN (Fig. 2b) during flower development.

Thus, while a canonical GRN provides a common mechanism for separating adjacent organs within the same whorl in both embryos and flowers, regulation by organ-specific genes acting upstream (e.g. *PTL*, *RBE*, *SUP*; Fig. 2) provides opportunities for fine-tuning morphology. While *PTL* is expressed between sepal primordia and plays a role in sepal and petal boundary formation, *RBE* is expressed in petal primordia and plays a role in interpetal boundary formation (Huang *et al.*, 2012). The transcriptional repressor *SUPERMAN* (*SUP*), also a zinc finger protein, appears to regulate boundary formation between stamen and carpel whorls (Sakai *et al.*, 1995) and may function in a similar manner to *RBE* by regulating expression of miR164 (Huang *et al.*, 2012). Both *RBE* and *SUP* have been linked with the control of floral organ identity through their influence on gene expression of the B and C class MADS box genes (Sakai *et al.*, 1995; Takeda *et al.*, 2004; Krizek *et al.*, 2006; Wuest *et al.*, 2012), demonstrating the link between the formation of individual primordia via organ boundary formation and the subsequent regulation of organ morphology and identity. In investigating specific boundary mutants, it also becomes clear that fusion is not a simple process that can be shifted from one organ whorl to another without consequences in other organs and that positional control is essential to maintaining organ form and function beyond boundary definition.

Parallel in the role of boundary formation to the *miR164*/*CUC* network, *LATERAL ORGAN FUSION 1* (*LOF1*) and *LATERAL ORGAN FUSION 2* (*LOF2*) are two MYB TFs that function in lateral organ separation and may be involved in floral organ differentiation as well (Lee *et al.*, 2009). Likewise, the boundary-specific gene *ORGAN BOUNDARY1* (*OBO1*/*LSH3*) belongs to a plant-specific gene family (*ALOG*) distinguishable by the presence of a single small domain of unknown function. *OBO1* overexpression causes petal–stamen fusions, while genetic ablation of cells expressing *OBO1* results in a loss of SAM and lateral organs, suggesting that *OBO1* plays an important role in meristem maintenance in addition to organogenesis (Cho & Zambryski, 2011). Recent studies indicate that OBO1/LSH3 are direct targets of CUC1 (Takeda *et al.*, 2011) and that, together with other ALOG genes, appear to suppress organ differentiation in the boundary regions, with overexpression resulting in either organ fusion or additional organ formation. It is unclear if they have action independent of the CUC pathway, but regardless they provide another family of genes and another set of gene interactions that provide targets for evolutionary analyses of fusion within and between floral whorls. While certain genes, such as *LATERAL ORGAN BOUNDARY* (*LOB*), have thus far only been demonstrated to be expressed in the boundary domain between the SAM and the emergent lateral organ and thus may not play a role in floral organ fusion, boundary genes *LOF1/2*, *OBO1/LSH3*, *JAGGED LATERAL ORGANS* (*JLO*), *AS2/1* and the *CUC* genes have been demonstrated to be expressed at the base of floral organs and/or in the boundary region between newly arising primordia, and they differentially appear to restrict cell division to effect boundary formation within and between whorls during flower development (Fig. 2c,d).

Various 'boundary genes' function to ensure proper organ initiation and placement across the shoot apex; for example, *BLADE ON PETIOLE 1* and *BLADE ON PETIOLE 2* (*BOP1/2*) TFs are expressed in the meristem-to-organ boundary and at the base of lateral organs to enable proper primordia development (Ha *et al.*, 2010; Žádníková & Simon, 2014). However, BOP1/2 also negatively regulate *YABBY* gene expression. *YABBY* genes are associated with polarity (adaxial–abaxial boundary definition) and subsequent laminar outgrowth (Fig. 3), developmental processes required for 'petaloidy' of floral organs. The use of the same genes or network components in these two developmental processes, boundary formation and laminar growth, demonstrates a connection among the processes involved in proper organ formation and provides a framework for understanding developmental constraints as well as opportunities for diversification that may be important in the evolution of organ morphology (Fig. 3). Likewise, *JAGGED LATERAL ORGANS* (*JLO*), a member of the LATERAL ORGAN BOUNDARY DOMAIN (LBD) gene family, is required for coordinated organ development in floral meristems: JLO acts in a

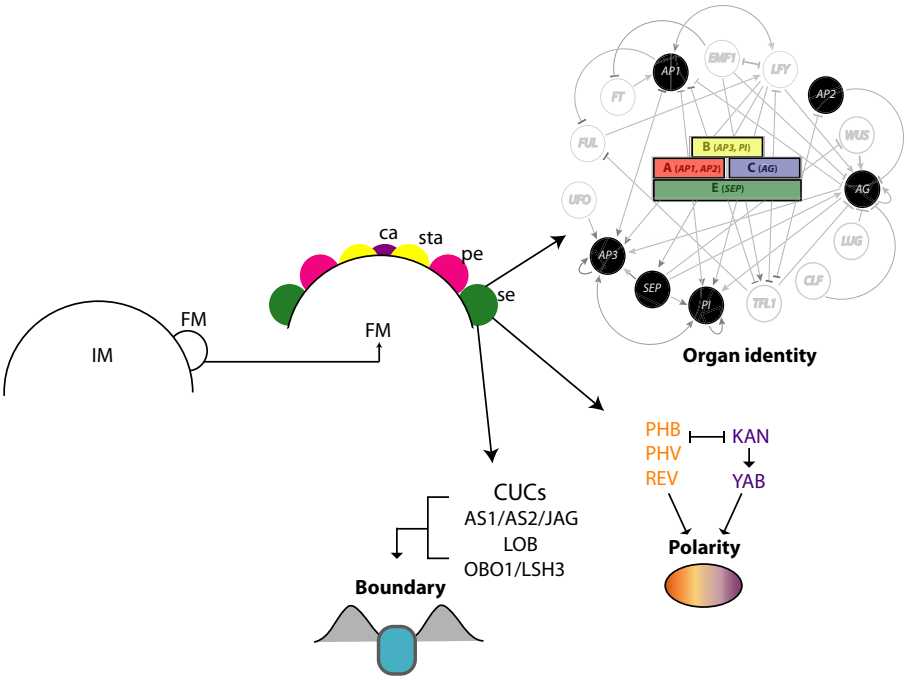

Fig. 3 Gene regulatory networks (GRNs) and the developmental processes leading to flower organ fusion. Many different developmental processes are responsible for flower morphology related to organ fusion. The flower meristem (FM) must first be defined from the inflorescence meristem (IM), both of which constitute particular types of shoot apical meristems (SAMs). Lateral organs on the FM are sepals (se), petals (pe), stamens (sta) and carpels (ca), all of which are defined by GRNs that can be contextualized as 'organ identity' (GRN involving the MADS-box homeotic genes); 'polarity', involving genes that result in abaxial/adaxial identity and lateral outgrowth leading to laminarity of polarized primordia; and 'boundary', resulting in the definition of individual primordia via suppression of cell division between centres of organogenesis.

trimeric protein complex with AS1 and AS2 to suppress expression of the class 1 KNOX gene *BREVIPEDICELLUS* (*BP*) in lateral organs (Rast & Simon, 2008, 2012). The AS1/AS2 complex is also known to repress *FILAMENTOUS FLOWER* (*FIL*) and the *KANADI*s and thus, like BOP1/2, to play a role in adaxial–abaxial polarity (see Fig. 3). Proper function of these genes is required to define separate floral primordia, and any functional or regulatory mutation that alters their expression in a whorl-specific manner could result in very specific organ fusion. Furthermore, these genes, together with the CUC boundary network, are candidates for fusions that alter floral symmetry, as interaction of the TCP genes with *AS1* and miR164 (Koyama *et al.*, 2010) could result in differential regulation of boundary formation among organs within a whorl, leading to a symmetric organ fusion, such as that found between five (of six) stamens forming the labellum of the Costaceae (Fig. 2d).

In *Petunia* × *hybrida* flowers, a member of the WOX1 subfamily of WUSCHEL-like homeodomain TFs, *MAEWEST* (*MAW*), is responsible for lateral expansion of petal primordia and the ultimate fusion of the developing petals (Vandenbussche *et al.*, 2009). Petal and carpel fusions is further diminished in *CHORI-PETALA SUZANNE* (*CHSU*) mutants. Based on mutant analyses, *MAW* appears to be involved in laminar growth and dorsoventral patterning of leaves as well as floral organs (Vandenbussche *et al.*, 2009). The roles of these two genes in organ polarity and laminar growth, as well as the fusion of floral organs that occurs in wildtype *Petunia*, further demonstrates the mechanistic link among organ polarity, laminar outgrowth and boundary formation in organ fusion. It is possible that genes involved in boundary formation are responsible for early congenital fusion, while genes responsible for organ polarity and laminar growth could play a role in late congenital fusion and even postgenital fusion, depending on timing and position of expression.

In summary, while connation or adnation may be mediated by different gene expression patterns and gene products, both spatially and temporally, an underlying common GRN may function as a conserved boundary-specification mechanism (Fig. 2, blue). In addition to being evolutionarily conserved, the organ boundary GRN is likely to be conserved within a given species, such that the same GRN is used for the process of boundary formation between the SAM and lateral organs and among lateral organ primordia whether in the vegetative or reproductive state. By having a redundant GRN that is regulated by upstream elements that can be expressed in spatially and temporally restricted manners, the boundary GRN can be evoked (or not) to differentially regulate the separation or fusion of individual organ primordia throughout the life of the plant. The presence of miRNA-based regulators in this robust pathway further contributes to the ability to fine-tune organ morphogenesis. In addition to resulting in organ fusion, boundary mutations can also cause primordia to incorporate cells from whorls that are normally distinct, creating chimeric organs (Huang *et al.*, 2012). Such chimeras could provide the basis for novel organ formation, such as coronas in *Narcissus* or *Asclepias*. An investigation of the many forms of fusion across flowers may demonstrate the importance of a genetic 'bricolage' involving both reuse (cooption) and reassembly of ancestral genetic networks (Fig. 3; Pires & Dolan, 2012).

VIII. Conclusion

Adaptation can be defined as the heritable change in the traits of a system in order to maintain or improve fitness within novel environments. Evolvability is the propensity for a system to discover adaptations (= heritable beneficial phenotypes). Thus the ability to discover distinct heritable phenotypes and the ability to transform novel traits into useful innovations during development

within a particular environment are essential elements of adaptation. As GRNs and their interactions provide evolvability to the process of organismal development, understanding how networked genes and/or their interactions are selected upon to yield novel outputs is the future for evodevo, especially for researchers interested in understanding how particular adaptive traits might have evolved.

By considering entire GRNs involved in key developmental processes and investigating changes in those GRNs across multiple evolutionary lineages, researchers can begin to piece together the fundamental genetic and genomic principles underlying the conservation and evolution of form.

First we must characterize, for any particular morphological trait, the underlying developmental processes that give rise to that morphology. For example, is the process that makes a laminar stamen in *Canna* (Zingiberales) the same as that which makes the laminar petal? Is the process that makes the laminar stamen in *Canna* (Zingiberales) the same as that makes a laminar stamen in *Nelumbo* (Nymphaeles)? In the first case, we are looking at a potentially homologous process ('laminarity') across different organs of the same species; in the second we are interested in a potentially homologous process in the same organ type but in different lineages. The common process is 'laminarity', which we can investigate by characterizing the GRN for these disparate laminar organs, comparing it with closely related organs that retain an ancestral nonlaminar form. We can then investigate how the underlying patterns of gene expression and gene interactions evolve (phylogenetic comparison) and change (within a plant) to give rise to diverse organ structures. Once we understand how evolution of the GRN influences the evolution and diversification of phenotypic traits, we can begin to look for signatures of selection on components of the GRN that we know influence morphological diversification. Thus, by studying the underlying developmental processes that give rise to particular morphologies, investigations of convergence in phenotype can help us to understand the correlations among GRNs, developmental processes and derived phenotypes. We can ultimately investigate such changes within a population or closely related taxa, enabling us to understand how patterns of selection might be influencing evolution, and if adaptation on those particular phenotypes is playing a role in driving diversification.

Despite conservation of developmental processes and their underlying GRNs across flowering plants, rampant gene and genome duplication provide the raw material for new network modules that enable diversification of form in the face of conservation of processes. Targets of some TFs, particularly those involved in specifying organ identity, are more numerous than had been thought even within model systems (Kaufmann *et al.*, 2010). Conversely, not all known TF binding events seem to affect gene expression (Schmidt *et al.*, 2010), and certain binding sites may only regulate gene expression in certain genetic backgrounds or under certain environmental conditions. In addition, certain genes characterized for a particular discrete role, such as *AP1/FUL* as a mediator of floral organ identity, might in fact act at many levels within the network and at different times during development, making the networks challenging to assemble without a tissue-specific understanding of timing and placement of expression patterns for each of the coacting factors (Wellmer & Riechmann, 2010; O'Maoileidigh *et al.*, 2014). As genes that are part of large families tend to be at least partially redundant in function, they are often recalcitrant to observation in typical mutant genetic screens. New approaches for assessing gene function and network connectivity using more detailed and quantifiable phenotypic analysis will be needed to provide candidate functions for evolutionary changes. Using functional analyses combined with RNA-seq to predict networks and network interactions shows promise for understanding not just how the networks function in a single organism or tissue, but how those networks do and can evolve to produce novel developmental consequences (Filkov, 2006; Palatnik *et al.*, 2007; Wollmann *et al.*, 2010).

Convergent evolution offers an excellent opportunity to test for the repeatability of genetic changes during adaptive evolution. While certain aspects of plant morphology, such as the formation of leaves, appear to evoke the same genetic mechanisms each time they evolve, regardless of the evolutionary distances of the lineages in which they appear (Harrison *et al.*, 2005), divergent genetic mechanisms, at least based on expression patterns, are known to underlie changes in floral morphology; this may be particularly true when considering reversals (Zhang *et al.*, 2013). The occurrence of convergent traits evolving multiple times independently indicates that internal genetic constraints could shape the ability of a plant to adapt to environmental selection.

As we begin to uncover networks involved in the developmental processes giving rise to diverse morphologies, we find that both genetic pathways and the processes they evoke are highly integrated (Posé *et al.*, 2012). Certain genes, such as LFY, may have multiple targets that control processes as disparate as plant immunity and floral transition (Winter *et al.*, 2011), and these processes might in fact have evolved to be highly coordinated aspects of plant development. Fusion is a highly adaptive feature of the flower, but its underlying genetics involves organ boundary formation, polarity and identity – all interacting networks that must ultimately coordinate to make a functional flower. In order to understand how evolution processes adaptive responses, we can determine how different TFs interact to regulate multiple, integrated developmental processes. This degree of 'process integration' (Posé *et al.*, 2012; Yant, 2012) indicates that major biological processes utilize overlapping components of GRNs to coordinate organismal development and provide robust developmental processes on which adaptive forces can act.

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