Journal of Systematics and Evolution 48 (2): 94–101 (2010) doi: 10.1111/j.1759-6831.2010.00066.x

Functional evolutionary developmental biology (evo-devo) of morphological novelties in plants

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Abstract The origin of morphological and ecological novelties is a long-standing problem in evolutionary biology. Understanding these processes requires investigation from both the development and evolution standpoints, which promotes a new research field called "evolutionary developmental biology" (evo-devo). The fundamental mechanism for the origin of a novel structure may involve heterotopy, heterochrony, ectopic expression, or loss of an existing regulatory factor. Accordingly, the morphological and ecological traits controlled by the regulatory genes may be gained, lost, or regained during evolution. Floral morphological novelties, for example, include homeotic alterations (related to organ identity), symmetric diversity, and changes in the size and morphology of the floral organs. These gains and losses can potentially arise through modification of the existing regulatory networks. Here, we review current knowledge concerning the origin of novel floral structures, such as "evolutionary homeotic mutated flowers", floral symmetry in various plant species, and inflated calyx syndrome (ICS) within Solanaceae. Functional evo-devo of the morphological novelties is a central theme of plant evolutionary biology. In addition, the discussion is extended to consider agronomic or domestication-related traits, including the type, size, and morphology of fruits (berries), within Solanaceae.

Key words biodiversity, development, evolution, functional evo-devo, heterochrony, heterotopy, morphological novelty.

An ever-increasing level of diversity is observed in the evolution of the life body plan, which becomes apparent in many traits among higher plants, including the increasing complexity of leaf morphology (Bharathan et al., 2002; Harrison et al., 2005), the origin of floral morphological novelties (He et al., 2004), and the evolution of fruit size and shape (Frary et al., 2000; Cong et al., 2008; Xiao et al., 2008). The origin of the morphological novelties is a long-standing problem in evolutionary biology. A better understanding of this problem demands elucidation of developmental and genetic mechanisms that generate such new structures. In addition, transference of this knowledge to higher taxonomic levels in a phylogenetic context is required to reveal the evolution of the novel traits within a broad range of taxa. The field of evolutionary development biology (evo-devo), resulting from the marriage of developmental and evolutionary biology, is particularly well suited for this purpose.

Morphological novelties reflect a part of biodiversity and are often characteristic of a species or higher-level taxon. Biodiversity, as a consequence of evolution, is therefore the arsenal for evo-devo, whereas knowledge from evo-devo could shed more light on evolutionary mechanisms. Here, we briefly review the advent and advance of evo-devo, its fundamental methodology and the major issues it addresses, which will be further exemplified by discussing the current understanding of evolution and diversity of several floral traits.

1 The origin and principles of evo-devo

Evo-devo, a new area of biology concerned with relationships between evolution and development, was established as a novel discipline to identify the developmental mechanisms that brought about evolutionary changes in organisms. It has been recognized as a valid, independent research area since 2000 (Goodman & Coughlin, 2000). However, evo-devo is deeply rooted in the comparative evolutionary embryology that emerged with the publication of *On the Origin of Species* (Darwin, 1859) and was promoted by the publication of *Ontogeny and Phylogeny* (Gould, 1977). The term "heterochrony" was coined by Ersnt Haeckel and its importance as a mechanism for evolutionary change was recognized (Gould, 1977). As evo-devo research progressed, more mechanisms, such as heterotopy, ectopic expression, and gene loss, have been shown to be fundamental to the process. These mechanisms occur as

Received: 1 August 2009 Accepted: 26 November 2009

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a result of mutations of transcription factors, including gain-of-function (ectopic expression, heterochrony, and heterotopy) and loss-of-function, during evolution. Any of these mutations, if adaptive, may spread rapidly through populations and become fixed later when a novel selection acts on a population, which is manifested by phenotypic diversities. The recruitment of an existing transcription factor into a particular functional context is the basic and common mechanism for the origin of a new body plan in both animals and plants (Gould, 1977; Wake & Roth, 1989; Raff et al., 1990; Keys et al., 1999; Wang et al., 1999; Frary et al., 2000; Carroll et al., 2001; Cong et al., 2002; Lee et al., 2003; Kanno et al., 2003; He & Saedler, 2005; Carroll, 2008; Jeong et al., 2008). In this scenario, ectopic, heterotopic, or heterochronic expression is due to mutations in *cis*-elements or in *trans*-regulators, although loss of function through mutation or complete deletion of a gene could also act as an impetus for evolutionary change, especially in the case of an adaptive loss of a feature (Kitahara & Matsumoto, 2000; He et al., 2004; Wang et al., 2005; Konishi et al., 2006; Khan et al., 2009). Both scenarios could occur more frequently following gene duplications (He et al., 2004; Irish & Litt, 2005; Khan et al., 2009).

Currently, a central theme of evo-devo is to address the molecular mechanisms underlying eye-catching morphological changes, for example the origin of floral novelties, during evolution. Three key questions regarding the evolution of a particular morphological novelty can be answered through evo-devo, namely: (i) what is the genetic architecture for the development of a particular novel structure; (ii) how does the knowledge from development hold for a broad range of taxa; and (iii) what is the selective value of the novel trait? The first question can be addressed through molecular developmental biology, whereas the latter two can be addressed only when knowledge from the first is framed into a phylogenetic framework integrated with ecological and geographic information.

2 How to do evo-devo?

A standard protocol for conducting eve-devo does not exist. However, several common steps are involved, including revealing trait evolution and functions (ecological or adaptive) in a phylogenetic context, selection of a trait-controlling candidate gene, and confirmation of developmental functions. The comparative approaches are keys to evo-devo in all steps.

2.1 Evo-devo in a phylogenetic context

Morphological comparison can reveal traits of interest that may include flower, fruit, leaf, and plant architecture, among others. Once a trait is chosen, a phylogenetic reconstruction based on molecular markers and morphological indices is the first and essential step in defining the non-trait closest relative(s). More detailed evolutionary questions, such as whether a particular morphology is ancestral (plesiomorphic) or derived, whether a morphology has evolved once or many times, whether a new trait within a family is due to the gain or loss of a feature, or what the adaptive function of the trait may be, cannot be properly addressed unless an accurate phylogeny is determined. Biogeographic, phylogeographic, habitat, and fitness analyses could set a logical context within which to infer the ecological functions of the trait maintained by natural selection during evolution. This inference could be further confirmed by studying candidate genes controlling the trait.

2.2 Selection of candidate genes

The selection of candidate genes for a particular trait is the greatest challenge in evo-devo and requires a considerable number of pioneer studies. In addition to transferring knowledge from model species, the characterization of evolutionary informative mutants or the differential display of transcriptomes is efficient. In the genomics era, some high-throughput approaches have emerged for this purpose. Comprehensively comparing the components (or their sequences) of known trait-related interacting networks, functional complexes, or certain pathways could reveal some potentially new candidates for natural variation of the trait among species. Computational analysis of a certain gene family in the entire genome and of the components in a certain pathway among species may offer another promising approach to find candidate genes playing important roles in the control of the trait or the special developmental process of interest (e.g. in bacteria, Ma et al., 2009).

Very often, the major players in the body plan are transcription factors (Doebley & Lukens, 1998). Therefore, any potential correlation between a transcription factor and the occurrence of a particular trait provides a clue to the trait-controlling candidate gene. Several levels of correlations are important and welcome. Phylogenetic trees of candidate genes could show a certain correlation with the distribution of the trait within the family. Molecular evolutionary analyses of trait-controlling candidate genes are the next step. Population genetics of the molecules within a large population in the family with a marked variation of the trait of interest could reveal how the selection may act, during evolution, on these genes to generate natural variation of the

trait under investigation. Correlation of candidate gene expression with the development of the trait in individuals and with the absence or presence of the trait within a genus or even family could also be evidence supporting the candidacy of the genes. Predictions of *cis*-elements in promoter or introns, or of *trans*-regulators, are primarily involved in this scenario. Although several tools are available, it is not easy to perform such analyses. The difficulties mainly arise because of a lack of a suitable "language" with which to decode the secret of *cis*-regulatory elements (i.e. in promoters or introns). Nonetheless, predictions can still be made in a meaningful way.

However, precautions must be taken during these processes. It is quite obvious that most of the outcomes at this stage are not observations, but hypotheses that need to be tested experimentally.

2.3 Functional evo-devo

Once a type of correlation has been established, detailed experimental analysis will be conducted to reveal the molecular mechanisms underlying the differential expression of orthologous transcription factors, to establish protein–protein interacting networks (thus providing an understanding as to how the transcription factor/s exerts its role/s in a pathway or network), to modulate the trait by manipulating trait-controlling candidate genes, or to demonstrate the ecological function of the innovative structure. These studies, as an essential part of evo-devo, are often referred to as "functional evo-devo". Modern molecular biology and functional genomics could further substantiate the results obtained.

Changes to the *cis*-regulatory elements in the promoter or changes to the *trans*-acting factors may lead to a distinct expression profile between orthologous genes, thus producing a novelty in a new context or losing a certain structure. Evidence could be provided by detailed comparative gene expression studies in conjunction with functional promoter analysis based on computer predictions, in which the putative promoters or any DNA fragments containing *cis*-elements are fused with any reporter genes in a proper host. If the promoters are identical either in sequence or function, changes to the *trans*-acting factors may account for the divergent expression profiles of the orthologous transcriptional factors and these factors could be evaluated experimentally.

Because transcription factors usually form dimers and even higher-order complexes to function, the establishment and comparison of their interacting networks and revealing their regulatory complexes could provide essential functional evidence to establish the functional networks or pathways controlling the novel trait. Further genetic analysis of the corresponding mutants could provide compelling evidence, but this is largely limited by the availability of mutants.

The real beauty of evo-devo is functional confirmation of the candidate genes and the ability to reveal the ability of these genes to control the trait *in planta* via reverse genetics mediated by plant transformation. The synthesis of a particular trait in a non-trait close relative, achieved by manipulating the trait-determining genes, is crucial evidence. Alternatively, knockdown or knockout of the corresponding gene leading to elimination of the trait is required. The selective values of the trait could be inferred accordingly and further verified experimentally.

3 Examples in plant evo-devo

Mechanisms underlying the evolution of a novel trait and its diversity have largely remained obscure in plants. Only recently have we started to understand some of the molecular details of these processes in which the activities of transcription factors, such as MADS-box genes, TCP-like genes and MYB-like genes, are involved. In this section, we focus on the evolution of floral novelties, including changes in the identity, symmetry, and morphology of floral organs, observed throughout the higher plants.

3.1 Origin of "evolutionary homeotic mutated flowers"

Some "fixed" floral homeotic mutations that are often observed in nature could be considered as "evolutionary homeotic mutated flowers", such as in species in Liliaceae, Iridaceae, and Orchidaceae that feature two whorls of petals, the so-called tepals in their perianth. This novelty arises from the replacement of sepals by petals and the underlying molecular mechanism becomes apparent as various floral homeotic mutants are studied in the laboratory.

Floral homeotic mutations, in which one type of floral organ is replaced by another, have been described in various species (He et al., 2004). The molecular basis for floral homeotic changes has been demonstrated as the ABC model of floral organ identity (Coen & Meyerowitz, 1991; Weigel & Meyerowitz, 1994). According to this model, an A-function is responsible for sepal formation, A- and B-function for petals, B- and C-function for stamen, and C-function for carpel formation. With the exception of *AP2*, other ABC-function genes encode MADS-box transcription factors. These proteins cannot function alone and instead often form dimers and/or tetramers of various composition

(Theissen & Saedler, 2001). As proposed in the "Floral Quartets" model, different "quartets" are involved in the establishment of different floral organ identity (Theissen & Saedler, 2001). The composition of new "quartets" in a given whorl could lead to a change in the organ identity of that whorl. The synthesis of two whorls of petals through ectopic expression of B-function genes in the first whorl (Davies et al., 1996) is reminiscent of the tepals. Therefore, an obvious notion in the evolution of the Liliaceae, Iridaceae, and Orchidaceae is that these tepals may have resulted from the ectopic expression of B-function genes in the outermost whorl. This is apparently the case in *Tulipa gesneriana* (Kanno et al., 2003), *Crocus sativas* (Tsaftaris et al., 2006), and *Habenaria radiata* (Kim et al., 2007). Furthermore, distinct sepal and petal morphologies in *Tradescantia reflexa* and *Commelina communis* from Commelinaceae are correlated with the expression of B-function genes (Ochiai et al., 2004).

Similar cases are found in horticultural varieties such as roses and peonies. In some roses, very dense petals are observed. This phenotype partially mimics the mutation of the C-function gene *AGAMOUS* in *Arabidopsis* (Yanofsky et al., 1990). Accordingly, evidence has been provided to show that the morphology of the double flower in the modern rose is due to mutations in the *AGAMOUS* homolog in these plants (Kitahara & Matsumoto, 2000). In peonies, floral homeotic variation is relatively rich, and various types of transient or stable floral homeotic alterations are often observed. In the most extreme case, one type of floral organs is completely abolished (F.Y. Chen, pers. comm., 2009). These may occur in response to the environment. One could assume that these homeotic variations could result from changes in some homeotic genes, including the epigenetic modification of these genes; as a consequence, the "quartet" is altered in a given whorl, thus giving rise to the diversity of floral homeotic alterations in these plants. However, evidence of variations in gene sequences and/or expressions is needed to verify this speculation.

Thus, in the evolution of homeotic mutated floral structures such as in the Liliaceae, Rosaceae, and Paeoniaceae, the presence, absence, or modification of particular floral quartets has engineered the novel floral morphology. The most likely selective pressures for these ornamental features may have been from horticulturists.

3.2 Evolution of floral symmetry

Floral symmetry, a key evolutionary trait, has arisen many times independently during evolution (Donoghue et al., 1998). In Antirrhinum, TCP-like transcription factors, such as *CYCLOIDEA* (Luo et al., 1996) and *DICHOTOMA* (Luo et al., 1999), and MYB family factors, such as *RADIALIS* (Corley et al., 2005; Baxter et al., 2007) and *DIVARICATA* (Galego & Almeida, 2002), are involved in determining floral symmetry, especially the symmetry of the corolla. Mutations in one of the *CYC* and *DICH* genes lead to semiradial flowers, whereas the double mutants are perfectly radial (Luo et al., 1996, 1999). Expression of *RAD* is absent in *cyc dich* double mutants and *CYC* expression is not altered in *rad*, which suggests that CYC and DICH exert their effects by directly or indirectly switching on *RAD*. As expected, the *rad* mutant, like *cyc dich*, has almost fully ventralized flowers (Luo et al., 1996; Corley et al., 2005). RAD and DIV antagonize each other to promote ventral identity of floral development (Galego & Almeida, 2002; Corley et al., 2005).

These findings from *Antirrhinum* are supported by observations from various plant species. Radial flower formation in peloric *Linaria* is the result of inactivation of the *CYC* gene by methylation (Cubas et al., 1999). A variation in the *DICH* expression pattern compared with that in *Antirrhinum* is responsible for the more actinomorphic flower of *Mohavea* (Hileman et al., 2003). Within the Brassicaceae, the expression of a *CYC*-like gene seems to be responsible for the bilateral petal symmetry of *Iberis amara*, which deviates from the radial petal symmetry in *Arabidopsis* (Busch & Zachgo, 2007). In Gesneriaceae, altered expression patterns of the *TCP* and *MYB* genes are related to the floral developmental transition from initial zygomorphy to actinomorphy in *Bournea* (Zhou et al., 2008) and differential expression of four *CYC*-like genes may play a role in establishing the floral dorsoventral asymmetry in *Chirita heterotricha* in response to different selective pressures after gene duplication (Gao et al., 2008). *Gerbera* and *Senecio* from the sunflower family (Asteraceae) share the composite head inflorescence. In *Gerbera hybrid*, overexpressing *GhCYC2* results in disk flower morphologies similar to ray flowers. In corroboration with the gene expression patterns, these data suggest that *GhCYC2* is involved in differentiation among *Gerbera* flower types, providing the first molecular evidence that CYC-like TCP factors take part in defining the complex inflorescence structure of the Asteraceae (Broholm et al., 2008). In *Senecio*, a natural polymorphism arose by introgression of a cluster of regulatory genes, the *RAY* locus encoding TCP transcription factors, and its influence on inflorescence head development was analyzed (Kim et al., 2008). The *RAY* genes apparently play a key role in promoting flower asymmetry, thus leading to an increase in the rate of outcrossing, and are responsible for the natural variations

of the inflorescence development in *Senecio* (Kim et al., 2008). Two TCP transcription factors, namely LOBED STANDARD 1 (LST1) and KEELED WINGS (K), have divergent functions to constitute the dorsoventral (DV) asymmetry, whereas SYMMETRIC PETALS 1 (SYP1) controls organ internal (IN) asymmetry; their interactions help specify floral zygomorphy in the pea (Wang et al., 2008).

The mechanism underlying the flower symmetry in grasses remains unclear. A recent study has reported that *CYCLOIDEA* (*CYC*)-like homolog *RETARDED PALEA*1 (*REP*1) determines floral zygomorphy along the lemma–palea axis in rice (*Oryza sativa*), thus confirming a common mechanism controlling floral zygomorphy by *CYC*-like genes in both eudicots and grasses (Yuan et al., 2009).

In addition to the symmetry of petals, lateral stamen abortion in *Mohavea* is correlated with an expansion of *CYC* and *DICH* expression (Hileman et al., 2003) and the expression of *OpdCYC* is well correlated with both dorsal and ventral stamen abortion in *Opithandra* (Song et al., 2009).

These comparative investigations, mutant analyses, and functional conformation via genetic manipulations reveal that the same set of genes and associated molecular networks, including TCP-like (CYC, DICH) and MYB-like (RAD, DIV) transcription factors, have been independently recruited to establish the diverse patterns of floral morphology by altering petal and/or stamen symmetry in different plant families. Such diversity may have originated in response to selection for pollination efficiency.

3.3 Evo-devo of the inflated calyx syndrome within Solanaceae

The calyx diversification of Solanaceae seems to be a playground in the evolution of floral morphological novelties (He et al., 2004; He & Saedler, 2005; Hu & Saedler, 2007; Khan et al., 2009). Inflated calyx syndrome (ICS) or "Chinese lantern" is characteristic for a number of species within several genera of the Solanaceae (D'Arcy, 1991; He et al., 2004; He & Saedler, 2005; Hu & Saedler, 2007). A comparative study of *Physalis floridana* and *Solanum tuberosum* revealed that one key to the genetic architecture for ICS formation is heterotopic expression of *MPF2*, a homolog of *Tunicate* in *Zea mays*, which plays multiple roles in calyx development and male fertility in *Physalis* (He et al., 2004; He & Saedler, 2005).

The ICS genetic pathway may involve an interplay between MADS transcription factors and hormonal signals. The cytokinin signal facilitates the transport of MPF2 to the nucleus of calyx cells. Thus, MPF2, in conjunction with its interacting proteins, promotes cell division, which, in the presence of gibberellins, enlarges and ultimately allows the formation of the ICS (He & Saedler, 2005, 2007; He et al., 2007). The synthesis of an ICS-like structure in *Solanum tuberosum* confirmed the proposed pathway (He & Saedler, 2007). However, MPF2 also interacts with PFMAGO, an ortholog of mago nashi from *Drosophila* known to control the formation of gametes (Boswell et al., 1991; Newmark & Boswell, 1994). These findings also hint of a possible coupling between ICS formation and fertility development in *Physalis*, although the mechanism is not yet clear (He et al., 2007).

Thus, in *Physalis*, the ICS is an apparently simple trait controlled by a complicated pathway consisting of multiple components, including some unknown factors.

Given the complex genetic architecture of the ICS, one would expect to see this trait within Solanaceae only rarely. However, several genera feature ICS, including *Physalis*, *Magarantha*, *Withania*, and *Przewalskia*. This raises questions concerning single versus multiple origins of ICS. Phylogeny suggests a polyphyly of taxa with ICS. However, floral expression of *MPF2*-like genes in most of Physaleae and most of Solanaceae is plesiomorphic, indicating an ancestral status of ICS (Hu & Saedler, 2007). Considering the complex genetic architecture of ICS, any mutations in the pathway could lead to the loss or modification of ICS. Therefore, the seemingly polyphylic origins of ICS currently observed within Solanaceae may actually be the result of secondary loss or modification of ICS.

Very recently, in tetraploid Withaninae, gain and loss of *MPF2*-like genes (*MPF2-like-A* and *MPF2-like-B*) and sub-functionalization of their proteins has been assessed in relation to ICS formation. The presence of a conserved CArG-box in their promoters, their expression pattern, and correlation with ICS evolution was elegantly determined in a phylogenetic context (Khan et al., 2009). It was shown that a novel *MPF2-like-A* gene was positively selected for ICS formation in *Withania* and that its loss could lead to ICS deficiency in *Tubocapsicum*, the closest relative of *Withania* in Withaninae.

The function of the ICS in Solanaceae is not entirely clear. As in *Przewalskia tangutica*, the ICS of *Przewalskia*, a genus endemic to China (Zhang et al., 1994), seems to facilitate wind dispersal of the fruits (Knapp, 2002). In Withanianae, the ICS may function to provide a microclimate that maintains the humidity necessary for berry development in the dry environment (Khan et al., 2009). In *Physalis*, the ICS may be a byproduct of fertility evolution because one of its controlling factors, MPF2, has become an integral part of

the male fertility system of *P. floridana* (He & Saedler, 2005; He et al., 2007). Calyx ablation analyses hint that the ICS may also serve as a source of photosynthesis or have a protective role in *Physalis* in the earlier developmental stage (He & Saedler, 2007). Understanding the adaptive values of the ICS in Solanaceae requires careful ecological investigation.

4 Outlooks

The molecular developmental mechanisms underlying the origin of novel structures are not well understood. Nonetheless, the studies described above have shown that the recruitment of an existing transcription factor into a different functional context or the loss of function play a pivotal role in the evolution of floral morphological novelties, as well as in the evolution of biodiversity. From our point of view, revealing the evolutionary mechanisms of the novel morphologies is still the major goal of evo-devo, especially for floral novelties in plants and the mechanisms for the domestication of agronomic traits in crops (Li et al., 2006).

It is worth mentioning that Solanaceae is currently on its way to becoming a model system for evo-devo owing to its richness in species diversity and morphological variation, the presence of many economically important plants in the family, and the feasibility of gene transformation. As a key agronomic trait, fruit is a post-floral organ and its type (Knapp, 2002), size (Frary et al., 2000; Cong et al., 2008), and morphology (Xiao et al., 2008) are polymorphic in Solanaceae, as shown in a phylogenetic perspective on fruit diversity within the family (Knapp 2002). Most of the edible Solanaceae bear berries, such as the tomato, eggplant, pepper, and *Physalis*. In tomato, several genes have been shown to play a role in the evolution of tomato size (Frary et al., 2000; Cong et al., 2008). Do the same genes or similar networks contribute to the diversity of berry size in different genus? Quantitative trait loci mapping analysis suggests that a synteny of genes controls fruit size among tomato, eggplants, and pepper (Doganlar et al., 2002; Paran & van der Knapp, 2007). In *Physalis*, the fruit size varies markedly and current investigations into the evolution of berry size in the genus are underway in our laboratory. Further investigations into the molecular basis of evolutionary innovations require an integrative approach involving developmental biology, evolutionary biology, molecular genetics, ecology, and bioinformatics. With rapid technical advances, especially in functional genomics, and with the expansion of the research community, the still young field of evo-devo will grow and bear fruit.

Acknowledgements The authors thank CAS Academia De-Yuan HONG and Drs Xiao-Quan WANG and Hong-Zhi KONG (Institute of Botany, Chinese Academy of Sciences) for their valuable suggestions to improve the manuscript. The authors also acknowledge Dr. Tao SANG (Michigan State University, East Lansing, MI, USA) and two anonymous reviewers for their constructive comments on the manuscript. The authors' work described herein was supported by a grant (30870175) from the National Natural Science Foundation of China (to CYH) and by the Hundred Talents Project of the Chinese Academy of Sciences (to CYH).

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