

REVIEW ARTICLE

Transcriptional Regulators and the Evolution of Plant Form

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INTRODUCTION

The modern or neo-Darwinian theory of evolution was formulated well over half a century ago at a time when genes were abstractions and development was understood only as the transition between a series of phenotypic states. Since that time, our knowledge of the gene, the diversity of types of genes, the interactions among genes, and the organization of genes into the networks and hierarchies that regulate development has grown enormously. Although still incomplete, this burgeoning knowledge of the molecular mechanisms that regulate ontogeny compels a continual reassessment of the question: How did the diversity of forms of life on earth evolve? Darwin resolved this question at the ecological level by explaining how natural selection would favor the “fittest” forms within a specific environmental context. The neo-Darwinian synthesis resolved this question at the genetic level by showing how the mechanisms of mutation, selection, and drift produce the directional changes in gene frequencies that underlie the evolution of novel forms (Huxley, 1963). The challenge facing the current generation of evolutionary biologists is to resolve this question at the molecular and developmental levels.

In this article, we explore how recent advances in the understanding of gene structure, function, and interactions have fueled the creation of new ideas on the molecular mechanisms underlying the evolution of plant form. We summarize evidence for our view that change in the *cis*-regulatory elements of transcriptional regulators provides a predominant mechanism for the generation of novel phenotypes. We end with a discussion of the opportunities for testing this idea empirically by establishing causal linkages between specific changes in the molecular structure of genes and the production of novel phenotypes in naturally occurring plant species.

“REGULATORY GENES” AND EVOLUTION

After the synthesis of genetics and evolution in the 1920s, several authors hypothesized that genes regulating the tim-

ing or rate of development must be required for the evolution of form (Haldane, 1932; Huxley, 1932; Goldschmidt, 1940). Exactly how genes could regulate development was a mystery. The decoding of DNA and illumination of the regulation of gene expression (Jacob and Monod, 1961) set the stage for another round of hypothesis development about the underlying molecular mechanisms for both physiological and morphological evolution.

Around 1970, Britten and Davidson (1969, 1971) presented a compelling theory for the molecular basis of evolutionary change. Although their terminology and some of the specifics of their model have not survived, the logic and the general outlines of their model have. Britten and Davidson distinguished between “producer” (or structural) genes and “receptor” (or regulator) genes, which are now called coding regions and promoters, respectively. They further identified “integrator” genes whose product, “activator RNA,” binds to receptor genes and thereby regulates the transcription of the producer gene. Integrator genes are equivalent in spirit to transcriptional regulators. The model proposed a hierarchical structure, with single integrators providing for the coordinate expression of multiple downstream producers. With this model of gene regulation, Britten and Davidson speculated that evolution would principally involve changes in the “regulatory genes,” or what we now call *cis*-regulatory elements.

EARLY EVIDENCE FOR REGULATORY EVOLUTION

The concept that the regulatory regions of genes have a predominant role in evolution won early but inconclusive support. Ohno (1971) argued that because the same set of structural genes are apt to be involved in the formation of the digits of a horse and a human, functionally and anatomically very different structures, changes in the regulation of these genes can best explain the differences in these structures. Wilson and his colleagues (Wilson et al., 1974; Wilson, 1976) noted the uncoupling of protein and morphological evolution and argued that extreme morphological divergence in the absence of a corresponding level of protein divergence could be reconciled if regulatory gene mutations (i.e., in *cis*-regulatory

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regions) rather than structural gene mutations (i.e., in protein coding regions) were the principal mechanism of evolutionary change. For example, these authors note that there is relatively little protein variation among mammals, despite considerable anatomical diversity, whereas considerable protein variation exists among frogs with little corresponding morphological diversity.

The neutral theory of molecular evolution, as formulated by Kimura (1968, 1983), also proposes that the vast majority of protein evolution does not affect the morphology of organisms. Because changes in morphology are often adaptive, neutral theory therefore implies that most protein evolution will not lead to adaptive changes in the phenotype. More recently, Endo et al. (1996) followed up on Kimura's conclusions by performing a global search for the signature of positive selection on proteins. Their logic was that for neutral evolution, the ratio of nonsynonymous substitutions (per nonsynonymous site) to synonymous substitutions (per synonymous site) will equal one (or less if deleterious amino acid changes are selected against). However, if positive selection is causing the fixation of advantageous amino acid substitutions, this ratio should be greater than one. For 3595 groups of proteins examined, only 17 showed evidence for positive selection. Again, this result is concordant with the view that the portion of morphological evolution that is adaptive in nature is not driven by changes in protein function.

This early evidence for the importance or predominance of regulatory evolution over protein evolution is at best suggestive. For example, although the level of protein differentiation between humans and chimpanzees may not be commensurate with their behavioral and morphological differences, a small number of effective amino acid substitutions could be sufficient to control these differences. In fact, under the model that protein evolution explains most morphological evolution, a reasonable expectation would be that a small number of effective changes in protein function should exist amid a much larger number of ineffective (or neutral) changes.

CONSERVATION OF PROTEIN FUNCTION

If most proteins are not evolving in a positive way under the guidance of natural selection, then their functions are apt to be conserved over long periods of evolutionary time. This, in fact, appears to be the case when one considers the common observation that a protein from one species can often complement a mutant or produce a similar phenotype in a second organism, even when the two species have been separated for long evolutionary periods. One of the most striking cases is the ability of the mouse *Small-eye* (*Pax-6*) gene, which controls eye formation, to induce ectopic eye formation in *Drosophila*, indicating that the function of this protein has been conserved for the 500 million years since the divergence of arthropods and vertebrates (Halder et al.,

1995). In another example, the chicken *gHoxb-1* gene can substitute for a *Drosophila Hox* mutant (*lab*), which functions in head involution, when linked to the appropriate *Drosophila* regulatory sequences (Lutz et al., 1996). The *Drosophila paired* (*prd*) gene can rescue a *gooseberry* (*gsb*) mutant when the *gsb* control region is fused with *prd* coding regions, despite the fact that these two homeobox genes serve distinct roles in *Drosophila* development (Li and Noll, 1994). Similarly, the *cdc2* homolog of maize complements the *cdc28* mutant of *Saccharomyces*, despite a divergence time of at least 600 million years (Colasanti et al., 1991). Finally, the DEF protein of snapdragon fully complements an *ap3-1* mutation and partially complements the *ap3-3* mutation of *Arabidopsis* (Irish and Yamamoto, 1995).

Evidence for the conservation of protein function over long evolutionary periods notwithstanding, proteins do evolve in function, and change in protein function is a crucial component of evolution. Indeed, there are a few cases in which the functional evolution of proteins involved in morphogenesis has been documented. For example, among members of the MADS box gene family of *Arabidopsis*, differences in three separate regions of the protein are involved in conferring the functional specificities that distinguish family members (Krizek and Meyerowitz, 1996). In this case, homologous proteins cannot substitute for other members of the same protein family. Because these different functions are required for normal flower development, one might infer that floral evolution has involved changes in protein function.

When one observes full complementation of function by a protein from another species, the interpretation is clear. Function has been conserved. When partial or noncomplementation is observed, the interpretation is more complex. One possibility is that the protein has specifically evolved to interact with a different set of partners or bind to a different set of downstream genes in ways that alter the phenotype. A second possibility is that the protein has changed in "specificity" but not in "function as related to the phenotype." For example, a pair of interacting proteins in one species may have coevolved on a particular path, whereas in a second species the orthologous pair of proteins has evolved independently along a different path. These pairs may fulfill the same function (e.g., they may activate the same set of downstream genes) in both species, but the individual proteins of one species may not be able to complement the "function" of their orthologs in the other species because of changes that specify the interaction with the particular partner. Function is contextual, and one cannot infer a change in function (as related to the phenotype) from failure of a protein to complement its ortholog in another species.

EVOLUTION BY CHANGES IN GENE EXPRESSION

The view that evolution often proceeds via changes in the spatial-temporal patterns of gene expression has been

embraced by diverse authors (see Raff, 1996). Evolutionary tinkering with promoters and enhancers could readily generate variation in the pattern of gene expression and thereby produce novel phenotypes upon which natural selection could act. The fact that plants readily tolerate insertions, rearrangements, and other forms of sequence evolution in *cis*-regulatory regions implies that these would be likely "hot spots" for the generation of novelty (Wessler et al., 1995). Two lines of evidence support the view that changes in gene expression are important evolutionary mechanisms.

The first line of evidence comes from studies of natural species that reveal correlations between the spatial patterns of gene expression and phenotype. For example, the formation of eye spots on butterfly wings is correlated with the domains of *Distal-less* expression (Brakefield et al., 1996), so a change in the spatial pattern of *Distal-less* expression could confer a change in the size and distribution of eye spots. Comparison of *Hox* gene expression in primitive wingless and advanced winged insects suggests that genes involved in wing formation acquired *cis*-regulatory elements that brought them under the negative control of *Hox* genes in some body segments (Carroll et al., 1995). Changes in *Hox* gene expression patterns are also associated with the transformation of thoracic limbs into feeding appendages in crustaceans (Averof and Patel, 1997). In tomato, expression of a homeobox-containing gene in the leaf primordia is associated with the formation of compound rather than simple leaves (Hareven et al., 1996). There are also several cases in the evolution of physiological traits in which specific adaptations are correlated with changes in the pattern of gene expression (Dudareva et al., 1996; Sinha and Kellogg, 1996). For some physiological traits, shifts in gene expression have been mapped to 5' regulatory regions (Schulte et al., 1997; Stockhaus et al., 1997). It seems reasonable that morphological evolution could occur by the same mechanism.

A second line of evidence comes from mutations that reveal how rearrangements in the nontranscribed flanking regions of genes can produce novel patterns of gene expression and/or novel phenotypes. In tomato, Chen et al. (1997) demonstrated how a chimeric gene resulting from the insertion of a duplicate copy of a metabolic enzyme into the 5' end of the homeobox-containing gene *LeT6* resulted in overexpression of the homeodomain protein and the conversion of unipinnately compound leaves into three- or fourfold pinnately compound leaves. Thus, changes in regulation associated with alterations of 5' regulatory sequences can produce novel phenotypes. More importantly, the novel phenotype is not simply a monstrous disruption of normal development but a well-integrated form of the pinnately compound leaf not uncommon among flowering plants. In another example, transposable element-induced alterations in the promoter of the maize alcohol dehydrogenase gene resulted in quantitative, organ-specific changes in its expression (KloECKener-Gruissem and Freeling, 1995).

Modular organization of promoters may contribute to their evolutionary flexibility (Kirchhamer et al., 1996). Distinct pro-

motor elements that drive expression in different cell, tissue, or organ types can be added in a stepwise fashion during evolution without the disruption of preexisting promoter elements.

SIGNALS, TRANSDUCERS, REGULATORS, AND TARGETS

Britten and Davidson's model for regulatory evolution was developed at a time when little was known about the diversity of gene functions and the interactions among genes. Although this knowledge is still incomplete, a far better catalog of the components is now available as well as substantial, if imperfect, knowledge of the interactions between these components. Some systems, such as vulval development in *Caenorhabditis elegans* and the pheromone response pathway in yeast, are understood at impressive levels of detail (Kayne and Sternberg, 1995; Schultz et al., 1995). Thus, one can now ask: Are some classes of proteins more apt to contribute to the evolution of form than are others?

In broad outline, developmental pathways are composed of individual modules that are arranged in a semihierarchical system (Raff, 1996). We define individual modules as consisting of signal(s), transducers, transcriptional regulator(s), and targets (Figure 1). The signals may be environmental (e.g., photoperiod) or internal (e.g., auxin). The transducers compose the signal transduction pathway and include those gene products that participate in signal production, perception, transmission, and modification (e.g., ligands, receptor kinases, phytochromes, and proteins/enzymes involved in hormone synthesis/deactivation). Transcriptional regulators would be the genes involved specifically in the regulation of gene expression through direct binding to *cis*-regulatory elements. Finally, target genes can be any type of gene. For modules placed near the endpoints of development, the targets are apt to be genes whose actions do not affect other genes directly (e.g., metabolic enzymes). For modules higher up in the cascade, the targets would include genes involved in signaling and in transcriptional regulation.

Viewed this way, plant development is a cascade of events that begins with the internal signals that initiate embryogenesis, followed by continual external and internal input directing the orderly activation of the hierarchically arranged modules. The modules themselves are visible in the processes, cell types, tissues, organs, and organ systems that we see. For example, one module may regulate trichome formation, another petal development, and a third seed maturation.

TRANSCRIPTIONAL REGULATORS AND EVOLUTION

At what point(s) in the genetic hierarchy can nature best experiment with change to produce novel phenotypes? One

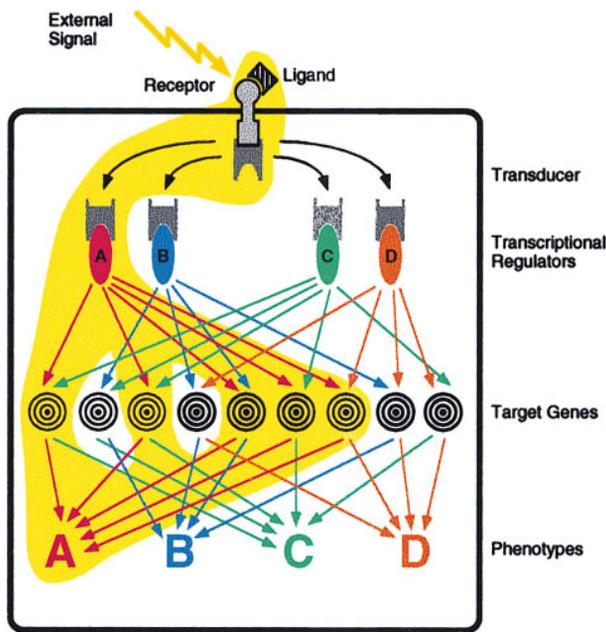


Figure 1. Simplified Representation of the Interactions among Genes in a Developmental Pathway.

Either an internal or external signal activates the receptor. The signal is transmitted by a transducer gene, which turns on the transcriptional regulators. The transcriptional regulators activate the target genes to produce a specific phenotype. As diagrammed, changes in the ligand, receptor, transducers, or target genes will be pleiotropic, altering multiple phenotypes (A, B, C, and D). However, alterations in a transcriptional regulator would affect only one phenotype. The yellow area represents a single developmental module.

criterion relevant to this question is the level of pleiotropy exhibited by genes at different hierarchical levels. Genes integrated into multiple modules will have broadly pleiotropic effects, thus limiting the extent to which they can be modified without disturbing the overall development of the plant. In contrast, genes whose actions are restricted to a single module could change with only limited impact on the organism overall.

We compiled a list of cloned genes from Arabidopsis with mutant phenotypes that altered the morphology of the plant. We asked a colleague to categorize the severity of the mutant phenotype as (1) affecting a single organ or organ system (not pleiotropic), (2) affecting several related organ systems (weakly pleiotropic), (3) affecting multiple organ systems (moderately pleiotropic), or (4) strongly disrupting overall development (strongly pleiotropic). We also categorized the genes as transcriptional regulators or signaling system genes. Tallying the data revealed (Figure 2) that mutations in transcriptional regulators typically have less severe phenotypic effects (mostly classes 1 and 2) than do

mutations in signaling genes (all classes 3 and 4). Using the criterion outlined above, transcriptional regulators should be more easily subject to evolutionary modification because their alteration would be less likely to have a major disruptive effect on development. Genes involved in signaling, on the other hand, appear to be integrated into diverse aspects of development, suggesting that nondisruptive alterations in their function and/or expression would be difficult to orchestrate.

The inference that transcriptional regulators play a central role in evolution has been advocated by one of us previously (Doebley, 1993), and it appears to reflect the communal wisdom of evolutionary-developmental biologists. For animal systems, most evolutionary interest has focused on homeo-domain-containing transcriptional regulators (Warren and Carroll, 1995). For plants, the MADS box-containing transcriptional regulators have been the focus of evolutionary studies (Purugganan et al., 1995; Münster et al., 1997). The reasons for this interest are clear. Transcriptional regulators frequently act as switches between phenotypic states that duplicate or closely approximate those that distinguish natural species or higher level taxonomic categories. Transcriptional regulators could act as evolutionary switches by providing for the coordinate expression of their targets in new spatial or temporal patterns as required to generate functional and well-integrated, yet novel, phenotypes (Goodrich et al., 1992; Byrne et al., 1996; Hanson et al., 1996).

The inference that signaling genes are less likely contributors to evolution is not intuitive. In principle, modification of signaling genes could also provide for the coordinate expression of target genes in new temporal or spatial contexts. Some genes involved in signaling, such as *tasselseed2* of maize (DeLong et al., 1993), would appear to be good candidates for evolutionary building blocks. Moreover, signaling

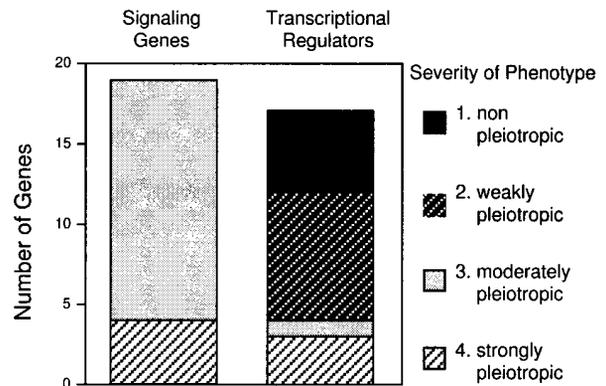


Figure 2. Classification of Cloned Arabidopsis Genes with Morphological Phenotypes.

Signaling genes tend to have broad pleiotropic effects, whereas transcriptional regulators are more narrowly pleiotropic.

genes, as we have broadly defined them, should represent a substantial portion of the genome, providing ample opportunity for their contribution to evolution. Perhaps signaling genes have a more restricted role in the evolution of form because signaling pathways tend to be shared among multiple developmental modules (see Figure 1). If this is true, then changes in signaling genes would be highly pleiotropic, increasing the likelihood that a positive alteration in one module could be accompanied by a detrimental alteration in another.

DEVELOPMENTAL PATHWAYS AND PLEIOTROPY

The structure of two known developmental pathways provides some support for our arguments (1) that transcriptional regulators are apt to have less pleiotropic mutant phenotypes than their upstream signaling genes and (2) that signaling pathways tend to be shared among multiple developmental modules. First, Okamoto et al. (1996) examined the role of hormonal signaling in the maintenance of floral meristem identity in *Arabidopsis*. These authors proposed that *LEAFY* (*LFY*), a transcription factor contributing to the maintenance of floral meristem identity, is a target of gibberellin (GA) signaling. Plants heterozygous for a *lfy* mutation can exhibit a reversion from floral to inflorescence meristem identity under some conditions. Application of exogenous GA blocks this reversion, as does a mutation at *SPINDLY* (*SPY*), a signaling gene whose wild-type function is to repress the GA signal transduction pathway. Their analyses show that *SPY*, which has broadly pleiotropic effects, acts upstream of *LFY*, which has more narrowly pleiotropic effects. If nature should care to tinker with floral meristem identity, *LFY* would represent a more promising target than does *SPY*. The fact that *spy* mutations exhibit a variety of defects beyond floral meristem identity also indicates that meristem identity is only one of multiple developmental modules that respond to the GA pathway.

A particularly clear example that transcription factors are apt to have more limited phenotypic effects than their upstream signaling genes comes from the Ras signal transduction pathway in *C. elegans* (Kayne and Sternberg, 1995; Greenwald, 1997). The Ras pathway influences vulval development, male spicule development, viability, and other processes. The pathway is activated via a ligand (LINEAGE ABNORMAL-3; *LIN-3*) and a receptor (LETHAL-23; *LET-23*), and the transcription factors *LIN-1* and *LIN-31* are known to be activated by the Ras pathway. Mutations in genes at the top of this pathway, such as *LIN-3*, *LET-23*, or *LET-60*, are lethal and produce multiple developmental defects. However, mutants with defects in the transcription factor *LIN-1* are viable and exhibit the specific defect of multivulva. Similarly, *LIN-31* mutants are also viable but exhibit multivulva or cells with confused identities. Thus, multiple developmental modules respond to the Ras pathway so

that mutations affecting it are highly pleiotropic, whereas the transcription factors activated by the Ras pathway have more limited effects.

BEYOND THEORY: GENETICS AND TRANSGENICS

We have discussed two molecular mechanisms by which plant form may evolve: (1) changes in *cis*-regulatory elements of genes and (2) changes in transcriptional regulators. We find it attractive to combine these two mechanisms and propose that modifications in the *cis*-regulatory regions of transcriptional regulators represent a predominant mode for the evolution of novel forms. This model has a statistical prediction. Taken over a large number of cases in which the molecular mechanisms of new morphologies have been clearly documented, *cis*-regulatory changes in transcriptional regulators will be significantly overrepresented. How can this model be tested?

Identification of the molecular mechanisms underlying the morphological differences between species is not a simple task. Genetics provides one opportunity. Distinct yet cross-compatible pairs of species that differ in major morphological traits are common among flowering plants (Figure 3; Gottlieb, 1984). The genes controlling these differences can be studied genetically in crosses and mapped using molecular markers. At present, the most promising systems for this approach are found among species related to model organisms such as maize, rice, or *Arabidopsis*. Extensive colinear gene order between model organisms and related species would allow one to relate genes controlling morphological differences between natural species to cloned genes in the model species (Moore, 1995). Once a candidate gene is identified, transgenics or genetic means could be employed to confirm that it controls the trait and to identify the molecular polymorphism underlying the phenotypic differences. This approach will be particularly powerful once the sequence of the entire genome of the model species is known (Ecker, 1997).

Although a genetic approach has advantages, the morphological traits of most interest to many evolutionists are often those that distinguish cross-incompatible species or higher level taxonomic groups (Kellogg, 1996). Transgenic approaches allow a way around this problem in some cases (Kellogg, 1996; Baum, 1998). Candidate genes (including *cis*-regulatory elements) from a species exhibiting a derived trait could be transformed into a related species exhibiting the ancestral state. If the transformants exhibit the derived trait, then one can infer that the candidate gene is responsible for the difference between the species. For example, if the *Arabidopsis* gene *PERIANTHIA*, which has a wild-type function of specifying cruciform flowers (Running and Meyerowitz, 1996), were solely responsible for the evolution of the cruciform flower of mustards from a pentamerous ancestor, then transformation of related pentamerous species

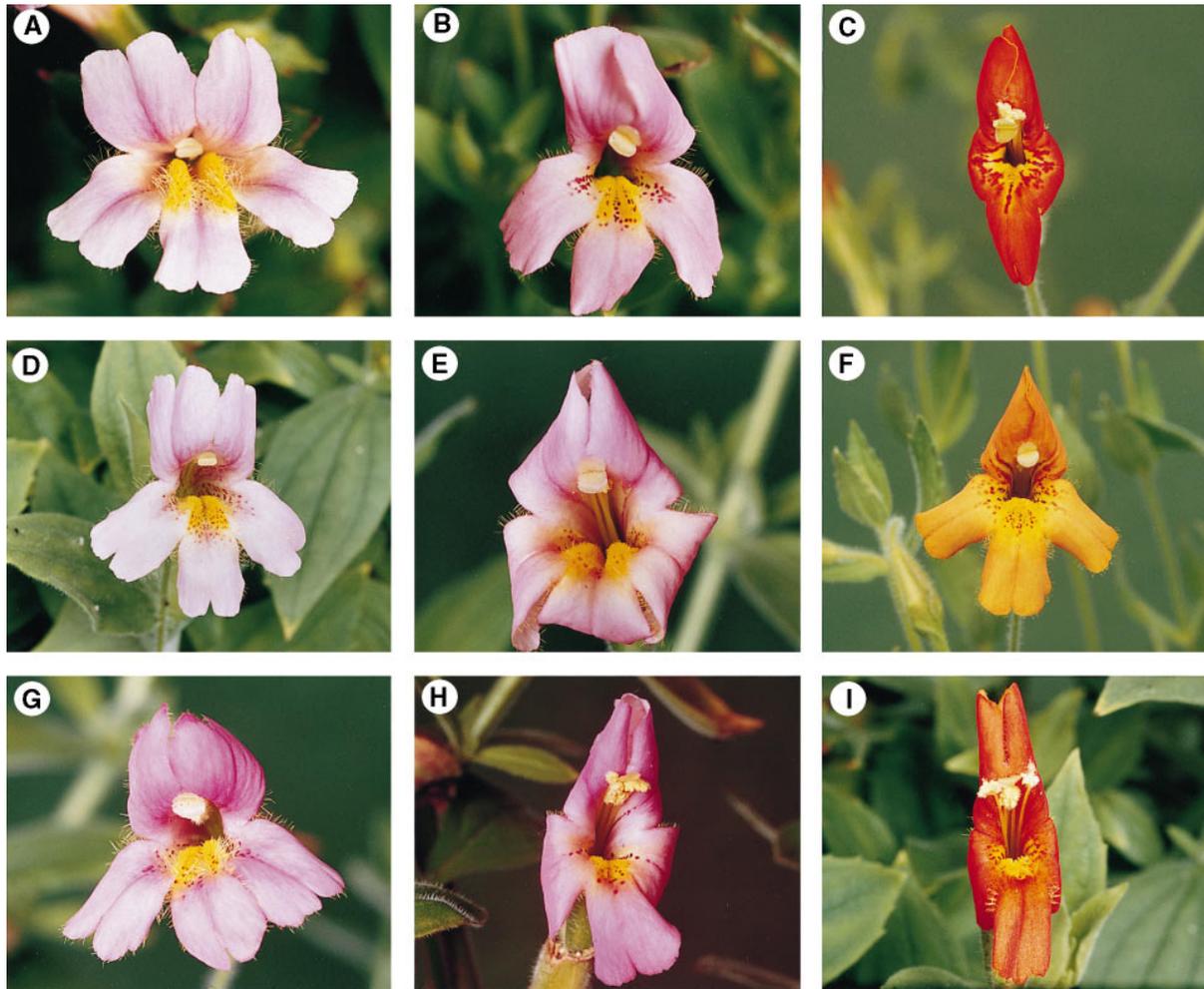


Figure 3. Variation among the Progeny of a Cross of Two Species of *Mimulus*.

Among the flowering plants, there are many examples of cross-compatible yet morphologically and adaptively well-differentiated species. One striking example is shown here.

(A) to (C) The bumblebee-pollinated *Mimulus lewisii* (A), the hummingbird-pollinated *M. cardinalis* (C), and their F_1 hybrid (B). The hybrid (B) has an intermediate phenotype.

(D) to (I) F_2 progeny from the above cross. Both parental [(D), (F), (G), and (I)] and recombinant [(E) and (H)] phenotypes are found. Genetic mapping has identified a small number of loci of large effect that control these phenotypic differences. Reprinted by permission from Nature (Bradshaw et al., 1995). Copyright (1995) Macmillan Magazines Ltd.

with the *Arabidopsis* *PERIANTHIA* gene may be sufficient to confer the cruciform condition. Subsequent analyses could identify genic regions (i.e., promoter or coding) that control the trait difference. Experiments of this nature clearly need to be tried, even though there is some risk that the candidate gene alone may not be sufficient to establish trait expression in a foreign context. This is especially true if multiple genes were involved in the evolution of the derived condition for the trait.

CONCLUSION

When Darwin proposed the theory of evolution by natural selection, genetics did not exist, and although it was understood that "like begets like," the mechanism of inheritance and therefore the mechanism of evolution was a mystery. The rediscovery of Mendelian genetics in the early part of this century identified the gene as the currency of inheri-

tance and provided the foundation for modern evolutionary theory based on how the forces of mutation, selection, and drift produce evolutionary change via their action on genes. However, still missing from the equation was an understanding of genes, their structure, and the diversity of their functions. Also lacking was an understanding of the interactions among genes and their organization into the networks and hierarchies that control ontogeny. Over the past several decades, many of these missing elements in the equation have been illuminated, creating the opportunity for both developing more realistic models of morphological evolution and conducting empirical tests of them.

In this review, we have presented some inferences that we and others have drawn from the modern understanding of gene structure, function, and interactions. These include (1) that development is modular in organization, (2) that protein function tends to be conserved over long evolutionary periods, (3) that plant promoters are labile in nature, (4) that signaling genes tend to have strongly pleiotropic effects, and (5) that transcriptional regulators act as key switches in plant development. The conclusion we draw from these inferences is that the evolution of plant form will be most readily accomplished by changes in the *cis*-regulatory regions of transcriptional regulators. The challenge now is to test this model by identifying the molecular mechanisms underlying specific events in the evolution of plant form among natural species. Genetics and transgenic plants will provide the necessary tools.

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