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REVIEW: PART OF A SPECIAL ISSUE ON EVOLUTION AND DEVELOPMENT

Robustness and evolvability in the B-system of flower development

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• *Background* Gene duplication has often been invoked as a key mechanism responsible for evolution of new morphologies. The floral homeotic B-group gene family has undergone a number of gene duplication events, and yet the functions of these genes appear to be largely conserved. However, detailed comparative analysis has indicated that such duplicate genes have considerable cryptic variability in their functions. In the Solanaceae, two duplicate B-group gene lineages have been retained in three subfamilies. Comparisons of orthologous genes across members of the Solanaceae have demonstrated that the combined function of all four B-gene members is to establish petal and stamen identity, but that this function was partitioned differently in each species. These observations emphasize both the robustness and the evolvability of the B-system.
• *Scope* We provide an overview of how the B-function genes can robustly specify petal and stamen identity and at the same time evolve through changes in protein–protein interaction, gene expression patterns, copy number variation or alterations in the downstream genes they control. By using mathematical models we explore regulatory differences between species and how these impose constraints on downstream gene regulation.
• *Conclusions* Evolvability of the B-genes can be understood through the multiple ways in which the B-system can be robust. Quantitative approaches should allow for the incorporation of more biological realism in the representations of these regulatory systems and this should contribute to understanding the constraints under which different B-systems can function and evolve. This, in turn, can provide a better understanding of the ways in which B-genes have contributed to flower diversity.

Key words: Robustness, evolvability, flower development, B-function, mathematical modelling.

ROBUSTNESS, EVOLVABILITY AND TRANSCRIPTIONAL REGULATION IN PLANTS

Robustness versus evolvability

Most developmental systems are extraordinarily robust to genetic or environmental perturbations; nonetheless, such systems evolve into new, and potentially more complex, systems. Robustness allows for a developmental network to accumulate cryptic genetic variation, and thus provide the potential for evolutionary change (Rutherford and Lindquist, 1998; Specchia *et al.*, 2010). One common form of such robustness is genetic redundancy; this is common in plant systems, with the *Arabidopsis* genome, for instance, displaying a considerable degree of redundancy (Martienssen and Irish, 1999). However, other types of genetic robustness can also promote the maintenance of a specific developmental outcome; this is often referred to as canalization (Waddington, 1957; Gibson and Wagner, 2000; Flatt, 2005). Evolvability, on the other hand, refers to the capacity of a system, and its inherent variation, to be adaptive under the right conditions. The processes of robustness and evolvability, then, appear to contradict each other, but a considerable literature has suggested that, paradoxically, robustness can promote evolvability (reviewed in Wagner, 2005). This can be understood by taking into account the many solutions possible to solve a single problem. Mechanisms that are functionally distinct at the molecular level can result in the same developmental outcome. This is the case for the B-class gene network in angiosperms. B-genes are known to be key regulators of petal and stamen identity in distantly related flowering plant species such as *Arabidopsis*, *Antirrhinum*, *Petunia* and *Papaver*, and this function appears to be conserved (Coen and Meyerowitz, 1991; Vandenbussche *et al.*, 2004; Drea *et al.*, 2007; Kramer *et al.*, 2007; Benlloch *et al.*, 2009). Yet it has now become clearer that related species of Solanaceae have evolved qualitatively different systems but that those systems have a conserved role in establishing petal and stamen identity (Geuten and Irish, 2010). Here we review, using the B-system as an example, the different ways such a system can be both robust and evolvable at the same time and we explore the constraints of such systems using theoretical models.

Evolution of transcriptional regulation in plants

The question of robustness versus evolvability is being addressed for several animal and microbial systems (Wagner, 2005). However, as plants utilize distinct molecular machinery to control their development and physiology, it appears that the specifics of how plants have responded to selective pressures may be different. The evolution of land plants, and in particular angiosperms, has been associated with MYB and MADS

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transcription factor family amplification (Riechmann *et al.*, 2000). Moreover, flowering plant genome sizes vary over a much wider range than other taxonomic groups, which is probably a consequence of differences in ancestral polyploidy and the amplification of retrotransposons (Bennetzen, 2005; Vitte and Panaud, 2005; Barker *et al.*, 2008). Although genomes can diminish in size after duplication (Devos *et al.*, 2002), the requirement for a consistent stoichiometry of regulatory complexes potentially explains why transcriptional regulators are often retained (Blanc and Wolfe, 2004; Freeling and Thomas, 2006; Birchler *et al.*, 2007). Because duplicate transcription factor genes can undergo sub- or neofunctionalization, they can lose redundancy, and so the absolute number of regulators per genome can be variable and can differ in complex ways between species.

Plants offer a number of interesting features pertinent to the study of the evolution of gene regulation, as compared with animal and microbial systems. First, the evidence for long-range 'enhancers', transcriptional control regions located at a distance from the coding region, is much more scarce for plants. To our knowledge only a few cases of distant *cis*-regulatory control elements have been found in plants; for instance, the maize *tb1* gene possesses upstream enhancer elements that reside more than 41 kb away from the coding sequences (Clark *et al.*, 2006). The presumed more restricted spatial localization of transcriptional control in plants could both facilitate their study as well as reflect salient differences in the ways in which gene regulation has evolved in plants. Second, the variation in genome sizes, usually seen as a difficulty, can also be an advantage in understanding regulatory evolution, because regulatory DNA may be relatively conserved between species, while non-functional DNA may accumulate mutations more rapidly (Peterson *et al.*, 2009). The potential of these advantages remains to be explored more fully.

ROBUSTNESS IN CLASS B GENES THROUGH MULTIPLE COPIES

One commonly cited mechanism for robustness is gene redundancy. This is the idea that more than one gene performs the same function, and so any mutation in one gene is compensated for by the additional gene copy (Gu *et al.*, 2003; Wagner, 2005). In the Solanaceae, for instance, duplicate copies of the MADS box B-gene lineages exist, and can, in some cases, compensate for the mutational loss of a redundant gene copy in specifying petal and stamen development (Fig. 1; Geuten and Irish, 2010). However, complete elimination of B-gene activity in these species indicates that this system is not robust against complete elimination in that evolutionarily unrelated developmental mechanisms cannot compensate for its absence. This is in contrast to other developmental genetic systems such as flowering time, for which backup regulatory mechanisms do exist. Flowering will occur even after a single pathway is eliminated as several other interacting but not fully redundant pathways can still promote the floral transition (Koorneef *et al.*, 1998; Wang *et al.*, 2009). However, the evolutionary history of class B genes throughout angiosperm diversification reveals another level of robustness in that multiple copies of these genes were generated and functions were partitioned throughout evolution.

The molecular evolution of the B-system illustrates its robustness against changes in copy number throughout angiosperm evolution

B-gene copy number can vary between species; phylogenetic analyses of such duplication events can also provide a relative timing for the evolutionary origin of paralogues (Kramer *et al.*, 1998, 2006, 2007; Becker and Theissen, 2003; Aoki *et al.*, 2004; Kim *et al.*, 2004; Stellari *et al.*, 2004; Hernández-Hernández *et al.*, 2007; Jaramillo and Kramer, 2007; Mondragón-Palomino *et al.*, 2009; Viaene *et al.*, 2009). Although *Antirrhinum* and *Arabidopsis* appear each to possess only a single *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) lineage gene, phylogenetic studies have shown that a duplication in the *DEF* lineage at the base of the core eudicots has resulted in the presence of a third B-class gene belonging to the *TOMATO MADS6* (*TM6*) lineage in most core eudicot species (Kramer *et al.*, 1998). Because the genome of *Arabidopsis* is sequenced and the transcriptome of *Antirrhinum* has been thoroughly sampled (Bey *et al.*, 2004), it is very likely that these two model species have independently lost their *TM6* lineage gene. Similarly, it is now clear that the core eudicot *GLOBOSA* lineage also underwent a duplication event, probably early in asterid diversification (Vandenbussche *et al.*, 2004; Viaene *et al.*, 2009; Geuten and Irish, 2010). This means that the B-system in *Arabidopsis* and *Antirrhinum* does not represent the same derivation of an ancestral regulatory system. These observations of gain and loss are an indication of the robustness of the B-system against copy number variation, as their roles in petal and stamen formation are conserved in all these species, despite the different gene duplication and gene loss events.

The function of B-genes can be redistributed over multiple paralogues when comparing related species

The B-system could further obtain robustness against elimination by distributing its functions over multiple fully redundant genes. However, there is little evidence for such redundancy in which B-gene paralogues share completely identical functions. Rather, duplicate B-genes tend to diversify in their function through changes at the level of gene expression, in their protein interaction specificity or potentially in the set of downstream genes they control. Robustness in this sense would reflect the partitioning of an ancestral function over multiple partially redundant paralogues. This was first indicated by the comparison of the *DEF* and *TM6* lineages in *Petunia* and tomato, two species belonging to the family Solanaceae (Fig. 2). Although the B-system as a whole is still responsible for petal and stamen development, similar to the situation in *Arabidopsis* and *Antirrhinum*, the *DEF* and *TM6* lineage genes do not have the same loss-of-function phenotypes in *Petunia* as compared with tomato. While *DEF* has a function necessary for petal development in *Petunia*, it is required for both petal and stamen development in tomato (de Martino *et al.*, 2006; Rijpkema *et al.*, 2006). It appears that the *TM6* gene is not required for petal or stamen

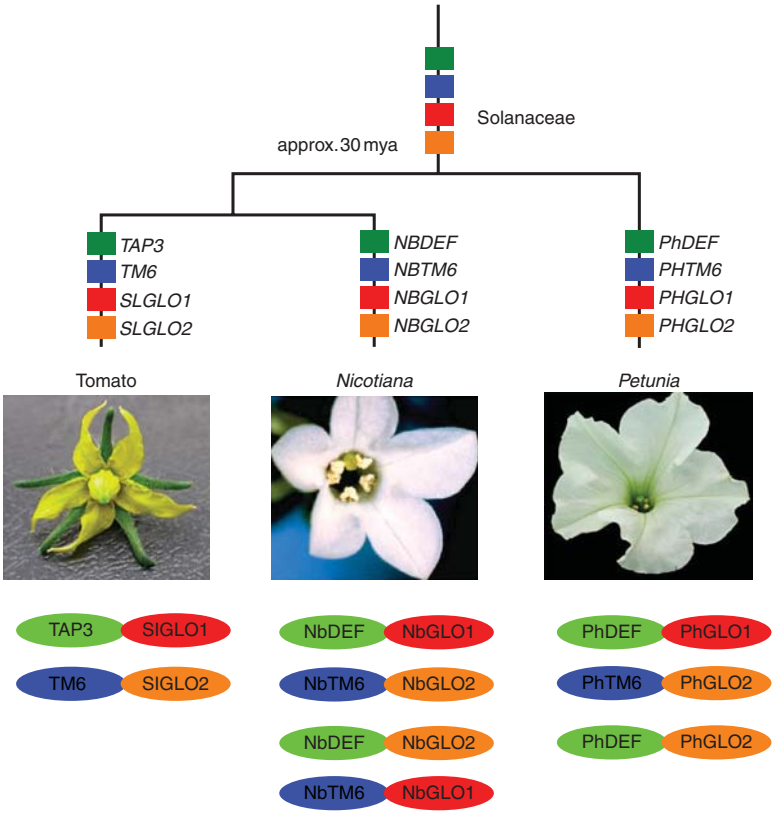


FIG. 1. Origin and evolution of B-function genes in Solanaceae. The four lineages, indicated in different colours, are present in tomato, *Nicotiana* and *Petunia*. Plausible relationships between these species are shown and their floral morphology is shown in the pictures. Below are a summary of yeast-two-hybrid studies by showing dimeric protein interactions. Most stringent are the interactions in tomato, followed by those in *Petunia*. Interactions showed least specificity in *Nicotiana* but N-terminally truncated constructs were used.

development in *Petunia* as its loss of function produces no phenotype. However, the knock-down phenotype of *TM6* in tomato affects stamen development. In *Nicotiana benthamiana* (also belonging to the Solanaceae), knock-down of both *TM6* and *DEF* has been shown to affect both petal and stamen development (Geuten and Irish, 2010; Liu *et al.*, 2004). Similar observations can be made for the two GLO lineages present in Solanaceae. While *GLO1* and *GLO2* knock-downs in *N. benthamiana* result in phenotypes in petals and stamens, knock-down of either one of these genes in tomato results only in defects in stamen development. Furthermore, knock-out of the *Petunia GLO1* gene weakly affects petal and stamen development, while knock-out of the *GLO2* gene has no characterized phenotype (Vandenbussche *et al.*, 2004). Together, this indicates that B-genes when present in multiple copies can fairly rapidly partition their function over multiple paralogues yet overall maintain their joint function in establishing petal and stamen identity. So in tomato, *Petunia* and *N. benthamiana* a full conversion of petal and stamens is the result when the function of both *DEF* and *TM6* or *GLO1* and *GLO2* paralogues are lost (Vandenbussche *et al.*, 2004; de Martino *et al.*, 2006; Rijpkema *et al.*, 2006; Geuten and Irish, 2010). In core eudicots, it is possible that this is a consequence of the B-system being more constrained by its autoregulation and obligate heterodimerization. However, in the case of opium poppy (*Papaver somniferum*), basal to core eudicots, there is also a partially redundant role for *DEF* paralogues in establishing petal and stamen identity rather than in the recruitment of novel functions for this biological process (Drea *et al.*, 2007). Functional analyses in more species will clarify whether this is the case across angiosperms.

ROBUSTNESS AND EVOLVABILITY OF *CIS*-REGULATION

Evolvability of B-gene expression

Several instances of expression divergence of B-genes have been described (reviewed in Zahn *et al.*, 2005; Hileman and Irish, 2009; Litt and Kramer, 2010). The fact that B-genes show variation in expression between species may be an inherent property related to the architecture of their promoter sequences. Recent studies in yeast have suggested that not all genes possess the same inherent capacity to evolve in terms of their expression patterns and that promoter architecture would be one determining factor in explaining this differential evolvability (Landry *et al.*, 2007; Tirosh *et al.*, 2009). Genes with TATA boxes, genes occupied by more nucleosomes, genes having more *trans*-acting factors that regulate their expression or genes that show unstable tandem repeats in their *cis*-regulatory regions appear to be more evolvable in yeast. To understand the robustness and evolvability of *cis*-regulation of the B-system genes, it would be important to

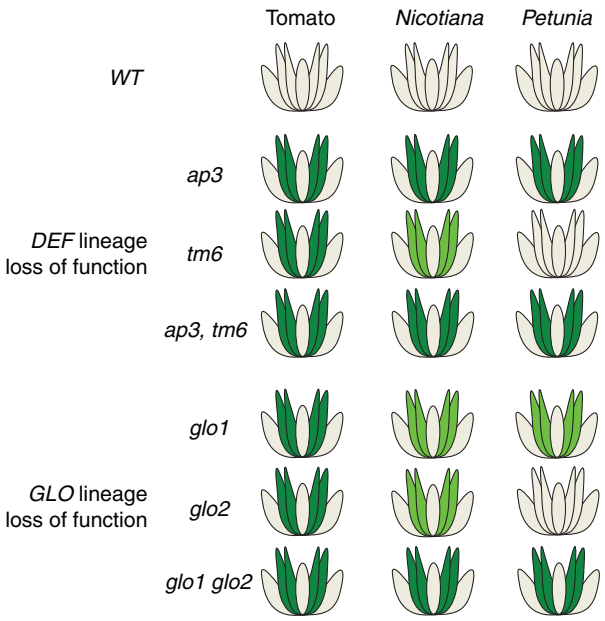


FIG. 2. Phenotypes observed in loss-of-function lines of three Solanaceae species, tomato, *Nicotiana benthamiana* and *Petunia*. Flowers appearing as per the wild-type show no colouring; whorls phenotypes are shown in green. Less severe phenotypes are indicated in transparent green.

investigate whether the same expression pattern can result from different functional architectures of promoter sequences in different species or whether the functional architecture is similar throughout evolution. This would require the functional analysis of B-gene promoter sequences in more than one species.

Does evolvability of B-gene expression contribute to evolutionary novelties?

Although expression patterns of B-genes have revealed differences between species, it has not yet been demonstrated through knock-down approaches that this variation is relevant to the origin of the identity of novel organs in evolution. Some already well-documented cases could provide such evidence in the future. Both in Ranunculales and orchids the gene expression patterns of paralogous B-genes correlate with different types of petals (Kramer *et al.*, 2007; Mondragón-Palomino and Theissen, 2008). In orchids, B-genes belonging to the *DEF* lineage have acquired different but overlapping expression patterns and this could indicate that the different *DEF* genes acquired complementary roles to establish the identity of the different petaloid organs (Tsai *et al.*, 2004; Mondragón-Palomino and Theissen, 2008; Chang *et al.*, 2009). Functional evidence to support this claim is hard to obtain, however, because transgenic strategies are not straightforward. Also in Ranunculales, diversification of *DEF* genes has been implicated in the diversification of different petal types. Specifically, the AP3-III lineage acquired a petal-specific expression pattern within the order (Kramer *et al.*, 2007; Rasmussen *et al.*, 2009). Further functional analysis may confirm a function of *AP3-III* to be restricted to petal development in Ranunculales. Although these B-genes may be involved in the development of such organs, it will be difficult to distinguish between mutations in *cis*-regulation of B-genes or whether mutations in *trans*-acting upstream regulators can best explain these altered expression patterns. An alternative view to interpret these observed expression patterns is that they reflect the partitioning of function after duplication through changes in expression patterns of B-genes to establish petal and stamen identity. Such interpretation emphasizes robustness rather than a role for B-genes in contributing to diverse flowering plant morphologies. Again, stable transformation techniques may help to clarify these issues but need to be developed in these species.

This area of research is important because variation in the organs that plants use to attract pollinators is a frequent observation for flowering plants and is of ecological importance. One of the first and seemingly obvious research strategies in plant evo-devo was to investigate these instances for heterotopic expression of B-genes (e.g. Albert *et al.*, 1998). Several instances have now been studied, but for none of these cases was hard evidence for a role of B-genes obtained (reviewed in Kanno *et al.*, 2007; Hileman and Irish, 2009; Litt and Kramer, 2010). Interesting cases for future research may be the petaloid bracts of dogwoods (*Cornus* sp.), the petaloid sepals of *Impatiens* (Geuten *et al.*, 2006) or the first whorl tepals from *Tricyrtis* (Liliales). These species have indeed been reported to be stably transformable (Adachi *et al.*, 2005; Nakano *et al.*, 2007; Feng *et al.*, 2009; Dan *et al.*, 2010).

ROBUSTNESS THROUGH PROTEIN–PROTEIN INTERACTIONS AND GENE BALANCE

Diversity of protein–protein interactions suggests evolution towards robustness of the B-system

B-proteins are best known to function as heterodimers. For instance, the interaction of *Arabidopsis* B-proteins AP3 and PI is necessary to enter the nucleus (McGonigle *et al.*, 1996). When reviewing protein–protein interactions of MADS-domain proteins in general, it is important to distinguish between the properties of protein–protein interaction and whether such interacting proteins also have the capacity to bind DNA. Obligate heterodimerization and DNA binding of B-proteins appears to be acquired by core eudicots and conserved throughout this taxonomic group (Davies *et al.*, 1996; Riechmann *et al.*, 1996; Egea-Cortinez *et al.*, 1999; Hernández-Hernández *et al.*, 2007; Immink *et al.*, 2010). Outside these core eudicots, B-class proteins have been shown to form homodimers or heterodimers in monocot species, this process being termed facultative heterodimerization (Winter *et al.*, 2002; Tzeng *et al.*, 2004). Recent new data are now also available for gymnosperms, in which it was shown that the *Gnetum gnemon* B-proteins form homodimers to bind DNA (Winter *et al.*, 2002), but have now been shown to equally heterodimerize and bind DNA with several other MADS-domain proteins (Wang *et al.*, 2010). Even more unexpected is that *Gnetum* B-proteins have been shown to loop DNA in tetrameric complexes, but only for a dimer of two homodimers binding DNA. It remains to be tested whether the capacity of *Gnetum* B-proteins to form higher order complexes will be extended to complexes involving

also other MADS-domain proteins, such as AGL6 homologues (Rijpkema *et al.*, 2006; Ohmori *et al.*, 2009; Viaene *et al.*, 2010). This is important to understand how the interaction network of MADS-domain proteins was remodelled around the time the angiosperm flower originated (Melzer and Theißen, 2009). An important advance in this area would be to obtain an accurate picture of the amino acid changes that result in changes in protein interaction specificity of B-proteins. One possibility is to study interaction specificity in more species (e.g. Liu *et al.*, 2010). This should ultimately result in the ability to predict interactions from sequence information. A technical problem that slows down such accumulation of data is the cross-comparison of protein–protein interaction data between experiments and labs. A second step would be to establish ways to investigate MADS-domain protein–protein interaction *in vivo*. Efforts towards this goal have for now been mostly directed at other MADS-domain protein complexes (reviewed in Immink *et al.*, 2010).

The evolution of protein interaction specificity of B-proteins apparently involves specialization as interactions and DNA binding starts off to be promiscuous and evolves to be more specific (Winter *et al.*, 2002). This could be a recurring theme as B protein–protein interactions also in Solanaceae seem to show different specificity between species (Fig. 1; Vandenbussche *et al.*, 2004; de Martino *et al.*, 2006; Leseberg *et al.*, 2008; Geuten and Irish, 2010). The answer to why such specialization could be advantageous has recently been proposed to be robustness. When using a stochastic model, the case of obligate heterodimerization provides a more certain decision behaviour than the case in which heterodimerization is only facultative (Lenser *et al.*, 2009).

Robustness and evolvability of B-genes can be understood through gene balance

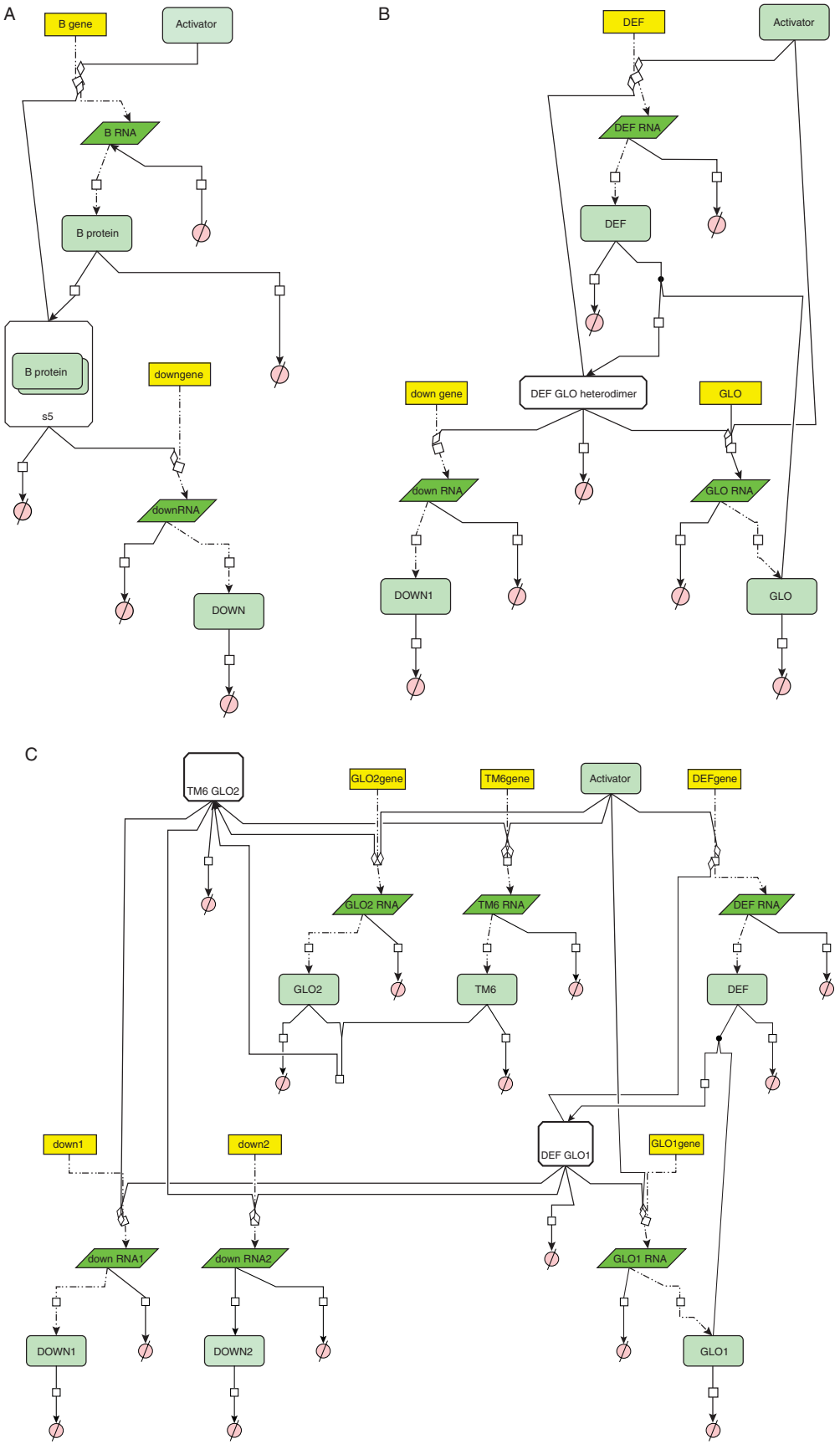
The gene balance hypothesis was formulated to understand dosage effects of genes in genomes. Effects of aneuploidy are more severe than those of polyploidy on phenotypes and gene expression levels. This can be explained by the fact that gene products often function as units of multiprotein complexes, and mutations that affect the stoichiometry of such a complex affect its function (reviewed in Birchler and Veita, 2007, 2010). The fact that B-proteins dimerize and multimerize implies that for B-genes dosage effects can also be expected. Such effects have indeed been demonstrated for duplicate B-genes in *Petunia* (Vandenbussche *et al.*, 2004). The gene balance hypothesis also explains why B-gene lineages have survived rounds of whole genome duplication (Blanc and Wolfe, 2004; Freeling and Thomas, 2006; Birchler and Vieta, 2007). Because they function in protein complexes and their interaction partners in the complex have also duplicated, B-genes have to be retained after duplication. As such, gene balance could provide longer periods of time in which adaptive evolution can act on B-genes after duplication (Birchler and Veita, 2010; Geuten and Irish, 2010).

The study of the B-system also provides interesting information relevant to questions implicated by the gene balance hypothesis. An important outstanding issue is the molecular details by which multiprotein complexes can evolve when under the constraint of dosage balance (Birchler and Veita, 2010). Data in recent years suggest that B-proteins indeed evolve adaptively and this may relate to protein–protein interaction specificity (Martinez-Castilla and Alvarez-Buylla, 2003; Hernández-Hernández *et al.*, 2007; Mondragón-Palomino *et al.*, 2009; Viaene *et al.*, 2009; Geuten and Irish, 2010). Gene balance could provide the necessary time to evolve specific protein–protein interactions that install improved robustness in the system. This would require detailed study comparing protein–protein interaction specificity between many relatively closely related species using a single experimental system while tracing adaptive evolution to test this hypothesis.

COOPERATIVITY IS ACQUIRED THROUGH OTHER FACTORS AND PROBABLY CONTRIBUTES TO ROBUST GENE EXPRESSION

Several mechanisms at the level of transcriptional regulation can contribute to robustness of the B-system. One of these mechanisms is cooperativity, which is defined as the facilitated binding to additional binding sites by binding a first site. This provides robustness of gene expression because sharp transitions in expression level can thus be established with small changes in regulator concentration. B-proteins have been illustrated to bind cooperatively to their DNA binding sequences, although probably not alone but in combination with other MADS-domain proteins such as SEPALLATA and APETALA1 (Egea-Cortines *et al.*, 1999; Melzer and Theißen, 2009; Melzer *et al.*, 2009; Immink *et al.*, 2010). Binding of MADS-domain proteins in general has been shown to show specificity for the CC-$(A/T)_6$-GG sequence but no studies have revealed a specific preference of B-protein dimers for a subset of this canonical sequence. Binding site selection studies such as performed for AGL15, another MADS-domain protein, could help to understand binding specificity of B-proteins and this could aid in defining position weight matrices to explore binding specificity for sites in genomes (Stormo, 2000; Tang and Perry, 2003). One application of this type of data could be to complement experimental approaches in sequenced genomes to find downstream targets (Zik and Irish, 2003; Bey *et al.*, 2004). Chromatin immunoprecipation combined with microarray or next-generation sequencing should further reveal binding sites experimentally as has recently been performed for SEPALLATA3 and APETALA1 (Kaufmann *et al.*, 2009, 2010*a*, *b*).

The presence of multiple conserved CArG boxes in promoter sequences of B-genes suggests also that binding of upstream regulators to B-gene promoters can be cooperative (Hill *et al.*, 1998; Tilly *et al.*, 1998; Koch *et al.*, 2001; Rijpkema *et al.*, 2006). An indication of the possible extent of this upstream cooperativity can be derived from quantitative measurements for the binding of SEP3 quartets to perfectly spaced consensus CArG sites. The relative affinity for binding of a SEP3 dimer when a first dimer was already bound increases around 80-fold (Melzer *et al.*, 2009). A promising additional tool in this respect could be surface plasmon resonance, which has been illustrated to work with longer *cis*-regulatory sequences and has the power of providing quantitative measurements (Moyroud *et al.*, 2009).


A
B gene
Activator
B RNA
B protein
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MATHEMATICAL MODELS DESCRIBING THE GENE REGULATORY NETWORK OF FLOWER DEVELOPMENT

Models are commonly used in biology to describe the current understanding of a regulatory system explicitly and to indicate the gaps in our understanding. In flower development, two such models are the ABC model and the floral quartet model, which represent compatible genetic and molecular models of floral organ identity specification (Coen and Meyerowitz, 1991; Theißen and Saedler, 2001). Extensions to such descriptive models are now possible through mathematical modelling. Computerized modelling approaches have several advantages in that (1) they allow more complexity, (2) they can incorporate quantitative experimental data and (3) they allow *in silico* hypothesis testing through simulation.

To represent the gene regulatory networks to be analysed, a mathematical formalism needs to be chosen (Karlebach and Shamir, 2008). Different formalisms have been used that represent different trade-offs between the type of input data available and biological realism. The most often used formalism in biological modelling is the use of ordinary differential equations (ODEs), because these allow precise numerical prediction of the behaviour of a biological system (e.g. Conrad and Tyson, 2006; Alon, 2007).

ODE modelling assumes quantitative knowledge of the kinetics of the biological system, information that is mostly absent. Therefore, more qualitative approaches have been explored first in the modelling of flower development and can be sufficient to capture many aspects (e.g. De Jong and Ropers, 2006). One such more qualitative approach is the use of a Boolean logical network. These models use discrete representations of the biological data (such as expression absence or presence) and incorporate logical rules grounded in experiments to connect these data (Mendoza *et al.*, 1999; Espinosa-Soto *et al.*, 2004). These models have illustrated the robustness of flower development to different initial conditions and to stochastic noise. An extension of these models in the form of discrete abstraction of ODEs, Glass equations, has also been used (Alvarez-Buylla *et al.*, 2008).

A second type of formalism has used Petri nets and its extensions to describe the *Arabidopsis* flower development gene regulatory network (Kaufmann *et al.*, 2010*a*, *b*). Petri nets have been chosen to represent biological reactions because they allow an intuitive graphical representation of biological processes and a well-defined mathematical formalism. An important advantage is that this is implemented in the Cell Illustrator software, which facilitates the user-friendly construction, visualization and simulation of the network. A main contribution of this model is the complexity captured through the many genes and genetic interactions incorporated. Simulations of this model also highlight the importance of heteromeric higher-order complexes and autoregulatory feedback loops for the generation of robust organ-specific gene expression.

FIG. 3. CellDesigner4·1 models for B-gene systems. Yellow blocks represent genes, pale green blocks proteins, parallelograms mRNA and pink discs as degradation. (A) Model in which a single activated B-gene of which the protein product homodimerizes and autoactivates. The homodimer regulates a single downstream gene. (B) Model in which a *DEF* and *GLO* gene produce protein products that obligately heterodimerize to autoactivate and regulate a downstream gene. (C) Model in which protein products of *DEF*, *TM6*, *GLO1* and *GLO2* genes obligately heterodimerize, cross-regulate and autoregulate and activate two downstream genes.

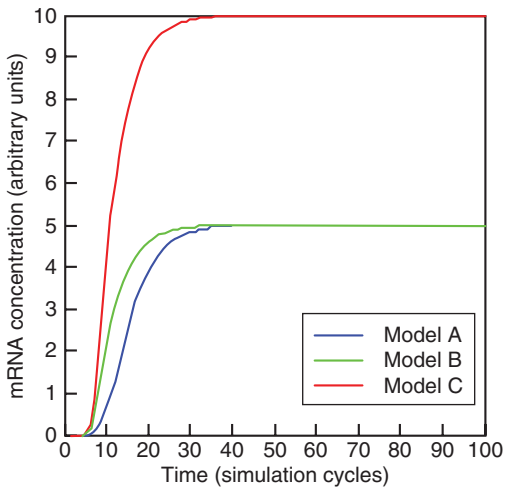

FIG. 4. Activation over time of a single downstream gene by three different B-systems, corresponding to Fig. 3. The same parameters and initiating values were taken for each model.

The classical ODE modelling strategy for biological systems has only recently been applied to understand flower development (Van Moorik *et al.*, 2010). ODE modelling requires (1) a diagram to represent the different molecular interactions, (2) assigning a set of kinetic rate equations that relate the rate of a reaction to the concentrations of substrates and (3) values for the parameters in the rate reactions. The first two elements are not problematic, because diagrams can be derived from the literature and the kinetics of the reactions in gene regulatory networks such as cooperative gene regulation are well understood. Estimating parameters, however, is rarely possible although plausible guesses can be made or parameter ranges can be tested. Simulations of the Van Moorik model have equally stressed the importance of dimers in the gene regulatory network and generated predictions for mutations yet to be made, offering further tools for model validation.

Finally, simulation of evolutionary different B-systems has been undertaken using the Gillespie algorithm, which is a general and exact way to take into account stochasticity (Lenser *et al.*, 2009). Stochastic behaviour is the random unpredictable behaviour that can occur when only a few molecules are involved and is relevant and inherent to many biological systems. A major conclusion of this study is that obligate heterodimerization of B-proteins, as observed in core eudicots, has the evolutionary advantage of being more robustly able to control cell-identity switching under stochastic conditions. This provides an explanation as to why in evolution obligate heterodimerization would have evolved.

Mathematical models to explore robustness and evolvability of B-systems

To explore the use of mathematical modelling for our understanding of the evolution of the B-system, we built gene

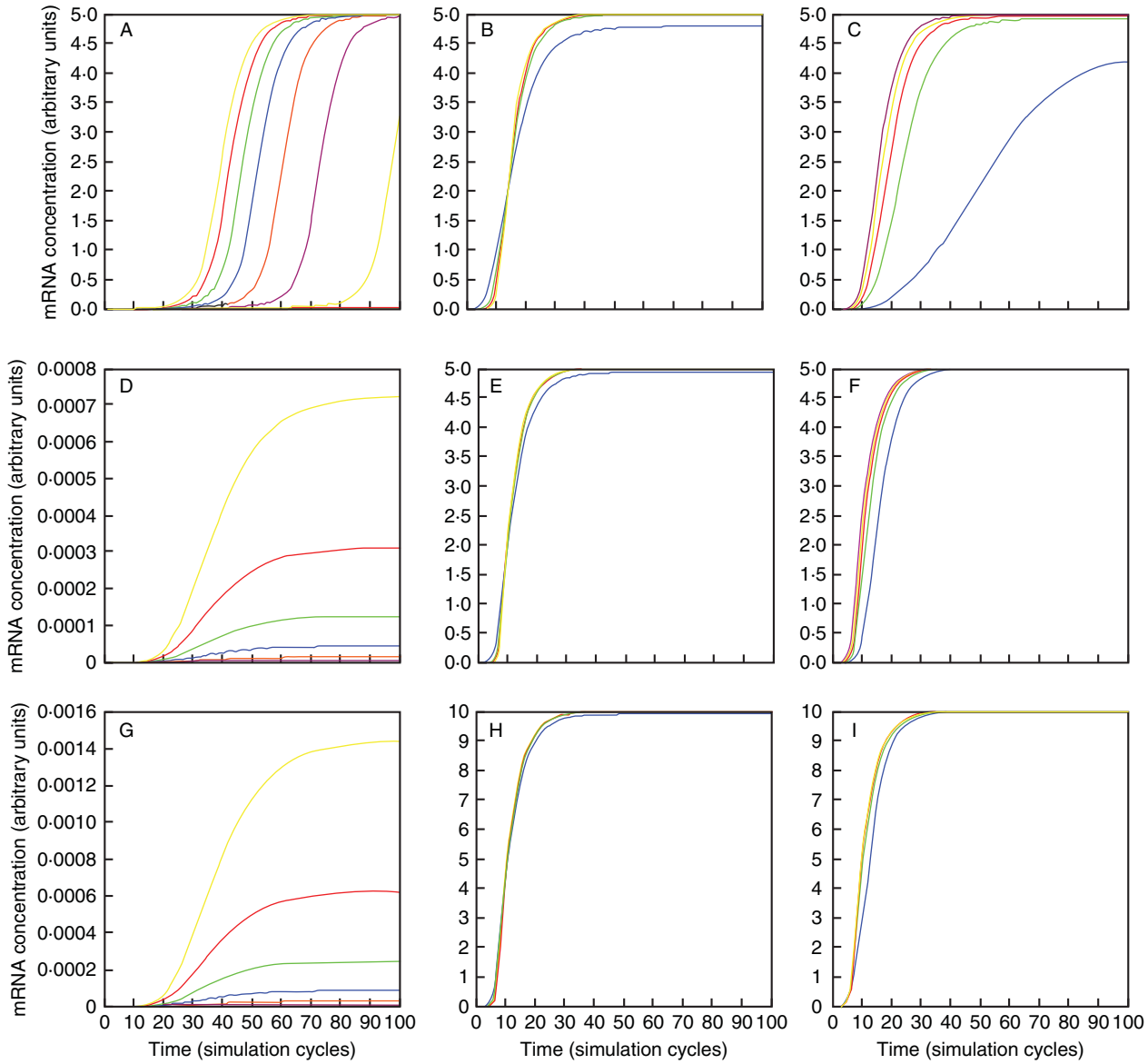

FIG. 5. Activation of a single downstream gene by B-systems. (A,D,G) Model A, (B,E,H) model B, (C,F,I) model C. (A–C) Activator concentration varies over a 100-fold range. (A) Model A can activate a downstream gene over a wider range of activator concentrations. (B,C) Models B and C require stronger concentrations of activator to activate a downstream gene. (D–F) Hill coefficient of cooperativity in the activation of the downstream gene as shown in the figure varies over a 4-fold range. (D) In model A the downstream gene is most sensitive to variation of the cooperativity coefficient. Model C is most robust because a second complex can activate the same downstream gene independently. (G,H) Rate constant of association for homodimerization (G), heterodimerization (H) or heterodimerization of one or both heterodimers varies over a 100-fold range. Model A using homodimerization is more sensitive to this parameter than model B or C.

regulatory models. The formalism used here is to automatically generate a set of ODEs for graphically designed models in CellDesigner4·1. Our goal is to examine, in theory, the possible effects of evolutionary changes to different regulatory systems, rather than to provide an encompassing model that is predictive and can be experimentally validated for a single species. This approach illustrates (1) how different theoretical regulatory systems show a different sensitivity to parameter changes and acquire robustness and (2) how user-friendly software permits biologists to incorporate mathematical modelling in thinking and experimenting.

We present three models that differ in the number of B-genes involved, their protein interactions and cross-regulatory interactions. They represent cases which may be similar to B-gene regulation in gymnosperms (Fig. 3A), *Arabidopsis* and *Antirrhinum* (Fig. 3B) or tomato (Fig. 3C; see Appendix for the methods employed). For each of these models, Hill functions were taken to represent transcriptional regulation and homo- and heterodimerization was taken as a reversible process modelled by mass action kinetics. Decay of molecules in the model was assumed to be linearly proportional to the species concentration. With the same parameters taken for the same kinetic equations in all three models, they produce a similar outcome for the activation of the gene downstream (Fig. 4). The third model results in double the absolute expression of

downstream genes and this is because in this model downstream genes are activated by two heterodimers of B-proteins rather than one in the first two models and this activation is independent (Fig. 3), making this model more robust in the sense of downstream activation. To illustrate the robustness of these models, we will study the behaviour of each model in response to changes in parameter values, which can be interpreted as evolutionary perturbations. We varied activator concentration, cooperativity of transcriptional regulation as measured by the Hill coefficient and the rate constant for dimerization.

The simplest model represents an autoregulatory circuit in which a stable activator concentration is responsible for initiating transcription of a single B-gene. The protein product homodimerizes and autoregulates its own expression and the expression of a single downstream gene (Fig. 3A). The parameters that have the strongest influence over the switching behaviour of the downstream gene are the activator concentration (Fig. 5A), the rate constant for homodimerization (Fig. 5B) and the cooperativity of direct regulation of the downstream gene (Fig. 5C). The cooperativity of activation of the single B-gene and autoregulation have only minimal impact on the switch to ON of the downstream gene (data not shown).

The second model uses obligate heterodimerization of two B-proteins rather than homodimerization (Fig. 5B). This implies that two B-genes need to be activated, which then can only autoregulate their expression and the expression of a downstream gene through heterodimerization. Transcription of the downstream gene in this model is more sensitive to low activator concentrations than is the case in the first model (Fig 5D). Thus, in species such as *Arabidopsis* and *Antirrhinum*, which may represent real-life examples of this model, one would expect a more elaborate control of the activation of the B-system than in species which use a conceptually simpler system of homodimerization of a single B-protein. Transcription of the downstream gene is also somewhat less sensitive to variations in association rate between the two different B-proteins (compare Fig. 5B and 5E). This becomes intuitive when considering that two independently regulated genes provide the proteins to interact, rather than a single gene in the homodimerization model. Apparently, using two different genes that obligately heterodimerize can be more robust to changes in interaction strength of the proteins. Similar to the previous model, cooperativity in activating the B-genes or in autoregulating the B-genes has only a minor impact on downstream transcription, while cooperativity of the direct regulation of the downstream gene does affect its transcription (Fig. 5F). This illustrates the intuitive point that the transcription of the downstream genes is more robust to changes in cooperativity of activation when these changes occur upstream in the regulation cascade, but is most sensitive to changes in its direct regulation, such as mutations in the *cis*-regulatory binding sites in its promoter.

The third model incorporates four different genes that form two types of heterodimer (Fig. 3C). Such regulation may be similar to that found in tomato (Leseberg *et al.*, 2008), but may be different in related species such as tobacco and *Petunia* for which interaction specificity was not found to be as exclusive (Vandenbussche *et al.*, 2004; de Martino *et al.*, 2006; Rijpkema *et al.*, 2006; Geuten and Irish, 2010). In this model, activator concentration affecting downstream genes varies over the same range as in the second model (compare Fig. 3D and 3G). This may be somewhat surprising as activation molecules now have to activate four genes rather than two, but this effect may be compensated for by the fact that each B-gene is both autoregulated and cross-regulated by a second B-dimer. This third model is also most robust to changes in the association rate of one of the dimers and to changes in the cooperativity of direct activation of the downstream gene (Fig. 5H, I). This is trivial as a second and largely independent system is redundant. In the absence of one of both B-protein interactions, expression of downstream genes reduces to only half, further illustrating that being regulated by two fully redundant dimers makes downstream gene expression partially robust when this direct regulation is independent (data not shown).

CONCLUSIONS

Transcriptional regulation of the B-system is only well characterized in a single model species, *Arabidopsis thaliana*. Comparative studies including more species indicate that the evolution of B-proteins is characterized by duplication, retention through gene balance and adaptive evolution that may result in specific protein–protein interactions or altered *cis*-regulation. Overall, the experimental evidence demonstrates that the B-system can be robust in different functional ways. It is unclear whether the molecular evolution of the B-system has been instrumental in the origin of novelties in plant evolution. Although expression suggests that the B-system is involved in establishing the identity of organs that are evolutionarily derived from petals, this function has yet to be experimentally demonstrated. In addition, it will be difficult to test whether mutations in B-genes were causative in the origin of these organs or whether B-genes were recruited through changes in upstream regulation.

To understand in detail the constraints of evolvability and the different ways in which the B-system can be robust will require more extensive functional characterization in more species. In addition, quantitative measurements will add more biological realism into models that try to capture the molecular details of transcriptional regulation. These details are important to fully test our understanding of the B-system and the way it is constrained in evolution.

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APPENDIX

To model the gene regulatory interactions involving B-genes as depicted in Fig. 3, we used the CellDesigner4·1 software. This software has as the main advantage that it allows the easy graphical construction of a gene regulatory network and supports the SBML format (Systems Biology Markup

Language). This in turn allows communication and reuse of models in different software programs. Automatic generation and simulation of ODEs associated with the network kinetic rate equations is possible through the SBML ODE Solver (Machne *et al.*, 2006). All reactions were taken as irreversible, except for protein dimerization. The kinetics of transcriptional activation were modelled using a Hill function. The Hill equation has three parameters n, K and β. Cooperativity n was initiated as $n = 2$, the activation coefficient K was initiated as $K = 0{\cdot}1$ and the maximal rate of expression was initiated as $\beta = 1$. The integration of multiple input signals was achieved by taking the weighted sum of multiple input signals with different regulators taking equal weights. Protein dimerization was assumed to be reversible and followed mass action kinetics. Translation and degradation was assumed to be not affected by regulation and followed zero-order mass-action kinetics. All kinetic parameters were initiated as $k = 0{\cdot}1$.