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Evolutionary complexity of MADS complexes

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Developmental programs rely on the timely and spatially correct expression of sets of interacting factors, many of which appear to be transcription factors. Examples of these can be found in the MADS-box gene family. This gene family has greatly expanded, particularly in plants, by a range of duplications that have enabled the genes to diversify in structure and function. MADS-box genes appear to have been instrumental in shaping one of the great evolutionary innovations, the true flower, which originated around 120–150 million years ago and led to the enormous radiation of the angiosperms. We propose a shift from analyzing individual gene functions towards studying MADS-box gene function at the subfamily level. This will enable us to distinguish subfunctionalization events from the evolutionary changes that defined floral morphology.

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Introduction

Evolutionary developmental biology (evo-devo) tries to explain the diversity in animal and plant body plans. Changes in the expression pattern or function of homeotic selector genes — genes that determine how the different regions of an organism develop — are especially important in generating morphological novelty. Homeobox genes are crucial in patterning the body axis in animals, but it is another transcription factor family, the MADS-box gene family, that has attained a very important developmental role in plants. Unlike animals and fungi, which contain only a few copies of MADS-box genes, many plant species harbor over a hundred MADS-box genes, belonging to a range of functionally diverged subfamilies. Many of the MIKC-type MADS-box genes (so-called because they contain a MADS, I, K and C domain) play an essential role in the determination of floral meristem and floral organ identity ([1,2]; Table 1). The evolution of the MADS-box gene family may therefore have played a central role in creating the enormous diversity in the body plans of extant plants.

One of the key driving forces in evolution is gene duplication. Indeed, extensive duplications within the MADS-box gene family have been essential in forming the intricate regulatory network that is involved in present-day floral development. The fate of duplicated gene copies ranges from the entire loss of one of the copies, through subfunctionalization, to (much rarer) neofunctionalization. The results of almost two decades of MADS-box research illustrate that the full plethora of these possibilities has been employed in the evolution of the MADS-box gene family.

In this review, we highlight several examples of non-functionalization, redundancy and different types of functional diversification of duplicated MADS-box genes. We also discuss how differential gene duplications between even closely related species and subsequent random functional diversification make it hard to distinguish simple, often species-specific, subfunctionalization events from the creation of evolutionary novelties. Such analyses are complicated also because many MADS-box genes regulate their own expression by participating in higher-order protein complexes. We argue that a shift from analyzing individual gene functions towards studying MADS-box gene function at the subfamily level should make analyses of the function and evolution of MADS-box genes easier.

An evolutionary driving force: gene duplication

Whole-genome sequencing and comparative genome analyses provide us with a lot of information on the occurrence and origin of duplicate genes and their loss or retention, which enhances our insight into the dynamics of the evolution of complete gene families. Duplicate genes can originate from large- or small-scale duplications. In many eukaryotic organisms (including *Arabidopsis* and poplar), several complete genome duplications have taken place [3,4]. A gene duplication event in its simplest form produces two functionally redundant, paralogous genes (a small-scale duplication could theoretically lead to changes in the expression pattern of the duplicate gene). Assuming that the selective pressure is low for either one of the duplicate genes immediately upon duplication, the odds are that one of the duplicate genes is neutralized because of the accumulation of deleterious mutations. Indeed, many non-functionalization events for MADS-box gene duplicates, either by point mutations creating a stop codon or by elimination

Table 1

Major MIKC-type MADS-box gene subfamilies with functions characterized for (mostly *Arabidopsis*, *Antirrhinum* and *Petunia*) members of these subfamilies

Subfamily	Function(s)	Reference(s)
AGL2/SEP	Development of all floral whorls, floral meristem development	[11,41,42]
AGL6	Not studied thoroughly yet, might be involved in flowering time	[55]
SQUA/AP1	Sepal and petal development, floral meristem development, fruit development, flowering time	[17••,45,56]
AG	Stamen, carpel, ovule and fruit development, floral meristem development	[8,9,18•,19••,20,40,43,44,50]
AGL11	Ovule development	[8,57]
GLO/PI	Petal and stamen development	[14,16,31,36,37]
DEF/AP3	Petal and stamen development	[14,16,31,36]
Bsister	Seed coat development	[58]
AGL17	Root development	[59,60]
TM3/SOC1	Flowering time, flowering activator (floral pathway integrator)	[61–63]
AGL15	Might be involved in promotion of embryo development	[64]
FLC	Flowering time, flowering repressor	[65]
StMADS11	Flowering time, flowering repressor and flowering activator	[66,67]

of gene parts or complete genes, have been identified in genome-wide analyses of *Arabidopsis*, rice and poplar [5–7].

Redundancy, subfunctionalization and neofunctionalization

Redundancy, the existence of paralogous genes that perform the same function, is common in the MADS-box gene family [8–11]. Redundant gene copies can apparently be maintained for some time by purifying selection, because their functional redundancy guards against deleterious mutations and contributes to the genetic robustness of an organism [12]. Moreover, redundancy is thought to create an advantage, especially for genes that encode a product that is 'beneficial' in larger quantities [13].

Although we do encounter a high degree of partial or full redundancy within the MADS-box gene family, especially in recently duplicated clades, there are also many examples of the diversification of the functions of duplicate genes (e.g. [14–16,17••]).

The most common mechanism for diversification in function after a gene duplication event is subfunctionalization, as seen for the rice *AGAMOUS* (*AG*)-clade genes *OsMADS3* and *OsMADS58* [18•]. Together, these two genes fulfill the complete ancestral role as defined for the *Arabidopsis AG* gene: regulating the organ identity of stamens and carpels and regulating floral meristem determinacy. However, *OsMADS3* and *OsMADS58* have divided these tasks: *OsMADS58* is mainly involved in floral meristem determinacy and has a predominant role in carpel morphogenesis, whereas *OsMADS3* is more important in inhibiting lodicule development and in specifying stamen identity [18•].

Subfunctionalization is a random process and happens independently in different species. Owing to the occurrence of species-specific subfunctionalization processes, orthologs do not necessarily have the same function, and conversely homologs that have the same function are not necessarily orthologs. This is nicely illustrated by the functionally equivalent homologs *PLENA* (*PLE*) from *Antirrhinum* and *AG* from *Arabidopsis*, which turned out to be paralogs [19••,20]. After the gene duplication in a common ancestor, different members of the duplicated gene pair have retained the primary homeotic functions in different lineages (*PLE* in *Antirrhinum* and *AG* in *Arabidopsis*), while their respective orthologs (*SHATTERPROOF* [*SHP*] in *Arabidopsis* and *FARINELLI* [*FAR*] in *Antirrhinum*) have undergone independent (and quite divergent) subfunctionalization processes [19••,20].

Even though subfunctionalization is more likely to happen than neofunctionalization, completely new gene functions do arise occasionally. A clear example of the neofunctionalization of a MADS-box gene resulting in a morphological novelty is found in the Solaneaceous species *Physalis*. An eye-catching characteristic of *Physalis* is its 'Chinese lantern', which is formed when the sepals resume growing after pollination to encapsulate the mature fruit. In an elegant study, He and Saedler [21••] demonstrated that it is the heterotopic expression of the *MPF2* MADS-box gene in the flower that provided the gene with a function in the development of this new morphological trait.

MADS-domain protein complexes and (auto)regulatory loops

MADS-domain proteins form multimeric protein complexes that interact with promoter sequences of their target genes [22,23]. Different complexes act on different sets of target genes, and thus bring about different developmental processes (e.g. [24••,25]). The majority of interactions of MADS-domain proteins reported to date are between different MADS-domain proteins, but non-MADS-domain protein components of these complexes

are also being identified [26,27]. Recently, a direct physical interaction between SEUSS (SEU), a transcriptional repressor of *AG*, and the carboxy-terminal (C-terminal) domain of SEPALLATA3 (SEP3) and APETALA1 (AP1) was demonstrated in *Arabidopsis* [28••]. This suggests that AP1 and SEP3 might function as both activators and repressors, depending on their interactions with co-activators (such as AG for SEP3 [24••]) or co-repressors (such as SEU and LEUNIG [28••]).

Both the K-domain (keratin-like domain, located downstream of the DNA-binding MADS domain) and the C-terminal domain are involved in the formation of (higher order) protein complexes [22,29–31]. Therefore, mutations in these regions can affect either partner affinity or the specificity of protein–protein interactions. A recent study on *AP1* and *CAULIFLOWER* (*CAL*), two *Arabidopsis* genes that have very similar sequences and expression patterns but partially diverged functions, showed that differences in the K and C-terminal domains of these genes were crucial for the unique and indispensable roles of AP1 during floral organ and meristem fate determination [17••]. AP1 (and not CAL) interacts with several specific proteins that are known to be involved in floral organ fate determination [32], and so it seems that the interaction of these proteins with the AP1 K and C-terminal domain regions determines its specific function [17••].

MADS-domain proteins can form complexes that often interact with their own and orthologous/paralogous promoters to regulate their own and each other's expression [24••,28••,33•,34–39]. Generally, the molecular origin of diversification in function (be it in unique or in redundant genes) is considered to be due to either changes in the coding sequence or changes in the regulatory circuit of the gene, which result in a shift (either restriction/expansion or reduction/enhancement) of its expression pattern. In the case of *AP1* and *CAL*, the diversification in function is clearly due to changes in the coding sequence, but the source of functional diversification will probably be more problematic to determine for many of the other MADS-box genes. As changes in the protein sequence can affect partner-specificity and as MADS-domain protein complexes are often part of autoregulatory loops, it is not unlikely that changes in protein sequence could also lead to changes in expression pattern. Such an effect would be completely masked if constructs were tested only under the control of constitutive promoters.

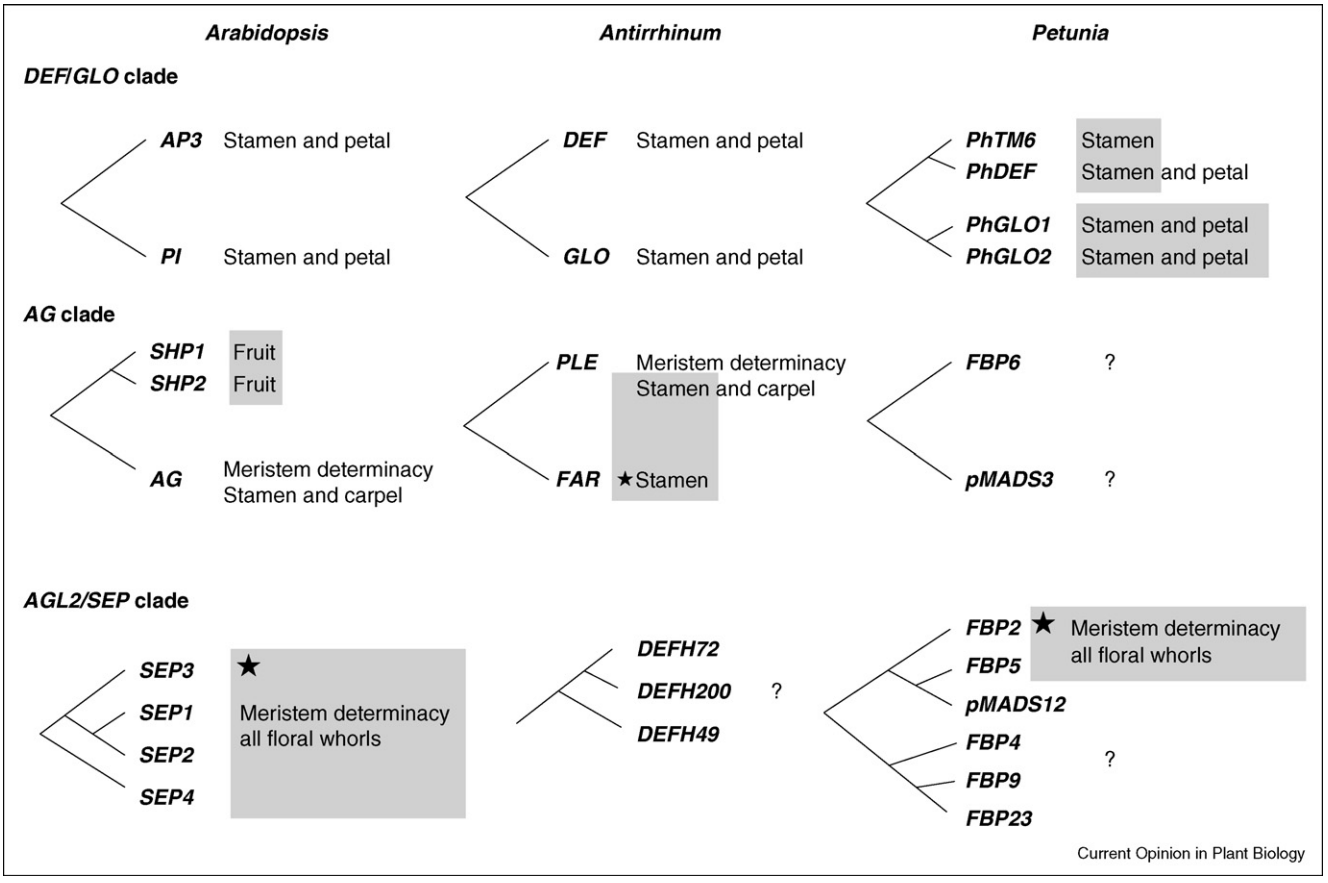
Shifting from the gene to the subfamily and family level

The random nature of subfunctionalization following duplication is becoming more and more apparent. This is reflected in the differences in number of particular subfamily members, even between closely related species, and in the differences in the degree of redundancy and subfunctionalization between these genes. Clear examples have been found already for class B-, C- and E-function MADS-box genes ([9,16,36,37,40–44]; Figure 1). When the full functional palette of a set of subfunctionalized genes is considered together, however, it turns out that the complete set of functions is fairly well conserved between species. In such cases, differences in gene function between individual orthologs from these species do not necessarily imply a fundamental difference in function. Therefore, when comparing individual gene functions between species in isolation from their specific gene subfamily context, it is difficult to distinguish true differences in gene function from differences caused by redundancy and divergent subfunctionalization. To facilitate the identification of differences in gene function between species, we therefore will have to analyze fully all members of entire subfamilies. For this reason, it is of crucial importance to consider the full array of MADS-box genes of the relevant subfamily in the species being studied. Large-scale expressed sequence tag (EST) sequencing, combined with genomic screens for MADS-box genes, expression analyses, and whole-genome sequencing efforts [5,45–50,51•], will certainly help to assign gene functions to all members of a specific gene family.

A clear example in which only analysis of all subfamily members provided the full answer is the analysis of the B-function in *Petunia*. In both *Arabidopsis* and *Antirrhinum*, knockouts of the eu*AP3* lineage gene result in the homeotic conversion of petals to sepals in the second whorl and of stamens to carpels in the third whorl. Knocking out the eu*AP3* gene in *Petunia* leads only to the homeotic conversion of petals to sepals, while stamen development is unaffected. This prompted the suggestion that the *Petunia* eu*AP3* function is different from that of its orthologs in other species [52]. The analysis of the entire B-function subfamily in *Petunia* revealed, however, that *Petunia hybrida TOMATO MADS-BOX GENE6* (*PhTM6*), a paleo*AP3* lineage gene copy that has been lost in *Arabidopsis*, was responsible for the one-whorl-only phenotype of *P. hybrida deficiens* (*phdef*) mutants: it acts redundantly with *PhDEF* in anther formation [16]. *PhDEF* thus displays all of the characteristics that are typically associated with normal eu*AP3* gene function as described for *DEF* and *AP3*.

MADS-box proteins seem to function mostly as subunits of larger protein complexes. Changes in MADS-box protein function can thus cause changes in the function of the particular complex that they are part of, and thus might be accompanied by co-evolutionary changes in other components of the complex. An in-depth analysis of the complete MADS-box gene family in a limited number of model species, including family-wide protein–protein interaction screens [32], will provide a deeper insight into these processes. The functioning of

Figure 1



Schematic depiction of the functions defined for representatives of the best-characterized MADS-box gene subfamilies in *Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia hybrida* [9,16,36,37,40–44]. Relationships between genes are indicated by branch points but lines are not to scale. Functional redundancy between two or more genes is indicated by a grey block. The stars indicate which of the partly redundant genes is most crucial for a particular function. For instance, *FAR* is more crucial to stamen development than *PLE*, and *SEP3* is more crucial for meristem determinacy and floral organ development than the other *Arabidopsis SEP* genes. The question marks indicate genes that have not yet been completely analyzed.

MADS-box proteins as components of protein complexes also clearly asks for functional analyses to be performed in the natural context of the gene examined, arguing for a functional analysis in the species under research rather then using heterologous systems. Unfortunately, many potential model species that are of interest from the morphological point of view are not amenable to the desired functional analyses. Applying techniques such as virus-induced gene silencing (VIGS) [53] or TILLING (Targeting Induced Local Lesions In Genomes [54]) might, however, provide research opportunities for such species in at least some cases.

Conclusions

Studies of MADS-box genes are beginning to cover an increasingly wide array of species across all major plant taxa, and thus we are gaining a truly evolutionary view of how these genes can change function upon duplication and of how the flower in its present form has emerged. The MADS-box genes offer exciting opportunities, not only in molecular research but also for understanding fundamental aspects of (co)-evolution and the background of morphological innovations in plants.

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- •• of outstanding interest

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