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MADS-box genes and floral development: the dark side

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Abstract

The origin of the flower during evolution has been a crucial step in further facilitating plants to colonize a wide range of different niches on our planet. The >250 000 species of flowering plants existing today display an astonishing diversity in floral architecture. For this reason, the flower is a very attractive subject for evolutionary developmental (evo-devo) genetics studies. Research during the last two decades has provided compelling evidence that the origin and functional diversification of MIKCc MADS-box transcription factors has played a critical role during evolution of flowering plants. As master regulators of floral organ identity, MADS-box proteins are at the heart of the classic ABC model for floral development. Despite the enormous progress made in the field of floral development, there still remain aspects that are less well understood. Here we highlight some of the dark corners within our current knowledge on MADS-box genes and flower development, which would be worthwhile investigating in more detail in future research. These include the general question of to what extent MADS-box gene functions are conserved between species, the function of TMI8-clade MADS-box genes which so far have remained uncharacterized, the divergence within the A-function, and post-transcriptional regulation of the ABC-genes.

Key words: ABC model, floral architecture, floral evolution, flower development, MADS-box gene family, Petunia.

MADS-box gene function and black boxes

MADS-domain proteins are transcription factors that are present throughout the eukaryotic section of the tree of life. Mammals possess several MADS-box genes, but in plants this family has greatly expanded and 107 members have been identified in Arabidopsis (Parenicova et al., 2003). In this species, 46 members belong to the type II subfamily of MADS-box genes, whereas 61, the majority, belong to the type I subfamily, which is very poorly characterized in comparison with the type II subfamily. In fact, only five type I genes have been functionally characterized and all of them play a role in the development of the female gametophyte and/or the early seed (Köhler et al., 2003; Portereiko et al., 2006; Bemer et al., 2008; Colombo et al., 2008; Kang et al., 2008; Steffen et al., 2008), and in both of these tissues, most type I genes are preferentially expressed (Bemer et al., 2010). All MADS-box proteins possess the eponymous MADS-box domain, which is the domain responsible for binding to the target DNA element [the CArG-box, CC(A/T)6GG], carries a nuclear localization signal and is involved in dimerization and binding of other factors (De Bodt et al., 2003). Type II MADS-box genes can be further subdivided into MIKCc* (also called M6) and MIKCc classes (the * stands for classic) (Parenicova et al., 2003; Gramzow and Theissen, 2010). These two classes are distinguished via structural differences in their K-domains and the length of the I-domain, which is longer and encoded by more exons in the MIKCc class proteins (Henschel et al., 2002). Unlike the type I subfamily, MIKCc proteins carry three additional characteristic regions: a weakly conserved intervening region (I), a well-conserved keratin-like domain (K), both of which are involved in protein interactions, and a more variable C-terminal region (C) which mediates higher order formation and confers regulatory activity on the functional complex of which the MADS-domain proteins are part. In plants, the MIKCc MADS-box genes are most famous for their roles in flower development, and members of this type spell out the ABC of floral development (Schwarz-Sommer et al., 1990; Coen and Meyerowitz, 1991). The phylogeny of MIKC genes has been
extensively studied, and shows that they can be subdivided into 13 major gene clades, of which seven probably existed already in the most recent common ancestor of angiosperms and gymnosperms ~300 million years ago (Becker and Theissen, 2003). Due to its undeniable advantages as a plant model species, MIKC$^c$ genes have been most extensively studied in Arabidopsis, for which for many clades all members have been functionally characterized, while for others at least one or more members have been analysed. In angiosperm genomes, one or more representatives of each clade are usually found, suggesting their functional conservation and importance for the plant life cycle.

A simplified phylogeny (using only one representative gene per species per clade) based on MADS-box proteins from Arabidopsis and Petunia is shown in Fig. 1 to illustrate this. Remarkably, however, Arabidopsis seems to have lost one of the ancient clades called the TM8 lineage (Fig. 1). This has been known for a while (Becker and Theissen, 2003), but now that more and more plant genomes are available, it becomes clear that the absence of a TM8 homologue as in Arabidopsis is an exception rather than a rule. Gene loss is not uncommon among the angiosperm MADS-box genes. For example, Arabidopsis seems to have lost the paleoAP3 subclade of the DEF/GLO clade (Kramer and Irish, 2000), and the lineage that led to extant gymnosperms may have lost the SEPALLATA (SEP)-clade genes (Becker and Theissen, 2003). However, the TM8 clade represents a special case since Arabidopsis contains no members of this clade at all and none of the TM8-clade genes has been functionally characterized in any species, whereas we currently know the function of at least one representative from all the other MIKC$^c$ clades (from Arabidopsis, and only in some instances from multiple other species). Therefore, the functional characterization of this gene clade is of special interest in order to ‘complete’ our understanding of MIKC$^c$ gene function. While so far no loss-of-function phenotypes have been described, at least the expression data available for tomato and grapevine TM8 genes suggest a role during flower development (Pnueli et al., 1991; Díaz-Riquelme et al., 2009). In addition, one should keep in mind that to a large extent, our knowledge on MIKC$^c$ gene function is based primarily

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**Fig. 1.** Simplified phylogeny of the major MIKC$^c$ clade genes in Arabidopsis and Petunia. Neighbor–Joining tree of MIKC$^c$ proteins from Arabidopsis thaliana and Petunia×hybrida (prefix Ph- and shaded grey). The tree has been constructed using one representative of each clade in both species. For the TM8 clade, representatives have been added from some other species to illustrate its wide taxonomic distribution (Sl, Solanum lycopersicon; Am, Antirrhinum majus; Pt, Populus trichocarpa; Vv, Vitis vinifera). Numbers along the branches are bootstrap values exceeding 50%, and branches with <50% bootstrap support were collapsed. Black boxes represent clades of which there are, to our knowledge, no functional data from species other than the Brassicaceae, or of which there are no functional data at all (in the case of the TM8 clade). The Arabidopsis type I MADS-box protein AGL64 was used as an outgroup. The tree was generated as described previously (Vandenbussche et al., 2003).
upon research in *Arabidopsis*, and functional data from other species are often lacking. Of course, in some instances, gene function has been shown to be relatively conserved (as in the case of the B- and C-function genes in flower development) but, in other cases, data from *Arabidopsis* do not necessarily translate directly to other species, as illustrated below. Therefore, to truly get a grasp on a gene’s function in plants, functional data from multiple phyla are highly desirable, and of course necessary to assess a gene’s functional conservation or divergence. As shown by the black boxes in Fig. 1, there is still some work to do in this respect. Moreover, the majority of the MADS-box subfamilies have been shaped by a complex history of gene duplications followed by random subfunctionalization events, of which the final outcome might differ significantly, even between closely related species. Therefore, when performing an interspecies comparison of individual gene functions isolated from their own specific gene subfamily context, it might be very hard to distinguish true fundamental differences in gene function from differences simply caused by a divergent degree of redundancy and subfunctionalization with the remaining subfamily members. Consequently, the function of all subfamily members should be determined and any possible redundancies uncovered (by multiple mutant analysis) before any hard conclusions can be drawn.

The dark side of the ABC model

A typical dicotyledonous flower, such as an *Arabidopsis* flower, consists of four different organs, arranged in four whorls or concentric rings of tissue, from outside to inside; the sepals, petals, stamens, and the carpels. The ABC model, initially based on floral mutant phenotypes in *Arabidopsis* and *Antirrhinum*, postulates that three different gene classes, or functions, specify the identity of these organs in the four floral whorls. In the outermost whorl, the A-function specifies sepal identity, while the A- and B-function genes together control petal identity in the second whorl. In the third whorl, the B- and C-function genes are required for stamen development, while the C-function by itself specifies carpel identity in the inner whorl and terminates floral meristem activity. In addition, the A- and C-functions antagonize each other, enforcing the proper domains of activity. A later addition to the model are the E-function genes, which are required for all the other functions, since they encode proteins necessary for the formation of the higher order complexes in which most of the ABC-function proteins are active. Interestingly, except for one of the A-function genes, *APETALA2 (AP2)*, all the ABCE-functions are encoded by one or more type II MADS-box genes, and a detailed overview of their function and regulation is given in Krizek and Fletcher (2005).

Functional analyses of B- and C-lineage MADS-box protein genes in a diverse range of species (covering several dicot and monocot species) show that in general the B- and C-functions are very well conserved at the molecular level. If there are differences at all, they are usually found at the level of subfunctionalization.Specialization between paralogues in the same gene lineage. In contrast, how the A-function is encoded in species other than *Arabidopsis* still remains one of the very dark corners of flower development.

The A-function, required for the specification of sepals and petals (together with B-function genes) and for restriction of C-function genes to the inner whorls, is proposed to be composed of two genes in *Arabidopsis*: *APETAL1 (AP1)* (Irish and Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993) and *AP2* (Komaki et al., 1988; Bowman et al., 1989, 1991; Kunst et al., 1989; Drews et al., 1991; Jofuku et al., 1994). While different *ap1* and *ap2* mutants show highly variable phenotypes, in a broad sense they follow the predictions of the ABC model, since perianth organ identity is generally disrupted.

Recently, however, researchers have started to question if these genes really should be thought of as perianth organ identity genes as defined in the classical ABC model, in the sense that they actively direct the formation of the perianth organs (Litt, 2007); perhaps more probably, their activity indirectly leads to sepal and petal development by establishing the floral meristem [which is in fact another function of these genes (Schultz and Haughn, 1993)] and restricting the C-function to the two inner whorls. This view is corroborated by the phenotypes of some *ap1* and *ap2* flowers. For both mutants, sepals can be converted to leaves or bracts, suggesting loss of floral meristem identity, or to carpels, indicating ectopic expression of the *Arabidopsis* C-gene AGAMOUS (*AG*). In the second whorl, secondary flowers may arise, also suggesting a loss of floral meristem identity, or petals are converted to stamens, as a result of ectopic expression of *AG4*. In other words, within the context of floral organ specification (as opposed to floral meristem specification), the A-function genes may have no function other than repressing the C-function.

In recognition of this fact, Wollmann et al. (2010) propose a scenario, based on detailed expression analyses of *miR172, AP2*, and *AG*, in which *AP2* acts predominantly as a cadaster gene, indirectly influencing organ fate, which is dependent upon the balance between *AG* and the AG-repressing activity of *AP2*. Thus, we will focus on their role in C-gene regulation when discussing the A-function genes in other species below.

Homologues of *AP2* have been found in a broad range of species, including, but not limited to, *Antirrhinum, Amborella, Petunia*, tobacco, tomato, maize, rice, Norway spruce, pine, and hybrid larch, and this gene may very well be ubiquitous within the seed plants. Although *AP2* genes from other species are poorly characterized, the available data suggest that its function has been conserved to some extent. In maize, two *AP2* homologues, *INDETERMINE SPIKELET1 (IDS1)* and *SISTER OF INDETERMINE SPIKELET1 (S1D1)*, play a role in floral meristem development and spikelet determinacy, but also in repression of C-function, since loss of function of these genes leads to the formation of carpelllloid bracts (Chuck et al., 2008), indicating a high degree of conservation between *Arabidopsis* and maize. When a rice *AP2* homologue is silenced, flowers carry fewer stamens and multiple stigmas, which is also observed in some *Arabidopsis ap2* alleles (Zhao et al., 2006). Via heterologous expression, a spruce *AP2*-like gene has been shown to delay flowering and lead to a loss of determinacy in *Arabidopsis* (Nilsson et al., 2007), which also occur when the endogenous *AP2* is overexpressed in *Arabidopsis*. Interestingly, orthologues of *AP2* from either *Petunia* or *Antirrhinum* do not appear to play a role in repression of C-class genes in the outer whorls, but the *Antirrhinum AP2*-like genes do play a role in floral development.
as loss of function of two AP2 homologues, LIPLESS1 (LIP1) and LIPLESS2 (LIP2), leads to sepal-to-leaf transformations and defects in petal development (Keck et al., 2003). Loss of function of an AP2 homologue in Petunia did not result in a floral phenotype (Maes et al., 2001). However, the genome sequence of Petunia (and Antirrhinum) was not known at the time of analysis, so it cannot yet be excluded that some of the floral functions are redundantly encoded by unknown closely related AP2 homologues, and therefore have been missed. So while part of the function of AP2 has been shown to be conserved in some species, especially with respect to its role in floral meristem identity specification, its role in C-gene repression remains to be further investigated.

Functional conservation of API is similarly limited. PROLIFERATING INFLORESCENCE MERISTEM (PIM) from pea, LeMADS_MC from tomato, and SQUAMOSA (SQUA) from Antirrhinum all appear to be involved in floral meristem identity establishment (Huijser et al., 1992; Berbel et al., 2001; Taylor et al., 2002; Vrebalov et al., 2002), but the role of API in C-gene repression in Arabidopsis (Gregis et al., 2006) does not seem to be conserved in other species (as reviewed in Litt, 2007). In addition to API and AP2, several other genes in Arabidopsis have a role in repressing AG, and thus may be argued also to contribute to the A-function: STERILE APETALA, RABBIT EARS, BELLRINGER, CURLY LEAF, AINTEGUMENTA, LEUNIG, SEUSS, SHORT VEGETATIVE PHASE, and AGAMOUS-LIKE24 (Liu and Meyerowitz, 1995; Goodrich et al., 1997; Byzova et al., 1999; Krizek et al., 2000, 2006; Franks et al., 2002; Bao et al., 2004; Gregis et al., 2006). Whereas STYLUSA, the Antirrhinum homologue of LEUNIG, has been shown to play a role in C-gene regulation (Motte et al., 1998), for other genes and species, these genes constitute largely unknown territory.

Nevertheless, mutants with a partial A-function phenotype do exist in Petunia and Antirrhinum, called blind (bl) and fistulata (fis), respectively. In both mutants, the petals are partially transformed into antheroid tissues (the petal tubes develop normally), whereas the sepals are often wild type in appearance, only occasionally carrying carpeloid tips (Vallade et al., 1989), whereas the sepals are often wild type in appearance, only occasionally carrying carpeloid tips (Vallade et al., 1989), for other genes and species, these genes constitute largely unknown territory.

To conclude, while the B- and C-function genes have been shown to be highly conserved in many angiosperms, the A-function may tell a different story. On the one hand, there simply is a lack of data. A systematic comparative analysis of ‘A’-function genes in other species is therefore needed. On the other hand, the data available suggest that the regulation of the C-function genes is controlled by different mechanisms, not only within one species (as indicated by the many C-gene regulators in Arabidopsis), but, more interestingly, also between different species, as illustrated by the examples of Petunia and Antirrhinum. Finally, not only are there differences in the set of genes involved, there are also differences with respect to the regulatory logic employed. While AP2-mediated control of the C-function primarily prevents expression from the outer whorls of the flower, the BLIND–FIS/NF-YA module seems to control the C-function primarily within its native expression domain.

Based on the difficulties in defining a general A-function discussed above, Causier et al. (2009) have recently proposed a revised (A)BC model in which the (A)-function comprises a plethora of genes that ultimately allow the B- and C-genes to determine floral organ identity. In this model, the (A)-function is composed of such disparate genes as C-gene repressors, SEP
genes, LEAFY, WUSCHEL, and others. On one hand, this model
succeeds much better than the traditional model in incor-
porating the variability observed within the classical A-function
and in recognizing that only the B- and C-function genes seem to
be bona fide, well-conserved organ identity genes. On the other
hand, one might argue that defining the (A)-function as a func-
tion that sets the stage for the organ identity genes to function
remains somewhat too vague to be useful.

Nevertheless, this model, which is essentially an expanded
version of an old BC model (then referred to as the AB model)
proposed by Schwarz-Sommer et al. (1990), seems better suited
to the current data.

Post-transcriptional control of the MADS-box ABC-genes?

Given that the ABC-genes control the identity of the floral
organs, their expression patterns, both in time and in space,
are of prime importance, and, as such, they are subject to tight
regulation. Most research in this regard has focused on tran-
scriptional regulation. It is still unclear, however, to what extent
post-transcriptional control directly influences the activity of the
MADS-box genes. Some observations suggest that at least some
of the type II MADS-box genes themselves might be directly
regulated at the post-transcriptional level.

Jack et al. (1994) have described 35S:AP3 lines in Arabidopsis,
which, in accordance with the predictions of the ABC model,
develop stamens in their fourth whorl. However, because AP3
functions in a complex with PISTILLATA (PI), the predicted
transformation from sepals to petals does not occur, since PI is
not expressed in the first whorl (Goto and Meyerowitz, 1994).
Interestingly, the authors have noted that while AP3 mRNA is
present in the first whorl of 35S:AP3 flowers (as expected), the
protein product is not. This discrepancy between mRNA and
protein levels could suggest that post-transcriptional regulation
of AP3 is taking place. Alternatively, the turnover rate of the
protein may be very high in the first whorl, possibly because of
the absence of PI, which might stabilize AP3 levels via protein–
protein interaction. Such a view would also correspond to the
discrepancy observed between AP3 mRNA and AP3 protein lev-
els in pi mutants, in which AP3 mRNA is present in the second
whorl of stage 5–10 flowers, while the protein is not (Jack et al.,
1992, 1994). Nevertheless, to our knowledge, it has never been
actually established whether these observations reflect active,
PI-dependent, post-transcriptional regulation of AP3, or whether
they are simply a result of reduced stability of AP3 in the absence
of PI.

Indirect indications of post-transcriptional regulation of
ABCs come from studies in Petunia. Whereas Arabidopsis
35S:AG flowers show transformations as predicted by the ABC
model, a conversion of petals to stamens and sepals to carpels
(Mizukami and Ma, 1992), analogous experiments in Petunia
produce very different results. Ectopic overexpression of either
PMADS3 or FBP6, the two Petunia C-function genes, results in
only partial petal-to-stamen transformation, while sepals remain
virtually unchanged, only sometimes producing carpelloid tips
(Tsuchimoto et al., 1993; and unpublished results). In contrast,
when PMADS3 is expressed in tobacco via the 35S promoter,
complete petal-to-stamen and sepal-to-carpel transformations
occur, showing that this gene is capable of specifying reproduc-
tive organ fate in the perianth whorls (Tsuchimoto et al., 1993).
Since RNA of the transgenes is present throughout the flower
in the Petunia overexpression lines, the lack of full homeotic
conversion may suggest that the Petunia C-function genes are
post-transcriptionally regulated in the two outer floral whorls.
Alternatively, cofactors or protein interaction partners required
for the formation of a functional complex may be absent from
these tissues. However, this is seemingly contradicted by the
fact that ectopically expressing an AG homologue of cucumber
in Petunia produces nearly complete petal-to-stamen and very
strong sepal-to-carpel transformations (Kater et al., 1998). On
the other hand, the two Antirrhinum C-genes differ in their abili-
ties to interact with E-function proteins (SEPs) (Airoldi et al.,
2010). A single amino acid insertion in FARINELLI (FAR), one
of the Antirrhinum C-function proteins, limits its interactions
with the Arabidopsis SEPs to just SEP3, which is not expressed
in the first whorl. As a result, ectopic expression of FAR in
Arabidopsis flowers does not result in sepal-to-carpel transfor-
mation in Arabidopsis. Such a scenario may also play a role in
Petunia, especially considering the fact it harbours six SEP
genes with divergent expression patterns. However, this particu-
lar polymorphism does not play a role in Petunia, since neither
of the two C-function genes contains this amino acid insertion.
Nevertheless, these data show that small changes in protein
sequence can dramatically alter protein function and perhaps
the cucumber C-function protein is able to form a functional com-
xplex in the perianth whorls of Petunia, while the endogenous pro-
teins are not, due to specialization among interaction partners.

Another interpretation of this data is that some post-
transcriptional repression mechanism in Petunia recognizes
the mRNAs of its own C-function genes and subsequently
represses them in the sepals and petals. The cucumber homolo-
gue may be sufficiently divergent to avoid recognition by this
repression mechanism, and would thus be able to accumulate
as a protein and specify reproductive organ fate in the perianth
whorls. Whatever the scenario, phenomena like these deserve
further study, since it could lead to the discovery of novel regu-
laratory mechanisms.

A flourishing future for floral research?

Over the years, enormous progress has been made in our under-
standing of floral development in a handful of model species.
In general, these model species have quite simple flower struc-
tures, and they do not cover the mind-blowing diversity of flo-
ral architecture displayed in the >250 000 species of flowering
plants. We are thus still far from identifying the key evolution-
ary molecular changes that generated this feast for the eye. So
far, for technical reasons (e.g. no functional analysis possible),
its has often been too challenging to start to work with more
exotic non-model plants. However, we might have good hopes
that this will change, perhaps already within the coming dec-
ade. Due to the unprecedented and continued progress made in
‘next-generation’ sequencing technologies, sequencing the entire
genome of large series of individual plants might become almost
as trivial as sequencing a single gene. In such a case, it will become feasible to start forward and reverse genetics screens in any species that have a reasonable generation time and the possibility to self-fertilize. It will be sufficient to mutagenize a seedstock, grow populations, screen for interesting phenotypes, recover several independent alleles, and simply sequence the entire genome of these individuals to identify the mutated gene. Likewise, reverse genetics screens might be performed in silico by blasting a database composed of the individual genome sequences of all members of a mutagenized population. By starting to analyse a new series of plants species with interesting floral architectural characteristics, we can certainly hope for a flourishing future for floral research.

References


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