Beyond the ABCs: ternary complex formation in the control of floral organ identity

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The production of a flower requires several events to occur. A floral meristem must form, boundaries must be set to enable discrete primordia to arise and the primordia must adopt the correct organ identity. Homeotic mutants, whose organs adopt inappropriate identities for their position within the flower, have helped the construction of a simple combinatorial model to explain how floral organ identity is defined. However, recent experiments suggest that the regulation of floral organ identity is more complex than was previously apparent. The simple interactions are becoming more complex and the universal applicability of the model less clear.

The past decade has seen great advances in our understanding of flower development. The key to much of the progress has been the availability of informative developmental mutants, primarily in the two model species *Antirrhinum* and *Arabidopsis*. Morphological and genetic analysis of homeotic floral mutants in these species led to the development of several broadly similar genetic models[1–3]. For reasons of pragmatism, the most apparently inclusive of these models has been universally adopted and has become known as the ABC model of flower development. This elegantly simple model (Fig. 1) has now become widely known and has even found its way into modern textbooks. It predicts the existence of three genetic 'functions', A, B and C, which, alone or in combination, can specify the identity of the organs formed in each of the four whorls of the flower. Expression of the A function alone leads to the production of sepals. Coexpression of the A and B or B and C functions leads to the formation of petals and stamens, respectively. Expression of the C function alone causes the formation of carpels.

For every complex problem, there is a solution that is simple, neat, and wrong. Henry Louis Mencken (1880–1956)

The application of molecular biology to the study of the floral homeotic mutants resulted in the identification of most of the genes that control these functions in both model species[4] (Fig. 2). Almost all of these genes belong to one conserved family of transcription factors, called the MADS-box factors[2]. MADS-box factors are not confined to the plant kingdom and are found in animals and yeast, where they control a variety of important processes such as the differentiation of muscle lineages and mating-type-specific gene expression[5]. However, although animals and yeasts each appear to have several MADS-box genes, the final tally for *Arabidopsis* might exceed 100. This extensive duplication of regulatory genes is common in plants[6].

As more and more of the plant MADS-box genes begin to be ascribed a function, it is becoming clear that they play a central role in many aspects of plant development. MADS-box genes have already been shown to be involved in meristem identity, lateral root development and the control of flowering time[7–10], in addition to their roles in defining floral organ identity and more subtle aspects of floral organ development[11,12]. As might be expected in such a large family of transcription factors, there are already several examples of genetic redundancy and complex regulatory interactions among plant MADS-box genes, and it is likely that these difficulties will complicate their future functional analysis. Nonetheless, given their apparent role as primary regulators of different aspects of plant developmental pathways, they represent attractive candidates for further investigation by, for example, reverse genetic screens. If we are to understand how different developmental fates are specified, we must also discover how the MADS-box genes interact with each other, with other factors and with DNA to regulate the expression of their specific target genes.

Problems with the ABC model

From the beginning, it was apparent that, although the ABC model provides a useful starting point, there are some clear anomalies. *Antirrhinum* and *Arabidopsis* B- and C-function mutants differ slightly in phenotype and the genes are regulated differently, most of which can probably be accounted for by the relative position of each gene in their respective regulatory networks[13]. However, in spite of these differences, the B and C functions of *Antirrhinum* and *Arabidopsis* are clearly comparable between species.

However, it is more difficult to make comparisons between the A functions of *Antirrhinum* and *Arabidopsis*[14]. In *Arabidopsis*, the A-function includes two genes: *APETALA1* (*AP1*) and *APETALA2* (*AP2*). Like the B- and C-function genes, *AP1* is a member of the MADS-box gene family, but *AP2* is not[15,16]. The *Antirrhinum* orthologue of *AP1* is considered to be *SQUAMOSA* (*SQUA*)[7] but *ap1* and *squa* mutants have different phenotypes. In *squa* mutants, flowers develop as inflorescence shoots, thus indicating that *SQUA* plays a role in determining floral meristem identity. Studies have so far been unable to demonstrate any effect of *SQUA* expression on the specification of sepal and petal organ identity, distinct from its role in promoting the establishment of floral meristems, as would be required for an A-function gene. Indeed, *Arabidopsis* currently remains the only species in which organ identity defects have been linked with A-function genes.

Even in *Arabidopsis*, the phenotypes of *ap1* mutants do not correspond precisely to the carpel–stamen–stamen–carpel pattern predicted by the model (Fig. 1). Indeed, it has been suggested that,

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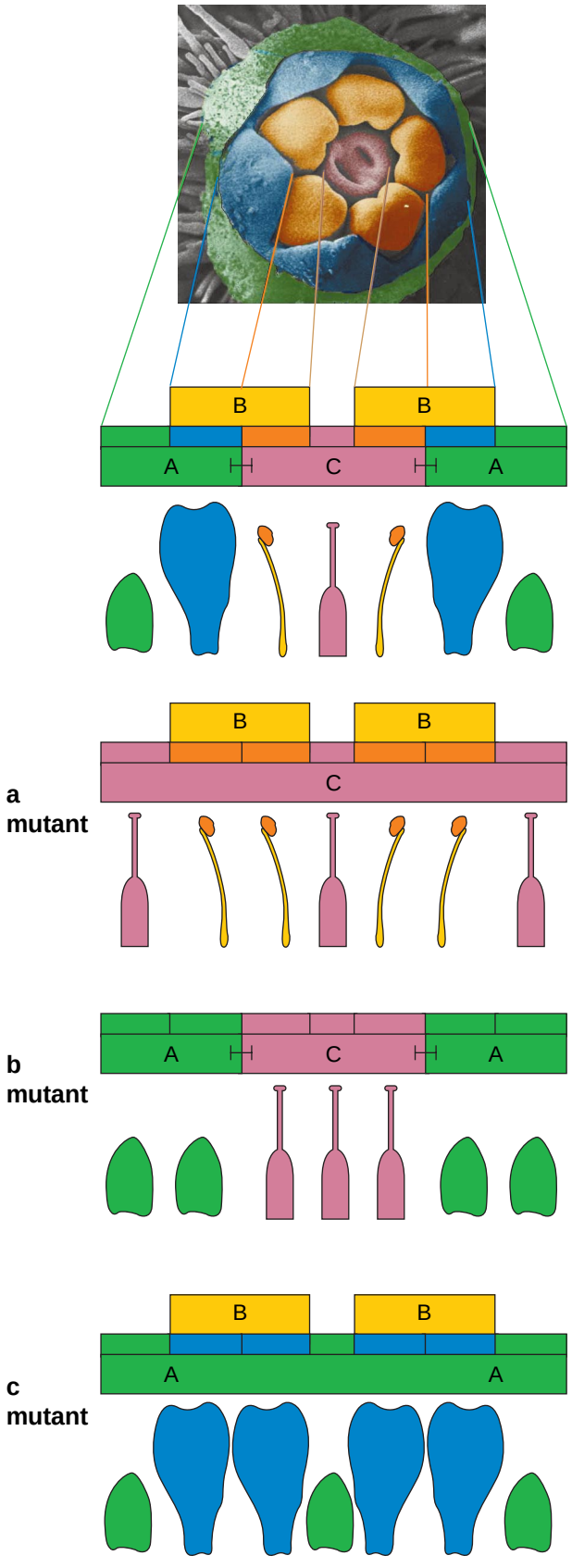

Fig. 1. The ABC model of flower development. The model shows how three genetic functions can specify four distinct organ types. The electron micrograph shows a tobacco flower shortly after all the floral organs have begun to develop; the four whorls have been artificially coloured. The sepals in the first whorl are green, the petals in the second whorl are blue, the stamens in the third whorl are orange and the carpels in the fourth whorl are pink. Beneath the photograph, a series of boxes illustrates the expression patterns of three genetic functions, called A (green), B (yellow) and C (pink), and below the boxes is a diagram of the mature organ type produced. Lines link the expression domains of the functions and combinations of functions to the whorls in the photograph. Function A is expressed alone in whorl 1 (green) and causes the formation of sepals. Functions A and B are expressed together in whorl 2 (green + yellow = blue), resulting in the formation of petals. Functions B and C are expressed together in whorl 3 (yellow + pink = orange), directing the formation of stamens. Function C is expressed alone in whorl 4 (pink), leading to the formation of carpels and the termination of floral organ development. The A and C functions are shown as mutually repressing, although the experimental evidence suggests that this is only partly true[33]. The idealized phenotypes predicted by the model for mutants in each of the functions are also shown. For example, in the b mutant, there is no B function and this results in whorls 1 and 2 both expressing only the A function, and whorls 3 and 4 expressing only the C function. As a consequence, a flower is produced that consists of sepals, sepals, carpels and carpels. It is important to realize that this represents an idealized phenotype and that there are many deviations from this in the corresponding mutants in different species.

in *Arabidopsis* as in *Antirrhinum*, the A function is required simply to establish floral meristems, destined to produce sepals; the B- and C-function genes would then act on these to specify the petals, stamens and carpels. This role for the A function might be obscured by the presence of three *AP1*-like MADS-box genes (*AP1*, *CAL* and *FUL*)[6]. Thus, in a strict sense, the A function might be unlike the B and C functions, in that it is not required for the control of floral organ identity.

ABC genes are necessary but not sufficient

Several lines of evidence show that the A, B and C functions are not the sole determinants of floral organ identity. According to the ABC model, expression of the C-function gene in the absence of B-function-gene expression should cause carpels to form (Fig. 1). This is supported by the phenotypes of B-function mutants in *Antirrhinum* and *Arabidopsis*, both of which have third whorls composed solely of carpels. Furthermore, a transposon-induced dominant allele of the *Antirrhinum* C-function gene *PLENA* (*PLE*), which results in ectopic expression of *PLE* throughout the plant, produces carpels in the first whorl[17].

This phenotype can be duplicated in other species by ectopic expression of C-function genes using the CMV 35S promoter[18–22]. However, it is noticeable that in all these cases the effects of ectopic C-function expression outside the flower are confined to slight alterations in bract morphology and leaf curling. In no cases are ectopic carpels produced outside the flower structure. This is also true of the *Arabidopsis* mutant *curly leaf* (*clf*), in which *AG* is ectopically expressed[23].

Similarly, there have been several reports of artificial ectopic expression of B-function genes in different species[22,24,25]. With the exception of some petalody of the cauline leaves in *Arabidopsis*, expression of the B-function genes outside the flower has failed to convert leaves to petals. These results imply that the organ identity genes are necessary and sufficient to specify organ identity within a floral or inflorescence context, presumably defined by the presence of one or more additional flower-specific factors.

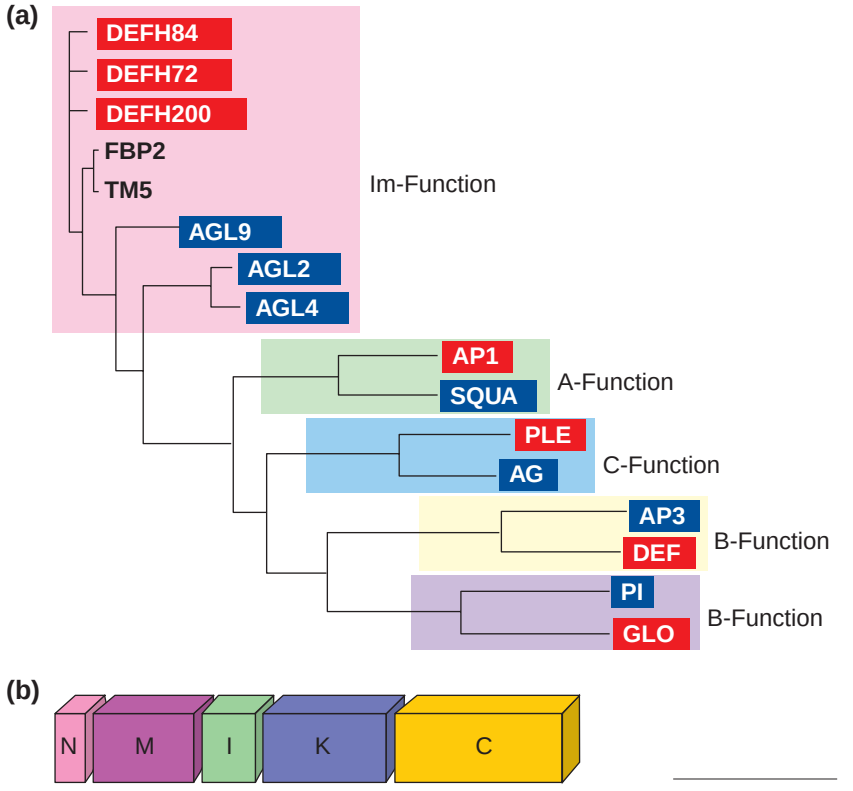

Fig. 2. Related MADS-box genes in different species. (a) Phylogenetic reconstruction of the MADS-box genes described in this review. Protein products of the genes were aligned using the ClustalW program and the resulting output was used as input for the Easytree phylogenetic program. Members of the same subgroups are boxed and labelled as having A, B, C or Im functions. *Antirrhinum* factors are shaded in red, *Arabidopsis* factors in blue. *FBP2* is from petunia and *TM5* from tomato. (b) Domain structure of plant MADS factors. All the above MADS factors, in common with most characterized plant MADS factors, can be divided into four or five domains. The MADS box (M) is always at or near to the N terminus and is mainly involved in DNA binding. The I region (I), which is of variable length and sequence composition, and the K box (K), which is predicted to form two or three amphipathic α helices, are both involved in protein–protein interactions. The C terminal domain (C) is conserved only in members of the same subfamily; experimental evidence suggests that this domain is essential for activity[30]. Certain MADS factors, such as those with the C function, have a variable number of amino acids N-terminal to the MADS box (N). The function of this N-terminal extension is unknown.

Further circumstantial evidence in support of the idea that other factors are required for the activity of the A, B and C functions came from domain-swap experiments. It is attractive to assume that the C-function factors specifically recognize sites in the promoters of C-function-specific genes. Thus, expression of the C function might have the effect of turning on genes required to make a carpel. This belief is supported by the fact that the MADS boxes of genes that have been shown to perform identical or similar developmental roles in different species have characteristic amino acids at certain positions within the MADS box. Because the MADS-box is the primary DNA-binding domain it might be reasonable to assume that these conserved positions are influential in determining the specificity of the target genes that are recognized. However, although the A-, B- and C-factors bind different sites with slightly different affinities, their MADS boxes seem to be interchangeable *in vivo*. A series of domain swap experiments showed that even the MADS boxes of mammalian and yeast MADS-box factors could be used to replace those of certain plant MADS-box factors with no apparent adverse effect on the function of the plant factor[4]. The most plausible explanation for this is that target gene specificity is determined by a complex combination of variables that affect promoter topology. One of these variables is likely to be the ability of any given transcription factor to interact with other factors that might, in turn, contact other factors and/or the DNA. All these studies point to the conclusion that the A, B and C factors do not act in isolation but require other factors to exert their effects.

Good candidates for such factors have been known for several years. In 1994, two independent groups used different approaches to investigate the function of related MADS-box genes from different species. The *FBP2* gene of petunia and the *TM5* gene of tomato are members of the same subfamily of MADS-box genes[26–28] (Fig. 2). Remarkably similar results were obtained following cosuppression of *FBP2* (the re-introduction of a gene into a plant in such a way that both the trans-gene and the pre-existing endogenous gene are silenced) and antisense expression of *TM5* (the expression of an antisense copy of a gene to prevent activity of the endogenous copy) (Fig. 3). In both cases, homeotic changes in organ identity were observed in the inner three whorls of the flower, accompanied by a loss of determinacy, which is consistent with a decrease in the influence of both B and C functions. These experiments clearly indicate a role for this class of MADS-box genes in the determination of floral organ identity and hence suggest that they constitute all or part of the 'floral context' required for the activity of the A, B and C functions. In view of this, we suggest that this class of MADS-box genes, which were previously called intermediate MADS-box genes[28], should be referred to as 'identity mediating' (Im) genes.

Multimerization of MADS-box factors

The early cosuppression and antisense experiments did not result in the widespread incorporation of the Im MADS-box genes into the ABC model, probably because *in vitro* analysis showed that the B-function factors could heterodimerize with each other and that the C-function factors could homodimerize, making it unclear how the Im MADS factors would exert their effects. Furthermore, in both cosuppression and antisense approaches, there is a possibility that other genes could be simultaneously affected.

Further support for the essential role of the Im genes in the establishment of floral organ identity came from analysing protein–protein interactions of the organ identity MADS factors using the yeast two-hybrid system (Fig. 4). Animal and yeast MADS-box factors are known to interact with a wide variety of other factors [ternary complex factors (TCFs)] to regulate gene expression[5]. In an attempt to identify plant TCFs, a two-hybrid screen was performed on the *Antirrhinum* C-function factor PLE. Surprisingly, although no evidence was found for the homodimerization of PLE, interactions were detected with two previously

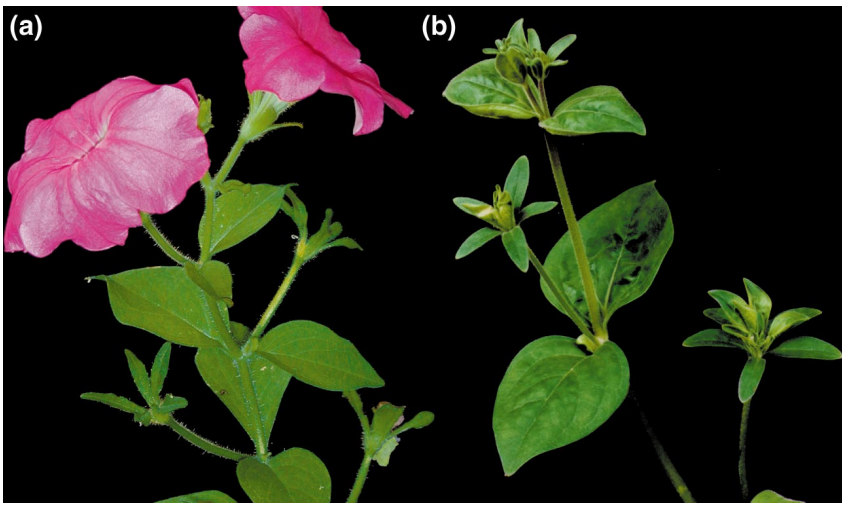

Fig. 3. Cosuppression of *FBP2* in petunia. (a) A normal petunia inflorescence with two mature flowers clearly visible. (b) A petunia inflorescence from a plant in which *FBP2* is cosuppressed and that carries flowers of approximately the same age. The replacement of petals by sepaloid organs is clear, as is the lack of reproductive organs within the flowers of the cosuppressed plant. A fuller description of the phenotype can be found in Ref. 26. *Image of the FBP2 cosuppression plant courtesy of Gerco Angenent*.

uncharacterized MADS-box factors, DEFH200 and DEFH72 (Ref. 28). Sequence and expression analyses of the genes encoding these newly identified factors showed that they are members of the Im class of MADS-box genes. Northern-blot analysis showed that the Im MADS-box genes are expressed in C-function mutants and so the Im genes are unlikely to act as essential downstream targets of the C-function genes.

Taken together, these results suggested that the Im MADS-box factors are essential for the activity of the C factors and that they act by forming Im–C heterodimers. Sequence comparisons between Im factors in *Antirrhinum* and other species have identified three likely candidates in *Arabidopsis*: AGL2, AGL4 and AGL9 (Ref. 28) (Fig. 2). Subsequent two-hybrid screens for proteins that could interact with AG, the *Arabidopsis* C factor, duly identified AGL2, AGL4 and AGL9 as interactors[29].

Although these studies provided evidence for direct interactions between the Im and C factors, there was still an inconsistency between the interaction studies and the transgenic analysis of Im genes. Cosuppression and antisense analysis of the petunia and tomato Im MADS-box genes not only affected the reproductive organ identity and the determinacy of the flowers, as would be expected, but also converted petals into sepals. This implies that the Im factors are essential for B-factor activity as well as C-factor activity. No evidence was found in yeast two-hybrid screens for any protein–protein interactions between *Antirrhinum* B-function factors and any other proteins, with the sole exception of the other B-function factor[28]. This suggested that, although the C-function factors can interact directly with the products of the Im MADS-box genes, the same is not true for the B-function factors.

The fact that B-function factors were only observed to interact with each other and not with any other class of MADS-box factors suggested that the combinatorial interaction between the B factors and the C, A or Im factors could not be at the level of heterodimerization. Or could it? The yeast two-hybrid experiments, although ideal for studying interactions between individual proteins, could not be used to study further interactions of the B-function DEF–GLO heterodimer. To test this, a modification of the two-hybrid experiment, known as the ternary-factor trap[30], was required. In this experiment, DEF and GLO were expressed together with a third protein or cDNA expression library (Fig. 4). This approach was initially carried out to look for interactions between the B-function proteins and the candidate *Antirrhinum* A-function factor SQUAMOSA. When all three proteins were expressed it became clear that, although SQUA could not detectably interact with either DEF or GLO alone, it could interact with the DEF–GLO heterodimer[30]. Furthermore, this ternary complex increased the level of DNA-binding seen in bandshift assays.

This result raised, for the first time, the possibility that the organ identity MADS-box factors were actually interacting as complex multimers to regulate target gene expression. Subsequent experiments using the DEF–GLO heterodimer as bait in the ternary factor trap to screen cDNA expression libraries have revealed that the DEF–GLO heterodimer can also interact with the Im factors DEFH200 and DEFH72 (M. Egea-Cortines and H. Sommer, unpublished). Thus, the ternary factor trap analysis shows that the Im MADS-box factors can interact with the B-function heterodimer and reveals a possible mechanism for the observed effect of cosuppression and antisense analysis of petunia and tomato Im genes on processes controlled by the B function.

The final proof that the Im MADS-box genes are indeed essential for the activity of the organ identity genes has now been obtained in *Arabidopsis*. Individual loss-of-function mutants in each of the three known *Arabidopsis* Im MADS-box genes, *AGL2*, *AGL4* and *AGL9* [now renamed *SEPALLATA* (*SEP*) *1*, *2* and *3*, respectively] have been identified[31]. Individual single mutants have subtle phenotypes but the *sep1 sep2 sep3* triple mutant has a phenotype similar to those found in cosuppression and antisense analysis of *FBP2* and *TM5*. Thus, the flowers of the triple mutant consist of indeterminate repeats of whorls containing four sepals, four sepals, six sepals and a new mutant flower. Although the two-hybrid and ternary factor trap analyses cited above provided a likely explanation for the phenotype observed in the Im MADS-box mutants, the availability of mutants allowed other formal possibilities to be ruled out. Expression of the organ identity genes is unaltered at early stages in the development of *sep1 sep2 sep3* triple mutants, showing that they are not induced by the Im genes[31]. Analysis of the expression of the *SEP* genes in *b* and *c* mutants also confirms the *Antirrhinum* data, that the Im MADS-box genes are unlikely to act as primary downstream targets of the organ identity genes.

Everything is interaction

All the evidence to date suggests that the B- and C-factor organ identity genes require the activity of the Im factors to specify organ identity. Furthermore, it seems likely that the Im function is needed because the B and C factors act as part of various complexes formed by the interactions between A, B, C and Im factors (Fig. 5). Formation of these different complexes might confer added specificity, which could be manifest at several different levels. There is evidence that different MADS-box factors induce differing degrees of DNA bending on binding[32]. The ability to form different complexes would offer the opportunity to modify promoter architecture by inducing bends in the DNA that could

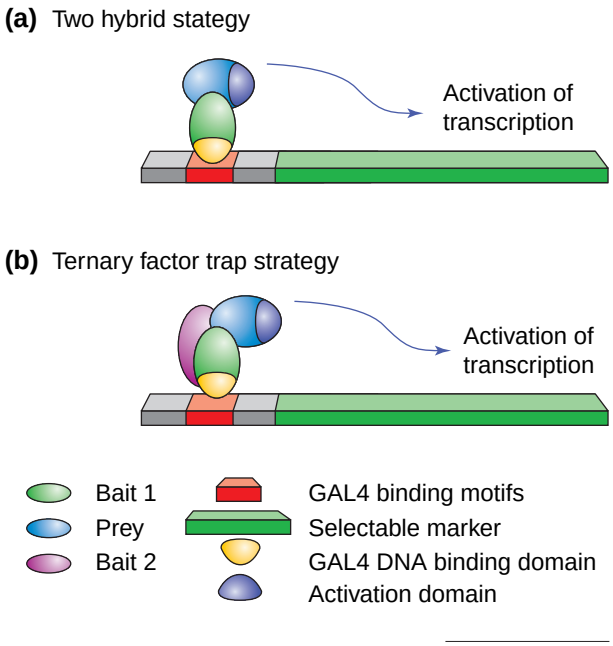

Fig. 4. Yeast two-hybrid and ternary factor trap protein–protein interaction screens. The yeast two-hybrid strategy (a) allows the identification and cloning of genes encoding proteins that interact with a known protein. The known protein (bait 1) is expressed in yeast as a fusion with a transcription factor DNA-binding domain. The proteins to be tested (prey), which can be the products of a cDNA library, are expressed as fusion proteins with a transcription activation domain. If the bait and prey interact in the yeast cell, the activation domain is brought to the promoter of the selectable marker and activates transcription of the marker gene. A limitation of this technique is that interactions are only detected if the prey interacts with the bait alone. The ternary factor trap (b) was developed to screen for interactions between preys and a heterodimer bait. In the ternary factor trap, bait 1 is expressed as in the two-hybrid screen. However, an additional protein, bait 2, is also expressed in the yeast cells. Baits 1 and 2 form a heterodimer that binds to the promoter of the selectable marker through the binding domain fused to bait 1. In this system, it is possible to identify proteins interacting with the bait-1–bait-2 heterodimer.

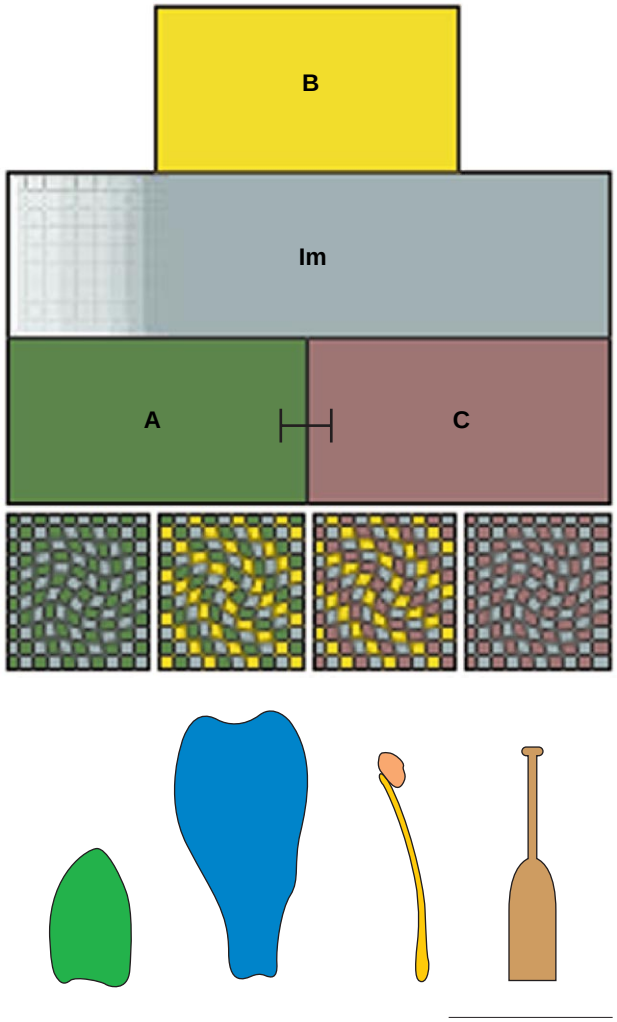

Fig. 5. Revised ABC model showing the Im function. The rectangles show the expression patterns of the A, B, C and Im functions in half a flower. A is expressed in whorls 1 and 2, B in whorls 2 and 3, C in whorls 3 and 4, and Im mainly in whorls 2, 3 and 4. The shading in whorl 1 in the Im rectangle represents the restricted expression of Im genes reported in the first whorl of some species[28,31]. The four squares show the combination of factors present in each whorl. Thus, in whorl 3, the B, C and Im factors are all present, resulting in the formation of stamens.

favour or preclude other protein–protein or protein–DNA interactions. It is also possible that ternary complexes can make further interactions that would not be possible for simple dimers and heterodimers. Indeed, some MADS-factor ternary complexes can activate transcription in plants, whereas the constituent lower-order heterodimers do not (K. Goto, pers. commun.). It will be interesting to discover whether coexpression of the Im factors with the B and C factors will be sufficient to produce reproductive organs outside the flower. Alternatively, because the A factors are also involved in ternary complex formation with the B, C and Im factors[28–31], it remains possible that expression of A-function genes such as *SQUA* or *AP1* will also be required.

In contrast with what is known about animal and yeast MADS-box factors, there is currently no well characterized report of an interaction between any plant MADS-box factor and another non-MADS factor. The ternary interactions between plant MADS-box factors seem to require a region of the protein not previously known to be required for heterodimerization or ternary factor interaction (Fig. 2). DNA-binding experiments and two-hybrid studies have identified the K box and the I region as the crucial domains involved in MADS-factor heterodimerization[4,28]. The C-terminal region, which is required for activity but whose function has not yet been determined, is not needed to form any of the heterodimers studied to date. However, the ternary interaction between DEF, GLO and SQUA is mediated by the C-terminal domain[30]. This suggests a different mechanism for the formation of the ternary complex and explains why specific interactions can occur at the ternary complex level that would not be seen at the level of heterodimerization.

It is apparent that we still have much to learn about the working of the floral organ identity MADS-box factors. We now need to look beyond our initial models involving homodimers and heterodimers to consider the possibility that more complex interactions are involved. Future studies will need to address the mechanisms of heteromultimerization and DNA binding, and the roles they play in the regulation of target gene expression. This

also has implications for the role of other MADS-box genes in plant development. The MADS-box gene family appears to suffer as much as any other from that blight of genetics, redundancy. The Im MADS-box genes are a case in point, comprising as they do at least three distinct genes in *Arabidopsis*, *Antirrhinum* and petunia[28,31] (B. Davies and H. Sommer, unpublished, G. Angenent, unpublished). Another unresolved issue concerns the A-function factors, which have also been identified as interactors with the organ-identity and Im factors, and therefore might still be involved in facilitating the action of the B and C factors. To understand these complex regulatory networks the related series of MADS-box genes as well as the direct and ternary interactions of their products will need to be investigated.

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