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Evolution in Action: Following Function in Duplicated Floral Homeotic Genes

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Summary

Gene duplication plays a fundamental role in evolution by providing the genetic material from which novel functions can arise [1, 2]. Newly duplicated genes can be maintained by subfunctionalization (the duplicated genes perform different aspects of the original gene's function) and/or neofunctionalization (one of the genes acquires a novel function) [3–8]. *PLENA* in *Antirrhinum* and *AGAMOUS* in *Arabidopsis* are the canonical C-function genes that are essential for the specification of reproductive organs [9, 10]. These functionally equivalent genes encode closely related homeotic MADS-box transcription factors. Using genome synteny, we confirm phylogenetic analyses [11] showing that *PLENA* and *AGAMOUS* are nonorthologous genes derived from a duplication in a common ancestor. Their respective orthologs, *SHATTERPROOF* in *Arabidopsis* [12] and *FARINELLI* in *Antirrhinum* [13], have undergone independent subfunctionalization via changes in regulation and protein function. Surprisingly, the functional divergence between PLENA and FARINELLI, is morphologically manifest in both transgenic *Antirrhinum* and *Arabidopsis*. This provides a clear illustration of a random evolutionary trajectory for gene functions after a duplication event. Different members of a duplicated gene pair have retained the primary homeotic functions in different lineages, illustrating the role of chance in evolution. The differential ability of the *Antirrhinum* genes to promote male or female development provides a striking example of subfunctionalization at the protein level.

Results and Discussion

C-Function Genes in *Arabidopsis* and *Antirrhinum*

The MADS-box family of transcription factor encoding genes consists of 107 members in *Arabidopsis thaliana* [14]. The establishment of floral organ identity was the first biological function to be experimentally assigned to this gene family in plants [15], leading to the formulation of the textbook "ABC" model of flower development [16]. In this model, the C-function MADS-box genes are responsible for the specification of male and female reproductive organ identity. Mutants in the C-function genes show homeotic conversion of reproductive organs into nonreproductive, perianth organs. *AGAMOUS* (*AG*) is an archetypal C-function gene that determines reproductive organ development in *Arabidopsis*; *ag* mutants lack reproductive organs and have petals and indeterminate mutant flowers in the third and fourth whorls, respectively [9]. In the snapdragon, *Antirrhinum majus*, mutations in a related MADS-box gene, *PLENA* (*PLE*), confer almost identical phenotypes [10]. In both species *ag* and *ple* are the only known mutants that display these characteristic homeotic changes. Despite this, other closely related genes exist in both species; *FARINELLI* (*FAR*) in *Antirrhinum* and the *SHATTERPROOF* genes (*SHP1* and *SHP2*) in *Arabidopsis* [12, 13]. In *Antirrhinum*, *far* mutants affect the male reproductive organs, causing partial male sterility. In contrast, the *SHP* genes of *Arabidopsis* redundantly affect fruit development in the female reproductive organs. To definitively establish their evolutionary relationship, irrespective of any functional constraints, we used synteny to elucidate the history of these genes.

Comparison of Gene Synteny at C-Function Loci

An *Antirrhinum* BAC library was constructed, and BAC clones containing the *PLE* and *FAR* loci were sequenced and compared to the *Arabidopsis* genome. Probably because of the prevalence of whole-genome-duplication events in the plant kingdom, the identification of syntenic regions between distant plant species has proved to be more problematic than is the case with vertebrate genomes [17]. For the C-function loci, sufficient synteny was observed to determine clearly the evolutionary relationship between these genes. Despite the similarity of the *Arabidopsis ag* and *Antirrhinum ple* mutant phenotypes, BLAST searches revealed that the *Antirrhinum* BAC carrying *PLE* showed the greatest homology to an *Arabidopsis* BAC containing *SHP1*. More detailed analysis was performed by directly comparing genes predicted on each *Antirrhinum* BAC with the regions of the *Arabidopsis* genome containing the *AG* and *SHP* genes. These comparisons corroborated the absence of synteny between the *PLE* and *AG* loci. However, striking synteny was found between the *PLE* and *SHP1* loci and, to a lesser extent, the *SHP2* locus (Figure 1). *SHP1* is flanked by a gene encoding a glycosyl transferase (*GTase*) downstream and an expressed gene (*EXP*) upstream. In addition, a haloacid dehalogenase (*HAD*) is predicted five genes downstream. In *Antirrhinum*, *PLE* is also adjacent to a *GTase* (downstream) and is separated from a gene with homology to *EXP* (upstream) by a putative transposase

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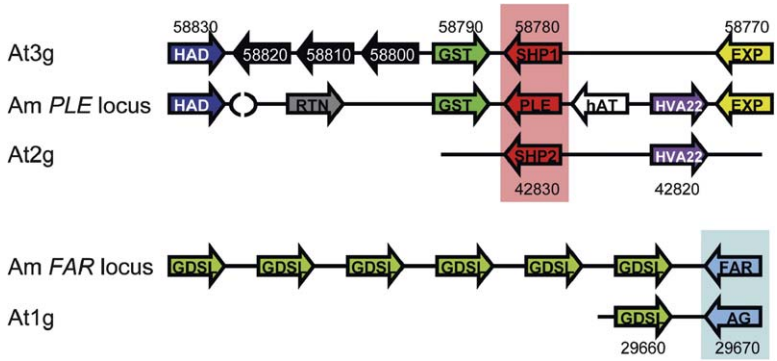

Figure 1. Synteny at the C-Function Loci

Schematic presentation of the predicted gene order and orientation on the *Antirrhinum* (Am) *PLE*- and *FAR*-containing clones aligned with the most similar regions of the *Arabidopsis* (At) genome. Red and blue shading show the *PLE/SHP* and *AG/FAR* genes, respectively. *Antirrhinum* BAC sequence accession numbers: AY935269 and AY935268.

(*Tase*) and a gene with similarity to *Arabidopsis HVA22*. Furthermore, EST hybridization experiments with a second *PLE*-containing BAC as probe identified a homolog of *HAD* farther downstream of *PLE* (Figure 1). *SHP1* acts redundantly with a closely related gene, *SHP2* [12]; the two genes being products of a relatively recent gene-duplication event [18]. Comparison of the *PLE*-containing BAC with the *SHP2* locus also reveals some colinearity. *SHP2* lies adjacent to an *HVA22* gene homologous to the one adjacent to *PLE* (Figure 1). Similarly, the *Antirrhinum FAR* locus shares some colinearity with the *AG* region of the *Arabidopsis* genome (Figure 1); both *AG* and *FAR* being adjacent to homologous GDSL lipase genes. Thus, the *FAR*- and *PLE*-containing regions of the *Antirrhinum* genome show similarity, in terms of gene identity, order, and orientation to the *Arabidopsis AG* or *SHP1/SHP2* loci, respectively, providing unambiguous evidence for the orthologous relationship between the *PLE/SHP* genes and the *FAR/AG* genes. Our analysis utilized a measure of relatedness that is independent of functional constraints on the evolution of the *AG*, *SHP*, *PLE*, and *FAR* genes. It is in agreement with phylogenic reconstructions based on protein sequence that, in apparent contradiction to mutant analysis, suggest orthology between the pairs *AG/FAR* and *PLE/SHP* [11].

Evolution of C-Function Genes

These results are best explained in the model presented in Figure 2A. An ancestral gene, C^a, essential for the formation of reproductive organs, became duplicated in a common ancestor, before the divergence of *Arabidopsis* and *Antirrhinum* (≈ 120 MYA), to form AG^a and PLE^a. Because the duplication partially maintained synteny, it may have resulted from a whole-genome duplication predicted to have occurred in dicots around this time [17]. Subsequently, the *Arabidopsis* and *Antirrhinum* lineages diverged, with both lineages maintaining the independent AG^a and PLE^a genes (Figure 2A). However, the primary responsibility for specifying reproductive organs was retained by a different member of the gene pair in the *Arabidopsis* and *Antirrhinum* lineages (white outlined circles in Figure 2A), suggesting that the newly duplicated genes were initially redundant in the common ancestor and demonstrating the role of chance in evolution. A subsequent gene duplication in *Arabidopsis* led to the formation of the two *SHP* genes from PLE^a. Synteny observed between the *SHP1* and *SHP2* loci (Figure 2B) and the timing of this duplication are consistent with it being part of the last whole-genome-duplication event in *Arabidopsis* (≈ 86 MYA) [17]. If that were the case, *AG* would also have been duplicated at the same time. Indeed, a syntenic locus lacking the *AG* gene, which could have been lost later, is detectable in the *Arabidopsis* genome (Figure 2B).

Subfunctionalization after Gene Duplication

If the primary homeotic role was retained by the nonorthologous genes *AG* and *PLE*, what became of their orthologs in *Antirrhinum* and *Arabidopsis*? *AG*, *PLE*, and *FAR* all display very similar expression patterns in the developing male and female reproductive organs (Figure 2A). In contrast, the *SHP* genes are only expressed in the fourth whorl [12] indicating subfunctionalization by alterations in gene expression patterns. Regulatory changes also occurred in the *Antirrhinum* genes *PLE* and *FAR*, although these differ from those that affected the *SHP* genes in *Arabidopsis* [13]. Previous work has identified conserved motifs in a large intron known to exert a regulatory influence over *AG* and *PLE* [19]. Two motifs, the aAGAAT and CCAATCA boxes, were shown to be present in the large intron of both *AG* and *FAR*. However, only the CCAATCA boxes are also found in *PLE*, and neither motif is found in the corresponding introns of the *SHP* genes, which are considerably shortened. It is possible that these differences play some part in the differential regulation of these genes.

AG and *SHP1/SHP2* are functionally equivalent and can direct both male and female organogenesis when ectopically expressed in transgenic *Arabidopsis* plants [20–23]. To test whether *PLE* and *FAR* also have the capacity to perform similar functions, we generated transgenic *Antirrhinum* plants. Ectopic expression of *FAR* in *Antirrhinum* (Figure 3) transformed petals to structures resembling male reproductive organs (stamens) but hardly influenced sepal identity. Conversely, ectopic expression of *PLE* resulted in a conversion of sepals into female organs (carpels) but less apparent transformation of petals to male organs (Figure 3). This demonstrates that after duplication, *PLE* and *FAR* have developed a differential ability to promote the formation of female and male reproductive organs, respectively. Thus, subfunctionalization in *Antirrhinum* resulted, in

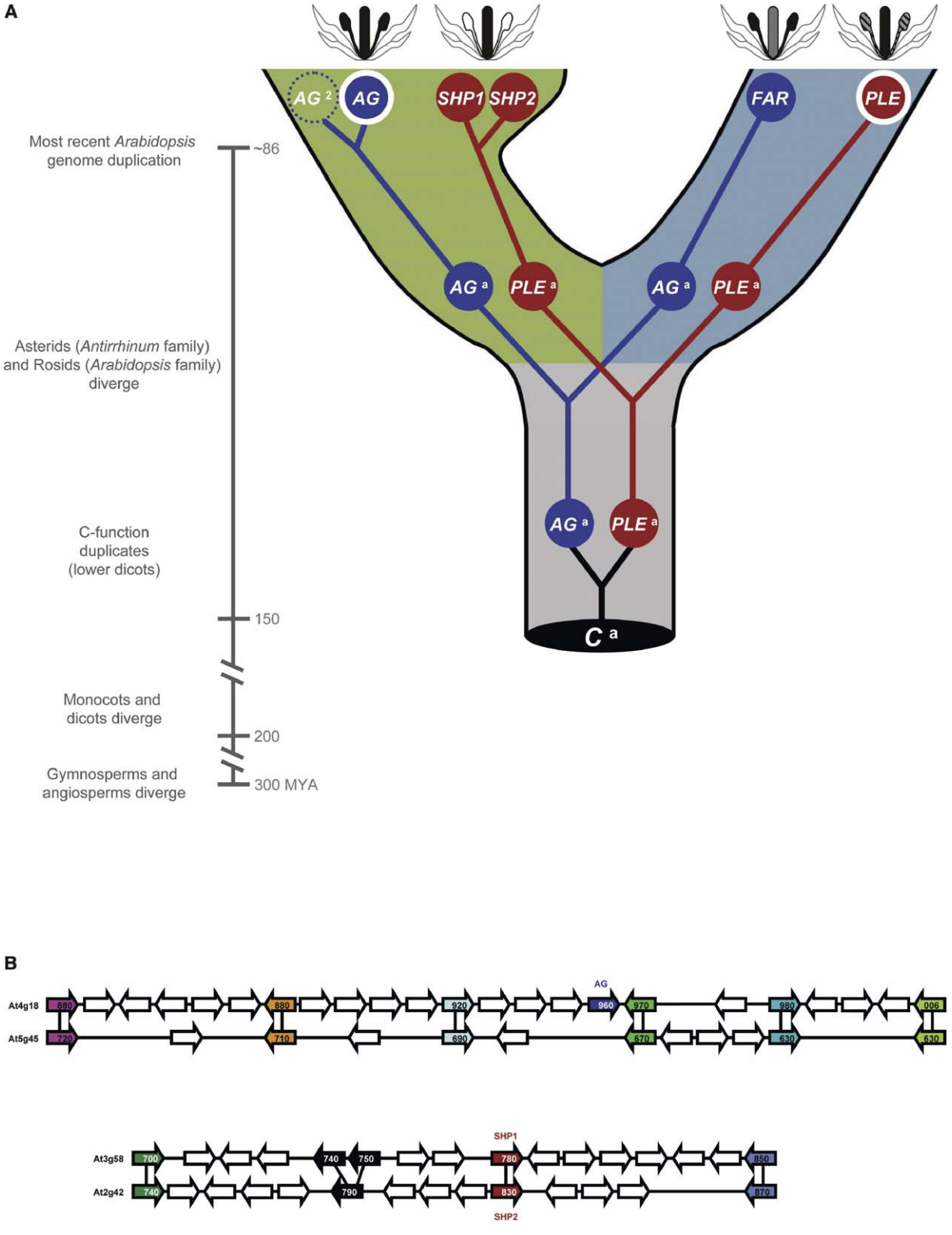

Figure 2. Evolution of the C Function

(A) C^a represents an ancestral C-function gene, which became duplicated after the divergence of monocots and dicots (see Figure S2) but before the divergence of *Arabidopsis* and *Antirrhinum* to create the ancestral AG^a and PLE^a gene lineages. After speciation (indicated by blue and green shading), both the *Arabidopsis* (green shading) and the *Antirrhinum* (blue shading) lineages received a copy of AG^a and PLE^a. However, different genes retained the primary C-function role in the two species (thick white circles). *AG* became primarily responsible for the specification of reproductive organ identity in *Arabidopsis*, whereas *PLE* adopted the same primary role in *Antirrhinum*. The approximate timescale of these events is shown by the timeline on the left. The current expression patterns and domains of function are illustrated

Figure 3. Ectopic Expression of *PLE* and *FAR* in *Antirrhinum* and *Arabidopsis*

Wild-type, 35S::*FAR*, and 35S::*PLE* flowers in *Antirrhinum* (top row) and *Arabidopsis* (bottom row). Whole flowers are shown except in the final column, which shows a detailed view of the first whorl organs containing ovules. Arrows show stamenoid organs in the second whorl (35S::*FAR*) and carpeloid organs in the first whorl (35S::*PLE*). Scale bars: *Antirrhinum*, 5 mm; *Arabidopsis*, 100 μm.

part, from changes in the capacity of the proteins to perform aspects of their original function. This subfunctionalization is not apparent in the single mutants, in which no reproductive organs form in *ple* mutants, despite an unaltered *FAR* gene, because of the relative position of the two genes in the regulatory heirarchy. Expression of *FAR* is reduced in *ple* mutants and expression of *PLE* is enhanced in *far* mutants, providing an explanation for *PLE*'s ability to partially complement loss of *FAR*, whereas *FAR* cannot complement loss of *PLE* [13].

Unexpectedly, although *Arabidopsis* does not appear to utilize this type of subspecialized C-function activity, it maintains the ability to respond to it. Ectopic expression of the devolved *Antirrhinum* C-function genes, *PLE* and *FAR*, in *Arabidopsis* mimicked the effects observed in transgenic *Antirrhinum* (Figure 3). So, even in *Arabidopsis*, the separate pathways leading to specification of male and female reproductive organs can be triggered jointly by one protein (AG) or individually by two proteins (PLE and FAR). The differences between the FAR and PLE proteins and their respective protein-protein [13] and protein-DNA interactions will help to elucidate the initiation of the male and female reproductive pathways.

Experimental Procedures

Identification and Analysis of BAC and TAC Clones

Antirrhinum BAC and TAC libraries were screened by hybridization with the appropriate cDNA clones lacking the MADS domain or by PCR with oligonucleotide primers, as described elsewhere [24]. The *PLE* BAC and the *FAR* TAC were sequenced commercially (MWG Biotech) to obtain single contigs of 85 kb and 54.3 kb, respectively.

Open reading frames were predicted with: Genscan (http://genes.mit.edu/GENSCAN.html) [25], GeneMark.hmm (http://opal.biology.gatech.edu/genemark/eukhmm.cgi) [26] (each with the *Arabidopsis* dataset), and FGENESH (http://www.softberry.com/) (with both *Arabidopsis* and tobacco datasets). BLAST homology searches were used to identify *Antirrhinum* ESTs (http://www.ebi.ac.uk/blast2/nucleotide.html and http://www.antirrhinum.net/blast/blast.html, with the Plant ESTs database and default settings). Where appropriate, the EST sequences were used to correct gene predictions. Comparisons with the *Arabidopsis* genome were made in a number of ways. First, each sequence was compared directly to *Arabidopsis* BAC sequences with the WU-BLAST2 algorithm at http://www.arabidopsis.org/ (default settings). Second, predicted peptides were subjected to BLAST homology searches against both the *Arabidopsis* protein dataset at http://www.arabidopsis.org/ (WU-BLAST2 algorithm) and the *Viridiplantae* dataset at http://www.ncbi.nlm.nih.gov/BLAST/ (BLASTP) (default settings). Finally, gene order and orientation were compared by manually mapping the predicted peptides against their *Arabidopsis* counterparts, facilitated by SeqViewer (http://www.arabidopsis.org/). Figure S1, available with this article online, summarizes gene prediction and identification for the *PLE* and *FAR* loci.

Transgenic Plants

Agrobacterium strain GV3101 was transformed with pB.10 and pB.12 binary vectors containing the *FAR* and *PLE* genes, respectively, under the control of the CaMV 35S promoter [13, 27]. *Arabidopsis* (Columbia ecotype) plants were transformed by the floral dip technique [28], and *Antirrhinum* plants were transformed as described previously [29].

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.current-biology.com/cgi/content/15/16/1508/DC1/.

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by flower diagrams above each gene. The four floral organs (sepals, petals, stamens, and carpels) are shown with shading to illustrate expression and function. Black shading indicates tissue-specific expression and function, gray shading denotes expression without apparent function, and gray and black stripes show expression with reduced function. Thus, the *SHP* genes are expressed and functional only in the carpels, whereas *FAR* is expressed in both stamens and carpels, but a function is only apparent in the stamens.

(B) Synteny between the *SHP* loci on chromosomes 2 and 3 is illustrated schematically with homologous genes shaded in the same colors and orientation shown by arrowheads. The *AG* locus on chromosome 4 and its most syntenic relative on chromosome 5 are compared in the same way. Although these loci share homologous genes, an *AG*-like gene is absent from the chromosome 5 locus.

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Accession Numbers

The DNA sequences of the *PLE*- and *FAR*-containing BACs have been deposited in the GenBank database under the accession numbers AY935269 and AY935268.

Note Added in Proof

During the publication of this manuscript, a general review of MADS-box gene duplication in plant evolution was published [30].