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Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations

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ABSTRACT

Frameshift mutations generally result in loss-of-function changes since they drastically alter the protein sequence downstream of the frameshift site, besides creating premature stop codons. Here we present data suggesting that frameshift mutations in the C-terminal domain of specific ancestral MADS-box genes may have contributed to the structural and functional divergence of the MADS-box gene family. We have identified putative frameshift mutations in the conserved C-terminal motifs of the B-function *DEF/AP3* subfamily, the A-function *SQUA/AP1* subfamily and the E-function *AGL2* subfamily, which are all involved in the specification of organ identity during flower development. The newly evolved C-terminal motifs are highly conserved, suggesting a *de novo* generation of functionality. Interestingly, since the new C-terminal motifs in the A- and B-function subfamilies are only found in higher eudicotyledonous flowering plants, the emergence of these two C-terminal changes coincides with the origin of a highly standardized floral structure. We speculate that the frameshift mutations described here are examples of co-evolution of the different components of a single transcription factor complex. 3′ terminal frameshift mutations might provide an important but so far unrecognized mechanism to generate novel functional C-terminal motifs instrumental to the functional diversification of transcription factor families.

INTRODUCTION

Plants exhibit a wide range of ornamental and functional differences in number and appearance of the organs that constitute their flowers. In general, such differences may be ascribed to variations in a basic set of key developmental regulators (called homeotic selector genes). These variations may simply represent differences in the expression patterns of an otherwise standard set of genes that determine the underlying morphogenetic processes. On the other hand, changes in the coding sequence might also lead to changes in gene function. Extensive analysis of plant floral developmental mutants during the last decade has revealed the importance of the MADS-box transcription factor family in flower development and plant architecture. The identity of the floral organs has been shown to be governed by the combined activity of specific MADS-box floral homeotic genes and it has been suggested that gene duplications followed by functional diversification within the MADS-box gene family must have been key processes in floral evolution (1–3). Phylogenetic studies of the MADS-box gene family thus have the potential to correlate differences in floral organ morphology with molecular and functional changes in MADS-box genes. The best-known subfamilies are the A (*SQUA/AP1*), B (*DEF/AP3* and *GLO/PI*) and C function (*AG*) MADS-box subfamilies, representing the basic players in the historical ABC model of flower organ identity.

Recent progress by reverse genetics strategies has uncovered redundant functions (4,5) that obviously have been missed by classical forward genetics approaches (6–13). Combined with the elucidation of protein–protein interactions between the different MADS-box genes, these results have led to extensions of the ABC model towards models with a higher complexity (14–18). All data together presently suggest a quartet model (14) in which the identity of the four different floral organs, sepals, petals, stamens and carpels, is specified by four different protein complexes consisting of various combinations of MADS-box proteins and yet unknown factors.

All MADS-box genes discussed here belong to the Type II class MADS-box genes; the proteins encoded by these genes share a conserved modular organization, called the MIKC type domain structure, consisting of a MADS (M), intervening (I), keratin-like (K) and C-terminal domain (2,19–21). The MADS-domain is responsible for DNA binding, but it is also involved in dimerization and accessory factor-binding functions (21). The K-domain seems to be plant-specific (2)

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and is involved in protein dimerization (19,21). Several lines of evidence demonstrate the functional importance of the C-terminal domain. Loss-of-function alleles may carry mutations in the C-terminus and dominant-negative phenotypes can be generated by overexpressing MADS-box genes lacking the C-terminus (summarized in 16). The first half of the C-terminal domain of *DEF* and *GLO* proteins appears to be essential for ternary complex formation between *SQUA* (A-function) and *DEF* and *GLO* (B-function) MADS-domain proteins *in vitro* (16). Several reports suggest the presence of a C-terminal transcriptional activation domain in proteins encoded by genes belonging to different MADS-box subfamilies (18,22–24). Recently, it was demonstrated that truncated versions of the *Arabidopsis* B-function genes *AP3* and *PI*, only lacking the characteristic C-terminal eu*AP3* and *Pi* motif, respectively, were unable to rescue the corresponding *ap3* and *pi* mutants (25). This implies that the C-terminal motifs are essential for the full function of these proteins. Finally, although the C-terminus is overall the most divergent region among the different MADS-domain proteins, members of the same subfamily usually contain highly conserved C-terminal motifs (26). This suggests that the C-terminus may have played an important role in the functional diversification of the major MADS-box gene subfamilies. Because a less-conserved region of variable length often precedes these highly conserved motifs, the C-terminal region has mostly been excluded from phylogenetic analyses. While the high sequence similarity in the MADS- and K-domains of all MIKC type MADS-domain proteins strongly suggests that they are derived from a common ancestor, and differences in the MIK domains between the different subfamilies can be attributed to mutational events like single amino acid substitutions in combination with small in-frame insertions or deletions, the origin of the highly divergent C-terminal motifs remains obscure. The goal of the present study was to obtain a better understanding of how these putatively functionally important C-terminal motifs may have originated at the DNA level.

MATERIALS AND METHODS

Assembling the MADS-box sequence dataset

We screened the available nucleotide (non-redundant and EST) and protein databases with a diverged set of sequences containing representatives of all known MIKC type MADS-box gene subfamilies, resulting in a collection of over 400 unique plant MIKC type MADS-box sequences from over 100 plant species. More details about the pursued approach are provided in the Supplementary Material. For expressed sequence tag (EST) sequences included in the phylogenetic analysis, consensus sequences covering the full coding sequence were derived from several overlapping ESTs (indicated with 'merge' in Figure 2).

Sequence alignments

Full-length sequences were aligned using the PILEUP function, followed by a manual alignment of the C-terminal regions using the Seqlab Editor of the GCG software package [Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI, USA]. For each gene, the cDNA sequence and the corresponding putative protein sequence were coupled and for both, the C-terminal domains were aligned manually.

Phylogenetic analysis

For simplicity reasons, the Neighbor Joining tree (Fig. 2) has been constructed using a representative subset of 97 sequences from the total collection of available plant MIKC type MADS-box sequences. These sequences have been selected as follows: subclasses within subfamilies were determined based on the presence of deviating but conserved C-terminal motifs. For each subclass, one to three representative sequences from each major plant group (when available) were selected. The MIK domains of the selected MADS-box genes were aligned using ClustalW (27) and subjected to a phylogenetic analysis. Phylogenetic trees were computed using the TREECON program (28) according to the neighbor-joining algorithm (29), based on Poisson and Tajima and Nei (30) corrected evolutionary distances.

RESULTS

The DEFICIENS (DEF)/AP3 subfamily

So far, only for B-function MADS-box genes has a detailed sequence analysis of the C-terminus been performed for a diverged set of species (31,32). Although protein sequences belonging to the *DEF/AP3* subfamily share extensive similarity, two lineages can clearly be distinguished on the basis of their completely different C-terminal motifs (31). The first motif is referred to as the paleo*AP3* motif and is found in *DEF/AP3* proteins from lower eudicots, magnoliid dicots, monocots and basal angiosperms, while a second type, named the eu*AP3* motif is uniquely present in *DEF/AP3* proteins from higher eudicots. In addition, some higher eudicots possess both the eu*AP3* and paleo*AP3* type (*TM6* lineage). Recently, Lamb and Irish published data on C-terminal motif swapping experiments involving eu*AP3* and paleo*AP3* motifs, and demonstrating that these two motifs clearly encode a diverged function (25): a chimeric construct in which the eu*AP3* motif of the *Arabidopsis AP3* gene was replaced by a paleo*AP3* motif displayed differential rescue of the second and third whorls of the *ap3-3* mutant: second whorl organs remained fully sepaloid while stamen formation was partially rescued. These results indicate that the C-terminal motif of paleo*AP3* proteins promote stamen but not petal formation in higher eudicots. Our own attention was initially drawn to paleo*AP3* B-function MADS-box genes while analyzing the *Petunia* B-function family (manuscript in preparation). The paleo*AP3* motif containing *PhTM6* gene of *Petunia* exhibits some atypical characteristics compared to the classical eu*AP3* B-function MADS-box genes. During later stages of floral development, *PhTM6* mRNAs are abundantly present in carpels [similar to the tomato *TM6* gene (33)], to a lesser extent in stamens and to even lower levels in petals and sepals. Also, the *Petunia Green Petals* (*GP*) mutant (a null mutant for the eu*AP3 Pmads1* gene) displays a homeotic conversion of petals to sepals, while the formation of stamens remains unaffected (34), suggesting that *PhTM6* cannot substitute the eu*AP3 Pmads1* gene in petal formation, but most likely can complement its function in stamen development. These

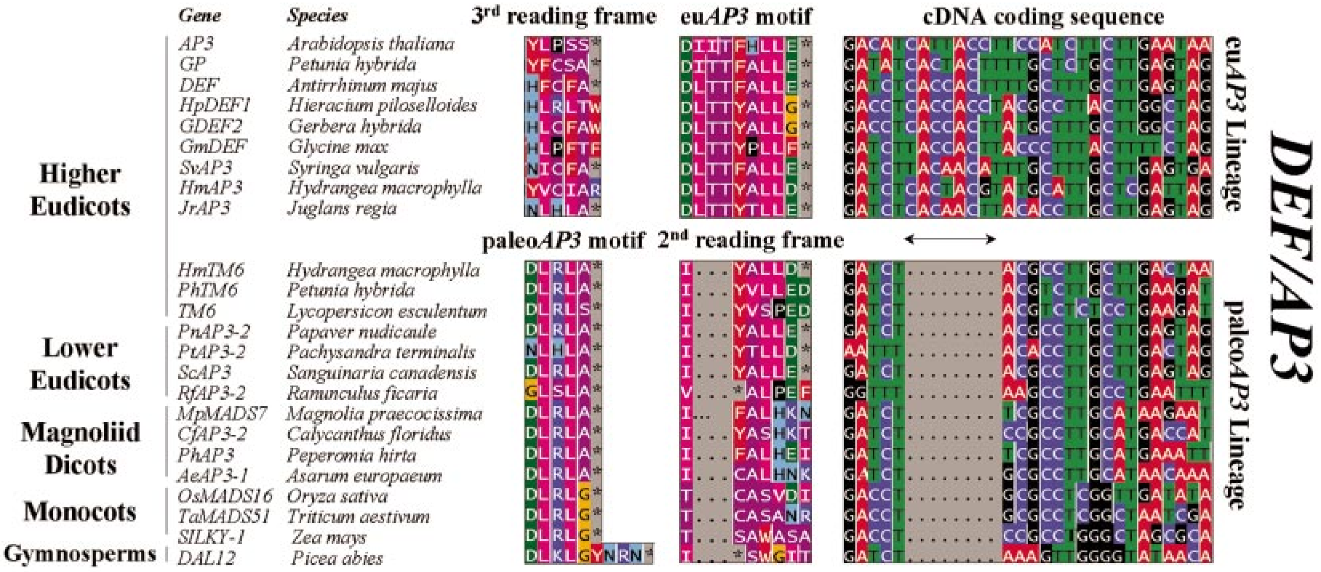
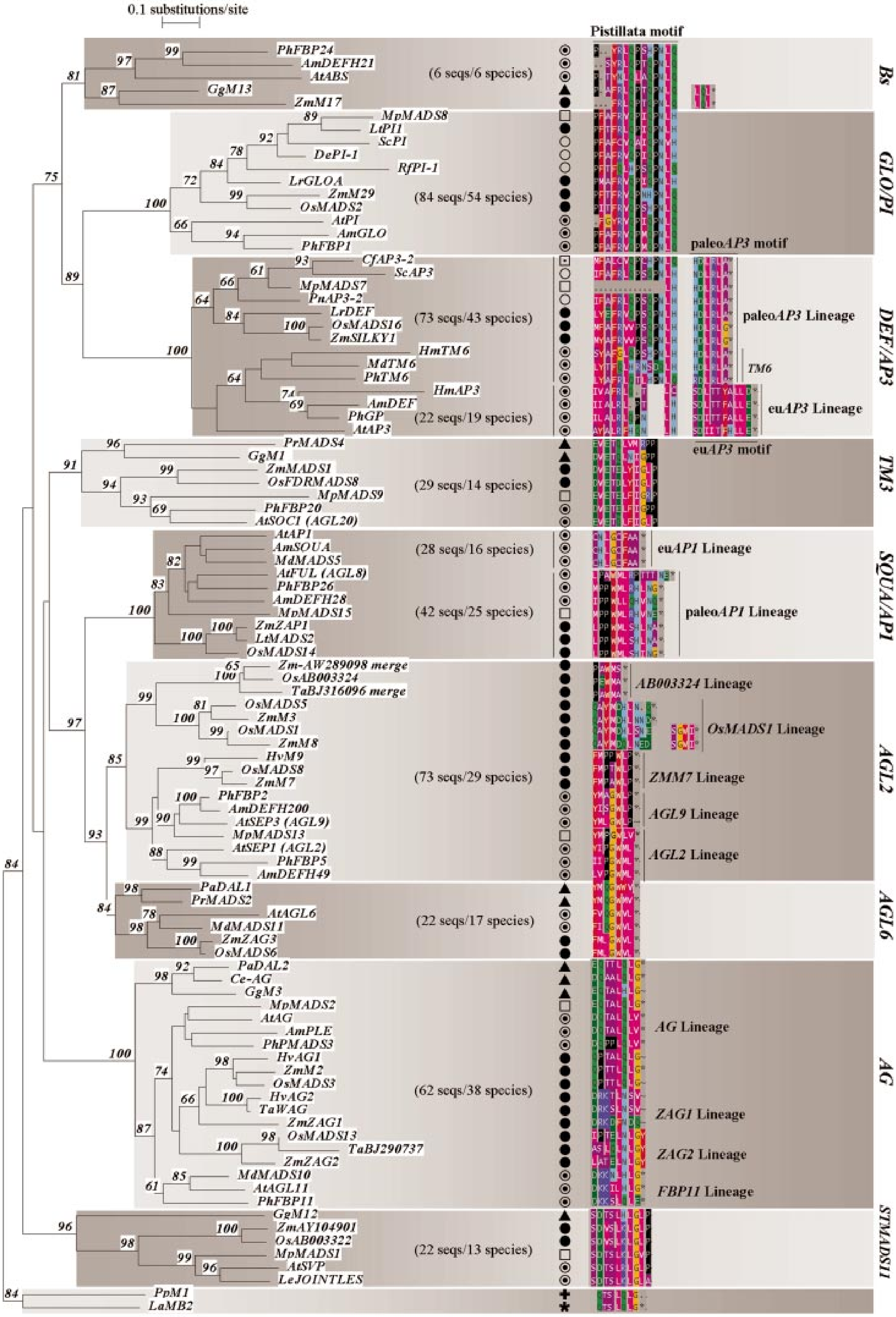


Figure 1. Alignment of paleo*AP3* and eu*AP3* C-terminal motifs present within the *DEF/AP3* subfamily. Although protein sequences belonging to the *DEF/AP3* subfamily display extensive homology almost along their entire length (not shown), two lineages can be distinguished on the basis of their completely different C-terminal motifs (columns indicated with paleo*AP3* and eu*AP3* motifs). In contrast, the cDNA fragments encoding the conserved motifs align very well (right column) upon the introduction of a gap of eight base pairs in the coding sequences of paleo*AP3* lineage members. The eu*AP3* motif, which is uniquely present in *DEF/AP3* subfamily members isolated from higher eudicots, may thus have originated by a frameshift mutation caused by the eight base pair insertion (indicated by a double headed arrow) into a paleo*AP3* ancestral gene. This is illustrated by the second reading frame translation of paleo*AP3* members (indicated with 2nd reading frame), which resembles the eu*AP3* motif. For details on the 3rd reading frame of the eu*AP3* motif, we refer to the text. A full set of analyzed sequences is presented in the Supplementary Material.

findings suggest that sequence diversification at the C-terminus may be responsible for differences in function between the *AP3* genes in higher eudicots as compared to other angiosperms and thus reflect part of the species diversification at the level of floral organ determining genes.

To understand how these different peptide motifs may have arisen at the molecular level during evolution, we compared the coding sequences of paleo*AP3* and eu*AP3* motif-encoding MADS-box genes in detail. To our surprise, we discovered that the C-terminal eu*AP3* motif can simply be explained by an eight base pair insertion in the C-terminus of paleo*AP3* genes, thus causing a frameshift mutation beyond the insertion site in eu*AP3* genes, when compared to the original reading frame of paleo*AP3* genes. A subset of the alignment of paleo*AP3* and eu*AP3* genes is shown in Figure 1. In a number of cases, translation of the C-terminus of paleo*AP3* genes according to the second reading frame indeed yields a motif that closely resembles the eu*AP3* motif (Fig. 1). It is interesting to note that although the paleo*AP3* motif is highly conserved among paleo*AP3* members, frameshift translations of the lower eudicot and *TM6* members resemble the eu*AP3* motif most, in contrast to frameshift translations of monocot paleo*AP3* genes, thus reflecting the phylogenetic relationships of the host species involved. Furthermore, the majority of paleo*AP3* members contain a clearly recognizable internal *PI* motif, while in eu*AP3* proteins this motif is degenerating (Fig. 2), suggesting that recruitment of the novel eu*AP3* motif may have been accompanied by a subsequent loss of the internal *PI* motif in eu*AP3* B-function proteins. The fact that both paleo*AP3* (*TM6* lineage) and eu*AP3* genes have been isolated from several higher eudicots suggests that eu*AP3* genes have originated after duplication of a paleo*AP3* ancestral gene, followed by a frameshift mutation in one of the copies. Species such as *Petunia*, tomato and *Hydrangea macrophylla* have retained both copies, while *Arabidopsis* apparently has lost the paleo*AP3* copy. Although the overall sequence analysis clearly points towards an eight base pair insertion in the eu*AP3* lineage, its exact origin remains elusive, because most likely it may have evolved further. We can presently envisage two putative mechanisms for this event: the insertion can be the result of a footprint left behind upon transposon excision or it may result from DNA polymerase slippage.

It is quite remarkable that a frameshift mutation just upstream of a highly conserved motif would yield a new, equally highly conserved motif. However, the data presented here are based on MADS-box sequences isolated from different species by different laboratories, rendering the possibility of sequencing mistakes unlikely. In addition, paleo*AP3* and eu*AP3* genes have been aligned in two different classes, solely based on the comparison of the non-C-terminal sequences (31). Finally, evolutionary conservation of the newly evolved frameshifted motif at the amino acid level changes the position of degenerate nucleotides compared to the original codon triplets. As a consequence, nucleotide substitutions, which may be silent in the new motif, may hamper the recognition of the original protein motif when translated according to the progenitor reading frame. This is in accordance with our observations that translating eu*AP3* genes according to the progenitor reading frame (third reading frame of the eu*AP3* Lineage in Figure 1) yields in the best cases only a highly diverged paleo*AP3* motif. If paleo*AP3* and eu*AP3*


0.1 substitutions/site
Pistillata motif
Bs
PhFBP24
AmDEFH21
AtABS
GgM13
ZmM17
(6 seqs/6 species)
GLO/PI
MpMADS8
LtPI1
ScPI
DePI-1
RfPI-1
LrGLOA
ZmM29
OsMADS2
AtPI
AmGLO
PhFBP1
(84 seqs/54 species)
paleoAP3 motif
DEF/AP3
CfAP3-2
ScAP3
MpMADS7
PnAP3-2
LrDEF
OsMADS16
ZmSILKY1
HmTM6
MdTM6
PhTM6
HmAP3
AmDEF
PhGP
AtAP3
(73 seqs/43 species)
(22 seqs/19 species)
paleoAP3 Lineage
TM6
euAP3 Lineage
euAP3 motif
TM3
PrMADS4
GgM1
ZmMADS1
OsFDRMADS8
MpMADS9
PhFBP20
AtSOC1 (AGL20)
(29 seqs/14 species)
SQUA/AP1
AtAP1
AmSQUA
MdMADS5
AtFUL (AGL8)
PhFBP26
AmDEFH28
MpMADS15
ZmZAP1
LtMADS2
OsMADS14
(28 seqs/16 species)
(42 seqs/25 species)
euAP1 Lineage
paleoAP1 Lineage
AGL2
Zm-AW289098 merge
OsAB003324
TaBJ316096 merge
OsMADS5
ZmM3
OsMADS1
ZmM8
HvM9
OsMADS8
ZmM7
PhFBP2
AmDEFH200
AtSEP3 (AGL9)
MpMADS13
AtSEP1 (AGL2)
PhFBP5
AmDEFH49
(73 seqs/29 species)
AB003324 Lineage
OsMADS1 Lineage
ZMM7 Lineage
AGL9 Lineage
AGL2 Lineage
AGL6
PaDAL1
PrMADS2
AtAGL6
MdMADS11
ZmZAG3
OsMADS6
(22 seqs/17 species)
AG
PaDAL2
Ce-AG
GgM3
MpMADS2
AtAG
AmPLE
PhPMADS3
HvAG1
ZmM2
OsMADS3
HvAG2
TaWAG
ZmZAG1
OsMADS13
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ZmZAG2
MdMADS10
AtAGL11
PhFBP11
(62 seqs/38 species)
AG Lineage
ZAG1 Lineage
ZAG2 Lineage
FBP11 Lineage
STMADS11
GgM12
ZmAY104901
OsAB003322
MpMADS1
AtSVP
LeJOINTLES
(22 seqs/13 species)
PpM1
LaMB2
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motifs had originated artificially from simple sequencing errors, the asymmetry in degree of conservation between correct and alternative reading frames would not be observed.

Intrigued by such a simple frameshifting mechanism, we were curious to find indications for a similar scenario in other subfamilies of the MADS-box gene family. Since only the C-terminus of the B-function subfamily has been analyzed in greater detail in a wide range of species (31,32,35), we first determined whether conserved C-terminal motifs existed in other major subfamilies as well. Therefore, we analyzed over 400 MIKC type MADS-box genes covering a wide range of species and representing all major subfamilies. Sequences were first grouped in subfamilies based on sequence homology in the MIK region. Once grouped, the C-terminal regions were aligned manually to determine C-terminal motifs. To illustrate this, we selected a representative set of sequences from each subclass for a diverged set of species, and performed a phylogenetic analysis to map the corresponding C-terminal motifs on the tree (Fig. 2). For simplicity reasons, the complexity of Figure 2 has been reduced in several ways. A number of subfamilies contain several conserved motifs separated by less conserved patches in the C-domain; we only show the conserved residues closest to the C-terminus. Monophyletic clades (e.g. the *AGL12*, *AGL15* and *AGL17* subfamilies) for which only a limited set of family members has been isolated, or that contain sequences from just a few species, were not included in the analysis. For these clades, sample numbers and/or species diversity were too low to allow a reliable identification of C-terminal conserved motifs.

For the majority of the subfamilies, we could identify subfamily specific C-terminal motifs. In an increasing level of detail, a number of subfamilies (e.g. the *AGAMOUS* subfamily) can be further divided into subclasses displaying distinct but related C-terminal motifs of which the differences can be attributed to normal nucleotide substitutions. On the other hand, we found that some subfamilies (e.g. the *SQUA/AP1* and *AGL2* subfamilies) could be further divided into subclasses displaying completely different but highly conserved C-terminal motifs, comparable to the situation found in the *DEF/AP3* subfamily. Other clades (e.g. *TM3* and *STMADS11* subfamilies) display C-terminal motifs that are highly conserved among protein sequences isolated from distantly related species such as angiosperms versus gymnosperms, suggesting that these C-terminal motifs were already fixed in the ancestors preceding the split of angiosperms and gymnosperms. It has been estimated that the lineages that led to extant gymnosperms and angiosperms probably separated about 300 million years ago, while the lineages that led to extant monocots probably separated from the lineage that led to extant eudicots about 160–200 million years ago (1 and references therein). A number of these small C-terminal peptide motifs thus have been preserved for several hundreds of millions of years. Similarly, it is remarkable that the *AGAMOUS* (*AG*) type C-terminal motif can be clearly recognized in the two MADS-box genes *PpM1* and *LaMB2* isolated from the moss *Physcomitrella patens* and clubmoss *Lycopodium annotinum*. C-terminal motifs of the full MADS-box gene dataset have been added in the Supplementary Material. In Figure 2, we have indicated the total number of analyzed sequences and the number of species from which genes belonging to a particular class have been isolated (in parentheses).

A minority of the analysed sequences did not exhibit the C-terminal peptide motif(s) as identified in the majority of the members of that subfamily or subclass. With the currently available data, we cannot exclude that at least some of these aberrant proteins represent the first isolated members of new classes of variants, perhaps only present in a subset of species of the plant kingdom.

However, for a substantial part of the sequences that did not exhibit the sub(class)family-specific motif, we were able to demonstrate extensive homology and the appearance of the sub(class)family-specific motif in either one of the three different reading frames downstream of the K-region, often beyond the proposed stop codon. Thus, the latter sequences presumably contain sequencing mistakes. Alternatively they might represent degenerating copies of recently duplicated genes. Besides a complete loss of the conserved C-terminal epitope, we also found pairs of recently duplicated paralogs of which one copy contained the consensus C-terminal motif, while the second copy displayed a more diverged motif. A clear example of such a case is the *Arabidopsis AGL13* gene, a member of the *AGL6* subfamily. The putative AGL13 protein terminates prematurely after only the first three amino acid residues of the *AGL6* motif, but still displays homology beyond the stopcodon (see Supplementary Material).

Having defined C-terminal motifs for the major subfamilies, we specifically searched for further examples of putative

Figure 2. (Opposite) Neighbor-joining tree of the MIKC type MADS-box gene family. The Neighbor-joining tree has been constructed using the MIK domains of a representative subset of 97 sequences from the total collection of available plant MIKC type MADS-box sequences (see Supplementary Material). These 97 sequences have been selected as follows: subclasses within subfamilies were determined based on the presence of deviating but conserved C-terminal motifs. For each subclass, one to three representative sequences from each major plant group (when available) were selected. The tree was rooted with two MIKC type MADS-box genes from the moss *Physcomitrella patens* and the clubmoss *Lycopodium annotinum.* To assess support for the inferred relationships, 1000 bootstrap samples were generated. In a final step, we mapped C-terminal conserved epitopes on the tree. Local bootstrap probabilities are indicated for branches supported with more than 60%. Asterisks behind protein motifs represent stop codons. Motifs not terminating with an asterisk are followed by a variable number of non-conserved residues (not shown). A two-letter code preceding the gene names as found in the database indicates the species involved. Species names and taxa are indicated as follows. Angiosperms: Higher eudicots (open circles with inner filled circles): *Am: Antirrhinum majus; At: Arabidopsis thaliana; Hm: Hydrangea macrophylla; Le: Lycopersicon esculentum; Md: Malus domestica; Ph: Petunia hybrida;* Basal eudicots (open circles): *De: Dicentra eximia; Pn: Papaver nudicaule; Rf: Ranunculus ficaria; Sc: Sanguinaria canadensis;* Monocotyledons (filled circles): *Hv: Hordeum vulgare; Lr: Lilium regale; Lt: Lolium temulentum; Os: Oryza sativa; Ta: Triticum aestivum; Zm: Zea mays;* Others: *Mp: Magnolia praecocissima (Magnoliales)* (open squares), *Cf: Calycanthus floridus (Laurales)* (open square with inner filled square). Gymnosperms (filled triangles): *Pa: Picea abies (Coniferales); Pr: Pinus radiata (Coniferales); Gg: Gnetum gnemon (Gnetales); Ce: Cycas edentata (Cycadales).* Outgroup: *La: Lycopodium annotinum (Lycopodiophyta)* (filled star)*; Pp: Physcomitrella patens (Bryophyta)* (plus sign). For each subfamily, the total number of analyzed sequences and different species is indicated in parentheses (no. sequences/no. species).

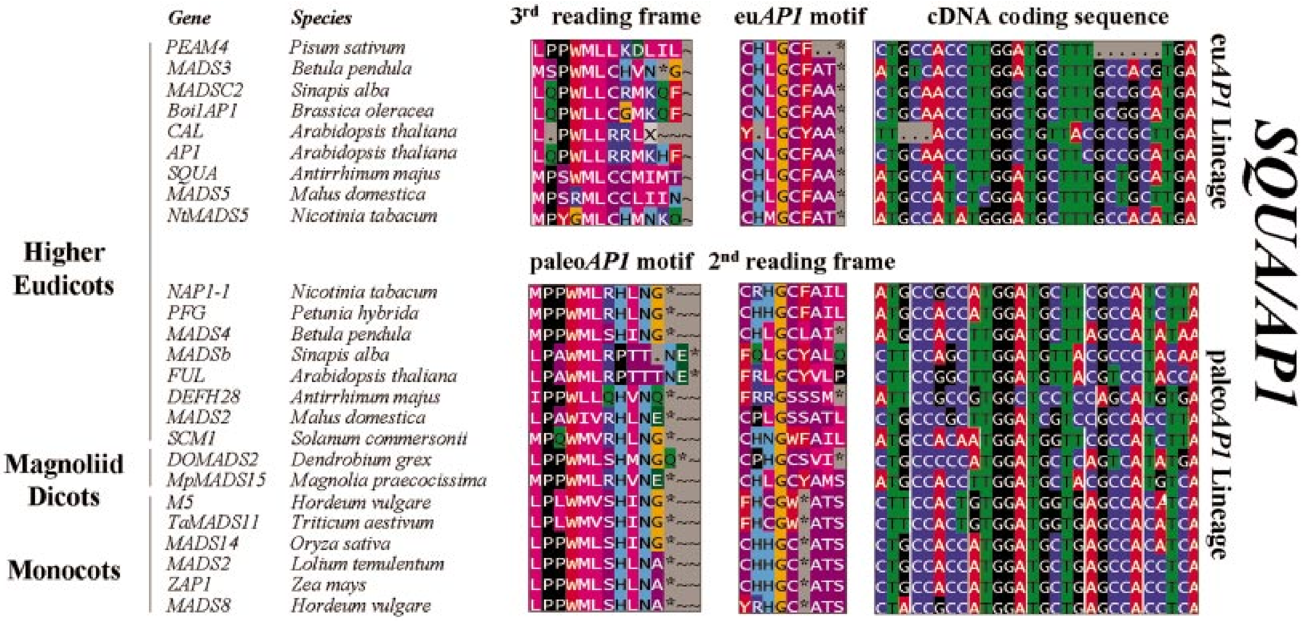

Figure 3. Alignment of paleo*AP1* and eu*AP1* C-terminal motifs present within the *SQUA/AP1* subfamily. Within the *SQUA/AP1* subfamily, two distinct lineages (eu*AP1* and paleo*AP1* lineages) can be distinguished, each displaying highly conserved but completely different C-terminal motifs (columns indicated with paleo*AP1* and eu*AP1* motifs). Representatives of both lineages have been isolated from a number of higher eudicot species, while magnoliid dicot and monocot species appear to yield only the paleo*AP1* type. Although these two types of C-terminal motifs are totally unrelated at the protein level, the cDNA fragments encoding these conserved motifs align surprisingly well (right column). This suggests that the eu*AP1* motif may have originated by a frameshift mutation in a paleo*AP1* ancestral gene at a position upstream of the paleo*AP1* motif. To illustrate this, we have shown frameshift translations of paleo*AP1* members (column indicated with 2nd reading frame) and of eu*AP1* members (column indicated with 3rd reading frame), which resemble the eu*AP1* motif and the ancestral paleo*AP1* motif, respectively. A full set of analyzed sequences is presented in the Supplementary Material.

frameshift mutations in these regions. Much to our surprise, we found additional examples in the *SQUAMOSA/AP1* and *AGL2* subfamilies.

The SQUAMOSA (SQUA)/AP1 subfamily

The majority of protein sequences belonging to the *SQUA/AP1* subfamily display either one of two highly conserved C-terminal motifs (Figs 2 and 3). We have designated these motifs the paleo*AP1* and eu*AP1* motifs, respectively. The highly conserved paleo*AP1* motif is present in *AP1* homologs from magnoliid dicots, monocots and higher eudicots. So far, no paleo*AP1* like proteins have been isolated from gymnosperm species. Note that the *Arabidopsis FRUITFULL* gene and *SAMADSB* from white mustard display a quite diverged paleo*AP1* motif compared to the other paleo*AP1* genes. The C-terminal eu*AP1* motif as found in the *Arabidopsis AP1* and *Antirrhinum SQUA* proteins seems to be restricted to the higher eudicots, since we extensively screened the available monocot EST databases without finding them. However, we also found higher eudicot sequences that displayed a more diverged eu*AP1* motif (e.g. the pea protein PEAM4 in Fig. 3). Although the two *AP1* subclasses exhibit a divergent C-terminal peptide motif, cDNA sequences encoding the terminal eu*AP1* and paleo*AP1* motifs align very well. Indeed, translation of the C-terminal part of paleo*AP1* genes according to the second reading frame yields motifs that closely resemble the eu*AP1* motif, and translation of the C-terminal part of eu*AP1* genes according to the third reading frame yields motifs that closely resemble the paleo*AP1* motif (Fig. 3). Similar to the situation in the *DEF/AP3* subfamily, frameshift translations of paleo*AP1* genes from dicot origin resemble the eu*AP1* motif most, which reflects the phylogenetic origin of the eu*AP1* genes. Also, correct reading frame translations yield motifs that are more rigidly conserved than frameshift translations, suggesting that the presence of these two different motifs have not originated from sequencing errors. Because the coding sequence preceding the terminal motifs appeared to be too divergent between paleo*AP1* and eu*AP1* genes to align, we could not determine the nature or the exact position of the putative frameshift mutation. The restriction of eu*AP1* type genes to the higher eudicots suggests that eu*AP1* type genes have originated after duplication of a paleo*AP1* type gene followed by a mutational event creating a frameshift in the C-terminus of one of two copies. In addition, higher eudicots such as *Arabidopsis*, snapdragon, tobacco, apple, birch and cauliflower have retained both variants. The taxonomic distribution suggests that the gene duplication happened close to or at the base of the higher eudicots.

The AGL2 subfamily

In higher eudicots, two closely related types of *AGL2* genes can be distinguished, each displaying a distinct but related C-terminal motif, represented by *AGL2* (*SEP1*) and *AGL9* (*SEP3*) types, respectively (Fig. 2). For monocots, we have identified three clearly divergent types of *AGL2*-like genes (Fig. 2). A first group, the *ZMM7* type, has a C-domain that is closely related to the *AGL9* type from higher eudicots. The other two groups exhibit very divergent C-domains. For the

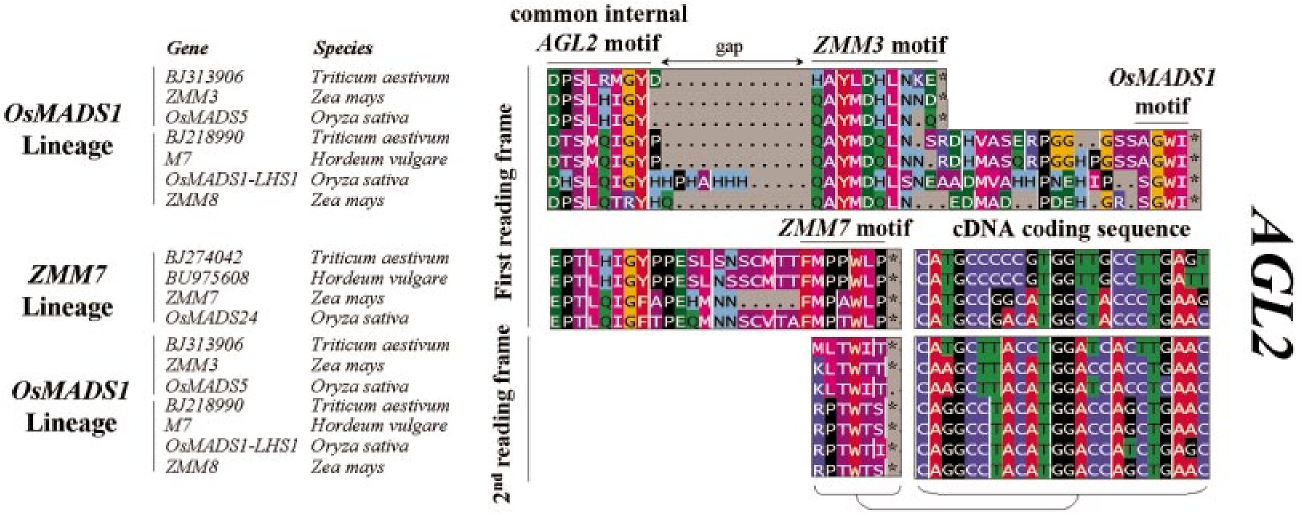

Figure 4. Alignment of C-terminal motifs of monocot *OsMADS1* and *ZMM7* type *AGL2* like subfamily members. In monocot species, we have identified three distinct types of *AGL2* like subfamily members, each displaying different C-terminal motifs (Fig. 2). Here we show part of the C-terminal domain alignment of *OsMADS1* and *ZMM7* type sequences. Both types have an internal motif in common (indicated with common internal *AGL2* motif), while their C-termini have fully diverged at the protein level. Sequences belonging to the *OsMADS1* type can be further subdivided into two classes: a short version terminating with a ZMM3 motif, and a longer version with a C-terminal extension terminating with a short conserved *OsMADS1* motif. As in the previous cases, we found that the cDNA fragments encoding the ZMM3 motif of the *OsMADS1* type align with those encoding the *ZMM7* motif by introducing a gap representing a frameshift mutation in the *OsMADS1* type sequences. The alignment of the cDNA fragments encoding these *ZMM3* and *ZMM7* motifs is shown on the right and the 2nd reading frame translation of the *ZMM3* motif is shown below the *ZMM7* motif.

first of these, designated the *AB003324* type, only the rice cDNA sequence *AB003324* was found in the nucleotide database at the time of analysis; a search in EST databases with this gene revealed highly homologous copies from maize and wheat, indicating that this type of *AGL2*-like gene may be functionally conserved among monocots (Fig. 2). Recently, the maize *ZMM24* and *ZMM31* MADS-box genes have been published (36) that correspond with the identified EST sequences. A second group with a deviating but conserved C-domain, named the *OsMADS1* lineage, contains two homologous types of MADS-box genes, exhibiting a difference in length. Both types contain a *ZMM3* motif; the long variant in addition contains a conserved C-terminal motif named the *OsMADS1* motif. Short and long version sequences have been isolated from both maize and rice, and searching the EST databases with these sequences showed the presence of both types in wheat. This indicates that both forms are conserved among monocots. To investigate the molecular origin of the divergent C-terminus of *AB003324* and *OsMADS1* types, we aligned these sequences with the *ZMM7* type *AGL2*-like genes. For the *AB003324* type, we were unable to find convincing C-terminal homologies with any of the other monocot *AGL2*-like genes. For the *OsMADS1* type genes, the sequences encoding the *ZMM3* motif clearly align with the C-terminus of *ZMM7* type genes by introducing an internal gap causing a downstream frameshift in the coding sequence of the *OsMADS1* type genes compared to the reading frame of the *ZMM7* type (Fig. 4). These results suggest that the C-terminal *ZMM3* motif of the *OsMADS1* type genes has originated after duplication of a monocot *ZMM7* type ancestral gene followed by a small deletion immediately downstream of the common internal motif. The C-terminus of the long versions may have been recruited from a sequence beyond the original stop codon of the *ZMM7* type genes.

DISCUSSION

Towards a model for neo-functionalization by C-terminal motif selection

While basic features such as DNA binding domains and motifs necessary for protein–protein interactions must be rigidly conserved in order to maintain the basic capacity to function as a transcription factor, generation of novel C-terminal motifs may have been of major importance for functional diversification. C-terminal domains may play a key role in determining partner specificity in higher order complex formation, may contain activation domains, or may be subject to post-translational modifications that may influence DNA binding specificity, subcellular localization or the ability to attract interacting partners. Although the exact role of these small peptide motifs residing in the C-domain largely remains to be determined (25), the fact that a number of these motifs has been preserved for hundreds of million of years of evolution (see above) strongly suggests that they may have been instrumental in the functional diversification of the MADS-box gene family. Furthermore, we found a comparable C-terminal domain conservation in the *WUSCHEL*, the *NAM* and the *AP2* transcription factor families. Members of these transcription factor families all possess a highly conserved DNA binding domain while their C-terminus is strongly divergent between different subfamilies. Within subfamilies however, small motifs of variable length occur that are highly conserved even between proteins isolated from distantly related species (unpublished results).

Based on these observations and the results presented here, we propose a model for the functional diversification of duplicated members of transcription factor families (Fig. 5). After duplication of an ancestral gene X, one of the copies (Y) may accumulate mutations in the C-terminus, while retaining

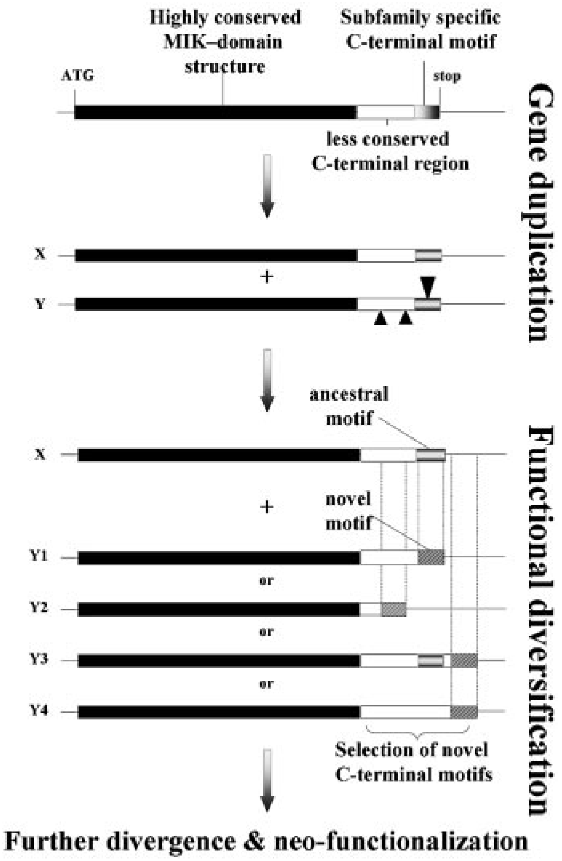

Figure 5. Model for the generation of novel C-terminal motifs within the MADS-box gene family. After duplication of an ancestral gene X, the Y copy accumulates mutations in the C-terminal domain, while retaining the essential MIK domain. Insertions or deletions will cause a frameshift in the coding sequence. Rarely, these frameshift mutations may yield novel functional motifs that consequently will be conserved. In cases where the novel motif is recruited from poorly conserved regions (e.g. Y 2–4) in the ancestral sequence, the sequence relation with the ancestral gene X will become unclear after a period of independent evolution. In the Y copy, new motifs may be added downstream of the ancestral motif as an extra feature, with retention of the ancestral motif which in this case becomes internal (e.g. Y3); or with subsequent loss of the ancestral motif (all other cases).

features such as DNA binding, essential for its function as a transcription factor, in the upstream coding regions. Apart from in frame insertions/deletions and single nucleotide substitutions, mutations in the coding sequence at the 3′ end will also induce frameshifts, as such masking the ancestral origin of the motif at the protein level. While most frameshift mutations will be deleterious for the existing function, in specific cases they may yield novel functional C-terminal motifs. The three cases we have described are perfect examples of such a neo-functionalization process. This widens the emerging view that plant transcription factors evolve mainly by changes in *cis*-regulatory elements that affect their expression pattern (37,38), and that after gene duplication, mainly degeneration and selection of complementary functioning, i.e. sub-functionalization occurs (39,40). At first sight, it may seem extraordinary that in all three cases, frameshift mutations of highly conserved motifs yielded novel highly conserved motifs. However, this specific situation is the only type of motif generation that can still be recognized after millions of years of independent evolution of both copies. If the new motif had been recruited from a sequence in a non-conserved (Y3 and Y4, Fig. 5) or less conserved region of the C-terminus (e.g. Y2), it would be impossible to trace back the ancestral motif. Equally important, either the new or the ancestral motif must contain amino acid residues that are not too highly degenerate in order to be able to recognize the related motif after frameshifting. Thus, the only cases of frameshift mutations that we still can recognize are those in highly conserved motifs that yield novel highly conserved motifs. Finally, novel motifs may be acquired in an additive way downstream of existing motifs as an extra feature, with retention of the ancestral motif that in such a case becomes internal (e.g. Y3); or with subsequent loss of the ancestral motif (all other cases).

Higher order complex formation and importance for flower evolution

We have identified drastic changes in the conserved C-terminal motifs of the core eudicot B-function subfamily (*DEF/AP3*), the *SQUA/AP1* subfamily and the *AGL2* subfamily. These mutations appear to be associated with changes in gene function. The apparent coincidence between the origin of eu*AP1* (A) and eu*AP3* (B) motifs, and the origin of the higher eudicots is remarkable. Higher eudicots show a characteristic canalization of floral development and thus a standardization of floral architecture (41 and references cited therein). Moreover, although petaloid organs may have evolved several times independently during evolution, the higher eudicot petals seem to be homologous organs that trace back to a single origin at the base of higher eudicots (30,31,33,36). Strikingly, higher eudicot petal identity is specified by A+B function genes encoding eu*AP1* and euAP3 motifs, respectively. It is conceivable, therefore, that there is a causal relationship between the parallel frameshift mutations described here and both the canalization of floral structure and the origin of a certain type of petals at the base of higher eudicots.

Recently, it has been demonstrated that B-function MADS-box proteins may form higher order complexes with SQUA in *Anthirrinum* and with SEP3 and AP1, and, alternatively, with SEP3 and AG in *Arabidopsis* (16,18). We speculate therefore, that the frameshift mutations represent an example of co-evolution between different components of a single transcription factor complex and that these mutations may have modulated the function. Clearly, in a next step, complex formation and function of complexes consisting of paleoAP3 and paleoAP1 proteins have to be studied in monocot and basal angiosperm species in comparison to eudicots.

CONCLUSIONS

The data presented here indicate an excitingly rapid mode of protein evolution: novel, highly conserved motifs at the C-terminus may originate by frameshift mutations in the existing coding sequence. This phenomenon may explain a substantial part of the high sequence divergence in the C-terminal region between and within the different MADS-box gene subfamilies. It will be interesting to see how general the mechanism of protein evolution by novel motif selection (whether or not induced by frameshift mutations) at the C-terminus will appear to be. There is evidence, however, that at least some aspects of it apply not only to plants.

The *Ultrabithorax* protein acquired a poly-ala tail in the lineage that led to *Drosophila*, but only after Crustaceans had branched off (42,43). This poly-ala tail is involved in suppressing abdominal leg development. It thus appears that a change in a C-terminal sequence motif of a homeodomain transcription factor can be correlated with a neo-functionalization event affecting the arthropod body plan.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

REFERENCES

1. Theissen,G., Becker,A., Di Rosa,A., Kanno,A., Kim,J.T., Munster,T., Winter,K.U. and Saedler,H. (2000) A short history of MADS-box genes in plants. *Plant Mol. Biol.*, **42**, 115–149.
2. Theissen,G., Kim,J.T. and Saedler,H. (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. *J. Mol. Evol.*, **43**, 484–516.
3. Theissen,G. (2001) Development of floral organ identity: stories from the MADS house. *Curr. Opin. Plant Biol.*, **4**, 75–85.
4. Pelaz,S., Ditta,G.S., Baumann,E., Wisman,E. and Yanofsky,M.F. (2000) B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature*, **405**, 200–203.
5. Liljegren,S.J., Ditta,G.S., Eshed,Y., Savidge,B., Bowman,J.L. and Yanofsky,M.F. (2000) SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature*, **404**, 766–770.
6. Bradley,D., Carpenter,R., Sommer,H., Hartley,N. and Coen,E. (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. *Cell*, **72**, 85–95.
7. Carpenter,R. and Coen,E.S. (1990) Floral homeotic mutations produced by transposon-mutagenesis in Antirrhinum majus. *Genes Dev.*, **4**, 1483–1493.
8. Coen,E.S. (1992) Flower development. *Curr. Opin. Cell Biol.*, **4**, 929–933.
9. Coen,E.S. and Meyerowitz,E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31–37.
10. Jack,T., Brockman,L.L. and Meyerowitz,E.M. (1992) The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell*, **68**, 683–697.
11. Schwarz-Sommer,Z., Hue,I., Huijser,P., Flor,P.J., Hansen,R., Tetens,F., Lonnig,W.E., Saedler,H. and Sommer,H. (1992) Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.*, **11**, 251–263.
12. Schwarz-Sommer,Z., Huijser,P., Nacken,W., Saedler,H. and Sommer,H. (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. *Science*, **250**, 931–936.
13. Yanofsky,M.F., Ma,H., Bowman,J.L., Drews,G.N., Feldmann,K.A. and Meyerowitz,E.M. (1990) The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. *Nature*, **346**, 35–39.
14. Theissen,G. and Saedler,H. (2001) Plant biology. Floral quartets. *Nature*, **409**, 469–471.
15. Theissen,G. (2001) Genetics of identity. *Nature*, **414**, 491.
16. Egea-Cortines,M., Saedler,H. and Sommer,H. (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. *EMBO J.*, **18**, 5370–5379.
17. Jack,T. (2001) Relearning our ABCs: new twists on an old model. *Trends Plant Sci.*, **6**, 310–316.
18. Honma,T. and Goto,K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature*, **409**, 525–529.
19. Ma,H., Yanofsky,M.F. and Meyerowitz,E.M. (1991) AGL1-AGL6, an Arabidopsis gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.*, **5**, 484–495.
20. Munster,T., Pahnke,J., Di Rosa,A., Kim,J.T., Martin,W., Saedler,H. and Theissen,G. (1997) Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. *Proc. Natl Acad. Sci. USA*, **94**, 2415–2420.
21. Shore,P. and Sharrocks,A.D. (1995) The MADS-box family of transcription factors. *Eur. J. Biochem.*, **229**, 1–13.
22. Cho,S., Jang,S., Chae,S., Chung,K.M., Moon,Y.H., An,G. and Jang,S.K. (1999) Analysis of the C-terminal region of Arabidopsis thaliana APETALA1 as a transcription activation domain. *Plant Mol. Biol.*, **40**, 419–429.
23. Moon,Y.H., Jung,J.Y., Kang,H.G. and An,G. (1999) Identification of a rice APETALA3 homologue by yeast two-hybrid screening. *Plant Mol. Biol.*, **40**, 167–177.
24. Lim,J., Moon,Y.H., An,G. and Jang,S.K. (2000) Two rice MADS domain proteins interact with OsMADS1. *Plant Mol. Biol.*, **44**, 513–527.
25. Lamb,R.S. and Irish,V.F. (2003) Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. *Proc. Natl Acad. Sci. USA*, **100**, 6558–6563.
26. Davies,B. and Schwarz-Sommer,Z. (1994) Control of floral organ identity by homeotic MADS-box transcription factors. *Results Probl. Cell Differ.*, **20**, 235–258.
27. Thompson,J.D., Higgins,D.G. and Gibson,T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
28. Van De Peer,Y. and De Wachter,R. (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput. Appl. Biosci.*, **10**, 569–570.
29. Saitou,N. and Nei,M. (1987) The neighbour-joining method: a new method for constructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.
30. Tajima,F. and Nei,M. (1984) Estimation of evolutionary distance between nucleotide sequences. *Mol. Biol. Evol.*, **1**, 269–285.
31. Kramer,E.M., Dorit,R.L. and Irish,V.F. (1998) Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. *Genetics*, **149**, 765–783.
32. Kramer,E.M. and Irish,V.E. (2000) Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *Int. J. Plant Sci.*, **16**, 29–30.
33. Pnueli,L., Abu-Abeid,M., Zamir,D., Nacken,W., Schwarz-Sommer,Z. and Lifschitz,E. (1991) The MADS box gene family in tomato: temporal expression during floral development, conserved secondary structures and homology with homeotic genes from Antirrhinum and Arabidopsis. *Plant J.*, **1**, 255–266.
34. Van Der Krol,A.R., Brunelle,A., Tsuchimoto,S. and Chua,N.H. (1993) Functional analysis of petunia floral homeotic MADS box gene pMADS1. *Genes Dev.*, **7**, 1214–1228.
35. Kramer,E.M., Di Stilio,V.S. and Schluter,P.M. (2003) Complex patterns of gene duplication in the *APETALA3* and *PISTILLATA* lineages of the Ranunculaceae. *Int. J. Plant Sci.*, **164**, 1–11.
36. Munster,T., Deleu,W., Wingen,L.U., Cacharrón,J., Ouzunova,M., Faigl,W., Werth,S., Kim,J.T.T., Saedler,H. and Theissen,G. (2002) Maize MADS-box genes galore. *Maydica*, **47**, 287–301.
37. Doebley,J. and Lukens,L. (1998) Transcriptional regulators and the evolution of plant form. *Plant Cell*, **10**, 1075–1082.
38. Wang,R.L., Stec,A., Hey,J., Lukens,L. and Doebley,J. (1999) The limits of selection during maize domestication. *Nature*, **398**, 236–239.
39. Force,A., Lynch,M., Pickett,F.B., Amores,A., Yan,Y.L. and Postlethwait,J. (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*, **151**, 1531–1545.
40. Prince,V.E. and Pickett,F.B. (2002) Splitting pairs: the diverging fates of duplicated genes. *Nat. Rev. Genet.*, **3**, 827–837.
41. Winter,K.U., Weiser,C., Kaufmann,K., Bohne,A., Kirchner,C., Kanno,A., Saedler,H. and Theissen,G. (2002) Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. *Mol. Biol. Evol.*, **19**, 587–596.
42. Galant,R. and Carroll,S.B. (2002) Evolution of a transcriptional repression domain in an insect Hox protein. *Nature*, **415**, 910–913.
43. Levine,M. (2002) How insects lose their limbs. *Nature*, **415**, 848–849.