The MADS and the Beauty: Genes Involved in the Development of Orchid Flowers

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Abstract: Since the time of Darwin, biologists have studied the origin and evolution of the Orchidaceae, one of the largest families of flowering plants. In the last two decades, the extreme diversity and specialization of floral morphology and the uncoupled rate of morphological and molecular evolution that have been observed in some orchid species have spurred interest in the study of the genes involved in flower development in this plant family. As part of the complex network of regulatory genes driving the formation of flower organs, the MADS-box represents the most studied gene family, both from functional and evolutionary perspectives. Despite the absence of a published genome for orchids, comparative genetic analyses are clarifying the functional role and the evolutionary pattern of the MADS-box genes in orchids. Various evolutionary forces act on the MADS-box genes in orchids, such as diffuse purifying selection and the relaxation of selective constraints, which sometimes reveals a heterogeneous selective pattern of the coding and non-coding regions. The emerging theory regarding the evolution of floral diversity in orchids proposes that the diversification of the orchid perianth was a consequence of duplication events and changes in the regulatory regions of the MADS-box genes, followed by sub- and neo-functionalization. This specific developmental-genetic code is termed the “orchid code.”

Keywords: Flower development, MADS-box genes, Orchidaceae, Orchid code.

THE ORCHIDACEAE: FLOWER MORPHOLOGY, PHYLOGENY AND EVOLUTIONARY ORIGIN

Among the flowering plants, the Orchidaceae family is one of the largest and includes species with greatly diversified and specialized floral morphology. Orchids have successfully spread to almost every habitat, exhibiting a broad assortment of epiphytic and terrestrial adaptations. The causes determining the wide species diversity in orchids have not been fully elucidated. However, relevant roles have been attributed to epiphytism, highly diversified pollination strategies [1], natural selection and genetic drift [2].

Universally known for its beauty and fascinating complexity, the orchid flower is bilaterally symmetrical (zygomorphic) and includes three outer tepals (sepals), two lateral inner tepals (petals) and a highly modified median inner tepal (lip or labellum) (Fig. 1). The gynostemium or column is the orchid’s reproductive structure and consists of fused male (stamen/anther) and female (pistil/stigma) tissues. At the top of the column are the pollinia, packets of mature pollen grains, and at the base of the column is the ovary, which develops when triggered by pollination [3-5]. The outer tepals either can be unlobed and without ornamentations, can resemble the lateral inner tepals, or can form nectar spurs. The lip generally exhibits a distinctive shape and color pattern different from that of the other tepals and can be decorated with calli, spurs and glands. The lip is considered homologous to the adaxial tepal of other monocots and, therefore, should be the uppermost one; however, the 180° rotation in floral orientation that occurs during the development of the orchid flower (resupination) shifts the lip to the lowest tepal position. The orientation of the lip after resupination and its collocation opposite to the fertile anther suggest that its highly diversified shape and pigmentation are the result of adaptations to specific pollinators [6].

Before the recombinant DNA era, the phylogeny of Orchidaceae was based on a relatively small set of morphological characteristics. However, many phenotypic traits, especially the morphology of some floral structures involved in the interactions between orchids and pollinators, are of an adaptive nature and may not, therefore, reflect the family’s actual phylogeny. As a consequence, several contradictory taxonomic and phylogenetic reconstructions of the Orchidaceae have been proposed, which are reviewed in another article [7]. In recent years, data from molecular markers have been progressively added to the morphological ones, expanding and improving the body of research concerning the phylogeny of orchids. The current classification system designates five subfamilies within the Orchidaceae: Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae and Vanilloideae (Fig. 2). Each subfamily includes a large number of tribes and subtribes [8, 9].

In addition to the debate on the phylogeny of orchids, the last decade has witnessed intense disagreement regarding the temporal origin and diversification of this plant family. The origin of the modern orchid lineage has been placed within a wide time period ranging from ~26 to ~110 million years ago (Mya) [10-13]. The recent discovery of a fossil of Proplebeia dominicana, an extinct stingless bee dated 15-20
Fig. (1). Schematic diagram of an orchid flower.

Fig. (2). Time-calibrated phylogenetic relationships of the five sub-families of Orchidaceae, modified from Gustafsson et al. [14]. The numbers below the branches indicate the divergence time, as expressed in millions of years ago (Mya). On the right are the images of orchid species that are representative of each subfamily.

Mya, covered with pollinia from the orchid species *Meliorchis caribea* has enabled researchers to narrow the timeframe of the orchid family’s origin, estimating it at 76-84 Mya in the Late Cretaceous [12]. A more recent study [14], which includes two new orchid fossils assigned to genera *Dendrobium* and *Earina* [15], confirms the ancient origin of the orchids’ most recent common ancestor in the Late Cretaceous (~77 Mya), although the origin of the five orchid subfamilies is dated ~1-8 Mya prior to the previous estimates [14] (Fig. 2). Both calibration analyses [12, 14] were conducted using
molecular phylogenetic reconstructions based on plastid DNA sequences (matK and rbcL), highlighting the relevance of molecular analyses in the study of the origin and evolution of orchids.

Although orchids possess many traits that are “unique” in the plant kingdom, such as highly specialized pollination strategies, diversified flower morphology, peculiar ecological strategies and developmental reproductive biology, molecular studies on this family are scarce when compared with those of other species-rich plant groups [16]. The genome projects of two orchid species, Phalaenopsis aphrodite (Project ID 53151) and P. equestris (Project ID 53913), are under development, although they are not yet available for release. The OrchidBase is a freely available collection of expressed nucleotide sequences that provides integrated information on ESTs from Phalaenopsis orchids (http://lab.fhes.tn.edu.tw/est) [17]. The establishment of such public resources is important, as it can facilitate the experimental design of studies on orchid biology.

In this review, we will examine the molecular mechanisms underlying the development of the flower, which is the most specialized and diversified orchid structure, with a particular emphasis on the role played by the MADS-box genes in the formation and evolution of the floral organs.

THE GENETICS OF FLOWER DEVELOPMENT:
THE MADS-BOX GENES FAMILY

The acronym MADS box is derived from the initials of four loci, MCM1 of Saccharomyces cerevisiae, AG of Arabidopsis thaliana, DEF of Antirrhinum majus and SRF of Homo sapiens, all of which contain the MADS-box domain, a conserved 56-amino-acid DNA-binding domain [18]. The MADS-box family has evolved from a region of the topoisomerase II subunit A [19] and includes genes encoding transcription factors. The MADS-box genes are present in nearly all major eukaryotic groups, although they constitute a large gene family only in land plants. A gene duplication preceding the divergence of plants and animals gave rise to two main groups of MADS-box genes: type I and type II, which are distinguished on the basis of genomic organization, evolutionary rate, developmental function and level of functional redundancy [20]. The type I genes are divided into three groups, Malph, Mbeta and Mgamma [21], and are involved predominantly in development of seed, embryo and female gametophyte [22]. The type II genes share a conserved MIKCC structure and encode proteins bearing the highly conserved DNA-binding MADS domain (M) at the amino terminus, a poorly conserved I domain and a moderately conserved K domain in the central portion, which are important for protein–protein interactions and the formation of coiled-coil structures, and a variable carboxyl-terminal (C) region that may function as a transactivation domain [23, 24]. The type II genes can be further divided into MIKC and MIKC* genes, which are distinguished by their various intron/exon structures in the I domain [25]. Functional studies have suggested a major specialization of the MIKC* genes in the development of the male gametophyte [26], whereas the MIKC* genes, the best-characterized group of MADS-box genes, are often referred to simply as the MIKC genes, are involved in many functions related to plant growth and development and are closely linked to the origin of the floral organs and fruits of angiosperms. The genomic organization of the MIKC* genes is generally consistent, with the presence of seven introns and eight exons [24, 27-31].

The regulatory systems controlling the expression of the MADS-box genes include complex feedback and feed-forward networks, which are often integrated in a complex cascade of events [24, 29, 32]. In addition, more specialized mechanisms, such as regulation by small RNAs [33] and epigenetic control [34], have evolved to control the expression of the MADS-box genes. In the future, more data from genome projects and reverse genetic studies will allow us to understand in greater detail the origin and functional diversification of members of this dynamic family of transcription factors [35].

The spatial and functional activity of the floral homeotic genes is exemplified by the elegant ABCDE model of flower development (Fig. 3A) [36, 37]. This model was initially developed on the basis of mutant analyses of the model species Arabidopsis thaliana, which exhibits a flower consisting of four concentric whorls of floral organs. With the exception of APETALA2 (AP2), all genes involved in the ABCDE model are MADS-box genes belonging to various functional classes. In Arabidopsis, the expression of the class A genes (APETALA1, AP1) controls the sepal development in whorl 1 and, together with the expression of the class B genes (e.g., PISTILLATA, PI, and APETAL3, AP3) in whorl 2, regulates the formation of petals. The expression of the class B genes in whorl 3, together with the expression of the class C genes (e.g., AGAMOUS, AG), mediates stamen development. The expression of the class C genes alone in whorl 4 determines the formation of carpel. The class D genes (e.g., SEEDSTICK, STK and SHATTERPROOF, SHP) specify the identity of the ovule within the carpel, and the class E genes (e.g., SEPALLATA, SEP), expressed in the entire floral meristem, are necessary for the correct formation of all of the floral organs.

The activity of the MADS-box transcription factors requires the formation of homo- and heterodimers that recognize the conserved nucleotide CC(A/T)6GG DNA sequences, which are known as the CARG boxes [23]. After the formation of dimers, MADS-box proteins further interact, leading to the formation of the “floral quartets”, complexes that activate floral organ-specific expression programs [38]. For example, the quartet model predicts that the complexes AP1/AP1/SEP/SEP, AP1/SEP/AP3/PI, AG/SEP/AP3.PI and AG/AG/SEP/SEP are present within whorls 1, 2, 3 and 4, respectively, to induce the formation of floral organs (Fig. 3A) [38, 39].

Although the ABCDE model is generally conserved [40-44], the increasing identification and functional and evolutionary analysis of the MADS-box genes is highlighting relevant differences in the mechanisms leading to flower development in non-model species, such as orchids, often emphasizing instances in which the MADS-box gene’s function could not be extrapolated from structural orthology [45]. Table 1 lists most of the MADS-box genes characterized in orchids, in Fig. (4) their evolutionary relationships are presented, and functions are described in the following sections.
THE ORCHID MADS-BOX GENES OF THE AP1/AGL9 GROUP

The AP1/AGL9 group includes the phylogenetically related MADS-box genes of class A and class E [29, 38], which originated during evolution after several duplication events [46, 47]. Class A genes belong to the AP1/SQUA-like subfamily (from the APETALA1 and SQUAMOSA locus of Arabidopsis thaliana and Antirrhinum majus, respectively), which is further divided into the paleoAP1-like and the euAP1-like clades [48, 49]. The C-terminal region of the AP1/SQUA-like proteins exhibits conserved motifs. The paleoAP1 motif L/MPPWML (also known as the FUL-like motif, from the FRUITFULL locus of A. thaliana) is typical of the paleoAP1-like clade, whereas the euAP1-like clade is characterized by two alternative motifs, RRNaLaLT/NLa (the euAP1 motif) and CFAT/A (the farnesylation motif), the latter evolved from the paleoAP1 motif through a frameshift mutation [48]. The role of the paleoAP1 and farnesylation motifs is not clear, and the absence of both motifs in some AP1/SQUA-like genes does not affect their function [50, 51].

As the AP1/SQUA-like genes are present only in angiosperms, their origin might be related to the emergence of the floral perianth. The euAP1-like clade is typical of the higher eudicots, while the paleoAP1 clade is present in both monocots and dicots [48, 49].

The E-function genes belong to the SEP-like subfamily (from the SEPALLATA locus of A. thaliana), which are divided into SEP3 and SEP1/2/4 clades (previously known as AGL9 and AGL2/3/4 clades, respectively) [29, 46, 47, 52]. A third clade, AGL6, also belongs to the AP1/AGL9 group [53-56]. In addition to their role in determining floral organs, almost all of the members of the AP1/AGL9 group of MADS-box genes are also involved in the floral meristem’s initiation and development [57]. This finding suggests that the genes of the AP1/AGL9 group could function at the top of the regulatory hierarchy of the MADS-box genes involved in flower development [58-60].

In orchids, a number of genes belonging to the AP1/AGL9 group have been identified and functionally char-
Table 1. MADS-Box Genes Identified in Orchids

| Locus    | Species                        | Clade            | Class                 | Genbank Accession Number |
|----------|--------------------------------|------------------|-----------------------|--------------------------|
| DthyrFL1 | Dendrobium thyrsiflorum        | AP1/SQUA -like   | A                     | AY927236                 |
| DthyrFL2 | Dendrobium thyrsiflorum        | AP1/SQUA -like   | A                     | AY927237                 |
| DthyrFL3 | Dendrobium thyrsiflorum        | AP1/SQUA -like   | A                     | AY927238                 |
| DOMADS2  | Dendrobium Madame Thong-In     | AP1/SQUA-like    | A                     | AF198175                 |
| OMADS10  | Oncidium Gower Ramsey          | AP1/SQUA-like    | A                     | HM140846                 |
| ORAP11   | Phalaenopsis amabilis          | AP1/SQUA-like    | A                     | DQ104328                 |
| ORAP13   | Phalaenopsis amabilis          | AP1/SQUA-like    | A                     | DQ104327                 |
| MADS1    | Cymbidium hybrid cultivar.     | AP3/DEF-like     | B                     | DQ683575                 |
| DoOAP3A  | Dendrobium crumenatum          | AP3/DEF-like     | B                     | DQ119838                 |
| DoOAP3B  | Dendrobium crumenatum          | AP3/DEF-like     | B                     | DQ119839                 |
| DMAMDS4  | Dendrobium moniliforme         | AP3/DEF-like     | B                     | GU132995                 |
| GogalDEF1| Gongora galeata                | AP3/DEF-like     | B                     | FJ804097                 |
| GogalDEF2| Gongora galeata                | AP3/DEF-like     | B                     | FJ804098                 |
| GogalDEF3| Gongora galeata                | AP3/DEF-like     | B                     | FJ804099                 |
| HrDEF    | Habenaria radiata              | AP3/DEF-like     | B                     | AB232663                 |
| OMADS3   | Oncidium Gower Ramsey          | AP3/DEF-like     | B                     | AY196350                 |
| OMADS5   | Oncidium Gower Ramsey          | AP3/DEF-like     | B                     | HM140840                 |
| OMADS9   | Oncidium Gower Ramsey          | AP3/DEF-like     | B                     | HM140841                 |
| PeMADS2  | Phalaenopsis equestris         | AP3/DEF-like     | B                     | AY378149                 |
| PeMADS3  | Phalaenopsis equestris         | AP3/DEF-like     | B                     | AY378150                 |
| PeMADS4  | Phalaenopsis equestris         | AP3/DEF-like     | B                     | AY378147                 |
| PeMADS5  | Phalaenopsis equestris         | AP3/DEF-like     | B                     | AY378148                 |
| PhlonDEF2| Phragmipedium longiflorum      | AP3/DEF-like     | B                     | FJ804106                 |
| PhlonDEF1| Phragmipedium longiflorum      | AP3/DEF-like     | B                     | FJ804105                 |
| PhlonDEF3| Phragmipedium longiflorum      | AP3/DEF-like     | B                     | FJ804107                 |
| PhlonDEF4| Phragmipedium longiflorum      | AP3/DEF-like     | B                     | FJ804108                 |
| SpodoDEF2| Spiranthes odorata             | AP3/DEF-like     | B                     | FJ804111                 |
| SpodoDEF1| Spiranthes odorata             | AP3/DEF-like     | B                     | FJ804110                 |
| SpodoDEF3| Spiranthes odorata             | AP3/DEF-like     | B                     | FJ804112                 |
| VaplaDEF1| Vanilla planifolia             | AP3/DEF-like     | B                     | FJ804115                 |
| VaplaDEF3| Vanilla planifolia             | AP3/DEF-like     | B                     | FJ804117                 |
| VaplaDEF2| Vanilla planifolia             | AP3/DEF-like     | B                     | FJ804116                 |
| EpalPI   | Epipactis palustris            | PI/GLO-like      | B                     | DQ005588                 |
| GogoGLO1 | Gongora galeata                | PI/GLO-like      | B                     | FJ804100                 |
| HrGLO1   | Habenaria radiata              | PI/GLO-like      | B                     | AB232665                 |
| HrGLO2   | Habenaria radiata              | PI/GLO-like      | B                     | AB232664                 |
| OMADS8   | Oncidium Gower Ramsey          | PI/GLO-like      | B                     | HM140842                 |
acterized (Table 1, Fig. 4). The identification of genes that function early during floral transition is the first step toward the elucidation of the molecular mechanisms of floral transition in orchids.

In the orchid Dendrobium Madame Thong-In, the MADS-box genes DOMADS1, DOMADS2 and DOMADS3 are homologous to SEP1, AP1/SQUA and SEP3, respectively. These genes are successively activated during the floral transition and continue to be expressed later in mature flowers [58]. Their expression pattern is quite different when compared with the transcriptional profile of the homologous genes of Arabidopsis, revealing an absence of functional conservation in MADS-box genes functioning during floral transition in flowering plants.

DOMADS1, DOMADS2 and DOMADS3, in accordance with almost all of the MADS-box genes involved in the regulation of floral transition, also function in the later stages of flower development [61-64]. DOMADS1 transcripts are present in the inflorescence meristem, in the floral primordium and in all of the floral organs. The same expression pattern in floral organs is shared by the DOMADS1 ortholog DcOSEP1 of Dendrobium crumenatum [65]. DOMADS2 is expressed early in the apical meristem of the shoot and throughout the process of floral transition; later, its expression is restricted to the column. The transcription of DOMADS3 is detectable before the differentiation of the flower primordium, and its expression in floral organs is only detectable in the pedicel tissue.

Compared with the activities in the floral transition of the Arabidopsis orthologs AP1, AGL8 and CAULIFLOWER (CAL) [61, 66, 67], followed by the activation of SEP1, SEP4 and SEP3 in stage 2 of the flower primordium [68-70], DOMADS2 and DOMADS3 are activated much earlier. Differences are also observed in the spatial expression pattern, as the transcripts of DOMADS1 and DOMADS2 accumulate in both the inflorescence and the floral meristem, whereas in Arabidopsis, the expression of AGL8 is restricted to the former region and that of AP1 and CAL is confined to the latter region. These differences indicate the evolution of specific regulatory systems controlling the activity of MADS-box genes involved in floral transition in various plant families.

The promoter region of the DOMADS1 gene contains multiple cis-acting elements that regulate the expression of DOMADS1 in the orchid’s reproductive organs and, at low levels, in the stem [71]. This promoter contains six CArG-box sequences, which are the binding sites of diverse MADS-box genes and are crucial modulators of their expression [72-75]. The presence of the CArG-boxes within the
**DOMADS1** promoter, as well as in the promoters of MADS-box genes of distantly related species (e.g., *Arabidopsis*), implies that the basic mechanism of regulation of the MADS-box genes through binding to the CArG-box sequences are conserved during the flowering process. In addition, within the promoter of the **DOMADS1** gene, there are five DNA-binding sites of the class 1 *knox* gene **DOHI** [71], which is a negative regulator of the expression of **DOMADS1** during floral transition that may directly interact with its binding sites to mediate the regulation of **DOMADS1** expression [76].

In addition to **DOMADS1-3**, three other MADS-box genes belonging to the **AP1/AGL9** group have been characterized in *Dendrobium*. The DthyFL1-3 genes of *D. thyrsiflorum* are paleo**AP1-like** genes within the **AP1/SQUA-like** subfamily, which evolved from a single ancestor common to all monocots [51]. Similarly to the events driving the evolution of several MADS-box gene lineages [48, 77-79], a frameshift mutation is considered responsible for the absence within the **DthyFL3** locus of the paleo**AP1-like** motif present in both **DthyFL1** and **DthyFL2**. All three of the genes are transcribed at low levels in vegetative root and leaf tis-
sues, at higher levels in ovules and at much higher levels in inflorescences, with increasing transcription levels of DihyrF1L1 and DihyrF1L2 observed from small to large floral buds [51]. This expression pattern may indicate that the genes are involved in different mechanisms controlling the development of orchid inflorescence.

In the orchid Phalaenopsis amabilis, the genes ORAP11 and ORAP13 belong to the AP1/SQUA-like subfamily, exhibit the typical paleo-AP1-like motif and lack the farnesylat-

ion motif [80]. Both genes possess a role in the establish-
ment of meristem identity, with initial expression in the in-
florescence and floral meristems that is similar to the early functions of FUL in A. thaliana [81] and OsMADS18 in rice [82]. Later expression of both ORAP11 and ORAP13 in the primordia of all floral organs is consistent with the transcriptional profile of the genes OsMADS18 [82] and LtMADS1 of the monocots Oryza sativa and Lolium temulentum [83], respectively, but not with that of the AP1 and FUL genes of Arabidopsis [66, 84]. Subsequently, both ORAP genes participate to the development of petals, lips, columns and ovules, with the last role also described for DihyrF1L1-3 in D. thyrsiflorum [51] and PFG in petunia [85]. The presence of ORAP11 transcripts in the columns of mature flowers is con-
sistent with the expression pattern of the DOMADS2 gene in Dendrobium Madame Thong-In [58]. ORAP genes are also expressed in vegetative tissues, such as the root and procambial strand region, thereby resembling the FUL, PFG, Os-
MADS18 and LtMADS1 genes more than the AP1, SQUA or PEAM4, the AP1 homolog of pea [83-89]. The expression profile of the ORAP genes suggests that the orchid AP1/SQUA-like genes have retained an ancestral role in the determination of meristem identity, but they have functions that are quite different from those of the “classic” class A genes.

In the orchid Oncidium Gower Ramsey, the OMADS1 gene belongs to the AP1/AGL9 group; in particular, it be-
longs to the AGL6 clade [90]. OMADS1 is transcribed early in the apical meristem of the orchid, and its role in regulating floral initiation is functionally similar to that of other mem-
ers of the AP1/AGL9 group, such as AP1 and SEP3 [50, 91-
93]. The OMADS1 protein is able to form heterodimers with OMADS3, a class B orchid MADS-box protein also in-
volved in the process of floral initiation [94]. However, the expression pattern of OMADS1 in the mature flower, which is restricted to the lip and carpel, does not overlap with that of its orthologs AGL6 of Arabidopsis and ZAG3 of Zea mays, which are expressed in all four flower organs and ovules [53, 54]. The heterodimerization activity of OMADS1 is also achieved with OMADS2, an Oncidium class D MADS-box protein that is expressed in the stigmatic cavity and ovary [95]. OMADS1 may represent a class of MADS-box genes with a function similar to that of the car-
pel-specific MADS-box genes in regulating floral initiation and ovary development in orchids.

In Oncidium, four additional AP1/AGL9-like genes, OMADS6, OMADS7, OMADS10 and OMADS11, have been characterized [96]. Specifically, OMADS6 is a SEP3 ortholog, OMADS11 is closely related to the SEP1/2 orthologs and OMADS7 is closely related to AGL6-like genes within the E-function genes; furthermore, OMADS10 is a paleoAP1 ortholog of orchid. OMADS6, OMADS7 and OMADS11 exhibit a similar expression pattern, whereas OMADS10 has a completely different profile. In contrast with the expression profile of the SEP3 gene and many of its orthologs, which are transcribed only in the three inner whorls of the flower [27, 34, 47, 69, 97-100], the expression of OMADS6 is observed in all four floral whorls, exhibiting relatively low levels in stamens. This pattern is similar to that of the SEP1/2 genes [27, 70, 100] and of LMADS3, a Lilium SEP3 ortholog [101], and could be explained by the significant morphological similarities between sepals and petals (tepals) in orchids and lilies. The expression of OMADS11, which is absent in the stamens, resembles that of OMADS6. The expression pattern of OMADS7 overlaps with that of OMADS6 and is similar to that of AGL6 of A. thaliana and ZAG3 of maize [54]. However, the expression profile of OMADS7 is divergent from that of OMADS1, which exhibits an expression pattern restricted to the lip and carpel [90]. Although not identical, the similar expression patterns of the OMADS6, OMADS7 and OMADS11 genes suggest a possible evolutionary conservation of their tran-
scriptional regulation.

Even though most genes of the AP1/SQUA-like subfamily are generally expressed in the early floral meristem and in floral organs and are absent in vegetative tissues [58, 66, 67, 86, 102, 103], some AP1/SQUA-like genes in monocots are also expressed in leaves [82, 104, 105]. OMADS10 is only expressed in the leaves, lips and carpels, and this expression pattern indicates a possible functional conservation for spe-
cific lineages of AP1/SQUA-like genes in monocots.

THE ORCHID CLASS C AND D MADS-BOX GENES

Within the ABCDE model of flower development, the class C genes regulate the development of carpels and, to-
gether with the class B genes, of stamens. The class D genes are primarily involved in the development of ovules. The class C and D genes are sister clades, which appeared after an early duplication event during angiosperm evolution [78]. Two motifs at the C-terminus, the AG motifs I and II, are common to all of the class C and D gene products [78].

In orchids, the number of characterized genes belonging to the C and D classes is smaller than those of the other classes (Table 1, Fig. 4).

In Dendrobium crumenatum, the DcOAG1 and DcOAG2 genes belong to the class C and class D MADS-box genes, respectively [65]. DcOAG1 is an ortholog of AG of A. thaliana, presents an N-terminal extension preceding the MADS domain, which is typical of the class C genes, and its genomic sequence contains the intron 8, which is common in several AG-like genes of class C and was possibly lost in the class D lineage after the divergence of Nymphaeales from the other angiosperms [78, 106]. DcOAG2 is a SEEDSTICK (STK) homolog and is specifically expressed in the ovary. The expression of DcOAG1 is detectable in all of the floral organs and, in accordance with the expression of the AG orthologs observed in some basal angiosperms, is not con-
fined to the reproductive organs [43]. This common expres-

sion pattern shared between the AG orthologs of orchids and basal angiosperms indicates that the regulatory mechanisms involved in the expression of these class C genes may have evolved independently.
In *Dendrobium thyrsiflorum*, DthyrAG1 is a class C gene and DthyrAG2 belongs to class D [107]. Both genes encode the conserved AG motifs at the C-terminus of the protein, and DthyrAG2 encodes an extension of the AG motif, the MD motif YET/AAK/DDXX, which is typical of the monocot D lineage genes and may be involved in determining their interaction with specific protein partners [78]. The DthyrAG1 gene presents an intron 8 located before the stop codon. Both DthyrAG1 and DthyrAG2 are expressed during ovule and flower development, specifically in the rostellum, stigma and stylar canal. In monocots, class D orthologs are generally expressed in ovules [108, 109], and the dicots exhibit a similar expression pattern; however, some exceptions have been reported, such as the *LMADS2* gene of *Lilium longiflorum*, which is expressed in the stylum [110], and the *ZmZAG2* of *Zea mays*, which is expressed in the stigma [111]. The differences in expression patterns between the class D lineage genes in monocots and dicots, together with the presence in monocots of the extension of the AG motif, could be related to the acquisition of a novel function for class D genes within monocots. Both the DthyrAG1 and DthyrAG2 genes are also expressed during ovule development, in agreement with the genes of class C and D in other species [27, 103, 108, 112, 113]. However, DthyrAG1 is only transcribed early, whereas DthyrAG2 is expressed throughout the process of ovule development, suggesting a prominent role for DthyrAG2 in late ovule development [107].

In *Phalaenopsis*, the products encoded by the genes PhalAG1 and PhalAG2 contain the AG I and II motifs in their C-terminal regions, and PhalAG2 also exhibits the MD motif [114]. PhalAG1 and PhalAG2 belong to the class C and D MADS-box genes, respectively. Both are genes are expressed in all floral organs at the earliest stage of floral development and, later, in the lip and column. Although these genes belong to different classes of AG-like genes, their similar expression patterns strongly suggest a sub-functionalization of the two genes. In contrast to the AG-like genes of the other monocots, which are generally involved in stamen and carpel development and are not expressed in whorls 1 and 2, PhalAG1 and PhalAG2 are also involved in the lip formation [114].

In *Oncidium* Gower Ramsey, the genes OMADS4 and OMADS2 belong to classes C and D, respectively [95]. Both of their encoded proteins present the AG motifs I and II, and OMADS2 also contains the conserved MD motif, which is specific to class D proteins of monocots. OMADS4 is specifically expressed only in stamens and carpels, thus resembling the expression patterns of other class C genes [111, 115, 116]. OMADS2 is only expressed in carpels, in accordance with other class D genes [117, 118]. Despite the sequence similarity, the expression patterns of OMADS4 and OMADS2 are quite divergent when compared with those of PhalAG1 and PhalAG2 and may reflect a functional evolutionary divergence of the class C/D genes in *Oncidium* and *Phalaenopsis*, with a more redundant role in the latter species than in *Oncidium* [95].

Although only one class C gene has been identified in *Dendrobium thyrsiflorum* (DthyrAG1), *D. crumenatum* (DcOAG1), *Oncidium* (OMADS4) and *Phalaenopsis* (PhalAG1), a duplication event generated two class C MADS-box genes in the orchid *Cymbidium ensifolium* (*CeMADS1* and *CeMADS2*), both of which are involved in regulating the development of the gynostemium [87]. Despite their redundant function in the meristem tissue, these two paralogs exhibit temporal and spatial differences in their expression pattern in floral organs, leading to the hypothesis of sub- and neo-functionalization during the evolution of the *CeMADS1* and *CeMADS2* genes [87]. According to the floral quartet model, the function of *CeMADS1* and *CeMADS2* genes in column development is enabled through the formation of the tetrameric protein complexes *CeMADS1-CeMADS1-class E-class E* and/or *CeMADS1-CeMADS2-class E-class E*. *CeMADS1* has a pivotal role in stamen and carpel development; in fact, the *Cymbidium* naturally occurring mutant *multitepal*, in which the column is subtended by tepals, continues to express *CeMADS2* but not *CeMADS1*. The transcription of *CeMADS1* enhances the formation of the column, followed by the expression of *CeMADS2* to complete development correctly. The function of *CeMADS2* is primarily maintenance, rather than initiation, and its expression alone is not sufficient to mediate the formation of the column [87].

**THE ORCHID CLASS B MADS-BOX GENES**

Based on the ABCDE model, the class B MADS-box genes are necessary for the correct development of petals and stamens and include two major lineages, the *AP3/DEF*-like genes (from the *APETALA3* and *DEFICIENS* loci of *A. thaliana* and *A. majus*, respectively) and the *PI/GLO*-like genes (from the *PISTILLATA* and *GLOBOSA* loci of *A. thaliana* and *A. majus*, respectively), which appeared after a duplication of an ancestral gene containing a paleoAP3 motif [77, 119]. The *AP3/DEF*-like genes include the paleoAP3 clade and two further clades, TM6 and euAP3, which originated after a second duplication event [77].

The class B genes characterized in orchids are the most numerous and thoroughly studied compared with those of the other classes (Table 1, Fig. 4). A feature common to a high number of the class B MADS-box orchid genes is the expansion of their expression profile into the first whorl of floral organs that may be responsible for the development of petaloid sepals in orchids (Fig. 3B).

In *Phalaenopsis equestris*, the four class B genes, PeMADS2-5, are *AP3/DEF*-like paralogs that are expressed during developmental stages ranging from early to late inflorescence [120]. Their organ-specific expression pattern demonstrates an absence of functional redundancy. In fact, *PeMADS2* is strongly expressed in the outer and inner tepals and, at lower levels, in the column; *PeMADS3* is strongly expressed in the inner tepals and lips and, to a lesser extent in the column; *PeMADS4* is expressed only in the lips and the column; *PeMADS5* is expressed in the outer and inner tepals, lips and the column. The expression pattern of these *AP3/DEF*-like genes in the naturally occurring *Phalaenopsis* peloric mutant reveals that *PeMADS2*, *PeMADS4* and *PeMADS5* is involved in specifying the development of the outer tepals, lip and inner tepals, respectively. In addition, *PeMADS4* is also involved in column development, and *PeMADS5* is important for the initiation of stamens [120].
In contrast to its complement of four AP3/DEF-like genes, the genome of _P. equestris_ contains only one _PI/GLO_-like gene, _PeMADS6_ [121]. The expression of _PeMADS6_ in the inflorescence meristem and floral primordium highlights its role in initiating floral development. The expression pattern of _PeMADS6_ in the outer and inner tepals, lip, column and ovary demonstrates its involvement in the development of these floral organs. Furthermore, the persistence of _PeMADS6_ transcripts in the flower until senescence might correlate the activity of this gene to the flower longevity of orchids [121]. The _PeMADS2-5_ proteins can interact with _PeMADS6_ to mediate the development of specific organs [122]. In addition, _PeMADS4_ and _PeMADS6_ can form homodimers, and both the _PeMADS4_ homodimer and the _PeMADS6_ homodimer/homomultimer can bind the CArG boxes, which are the MADS-box protein-binding motif. Also, the heterodimers _PeMADS2–PeMADS6_, _PeMADS4–PeMADS6_ and _PeMADS5–PeMADS6_ are able to bind the CArG boxes, indicating that, in orchids, the _AP3/DEF_-like clades and _PI/GLO_-like genes interact in different combinations and revealing the notable complexity of their regulatory functions [122].

In _Dendrobium crumenatum_, _DcOPI_ is a class B gene belonging to the _PI/GLO_-like lineage, whereas _DcOAP3A_ and _DcOAP3B_ belong to the paleo_AP3_ lineage of the _AP3/DEF_-like genes [65]. Both _DcOPI_ and _DcOAP3A_ are expressed in all whorls of the floral organs. _DcOAP3B_ is expressed in inner tepals and lip, in pollinia and in the column. These three genes are also expressed in the ovary [65].

In _Habenaria radiata_, three class B MADS-box genes have been identified: _HrGLO1_ and _HrGLO2_, which are two _PI/GLO_-like genes, and _HrDEF_, which is an _AP3/DEF_-like gene [123]. _HrGLO1_ and _HrGLO2_ are expressed in the outer and inner tepals and in the column, whereas _HrDEF_ is expressed only in the inner tepals and the column [123].

In _Oncidium_ Gower Ramsey, _OMADS3_, _OMADS5_ and _OMADS9_ are class B MADS-box genes belonging to the _AP3/DEF_-like lineage, whereas _OMADS8_ is a _PI/GLO_-like gene [94, 124]. _OMADS8_ is expressed in all of the floral organs and leaves. _OMADS3_ is expressed in all four flower organs and in leaves, exhibiting an expression pattern similar to that of a number of the _AP3/DEF_-like genes of the _TM6_ clade [125]. _OMADS5_ is only expressed in the outer and inner tepals, in accordance with _PeMADS2_ of _Phalaenopsis_ [120]. _OMADS9_ is transcribed in the inner tepals and lip, in agreement with the expression patterns of _DcOAP3B_, _PeMADS3_ and _HrDEF_ [65, 120, 123]. Both _OMADS5_ and _OMADS9_ are not expressed in stamens and leaves, and their expression profiles are different from that of _OMADS3_, which is expressed in all flower organs and leaves [94], indicating a functional diversification of _OMADS5_, _OMADS9_ and _OMADS3_ [124]. _OMADS5_ can form homodimers and heterodimers with _OMADS3_ and _OMADS9_, whereas _OMADS8_ forms heterodimers only with _OMADS3_, while _OMADS3_ can form homodimers and heterodimers with _OMADS8_. _OMADS9_ can form homodimers and heterodimers with _OMADS3_ and _OMADS5_ [124].

In _Orcis italic_, _OrcPI_ is a class B _PI/GLO_-like gene [126]. _OrcPI_ transcripts are detectable in all floral organs, and the maintenance of _OrcPI_ transcripts in the flower through anthesis to senescence confirms the relationship between the _PI/GLO_-like genes and the long-persisting flower longevity of orchids, as also described in _Phalaenopsis_ [127]. The high number of MADS-box gene sequences publicly available has enabled comparative evolutionary studies to determine the selective constraints acting on coding and/or non-coding regions and the eventual traces of adaptive, purifying and neutral selection. Different evolutionary constraints act on the coding and non-coding regions of _OrcPI_, suggesting a heterogeneous selective pattern of the _OrcPI_ locus [126, 127]. Phylogenetic footprinting analysis detected conserved regions within the _5'_ regulatory sequence of _OrcPI_ and the homologous regions of _Oryza sativa_, _Lilium regale_ and _Arabidopsis thaliana_, confirming the wide conservation of regulatory signals required during flower development [128]. A paralog copy of _OrcPI_, _OrcPI2_, has been recently identified in _O. italic_ and other members of the Orchidoideae subfamily. The two _PI/GLO_-like genes exhibit different selective pressures, particularly on the synonymous sites, and seem to have experienced sub-functionalization [129]. In _O. italic_, four _AP3/DEF_-like genes are also present (Aceto et al., unpublished data).

Recently, the evolutionary analysis of a number of class B genes from the major subfamilies of Orchidaceae indicated the presence of four distinct clades (from 1 to 4) of _AP3/DEF_-like orthologs, while the _PI/GLO_-like genes seem to form a single ancient clade with recent paralogs present only in the Orchidoideae subfamily [123, 129, 130]. Within the four _AP3/DEF_-like clades, the genes belonging to clade 2 exhibit relaxation of purifying selection when compared with the other orchid _AP3/DEF_-like clades and with the _PI/GLO_-like genes. In Orchidaceae, gene duplication followed by sub- and neo-functionalization, particularly within the class _B_ _AP3/DEF_-like genes, seems to have played a crucial role in the morphological evolution that resulted in the extreme specialization of the floral perianth [130].

**THE ORCHID CODE**

In contrast to _Arabidopsis_ and the other eudicots, which exhibit sepals (whorl 1) and petals (whorl 2) with clearly different morphologies, the flowers of orchids and a number of other monocots have phenotypically similar organs (tepals) in the outer whorls 1 and 2. The modification of the ABCDE model attributes this difference to the extension of the expression of the class B genes into the whorl 1, in addition to the expression in whorls 2 and 3 [131, 132] (Fig. 3B). However, orchid tepals are distinguished in the outer and inner tepals and, among the latter, the lip has a highly diversified morphology, which is a feature that cannot be satisfactorily explained by the modified ABCDE model.

A decade of molecular studies on the orchid MADS-box genes has strongly enhanced understanding of the mechanisms underlying flower development in this plant family. However, questions remain regarding the evolution and diversification of flower morphology in orchids. Can all of the data obtained from various orchid species be integrated into an evolutionary model to explain the uniqueness of the orchid flower? The recent theory known as “the orchid code” proposes an elegant model describing the development and evolution of the orchid perianth [6, 133, 134].
The orchid code theory illustrates a developmental-genetic code that attributes to the class B AP3/DEF-like genes a pivotal role in tepal and lip identity and leaves unchanged the function of the class B PI/GLO-like genes and the functions of the A, C, D and E class genes with respect to the modified ABCDE model.

In contrast to eudicot model species, such as Arabidopsis, in which the identity of petals is realized through the interaction of one AP3/DEF-like and one PI/GLO-like gene product, the orchid code theory suggests that the identity of orchid tepals and lips is determined by the interactions of the products of four paralogous AP3/DEF-like genes belonging to four different clades with the product of one PI/GLO-like gene. The orchid AP3/DEF-like genes are grouped into four well-defined clades: clade 1 (PeMADS2-like) is sister to clade 2 (OMADS3-like), while clade 3 (PeMADS3-like) is sister to clade 4 (PeMADS4-like). Each clade is characterized by a specific expression pattern [133, 134].

Under the assumptions of the orchid code theory, the interactions of the clade 1 and clade 2 gene products mediates the development of the outer tepals (whorl 1). The formation of the two lateral inner tepals (whorl 2) is specified by the interaction of high levels of the clade 1 and 2 and low levels of the clade 3 and 4 gene products, whereas the development of the lip, which is a highly modified inner tepal, is determined by the expression of high levels of the clade 3 and 4 gene products, in addition to low levels those of clades 1 and 3 (Fig. 5). Thus, the expression of clade 3 genes differentiates between the inner and outer tepals, whereas the expression of clade 4 genes distinguishes between the two lateral inner tepals and the lip [6, 133].

This proposed scheme can also explain the evolution of the zygomorphic orchid flower, starting from an actinomorphic flower composed of six nearly identical tepals in which the ancestor of the current AP3/DEF-like genes was equally transcribed. The duplication and evolution of different cis-regulatory elements played a fundamental role in the functional diversification of the four AP3/DEF-like orchid clades. An initial duplication event produced the ancestor of the clade 1 and clade 2 genes and the ancestor of the clade 3 and clade 4 genes. At this stage, the evolution of a more specialized expression of the ancestor of the clade 3 and 4 genes, which was excluded from the outer tepals, might have established an intermediate flower structure, with distinctive outer and inner tepals (Fig. 5). After a second duplication round, clade 3 and clade 4 genes differentiated, and the modularization of their expression led to the evolution of the lip [133, 134].

CONCLUSIONS

The ancient scientific interest in the “mystery” of the orchid flower has greatly expanded since the advent of the EVO/DEVO molecular approach. Certainly, the future of studies on the flower development genes in orchids will...
greatly benefit from the completion of the genome projects currently in progress and from new and challenging transcriptomic projects, such as the analysis of microRNAs. The continuous and increasing characterization of genes involved in flower development in orchids has clarified many functional and evolutionary aspects of orchid development. The complexity of the expression pattern of the class B MADS-box genes of the Orchidaceae has successfully been simplified and integrated in the specific developmental-genetic code of the orchid perianth. Even if the evolutionary role of the recently discovered paralogs of the PI/GLF0-like genes still remain to be clarified. A more extensive and detailed analysis of the orchid MADS-box genes belonging to classes A, C, D and E will allow the proposal of a more exhaustive model that could also explain the evolution and diversification of the orchid’s reproductive structures. In this context and in the presence of a growing number of characterized loci, it is particularly important to establish a gene nomenclature system that is less ambiguous than the existing one to identify clearly homolog and paralog genes. 

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