

EVOLUTION AND CONTRIBUTION OF MADS-BOX GENES IN RELATION TO FLORAL DIVERSITY IN ORCHIDS

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Abstract

Orchid flowers with myriad shapes, sizes and colors have always fascinated the scientists, growers, entrepreneurs, and common men. This peculiar floral organ identity control is attributed to MADS-box genes which form the foundation of the much accepted ABCDE model of orchid floral organ identity specification. In angiospermic ABC model, the transcriptional factors encoded by Class-B floral homeotic genes, encode petal and stamen identity of flowers. These have two ancient clades: *DEF* (*DEFICIENS*)-like and *GLO* (*GLOBOSA*)-like genes with one copy each. However, in orchids four copies of *DEF*-like floral homeotic genes are present as a result of two rounds of duplication, and subsequent sub-functionalisation followed by neo-functionalisation (ancient gene Clade 1, 2, 3 and 4). A combinatorial differential expression of genes belonging to these clades is responsible for formation of specialised labellum (inner median perianth) and petaloid whorls (outer tepals and lateral inner tepals). Multiple studies have time and again refined and given inputs to resolve the complexities in orchid floral morphogenetic networks. Orchid Code remains a tested, validated and till date, widely accepted model that determines orchid perianth organ identity and puts light on evolutionary track of lip-development in orchids. However, still much needs to be learnt in the coming years to trace the underlying molecular and genetic mechanisms controlling the floral development in orchids.

Introduction

ORCHIDS HAVE always been tremendously fascinating, to some extent mysterious, to scientists, growers, entrepreneurs, and common men. These were once referred to as *gorgeous floral parasites* blazing on tree tops (Fitz-Gerald, 1906). Orchidaceae is one of the largest angiosperm family in terms of number of accepted species which counts more than 25,000, distributed in around 880 genera (Cameron *et al.*, 1999; Chase *et al.*, 2003; Gorniak *et al.*, 2010; Hsu *et al.*, 2015; Mondragón-Palomino and Theißen, 2011; Roberts and Dixon, 2008), representing approximately 10 per cent of angiosperms. Owing to tremendous diversity, distribution and specialised set of floral traits, that include zygomorphy, presence of perianth organs *i.e.*, three outer tepals (petal-like, ornamental, brightly coloured sepals to attract insects), two inner lateral tepals (petals) and one highly modified inner median tepal (labellum, main attracting organ) (Gravendeel and Dirks-Mulder, 2015), this plant family has managed to capture the imagination of people worldwide, for hundreds of years (Albert and Carretero-Paulet, 2015; Rudall and Bateman, 2002).

Owing to multiple architectural innovations in flowers, orchids are often considered to be epitome of plant evolution. In a typical orchid flower, stamens and pistil are partly/completely fused to form gynostegium, stamens are on abaxial side, pollen grains are coherently present in masses as pollinia, labellum is present opposite to fertile stamens, flowers are resupinate, and

thousands of small seeds are produced per ovary (Roberts and Dixon, 2008). These features provide special advantage to these fascinating flowers to form unusual relationships with pollinators and achieve reproductive assurance. Extreme novelties/synapomorphies have been associated with orchid floral forms, some of which include bilateral symmetric flowers due to enormous modifications in perianth, formation of gynostegium by fusion of pistil and stamens, and resupination of pedicel (Rudall and Bateman, 2002). The existence and appearance of labellum is thought to be a novel device to attract pollinators. In addition, frequent variations in colour, texture, ridges, outgrowths, blotches, wax and nectar glands, fragrance and position of stamens and stigma are leading to newer and specialised pollinator interactions by constant coupling and de-coupling processes within pollination networks (Madan *et al.*, 2013). Due to massive investment in floral display, orchids have been successful in attracting pollinators from diverse guild, including beetles, butterflies, ants, wasps, moths, bees, flies, geckos as well as birds. For sustenance in these networks and for ensuring effective pollination, diverse ecological adaptations have been adopted by orchids that include food and sexual deceit, mutualism, exclusive pollination guilds, and association with non-rewarding magnet species (Roberts and Dixon, 2008).

Orchid Evolution

After the dominance of angiosperms on earth, Late Cretaceous (76-84 mya, Million years ago) marks the evolution of orchids and their radiation occurred 33-

57 mya which was in congruence with that of the insects (Ramirez *et al.*, 2007), rendering these as one of the most advanced of the families among angiosperms, consisting of 5 sub-families i.e., Apostasioideae (most basal), Vanillioideae, Cyripedioideae, Orchidoideae and Epidendroideae. Interestingly, it is an amazingly remarkable fact that no orchid can persist without its associate pollinators and mycorrhizal fungi. Vivid and diverse mechanisms of mimicking and temporarily trapping pollinators, epiphytism, succulent and leafless body-plan (Albert and Carretero-Paulet, 2015) are some highly sophisticated floral organizational features that open the doors to discovery of newer morphogenetic networks because of exceptionally high speciation rates in orchids (Gill, 1989). Unfortunately, the unusual set of interspecific interactions with mycorrhizal fungi as well as pollinators, exceptionally unconventional nuclear genome, are some traits that are yet to be ascertained holistically (Albert and Carretero-Paulet, 2015; Bronstein *et al.*, 2014).

It is well-established by now that Orchidaceae display unparalleled diversity in terms of floral, vegetative and physiological adaptations and is therefore, of tremendous horticultural importance (Albert and Carretero-Paulet, 2015). The specialised floral perianth acts as a selection advantage, especially to rewardless orchids that constitute one-third of the family and are scattered throughout its many unrelated clades. Exemption of rewards for pollinators may turn into a disadvantage as gradually the pollinator may learn to avoid such flowers. But orchids still continue to evolve and tempt and dupe the pollinators. Mechanisms behind evolution of such extra-ordinary strategies are hidden in the floral genetic code (Gravendeel and Dirks-Mulder, 2015). Also, it becomes imperative to decode such evolutionary mysteries because evolution is trending towards increased orchid specialisation by reduction in number of pollinator species per orchid species, thus making orchids more dependent on their corresponding pollinators and not the vice-versa (Roberts and Dixon, 2008).

The Orchid Code: Genetic Basis of Floral Structure

ABCDE model of floral organ identity in orchids is an extension of ABC model that is applicable to rest of the angiosperms. According to the latter, A class of genes alone code for the formation of sepals, Class A and B together code for petals, Class B and C for stamens, while Class C alone codes for carpels. Class A and C are mutually repressive functionally (Theissen *et al.*, 2000). Later, ABCD model was put forward,

based on studies on *Petunia*, wherein Class D specified ovule development (Angenent and Colombo, 1996). Class B floral organ identity genes that specify petal and stamen identity encode MADS domain transcription factors (Mondragon-Palomino and Theißen, 2011). Two ancestral clades of these B Class genes include *DEFICIENS*-like (*DEF*) and *GLOBOSA*-like (*GLO*) genes, single copies of which are present in angiosperms, as revealed in *Antirrhinum majus* and *Arabidopsis thaliana* (Zahn *et al.*, 2005). However in case of orchids, *DEF*-like genes underwent two rounds of duplication to produce four gene copies whose functions later diverged to produce various specialized tepals in orchids. This led to emergence the ABCDE model of orchid perianth identity also well known as the Orchid Code which is rigorously tested and can be applied to most orchids (Mondragon-Palomino and Theißen, 2007, 2008, 2009). According to this code, Class A and E MADS-box proteins specify sepals, and Class A, B and E control petals, stamens are controlled by Class B, C and E gene activity, carpels by Class C and E, and ovules by Class C, D and E gene expression. And the quartet model says that a unique combinatorial result of activation and silencing of genes coding four *DEF*-like MADS-box proteins specify the fate of each whorl (Gravendeel and Dirks-Mulder, 2015; Mondragon-Palomino and Theißen, 2007). These combinatorial protein-protein interactions form multimeric regulatory complexes which specifically recognize cis-regulatory elements of target genes. This further stimulates or represses these target genes to form a specific organ. More recently, Su *et al.* (2013) reported modified molecular model of flower development based on functional analysis of gene expression profiles in *Phalaenopsis aphrodite* and identified floral organ specific genes and reported that Classes A and B in this species have novel functions due to evolutionary diversification and display differential expression patterns.

Infact, the body plan of orchid flower is decided in a founder cell where the combinatorial activity of homeotic selection genes is initiated. These encode MIKC-type MADS-box domain proteins to specify and dictate the expression of all the genes encoding the proteins required for identity, formation and development of each floral organ. Once these proteins are expressed, their differential combination forms distinct whorls as outer tepals, inner lateral tepals and inner median labellum (Mondragon-Palomino and Theißen, 2007; 2011). B Class MADS domain proteins underwent first round of duplication to produce a lineage of two sister clades encoding *DEF*-like proteins and *GLO*-like proteins (Kramer *et al.*, 1998), whose representatives are APETALLA (AP3) and PISTILLATA

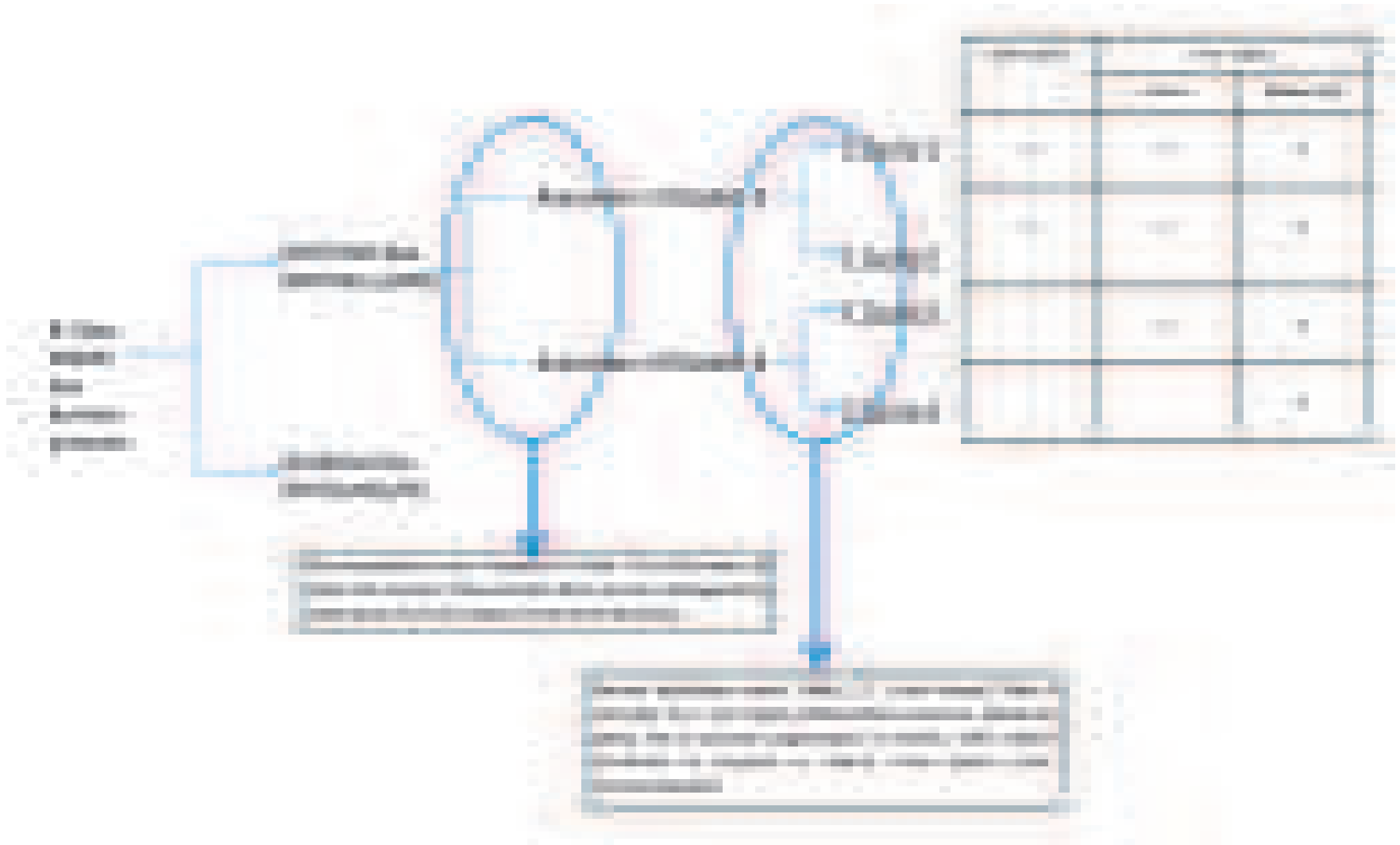


Fig. 1. Evolution of B-Class *DEF*-like MADS-box genes by duplication and functional diversification giving rise to four clades specifying unique perianth of orchids (Based on Mondragón-Palomino and Theißen, 2007).

(PI), respectively. These undergo obligate heterodimerisation for petal development. Resultant four orchid clades are the drivers to regulate their own expression and that of downstream target genes. These genes later complex with Class A and E proteins to give rise to perianth initials. Outer tepals are determined by heterotropic expression of Clade 1 and 2 genes. While, Clade 1, 2 and 3 are responsible for formation of lateral inner tepals and expression of clade 1, 2 and 4 forms labellum. In short, Clade 1 and 2 are expressed in all the tepals and are considered to be responsible for petaloid nature of the tepals. Identity to inner tepal is provided by Clade 3 and that of labellum is designed by Clade 4. This combinatorial expression is the essence of beautiful orchid floral architecture (Mondragon-Palomino and Theißen, 2007) (Fig. 1).

Molecular foundation of orchid lip development lies in MADS-box gene family. According to Wagner (2008), during the entire course of evolution, gene duplications substantially facilitate evolutionary innovations and novelties in plant structures by enhancing the chances of mutational robustness. Therefore, orchids offer multiple avenues in molecular developmental and physiological research (Albert and Carretero-Paulet, 2015).

Evolution of the Code

From an evolutionary perspective, the most basal orchid sub-family Apostosoideae is considered least diverse, without a pronounced lip. Dating studies revealed that diversification and speciation of orchids was triggered by lip evolution, due to duplication of deeply rooted *DEF*-like MADS-box genes around 60-70 mya and subsequent speciation by adoption of new diverse functions by the newly formed gene copies or orthologs (Chang *et al.*, 2010; Gravendeel and Dirks-Mulder, 2015; Hsu and Yang, 2002; Kim *et al.*, 2007; Mondragon-Palomino and Theißen, 2007; Tsai *et al.*, 2004, 2005; Xu *et al.*, 2006). However, *GLO*-like gene exists as a conserved single copy, not contributing to tepal distinction (Kim *et al.*, 2007; Ramirez *et al.*, 2007).

The striking floral morphological novelties in orchids are a result of lineage specific expansions and contractions in MADS-box gene subfamilies that underlie protein functional diversification and generate unique regulatory interaction networks (Albert and Carretero-Paulet, 2015).

Modularization of orchid perianths forming dramatically different floral structure, leading to floral diversification, is the basis of evolutionary

developmental biology (evo-devo) of orchids. This makes orchids a well suited system to link evolutionary and phylogenetic development with morphological speciation (Lu *et al.*, 2007). ABCDE model persists in scientific community as the most dominant concept determining bipartite perianth and labellum in orchids.

Evolutionarily, the genes belonging to Clade 1 and 2 follow the ancestral pattern of gene expression because these are expressed in all the perianth organs, while those belonging to Clade 3 and 4 are the derived states. In details, these four clades are two pairs of sister clades where the paralogs, even after duplication, retained their regulatory elements and are still controlled by similar upstream factors (Mondragon-Palomino and Theißen, 2011). On expression in their separate domains, these genes respond independently to natural selection, leading to evolutionary divergence of initially identical structures (Mondragon-Palomino and Theißen, 2007). Orchids have pushed the limits of evolution in a number of ways; therefore, an understanding of these limits may reveal the factors behind the key innovations exclusive to orchids. Certainly, with advent of evo-devo molecular approach, the complexities of floral development in orchids have been simplified to a greater extent (Aceto and Gaudio, 2011).

Conclusion

This highly specialized code bridges the gaps between the orchid diversification and phylogenetic basis. It would provide a rational framework not only in understanding the evolution and function of floral ontogeny genes, but it also gives identity to the appearance and diversification of such enigmatic floral innovations and evolution of arms-race concept (Mondragon-Palomino and Theißen, 2007). This code has managed to resolve uncertainties regarding evolutionary ancestries of orchid floral architecture (Tsai *et al.*, 2014). Time and again revisions, refinements and inputs have been added to the basic Orchid Code, but its applicability is widely tested in diverse genera across the five sub-families and is accepted validly. Mondragon-Palomino and Theißen (2011) performed expression based experiments to establish that organ identity is not defined as a simple ON and OFF pattern of *DEF*-like genes, instead it is due to the distinct mRNA levels from combinatorial expression of each of the four copies of *DEF*-like genes. Similarly, Hsu *et al.* (2015) comprehensively linked petal identity to gene expression and explained the concept of 'Perianth Code' in highly specialized orchids, owing to the tissue specific expression of *AP3* (B Class) and *AGL6* (E Class) that are duplicated MADS-box gene

copies. According to Perianth Code, there exists a competition MADS-box gene protein L-complex, to promote lip expression and SP-complex, to promote petal expression. These competitive/antagonistic protein interactions have been validated by FRET analysis and also using virus-induced gene-silencing. Down-regulation of *OAGL6* gene in L-complex resulted in conversion of lips to petal-like structures in *Onicidium* and *Phalaenopsis* orchid mutants (Gravendeel and Dirks-Mulder, 2015). Such detailed and comprehensive studies to elucidate floral developmental steps are much needed in the coming years.

Future Prospects

Detailed studies validate the fact that this unusual plant family can serve as a well-tested and accepted model system and will be successful to address radical questions, especially those related to interspecific interactions, physiological adaptations and evolutionary ecology, including evolutionary arms race with pollinators (Albert and Carretero-Paulet, 2015; Gravendeel and Dirks-Mulder, 2015; Mondragon-Palomino and Theißen, 2007). Molecular and genome based studies have provided ample evidence regarding this exceptionally different model for perianth formation and is still a potentially alluring area of future researches (Bronstein *et al.*, 2014). A renewed interest has been developed amongst the researchers associated with studying evo-devo studies based on floral ontogeny. Exposing the intricacies at different levels of orchid floral development may help in a better understanding of the process of natural selection that played an important role in radiation of orchids. Expression based studies are still a nascent area which needs to be taken up to sort out the mysteries behind floral innovations and associated biological diversity (Mondragon-Palomino and Theißen, 2007). During the past decade, there is an increasing focus observed towards efforts in ascertaining and resolving the evolutionary mysteries and ecological novelties in orchids using rigorous and advanced phylogenetic methods and molecular techniques (Mondragon-Palomino, 2013; Tsai *et al.*, 2014).

Highly specialized adaptations have acted as boon, as well as proved to be a bane, towards a clearer understanding of molecular basis of orchid floral ontogeny. Because orchids have long life-cycle, large genome size, and inefficient transformation system, extensive studies on classes A, C, D and E are still in infancy. Development of an exclusive and specialized gynostegial structure and ovule development provide tremendous opportunities to address the evolutionary queries because these form the potential areas

accessible to many researchers and may lead to new discoveries of genetic variants in terms of floral architecture (Hsiao *et al.*, 2011). Sequence data and other genomics tools (transformation and virus-induced gene silencing) may lead to a better understanding of more promising areas such as that of reverse genetics (Lu *et al.*, 2007). However, still much needs to be learnt to trace the underlying molecular and genetic mechanisms controlling the floral development in orchids as also indicated earlier by Albert and Carretero-Paulet (2015).

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