Floral organ identity genes in the orchid *Dendrobium crumenatum*

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**Summary**

Orchids are members of Orchidaceae, one of the largest families in the flowering plants. Among the angiosperms, orchids are unique in their floral patterning, particularly in floral structures and organ identity. The ABCDE model was proposed as a general model to explain flower development in diverse plant groups, however the extent to which this model is applicable to orchids is still unknown. To investigate the regulatory mechanisms underlying orchid flower development, we isolated candidates for A, B, C, D and E function genes from *Dendrobium crumenatum*. These include *AP2*, *PI*/GLO*, *AP3*/DEF*, *AG* and *SEP*-like genes. The expression profiles of these genes exhibited different patterns from their Arabidopsis orthologs in floral patterning. Functional studies showed that *DcOPI* and *DcOAG1* could replace the functions of *PI* and *AG* in Arabidopsis, respectively. By using chimeric repressor silencing technology, *DcOAP3A* was found to be another putative B function gene. Yeast two-hybrid analysis demonstrated that *DcOAP3A*/B and *DcOPI* could form heterodimers. These heterodimers could further interact with *DcOSEP* to form higher protein complexes, similar to their orthologs in eudicots. Our findings suggested that there is partial conservation in the B and C function genes between Arabidopsis and orchid. However, gene duplication might have led to the divergence in gene expression and regulation, possibly followed by functional divergence, resulting in the unique floral ontogeny in orchids.

Keywords: ABCDE model, flower development, functional divergence, gene duplication, orchid, organ identity.

**Introduction**

Molecular and genetic regulation of flower development has been extensively investigated in the last two decades (Jack, 2004; Theissen, 2001). As a result, the classical ABC model has been proposed to explain the specification of floral organ identity in Arabidopsis and *Antirrhinum* (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Based on this model, three classes of functions, A, B and C with each consisting of one or more genes, determine various floral organ identities. Function A alone controls the development of sepals in the first whorl of a flower. Functions A and B specify petal development in the second whorl, while B and C functions control stamen development in the third whorl. Function C alone determines carpel development in the fourth whorl. Functions A and C are considered mutual antagonists and the expression of one represses the other (Bowman et al., 1991; Drews et al., 1991; Gustafson-Brown et al., 1994). The model has been used to explain floral organ development in a wide range of plant species (Ambrose et al., 2000; Kang et al., 1998; Kramer et al., 1998; Kyoizuka and Shimamoto, 2002; Mena et al., 1996; Whipple et al., 2004). Recently, two classes of new functions, namely D and E, were added, resulting in the revised ABCDE model (Pelaz et al., 2000, 2001; Pinyopich et al., 2003). In the revised model, function D determines ovule development, while function E is required for development of all the floral organs (Ditta et al., 2004; Pelaz et al., 2000, 2001; Pinyopich et al., 2003).

With the exception of the function A gene *APETALA2* (AP2), all known homeotic genes in the ABCDE model belong to the MADS-box family of transcription factors. In
plants, MADS-box genes are important regulators controlling developmental programs from vegetative growth, flowering process, to seed development (Theissen, 2001). To date, five types (Mø, Mj, Mγ, Mδ and MIKC) of MADS-box genes have been identified in Arabidopsis (Parenicova et al., 2003; Riechmann and Meyerowitz, 1997). Studies on the plant MADS-box genes have been mainly focused on the MIKC type, whose members have a similar modular structure. This includes a highly conserved domain of approximately 56 amino acids called MADS-box (M), a weakly conserved intervening region (I), a moderately conserved K domain (K) and a variable carboxyl-terminal (C; Kaufmann et al., 2005). MIKC-type MADS-box transcription factors are specific to plants and are essential for plant growth and development (Becker and Theissen, 2003).

AP2, on the other hand, encodes a transcription factor with two continuous AP2 domains, which are specific to plants. AP2 confers function A in Arabidopsis and represses AG in floral whorls 1 and 2 (Drews et al., 1991; Jofuku et al., 1994). Although the transcripts of AP2 can be detected in both reproductive and vegetative tissues (Drews et al., 1991), the function of AP2 is restricted to whorls 1 and 2, which is caused by miRNA-mediated translational regulation (Chen, 2004).

The above-mentioned mechanisms of floral patterning may be applicable to diverse plant species, while unique flower structures in some species may indicate the divergence of the genetic mechanism involved (Irish and Litt, 2005; Zahn et al., 2005). Some monocot species possess distinct floral structures. These include the grass family (e.g. rice and maize) and the orchid family. Floral development has been extensively studied in two typical monocotyledonous crop species, namely, rice and maize. The B and C function genes are conserved in the grass family and Arabidopsis (Ambrose et al., 2000; Kyoizuka and Shimamoto, 2002; Schmidt et al., 1993; Whipple et al., 2004). However, floral patterning in grasses may not be comparable to those from other flowering plants, as they usually possess highly reduced floral organs. Orchids belong to Orchidaceae, one of the largest families of flowering plants. As a group of important ornamental plants with great diversity and specialization in floral morphology, orchids are one of the desirable monocot species for studying the genetic basis of flower development. Although there have been many reports on the physiological and horticultural aspects of orchids, only a limited number of reports on molecular genetic analysis of orchid flower development are available (Hsu and Yang, 2002; Tsai et al., 2004; Yu and Goh, 2000, 2001).

In orchids, the only functional study of a putative floral organ identity gene OMADS3 (a paeloAP3 gene from Oncidium) indicated that it may be an A function gene involved in flower formation and floral initiation, despite its sequence similarity to the known B function genes (Hsu and Yang, 2002). Recently, four APETALA3 (AP3)/DEFICIEN (DEF)-like MADS box genes have been isolated from the Phalaenopsis orchid (Tsai et al., 2004). These genes demonstrated distinct expression profiles in wild-type orchids and the peloric mutants with lip-like petals, indicating that the regulation of AP3/DEF-like genes in orchids may be more complicated than those in eudicots and grass species, where only one AP3/DEF-like gene is usually found in each species (Ambrose et al., 2000; Jack et al., 1992; Moon et al., 1999). Thus, it is so far unknown if the counterparts of the floral organ identity genes are functional in the highly specialized structures of orchid flowers.

In our efforts to investigate the unique features in orchid flowers, such as the modifications of perianth organs and the fusion of reproductive organs, we systematically isolated and characterized a group of putative floral organ identity genes from the orchid Dendrobium crumenatum. Investigation of their expression profiles in the orchid and functional characterization of these genes in Arabidopsis revealed that DcOAP3A/B, DcOPI and DcOAG1 are B and C function genes, respectively. Our studies suggested that gene duplication followed by the divergence in expression patterns or regulatory mechanisms may be responsible for the diverse floral morphology in orchids.

Results

Floral morphology in D. crumenatum

Among the numerous orchid species and hybrids, floral characteristics of Dendrobium species are comparatively constant throughout the genus despite their variations at vegetative stages. D. crumenatum has typical orchid flowers that develop from young floral buds at the nodes of an inflorescence axis (Figure 1a,b). Like most of the angiosperms, D. crumenatum flowers are made up of four whorls, namely sepals, petals, anthers and pistils (Figure 1c). However, there are several unique features in orchids. First, the first whorl of orchid flowers is made up of one dorsal and two lateral petaloid sepals (Figure 1c). Even though the petals in the second whorl are usually broader than sepals, the general appearance of the two lateral petals is very similar to the petaloid sepals. Secondly, the median petal is modified into a lip with different size, shape and color from other petals, which generally serves as a ‘landing platform’ for insect pollinators (Figure 1c). Lastly, unlike the configuration of separate male and female organs in most other flowers, the stigmatic region (part of female organs) and the anther (male organs) of orchid flowers are fused into a finger-like structure located at the tip of a column (Figure 1c,d). The anther under the anther cap contains clusters of pollen grains called pollinia. Closer to the base of the anther is the inferior ovary located at the flower stalk. After successful pollination, the ovary develops into a capsule containing numerous seeds.
Identification of floral organ identity genes from D. crumenatum

To identify genes involved in the development of orchid flowers, we isolated candidates for A, B, C, D and E function genes from D. crumenatum. A group of MADS-box genes and one AP2-like gene were isolated from dormant flower buds or newly open flowers using PCR amplification of the cDNAs with degenerate primers as described in Experimental procedures. The rapid amplification of cDNA ends (RACE) method was employed to further isolate full-length cDNAs of AP2, AP3, PISTILLATA (PI), AGAMOUS (AG) and SEPALLATA (SEP) orthologs. These orthologs were named DcOAP2 (D. crumenatum AP2-like gene), DcOAP3A, DcOAP3B, DcOPI, DcOAG1, DcOAG2 and DcOSEP1 (GenBank accession numbers DQ119837, DQ119838, DQ119839, DQ119840, DQ119841, DQ119842 and DQ119843, respectively).

DcOAP2 cDNA is 1711 bp long with a 1338-bp coding region (Figure S1). Similar to the known AP2-like genes, the predicted amino acid sequence of DcOAP2 contains two AP2 domains, which bear a high similarity with other AP2-like proteins (Figure 2a; Okamuro et al., 1997). This suggests that DcOAP2 is an AP2-like transcription factor, which may play a role in orchid flower development.

All of the predicted protein sequences of MADS box genes cloned contain the conserved MIK region and a divergent C-terminal region. DcOPI possesses a highly conserved PI motif at the C-terminal region, which is characteristic of PI lineage and shared by other members in this lineage from both dicots and monocots (Figure 2b; Kramer et al., 1998). In contrast, AP3/DEF-like genes are separated into euAP3, TM6 and paleoAP3 lineages (Kramer et al., 1998). Proteins of euAP3 lineage in core eudicot species possess a euAP3 motif and a PI-derived motif, while members of the paleoAP3 lineage in basal eudicots, magnoliids and monocots, including DcOAP3A and DcOAP3B of D. crumenatum, contain a paleoAP3 motif and a distinct PI-derived motif (Figure 2c; Kramer et al., 1998; Moon et al., 1999; Tseng and Yang, 2001). Sequence analysis revealed that both DcOAG1 and DcOAG2 belong to AG lineage (Figure 2d). DcOAG1 encodes a protein containing an N-terminal region upstream of the MADS-box domain. In addition, it contains the eighth intron (Figure 2e), which is present in the members of the function C lineage, while most of the genes reported in the D lineage from core eudicots and grass species lack this intron (Kramer et al., 2004).

Phylogenetic analysis further confirmed that these orchid MADS box genes belong to paleoAP3, PI, SEP and AG lineages (Figures 3 and 4). DcOAP3A and DcOAP3B were found to be members of the paleoAP3 lineage (Figure 3), which was consistent with the sequence alignment shown in Figure 2c. The paleoAP3 genes from the orchids Phalaenopsis, Oncidium and Dendrobium were subdivided into two subclusters. DcOAP3A belongs to the subcluster containing OMADS3, PeMADS2 and PeMADS5. The transcripts of PeMADS2 and PeMADS5 were found in all floral organs except the pollinia (Tsai et al., 2004). PeMADS2 might be required for sepal development, while PeMADS5 might be...
involved in petal and stamen development. **OMADS3** was ubiquitously expressed and could confer function A in Arabidopsis (Hsu and Yang, 2002). **DcOAP3B** was clustered with **PeMADS5** of Phalaenopsis and **OMADS3** of Oncidium, which was detectable in the lip and column with unknown functions (Tsai et al., 2004). **DcOPI** was within the PI/GLOBOSA (GLO) cluster (Figure 3). In this cluster, **DcOPI** and other orthologs from monocots formed one subcluster, which was separated from the members of eudicots. Compared with the PI/GLO and AP3/DEF lineages, the members of the SEP lineage were more diversified in protein sequences. **DcOSEP1** (Figure S2) was clustered with other SEP-like genes from orchids, including **OM1** and **DOMADS1** (Figure 3).

In the AG subfamily, phylogenetic analyses of 96 previously released AG-like sequences and two orchid AG-like sequences obtained in our study revealed the division of the
angiosperm sequences into two major clades, termed the C and D lineages (Kramer et al., 2004; Figure 4). DcOAG1 was clustered in the C lineage, while DcOAG2 was in the D lineage.

Expression patterns of orchid floral organ identity genes

In order to investigate the function of orchid floral organ identity genes, their expression patterns in vegetative and reproductive organs were examined by reverse transcription-PCR and in situ hybridization. With the exception of the function A ortholog DcOAP2 and the function B ortholog DcOAP3A, other floral organ identity genes were expressed specifically in Dendrobium flowers (Figure 1e). Transcripts of DcOAP2 and the function E ortholog DcO-SEP1 were detected in all the floral organs (Figure 1e), as with their orthologs in Arabidopsis (Drews et al., 1991; Pelaz et al., 2000). However, the putative function B genes of Dendrobium demonstrated different expression patterns from their counterparts in angiosperms, where the expression is usually restricted to whorls 2 and 3 of floral organs. DcOPI was expressed equally in all floral organs, including sepal, petals, lips, male organs (anther caps and pollinia) and female organs (lower part of column with ovary; Figures 1e and 5a–f). The two orchid AP3/DEF orthologs showed distinct expression patterns from each other. The transcripts of DcOAP3A were present in all the floral organs and vegetative tissues (Figures 1e and 5g–i), whereas DcOAP3B was only strongly expressed in the second whorl of petals and lips and in the reproductive organs including pollinia and columns (Figure 1e). Thus, the expression patterns of function B genes in Dendrobium showed two features. First, DcOPI and DcOAP3A were clearly detectable in the first whorl of sepal. Secondly, all the putative function B genes, including DcOPI, DcOAP3A and DcOAP3B, were expressed at various levels in the lower part of the column (ovary), which was equivalent to the fourth whorl (carpels) in Arabidopsis. In contrast to its orthologs in most of the angiosperms (Benedito et al., 2004; Kang et al., 1998; Kater et al., 1998; Kim et al., 2005; Pnueli et al., 1994), the putative function C gene DcOAG1 was expressed in all floral organs (Figures 1e and 5j–l). However, the function D gene DcOAG2 was expressed mainly in the lower part of columns (ovary) and in the envelop cells of pollinia (Figures 1e and 5m,n).

Analysis of putative B function genes of D. crumenatum

To study the biological function of DcOPI, we overexpressed the DcOPI cDNA under the control of the constitutive CaMV 35S promoter in Arabidopsis. Among 33 independent lines of kanamycin-resistant transgenic plants, 10 lines showed partial transformation of sepal into petaloid structures in the first whorl (Figure 6a,b), which resembled the homeotic transformation in the flowers of 35S::PI lines (Krizek and Meyerowitz, 1996). SEM analysis further showed that the adaxial surface of the base and margin of the petaloid sepal contained regular rounded cells, which was characteristic of the adaxial surface of wild-type petals (Figure 6e,g,h), while irregular rectangular cells interspersed with stomata were arranged in the upper center region, which was characteristic of the adaxial surface of wild-type petals (Figure 6e,i,j). Similarly, the abaxial surface of the petaloid sepal also showed the mixed features of sepals and petals (Figure 6f,k–n).

A complementation study was further performed to examine if DcOPI could provide the same activity as endogenous PI in Arabidopsis flower development. In Arabidopsis loss-of-function mutant pi-1, flowers consist of two outer whorls of sepals and an abnormally large gynoe- cium in the center (Figure 6c; Bowman et al., 1991; Hill and Lord, 1989). Similar to 35S::PI (Krizek and Meyerowitz, 1996),
35S::DcOPI could largely, but not fully, rescue pi-1 (Figure 6d). 35S::DcOPI pi-1 flowers developed petaloid sepals in the first whorl, petals in the second whorl, stamens, carpeloid stamens or pistils in the third whorl, and normal gynoecium in the fourth whorl (Figure 6d). Compared with the full rescue of petals in the second whorl, the rescue of organ defects in the third whorl was weaker, because many carpeloid organs remained. These patterns mimicked...
overexpression of PI in pi-1, suggesting that DcOPI was able to substitute PI in Arabidopsis.

As suggested by sequence analysis, DcOAP3A and DcOAP3B belonged to paleoAP3 lineage and had high sequence similarity in the predicted protein sequences. To address if orchid B-function genes contribute to the identity of petaloid sepals, DcOAP3A was further characterized, because only DcOAP3A was detectable in the first whorl of orchid flowers. However, the phenotype of all 43 transgenic Arabidopsis lines overexpressing DcOAP3A was indistinguishable from that of wild-type plants (data not shown). In Arabidopsis, flowers of function B mutant ap3-3 contained sepals in the outer two whorls and carpels in the center (Figure 7a; Jack et al., 1992). Previous reports have suggested that the paleoAP3 motif was able to promote stamen identity in the fourth whorl and partially rescue the third whorl into stamen-like organs in ap3-3 (Lamb and Irish, 2003). Therefore, we further examined if 35S::DcOAP3A was able to rescue ap3-3. Consistent with void effects in wild-type plants, 35S::DcOAP3A in ap3-3 also showed indistinguishable phenotypes from ap3-3 (data not shown). To test if DcOAP3A could function in the presence of its potential partner DcOPI, we produced double transgenic plants 35S::DcOAP3A 35S::DcOPI and also introduced both transgenes into ap3-3. The double transgenic plants showed the same phenotypes as 35S::DcOPI (Figure 6b), and double transgenes could not rescue floral defects in ap3-3 (data not shown). Taken together, these results demonstrated that DcOAP3A cannot function as AP3 in Arabidopsis.

In order to further clarify the roles of DcOPI and DcOAP3A, we applied a chimeric repressor silencing technology by using the SRDX domain, a super repression motif derived...
from the EAR-motif of the repression domain of tobacco ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR 3 (RD) and SUPERMAN (SUPRD) (Hiratsu et al., 2003). When it is fused translationally with the genes of interest, it can efficiently convert transcription activators into dominant repressors, which suppress the expression of target gene(s).

early flowering phenotype of 35S::DcOAG1 Arabidopsis. The second whorl cannot be clearly identified and the third whorl stamens are absent. In several strong transgenic lines, ovule-like structures were observed in the third whorl (Figure 7c). These phenotypes resembled those of the function B mutant ap3-3 (Figure 7a) and pi-1 (Figure 6c). It was surprising that introduction of DcOAP3A-SRDX into Arabidopsis could also cause similar phenotypes as function B mutants in transgenic flowers (25 out of 89 lines; Figure 7d–g), indicating that DcOAP3A might regulate the same target genes as AP3 in orchid flower development.

Several lines of evidence from Arabidopsis and Antirrhinum suggested that MADS-box proteins function by forming higher order complexes in flower development (Honna and Goto, 2001; Theissen, 2001; Theissen and Saedler, 2001). In Arabidopsis and Antirrhinum, AP3 and PI, or DEF and GLO were known to interact with each other to form heterodimers, which in turn formed higher complexes with SEP lineage proteins (Honma and Goto, 2001; Theissen, 2001; Theissen and Saedler, 2001). In monocot species, it has been reported that TGGLO of tulip bound to DNA as a homodimer (Kanno et al., 2003). To examine if orchid floral organ identity regulators can form similar protein complexes, yeast two-hybrid analyses were performed using the two-hybrid MATCHMAKER II system (Clontech, Palo Alto, CA, USA). The coding regions of DcOPI, DcOAP3A, DcOAP3B and DcOSEP1 were cloned into the GAL4 DNA activation domain (AD) vector pACT2 and the GAL4 DNA-binding domain (BD) vector pAS2-1. Yeast two-hybrid analyses showed that in contrast to their orthologs in tulip, DcOAP3A/B and DcOPI formed heterodimers rather than homodimers (Table 1). In addition, DcOAP3A and DcOAP3B were not able to form dimers. Similar to their orthologs in Arabidopsis and Antirrhinum, DcOAP3A/B and DcOPI alone were not able to form heterodimers with DcOSEP1, but the heterodimer of DcOAP3A or DcOAP3B and DcOPI could interact with DcOSEP1 to form a higher order protein complex (Table 1). Thus, the patterns of protein–protein interactions of MADS-box proteins in orchid and Arabidopsis flowers may resemble each other.
interactions between orchid B-function regulators were comparable to those of their counterparts in Arabidopsis and Antirrhinum.

Analysis of a putative function C gene of D. crumenatum

To elucidate the role of the putative function C gene DcOAG1, we overexpressed it in Arabidopsis. Out of 35 independent transgenic lines obtained, 12 lines showed early flowering with the phenotype of curly leaves (Figure 7j), and seven lines showed defects in the first and second whorls of floral organs. The strong lines showed typical ap2-like phenotypes with reduced height, small and curled leaves, loss of inflorescence indeterminacy (Figure 7j,k), and homeotic transformation in the first and second whorls of flowers (Figure 7l). In these flowers, the sepals were modified into carpel-like structures with stigmatic papillae at the apex, and the petals were either absent or transformed into stamen-like structures (Figure 7l). These results suggested that DcOAG1 had the same function as AG in Arabidopsis.

Discussion

Although several MADS-box genes have been isolated from orchids (Hsu and Yang, 2002; Tsai et al., 2004; Yu and Goh, 2000), molecular mechanisms underlying floral organ development in this group of plants are unknown. In this study, we isolated putative function A, B, C, D and E genes from D. crumenatum orchid and carried out functional characterization on the B and C function genes.

The B function, conferred collectively by the members of the AP3/DEF and PI/GLO lineages, is necessary for the formation of petals and stamens in flowers. Extensive studies in the past decade have proven that AP3/DEF and PI/GLO lineages are highly conserved in diverse plant species. Gene duplication and divergence in these two lineages have been known throughout the history of the evolution of angiosperms (Kramer et al., 1998; Zahn et al., 2005). While PI/GLO-like genes are highly conserved, AP3/DEF-like genes have three distinct lineages, paleoAP3, TM6 and euAP3 (Kramer and Irish, 2000; Kramer et al., 1998). It has been proposed that these genes may have evolved from an ancestral gene possessing a paleoAP3 motif after two major duplications (Kramer et al., 1998; Purugganan, 1997). The first duplication generated the PI and paleoAP3 lineages. In the second duplication, the paleoAP3 lineage remained in lower eudicots, magnolid dicots and monocots, but further divided into the euAP3 and TM6 lineages in core eudicots (Kramer et al., 1998).

We have isolated three putative B function genes, DcOPI, DcOAP3A and DcOAP3B, from the orchid D. crumenatum. DcOPI belongs to the PI lineage, while DcOAP3A and DcOAP3B are typical genes in the paleoAP3 lineage. This result indicates that gene duplication has at least taken place in the AP3/DEF clade in the monocots. Overexpression of DcOPI in Arabidopsis wild-type plants and pi-1 null mutants have shown that DcOPI could provide similar regulatory effects as PI in flower development, indicating that DcOPI is an ortholog of PI in orchids. In contrast, overexpression of DcOAP3A in wild-type or ap3-3 plants failed to cause significant phenotypic changes relevant to B function genes. Compared with the PI lineage, the AP3/DEF lineage is less conserved in protein sequences. DcOAP3A and AP3 belong to paleoAP3 and euAP3 lineages, respectively. AP3 contains a distinct euAP3 motif in its C-terminal region (Figure 2a) resulting from a frame-shift mutation in one of the copies of a duplicated ancestral paleoAP3-type gene (Vandenbussche et al., 2003). It has been shown that the paleoAP3 and euAP3 motifs within the eudicot lineage may have different functions (Lamb and Irish, 2003; Vandenbussche et al., 2004). A chimeric construct for the Arabidopsis AP3 gene, in which the C-terminal euAP3 motif was replaced by a paleoAP3 motif, could partially rescue stamen development in ap3-3 (Lamb and Irish, 2003). This result indicates that the paleoAP3 motif can only promote stamen but not petal development in core eudicots. However, another study has argued that the paleoAP3 genes have a similar function to their euAP3 orthologs. Overexpression of maize Silky under the control of the AP3 promoter can partially rescue ap3-3 in Arabidopsis (Whipple et al., 2004). Our study suggests that the paleoAP3 gene may have different functions from the euAP3 gene, demonstrating the functional diversity of the AP3/DEF-lineage genes in different plant species.

We have further tested whether DcOPI and DcOAP3A are function B orthologs in orchids by using a chimeric
The dominant-negative phenotypes as those in B function and AP3 possibility that did not produce any dominant-negative phenotypes, the DcOAP3A-SRDX expressing repressor silencing technology. The transgenic plants Yifeng Xu the phenotypes of B function mutants. In either case, critical regulatory protein complexes, thus indirectly causing low. DcOAP3A-SRDX oopsis, 35S::35S::TGDEFA/B function B genes expressed in whorls 1 and 2 (Kanno et al., 1999), function as a heterodimer as indicated by in vitro DNA-binding and yeast two-hybrid assays. However, it has been reported that in tulip and lily, their PI/GLO-like proteins are able to form homodimers (Kanno et al., 2003). In our study, yeast two-hybrid analyses have shown that AP3/DEF-like protein, DcOAP3A or DcOAP3B, form heterodimers with DcOPI. All these function B proteins are not able to form homodimers. Also, DcOAP3A and DcOAP3B do not interact with each other. Furthermore, the heterodimers formed by DcOPI and DcOAP3A/B can form higher order complexes with DcOSEP1. These results strongly suggest that the heterodimerization of B function proteins is at least conserved among orchids, grasses and eudicots.

The unique floral structures in orchids can be partially explained by the conserved role of function B genes. RT-PCR and in situ analyses have shown that both DcOPI and DcOAP3A are expressed in all whorls of floral organs. These expression patterns, together with the shown functions in transgenic Arabidopsis, suggest that the expansion of function B genes, represented by DcOPI and DcOAP3A, into the first whorl may be responsible for the petaloid sepals in orchids. Our results are consistent with the modified ABC model (van Tunen et al., 1993). In Tulipa (Liliaceae), the function B genes TGDEFA/B and TGGLO were also expressed in whorls 1 and 2 (Kanno et al., 2003). On the other hand, the mRNA expression of an AP3/DEF-like gene, LMADS1 of Lilium longiflorum, was detectable strongly in whorls 2 and 3, but weakly in whorls 1 and 4. In addition, LMADS1 protein was only detectable in whorls 2 and 3, suggesting that the outer two whorls of tepals in lily are regulated by a different mechanism despite their similarity in morphology (Tzeng and Yang, 2001). The above contradictory results may indicate the complexity in the role of function B genes in the development of petaloid sepals.

In the second whorl of orchid flowers, multiple polymorphisms are widely observed in wild-type plants and naturally occurring peloric mutants. The median petal of a wild-type orchid flower is specialized as a lip, which may be differently colored, shaped, or decorated with crests, tails, horns or other accessories attractive to potential pollinators. In natural peloric orchid mutants, a specific type of organs such as petals, lips or transformed sepals in the second whorl can be transformed into another type without affecting the identities of other whorls of floral organs (Rudall and Bateman, 2002). This subtle regulation should require more regulatory genes to be involved, which is consistent with the isolation of at least two AP3/DEF orthologs from D. crumenatum. Recently, four AP3/DEF-like MADS-box genes were also identified in Phalaenopsis orchid (Tsai et al., 2004). Comparison of their expression patterns between wild-type plants and peloric mutants has shown the functional specialization of these paralogous genes in perianth whorls. The above observations suggest that the exquisite regulation of the second whorl in orchid flowers may need the coordinated functions of several AP3/DEF-like genes.

There are several lines of evidence suggesting that two AG-like genes from D. crumenatum may have different biological functions. First, an N-terminal extension preceding the MADS domain found only in C-lineage members is present in DcOAG1, but not in DcOAG2. Secondly, the genomic sequence of DcOAG1 contains the intron 8, which is absent in DcOAG2 and most other known function D genes (Kramer et al., 2004; Zhang et al., 2004). Thirdly, phylogenetic analyses show that DcOAG1 and DcOAG2 belong to function C and D genes in orchids, respectively (Figure 4). Lastly, overexpression of DcOAG1 in Arabidopsis causes early flowering, curly leaves and, in particular, the homeotic transformation of floral organs as observed in overexpression of AG and AG orthologs in other plant species. These results strongly support that DcOAG1 is the orchid ortholog of AG, while DcOAG2 is a putative function D gene according to its specific expression in the ovary and its clustering with D-lineage genes by phylogenetic analysis.

While the sequence analyses and functional studies have suggested that DcOAG1 is a putative C function gene in orchids, its expression is detectable in all the floral organs. Recently, it has been reported that, in some basal angiosperm species, the expression of the AG orthologs was also not restricted to the reproductive organs (Kim et al., 2005). The Illicium AG ortholog is expressed strongly in the inner tepals, while the Persea AG orthologs are detectable in all floral organs. The broader expression of AG orthologs in basal angiosperms Illicium and Persea as well as the
monocot orchids indicates that the expression of AG orthologs or the regulatory mechanisms involved can be independently derived. In orchids, the concurrence of the expression of DcOAP2, a putative AP2 ortholog and DcOAG1 in all floral organs implies that the mechanism underlying the regulation of AG orthologs may be different from that in Arabidopsis, as observed in Antirrhinum and petunia (Keck et al., 2003; Maes et al., 2001).

The column of an orchid flower is thought to be the fused structure of male and female organs. The finding of the co-expression of orchid B, C and D function genes in the column (Figure 4), provides molecular evidence to support the chimeric nature of the column. Because the expression of B function genes in female organs has been reported in many angiosperm lineages (Zahn et al., 2005), the unique structure of orchid reproductive organs may indicate a hitherto unidentified function of B function genes in flower development. For example, it will be interesting to explore if expression of function B genes in the column causes the fusion of male and female organs in orchids.

In conclusion, our study demonstrates that the molecular mechanisms underlying flower development are partly conserved between Arabidopsis and orchid despite extensive differences in their floral morphology. During floral evolution, gene duplication events as revealed in this study may have given rise to a diversity of gene functions or evolutionary innovations. Further functional analyses of orchid floral organ identity genes are necessary to achieve a better understanding of the evolution of the unique structures in orchid flowers and to provide a mechanistic basis for genetic engineering of orchids.

Experimental procedures

Plant materials and growth conditions

Dendrobium crumenatum plants used in this study were grown in the garden of the Department of Biological Sciences, National University of Singapore, Singapore. Arabidopsis mutants of pi-1 and ap3-3 as well as transgenic plants were grown in growth chambers (22°C, 16 h of light/8 h of darkness).

Molecular cloning of MADS-box genes

Total RNA from different floral tissues and vegetative parts of D. crumenatum was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). To isolate the putative AP3/DEF and PI/GLO orthologs, fragments around 850 bp were amplified by one degenerate primer dMADS1 and 5' RACE with SMART RACE cDNA Amplification Kit (Clontech). Comparison of sequences in the database revealed two fragments corresponding to AP3/DEF and PI/GLO homologs. To get the complete cDNAs, 5' RACE was performed with the gene-specific primers 5RACEOAP3A and 5RACEOPI. To isolate more AP3/DEF-like genes, a degenerate primer DODEF1 corresponding to the motif specifically conserved among AP3/DEF-lineage members was designed. Using this degenerate primer, 3'-RACE PCR was performed to obtain another AP3/DEF gene fragment with the partial K domain, C region and 3'-UTR. The upstream sequence of this cDNA was isolated by 5'-RACE PCR with the gene-specific primer 5RACEOAP3B. During isolation of the full length of DcOAP3B, a cDNA fragment containing the 3'-terminal region of DcOAP2 was obtained, 5'-RACE was performed to isolate the upstream cDNA fragment with the gene-specific primer 5RACEOAP2. Partial cDNAs of AG orthologs were isolated by RT-PCR using two degenerate primers dgAGF and dgAGR, which correspond to the conserved sequence of MADS-box regions. This led to the isolation of two fragments corresponding to DcOAG1 and DcOAG2. To obtain the full cDNA sequences, 5'- and 3'-RACEs were performed respectively with the internal gene-specific primers: 5RACEOAG1, 3RACEOAG1, 5RACEOAG2 and 3RACEOAG1. The partial genomic sequences of DcOAG1 and DcOAG2 were isolated using primers GDOAG1-F1, GDOAG1-R1, GDOAG2-F1 and GDOAG2-R1, which were designed near the last codon of their protein sequences to examine the existence of intron 8. A partial cDNA fragment of DcOSEP1 was amplified by degenerate primers dgSEP1F and dgSEP1R based on the conserved sequence of the AP1/AGL9-lineage genes. 5'- and 3'-RACEs were further performed to obtain upstream and downstream fragments with the internal gene-specific primers 5RACEOSEP1 and 3RACEOSEP1. All the primers used for cloning are listed in Table S3.

Sequence analysis

Sequencing was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence data were compared with the relevant sequences retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) database.

Phylogenetic analyses for B and E class genes were carried out using the program PAUP version 4.0b4 (Thompson et al., 1997). DEFICIENS-AGAMOUS-LIKE2 (DAL2) (X79280) of Picea abies was chosen as an outgroup. The sequence used for phylogenetic analysis included the MADS-box, I-region and K-box of MADS-box genes. These sequences were aligned using CLUSTALW and then used for neighbor-joining and bootstrapping analyses.

Phylogenetic analyses for AG-like genes were performed as the published protocols (Kramer et al., 2004) with some modifications. The amino acid sequences of putative orchid AG-like genes DcOAG1 and DcOAG2 and 96 previously published AG-like genes from angiosperms and gymnosperms were aligned by MUSCLE (Edgar, 2004) and manually adjusted. Their nucleotide sequences were aligned through the protal2dna server (http://bioweb.pasteur.fr/seqanal/interfaces/protal2dna.html), and this alignment was further adjusted based on the alignment of amino acid sequences. These aligned nucleotide sequences were used for phylogenetic analysis by using the Neighbor-joining program in the PHYLGENY INFERENCE PACKAGE, version 3.65 (PHYLIP 3.65; Department of Genome Sciences, University of Washington, Seattle, WA, USA). The statistical significance was tested by bootstrap analysis for 10 000 replicates.

RT-PCR analysis and in situ hybridization

Total RNA extracted from different tissues served as the template for first strand cDNA synthesis using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Orchid polyubiquitin (DcOUPQ1) was amplified as a quantitative
control for RT-PCR. RT-PCR was preformed using gene-specific primers listed in Table S4. In situ hybridization was preformed according to Long and Barton (1998) with minor modifications. In brief, the probe fragments were amplified by the gene-specific primers and cloned into pGEM-T Easy vectors (Promega, Madison, WI, USA). The single-stranded antisense and sense RNA probes were transcribed in vitro with T7 or SP6 polynucleases using the DIG RNA Labeling Kit (Roche Diagnostic, Mannheim, Germany).

Construction of binary vectors

The constructs used for overexpression of DcOPI and DcOAP3A were prepared from pBI121 binary vector (Clontech). The open reading frames (ORFs) of cDNAs were amplified using the proof-reading Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) with primers incorporating relevant restriction sites. The primers used in PCR were OPI-F(BamHI), OPI-R(XbaI), OAP3A-F(BamHI) and OAP3A-R(XbaI). The PCR products were digested with XbaI and BamHI, and cloned into the pBI121 binary vector, where the 4'-glucuronidase (GUS) gene has been removed. For the preparation of 35S::DcOAG1 construct, the primers OAG1-F(Xhol) and OAG1-R(BamHI) were used to amplify the DcOAG1 coding region. The PCR products were digested with Xhol and BamHI, and inserted into the corresponding sites of a pGreen 0229-35S binary vector (Yu et al., 2004). For chimeric repressor constructs of DcOAP3A and DcOPI, a binary vector pGreen-SRDX with SRDX motif, a derived motif of SUPERMAN, was first prepared. SRDX was amplified from Arabidopsis genomic DNA using the primers SUPRD1(BamHI) and SUPRD2(XbaI). Because the resulting DNA fragment contained one internal XbaI restriction site, partial digestion with XbaI was preformed after complete digestion with BamHI. The resulting DNA fragment with the expected size of 89 bp was inserted into BamHI and XbaI sites of pGreen 0229-35S binary vector. cDNA fragments of DcOPI and DcOAP3 were amplified by primers OPI-Fus1(Xhol), OPI-Fus2(BamHI), OAP3A-Fus1(Xhol) and OAP3A-Fus2 (BamHI). After restriction digestion, the resulting fragments were inserted into Xhol and BamHI of pGreen-SRDX to be translationally fused with SRDX domain at the C terminus. Primers used for construction of the above vectors are listed in Table S3.

Arabidopsis transformation

Agrobacterium tumefaciens-mediated plant transformation was performed by the floral dipping method (Clough and Bent, 1998). Seeds of 35S::DcOAP3A and 35S::DcOPI transgenic plants were selected on MS medium supplemented with kanamycin, and placed at 23°C in a growth room under long-day conditions (16 h light). Seeds of 35S::DcOAG2, 35S::DcOAP3-SRDX and 35S::DcOPI-SRDX were sown directly on soil and screened by Basta selection.

Yeast two-hybrid analysis of protein–protein interaction

Two-hybrid analyses were preformed by the MATCHMAKER II system according to the manufacturer’s recommendation. Full-length cDNAs of DcOAP3A, DcOAP3B, DcOPI and DcOAP2 were cloned into pACT2 and pAS2-1 vector. Yeast strain Y190 was used. Selection for interaction was performed on medium lacking His and Leu, and supplemented with 10 mM 3-amino-1,2,4-triazole (Sigma-Aldrich, St. Louis, MO, USA). The interaction activity was quantified by β-galactosidase assay in liquid culture using o-nitrophenyl-β-D-galactoside (Sigma-Aldrich) as the substrate. Scanning electron microscopy

Freshly harvested plant materials were flash-frozen in liquid nitrogen. Examination of specimens was carried out with the (JSM-5600LV) electron microscope at 10 kV.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. cDNA and predicted protein sequence of DcOAP2.

Figure S2. cDNA and predicted protein sequence of DcOAP1.

Table S3 Primers used for gene cloning and construction of binary vectors

Table S4 Primers used for RT-PCR and generating probes for in situ hybridization

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References


Flower development in orchids


