The Duplicated B-class MADS-Box Genes Display Dualistic Characters in Orchid Floral Organ Identity and Growth

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Orchidaceae are an excellent model to examine perianth development because of their sophisticated floral architecture. In this study, we identified 24 APETALA3 (AP3)-like and 13 PISTILLATA (PI)-like genes from 11 species of orchids and characterized them into four AP3- and two PI-duplicated homologs. The first duplication event in AP3 homologs occurring in the early evolutionary history of the Orchidaceae gave rise to AP3A and AP3B clades. Further duplication events resulted in four subclades, namely AP3A1, AP3A2, AP3B1, and AP3B2, during the evolution of Orchidaceae. The AP3 paralogous genes were expressed throughout inflorescence and floral bud development. From the in situ hybridization results, we noticed that the transition timings from ubiquitous to constrained expression in floral organs for both clades are different. The transition point of expression from ubiquitous to constrained expression in floral organs (clades 1 and 2) was not observed until the late inflorescence and floral bud stages. In contrast, that for the AP3B1 clade (clades 3 and 4) was at the late floral organ primordia stage. In contrast, that for the AP3B1 clade (clades 3 and 4) was at the late floral organ primordia stage. In contrast, that for the AP3B1 clade (clades 3 and 4) was at the late floral organ primordia stage.

Keywords: B-class MADS-box • Floral growth • Floral organ identity • Gene duplication • Orchidaceae.

Abbreviations: AP3, APETALA3; BAC, bacterial artificial chromosome; FISH, fluorescence in situ hybridization; HOT, Homeotic Orchid Tepal; PI, PISTILLATA; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR

The nucleotide sequences reported in this paper have been submitted to the NCBI database under the following accession numbers: AfAP3-1, EU444018; AfPI-1, EU444019; AfPI-2, EU444020; BnAP3-1, EU444021; BnAP3-2, EU444022; BnAP3-3, EU444023; BnPI, EU444024; DenAP3-1, EU444025; DenAP3-2, EU444026; DenAP3-3, EU444027; DenPI, EU444028; GalAP3-1, EU444029; GalAP3-2, EU444030; GalAP3-3, EU444031; GalPI, EU444032; HpaPI-1, EU444033; HpaPI-2, EU444034; HpaPI-3, EU444035; LdAP3-1, EU444036; LdAP3-2, EU444037; LdPI, EU444038; LudPI-1, EU444039; LudPI-2, EU444040; OncAP3-1, EU444041; OncAP3-2, EU444042; OncAP3-3, EU444043; OncAP3-4, EU444044; OncPI, EU444045; PaphAP3-1, EU444046; PaphAP3-2, EU444047; PaphAP3-3, EU444048; PaphPI, EU444049; PtAP3-1, EU444050; PtAP3-2, EU444051; PtAP3-3, EU444052; PiPI, EU444053.

Introduction

Although the floral ABCDE model is generally thought to be ubiquitous in the core eudicots, studies of basal angiosperms, monocots and basal eudicots have demonstrated large variations in the patterns of gene expression (Stellari et al. 2004, Kramer and Hall 2005, Zahnd et al. 2005). A ‘sliding-boundary’ model and modified ABC model have been proposed to explain the development of petaloid sepals caused by an outward shift of gene expression (Kanno et al. 2003, Kramer and Jaramillo 2005). However, in some cases, variations in gene expression cannot be easily explained. For example, the expression of B-class genes extends to whorl 1 of the flowers in Asparagales species such as Agapanthus praecox (Nakamura et al. 2005),
Muscaria armeniacum (Nakada et al. 2006) and Phalaenopsis equestris (Tsai et al. 2004), whereas AOGLOA and AOGLOB (B-class genes) are expressed only in whorl 2 in Asparagus officinalis, which produces undifferentiated tepals (Park et al. 2004).

The Orchidaceae are divided into five subfamilies based on robust molecular evidence: Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae and Epidendroideae (Cameron 2004). Because members of the Apostasioideae largely develop lily-like flowers with three anthers, but without distinct lips, the family is sometimes considered to be a more primitive and separate subfamily. Orchids make up nearly 10% of angiosperms, and the high species diversity in orchids is largely due to their adaptation to specialized insect pollination (Dressler 1993). The family Orchidaceae is characterized by a fused gynoecium and androecium (the gynostemium or the column). In addition, two whorls of the perianth are sometimes indistinguishable, although in most cases one of the inner three petals becomes a highly modified organ known as a labellum or lip. Lips offer a landing platform for pollinators or function in mimicry (Dressler 1993, Rudall and Bateman 2002, Cozzolino and Widmer 2005).

The highly specialized and diverse forms of flowers in orchids have been excellent models for examining the complicated network of regulatory genes in floral morphogenesis. Three types of perianths of orchid flowers have been discovered, including normal (non-pelor), semi-pseudopelor and pseudopelor (Fig. 1) according to the similarities between the sepal and lateral petals in both naturally occurring and micropropagated populations. Although the perianth of orchids should be generally called tepals, the sepal and petal can be easily distinguished in most of the normal orchid perianth (Fig. 1A). A complete homeotic transformation and only a mild change can be observed in pseudopelor (Fig. 1C) and semi-pseudopelor (Fig. 1B), respectively. In pseudopeleric flowers, petals are transformed into organs that resemble lateral petals (Fig. 1C) defined as Type D pseudopelor previously (Mondragon-Palomino and Theissen 2009). However, perianth organs showing a mild transformation of sepal to lateral petals at a gradually changing level are found in the semi-pseudopelor flowers (Fig. 1B). The morphological features of sepal and petals are very similar; moderate differences in coloration, corrugation, shape and cell type distinguish these two floral organs. In orchids, a floral mutant with the morphological features of lateral petals with a keeled callus similar to that of a lip is referred as a peloric mutant (Rudall and Bateman 2002, Cubas 2004).

A set of biological processes, such as organ positioning, organ identity specification, cell proliferation, cell expansion, and cell differentiation and maturation, are coordinated by many regulators, including auxin, MADS-box transcription factor and TCP transcription factor in Arabidopsis (Dornelas et al. 2010). Members of the B-class MADS-box genes are reportedly involved in the continuous developmental program in orchids (Tsai et al. 2004, Tsai et al. 2005, Xu et al. 2006, Kim et al. 2007, Chang et al. 2010). So far, orchid B-class MADS-box genes have been characterized for P. equestris (Epipendroideae) (Tsai et al. 2004, Tsai et al. 2005), Dendrobium crumenatum (Epidendroideae) (Xu et al. 2006), Habenaria radiata (Orchidoideae) (Kim et al. 2007) and Oncidium Gower Ramsey (Chang et al. 2010). Members of MADS-box genes in P. equestris participate in various processes related to organ development. A PI-like gene, PeMADS6, is ubiquitously expressed in petaloid sepal, petals, column and ovary (Tsai et al. 2005). In contrast, four APETALLA3 (AP3)-like genes, PeMADS2, PeMADS3, PeMADS4 and PeMADS5 exhibit different expression patterns [e.g. PeMADS4 is expressed in the lip of orchid perianth (Tsai et al. 2004)]. These differences provide the first indication that at least four AP3-like paralogs are involved in a single species of monocots (Tsai et al. 2004). In addition, one PISTILLA (PI)-like homolog (DcOPI) and two AP3-like homologs (DcOAP3A/B) are identified in D. crumenatum (Xu et al. 2006), two PI-like homologs (HrGLO1 and HrGLO2) and one AP3-like homolog (HrDEF) in H. radiata (Kim et al. 2007), and one PI-like holog (OMADS8) and three AP3-like homologs (OMADS3, OMADS5 and OMADS9) in O. Gower Ramsey (Hsu and Yang 2002, Chang et al. 2010). Extended expression of HrDEF is detected in petaloid sepal of the petaloid-sepal mutant but not in wild-type sepal, so ectopic expression of a B-class MADS-box gene can change the organ identity of whorl 1 into whorl 2 in orchid flowers (Kim et al. 2007). In flowers of the peloric mutant of P. equestris, the appearance of the two lateral petals is completely converted...
into lip-like petals (Tsai et al. 2004). The changed expression of PeMADS4 in the lip-like petals of peloric mutants is always accompanied by the conversion of petals into lips within the same whorl in *Phalaenopsis* (Tsai et al. 2004). Moreover, an ‘orchid code’ was proposed, theorizing the combinational interaction of four DEF-like genes for the organ identity of the different perianth organs (Mondragon-Palomino and Theissen 2008, Mondragon-Palomino and Theissen 2009), and shifted expression of PeMADS4 in lip-like petals has also been reported in peloric *Phalaenopsis* (Mondragon-Palomino and Theissen 2011). The AP3B2 orthologous gene in *O. Gower Ramsey, OMADS3*, plays a potential role in cell proliferation and repressing lip development (Chang et al. 2010). However, the function of these genes in orchid floral organ development can only be revealed by addressing their expression patterns at early stages of development by in situ hybridization. Yet, the expression of B-class genes per se cannot be simply equated with their functions.

Previous studies have uncovered multiple paralogs and complex expression patterns of B-class genes in *Phalaenopsis* orchids, which led us to wonder what the roles for these genes really are. To assess whether the duplication of B-class genes detected in *P. equestris* is also true for all orchids for dictating their spectacular floral morphology, we identified and characterized orthologs of B-class genes in subfamilies of Orchidaceae. AP3-like and PI-like MADS-box genes from four subfamilies of Orchidaceae were identified and characterized. A total of 37 orchid B-class genes were isolated from 11 species collected from the field or from orchid nurseries and subjected to extensive phylogenetic analyses. Four AP3 paralogs in orchids resulted from two runs of duplication events in the ancestral AP3-like genes. Strong purifying selection was detected in both B-class genes. A dualistic model of perianth development addressing differences in the expression patterns of the homeotic B-class genes may have influenced the identity and growth of the floral organs, which is required if evolution in orchids is to keep pace with that in their pollinators.

### Results

#### Identification of four subclades of AP3-like genes and two clades of PI-like genes in Orchidaceae

A total of 24 AP3-like and 13 PI-like genes were identified from 11 species of orchids distributed in four subfamilies (Supplementary Table S1, Fig. S1). Multiple alignments of protein sequences of AP3 and PI homologs from Orchidaceae, Poaceae, Liliaceae, Arecales and the Eudicots showed that they have distinct C-terminal domains (Supplementary Fig. S2). The orchid AP3 homologs were divided into four subclades, namely AP3A1 (containing PeMADS3), AP3A2 (containing PeMADS4), AP3B1 (containing PeMADS2) and AP3B2 (containing PeMADS5) (Table 1; Supplementary Fig. S2A). Except for the AP3B2 orthologs with truncated ends, all the AP3 homologs contained both a PI-derived motif and a paleoAP3 motif at the end of the C-terminal region (Supplementary Fig. S2A). In contrast, the PI homologs shared a highly conserved sequence identity (86.0–99.0%) with the PI motif (Supplementary Fig. S2B). An insertion/deletion phenomenon was detected in four PI homologs in the subfamily Orchidoideae: a deletion of one amino acid at residue 166 of AfPI-1, LudPI-1 and HrGLO2, and an insertion of one amino acid at residue 193 of HrPI-2 and HrGLO2 (Supplementary Fig. S2B). Together, these results demonstrate the presence of four subclades of the AP3 homologs and two PI homologs in Orchidaceae.

### Supplementary Table S1

<table>
<thead>
<tr>
<th>Lineages</th>
<th>B-class MADs-box genes</th>
<th>AP3A1</th>
<th>AP3A2</th>
<th>AP3B1</th>
<th>AP3B2</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanilla pilifera (VA)*</td>
<td>GalAP3-2</td>
<td>GalAP3-3</td>
<td>GalAP3-1</td>
<td>ND*</td>
<td>GalPI</td>
<td></td>
</tr>
<tr>
<td>Vanilla pilifera (VA)</td>
<td>PaphAP3-1</td>
<td>PaphAP3-2</td>
<td>PaphPe2</td>
<td>ND</td>
<td>PaphPI</td>
<td></td>
</tr>
<tr>
<td>Paphiopedium Macabre (CY)</td>
<td>AfnAP3-1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>AfnPI-1, AfnPI-2</td>
<td></td>
</tr>
<tr>
<td>Anoectochilus formosanus (OR)</td>
<td>LdAP3-1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>LdPI-1, LdPI-2</td>
<td></td>
</tr>
<tr>
<td>Ludisia discolor (OR)</td>
<td>HrAP3-1</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>HrAP3-1, HrAP3-2</td>
<td></td>
</tr>
<tr>
<td>Habenaria petelotii (OR)</td>
<td>OncAP3-2</td>
<td>OncAP3-1</td>
<td>OncAP3-1</td>
<td>OncAP3-3-4</td>
<td>OncPI</td>
<td></td>
</tr>
<tr>
<td>Oncidium Gower Ramsey (EP)</td>
<td>LdAP3-2</td>
<td>ND</td>
<td>LdAP3-1</td>
<td>ND</td>
<td>LdPI</td>
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<td>Liparis distans (EP)</td>
<td>PeMADS4a</td>
<td>PeMADS4c</td>
<td>PeMADS2d</td>
<td>PeMADS5d</td>
<td>PeMADS6d</td>
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</tr>
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<td>Phalaenopsis equestris (EP)</td>
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<td>PtAP3-3</td>
<td>PtAP3-1</td>
<td>ND</td>
<td>PePI</td>
<td></td>
</tr>
<tr>
<td>Brassavola nodosa (EP)</td>
<td>BnAP3-3</td>
<td>BnAP3-1</td>
<td>BnAP3-2</td>
<td>ND</td>
<td>BnPI</td>
<td></td>
</tr>
<tr>
<td>Dendrobium Spring Jewel (EP)</td>
<td>DenAP3-2</td>
<td>DenAP3-3</td>
<td>DenAP3-1</td>
<td>ND</td>
<td>DenPI</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations are of subfamilies of Orchidaceae: CY, Cypripedioideae; EP, Epidendroideae; OR, Orchidoideae; and VA, Vanilloideae.
* An asterisk indicates orthologs not surveyed in this study.
* Orthologs cannot be identified by RACE and RT–PCR with degenerated primers.
* Sequence identified by Tsai et al. (2004, 2005).
Phylogenetic analyses of B-class MADS-box genes in Orchidaceae

To verify the phylogenetic relationships of orchid B-class genes, we analyzed the newly identified orchid B-class genes along with the homologs of other seed plants (mainly monocots) reported earlier (Supplementary Table S2) for resolving their phylogenetic relationships. Different analytical procedures, including Bayesian inference, maximum parsimony and Neighbor–Joining analyses, yielded consistent results (Fig. 2). The orchid AP3 homologs were separated into two major clades, AP3A and AP3B (Fig. 2A), and each was subdivided further into two subclades, AP3A1/AP3A2 and AP3B1/AP3B2 (Fig. 2A), in line with the results of sequence analysis shown in Supplementary Fig. S2. The classification of four subclades corresponds to the data described by Mondragon-Palomino and Theissen (2008, 2009). The subclades AP3A1, AP3A2, AP3B1 and AP3B2 correspond to orchid clades 3, 4, 1 and 2, respectively. To emphasize the relationship among these orthologs according to their origins resulting from duplication, we preferred to use the subclades AP3A1, AP3A2, AP3B1 and AP3B2 for the following. The four paralogous subclades could be the result of two duplication events throughout the evolution of the monocots and show no robust support for the Monocot AP3A and Monocot AP3B clades. The phylogenetic tree indicates that the AP3 duplications pre-dated the Orchidaceae family and may have pre-dated the origin of Asparagales. The secondary duplication probably occurred at or after the diversification of the orchids. According to the molecular phylogeny of the AP3A clade, orchid AP3A was shown as a sister group of AP3-like genes in Poaceae and Liliaceae families (Fig. 2A). However, clade 1/2 (AP3B clade) is clustered with Poales SILKY1-like genes (Mondragon-Palomino et al. 2009). The PI ortholog was present as a single-copy gene in Vanilloideae (GalPI and ViPI), and a monophyletic group of the Vanilloideae. PI genes appeared outside of other orchid PI orthologs. A duplication on the node gave rise to all other Orchidaceae PI genes (orchid PI-1 and PI-2), after which both Cypripedioideae and Epidendroideae probably lost one duplicated PI copy (orchid PI-1) (Fig. 2B). The topology of Cypripedioideae PI (PaphPI) seems unlike that of PhlonGLO1 (Mondragon-Palomino et al. 2009); PaphPI appeared as a group with Epidendroideae and Epidendroideae PI orthologs in the

Fig. 2 Phylogenetic trees of B-class MADS-box genes for the AP3 and PI lineages from Orchidaceae. Phylogenetic trees based on Bayesian inference (BI) were constructed for (A) PI and (B) AP3 genes, from Orchidaceae, other monocots, basal angiosperms and gymnosperms. The numbers above the branches are posterior probabilities from BI analysis (only posterior probabilities >0.50 are shown) and the numbers below the branches are Neighbor–Joining (NJ) analysis (only bootstrap values >50 are shown). Circles mark the lineages, clades and subclades of AP3-like genes as well as the clades of PI-like genes. Abbreviations to the right of the tree are of subfamilies of Orchidaceae: CY, Cypripedioideae; EP, Epidendroideae; OR, Orchidoideae; and VA, Vanilloideae.
PI-2 clade. Within the orchid PI-1 clade, all members were PI orthologs of Orchidoideae (Fig. 2B).

**Maximum-likelihood tests of selection for the B-class genes in orchids**

To assess selection pressure during the evolution of the lineage, we performed statistical tests for the ratio of non-synonymous (dN) substitutions to synonymous (dS) substitutions (ω = dN/dS). Maximum-likelihood tests revealed that the ω ratio changed significantly between the Orchidaceae and the rest of the angiosperm lineage. The ω ratio is close to 1 under neutral evolution, and divergence of the expected ratio points to purifying selection (ω < 1) or positive selection (ω > 1) (Yang and Bielawski 2000). For the AP3 lineage, we performed the maximum-likelihood test between the clades AP3A and AP3B (Table 2). The model that assumed different selection pressures for AP3A, AP3B and all other AP3 genes performed better than the model that assumed the same ω value (P < 0.001) for all. This test revealed that orchid AP3A and AP3B are under purifying selection. To be specific, the AP3B clade evolved under a more relaxed selection pressure (ω_{orchidAP3B} = 0.2275) than the AP3A clade (ω_{orchidAP3A} = 0.0353) did. Within clade AP3A, both AP3A1 and AP3A2 do not differ significantly in their ω ratios (Table 2). Similar results were obtained with the two subclades of the AP3B homologs, orchid AP3B1 and orchid AP3B2. Furthermore, the two-ratio model tests, which assume different values of ω between orchids and other PI-like genes, fitted the data significantly better than the one-ratio model, which assumes a constant selection pressure across all the B-class genes (ω). The two orchid PI subclades, orchid PI-1 (ω_{orchidPI-1} = 0.1175) and orchid PI-2 (ω_{orchidPI-2} = 0.0361) showed a significant difference in ω ratios (P < 0.001) (Table 2).

**Fluorescence in situ hybridization of the four subclades of orchid AP3 homologs on chromosomes of the Phalaenopsis genome**

To study whether the four duplicated AP3 homologs were located on the same or different chromosomes, each subclade of the orchid AP3 homologs was localized by fluorescence in situ hybridization (FISH) in the P. equestris genome. FISH was carried out with bacterial artificial chromosome (BAC) probes, and each probe corresponded to a distinct BAC representing one PeMADS gene. In the nucleus at the G2 stage, signals are presented as paired dots (Fig. 3). The results of FISH revealed that the four subclades of the orchid AP3 homologs were located on different chromosomes because various PeMADS genes were detected as separate pairs of signals in the bi-colored FISH (PeMADS2 and PeMADS3, Fig. 3A; PeMADS2 and PeMADS4, Fig. 3B; PeMADS2 and PeMADS5, Fig. 3D; and PeMADS4 and PeMADS5, Fig. 3G). In addition, AP3 and PI homologs were located on different chromosomes (PeMADS5 and PeMADS6, Fig. 3J, K). These results suggest that the four orchid AP3 homologs may result from genome duplications, and yet tandem duplications cannot be ruled out as their original source during the evolution of orchids.

**In situ hybridization of PeMADS2–PeMADS5 at the early floral development stage of Phalaenopsis**

To study the spatial expression of these genes, we dissected the inflorescence tip of P. equestris and performed detailed analysis of various floral development stages. In accordance with the floral organ developmental program described in Arabidopsis, *Phalaenopsis* shows fundamental similarities in inflorescence and floral development from early to late stages. Flowers originate from the shoot apex and undergo an exceedingly long period of identity, development and growth in most orchids. The *Phalaenopsis* inflorescence is racemose, and each flower subtended by its bract is axially on the rachis from the base upward (Fig. 4A). Floral primordia, floral buds and flowers are arranged in a spiral pattern on the twig (Fig. 4A).

On dissecting the tip of the inflorescence, about 11 floral primordia and floral buds constitute the inflorescence in *P. equestris*, and three major stages of inflorescence development are defined according to their various traits (Fig. 4B, C). Stage 1 corresponds to the floral primordia stage in which floral primordia initiate from the inflorescence meristem. Stage 2 corresponds to the early floral organ primordia stage, and the formation of sepal, petal, lip and column primordia occurs gradually (Fig. 4B, C). Stage 3 corresponds to the late stage of floral organ primordia formation. At stage 3 of inflorescence development, pollinia and callus have developed on the column and lip primordia, respectively (Fig. 4B, C). After the maturation of floral organ primordia, floral organs enlarge in size and protrude out of their accompanying bracts because of cell proliferation and expansion (Fig. 4A). At stage 4, five sub-stages of the floral bud stage are assigned according to the sizes of floral buds as previously described (Tsai et al. 2004).

The spatial expression patterns of PeMADS2–PeMADS5 were studied by in situ hybridization on longitudinal or transverse sections during floral primordia and floral organ primordia stages of the *Phalaenopsis* inflorescence (Fig. 4D–Z). Interestingly, the distinct expression patterns of the four AP3 orthologs in the late floral bud stage (Tsai et al. 2004) were not found for those in the early floral primordia stage and the floral organ primordia stage. Instead, the four duplicated AP3 paralogous genes were expressed throughout inflorescence development and floral growth from stage 1 to stage 3 (Fig. 4D–F, H–K, N–P, S, U, W–Y). At the beginning, signals of PeMADS genes were detected after the earliest stage of floral primordia emergence (stage 1) (Fig. 4D, I–J, O, U). Then, the transcripts of PeMADS2, PeMADS3, PeMADS4 and PeMADS5 were widely detected in both floral primordia and floral organ primordia developmental stages 2 and 3 (PeMADS2: Fig. 4F, H; PeMADS3: Fig. 4K, M, N; PeMADS4: Fig. 4P, Q, S and PeMADS5: Fig. 4W–Z). At stage 3, PeMADS3 was expressed in each floral organ primordium, including sepal, petal and lip (Fig. 4S). Expression of PeMADS3 (AP3A1 subclade) in lip and column primordia was first...
distinguished at the late floral organ primordia stage (stage 3) ([Fig. 4T](#)) and was very weakly expressed in sepals. In addition, restricted expression of PeMADS3 in the lip could only be found at the last floral organ primordia stage (stage 3) ([Fig. 4T](#)). The expression restricted to the lip and column of PeMADS4 was detected at the last floral organ primordia stage (stage 3) ([Fig. 4Z](#)). Moreover, PeMADS2–PeMADS5 transcripts accumulated predominantly in the epidermal cells during the late floral organ primordial stage ([Fig. 4H, N, T, Y](#)). The rearranged expression patterns of AP3 genes in Phalaenopsis showed asynchrony transition points from floral formation through floral organ differentiation and growth, and finally floral organ formation.

### Table 2: Maximum likelihood tests of selection for the B-class genes

<table>
<thead>
<tr>
<th>B-class genes</th>
<th>-ln likelihood</th>
<th>Γ = 2(lnL0 − lnL1)</th>
<th>P-value</th>
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<td><strong>AP3 lineage</strong></td>
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<td></td>
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<tr>
<td>One ratio</td>
<td>-2924.85 ((o = 0.103))</td>
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<td></td>
</tr>
<tr>
<td>Two ratio (orchid vs. all others)</td>
<td>-2923.204</td>
<td>3.29 (vs. AP3 one ratio)</td>
<td>&lt;0.1</td>
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<tr>
<td>All others (oAllotherAP3 = 0.1162)</td>
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<td>Orchid AP3 (oorchidAP3 = 0.0746)</td>
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<td><strong>Orchid AP3 A vs. B</strong></td>
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<tr>
<td>A = B (\neq) all others</td>
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<td><strong>Orchid AP3 A1 vs. A2</strong></td>
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<tr>
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<td>18.36 (vs. AP3 one ratio)</td>
<td>&lt;0.001</td>
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<td>OrchidAP3A1 = A2 (oorchidAP3A1 = 0.0353)</td>
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<tr>
<td>A1 (\neq) A2 (\neq) all others</td>
<td>-2915.518</td>
<td>0.304 (vs. A1 = A2 (\neq) all others)</td>
<td>&gt;0.5</td>
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<td>All others (oAllotherAP3 = 0.1258)</td>
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<td></td>
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<tr>
<td>OrchidAP3A1 (oorchidAP3A1 = 0.0396)</td>
<td></td>
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<tr>
<td>OrchidAP3A2 (oorchidAP3A2 = 0.0276)</td>
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<td><strong>Orchid AP3 B1 vs. B2</strong></td>
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<tr>
<td>B1 = B2 (\neq) all others</td>
<td>-2921.231</td>
<td>7.238 (vs. AP3 one ratio)</td>
<td>&lt;0.01</td>
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<tr>
<td>All others (oAllotherAP3 = 0.0922)</td>
<td></td>
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<tr>
<td>OrchidAP3B1 = B2 (oorchidAP3B1 = 0.2315)</td>
<td></td>
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<tr>
<td>B1 (\neq) B2 (\neq) all others</td>
<td>-2920.639</td>
<td>1.184 (vs. B1 = B2 (\neq) all others)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>All others (oAllotherAP3 = 0.0922)</td>
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<tr>
<td>OrchidAP3B1 (oorchidAP3B1 = 0.1866)</td>
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<tr>
<td>OrchidAP3B2 (oorchidAP3B2 = 0.3965)</td>
<td></td>
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<tr>
<td><strong>PI lineage</strong></td>
<td></td>
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<tr>
<td>One ratio</td>
<td>-9578.499 ((o = 0.082))</td>
<td></td>
<td></td>
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<tr>
<td>Two ratios</td>
<td>-9574.21</td>
<td>8.578 (vs. PI one ratio)</td>
<td>&lt;0.01</td>
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<tr>
<td>Orchid (\neq) monocots (=) all others</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>All others (oAllotherPI = 0.0875)</td>
<td></td>
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<tr>
<td>Orchid PI (oorchidPI = 0.0515)</td>
<td></td>
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<tr>
<td><strong>Three ratios</strong></td>
<td></td>
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<tr>
<td>Monocots (\neq) orchids</td>
<td>-9566.119</td>
<td>16.18 (vs. PI one ratio for orchids)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PI-1 (\neq) orchids (oMonocotsPI = 0.0874)</td>
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<tr>
<td>PI-2 (\neq) Vanilloideae (oorchidPI-1 = 0.0175)</td>
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<td></td>
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<tr>
<td>(oorchidPI-2 = 0.0361)</td>
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The rearranged expression patterns of AP3 genes in Phalaenopsis showed asynchronous transition points from floral formation through floral organ differentiation and growth, and finally floral organ differentiation and growth.
extension (Fig. 5). The transition point of PeMADS3 and PeMADS4 genes took place at the floral organ primordia stage (stage 3) (Fig. 5C, D, arrows), whereas that of PeMADS2 and PeMADS5 was at the floral bud stage (stage 4) (Fig. 5A, B, arrows). The dualistic transcriptional patterns may indicate the differentiated characters of AP3 homologs in floral organ identities and in floral development and growth.

Spatial expression profiles of orchid B-class MADS-box homologs at floral bud stages

Whether the duplicated orchid B-class MADS box genes that belong to the same subclade show similar expression profiles across the four subfamilies is of interest. For this purpose, we examined the expression patterns of the four subclades of AP3-like (AP3A1/3A2 and AP3B1/3B2) and PI-like genes in floral bud stages by semi-quantitative reverse transcription–PCR (RT–PCR) and real-time RT–PCR. As expected, similar to that of *P. equestris*, the orchid AP3 orthologous genes displayed diverse expression patterns at late floral bud stages. The expression patterns of the AP3A1 subclade were correlated with the perianth types (Fig. 6). According to similarities between the sepals and lateral petals, perianth types of *Paphiopedilum Macabre*, *Anoectochilus formosanus* and *Habenaria petelotii* are classified as normal (non-peloric), whereas that of the rest of the species in this study are semi-pseudopeloric or pseudopeloric (Supplementary Fig. S1). First, most AP3A1 orthologs (*OncAP3-2*, *DenAP3-2*, *PtAP3-2*, *LdAP3-2* and *BnAP3-2*) show extremely faint signals in the sepals of semi-pseudopeloric, and pseudopeloric tepals (Fig. 6A, rows 4–8). Therefore, AP3A1 orthologs may play a part in the homeotically transformed petaloid sepals of semi-pseudopeloric and pseudopeloric flowers and in lateral petals and lip but not sepals of...
Fig. 4 RNA in situ hybridization analysis of PeMADS2–PeMADS5 transcripts in the inflorescence stage of *Phalaenopsis*. Inflorescence and flower buds of *Phalaenopsis* (A–C). Photograph of a *Phalaenopsis* inflorescence and developing floral buds (A). The blue box represents the inflorescence stage (stages 1–3), the white arrow indicates the first floral bud (B1) at stage 4, and the green arrow indicates its bract (scale bar = 1 mm). (B) Schematic representation of a *Phalaenopsis* inflorescence. The light yellow color symbolizes inflorescence meristem; the light blue, blue, dark...
normal plants. Secondly, the expression of only two AP3A2 orthologs, DenAP3-3 and PtAP3-3, was limited in the lip and was absent in all other floral organs (Fig. 6B, rows 3 and 4). However, the other AP3A2 homologs showed varied expression profiles, either in columns or in sepals and petals (Fig. 6A, rows 1, 2, and 5). Alternatively, AP3B clade genes were expressed mainly in sepals and lateral petals, with occasional exceptions (Fig. 6C, rows 1–6). Three AP3B homologs, LdAP3-1, OncAP3-1 and BnAP3-2, were expressed in sepals and/or petals (Fig. 6C, rows 4–6), whereas the expression of the remaining three AP3B homologs (PaphAP3-3, OncAP3-4 and DenAP3-1) was relatively constant in all floral whorls during the development of floral buds (Fig. 6C, rows 1–3). All the PI orthologs (Fig. 6D), displaying extremely high similarity (86.0–99.0%), show uniformly and highly conserved expression profiles in all four floral organs, similar to that of PeMADS6 (Tsai et al. 2005). The expression profiles of orchid B-class MADS-box homologs in various floral organs at stage 4 of the floral bud stage were also confirmed by real-time quantitative RT–PCR. The transcript profiles of PaphAP3-1, OncAP3-2, DenAP3-2, BnAP3-3, OncAP3-1, BnAP3-2, OncPi and BnPi determined by real-time quantitative RT–PCR were consistent with those by semi-quantitative RT–PCR (Supplementary Fig. S3). Weak expression of OncAP3-2, DenAP3-2 and BnAP3-3 (Supplementary Fig. S3B–D) was detected in the sepals of semi-pseudopeloric and pseudopeloric flowers, whereas PaphAP3-1 showed extremely faint transcript levels in the sepals of normal (non-peloric) perianth (Supplementary Fig. S3A). For orchid AP3B homologs, we found relatively high expression of OncAP3-1 (Supplementary Fig. S3E) in both sepals and petals, and of BnAP3-2 only in sepals (Supplementary Fig. S3F).

**Analyses of homeotic changes in expression profiles of B-class MADS-box homologs associated with the development of peloric flowers**

Our earlier study showed complex protein–protein interactions among the four AP3s and one PI in various floral organs of *P. equestris* (Tsai et al. 2008). We wondered whether the expression profiles are altered and lead to the conversion of lateral petals into lip-like petals in the peloric mutants (Fig. 7). In three different pairs of normal *Phalaenopsis* plants and their peloric mutants, we compared the expression of PeMADS2–PeMADS6 in various floral organs of stage 4 of floral buds using real-time quantitative RT–PCR analysis (Fig. 7A–F). In normal plants, PeMADS2 and PeMADS5 are expressed in all floral organs, whereas signals of PeMADS5 are weaker than those of PeMADS2. In addition, we detected slightly higher transcript levels in sepals and petals than in the lip and column. PeMADS3 was expressed in lateral petals, lip and column, but was undetectable or negligible in sepals. PeMADS4 was expressed in the lip and column but was undetectable or negligible in the first two whorls. Moreover, transcripts of PeMADS6 are almost ubiquitous in all floral organs. Although all the PeMADS2–PeMADS6 genes are expressed in the lip and column, the expression of PeMADS3 was significantly higher in the lip than in the column. In the *Phalaenopsis* peloric mutants, the transcriptional patterns of these five genes in sepals, lip and column were not affected, which is consistent with the observation that the morphological features of these organs are nearly unaffected in the peloric mutants. Nevertheless, the expression of PeMADS4 was detected in lip-like petals (arrows, in Fig. 7B, D, F). In summary, even though the morphology of sepals and lateral petals is similar in orchids, the differential expression of PeMADS3 (AP3A1 subclade) between sepals and petals is crucial. In addition, the modified petal of the lip shows the differential expression of PeMADS4 (AP3A2 subclade) in lateral petals distinct from that in the lip. To test the strong correlation of AP3A2 genes and lip formation and development in Orchidaceae, we further examined the expression patterns of the AP3A2 ortholog in a pair of normal *Cymbidium ensifolium* plants and its peloric mutant at stage 4 of the floral bud stage (Fig. 7G, H). The AP3A2 subclade gene is expressed only in the lip of normal *Cymbidium* tepals (Fig. 7G, lane 3). As expected, the expression of the AP3A2 subclade gene extended to the lip-like petals in the peloric mutant of *Cymbidium* (Fig. 7H, lane 6). This result strongly supports our previous observations in *Phalaenopsis* of the involvement of AP3A2 in orchid lip morphogenesis. Therefore, AP3A2 orthologs playing a notable role in lip formation was strongly supported by further evidence of their specific transcripts in both lip and lip-like petals during floral bud growth in *Phalaenopsis* and *Cymbidium* peloria mutants.
Discussion

Detecting the selection of orchid B-class MADS genes

The orchid Pi-like homologs show greater similarities in their sequences than do AP3-like homologs. Both Pi-like and AP3-like homologs have evolved under a different selection pressure (measured by ratios), observations that are consistent with earlier studies of Pi-like genes in general (Hernández-Hernández et al. 2007). Furthermore, both orchid AP3A and AP3B clades show significantly different selection pressures, which suggests that these two duplicated lineages are selected for different functions. However, we detected no differential selection pressure within the two subclades of orchid AP3A (AP3A1 and AP3A2) or AP3B (AP3B1 and AP3B2), so that the two pairs of AP3 subclade paralogs may have a high functional redundancy or overlapping expression domains. These selection results at the subclade level are inconsistent with those estimated in the literature (Hernández-Hernández et al. 2007). Our results show the same selection pressure at the subclade level (i.e. between AP3A1 and AP3A2, and between AP3B1 and AP3B2).

Heterologous systems are an alternative method for non-model plants for indirectly studying functional roles of orchid B-class genes. In previous studies, the functions of various orchid B-class genes identified in *Phalaenopsis*, *Oncidium* and *Dendrobium* have been examined by heterologous expression in other plants. For example, overexpression of *PeMADS6* in Arabidopsis which showed flowers with petaloid sepals, and with a 3- to 4-fold increase in flower longevity. Concomitantly, fruit maturation was also delayed in the transgenic Arabidopsis (Tsai et al. 2005). However, ectopic overexpression of *PeMADS2, PeMADS3, PeMADS4* and *PeMADS5* did not produce any morphological changes in transgenic Arabidopsis plants (W. C. Tsai et al. unpublished data). Overexpression of *PhPI15* in tobacco plants produced morphological changes with male-sterile phenotypes (Guo et al. 2007). In the case of *Oncidium*, heterologous expression of *OMADS3* and *OMADS8*, paralogous genes of *PeMADS5* and *PeMADS6*, was studied in Arabidopsis. 35S::*OMADS3* transgenic plants show novel phenotypes by producing terminal flowers at the end of the
inflorescence (Hsu and Yang 2002). Similar to the phenotype that is observed in the ectopic expression of PeMADS6 in transgenic Arabidopsis, homeotic conversion of the first whorl of green sepals into white petal-like structures was found in these 35S::OMADS8 flowers (Chang et al. 2010). In Dendrobium, overexpression of DcOPI and DcOAP3A has been performed in wild-type and mutant Arabidopsis (Xu et al. 2006). Rescue of pi-1 by 35S::DcOPI reveals that the sepals are transformed into petaloid structures, petals are rescued, and organs in the third and the fourth whors are transformed into normal stamens and carpeloid stamen or pistils, respectively. However, 35S::DcOAP3A in ap3-3 also showed phenotypes indistinguishable from ap3-3. Dominant repression transgenic lines DcOPI-SRDX and DcOAP3A-SRDX show various degrees of phenotypes like the function B null mutants in Arabidopsis (Xu et al. 2006). These observations in these studies indicated that orchid B-class genes may specify floral organ identity in orchids. However, the changes in floral morphology of heterologous plants may not represent the actual developmental events in orchid due to the lack of other orchid MADS-box proteins to make up orchid multimeric protein complexes. Further functional analysis of genes involved in regulating the identity of floral organs in orchids by using loss-of-function mutations or virus-induced gene silencing (VIGS) is necessary. Such an analysis can elucidate the regulatory network at the molecular level that governs morphogenesis in orchids and provide a physical basis for genetic engineering in orchids. Knockdown expression of floral MADS-box genes simultaneously in Phalaenopsis by inoculating PeMADS6 at a conserved region among MADS-box genes with Cymbidium mosaic virus (CymMV) transcripts indicates that the flower organ shows greenish streaks and patches in the silenced plants which suggests a conversion of reproductive tissue to vegetative tissue (Lu et al. 2007). Moreover, down-regulation of PeMADS6 expression leads to severe phenotypic effects in the floral perianth of Phalaenopsis by using a modified optimal method in the VIGS plants (M. H. Hsieh et al. unpublished data). Homeotic developmental changes of the perianth with a tendency toward phyllody representing an alteration of floral organ identities were supported by the reduced expressions of target (PeMADS6) and

Fig. 6 Expression patterns of AP3- and PI-like genes in orchids at the floral stage. Semi-quantitative RT–PCR was performed for analysis of expression of the B-class homologs of AP3A1 (A), AP3A2 (B), AP3B1/2 (C) and PI (D) in various floral organs. Actin (E) was an internal control. Se, sepal; Pe, lateral petal; Li, lip; Co, column.
Fig. 7 Ectopic expression of AP3A2 orthologs in peloric flowers of Phalaenopsis and Cymbidium. (A–F) Three pairs of normal plants and their peloric mutant flowers of P. equestris. (A, B) Diploid P. equestris (S82-159) and its peloric mutant (S85-355); (C, D) Tetraploid P. equestris ‘King Car’ and its peloric mutant. (D) One pair of C. ensifolium and its peloric mutant flower (scale bar = 1 cm). Real-time quantitative RT–PCR analysis of PeMADS2–PeMADS6 in three pairs of dissected floral organs at stage 4 of the floral bud stage in Phalaenopsis. Green bar, PeMADS2; grass green bar, PeMADS5; yellow bar, PeMADS3; red bar, PeMADS4; blue bar, PeMADS6. Arrows in B, D and (F) indicate the ectopic expression of PeMADS4 in the lip-like petals of peloric mutants. (G, H) RT–PCR analysis of the transcript levels of the AP3A2 clade gene expressed in the lip (lane 3) of wild-type Cymbidium ensifolium tepals at stage 4 of the floral bud stage, and extending to the lip-like petals in the peloric mutant of Cymbidium (lane 6). Se, sepal; Pe, lateral petal; Li, lip; Co, column; Lp, lip-like petal.
non-target B-class MADS-box genes (PeMADS2-5) (M. H. Hsieh et al. unpublished data).

Dualistic transcriptional patterns of AP3-like genes in orchids

The fact that the combinatorial expression profiles of orchid AP3-like genes span the whole floral organ development program may indicate their roles in biological processes, through organ identity specification, cell proliferation, cell expansion, and cell differentiation and maturation (Dornelas et al. 2010). Of note, the transition points of transcripts from ubiquitous expression patterns to confined floral organs differ among the duplicated AP3 paralogs in Phalaenopsis. In this paper, results from in situ hybridization and Northern blots (Tsai et al. 2004) revealed that Phalaenopsis AP3-like genes are expressed regionally in particular floral organs to determine floral organ identity at the floral organ primordia stage and to promote floral growth and development at the floral bud stage. The expression of AP3A genes was confined to the inflorescence stage when floral formation begins, whereas that of AP3B homologs was detected at the floral bud stage that occurs after the onset of floral organ differentiation and volume expansion. The major critical transition points represented by the asynchrony of relocated expression in duplicated AP3 paralogs implies their dualistic roles in floral organ specification and indicates that the shifting patterns of AP3 genes may determine the fate of orchid perianth growth and development both temporally and spatially.

The specific tendency of AP3 genes in orchid perianth development

The morphological features of the orchid perianth have developed into five tepal-like floral organs and a distinct lip during evolution. The as yet unknown molecular mechanism in regulation of specific floral organ formation may be uncovered by gene duplication and subsequent sub- and/or neofunctionalization. The duplicated and divergent genes may result from alteration of the promoter region and/or changes in the coding sequence by mutation following a shift in regulation of expression (Force et al. 1999, Moore et al. 2005). Our results revealed that the orchid duplicated AP3A and AP3B show significantly different selection pressure and distinct expression profiles. Through this, gene functions can evolve to regulate floral organ morphogenesis; for example, the formation of the lip may be contributed by the duplication of B-class MADS-box genes in orchids. Although the orchid tepal may seem to have extremely similar organs, results from in situ hybridization show that the dualistic patterns of AP3A genes discriminated the intrinsic qualities between the sepals and the petals since the early floral development stage.

This study revealed that the shifted homeotic and distinct expression of AP3A2 homologs was detected in the lip-like petals of peloric mutants of *P. equestris* and *C. ensifolium*. Similar observations were reported in the petaloid-sepal mutant of *H. radiata*, whereby greenish sepals were changed to white lip-like organs because of the extended expression of *HrDEF*, an AP3A2 homolog (Kim et al. 2007). In addition, shifted expression of PeMADS4 is detected in lip-like petals of peloric Phalaenopsis (Mondragon-Palomino and Theissen 2011). Complete peloric mutants have been reported in natural populations of both Rhipoepedium rothschildianum (Cypripedioideae) and Cattleya (Epidendroideae) (Rudall and Bateman 2003). Peloric mutants seem to broadly exist in natural populations of the above subfamilies of Orchidaceae, and they may indeed result from ectopic expression of the single orchid AP3A2 homologs. These observations set the foundations of the subfunctionalization of orchid AP3A genes by extending their expression. The diversification of PeMADS4 (AP3A2 subclade) during evolution may have been a major driving force behind the development of different types of petal organs. Therefore, the orchid AP3A2 gene plays an essential role in specifying the development of the lip. The zygomorphic expression of the AP3A2 homologs is associated with the highly modified perianth development in Orchidaceae. These results indicate that the duplicated factors modify the expression patterns of original B-class genes in species with tepal flowers and represent the restoration of their function in orchid perianth development.

‘Homeotic Orchid Tepal’ model: a molecular regulatory mechanism of orchid floral development.

In model plants, such as Arabidopsis and Antirrhinum, transcripts of AP3 and PI are typically expressed in petals and stamens, following the well-known ‘ABC model,’ in which homologs of B-class genes are broadly expressed in tepals, stamens and carpels in basal angiosperms (Soltis et al. 2007). The AP3-like genes from tepal-like monocots such as lily and tulips also show similar expression patterns in the outer three whorls, which supports the ‘modified ABC model’ (Tseng and Yang 2001). However, floral development of all angiosperm species cannot be commonly and perfectly explained by these two models. For investigation of the evolutionary history of the perianth, developmental genetic data were used to infer the ancestral perianth character state for different angiosperm clades (Hileman and Irish 2009). The monocot ancestor of the AP3 homolog may have been regionally but weakly expressed in perianth organs or ubiquitous in perianth organs from early through late stages of floral development (Hileman and Irish 2009). The results of our study show that the expression profiles of AP3A-like genes in orchids are restricted to specific floral organs and different stages, whereas AP3B-like genes transiently show ubiquitous expression in the inflorescence stage and are expressed in distinct floral organs only in the floral bud stage. This finding raised the discrepancy between the transition time for determination of floral organ identity (stage 3 of the inflorescence stage) and for the differential expression of AP3 genes. Two possibilities are evoked to
elucidate the asynchronous phenomenon of re-localized transcripts of AP3-like genes following the various floral identity stages. First, except for AP3-like MADS-box genes, other factors specifically determined the floral organ identities in the orchid perianth. Based on this concept, a gene of the AP1/AGL9 group of MADS-box genes, OMADS1, showing high homology to AGL6, is expressed in the apical meristem, lip and carpel (Hsu et al. 2003). Moreover, OMADS1 can interact with OMADS3, which belongs to the AP3B2 subclade (Hsu et al. 2003). In addition, a putative paleoAP1 gene, OMADS10, showed a specific expression pattern in the lip and carpel (Chang et al. 2009). Further complications arise from the regulatory functions of the five B-class PeMADS proteins involved in the formation of complexes with various combinations of the duplicated homologs (Tsai et al. 2008). These reports suggest that specific expression patterns for the duplicated AP3 and PI homologs and other AGL6-like genes may account for the multiple protein–protein interactions, which specify the identity of different floral organs, especially petals and lips, in orchids. Thus, the lip growth in the floral bud stage may not be completely determined by AP3A2 genes.

The other possibility explains that the floral organ identities of orchid perianth are determined by two gradual progressions from inflorescence through floral bud stages. The floral primordia are not discriminated into sepal primordia and inner tepal primordia until the first transition point (AP3A) during the late inflorescence stage, whereas the petal and lip tissue are categorized after the second transition point (AP3A2 and AP3B) in the floral bud stage. If this is the case, the differences in morphological features among sepals, lateral petals and the lip in Orchidaceae lie in the combination of the duplicated B-class genes and other factors. Thus, a ‘Homeotic Orchid Tepal’ (HOT) model is proposed to explain the regulation of perianth morphogenesis in Orchidaceae in both spatial and temporal conditions (Fig. 8A, B). In this model, various floral identities of the orchid perianth are contributed by all members of B-class MADS-box genes at the early inflorescence stage (Fig. 8A). However, the fact that the distinct expression of B-class MADS-box genes occurs at the late inflorescence and floral bud stages was denoted by restricted transcripts of PeMADS3 and PeMADS4 after the transition point (Fig. 8B). PI and AP3B clades determine the formation of sepals. The combination of PI and both AP3A1 and AP3B controls the formation of the lateral petals. Both PI and AP3A2 clade genes (and/or other MADS-box genes, such as AGL6-like, SQUAMOSA (SQUA)-like or unknown genes) control the formation of the lip. Green, AP3B1; grass green, AP3B2; yellow, AP3A1; red, AP3A2; blue, PI-like genes; purple, C- and D-class genes; and pink, other MADS genes. Se, sepal; Pe, lateral petal; Li, lip; Co, column.

Phalaenopsis (Chen et al. 2007) have been identified, but these genes do not perform the A-function role in determining the identities of floral organs. Instead, they maintain their ancestral role of determining the identity of the meristem (Chen et al. 2007).

In general, the expression patterns in the flower bud stage of the HOT model (Fig. 8B) we propose were consistent with those of the revised ‘orchid code’ hypothesis based on the patterns at the floral bud stage (Mondragon-Palomino and Theissen 2011). Yet, the HOT model focuses not only on the flower bud stage but also on the floral primordia stage. According to the proposed HOT model, the combinatorial effects of homeotic proteins can form high-order complexes of multimers with more components than the proposed quartet model for ABCDE MADS-box proteins (Theissen 2001). This novel multimerization model appears to demonstrate the extraordinary evolutionary history of floral zygomorphic morphogenesis in orchids. Yet, there are still missing components waiting to be identified and their functions elucidated.

Materials and Methods

Plant materials

Eleven species of orchids were selected from four subfamilies of Orchidaceae collected from orchid nurseries and from the wild
for de novo identification of B-class MADS box genes: Vanilloideae (VA; Galeola falconeri, Vanilla pilifera Holttum); Cypripedioideae (CY; Paphiopedilum Macabre); Orchidoideae (OR; Anoectochilus formosanus, Ludisia discolor and Habenaria petelotii); and Epidendroideae (EP; Oncidium Gowen Ramsey, Liparis distans, Phaius tankervilliae, Brassavola nadora and Dendrobium Spring Jewel). Each of the species is shown in Supplementary Fig. S1, and voucher information is given in Supplementary Table S1.

Normal plants of P. equestris and its somaclonal variants of the peloric mutant were analyzed for gene expression patterns of the five B-class MADS box genes. Three pairs of wild-type and peloric mutant plants were recruited, namely a diploid wild-type P. equestris (S82-159), with red petals and an orange lip, and its peloric mutant (S85-355); a tetraploid P. equestris ‘King Car’ and its peloric mutant; and P. sp. ‘A3947’ and its peloric mutant (Fig. 7A–F). In addition, one pair of normal plants of C. ensifolium and its peloric mutant were analyzed for AP3A2 ortholog expression (Fig. 7G, H). All the peloric mutant flowers show the phenotypic conversion of lateral petals into lip-like petals. Detailed information on plant collections and growth conditions is given in the Supplementary text. All plants were grown in a greenhouse at the National Cheng Kung University (NCKU) under natural light (photosynthetic photon flux density of 90 μmol m⁻² s⁻¹) and controlled temperature (23–27°C).

Cloning and characterization of the orchid AP3-like and PI-like homologs

Total RNA was extracted as described earlier (Tsai et al. 2004). For the small samples of plant materials such as A. formosanus, L. discolor and H. petelotii, RNA was extracted by use of the RNeasy Plant Mini Kit (QIAGEN). To identify B-class MADS-box genes from various orchids, we used rapid amplification of cDNA ends (RACE) and RT–PCR with degenerated primers (Supplementary Table S3) to isolate partial or full-length cDNAs. The methods and primers used for 3'–RACE and PCR are described in the Supplementary text. The PCR-amplified products were cloned into a pGEM-T Easy vector (Promega). For DNA sequencing, 10–12 positive clones were randomly selected.

Phylogenetic analyses

Two data sets for B-class genes were constructed. The PI data set comprised 74 sequences and the AP3 data set 63 sequences (Supplementary Table S2). Full-length (and some partial length) DNA sequences were aligned by use of CLUSTALX (Thompson et al. 1997) (Supplementary Figs. S4, S5) with the default options and then used for nucleotide alignment justification by RevTrans (Wernersson and Pedersen 2003). Phylogenetic analyses involved Bayesian inference with MrBayes 3.0b4 (Huelserbeck and Ronquist 2001), and Neighbor–Joining and maximum parsimony methods involved the use of PAUP* 4.0b10 (Swofford 2002). Detailed information is provided in the Supplementary text.

Maximum-likelihood tests of selection

The branch lengths with maximum likelihood were calculated by use of PAUP* 4.0b10 (Swofford 2002) under the GTR + I + G model on the basis of the Bayesian inference tree obtained. A tree-base maximum-likelihood approach was used, as implemented in phylogenetic analysis by maximum likelihood (PAML) (Yang 1997), to test changes in selection in Orchidaceae. Comparative sets are described in the Supplementary text.

Fluorescence in situ hybridization

Chromosome preparation is described in the Supplementary text. FISH was performed as described (Chung et al. 2008), with minor modifications. Chromosome preparation consisted of treatment with pepsin (12.5 mg ml⁻¹ in 10 mM HCl) at room temperature for 60 min to remove the cell debris covering the chromosomes. Individual BAC clones (1045F09, 2050C15, 2053A18, 2016E10-B and 1031M24) representing each AP3 paralog (PeMADS2, PeMADS3, PeMADS4, PeMADS5 and PeMADS6) were used as probes (provided by Dr. W. L. Wu, Department of Life Sciences, NCKU). Detailed information on FISH is provided in the Supplementary text.

RNA in situ hybridization

Phalaenopsis equestris inflorescences, excluding floral buds, were fixed in fixation buffer (4% paraformaldehyde and 0.5% glutaraldehyde) at 4°C for 16–24 h. Inflorescence materials were dehydrated through a graded ethanol series, embedded in Histoplast and sectioned at 6–8 μm with use of a rotary microtome (MICROM, HM 310, Walldorf). Tissue sections were deparaffinized with xylene, rehydrated through an ethanol series, and pre-treated with proteinase K (2 μg ml⁻¹) in 1 x phosphate-buffered saline (PBS) at 37°C for 30 min. Pre-hybridization and hybridization followed previous protocols (Tsai et al. 2005). DNA substrates containing a partial C-terminal region and the 3'-untranslated region were used for riboprobe synthesis. PCR products amplified with primers are listed in Supplementary Table S3. The resulting PCR fragments were used as templates for synthesis of both antisense and sense riboprobes with digoxigenin-labeled UTP-DIG (Roche Applied Science) and the T7/SP6 Riboprobe in vitro Transcription System (Promega) following the manufacturer’s instructions.

RT–PCR analyses

RNA samples were extracted from different floral organs at the floral bud stage. Total RNA was treated with RNase-free DNase (NEB) to remove contamination with genomic DNA. First-strand cDNA was synthesized by use of SuperScript III reverse transcriptase (Invitrogen) from total RNA of the dissected floral organs, including sepals, lateral petals, lip and column. Semi-quantitative PCR amplification involved 25 and 30 cycles with specific forward and reverse primers located within the partial C-terminal region (Supplementary Table S3).
Real-time quantitative RT–PCR

The primers for real-time RT–PCR were designed by use of Primer Express (Applied Biosystems); gene-specific primers for PeMADS2, PeMADS3, PeMADS4, PeMADS5 and PeMADS6 are given in Supplementary Table S3. Real-time RT-PCR used SYBR GREEN PCR Master Mix (Applied Biosystems) with the ABI 7500 Real-Time PCR Instrument (Applied Biosystems). Experiments were carried out in triplicate and repeated at least three times independently. Data were analyzed by use of the sequencing detection software (Version 1.2.2, Applied Biosystems).

Supplementary data

Supplementary data are available at PCP online.

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References


