Characterization of the Possible Roles for B Class MADS Box Genes in Regulation of Perianth Formation in Orchid[1][C]

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To investigate sepal/petal/lip formation in *Oncidium* Gower Ramsey, three paleo*APETALA3* genes, *O*. Gower Ramsey *MADS box gene5* (*OMADS5*; clade 1), *OMADS3* (clade 2), and *OMADS9* (clade 3), and one *PISTILLATA* gene, *OMADS8*, were characterized. The *OMADS8* and *OMADS3* mRNAs were expressed in all four floral organs as well as in vegetative leaves. The *OMADS9* mRNA was only strongly detected in petals and lips. The mRNA for *OMADS5* was only strongly detected in sepals and petals and was significantly down-regulated in lip-like petals and lip-like sepals of peloric mutant flowers. This result revealed a possible negative role for *OMADS5* in regulating lip formation. Yeast two-hybrid analysis indicated that OMADS5 formed homodimers and heterodimers with OMADS3 and OMADS9. OMADS8 only formed heterodimers with OMADS3, whereas OMADS3 and OMADS9 formed homodimers and heterodimers with each other. We proposed that sepal/petal/lip formation needs the presence of *OMADS3/8* and/or *OMADS9*. The determination of the final organ identity for the sepal/petal/lip likely depended on the presence or absence of *OMADS5*. The presence of *OMADS5* caused short sepal/petal formation. When *OMADS5* was absent, cells could proliferate, resulting in the possible formation of large lips and the conversion of the sepal/petal into lips in peloric mutants. Further analysis indicated that only ectopic expression of *OMADS8* but not *OMADS5/9* caused the conversion of the sepal into an expanded petal-like structure in transgenic Arabidopsis (*Arabidopsis thaliana*) plants.

The ABCDE model predicts the formation of any flower organ by the interaction of five classes of homeotic genes in plants (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994; Jofuku et al., 1994; Pelaz et al., 2000, 2001; Theißen and Saedler, 2001; Pinyopich et al., 2003; Ditta et al., 2004; Jack, 2004). The A class genes control sepal formation. The A, B, and E class genes work together to regulate petal formation. The B, C, and E class genes control stamen formation. The C and E class genes work to regulate carpel formation, whereas the D class gene is involved in ovule development. MADS box genes seem to have a central role in flower development, because most ABCDE genes encode MADS box proteins (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Purugganan et al., 1995; Rounsley et al., 1995; Theißen and Saedler, 1995; Theißen et al., 2000; Theißen, 2001).

The function of B group genes, such as *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), has been thought to have a major role in specifying petal and stamen development (Jack et al., 1992; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996; Kramer et al., 1998; Hernandez-Hernandez et al., 2007; Kanno et al., 2007; Whipple et al., 2007; Irish, 2009). In Arabidopsis (*Arabidopsis thaliana*), mutation in *AP3* or *PI* caused identical phenotypes of second whorl petal conversion into a sepal structure and third flower whorl stamen into a carpel structure (Bowman et al., 1989; Jack et al., 1992; Goto and Meyerowitz, 1994). Similar homeotic conversions for petal and stamen were observed in the mutants of the *AP3* and *PI* orthologs from a number of core eudicots such as *Antirrhinum majus*, *Petunia hybrida*, *Gerbera hybrida*, *Solanum lycopersicum*, and *Nicotiana benthamiana* (Sommer et al., 1990; Tröbner et al., 1992; Angenent et al., 1993; van der Krol et al., 1993; Yu et al., 1999; Liu et al., 2004; Vandenbussche et al., 2004; de Martino et al., 2006), from basal eudicot species such as *Papaver somniferum* and *Aquilegia vulgaris* (Drea et al., 2007; Kramer et al., 2007), as well as from monocot species such as *Zea mays* and *Oryza sativa* (Ambrose et al., 2000; Nagasawa et al., 2003; Prasad and Vijayraghavan, 2003; Yadav et al., 2007; Yao et al., 2008). This indicated that the function of the B

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class genes *AP3* and *PI* is highly conserved during evolution.

It has been thought that B group genes may have arisen from an ancestral gene through multiple gene duplication events (Doyle, 1994; Theißen et al., 1996, 2000; Purugganan, 1997; Kramer et al., 1998; Kramer and Irish, 1999; Lamb and Irish, 2003; Kim et al., 2004; Stellari et al., 2004; Zahn et al., 2005; Hernandez-Hernandez et al., 2007). In the gymnosperms, there was a single putative B class lineage that duplicated to generate the paleo*AP3* and *PI* lineages in angiosperms (Kramer et al., 1998; Theißen et al., 2000; Irish, 2009). The paleo*AP3* lineage is composed of *AP3* orthologs identified in lower eudicots, magnolid dicots, and monocots (Kramer et al., 1998). Genes in this lineage contain the conserved paleoAP3- and PI-derived motifs in the C-terminal end of the proteins, which have been thought to be characteristics of the B class ancestral gene (Kramer et al., 1998; Tzeng and Yang, 2001; Hsu and Yang, 2002). The *PI* lineage is composed of *PI* orthologs that contain a highly conserved PI motif identified in most plant species (Kramer et al., 1998). Subsequently, there was a second duplication at the base of the core eudicots that produced the eu*AP3* and *TM6* lineages, which have been subject to substantial sequence changes in eudicots during evolution (Kramer et al., 1998; Kramer and Irish, 1999). The paleoAP3 motif in the C-terminal end of the proteins was retained in the *TM6* lineage and replaced by a conserved euAP3 motif in the eu*AP3* lineage of most eudicot species (Kramer et al., 1998). In addition, many lineage-specific duplications for paleo*AP3* lineage have occurred in plants such as orchids (Hsu and Yang, 2002; Tsai et al., 2004; Kim et al., 2007; Mondragón-Palomino and Theißen, 2008, 2009; Mondragón-Palomino et al., 2009), Ranunculaceae, and Ranunculales (Kramer et al., 2003; Di Stilio et al., 2005; Shan et al., 2006; Kramer, 2009).

Unlike the A or C class MADS box proteins, which form homodimers that regulate flower development, the ability of B class proteins to form homodimers has only been reported in gymnosperms and in the paleo-*AP3* and *PI* lineages of some monocots. For example, LMADS1 of the lily *Lilium longiflorum* (Tzeng and Yang, 2001), OMADS3 of the orchid *Oncidium* Gower Ramsey (Hsu and Yang, 2002), and PeMADS4 of the orchid *Phalaenopsis equestris* (Tsai et al., 2004) in the paleo*AP3* lineage, LRGLOA and LRGLOB of the lily *Lilium regale* (Winter et al., 2002), TGGLO of the tulip *Tulipa gesneriana* (Kanno et al., 2003), and PeMADS6 of the orchid *P. equestris* (Tsai et al., 2005) in the *PI* lineage, and GGM2 of the gymnosperm *Gnetum gnemon* (Winter et al., 1999) were able to form homodimers that regulate flower development. Proteins in the eu*AP3* lineage and in most paleo*AP3* lineages were not able to form homodimers and had to interact with PI to form heterodimers in order to regulate petal and stamen development in various plant species (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Riechmann et al., 1996; Moon et al., 1999; Winter et al., 2002; Kanno et al., 2003; Vandenbussche et al., 2004; Yao et al., 2008). In addition to forming dimers, AP3 and PI were able to interact with other MADS box proteins, such as SEPALLATA1 (SEP1), SEP2, and SEP3, to regulate petal and stamen development (Pelaz et al., 2000; Honma and Goto, 2001; Theißen and Saedler, 2001; Castillejo et al., 2005).

Orchids are among the most important plants in the flower market around the world, and research on MADS box genes has been reported for several species of orchids during the past few years (Lu et al., 1993, 2007; Yu and Goh, 2000; Hsu and Yang, 2002; Yu et al., 2002; Hsu et al., 2003; Tsai et al., 2004, 2008; Xu et al., 2006; Guo et al., 2007; Kim et al., 2007; Chang et al., 2009). Unlike the flowers in eudicots, the nearly identical shape of the sepals and petals as well as the production of a unique lip in orchid flowers make them a very special plant species for the study of flower development. Four clades (1–4) of genes in the paleo*AP3* lineage have been identified in several orchids (Hsu and Yang, 2002; Tsai et al., 2004; Kim et al., 2007; Mondragón-Palomino and Theißen, 2008, 2009; Mondragón-Palomino et al., 2009). Several works have described the possible interactions among these four clades of paleo*AP3* genes and one *PI* gene that are involved in regulating the differentiation and formation of the sepal/petal/lip of orchids (Tsai et al., 2004; Kim et al., 2007; Mondragón-Palomino and Theißen, 2008, 2009). However, the exact mechanism that involves the orchid B class genes remains unclear and needs to be clarified by more experimental investigations.

O. Gower Ramsey is a popular orchid with important economic value in cut flower markets. Only a few studies have been reported on the role of MADS box genes in regulating flower formation in this plant species (Hsu and Yang, 2002; Hsu et al., 2003; Chang et al., 2009). An *AP3*-like MADS gene that regulates both floral formation and initiation in transgenic Arabidopsis has been reported (Hsu and Yang, 2002). In addition, four *AP1/AGAMOUS-LIKE9* (*AGL9*)-like MADS box genes have been characterized that show novel expression patterns and cause different effects on floral transition and formation in Arabidopsis (Hsu et al., 2003; Chang et al., 2009). Compared with other orchids, the production of a large and well-expanded lip and five small identical sepals/petals makes *O.* Gower Ramsey a special case for the study of the diverse functions of B class MADS box genes during evolution. Therefore, the isolation of more B class MADS box genes and further study of their roles in the regulation of perianth (sepal/petal/lip) formation during *O.* Gower Ramsey flower development are necessary. In addition to the clade 2 paleo*AP3* gene *OMADS3*, which was previously characterized in our laboratory (Hsu and Yang, 2002), three more B class MADS box genes, *OMADS5*, *OMADS8*, and *OMADS9*, were characterized from *O.* Gower Ramsey in this study. Based on the different expression patterns and the protein interactions among these four orchid B class genes, we propose that the presence of *OMADS3/8* and/or *OMADS9* is required for sepal/petal/lip for-

mation. Further sepal and petal formation at least requires the additional presence of *OMADS5*, whereas large lip formation was seen when *OMADS5* expression was absent. Our results provide a new finding and information pertaining to the roles for orchid B class MADS box genes in the regulation of sepal/petal/lip formation.

RESULTS

Isolation of *OMADS5*, *OMADS8*, and *OMADS9* cDNAs from *O.* Gower Ramsey

To isolate MADS box genes from *O.* Gower Ramsey, a strategy that combined reverse transcription (RT)-PCR and 5′ and 3′ RACE was used. A DNA fragment was amplified by RT-PCR using total RNA from young floral buds as a template. Sequence comparison led to the identification of partial sequences for several MADS box genes. The full-length cDNA sequences for two *AP3*-like B class genes, *OMADS5* and *OMADS9*, and one *PI*-like B class gene, *OMADS8*, were isolated.

OMADS5 and *OMADS9* encoded 227- and 222-amino acid proteins, respectively, that showed high sequence identity to the paleo*AP3* lineage of B group MADS box genes. *OMADS5* is closely related to clade 1 paleo*AP3* genes of orchids, such as *PeMADS2* of *Phalaenopsis* (Tsai et al., 2004) and *DcOAP3A* of *Dendrobium* (Xu et al., 2006), and also showed high sequence identity to another *O.* Gower Ramsey clade 2 paleo*AP3* gene, *OMADS3*, which was previously reported by our laboratory (Hsu and Yang, 2002; Figs. 1 and 2). *OMADS9* is closely related to clade 3 paleo*AP3* genes, such as *DcOAP3B* of *Dendrobium* (Xu et al., 2006) and *PeMADS3* of *Phalaenopsis* (Tsai et al., 2004; Figs. 1 and 2). Within their K boxes, a sequence (QYQRM) matching a conserved motif (Q/HYExM) in *AP3* homologs (Kramer et al., 1998) was found (Fig. 1). In their C-terminal regions, two completely consensus PI-derived (FxFRLOPSQPNLH) and paleoAP3 (D[L/I]ITTFALLE) motifs that are unique to B class genes of monocots (Kramer et al., 1998; Moon et al., 1999; Tzeng and Yang, 2001) were identified (Fig. 1). Sequence identity between *OMADS5* and *OMADS9* and paleo*AP3* orthologs indicates that *OMADS5* and *OMADS9* are *O.* Gower Ramsey B group MADS box paleo*AP3* genes.

The *OMADS8* cDNA encoded a 210-amino acid protein that showed high sequence identity to the B class gene *PI* of monocots (Figs. 1 and 2). In its C-terminal region, a consensus PI motif (MPFxFRVQPxQPNLQE), which is unique to B class *PI* genes (Kramer et al., 1998; Moon et al., 1999), was identified. Sequence identity between *OMADS8* and *PI* homologs indicates that *OMADS8* is an *O.* Gower Ramsey B group MADS box *PI* gene.

The alignment of amino acid sequences shown in Figure 1 and sequences for several other MADS box genes were used to construct a phylogenetic tree for B class genes (Fig. 2). Based on this analysis, *OMADS8* was in the *PI* lineage, whereas *OMADS5* and *OMADS9* were in the paleo*AP3* lineage.

OMADS8 Is Expressed in All Four Floral Organs as Well as in Vegetative Leaves

To explore the relationships between sequence identity and expression pattern for *OMADS8*, *OMADS8* expression was detected by RT-PCR analysis. As shown in Figure 3D, *OMADS8* mRNA was detected in vegetative leaves, roots, and flowers, indicating that the expression of *OMADS8* was not flower specific. When floral organs from 8- to 10-mm mature floral buds (Fig. 3, A–C) were examined, *OMADS8* was expressed in all floral organs, with relatively higher expression in the sepal, petal, and lips than in the stamen and carpel (Fig. 3D). When the expression of *OMADS8* in sepal/petal/lip of flower buds in different early developmental stages (2, 3, and 5 mm in length; Fig. 4, A–C) was further analyzed, *OMADS8* mRNA was consistently detected in sepals, petals, and lips of all three early flower buds (Fig. 4D), similar to that observed in mature flower buds (Fig. 3D). This expression pattern was different from that observed for *PI* orthologs of Arabidopsis and other orchids, which was floral specific and was only expressed in stamens and petals (Jack et al., 1992; Rounsley et al., 1995; Moon et al., 1999; Tsai et al., 2005; Xu et al., 2006; Guo et al., 2007; Kim et al., 2007). Interestingly, the expression pattern for *OMADS8* was very similar to that observed for the clade 2 paleo*AP3* gene *OMADS3* (Fig. 3D; Hsu and Yang, 2002). *OMADS3* mRNA was also consistently detected in sepals, petals, and lips of all three early flower buds (Fig. 4E), although it showed a relatively lower expression in lips than in sepal/petal (Fig. 4E). This result indicated that *OMADS8* and *OMADS3* possibly play similar roles in regulating the formation of the sepal, petal, and lip in *O.* Gower Ramsey.

OMADS5 and *OMADS9* Showed Different Expression Patterns in Floral Organs

To explore the relationships between sequence identity and expression pattern for *OMADS5* and *OMADS9*, expression of *OMADS5* and *OMADS9* was detected by RT-PCR. As shown in Figure 3D, the *OMADS5* mRNA was flower specific and absent in vegetative leaves and roots. When floral organs from 8- to 10-mm mature floral buds (Fig. 3, A–C) were examined, *OMADS5* was only expressed in sepals and petals (Fig. 3D). Similar to *OMADS5*, *OMADS9* was also strongly detected in flowers and absent in vegetative leaves and roots (Fig. 3D). In flowers, the *OMADS9* mRNA was also highly expressed in petals (Fig. 3D). However, in contrast to *OMADS5*, *OMADS9* was highly expressed in the lip and was not detected in the sepal (Fig. 3D). To examine the consistency of

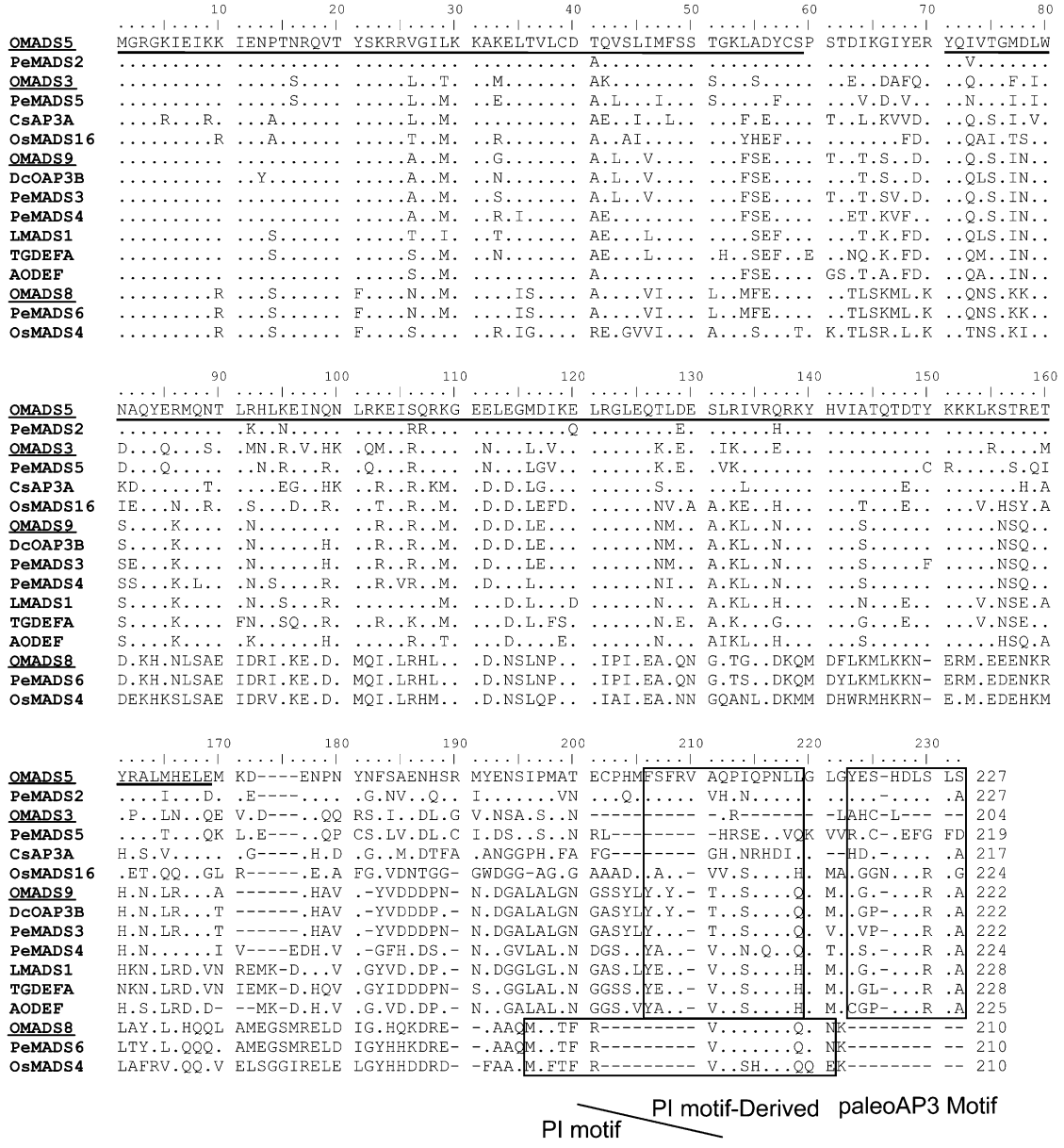


Figure 1. Sequence comparison of OMADS3, OMADS5, OMADS8, and OMADS9 and the related B class MADS domain proteins. The functional MADS box proteins include PeMADS2, PeMADS3, PeMADS4, PeMADS5, and PeMADS6 (*P. equestris*), LMADS1 (*L. longiflorum*), OsMADS16 and OsMADS4 (*O. sativa*), CsAP3A (*Crocus sativus*), DcOPA3B (*Dendrobium crumenatum*), TGDEFA (*T. gesneriana*), and AODEF (*Asparagus officinalis*). The first and second underlined regions represent the MADS domain and the K domain, respectively. The three blocks in the C-terminal region represent the three motifs conserved among B class MADS box proteins. The paleoAP3 and PI-derived motifs are the two highly conserved motifs for paleoAP3 proteins of monocots. The PI motif is a highly conserved motif for PI orthologs. Amino acid residues identical to OMADS5 are indicated as dots. To improve the alignment, dashes were introduced into the sequence. The names of the OMADS3, OMADS5, OMADS8, and OMADS9 proteins are underlined. This sequence alignment was generated by the ClustalW Multiple Sequence Alignment Program at the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/top-e.html).

the *OMADS5* and *OMADS9* expression, the expression of *OMADS5* and *OMADS9* in sepal/petal/lip of flower buds in different early developmental stages (2, 3, and 5 mm in length; Fig. 4, A–C) was further analyzed. The result indicated that *OMADS5* mRNA was consistently only expressed in sepals and petals and was absent in lips of all three early flower buds (Fig. 4F). Similar to that observed in mature flower buds, *OMADS9* mRNA was also only expressed in petals and lips and was not detected in the sepals of all three early flower buds (Fig. 4G). This result indicated that *OMADS5* and *OMADS9* may play different roles in controlling the formation of the sepal, petal, and lip in *O.* Gower Ramsey. The formation of the sepal and

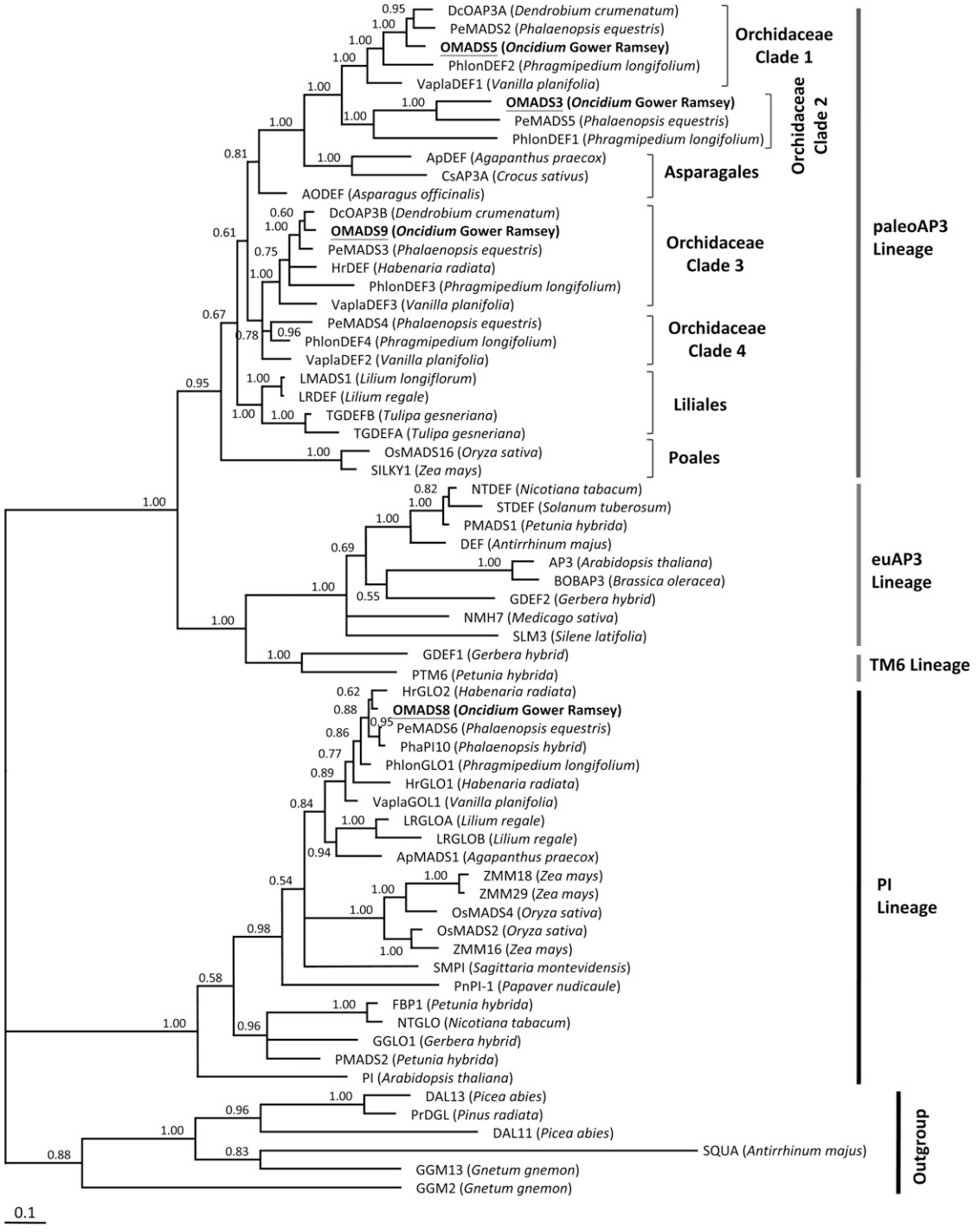


Figure 2. Phylogenetic analysis of B class MADS domain proteins. Based on the amino acid sequence of the full-length protein, *OMADS8* was closely related to *PeMADS6* and *OsMADS4* in the *PI* group of MADS box genes in monocots. *OMADS3*, *OMADS5*, and *OMADS9* were closely related to genes in the paleo*AP3* lineage of monocots. *OMADS5* belongs to clade 1, *OMADS9* belongs to clade 3, and *OMADS3* belongs to clade 2 of paleo*AP3* genes of orchids. The names of the OMADS3, OMADS5, OMADS8, and OMADS9 proteins are shown in boldface and underlined. The names of the plant species for each MADS box gene are listed behind the protein names. Amino acid sequences of B class MADS box genes were retrieved via the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree was generated using Bayesian analysis as described in "Materials and Methods."

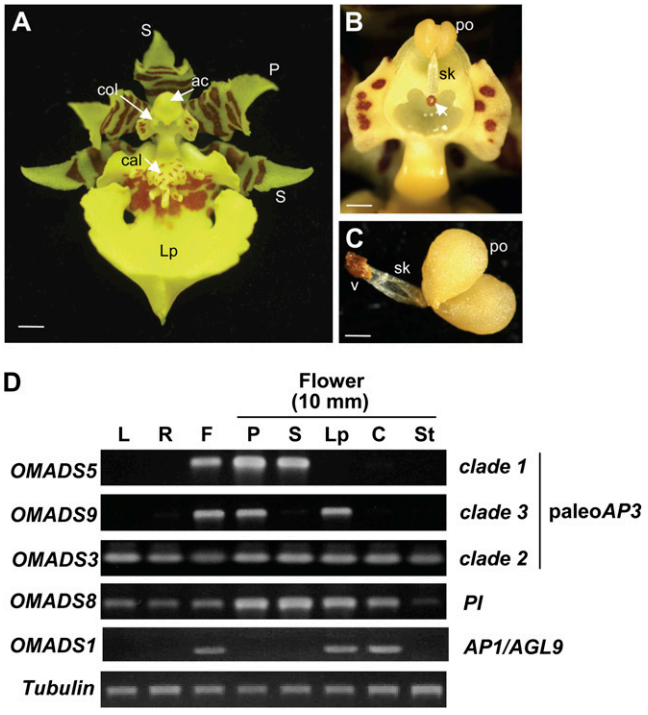


Figure 3. Detection of expression of *OMADS3*, *OMADS5*, *OMADS8*, *OMADS9*, and *OMADS1* in *O.* Gower Ramsey. A, An *O.* Gower Ramsey mature flower bud (10 mm) consisting of three sepals (S), two petals (P), a lip (Lp) with red-brown around the callus (cal), and a reproductive organs column (col). Sepals and petals are yellow with red-brown bars and blotches toward the base of the segment. ac, Anther cap. Bar = 2 mm. B, Closeup of the column. The anther cap, which covers the reproductive organs column (col in A), was removed, revealing the pollinarium (male reproductive organ), which consists of two pollinia (po), a stalk (sk), and the brown viscidium (arrowed). Bar = 0.5 mm. C, Closeup of the pollinarium. po, Pollinia; sk, stalk; v, viscidium. Bar = 0.2 mm. D, Total RNAs isolated from leaves (L), roots (R), and the flower organs sepal (S), petal (P), lip (Lp), stamen (St), and carpel (C) of 10-mm-long floral buds (F) were used as templates to detect the expression of *OMADS3*, *OMADS5*, *OMADS8*, and *OMADS9* by RT-PCR. In this study, two pollinia, a stalk of pollinarium, and the viscidium from the column were isolated as male reproductive organs (indicated as stamen). The remaining tissues of the column were used as female reproductive organs (indicated as carpel). The results indicated that *OMADS8* and *OMADS3* were expressed in all four floral organs as well as in vegetative leaves and roots. The mRNA for *OMADS5* was only strongly detected in sepals and petals, whereas *OMADS9* was only strongly detected in petals and lips. The *AGL6*-like gene *OMADS1* was only expressed in lips and carpels. Each experiment was repeated twice with similar results. A fragment of the *α-tubulin* gene was amplified as an internal control.

petal at least requires the presence of *OMADS5*, whereas the formation of lips requires no less than the presence of *OMADS9* and the absence of *OMADS5*. The expression patterns in the sepal/petal/lips and the phylogenetic relationship between the four B class genes *OMADS3*, *OMADS5*, *OMADS9*, and *OMADS8* and one AGL6-like gene, *OMADS1*, of *O.* Gower Ramsey are illustrated in Figure 5.

OMADS5 Was Significantly Down-Regulated in Lip-Like Petals/Sepals in the Peloric Mutants

To further explore the roles of *OMADS5*, *OMADS9*, *OMADS8*, and *OMADS3* in regulating sepal, petal, and lip formation in *O.* Gower Ramsey, the expression of these four genes in peloric mutants of *O.* Gower Ramsey with conversions of either petal to lip (Fig. 6A, middle) or the lateral sepal to lip (Fig. 6A, right) was examined by real-time PCR. As shown in Figure 6B, the *OMADS5* mRNA was undetectable in lips and was highly expressed in normal sepals and petals of wild-type and both peloric mutant flowers. The expression of *OMADS5* was significantly reduced (Fig. 6B) in lip-like petals of peloric mutant flowers (Fig. 6A, middle). A similar reduction of the *OMADS5* mRNA was also observed (Fig. 6B) in lip-like lateral sepals of peloric mutant flowers (Fig. 6A, right). This result clearly indicated a strong correlation between lip formation and reduction in *OMADS5* expression. Since the sizes of both lip-like petals and lip-like lateral sepals in peloric mutant flowers were relatively smaller than normal lips (Fig. 6A), lower *OMADS5* expression also associated with the larger size of the lips produced.

When the expression of *OMADS9* was examined, its mRNA was not detected (Fig. 6C) in normal sepals of either wild-type or peloric mutant flowers with lip-like petals (Fig. 6A, middle). In contrast, the *OMADS9* mRNA was increased (Fig. 6C) in the lip-like lateral sepals of peloric mutant flowers (Fig. 6A, right). Interestingly, the increase in *OMADS9* expression was also observed in the normal dorsal sepal (Fig. 6C) of peloric mutant flowers (Fig. 6A, right). This result revealed that the increase in *OMADS9* expression is not absolutely linked to the formation of lips.

Furthermore, the expression of *OMADS8* in lip-like petals (Fig. 6A, middle) or lip-like lateral sepals (Fig. 6A, right) of peloric mutant flowers was similar to that observed in normal sepals or petals, respectively (Fig. 6D). The expression of *OMADS3* was also detected in lip-like petals (Fig. 6A, middle) or lip-like lateral sepals (Fig. 6A, right) of peloric mutant flowers and was slightly reduced to a level similar to that observed in normal lips (Fig. 6E). When the expression of *OMADS1*, an *AGL6*-like gene in *O.* Gower Ramsey that is specifically expressed in the lip and carpel of flowers (Fig. 3; Hsu et al., 2003), was examined, an increase in expression (Fig. 6F) was observed in both lip-like petals (Fig. 6A, middle) and lip-like sepals (Fig. 6A, right) of peloric mutant flowers.

The Formation of Larger Lips Is Due to an Increase in Cell Proliferation Rather Than Cell Expansion

A major difference between the lip and sepal/petal in *O.* Gower Ramsey is size. The well-expanded large lip in a mature *Oncidium* flower is about 15- to 20-fold larger than the sepal and petal (Figs. 6A, left, and 7B). It is interesting to investigate whether the increased size of the lips, possibly caused by the absence of

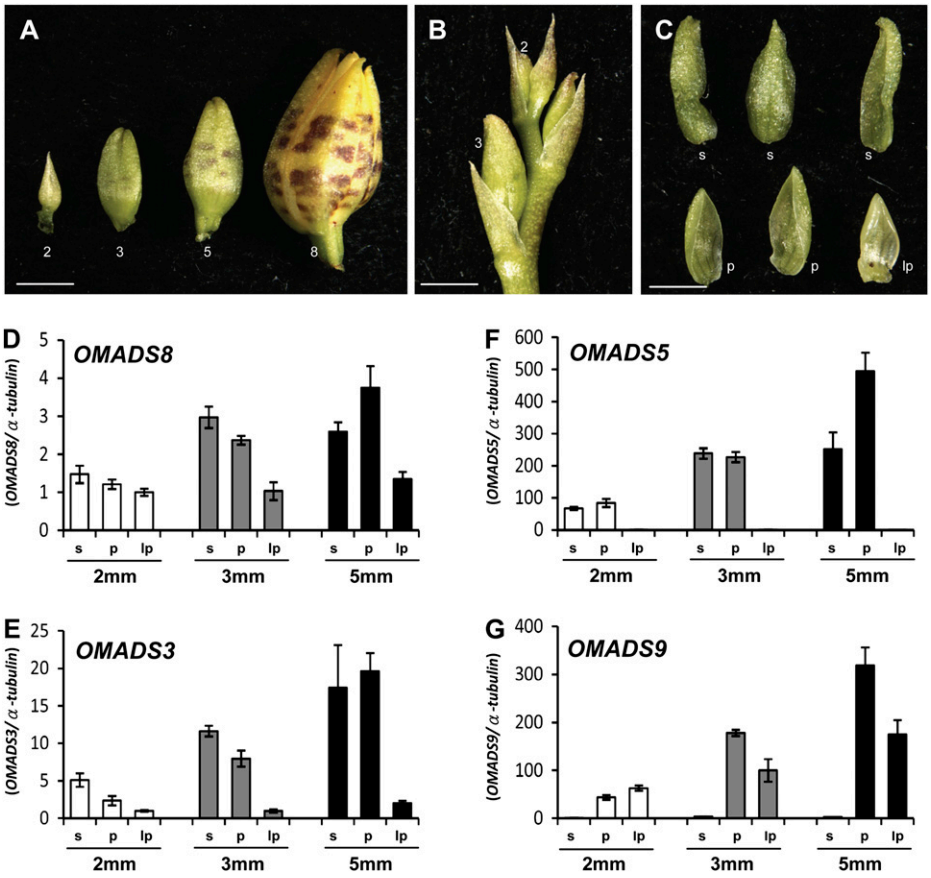


Figure 4. Detection of expression of *OMADS3*, *OMADS5*, *OMADS8*, and *OMADS9* in sepal/petal/lip during early flower development in *O.* Gower Ramsey. A, Flower buds of *O.* Gower Ramsey at different developmental stages (2, 3, 5, and 8 mm long). Bar = 2 mm. B, Closeup of the young flower buds on the top of the inflorescence in *O.* Gower Ramsey. The positions of the 2- and 3-mm-long flower buds are indicated by the numbers. Bar = 2 mm. C, A 2-mm-long *O.* Gower Ramsey flower bud consisting of three sepals (s), two petals (p), and a lip (lp). In this stage, the lip is small and morphologically similar to the petals. Bar = 1 mm. D to G, Total RNAs isolated from the sepal (s), petal (p), and lip (lp) of 2-, 3-, and 5-mm-long young flower buds from A were used as templates to detect the expression of *OMADS8* (D), *OMADS3* (E), *OMADS5* (F), and *OMADS9* (G) by real-time PCR. The results indicated that *OMADS8* mRNA was consistently detected in sepals, petals, and lips of all three early flower buds. *OMADS3* mRNA was also consistently detected in sepals, petals, and lips of all three early flower buds with a relatively lower expression in lips than in sepal/petal. *OMADS5* mRNA was consistently expressed only in sepals and petals and was absent in lips, whereas *OMADS9* mRNA was only expressed in petals and lips and was not detected in the sepals of all three early flower buds. In quantitative real-time PCR, the columns represent the relative expression of these genes. Transcript levels of these genes were determined using two to three replicates and were normalized using *α-tubulin*. Error bars represent SD. Each experiment was repeated three times with similar results.

OMADS5 expression, was due to a difference in the total number of cells produced or the increased size of each individual cell. Therefore, the epidermal cells in these three organs were examined by confocal laser scanning microscopy. In flower buds (Fig. 7A), the epidermal cells in the sepal (Fig. 7C) and petal (Fig. 7D; $100 \times 40\ \mu m^2$) were about eight times larger than those observed in lips ($25 \times 20\ \mu m^2$; Fig. 7E). In this stage, the size of the lips was only approximately twice that of the sepal and petal (Fig. 7A). This indicated that there were about 16 times more cells in the lips than in the sepal and petal during the floral bud stage. When the epidermal cells in the lips (Fig. 7, L and M) of a mature flower (Fig. 7B) were examined, they were almost the same size ($100 \times 40\ \mu m^2$) as those of the sepal (Fig. 7, F and G) and petal (Fig. 7, I and J) cells, although these papillate cells showed a cobblestone-like appearance with a protuberance on the top (Fig. 7, M and N) that was morphologically different from the irregularly shaped flat sepal (Fig. 7, G and H) or petal (Fig. 7, J and K) cells. Interestingly, these papillate cells found in the lip epidermis are actually the characteristic of petals for plants such as Arabidopsis and *Petunia*. This result indicated that the total number of cells in the lips during early flower development was already about 16 times more than in the sepal and petal. These epidermal cells in the lips grew rapidly during maturation, reached the same size as sepal/

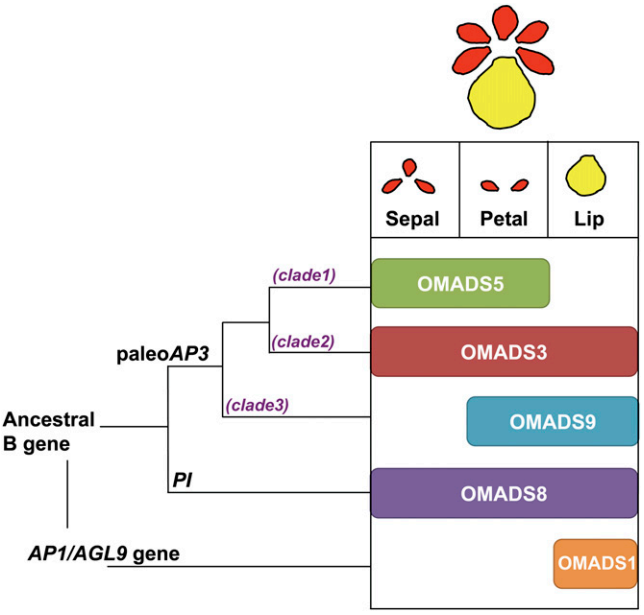


Figure 5. Gene duplications and expression patterns of the B class genes in *O.* Gower Ramsey. A major duplication event from an ancestral B gene generated the paleo*AP3* and *PI* lineages. In orchid *O.* Gower Ramsey, there is only one *PI* gene, *OMADS8*, and two more duplications occurred in the paleo*AP3* gene that generated at least three paleo*AP3*-like genes, *OMADS5* (clade 1), *OMADS3* (clade 2), and *OMADS9* (clade 3). *OMADS8* and *OMADS3* were expressed in the sepal/petal/lip, *OMADS5* was expressed in the sepal/petal, while *OMADS9* was expressed in the petal/lip. The *AGL6*-like gene *OMADS1* was expressed in lip and carpel. [See online article for color version of this figure.]

petal cells, and caused the 15 to 20 times larger size of the lip than the sepal and petal. Therefore, it is clear that the increase in cell proliferation was the main reason for the larger size of the lips in *Oncidium*. When the epidermal cells in lip-like petals (Fig. 6A, middle) of peloric mutant flowers were examined, they were morphologically identical to the wild-type lip epidermis (Fig. 7, E and L–N) in either the floral bud (Fig. 7O) or the mature flower (Fig. 7, P–R) and distinct from the wild-type sepal/petal epidermis.

Homodimer and Heterodimer Formation of the OMADS5, OMADS9, OMADS8, and OMADS3 Proteins

LMADS1, the AP3 homolog from the monocot lily, has been reported to be able to form homodimers (Tzeng and Yang, 2001; Tzeng et al., 2004). Since OMADS5 and OMADS9 showed high sequence identity to monocot AP3 homologs, it was interesting to study the possible interaction between OMADS5 and OMADS9. To achieve this, a yeast two-hybrid analysis was performed. Similar to that observed for LMADS1 (Tzeng and Yang, 2001), our result indicated that the OMADS5 and OMADS9 proteins were also able to form strong homodimers (Fig. 8, A and B). When the interaction between OMADS5 and OMADS9 was analyzed, a high level of β-galactosidase activity was also detected (Fig. 8, A and B). This indicated that OMADS5 and OMADS9 are not only able to form homodimers but also formed heterodimers with each other.

When OMADS8 was analyzed in the yeast two-hybrid analysis, it was not only unable to form homodimers itself but also unable to form heterodimers with either OMADS5 or OMADS9 (Fig. 8, A and B). Interestingly, OMADS8 was able to form strong heterodimers with OMADS3 (Fig. 8, A and B). When OMADS3 was further analyzed, it showed a completely different pattern from OMADS8, since it was able to form strong homodimers and heterodimers with OMADS5, OMADS9, and OMADS8 (Fig. 8, A and B). The interactions among the four orchid B class proteins are illustrated in Figure 8C.

Ectopic Expression of *OMADS8* Converted Sepals into Petal-Like Structures in Transgenic Arabidopsis Plants

To further investigate the functions of OMADS5, OMADS9, and OMADS8, ectopic expression of these genes in transgenic plants was necessary. cDNAs for these three genes driven by the cauliflower mosaic virus 35S promoter were transformed into Arabidopsis plants for functional analysis.

Twenty independent *35S*::*OMADS8* transgenic Arabidopsis plants were obtained. Nine plants were phenotypically indistinguishable from wild-type plants, whereas the other 11 plants showed identical novel phenotypes in both vegetative and reproductive development. These plants flowered earlier than wild-type plants by producing fewer rosette leaves. Flowers produced in the inflorescence of these plants (Fig. 9C) were different from those observed in wild-type plants (Fig. 9A). Unlike wild-type flowers (Fig. 9B), homeotic conversion of first whorl green sepals into white petal-like structures (Fig. 9, D and E) was observed in these *35S*::*OMADS8* flowers. When the epidermal cells (Fig. 9F) in these first whorl petal-like structures were examined, they were morphologically similar to wild-type petal epidermal cells (Fig. 9G) and distinct from the wild-type sepal epidermis (Fig. 9H). To explore whether the severe phenotype correlated with *OMADS8* expression in the transgenic plants, RT-PCR analysis was performed. As shown in Figure 9I, higher *OMADS8* expression was observed in the transgenic plants with the severe phenotype compared with transgenic plants with less severe phenotypes or phenotypes indistinguishable from wild-type plants. This result clearly indicated that the phenotypes generated in the *35S*::*OMADS8* transgenic Arabidopsis were due to the ectopic expression of the orchid *OMADS8* gene.

Twenty and 29 independent *35S*::*OMADS5* and *35S*::*OMADS9* transgenic Arabidopsis plants were obtained, respectively. In contrast to the flowers in *35S*::*OMADS8* described above, no homeotic conversion in

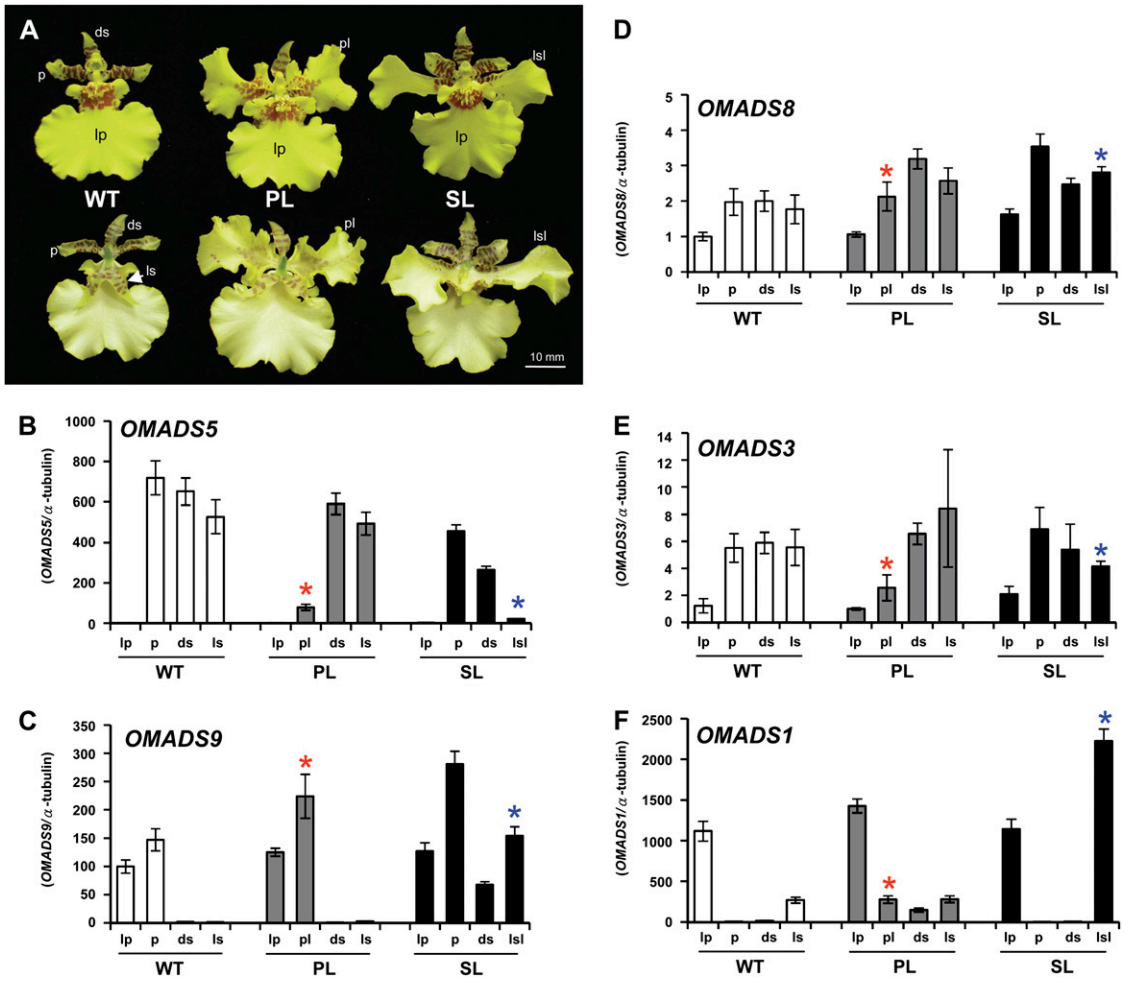


Figure 6. Detection of expression of *OMADS1*, *OMADS3*, *OMADS5*, *OMADS8*, and *OMADS9* in peloric mutants of *O.* Gower Ramsey. A, Flower phenotypes of wild-type plants (WT), peloric mutants with two petals converted into lips (PL), and peloric mutants with two lateral sepals converted into lips (SL) of *O.* Gower Ramsey. The top row represents the front side and the bottom row represents the back side of the flower. ds, Dorsal sepal; ls, lateral sepal; p, petal; lp, lip; pl, lip-like petal; lsl, lip-like lateral sepal. Bar = 10 mm. B to F, Total RNAs isolated from the dorsal sepal (ds), lateral sepal (ls), petal (p), and lip (lp) of mature flowers from A, the wild type (WT), peloric mutants with petals converted into lips (PL), and peloric mutants with lateral sepals converted into lips (SL), were used as templates to detect the expression of *OMADS5* (B), *OMADS9* (C), *OMADS8* (D), *OMADS3* (E), and *OMADS1* (F) by real-time PCR. The level of gene expression in lip-like petals is marked with red stars, and expression in the lip-like lateral sepal (lsl) is marked with blue stars. The results indicated that *OMADS5* was significantly down-regulated in both lip-like petals and lip-like lateral sepals in peloric mutants. *OMADS9* was significantly up-regulated in both lip-like lateral sepals and normal dorsal sepals in peloric mutants (A, right). *OMADS1* was clearly up-regulated in both lip-like petals and lip-like lateral sepals in peloric mutants. In quantitative real-time PCR, the columns represent the relative expression of these genes. Transcript levels of these genes were determined using two to three replicates and were normalized using *α-tubulin*. Error bars represent SD. Each experiment was repeated three times with similar results.

the floral organs was observed in the flowers of these *35S*::*OMADS5* and *35S*::*OMADS9* plants.

DISCUSSION

To investigate the role of B class MADS box genes in the regulation of flower development in the orchid *O.* Gower Ramsey, three genes, *OMADS5*, *OMADS8*, and *OMADS9*, were identified and characterized in this study. Based on sequence alignment, the conserved motifs in the C-terminal regions of the proteins, and phylogenetic tree analysis, *OMADS5* belongs to clade 1 whereas *OMADS9* belongs to clade 3 of paleo*AP3* genes of orchids. *OMADS8* is a *PI*-like gene and is closely related to monocot *PI* orthologs (Figs. 1 and 2).

Like the other clade 1 paleo*AP3* genes, such as *PeMADS2* of *Phalaenopsis* (Tsai et al., 2004), the mRNA for *OMADS5* was only strongly detected in sepals and petals. The *OMADS9* mRNA was only strongly de-

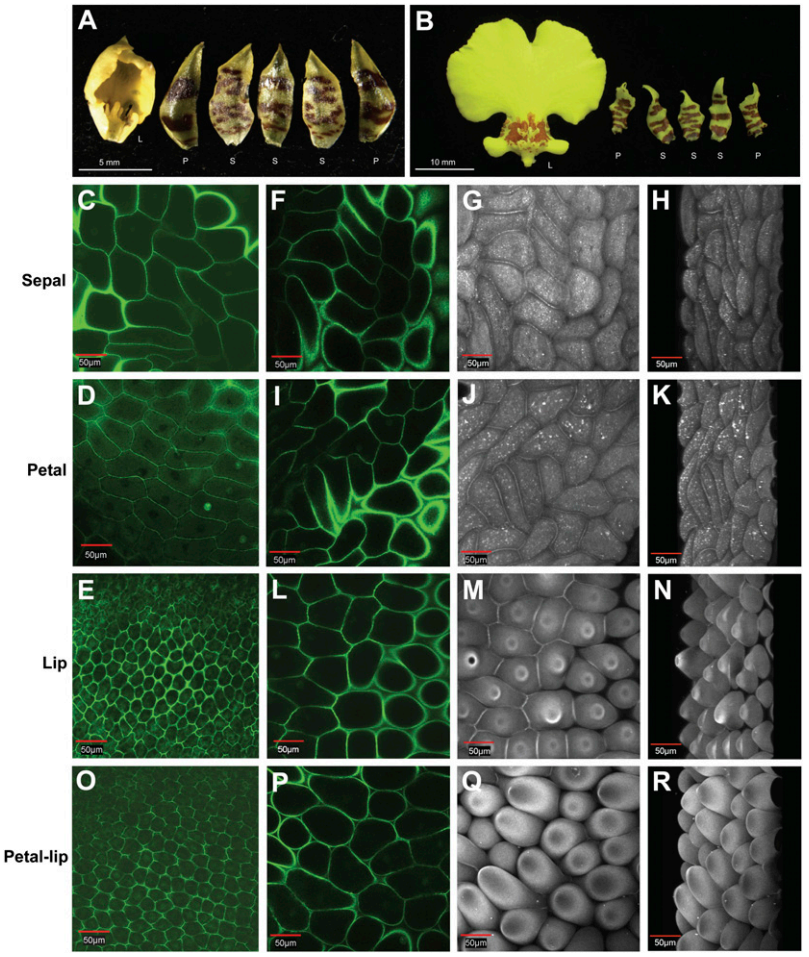
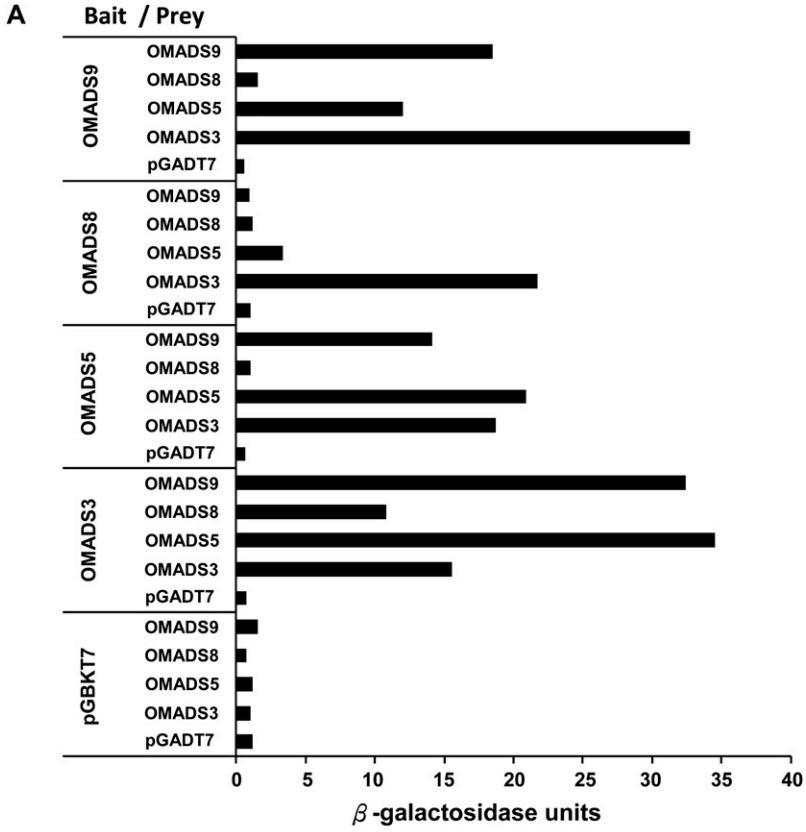


Figure 7. Confocal laser scanning microscopy of various floral organs in *O.* Gower Ramsey. A, A lip (L), two petals (P), and three sepals (S) of a 10-mm *O.* Gower Ramsey floral bud. The sepals and petals look the same. Bar = 5 mm. B, A lip (L), two petals (P), and three sepals (S) of an *O.* Gower Ramsey mature flower. The sepals and petals look the same. Bar = 10 mm. C, Irregularly shaped cells in the epidermis of sepals in the wild-type floral bud in A. Bar = 50 μm. D, Irregularly shaped cells in the epidermis of petals in the wild-type floral bud in A. Bar = 50 μm. E, Small cobblestone-like cells in the epidermis of lips in the wild-type floral bud in A. Bar = 50 μm. F to H, Irregularly shaped cells in the epidermis of sepals in the wild-type mature flower in B. Bar = 50 μm. I to K, Irregularly shaped cells in the epidermis of petals in the wild-type mature flower in B. Bar = 50 μm. L to N, Cobblestone-like cells in the epidermis of lips in the wild-type mature flower in B. Bar = 50 μm. O, Small cobblestone-like cells in the epidermis of lip-like petals in a peloric mutant floral bud (Fig. 3A, middle). Bar = 50 μm. P to R, Cobblestone-like cells in the epidermis of lip-like petals in a peloric mutant mature flower. Bar = 50 μm. The cell wall was counterstained with propidium iodide. C to F, I, L, O, and P are single slices to indicate the cell outline; G, J, M, and Q are Z-stacks to present the cell morphology; and H, K, N, and R are three-dimensional rotated images from the Z-stack images using the Fluoview 1000 software.

tected in petals and lips, similar to the clade 3 paleo-*AP3* genes *DcOAP3B*, *PeMADS3*, and *HrDEF* (Tsai et al., 2004; Xu et al., 2006; Kim et al., 2007). The expression of both *OMADS5* and *OMADS9* was absent in stamens and leaves. Interestingly, the expression patterns of *OMADS5* and *OMADS9* were largely different from *OMADS3*, which was expressed in all flower organs and leaves (Fig. 3; Hsu and Yang, 2002). This indicated the possibility of functional and transcriptional diversification of *OMADS5*, *OMADS9*, and *OMADS3*. *OMADS9* and *OMADS5* may have experienced significant expression diversification from *OMADS3* after the gene duplication in the orchid during evolution (Fig. 5).

The possible role for *OMADS5* and *OMADS9* in regulating perianth formation is interesting. The detection of only *OMADS9* (clade 3) expression in lips suggests that *OMADS9* may have a positive role in lip formation. However, it is unclear why the petals did not convert into lips, since *OMADS9* was also expressed in petals. In addition, a high amount of *OMADS9* expression was also observed in the normal dorsal sepal of peloric mutant flowers (Fig. 6C). This dorsal sepal could be converted into a lip-like structure. This result revealed that the presence of *OMADS9* expression is not necessarily associated with the formation of lips. One possibility is that the increase of *OMADS9* alone is necessary but not sufficient for the transformation of sepals or petals into lips. It has been proposed that a lip-specific clade 4 gene, such as *PeMADS4* of *Phalaenopsis*, was responsible for lip formation (Tsai et al., 2004; Mondragón-Palomino and Theißen, 2008, 2009). *OMADS9* may need to work together with a clade 4 gene that remains to be identified in *O.* Gower Ramsey to control lip formation.

Alternatively, the difference for petal and lip formation may be due to the expression of *OMADS5* in the petal and the absence of *OMADS5* expression in the lip. This suggested a possible negative role for *OMADS5* in regulating lip formation. Based on this assumption, in the presence of *OMADS5* expression, lip formation will be suppressed and the organs will be converted into sepals/petals, no matter whether *OMADS9* expression is present or absent. Thus, a high amount of *OMADS9* expression did not convert normal dorsal sepal of peloric mutant flowers into lips, since a certain high amount of *OMADS5* expression was also detected (Fig. 6, B and C). In the absence of *OMADS5* expression, the organs will be converted into



B

AD / BK	O3	O5	O8	O9
O3	+	+++	+	+++
O5	++	++	-	+
O8	++	-	-	-
O9	+++	+	-	++

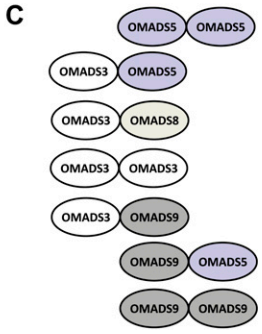


Figure 8. Protein interactions among B class proteins of *O.* Gower Ramsey in a yeast two-hybrid assay. A, β-Galactosidase activity in yeast cells transformed with combinations of truncated OMADS3, OMADS5, OMADS8, and OMADS9 in either the binding domain plasmid (Bait) or the activation domain plasmid (Prey) calculated according to Miller (1992). Yeast cells transformed with OMADS3, OMADS5, OMADS8, and OMADS9 in the binding domain plasmid or the activation domain plasmid alone were used as controls for background activity. B, A summary of the strength of the β-galactosidase activity obtained in A. The number of + signs indicates the relative strength of the activity detected in each reaction. The – sign indicates that no activity was detected. AD, Activation domain in plasmid pGADT7; BK, binding domain in plasmid pGBKT7. C, A summary of the homodimers and heterodimers among the four B class proteins of *O.* Gower Ramsey obtained in A. [See online article for color version of this figure.]

lips in the presence of *OMADS9* expression. This assumption was supported by the expression analysis of *OMADS5* and *OMADS9* in peloric mutants of orchid. *OMADS5* mRNA was significantly reduced in both lip-like petals and lip-like sepals of peloric mutant flowers (Fig. 6B). Taken together, these results support that the loss of *OMADS5* (clade 1) expression is likely the main reason for the lip specification and for the conversion of the sepals/petals into lips in *O.* Gower Ramsey and perhaps also in other orchids with lips and petals in different sizes. Interestingly, these peloric mutants of orchid are also likely affected in floral symmetry, which was regulated by two duplicated TCP transcription factors, *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*; Mondragón-Palomino and Theißen, 2009; for review, see Preston and Hileman, 2009). It has been reported that the expression of CYC depends on the transcription of the B class gene *DEF* in *A. majus* (Clark and Coen, 2002). Therefore, the change of expression for B class genes *OMADS5*/*OMADS9* in orchid may cause the alteration for *CYC/DICH* expression and result in the changes in floral symmetry. However, it has been shown that there are no true orthologs of the floral symmetry genes *CYC* and *DICH* outside core eudicots (for review, see Preston and Hileman, 2009). This implies a possible different genetic mechanism underlying dorsoventral asymmetry in monocot orchids.

Similar to *OMADS3*, the mRNA for *OMADS8* was detected in all four floral organ whorls as well as in vegetative leaves and roots (Fig. 3). The expression in organs other than flowers has also been reported in some B class genes such as *GDEF1* (*G. hybrida*), *TGGLO* (*T. gesneriana*), *ZMM16* (*Z. mays*), and *EgGLO2*

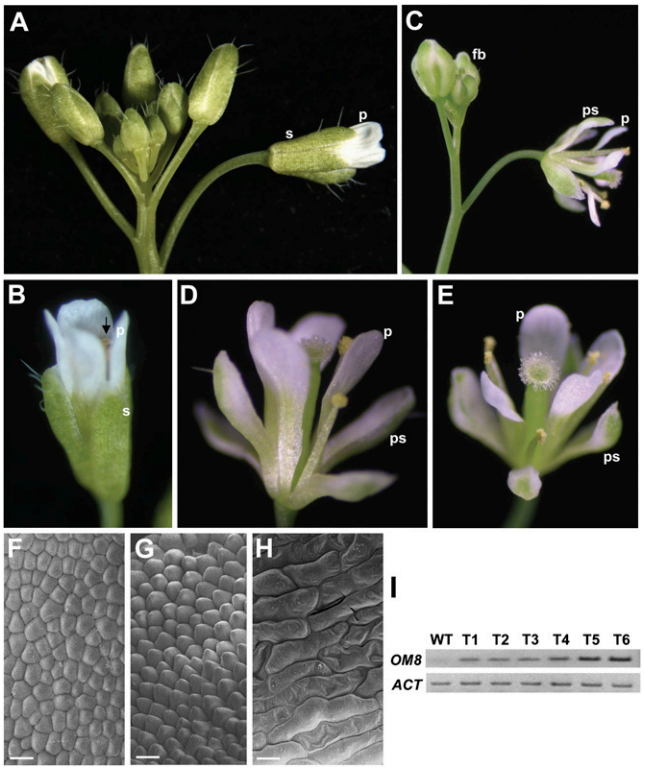


Figure 9. Phenotypic analysis of transgenic Arabidopsis plants ectopically expressing *OMADS8* or *OMADS8*-ΔMADS. A, A wild-type inflorescence contained flower buds and mature flowers with normal sepals (s) and petals (p). B, A mature wild-type Arabidopsis flower consisted of four whorls of organs, including four sepals (s), four elongated petals (p), six stamens (arrow), and two fused carpels. The sepals had not opened in this stage. C, A *35S*::*OMADS8* inflorescence contained flower buds (fb) with green/white sepals in the first whorl, fully opened mature flowers with white elongated petal-like sepals (ps) in the first whorl, and normal petals (p) in the second whorl of the flower. D and E, Closeup views of the *35S*::*OMADS8* transgenic Arabidopsis flowers. White elongated petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. F, Scanning electron micrograph of the surface cells of the epidermis of the first whorl petal-like sepal of a *35S*::*OMADS8* flower was similar to the mature wild-type petal epidermis in G. Bar = 10 μm. G, Scanning electron micrograph of the surface cells of the epidermis of a mature wild-type petal. Bar = 10 μm. H, Scanning electron micrograph of the surface cells with irregular shapes in the epidermis of wild-type sepals. Bar = 10 μm. I, Total RNA isolated from two severe (T5 and T6) and four less severe (T1–T4) 14-d-old *35S*::*OMADS8* transgenic Arabidopsis plants and from one untransformed wild-type plant (WT) used as a template. The results indicated that *OMADS8* (*OM8*) was clearly expressed higher in the T5 and T6 than in the T1 to T4 transgenic plants. A fragment of the *ACTIN* (*ACT*) gene was amplified as an internal control.

(*Elaeis guineensis*) that showed a similar expression pattern to *OMADS8/3* by expressing in both flower organs and leaves (Yu et al., 1999; Münster et al., 2001; Kanno et al., 2003; Adam et al., 2007). This revealed that in addition to regulating flower organ formation, B class genes such as *OMADS3/8*, *GDEF1*, *TGGLO*, *ZMM16*, and *EgGLO2* are also possibly involved in the regulation of leaf development. However, this assumption still requires further investigation. The expression of *OMADS3* and *OMADS8* in sepals, petals, and lips suggested that *OMADS8* and *OMADS3* may play similarly general and necessary roles in regulating the formation of these three flower organs in *O.* Gower Ramsey. Thus, we proposed that the presence of at least *OMADS3/8/5* and/or *OMADS9* is required for sepal and petal formation, whereas the presence of *OMADS3/8/9* and the absence of *OMADS5* are likely required for lip formation in *O.* Gower Ramsey (Figs. 5 and 10).

This assumption was further examined using yeast two-hybrid analyses of the interactions among the orchid B class proteins OMADS3, OMADS5, OMADS8, and OMADS9. The result indicated that OMADS3 formed homodimers and heterodimers with OMADS5, OMADS8, and OMADS9. OMADS8 only formed heterodimers with OMADS3, whereas OMADS5 and OMADS9 formed homodimers individually and heterodimers with each other and with OMADS3 (Fig. 8). The ability to form homodimers supported the possibility that *OMADS3*, *OMADS5*, and *OMADS9* also likely retained the characteristic of ancestral forms of the B genes for the paleo*AP3* lineage in monocots, such as LMADS1 in lily (Tzeng and Yang, 2001). This result revealed a possible model for the interaction of these four B class proteins in regulation of the formation of sepals, petals, and lips in *O.* Gower Ramsey. As illustrated in Figure 10, a complex composed of at least OMADS3/8 and/or OMADS9 is needed for the formation of sepals, petals, and lips. Adding OMADS5 to the complex may convert organs into sepals/petals; any complex without OMADS5 controls lip formation. In addition to these B class proteins, other A/E class proteins are expected to participate in these complexes. For example, *OMADS1*, an *AGL6*-like gene of *O.* Gower Ramsey, is predominantly expressed in lips (Fig. 3) and could form heterodimers with OMADS3 (Hsu et al., 2003). High expression of *OMADS1* is clearly needed for lip formation and was supported by the up-regulation of *OMADS1* in both lip-like petals and lip-like sepals of peloric mutant flowers (Fig. 6F). This assumption was further supported by previous work demonstrating that the B class proteins DcAP3A/B and DcOPI of *Dendrobium* formed higher order complexes with the E class protein DcOSEP1 (Xu et al., 2006).

One interesting and critical question is what is the possible role for *OMADS5* (clade 1) in negatively regulating lip formation. Since an increase in cell proliferation rather than cell expansion was the main reason for the large size of the lips in *Oncidium* (Fig. 7), it is reasonable to propose that a gene such as *OMADS5* may play a role in suppressing lip formation by inhibiting cell proliferation in orchids like *Oncidium* (Fig. 10). Any protein complex that contains OMADS5 may inhibit cell proliferation and result in the formation of short organs, such as sepals and petals

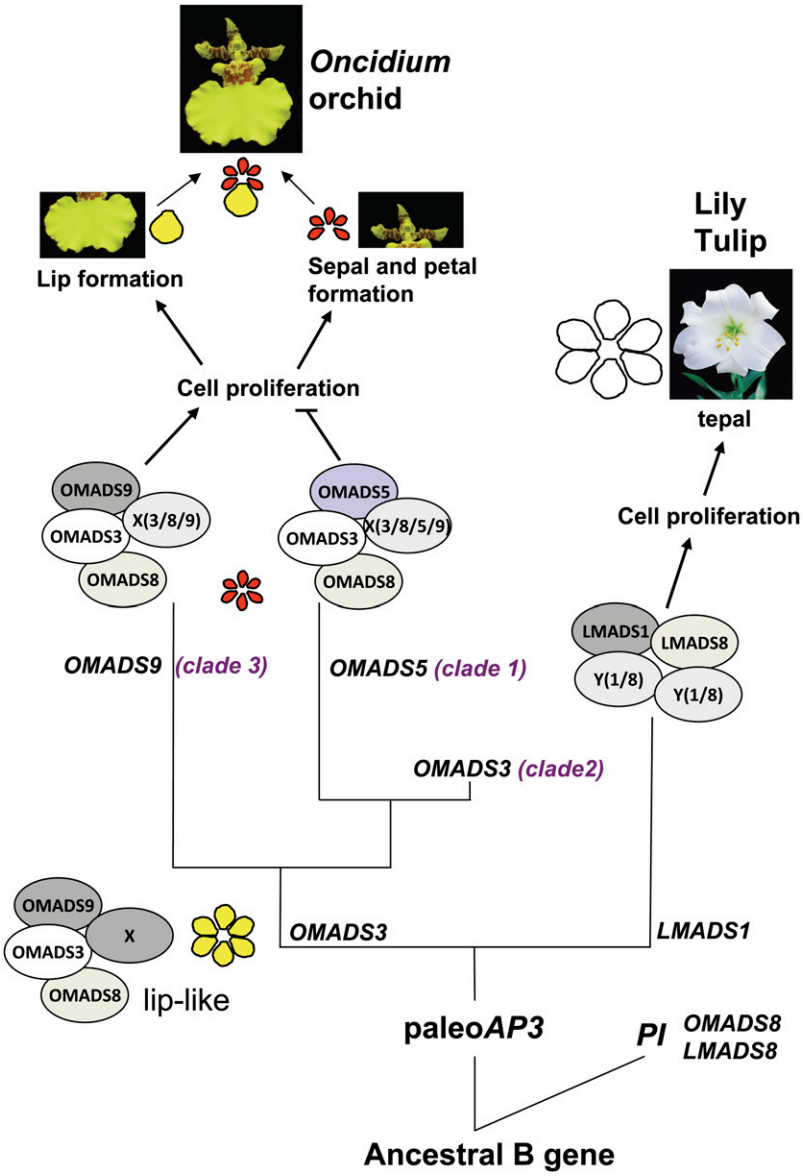


Figure 10. Possible evolutionary relationships between B class genes in the regulation of sepal/petal/lip formation for *O.* Gower Ramsey. B group genes in monocots are thought to have been produced by a major duplication event from an ancestral gene that generated the paleo*AP3* and *PI* lineages. In orchid *O.* Gower Ramsey, there is only one *PI* gene, *OMADS8*, and at least two more duplications occurred in the paleo*AP3* gene *OMADS3* that generated the paleo*AP3*-like genes *OMADS5* and *OMADS9*. The ancestral complex containing OMADS3/8 and other A/E proteins (X) is sufficient to convert the first and second whorl organs into well-expanded lip-like sepals/petals (in yellow). After gene duplication, *OMADS9* may retain the ancestral B gene function of possibly promoting cell proliferation, whereas *OMADS5* might have been altered in relation to this function. This caused the possible suppression of cell proliferation for any complex that includes OMADS5 (e.g. OMADS3/8/5/9/X) and might result in the formation of six short sepals/petals (in red) in the orchid flowers. During evolution, one of the short sepals/petals was converted into a well-expanded lip structure (in yellow) due to the loss of *OMADS5* expression, resulting in the formation of one expanded lip (in yellow; OMADS3/8/9/X) and five short sepals/petals (in red; OMADS3/8/5/9/X), as seen in the modern *O.* Gower Ramsey. Unlike *O.* Gower Ramsey, there is only one paleo*AP3* ortholog, *LMADS1*, and one *PI* ortholog, *LMADS8*, in lily. The complex containing LMADS1/8 and other A/E proteins (Y) may have the ability, similar to OMADS3/8/X, to convert the first and second whorl organs into well-expanded tepals (in white) in lily. The production of short tepals by suppressing cell proliferation has not been seen in lily, since no other paleo*AP3* gene, such as *OMADS5*, has been identified in lily. [See online article for color version of this figure.]

of *Oncidium*. When OMADS5 was absent in these complexes, the cells proliferated, resulting in the formation of large lips (Fig. 10). Based on this assumption, there were likely six lip-like organs in the ancestral *O.* Gower Ramsey before the gene duplication that generated *OMADS5*. It has also been proposed that the perianth of the most recent common ancestor of orchids and the rest of the Asparagales possibly is composed of six almost identical tepals in which an ancestral paleo*AP3*-like gene was uniformly expressed (Mondragón-Palomino and Theißen, 2008, 2009). In this case, the ancestral complex containing OMADS3/8 and other A/E proteins is sufficient to convert the first and second whorl organs into a well-expanded lip-like sepal/petal. After gene duplication, *OMADS5* (clade 1) might be functionally altered (Fig. 10). This would have caused a possible reduction or loss of the ability to promote cell proliferation for any complex that includes OMADS5 and resulted in the formation of short sepals/petals in the flowers (Fig. 10). Later, *OMADS5* expression in one of the short sepals/petals was altered and eliminated, resulting in the conversion of this organ into a well-expanded lip structure, as seen in the modern *O.* Gower Ramsey. One can expect that any one of the sepals or petals will be converted into a lip-like structure once expression of *OMADS5* was reduced or eliminated, as seen in the peloric mutant flowers (Fig. 6B). Furthermore, the degree of the conversion was clearly correlated with the level of the reduction of *OMADS5* expression, since *OMADS5* expression was completely eliminated in the normal lips, which were relatively larger than both the lip-like petals and the lip-like lateral sepals in the peloric mutant flowers.

This assumption can be further supported by the analysis of lily flowers that contained extremely similar sepals and petals, known as perianth segments or tepals, which are fully expanded organs (Fig. 10). There is only one paleo*AP3* ortholog, *LMADS1* (Tzeng and Yang, 2001; Tzeng et al., 2004), and two nearly identical *PI* orthologs, *LMADS8* and *LMADS9* (M.-K. Chen, W.-P. Hsieh, and C.-H. Yang, unpublished data), that have been identified and characterized in *L. longiflorum* so far. Interestingly, both lily *LMADS1* (Tzeng and Yang, 2001) and *LMADS8* and *LMADS9* (M.-K. Chen, W.-P. Hsieh, and C.-H. Yang, unpublished data) were expressed in all four flower organs, similar to the orchid orthologs *OMADS3* and *OMADS8* (Fig. 3). LMADS1 was also able to form homodimers (Tzeng and Yang, 2001) and heterodimers with LMADS8 (M.-K. Chen, W.-P. Hsieh, and C.-H. Yang, unpublished data). This indicated that the lily paleo*AP3* (*LMADS1*) and *PI* orthologs (*LMADS8*) retained similar functions and regulation as the ancestral B gene and the orchid orthologs *OMADS3* and *OMADS8* (Fig. 10). Therefore, the LMADS1/8 complex may have an ability similar to OMADS3/8 to convert the first and second whorl organs into well-expanded tepals in lily. Since there is no evidence for gene duplication to generate other paleo*AP3* genes, such as *OMADS5* in lily, the production of short tepals by possibly suppressing cell proliferation was not seen in lilies.

Not surprisingly, functional analysis produced useful results for interpretation of the possible roles of *OMADS5*, *OMADS8*, and *OMADS9* in flower formation. The production of elongated petal-like sepals in *35S*::*OMADS8* transgenic Arabidopsis plants was similar to that observed in transgenic Arabidopsis plants ectopically expressing *PI* (Lamb and Irish, 2003) or its orthologs, such as *DcOPI* of *Dendrobium* (Xu et al., 2006) and *LMADS8* and *LMADS9* of lily (M.-K. Chen, W.-P. Hsieh, and C.-H. Yang, unpublished data). This result provides further evidence to support the assumption that *OMADS8* simply functions as a B gene in the *PI* lineage. By contrast, no homeotic conversion in the floral organs was observed in the flowers ectopically expressing either *OMADS9* or *OMADS5*. One explanation is that the orchid OMADS9 and OMADS5 proteins are unable to interact with or insufficient for interaction with the Arabidopsis B/A/E class protein complex that regulates sepal and petal formation. This assumption might be supported by PI orthologs being more conserved in their protein sequences than AP3 orthologs in the orchid and Arabidopsis. When the sequence was compared, OMADS8 showed 59% identity to Arabidopsis PI, with 76% identity in the MADS box domain. OMADS5 and OMADS9 showed less than 47% identity to Arabidopsis AP3, with less than 71% identity in the MADS box domain. Furthermore, it has been reported that a mutant form of the lily paleo*AP3* ortholog *LMADS1* was able to form a heterodimer with Arabidopsis PI and generated an *ap3*-like dominant negative phenotype in transgenic Arabidopsis (Tzeng and Yang, 2001). When the sequences were compared, LMADS1 only showed 78%, 64%, and 61% identity to OMADS9, OMADS5, and OMADS3, respectively. This sequence diversity might result in the difference between the interactions of lily LMADS1 and orchid OMADS9 and OMADS5 proteins with the Arabidopsis B/A/E class protein complex that regulates sepal and petal formation.

In summary, in addition to the clade 2 paleo*AP3* gene *OMADS3* characterized previously in our laboratory (Hsu and Yang, 2002), two more paleo*AP3* genes, *OMADS5* (clade 1) and *OMADS9* (clade 3), and a *PI* gene, *OMADS8*, that specify flower development were characterized from the orchid *O.* Gower Ramsey in this study. Based on the expression patterns and the protein interactions among these four orchid B class genes, we propose that sepal and petal formation at least requires the presence of *OMADS3/8/5* and/or *OMADS9*, whereas lip formation at least requires the presence of *OMADS3/8/9* and the absence of *OMADS5* in *O.* Gower Ramsey (Fig. 10). The characteristics of these four genes provide useful information for understanding the relationships among the orchid B class MADS box genes as well as their roles in regulating sepal/petal/lip formation. Since most of the A/E genes of *O.* Gower Ramsey have been isolated and characterized in our laboratory (Chang et al., 2009), further investigation of the role for these A/E genes in the interaction with the B genes characterized in this study should lead to deeper understanding of sepal/petal/lip formation in the orchid *O.* Gower Ramsey.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type and peloric mutant orchid plants used in this study were *Oncidium* Gower Ramsey, a hybrid (*O. goldiana* × *O. guinea gold*) introduced by Koh Keng Hoe in 1977 (Royal Horticultural Society, 1998; Fitch, 2004). Both *O. goldiana* (*O. flexuosum* × *O. sphacelatum*) and *O. guinea gold* (*O. sphacelatum* × *O. varicosum*) are also hybrids. Orchid plants were grown in the Tzu-Fu Chou orchid field in Houli, Taichung County, Taiwan. Seeds of Arabidopsis (*Arabidopsis thaliana*) were sterilized and placed on agar plates containing half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) at 4°C for 2 d. The seedlings were then grown in growth chambers under long-day conditions (16 h of light/8 h of dark) at 22°C for 10 d before being transplanted into soil. The light intensity of the growth chambers was 150 μE m^{-2} s^{-1}.

Cloning of *OMADS5*, *OMADS8*, and *OMADS9* cDNAs from *O.* Gower Ramsey

Total RNA was isolated from 5-mm-long floral buds as described by Hsu and Yang (2002). Flower bud RNA (1 μg) was used to synthesize cDNA using the ImProm-II RT System (Promega). B class gene-specific MADS box degenerate 5′ primer Kram1 (5′-GGGGTACCAAYMGICARGTIACITAYTCIAAGM-GIMG-3′) and 3′ primers Kram2 (5′-TGIARRTTIFFITGIAWKGGITG-3′) and Kram3 (5′-CIAGICGIAGRTCRT-3′) were used for PCR amplification (Kramer et al., 1998). PCR products of 500 to 600 bp were cloned into the pGEM-T Easy vector (Promega) and sequenced. Partial sequences for *OMADS5*, *OMADS8*, and *OMADS9* that showed high identity to B group MADS box genes were identified. The cDNAs containing the 5′ and 3′ ends of *OMADS5*, *OMADS8*,

and *OMADS9* were obtained by 5′ RACE and 3′ RACE using the SMART cDNA Amplification Kit (Clontech) and the following primers: internal 3′ gene-specific primers OM5-3 (5′-CTTGTAAGTGTCAGTTTGTGTG-3′) for *OMADS5*, OPI-3-6 (5′-CTCTGCGTTGATACCACTGCT-3′) for *OMADS8*, and OAP3-3-4 (5′-CCGATTCCTTACAAGCTTGAGGGCCTCA-3′) for *OMADS9*; internal 5′ gene-specific primers OM5-5 (5′-GATATATGAGAGGTACCAGATT-3′) for *OMADS5*, OPI-5-5 (5′-TGGTATCAACGCAGAGTACG-3′) for *OMADS8*, and OAP3-5-2 (5′-TCGAAGAGGAGAGCTGGGATCATGAAGAA-3′) for *OMADS9*. The longest cDNAs for *OMADS5*, *OMADS8*, and *OMADS9* were obtained by PCR amplification using the following primers: *OMADS5*, p5-5 (5′-GGATCCGCAGAACAGAGCACGACAGAT-3′) and p5-3 (5′-GGATCCCCCATTAAATACAGCAACAA-3′); *OMADS8*, p8-5 (5′-TCTAGAATGGGGCGCGGAAAGATAGAG-3′) and p8-3 (5′-CTCGAGACCAACAAGGAGGAGTACAAT-3′); and *OMADS9* p9-5 (5′-TCTAGAATGGGAAGAGGGAAGATTGAA-3′) and p9-3 (5′-CTCGAGTACTCAATGATGATGGTTCGC-3′). The specific 5′ and 3′ primers for *OMADS5*, *OMADS8*, and *OMADS9* contained the *Bam*HI (5′-GGATCC-3′; underlined), *Xba*I (5′-TCTAGA-3′; underlined), or *Xho*I (5′-CTCGAG-3′; underlined) recognition sites to facilitate cloning of the cDNAs.

RT-PCR

Total RNA isolated from various organs of the orchid or from leaves of Arabidopsis plants were used for cDNA synthesis. Five microliters of cDNA sample from the RT reaction was used for 25 cycles of PCR consisting of denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (60 s). The PCR product (25 μL) in each reaction was analyzed by electrophoresis on 1.5% agarose gels. Primers specific for *OMADS1*, *OMADS3*, *OMADS5*, *OMADS8*, and *OMADS9* used in RT-PCR are as follows: *OMADS1*, OAGL6-8-1 (5′-GAGCAAATGGTGGGTCATCG-3′) and O1RT-2 (5′-ATGGTTGCTTCAGAAGAGACATATTG-3′); *OMADS3*, OAP3-5-1 (5′-AAGGAGCTGCGCGGTCTTGA-3′) and OAP3-3-1 (5′-ATAGGGCTGGGTACATTTCTCTAG-3′); *OMADS5*, OM5-3 and OM5-5; *OMADS8*, OPI-3-6 and OPI-5-5; and *OMADS9*, OAP3-3-4 and OAP3-5-2. The *O.* Gower Ramsey *α-tubulin* gene and the Arabidopsis *ACTIN* gene were used as internal controls for the orchid tissue and Arabidopsis tissue, respectively, with the corresponding primers as follows: *α-tubulin* forward primer (5′-AGGGCTTTCTGGTTTTCAATGCTGT-3′) and reverse primer (5′-CGGCGACGGCAGTGTTGTT-3′); *ACTIN* forward primer (5′-ATGAAGATTAAGGTCGTGGCA-3′) and reverse primer (5′-TCCGAGTTTGAAGAGGCTAC-3′).

Quantitative Real-Time PCR

Quantitative real-time PCR was conducted using a Mini Opticon Real-Time PCR Detection System and the Optical System Software version 3.0a (Bio-Rad Laboratories) and the SYBR Green Master Mix (Toyobo) for transcript measurements. The reaction mixture was cycled as follows: one cycle at 95°C for 1 min, then 40 cycles of 95°C (15 s), 58°C (15 s), and 72°C (30 s), and plate reading after each cycle. The following gene-specific primers were used: *OMADS1*, O1-RT-1 (5′-TGGAGACTTTTCGTAATCATTCAAATAAC-3′) and O1-RT-2 (5′-ATGGTTGCTTCAGAAGAGACATATTG-3′); *OMADS3*, O3-RT-1 (5′-GCGAATCAGAGGTTAGCACATTG-3′) and O3-RT-2 (5′-AAATTACTGGTCTATACATGAGGAAAGG-3′); *OMADS5*, O5-RT-1 (5′-ACGAACTGGAGATGAAAGACGAG-3′) and O5-RT-2 (5′-GTAGATTGGGTTGAATTGGCTGAG-3′); *OMADS8*, O8-RT-1 (5′-ATGGAAGGCAGCATGAGAGAAC-3′) and O8-RT-2 (5′-AAAGCGTTAGCATTGTTACTTGTTT-3′); and *OMADS9*, O9-RT-1 (5′-TGATGATCCGAACAATTATGATGGTG-3′) and O9-RT-2 (5′-TTTGGCTGGCTTGGTTGGG-3′). The *O.* Gower Ramsey *α-tubulin* gene was used as a normalization control with the primers OnAT-RT-3 (5′-GGATTAGGCTCTCTGCTGTTGG-3′) and OnAT-RT-4 (5′-GTGTGGATAAGACGCTGTTGTATG-3′). Data were analyzed using the Gene Expression Macro software (version 1.1; Bio-Rad).

Plant Transformation and Transgenic Plant Analysis

The full-length cDNAs of *OMADS5*, *OMADS8*, and *OMADS9* were cloned into binary vector PBI121 (Clontech) under the control of the cauliflower mosaic virus 35S promoter. These constructs were transformed into Arabidopsis plants using the floral dip method as described elsewhere (Clough and Bent, 1998). Transformants were selected in medium containing 50 μg mL^{-1} kanamycin and were further verified by PCR and RT-PCR analyses.

Yeast Two-Hybrid Analysis

The cDNA truncations without the MADS box regions for *OMADS3*, *OMADS5*, *OMADS8*, and *OMADS9* were amplified by PCR. The primers for *OMADS3* were O3-Y-5 (5′-CATATGCCTTCTACAGAAATCAAAGATGCG-3′) and O3-Y-3 (5′-GGATCCTATCCACAGGCAATGTAAAAT-3′); for *OMADS5* were O5-Y-5 (5′-CATATGTACCAGATTGTAACTGGTAT-3′) and O5-Y-3 (5′-GGATCCCCCATTAAATACAGCAACAA-3′); for *OMADS8* were O8-Y-5 (5′-CATATGCCATCGACAACGTTGTCGAAGATGT-3′) and O8-Y-3 (5′-GGATCCACCAACAAGGAGGAGTACAAT-3′); and for *OMADS9* were O9-Y-5 (5′-CATATGACTACTGACACGAAGAGCATA-3′) and O9-Y-3 (5′-GGATCCTACTCAATGATGATGGTTCGC-3′). Specific 5′ and 3′ primers contained the *Nde*I (5′-CATATG-3′; underlined) or *Bam*HI (5′-GGATCC-3′; underlined) recognition site to facilitate cloning of the cDNAs. PCR fragments were ligated into the plasmid pGBKT7 (binding domain vector) or pGADT7 (activation domain vector) provided by the Matchmaker Two-Hybrid System 3 (Clontech). Recombinant plasmids were transformed into yeast using the lithium acetate method (Gietz et al., 1992). The transformants were selected on selection medium according to the manufacturer's instructions. β-Galactosidase activity in the transformants was analyzed as described by Tzeng and Yang (2001) and calculated according to Miller (1992).

Confocal Laser Scanning Microscopy

Flower tissues were imaged by an Olympus FV1000 confocal microscope. The plant cell wall was stained with 40 μg mL^{-1} propidium iodide (Molecular Probes). Propidium iodide was excited by a 543-nm helium/neon laser line, and emission was collected at 555 to 655 nm. Three-dimensional reconstruction was performed using the Fluoview 1000 software.

Scanning Electron Microscopy

Scanning electron microscopy was performed according to the methods of Hsu and Yang (2002) and Tzeng and Yang (2001). Various floral organs were fixed in 2% glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8) at 4°C overnight. After dehydration in a graded ethanol series, specimens were critical point dried in liquid CO_2. The dried materials were mounted and coated with gold-palladium in a JBS sputter coater (model 5150). Specimens were examined with a Topcon scanning electron microscope (model ABT-150S) with an accelerating voltage of 15 kV.

Phylogenetic Analyses

Predicted amino acid sequences of B class genes were obtained from the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) database. These were aligned using ClustalW, and a phylogenetic tree was constructed using Bayesian analyses. To select an appropriate protein model for subsequent phylogenetic analyses, the program ProtTest (Abascal et al., 2005) was used. The JTT + G + I model with among-site rate heterogeneity (α = 1.58) and invariable sites (pinvar = 0.05) was selected as the most fit model using a test of the Akaike Information Criterion framework (Abascal et al., 2005). Phylogenetic trees were reconstructed with Bayesian, and the numbers on every node indicate the Bayesian posterior probabilities.

The Bayesian method was conducted with the program MrBayes (Huelsenbeck and Ronquist, 2001). In the Bayesian analysis, one million generations of Markov chains were run. Trees were saved every 100 generations for a total size of 20,000 in the initial samples. Stationary phase of log likelihood was reached within 500,000 generations. Thus, burin-in (numbers of the initial trees were discarded) was set to 5,000. A majority rule consensus tree was constructed from the 15,000 remaining trees.

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