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

Yu-Yun Chang, Nai-Hsuan Kao, Jen-Ying Li, Wei-Han Hsu, Yu-Ling Liang, Jia-Wei Wu, and Chang-Hsien Yang*

Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan 40227, Republic of China

To investigate sepal/petal/lip formation in Oncidium Gower Ramsey, three paleoAPETALA3 genes, O. Gower Ramsey MADS box gen5 (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 sepal and petals and was significantly down-regulated in lip-like petals and lip-like sepal 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 Saudler, 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 Saudler, 1995; Theißen et al., 2000; Theißen et al., 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
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 paleoAP3 and PI lineages in angiosperms (Kramer et al., 1998; Theißen et al., 2000; Irish, 2009). The paleoAP3 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; Tsai et al., 2004; Kim et al., 2007; Mondragón-Palomino and Theißen, 2008, 2009). Several works have described the possible interactions among these four clades of paleoAP3 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 paleoAP3 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/OMADS5/OMADS8 and/or OMADS9 is required for sepal/petal/lip for-
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 paleoAP3 lineage of B group MADS box genes. OMADS5 is closely related to clade 1 paleoAP3 genes of orchids, such as PeMADS2 of Phalaenopsis (Tsai et al., 2004) and DeoAP3A of Dendrobium (Xu et al., 2006), and also showed high sequence identity to another O. Gower Ramsey clade 2 paleoAP3 gene, OMADS3, which was previously reported by our laboratory (Hsu and Yang, 2002; Figs. 1 and 2). OMADS9 is closely related to clade 3 paleoAP3 genes, such as DeoAP3B of Dendrobium (Xu et al., 2006) and PeMADS3 of Phalaenopsis (Tsai et al., 2004; Figs. 1 and 2). Within their K boxes, a sequence (QYQRQ) 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 (FxRLOPSQPNLH) and paleoAP3 (DL/IITTFALLE) 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 paleoAP3 orthologs indicates that OMADS5 and OMADS9 are O. Gower Ramsey B group MADS box paleoAP3 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 (MPFxFRVQPxQPNNLQE), 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 paleoAP3 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 paleoAP3 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
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 paleoAP3 lineage of monocots. OMADS5 belongs to clade 1, OMADS9 belongs to clade 3, and OMADS3 belongs to clade 2 of paleoAP3 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.”
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 sepal 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 sepal 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 OMADS5 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
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 × 40 μm²) were about eight times larger than those observed in lips (25 × 20 μm²; 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 × 40 μm²) 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/
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) 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...
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 paleoAP3 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 paleoAP3 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-
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
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 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-GMADS. 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 transgenic 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.
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 paleoAP3-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 tepal-like structure, as seen in the modern *O.* Gower Ramsey. Unlike *O.* Gower Ramsey, there is only one paleoAP3 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 a well-expanded tepal-like 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 has not been seen in lily, since no other paleoAP3 gene, such as OMADS5, has been identified in lily.

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 paleoAP3 and PI lineages. In orchid *O.* Gower Ramsey, there is only one PI gene, OMADS8, and at least two more duplications occurred in the paleoAP3 gene OMADS3 that generated the paleoAP3-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, OMADS5 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 tepal-like 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 paleoAP3 ortholog, LMADS1, and one PI ortholog, LMADS8, in lily.

[See online article for color version of this figure.]
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 paleoAP3 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 paleoAP3 (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 paleoAP3 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 DeOPI 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 paleoAP3 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 paleoAP3 gene OMADS3 characterized previously in our laboratory (Hsu and Yang, 2002), two more paleoAP3 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. verricosum*) 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 μmol 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-ll RT System (Promega). B class gene-specific MADS box degenerate 5’ primer Kram1 (5’-GGGTTACTCAAAAYCIGARCIGACIATAYCTYCTAAGMIGM-3’) and 3’ primers Kram2 (5’-TCIARRTTIFIFITGIAWGKGGTTC-3’) and Kram3 (5’-CIAIGCGGAAGTCTT-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 used to design primer pairs for specific amplification of cDNAs from *O. Gower Ramsey*.
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 OMS-3 (5′-CGAAGAGACATATTG-3′) for OMADS5, OPI-3-6 (5′-CCTCGCTTTGTGATCACCCTGTC-3′) for OMADS8, and OAP3-3-4 (5′-CCGATTCCTTACAGGGCTGCTCA-3′) for OMADS9, internal 5′ gene-specific primers OMS-5 (5′-GATATAGGAGTACACGATATG-3′) for OMADS3, OPI-5-5 (5′-TGGTAACAAGCAGTACTGACG-3′) for OMADS8, and OAP3-5-2 (5′-TCGAAAGACAGCTGATGACGA-3′) for OMADS9. The longest cDNAs for OMADS5, OMADS8, and OMADS9 were obtained by PCR amplification using the following primers: OMADS5, p5-5 (5′-GGAATTCGGCAACAAGACAGCAGATATG-3′) and p5-3 (5′-GGATCCGCCCCCATTAATTACACCAAA-3′); OMADS8, p8-5 (5′-TCTAGATTGGGGCCGGAGAAAGTATAGAGGCTGCA-3′) and p8-3 (5′-TCGAAGAGGAGAGCTGGGATCATGAAGAA-3′); and OMADS9, p9-5 (5′-TCTAGATCTCAGGAAAGGAGAAGTATAGGACA-3′) and p9-3 (5′-TCGAAAGACAGCTGATGACGA-3′). The specific 5′ and 3′ primers for OMADS5, OMADS8, and OMADS9 contained the BamHI (5′-GGAATTC-3′; underlined), XhoI (5′-TCTAGA-3′; underlined), and XhoI (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 for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 1 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′-GACGACAAATTGGTGGTCATG-3′) and OIRT-2 (5′-ATGGTCTTCGAGAACAGACATATTG-3′); OMADS3, OAP3-5-1 (5′-AAGAGTCTGAGGCTGTCCGA-3′) and OAPS-3-4 (5′-ATATCGGCTGTGATCTTCATG-3′); OMADS5, OMS-5-3 and OMS-5-6: OMS8, OPI-3-6 and OPI-5-5: and OMADS9, OAP3-3-4 and OAP3-5-2. The O. Gower Ramsey *a-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: *a-tubulin* forward primer (5′-AGGCTCTTCTTTGTTTACATGTC-3′) and reverse primer (5′-GCGGACCCGCGCTGTGTTG-3′); *ACTIN* forward primer (5′-AGAGATGAAATGCTGTCGGCA-3′) and reverse primer (5′-TCGGAGTTGAGAGGCTGAC-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 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′-GACGACAAATTGGTGGTCATG-3′) and OIRT-2 (5′-ATGGTCTTCGAGAACAGACATATTG-3′); OMADS3, OAP3-5-1 (5′-AAGAGTCTGAGGCTGTCCGA-3′) and OAPS-3-4 (5′-ATATCGGCTGTGATCTTCATG-3′); OMADS5, OMS-5-3 and OMS-5-6; OMS8, OPI-3-6 and OPI-5-5; and OMADS9, OAP3-3-4 and OAP3-5-2. The O. Gower Ramsey *a-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: *a-tubulin* forward primer (5′-AGGCTCTTCTTTGTTTACATGTC-3′) and reverse primer (5′-GCGGACCCGCGCTGTGTTG-3′); *ACTIN* forward primer (5′-AGAGATGAAATGCTGTCGGCA-3′) and reverse primer (5′-TCGGAGTTGAGAGGCTGAC-3′).

**Scanning Electron Microscopy**

Scanning electron microscopy was performed according to the methods of Hsu and Yang (2002) and Tseng and Yang (2001). Various floral organs were fixed overnight. After dehydration in a graded ethanol series, specimens 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 8 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 the program MrBayes (Abascal et al., 2005) was used. The JTT + G + I model with among-site rate heterogeneity (ω = 1.58) and invariable sites (π = 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 probability.

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, burn-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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**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 OMS-3-5 (5′-CATATGCTTCTAACGAAATAAAC-3′); OMS-3-6 (5′-GAAATTCGGCAACAAGACATATTG-3′); and OMS-3-7 (5′-GAAATTCGGCAACAAGACATATTG-3′); for OMADS5, OMS-5-5 (5′-CATATGCGGCGCTGATGACGA-3′); OMS-5-6 (5′-GAAATTCGGCAACAAGACATATTG-3′); and OMS-5-7 (5′-GAAATTCGGCAACAAGACATATTG-3′); for OMADS8, OMS-8-3-5 (5′-CATATGCGGCTGATGACGA-3′); and OMS-8-3-6 (5′-GAAATTCGGCAACAAGACATATTG-3′); and for OMADS9, OMS-9-3-5 (5′-CATATGCGGCTGATGACGA-3′); and OMS-9-3-6 (5′-GAAATTCGGCAACAAGACATATTG-3′). The selected 5′ and 3′ primers contained the NdeI (5′-CATATG-3′; underlined) or BamHI (5′-GGAATTC-3′; underlined) recognition site to facilitate cloning of the cDNAs. PCR fragments were ligated into the plasmid pGBK7 (binding domain vector) or pGAD7 (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.
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