Characterization of C- and D-Class MADS-Box Genes in Orchids¹[OPEN]

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Orchids (members of the Orchidaceae family) possess unique flower morphology and adaptive reproduction strategies. Although the mechanisms underlying their perianth development have been intensively studied, the molecular basis of reproductive organ development in orchids remains largely unknown. Here, we report the identification and functional characterization of two AGAMOUS (AG)-like MADS-box genes, Dendrobium ‘Orchid’ AG1 (DOAG1) and DOAG2, which are putative C- and D-class genes, respectively, from the orchid Dendrobium ‘Chao Praya Smile’. Both DOAG1 and DOAG2 are highly expressed in the reproductive organ, known as the column, compared to perianth organs, while DOAG2 expression gradually increases in pace with pollination-induced ovule development and is localized in ovule primordia. Ectopic expression of DOAG1, but not DOAG2, rescues floral defects in the Arabidopsis (Arabidopsis thaliana) ag-4 mutant, including reiteration of stamenoid perianth organs in inner whorls and complete loss of carpels. Downregulation of DOAG1 and DOAG2 in orchids by artificial microRNA interference using L-Met sulfoximine selection-based gene transformation systems shows that both genes are essential for specifying reproductive organ identity, yet they, exert different roles in mediating floral meristem determinacy and ovule development, respectively, in Dendrobium spp. orchids. Notably, knockdown of DOAG1 and DOAG2 also affects perianth organ development in orchids. Our findings suggest that DOAG1 and DOAG2 not only act as evolutionarily conserved C- and D-class genes, respectively, in determining reproductive organ identity, but also play hitherto unknown roles in mediating perianth organ development in orchids.

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pertaining to flower development. By now, some orchid floral homeotic genes have been investigated (Yu and Goh, 2000; Hsu et al., 2003, 2010; Tseng et al., 2003; Tsai et al., 2004, 2008b; Skipper et al., 2005; Xu et al., 2006; Chen et al., 2007, 2012; Chang et al., 2010; Teo et al., 2019), and the relevant mechanisms have been explored to explain the unique morphology of orchid flowers (Aceto and Gaudio, 2011; Mondragón-Palomino, 2013; Tsai et al., 2014). Among different floral homeotic genes, B- and E-class genes have been intensively studied in orchids, resulting in two proposed principles, the “orchid code” and the “perianth code”, which explain diverse patterns of perianth organ formation in orchids (Mondragón-Palomino and Theissen, 2008; Aceto and Gaudio, 2011; Hsu et al., 2015). The orchid code suggests that the identity of tepals and lips is determined by the interaction between one PISTILLATA/GLOBOSA (PI/GLO)-like gene and four paralogous APETALA3/DEFICIENS (AP3/DEF)-like genes belonging to four different clades (Mondragón-Palomino and Theissen, 2011). The interaction of clade 1 and 2 DEF-like genes determines sepal formation. High expression of these genes, together with low expression of clade 3 and 4 DEF-like genes, determines petal development, whereas the reverse expression pattern determines lip development (Aceto and Gaudio, 2011; Mondragón-Palomino and Theissen, 2011; Wang et al., 2019). The GLO-like genes that form a single clade are expressed in all four whorls of floral organs (Mondragón-Palomino and Theissen, 2011; Wang et al., 2019). The perianth code suggests that a competition between two protein complexes containing different AP3/AGAMOUS-LIKE 6 (AGL6) homologs of the B- and E-class genes determines the complex perianth patterns (Hsu et al., 2015). In this model, the sepal/petal tetrameric protein complex (OAP3-1/OAGL6-1/OAGL6-1/OPI) and the lip tetrameric protein complex (OAP3-2/OAGL6-2/OAGL6-2/OPI) specify the formation of sepals/petals and lips, respectively (Hsu et al., 2015). Despite the intensive research carried out on tepal and lip formation, the molecular mechanisms of gynostemium and ovule development in orchids remain largely unknown.

According to the ABCDE model, C- and D-class genes play conserved roles in the control of reproductive organ development (Theissen, 2001); they are thought to have arisen from gene duplications during the evolution of angiosperms (Theissen et al., 2000; Kramer et al., 2004). AGAMOUS (AG) is the only C-class homeotic gene found in the model plant Arabidopsis (Arabidopsis thaliana; Bowman et al., 1991; Drews et al., 1991), while redundant and complementary AG orthologs have been identified in other species, for example, PLENA (PLE) and FARINELLI (FAR) in snapdragon (Antirrhinum majus) and Floral Binding Protein gene6 (FBP6) and pMADS3 in Petunia hybrida (Kater et al., 1998; Davies et al., 1999). In addition to their conserved function in specifying the identity of stamens and carpels, AG and its orthologs also play a role in floral meristem determinacy (Mizukami and Ma, 1997; Kater et al., 1998). Similar results have been reported in some monocot plants. In rice (Oryza sativa), the two duplicated C-class genes, OsMADS3 and OsMADS58, act redundantly, with the former as a more important regulator, to specify reproductive organ identity and floral meristem determinacy (Yamaguchi et al., 2006; Dreni et al., 2011). In maize (Zea mays), three C-class genes, ZMM2, ZMM23, and ZAG1, have been identified. Functional analysis has shown that ZAG1 determines the floral meristem, while ZMM2 may play a role in regulating the formation of stamens and carpels (Mena et al., 1996). The best known D-class genes are petunia FBP7 and FBP11, both of which are involved in ovule development (Colombo et al., 1997). There are two duplicated D-lineage genes, OsMADS13 and OsMADS21, in rice. OsMADS13 is involved in determining ovule identity, whereas OsMADS21 does not contribute to ovule identity determination, possibly due to its low expression (Yamaguchi et al., 2006; Dreni et al., 2011). Of the two D-class genes, ZAG2 and ZMM1, identified in maize (Li et al., 2014), ZAG2 is a floral-specific gene whose expression is largely restricted to the developing ovules and the inner face of the carpels (Schmidt et al., 1993). So far, although several C- and D-class genes have been reported in different orchid species (Skipper et al., 2006; Song et al., 2006; Xu et al., 2006; Hsu et al., 2010; Wang et al., 2011; Chen et al., 2012; Salemme et al., 2013), functional analyses of these genes were mostly performed by ectopic overexpression in heterologous systems such as Arabidopsis or Nicotiana tabacum (tobacco). Thus, the endogenous functions of C- and D-class genes in orchids are so far largely unclear.

In this study, we have isolated the C- and D-class genes Dendrobium ‘Orchid’ AGAMOUS 1 (DOAG1) and DOAG2, respectively, from Dendrobium ‘Chao Praya Smile’. We characterized transgenic orchids in which DOAG1 and DOAG2 were downregulated by artificial microRNA interference (AmiR) and also investigated the effects of overexpression of DOAG1 and DOAG2 in Arabidopsis in both wild-type and ag-4 mutant backgrounds. These phenotypic analyses, together with the expression patterns of DOAG1 and DOAG2, suggest that while DOAG1 and DOAG2 share a similar function in Dendrobium ‘Chao Praya Smile’ in specifying reproductive organs, they exert different roles in that the former regulates floral meristem determinacy while the latter specifies ovule development.

RESULTS

Isolation of DOAG1 and DOAG2 from Dendrobium Orchids

To isolate C- and D-class genes from Dendrobium ‘Chao Praya Smile’, we designed degenerate primers based on the conserved regions of C- and D-class AG-like genes, respectively, in various flowering species. The full-length complementary DNA (cDNA) sequences of a putative C-class gene, DOAG1 (GenBank accession
no. MF363051) and a putative D-class gene, DOAG2 (GenBank accession no. MF363052) were obtained through a combined strategy of reverse-transcription PCR and rapid amplification of cDNA ends (RACE). DOAG1 cDNA is 1,205 bp in length and comprises a 705-bp coding sequence that encodes a protein with 235 amino acids and an estimated molecular mass of 27.2 kD. DOAG2 cDNA contains a 669-bp coding sequence that encodes a protein with 223 amino acids and an estimated molecular mass of 25.8 kD. Sequence analysis revealed that both DOAG1 and DOAG2 showed high sequence similarity with other AG-like proteins (Supplemental Fig. S1). Both proteins contain a highly conserved MADS-box domain, less conserved I and K domains, and divergent N and C termini (Supplemental Fig. S1; Yang et al., 2003; Kaufmann et al., 2005), while the conserved AG motifs I and II, which are located in the C-terminal regions of AG-like proteins (Kramer et al., 2004; Yun et al., 2004), are also present in DOAG1 and DOAG2 (Supplemental Fig. S1). We further constructed a phylogenetic tree based on DOAG1, DOAG2, and other known C- and D-class MADS-box genes, and revealed that DOAG1 and DOAG2 were clustered with other C- and D-class genes and closely related to DthyrAG1 from Dendrobium thyrsiflorum and DcOAG2 from Dendrobium crumenatum, respectively (Supplemental Fig. S2). In addition, intron 8, which is present in C-lineage genes but absent in most of the D-lineage genes from core eudicots and grass species (Kramer et al., 2004), is located only in the nucleotides encoding the last codon of AG motif II in DOAG1; it is absent in DOAG2. Taken together, these results suggest that DOAG1 and DOAG2 are C- and D-class genes, respectively, in Dendrobium ‘Chao Praya Smile’.

To examine the genomic organization of DOAG1 and DOAG2 in Dendrobium spp. orchids, we performed Southern blot analysis of genomic DNA digested with several restriction enzymes using digoxigenin-labeled probes synthesized from the C-terminal-specific regions of DOAG1 and DOAG2. DNA blot analyses revealed a single strong band in all of the digests for DOAG1 and DOAG2 (Supplemental Fig. S3), indicating that both genes likely exist as single-copy genes in the genome of Dendrobium ‘Chao Praya Smile’.

Expression of DOAG1 and DOAG2 in Dendrobium ‘Chao Praya Smile’

To understand the function of DOAG1 and DOAG2, we first examined the spatial expression patterns of DOAG1 and DOAG2 in various orchid organs (Fig. 1, A–G) by quantitative real-time PCR (qPCR). The expression of both DOAG1 and DOAG2 was relatively high in reproductive organs including inflorescence apices, flower buds, and open flowers, but low in vegetative organs like roots, leaves, and stems (Fig. 1, A, H, and I). However, their spatial expression patterns exhibited two different features. First, expression levels of DOAG1 in reproductive organs were much higher than those of DOAG2 (Fig. 1, H and I). Second, DOAG1 expression was highest in the inflorescence apices bearing differentiating floral meristems, but subsequently decreased in developing floral buds and open flowers, whereas DOAG2 expression displayed the opposite trend (Fig. 1, H and I).

A Dendrobium ‘Chao Praya Smile’ flower consists of three sepals at the first whorl, two petals and a lip at the second whorl, and a fused reproductive organ, the column, in the center containing two pollinia (male reproductive organs) covered by an anther cap and stigmatic cavity linked with the ovary (female reproductive organ; Fig. 1, B–F). Further expression analysis in Dendrobium spp. flowers revealed that DOAG1 was expressed in all floral organs, with the highest level in pollinia, but with the minimal level in the ovary (Fig. 1J), while DOAG2 was mainly expressed in the whole column, with the highest level in the ovary (Fig. 1K). These expression patterns are only partially similar to those observed for most AG-like genes that are expressed in the inner reproductive organs, but usually absent in sepals and petals (Kempin et al., 1993; Schmidt et al., 1993; Pnueli et al., 1994; Kang et al., 1995; Tzeng et al., 2002; Yun et al., 2004). Detectable expression of DOAG1 and DOAG2 in perianth organs, as similarly exhibited by a few AG-like genes in the basal angiosperm Illicium spp. (Kim et al., 2005) and the orchids Dendrobium crumenatum (Xu et al., 2006) and Phalaenopsis spp. (Song et al., 2006), implies that these AG-like genes may have divergent functions in floral organ development.

Several studies have reported that C- and D-class MADS-box genes are involved in ovule development (Aceto and Gaudio, 2011; Su et al., 2013). Since our analysis also revealed expression of DOAG1 and DOAG2 in the ovary (Fig. 1, J, and K), we further examined their temporal expression in developing ovules of Dendrobium ‘Chao Praya Smile’. To this end, we first investigated ovule development (Fig. 1G) in Dendrobium orchids using scanning electron microscopy (SEM; Supplemental Fig. S4). The whole course of ovule development in Dendrobium ‘Chao Praya Smile’ usually lasted for ~90 d after it was triggered by pollination. Before pollination, the ovary developed poorly, with placental protuberances differentiated from a single epidermal layer of the placenta (Supplemental Fig. S4, A and B). At 6 d after pollination (DAP), the placenta did not show any obvious change (Supplemental Fig. S4C), although the upper part of the ovary had started to bulge (Fig. 1G). At 16 DAP, the placental ridges continued elongating and branching to form thousands of finger-like ovule primordia (Supplemental Fig. S4, D–F), which is consistent with the progressively swollen appearance of the ovary (Fig. 1G). The outer and inner integuments of ovules extended to almost enclose the nucellus tip (Supplemental Fig. S4, G–I) at 32 DAP when the swollen ovary was clearly observable (Fig. 1G). At 64 DAP, double fertilization had already occurred to generate immature seeds (Supplemental
Figure 1. Quantitative analyses of $DOAG_1$ and $DOAG_2$ expression in *Dendrobium* 'Chao Praya Smile'. A, A *Dendrobium* 'Chao Praya Smile' plant with inflorescences and flowers. FB, Floral bud; IA, inflorescence apex; Lf, leaf; OF, open flower; Rt, root; Sm, stem. B, Close-up view of the inflorescence bearing open flowers of *Dendrobium* 'Chao Praya Smile'. C, A *Dendrobium* 'Chao Praya Smile' flower comprising three sepals (Se), two petals (Pe), one lip (Li), and the reproductive organ column (Co). D, Close-up view of the inflorescence bearing open flowers of *Dendrobium* 'Chao Praya Smile'. E, A *Dendrobium* 'Chao Praya Smile' flower comprising three sepals (Se), two petals (Pe), one lip (Li), and the reproductive organ column (Co). D, Close-up view of the inflorescence bearing open flowers of *Dendrobium* 'Chao Praya Smile'. E, A *Dendrobium* 'Chao Praya Smile' flower comprising three sepals (Se), two petals (Pe), one lip (Li), and the reproductive organ column (Co). F, Close-up view of the column showing the anther cap (Ac), stigmatic cavity (Sc), and ovary (Ov). G, Seed pods of *Dendrobium* 'Chao Praya Smile' at different DAP. Scale bars = 1 cm (A–D and G) and 500 μm (E and F). H and I, Expression of $DOAG_1$ (H) and $DOAG_2$ (I) in various tissues. J and K, Expression of $DOAG_1$ (J) and $DOAG_2$ (K) in different flower organs. The anther cap and pollinia from the column were collected as male reproductive organs (indicated as Po), whereas the remaining tissue of the column surrounding the stigmatic cavity and the ovary were collected as female reproductive organs. L and M, Expression of $DOAG_1$ (L) and $DOAG_2$ (M) in developing seed pods at different DAP, as shown in G. In H to M, expression of $DOAG_1$ and $DOAG_2$ was examined by qPCR analysis of three independent biological samples. The levels of $DOAG_1$ and $DOAG_2$ expression were normalized to the expression of the orchid polyubiquitin gene $DOUbi$. Error bars indicate the mean ± sd.
Fig. S4, J–L), and the ovary had almost reached maximum size (Fig. 1G). We found that DOAG1 expression levels remained constantly low during ovule development (Fig. 1L). In contrast, DOAG2 expression in ovule development was much higher. It exhibited a clearly increasing trend in developing ovules from 0 to 32 DAP, and remained at high levels afterward (Fig. 1M). These observations indicate that DOAG2 activity could be closely relevant to ovule development in *Dendrobium ‘Chao Praya Smile’*

**In Situ Localization of DOAG1 and DOAG2 in Orchid Reproductive Organs**

As both DOAG1 and DOAG2 were highly expressed in reproductive organs (Fig. 1, H and I), we then examined their detailed expression patterns in inflorescence apices, flowers, and developing ovules by in situ hybridization using their specific antisense versus sense RNA probes (Fig. 2; Supplemental Figs. S5 and S6). Both genes were detected in floral primordia and developing floral buds (Fig. 2, A–H; Supplemental Fig. S5, A–H). In young floral buds, DOAG1 and DOAG2 expression was relatively high in pollinia and the supporting rostellum compared to other floral organs (Fig. 2, B, C, F, and G). Strong expression of these two genes in the pollinia and rostellum was more apparent in old flowers (Fig. 2, D and H). Since increased DOAG2 expression is associated with ovule development (Fig. 1M), we further detected its localization in developing ovules after pollination by in situ hybridization. DOAG2 expression was barely visible in ovaries at 0 and 6 DAP (Fig. 2, I and J), but was clearly detectable in ovule primordia at 16 DAP (Fig. 2K). In contrast, DOAG1 expression was undetectable in ovaries at 0, 6, and 16 DAP (Supplemental Fig. S6, A–C). These results are generally in agreement with the quantitative expression data (Fig. 1, H–M), suggesting that although both DOAG1 and DOAG2 could be involved in development of floral organs, particularly reproductive organs, DOAG2 expression might be closely relevant to ovule development.

**Ectopic Expression of DOAG1 and DOAG2 in Arabidopsis**

To elucidate the biological function of DOAG1, we generated transgenic Arabidopsis plants (35S:DOAG1) in which DOAG1 was driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. Among 30 independent 35S:DOAG1 transgenic plants generated at the T1 generation, 22 lines showed curled leaves, early flowering, and defects in the second whorl of floral organs to different extents. In particular, strong 35S:DOAG1 lines displayed severely curled small leaves, stamenoid petals, and very early flowering with only three to five rosette leaves compared to wild-type plants (Fig. 3, A–E). All these phenotypes are similar to those of overexpression of AG and AG orthologs in other species (Schmidt et al., 1993; Rutledge et al., 1998; Tzeng et al., 2002). We further crossed a strong 35S:DOAG1 line (no. 1) with the Arabidopsis ag-4 mutants (Sieburth et al., 1995) to examine whether DOAG1 can compensate for the loss of AG function. ag-4 35S:DOAG1 flowered early with only four to five rosette leaves (Fig. 3F). Under our growth conditions, ag-4 flowers displayed stamens at the third whorl and a reiteration of stamenoid perianth.

**Figure 2.** In situ localization of DOAG1 and DOAG2 transcripts in *Dendrobium ‘Chao Praya Smile’*. Sections in this figure were hybridized with DOAG1 and DOAG2 gene-specific antisense RNA probes or the corresponding sense probes, which served as negative controls (Supplemental Fig. S5). A to D, DOAG1 mRNA localization in an inflorescence apex (A), young floral buds (B and C), and an old floral bud (D). E to H, DOAG2 mRNA localization in an inflorescence apex (E), young floral buds (F and G), and an old floral bud (H). I to K, DOAG2 mRNA localization in ovules at different developmental stages. DOAG2 is barely detectable in sections of ovaries at 0 DAP (I or 6 DAP J), but is expressed in ovule primordia at 16 DAP (K). Red arrows indicate ovule primordia. The inset in K shows an enlarged view of the ovule primordium in the dotted square. Scale bars = 200 μm. Ac, Anther cap; Co, column; FP, floral primordium; IM, inflorescence meristem; Li, lip; Pe, petal; Po, pollinia; Ro, rostellum; Se, sepal.
organs without carpels in the center (Fig. 3G), suggesting an indeterminate proliferation of the floral meristem. These phenotypes were fully rescued in ag-4 35S:DOAG1 flowers, which exhibited a determinate generation of carpels at the fourth whorl (Fig. 3H). Quantitative expression analysis showed that DOAG1 expression was remarkably high in representative 35S:DOAG1 transgenic lines and ag-4 35S:DOAG1 compared to wild-type plants (Fig. 3I). These results suggest that DOAG1 plays a conserved role as AG in specifying reproductive organ identity and floral meristem determinacy in Arabidopsis (Mizukami and Ma, 1995, 1997).

We also created 35S:DOAG2 transgenic Arabidopsis plants, and found that 19 of 28 independent transgenic lines displayed phenotypes similar to those observed in 35S:DOAG1, including curled leaves and early flowering (Fig. 4A). However, their phenotypes were generally much weaker than those of 35S:DOAG1. 35S:DOAG2 displayed the curled-leaf phenotype in fewer rosette leaves (Fig. 4B), produced flowers with normal identity in four whorls (Fig. 4C–E), and exhibited a weaker early-flowering phenotype than 35S:DOAG1 plants (Figs. 3E and 4F), although DOAG2 expression was very high in 35S:DOAG2 plants (Fig. 4G). We subsequently crossed a strong 35S:DOAG2 line (no. 1) with ag-4 to examine whether DOAG2 also confers the AG function. ag-4 35S:DOAG2 showed growth phenotypes similar to those of ag-4 (Supplemental Fig. S7). In particular, the floral defects of ag-4, including lack of carpels and indeterminate floral meristems, remained unchanged in the 35S:DOAG2 background (Supplemental Fig. S7, B–E), indicating that DOAG2 does not compensate for the loss of AG in Arabidopsis. These observations suggest that DOAG2 and DOAG1 may play different roles in floral organ development.

**Figure 3.** Phenotypic analyses of transgenic Arabidopsis plants over-expressing DOAG1. A, A 35S:DOAG1 plant displays early flowering and curled leaves compared to a wild-type (WT) plant. B, Close-up view of a wild-type Arabidopsis flower consisting of four whorls of floral organs, including sepals, petals, stamens, and carpels. C, Inflorescence apex of a 35S:DOAG1 plant. D, A 35S:DOAG1 flower showing stamenoid petals. E, Flowering time distribution of wild-type and 35S:DOAG1 plants under long-day conditions. F, ag-4 35S:DOAG1 flowered earlier with fewer rosette leaves than ag-4. G and H, Close-up views of ag-4 (G) and ag-4 35S:DOAG1 (H) flowers. I, Quantitative analysis of DOAG1 expression in representative 35S:DOAG1 transgenic plants and ag-4 35S:DOAG1. Results are normalized against the expression levels of TUB2 and shown as relative values to the highest level set to 100%. Error bars indicate the mean ± SD. Asterisks indicate significant differences in DOAG1 expression levels between 35S:DOAG1 and wild-type plants or between ag-4 35S:DOAG1 and ag-4 using two-tailed paired Student’s t test (*P < 0.05). Scale bars = 1 cm (A and F) and 0.5 mm (B–D, G, and H).

**Knockdown of DOAG1 in Dendrobium ‘Chao Praya Smile’**

To understand the endogenous function of DOAG1, we created AmiR-doag1 transgenic orchids using particle bombardment or Agrobacterium-mediated transformation coupled with an in vitro tissue culture system (Supplemental Fig. S8; Yu and Goh, 2000; Yu et al., 2001; Chai et al., 2007; Ding et al., 2013; Wang et al., 2017). Putative transgenic *Dendrobium* orchids were screened by L-Met sulfoximine (MSO) as an agent for selection of transgenic lines bearing the *bialaphos resistance* (bar) gene as a selectable marker (Chai et al., 2007). The presence of the *AmiR-doag1* transgene in all putative lines was first examined by PCR genotyping, and further confirmed by Southern blot analysis of representative lines (Fig. 5). We created seven independent AmiR-doag1 transgenic orchid lines, and all proceeded to the reproductive stage except for line 5, which exhibited floral abortion. Among the six lines that generated flowers, lines 1, 2, and 4 exhibited the same floral phenotypes, showing an increased number of floral organs at all whorls. For example, compared to a wild-type flower consisting of three sepals, two petals plus one lip, and one gynostemium (column; Fig. 6A), a typical flower of *AmiR-doag1* line 1 displayed five sepals, five petals plus one lip, and two pieces of gynostemium supported by one small leaf-like bract (Fig. 6, B and C). These lines generated poorly developed pollinia (Fig. 6, D and E), which were dysfunctional in pollinating orchid flowers under tissue culture conditions. Notably, *AmiR-doag1* line 3 produced floral structures with all leaf-like reiterated tissues but without any reproductive organs (Fig. 6F). The other two lines (lines 6 and 7) produced flowers with normal floral organ identity, like wild-type plants.
To evaluate whether the phenotypes exhibited by different AmiR-doag1 orchid lines are associated with downregulation of DOAG1, we examined DOAG1 expression levels in reproductive tissues from AmiR-doag1 lines 1, 2, 3, and 6. DOAG1 expression was downregulated in lines 1 and 2, and greatly reduced, by two-thirds, in line 3, whereas its expression in line 6 remained unchanged compared to that in wild-type plants (Fig. 6G). These results demonstrate that changes in DOAG1 expression are associated with floral phenotypes to different extents in AmiR-doag1 orchid lines. Downregulation of DOAG1 in different AmiR-doag1 lines resulted in an increase in floral organs at all whorls (Fig. 6, B, C, and F), which implies a compromise in floral meristem determinacy. In addition, dramatic reduction of DOAG1 expression abolished the formation of all floral organs, including complete loss of male and female reproductive organs (Fig. 6F). Interestingly, we found that in AmiR-doag1 orchid lines 1 to 3 where DOAG1 was downregulated, DOAG2 expression was slightly upregulated (Fig. 6H), implying a potential effect of DOAG1 on DOAG2 expression during flower development.

Knockdown of DOAG2 in Dendrobium ‘Chao Praya Smile’

We also created independent AmiR-doag2 transgenic orchid lines to investigate the endogenous function of DOAG2. The presence of the AmiR-doag2 transgene in all eight putative lines was examined by PCR genotyping, and substantiated by Southern blot analysis of two representative lines (Fig. 5). All of these AmiR-doag2 lines proceeded to the reproductive stage and produced inflorescences bearing flowers with different phenotypes. Lines 6 to 8 generated flowers with normal floral organ identity, like wild-type plants, whereas
lines 1 to 5 generated floral structures with only two or three perianth organs and no gynostemium (Fig. 7, A–F). These perianth organs were mostly greenish sepal-or petal-like structures, many of which showed the purple color at the base near the pedicel (Fig. 7, B and D), which is characteristic of perianth organs in wild-type *Dendrobium* ‘Chao Praya Smile’ flowers (Fig. 7A).

We also measured DOAG2 expression in reproductive tissues of the AmiR-doag2 lines to investigate the relationship between DOAG2 expression levels and the floral organ defects in these lines. Compared to its expression in wild-type plants, DOAG2 expression was not significantly altered in reproductive tissues from lines 6 to 8, which produced normal flowers (Supplemental Fig. S9A). In contrast, DOAG2 expression was significantly downregulated in AmiR-doag2 lines 1 to 5 (Fig. 7G), suggesting that downregulation of DOAG2 has a causal link with the defects in the formation of gynostemium and perianth organs in *Dendrobium* orchids. Furthermore, DOAG2 expression was much lower in lines 1 and 2 than in lines 3 to 5 (Fig. 7G). This result is associated with the observation that fewer perianth organs were generated in AmiR-doag2 lines 1 and 2 than in lines 3 to 5 (Fig. 7, B–F), indicating that the degree of downregulation of DOAG2 influences the development of perianth organs. Although both DOAG1 and DOAG2 affect perianth organ development, knockdown of these genes did not alter the expression of DOAPI (Supplemental Fig. S10), an ortholog of a class A Arabidopsis gene, APETALA1 (AP1), in *Dendrobium* ‘Chao Praya Smile’ (Sawettalake et al., 2017). In addition, we also found that in AmiR-doag2 orchid lines 1 to 5, where DOAG2 was downregulated, DOAG1 expression was upregulated to different extents (Fig. 7H), whereas in AmiR-doag2 lines 6 to 8, which showed insignificant changes in DOAG2 expression, DOAG1 expression remained unchanged (Supplemental Fig. S9B). These results, together with the reverse trend (Fig. 6H), imply that DOAG1 and DOAG2 mutually influence each other’s expression during flower development.

**DISCUSSION**

In the ABCDE model of flower development, C- and D-class genes regulate the development of reproductive organs (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). It has been suggested that C-class genes determine the identity of stamens and carpels, while D-class genes are primarily involved in ovule development. As members in one of the largest and most widespread families of flowering plants, orchids display unique characteristics in reproductive organ development, including fusion of male and female reproductively organs into one gynostemium (or column;
Second, the presence of intron 8 in the last codon of the DOAG1 but not in DOAG2 (Supplemental Fig. S1). In particular, an N-terminal extension preceding the MADS domain, found only in C-lineage genes, is present in DOAG1 and other known D-class genes, respectively (Supplemental Fig. S1). In parthenocarpic orchids, lines 1 (B) and 2 (C), develop only two perianth organs without the gynostemium. D to F, AmiR-doag2 transgenic orchids, lines 3 (D), 4 (E), and 5 (F), develop three perianth organs without the gynostemium. Scale bars = 0.5 cm. G and H, Quantitative analysis of DOAG2 (G) and DOAG1 (H) expression in wild-type and representative AmiR-doag2 transgenic orchids. Total RNA extracted from open flowers and inflorescence apex was used for expression analysis. Results are normalized against the expression levels of DOUbi and showed as relative values to the wild-type level set at 100% (G) or 1.0 (H). Error bars indicate the mean ± sd. Asterisks indicate significant differences in expression levels of DOAG2 (G) and DOAG1 (H) between specified transgenic lines and wild-type plants using two-tailed paired Student’s t test (*P < 0.05).

Sequence analysis of DOAG1 and DOAG2 have provided several pieces of evidence supporting the hypothesis that DOAG1 and DOAG2 are putative C- and D-class genes, respectively. First, multiple sequence analysis has revealed that DOAG1 and DOAG2 share high sequence similarity with C- and D-class proteins, respectively (Supplemental Fig. S1). In particular, an N-terminal extension preceding the MADS domain, found only in C-lineage genes, is present in DOAG1 but not in DOAG2 (Supplemental Fig. S1). Second, the presence of intron 8 in the last codon of the DOAG1 genomic sequence, which is absent in DOAG2 and other known D-class genes, also suggests that DOAG1 is a function C gene in Dendrobium orchids (Kramer et al., 2004). Lastly, phylogenetic analyses of C- and D-class MADS-box genes have also shown that DOAG1 and DOAG2 belong to C- and D-lineage genes, respectively (Supplemental Fig. S2).

In addition to the results from sequence analyses, further characterization of these two genes has provided other lines of evidence to support that DOAG1 and DOAG2 act as C- and D-class genes, respectively, in Dendrobium orchids. First, although both DOAG1 and DOAG2 are relatively highly expressed in the reproductive organ (the column) versus the perianth organs (Fig. 8B), DOAG2 expression is much higher than that of DOAG1 in ovules and gradually increases in pace with ovule development. These expression patterns were similar to those of C- and D-class genes from other orchid species. For instance, several D-class genes, including PeMADS7 from Phalaenopsis equestris (Chen et al., 2012), OMADS2 from Oncidium ‘Chao Praya Smile’ (Hsu et al., 2010), PhalAG1 and OMADS hybrid (Chen et al., 2006), OitaSTK from Orchis italica (Sallemme et al., 2013), and DthyrAG1 from D. thyrsiflorum (Skipper et al., 2006), show higher expression in developing ovules than their corresponding C-class genes, PeMADS1, OMADS4, PhalAG1, OitaAG, and DthyrAG1, respectively. These observations suggest that orchid D-class genes, including DOAG2, likely function in mediating ovule development. Second, floral defects in the Arabidopsis ag-4 mutant, including reiteration of stigmatic perianth structures in inner whorls and complete loss of carpels, are rescued only by overexpression of DOAG1, not by overexpression of DOAG2. These results suggest that DOAG1 itself is sufficient to exert the C-class gene role similar to that of AG in specifying reproductive organ identity and floral meristem determinacy in Arabidopsis. This is in agreement with the observation that only overexpression of DOAG1 results in the formation of stamenoid petals, whereas overexpression of DOAG2 does not influence floral organ identity in Arabidopsis.
Third, downregulation of DOAG1 in *Dendrobium* ‘Chao Praya Smile’ results in floral meristem indeterminacy and defects in reproductive organ formation (Fig. 8C), which are comparable to the phenotypes caused by loss of *AG* activity in Arabidopsis (Mizukami and Ma, 1997; Kater et al., 1998) or *AG* orthologs in monocots, such as rice and maize. In rice, two duplicated C-class genes, OsMADS3 and OsMADS558, act redundantly to specify reproductive organ identity and floral meristem determinacy (Dreni et al., 2011). Among the C-class genes in maize, ZAG1 is required for floral meristem determinacy, while ZMM2 may participate in regulating the formation of stamens and carpels (Mena et al., 1996). In contrast, knockdown of DOAG2 mainly influences reproductive organ development, but does not affect floral meristem determinacy in *Dendrobium* ‘Chao Praya Smile’ (Fig. 8C). Taken together, these results suggest that although both DOAG1 and DOAG2 are required for specifying reproductive organ identity in orchids, they also play evolutionarily conserved, but different, roles, like C- and D-class genes, respectively.

C- and D-class genes are thought to have arisen from gene duplication during the evolution of angiosperms and may function redundantly in the control of reproductive organs (Kramer et al., 2004; Mondragón-Palomino, 2013). This is supported by extensive sequence similarity, overlapping expression profiles, and functional similarity between C- and D-class genes. In Arabidopsis, C- and D-class genes in the AG clade, including *AG*, *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*), play essential yet partially redundant roles in specifying carpel and ovule identity (Pinyopich et al., 2003). Similarly, most of the C- and D-class genes so far identified in different orchid species, including DOAG1 and DOAG2 in this study, share overlapping gene expression patterns in reproductive organs (Skipper et al., 2006; Song et al., 2006; Xu et al., 2006; Hsu et al., 2010; Wang et al., 2011; Chen et al., 2012; Salemme et al., 2013). While these expression patterns indicate possible functional redundancy among orchid C- and D-class genes, our studies in *Dendrobium* orchids have surprisingly revealed nonredundant roles of DOAG1 and DOAG2 in specifying column identity. Knockdown of DOAG1 to different extents causes a range of reproductive defects from poorly developed pollinia to complete loss of the column, while knockdown of DOAG2, even at mild levels, completely abolishes column formation (Fig. 8C), suggesting that both DOAG1 and DOAG2 are essential for specifying column identity.

Notably, in addition to their regulatory roles in reproductive organ development, both DOAG1 and DOAG2 affect the development of perianth organs in *Dendrobium* orchids (Fig. 8C). Strong knockdown of DOAG1 results in leaf-like reiterated structures (green leaves). Knockdown of DOAG2 abolishes the generation of reproductive organs and produces greenish chimeric perianth organs (light green leaves).
plants were grown under long-day conditions (16 h light/8 h dark) at 23°C. Daylight fluorescent lamps (Ding et al., 2013). Arabidopsis (Arabidopsis thaliana) plants were grown under long-day conditions (16 h light/8 h dark) at 23°C ± 2°C. Calli that developed from seeds were cultured at 24°C under a 16-h photoperiod of 35 μmol m⁻² s⁻¹ from daylight fluorescent lamps (Ding et al., 2013). Arabidopsis (Arabidopsis thaliana) plants were grown under long-day conditions (16 h light/8 h dark) at 23°C ± 2°C. ag-4 mutants are in the Landsberg erecta (Ler) background.

CONCLUSION

In conclusion, through functional characterization of two AG-like genes, DOAG1 and DOAG2, using MSO selection-based gene transformation systems in Dendrobium ‘Chao Praya Smile’, we have revealed hitherto unknown endogenous functions of the C- and D-class genes in orchids. DOAG1 and DOAG2 are both required for specifying reproductive organ identity, yet their roles in mediating floral meristem determinacy and ovule development, respectively, suggest that they act as evolutionarily conserved C- and D-class genes in orchids. In addition, both genes are expressed in perianth organs and petal development, implying functional divergence of AG orthologs in orchids compared to other flowering plants. Taken together, our findings contribute to elucidation of the underlying molecular mechanisms of flower development in one of the largest angiosperm families and shed light on the functional conservation and divergence of floral homeotic genes during plant evolution.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Dendrobium ‘Chao Praya Smile’, a hybrid of Dendrobium ‘Pinkly’ and Dendrobium ‘Kiyomi Beauty’, were grown in the greenhouse under long-day conditions (16 h light/8 h dark) at 25°C ± 2°C. Calli that developed from seeds were cultured at 24°C under a 16-h photoperiod of 35 μmol m⁻² s⁻¹ from daylight fluorescent lamps (Ding et al., 2013). Arabidopsis (Arabidopsis thaliana) plants were grown under long-day conditions (16 h light/8 h dark) at 23°C ± 2°C. ag-4 mutants are in the Landsberg erecta (Ler) background.

Plant Transformation

Agrobacterium tumefaciens-mediated transformation of Arabidopsis plants was carried out using a floral dipping method (Clough and Bent, 1998). All transgenic Arabidopsis plants described in this article were selected by Basta (glufosinate ammonium) on soil. Genetic transformation of Dendrobium ‘Chao Praya Smile’ was performed utilizing particle bombardment and A. tumefaciens-mediated transformation coupled with our established in vitro tissue culture and MSO selection systems (Supplemental Fig. S7; Yu et al., 2001; Chai et al., 2007; Ding et al., 2013; Wang et al., 2017). A tumefaciens-mediated transformation was carried out when the A. tumefaciens OD600 reached 0.4. The transformed orchid thin sections were cocultured with A. tumefaciens for 2 h at 24°C with shaking at 120 rpm, and thin sections then were transferred to solid medium for further screening.

Cloning of DOAG1 and DOAG2 cDNAs from Dendrobium ‘Chao Praya Smile’

Total RNA was isolated from inflorescences of Dendrobium ‘Chao Praya Smile’ using the RNeasy Plant Mini Kit (Qiagen). Specific AG-like cDNA fragments were amplified with one pair of degenerate primers. The resulting cDNA fragments of ~300 bp were cloned into the pGEM-T Easy vector (Promega). After sequencing, the fragments were identified as the partial sequences of AG orthologs, namely DOAG1 and DOAG2. To obtain the full-length cDNA sequences of DOAG1 and DOAG2, 3’-RACE and 5’-RACE were performed with gene-specific primers using the SMART RACE cDNA Amplification Kit (BD Biosciences Clontech). All primers used for gene cloning are listed in Supplemental Table S1.

Generation of Knockdown and Overexpressing Constructs

For the overexpression constructs, the coding regions of DOAG1 and DOAG2 were ligated into a binary vector (pGreen 0229-35S) and driven by the CaMV 35S promoter (Yu et al., 2004), which permitted MSO selection for the bar gene (Chai et al., 2007). To construct AmiR-daog1 and AmiR-daog2 for down-regulation of DOAG1 and DOAG2, AmiRs were designed using the software on the Web MicroRNA Designer site (http://www.wmd3.weigelworld.org). A set of four primers based on DOAG1 and DOAG2 sequences were generated and used for PCR amplification according to the published protocol (Schwab et al., 2010). The amplified PCR fragments were digested with SpeI and EcoRI and cloned into the pGreen 0229-35S vector.

Sequence Analysis

Alignment of the deduced amino acid sequences was performed using the software MEGA X and BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The protein sequences of AG orthologs from various plant species aligned in this study were retrieved from the National Center for Biotechnology Information database.

Phylogenetic Analysis

The 59-nucleotide sequences of C- and D-class MADS-box genes from various plant species aligned in this analysis were retrieved from the National Center for Biotechnology Information database. The phylogenetic tree was constructed by the maximum-likelihood framework using an alignment of nucleotides by MEGA X.

Southern Blot Analysis

Genomic DNA was isolated from leaves of Dendrobium ‘Chao Praya Smile’ following the method described by Carlson et al. (1991). Twenty micrograms of genomic DNA was digested with different restriction enzymes, electrophoresed on a 0.7% (w/v) agarose gel and subsequently blotted onto a nylon membrane. The blot was hybridized overnight with the specific digoxigenin-labeled DNA, washed, and detected as previously described (Yu and Goh, 2000).

Expression Analysis

Total RNA was extracted from either orchid or Arabidopsis using the FavorPrep Plant Total RNA Mini Kit (Favorgen) and reverse transcribed by M-MLV Reverse Transcriptase (Promega) based on the manufacturers’ instructions. qPCR was performed on three biological replicates, each with three technical replicates, on the CFX384 Real-Time PCR Detection System (Bio-Rad) with SYBR Green Master Mix (Toyobo). The relative gene expression levels were calculated as previously reported (Liu et al., 2007). The normalization controls for expression analyses in orchid and Arabidopsis were the orchid polyubiquitin gene DOUBI and the Arabidopsis TUBULIN 2 (TUB2) gene, respectively. Primers used for real-time PCR gene are listed in Supplemental Table S1.
In Situ Hybridization

We performed nonradioactive in situ hybridization on sections of inflorescences apices, floral buds, and ovaries harvested from Dendrobium orichs as previously described (Yu and Goh, 2000). For synthesis of DOAG1 and DOAG2 antisense and sense RNA probes, the 3‘-end gene-specific regions of DOAG1 and DOAG2, respectively, were amplified and cloned into the pGEM-T Easy vector (Promega). The resulting vectors were then used as the templates for in vitro transcription by the DIG RNA Labeling Kit (Roche).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: MF363051 (DOAG1) and MF363052 (DOAG2).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Alignment of amino acid sequences of DOAG1, DOAG2, and other AG-like proteins.

Supplemental Figure S2. Phylogenetic analysis of DOAG1 and DOAG2 with other C-end class MADS-box genes.

Supplemental Figure S3. Southern blot analysis of DOAG1 and DOAG2 genomic organization in Dendrobium ‘Chao Praya Smile’.

Supplemental Figure S4. Ovule development in Dendrobium ‘Chao Praya Smile’.

Supplemental Figure S5. In situ control sections from Dendrobium ‘Chao Praya Smile’ hybridized with DOAG1 and DOAG2 sense RNA probes.

Supplemental Figure S6. In situ localization of DOAG1 transcripts in ovules at different developmental stages in Dendrobium ‘Chao Praya Smile’.

Supplemental Figure S7. Overexpression of DOAG2 does not rescue ag-4 phenotypes in Arabidopsis.

Supplemental Figure S8. Generation of transgenic Dendrobium ‘Chao Praya Smile’ via Agrobacterium-mediated transformation and particle bombardment.

Supplemental Figure S9. Quantitative analysis of DOAG1 and DOAG2 expression in wild-type and several Amlr-doag2 transgenic orchids (lines 6–8).

Supplemental Figure S10. Quantitative analysis of DOAP1 expression in wild-type and representative Amlr-doag1 and Amlr-doag2 transgenic orchids.

Supplemental Table S1. List of primers used in this study.

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