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Original Paper

Ectopic expression of an *AP3*-like and a *PI*-like genes from 'Sekkoku' orchid (*Dendrobium moniliforme*) causes the homeotic conversion of sepals to petals in whorl 1 and the suppression of carpel development in whorl 4 in *Arabidopsis* flowers

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Abstract Three class-B MADS-box genes, the paleotype *AP3* genes *DMAP3A* and *DMAP3B,* and the *PI*-like gene *DMPI*, were isolated from a native orchid in Japan named 'Sekkoku' (*Dendrobium moniliforme*) and ectopically expressed in *Arabidopsis*. *DMAP3A* and *DMPI* were expressed in all four floral whorls in the orchid, whereas *DMAP3B* was expressed only in whorls 2, 3, and 4. The ectopic expression of 35S::*DMPI* caused a partial sepal-to-petal conversion in whorl 1, increased the longevity of the flowers and delayed silique maturation in the transgenic plants, compared with wild-type plants. Transgenic *Arabidopsis* plants over-expressing 35S::*DMAP3B*/35S::*DMPI* exhibited a vegetative phenotype with leaf curling, a near-complete sepal-to-petal conversion in whorl 1, and the suppression of carpel development in whorl 4. Our results indicate that *DMAP3B* and *DMPI* are major class B MADS-box genes in *D. moniliforme* and may play an important role in the development of petals in the second floral whorl of the orchid. In addition, our results also suggest that these genes can be used for the genetic engineering of flower shape in plants.

Key words: Class B MADS-box, *Dendrobium moniliforme*, floral whorls, longevity of flower, sepal-to-petal.

Floral organ development is an important part of the plant life cycle. In angiosperms, floral organs are formed in concentric rings, called whorls. Sepals are formed first in the outer whorl (whorl 1), followed by petals, stamens, and carpels in whorl 2, 3 and 4 of flower, respectively. According to the ABC model of floral patterning that has been proposed by Coen and Meyerowitz in 1991, class B genes are important for establishing petals (together with class A genes) and stamens (together with class C genes) (Coen and Meyerowitz 1991). All of class B genes belong to the MIKC-type MADS-box gene family of transcription factors, which include a highly conserved region called a MADS domain, an intervening (I) domain, a keratin-like coiled-coil (K) domain, and a C-terminal domain (Shore and Sharrocks 1995). Duplication of an ancestral B gene during plant evolution gave rise to two class B subfamilies, *APETALA3/DEFICIENS* (*AP3/DEF)* and *PISTILLATA/GLOBOSA (PI/GLO)* (Goto and Meyerowitz 1994; Jazk et al. 1994; Kramer et al. 1998; Schwarz-Sommer et al. 1992). The multiple gene duplication took place in the *AP3/DEF* lineage, causes separation of three distinct clades with divergent C-terminal motifs: eu*AP3*, paleo*AP3*, and *TOMATO MADS-BOX GENE6* (*TM6*) (Kramer et al. 1998; Theissen et al. 1996). The eu*AP3* clade is composed of *AP3/DEF*-like genes isolated from higher eudicots, whereas genes belonging to the paleo*AP3* clade have been identified in lower eudicots, magnolid dicots, monocots, and basal angiosperms (Kramer and Irish 2000). The genes in the *TM6* clade are present in higher dicots and monocots, the expression patterns and functions of the genes in this clade are more diverse than those in the class-B MADS-box family (Pnueli et al. 1991; Yu et al. 1999). In *Antirrhinum*, DEF and GLO proteins are function in cells as heterodimeric complexes (Tröbner et al. 1992) and can be integrated into a tetrameric protein complex together with a class E protein; SEPALLATA 3 (SEP3) (Melzer et al. 2009). Functions of class B genes have been largely confirmed in several species of core-eudicot plants, the results

Abbreviations: MS medium, Murashige and Skoog medium; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SEM, scanning electron microscopy
This article can be found at http://www.jspcmb.jp/

indicated that the expression pattern and function of B genes are similar among the plant species (Bowman 1997; Hernández-Hernández et al. 2007; Kramer and Irish 1999).

Unlike the eu-dicot plants which the floral morphology in whorl 1 and whorl 2 are clearly different, most of plants in basal angiosperm, magnolids and non-grass monocot have undifferentiated perianths which are referred to as tepals. To explain the molecular mechanism of tepal formation in non-grass monocots belonging to the Liliaceae family, van Tunen et al. (1993) proposed the modified ABC model. In this model, class B genes are expressed in both whorls 1 and 2. In connection with the modified ABC model, the expression of two *DEF*- and one *GLO*-like genes isolated from tulips was detected in whorls 1 and 2 in flowers (Kanno et al. 2003).

In orchid, the mechanisms of tepal arrangement seem to be more complicated than other monocots as flowers of orchids are zygomorphic and it obtains three kinds of perianths in a flower i.e. three outer tepals (often also termed 'sepals') located in the whorl 1 of flower, and two lateral inner tepals (petals) and a median inner tepal called lip or labellum arranged in the second floral whorl (Mondragon-Palomino and Theissen 2008; Mondragon-Palomino et al. 2009). The molecular mechanisms of perianth formation in orchid flower was found to be involved with combinational interaction of four *DEF*-like genes with *GLO*-like genes based on the studies in several orchid species i.e. *Oncidium* Gower Ramsey, *Phalaenopsis equestris*, *Habenaria radiata* and *Dendrobium crumenatum* (Hsu and Yang 2002; Kim et al. 2007; Mondragon-Palomino and Theissen 2008; Mondragon-Palomino et al. 2009; Tsai et al. 2004; Xu et al. 2006). Given the *DEF*-like genes phylogeny of those genes, orchid *DEF*-like genes have been separated into four major clades (Mondragon-Palomino and Theissen 2008; Mondragon-Palomino et al. 2009).

Clade 1 contains *PeMADS2*-like genes, including *PeMADS2* (*P. equestris*) and *DcOAP3A* (*D. crumenatum*). The ubiquitous expression of these genes has been detected in outer tepals, suggesting that the genes in this clade are responsible for the development of tepals in the whorl 1. However, the over-expression of *DcOAP3A* and *DcOPI*, a *PI/GLO*-like gene from *D. crumenatum*, in *Arabidopsis*, did not result in a complete sepal-to-petal conversion in whorl 1 or a carpel-to-stamen conversion in whorl 4, like its orthologs (e.g., *AP3* and *PI* from *Arabidopsis*) (Xu et al. 2006). Clade 2 is composed of *OMADS3*-like genes, including *OMADS3* (*Oncidium*) and *PeMADS5* (*P. equestris*) (Tsai et al. 2004). Like the first clade, the expression of these genes has been detected in outer tepals. However, function of these genes may have a different mechanism of regulation than other *DEF*-like genes since transgenic *Arabidopsis* over-expressing *OMADS3* truncated the MADS domain had a similar phenotype to plants ectopically expressing class A genes (Hsu and Yang 2002). Clade 3 contains such *PeMADS3*-like genes as *PeMADS3* (*P. equestris*), *DcOAP3B* (*D. crumenatum*), and *HrDEF* (*H. radiata*). The expression of genes in this clade has only been reported in whorls 2, 3, and 4 in flowers but not in whorl 1 suggesting that the genes in this clade may involve with the development of inner tepals, the second floral whorl. Finally, clade 4 contains *PeMADS4*-like genes such as *PeMADS4* (*P. equestris*) and *DMMADS4 (D. moniliforme)*, and their expression has been detected exclusively in the lip and the column in orchid flowers (Tsai et al. 2004), suggesting that this gene may specifically responsible for the development of orchid lip and column.

As the expression patterns of the *DEF*-like genes in monocot and basal angiosperm are various, the functions of these genes may not be strictly conserved throughout angiosperm (Hsu and Yang 2002; Kim et al. 2005; Kramer and Irish 2000; Su et al. 2008; Whipple et al. 2007). For example, a paleo*AP3*-line gene (Silky1) in maize can complement *Arabidopsis ap3* mutant, suggesting that *Siky1* has a conserved function to *Arabidopsis AP3* gene in regulating of petal and stamen identity (Su et al. 2008; Whipple et al. 2004).

In orchid, although the functional analysis of class B genes has been performed in some *AP3/DEF*-like genes especially, in the clades 1 and 2 (Hsu and Yang 2002; Xu et al. 2006), functions of the genes to control petal and stamen development were still not clear. To clarify the functions and combinatorial interactions between orchid *AP3/DEF*- and *PI/GLO*-like genes, in present study, a native orchid in Japan named 'Sekkoku' (*Dendrobium moniliforme)* was used as plant material since this species of orchid includes several natural floral mutants which can be utilized as important genetic resources for further study on orchid floral morphology. We have isolated three class-B MADS-box genes in *D. moniliforme*: *DMAP3A*, *DMAP3B*, and *DMPI*. *DMAP3A* is a member of clade 1 (*PeMADS2*-like genes), whereas *DMAP3B* belongs to clade 3 (*PeMADS3*-like genes). As the function of the genes in clade 3 has not yet been clarified in orchids, we over-expressed *DMAP3B* in *Arabidopsis* under the control of the CaMV 35S promoter and crossed the resulting plants with transgenic *Arabidopsis* over-expressing *DMPI*, a *PI/GLO*-like gene from *D. moniliforme*. These results will increase further our understanding of the molecular mechanism underlying petaloid organ development in orchids and help elucidate the functional conservation of class B genes between non-grass monocots such as orchids and dicotyledonous plants, such as *Arabidopsis.* As orchid is one floral species of high economic value, the knowledge on genes controlling floral shape formation will help to improve

flower quality and generate new traits of orchid for flower market in future.

Materials and methods

Plant materials

'Sekkoku' orchids (*Dendrobium moniliforme* L. Sw.) were grown in a greenhouse at the Agriculture and Forestry Research Center, University of Tsukuba. For RT-PCR analysis, floral buds and leaves were collected and stored at −80°C until use.

Wild-type *Arabidopsis* (ecotype Columbia) seeds were grown on sterilized soil and kept in growth chambers under long-day conditions (16-h light/8-h dark) at 25°C. After four weeks, plants at the floral bud stage were used for genetic transformation. Seeds from the transgenic plants were sterilized and cultured on MS medium containing 100 mg L^{-1} kanamycin at 25°C for ten days under long-day conditions before being transferred to soil.

Isolation of class B MADS-box genes from the floral buds of D. moniliforme

Total RNA was isolated from the floral buds of wild-type *D. moniliforme* using an RNeasy plant mini kit (Qiagen, Hilden, Germany). The RNA (2 μg) was used as template for cDNA synthesis SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the supplier's instructions. Prior to PCR analysis, the cDNA was treated with RNaseH to remove left over RNA template. To isolate *AP3/DEF*-like genes, two degenerate primers (*DEF-F1*, 5′-ACDGTKCTCTGYGAYGCT-3′, and *DEF-F2*, 5′-ACAGTTCTSTGYGACGCT-3′), designed based on conserved sequences in the MADS-box region of *Oncidium*, *P. equestris*, and *D. crumenatum* (Hsu and Yang 2002; Tsai et al. 2004; Xu et al. 2006) were used with an anchored reverse primer (a polyT primer) for first-stand cDNA synthesis using a first strand cDNA synthesis kit (Takara, Otsu, Japan). The full-length genes were obtained using the primers *AP3AF* (5′-ATGGGGAGGGGGAAGATAGAG-3′) and *AP3R* (5′-TGATCTTAGCCTCGCATGAT-3′) for *DMAP3A*, and *AP3BF* (5′-ATGGGGAGGGGGAAGATCGAGATAA-3′) and *AP3BR* (5′-TCAAGCGAGACGTAGATCATGAGGGC-3′) for *DMAP3B*. To amplify *PI/GLO*-like genes, the primers *DMPI-F* (5′-ATGGGCGGTAAGATAGAGAT-3′) and *DMPI-R* (5′-TTACTTATTTCCCTGCAAGTTTG-3′) were designed, based on the sequences of *PeMADS6* (*P. equestris*) and other *PI*-like genes. Amplification was performed under the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The products were cloned into pGEM-Teasy (Promega, Madison, WI) for identification and sequencing. Three cDNA clones per gene were sequenced and analyzed. In this study, two *AP3/DEF*-like genes and one *PI/GLO*-like gene were isolated; they are referred to as *DMAP3A*, *DMAP3B*, and *DMPI*, respectively. Sequences were aligned by using the clustalW alignment program of The European bioinformatics institute (EBI) and a CLC sequence viewer version 6 (CLC bio, Cambridge, MA). The full-length amino acid sequences of several plants class B MADS-box genes were used to create a phylogenetic tree using the PAUP version 4.0 software (http://www.sinauer.com/detail.php?id=8060) by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method. Boot-strap values were derived from 2000 replicate runs. The accession numbers of sequences used in the analysis are: *GogalDEF1 (ACR16036), MADS1 (ABG78568), DcOAP3A (DQ119838), DMAP3A (EU056327), PeMADS2 (AY378149), VaplaDEF1 (ACR16054), GogalDEF2 (ACR16037), OMADS3 (AY196350), PeMADS5 (AY378148), SpodoDEF2 (ACR16050), DMAP3B (EU056328), DcOAP3B (DQ119839), GogalDEF3 (ACR16038), PeMADS3 (AY378150), SpodoDEF1 (ACR16049), HrDEF (AB232663), VaplaDEF3 (ACR16056), DMMADS4 (GU132995), PeMADS4 (AY378147), PhlonDEF4 (ACR16047), VaplaDEF2 (ACR16055), SpodoDEF3 (ACR16051), PhlonDEF3 (ACR16046), LRDEF (AB071378), LMADS1 (AF503913), AlsDEFa (AB267842), TGDEFA (AB094965), TGDEFB (AB094966), AlsDEFb (AB267843), SILKY1 (AF181479), OsMADS16 (AF077760), PhlonDEF1 (FJ804105),AP3 (M86357), DEF (X52023), OrcP1 (AB094985), HrGLO1 (AB232665), GogalGLO1 (FJ804100),DMPI (EU056326), PeMADS6 (AY678299), PhPI15 (AY771992), SpodoGLO1 (FJ804114), PhlonGLO1, HrGLO2 (AB232664), VaplaGLO1 (FJ804118), LRGLOA (AB071379), TGGLO (AB094967), AOGLOB (AB103466), AOGLOA (AB103456), OsMADS2 (L37526), ZMM16 (AJ292959), FBP1 (M91190), GLO (X68831), PI (D30807), SQUA (X63701),* and *AP1 (Z16421).*

Expression analysis

Floral buds of *D. moniliforme* were carefully separated into sepals, petals, lips and columns. Total RNA was extracted from the floral organs and leaves using an RNeasy plant mini kit (Qiagen). First-strand cDNA was synthesized from 2 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen) according to the supplier's instructions. The cDNA was treated with RNaseH to remove left over RNA template. cDNA (500 ng) was used as template for a 20-μL PCR with gene-specific primers for *DMAP3A* (*RTDMAP3A-F*, 5′-AAGCACCAGGGAGACCTACA-3′, and *RTDMAP3A-R*, 5′-TGCGAGGCTAAGATCATGTG-3′), *DMAP3B* (*RTDMAP3B-F*, 5′-CGCAGTACGAGAAGATGCAG-3′, and *RTDMAP3B-R*, 5′-GTATCGGTCTGGGTGCTGAT-3′), *DMPI* (*RTDMPI-F*, 5′-GCAGATCGAACTCAGGCATT-3′, and *RLDMPI-R*, 5′-TCC-CTTCCATTGCTAGTTGC-3′) and *DMACT* (*RTACT-F*, 5′-G-CTGGTCGTGACCTGACTGA-3′, and *RLACT-R*, 5′-ACGG-AACCTCTCAGCTCCAA-3′). The reaction conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. RT-PCR analysis were performed at least twice with independent RNA sample.

Transformation and analysis of transgenic Arabidopsis containing the 35S::DMAP3B or 35S::DMPI sense construct

Full-length coding region of *DMAP3B* or *DMPI* cDNA was cloned into the binary vector pGWR1 (In planta Innovations Inc., Yokohama, Japan) under the control of the CaMV 35S promoter. *Agrobacterium tumefaciens* strain GV3101 containing 35S::*DMAP3B* or 35S::*DMPI* was transformed into *Arabidopsis* plants by the floral dip method (Clough 1998). Seeds from the transgenic plants were selected on MS medium containing 100 mg L^{-1} kanamycin. PCR and RT-PCR were

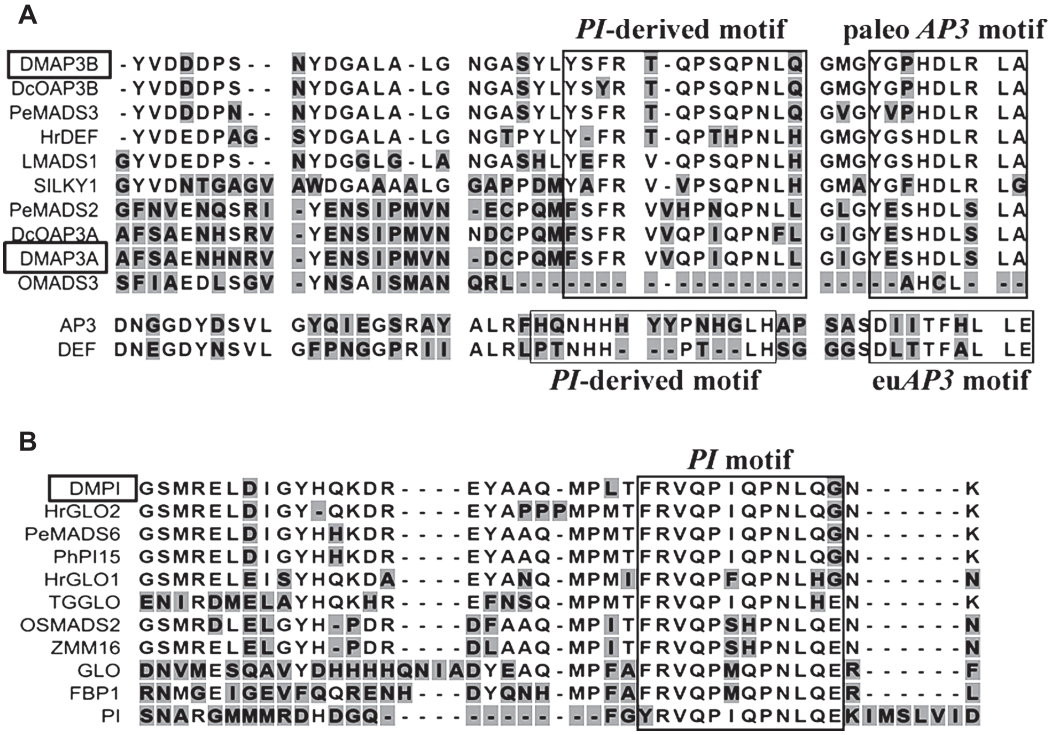

Figure 1. Alignment of the deduced amino acid sequences of C-terminal regions of DMAP3A, DMAP3B and DMPI from *Dendrobium moniliforme* with those of AP3/DEF and PI/GLO orthologs from other plant species. (A) Both of DMAP3A and DMAP3B contain the PI-derived and paleoAP3 motifs, which are commonly found in monocots and basal angiosperms. However the PI-derived motif and the euAP3 motif are uniquely present in AP3/DEF proteins from eudicotyledons. (B) DMPI contains the PI motif that is conserved in all PI/GLO orthologs. The background color showed different residues. Sequences were aligned by using the clustalW alignments in a CLC sequence viewer version 6.

performed to confirm the presence and expression levels of the transgenes. Morphological observation was carried out at the T_1 generation and cross-pollinated T_2 generation.

Scanning electron microscopy (SEM)

Floral organs, including sepals and petals, from three independent transgenic lines of *Arabidopsis* per construct were collected and cut so that they would fit in the specimen chamber of a scanning electron microscope (KEYENCE model VE-8000 real-3D system).

Statistics

Floral longevity and the time to silique maturation in wild-type plants, 35S::*DMAP3B* plants, 35S::*DMPI* plants and 35S::*DMAP3B*/35S::*DMPI* plants were given as the mean of at least four replicate samples ±SD. Data were subjected to one-way analysis of variance and mean separation was performed with LSD test. Differences at $P<0.05$ were considered significant.

Results

Isolation of two AP3/DEF-like genes and a PI/GLO-like gene in D. moniliforme

We obtained two *AP3/DEF*-like genes, *DMAP3A* and *DMAP3B*, in *D. moniliforme* using RT-PCR. The full-length genes were generated using primers specific for the start codon of *AP3/DEF*-like genes from *D. crumenatum* and *P. equestris*. The full-length cDNAs of *DMAP3A* and *DMAP3B* were 684 and 669 bp in length, and encoded 268 and 223 amino acids, respectively (Figure 1A). The sequence data was deposited in GenBank under accession numbers EU056327 and EU056328, respectively. Both sequences contain the conserved MIK region and C-terminal region of MADS box genes. In the I-box region, a highly conserved sequence in *AP3* homologs ([H/Q]YExM) (Kramer et al. 1998) was observed in *DMAP3A* and *DMAP3B*. Furthermore, two consensus motifs found in B functional genes, a *PI*-derived motif (FxFRLOPSQPNLH) (Kramer et al. 1998) and paleo*AP3* motif (YGxHDLRLA) (Kramer et al. 1998; Moon et al. 1999) were identified in the C-terminal region of each gene. The existence of a paleo*AP3* motif in the C-terminal domains of the genes indicates that *DMAP3A* and *DMAP3B* belong to paleotype *AP3* genes, like those found in lower eudicots, magnolid dicots, and monocots (Kramer et al. 1998; Lamb and Irish 2003). Although they are both paleotype *AP3* genes, the sequences of *DMAP3A* and *DMAP3B* are only 64% identical (data not shown).

Among the four clades of *AP3/DEF* that have been identified in orchids (Mondragon-Palomino and Theissen 2008; Mondragon-Palomino et al. 2009; Tsai et al. 2004), *DMAP3A* was clustered in the same group with *DcOAP3A* (*D. crumenatum*), *MADS1* (Cymbidium spp.) and *GogalDEF1* (*Gongora galeata*), which are 96, 89

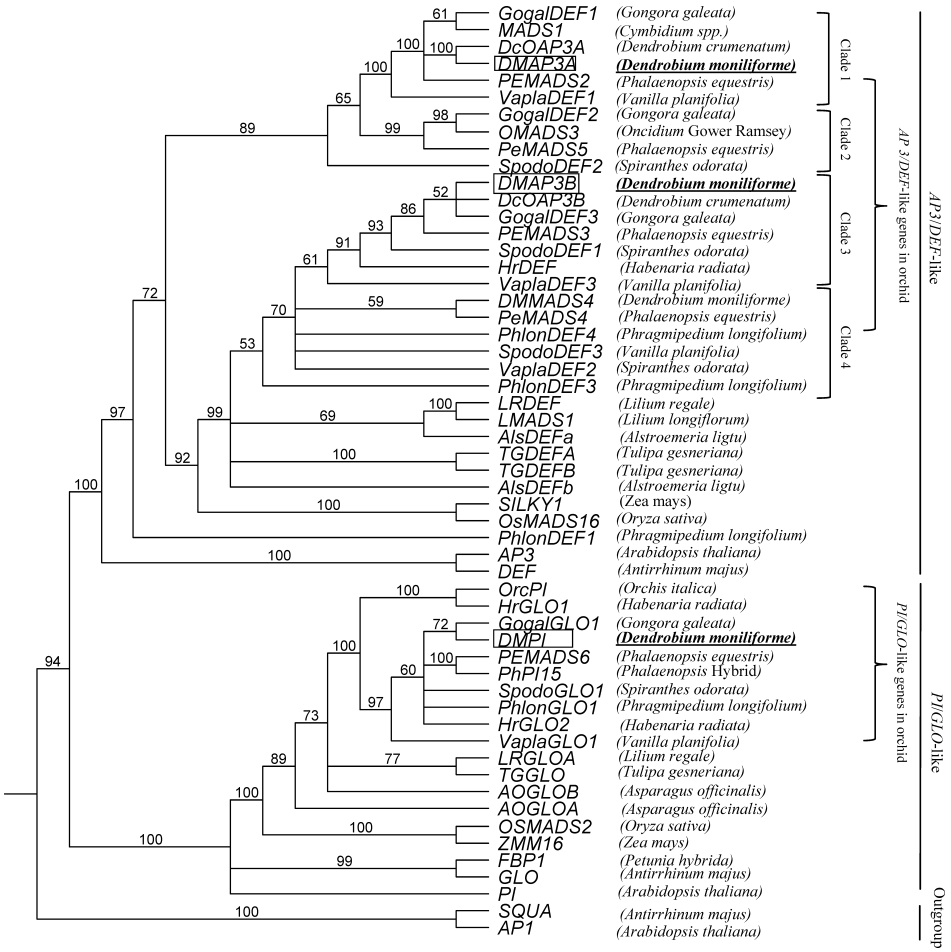

Figure 2. Phylogenetic analysis of *A*P3/DEF-like and PI/GLO-like MADS-box proteins in plant species. In the AP3/DEF lineage, the four orchid AP3/DEF-like clades are indicated by brackets. The B-class sequences isolated from *Dendrobium moniliforme* in this study are boxed. Numbers above the branches represent bootstrap values from 2000 replicates.

and 88% identical, respectively (Figure 2). *DMAP3B* was present in the same monophyletic group as *DcOAP3B* (*D. crumenatum*), *GogalDEF3* (*Gongora galeata)* and *PeMADS3* (*P. equestris*) with 96, 95, and 94% similarity, respectively. The full-length cDNA of *DMPI* was 633 bp in length and encoded 211 amino acids (Figure 1B). The sequence data have been deposited in GenBank under accession number EU056326. Within the C-terminal region, *DMPI* contains the highly conserved *PI* sequence motif (MPFxFRVQPxQPNLQE). These results confirm that *DMPI* is a *PI/GLO*-like MADS-box gene. Recently, two *PI/GLO*-like genes, *HrGLO1* and *HrGLO2* (Kim et al. 2007), were found in the genome of *H. radiata*; *DMPI* belongs to the same monophyletic group as *GogalGLO1 (Gongora galeata)*, *PeMADS6* (*P. equestris*) and *HrGLO2*, which are 98, 96 and 92% identical, respectively.

Expression patterns of DMAP3A, DMAP3B, and DMPI in the floral and vegetative organs of D. moniliforme

To investigate the expression profiles of DMAP3A, DMAP3B and DMPI in floral and vegetative organs of *D. moniliforme*, RT-PCR was performed. Leaves, immature ovaries and floral organs including sepals, petals, lips and columns, were used in the analysis (Figure 3A, B). DMAP3A expression was detected in all four whorls of the flowers as well as in the immature ovaries and leaves (Figure 3C). DMAP3B was expressed in whorl 2 of the flowers (petal and lip) and column. No signal was detected in the immature ovaries or leaves. Similar to DMAP3A, DMPI was expressed in all floral organs and leaves. The expression of DMAP3A and DMPI, but not DMAP3B, is consistent with the modified ABC model in which class B genes are expressed in whorls 1 and 2.

Ectopic expression of DMAP3B and DMPI in Arabidopsis

Although the modified ABC model predicts the expression of paleotype *AP3*-like genes in whorls 1 and 2 in non-grass monocots, the expression of *DMAP3B* and its homolog from clade 3 (Figure 2) was detected only in whorl 2 and the column of orchid flowers. This

expression pattern is similar to that of eu*AP3*-like genes in dicotyledonous plants. To investigate the potential roles of these genes in the control of flower development and to clarify the functional conservation of *DMAP3B* between orchid and eu*AP3*-like genes, we over-expressed *DMAP3B* and *DMPI* in *Arabidopsis* under the control of the CaMV 35S promoter using *Agrobacterium*-mediated transformation. We obtained 18 and 15 independent kanamycin-resistant T_1 plants expressing 35S::*DMAP3B* and 35S::*DMPI*, respectively. We further confirmed the insertion and transcription of the genes by PCR and RT-PCR, respectively (data not shown).

The phenotypes of the 35S::*DMAP3B* plants were identical to those of wild-type plants in both the vegetative and reproductive phases. In contrast to the ectopic expression of *AP3* in *Arabidopsis*, in which the carpels were converted to stamen-like structures (Jack et al. 1994), a carpel-to-stamen conversion in floral whorl 4 was not detected in any of the 35S::*DMAP3B* lines. In *Arabidopsis* plants over-expressing *DMPI*, nine of 15 lines showed a partial conversion of whorl 1 to petals. Patches of white and petal cells were observed inside and at margin of the petaloid sepals of the transgenic plants (Figure 4C). SEM revealed rounded petal cells at the margin and inside the sepal in 35S::*DMPI* plants (Figure 5B).

To investigate the function of *DMAP3B* in cooperation with its potential partner *DMPI,* transgenic plants ectopically expressing *DMAP3B* and *DMPI* were generated by crossing 35S::*DMAP3B* and 35S::*DMPI* plants, in which flowers of 35S::*DMAP3B* plants were pollen receptor while the flowers of *35S::DMPI* plants were pollen donor. We obtained eight independent lines expressing 35S::*DMAP3B/*35S::*DMPI*. Five of eight lines showed a phenotype different from that observed in wild-type *Arabidopsis* and plants expressing *DMPI* alone, in particular, the flower buds in the inflorescences of the 35S::*DMAP3B/*35S::*DMPI* plants were clearly opened (Figure 4D), and the sepals and petals were similar in shape and size (Figure 4D, E). A sepal-to-petal conversion in whorl 1 was observed in the plants expressing 35S::*DMAP3B/*35S::*DMPI* beginning at the immature floral stage (Figure 4E); the conversion was more complete than that seen in plants expressing 35S::*DMPI* (Figure 4C, F). Additionally, SEM showed that the epidermal cells at the adaxial surface of the petaloid sepals in 35S::*DMAP3B/*35S::*DMPI* plants were similar to the epidermal cells of the petals (Figure 5C, F). Thus, *DMAP3B* and *DMPI* may play important roles in regulating the development of petals in plants.

Unlike transgenic *Arabidopsis* plants ectopically expressing *AP3/PI* in which the number of stamens was increased due to the addition of a stamen in whorl 4, in 35S::*DMAP3B/*35S::*DMPI* plants the number of stamens in whorl 3 was equal to that in wild-type (6 stamens), and no carpel-to-stamen conversion was noted in whorl 4. However, the carpels and ovaries of the transgenic plants were poorly developed, leading to the production of short and rough siliques compared to wild type (Figure 4I). Additionally, the seeds within the siliques of the 35S::*DMAP3B/*35S::*DMPI* plants were tightly packed (Figure 4J), and the number of seeds per silique in the 35S::*DMAP3B/*35S::*DMPI* plants was reduced by 10–50% (data not shown), compared with wild-type plants. However, the seeds were fertile and had the same characteristics as the seeds of the wild-type plants.

Discussion

Although several class-B genes have been isolated from orchids, there have been a limited number of studies covering the functional analysis of those genes in the control of petaloid organ development, especially for those genes in the *AP3/DEF*-like lineage. To increase further our understanding of the roles of these genes, a functional analysis of the *AP3/DEF*-like lineage was conducted. In *Oncidium*, the over-expression of *OMADS3*, a *DEF*-like gene from clade 2, revealed that this gene was involved in the formation and initiation of flowers, and the phenotype of *OMADS3*-transgenic *Arabidopsis* plants was similar to transgenic *Arabidopsis* over-expressing class-A genes (Hsu and Yang 2002). In *D. crumenatum*, the ectopic expression of *DcOAP3A*, a *DEF*-like gene from clade 1, with its suggested partner *DcOPI*, a *PI*-like gene, resulted in plants the phenotype of which was indistinguishable from that of plants expressing *DcOPI* alone, suggesting that the *DcOAP3A* cannot function as *AP3* in *Arabidopsis* (Xu et al. 2006). As there are at least four types of *AP3/DEF*-like genes in the genome of *Phalenopsis* orchid (Tsai et al. 2004), in this study we investigated the possible functions of orchid *AP3/DEF*-like genes of clade 3 and examined the relationship between *AP3/DEF*- and *PI/GLO*-like genes in the control of floral organ identity in *Dendrobium* orchid.

Two major duplication events have occurred among class B MADS-box genes, the first of which caused the separation of the *AP3/DEF* and *PI/GLO* lineages. The second duplication event arose in the *AP3/DEF* lineage, and resulted in the generation of three distinct lineages: paleo*AP3*, *TM6* and eu*AP3* (Kramer et al. 1998). In this study, *DMAP3A* and *DMAP3B* were found to belong to the paleotype *AP3* lineage as same as its homolog *DcOAP3A* and *DcOAP3B* (Xu et al. 2006), which were common among monocotyledonous plants, because both sequences contained paleo*AP3* and *PI*-derived motifs at their C-terminal ends. In contrast to eudicots, at least two homologs of *PI/GLO*-like genes have been detected in some monocots, such as rice (Yao et al. 2008), lily (Winter et al. 2002), asparagus (Park et al. 2004) and

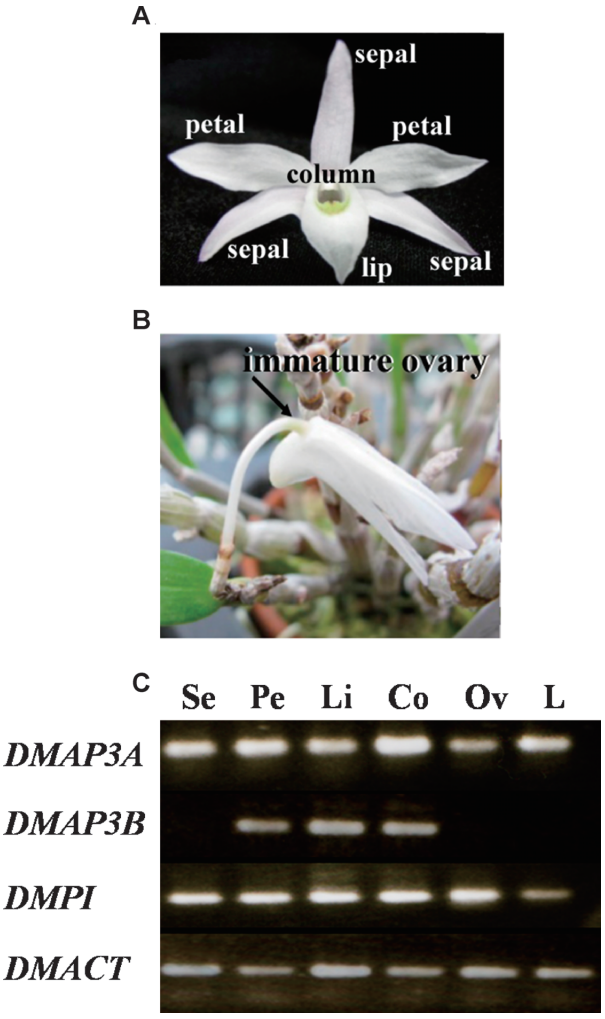

Figure 3. (A) Flower morphology of *Dendrobium moniliforme*. (B) The position of immature ovary in a flower of *D. moniliforme*. (C) Expression patterns of *DMAP3A*, *DMAP3B* and *DMPI* in floral organs and vegetative tissues were detected by RT-PCR. *DMACT* was used as the control. Total RNA was extracted from sepal; Se, petal; Pe, lip; Li, column; Co, immature ovary; Ov and leaves; L. Scale bar = 1 cm in (A) and (B). The experiment was repeated at least twice with similar results.

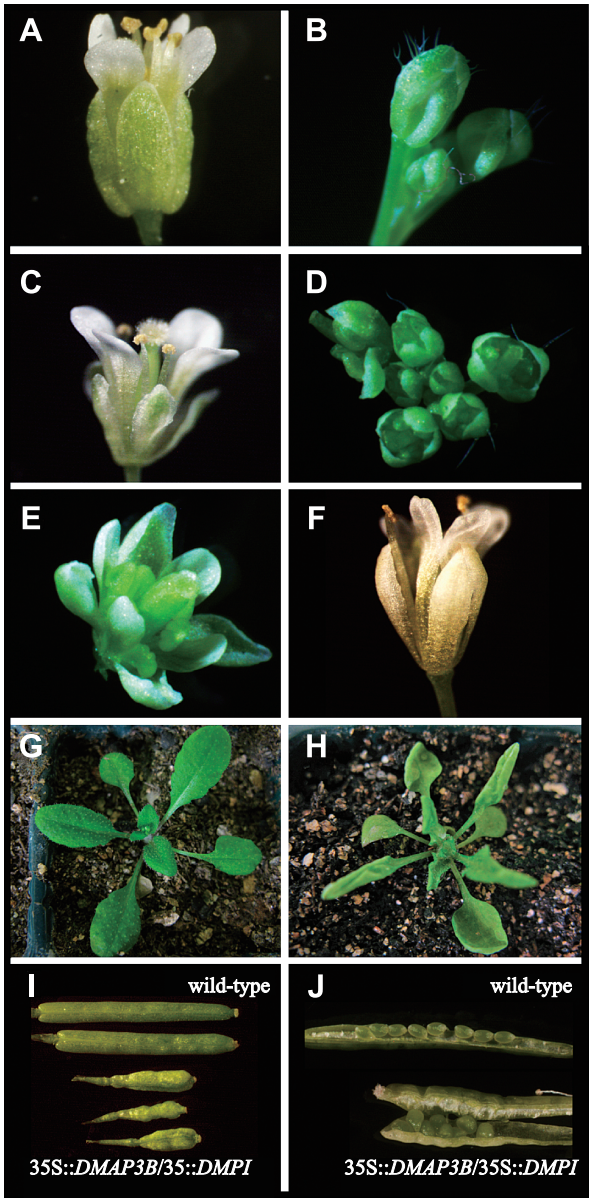

Figure 4. Phenotypes of wild-type and transgenic *Arabidopsis* plants introducing 35S::*DMPI* and 35S::*DMAP3B*/35S::*DMPI*. (A) Flower of wild-type *Arabidopsis* consists of four floral whorls of sepals, petals, stamens and carpel. (B) Flower bud of 35S::*DMPI*. (C) Partial transformation of sepal into petal-like organ in whorl1 of the 35S::*DMPI* flower. (D) Flower bud of the 35S::*DMAP3B*/ 35S::*DMPI* plant had opened prematurely. (E, F) Near-complete sepal-to-petal transformation in whorl 1 was observed in both of immature and mature stages of flowers of 35S::*DMAP3B*/ 35S::*DMPI*, respectively. (G) Leaf morphology of a wild-type plant. (H) Leaf-curling phenotype was observed in 35S::*DMAP3B*/35S::*DMPI* plants. (I) Production of short and rough siliques in 35S::*DMAP3B*/35S::*DMPI* compared to wild type siliques. (J) Seeds within the siliques of the 35S::*DMAP3B*/35S::*DMPI* plants were tightly packed, and the number of seeds per silique in the 35S::*DMAP3B*/35S::*DMPI* plants was reduced compared with wild-type plant. For morphological observation of transgenic plants, three independent transgenic *Arabidopsis* lines ectopically expressed *DMAP3B* or *DMPI* at T_1 generation, and cross-pollinated T_2 generation of 35S::*DMAP3B* and 35S::*DMPI* were used, and similar results were obtained within the same construct.

Habenaria orchid (Kim et al. 2007). However, phylogenetic analysis of *GLO*-like sequences in several orchid species showed that all of those sequences only form in a single clade (Mondragon-Palomino et al. 2009). In this study, between the two *GLO*-like genes from *Habenaria* orchid, *DMPI* is more closely related to *HrGLO2* and *PeMADS6* than to *OrPI* and *HrGLO1* (Figure 2).

To explain the molecular mechanism underlying the control of floral morphology in non-grass monocots, the modified ABC model has been proposed (Kanno et al. 2003; Van Tunen et al. 1993). Our RT-PCR results revealed that the expression of *DMAP3A* was in accordance with the modified ABC model. Additionally, *DMAP3A* was also expressed in leaves, similar to its homolog *DcOAP3A* from *D. crumenatum* (Xu et al.

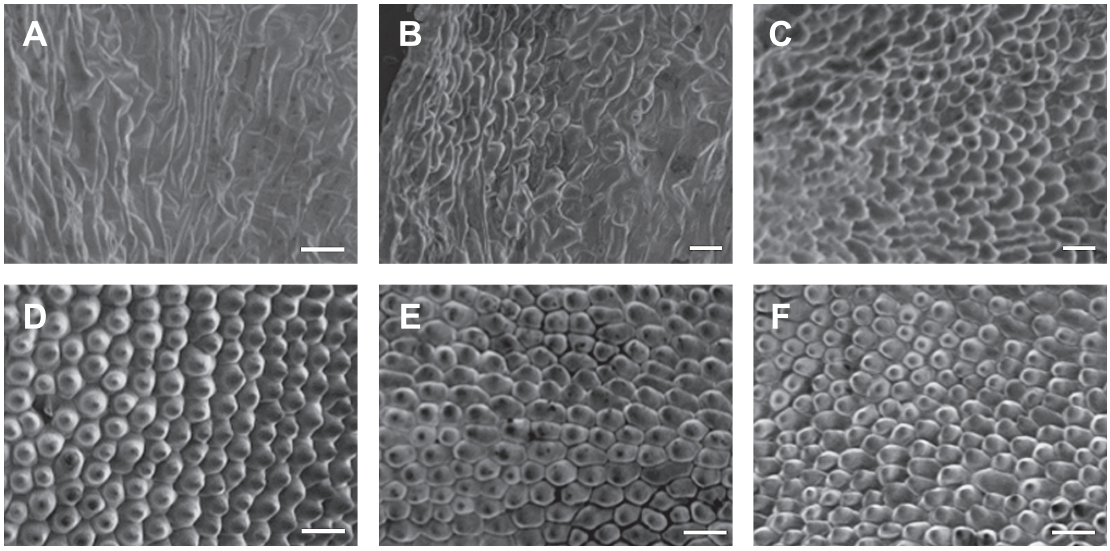

Figure 5. Cell morphology of sepal (upper) and petal (lower) in wild-type (left), 35S::*DMPI* (center) and 35S::*DMAP3B*/35S::*DMPI* (right). (A) Adaxial surface of wild-type sepal. (D) Rounded cells were presented in entire area of adaxial surface of wild-type petal. (E) Rounded cells of petal were detected at margin of the petaloid-sepal in 35S::*DMPI* plants. (C) Regular rounded cells of petal were detected in most area of the petaloid-sepal in 35S::*DMAP3B*/35S::*DMPI* plants. The cell morphology at the adaxial surface of 35S::*DMPI* and 35S::*DMAP3B*/35S::*DMPI* were similar to the adaxial surface of wild-type petal. Scale bars= 25 μm (A, B and C), 20 μm (D, E and F). Floral organs include sepals and petals from three independent transgenic lines per construct were used, and similar results were observed in samples derived from the same construct.

2006). However, in *Phalaenopsis*, *PeMADS2* expression was not detected in the leaves. This suggests that the expression pattern of the *DMAP3A* homolog may be conserved in the genus *Dendrobium*. Although most paleotype *AP3* genes in monocotyledonous plants are expressed in whorls 1 and 2, *DEF*-like genes in the clade 3 of orchid are expressed only in whorl 2, such as *PeMADS3* of *P. equestris* (Tsai et al. 2004), *DcOAP3B* of *D. crumenatum* (Xu et al. 2006) and *HrDEF* of *H. radiata* (Kim et al. 2007). In *H. radiata*, the expanded expression of *HrDEF* into whorl 1 was detected in petaloid-sepal mutant of *H. radiata* in which the floral organ in whorl 1 was morphologically indistinguishable from the petals in whorl 2. Thus, this group of genes was reported to be correlated with the differentiation of whorls 1 and 2 in non-grass monocots. As expected, the expression pattern of *DMAP3B* was similar to that of its homologs *PeMADS3*, *DcOAP3B* and *HrDEF* (i.e., no signal in whorl 1). As only a few studies on functional analysis of orchid *AP3*-like genes have been conducted, it is of interest to determine the function of the gene and the functional similarity between paleotype *AP3* and eu*AP3* because their expression patterns are similar. To investigate the roles of *DMAP3B* and *DMPI*, we over-expressed them in *Arabidopsis* plants.

The phenotype of transgenic *Arabidopsis* over-expressing *DMAP3B* was indistinguishable from that of wild-type plants, in contrast to *Arabidopsis* plants ectopically expressing *AP3*, which showed a carpel-to-stamen conversion in floral whorl 4 (Jack et al. 1994). These results suggest that *DMAP3B*, a paleotype *AP3* gene, might not function in accordance with eu*AP3* in *Arabidopsis* to generate the phenotype seen in the over-expresser, because the differentiation between sequences of *DMAP3B* and *AP3* especially in the conserved C-terminal sequence of eu*AP3,* the sequence is distinct and thought to have a different function from paleo*AP3* (Lamb and Irish 2003; Vandenbussche et al. 2004). However, the MIK region rather than the C-terminal domain of *AP3*-like class B floral homeotic proteins has been suggested to play action dominated in the development of floral organs particularly in promotion of petal identity (Su et al. 2008). Therefore another possible reason is that *DMAP3B* may play only a minor role in the control of stamen development in orchids; thus, its expression level might not be sufficient to promote the conversion of carpels to stamens in *Arabidopsis.* A similar result was found in *Arabidopsis* over-expressing *MASAKO B3*, a rose paleotype *AP3* gene (Hibino et al. 2006).

Ectopic *DMPI* expression caused the partial conversion of sepals to petal-like organs in *Arabidopsis*. This phenotype is similar to that observed in *Arabidopsis* ectopically expressing *PI* (Krizek and Meyerowitz 1996) and in *Arabidopsis* ectopically expressing *DcOPI* (Xu et al. 2006) or *PeMADS6* (Tsai et al. 2005), suggesting that the function of *PI* in *Arabidopsis* and orchids are similar. Interestingly, besides petals and stamens, *DMPI* expression was also detected in the immature ovaries of orchids similar to its homolog *PeMADS6*, suggesting that *DMPI* may plays an additional role in ovarian development. Although the function of *PI/GLO*-like genes is conserved across species of *D.moniliforme* and *P. equestris*, the results of a recent yeast two-hybrid

analysis showed that *PeMADS6* could form homodimers and heterodimers (Tsai et al. 2008), whereas *DMPI* can form only heterodimers with other paleotype *AP3* (Sirisawat et al. 2009).

Corresponding to the function of class-B MADS-box genes, the over-expression of *DMAP3B/DMPI*, which was achieved by crossing 35S::*DMAP3B* plants with 35S::*DMPI* plants, caused the replacement of sepals with petal-like structures, resembling the phenotype of transgenic *Arabidopsis* plants over-expressing *AP3/PI* (Krizek and Meyerowitz 1996), *MASAKO B3*, a paleotype *AP3*, and *MASAKO BP*, a *PI*-like gene in rose (Hibino et al. 2006). Additionally, the maize mutant *silky* exhibited the homeotic conversion of lodicules into palea/lemma-like structures and stamens into carpel-like organs (Ambrose et al. 2000). These results suggest that the function of *DMAP3B* in the control of petal development is conserved with other dicot- and monocot-paleotype *AP3* genes and eu*AP3*.

Nevertheless, over-expression of *DMAP3B/DMPI* did not have a dominant effect in whorl 4 of the flowers in the transgenic plants, in which the homeotic conversion of carpels to stamens was not detected. It is possible that the level of *DMAP3B/DMPI* expression in *Arabidopsis* was not high enough to promote the phenotype in whorl 4. However, the inhibition of silique elongation was observed in transgenic plants expressing both genes, resulting in the production of short, rough siliques with a decreased number of seeds. As this phenotype has not been reported in other plants over-expressing class-B genes, it is possible that the inhibition of silique elongation was due to the moderate suppression of carpel development in floral whorl 4 in 35S::*DMAP3B*/35S::*DMPI* plants.

Additionally, the transgenic plants showed a vegetative phenotype similar to the leaf-curling phenotype observed in *Arabidopsis* plants ectopically expressing *AP3/PI*; however, the curling of leaves was not detected in plants expressing *DMAP3B* or *DMPI*. This suggests that leaf curling is a general phenomenon that occurs in response to the over-expression of B MADS-box genes in *Arabidopsis*.

In summary, the results from genetic transformation in this study show that the combination of *DMAP3B*/*DMPI* isolated from *Dendrobium* orchid are able to promote phenotype of petals instead of sepals in the first floral whorl even in the heterologous transformation system. Therefore these two class B MADS-box genes may be utilized as a potential genetic resource to produce new floral traits especially, flower that obtain double number of petals without drastic disturbance of the stamen and carpel development so flowers can fertilize as normal. However, the functional analysis of both genes by homologous transformation system in orchid is also necessary to confirm in further study to elucidate how *DMAP3B/DMPI* is involved in stamen and carpel development. Because the male (stamen) and female (carpel) reproductive organs in orchids are fused together, forming a single unit called a column, and given that most class-B genes are expressed in this organ, the functions of class-B proteins as they relate to column development should be clarified.

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