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Genetic Transformation and Analysis of Protein-Protein Interaction of Class B MADS-Box Genes from *Dendrobium moniliforme*

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1. Introduction

In angiosperm, flower formation has been initiated by transition of adult vegetative phase to reproductive phase under controlling of plant endogenous signals like hormone and circadian rhythm, and external factors such as photoperiod and temperature (Taiz and Zeiger, 2010). Floral organs of angiosperm are generally arranged in four whorls from outer part into inner part of a flower comprise; sepal-petal-stamen-carpel respectively. Analysis of molecular mechanism controlling flower development reveals that the formation of floral organs concerns functions of a group of transcription factors namely the ABC genes family. Coen and Meyerowitz (1991) have formulated the classical ABC model to explain function of these floral organ identity genes. Based on the classical ABC model, class A gene consists of *APETALA1* (*AP1*) and *APETALA2* (*AP2*) from *Arabidopsis*, act to specify sepal in whorl 1. Combination of class A and class B genes, such as *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum*, is necessary for petal and stamen development in whorls 2 and 3, respectively. A combination of class B and class C genes including *AGAMOUS* (*AG*) in *Arabidopsis* controls stamen development in whorl 3. The class C gene alone specifies carpel development in the forth floral whorl.

During the timeline of evolution, duplication event has once occurred in the ancestral B genes and gave rise of two gene lineages includes AP3/DEF and PI/GLO (Goto and Meyerowitz, 1994; Jack et al., 1994 and Kramer et al., 1998). The second duplication has taken place in the AP3 lineages causes separation of the B function genes into three sub-clades; euAP3 clade generally presents in higher eudicots, paleo AP3 clade exists in lower eudicots, magnolid dicot, monocot and basal angiosperm (Kramer and Irish, 2000), and an additional sub-clade of paleo AP3 named *TOMATO MADS BOX GENE6* (*TM6*) (Pnueli et al., 1991, Yu et al., 1999 and Hsu and Yang, 2002). Expression pattern and function of genes in the *TM6-*

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lineage are diverse than other class B gene lineages. In petunia, expression of *Petunia TM6* was detected in whorl 3 and 4 of flower and it plays a role like function of the C-class gene (Rijpkema et al., 2006). Recently, numerous of the class B genes have been widely proved for gene expression pattern and functions in several flowering plant species. In Orchidaceae, at least four AP3/DEF-like and one PI/GLO-like class B genes have been reported to contribute the developmental mechanism of perianth and reproductive organ development.

2. Molecular mechanism regulating orchids floral morphogenesis

Orchidaceae is one of the largest families in angiosperm containing more than 24,000 plant species (Fay and Chase, 2009). Flowers have been admired by people due to the great diversity of flower color and morphology. An orchid flower is usually comprised of 3 types of perianth arrange in 4 floral whorls include whorl 1 of three outer tepals (tepals is a technical term for sepals and petals that the floral morphology is similar), whorl 2 of inner tepals and whorl 3 and 4 of ‘gynostemium’ or ‘column’ which is a reproductive organ where the male and female are fused into a single unit. One tepal in the second floral whorl called ‘labellum’ or ‘lip’ is typically large and colorful, which provides for trapping insect to help pollination. Another two inner petals are usually smaller than the lip and have morphologically similar structure of the three outer tepals.

Similarity between sepal and petal morphology is a dominant character in non-grass monocotyledonous plants such as lily, tulip, asparagus and orchids. van Tunen et al. (1993) has proposed a modified ABC model to explain the similarity of outer and inner tepals in tulip which is due to expanded expression of class B genes to the first floral whorl. The hypothesis has been confirmed by Kanno et al. (2003) that the identical of outer and inner tepals formation of tulip concerns regulatory mechanisms of two DEF- and GLO-like genes, in which all genes are expressed in both of the first and second floral whorls.

Formation of all type of perianth in orchid flower is regulated by the two lineages of class B-function genes: AP3/DEF-like and PI/GLO-like lineage associated with the E-function genes (Xu et al., 2006). While only a DEF-like gene generally present in genome of dicotyledonous plants, at least four DEF-like genes have been detected in genome of *Phalaenopsis* orchids (Tsai et al., 2004). Therefore the chance to form heterodimeric interaction between DEF-like gene and GLO-like gene in orchid genome may help to generate different morphology of the petaloid organs.

Recently, several DEF- and GLO-like genes were isolated from orchid species. Given the DEF- and GLO-like genes phylogenic analysis, orchid DEF-like genes are the paleo AP3 type. Mondrago´n-Palomino and Theißen (2008, 2009 and 2011) have formulated the ‘orchid code’ to explain orchid’s perianth formation. In the orchid code, there are four clades of DEF-like and a clade of GLO-like lineage. Based on overall current research of DEF-like genes, clade 1 contains PeMADS2-like genes, including PeMADS2 (*Phalaenopsis equestris*), DcOAP3A (*Dendrobium crumenatum*) and OMADS5 (*Oncidium Gower Ramsey*). Clade 2 consists of OMADS3-like genes, including OMADS3 (*O. Gower Ramsey*) and PeMADS5 (*P. equestris*). Clade 3 contains PeMADS3-like genes, including PeMADS3 (*P. equestris*), DcOAP3B (*D. crumenatum*) and HrDEF (*Habenaria radiata*). Clade 4 contains PeMADS4-like genes such as PeMADS4 (*P. equestris*).

Results from phylogenic and gene expression analysis suggested that another gene duplication may be taken placed within the group of paleo AP3-like genes of orchid and...
cause partition into two sister clades. Clades 1 and 2 are considered as a first sister clade in which gene expression is detected in both of outer and inner tepal formation. Clade 3 and 4 are another sister clade specifying only inner tepal development, but excluding outer tepal (Mondragó´n-Palomino and Theißen 2009, 2011).

In the GLO-like lineage, only one GLO-like gene was found in most of orchid genomes. However H. radiata has two GLO-like genes (Kim et al., 2007). Orchid GLO-like gene expression in all floral whorls of outer and inner tepals (Xu et al., 2006, Kim et al., 2007 and Sirisawat et al., 2010).

3. Isolation of class B MADS-box genes from genome of Dendrobium moniliforme

Dendrobium is a huge genus of orchid containing more than 1,200 species (Adams, 2011). Most of species are epiphytic and occasionally lithophytic. Flower of Dendrobium orchids usually has 3 outer tepals, 3 inner tepals in which one of them has developed to be labellum or lip that usually large, colorful to trap for pollination purpose. Female and male reproductive organ of Dendrobium orchid has fused as a single unit called column.

Dendrobium moniliforme or “SEKKOKU” in Japanese is a native orchid of Japan, and historical story about this orchid has been recorded since the Edo period. This species have a great variety of floral mutant phenotypes such as the double-petal mutant which lip has converged to be normal petal, and the peloric mutant which flower has 3 outer tepals and 3 lips replaced of normal inner tepals (Figure 1). Therefore D. moniliforme serves as a good source for genetic analysis of floral organ identity. Perianth morphology of the double-petal mutant is similar to flower of Apostasia orchid, a genus of primitive orchid which has very simple gynostemium. Perianth forms in dimensional symmetry in which outer and inner tepals are similar in shape and color. This symmetrical flower is supposed to be a character of ancestral orchids (Mondragó´n-Palomino and Theißen 2008). Since lip development requires more heterodimeric complex between the DEF-and GLO-like genes than petal in Phalaenopsis (Tsai et al., 2004), lip is considered as an elevated perianth organ during evolution of orchid flower. Based on this hypothesis, the peloric mutant of D. moniliforme may be developed after the wild-type one.

Recently, at least 17 floral organ identity genes were isolated from several species of Dendrobium orchids comprising D. Madame Thong-In, D. thrysiflorum (Reichb. f.), D. crumenatum and D. moniliforme (Yu and Goh, 2000, Skipper et al., 2006, Xu et al., 2006, Sirisawat et al., 2009, Sirisawat et al., 2010). Seven from 12 genes are members of class B MADS-box genes named DcOAP3A, DMAP3A, DcOAP3B, DMAP3B, DMMADS4, DcOPI and Dmpi (Table 1).

In D. moniliforme, three paleo AP3-like and one PI-like genes were isolated and classified (Sirisawat et al., 2009 and 2010). Phylogenetic analysis using amino acid sequences showed that DMAP3A was a member of clade 1 PeMADS2-like genes and which was 96 and 88% identical to DcOAP3A and PeMADS2, respectively. DMAP3B was clustered in the clade 3 of PeMADS3-like genes and which was 96 and 88% identical to DcOAP3A and PeMADS3 respectively. As a representative of PeMADS4-like genes in Dendrobium orchid, DMMADS4 showed 87% identical to PeMADS4 which belonged to clade 4 of AP3/DEF-like lineage of orchid (Figure 2).
Apart from *Habenaria radiata* which has two GLO-like genes, most of orchids have only one GLO-like gene in genome and the sequences are greatly similar. In particular, *DMPI* showed 96 and 93% identical to *PeMADS6* and *HrGLO2*, respectively (Figure 3). The great similarity of GLO-like sequences among *Orchidaceae* (more than 90%) indicated that functions of the group genes may also be highly conserve.

Fig. 1. Flower morphology of *Dendrobium moniliforme* wild-type (A), the double petal mutants (B) and the peloric mutant (C).

<table>
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<th>Class</th>
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<th>Species</th>
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<th>Reference</th>
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Table 1. List of floral organ identity genes isolated from genus *Dendrobium*
Genetic Transformation and Analysis of Protein-Protein Interaction of Class B MADS-Box Genes from *Dendrobium moniliforme*

Fig. 2. The most parsimonious tree of AP3/DEF-like genes in plants based on an alignment of the full-length amino acid sequence using UPGMA method. Numbers are bootstrap values after 100 replicate runs. The four orchid AP3/DEF like clades were indicated by brackets. The B-class sequences isolated from *D. moniliforme* in this study were bolded. Two class A MADS-box genes were used as outgroup.
Fig. 3. The most parsimonious tree of PI/GLO-like genes in plants based on an alignment of the full-length amino acid sequence using UPGMA method. Numbers are bootstrap values after 100 replicate runs. The PI/GLO-like genes from orchid were indicated by brackets. The B-class sequences isolated from *D. moniliforme* in this study were boxed. Two class A MADS-box genes were used as outgroup.

### 4. Expression analysis of orchid class B-function genes by RT-PCR and Quantitative real-time PCR

Expression of *AP3/DEF*- and PI/GLO-like class B genes in angiosperm is usually detected in the second and third floral whorls of petal and stamen, respectively. Formation of double petals is a typical character in basal angiosperm and monocot plants. In non-grass monocots, expanded expression of the B function genes to the first floral whorl was found in tulip (*Kanno et al.*, 2003), *Habenaria radiata* (*Kim et al.*, 2007), *D. crumenatum* (*Xu et al.*, 2006) and *P. equestris* (*Tsai et al.*, 2004) which contributes the petaloid-sepal formation in the first floral whorl in those plants.

Diverse expression pattern of DEF-like genes was found in orchid (Table 2). The combinational interaction between orchid DEF-and GLO-like genes is associated with distinct morphology of...
all perianth organs such as outer tepals, two inner tepals and lip in orchid. In particular, expression of GLO-like genes is strongly detected in all floral whorls and highly conserved gene expression pattern is shown throughout Orchidaceae (Table 2). Similar to expression pattern of GLO-like genes, DEF-like genes in the clade 1 and 2 are strongly expressed in both of whorl 1 and whorl 2, whereas the signals of DEF-like genes of clade 3 and clade 4 are not detected in whorl 1 (Table 2). The results suggest that development of outer tepals in whorl1 of orchid is regulated by combinational interaction between DEF- and GLO-like genes of clade 1 and 2 (Mondragón-Palomino and Theißen, 2011).

<table>
<thead>
<tr>
<th>Sub family</th>
<th>Gene name</th>
<th>Species</th>
<th>Sepal</th>
<th>Petal</th>
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<th>Column</th>
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<td>ND</td>
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</tbody>
</table>

| PI/GLO     | DcOPI<sup>e</sup> | Dendrobium crumenatum | +++  | +++  | +++  | +++  | ND    |
|            | DcMPP<sup>e</sup> | Dendrobium moniliforme | +++  | +++  | +++  | +++  | ND    |
|            | HrGLO1<sup>e</sup> | Habenaria radiata | +++  | +++  | +++  | +++  | ND    |
|            | HrGLO2<sup>e</sup> | Habenaria radiata | +++  | +++  | +++  | +++  | ND    |
|            | OMADS8<sup>e</sup> | Oncidium Gower Ramsey | +++  | +++  | +++  | ND   | ND    |
|            | PeMADS6<sup>e</sup> | Phalaenopsis equestris | +++  | +++  | +++  | +++  | ND    |
|            | PhlonGLO<sup>e</sup> | Phragmipedium longifolium | +++  | +++  | +++  | +++  | ND    |
|            | VaplaGLO<sup>e</sup> | Vanilla planifolia | +++  | +++  | +++  | +++  | ND    |

The - sign indicates non of gene expression, + sign indicate level of gene expression, ND indicates data is not applicable.

<sup>a</sup>Data from Xu et al., 2006, <sup>b</sup>Data from Sirisawat et al., 2010, <sup>c</sup>Data from Tsai et al., 2004, <sup>d</sup>Data from Mondragón-Palomino and Theißen, 2011, <sup>e</sup>Data from Chang et al., 2010, <sup>f</sup>Data from Hsu et al., 2002, <sup>g</sup>Data from Kim et al., 2005, <sup>h</sup>Data from Sirisawat et al., 2009, <sup>i</sup>Data from Tsai et al., 2005.

Table 2. Summary of expression patterns of AP3/DEF-like and PI/GLO-like genes during the development of flower buds in different orchid tissues.

Strong expression of PeMADS4, a clade 4 DEF-like gene, in lip of orchid flowers indicates that this gene is a key gene to specify lip formation. However, the expression of other genes in the clade 4 such as DMMADS4 (D. moniliforme), PhlonDEF4 (Phragmipedium longifolium) and VaplaDEF2 (Vanilla planifolia) is found in both whorls of petal and lip similar to
expression pattern of the clade 3 DEF-like genes (Table 2). This result suggests that the clade 3 and 4 DEF-like genes regulated both of petal and lip development. In particular, clade 3 and 4 DEF-like genes from *P. longifolium* and *V. planifolia* were strongly expressed in labellum rather than in petals (Mondrago´n-Palomino and Theißen, 2011). Additionally, expression of the four clades DEF-and GLO-like genes also associate with development of reproductive organs in the whorl 3 and 4 floral whorls.

Modulated signal of some DEF- and GLO-like gene expression was also found in immature ovary of orchid flower. Generally, ovary development in angiosperm is regulated by function of class D MADS-box genes (Lopez-Dee et al. 1999; Favaro et al. 2002), however molecular mechanism of class B MADS-genes related to ovary development is not well understood. In *Phalaenopsis*, expression of PeMADS6, a GLO-like gene, was strongly detected in immature ovary and the signal was decreased after pollination. Therefore expression of class B MADS-box genes in ovary is supposed to be regulated by pollination (Tsai et al., 2005).

5. Analysis of protein-protein interactions by the yeast 2-hybrid system


Homodimeric formation of protein is supposed to be primarily characteristic of the class B floral homeotic proteins. In the gymnosperm *Gnetum gnemon*, GGM2 is able to bind DNA in a sequence-specific manner as a homodimer (Winter et al., 2002). In angiosperm, there are three sub-lineage of AP3/DEF-like proteins include paleo AP3, TM6 and euAP3 lineage. Some paleo AP3- and TM6-like proteins maintain homodimeric configuration, however most of protein in euAP3 lineage that generally present in dicotyledonous plants lost the ability of homodimeric formation and they need forming heterodimer with protein in the PI lineage (Winter et al., 2002, Hsu and Yang, 2002).

In non grass monocots, some class B function proteins are able to make homodimeric formation such as LMADS1 (*Lilium longiflorum*) (Tzeng and Yang, 2001) and TGGLG (*Tulipa gesneriana*) (Kanno et al., 2003). To confirm suspected interactions of DEF-and GLO-like proteins in *D. moniliforme*, we performed the Matchmaker Two-Hybrid assay (Clontech Co. Ltd.) according to the supplier’s instruction. The entire coding sequence of DMMADS4 gene was ligated to the pGBKKT binding domain vector (pGBKKT-DMMADS4) or the pGADT7 activation domain vector (pGADT7-DMMADS4). In the same way, the PCR fragments of DMPI were ligated to the pGBKKT binding domain vector (pGBKKT-DMPI) or the pGADT7 activation domain vector (pGADT7-DMPI). Several possible protein-protein interaction of the DMMADS4 and DMPI were screened by cotransformation to yeast using the lithium acetate method (Gietz et al., 1992). The transformants were selected on selection medium and then analyzed for the β-galactosidase activity. The results showed that the DMMADS4 was strongly interacted with the DMPI as heterodimer formation. Additionally, DMMADS4 is able to form homodimer weakly, whereas DMPI could not make the homodimeric interaction (Figure 4).
Fig. 4. Homodimeric interaction between pGBKTDMMADS4 and pGADTDMMADS4 (A) and heterodimeric interaction between pGBKTDMMADS4 and pGADTDMP1 (B) (Sirisawat et al., 2009). Strong signal was detected when pGBKTDMMADS4 and pGADTDMP1 form as heterodimer.

<table>
<thead>
<tr>
<th>Sub family</th>
<th>Protein name</th>
<th>Species</th>
<th>Homodimer formation</th>
<th>Heterodimer formation with PI/GLO</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade 1</td>
<td>DcOAP3A</td>
<td>Dendrobium crumenatum</td>
<td>-</td>
<td>+++ (DcOPI)</td>
<td>Xu et al., 2006</td>
</tr>
<tr>
<td></td>
<td>PeMADS2</td>
<td>Phalaenopsis equestris</td>
<td>-</td>
<td>++ (PeMADS6)</td>
<td>Tsai et al., 2008</td>
</tr>
<tr>
<td>Clade 2</td>
<td>OMADS3</td>
<td>Oncidium Gower Ramsey</td>
<td>++</td>
<td>- (OMADS8)</td>
<td>Chang et al., 2010</td>
</tr>
<tr>
<td>Clade 3</td>
<td>DcOAP3B</td>
<td>Dendrobium crumenatum</td>
<td>-</td>
<td>+++ (DcOPI)</td>
<td>Xu et al., 2006</td>
</tr>
<tr>
<td>Clade 4</td>
<td>DMMADS4</td>
<td>Dendrobium moniliforme</td>
<td>+</td>
<td>+++ (DMPI)</td>
<td>Tsai et al., 2008</td>
</tr>
</tbody>
</table>

Table 3. Protein-protein interaction of class B MADS-box protein in Orchidaceae

The – sign indicates no interaction between two proteins, + sign indicates level of protein-protein interaction

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Similar to other flowering plants, most of DEF-like proteins in orchid need to make heterodimer with the GLO-like protein to initiate flower organ development. Strong heterodimeric signal between the four clade DEF-and GLO-like proteins was detected in \textit{P. equestris}, \textit{D. crumenatum} and \textit{D. moniliforme} (Table 3). In clade 4 DEF-like proteins, although PeMADS4 or DMMADS4 is able to make homodimer, a stronger signal was detected when the protein form heterodimer with the orchid GLO-like protein. Interestingly, the clade 1 and 3 DEF-like proteins of \textit{O. Gower Ramsey} have also retained the ancestral characteristic of the B-MADS box protein since they are able to form as homodimer (Table 3). Several possibility of heterodimeric interaction between the DEF- and GLO-like protein may be help to improve the orchid flower diversity.

6. \textit{Agrobacterium} mediated-transformation of class B MADS-box genes from \textit{D. moniliforme} to \textit{Arabidopsis}

Genetic transformation is a general molecular basis to learn functions of genes. In orchids, genetic transformation usually limit by problems of low transformation efficiency, extensive regeneration time due to long juvenile period of orchid and labor consumption to generate transgenic plants. As the results, most of orchid class B MADS-box genes were clarified their function by ectopically expressed in \textit{Arabidopsis}. Additionally, rescue of \textit{Arabidopsis} mutant phenotype is another way to know orchid gene function.

Ectopic expression of AP3 in \textit{Arabidopsis} causes conversion of carpel to stamen-like structures in whorl 4 (Jack et al., 1994). In orchids, most of AP3/DEF-like genes are paleo AP3-type in which the sequence is greatly different from \textit{Arabidopsis} AP3, therefore over-expressing of several paleo AP3-like genes from orchids such as OMADS3 (Oncidium), DcOAP3A (\textit{D. crumenatum}), DMAP3B (\textit{D. moniliforme}) in \textit{Arabidopsis} do not affect the wild-type floral morphology (Hsu and Yang, 2002, Xu et al., 2006, Sirisawat et al., 2010). In contrast to AP3/DEF-like lineage, conserved function of the PI/GLO-like genes was found between \textit{Arabidopsis} and orchids since ectopic expression of PI/GLO-like genes from several species of orchids, including DcOPI, DMPI, PeMADS6 and OMADS8, causes partial transformation of sepal to petal-like organs in the first floral whorl (Figure 5A). This suggests that function of ancestral PI/GLO-like genes is slightly developing during the evolution of angiosperm.

In \textit{Arabidopsis}, ectopically expressing AP3/PI caused transition of the whorl 1 sepal to be petal and the whorl 4 carpel to be stamen (Jack et al., 1994), therefore heterodimeric formation of AP3 and PI play role to control petal and stamen development. In \textit{D. moniliforme}, although the DMMADS4 and DMAP3B were isolated from \textit{D. moniliforme} and had the similar gene expression pattern, DNA sequence of DMMADS4 was more related to PeMADS4 (\textit{P. equestris}), DcOAP3A (\textit{D. crumenatum}), DMAP3B (\textit{D. moniliforme}) in \textit{Arabidopsis} than DMAP3B. Therefore, the DMMADS4 is considered to be a member of the clade 4 PeMADS4-like genes rather than group of DMAP3B (Figure 2). Since functional analysis of orchid clade 4 DEF-like genes has not been confirmed in \textit{Orchidaceae}, we over-expressed the DMMADS4 in \textit{Arabidopsis} and verify whether it can make heterodimeric interaction to regulate flower organ identity with the DMPI, a PI/GLO-like protein of \textit{D. moniliforme}. F1 populations were generated by crossing the 3SS::DMMADS4 with the 3SS::DMPI. The results showed that plants of 3SS::DMMADS4 have the same floral characteristics with wild-type \textit{Arabidopsis}. Progenies derived from crossing population between 3SS::DMMADS4 and 3SS::DMPI showed the phenotype resembling to \textit{Arabidopsis} plants over-expressing AP3/PI (Krizek and Meyerowitz, 1996) in which the sepal in whorl 1 converted into petal-like organs resulting in double petal formation (Figure 5B). Scanning electron microscope has been performed to verify differentiation between epidermal cell morphology between flower organ in whorl 1
and whorl 2 of the wild-type and transgenic plants. The results showed that the epidermal cells at the adaxial surface of the petaloid sepals in 35S::DMMADS4/35S::DMPI were similar to the epidermal cells at the base of petals (Figure 6). Thus heterodimer formation of DMMADS4 and DMPI play roles in regulating the development of petals.

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::DMMADS4/35S::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 7D). Additionally, the seeds within the siliques of the 35S::DMMADS4/35S::DMPI plants were tightly packed (Figure 7D), and the number of seeds per siliques in the 35S::DMMADS4/35S::DMPI plants was reduced (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.

Functional analysis of clade 3 and 4 DEF-like genes; DMAP3B and DMMADS4 from D. moniliforme in Arabidopsis revealed that both genes are functional homology in order to control petal formation and regulation of ovary development (Figure 5B, 7A,7C, 7D). As the transgenic Arabidopsis obtains DMAP3B/DMPI or DMMADS4/DMPI showed indistinguishable phenotype of stamen and carpel compared to the wild-type plants, it is unclear how DMAP3B/DMPI and DMMADS4/DMPI are involved in stamen and carpel development. Because the male (stamen) and female (carpel) reproductive organs in orchids are fused together into a single unit named column, most of class-B genes are expressed in this organ. The functions of class-B proteins as they relate to column development should be clarified in further study.

Fig. 5. Phenotypes of transgenic Arabidopsis overexpressing class B MADS-box genes from orchids

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Fig. 6. Cell morphology of sepal and petal in wild-type, 35S::DMMADS4, 35S::DMPI and 35S::DMMADS4/35S::DMPI. Adaxial surface of sepal in 35S::DMMADS4 (B) was similar to the wild-type sepal (A). Rounded cells were detected at the adaxial surface of petal in wild-type (E), 35S::DMMADS4 (F), 35S::DMPI (G) and 35S::DMMADS4/35S::DMPI (H). The rounded cells were also presented at central region of the petaloid-sepal in 35S::DMPI plants (C). In 35S::DMMADS4/35S::DMPI, elongated cells of petal were observed over the entire area of the petaloid-sepal (D).

Fig. 7. Siliques of transgenic Arabidopsis overexpressing 35S::DMAP3B/35S::DMPI (A, C), and 35S::DMMADS4/35S::DMPI (D), and wild-type plants (B).

7. Conclusions

Three paleo AP3-like genes; DMAP3A, DMAP3B and DMMADS4 and a PI-like genes; DMPI were isolated from D. moniliforme and clarified for gene expression pattern, protein-protein interaction and the gene functions. The results showed that all four genes have similar expression patterns to their homolog from other orchids and most of them need to make heterodimeric interaction to drive their transcriptional output. Functional analysis of a clade 4-paleo AP3-like genes; DMMADS4 in Arabidopsis showed that it has functional homology with the clade 3 orchid paleo AP3-like genes (Xu et.al, 2006 and Sirisawat et.al.,2010) and also has highly conserved function to Arabidopsis AP3 in controlling of petal formation.
Genetic Transformation and Analysis of Protein-Protein Interaction of Class B MADS-Box Genes from Dendrobium moniliforme

Based on a systematic reviews drawing together with the results of several research studies on floral organ identity genes in orchids, Mondragón-Palomino and Theißen (2008-9, 2011) has defined ‘orchid code’ to explain molecular mechanism underlying orchid flower formation. In the ‘orchid code’, a duplication event is suggested to occur to an ancestral paleo AP3-like gene of orchid, this give rise of two sister clades in which the first sister clade consists of clade 1 and 2 paleo AP3-like genes and another sister clade includes clade 3 and 4 paleo AP3-like genes. Both sister clades have evolved under different rates of substitution (Mondragón-Palomino and Theißen, 2009). Genes in the clade 1 and 2 maintain some characters of ancestral AP3-like genes such as diverse expression pattern in all floral whorls, retaining its ability to form homodimeric of protein which most AP3-like genes of eudicot has lost the ability to form homodimers, they need to make heterodimeric interaction with PI (Hsu and Yang, 2002). Additionally, ectopic expression of DcOAP3A, a paleo AP3-like gene in clade 1 together with DcOPI, a PI-like gene, could not generate phenotypes to indicate the possible function of the heterodimeric interaction between these AP3 and PI-like genes from orchid (Xu et al., 2006).

In contrast to the function of clade 1 and 2, the clade 3 and 4 paleo AP3-like have similar expression pattern to eudicot AP3 in which the expression of genes was regularly found in whorl 2 and column, no signal was detected in the first floral whorl like that of the clade 1 and 3. Functional analysis suggests that clade 3 and 4 are functionally homolog in order to control petal development of D. moniliforme and show highly conserved function to AP3-like genes of dicotyledonous plants as Arabidopsis, suggesting that these two clade of paleo AP3-like genes are greatly elevated from the ancestral AP3 throughout evolution while the clade 1 and 3 retain ancestral characterization of the B functional gene.

Additionally, ectopic expression of paleo AP3-like genes in clade 3 and 4 i.e. DMAP3B and DMMADS4 with its potential partner DMP1 caused suppression of ovary development in transgenic Arabidopsis obtained DMAP3B/PI or DMMADS4/DMP1, although it could not been found when the AP3-or PI-like gene was expressed singly. The results suggesting that heterodimeric interaction between the paleo AP3-like genes in clade 3 or 4 with a PI-like gene from D. moniliforme is not only required for petals development, but also they play some role during growth of other part of flower including ovary.

Ovary of orchid usually stay in the immature stage throughout anthesis, development of mature ovary is initiated after pollination (Nadeau et al., 1996). Since expression of some paleo AP3-like genes and PI-like gene were also detected in the immature ovary of orchids (Table 2), orchid B-function genes may involve mechanism related prolongation of the immature ovary. As the result, production of undersize silique in transgenic Arabidopsis obtained DMAP3B/PI or DMMADS4/DMP1 may be due to functions of the heterodimeric interaction of those B-function genes in order to prolong immature stage of ovary growth.

8. References

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