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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 whorl1. 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 Myerowitz, 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; eu*AP3* 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 (tepal is a technical term for sepal and petal 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 (Mondrago´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 conversed 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 (Mondrago´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. thyrsiflorum* (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.

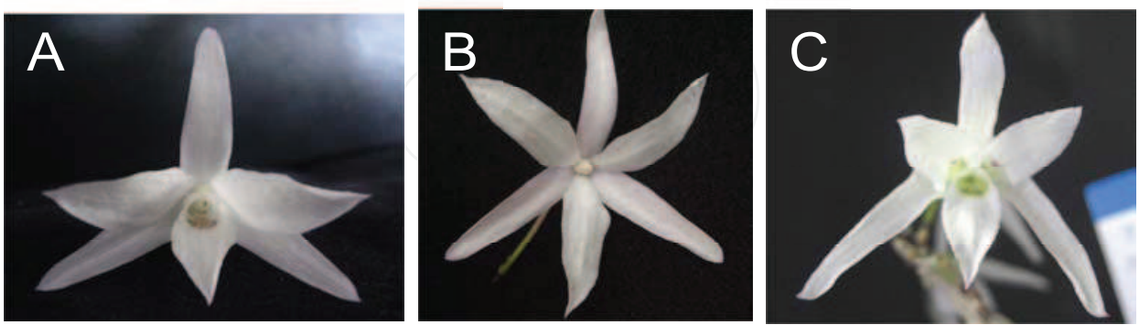

Fig. 1. Flower morphology of *Dendrobium moniliforme* wild-type (A), the double petal mutants (B) and the peloric mutant (C).

Class	Gene	Species	Sub Family	Accession No.	Reference
A	*DOMADS1*	*Dendrobium* Madame Thong-In	*AP1/AGL9*	AF198174	Yu and Goh, 2000
	DOMADS2	*D.* Madame Thong-In	*AP1/AGL9*	AF198175	Yu and Goh, 2000
	DOMADS3	*D.* Madame Thong-In	*AP1/AGL9*	AF198176	Yu and Goh, 2000
	*DthyrFL*1	*D. thyrsiflorum*	*AP1/FUL*	AY927236	Skipper et al., 2005
	*DthyrFL*2	*D. thyrsiflorum*	*AP1/FUL*	AY927237	Skipper et al., 2005
	*DthyrFL*3	*D. thyrsiflorum*	*AP1/FUL*	AY927238	Skipper et al., 2005
	DcOAP2	*D. crumenatum*	*AP2*	DQ119837	Xu et al., 2006
B	*DcOAP3A*	*D. crumenatum*	*AP3/DEF*	DQ119838	Xu et al., 2006
	DcOAP3B	*D. crumenatum*	*AP3/DEF*	DQ119839	Xu et al., 2006
	DMAP3A	*D. moniliforme*	*AP3/DEF*	EU056327	Sirisawat et al., 2010
	DMAP3B	*D. moniliforme*	*AP3/DEF*	EU056328	Sirisawat et al., 2010
	DMMADS4	*D. moniliforme*	*AP3/DEF*	GU132995	Sirisawat et al., 2009
	DcOPI	*D. crumenatum*	*PI/GLO*	DQ119840	Xu et al., 2006
	DMPI	*D. moniliforme*	*PI/GLO*	EU056326	Sirisawat et al., 2010
C	*DcOAG1*	*D. crumenatum*	*AG*	DQ119841	Xu et al., 2006
D	*DcOAG2*	*D. crumenatum*	*AG*	DQ119842	Xu et al., 2006
E	*DcOSEP1*	*D. crumenatum*	*SEP*	DQ119843	Xu et al., 2006

Table 1. List of floral organ identity genes isolated from genus *Dendrobium*

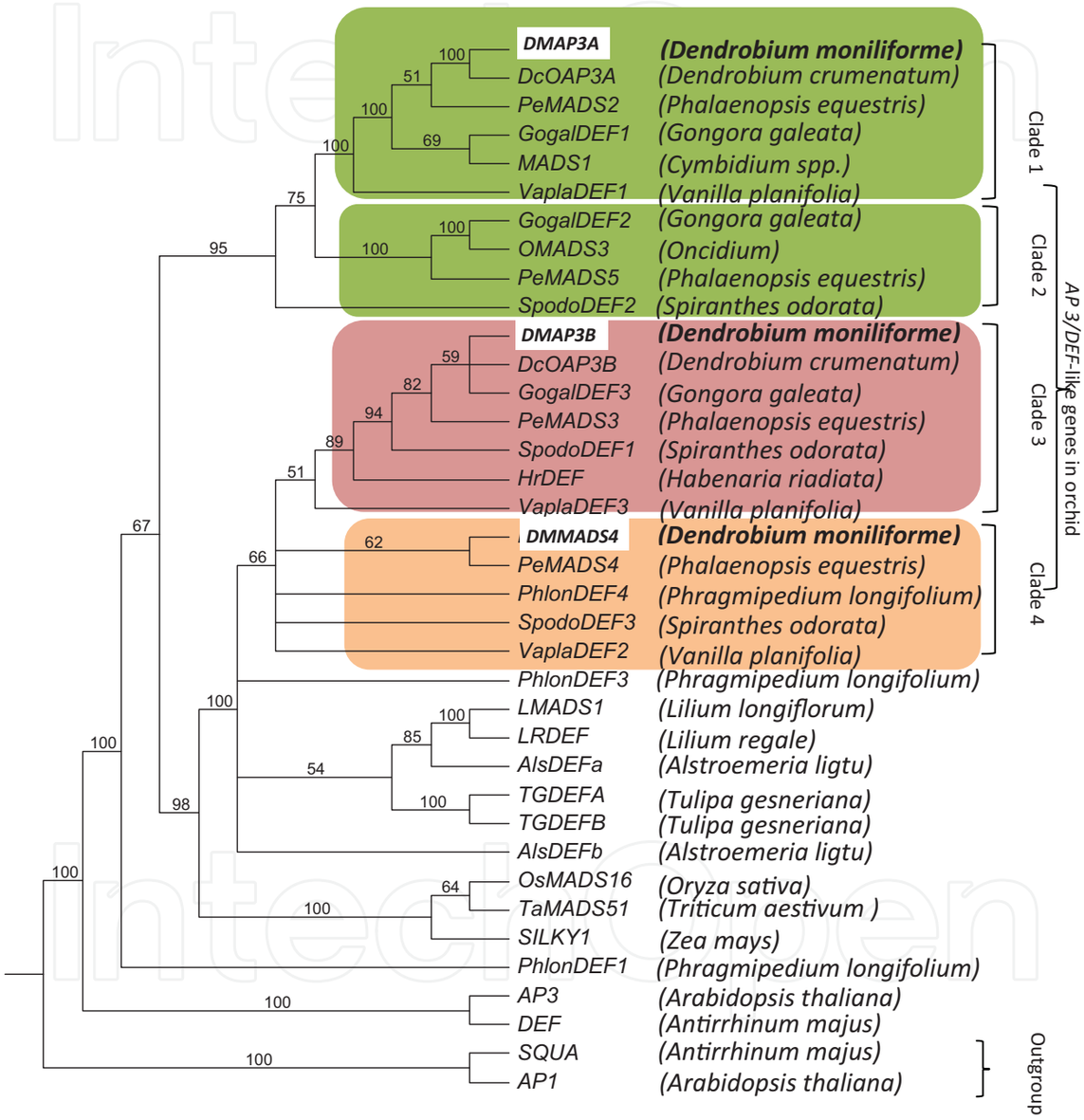

Fig. 2. The most parsimonious tree of *AP3/DEF*-like genes in plants based on an alignment of the full-length amino acid sequence using UPGMA method. Numbers are bootstrap values after 100 replicate runs. The four orchid *AP3/DEF* like clades were indicated by brackets. The B-class sequences isolated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

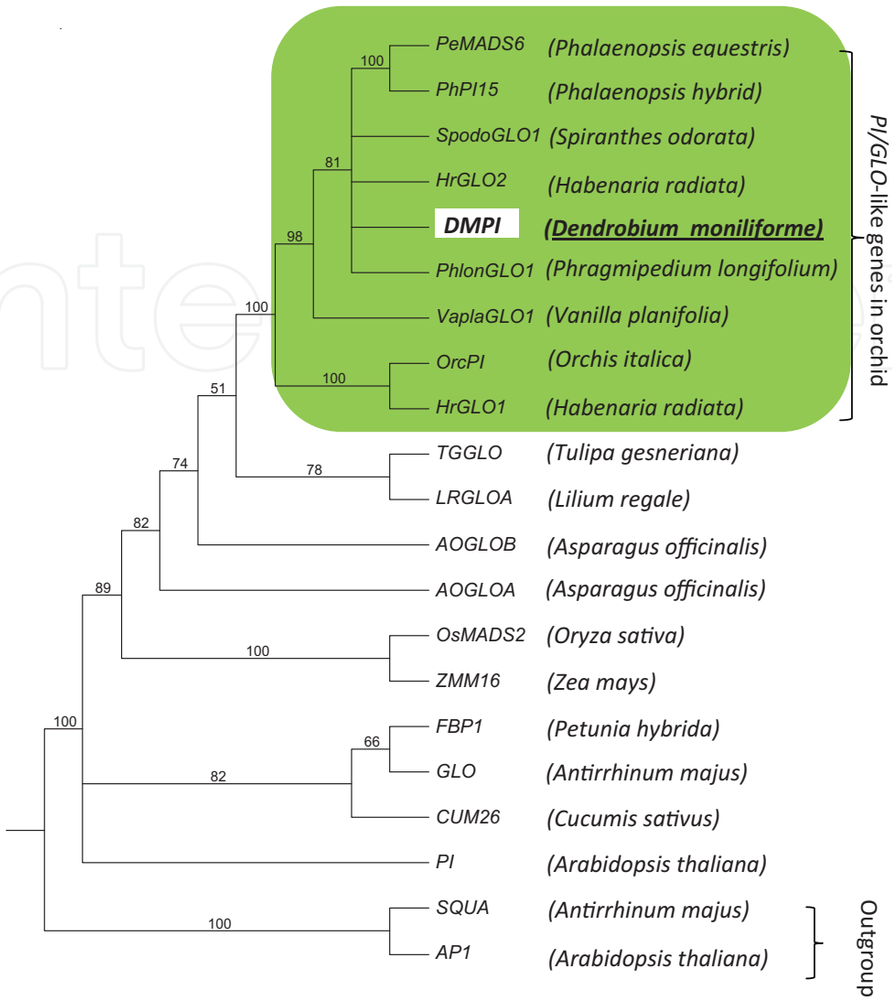

Fig. 3. The most parsimonious tree of *PI/GLO*-like genes in plants based on an alignment of the full-length amino acid sequence using UPGMA method. Numbers are bootstrap values after 100 replicate runs. The *PI/GLO*-like genes from orchid were indicated by brackets. The B-class sequences isolated from *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 (Mondrago´n-Palomino and Theißen, 2011).

Sub family	Gene name	Species	Sepal	Petal	Lip	Column	Ovary
AP3/DEF							
Clade 1	*DcOAP3A*[a]	*Dendrobium crumenatum*	+++	+++	+++	+++	ND
	DMAP3A[b]	*Dendrobium moniliforme*	+++	+++	+++	+++	++
	PeMADS2[c]	*Phalaenopsis equestris*	+++	+++	+	+	ND
	PhlonDEF2[d]	*Phragmipedium longifolium*	+++	+++	+	+++	++
	OMADS5[e]	*Oncidium* Gower Ramsey	+++	+++	++	ND	ND
	VaplaDEF1[d]	*Vanila planifolia*	++	++	+	+++	+
Clade 2	*OMADS3*[f]	*Oncidium* Gower Ramsey	+++	+++	+++	ND	ND
	PeMADS5[c]	*Phalaenopsis equestris*	++	+++	++	+	ND
	PhlonDEF1[d]	*Phragmipedium longifolium*	+++	+++	+	++	++
Clade 3	*DcOAP3B*[a]	*Dendrobium crumenatum*	-	+++	+++	+++	ND
	DMAP3B[b]	*Dendrobium moniliforme*	-	+++	+++	+++	-
	HrDEF[g]	*Habenaria radiata*	-	+++	+++	+	ND
	OMADS9[e]	*Oncidium* Gower Ramsey	-	+++	+++	ND	ND
	PeMADS3[c]	*Phalaenopsis equestris*	-	+++	+++	+	ND
	PhlonDEF3[d]	*Phragmipedium longifolium*	-	+++	+++	++	-
	VaplaDEF3[d]	*Vanila planifolia*	-	+++	+++	+++	-
Clade 4	*DMMADS4*[h]	*Dendrobium moniliforme*	-	+++	+++	+++	+++
	PeMADS4[c]	*Phalaenopsis equestris*	-	-	+++	+++	ND
	PhlonDEF4[d]	*Phragmipedium longifolium*	-	++	++	++	-
	VaplaDEF2[d]	*Vanila planifolia*	-	++	++	++	+
PI/GLO							
	DcOPI[a]	*Dendrobium crumenatum*	+++	+++	+++	+++	ND
	DMPI[b]	*Dendrobium moniliforme*	+++	+++	+++	+++	+++
	HrGLO1[g]	*Habenaria radiata*	+++	+++	+++	+++	ND
	HrGLO2[g]	*Habenaria radiata*	++	+++	+++	+	ND
	OMADS8[e]	*Oncidium* Gower Ramsey	+++	+++	+++	ND	ND
	PeMADS6[i]	*Phalaenopsis equestris*	+++	+++	+++	+++	+++
	PhlonGLO[d]	*Phragmipedium longifolium*	+++	+++	+++	+++	++
	VaplaGLO[d]	*Vanila planifolia*	++	++	++	++	++

The - sign indicates non of gene expression, + sign indicate level of gene expression, ND indicates data is not applicable.
[a]Data from Xu et al., 2006, [b]Data from Sirisawat et al., 2010, [c]Data from Tsai et al., 2004, [d]Data from Mondragón-Palomino and Theißen, 2011, [e]Data from Chang et al., 2010, [f]Data from Hsu and Yang et al., 2002, [g]Data from Kim et al., 2005, [h]Data from Sirisawat et al., 2009, [i] 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

Yeast two-hybrid screening is a method to examine interaction between protein-protein, protein-DNA by detecting binding property of the protein-protein, protein-DNA in yeast cells. Heterodimer formations between AP3/DEF- and PI/GLO- like proteins are required for function of class B MADS-box genes in angiosperm. In *Arabidopsis* and *Antirrhinum*, AP3/DEF- like proteins needs to make heterodimer with the PI/GLO-like proteins to function accurately as transcriptional regulators (Schwarz-Sommer et al. 1992, Honma and Goto 2001, Immink et al., 2003).

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 TGGLO (*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 pGBKT binding domain vector (pGBKT-DMMADS4) or the pGADT7 activation domain vector (pGADT7-DMMADS4). In the same way, the PCR fragments of *DMPI* were ligated to the pGBKT binding domain vector (pGBKT-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).

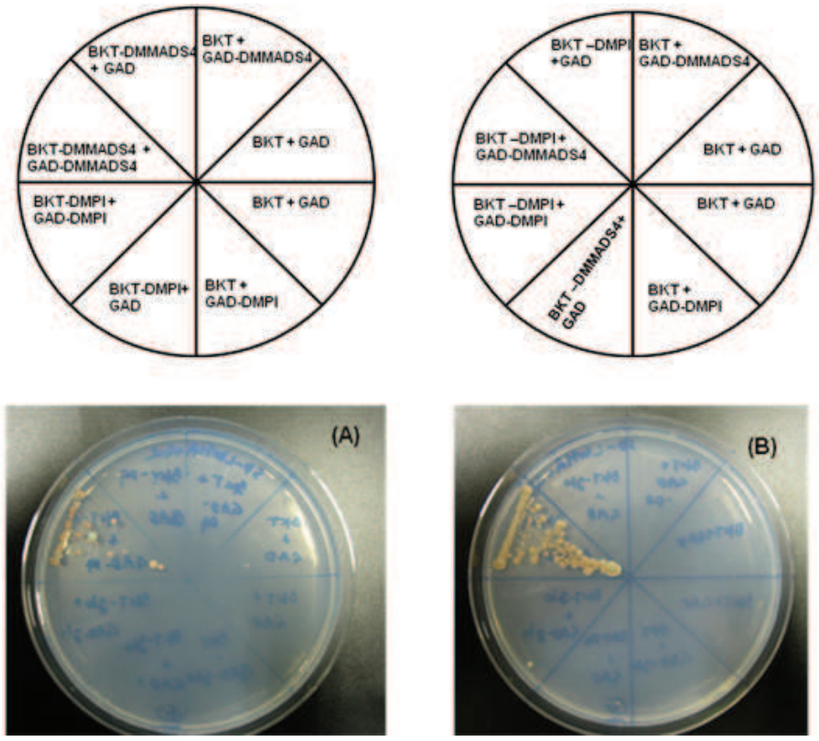

Fig. 4. Homodimeric interaction between pGBKT-DMMADS4 and pGADT-DMMADS4 (A) and heterodimeric interaction between pGBKT-DMMADS4 and pGADT-DMPI (B) (Sirisawat et al., 2009). Strong signal was detected when pGBKT-DMMADS4 and pGADT-DMPI form as heterodimer.

Sub family	Protein name	Species	Homodimer formation	Heterodimer formation with *PI/GLO*	Reference
AP3/DEF					
Clade 1	DcOAP3A	*Dendrobiun crumenatum*	-	+++ (DcOPI)	Xu et al., 2006
	PeMADS2	*Phalaenopsis equestris*	-	++ (PeMADS6)	Tsai et al., 2008
	OMADS5	*Oncidium* Gower Ramsey	++	- (OMADS8)	Chang et al., 2010
Clade 2	OMADS3	*Oncidium* Gower Ramsey	+	++ (OMADS8)	Chang et al., 2010
	PeMADS5	*Phalaenopsis equestris*	-	+ (PeMADS6)	Tsai et al., 2008
Clade 3	DcOAP3B	*Dendrobium crumenatum*	-	+++ (DcOPI)	Xu et al., 2006
	OMADS9	*Oncidium* Gower Ramsey	++	- (OMADS8)	Chang et al., 2010
	PeMADS3	*Phalaenopsis equestris*	-	+++ (PeMADS6)	Tsai et al., 2008
Clade 4	DMMADS4	*Dendrobium moniliforme*	+	+++ (DMPI)	Sirisawat et. al., 2009
	PeMADS4	*Phalaenopsis equestris*	++	+++ (PeMADS6)	Tsai et al., 2008
Sub family	**Protein name**	**Species**	**Homodimer formation**	**Heterodimer formation with** *AP3/DEF*	**Reference**
PI/GLO					
	DcOPI	*Dendrobium crumenatum*	-	+++ (DMOAP3A)	Xu et al., 2006
	DMPI	*Dendrobium moniliforme*	-	+++ (DMMADS4)	Sirisawat et al., 2009
	OMADS8	*Oncidium* Gower Ramsey	-	++ (OMADS3)	Chang et al., 2010
	PeMADS6	*Phalaenopsis equestris*	+	+++ (PeMADS3)	Tsai et al., 2008

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

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

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 *P. equestris*, *D. crumenatum* and *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 *O.* Grower 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. *Agrobacterium* mediated-transformation of class B MADS-box genes from *D. moniliforme* to *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 *Arabidopsis.* Additionally, rescue of *Arabidopsis* mutant phenotype is another way to know orchid gene function.

Ectopic expression of *AP3* in *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 *Arabidopsis AP3,* therefore over-expressing of several paleo *AP3*-like genes from orchids such as *OMADS3* (*Oncidium*), *DcOAP3A* (*D. crumenatum*), *DMAP3B* (*D. moniliforme*) in *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 *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 *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 *D. moniliforme*, although the *DMMADS4* and *DMAP3B* were isolated from *D. moniliforme* and had the similar gene expression pattern, DNA sequence of *DMMADS4* was more related to *PeMADS4 (P. equestris*) 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 *Orchidaceae*, we over-expressed the *DMMADS4* in *Arabidopsis* and verify whether it can make heterodimeric interaction to regulate flower organ identity with the *DMPI*, a PI/GLO-like protein of *D. moniliforme*. F1 populations were generated by crossing the 35S::*DMMADS4* with the 35S::*DMPI*. The results showed that plants of 35S::*DMMADS4* have the same floral characteristics with wild-type *Arabidopsis*. Progenies derived from crossing population between 35S::*DMMADS4* and 35S::*DMPI* showed the phenotype resembling to *Arabidopsis* plants over-expressing *AP3/PI* (Krizek and Meyerowitz, 1996) in which the sepal in whorl 1converted 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 silique 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.

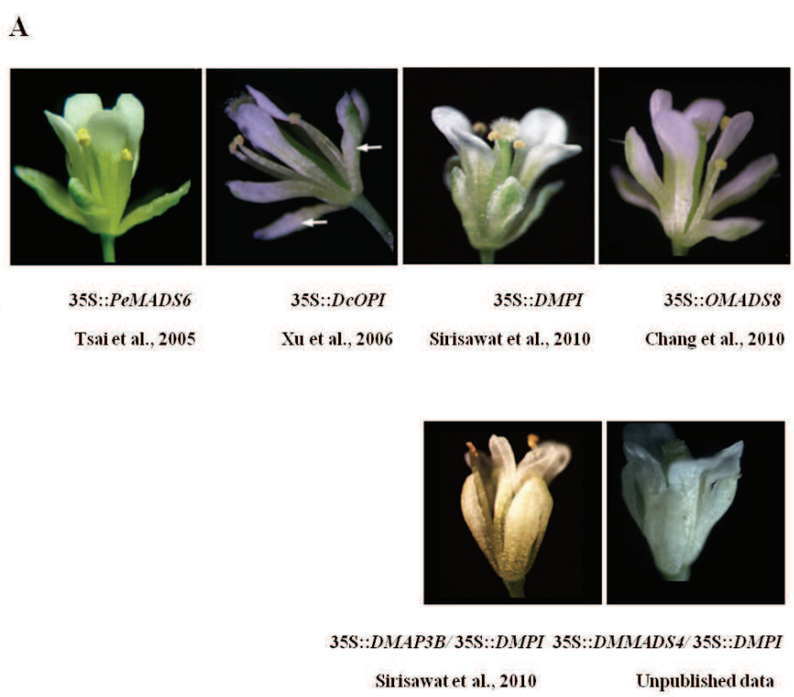

Fig. 5. Phenotypes of transgenic *Arabidopsis* overexpressing class B MADS-box genes from orchids

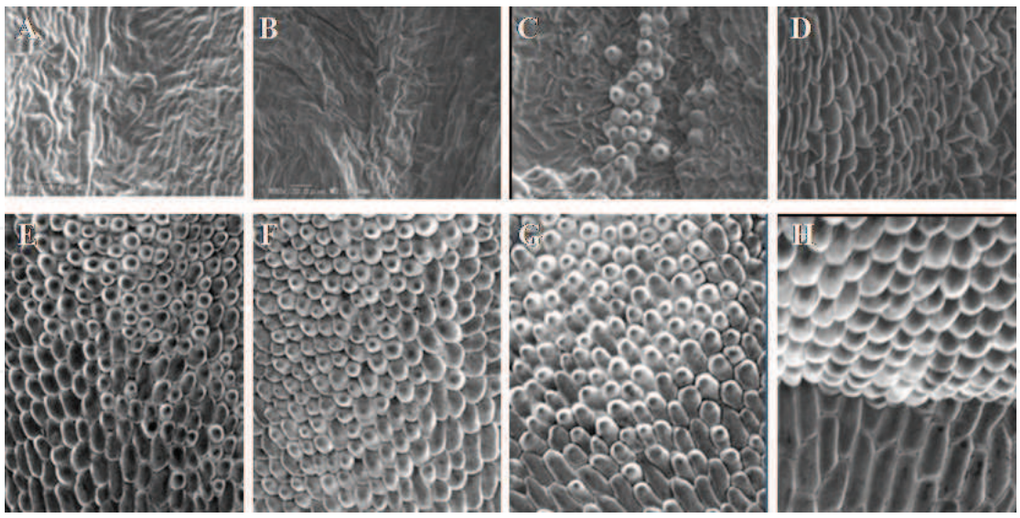

Fig. 6. Cell morphology of sepal and petal in wild-type, 35S::*DMMADS4*, 35S::*DMPI* and 35S::*DMMADS4*/35S::*DMPI*. Adaxial surface of sepal in 35S::*DMMADS4* (B) was similar to the wild-type sepal (A). Rounded cells were detected at the adaxial surface of petal in wild-type (E), 35S::*DMMADS4* (F), 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).

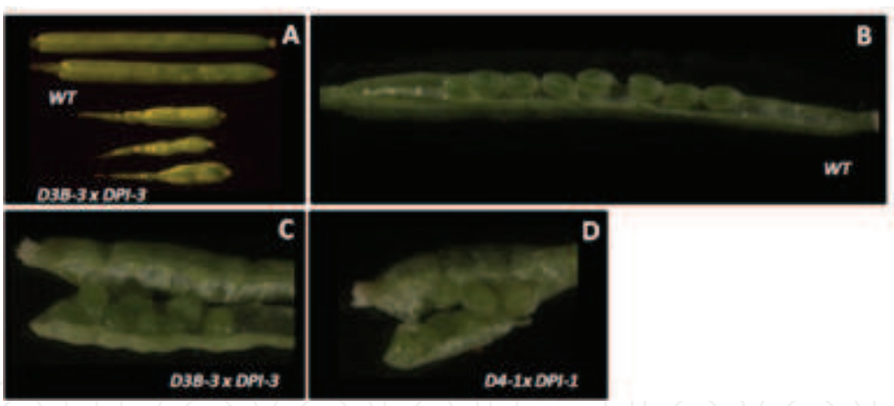

Fig. 7. Siliques of transgenic *Arabidopsis* overexpresing 35S::*DMAP3B*/35S:: *DMPI* (A, C), and 35S::*DMMADS4*/35S:: *DMPI* (D), and wild-type plants (B).

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.

Based on a systematic reviews drawing together with the results of several research studies on floral organ identity genes in orchids, Mondrago´n-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 (Mondrago´n-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 *DMPI* caused suppression of ovary development in transgenic *Arabidopsis* obtained *DMAP3B/PI* or *DMMADS4/DMPI*, 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/DMPI* may be due to functions of the heterodimeric interaction of those B-function genes in order to prolong immature stage of ovary growth.

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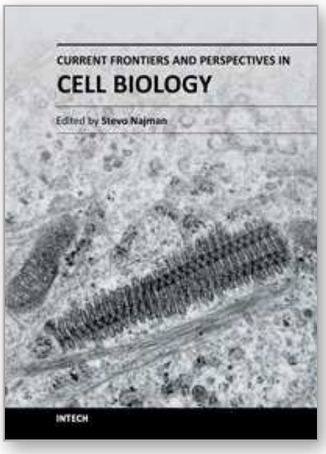

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