Title: Virus-induced Gene Silencing Unravels Multiple Transcription Factors Involved in Floral Growth and Development in *Phalaenopsis* Orchids

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Supplementary data: 3 tables and 11 figures

Table S1. Primers for semi-quantitative RT-PCR and real-time RT-PCR amplification

Primer name	Sequence (5'-3')
CymMV-CP-F	GAAATAATCATGGGAGAGCC
CymMV-CP-R	AGTTTGGCGTTATTCAGTAGG
ORSV-CP-F	ACGCACAATCTGATCCGTA
ORSV-CP-R	ATCCGCAGTGAAAACCC
PeActin-4-F	TTGTGAGCAACTGGGATGACAT
PeActin-4-R	GCCACGCGAAGTTCATTGT
PeMADS1-rt-F	AGCACAGCAGCAGCATCAGC
PeMADS1-rt-R	CCCTAAAACACCACAGCTTCCA
PeMADS6-rt-F	GCCCAACTTGCAGGGAAATA
PeMADS6-rt-R	ATTCTCACAGAACTGCCAAAAGTTAA
PeMADS7-rt-F	GAGATCATGGGGAGGGGAAAAATTGAGATC
PeMADS7-rt-R	GCTGCATTATATATGTCATTTGCTCC
PebHLH-rt-F	GGTTACCGCTGGTGGACGAA
PebHLH-rt-R	AACGGGGGCATTACGGAAAA
PebZIP-rt-F	GGGAGGAAGTTCACCGGCTCA
PebZIP-rt-R	AATCATGGGTGCTCCGGTCGC
PeHB-rt-F	AGAGGGGAAGATGGGCGGCG
PeHB-rt-R	AGTTTCGGCCTCCTCCCCACA
PebHLH-realtime-F	TGGGGAAAGCAGCAGAGTCC
PebHLH-realtime-R	AACGGGGGCATTACGGAAAA
PeMADS1-realtime-F	AGCACAGCAGCAGCATCAGC
PeMADS1-realtime-R	GTTATTCCCACCAGCAGCAAA

Table S2. Ratios of features of plant organs affected by transcription-factor silencing

Genes with silencing	Cultivar[a]	Replication[b]	Initial bud abortion	Abnormal flower with floral morphologic changes
PeMADS7	cv. 1	Exp. 1	5/5[c](14/44)[d]	0/5[e](0/44)[f]
		Exp. 2	5/5 (15/43)	0/5 (0/43)
PeHB	cv. 1	Exp. 1	5/5 (13/46)	0/5 (0/46)
		Exp. 2	5/5 (15/43)	0/5 (0/43)
PebZIP	cv. 1	Exp. 1	5/5 (15/45)	0/5 (0/45)
		Exp. 2	5/5 (14/44)	0/5 (0/44)
PeMADS1	cv. 1	Exp. 1	3/5 (3/46)	5/5 (15/46)
		Exp. 2	3/5 (3/48)	5/5 (15/48)
	cv. 2	Exp. 1	3/5 (3/47)	5/5 (15/49)
		Exp. 2	3/5 (3/49)	5/5 (15/47)
PebHLH	cv. 1	Exp. 1	4/5 (4/47)	4/5 (15/47)
		Exp. 2	3/5 (3/44)	5/5 (18/44)
PebHLH	cv. 2	Exp. 1	3/5 (3/48)	5/5 (19/48)
		Exp. 2	3/5 (3/46)	5/5 (18/46)
PeMADS6 (Control)	cv. 1	Exp. 1	2/5 (2/49)	4/5 (30/49)
		Exp. 2	3/5 (3/48)	5/5 (37/48)
	cv. 2	Exp. 1	4/5 (4/47)	5/5 (36/49)
		Exp. 2	3/5 (3/49)	5/5 (37/48)

[a] Index of the cultivars: cv. 1, *Dtps.* I-Shin Sunrise Cinderella 'OX1357'; cv. 2, *Phal.* Sogo Yukidian 'V3'

[b] Replications of experiments per treatment

[c] Number of affected plants to total number of inoculated plants

[d] Number of affected buds to total number of buds

[e] Number of affected plants to total number of inoculated plants

[f] Number of affected flowers to total number of blossomed flowers

Table S3. Ratios of features of plant organs affected by transcription-factor silencing in three cultivars

Treatments	Cultivar[a]	Initial bud abortion	Floral organs with morphological features changed		
			Tepals[b]	Column	Tepal and Column
PeMADS1-silenced	cv. 1	0/5[c] (3/46)[d]	0/5[e] (0/46)[f]	5/5 (15/46)	0/5 (0/46)
	cv. 2	0/5 (3/46)	0/5 (0/46)	5/5 (15/46)	0/5 (0/46)
	cv. 3	0/5 (3/48)	0/5 (0/48)	5/5 (15/48)	0/5 (0/48)
PeMADS6-silenced	cv. 1	3/5 (3/46)	5/5 (37/46)	0/5 (0/46)	0/5 (0/46)
	cv. 2	4/5 (4/47)	5/5 (36/47)	0/5 (0/47)	0/5 (0/47)
	cv. 3	3/5 (3/45)	5/5 (38/45)	0/5 (0/45)	0/5 (0/45)
Double silenced	cv. 1	0/5 (3/49)	5/5 (42/49)	2/5 (4/49)	2/5 (4/49)
	cv. 2	0/5 (3/48)	5/5 (40/48)	2/5 (4/48)	2/5 (4/48)
	cv. 3	0/5 (3/47)	5/5 (38/47)	3/5 (6/47)	3/5 (6/47)

[a] Index of the cultivars: cv. 1, *Dtps.* I-Shin Sunrise Cinderella ‘OX1357’; cv. 2, *Phal.* Sogo Yukidian ‘V3’; cv. 3, *Dtps.* OX Red Shoes ‘OX1407’

[b] “Tepals” are elements of the perianth, or outer part of a flower, which includes the petals, sepals and lip in orchid flower.

[c] Number of affected plants to total number of inoculated plants

[d] Number of affected buds to total number of buds

[e] Number of affected plants to total number of inoculated plants

[f] Number of affected flowers to total number of blossomed flowers

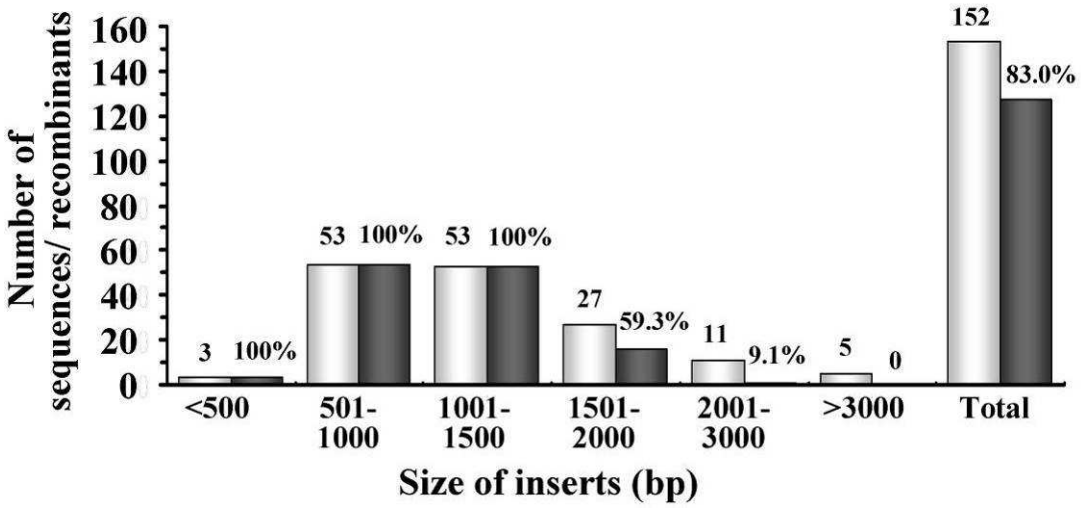


Figure S1. Effect of gene fragment length on successful insertion into the pCymMV-Gateway vector. Efficiency of cloning of expressed sequence tags (ESTs) into the pCymMV-Gateway vector with increasing insert size. The white bar and number represent the number of EST sequences. The black bars and percentages represent the success rate of recombination.

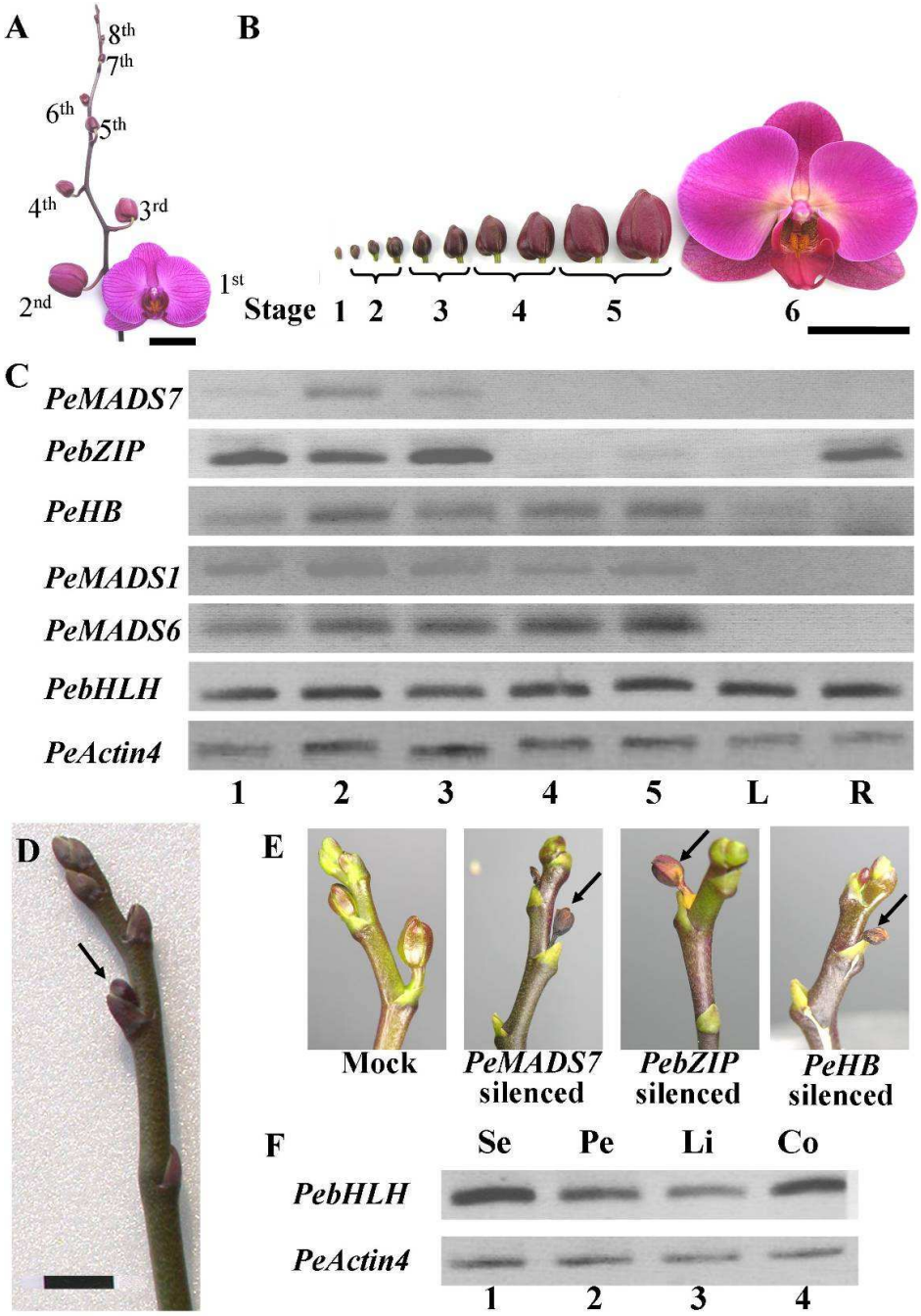


Figure S2. Analysis of *PeMADS7*, *PebZIP*, *PeHB*, *PeMADS1*, *PeMADS6*, *PebHLH* expression during flower development and in different tissues in *Phalaenopsis*. (A) The inflorescence of *Phalaenopsis* and (B) floral buds at different development stages (stage 1: floral meristem, stage 2: 1~8 mm, stage 3: 8~12 mm, stage 4: 12~18 mm, stage 5: 18~25 mm and stage 6: blooming flower). Bars, 5 cm. (C) Expression patterns of *PeMADS7*, *PebZIP*, *PeHB*, *PeMADS1*, *PeMADS6* and *PebHLH* at different developmental stages of floral buds and in the leaf (L) and root (R) . Lanes 1-5 represent floral bud stages 1 to 5. (D) Inflorescences containing eight nodes and one visible floral bud used for agro-infiltration. (E) The floral buds of mock-treated inflorescence and aborted floral buds of *PeMADS7*-, *PebZIP*- and *PeHB*-silenced inflorescence (black arrows). Bar, 1 cm. (F) Expression of *PebHLH* in various floral organs of the floral bud at development stage 3 in represent sepal (Se), petal (Pe), lip (Li) and column (Co). *PeActin4* was a loading control.

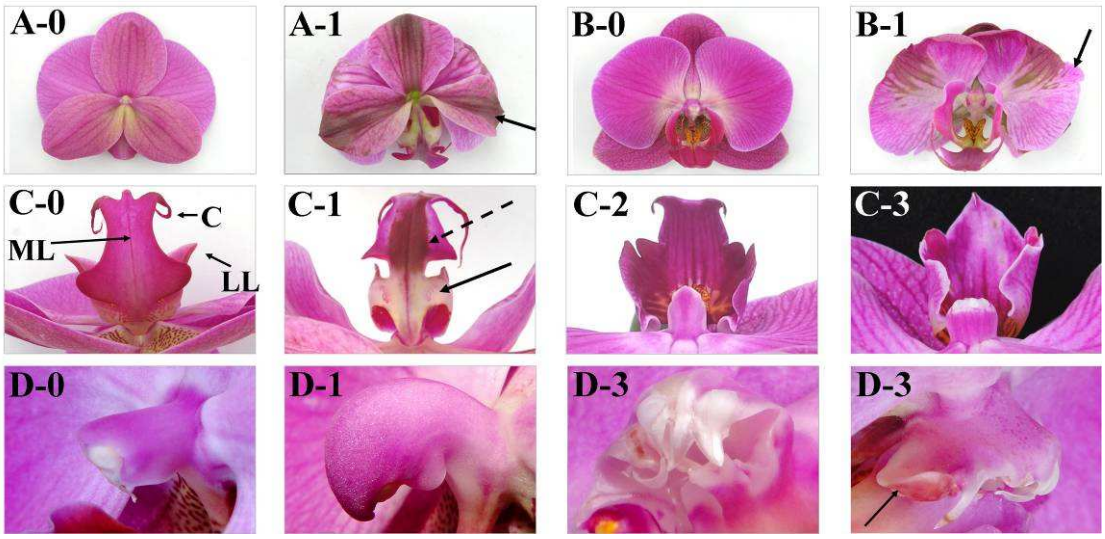


Figure S3. **Severity of floral organ morphologic changes in *PeMADS1*-, *PeMADS6*- and double-silenced flowers.** Severity index of sepal (A), petal (B), lip (C), and column (D) morphologic changes : (A-0) no phenotypic change in sepal, (A-1) greenish areas on the abaxial surface in sepal (black arrow indicates site), (B-0) no phenotypic change in petal, (B-1) greenish and discolored areas on the adaxial surface in petal (black arrow indicates site), (C-0) no phenotypic change in lip, (C-1) greenish (dashed black arrow) and discolored areas (solid black arrow) on the midlobe and lateral lobes abaxial surface in lip, (C-2) no lateral lobes developed in lip, (C-3) no cirrus or lateral lobes developed in lip. (D-0) no phenotypic change in column, (D-1) column became longer and curly, (D-2) column became wider and developed an abnormal stigmatic cavity (black arrow indicates site), (D-3) column developed an abnormal stigmatic cavity and an extra petal-like organ (black arrow).

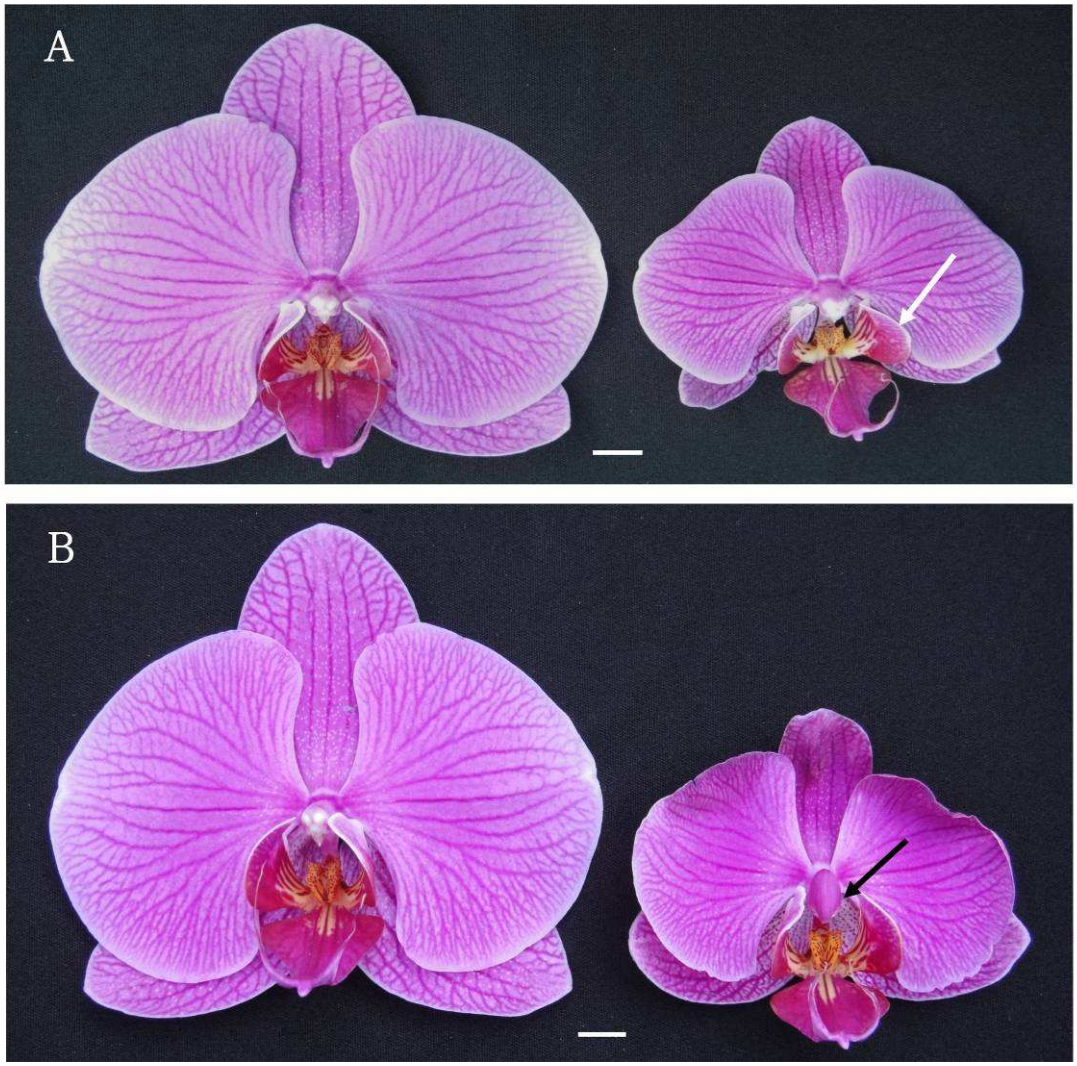


Figure S4. VIGS phenotypes of *PeMADS1*- and *PebHLH*-silenced plants in *Dtps.* OX Red Shoes 'OX1407'. (A) Front view of mock-treated (left) and *PebHLH*-silenced (right) flowers. White arrow indicates that the lateral lobes of the lip bowed outward. (A) Front view of mock-treated (left) and *PeMADS1*-silenced (right) flowers. Black arrow indicates that the column became longer and wider. Bars, 1 cm.

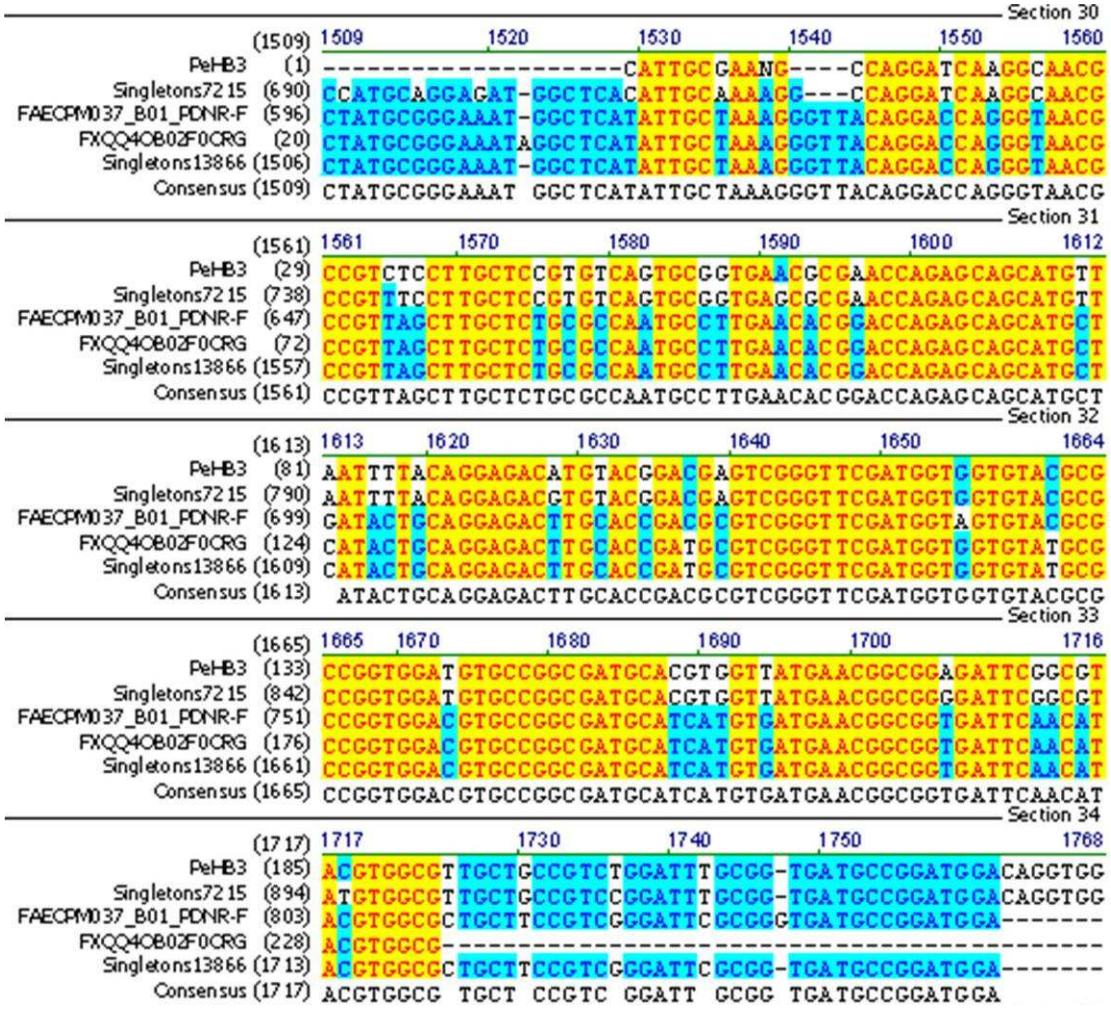
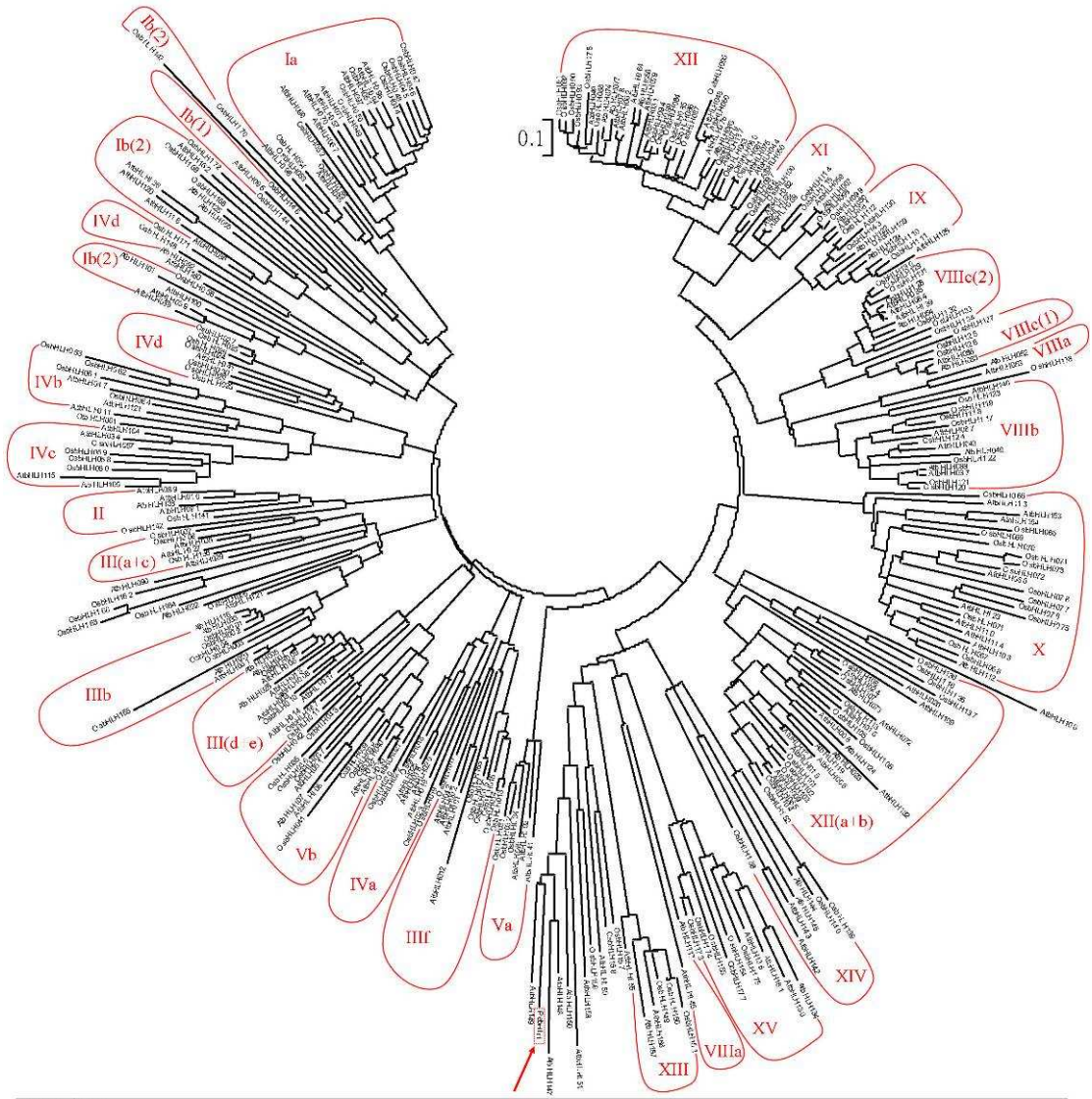


Figure S5. The multiple sequence alignment of homeobox (*HB*) transcription factor nucleotide sequences from Orchidbase 2.0. The result was obtained by doing BLASTn. As shown in the figure, these are the regions which have maximum nucleotide sequence similarity. BLAST information: gapped alignment BLASTn, Lambda=1.37, K=0.711, H=1.31; Gapped: Lambda=1.37, K=0.711, H=1.31, Matrix: blastn matrix:1 -3; Gap Penalties: Existence: 5, Extension: 2.



Subfamily	Proteins
Ia	AtbHLH045, AtbHLH057, AtbHLH067, AtbHLH070, AtbHLH071, AtbHLH094, AtbHLH096, AtbHLH097, AtbHLH098, AtbHLH099, OsbHLH044, OsbHLH045, OsbHLH046, OsbHLH047, OsbHLH048, OsbHLH049, OsbHLH050, OsbHLH051, OsbHLH052, OsbHLH053, OsbHLH054, OsbHLH055
Ib(1)	AtbHLH095, OsbHLH144, OsbHLH146, PpbHLH037, PpbHLH048, PpbHLH097, SmbHLH019, SmbHLH026, SmbHLH032, SmbHLH048, SmbHLH081
Ib(2)	AtbHLH096, AtbHLH038, AtbHLH039, AtbHLH055, AtbHLH100, AtbHLH101, AtbHLH118, AtbHLH120, AtbHLH125, AtbHLH126, AtbHLH160, AtbHLH162, OsbHLH056, OsbHLH147, OsbHLH168, OsbHLH169, OsbHLH170, OsbHLH172
II	AtbHLH010, AtbHLH080, AtbHLH091, AtbHLH138, OsbHLH141, OsbHLH142
III(a↑c)	AtbHLH027, AtbHLH030, AtbHLH035, OsbHLH006, OsbHLH007, OsbHLH156
IIIb	AtbHLH033, AtbHLH061, AtbHLH093, AtbHLH116, OsbHLH001, OsbHLH002, OsbHLH003, OsbHLH004, OsbHLH155
III(d↑e)	AtbHLH003, AtbHLH004, AtbHLH005, AtbHLH006, AtbHLH013, AtbHLH014, AtbHLH017, AtbHLH028, OsbHLH008, OsbHLH009, OsbHLH010, OsbHLH011, OsbHLH167
IIIf	AtbHLH001, AtbHLH002, AtbHLH012, AtbHLH042, OsbHLH012, OsbHLH013, OsbHLH014, OsbHLH015, OsbHLH016, OsbHLH017, OsbHLH165
IVa	AtbHLH018, AtbHLH019, AtbHLH020, AtbHLH025, OsbHLH018, OsbHLH019, OsbHLH020, OsbHLH021, OsbHLH022, OsbHLH023
IVb	AtbHLH011, AtbHLH047, AtbHLH121, OsbHLH061, OsbHLH062, OsbHLH063, OsbHLH064
IVc	AtbHLH034, AtbHLH104, AtbHLH105, AtbHLH115, OsbHLH057, OsbHLH058, OsbHLH059, OsbHLH060, OsbHLH003
IVd	AtbHLH041, AtbHLH092, OsbHLH024, OsbHLH025, OsbHLH026, OsbHLH027, OsbHLH028, OsbHLH029, OsbHLH030, OsbHLH148, OsbHLH171
Va	AtbHLH046, AtbHLH102, AtbHLH141, OsbHLH031, OsbHLH032, OsbHLH034
Vb	AtbHLH030, AtbHLH032, AtbHLH051, AtbHLH106, AtbHLH107, OsbHLH035, OsbHLH036, OsbHLH037, OsbHLH038, OsbHLH039, OsbHLH040, OsbHLH041, OsbHLH042, OsbHLH043
VII(a↑b)	AtbHLH008, AtbHLH009, AtbHLH015, AtbHLH016, AtbHLH023, AtbHLH024, AtbHLH026, AtbHLH056, AtbHLH065, AtbHLH072, AtbHLH073, AtbHLH119, AtbHLH124, AtbHLH127, AtbHLH132, OsbHLH101, OsbHLH102, OsbHLH103, OsbHLH104, OsbHLH105, OsbHLH106, OsbHLH107, OsbHLH108, OsbHLH113, OsbHLH116, OsbHLH135, OsbHLH136, OsbHLH137, OsbHLH152
VIIIa	AtbHLH052, AtbHLH053, AtbHLH117, AtbHLH146, OsbHLH178
VIIIb	AtbHLH037, AtbHLH040, AtbHLH043, AtbHLH087, AtbHLH088, AtbHLH140, OsbHLH117, OsbHLH118, OsbHLH119, OsbHLH120, OsbHLH121, OsbHLH123, OsbHLH129, OsbHLH134
VIIIc(1)	AtbHLH083, AtbHLH086, OsbHLH125, OsbHLH126, OsbHLH127
VIIIc(2)	AtbHLH004, AtbHLH084, AtbHLH085, AtbHLH139, OsbHLH128, OsbHLH129, OsbHLH130, OsbHLH131, OsbHLH132, OsbHLH133, OsbHLH134
IX	AtbHLH080, AtbHLH081, AtbHLH132, AtbHLH138, AtbHLH139, AtbHLH130, OsbHLH109, OsbHLH110, OsbHLH111, OsbHLH112, OsbHLH143
X	AtbHLH068, AtbHLH103, AtbHLH110, AtbHLH112, AtbHLH113, AtbHLH114, AtbHLH123, AtbHLH153, AtbHLH154, OsbHLH065, OsbHLH066, OsbHLH067, OsbHLH068, OsbHLH069, OsbHLH070, OsbHLH071, OsbHLH072, OsbHLH073, OsbHLH074, OsbHLH075, OsbHLH076, OsbHLH077, OsbHLH078
XI	AtbHLH007, AtbHLH059, AtbHLH066, AtbHLH069, AtbHLH082, OsbHLH096, OsbHLH097, OsbHLH098, OsbHLH099, OsbHLH100, OsbHLH114, OsbHLH115
XII	AtbHLH031, AtbHLH044, AtbHLH048, AtbHLH089, AtbHLH050, AtbHLH058, AtbHLH060, AtbHLH062, AtbHLH063, AtbHLH064, AtbHLH074, AtbHLH075, AtbHLH076, AtbHLH077, AtbHLH078, AtbHLH079, AtbHLH137, OsbHLH079, OsbHLH080, OsbHLH081, OsbHLH083, OsbHLH084, OsbHLH085, OsbHLH086, OsbHLH087, OsbHLH088, OsbHLH089, OsbHLH090, OsbHLH091, OsbHLH093, OsbHLH093, OsbHLH094, OsbHLH095, OsbHLH176
XIII	AtbHLH155, AtbHLH156, AtbHLH157, OsbHLH149, OsbHLH150, OsbHLH151
XIV	AtbHLH042, AtbHLH043, AtbHLH044, AtbHLH045, OsbHLH138, OsbHLH139, OsbHLH140
XV	AtbHLH134, AtbHLH135, AtbHLH136, AtbHLH161, OsbHLH153, OsbHLH154, OsbHLH173, OsbHLH174, OsbHLH175, OsbHLH177
Orphans	PebHLH, AtbHLH021, AtbHLH022, AtbHLH090, AtbHLH108, AtbHLH109, AtbHLH147, AtbHLH148, AtbHLH149, AtbHLH150, AtbHLH151, AtbHLH158, AtbHLH159, CreHLH0001, CreHLH0001, CreHLH0002, CreHLH0003, CreHLH0001, CreHLH0002, CreHLH0003, CreHLH0004, OsbHLH005, OsbHLH157, OsbHLH158, OsbHLH159, OsbHLH162, OsbHLH163, OsbHLH164, OsbHLH166

Figure S6. Phylogenetic analysis of *PebHLH* in *bHLH* gene families of *Arabidopsis* and rice. A phylogenetic tree was created using the MEGA5.0 program with 1,000 bootstraps and full-length sequences of 158 Arabidopsis and 173 rice bHLH proteins. The similar classification of *bHLH* subfamilies was cited from the classification of *bHLH* proteins in *Arabidopsis* and rice (Pires and Dolan, 2010).

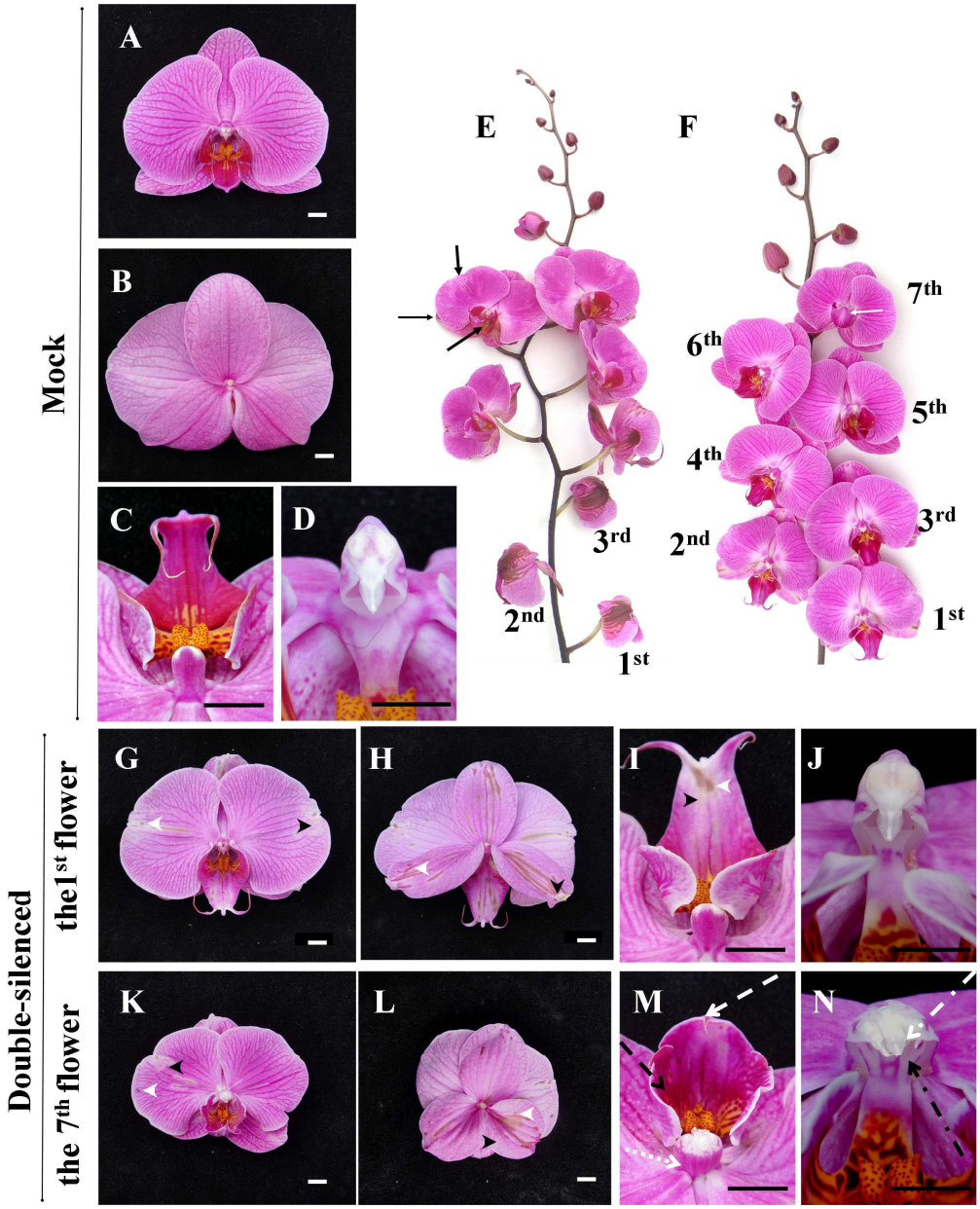


Figure S7. Concurrent silencing of both *PeMADS1* and *PeMADS6* with a pCymMV-Gateway vector in *Doritaenopsis* OX Red Shoes 'OX1407'. Top view (A), back view (B) of flower and front view of lip (C) and column (D) of mock-treated plants. Inflorescences of *PeMADS6* -silenced (E) and *PeMADS1* and *PeMADS6* double-silenced plants (F). Solid black arrows indicate that there were greenish and discolored areas in sepal, petal and lip. Solid white arrow indicates the petal-like lip. Top view (G), back view (H) and front view of lip (I) and column (J) of 1st blooming flower of double-silenced plants. Top view (K), back view (L) and front view of lip (M) and column (N) of 7st blooming flower of double-silenced plants. Dashed white arrow indicates that no cirrus developed in the tip of the midlobe. Dashed black arrow indicates that the lateral lobes and midlobe were fused into a petal-like lip. Dotted white arrow indicates that the base of the column became wider. Dotted black arrows indicate that the side of the column developed an extra petal-like organ. Dash-dotted white arrow indicates that the anther cap became wider and the appearance of more than one rostellum-like organ. Dash-dotted black arrow indicates that the stigmatic cavity did not develop normally. White bars and black bars, 1 cm.

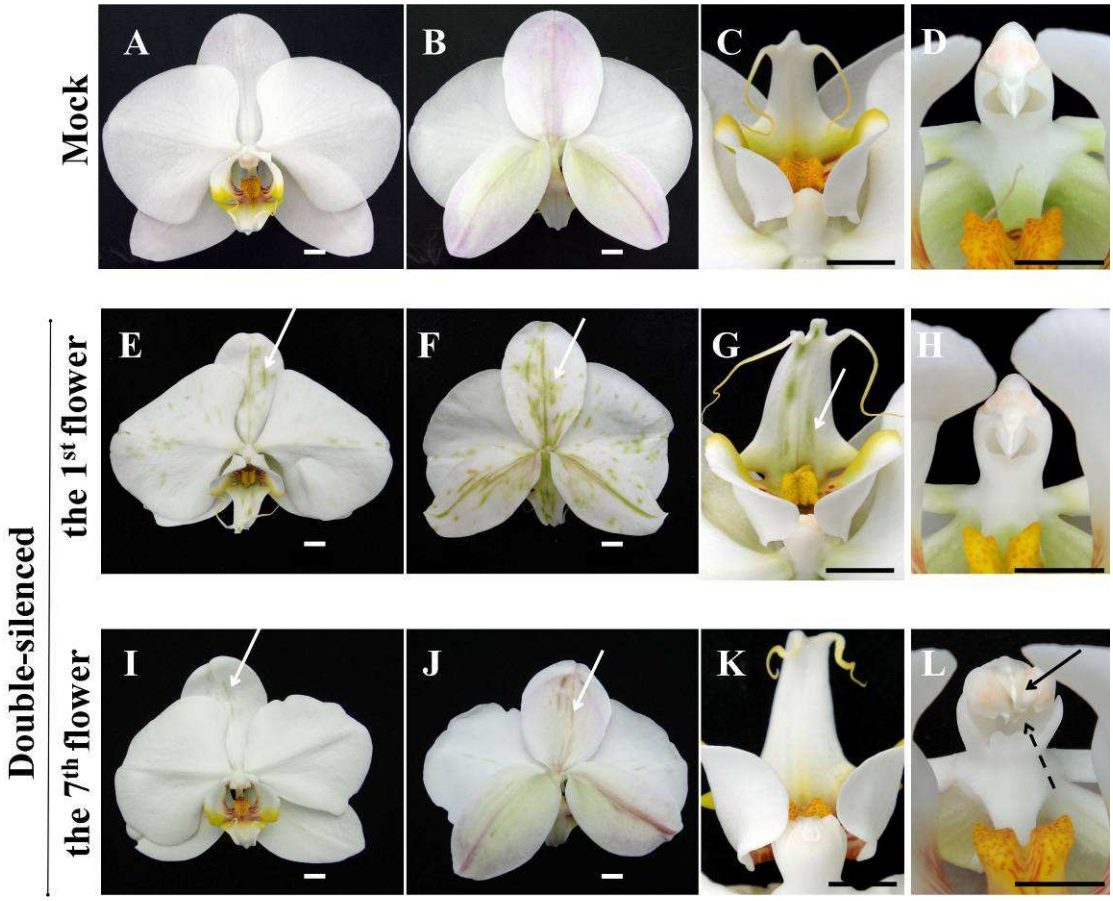


Figure S8. Concurrent silencing of both *PeMADS1* and *PeMADS6* with a pCymMV-Gateway vector in *Phalaenopsis* Sogo Yukidian 'V3'. Top view (A), back view (B) of flower and front view of lip (C) and column (D) of mock-treated plants. Top view (E), back view (F) and front view of lip (G) and column (H) of 1st blooming flower of double-silenced plants. Top view (I), back view (J) and front view of lip (K) and column (L) of 7th blooming flower of double-silencing plants. White arrows indicate dark green coloration (including leaf-like characteristics) of sepals, petals and lips. Solid black arrow indicates that the anther cap became wider and the appearance of more than one rostellum-like organ. Dashed black arrow indicates that the stigmatic cavity did not develop normally. White bars and black bars, 1 cm.

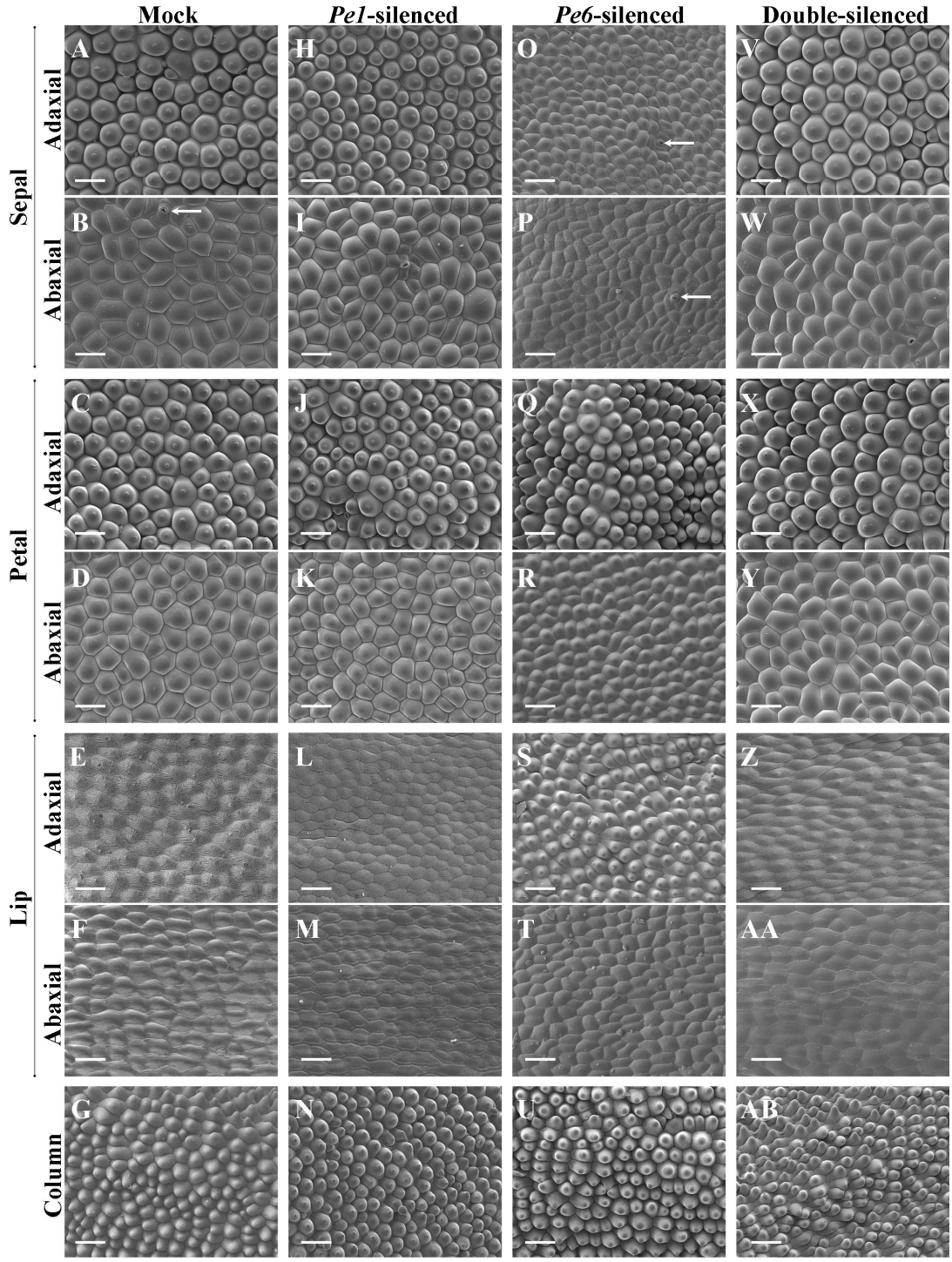


Figure S9. Cyro-SEM micrograph of epidermal cell arrangements of floral organs. The epidermal cells of mock-treated (A-G), *PeMADS1*-silenced (H-N), *PeMADS6*-silenced (O-U), and double-silenced floral organs (V-AB) **were** from the 7th blooming flowers. Sepal adaxial (A, H, O and V) and abaxial epidermal cells (B, I, P and W), petal adaxial (C, J, Q and X) and abaxial epidermal cells (D, K, R and Y) all had irregular arrangements. Lip adaxial epidermal cells (E, L, S and Z) had regular, irregular, irregular and regular arrangements. Lip abaxial epidermal cells (F, M, T and AA) all had regular arrangements. Column epidermal cells (G, N, U and AB) had irregular arrangements. White arrows indicate stomata. Bars, 100 μm. Materials were *Dtps.* OX Red Shoes 'OX1407'.

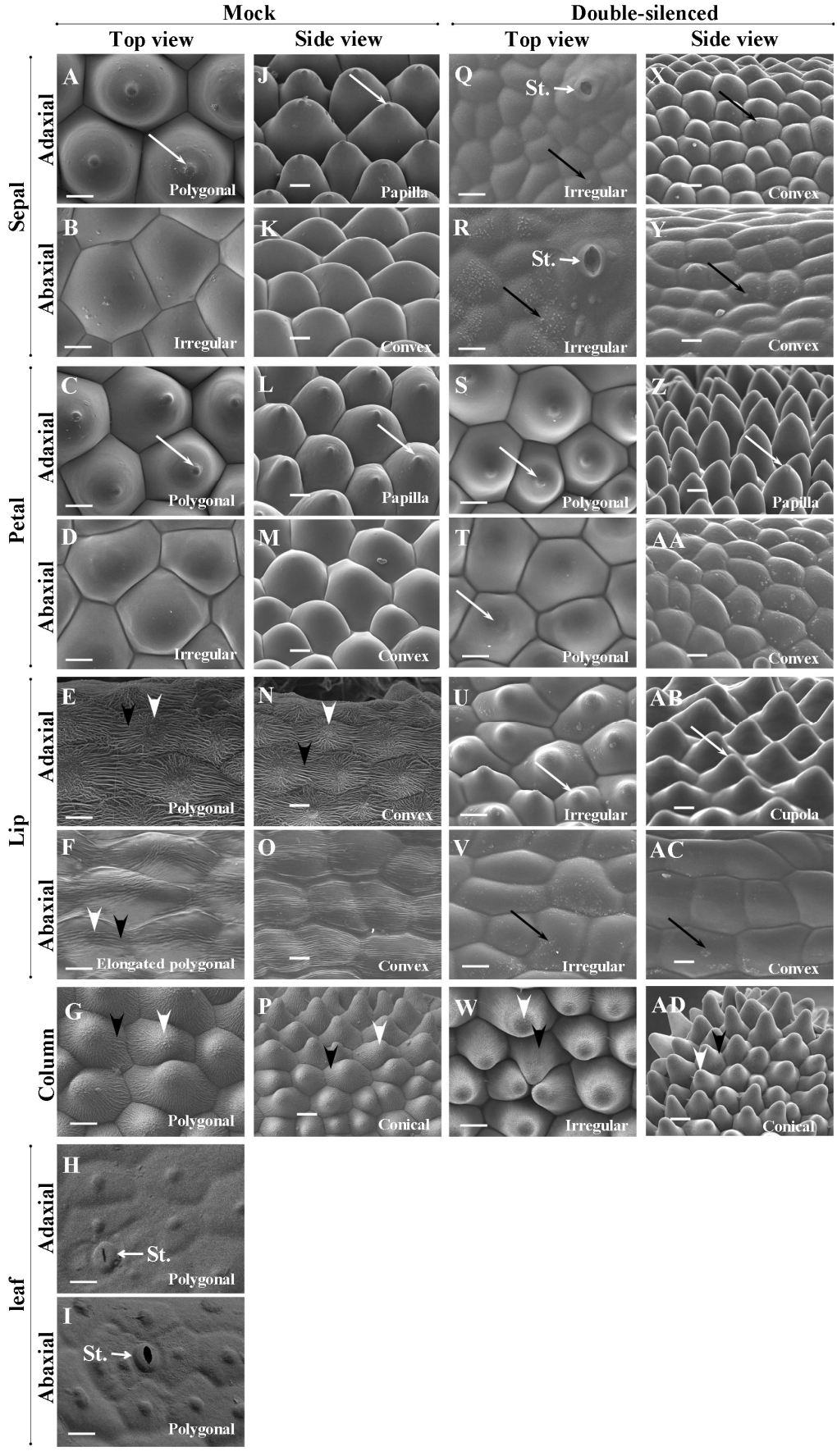

Mock
Double-silenced
Top view
Side view
Top view
Side view
Sepal
Petal
Lip
leaf
Adaxial
Abaxial
Column
A
Polygonal
J
Papilla
Q
St.
Irregular
X
Convex
B
Irregular
K
Convex
R
St.
Irregular
Y
Convex
C
Polygonal
L
Papilla
S
Polygonal
Z
Papilla
D
Irregular
M
Convex
T
Polygonal
AA
Convex
E
Polygonal
N
Convex
U
Irregular
AB
Cupola
F
Elongated polygonal
O
Convex
V
Irregular
AC
Convex
G
Polygonal
P
Conical
W
Irregular
AD
Conical
H
St.
Polygonal
I
St.
Polygonal

Figure S10. Cyro-SEM micrograph top and side view of adaxial and abaxial epidermal cells of *PeMADS1* and *PeMADS6* double-silenced floral organs from the 1st blooming flowers. The top views (A-I) and side views (J-P) epidermal cells of mock-treated floral and leaf organs, and the top views (Q-W) and side views (X-AD) epidermal cells of double silenced floral organs from the 1st blooming flowers. (A, J, Q and X) Sepal adaxial epidermal cells both had polygonal, papilla, irregular, and convex. (B, K, R and Y) Sepal abaxial epidermal cells both had irregular, convex, irregular, and convex. St. is the stomata abbreviation. (C L, S and Z) Petal adaxial epidermal cells had polygonal, papilla, polygonal, and papilla. (D M, T and AA) Petal abaxial epidermal cells all had irregular, convex, irregular, and convex.. (E N, U and AB) Lip adaxial epidermal cells had polygonal, convex, irregular and cupola. (F O, V and AC) Lip abaxial epidermal cells had elongated polygonal, convex, irregular and convex. (G P, W and AD) Column epidermal cells had polygonal, conical, irregular, and conical. (H and I) Leaf adaxial and abaxial epidermal cells had polygonal, flat. Solid white arrows indicate a protuberance on the top of the cell. Solid black arrow indicates epicuticular wax crystals. White arrowheads indicate irregular cuticular folds in the central field. Black arrowheads indicate parallel cuticular folds in the central field. Dashed white arrows indicate irregular cuticular folds in the anticline field. Dashed black arrows indicate parallel cuticular folds in the central field. Bars, 25 μm. Materials were *Dtps.* OX Red Shoes ‘OX1407’.

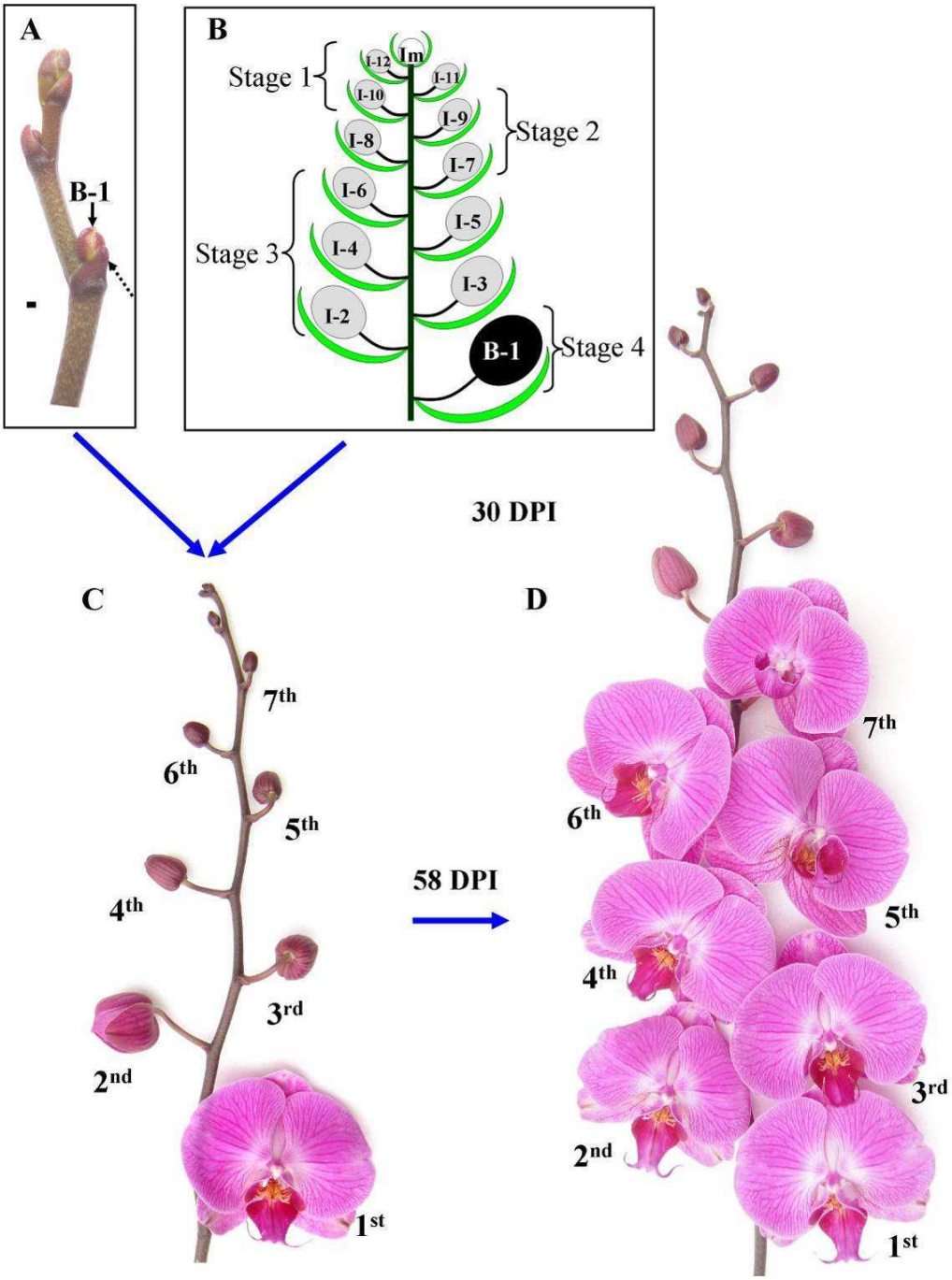


Figure S11. Estimated what period of floral development is most sensitive to loss of function by *PeMADS1* and *PeMADS6* silencing in orchid. (A) The tip of inflorescence including floral buds under differentiation, black arrow indicates the first visible floral bud (B1), and dashed arrow indicates its bract. Black bar, 1 mm. (B) Schematic diagram of a tip of a *Phalaenopsis* inflorescence, including one visible floral bud (modified from Pan *et al*., 2011). Im, inflorescence meristem; I-10 to I-12, stage 1, the formation of floral primordia; I-7 to I-9, stage 2, the formation of sepal, lateral petal, lip and column primordia; I-2 to I-6, stage 3, the formation of pollina and callus on the column and lip, respectively, B1, stage 4, the first visible floral bud. Inflorescence with the 1st flower blooming at 30 DPI (C) and the 7th flower blooming at 51 DPI (D) in *PeMADS1* and *PeMADS6* double-silenced plants. The major stages are defined as in Fig. 9B (modified from Pan *et al*., 2011)