Flower development of *Phalaenopsis* orchid involves functionally divergent SEPALLATA-like genes

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Summary

- The *Phalaenopsis* orchid produces complex flowers that are commercially valuable, which has promoted the study of its flower development. E-class MADS-box genes, SEPALLATA (SEP), combined with B-, C- and D-class MADS-box genes, are involved in various aspects of plant development, such as floral meristem determination, organ identity, fruit maturation, seed formation and plant architecture.
- Four SEP-like genes were cloned from *Phalaenopsis* orchid, and the duplicated *PeSEPs* were grouped into *PeSEP1/3* and *PeSEP2/4*.
- All PeSEPs were expressed in all floral organs. *PeSEP2* expression was detectable in vegetative tissues. The study of protein–protein interactions suggested that PeSEPs may form higher order complexes with the B-, C-, D-class and AGAMOUS LIKE6-related MADS-box proteins to determine floral organ identity. The tepal became a leaf-like organ when *PeSEP3* was silenced by virus-induced silencing, with alterations in epidermis identity and contents of anthocyanin and chlorophyll. Silencing of *PeSEP2* had minor effects on the floral phenotype. Silencing of the E-class genes *PeSEP2* and *PeSEP3* resulted in the downregulation of B-class *PeMADS2-6* genes, which indicates an association of *PeSEP* functions and B-class gene expression.
- These findings reveal the important roles of PeSEP in *Phalaenopsis* floral organ formation throughout the developmental process by the formation of various multiple protein complexes.

Introduction

A typical flower of an angiosperm contains four whorls of floral organs, including sepals, petals, stamens and carpels. A number of homeotic genes that associate with the floral organ identity determination have been identified, and the ‘ABC’ model proposes the combined effect of the A-, B- and C-function genes on floral organ identity determination. In brief, A- and B-function genes specify petal identity, B- and C-function genes specify stamen identity, and A- and C-function genes alone specify sepal and carpel identity, respectively.

*SEPALLATA* (SEP) genes are the E-class MADS-box genes involved in floral organ determination (Becker & Theissen, 2003). The unique interaction of E-function genes and various floral organ identity genes is required for the determination of floral organs (Stellarì et al., 2004; Kramer & Hall, 2005; Zahn et al., 2005). Phylogenetic analysis has revealed that the SEP subfamily is monophyletic and that multiple SEP homologs are present in distant angiosperm lineages (Zahn et al., 2005). The first duplication occurred before the origin of the extant angiosperms, which resulted in two clades, the AGL2/3/4 (SEP1/2) and AGL9 (SEP3) clades (Zahn et al., 2005). Subsequent duplications occurred within these clades in the eudicots and monocots (Zahn et al., 2005). The Arabidopsis SEP gene family has four members – SEP1, SEP2, SEP3 and SEP4 – from gene duplication (Zahn et al., 2005). Many SEP-like genes have been identified from monocots. Maize and rice have at least eight and five different SEP-like genes, respectively (Becker & Theissen, 2003; Zahn et al., 2005; Cui et al., 2010). However, recent findings of SEP-like genes in monocots were mainly restricted within the Poaceae.

The expression of SEP-like genes is floral specific in several angiosperm species, including core eudicots, such as Arabidopsis (Pelaz et al., 2000; Ditta et al., 2004), tomato (*Solanum lycopersicum*) (Uimari et al., 2004) and petunia (*Petunia hybrida*) (Ferrario et al., 2003; Vandenbussche et al., 2003; Matsubara et al., 2008), and monocots, such as rice (*Oryza sativa*) (Cui et al., 2010) and maize (*Zea mays*) (Lid et al., 2004). In addition
to their floral-specific expression, members of the SEP gene family are expressed during the development of vegetative tissues, seeds and fruits (Chung et al., 1994; Buchner & Boutin, 1998; Ampomah-Dwamena et al., 2002; Elitzur et al., 2010; Seymour et al., 2010; Ireland et al., 2013). These differential expression patterns suggest functional divergence of SEP-like genes (Malcomber & Kellogg, 2005).

In Arabidopsis, the multimeric complexes that contain MADS-box proteins can be formed via flexible combinations with various components (Smaczniak et al., 2012). The protein complexes that consist of at least one SEP protein and other combinational proteins function in the specification of floral organ identity and determination of flowering time. SEP3 is a ‘glue’ protein mediating the multimeric complex formation in various developmental processes in Arabidopsis (Immink et al., 2009). In other angiosperm species, SEP-like proteins are ‘hub’-like proteins, with their conservational tendency of protein–protein interacting behavior with AP1/FUL, B- and C/D-function as well as AGL6-like MADS-box proteins found in Gerbera (Ruokolainen et al., 2010), Petunia and rice (Malcomber & Kellogg, 2005). However, a slightly different situation is found in various species of angiosperm, especially those that are radically different from the studied models. Evidence includes the varied interacting patterns between homologs of floral organ identity proteins, which are produced after the duplication and subfunctionalization events.

The genome-wide binding patterns of Arabidopsis SEP3 reveal that it targets directly regulatory elements of both MADS and non-MADS transcription factors, including AP1, AG, AP3, PI, SEPs, SHP1, SHP2, bHLH/bZIP, TCP and ARF (Kaufmann et al., 2009). SEP3 controls Arabidopsis floral organ outgrowth and morphogenesis by integrating developmental and auxin signalling pathways (Kaufmann et al., 2009). In addition, SEP3 regulates flower size in aspects of cell proliferation and expansion by binding to gene sets of GFI1, GRF, JAG, TCP and ARF8, ARL, BPEp and MED8 (Krizek & Anderson, 2013). The tomato SEP4 co-orthologous protein, ripening inhibitor (RIN), has a transcription-activating function similar to that of Arabidopsis SEP proteins by binding to its target sites to regulate the expression of genes that control floral organ identity (Ito et al., 2008). Throughout tomato fruit ripening, RIN regulates directly the expression of genes involved in multiple ripening processes, including cell wall modification, aroma and flavor development (Ito et al., 2008; Fujisawa et al., 2011, 2012).

During the evolution of Orchidaceae, highly specialized floral morphological features were associated with its pollination strategies (Dressler, 1993). The perianth of the orchid flower is composed of three sepals, two petals and a highly diversified lip in the two outer floral whorls. The inner fertile organ is adapted to a gynostemium or column, which is fused by the gynoeicum and androecium (Dressler, 1993). Orchid A-, C-, D- and E-class MADS-box genes have been characterized, and gene duplications of each group have been analyzed (Mondragon-Palomino, 2013). Functions of B-, C/D- and E-class MADS-box genes in the specification of orchid floral organ development have been reported in only a few species, such as Phalaenopsis, Oncidium and Dendrobium (Tsai & Chen, 2006; Aceto & Gaudio, 2011). In the ‘Homeotic Orchid Tepal’ (HOT) model, the divergent orchid B-class genes (PI-like, AP3A1, AP3A2, AP3B1 and AP3B2 genes) combine with other classes of MADS genes to regulate the complexity of sepal, petal and lip identity, showing a conserved pattern during orchid evolution (Pan et al., 2011). The revised ‘orchid code’ hypothesized that relatively different quantities of high levels of B-class genes distinguished orchid tepal development, with high levels of genes in AP3A and AP3B subclades required for petal and labellum development, respectively (Mondragon-Palomino & Theissen, 2011). Higher order protein complexes formed by multiple homeotic MADS-box proteins have been proposed to decipher the orchestration of orchid tepal morphogenesis (Pan et al., 2011). The formation of heterodimeric complexes consisting of B-class PeMADS6/PeMADS2–5 (PI-like/AP3-like) in Phalaenopsis and multimeric proteins of DcOSEP1/DcOPI/DcOAP3A or DcOAP3B (SEP-like/PI-like/AP3-like) in Dendrobium has been reported (Xu et al., 2006; Tsai et al., 2008). However, how the heteromeric MADS-box proteins assemble to decipher various floral organs has not been studied extensively.

So far, several SEP-like genes have been identified from a few orchid species, including AdOM1 in Aranda (Lu et al., 1993), DcSEP1 in Dendrobium crumenatum (Xu et al., 2006), and DOMADS1 and DOMADS3 in Dendrobium greg Madame Thong-IN (Yu & Goh, 2000). AdOM1 is expressed in sepals and petals and DcSEP1 in sepals, petals, lips and column (Lu et al., 1993; Xu et al., 2006). Both DOMADS1 and DOMADS3 are expressed in the shoot apical meristem during the switch from vegetative to reproductive growth, and later in mature flowers, and may play an important role in floral transition and floral organ identity (Yu & Goh, 2000). However, although these orchid SEP-like genes show distinct expression patterns, none of their functions in floral organ identity has been investigated in orchids. Therefore, we need to address the developmental role and biochemical function of E-class genes in combination with other homeotic genes involved in floral organ identity in orchids.

The flower of the most popular model system in the orchid family, Phalaenopsis, is the classic model for highly differentiated and relatively complex petaloid monocots. Orchid evo–devo studies have reviewed the advantages and limitations of the comparative strategy used for analysis (Mondragon-Palomino, 2013). Methods for the study of functional genes by the creation of transgenic orchids have not been well developed. Only virus-induced gene silencing (VIGS) has been a useful method to obtain loss-of-function phenotypes in Phalaenopsis (Lu et al., 2007, 2012; Hsieh et al., 2013a,b) to compensate for difficulties in orchid research because of their long generation time. The silencing effect and transient efficiency of loss-of-function assays have limitations that can substantially complicate the functional analysis of redundant genes.

In this study, we performed a thorough, multifaceted experimental investigation of the developmental genetics of the SEP-like gene family (E-class) in Phalaenopsis orchid by the downregulation of individual or multiple PeSEP genes using VIGS. We demonstrate the conservation of PeSEP genes in floral organ identity, but also divergence in roles in vegetative growth,
floral initiation and ovule development, which indicates a diversification of SEP-like genes in the orchid family.

Materials and Methods

Plant materials

Two native Phalaenopsis species in Taiwan, P. equestris and P. aphrodite ssp. formosana, were from Taiwan Sugar Corporation (Tainan and Puli, respectively, Taiwan) and grown in the glasshouse at National Cheng Kung University (NCKU) under natural light (photosynthetic photon flux density, 90 μmol m⁻² s⁻¹) and controlled temperature (23–27°C).

Cloning and characterization of Phalaenopsis SEP-like genes

Total RNA was extracted from Phalaenopsis as described by Tsai et al. (2004). SEP-like genes from the inflorescence of P. equestris were identified by rapid amplification of cDNA ends (RACE) and reverse transcription-polymerase chain reaction (RT-PCR) with degenerated primers. Degenerated primers for PCR were designed in the conserved MADS-box and K-box regions, and are listed in Supporting Information Table S1. The amplified products were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). We randomly selected 10–12 positive clones for sequencing.

Phylogenetic analysis

A total of 100 SEP-like, 12 AGL6-like and three SQUA genes were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) for phylogenetic analysis. The AGL6 and SQUA family members were used as outgroups. Full-length amino acid sequences were first aligned using the default settings in ClustalW and MUSCLE implemented in MEGA v5.2 (Tamura et al., 2011), and then adjusted manually with the reference alignment provided by Zahn et al. (2005). The aligned sequences were back-translated to nucleotides. To infer the best nucleotide substitution model for the following phylogenetic analyses, the model (GTR + I + G) for our dataset was selected using the Akaike information criterion (AIC) in jModelTest v2 (Darriba et al., 2012). Maximum likelihood (ML) and Bayesian analysis trees were reconstructed with the obtained model parameters as input with the use of GARLI 2.0 (Zwickl, 2006) and MrBayes v3.1.2 (Huelsenbeck et al., 2001). To calculate ML bootstrap support (MLBS) values, 250 replicates were run under the same criteria. The Bayesian inference analysis was run for four exchangeable Markov chains (106 generations each) with trees sampled every 100 generations. The first 25% ‘burn-in’ phase trees were discarded and the rest were summarized in a 50% majority-rule consensus tree for the calculation of Bayesian branch support (Bayesian inference; BI).

Promoter analysis

The genomic DNA sequences of PeSEP1–4 were cloned using genome walking (primers are listed in Table S1). Two motif-finding programs were used for the analysis of the 2-kb promoter sequences upstream from the translation start site of PeSEPI–4 genes. The promoters were analyzed for patterns of CarG boxes (Tables S2, S3) with the ‘fuzznuc’ software from EMBoss (http://emboss.biocomputing.nl/cgi-bin/emboss/fuzznuc). The motif discovery tool of Multiple EM for motif Elicitation (MEME) was used to identify sets of over-represented consensus motifs. Motif widths ranged from 6 to 50 bp, and promoters were searched from both strands.

Real-time RT-PCR

RNA extraction, cDNA synthesis and real-time RT-PCR experiments were performed as described (additional methods in Methods S1). cDNAs for real-time RT-PCR analysis were obtained from vegetative and reproductive tissue, including two-leaf seedlings, leaf, root, floral stalk, floral bud and pedicel. Floral buds were divided into five developmental stages: B1, B2, B3, B4 and B5. Floral organs (sepals, lateral petals, lip, pollinaria and column) were collected. Samples were examined after pollination: ovules, embryos and mature dry seeds (Methods S1).

Yeast two-hybrid assay

Yeast two-hybrid analysis involved the use of the MATCHMAKER II system (Clontech, Palo Alto, CA, USA). Because of the autoactivation of the GAL4 reporter genes by intrinsic transcription activation domains in PeSEP, we used truncated PeSEPΔAC proteins containing the MADS, I and K regions (primers are listed in Table S1). Interactions between the tested proteins were determined by spotting assay. Trimeric protein–protein interactions were tested with PeMADS2–6 and PeSEP1–4AC proteins (Methods S1).

Virus-induced gene silencing (VIGS)

Phalaenopsis I-Hsin Sunrise Cinderella ’OX1357’ and P. OX Red Shoes ’OX1408’ were used in the functional analysis with VIGS assay as described by Hsieh et al. (2013a). Seedlings from Oxen Biotechnology (Tainan, Taiwan) showed a high proportion of virus-free plants after screening for the two prevalent viruses, Cymbidium mosaic virus and Odontoglossum ringspot virus, by RT-PCR. VIGS involved fragments of PeSEP2 and PeSEP3 (primers are listed in Table S1) constructed in a pCymMV-Gateway plasmid (Lu et al., 2012). The region from nucleotides 18–104 of the PeSEP3 coding sequence contains 98 nucleotides that are 93% identical to those of PeSEP1 (Fig. S1). The specific region of PeSEP2 was constructed from nucleotides 18–113, with 77% identity to that of PeSEP3. Transformed Agrobacterium (strain EHA105) was injected into inflorescence spikes with one visible floral bud and into the leaf right above the inflorescence. We generated eight independent PeSEP3- and eight PeSEP2-silenced plants in P. I-Hsin Sunrise Cinderella ’OX1357’. The same experiment was performed in P. OX Red Shoes ’OX1408’. Real-time RT-PCR was used to examine the knockdown expression of MADS-box genes in floral buds, 1.5 cm in length, at c. 0-2 days post-treatment (dpt)
Ectopic expression of PeSEP1 and PeSEP3 in Arabidopsis

cDNA fragments containing the coding regions of PeSEP1 and PeSEP3 were cloned into the pBI121 vector (primers are listed in Table S1) under the control of the constitutive CaMV 35S promoter (Methods S1). The constructs were then introduced into Agrobacterium tumefaciens (strain GV3101). Wild-type Arabidopsis thaliana ecotype Columbia plants were transformed by the floral dip method (Clough & Bent, 1998).

Results

Cloning and phylogenetic analysis of four SEP-like genes in Phalaenopsis

Four SEP-like genes isolated from *P. equestris* were named PeSEP1, PeSEP2, PeSEP3 and PeSEP4 (GenBank KF673857–KF673860, Table S4). The reconstructed phylogeny suggested that these four *P. equestris* SEPs, together with all available orchid SEP homologs, formed two separate monophyletic clades, in which PeSEP2 and PeSEP4 grouped to the orchid SEP1/2 (in the AGL2/3/4 or SEP1/2/4 clade), with PeSEP1 and PeSEP3 included in the orchid SEP3 (in the AGL9 or SEP3 clade) (Fig. 1). Therefore, apart from the major pre-angiosperm gene duplication event separating AGL2/3/4 and AGL9 clades, additional SEP duplications within the monocots and orchids were evident (blue bars in Fig. 1). Similar additional duplication events also pre-dated the rosid and asterid diversification within their lineages. All predicted amino acid sequences of PeSEP proteins shared 61–82% identity and 76–89% similarity, and possessed the conserved MIK domain and a divergent C-terminal domain with the conserved SEP I and SEP II motifs, whereas PeSEP2 has a partial deleted form of the SEP I motif (Figs 2a, S2).

Divergent distribution of cis-regulatory elements on the promoters of PeSEP genes

cis-Regulatory elements in the promoter region and/or second intron of MADS-box genes are required for appropriate regulation of expression by the cooperative binding of their activators and repressors to induce conformational changes in regulatory regions. To verify the conservation and divergence of the duplicated PeSEP paralogs, we analyzed the regulatory elements in the upstream regions, including the CarG boxes and over-represented conserved motifs in the PeSEP1–4 promoters (designated pPeSEP1–4). Ten to 20 CarG boxes were identified within the 2-kb fragment upstream from the start codon of pPeSEP (Fig. 2b). pPeSEP1–3 showed high CarG box density within the 1–2-kb region, whereas the CarG boxes for pPeSEP4 showed an even distribution. We predicted 84 cis-elements classified into ten motifs from the 2-kb upstream regions of pPeSEP1–4 (Figs 2c, S3). pPeSEP4 seemed to have hotspots for Motif 1 in the region of 1–1.5 kb. By contrast, Motif 2 was abundant in pPeSEP1, pPeSEP2 and pPeSEP3, and Motif 3 was abundant in pPeSEP2 and pPeSEP4. Motifs 4–10 were exclusive, with an E-value > 0.001 (Fig. S3). Overall, pPeSEP could be categorized into two unequally supported PeSEP gene topologies as (pPeSEP1/2/3, pPeSEP4) and (pPeSEP1/3, pPeSEP2/4) according to the MEME results.

Expression of PeSEPs, except for PeSEP2, is specific to floral organs

We investigated the expression profiles of the four PeSEP genes in *Phalaenopsis* (Fig. 3). All four genes were expressed in the flower buds (Fig. 3i). The expression of PeSEP1 and PeSEP3 was specific to reproductive tissues, whereas only the expression of PeSEP2 was detectable in vegetative tissues and significantly high in the floral stalk (Fig. 3i). By contrast, PeSEP4 expression was extremely low in all tissues. Furthermore, transcripts of PeSEPs persisted throughout the entire floral development (Fig. 3j). We further analyzed the expression of PeSEP1–3 in sepal, petal, lip and column; transcripts of PeSEP1–3 were widely detected in all floral organs (Fig. 3k). PeSEP2 was highly expressed in the column, and PeSEP3 expression was dominant in the petal. In addition to floral organs, we analyzed transcripts of PeSEPs in developing ovules, embryos and mature seeds after pollination; the expression of PeSEP1–3 was enriched in the near-mature ovule at 56 d after pollination (DAP), but was nearly undetectable in various developing embryo stages (embryo 1, 80 DAP and embryo 2, 100 DAP) and seeds (Fig. 3i). Although the PeSEP genes were produced by recent gene duplication events, they showed overall non-overlapping expression profiles, both temporally and spatially. The non-ubiquitous expression patterns of PeSEP genes suggest that these *SEP* genes probably have functional divergence in regulating *Phalaenopsis* vegetative tissue, flower and ovule development.

PeSEPs form protein complexes with various MADS-box proteins

Previously, the HOT model has proposed the formation of higher order protein complexes, consisting of B-, C-, D- and other classes of MADS-box proteins, for orchid tepal development (Pan et al., 2011). Although protein–protein interactions have been revealed among floral organ identity MADS-box proteins in *Phalaenopsis*, *Oncidium* and *Dendrobium* floral development, extensive studies are still needed to understand whether PeSEP proteins are involved in the formation of various higher order protein complexes with other *Phalaenopsis* floral organ identity MADS-box proteins. We comprehensively assessed the protein complex formation by GAL4-based yeast two-hybrid assay followed by spotting assay with serial dilutions of cultured yeast among 14 *Phalaenopsis* MIKC*-type* MADS-box proteins: four AP3 (*PeMADS2–5*) (Tsai et al., 2004), PI-like (*PeMADS6*) (Tsai et al., 2005), AG-like (*PeMADS1*) (Chen et al., 2012), STK-like (*PeMADS7*) (Chen et al., 2012), four SEP (*PeSEP1,*
Fig. 1 Phylogenetic tree of SEP family genes based on maximum-likelihood (ML) criteria from major seed plant clades, including gymnosperm, magnoliids plus basal angiosperms (purple), monocots (green), rosids (pink) and asterids (yellow). ML bootstrap support (MLBS) values and Bayesian posterior probabilities (Bayesian inference; BI) are indicated on each branch as MLBS/BI. The plus sign (+) represents MLBS = 100 or BI = 1.00. Thick branches indicate high support values with MLBS ≥ 70 and BI ≥ 1.00. Blue bars denote major duplication events, and four Phalaenopsis SEP copies are marked with blue stars.
PeSEP2, PeSEP3 and PeSEP4) and three AGL6-like (PeAGL6a, PeAGL6b and PeAGL6c) proteins (Y. R. Luo et al., Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan, unpublished). The four PeSEP proteins seemed to show comprehensive direct interactions with *Phalaenopsis* AP3/PI-like, C/D-class and AGL6-like proteins, but poor interactions among the four PeSEP members (Table 1). PeSEP1 showed extensive interactions with the *Phalaenopsis* MADS-box domain proteins in a heterodimerizing manner. By contrast, unlike its closest paralog PeSEP1, PeSEP3 showed relatively limited interactions. Similar to PeSEP1, PeSEP2 interacted with MADS-box proteins in other subfamilies, but was unable to form PeSEP dimers. PeSEP4 revealed a strong interaction with only one of the AGL6-like proteins, PeAGL6a, but restricted interactions with other MADS-box proteins.

Unexpectedly, the average poor dimeric interactions formed between PeSEP and other MADS-box proteins were not consistent with the role of SEP proteins as a glue to mediate complex assembly. These heterodimers may have unstable binding abilities relative to the trimeric protein complexes bridged by SEP proteins. We therefore tested whether the PeSEP proteins can function as a glue to mediate and strengthen the interactions between PeMADS6 (PI-like) and other MADS-box proteins (PeMADS2 and PeMADS4, AP3-like and PeMADS7, STK-like) by co-transforming three constructs in yeast. The original weak interactions between PeSEPs and PeMADS2, PeMADS4, PeMADS6 and PeMADS7 were greatly enhanced to strong and very strong levels (Fig. 4a,b) with the formation of PeSEP/PeMADS6/PeMADS2, 4 and 7 complexes. Heterotrimerizations were revealed by facilitating the interaction among the three *Phalaenopsis* MADS-box proteins in PI-like/SEP-like/AP3- or STK-like complexes mediated by PeSEP proteins.

**Expression of PeSEPs is associated with flower organ formation**

Given the close phylogenetic relationship and divergent expression patterns of *PeSEPs*, we explored the functions of these
PeSEP genes by VIGS. To assess the possible redundant functions among various PeSEP genes, we generated the PeSEP2/3-silenced line with simultaneously silenced transcripts of PeSEP2 and PeSEP3. We observed several pronounced phenotypes of morphological changes in blooming flowers at c. 45 d after infection (DAI), with no vegetative growth retardation and abnormalities in flowering time or inflorescence determinacy. Most flowers in PeSEP-silenced plants were abnormal to various degrees (Fig. 5).

The knockdown levels of PeSEP genes were strongly related to the degree of flower abnormality in PeSEP-silenced lines (see Figs 9, 10).

The major morphological changes in PeSEP-silenced plants were in the sepals and petals, with only minor alterations in the lip (Fig. 5a,b). In severe cases, the flowers remained enclosed into a bud shape after full maturation because of the tight connection between the anticlinal joint of the sepals and petals themselves (Fig. 5a). The dominant changes in the sepals and petals appeared to be a transformation into leaf-like tissues (Fig. 5a, yellow arrows) showing as chimeric green sepal/petal organs. The PeSEP3-silenced flowers showed a noticeable greenish coloration mainly from the tip to the central area of the sepals and petals (Fig. 5c) because of the abundance of chloroplast in epidermal and parenchyma cells (Fig. 5d). The chlorophyll content was increased by three- to seven-fold in PeSEP3- and PeSEP2/3-silenced flowers when compared with mock-treated flowers (Fig. 5h). The number of stomata was higher on the adaxial epidermal surface of the sepals and petals in PeSEP3- and PeSEP2/3-silenced flowers than in mock-treated flowers, similar to that in...
mock-treated leaves (data not shown), which suggests a homeotic transformation of tepal to leaf-like organ. Bleached patches against the purple–red background were clear in the sepals and petals (white arrowheads, Fig. 5c) and slight in the lip (white arrowheads, Fig. 5a,b). Total anthocyanin content was slightly decreased in PeSEP2- and PeSEP3-silenced flowers (Fig. 5i). Meanwhile, a deep purple–red pigment appeared at the tips of the sepals and petals (Fig. 5e, blue arrows). As the flowers proceeded to anthesis and senescence, the perianth, especially the lip of PeSEP3-silenced flowers (Fig. 5k, yellow arrowhead), remained fleshy and prominent even after 60 d of blooming, when compared with the senescent perianth in mock-treated flowers (Fig. 5j, yellow arrowhead). The venational pigmentation of anthocyanin still persisted in a distinct pattern in PeSEP3-silenced petals (Fig. 5k). In mild and weak mutants, these changes in floral morphological features were slight relative to those in the severe mutant (Fig. 5a,b).

Differential silencing effects in PeSEP-silenced flowers

The VIGS effect in Phalaenopsis was highest at c. 7 wk after viral inoculation and lasted for 9 wk with approximately eight bloomed flowers in an inflorescence. Because of the transient silencing nature of VIGS, the altered phenotypes in the PeSEP-silenced raceme inflorescence were usually unstable (Fig. 5l–o). Abnormal flowers on PeSEP3- and PeSEP2/3-silenced floral stalks displayed an acropetal gradient, and later emerged floral buds eventually reverted to a normal appearance (Fig. 5m,o). In PeSEP-silenced lines, most flowers usually showed morphological changes ranging from severe to mild and weak phenotypes, and were designated PeSEP-, PeSEP+-, PeSEP+-, and PeSEP+-silenced phenotypes, respectively (Fig. 5q–s,u–w). Mutant PeSEP3-silenced flowers showed mainly severe and mild phenotypes, occasionally with weak conversion. The mutant phenotype with double-silenced PeSEP2/3 appeared more frequently in PeSEP2/3- and PeSEP2/3-silenced flowers, but less frequently in PeSEP2/3-silenced flowers (Fig. 5o,u–w). By contrast, all flowers in the PeSEP2-silenced plants (Fig. 5n,t) were almost morphologically indistinguishable from mock-treated flowers (Fig. 5l,p).

To determine whether the identities of floral organs of the three PeSEP-silenced lines were affected, we quantified several characteristics, including flower size, cell density, shape, height and length (Fig. 6). The mean flower size was smaller in PeSEP3- and PeSEP2/3-silenced plants than in mock-treated and PeSEP2-silenced plants (Fig. 6a), whereas the epidermal cell size was decreased in both height and width in sepals and petals (Fig. 6c, d), together with increased cell density (Fig. 6b). Although the PeSEP2-silenced floral morphological features, especially the lip and column, were almost unaffected and indistinguishable from mock-treated features (Fig. 5p,t), we observed slight changes in the epidermis. Similar phenotype changes were observed in the independent experiment with P. I-Hsin Sunrise Cinderella (Figs S4, S5). Therefore, morphological changes were stronger for PeSEP3- than PeSEP2/3-silenced plants. No noticeable changes in PeSEP2-silenced plants suggested that PeSEP2 has redundant functions in floral organ identity.
Micromorphological changes in PeSEP-silenced floral organs

To determine whether floral organs of the PeSEP-silenced flowers were affected at the cellular level, we observed perianth epidermal cell shapes under cryo-SEM, indicated as the aspect ratio $\beta$, the ratio of the width to height of epidermal cell curvature (Fig. 6c). In addition to the decreased cell size, cell shape and several ultrastructures were altered in epidermal cells of PeSEP-silenced flowers (Figs 7, 8). Among the three PeSEP-silenced lines,

![Table and diagram](https://via.placeholder.com/150)

**Fig. 4** Analysis of protein–protein interactions between PeSEPs and other MADS-box proteins of Phalaenopsis by a GAL4 yeast two-hybrid system. Ten-fold serial dilutions of co-transformed yeast with three (a) and two (b) pGADT7 and pGBK7 constructs. Equivalent numbers of cells from $10^3$ to $10^6$ were diluted and spotted onto control SD–Leu/~Trp~ growth plates and selective SD–Leu/~Trp~/~His~ and SD–Leu/~Trp~/~His~/~Ade~ plates. ‘++++’, ‘+++’, ‘++’ and ‘+’ are very strong, strong, moderate and weak interactions, respectively, on selective plates. ‘−’, no interaction. The fusion of C-terminally truncated PeSEP (PeSEPΔC) proteins was used.
Fig. 5 Phenotype changes of PeSEP-silenced flowers in Phalaenopsis OX Red Shoes. (a, b) Flowers of PeSEP-silenced plants show morphological phenotypes of various degrees. From left to right: severe, mild, weak and normal. (c) Green leaf-like tissues and bleaching patches (white arrowheads) in sepals of PeSEP3-silenced flower. White arrowheads show bleaching in sepals and the central lobe of the lip. (d) Chlorophyll in epidermal and parenchyma cells of PeSEP3-silenced greenish perianth. (e) Anthocyanin accumulated at the tips of sepals and petals (blue arrows). (f, g) Lips showing purple–red color from normal flowers and bleached coloration (white arrowhead) from mutated flowers. (h, i) Increased total chlorophyll and slightly decreased anthocyanin contents of SEP3-, PeSEP2- and PeSEP2/3-silenced flowers. Senescent flowers from (j) mock-treated and (k) PeSEP3-silenced plants after 45 and 60 d of blooming. (l–o) Normal and gradually mutated flowers displaying normal, severe (s), mild (m) and weak (w) phenotypes on the floral stalks of mock-treated, SEP3-, PeSEP2- and PeSEP2/3-silenced plants. (p, t) Normal flowers observed on mock and PeSEP2-silenced plants. Severe, mild and weak flowers from (q–s) PeSEP3- and (u–w) PeSEP2/3-silenced plants. Yellow and blue arrows show greenish regions and anthocyanin accumulation in sepals and petals. Bars: 1 cm.
PeSEP3- and PeSEP2/3-silenced flowers showed the most severe defects in the overall cellular changes, whereas these changes in PeSEP2-silenced flowers were minor.

Knocked down expression of PeSEP and B-class MADS-box genes in PeSEP-silenced flowers

The polygonal-shaped epidermis on the adaxial side became flattened and changed from a papillae/cupola/conical to hemispherical shape in sepals and petals (Fig. 7b,d,i,l) of PeSEP3- and PeSEP2/3-silenced flowers, accompanied by an increased aspect ratio $\beta$ (Fig. 6e). When compared with the convex-shaped epidermal cells on the abaxial surface of the sepal and petal in mock-treated flowers (Fig. 7c,m), cells in PeSEP3- and PeSEP2/3-silenced flowers were flat (Fig. 7f,h,n,p). In addition, PeSEP-silenced flowers showed a protuberance in the central field of the petal epidermis on the abaxial side (arrows in Figs 7n,p, 8i) that resembled leaf abaxial epidermal cells (Fig. 7x). Moreover, several microgranules were secreted from the epidermis on the adaxial side of the sepal and petal in PeSEP-silenced flowers (Fig. 7b–d, j–l), unlike the smooth surface of the epidermis in mock-treated flowers (Figs 7a,i, 8a,b). These microgranules covered the protuberance and sometimes appeared in the central field of the epidermis (Fig. 8c,d). Furthermore, the greatly accumulated microsecretory granules on the protuberance of the adaxial epidermis may have contained lipids, as seen on histochemistry with Sudan IV staining (Fig. 8l,o). In addition, platelet-shaped epicuticular wax crystals accumulating on the bract epidermis on the abaxial surface of mock-treated plants (Figs 7y, 8j) appeared to aggregate randomly on the epidermal surface in all PeSEP2-, PeSEP3- and PeSEP2/3-silenced sepals (Figs 7f–h, 8h).

In the lip, the ultrastructural cuticular folding covered both surfaces of the epidermis in mock-treated flowers (Figs 7q,u, 8q, t), but became looser and thinner in PeSEP3-silenced flowers (Figs 7r,u, 8r,u). Therefore, all four floral organ identities, especially the two outer floral whorls, were homeotically transformed, at least in part, from tepal- to leaf-like in PeSEP-silenced plants. The altered characteristics, including flower morphological features, cell identities and ultrastructures of the epidermis, in PeSEP-silenced plants confirmed that abnormalities of the perianth could be caused by knocked down expression of PeSEP genes.

Knocked down expression of PeSEP and B-class MADS-box genes in PeSEP-silenced flowers

The PeSEP-silenced mutants exhibited a life-like phenotype whereby the perianth was partially transformed into leaf-like tissues, as observed in SEP mutants and B-class gene deficiencies in Arabidopsis. From the mutated phenotype, in addition to the PeSEP genes, off-target silencing pointing to the B-class MADS-box genes in Phalaenopsis may also result in PeSEP-silenced mutant flowers. Therefore, we analyzed the transcriptional levels of E-class PeSEP1–4 and B-class PeMADS2–6 genes to assess their expression profiles in sepals, petals and lip of PeSEP-silenced flowers (Figs 9, 10).

The comprehensive silencing effect of PeSEP genes was manifested more strongly in PeSEP2- than PeSEP3-silenced flowers. The transcript level of the target gene PeSEP2 was knocked down by 12–32% in PeSEP2-silenced perianth (Fig. 9b). The transcript level of off-target PeSEP genes, PeSEP1, PeSEP3 and PeSEP4, was not obviously changed in PeSEP2-silenced flowers (Fig. 9a,c, d). Therefore, the altered phenotypes observed in PeSEP2-
silenced flowers were specifically caused by the knockdown of PeSEP2 expression. In PeSEP3-silenced flowers, the expression of PeSEP3 was significantly downregulated by 26–55% and 73–93% in PeSEP2- and PeSEPm-silenced perianths, respectively (Fig. 9c); the silencing levels corresponded to the morphological phenotypes. However, the transcripts of PeSEP1 and PeSEP2 were also affected to various degrees in PeSEP3-silenced flowers (Fig. 9a,b). As expected, the transcript level of PeSEP4 remained extremely low and was unaffected (Fig. 9d). Therefore, the combined effect of specific knockdown of PeSEP3 and off-target silencing of PeSEP1 and PeSEP2 genes caused the leaf-like changes in PeSEP3-silenced plants. In PeSEP2/3-silenced flowers, transcripts of PeSEP1 and PeSEP2 were knocked down to c. 58% and 30%, but the expression of PeSEP3 was not decreased.
Thus, PeSEP2 and PeSEP3 were not equally effective in knocking down the intended targets in PeSEP2/3 double-silenced plants.

The varied decreased transcript levels of the five Phalaenopsis B-class MADS-box genes, PeMADS2–6, indicate a direct or indirect transcriptional regulatory effect related to the downregulated degree of PeSEP genes in PeSEP-silenced flowers. In PeSEP3-silenced flowers, the transcript levels of PeMADS3, PeMADS4, PeMADS5, and PeMADS6 were strongly downregulated (Fig. 10b–e), but that of PeMADS2 was only slightly decreased (Fig. 10a). Similar but mild patterns of reduced transcript levels were detected in PeSEP3m-silenced flowers. Under the silenced PeSEP2 background, the expression of PeMADS3, PeMADS4, PeMADS5, and PeMADS6 (Fig. 10b–e) was suppressed only moderately to weakly relative to that in mock-treated flowers, whereas the transcript level of PeMADS2 remained unchanged (Fig. 10a). Thus, the transcript level of PeSEP genes could be specifically knocked down by VIGS. Further expression analyses indicated that the transcript level of Phalaenopsis B-class genes may be differentially affected under the reduced transcript level of E-class genes through gene–gene interactions.

**Ectopic expression of PeSEP3 in Arabidopsis**

To further investigate the function of Phalaenopsis SEP-like genes in flower transition, floral determinacy, and organ identity, we ectopically expressed PeSEP1 and PeSEP3 using the cauliflower mosaic virus 35S promoter in Arabidopsis. Ectopic expression of PeSEP3 strongly affected the flowering time, plant architecture, and floral morphological features (Fig. 11). When compared with wild-type Arabidopsis (Fig. 11a), 35S:PeSEP3 transgenic plants with the severe phenotype had a much smaller plant size, with early flowering, whereby the terminal flower was formed after the production of a few rosette leaves (Fig. 11c). Early flowering is commonly observed when SEP genes are overexpressed in Arabidopsis (Ditta et al., 2004). However, 35S:PeSEP1 transgenic

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**Fig. 8** Ultrastructures on epidermal cells of flowers of Phalaenopsis OX Red Shoes. (a–j) Ultrastructures of protuberance on the adaxial conical-shaped epidermal cells (a–e) and epicuticular wax crystals on the surface of abaxial convex-shaped epidermal cells (f–i). (k–p) Sudan IV staining of secreted lipid granules on adaxial conical cells in sepal and petal of mock-treated and PeSEP-silenced flowers. (q–v) Cuticular folding on the lip epidermal cells. Bars: (a–j, q–v) 10 μm; (k–p) 100 μm.
plants showed no phenotypic changes (Fig. 11b). 35S:PeSEP3 transgenic plants flowered with only two emaciated rosette and cauline leaves, and three flowers were produced on the terminated inflorescence (Fig. 11c). In the first two flowers, sepals and petals showed abnormal morphological features, including small size and deteriorated shape (Fig. 11f). For the terminal flower, sepals showed a curly oval shape, and non-expending petals did not protrude out of the sepals (Fig. 11g). The stamens with short filaments were converted to organs classified as petaloid stamens. Their shape was like stamens, but with some white petal tissue.
usually near the top of the organ (Fig. 11g, arrowhead). The pistil was short, accompanied by a translucent ovary wall and ovules (Fig. 11g, arrow).

**Discussion**

**PeSEPs have an E-class homeotic gene function in Phalaenopsis**

By studying loss-of-function mutants, several species of dicots have shown a general conservation of SEP-like gene functions in the specification of floral organ identity. The *Arabidopsis* sep1sep2sep3 triple mutants have a phenotype of all sepals, petals, stamens and carpels converted to sepal-like organs, whereas the sep1sep2sep3sep4 mutant has the most striking changes, with all floral organs replaced by leaf-like organs (Pelaz *et al.*, 2000; Ditta *et al.*, 2004). Downregulation of SEP1/2-like genes in *tomato* (*LeTM29*) and *petunia* (*FBP2* and *FBP5*) produces sepallata-like flowers (Ampomah-Dwamena *et al.*, 2002; Vandenbussche *et al.*, 2003; Matsubara *et al.*, 2008). Similar phenotypes were found when silencing ripening-related genes: *FaMADS9* in strawberry and *MaMADS8* and *MaMADS9* in apple (Seymour *et al.*, 2010; Ireland *et al.*, 2013). The *Petunia* PhAGL6 shows similar expression patterns, but also redundant functions, to *FBP2/FBP5* (Rijpkema *et al.*, 2009). In monocots, grass species have been analyzed for loss of function of SEP-like genes in floral organ identity. Simultaneous silencing of *rice* *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8* transformed all floret organs into leaf-like structures (Cui *et al.*, 2010). Our analysis of *PeSEP* silencing is the first species investigated other than Poaceae.

Although functions of orchid SEP-like genes have been characterized by ectopic expression in *Arabidopsis* in two species, *Oncidium* and *Dendrobium* (Yu & Goh, 2000; Chang *et al.*, 2009), VIGS of *PeSEP* genes in *Phalaenopsis* allows for a much more comprehensive and detailed understanding of their functions. We found that the *PeSEP*-silenced perianth was unable to maintain its distinctive nature as an elaborate floral tepal, but rather converted to an organ with leaf-like appearance. Silencing of the *PeSEP* genes was sufficient to promote homeotic transformation with reduced flower size and delayed perianth senescence in leaf-like perianth. *PeSEPs* may trigger a change in the original cell identity from a leaf to perianth via broad changes in cellular levels by cell expansion, inhibition of chlorophyll accumulation, anthocyanin pigmentation, cutin formation and extracellular structures (Fig. 12).

**B-class genes are direct targets of SEP proteins in *Arabidopsis***

*Kaufmann *et al.*, 2009). Many floral size regulators, such as *ARF8*, *ARL*, *BPEp*, *MED8* and *MED25*, in *Arabidopsis* were identified as direct targets of AP3, PI and SEP3 proteins (Krizek & Anderson, 2013). MIXTA, controlling the conical-shaped epidermal cell formation, is a well-defined downstream target of a B-function protein in *Antirrhinum* (Noda *et al.*, 1994; Perez-Rodriguez *et al.*, 2005; Manchado-Rojo *et al.*, 2012). Indeed, we detected the downregulated transcripts of B-class genes (*PeMADS2*–6) in *PeSEP*-silenced flowers (Fig. 10). The *Phalaenopsis* PI-like gene, *PeMADS6*, was expressed in all floral organs, and *PeMADS6* showed a broad protein interaction network with other MADS-box proteins (Tsai *et al.*, 2005, 2008). *PeMADS6*-silenced flowers showed the greenish phenotype of the perianth (Lu *et al.*, 2007; Hsieh *et al.*, 2013a,b). The overlap...
between PeMADS6 and PeSEPs is demonstrated in aspects of the expression profile, protein–protein interactions and functional analysis. Thus, PeSEP genes may play a role in the control of floral morphogenesis directly and/or indirectly via the regulatory effect of Phalaenopsis B-class genes.

The conical epidermal cells of the petal, which are usually found on the adaxial side towards potential pollinators, have been used as markers of petal identity in angiosperms (Whitney et al., 2011). A mature orchid perianth shows the appropriate properties of shape, scent and pigmentation, which are usually visually and olfactorily attractive to its pollinators (Darwin, 1885; Dressler, 1993; Rudall & Bateman, 2002; Cozzolino & Widmer, 2005). For the mirror orchid, with a glossy blue appearance, the visual effects are achieved by multiple factors combined with biochemical pigmentation and a physically dedicated reflecting structure (Vignolini et al., 2012). Cuticle and epicuticular waxes on the surface of epidermal cells play an important role in cellular structuring and surface wettability (Koch et al., 2008). The heavy cuticle covering the free surface of epidermal cells is present only in the distinct lip, but absent from the sepals and petals of the Phalaenopsis flower. The cuticle probably characterizes the lip, playing an important role in visual attraction for visiting insects, and is distinct from other perianths in Phalaenopsis. Of note, PeSEP-silenced flowers showed a reduced amount of cuticular folding in the lip epidermis, which implies a loss of the typical lip features relative to sepals and petals, even though the lip did not show substantial changes. Transcriptional regulators belonging to the AP2/EREBP and R2R3 MYB families are tightly involved in cuticle biosynthesis in flowering plants (Oshima et al., 2013). An AP2/EREBP-like gene identified from Phalaenopsis transcriptomic analysis was differentially expressed in the lip relative to the petal (Hsiao et al., 2012). As for VIGS of PeSEPs, downregulation of this AP2/EREBP-like gene demonstrated reduced cuticular folding in the lip (P. H. Lai et al., Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan, unpublished). Thus, the AP2/EREBP regulator appears to be involved in cutin formation in the Phalaenopsis lip, probably as a downstream target of PeSEPs (Fig. 12).

In our model of the regulatory network in Phalaenopsis floral development, the role of PeSEP genes broadly covers E-class floral homeotic function in wide aspects of perianth development. PeSEP2 initially activates other PeSEP genes at the early inflorescence stage. PeSEP3 then crucially regulates its downstream genes involved in the stimulation of cell shape, cellular ultrastructures, floral pigmentation and cutin biosynthesis, but in the inhibition of leaf-like features in floral developmental stage (Fig. 12).

Fig. 11 Phenotype analysis of transgenic Arabidopsis plants ectopically expressing PeSEP1 and PeSEP3 genes; 30-d-old wild-type (a), 35S:PeSEP1 (b) and 35S:PeSEP3 (c) Arabidopsis plants. The inflorescence and flower of the wild-type are normal (d, e), whereas that of 35S:PeSEP3 has an abnormal stamen and ovule (f, g). Arrow and arrowhead indicate stamen and ovule, respectively. Bars: (a–d, f) 10 mm; (e, g) 1 mm.
Functional divergence and redundancy of PeSEP genes in *Phalaenopsis*

VIGS is a useful method to obtain loss-of-function phenotypes to compensate for difficulties in orchid research. However, the silencing effect and transient efficiency of loss-of-function assays have limitations that can complicate the functional analysis of redundant genes in *Phalaenopsis*. Thus, VIGS is a useful investigative technique, given that it can reveal constraints as well as benefits. Not least, its effects are clearly variable, both between individuals and within individuals through ontogenetic time. The PeSEP3-silenced fragment may affect PeSEP1 gene expression. The phenotype of the co-suppressed PeMADS1/6-silenced mutant was less severe than that of the single mutant, which suggests that double silencing by infection with two viral constructs may result in competition for viral replication (Hsieh et al., 2013b). Therefore, the phenotype observed in PeSEP2/3-silenced flowers was not further enhanced. With VIGS, the silencing phenotype was observed in the PeSEP3-silenced plant only when the PeSEP3 transcript level was mildly reduced. Meanwhile, the PeSEP2-silenced mutant did not show this alteration, even though the expression of PeSEP2 was strongly downregulated. This result illustrates a more essential role of PeSEP3 than PeSEP2 in flower development, and may suggest their divergent functions in flower development. Although PeSEP2 and PeSEP3 showed an extremely overlapping expression profile in flower development, they revealed an unequal ability to regulate *Phalaenopsis* floral organ specificity. The differential contribution of PeSEP2 and PeSEP3 to floral transition and floral organ identity reveals that the fates of the PeSEPs are not redundant. This comparison suggests that an ancestral function of E-class homeotic genes involved in floral organ identity is conserved, as in other species in angiosperms, with varied participation in floral transition, morphogenesis and ovule development among *Phalaenopsis* SEP paralogs. The evolutionary conservation in the specification of floral determinacy and organ identities and dynamics was revealed in monocot-specific neo- and subfunctionalization of SEP-like genes in rice (Cui et al., 2010). Unlike *Arabidopsis*, in which the four SEP genes are largely redundant, the downregulation of PeSEP3 has a significant effect on floral morphology. This result agrees with that from grasses, and lends strong support to the hypothesis that SEP proteins function differently in monocots than in eudicots.

Formation of multimeric protein complexes in orchid floral development

The divergence of five B-class MADS-box genes into *PI, AP3A1, AP3A2, AP3B1* and *AP3B2* subclades with the evolution of the orchid family involved the development of a highly modified perianth consisting of sepals, petals and lip (Pan et al., 2011). In addition, three AGL6-like genes were identified and displayed varied differential expression profiles in *Phalaenopsis* floral organs (Y. R. Lou et al., Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan, unpublished). Of note, PeSEP proteins, as potential partners, should be able to serve as glue factors to interact with the mass of multi-MADS-box proteins and facilitate varied protein complex formation in the regulation of sepal, petal and lip identities. Dimeric, trimeric and tetrameric protein–protein interactions formed between PeSEP and other MADS-box proteins imply that PeSEP proteins show high affinities and broad relationships in their interacting network. Therefore,
PeSEP proteins have important competence for complex maintenance, and the outcomes of the interactions to regulate varied Phalaenopsis orchid tepal morphogenesis may be deeply influenced by the diverse evolutionary dynamics of orchid floral MADS-box genes through the complicated co-evolutionary process.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Multiple alignment of regions in PeSEP genes used for virus-induced gene silencing (VIGS) experiment.

Fig. S2 Multiple alignments of SEP-like MADS-box proteins.

Fig. S3 Over-represented motifs discovered on the 2-kb promoters of PeSEP1–4 genes using the Multiple EM for motif Elicitation (MEME) program.

Fig. S4 Phenotypes of the PeSEP-silenced Phalaenopsis I-Hsin Sunrise Cinderella.

Fig. S5 Cell types of the PeSEP-silenced flowers in Phalaenopsis I-Hsin Sunrise Cinderella.

Table S1 Primers used in this study

Table S2 Consensus CArG-box sequences used for patterning search by the fuzznuc software

Table S3 Predicted CArG boxes on the promoters of PeSEP genes

Table S4 SEP-like MADS-box genes in Orchidaceae

Methods S1 Additional information for the Materials and Methods section.

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