Research on Orchid Biology and Biotechnology

Yu-Yun Hsiao1,2, Zhao-Jun Pan1, Chia-Chi Hsu1, Ya-Ping Yang1, Yi-Chin Hsu1, Yu-Chen Chuang1, Hsing-Hui Shih1, Wen-Huei Chen2, Wen-Chieh Tsai2,3,* and Hong-Hwa Chen1,2,3,*

1Department of Life Sciences, National Cheng Kung University, Tainan 701, Taiwan
2Orchid Research Center, National Cheng Kung University, Tainan 701, Taiwan
3Institute of Tropical Plant Sciences, National Cheng Kung University, Tainan 701, Taiwan
*Corresponding authors: Hong-Hwa Chen, E-mail, hhchen@mail.ncku.edu.tw; Fax, +886-6-235-6211; Wen-Chieh Tsai, E-mail, tsaiwc@mail.ncku.edu.tw; Fax, +886-6-274-2583
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Orchidaceae constitute one of the largest families of angiosperms. They are one of the most ecological and evolutionary significant plants and have successfully colonized almost every habitat on earth. Because of the significance of plant biology, market needs and the current level of breeding technologies, basic research into orchid biology and the application of biotechnology in the orchid industry are continually endearing scientists to orchids in Taiwan. In this introductory review, we give an overview of the research activities in orchid biology and biotechnology, including the status of genomics, transformation technology, flowering regulation, molecular regulatory mechanisms of floral development, scent production and color presentation. This information will provide a broad scope for study of orchid biology and serve as a starting point for uncovering the mysteries of orchid evolution.

Keywords: Oncidium • Orchidaceae • Orchid biology • Orchid biotechnology • Phalaenopsis.

Abbreviations: ANS, anthocyanidin synthase; BA, benzylaminopurine; BAC, bacterial artificial chromosome; BES, BAC end sequence; bHLH, basic helix–loop–helix; CymMV, Cymbidium mosaic virus; DMAPP, dimethylallyl diphosphate; EST, expressed sequence tag; GDP, geranyl diphosphate; GDPS, geranyl diphosphate synthase; GUS, β-glucuronidase; IPP, isopentenyl diphosphate; LD, long days; MEP, methylerythritol phosphate; PLB, protocorm-like body; PTGS, post-transcriptional gene silencing; PY5, phytene synthase; SD, short days; SSR, simple sequence repeat; VIGS, virus-induced gene silencing.

Introduction

With an estimated >25,000 species, orchids are the most species rich of all angiosperm families. They show a wide diversity of epiphytic and terrestrial growth forms and have successfully colonized almost every habitat on earth. The most recent common ancestor of extant orchids lived in the late Cretaceous (76–84 Mya) as dated by a fossil orchid and its pollinator (Ramirez et al. 2007). The radiation of the orchid family probably took place in a comparatively short period as compared with that of most flowering plant families, which suggests that their speciation rates are presumed to be exceptionally high (Gill 1989).

Associated with the enormous number of Orchidaceae species is extraordinary floral diversification. Orchids are renowned for an abundance of types, with a seemingly unending array of strange and often fantastic variations, and represent a highly advanced and terminal line of floral evolution in the monocotyledons. This spectacular diversification has been linked to the specific interaction between the orchid flower and pollinator (Cozzolino and Widmer 2005), sequential and rapid interplay between drift and natural selection (Tremblay et al. 2005), the role of obligate orchid–mycorrhizal interactions (Otero and Flanagan 2006), and Cuscutaceae acid metabolism and epiphytism (Silvera et al. 2009). In addition to the prosperity of their ecological manipulations, orchids have several unique reproductive strategies that contribute to their success. These include mature pollen grains packaged as pollinia, pollination-regulated ovary/ovule development, synchronized timing of micro- and mega-gametogenesis for effective fertilization and the release of thousands or millions of immature embryos (seeds without endosperm) in mature pods (Yu and Goh 2001).

Taiwan is one of the orchid cultivation and hybridization centers of the world, and the fine quality of Taiwan orchid hybrids has attracted consumers worldwide. Some of the most popular potted flowering plants in the floriculture market are Phalaenopsis hybrids. Their stylish, elegant appearance and extended longevity have boosted popularity among growers and consumers. Hybrids within the genus Oncidium are also some of the top-traded cut flowers and differ from other commercial genera by their predominant yellow coloration commonly accented with reds. Both the government and
entrepreneurs have high hopes for the orchid industry and intend it to become another commercial success story like the IT industry.

Because of the thriving and prosperous orchid breeding and industry, plant scientists in Taiwan are well placed to study orchid biology and develop orchid biotechnology to apply to the orchid industry. In this article, we have reviewed the scientific activities in orchid research including the status of genomics, transformation technology, flowering regulation and molecular regulatory mechanism of floral development, scent production and color presentation.

Genomics

Karyotypes of Phalaenopsis orchids

A better understanding of the karyotypes and DNA contents of orchid will aid in the development of new cultivars of orchids. All Phalaenopsis species have the same chromosome number (2n = 2x = 38), but their genomes vary considerably in size. Analysis of karyotypes of nine Phalaenopsis species and Doritis pulcherima by Feulgen- and 4',6-diamidino-2-phenylindole (DAPI)-stained somatic metaphase chromosomes (Hsu et al. 2006). A comparison of nucleotide substitutions between the plastid genome of P. aphrodite and the grasses indicates that only the plastid expression genes have a strong positive correlation between non-synonymous (Ka) and synonymous (Ks) substitutions per site, which provides evidence for a generation time effect, mainly across these genes (Chang et al. 2006). Furthermore, the transcripts of 74 protein-coding genes from the chloroplast genome of P. aphrodite subsp. formosana were used to study extensively the pattern of RNA editing in chloroplasts. A total of 44 editing sites are identified in the 24 transcripts of P. aphrodite chloroplast genes, and all are of the C-to-U conversion type (Zeng et al. 2007). In addition, the entire chloroplast genome of P. equestris has been sequenced and was found to be very similar to that of P. aphrodite subsp. formosana (C.C. Chang et al. unpublished data).

The chloroplast genome

The entire chloroplast genome of P. aphrodite subsp. formosana has been sequenced, with the generation of 148,964 bp sequences, which encode 110 different known genes, including 74 protein-coding genes, four tRNA genes, 30 tRNA genes and two conserved reading frames of unknown function (Chang et al. 2006). A comparison of nucleotide substitutions between P. aphrodite and the grasses indicates that only the plastid expression genes have a strong positive correlation between non-synonymous (Ka) and synonymous (Ks) substitutions per site, which provides evidence for a generation time effect, mainly across these genes (Chang et al. 2006). Furthermore, the transcripts of 74 protein-coding genes from the chloroplast genome of P. aphrodite subsp. formosana were used to study extensively the pattern of RNA editing in chloroplasts. A total of 44 editing sites are identified in the 24 transcripts of P. aphrodite chloroplast genes, and all are of the C-to-U conversion type (Zeng et al. 2007). In addition, the entire chloroplast genome of P. equestris has been sequenced and was found to be very similar to that of P. aphrodite subsp. formosana (C.C. Chang et al. unpublished data).

On the basis of the above information, the chloroplast genome of O. Gower Ramsey was sequenced by PCR and
Sanger-based ABI sequencing, and revealed 146,684 bp sequences, which encode 133 genes. A total of 7,042 bp sequences amplified from eight regions of the genome were used to identify the relationships at the species level between the 15 Oncidinae hybrids, which were supported by high bootstrap values (Wu et al. 2010).

**Expressed sequence tags (ESTs)**

A subtractive EST library was constructed from the pseudobulb of O. Gower Ramsey, which plays a key role in water, carbohydrate and other nutrition support during floral development, and 1,080 subtractive ESTs were obtained. Most ESTs were annotated as being involved in carbohydrate metabolism, in mannose, pectin and starch biosynthesis, transportation, and stress-related and regulatory function (Tan et al. 2005).

To study gene expression in Phalaenopsis reproductive organs, a cDNA library was constructed from mature flower buds of P. equestris; 5,593 ESTs were sequenced and assembled into 3,688 unigenes (including 732 contigs and 2,956 singletons) (Tsai et al. 2006). The Phalaenopsis flower bud cDNA library contains a significant proportion of ESTs encoding enzymes of primary and secondary metabolism, then subcellular organization-, transcription- and signal transduction-related genes. In addition, a cDNA library has been constructed from scented P. bellina flower buds with the column removed; 2,359 ESTs were sequenced and assembled into 1,187 unigenes (including 499 contigs and 688 singletons) (Hsiao et al. 2006). The set of floral scent-producing enzymes in the biosynthetic pathway from glyceraldehyde-3-phosphate to geraniol and linalool is recognized through these ESTs and distinguished by comparing their expression patterns in P. bellina and a scentless species, P. equestris (Hsiao et al. 2006). A similar strategy was adopted for Vanda Mimi Palmer principally to mine any potential fragrance-related EST-SSRs as markers in the identification of fragrant vandaceous orchids endemic to Malaysia (Teh et al. 2010).

Phalaenopsis ESTs derived from cDNA-amplified fragment length polymorphism (cDNA-AFLP) or randomly amplified polymorphic cDNAs (cDNA-RAPD) were also reported. These methods were used to systematically screen a large number of differentially expressed cDNA fragments in the wild type as well as somaclonal variants (Chen et al. 2005, Hsu et al. 2008). Several differentially expressed transcripts related to flower development and flower color were identified. Despite the limited number of ESTs generated by these two methods, they are still valuable for further studying their roles in orchid flower development and flower color regulation.

Recently, the OrchidBase has collected the transcriptome sequences from Phalaenopsis CDNA libraries and assembled them into 84,617 non-redundant transcribed sequences (including 8,501 contigs and 76,116 singletons) (Fu et al. 2011). The OrchidBase contains the transcript sequences derived from 11 Phalaenopsis orchid cDNA libraries, which were constructed from different species, including P. aphrodite subsp. formosana, P. equestris and P. bellina, and from different tissues, including developing seed, protocorm, vegetative tissue, leaf, cold-treated plantlet, pathogen-treated plantlet, inflorescence and flower buds (Fu et al. 2011, Hsiao et al. 2011). The EST sequences collected in OrchidBase were obtained through both deep sequencing with ABI 3730 and NGS Roche 454 and Illumina/Solexa. The OrchidBase is freely available at http://lab.tn.edu.tw/est and provides researchers with a high-quality genetic resource for data mining and efficient experimental studies of orchid biology and biotechnology.

Another orchid transcriptomic database, Orchidstra (http://orchidstra.abrc.sinica.edu.tw), was constructed from the 233,924 unique contigs of the transcriptome sequences of P. aphrodite subsp. formosana by use of a Roche 454 and Illumina/Solexa platform, and the genes of tissue-specific expression were categorized by profiling analysis with RNA-Seq (Su et al. 2011).

**Virus-induced gene silencing (VIGS) for functional validation of genes in orchids**

Because of the long life cycle and inefficient transformation system of orchids, the study of functional genomics of orchid genes is not feasible. Alternatively, VIGS analysis involving a symptomless Cymbidium mosaic virus (CymMV) for gene silencing was developed for Phalaenopsis orchids (Lu et al. 2007). In Phalaenopsis plants inoculated with CymMV transcripts containing 500 nt of PeMADS6, an orchid floral organ identity B-class GLOBOSA/PISTILLATA-like gene, the transcription level of PeMADS6 and the B- and C-class MADS-box genes was reduced by up to 97.8% and the flower morphology was affected. This in vivo experiment demonstrates an efficient way to study gene functions involved in the reproductive stage of orchid plants with a long life cycle.

Further application of this tool for Phalaenopsis functional genomics has been made possible by knocking down PeUFGT3 via VIGS in a Doritaenopsis hybrid derived from a cross of Dtps. ‘I-Hsin Lucky Girl’ × Dtps. ‘I-Hsin Song’.
The PeUFGT3-suppressed Doritaenopsis hybrids exhibited various levels of fading of flower color, which was well correlated with the extent of reduced PeUFGT3 transcriptional activity (Chen et al. 2011).

Transformation technology

Orchids are used for cut flowers and potted plants, and constitute an important ornamental industry in Taiwan because of their excellent properties, including long-lasting, fascinating flowers. Thus, the improvement of traits, such as flower shape, color, fragrance, longevity, architecture, and disease and stress resistance, and creation of novel variations are important to increase the commercial value of orchids. Traditional breeding can alter traits and produce new variants but is limited in the use of germplasm of the same or closely related species. Because of processing the long juvenile periods and reproductive cycles, farmers in orchid nurseries usually expend high cost, much effort and long-term culture to modify traits or produce new hybrids.

Orchid transformation

In past two decades, many researchers have devoted efforts to the study of gene transformation of orchids to improve orchid traits or create new orchid variants. Several factors are considered essential for the gene transformation method of orchids. How to choose what kinds of explants and regeneration capacity is important to increase the efficiency of transgenic orchid plants. Using unsuitable explants such as callus cultures as target materials for transformation may cause chimeric transgenic plants and often confuse the analysis because of difficulty in maintaining single-cell embryogenesis or a high incidence of somaclonal variation (Ishii et al. 1998, Chen et al. 1999). In general, protocorm-like bodies (PLBs) have been generally used as target tissues because of their higher regeneration capacity for gene transformation of orchids, especially in Oncidium, Dendrobium, Cymbidium and Phalaenopsis. In addition, protocorms could also be used as target tissues (Mishiba et al. 2005). So far, PLBs have been used as target tissues for orchid transformation in Taiwan with Oncidium orchid ‘Sherry Baby cultivar OM8’ (Liau et al. 2003, You et al. 2003, Li et al. 2005) and Phalaenopsis orchid ‘TS340’ (P. Taisuco Kochdiam × P. Taisuco Kaaladian) (Liao et al. 2004, Chan et al. 2005) as transgenic explants.

Selectable marker genes can be used to monitor transgenic events and manually separate transformants from non-transformants on medium containing the selective agent. Most orchid selection markers are antibiotic resistance genes encoding resistance proteins, such as neomycin phosphotransferase II, hygromycin phosphotransferase II and phosphinothricin acetyl transferase. In addition, to obtain more successful transformants, two reporter genes are widely used as selection markers: green fluorescent protein and β-glucuronidase (GUS) fusion genes. Expression of these marker genes detected at the same time demonstrates that the target gene is turned on after introducing new DNA into a cell. Three major methods for gene transformation include Agrobacterium tumefaciens-mediated transformation, microprojectile bombardment and direct gene transformation. Agrobacterium tumefaciens-mediated transformation is commonly used in dicots, whereas microprojectile bombardment and direct gene transformation are mainly used in monocots. Recently, several A. tumefaciens-mediated transformation events for monocotyledonous ornamental plants, including Phalaenopsis, Oncidium, Dendrobium, Anthurium and iris, have been reported (Mishiba et al. 2005, Sanjaya and Chan 2007).

Li and co-workers (2005) developed a highly efficient protocol for efficiently producing transgenic plants of O. ‘Sherry Baby cultivar OM8’. PLBs pre-treated with 0.5 M sucrose for 2 h were transformed by particle bombardment with the pfI gene: treated PLBs showed 3- to 4-fold increased single-cell embryogenesis and 14.8-fold increased GUS expression compared with untreated PLBs. Hence, sucrose-pre-treated Oncidium PLBs can increase single-cell embryogenesis and efficiency of transformation (Li et al. 2005).

Disease resistance

Maintaining or increasing the yield of orchid variants through classical breeding is difficult because productivity is limited by infection of the viral and bacterial pathogens that cause orchid diseases, particularly by Odontoglossum ringspot virus (ORSV), CymMV and Erwinia carotovora (Chia et al. 1992). To solve this problem, efforts have been made to establish a genetic transformation system in orchids for resistance to orchid diseases. In 2003, You and co-workers successfully developed a novel selection marker, the sweet pepper (Capsicum annum L.) ferredoxin-like protein (pfI) gene, for O. ‘Sherry Baby cultivar OM8’ transformation by A. tumefaciens and particle bombardment. The pfI gene is a disease resistance gene, which encodes a ferredoxin-like protein to decrease infection by E. carotovora pathogen for soft rot disease. The authors further used E. carotovora as a selection agent to screen transformants, thereby obtaining transgenic plants without antibiotic selection. The selection efficiency of PLBs transformed by E. carotovora was greater than by hygromycin. This selection marker can contribute to the transformation selection system. In addition, transgenic orchid plants with a pfI gene can enhance resistance to E. carotovora.

Liao and co-workers (2004) established a gene transformation system for Phalaenopsis orchids for resistance to viral disease. The authors used the concept of post-transcriptional gene silencing (PTGS) at the small interfering RNA (siRNA)-mediated RNA level. The constructs with a CymMV coat protein (CP) cDNA fragment and a nos terminator placed downstream of a maize ubiquitin promoter were transformed into Phalaenopsis orchid ‘TS340’ by particle bombardment. Transgenic orchid plants showed enhanced protection against CymMV infection because of RNA-mediated resistance through a PTGS mechanism (Liao et al. 2004). Furthermore,
the authors attempted to confer both viral and bacterial disease resistance on Phalaenopsis orchids by double transformation. CymMV CP and Pflp cDNA were transformed into PLBs of Phalaenopsis ‘TS340’ by A. tumefacien s transfection. Transgenic orchid plants showed enhanced resistance to CymMV and E. carotovora infection. This is the first report describing a transgenic Phalaenopsis orchid with dual resistance to phytopathogens (Liao et al. 2004).

**Flowering regulation**

**Flowering mechanism in Phalaenopsis and Oncidium**

The control of flowering time has been widely studied by genetic analyses in several plant species, especially Arabidopsis, and a genetic network including four main pathways—photoperiod, vernalization, autonomous and gibberellins (Blazquez et al. 2001)—has been developed. The photoperiod pathway senses seasonal changes in daylength, whereas the vernalization pathway monitors the prolonged exposure to low temperature. The gibberellin pathway promotes flowering under non-inductive photoperiods, and the autonomous pathway perceives the plant development status to mediate flowering (Shen et al. 2011).

Usually, plants flower only after they reach a certain stage of growth (i.e. maturity). For many orchids, 4–7 years are required to finish the juvenile stage and flower from seed (Goh and Arditti 1985). However, certain commercial hybrids can flower within 36 months from seeding (Hew and Yong 1997). Based on the study of orchid flowering regulation, various predominating pathways prevail for different species in Phalaenopsis orchids. For example, autonomous, temperature (cool or ambient temperature) or hormone (cytokinin) pathways may play an important role for P. aphrodite subsp. formosana.

Reproductive development in the flowering shoot of Phalaenopsis orchids begins with the transition of the dormant meristem from producing vegetative structures to producing inflorescence branches, floral bracts and finally flowers. Phalaenopsis orchids develop at least two undifferentiated bud primordia at each node, and then these buds will partially develop and become dormant to wait for proper conditions for flowering (Rotor 1959, Wang et al. 2002). When the weather becomes appropriate, the upper bud elongates and emerges through the epidermis of the stem and develops into an inflorescence (Wang 1995). Usually, the inflorescence emerges from the fourth node below the apical leaf (Sakanishi et al. 1980). Flower bud initiation occurs after the spike has reached about 5 cm long if the environment is favorable. Several previous studies of regulation of flowering in orchids are mainly of the effects of low temperature or daylength. Temperature has been reported to control flowering in several orchid genera such as Dendrobium (Rotor 1952), Miltoniopsis (Lopez and Runkle 2006), Phalaenopsis (Sakanishi et al. 1980, Blanchard and Runkle 2006) and Zygopetalum (Lopez et al. 2003). The promotion of flowering in these orchid genera by exposure to low temperature suggests that flowering in other orchid species could also be regulated by temperature.

The ambient temperature treatment of warm day and cool night (28°C day/20°C night) significantly enhances the transition to an inflorescence branch (spike) for P. aphrodites within 3 weeks (Chen et al. 2008). Uniform spiking can be reached in Phalaenopsis orchids through temperature control with a 25°C day/20°C night for 4–5 weeks. However, a cool temperature is required for continuing development of the inflorescence branch. The branch will become a vegetative shoot if it encounters high temperature.

At low temperature, blocking the sunlight with shields or keeping the plantlet under shelves to prevent exposure to sunlight could not promote inflorescence branches in Phalaenopsis (Liu et al. 2010). These results suggest that accumulation of sugar production through photosynthesis is important for orchid flowering. Flowering is also promoted when the plants are exposed to short photoperiods, although at low temperatures. The effects of daylength on protein synthesis and flowering in D. pulcherrima have been studied: 2–3 cm spikes were initiated under 9 h short-day (SD) conditions for 30 d with day/night temperature of 30°C/20°C, and spikes grew to 7–10 cm under SD conditions for 45 d (Wang et al. 2002). A cell division-related protein, p21, is more expressed in the SD condition than under the long-day (LD) condition (17 h) (Wang et al. 2003). Even though most Phalaenopsis species are SD plants, there are several species of Phalaenopsis, such as P. bellina and P. violacea, which flower in summer, suggesting they are LD plants.

In addition to ambient temperature, plant growth regulators (PGRs) also play important roles in inflorescence induction. A wide range of PGRs, including gibberellins, auxins, cytokinins and ABA, affect flowering in orchids. However, different experimental conditions and types of orchids may have various effects. For Phalaenopsis, cytokinins [e.g. benzylaminopurine (BA)] stimulate flowering, and auxin suppresses the BA effect; gibberellin is not effective when applied alone but when added in combination with BA seems to accelerate the BA effect slightly (Goh and Yang 1978, Hew and Clifford 1993). Cytokinins (e.g. BA) stimulate flowering in monopodial (e.g. Phalaenopsis) and sympodial orchids (e.g. Dendrobium) (Goh and Arditti 1985). For Dendrobium (Lee and Koay 1986), Doritaenopsis and Phalaenopsis (Blanchard and Runkle 2008), GA3 did not stimulate the BA effect. However, Doritaenopsis and Phalaenopsis sprayed with BA and kept at 29°C did not initiate an inflorescence. The promotion of flowering by BA application seems to suggest that cytokinins play a part in regulating the inflorescence initiation of Doritaenopsis and Phalaenopsis, but its promotion depends on certain conditions, and BA application is not an effective replacement for an inductive low temperature (Blanchard and Runkle 2008).

Although gibberellin is not effective at inducing flowering, it increases flower spike length and flower size. When a plant with
an inflorescence <10 cm is subsequently grown at ≥28°C for extended periods, a spike can form a vegetative air plantlet instead of flower buds, buds may abort, or the stem may elongate indefinitely without open flowers (Sakanishi et al. 1980, Wang 1995). The blockage of flower development under high temperature can be rescued by applying gibberellin exogenously (Su et al. 2001). Dormant buds of *Phalaenopsis* contain a relatively high level of free ABA, whereas detectable free or bound ABA was found in flowering shoots (Wang et al. 2002). A decrease in free ABA in buds may be associated with bud activation and the development of flowering shoots.

Another important orchid in the Taiwan floral industry is *Oncidium*, a thin-leaf, epiphytic sympodial orchid with an enlarged bulb-like structure called a pseudobulb. The pseudobulb serves for water, mineral and carbohydrate storage that supports both vegetative growth and reproduction (Herold and Lewis 1977, Zimmerman 1990, Stern and Morris 1992). The phase switch from vegetative to reproductive stage is defined by the bolting period from the pseudobulb base. The transition to bolting (flowering) requires a radical change in the apical meristem of the auxiliary shoot apex of *Oncidium*. Once the shoot meristem becomes committed to the new development program for bolting, it is considered the reproductive stage (Shen et al. 2009). Usually, *Oncidium* orchids flower twice per year from March to May, and from September to November. Cool nights are not required for *Oncidium* to produce flowers. Unlike *Phalaenopsis*, the regulation of *Oncidium* flowering is more via the autonomous pathway, and it is tightly linked to the nutrition status of the pseudobulb.

A large amount of mucilage composed mainly of mannan was found in the pseudobulb during the early inflorescence stage of *O. Gower Ramsey* (Wang et al. 2006). The ratio of mannose to total sugar is 96.4% and outcompetes that of other sugars such as arabinose, galactose and glucose by 1.5, 0.9 and 1.2%, respectively. Mannan will decrease gradually and convert to starch with the emergence of the inflorescence. The starch synthesized at the developing inflorescence stage will eventually be degraded at the floral development stage (Wang et al. 2008).

**Molecular mechanisms of flowering initiation in orchids**

Unlike Arabidopsis, in which research on flowering time has been done by examining the phenotypes of mutant plants, the only available strategy to study flowering time in orchid is to search homolog genes identified in other species and to overexpress or silence these genes to examine the phenotype changes. So far, several genes involved in flowering time regulation have been identified in *Oncidium*. OMADS1, an AGL6-like gene isolated from *Oncidium*, is able to up-regulate the expression of flowering time genes *FT* and *SOC1*, and the flower meristem identity genes *LFY* and *AP1* in transgenic Arabidopsis (Hsu et al. 2003). The late-flowering defect in *gi-1* or *co-3* can be compensated by ectopically expressing *OMADS1*, indicating that *FT* may be the target for *OMADS1*. In addition, four *AP1/AGL9* functional MADS-box genes, *OMADS6*, *OMADS7*, *OMADS10* and *OMADS11*, have also been characterized in *O. Gower Ramsey*. *OMADS6* is an *SEP3* ortholog that is expressed in sepal, petal, lip and carpel, but barely expressed in stamen. *OMADS11* is a *SEP1/2* ortholog with an expression pattern similar to that of *OMADS6*. *OMADS7* is an *AGL6*-like gene whose expression pattern is nearly identical to that of *OMADS6*. *OMADS10* is a putative *AP1* ortholog that is expressed only in vegetative leaf, lip and carpel of mature flowers. The similar expression patterns of *OMADS6, 11* and 7 indicate that their transcriptional regulation is highly conserved in orchids during evolution. Ectopic expression of these genes in Arabidopsis shows different phenotypes. Overexpression of *OMADS6, 11* or 7 causes extremely early flowering, whereas 35S::OMADS10 only causes moderate early flowering. Flower organ conversions have also been observed in transgenic Arabidopsis, including carpelloid sepal and staminoid petals found in 35S::OMADS6 and carpelloid sepal produced in 35S::OMADS7. However, flower organ conversions are not observed in 35S::OMADS11 or 35S::OMADS10 transgenic flowers, indicating that there might be a functional diversification in these genes in the regulation of flower transition and formation (Chang et al. 2009).

The orthologs of *FT* and *TFL1*, *OnFT* and *OnTFL1*, have also been isolated and characterized from *O. Gower Ramsey* (Hou and Yang 2009). The mRNA of *OnFT* is detected in axillary buds, leaves, pseudobulbs and flowers. Their expression remains at a lower level during the vegetative stage and increases during the reproductive stage. In flowers, *OnFT* is expressed more in young flower buds than in mature flowers and is expressed mainly in sepal and petals. Although they are highly expressed in axillary buds during vegetative stages, they are insufficient for the flower transition, indicating that the production of the pseudobulb before flower transition is also necessary. The expression of *OnFT* is regulated by photoperiod, with the lowest expression at daybreak and the highest from the eighth to the 12th hour of the light period. This pattern is slightly different from *FT* in Arabidopsis, with the highest level at the 16th hour of the light period and the lowest at dawn under LD (Corbesier et al. 2007, Fujiwara et al. 2008), or *Hd3a* of rice and *PnFT* of *Pharbitis*, which have the highest expression at dawn under SD (Ishikawa et al. 2005, Hayama et al. 2007, Tamaki et al. 2007). The difference may be due to *Oncidium* being a light-neutral plant (Hew and Yong 1997). *OnTFL1*, on the other hand, is expressed only in axillary buds and pseudobulbs and is not regulated by light. *OnTFL1* can suppress the flower transition during the vegetative stage with its high expression in axillary bud. Since the development of the pseudobulb is essential for *Oncidium*, *OnTFL1* also plays an important role in controlling the length of the vegetative stage because of its high expression in pseudobulbs. Flowering patterns are affected when overexpressing *OnFT* and *OnTFL1* in Arabidopsis. When *OnFT* is overexpressed, the expression of *AP1* is up-regulated and a strong correlation between the level of *AP1* and the expression of
OnFT is found, indicating that OnFT regulates flower transition similarly to Arabidopsis FT. Overexpression of OnFT in Arabidopsis ft-1 mutants promotes flowering, but 35S:OnFT/ft-1 transgenic plants still flower later than wild-type plants, suggesting that OnFT cannot completely complement Arabidopsis FT in regulating flowering time. On the other hand, delayed flowering and the production of more rosette leaves are observed in transgenic Arabidopsis overexpressing OnTFL1, and altered leaf and shoot morphology are also detected. AP1 is observed to be down-regulated in both 35S:OnTFL1 and 35S:OnTFL1/tfl1-11, indicating that OnTFL1 can prohibit flower transition and the development of floral meristem by negatively regulating AP1, similarly to Arabidopsis TFL1 (Hou and Yang 2009).

So far, flowering genes have been identified and characterized in Oncidium, but studies of flowering time genes in Phalaenopsis are deficient. Although genes such as CO and FLC cannot be detected, several genes including AP1, FT, LFY and SOC1 have been found in OrchidBase or in Orchidstra. These include vernalization insensitive 3 (PaVRN3-1 and PaVRN3-2) and vernalization 2 (PaVRN2) in the vernalization pathway. PaGI and PaFT were also identified. In addition, five autonomous pathway genes, PaF, PaFVE, PaSVP, PaSOC1 and PaFCA, have also been identified from Phalaenopsis EST libraries and were characterized under cool-night treatment (F.M. Tsao et al. unpublished data). With the information provided by the orchid database, more flowering time genes can be isolated for functional analysis, and their role in the control of flowering in orchid can be clarified.

**Molecular mechanisms of flower development**

According to the classical view, the orchid flower is composed of five whorls of three segments, each including two perianth whors, two staminal whors and one carpel whorl. This structure also conforms to the general flower structure of many other monocotyledons families. Within the monocots, only well-known crop species such as rice and maize have been studied thoroughly. However, their highly reduced flowers make them unsuitable for general floral development studies. All expected whors in the flowers are present in orchids, and their highly sophisticated flower organization offers an opportunity to discover new variant genes and different levels of complexity within morphogenetic networks. Thus, the Orchidaceae provide a rich subject for investigating evolutionary-relationships and developmental biology to verify the validity of the ‘ABCDE model’ in monocots and how MADS-box genes are involved in defining the different highly specialized structures in orchid flowers. Many orchid MADS-box genes related to floral development have been isolated (Table 1), and we focus on the discussion of the roles of MADS-box genes in *P. equestris*, O. Gower Ramsey and *C. ensifolium*, because these three species are major research targets in Taiwan (Fig. 1).

**A-class genes**

At present, no nomenclature system exists for the MADS-box genes. Many of them were named according to their order of identification. A-class MADS-box genes are placed in the AP1/AGL9 clade of MADS-box genes by phylogenetic analysis (Theissen 2001). The A-class SQUAMOSA (SQU) MADS-box gene lineage within the eudicots is recognized as two subclades (euAP1 and euFUL clades), whereas the non-core eudicots and monocots have sequences similar only to euFUL genes and are classified in the paleoAP1 subclade (Litt and Irish 2003). Two functions are attributed to AP1: specification of (i) floral meristem and (ii) perianth identity in Arabidopsis (Irish and Sussex 1990, Bowman et al. 1993).

So far, two A-class MADS-box genes, ORAP11 and ORAP13, have been identified from P. hybrida cv. Formosa rose (Chen et al. 2007), one (OMADS10) from O. Gower Ramsey (Chang et al. 2009) and four (DOMADS2, DthyrFL1, DthyrFL2 and DthyrFL3) from *Dendrobium* (Yu and Goh 2000, Skipper et al. 2005). Based on results of the expression patterns and over-expression in transgenic plants, orchid A-class MADS-box genes may be involved in the transition of flowering and floral organ development. However, unlike its homolog AP1 in Arabidopsis, orchid A-class MADS-box genes may not be associated with the development of the first two whors.

In Arabidopsis, another A-class gene without a MADS-box region is APETALA2 (AP2). AP2 encodes a transcription factor with two continuous AP2 domains. AP2 enables A-class function in Arabidopsis and represses AGAMOUS (AG) in the first and second floral whors (Jofuku et al. 1994). Only one AP2-like gene, named DcOAP2, from *Dendrobium* has been reported (Xu et al. 2006). Transcripts of DcOAP2 were detected in all the floral organs, as was AP2 in Arabidopsis. However, the concurrence of the expression of DcOAP2 and DcOAG1, a putative AG-like gene in all floral organs, implies that the mechanism underlying the regulation of AG orthologs may be different from that in Arabidopsis (Xu et al. 2006).

**B-class genes**

Both the developmental and biochemical aspects of B-class genes required to specify the identity of petals in whorl 2 and stamens in whorl 3 appear to be conserved in many core eudicots and monocots (Ambrose et al. 2000, Theissen et al. 2000, Whipple et al. 2004). So far, several members of APETALA3 (AP3)-like and PISTILLATA (PI)-like genes have been isolated from *P. equestris* and O. Gower Ramsey. These genes include four AP3-like genes and one PI-like gene identified in *P. equestris* and three AP3-like genes and one PI-like gene isolated from O. Gower Ramsey (Hsu and Yang 2002, Tsai et al. 2004, Tsai et al. 2005, Chang et al. 2010). In addition, two AP3- and one PI-like gene were also identified in *D. crumenatum* (Xu et al. 2006). All these AP3-like genes in orchids are members of the paleoAP3 lineage. The paleoAP3 genes identified in orchids were subdivided into four subclades (Tsai et al. 2004, Mondragón-Palomino and Theissen 2008, Pan et al. 2011). PeMADS2 and
<table>
<thead>
<tr>
<th>Clade</th>
<th>Subclade</th>
<th>B-class</th>
<th>AP3A1</th>
<th>AP3A2</th>
<th>AP3B1</th>
<th>AP3B2</th>
<th>PI</th>
<th>C-class</th>
<th>AP1/AGL9</th>
<th>AP1</th>
<th>LOFSEP</th>
<th>SEP3</th>
<th>AGL6</th>
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<tbody>
<tr>
<td>Phalaenopsis</td>
<td>equestris</td>
<td>PeMADS3</td>
<td>PeMADS4</td>
<td>PeMADS2</td>
<td>PeMADS5</td>
<td>PeMADS6</td>
<td></td>
<td>PeMADS1</td>
<td>PeMADS7</td>
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<tr>
<td>Phalaenopsis</td>
<td>hybrid</td>
<td>PhalAG1</td>
<td>PhalAG2</td>
<td>ORAP11</td>
<td>ORAP13</td>
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<tr>
<td>Oncidium</td>
<td>Gower Ramsey</td>
<td>OMADS9</td>
<td>OMADS5</td>
<td>OMADS3</td>
<td>OMADS8</td>
<td>OMADS4</td>
<td></td>
<td>OMADS2</td>
<td>OMADS10</td>
<td>OMADS11</td>
<td>OMADS6</td>
<td>OMADS1</td>
<td>OMADS7</td>
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<tr>
<td>Dendrobium crumenatum</td>
<td></td>
<td>DcOAP3B</td>
<td>DcOAP3A</td>
<td>DcOPI</td>
<td>DcOAG1</td>
<td>DcOAG2</td>
<td></td>
<td>DcOSEP1</td>
<td></td>
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<td>DOMADS3</td>
<td>DOMADS1</td>
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<tr>
<td>Dendrobium thyrisifbrum</td>
<td></td>
<td>DthyrPI</td>
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<td></td>
<td>DthyrAG1</td>
<td>DthyrAG2</td>
<td>DthyrFL1</td>
<td>DthyrFL2</td>
<td>DthyrFL3</td>
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<tr>
<td>Cymbidium ensifolium</td>
<td></td>
<td>CeMADS1</td>
<td>CeMADS2</td>
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<tr>
<td>Habenaria radiata</td>
<td></td>
<td>HrDEF</td>
<td></td>
<td></td>
<td>HrGLO1</td>
<td>HrGLO2</td>
<td></td>
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<tr>
<td>Spiranthes odorata</td>
<td></td>
<td>SpodoDEF1</td>
<td>SpodoDEF3</td>
<td>SpodoDEF4</td>
<td>SpodoDEF2</td>
<td>SpodoGLO1</td>
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<td></td>
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<tr>
<td>Gongora goreata</td>
<td></td>
<td>GogalDEF3</td>
<td>GogalDEF1</td>
<td></td>
<td>GogalDEF2</td>
<td>GogalGLO1</td>
<td></td>
<td></td>
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<tr>
<td>Phragmipedium longifolium</td>
<td></td>
<td>PhlonDEF3</td>
<td>PhlonDEF4</td>
<td>PhlonDEF2</td>
<td>PhlonDEF1</td>
<td>PhlonGLO1</td>
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<tr>
<td>Vanilla planifolia</td>
<td></td>
<td>VaplaDEF3</td>
<td>VaplaDEF2</td>
<td>VaplaDEF1</td>
<td></td>
<td>VaplaGLO1</td>
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<td>Orchis italica</td>
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</table>
OMADS5 belong to clade AP3B1 (the same as clade 1 proposed by Mondragón-Palomino and Theissen 2008). OMADS3 and PeMADS5 are included in clade AP3B2 (the same as clade 2). PeMADS3 and OMADS9 are classified in clade AP3A1 (the same as clade 3) and PeMADS4 is in clade AP3A2 (the same as clade 4).

Expression and protein–protein interaction studies suggest that the specific combination of duplicate gene expression and protein–protein interactions is responsible for the development of different floral organs (Tsai et al. 2008a, Tsai et al. 2008b, Chang et al. 2010). However, the proposed models derived from studies of Phalaenopsis and Oncidium to explain lip development are diverse. From study of Phalaenopsis, the PeMADS4 gene (clade AP3A2 or 4) is suggested to play a critical role in determining lip development because its transcripts are specifically detected in lip and in peloric mutant lip-like petals (Tsai et al. 2004, Tsai et al. 2008a). However, OMADS5 (clade AP3B1 or 1) may have a suppression effect on cell proliferation. Lack of expression of OMADS5 is necessary for the development of the lip or lip-like structures (Chang et al. 2010). Very recently, B-class genes were collected from 11 species in four orchid subfamilies except Apostasioideae to give a picture of the floral organ development controlled by B-class genes (Pan et al. 2011). After analyzing expression patterns of B-class genes by PCR-based methods and in situ hybridization, the ‘Homeotic Orchid Tepal (HOT) model’ was proposed to illustrate the regulation of perianth morphogenesis in orchids. In this model, PI and AP3B clades determine the formation of sepals. The combination of PI and both AP3A1 and AP3B clade genes and AGL6-like genes contribute to lip morphogenesis in the floral bud stage (Pan et al. 2011). The HOT model is somewhat similar to the ‘orchid code’ (Mondragón-Palomino and Theissen 2008) but more precisely points out the effect of PeMADS4 (AP3A2 clade) in determining lip morphogenesis at a relevant later floral development stage.

The overexpression of paleoAP3 genes from Oncidium, Phalaenopsis and Dendrobium under the control of the Cauliflower mosaic virus 35S promoter was examined in Arabidopsis (Hsu and Yang 2002, Tsai and Chen 2006, Xu et al. 2006). Consistently, the flower morphology of the transgenic Arabidopsis plants overexpressing the orchid paleoAP3 genes was indistinguishable from that of wild-type plants. The absence of any morphological changes in the floral organs of transgenic plants suggested that sequence diversification

![Floral diagrams of Phalaenopsis, Oncidium and Cymbidium. (A) Phalaenopsis. (B) Oncidium. (C) Cymbidium. (D) Diagram showing orchid flower organs (adapted from Tsai et al. 2005). Orchid flowers have three sepals and three petals. One of the petals is morphologically different in structure and is known as the labellum or lip. The male and female reproductive parts are fused in a structure, the gynostemium or column, in the center of the flower. The pollen grains stick together to form the pollinia located at the upper tip of the column under the anther cap.](image-url)
between orchid paleoAP3 and Arabidopsis AP3 genes causes some functional differences when placed into heterologous plants.

Only one PI-like gene was reported in *P. equestris, D. crumenatum* and O. Gower Ramsey: PeMADS6, DcOPI and OMADS8, respectively (Tsai et al. 2005, Xu et al. 2006, Chang et al. 2010). All of these three genes are expressed in all floral organs. However, OMADS8 is also expressed in vegetative roots and leaves, whereas both PeMADS6 and DcOPI are not expressed in vegetative tissues. In addition, PeMADS6 expressed in the undeveloped ovary suggests that the expression of PeMADS6 in the ovary has an inhibitory effect on the development of the ovary (Tsai et al. 2005). Ectopic overexpression of PeMADS6, DcOPI or OMADS8 in Arabidopsis demonstrated that both share the angiosperm PI function (Tsai et al. 2005, Xu et al. 2006, Chang et al. 2010).

In conclusion, the expression patterns of B-class genes in orchid floral organs nicely fit the ‘modified ABC model’ in that the expression of the B-class genes is extended to whorl 1 in plants possessing nearly identical morphology of sepals and petals (van Tunen et al. 1993). The paleoAP3 genes are highly duplicated in orchids. Diversification and fixation of both of these gene sequences and expression profiles might be explained by subfunctionalization and even neofunctionalization. The driving force behind the specialized labellum and diversified orchid flowers may be linked to the duplication and fast evolution rate of paleoAP3 genes. The final piece of the puzzle for the unique evolutionary trajectory of orchid B-class genes is still hidden in the primitive Apostasioideae. Further investigation of the B-class genes in Apostasioideae will undoubtedly shed light on the mystery of the great diversity of orchid flowers.

The models proposed to explain orchid floral development were reasoned from comparing expression patterns of B-class genes between wild-type and peloric flowers in orchids. Peloric flowers are actinomorphic mutants with lip-like petals (Tsai et al. 2004). Evidence that epigenetic mechanisms play a role in peloric flowers includes methylation pattern variation (unpublished data). Because altered B-class gene expression is linked to the peloric flower of orchids, it will be of interest to isolate *cycloidea*-like genes to study their roles on orchid flower zygomorphy and the regulation relationship between B-class genes.

**C- and D-class genes**

According to the genetics ABCDE model, C-class genes specify stamen and carpel development and function in meristem determination because C-class mutants are indeterminate in whorl 4 and form a new C-class mutant flower instead of carpels. The development of the column in orchids, which involves whorls 3 and 4, would be an interesting subject to elucidate the evolution of C-class genes. More recently, one C-class gene and one D-class gene were isolated from O. Gower Ramsey, and two C-class genes were isolated from *C. ensifolium* (Hsu et al. 2010, Wang et al. 2011). OMADS4, CeMADS1 and CeMADS2 were classified in the C-lineage of AG-like genes, whereas OMADS2 was in the D-lineage genes. Phylogenetic analysis showed that duplication of C-class genes may have occurred in Epidendroideae. Our current understanding of these two paralog functions in *Cymbidium* suggests that after the duplication event, the lineages underwent functional diversification to produce distinct functional repertoires. CeMADS1 may initiate the development of fused male and female reproductive organs and be involved in floral meristem determinancy. However, CeMADS2 may play a maintenance role to complete gynostemium morphogenesis and have a redundant function in floral meristem determinacy (Wang et al. 2011). Good examples of functional diversified genes are the rice C-class genes OsMADS3 and OsMADS58 (Yamaguchi et al. 2006). The specification of C function in rice is divided so that OsMADS3 is required for stamen identity, whereas OsMADS58 is mainly involved in floral meristem determinacy and carpel morphogenesis (Yamaguchi et al. 2006). Together these genes fulfill the functions that in Arabidopsis are accomplished by a single AG gene.

According to the expression patterns, OMADS4 is probably the CeMADS1 ortholog in *Oncidium*. However, OMADS4 appears not to be functionally equivalent to CeMADS1. Transgenic Arabidopsis with overexpressed OMADS4 shows an early flowering phenotype. This phenotype is not the same as ectopic expression of CeMADS1 in Arabidopsis presenting the primary inflorescence apices terminated with a cluster of flower buds. The protein behavior of OMADS4 is also of interest. OMADS4 and D-class protein OMADS2 can form homodimers and can form heterodimers with each other (Hsu et al. 2010). Interaction between C- and D-class proteins was still not observed in eudicots or in rice or maize. Investigating the interaction behavior between C- and D-class proteins in *Phalaenopsis* and *Dendrobium* is of interest, because C- and D-class genes have been isolated from these two species (Skipper et al. 2006, Song et al. 2006, Xu et al. 2006). The expression pattern of the D-class gene OMADS2 is similar to that of OMADS4. Flowering in Arabidopsis with overexpressed OMADS2 has a phenotype similar to that of overexpressed OMADS4, except that 35S::OMADS2 transgenic Arabidopsis flower earlier and produce curled leaves. Co-expression in the stigmatic cavity and ovary, the ability to interact with each other and the similar phenotypes when ectopically expressed in Arabidopsis suggest that OMADS2 and OMADS4 have a close relationship in regulating the formation of stigmatic cavity and ovary in *O. Gower Ramsey* (Hsu et al. 2010).

**E-class genes**

E-class MADS-box genes are placed into the SEPALLATA (*SEP*) clade. The clade is characterized by a duplication coinciding with the origin of the angiosperms that produced the SEP3 (*AGL9*) and LOFSEP or AGL2/3/4 (SEP1, SEP2, SEP4) subclades (Zahn et al. 2005). E-class genes are required for floral organ identity in all four floral organs and for floral determinacy (Ditta et al. 2004, Kaufmann et al. 2005). In addition, members in the
AGL6 subclade are placed in the AP1/AGL9 group between the SQUA-like and SEP-like subclade. Phylogenetic analysis of MADS-box genes and/or proteins has shown that AGL6-like genes are sister to the SEP-like genes (Zahn et al. 2005). Recently, an AGL6-like gene, OsMADS6, was found to have functions in regulating floral organ identity and meristem fate in rice (Ohmori et al. 2009, Li et al. 2010).

So far, two E-class MADS-box genes and two AGL6-like genes have been identified from O. Gower Ramsey (Hsu et al. 2003, Chang et al. 2009): OMADS11 in the LOFSEP subclade, OMADS6 in the SEP3 subclade, and OMADS1 and OMADS7 in the AGL6 subclade (Chang et al. 2009). OMADS1 is expressed in apical meristem and in the lip and carpel of flowers, but not in vegetative tissues. Ectopic-expressed OMADS1 in Arabidopsis showed an early flowering phenotype and homeotic conversion of flower organs, such as carpelloid sepals and staminoid petals (Hsu et al. 2003). Interestingly, 35S::OMADS1 transgenic Eustoma grandiflorum plants flowered significantly earlier and produced more flowers than did non-transgenic plants (Thiruvengadam and Yang 2009). The other three genes were discussed in the section ‘Flower regulation’ and are not mentioned here. Four E-class genes (OM1, DOMADS1, DOMADS3 and DcOSEP1) have also been identified from Dendrobium (Lu et al. 1993, Yu and Guo 2000, Xu et al. 2006): DOMADS3 in the LOFSEP subclade, and the other three genes in the SEP3 subclade. In addition, two E-class genes have also been identified from P. equestris (our unpublished data). In general, the fact that the expression pattern of E-class genes overlaps with that of ABCD genes in orchids suggests that the higher order MADS-box complexes are involved in all floral organ development in orchids as for Arabidopsis E-class genes. However, whether orchid E-class genes play a role on floral determinacy still needs to further investigated. Further investigation of these gene functions in orchids will provide useful information in understanding the evolutionary roles of E-class MADS-box genes in orchid flowering and/or flower development.

These findings are in line with current thoughts on how major evolutionary changes in the genetic basis of organ identity were established by gene duplication and the separation of functions. However, we cannot be sure that all the genes within each family have been isolated in orchids. Recently established orchid EST databases providing plentiful gene information, and integrated bioinformatic tools offer opportunities for finding A-, B-, C-, D- and E-function paralogous genes or additional genes related to flower development. The effort of many scientists will lead to a better understanding of the molecular and genetic mechanisms of orchid floral control.

Flower scent
Significance and distribution of the orchid floral scent
Floral scent volatiles and pigments have evolved to attract insect pollinators and enhance fertilization rates (Kaiser 1993, Pichersky and Gang 2000). In orchids, pollinators play an important role in orchid floral diversification, which is advantageous to the evolution of a successful family. Large quantities of pollen in masses are spread by bees, moths, flies and birds, and the floral scents serve as attractants for species-specific pollinators (van der Pijl and Dodson 1969). Floral signals from distinct modalities elicited both attraction and copulation behavior, as seen by continuous volatiles attractive to patrolling males and pollination by mimicking the pheromone and posture of female thynnine wasps in Chiloglottis orchids (Australian orchids) (Schiestl et al. 2003, Mant et al. 2005). An enormous variety of orchids pollinated by bees, wasps, flies and bumblebee species cover a whole range of scents, from rosy-floral and ionone-floral to aromatic-floral and spicy-floral (Arditti 1992).

Difficulties in orchid scent research
The global flower industry thrives on novelty. Domestication of wild species in conjunction with traditional breeding has long been the principle path for generation of novel flowers in the industry. For orchid, traits such as flower color, shape and fragrance are primary novel markers because they are key determinants of consumer choice. However, many modern floricultural varieties have lost their scent with traditional breeding programs. Breeders of orchids in cut-flower and ornamental markets have focused on producing plants with improved vase life, shipping characteristics and visual aesthetic values (i.e. color and shape).

Phalaenopsis Alliance is undoubtedly the most widely grown orchid in the world. Despite the sympatric speciation of orchids being linked to differences in floral odors, large genome size, long life cycle and regeneration time of these plants, together with inefficient transformation systems, the investigation of orchid scent biology is difficult. Furthermore, several scented and scentless species are cross-incompatible, which restricts the production of scented offspring by traditional breeding techniques. In some successful cases of cross-breeding, the progeny have a diluted scent or have lost the ability to produce scent. To date, the biosynthetic pathways of orchid flower fragrance have not been well understood. Little was known about the enzymes and genes controlling scent production in monocotyledons such as orchids.

Orchid floral scent biosynthesis pathway and related genes
Phalaenopsis bellina, classified in the subgenus Polychilos, is native to Malaysia, and numerous commercial varieties have been bred because of the orchid’s pleasant fragrance. In addition, the species has some native tetraploid species to breed scented commercial Phalaenopsis orchids and therefore is an important parent for breeding scented cultivars. Floral scent is a composite characteristic determined by a complex mixture of low molecular mass volatile molecules and dominated by monoterpenoid, sesquiterpenoid, phenylpropanoid and...
benzenoid compounds, and fatty acid derivatives. The floral scents in *P. bellina* are rich in monoterpenes, geraniol and linalool and their derivatives (Hsiao et al. 2006). They include geraniol, nerol, 2,6-dimethyl-octa-3,7-diene-2,6-diol, 2,6-dimethyl-octa-1,7-diene-3,6-diol, 3,7-dimethyl-2,6-octadienal, geranic acid and 2,6-dimethyl-octa-2,6-diene-1,8-diol (Table 2). In contrast, no monoterpenoid derivatives were emitted in scentless *P. equestris* flowers; fatty acid derivatives, phenylpropanoids and benzenoids were the major volatiles. These compounds are barely detectable by the human nose.

Research into plant scents has been hampered mainly by the invisibility of this character, its dynamic nature and complex mixtures of components that are present in very small quantities. Most progress in scent research, as in other areas of plant biology, has come from the use of molecular and biochemical techniques. A strategy combining chemical analysis, genomics and bioinformatics was adopted to uncover the scent biosynthesis pathway and the relevant genes in *P. bellina* flower (Hsiao et al. 2006; Fig. 2). According to volatile analysis, monoterpenoids are major compounds of scent (Table 2) and therefore are probably biosynthesized in these flowers.

From chemical and bioinformatics analyses, we deduced a monoterpenes biosynthesis pathway of 15 steps in the *P. bellina* flower leading from glyceraldehyde-3-phosphate to geraniol, linalool and their derivatives (Hsiao et al. 2006; Fig. 3). Comparisons of the most abundant ESTs by calculating the enrichment factor for different transcripts in the floral dbESTs of *P. bellina* and *P. equestris* facilitates the provisional identification of scent metabolism genes. The enrichment factor is obtained by dividing the proportion of a certain transcript in the scented species by that in the scentless species. Geranyl diphosphate synthase (GDPS), epimerase (EPI), lipoxxygenase and diacylglycerol kinase show 15-, 10-, 10- and 8.5-fold enrichment, respectively. Expression of the scent-related genes was confirmed by RNA blot hybridization (Hsiao et al. 2006).

### Critical enzyme for scent biosynthesis

Terpenoids belong to a large family of plant secondary metabolites, and their corresponding alcohols possess useful properties such as fragrance, flavor, insecticidal properties and characteristics that make them useful as pharmaceutical agents. All monoterpenes are derived from the same substrate, GDP (C10), which is catalyzed by GDPS, a member of the short-chain trans-prenyltransferase family, via the condensation of dimethylallyl diphosphate (DMAPP) with isopentenyl diphosphate.

#### Table 2 Major classes of volatiles emitted by *P. bellina* and *P. equestris*

<table>
<thead>
<tr>
<th>Class of volatiles</th>
<th>Amount (ng flower⁻¹ h⁻¹)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><em>P. bellina</em> (scent)</td>
</tr>
<tr>
<td>Monoterpenes</td>
<td>382.8 ± 16.1</td>
</tr>
<tr>
<td>Linalool</td>
<td>105.4 ± 15.3</td>
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<tr>
<td>Linalool derivaties</td>
<td>39.6 ± 20</td>
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<tr>
<td><em>trans</em>-Geraniol</td>
<td>163.4 ± 1.6</td>
</tr>
<tr>
<td>Geraniol derivaties</td>
<td>34.5 ± 5.4</td>
</tr>
<tr>
<td>Sequesterpene</td>
<td>ND</td>
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<tr>
<td>Phenylpropanoid</td>
<td>38.6 ± 17.1</td>
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<tr>
<td>Benzenoid</td>
<td>40.2 ± 20.8</td>
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<tr>
<td>Fatty acid derivatives</td>
<td>3.3 ± 1.8</td>
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</table>

![Diagram](image-url) Fig. 2 Strategy of identification of the *Phalaenopsis* scent metabolism pathway and scent candidate genes involved in the deoxysylulose-5-phosphate-geraniol-linalool pathway.
diphosphate (IPP). GDPs is differentially expressed in the scented species. All monoterpenes are formed from GDP, which is synthesized from DMAPP and IPP (Tholl et al. 2004).

The full-length cDNA of *P. bellina* GDPs (PbGDPS) was isolated from a *P. bellina* floral cDNA library (Hsiao et al. 2006) and sequenced. The PbGDPS lacks an aspartate-rich motif for scent production in the flower of *P. bellina*. Instead, a glutamate-rich
sequence (EAEVE) was identified in the SARM motif site to be able to catalyze the formation of both GDP and farnesyl diphosphate (FDP, C15) by site-directed mutagenesis (Hsiao et al. 2008). Spatial expression analyses revealed that PbGDPS is a flower-specific gene. Temporal expression analyses showed the highest expression of PbGDPS during flower development concomitant with the maximal floral emission of monoterpenes at day 5 post-anthesis, which suggests that PbGDPS plays a crucial role in scent production/emission in orchid flowers. Further evidence of the involvement of PbGDPS in floral scent emission came from the analysis of PbGDPS expression in various orchid species with different scent-producing abilities (Hsiao et al. 2008). Expression of GDPS was also analyzed in two varieties of P. equestris. The scented flowers of P. equestris ‘W-72’ emit volatiles that are not monoterpene, as do those of the scentless species P. equestris. GDPS was expressed at lower levels in the scented offspring than in the parental line, which is associated with the lower production of linalool in the former. In contrast, no GDPS expression was detected in P. equestris or the scentless offspring D. Kenneth Schubert (Hsiao et al. 2008). Thus, PbGDPS may play a key role in the regulation of scent production in P. bellina flowers.

Floral scent research has mainly concentrated on the isolation and characterization of enzymes and genes involved in the final steps (terpene synthase) of scent biosynthesis. However, scent production might not only be regulated by the level of these enzymes; the direct precursor (GDP) of the scent compounds could also contribute to the regulation (Guterman et al. 2006, Nogues et al. 2006). Meanwhile, several terpene synthases (TPSs) were identified from the P. bellina flower, which has a dual function to catalyze monoterpene and sesquiterpene products. In addition, the MYB transcription factors were also identified from Phalaenopsis TS-Oligo microarray, and they showed a similar expression pattern to PbGDPS, indicating the possibility of MYB transcription factors being involved in terpenoid biosynthesis regulation (unpublished data).

Metabolic engineering of flower scent

To date, successful transformations have been developed for several cut flowers, including commercial roses, chrysanthemums and carnations, but for most varieties work is still in progress. Overall, it is clear that genetic manipulation of floral scent is possible but will require a more rational design based on the correct choice of species. If we hope to create new scent varieties of Phalaenopsis, we need to understand scent components, the pathways involved, key enzyme regulation, flux-controlling steps, possible feedback control, judicious use of promoters and empirical testing in this candidate variety.

For genetic engineering, most of the scented F1 plants are sterile, even if a few of these interspecific hybrids are obtained. A high frequency of bivalents was observed in hybrids of both species with small chromosomes and between both species with large chromosomes, but a low frequency of bivalents in hybrids was discovered between a species with small chromosomes and one with large chromosomes. Doubling the chromosomes of diploid scent species to form tetraploid species is urgently needed to accelerate the improvement of scented commercial Phalaenopsis orchids.

Flower color

Flower pigments, including anthocyanins, carotenoids, betalains and Chl, contribute to varied flower color patterns. In addition to attracting pollinators, these pigments play roles in photosynthesis and the response to UV radiation in vegetative tissues (Davies 2004). Anthocyanins, carotenoids and Chl are major pigments found in Orchidaceae, and anthocyanins have the broadest distribution. In this section, we discuss floral color in Phalaenopsis and Oncidium.

Anthocyanin biosynthesis pathway

Enzyme and transcription factors involved in the anthocyanin biosynthesis pathway have been well characterized in different species, such as grapes (He et al. 2010) and Arabidopsis thaliana (Martens et al. 2010), and have been reviewed extensively (Grotewold 2006). The pathway starts with the condensation of coumaroyl-CoA and malonyl-CoA via a polyketide folding mechanism by chalcone synthase (CHS) to form the intermediate, chalcone, the primary precursor for all classes of flavonoids. After the action of chalcone isomerase (CHI), chalcone is modified to its isomer naringenin, which is further hydroxylated by a group of cytochrome P450-dependent monoxygenases to produce flavanones. Cytochrome P450-dependent monoxygenases include flavonoid 3-hydroxylase (F3H), flavonoid 3’-hydroxylase (F3’H) or flavonoid 3’5’-hydroxylase (F3’5’H) for dihydrokaempferol, eriodictyol or pentahydroxyflavanone, respectively. These three flavanones can be modified again by the catalysis of F3H, F3’H and F3’5’H to produce dihydrafлавanols, dihydrokaempferol, dihydroquercetin and dihydromyricetin. Dihydroflavanol 4-reductase (DFR) further reduces these dihydroflavanols to colorless leucoanthocyanidins, which are catalyzed by anthocyanidin synthase (ANS) to their corresponding anthocyanidins, pelargonidin, cyanidin and delphinidin.

A putative F3’5’H gene has been isolated from a Phalaenopsis flower petal cDNA library (Su and Hsu 2003). This gene contains a P450 conserved motif, Phe-X-X-Gly-Arg-X-Cys-X-Gly (where X is a non-conserved amino acid), that is homologous to a published P450 found in pollen tubes of other Phalaenopsis. Bombardment of the F3’5’H gene in Phalaenopsis petals altered the cell color from pink to magenta as compared with those transformed with an antisense fragment, so the putative F3’5’H influences anthocyanin synthesis. Four color-related genes in Oncidium, i.e. OgCHS, OgCHI, OgANS and OgDFR, have been identified (Chiou and Yeh 2008). These genes are expressed during floral development. Among them, OgCHI and OgDFR show especially low expression in...
yellow lip tissue but greater expression in the red part of *Oncidium* flowers. Transformation of OgCHI and OgDFR together by particle bombardment complements the absence of anthocyanin synthesis in lip tissue. Several together by particle bombardment complements the absence of anthocyanin synthesis in lip tissue. Several (unpublished data), and the function of these genes need to be further characterized.

The anthocyanins undergo further modification for hydrophilicity, such as glycosylation, methylation and acylation, and become stabilized as vacuolar anthocyanins (Grotewold 2006). UDP-glucose: anthocyanidin:flavonoid glucosyltransferase (UGFT) is responsible for O-glycosylation of anthocyanidins or anthocyanins. A UFGT isolated from the *P. equestris* flower buds dEST, PeUFGT3, showed high expression in red cultivars (Chen et al. 2011). Knockdown of the expression of PeUFGT3 by RNA interference resulted in various levels of fading color in *Phalaenopsis*, so PeUFGT3 may be associated with red color formation in *Phalaenopsis*.

### Regulation in the anthocyanin biosynthesis pathway

MYB transcription factors are involved in regulating flavonoid and anthocyanin biosynthesis (Sablowski et al. 1994, Allan et al. 2008). They contain a MYB DNA-binding domain composed of 51–52 amino acid residues to form a helix–turn–helix docking in a major groove of the DNA (Dubos et al. 2010). They are classified into three groups on the basis of repeat numbers, including MYB-related factors with one repeat, R2R3 MYB with two repeats and MYB3R factors with three repeats (Ito 2005). The first plant MYB gene, C1, a R2R3 MYB factor isolated from *Zea mays*, is related to anthocyanin biosynthesis (Paz-Ares et al. 1987). Extensive studies indicate that most R2R3 MYBs interact with basic helix–loop–helix (bHLH) factors to control anthocyanin accumulation. Examples are the R/B gene family in maize, which interacts with C1 (Mol et al. 1998), and TT8 in Arabidopsis, which, with TT2, controls flavonoid metabolism in the seed coat (Nesi et al. 2000). A motif [D/E]Lx2[R/K]x3Lx6Lx3R on the R3 domain is related to bHLH interaction (Zimmermann et al. 2004). Analysis of a R2R3 MYB isolated from *Oncidium*, OgMYB1, suggested that it is critical for the red color pattern formation in floral organs. OgMYB1 contains bHLH-interacting motifs and is expressed in red lip crests but not yellow lip tissues. Bombardment of OgMYB1 in lip tissue of *Oncidium* flowers regenerated red pigment spots in yellow lip tissue (Chiou and Yeh 2008).

In contrast to the uniform color performance of tepals discussed above, several *Phalaenopsis* cultivars are spotted flowers, especially for the harlequin flowers, which show denser coloration with fused dark spots (Chen et al. 2004). This pigment pattern is different from the central and large spots shown on *Clarkia gracilis* subsp. *Sonomensis* (Gottlieb and Ford 1988), suggesting the regulation mechanism is probably dissimilar, and could be an interesting subject.

### Carotenoids

The carotenoid biosynthesis pathway is well established (Christopher et al. 2010, Lu and Li 2010). Carotenoids are C10 isoprenoids synthesized via the methylethyltritol phosphate (MEP) pathway in plastids. The MEP pathway starts with the formation of a basic C5 unit, IPP and DMAPP. Three molecules of IPP are condensed with one molecule of DMAPP to form C10 diphosphate, and geranylgeranyl diphosphate (GGPP; Dudareva et al. 2004), the common precursor of all carotenoids. Phytoene synthase (PSY) catalyzes the condensation of two molecules of GGPP into phytoene, which is further saturated by two enzymes, phytoene desaturase and δ-carotene desaturase, into red-colored lycopene. Poly-cis-configured phytoene is transformed into all trans-form phytoene by δ-carotene isomerase (Z-ISO) and carotenoid isomerase. Lycopene ε-cyclase (LCYE) and/or lycopene β-cyclase (LYCB) catalyzes the cyclization of lycopene and is an important branch point of this pathway. The combination of LCYE and LYCB yielded α-carotene (β,ε-carotene), whereas LYCB alone produces β-carotene. α-Carotene and β-carotene are hydroxylated by the action of β-hydroxylase (HYB) to generate orange-colored lutein and zeaxanthin, respectively. Lutein is the most abundant form of carotenoids detected in leaf tissue. Zeaxanthin epoxidase (ZEP) further epoxidizes zeaxanthin to violaxanthin. The final step of the carotenoid biosynthesis pathway is the formation of neoxanthin by neoxanthin synthase (NXS). Chiou et al. (2010) isolated several carotenoid-related genes in three *Oncidium* cultivars, and four genes exhibited varied expression: OgHYB and OgZEP showed higher expression in yellow Gower Ramsey than in orange Sunkist, whereas OgZDS and OgLCY were more active in Sunkist, thus resulting in lower level of violaxanthin, 9-cis-violaxanthin and neoxanthin, and accumulation of β-carotene in Sunkist as mentioned above and orange color formation in Sunkist flowers. The white color pattern might result from the up-regulated expression of OgCCD1 (*carotenoid cleavage dioxygenase 1*). Both CCD and 9-cis-epoxycarotenoid dioxygenase catalyze the oxidative cleavage of carotenoids to produce a diversity of apocarotenoids and are involved in the catabolism of carotenoids, helping to regulate the level of carotenoids, generating substrates for ABA synthesis and signaling molecules (Lu and Li 2010). Methylation assay of the OgCCD1 promoter in white Jade and yellow Gower Ramsey indicated a higher level of methylation in Gower Ramsey. The function of OgCCD1 was demonstrated by bombardment of OgCCD1 in the yellow lip tissue of Gower Ramsey, which resulted in a snow-white spot (Chiou et al. 2010).

Carotenoids are synthesized de novo in plastids, but they accumulate in chloroplasts in leaves for function in photosynthesis and are present in chromoplasts of flowers and fruits. Chloroplasts and chromoplasts differ in sequestering carotenoids (Christopher et al. 2010). Carotenoids integrate with Chl-binding proteins in chloroplasts. In contrast, they are associated with polar lipids and carotenoid-associated protein, such
as CHRC and CHRD, to retain stability and reach a considerable level in chromoplast membrane (Vishnevetsky et al. 1999). Chiu et al. (2008) identified OgCHRC and its promoter (Pchrc) in Oncidium. OgCHRC is specifically expressed in flowers. Transient expression of Pchrc in different species by bombardment resulted in Pchrc-GUS mimicking the expression pattern of OgCHRC, expressed in conical papillate cells of adaxial epidermis of lip tissues, with accumulation of anthocyanins and carotenoids and higher expression in monocots. The tissue specificity of Pchrc could provide a convenient and useful tool in orchid breeding biotechnology, such as OgCHI and OgDFR ectopic transformation (Chiou and Yeh 2008).

Compared with the well-studied regulation mechanism of the anthocyanin pathway, little is known for the carotenoids. Several studies have shown that PSY expression is directly negatively regulated by phytochrome-interacting factor (PIF), a repressor of photomorphogenesis in A. thaliana (Toledo-Ortiz et al. 2010, Meier et al. 2011). The situation in Phalaenopsis and Oncidium is under investigation.

**Perspectives**

At present, the orchid industry in Taiwan faces several problems waiting to be resolved. The breeding process is too slow (3 years for one generation), and no marker systems are available for screening desirable varieties. Thus, orchid breeding is not efficient. A regular supply of Oncidium flowers to the market all year round has been constrained by lack of effective means for flowering modulation. Blue Phalaenopsis flowers, red Oncidium flowers, and perfect flower shape with a pleasant fragrance are the highly expections of consumers. In addition, poor quality of planting materials due to somaclonal variations generated by micropropagation constrains the full expansion of the orchid industry.

For an important floral crop, both physical and genetic maps are needed for developing markers linking economic traits. However, there are no maps available for orchids. Recently, we began a preliminary attempt to build the physical map of *P. equestris*. So far, about 20,000 BAC clones have undergone fingerprinted contig analysis, and several of them were assembled to be contiguous. Nevertheless, more vigorous inputs in terms of budget and workflow will be needed to complete the 120,000 BAC clones for 8.4-fold coverage to resolve a good-quality physical map. However, constructing the genetic map of Phalaenopsis orchids is hampered by their long life cycles and slow growth rate. Currently, a colleague at National Taiwan University has initiated the genetic mapping of Phalaenopsis. Hopefully, more updated statistics and available software may help resolve this problem with progeny from a few generations.

The next challenging problems to be solved are molecular networks of flowering and the regulation mechanism of flower color formation. These two fields are under study in Taiwan. On the application side, transformation and tissue culture technologies for Phalaenopsis and Oncidium are feasible, and new genetically engineered varieties with characters of adjustable flowering and new varieties of colors are promising areas. Genetic engineering of novel flower colors is now a practical technology, as typified by commercialization of a transgenic blue rose and blue carnation (Nishihara and Nakatsuka 2011). Blue Phalaenopsis and red Oncidium cannot be bred by traditional means. Through basic research into the formation and regulation of floral color of Phalaenopsis and Oncidium, genetically modified orchid flowers with designed colors will be genetically engineered, propagated by micropropagation and then distributed worldwide.

Although most of the Phalaenopsis spp. are scentless, some do have a pleasant scent. However, many modern Phalaenopsis cultivars have lost the ability for scent emission with traditional breeding programs. In order to revive fragrance, molecular breeding by genetic engineering for a gene network is currently underway for generating the scented *P. aphrodite* subsp. formosana. The prospective applications for restoring scent in Phalaenopsis follow the wishes of the consumer. Further study of key enzymes, such as GDPS and terpene synthase, involved in the scent biosynthesis pathway, are necessary for using metabolic engineering to improve the production of scent precursors to emit high-level scents. In general, consumers accept genetically modified flowers more readily than foods; therefore, many genetically modified floricultural plants are at advanced stages of development.

A much more urgent issue faced by the orchid industry is the decrease in or the early detection of somaclonal variations occurring during mass clonal micropropagation. Evidence that epigenetic mechanisms play a role in somaclonal variation includes activation of transposons and retrotransposons, putative silencing of genes, and methylation pattern variation of the genome. Understanding epigenetic regulation will have important implications in the orchid industry while increasing the consistency of floral products. Eventually, directed manipulation of epigenetic regulators will open the way to epigenetic engineering in orchids.

With the draft of the whole genome sequence of *P. equestris* in progress, the genetic blueprint of orchids will soon be available. In addition to providing a basic understanding of the genetic basis of orchids, this information will be useful in having significant effects on floral crop design. This information includes identifying genes that could improve crop value and our understanding of the evolution of the unique orchid biology. Application of these resources through the common language of nucleotide sequences will greatly enhance insights into orchid biology and biotechnology.

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