Orchid Biotechnology in Production and Improvement

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The Orchidaceae is one of the largest families of flowering plants, with several genera being used in cut flower and potted plant production. The molecular biology of orchids, last reviewed in Kuehnle (1997), covered research in phylogeny and systematics, floral physiology, and plant breeding. Only 10 orchid genes were cloned at that time. Since then, the total number of genes cloned from orchids has expanded considerably. The objective of this paper is to: 1) provide an overview of the function of some recently cloned genes; and 2) review advances made in other applications of biotechnology in orchid production and improvement.

Functions of some cloned orchid genes

About 70 genes have been cloned from seven orchid genera, namely *Dendrobium*, *Phalaenopsis*, *Doritaenopsis*, *Aranda*, *Bromheadia*, *Vanilla*, and *Cymbidium*. These genes can be divided into seven categories based on their presumptive functions (Table 1). Here we review genes affecting flower induction, flower color, flower senescence, and disease resistance. Other genes have been cloned that affect primary metabolism, ovule development, cell division, and cell structure (Table 1). At this time, the latter categories have had less direct impact on commercial orchid production and improvement.

Flower induction. The ability to time flowering for holidays and to hasten flowering in otherwise slow-maturing orchids is of keen interest to growers of commercially cropped orchids. Several research groups are examining the genetic aspects of flower induction and the transition of the apical meristem from a vegetative shoot apical meristem (VSAM) through a transitional shoot apical meristem (TSAM) to a reproductive meristem. Specifically, several orchid genes have been identified to be so-called homeobox and MADS box genes, which encode transcription factors and are preferentially expressed in vegetative or transitional stages (Table 1).

Homeobox genes. Five clones were identified by mRNA differential display as transcription factors involved in floral induction (Yu and Goh, 2000a). Of these, the partial cDNA clone *ovg2* showed significant similarity to homeobox genes, which are a universal group of transcription factors important in development. The

full-length single copy clone, *DOH1*, which was subsequently isolated from a *Dendrobium* cDNA library of VSAM, shared considerable similarity to a class I *knox* (knotted-like) genes (Yu et al., 2000). Northern analysis and *in situ* hybridization revealed that *DOH1/ovg2* mRNA accumulated in all meristem-rich tissues and its expression was down-regulated at the beginning of floral transition (Yu and Goh, 2000a; Yu et al., 2000). A *Dendrobium* hybrid transformed with sense constructs of *DOH1* gene driven by the cauliflower mosaic virus (CaMV) 35S promoter (Yu et al., 2000) revealed that the overexpression of *DOH1* completely suppresses shoot organization and development. Anti-sense plants formed multiple shoot meristems and precociously flowered. With these results, the authors concluded that *DOH1* is involved in controlling the formation and identity of the shoot apical meristem, and thereby involved in maintaining the basic plant architecture.

Another floral homeotic gene was isolated from red and white *Phalaenopsis* using Amplified Fragment Length Polymorphism (Liu and Chen, 1999). A fragment that was differentially amplified, AM1-3, has shown 87% similarity to the floral homeotic gene *AGL5* from *Arabidopsis thaliana* and 90% similarity to the *AG* gene of *Brassica napus* at the deduced amino acid sequence. *AGL5* is known to act downstream of *AG* in programming normal carpel and ovule development.

MADS box genes. MADS-box genes comprise another important family of transcription factors that regulate the transition of meristem identity. They are characterized by the presence of a highly conserved DNA binding domain (MADS-box) and a second conserved domain (K-box) involved in protein-protein interactions. The first flower-specific MADS-box gene from an orchid was isolated from the mature flower cDNA library of an orchid hybrid *Aranda* Deborah using an *agamous* cDNA from *Arabidopsis* as the probe (Lu et al., 1993). A single clone, *om1* was isolated, with an open reading frame (ORF) of 750 base pairs (bp) encoding a polypeptide of 250 amino acids. Expression of *om1* was limited to the petals and sepals of mature flowers but was not found in the column, early inflorescence, or floral buds, suggesting that *om1* might be playing an important role in petal development.

Another orchid MADS-box gene, *otg7*, expressed only in the TSAM was identified from *Dendrobium* (Yu and Goh, 2000a). Clone *otg7* was used later to isolate three new MADS box genes, *DOMADS1*, *DOMADS2*, and *DOMADS3*, all of which were expressed exclusively in floral tissues (Yu and Goh,

2000b). *DOMADS1* was expressed early in the developing inflorescence and in all floral organ primordia. Its high expression in pollinaria suggested it could be an early regulator of pollen mother cell development. *DOMADS2* was expressed early in the VSAM and increased in expression during the transition of the shoot apical meristem from the vegetative to reproductive phase, which might indicate a role in vegetative to reproductive phase change. The expression pattern of *DOMADS3*, as revealed by *in situ* hybridization, suggested that it might function as a regulatory factor in the formation of the TSAM and in the development of pedicel tissue.

Another transcription factor, *ovg27*, a homologue of a *Drosophila* shuttle-craft gene, was isolated from the VSAM of *Dendrobium* and appears to be important in maintaining the vegetative state of shoot apical meristem in plants (Yu and Goh, 2000a). Also, a cDNA clone, *otg16*, expressed only in the TSAM has shown significant similarity to an *Arabidopsis* casein kinase gene involved in protein phosphorylation and signal transduction (Yu and Goh 2000a). The complete signal transduction pathway in floral induction of orchids is yet to be discovered.

Flower color (flavonoid biosynthesis). The biosynthesis of anthocyanins is well characterized in many ornamental plants. The synthesis of flavonoids, including anthocyanins, occurs through the phenylpropanoid pathway, which starts with the stepwise condensation of three acetate units from malonyl-coA with 4-coumaroyl CoA to yield tetrahydroxychalcone (Heller and Forkmann, 1988). This reaction is catalyzed by the enzyme chalcone synthase (CHS), which is attractive to researchers for flower color manipulation. The magnificent spectrum of colors found in orchids is mainly due to the accumulation of anthocyanins in their flowers (Arditti and Fisch, 1977). Although carotenoids and chlorophylls contribute to flower color in many orchids, there are no detailed reports of cloned orchid genes associated with the biosynthesis of these compounds except for a brief mention of an amplified restriction fragment of *Phalaenopsis*, AM4-1, which showed similarity to the geranyl-geranyl pyrophosphate synthase gene (Liu and Chen, 1999). Genes for other important enzymes cloned in orchids are flavanone 3-hydroxylase (F3H) and dihydroflavonol 4-reductase (DFR).

Chalcone synthase genes. The earliest report of the isolation of flower color genes in orchids appeared as an abstract only by Yong and Chua (1990), in which four cDNA clones encoding CHS were isolated from a cDNA library of a *Dendrobium* hybrid. These DNA sequences are absent in all the searchable

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Table 1. Isolated orchid genes or cDNA clones and their presumptive function.

Gene or cDNA designation	Orchid genus	Presumptive function	Reference ^a and (GenBank accession numbers)
<i>Flower Induction</i>			
<i>ovg2/DOH1</i>	<i>Dendrobium</i>	Homeobox gene, class 1 knox gene	Yu and Goh, 2000a; Yu et al., 2000 (AF100326, AJ276389)
<i>om1</i>	× <i>Aranda</i>	Flower specific MADS box gene	Lu et al., 1993. (X69107)
<i>otg7</i>	<i>Dendrobium</i>	MADS box gene	Yu and Goh, 2000a. (AF107588)
<i>DOMADS1</i> , 2 and 3	<i>Dendrobium</i>	MADS-box genes in AP1/AGL9 subfamily	Yu and Goh, 2000b. (AF198174-b)
<i>ovg27</i>	<i>Dendrobium</i>	Transcriptional repressor	Yu and Goh, 2000a. (AF100331)
<i>otg 16</i>	<i>Dendrobium</i>	Casein kinase1, a homologue of serine/threonine protein kinase.	Yu and Goh, 2000a. (AF107592)
<i>Cell Division and Cell Structure</i>			
<i>Ovg14</i>	<i>Dendrobium</i>	Transcriptional regulator of cell cycle regulators.	Yu and Goh, 2000a. (AF100328)
<i>otg4</i>	<i>Dendrobium</i>	Cell division control protein, a homologue of yeast NDA4	Yu and Goh, 2000a. (AF107586)
<i>Ovg30</i>	<i>Dendrobium</i>	DNA binding protein, regulate cell cycle progression	Yu and Goh, 2000a. (AF100333)
<i>Ovg29</i>	<i>Dendrobium</i>	Putative 21D7 protein. Degradation of cell cycle regulatory proteins	Yu and Goh, 2000a. (AF100332)
<i>otg2</i>	<i>Dendrobium</i>	Myosin heavy chain	Yu and Goh 2000a. (AF107585)
<i>P-ACT1</i>	<i>Phalaenopsis</i>	Actin partial clone	Nadeau et al., 1996. (U18102)
<i>ACT2</i>	<i>Phalaenopsis</i>	Actin-like protein	Huang et al., 2000. (AF246715)
---	<i>Phalaenopsis</i>	Profilin, an actin binding protein	Lee et al., 1999b. (AF126263)
<i>Flower Senescence</i>			
<i>Ds-ACSI</i> , <i>Ds-ACS2</i>	× <i>Doritaenopsis</i>	ACC synthase	O'Neill et al., 1993 (L07882, L07883)
<i>ACS2</i> , <i>ACS3</i>		ACC synthase	Bui and O'Neill, 1998 (AF007213, AF00721)
<i>pOACS10</i>	<i>Phalaenopsis</i>	ACC synthase	Do and Huang, 1998. (Z77854)
---	× <i>Doritaenopsis</i>	ACC oxidase	Nadeau et al., 1993. (L07912)
---	× <i>Doritaenopsis</i>	ACC oxidase	Nadeau and O'Neill, 1995 (L37103)
<i>pPEFEA</i>	<i>Phalaenopsis</i>	ACC oxidase	Lee and Huang, 1995
<i>Petr1</i>	<i>Phalaenopsis</i>	Homologue of ethylene receptor	Do et al., 1999. (AF055894)
<i>Per1</i>	<i>Phalaenopsis</i>	Ethylene response sensor	Chai et al., 1999. (AF113541)
<i>POAC031/PACO1</i>	<i>Phalaenopsis</i>	Homologue of human Acyl-CoA oxidase	Do and Huang, 1996. (U66299)

Table 1 continues on next page.

databases. Later, three CHS cDNA clones, *OCHS3*, *OCHS4*, and *OCHS8*, isolated and characterized from the cDNA library of *Bromheadia finlaysoniana* flowers (Liew et al., 1998a), were shown to contain a single ORF of 1185 bp encoding a protein of 42.9 KD. CHS in *Dendrobium* and *Bromheadia* are encoded by a small multigene family and is expressed in high levels in flower buds and other tissues containing anthocyanin such as leaves with pigmented edges and roots (Liew et al., 1998a; Yong and Chua 1990). Another cDNA clone suggested to be a CHS homolog, *pOCHS01*, was isolated from a *Phalaenopsis* hybrid (Hsu et al., 1997). However, a recent BLAST search revealed that this cDNA clone is in fact 98% similar to bibenzyl synthase of *Phalaenopsis* and *Bromheadia*. Authors noted that there are at least 10 other sequences that hybridized with *pOCHS01* in southern analysis. It is difficult to distinguish genes encoding CHS, bibenzyl synthase and stilbene synthase due to their close similarity at the nucleotide and amino acid sequence levels (Liew et al., 1998a).

Flavanone 3-hydroxylase genes. Another key enzyme in flavonoid biosynthesis, flavanone 3-hydroxylase, catalyzes the formation of dihydroflavonols from (2S)-flavanones in plants. A full-length cDNA clone of 1393 bp encoding F3H was isolated from *Bromheadia finlaysoniana* (Liew et al., 1995). It encodes a protein of 41.5 KD with 464 amino acid residues and shared 52% to 59% and 70% to 76% homology with F3H of other plants

at the nucleotide and amino acid levels, respectively.

Dihydroflavonol 4-reductase (DFR) genes. The conversion of dihydroflavonols such as dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM) to the corresponding leucoanthocyanidin is the first committed step in anthocyanin biosynthesis and is catalyzed by the enzyme, DFR. The color of the anthocyanin produced depends on the dihydroflavonol substrate that is reduced by DFR. The substrate specificity of DFR explains the absence of certain colors from some ornamental plants, e.g., *Petunia hybrida* which does not catalyze the conversion of DHK to orange-colored pelargonidin (Meyer et al., 1987). In orchids, *Cymbidiums* are noticeably devoid of orange colored flowers (Johnson et al., 1999) and only a few pelargonidin accumulating flowers are found in *Dendrobium* (Kuehnle et al., 1997). This important feature of DFR makes this enzyme an important target for flower color manipulation through genetic engineering (Johan et al., 1995; Meyer et al., 1987).

In order to characterize DFR in orchids, full-length clones encoding DFR were isolated from petal cDNA libraries of *Bromheadia finlaysoniana* (Liew et al., 1998b) and a *Cymbidium* hybrid (Johnson et al., 1999). Southern analysis revealed that DFR is represented by a single copy gene in both orchid genera. A full-length DFR cDNA clone isolated from *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' using a RT-PCR based technique showed 87% homology

to *Bromheadia* and 84% homology to *Cymbidium* (Kuehnle and Mudalige, 2002). The substrate specificity of *Cymbidium* DFR was investigated by transforming a mutant *Petunia* line accumulating DHK as the major flavonol (Johnson et al., 1999). Chemical analysis of transformed lines revealed that *Cymbidium* DFR did not efficiently catalyze the reduction of DHK and preferred DHQ as a substrate, resulting in the production of pink cyanidin instead of orange pelargonidin. Introduction of a DFR from a plant that efficiently reduces DHK to pelargonidin (e.g., *Antirrhinum majus*, *Rosa hybrida*, *Gerbera hybrida*, and *Zea mays*) might be an attractive alternative to introduce orange color into orchids such as *Cymbidium* and *Dendrobium*.

Flower senescence. In the absence of pollination, many orchid flowers are known to have a very long life, some lasting up to 6 months (Goh and Arditti, 1985). This long life span may increase the chance of pollination by a highly specific insect pollinator. Pollination triggers a series of physiological and developmental changes, known collectively as "post-pollination syndrome", which mobilize nutrients out of nonessential parts (perianth) into essential parts, the developing ovule and ovary of a flower. The hormone ethylene plays a key role in the transduction of the pollination signal and the coordination of post-pollination development in many flowers including orchids (Porat et al., 1994a and b). The production of ethylene starts with the synthesis of S-adenosyl methionine (SAM) catalyzed

Table 1. Continued.

Gene or cDNA designation	Orchid genus	Presumptive function	Reference ² and (GenBank accession numbers)
<i>Ovule Development</i>			
<i>o39</i>	<i>Phalaenopsis</i>	Homeobox protein	Nadeau et al., 1996. (U34743)
<i>o40/CYP78A2</i>	<i>Phalaenopsis</i>	Putative cytochrome p-450 monooxygenase	Nadeau et al., 1996. (U34744)
<i>o108</i>	<i>Phalaenopsis</i>	Homologue of SKP1, a cell cycle regulator gene	Nadeau et al., 1996. (U34745)
<i>o126</i>	<i>Phalaenopsis</i>	Glycine rich cell wall structural protein	Nadeau et al., 1996. (U34746)
<i>o141</i>	<i>Phalaenopsis</i>	Cysteine proteinase	Nadeau et al., 1996. (U34747)
<i>o138</i>	<i>Phalaenopsis</i>	A new gene involved in embryo formation	Wang et al., 1999
<i>o38</i>	<i>Phalaenopsis</i>	Specific function unknown	O'Neill et al., 1996. (U78100)
<i>Flower Color</i>			
OCHS3,4, and 8	<i>Bromheadia finlaysoniana</i>	Chalcone synthase	Liew et al., 1998a. (CAF007097-9)
<i>Fht/pCF1</i>	<i>Bromheadia finlaysoniana</i>	Flavanone 3 hydroxylase	Liew et al., 1995. (X89199)
<i>ODFR</i>	<i>Bromheadia finlaysoniana</i>	Dihydroflavonol 4-reductase	Liew et al., 1998b. (AF007096)
<i>Dfr</i>	<i>Cymbidium</i> hybrid	Dihydroflavonol 4-reductase	Johnson et al., 1999. (AF017451)
<i>Disease Defense/ Stress Response</i>			
<i>Pal/OPAL1</i>	<i>Bromheadia finlaysoniana</i>	Putative phenyl alanine ammonia lyase (PAL)	Liew et al., 1996. (X99997)
<i>ovg43</i>	<i>Dendrobium</i>	PAL enzyme	Yu and Goh, 2000a. (AF100336)
<i>pBibSy811, pBibSy211</i>	<i>Phalaenopsis</i>	Bibenzyl synthase	Preisig-Müller et al., 1995. (X79904, X79903)
<i>pBBS1</i>	<i>Bromheadia finlaysoniana</i>	Bibenzyl synthase	Lim et al., 1999. (AJ131830)
<i>pAHH511</i>	<i>Phalaenopsis</i> spp	S-adenosyl homocysteine hydrolase	Preisig-Müller et al., 1995 (X79905)
<i>pOCHS 01</i>	<i>Phalaenopsis</i>	98% similar to <i>pBibSy811</i> , but labeled as a CHS homolog	Hsu et al., 1997. (U88077)
<i>ovg23</i>	<i>Dendrobium</i>	Putative copper chaperone of copper/zinc superoxide dismutase	Yu and Goh, 2000a. (AF100330)
<i>Primary Metabolism</i>			
<i>otg11</i>	<i>Dendrobium</i>	Putative fructose-bis-phosphate aldolase.	Yu and Goh, 2000a. (AF107590)
<i>otg9</i>	<i>Dendrobium</i>	Alternative oxidase	Yu and Goh, 2000a. (AF107589)
<i>otg6</i>	<i>Dendrobium</i>	NADH dehydrogenase intron region	Yu and Goh, 2000a. (AF107587)
<i>ovg41</i>	<i>Dendrobium</i>	formate dehydrogenase	Yu and Goh, 2000a. (AF100335)
<i>Pepe</i> gene, isoform 1-4	<i>Vanilla planifolia</i>	Phosphoenol pyruvate (PEP) carboxylase	Gehrig et al., 2001. (AJ312624, AJ312625, AJ312626, AJ312627)
<i>Ppc3</i>	<i>Vanilla planifolia</i>	PEP carboxylase	Gehrig et al., 1999 (AJ249988, AJ249989)
<i>Mdh1/pVM7</i>	<i>Vanilla planifolia</i>	NADP malate dehydrogenase	Gehrig et al., 2002. (AJ306489)
---	<i>Phalaenopsis</i>	Putative chlorophyll a/b binding protein	Lee et al., 1999a. (AF133340)
<i>Dcr1c1</i>	<i>Dendrobium crumenatum</i>	Isocitrate lyase	Vellupillai et al., 1999 (AF193815)
<i>ovg37</i>	<i>Dendrobium</i>	Putative acyl carrier protein,	Yu and Goh, 2000a. (AF100334)
---	<i>Dendrobium crumenatum</i>	Vacuolar H ⁺ ATPase proteolipid subunit	Liew et al., 1999. (AF193814)
<i>Other Genes</i>			
<i>ovg11, ovg15, ovg50, ovg14</i>	<i>Dendrobium</i>	Function unknown, involved in floral transition	Yu and Goh, 2000a. (AF100327, AF100329, AF100337, AF107591)
<i>cko1</i>	<i>Dendrobium</i>	Cytokinin oxidase	Yang et al., 2000. (AJ294542)

²Some genes are directly submitted to GenBank and the research is published later in journals. For these records, year of publication does not reflect the date of submission to GenBank. For the sequences that are only in GenBank, the date of submission is mentioned as the year of publication.

by SAM synthetase. Conversion of SAM to 1-aminocyclopropane-1-carboxylate (ACC) is catalyzed by ACC synthase, followed by the oxidation of ACC to ethylene by ACC oxidase. The rate-limiting step is believed to be the conversion of SAM to ACC. The physiology of post-pollination syndrome in terms of ethylene production and sensitivity in *Phalaenopsis* flowers (Porat et al., 1994b; Nadeau et al., 1993) and *Dendrobium* flowers (Porat et al., 1994a) has been well characterized. The exact nature of the primary pollination signal is still uncertain and speculated to be pollen-borne ACC or auxin (Bui and O'Neill, 1998; Wang et al. 2001).

ACC synthase genes. A total of six cDNA clones have been obtained from the pollinated flowers of different species of orchids (Table 1). Two ACC synthase clones, *Ds-ACS1* and *Ds-ACS2*, were isolated from *Doritaenopsis* (O'Neill et al., 1993). Northern analysis revealed that *Ds-ACS1* and *Ds-ACS2* mRNA accumulated in the stigma, ovary, and label-

lum (a modified petal also known as lip) but not in the perianth (petals and sepals). Both ACC synthase genes were shown to respond to ethylene produced in the stigma in response to the primary pollination signal. Three cDNA clones involved in regulation of the initiation and propagation of ethylene biosynthesis, *Phal-ACS1*, *Phal-ACS2*, and *Phal-ACS3* were isolated from stigma and ovary tissue of pollinated *Phalaenopsis* flowers (Bui and O'Neill, 1998). *Phal-ACS2* mRNA was apparent in the stigma while *Phal-ACS3* mRNA accumulated in the ovary as a response to pollination. *Phal-ACS1* was believed to be involved in amplification and transmission of the pollination signal among different floral organs.

Yang et al. (1996) isolated a clone, *DCACS*, from the senescing flowers of *Dendrobium crumenatum*. Two additional clones (*pOACS10* and *pOACS77*) were isolated from wilting petals of a *Phalaenopsis* hybrid (Huang et al., 1996).

ACC oxidase genes. Three ACC oxidase

genes have been reported as cloned (Table 1). Two genes, *OAO1* and *D-ACO2*, were isolated from cDNA libraries of gynoeceia and the senescing petals of pollinated *Doritaenopsis* flowers, respectively (Nadeau et al., 1993; Nadeau and O'Neill, 1995). Another ACC oxidase cDNA clone was isolated from senescing petals of *Phalaenopsis* (Lee and Huang, 1995). ACC oxidase activity increased significantly in the stigma of *Phalaenopsis* after pollination due to de novo synthesis of ACC oxidase mRNA and presumably the protein (Nadeau et al., 1993). Unlike ACC synthase, ACC oxidase mRNA accumulated in the perianth in response to pollination. Emasculatation and application of auxin or ethylene to the stigma were found to simulate the pollination signal, increasing the expression of the ACC oxidase gene. Application of ACC to petals of pollinated flowers increased the ACC oxidase expression in petals, suggesting that ACC itself can also act as a secondary signal in *Phalaenopsis*.

Other senescence-related genes. The binding of ethylene to its receptor is the first step in hormonal signal perception and transduction. Plant ethylene receptors are members of a multigene family, with highly conserved domains in the N-terminus of their deduced amino acid sequence and their putative histidine kinase regions (Do et al., 1999). A putative ethylene receptor cDNA clone was isolated from the pollination-induced senescent petals of *Phalaenopsis* (Table 1). It encodes a 71 KD polypeptide reported to be most similar to banana and rice (monocotyledons) ethylene receptors (75% to 79%) in deduced amino acid sequences. Another important gene associated with pollination-induced flower senescence, *PACO1*, was isolated from senescing *Phalaenopsis* petals (Do and Huang, 1996). It appeared to be a single copy gene whose expression is induced by exogenous application of ethylene. A genomic clone of *PACO1* was isolated from a genomic DNA library of *Phalaenopsis* with the intention to analyze the promoter sequence and further characterize the activity of this gene in orchids (Do and Huang, 1997).

Disease defense. The production of polyphenols is a defense response of plants towards wounding, microbial infection and stress. The first step in biosynthesis of polyphenols is the production of cinnamate from phenylalanine, catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). A full length cDNA clone, *OPAL1*, was isolated from the orchid *Bromheadia finlaysoniana* in order to understand the regulatory response of PAL toward wounding and fungal elicitors (Liew et al., 1996). The deduced amino acid sequence was 78% to 83% similar to PAL of other plant species. Another cDNA clone encoding PAL, *ovg 43*, was isolated from the vegetative shoot apical meristem (Yu and Goh, 2000a). Northern analysis indicated that expression of *ovg43* is down-regulated in the transition of the shoot apical meristem to a reproductive phase. However, the exact reason for this down-regulation and its function in shoot apical meristem during floral transition is unknown.

Phytoalexin production genes. In many orchid plants, the invasion of fungal parasites

induces the synthesis of specific chemical defense compounds, 9,10-dihydrophenanthrenes, through the dihydrophenanthrene pathway. A cDNA library prepared from mRNA of *Phalaenopsis* leaves inoculated with the conidia of *Botrytis cinerea* was used to isolate clones encoding for the enzyme bibenzyl synthase (Preisig-Müller et al., 1995). Two full-length cDNA clones encoding bibenzyl synthase, *pBibSy811* and *pBibSy212*, were isolated, as well as a clone encoding s-adenosylhomocysteine hydrolase (*pAHH511*). The bibenzyl synthases, *pBibSy811* and *pBibSy212* were verified by expression and production of two enzymatically active recombinant proteins in *Escherichia coli* with the same substrate specificity *in vitro* as that of the plant enzyme. In young *Phalaenopsis* plants, fungal infection induced a transient increase in expression of both bibenzyl synthases and s-adenosylhomocysteine hydrolase by 100-fold with a peak at 20 h after infection. A concomitant increase of PAL expression and the production of the phytoalexin hircinol were also observed with fungal infection (Reinecke and Kindl, 1994). Another full-length cDNA clone encoding bibenzyl synthase was isolated from a petal cDNA library of *Bromheadia finlaysoniana* (Lim et al., 1999, GenBank no. AJ131830).

Orchid genetic engineering and commercially applicable traits

Methods of transgenesis. Production of new varieties with improved characters such as new flower colors, flower morphology, plant stature, fragrance and increased vase-life is crucial for the growth of the floriculture industry. Plant breeding by sexual hybridization, selection of variants and polyploids have been essential parts of developing many improved traits and a large number of commercial orchid varieties. Interest in molecular breeding of orchids has increased considerably during the recent years to hasten selection and expand the gene pool available for improvement. Methods used in transformation of *Phalaenopsis*, *Dendrobium*, *Cymbidium*, and *Calanthe* have been extensively reviewed by Kuehnle (1997) and Chen et al. (2003). Here we discuss additional

papers appearing since these reviews on gene transfer methods (Table 2) and commercially applicable traits (Table 3).

The most widely used method of gene transfer in orchids is particle bombardment, with protocorms and protocorm-like-bodies (PLBs) being the most popular choices as target tissues (Table 2). Evaluation of antibiotic selective agents for *Dendrobium* transformation indicated hygromycin to be excellent and geneticin also effective (Ong et al., 2000). The herbicide bialaphos proved to be a viable selection agent in transformation of *Brassia*, *Cattleya* and *Doritaenopsis* (Knapp et al., 2000).

Agrobacterium-mediated transformation is also proving applicable to some orchid genera (Table 2). Wounded PLBs of *Dendrobium* were inoculated with *Agrobacterium* strain LBA4301 harboring a binary vector system (Nan et al., 1998), containing the hygromycin phosphotransferase (*HPT*) gene under the control of a nopaline synthase (*nos*) promoter and the *uidA* gene encoding GUS (β -glucuronidase) enzyme under a CaMV 35S promoter. Southern analysis and PCR confirmed the successful transformation and recovery of transgenic plants. Parallel work has shown the presence of coniferyl alcohol as the virulence gene inducer in *Dendrobium* (Nan et al., 1997). *Agrobacterium*-mediated gene transfer was also used to elucidate the function of class 1 knox gene, *DOH1*, in *Dendrobium* (Table 3; Yu et al., 2001). Selectable marker *NPTII* under the control of a *nos* promoter, and sense as well as antisense *DOH1* genes under CaMV 35S promoters were used to transform thin sections of *Dendrobium* PLBs. Molecular analysis by PCR and Southern hybridization revealed the successful integration of both sense and antisense constructs of the *DOH1* gene. Analysis of results and discussion of the role of *DOH1* gene in shoot apical meristem is discussed previously under floral induction.

Engineering disease resistance. Two common, readily transmitted viruses impacting cultivated orchids are Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV). Viral genomic RNA

Table 2. Examples of genetically engineered orchids: Means of transgenesis.

Method of transformation	Orchid genus	Gene(s) used ^a	Reference
Particle Bombardment	<i>Dendrobium</i> protocorms	NPT II, GUS, Papaya ringspot virus coat protein	Kuehnle and Sugii, 1992
	<i>Dendrobium</i>	GUS, NPT II	Nan and Kuehnle, 1995a
	<i>Dendrobium</i> PLBs	LUC	Chia et al., 1994
	<i>Dendrobium</i> PLBs	LUC	Chia et al., 2001
	<i>Dendrobium</i> protocorms	GUS, HPT	Yu et al., 1999
	<i>Cymbidium</i> PLBs	GUS, NPT II	Yang et al., 1999
	<i>Cymbidium</i> petals	GUS	Boase et al., 2001; Peters et al., 2001
	<i>Phalaenopsis</i> × <i>Doritaenopsis</i> , <i>Phalaenopsis</i>	Bar, NPT II, GUS, Soybean β -1,3-endoglucanase	Anzai and Tanaka, 2001
	<i>Doritaenopsis</i> × <i>Phalaenopsis</i> callus	GUS, NPT II, HPT	Belarmino and Mii, 2000
	<i>Dendrobium</i>	GUS, HPT	Nan et al., 1998
Pollen Tube Pathway	<i>Dendrobium</i>	GUS, NPT II	Nan and Kuehnle, 1995b
	<i>Phalaenopsis</i>	GUS, NPT II	Hsieh and Huang, 1995
Seed Imbibition; Electroporation	<i>Dendrobium</i>	GUS, NPT II	Nan and Kuehnle, 1995b
	<i>Calanthe</i>	GUS NPT II	Griesbach, 1994

^aNPT II = neomycin phosphotransferase; GUS = β -glucuronidase; LUC = luciferase; HPT = hygromycin phosphotransferase; Bar = Bialaphos resistance gene.

Table 3. Examples of genetically engineered orchids: potential commercial traits.

Purpose of transformation	Species	Gene(s) used	Reference
Disease resistance			
Virus	<i>Dendrobium</i>	CymMV coat protein	Chia, 1999
Fungus	<i>Phalaenopsis</i>	β -1,3-endoglucanase	Anzai and Tanaka, 2001
Flower wilting			
Long vase-life	<i>Dendrobium</i>	Sense and antisense ACC oxidase	Chia et al., 2001
Other interests			
Function of <i>DOH1</i>	<i>Dendrobium</i>	Sense and antisense DOH1 gene	Yu et al., 2001
Glow in the dark	<i>Dendrobium</i>	Firefly luciferase	Chia et al., 2001

was isolated from a field strain of CymMV, and a cDNA clone encoding the viral coat protein (CP) was synthesized from it (Chia et al., 1992a). This cDNA clone driven by CaMV 35S promoter was used to transform the model plant *Nicotiana benthamiana* to test the effectiveness of the CP gene in conferring viral resistance. At a low level of inoculum, transformed *Nicotiana* plants showed resistance to CymMV by preventing systemic infection completely and reducing the viral titer in the inoculated leaf. A coat protein gene was also used in antisense orientation under the CaMV 35S promoter to transform *Nicotiana occidentalis* (Lim et al., 1999a). Transformed *Nicotiana* plants were found to be highly resistant to CymMV virus. However, *Dendrobium* plants transformed with the CymMV CP gene showed only partial resistance; plants were susceptible when challenged with high viral titer (Chia, 1999).

Plants show various defense mechanisms to prevent infection from fungal pathogens. Release of elicitors from fungal cell walls by the plant β -1,3-endoglucanase enzyme is considered to be one of the earliest processes in host defense such as phytoalexin production. Anzai and Tanaka (2001) produced transgenic *Phalaenopsis* plants transformed with soybean β -1,3-endoglucanase (EG) gene under the control of a maize ubiquitin promoter. Two transformants showed incorporation of EG by Southern hybridization. However, Western analysis failed to detect the EG enzyme in either of the transformants.

Engineering novel flower colors. There are several research groups working on creating new flower colors in orchids through biotechnology in Singapore, Taiwan, South Korea, New Zealand and Hawaii. Research is still in progress and not yet published as completed work. Chia et al. (2001) reported successful production of transgenic *Dendrobium* plants carrying the firefly luciferase gene. When these plants are sprayed with the substrate luciferin, the flowers emit a soft light (glow-in-the-dark).

Engineering for increased vase life. As discussed under flower senescence, flower wilting of orchids is mediated by the phytohormone ethylene. An endogenous ACC oxidase gene cloned in sense and antisense orientation was used to transform *Dendrobium* plants to increase the vase-life of flowers (Chia, 1999). Transgenic plants showed normal morphology and growth without any aberrations due to the ACC oxidase insert. Authors reported a significant extension in vase-life in one out of four transformed lines (Chia et al., 2001).

Other biotechnology applications in production and improvement

Virus detection. Cultivated orchid plants that are infected by CymMV and ORSV can remain symptomless, making it difficult to diagnose and control the spread of the virus. A simple and sensitive technique known as nucleic acid spot hybridization (NASH) was used by Chia et al. (1990, 1992b) for the detection of these two viruses in picogram amounts. Two cDNA probes specific to CymMV and ORSV were used for hybridization and detection of viral nucleic acid in crude extracts from infected plants spotted onto a membrane. Barry et al. (1996) used two other methods, immunocapture-PCR (IC-PCR) and direct binding PCR (DB-PCR), for the detection of these two viruses. In IC-PCR, microcentrifuge tubes coated with polyclonal antibodies developed against the virus were used to capture viral particles from a crude plant extract. PCR was carried out directly in these tubes to detect the captured viral particles. In DB-PCR, crude plant extracts were incubated in tubes without the antibody trapping and subsequent PCR was performed to identify viral particles directly bound to the tubes. Both methods were shown to be easy, rapid and reliable and to eliminate the necessity of time-consuming nucleic acid extractions.

Antiviral strategies. A new molecular biology based technique known as population cloning was used to synthesize biologically active cDNA clones from a CymMV genomic RNA isolate (Yu and Wong, 1998). Three populations of overlapping RT-PCR products were ligated to form full-length cDNA clones. Infectious clones were identified by inoculating each clone onto *Nicotiana benthamiana* leaves. The infectivity of these clones was verified by western blotting, electron microscopy and PCR techniques. The authors have successfully demonstrated the appropriateness of this technique in synthesizing biologically active viral clones from positive-strand RNA viruses.

Flower color gene activity test. There are several regulatory genes that can affect flower pigmentation. Three regulatory genes, C1, B, and R, from *Zea mays* are suggested to encode transcriptional activators of pigment biosynthetic genes. Effects of B and C1 genes on the biosynthesis of anthocyanin in *Doritis pulcherrima* was investigated using particle bombardment (Griesbach and Klein, 1993). Petals from three different floral phenotypes, purple, white (alba), and white with a light purple lip (albescent), were used in the study. The authors concluded that the alba phenotype

was due to a mutation in an anthocyanin biosynthetic gene (structural gene) while the albescent form is due to a mutation in the anthocyanin regulatory genes. Testing of anthocyanin biosynthetic and regulatory genes using transient expression is an attractive rapid procedure for assessment of color gene complementation via sexual hybridization.

Transient expression was also used to assess the effectiveness of petal-specific promoters to drive the expression of reporter genes in *Cymbidium* petals compared to the most commonly used constitutive promoter, CaMV 35S sequence (Peters et al., 2001). Promoter regions from the CHS gene and *Adonis* ketolase (AKET) gene isolated from *Antirrhinum* and *Adonis aestivalis*, respectively, were used to drive the GUS reporter gene. Results indicated that the 35S-GUS construct produced the highest level of expression followed by CHS-GUS and AKET-GUS constructs.

Another short-term alternative to test color genes is to transform model crops with defined color mutations, such as transforming *Petunia* with orchid genes (Johnson et al., 1999; see previous section on cloned color genes).

DNA markers to improve breeding strategy. Orchid breeding is a lengthy, time consuming process due to the long life cycle of many commercially important genera. In order to understand the genetic basis underlying the quantitative traits (QTL) and to select for these traits at an early seedling stage, it is important to develop a DNA-based marker system. Chia (1999) communicated the identification of RAPD markers (Random amplified polymorphic DNA) for inflorescence length, flower shape and size in *Dendrobium* orchids. With such marker systems breeders will be able identify the superior off-spring and predict the productivity and flower qualities when the plants are still at the seedling stage.

Another DNA marker system known as DNA Amplification Fingerprinting (DAF) was developed by Chen et al. (1994) to identify different varieties developed at the Taiwan Sugar Corporation to protect the patent rights of their *Phalaenopsis* hybrids. Genomic DNA was amplified by PCR using different sets of primers to distinguish the clonal variation. In addition, authors discussed the importance of establishing a molecular marker system to identify important horticultural traits such as flower color, fragrance and disease resistance. DAF analysis was used to identify DNA markers associated with red flower color in *Doritis pulcherrima* and *Phalaenopsis equestris*. In addition to plant breeding, DNA markers (RFLP, RAPD) can also be used in plant systematics, evolution, and determining phylogenetic relationships.

A PCR-based DNA fingerprinting technique, amplified restriction fragment length polymorphism (AFLP), has been successfully used in the identification of different cultivars of *Aranda* and *Mokara* hybrids (Lim et al., 1999b). In AFLP, the genomic DNA is digested with restriction enzymes and the fragments are ligated to adapters. These fragments are amplified with different sets of standard primers, resulting in a distinct banding pattern. Repro-

ducibility of AFLP was tested with DNA from different tissues and same tissues collected at different times and the results were shown to be consistent. The authors also showed that the variation of polymorphic bands/total number of bands is 10% to 11% among siblings while it is less than 1% among the somatic mutants of the same clone (somaclonal variation). Moreover, subtle phenotypic differences of flower color can be directly linked to some of the polymorphic bands in the AFLP pattern. Therefore, AFLP can be used to identify flower color in segregating progenies in the seedling stage.

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