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Recent Advances in Transgenic Orchid Production

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ABSTRACT

With their attractive flowers and wildly diverse forms, orchids are a mainstay of the global floricultural trade. New varieties with improved floral characters and extended vase-life are continuously being generated by classical breeding techniques, though the selection process is necessarily time-consuming and remains a major obstacle to the rapid production of commercially-valuable orchids. Manipulation of specific floral traits and other desirable characteristics such as flowering time and vase-life by conventional sexual hybridization methods is also practically impossible. In the past decade, researchers have used molecular genetic techniques to revolutionize orchid biotechnology, which typically employs gene transformation systems coupled with rapid selection and regeneration methods for the production of new orchid varieties with the desired traits. Here, we summarize recent findings in transgenic orchid production, particularly with regards to improved transformation methods and the use of novel selectable markers.

Keywords: orchid, genetic transformation, regeneration
Abbreviations: ***bar***, bialaphos-resistance; **GFP**, green fluorescent protein; **GUS**, β-glucuronidase; ***hpt***, hygromycin phosphotransferase; ***luc***, luciferase; **mRNA**, messenger RNA; **MSO**, L-methionine sulfoximine; ***npt*II**, neomycin phosphotranferase type II; ***pflp***, sweet pepper ferredoxin-like protein; **PLBs**, protocorm-like bodies; **PPT**, phosphinothricin; **T-DNA**, transfer DNA; **Ti**, tumor-inducing

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INTRODUCTION

Orchids are members of the Orchidaceae, one of the largest families of flowering plants with an estimated 800 genera and 25,000 species (Atwood 1986). They inhabit a variety of ecological habitats in all continents except Antarctica, though they are most numerous in tropical and subtropical regions (Pridgeon *et al*. 1999). Orchids exhibit highly diverse morphological forms even within the same genus (Dressler 1990), and this diversity in form coupled with their attractive flowers have made numerous orchid species and hybrids extremely popular in the floricultural trade.

While orchids have the typical floral structure of flowering plants, i.e. four whorls of floral organs namely sepals, petals, anthers, and pistils, the sepals in orchids are modified to resemble petals, and are thus termed petaloid sepals (Goh and Arditti 1985). Also, the median petal is usually modified to form a brightly-colored labellum or lip, which varies greatly between species and is frequently the most distinguishable feature of an orchid genus, e.g. *Paphiopedilum*, the slipper orchid.

To satisfy consumer appetites for new and improved orchid varieties, the orchid industry employs classical breeding methods of sexual hybridization and selection to create new orchid hybrids. However, this traditional method is very time-consuming as orchids take at least 3-6 years from seed germination to flowering, and the protracted selection period remains a major hurdle to the rapid production of new orchid varieties. In addition, conventional orchid hybridization techniques are unable to easily manipulate specific floral traits and other desirable characteristics such as vase-life and flowering time to keep up with consumer demand.

With the advent of molecular biology in the last two decades, relatively rapid production and selection of genetically-modified orchids with desired traits now become

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feasible. Gene transfer technology available overcomes many limitations of traditional breeding methods, as it is able to change particular floral traits by genetically engineering specific genes into orchids. The relevant new molecular techniques allow for selection and regeneration of desired orchid plants in far less time than conventional hybridization methods.

Transgenic orchid research has, of late, focused on the establishment and optimization of gene transformation methods, with a particular emphasis on the efficacy of different selectable markers. Here, we summarize recent research in the application of various selectable markers and transformation protocols in orchid genetic engineering, with a discussion of plant regeneration techniques and parameters involved in transformation.

SELECTABLE MARKERS

The choice of an appropriate selectable marker is vital to the success of any plant transformation protocol, and orchids are no exception. Much effort has thus been spent on testing different selectable markers and their corresponding selection agents for efficacy and speed in the selection of transgenic orchids. Several selectable markers such as the neomycin phosphotransferase and hygromycin transferase genes that require the selection agents kanamycin and hygromycin, respectively, are currently popular. In addition, other markers like the bialaphos resistance (*bar*) gene (more commonly used in *Arabidopsis* research) and the sweet pepper ferredoxin-like protein (*pflp*) gene have shown their potential to be potent selectable markers for orchid transformation. Nevertheless, differences in orchid genotype, explant used, and transformation protocol can significantly affect the efficacy of the selectable marker chosen. Researchers therefore have to optimize transformation protocols for each orchid species or hybrid used.

Aminoglycoside antibiotic resistance

Thus far, aminoglycoside antibiotics such as kanamycin and neomycin are the most common type of selection agent used in orchid transformation. A corresponding selectable marker used is the *npt*II gene which encodes neomycin phosphotransferase type II (NPTII), a bacterial enzyme that inactivates several aminoglycoside antibiotics by phosphorylation. When expressed with the appropriate transcriptional promoters in some eukaryotes, NPTII confers resistance to several aminoglycoside antibiotics which would otherwise be lethal to these organisms (Hayford *et al*. 1988).

Kanamycin has been used in many orchid transformation studies, and was shown to be effective in selecting transformants from orchid genera such as *Cymbidium*, *Dendrobium*, and *Phalaenopsis* (Kuehnle and Sugii 1992; Anzai *et al*. 1996; Yang *et al*. 1999; Yu *et al*. 2001). This antibiotic works by affecting mRNA translocation during translation, causing lethal translational errors (Davies *et al*. 1964). However, because orchids have some level of endogenous resistance to kanamycin, high concentrations of the antibiotic ranging from 150-400 mg/l and long selection periods of 3.5 to 9 months are usually required to effectively distinguish transformants from non-transformants. As high antibiotic concentrations used are expensive and can inhibit plant regeneration and growth, and a long plant tissue culture period causes somaclonal variation (Chen *et al*. 1998), kanamycin and other similar aminoglycoside antibiotics are suboptimal for use in orchid transformation studies.

Resistance to other antibiotics

Hygromycin B is an aminocyclitol antibiotic that is also popular as a selection agent for plant genetic transformation. The selectable marker is the *hpt* gene encoding hygromycin phosphotransferase (HPT), an enzyme originally derived from *Escherichia coli*, which detoxifies the antibiotic. Like kanamycin and other aminoglycoside antibiotics, hygromycin B inhibits protein synthesis by interfering with mRNA translation and causing mistranslocation of mRNA (Cabanas *et al*. 1978).

Hygromycin B has been used in the transformation of several orchid genera like *Cymbidium*, *Dendrobium*, *Oncidium* and *Phalaenopsis* (Yu *et al*. 1999; Belarmino and Mii 2000; Liau *et al*. 2003; Men *et al*. 2003a; Chang *et al*. 2005; Chin *et al*. 2007). Various transformation methods were utilized, such as *Agrobacterium*-mediated transformation for *Phalaenopsis* (Belarmino and Mii 2000), *Oncidium* (Liau *et al*. 2003) and *Cymbidium* (Chin *et al.* 2007), and biolistic bombardment for *Dendrobium* (Yu *et al*. 1999; Men *et al*. 2003a).

In recent years, hygromycin B has been more widely used than kanamycin in orchid transformation studies, because of the lower concentrations needed for selection (5-50 mg/l for hygromycin as opposed to 150-400 mg/l for kanamycin) and shorter period (2-7 months) required for elimination of non-transformants.

Herbicide resistance

A less common selectable marker in orchid transformation is the *bar* gene from *Streptomyces hygroscopicus* encoding phosphinothricin acetyltransferase, an enzyme which confers resistance to phosphinothricin (PPT) by acetylating and inactivating the compound (Thompson *et al*. 1987). PPT kills plant tissue by inhibiting the action of glutamine synthetase which detoxifies ammonia released by nitrogen metabolism, resulting in the lethal accumulation of ammonia (de Block *et al*. 1987). Transgenic plants with the *bar* gene are resistant to the selection agents glufosinate and bialaphos, both of which are PPT derivatives found in the commercial herbicides Basta® and Finale®.

Far more commonly used as a selectable marker in the transformation of dicotyledonous and other monocotyledonous plants such as *Arabidopsis*, rice and wheat, the *bar* gene is nevertheless applied in the transformation of *Brassia*, *Cattleya*, and *Doritaenopsis* (Knapp *et al*. 2000). Little to no orchid endogenous resistance to PPT is a major advantage that allows selection of transgenic orchids with very low concentrations of selection agent, along with a relatively short selection period. However, non-specific growth inhibitors found in commercial herbicides prevent their use in solid tissue culture medium (Maughan and Cobbett 2003), and purified PPT and bialaphos are often prohibitively expensive, therefore being unfeasible for use in large-scale orchid transformation.

L-methionine sulfoximine (MSO) has recently been proposed as an alternative selection agent for the *bar* selectable marker in plants (Maughan and Cobbett 2003). MSO, like PPT, is a glutamate analogue which exerts an antibiotic effect by inhibiting the action of glutamine synthetase. Despite the significantly lower cost of MSO, PPT has traditionally been used as the selection agent for *bar* gene expression. However, a recent study has shown that MSO exhibits 40-fold more potency than PPT as a selection agent for *bar* resistance in agar medium (Maughan and Cobbett 2003), and is thus suitable for *in vitro* use. We have tested the use of MSO for selection of the *bar* gene in the orchid hybrids *Dendrobium* 'Madame Thong-In' and *Dendrobium* 'Chao Praya Smile' (Chai *et al*. 2007). This investigation found that only very small amounts of MSO were required for selection of *Dendrobium* orchids, with 5-10 µM MSO sufficient for selection of transgenic *D*. 'Madame Thong-In', and 0.5-2 µM MSO for *D*. 'Chao Praya Smile'. Because of substantial time and economic savings, the utility of MSO as a selection agent for the *bar* gene in *Dendrobium* hybrids will facilitate functional studies on orchid genes by genetic transformation methods.

Pathogen resistance

Antibiotic selection genes have been a source of some concern for some, and several groups have undertaken the

search for new types of markers. One of these is the *pflp* gene, which confers resistance to the selection agent *Erwinia carotovora*, a pathogen that causes soft-rot disease in orchids (You *et al*. 2003). The mechanism by which the sweet pepper ferredoxin-like protein causes disease resistance in plants has not been elucidated, but the protein has demonstrated anti-microbial activity against *Pseudomonas syringae* (Lin *et al*. 1997), and conferred resistance to *Xanthomonas oryzae* in transgenic rice overexpressing the *pflp* gene (Tang *et al*. 2001).

In orchids, the *pflp* gene has been successfully transformed into *Oncidium* and *Phalaenopsis* (You *et al*. 2003; Chan *et al*. 2005). These transgenic orchid plants survived selection with *E. carotovora*, while non-transformed plants could not survive on medium containing the pathogen. Advantages of this system over antibiotic- and herbicide-resistance gene selection are the short time required for identification of putative transformants after *E. carotovora* selection (2 weeks), and the less labor-intensive nature of the work due to fewer subcultures being needed. The *pflp* gene may also confer *E. carotovora* resistance on other orchids, though further studies need to be conducted to optimize this transformation system. Although the *pflp* system is faster than most antibiotic-resistance selection systems, the long *Agrobacterium* transformation period (5 weeks) used in the study (You *et al*. 2003) does not render it significantly superior to the antibiotic- and herbicide-resistance gene transformation systems in terms of time, particularly the *hpt* and *bar* gene selection systems.

Visual selection

Genes coding for green fluorescent protein (GFP), β-glucuronidase (GUS), and firefly luciferase have been used in the visual selection of transgenic orchids. However, it was found that *Dendrobium* tissues have endogenous GUS activity, making it difficult to distinguish transgenic and untransformed tissues (Kuehnle 1997). Furthermore, as the GUS histochemical assay is destructive, necessitating the killing of tissues to be tested (Tee and Maziah 2005), it is not used as the initial selection for orchid transformants, but as one of several assays for transgenic plants selected initially by antibiotics and other means. For example, two groups used the GUS assay as a confirmatory assay of transgenic orchids such as *Dendrobium* and *Phalaenopsis* following antibiotic selection (Men *et al*. 2003a; Liao *et al*. 2004). The following discussion therefore centers on the GFP and luciferase detection assays.

The GFP reporter system uses a fluorescence microscope with a GFP2 filter to visualize plant tissue by masking the red fluorescence of chlorophyll, so non-transformed tissue appear red, while transformed cells expressing GFP will fluoresce green (Tee and Maziah 2005). The firefly luciferase reporter gene (*luc*), however, is detected in transformed cells by the addition of non-toxic luciferin to transformed plant tissue, which is then monitored for bioluminescence using a low-light video microscope system with real-time photon imaging technology (Chia *et al*. 1994). GFP- and *luc*-positive cells can then be counted and recorded over a time period, or separated from non-transformed tissue for further growth.

Both the GFP and *luc* reporter gene systems have been successfully used to select for transgenic *Dendrobium*, and GFP is already widely used in the transformation of monocotyledons and dicotyledons. While these visualization systems are non-destructive and therefore technically better than the GUS histochemical assay, they are extremely time-consuming and labor-intensive, which make this technology clearly unsuitable for application in the orchid industry, though possibly still useful as confirmatory assays.

TRANSFORMATION METHODS

There are several transformation methods which have been employed for the transfer of exogenous genes into orchid tissues, including particle bombardment, *Agrobacterium* transformation, electrophoresis of orchid protoplasts/protocorms, seed imbibition, and transformation via the pollen tube pathway (Kuehnle 1997). The most widely-used method is particle bombardment, followed by *Agrobacterium*-mediated transformation (Mudalige and Kuehnle 2004), as orchids, being monocotyledons, are less amenable to transformation using *Agrobacterium tumefaciens*. Every transformation protocol has various parameters, which can significantly affect the transformation efficiency. Like selectable markers, these parameters need to be optimized for each particular orchid species under study, though parameters that work for one or two species can generally be applied to others in the same genus. The following discussion will focus on parameters involved in the success of particle bombardment and *Agrobacterium*-mediated transformation, with brief descriptions of the various protocols and explants used.

Particle bombardment

Particle bombardment, first described by Klein and co-workers in 1987, refers to the delivery of DNA into plant cells by coating the DNA onto metal microcarriers that are then driven into plant cells by gas acceleration using a particle gun or biolistic transformation system. Plant tissues are subsequently cultured and transformants were selected using various selectable markers as described in the previous section. Particle bombardment is commonly used in orchid transformation due to its relatively higher transformation efficiency over *Agrobacterium*-mediated transformation. The first reports of successful orchid transformation in the genera *Vanda* (Chia *et al*. 1990) and *Dendrobium* (Kuehnle and Sugii 1992) used particle bombardment, while the first example of *Agrobacterium*-mediated transformation in orchids surfaced only several years later (Nan *et al* 1998).

Physical parameters

Numerous factors contribute to the efficiency of transformation using particle bombardment. One group of factors comprises the physical parameters involved in the bombardment procedure, namely helium gas pressure, size and type of microcarriers, and distance between the target tissue and the stopping screen. It is a matter of some disagreement as to whether differences in the helium gas pressure used actually affect transformation efficiency in orchids, with some studies showing no substantial effect (Nan and Kuehnle 1995), and others showing a significant difference in transformation efficiency with varying gas pressures (Tee and Maziah 2005). However, variance in gas pressure has been found to considerably affect transformation efficiency of other plants such as *Eucalyptus* (Rochange *et al*. 1995) and sorghum (Casas *et al*. 1993); hence it is recommended that this parameter should be optimized for each orchid species or hybrid to be transformed.

Early particle bombardment protocols used gunpowder-driven apparatus with DNA coated onto tungsten microcarriers. This method was found to cause a significant amount of cell injury due to the trauma caused by acoustic shock and gas blast, and toxicity of the tungsten particles further resulted in poor transformation efficiency (Russell *et al*. 1992). Helium-driven biolistic devices are now used together with inert gold microcarriers for transformation as they are less injurious to the bombarded cells. Size of the gold particles used was generally found to affect transformation efficiency in orchid transformation studies. Gold particles with a size of 0.6 μm is most efficient for gene delivery into *Cymbidium* (Yang *et al*. 1999), while gold particles with a size of 1.0 μm resulted in increased transient expression of genes inserted in *Dendrobium* as compared to particles of 1.6 μm (Tee and Maziah 2005).

Like gas pressure, the distance between the plant tissue and the stopping screen (in helium-driven biolistic bombardment devices) has not been conclusively shown to af-

fect orchid transformation efficiency, though some studies have reported higher transformation efficiency with certain distances used. For example, Tee and Maziah (2005) found that greatest transient expression of inserted genes was obtained with the distances of 6 cm and 9 cm for two different types of calli. However, there was no statistically significant difference between the two distances used, which is different from our observation that a distance of 9 cm for *Dendrobium* calli results in highest transformation efficiency (Chai *et al*. 2007).

Biological parameters

Several biological factors also contribute to transformation efficiency. Amongst these are orchid genotype, type of plant tissue used for bombardment, and selection conditions. The particle bombardment protocols optimized by researchers for different orchid genera are significantly different, clearly demonstrating that orchid genotype has a significant impact on transformation efficiency. Transformation protocols for various orchid species and hybrids can differ even within a specific genus. For example, the highest GUS transient expression for six *Dendrobium* hybrids was achieved with a bombardment helium pressure of 900 psi (Nan and Kuehnle 1995), while 1100 psi with a target tissue distance of 6 cm was found to be optimal for the transformation of *D. nobile* and *D. phalaenopsis* (Men *et al*. 2003a), and 1350 psi with a target tissue distance of 9 cm for hybrids *D.* Madame Thong-In and *D.* Chao Praya Smile (Chai *et al*. 2007). It is thus recommended that the physical parameters involved in particle bombardment should be optimized for each orchid species and hybrid used (Nan and Kuehnle 1995), though identical parameters can probably be used for hybrids with very similar genetic backgrounds, such as *D.* 'Madame Thong-In' and *D.* 'Chao Praya Smile'. Teixeira da Silva and Tanaka (2008a, 2008b) also optimized the bombardment conditions for several *Cymbidium* hybrids, showing that for all of 8 hybrids, except one, that the optimum parameters were the use of gold particles (as opposed to tungsten), 1100 psi with a target tissue distance of 6 cm, although this varied on the initial tissue type. Expression was measured using GUS.

The orchid tissue type used in the bombardment process can also have a significant effect on transformation efficiency. Nan and Kuehnle (1995) found that the highest transient GUS activity after transformation was observed for protocorm-like bodies (PLBs), followed by etiolated shoots and protocorms. However, as chimerism could occur if orchid embryos and protocorms are used as target tissue (Kuehnle and Sugii 1992), most studies utilize PLBs or calli for bombardment. One study identified three distinct types of calli, and reported that type B callus (light yellow, nodular, and structurally compact) had significantly greater transient GFP expression after bombardment than type A (white or transparent, slightly friable) or type C (yellow and hollow-centered) calli (Tee *et al*. 2003). Choice of tissue type used is therefore important in optimization of a particle bombardment protocol for orchid transformation. In studies by Teixeira da Silva and Tanaka (2007a, 2007b) half-moon protocorm-like bodies or PLBs were important for maximizing transformation efficiency, and this was directly related to the regeneration efficiency. In general, for *Cymbidium* hybrids, the outer layers of the PLB appear to be the most important and totipotent, leading to the greatest level of regeneration, more than any other organ. This directly affects the number of transformants and regenerants that one can obtain, independent of the selection process.

The selection process is an integral part of any transformation protocol, and selection conditions can, to a large extent, determine the successful isolation of real transformants. In particular, selection stringency and the number of recovery days after transformation have a profound effect on transformation efficiency. For example, if selection is performed using very high amounts of the selection agent such as bialaphos, putative transformants may die together with non-transformants before being selected for. On the other hand, too-low amounts of selection agent will result in numerous false positive results from 'escapes'. Timing of selection is also crucial, as plant tissues require a healing period after bombardment on medium with no selection agent to recover from the damage. No transformants are obtained when selection is performed immediately after bombardment (Chai *et al*. 2007). Delayed selection has been shown to adversely affect transformation efficiency, e.g. when transformation frequency was reduced to 0% when selection was initiated 30 days after bombardment for *D. phalaenopsis*, but was as high as 14% when selection was performed 2 days after bombardment (Men *et al*. 2003a).

Agrobacterium-mediated transformation

Agrobacterium tumefaciens is a bacterium that is tumorigenic in plants. The tumor-inducing (Ti) plasmid of *A. tumefaciens* can transfer a portion of its T-DNA into the genome of an infected plant cell, and this ability has been exploited for use in plant genetic engineering by deletion of the tumor-inducing genes from the plasmid and replacing them instead with the DNA sequence (including a selectable marker) intended for transfer. The transformed plants are then grown on media with the appropriate selection agent, and cells without T-DNA integration into their genome will die.

Agrobacterium-mediated gene transfer has proven to be especially efficient for dicotyledonous plants, but is less efficient for monocotyledonous species like wheat and orchids due to their resistance to *Agrobacterium* infection. In recent years, *Agrobacterium* transformation protocols have been developed for rice, but major hurdles remain for other major cereal crops (Shrawat and Lorz 2006). The outlook is slightly more promising for orchids, with successful examples of *Agrobacterium*-mediated transformation for the genera *Dendrobium*, *Cymbidium*, *Oncidium*, and *Phalaenopsis* (Yu *et al*. 2001; Liau *et al*. 2003; Belarmino and Mii 2000; Chan *et al*. 2005; Mishiba *et al*. 2005; Chin *et al*. 2007).

As expected, the physical parameters for particle bombardment are not relevant for transformation using *A. tumefaciens*, while the biological parameters involved in the success of particle bombardment as described above still apply. In addition, the typical three-step protocol of *Agrobacterium* transformation, namely infection, co-cultivation, and selection, incorporates two steps that are absent from the particle bombardment protocol, which introduces additional biological parameters that can affect transformation efficiency.

The initial infection step typically incubates PLBs, or sometimes calli, with fresh overnight cultures of *A. tumefaciens*. The period of infection varies from protocol to protocol, with most studies immersing the plant tissue for 30 mins (Yu *et al*. 2001; Liau *et al*. 2003; Men *et al*. 2003b; Chan *et al*. 2005), and others for up to 10 hours (Belarmino and Mii 2000). However, one study found that a 30-min inoculation period resulted in higher transformation efficiency as compared to longer inoculation periods of 45 min and 60 min (Men *et al*. 2003b). At this stage, addition of a low amount of acetosyringone (100-200 μM) appeared to increase transformation efficiency during *Agrobacterium*-mediated transformation of orchids (Belarmino and Mii 2000; Liau *et al*. 2003; Men *et al*. 2003b), as acetosyringone can induce the virulence (*vir*) genes and facilitate uptake of the Ti plasmid into the plant cells. Acetosyringone is often also incorporated into the medium used in the co-cultivation step for the aforementioned reason.

Length of the co-cultivation period seems to be a source of disagreement amongst different research groups, as some reported higher transformation efficiency with a long co-culture period of 1-2 months (Yu *et al*. 2001; Liau *et al*. 2003), while others used 2-3 days for co-cultivation (Men *et al*. 2003b; Chin *et al*. 2007). As some orchid materials, such as *Phalaenopsis* calli, underwent necrosis and death when the co-cultivation period was too long (Belarmino and Mii

2000), an appropriate co-cultivation period should be optimized to achieve highest transformation efficiency, but least necrosis of transformed tissues.

Other transformation methods

There have been relatively few studies on other orchid transformation methods, particularly in recent years when the focus has been on particle bombardment and *Agrobacterium*-mediated transformation. These methods include seed imbibition, where dry seeds from capsules are imbibed in DNA solution and germinated on selection medium; electrophoresis of protoplasts; and pollen tube-mediated gene transfer, where DNA solution is applied to the stigma (sliced) of a pollinated flower (Kuehnle 1997).

These transformation methods are not popular possibly because of low transformation efficiency and non-optimized protocols. In addition, it is difficult to regenerate whole orchid seedlings from protoplasts, and the screening of tens of thousands of seeds from one capsule is also very tedious. Hence, the research on transgenic orchids looks to continue using the firmly-established methods of particle bombardment and *Agrobacterium*-mediated transformation for some time yet, until breakthroughs in novel transformation strategies appear.

REGENERATION

To be feasible for industrial application, genetic engineering of orchids must be able to produce whole plants. As such, methods of regenerating orchid seedlings from the tissue used for transformation are extremely important, and cannot be neglected in transformation protocols. Fortunately, myriad protocols have been developed for the regeneration of plants from various orchid genera including *Calanthe*, *Dendrobium*, *Oncidium*, *Phalaenopsis*, and *Vanda*, and from different tissue types such as protocorms, PLBs, calli, and other meristematic tissues (Arditti and Ernst 1993).

In one extensively studied orchid genus, *Phalaenopsis*, plantlets were regenerated from PLBs on Vacin and Went (VW; 1949) solid medium containing Nitsch microelements, 100 mg/l *myo*-inositol, 1 mg/l nicotinic acid, 2 g/l activated charcoal, and 3% sucrose, supplemented with 30 g/l potato extract (Tanaka 1992). However, *Cymbidium* PLBs were able to grow into plantlets on basic liquid Murashige and Skoog (MS; 1962) or VW medium, supplemented with nothing more than 10% coconut water (Wang 1988). *Dendrobium* plantlets were also successfully regenerated from PLBs on solid MS, VW, or Knudson C (Knudson 1946) medium with 0.5% sucrose, 5% (w/v) homogenized banana and 0.01% (w/v) activated charcoal (Chia *et al*. 2001).

An optimized protocol for regeneration of plantlets from calli has been developed for *Oncidium* Gower Ramsey, using 1/2 MS basal medium supplemented with 0.1 mg/l naphthaleneacetic acid (NAA) and 3 mg/l 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ; Chen and Chang 2000). Another chemical used to successfully regenerate orchid plantlets is triacontanol (TRIA), which was applied to regenerate shoots from thin shoot tip sections of *Dendrobium nobile* (Malabadi *et al*. 2005).

Recently, a novel method of hormone-free plant regeneration from *Phalaenopsis* protoplasts was developed (Shrestha *et al*. 2007). Cell suspension-derived protoplasts of *Phalaenopsis* Wataboushi were regenerated into calli on hormone-free 1/2 New Dogashima medium, which were further induced to form PLBs on New Dogashima medium with 10 g/l maltose. These PLBs regenerated into plantlets after the carbon source was changed to 10 g/l sorbitol.

Much work has been done on callus induction and plant regeneration from *Cymbidium* orchids lately (Huan and Tanaka 2004). Several studies found that medium composition, biotic factors, and abiotic factors have profound effects on callus induction and plantlet formation from a *Cymbidium* hybrid, *C*. Twilight Moon 'Day Light'. For example, rates of callus induction and PLB formation were highest when PLBs (whole, in segments, or as thin-cell layers) were used as explants, rather than the bases and tips of leaves or roots (Teixeira da Silva *et al*. 2006b). Addition of plant growth regulators such as NAA and kinetin, activated charcoal and coconut water was also found to increase PLB formation (Teixeira da Silva *et al*. 2006a), thus validating the use of these additives in callus and PLB induction and plantlet regeneration for other orchid genera by researchers in past decades.

Given the differences in plantlet regeneration protocols that have been formulated for various orchid genera, it is clear that there is no 'standard' regeneration protocol to use. Orchid tissues of different genera also have varying affinities for the various sugars (e.g. glucose and fructose) that can be used in culture (Hew and Yong 2004), further exacerbating the problem of trying to find a generic regeneration protocol useable for most orchid genera. However, nearly all regeneration protocols in orchid transformation studies use essentially the same medium as for post-transformation selection, without the selection of antibiotics or herbicides. This indicates that in most cases, the solid growth medium used for screening of transgenic tissues needs only to be slightly modified for further plantlet regeneration.

In addition, some recommendations can be made for optimization of the culture components for growth media prior to seedling transfer to the greenhouse or field. Recent findings indicate that hyperhydricity of *in vitro* plantlets is due to low light intensity, high humidity, and high levels of minerals, carbohydrates and plant hormones. These factors contribute to physical anomalies in plants that reduce their ability to survive outside of a tissue culture container (Hew and Yong 2004). As sugar present in the culture medium discourages photoautotrophy of plantlets, reducing or removing sugar and increasing light intensity to the cultures could increase photosynthetic rate of orchid seedlings, and therefore reduce vitrification.

CONCLUSION

Significant advances have been made in the area of transgenic orchid production, with novel selectable markers and ever-faster transformation protocols being developed; however, there remains much to be done. While the industrial relevance of this field cannot be denied, few transgenic orchid varieties produced have been available to the commercial market, and the gap between lab theory and field application still exists. Even so, the progress made in this field has been encouraging as orchid transformation protocols have already been developed and optimized for maximal efficacy. Researchers now need to incorporate potentially useful genes into their plasmid vectors, such as genes conferring virus resistance, early flowering, increased shelf-life, or novel flower colors and patterns. Meanwhile, application of orchid genetic transformation has greatly facilitated molecular genetic studies of orchid developmental genes, which can in turn provide more gene resources for genetic engineering of orchids. Thus, recent advances in transgenic orchid production have shed light on both industrial application and the advancement of fundamental knowledge.

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