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Optimization of Particle Bombardment Conditions for Hybrid *Cymbidium*

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ABSTRACT

The conditions to maximize β-glucuronidase (GUS) transient and stable expression in protocorm-like bodies (PLBs) and embryogenic callus of hybrid *Cymbidium* Twilight Moon 'Day Light' through particle bombardment have been optimized using two plasmids, pBI121 and pWI-GUS. Although callus tended to be much more sensitive to particle bombardment than PLBs, thus affecting regeneration, it was possible to obtain conditions for high level transient and stable GUS expression. The conditions for callus and PLBs were: microcarrier (gold for both explant types), number of shots (2 for both explant types), rupture disk pressure (1100 and 1350 psi), target distance (6 cm for both explant types), explant pre-culture (3, 6 or 9 days for both explant types) period, respectively.

Keywords: biolistics, GUS, microcarrier, rupture pressure, target distance
Abbreviations: NAA, α-naphthaleneacetic acid; **PLB**, protocorm-like body; **PGR**, plant growth regulator; **TDZ**, thidiazuron (*N*-phenyl-N-1,2,3-thidiazuron-5'-ylurea); **VW**, Vacin and Went

INTRODUCTION

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 (*npt*) and hygromycin transferase (*hpt*) 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 (reviewed in Chai and Yu 2007).

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). 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*. 2003). The *pflp* gene, which confers resistance to the selection agent *Erwinia carotovora*, a pathogen that causes soft-rot disease in orchids, has been successfully transformed into *Oncidium* and *Phalaenopsis* (You *et al*. 2003; Chan *et al*. 2005).

One of the most effective ways of achieving *Cymbidium* tissue culture is through the culture of protocorm-like bodies (PLBs). Embryogenic callus in *Cymbidium* has been induced either from PLB outer epidermal tissue (Begum *et al*. 1994b; Huan and Tanaka 2004a, 2004b; Huan *et al*. 2004), or inner PLB tissue (Begum *et al*. 1994a) in *Cymbidium* hybrids, or from pseudobulb sections, rhizomes and roots of seedlings of *C. ensifolium*, a terrestrial orchid species (Chang and Chang 1998), induction being rapid in the former but slow in the latter. PLB formation in *Cymbidium* hybrids using PLB thin cell layers, conventional PLB segments and other explant types has been studied (Teixeira da Silva and Tanaka 2006) to test the effect of medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a), vessel type (Teixeira da Silva and Tanaka 2009a), gelling agent and other media additives (Teixeira da Silva and Tanaka 2009b) on PLB and callus formation.

In this study, we establish the ideal conditions for the successful integration of the β-glucuronidase (GUS) gene (*uid*A) using particle bombardment through the use of PLBs and embryogenic callus from conventional PLB segments of epiphytic hybrid *Cymbidium* Twilight Moon 'Day Light', a popular hybrid. This serves as a first step to inducing disease resistance in *Cymbidium*. Since the GUS assay was used as a confirmatory assay of transgenic orchids such as *Dendrobium* and *Phalaenopsis* following antibiotic selection (Men *et al*. 2003; Liao *et al*. 2004), we too have used it here for hybrid *Cymbidium*.

To date, only two studies (Yang *et al*. 1999; Chen *et al*. 2007) have reported on the genetic transformation of *Cymbidium* using particle bombardment. In both cases, hybrids were not used. This study therefore constitutes the first report on the genetic transformation of *Cymbidium* hybrids by particle bombardment.

MATERIALS AND METHODS

Chemicals and reagents

All plant growth regulators (PGRs) were purchased from Sigma-Aldrich (St. Louis, USA) and were of tissue culture grade. All other chemicals and reagents were of the highest analytical grade available and were purchased from either Wako (Japan) or Nacalai Tesque (Japan), unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U,

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Japan) originated from shoot-tip culture on Vacin and Went (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium or VW_{PLB}) every two months on modified VW supplemented with 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA) and 0.1 mg l^{-1} kinetin, 2 g l^{-1} tryptone and 20 g l^{-1} sucrose, and solidified with 8 g l^{-1} Bacto agar (Difco Laboratories, USA). Callus induction and proliferation medium (VW_{CALLUS}) was similar to VW_{PLB}, except that thidiazuron (TDZ) was used instead of kinetin. All media were adjusted to pH 5.3 with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100 ml Erlenmeyer flasks, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 µmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally bisected PLB (3-4 mm in diameter) segments, 10 per flask, were used as explants for PLB induction and proliferation and for all experiments. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a) for PLB and callus induction, formation and proliferation.

Particle bombardment conditions

A series of six trials were conducted to select optimal particle bombardment conditions. PLBs or embryogenic callus derived from culture on VW_{PLB} or VW_{CALLUS} medium for 90 or 45 days, respectively were used as the material for bombardment using a Bio-Rad Biolistic PDS-1000/He® particle delivery system. All material tested in these trials was purchased from Bio-Rad and all experimental procedures followed the manufacturer's guidelines and recommendations. For all trials two plasmids were tested: pBI121 (Clontech) or pWI-GUS (see Anzai *et al*. 1996 for description) (plasmids kindly provided by Prof. Hiroyuki Anzai). Both plasmids contain the *npt*II and *uid*A genes, the former coding for kanamycin resistance. Explants were subjected to the following trials:

Trial 1: Effect of number of shots (either one or two rounds of particle bombardment).
Trial 2: Effect of microcarriers, which were either gold or tungsten at ~0.8 µg plasmid DNA/500 µg microcarriers.
Trial 3: Effect of rupture disk pressure (900, 1100, 1350 p.s.i.).
Trial 4: Effect of explant pre-culture period (3, 6 and 9 days).
Trial 5: Effect of target distance (3, 6 and 9 cm).

GUS testing

GUS testing was measured in freshly bombarded explants (PLBs or callus) following incubation overnight at 37°C in a histochemical GUS assay (Jefferson *et al*. 1987). Following incubation, GUS expression was recorded and measured as the number of GUS focal points (GFPs) 72 h after particle bombardment. Explant survival was also checked at the same time intervals.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 20 replicates per treatment for callus ad 60 replicates per treatment for PLBs (one replicate was either a 0.5 cm^2 callus piece or a PLB half-segment placed cut surface down on a single sterilized sheet of Whatman No. 1 filter paper in a circular area of 3 cm in diameter in Petri dishes overlaying VW_{PLB} or VW_{CALLUS} medium). Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's New Multiple Range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Hybrid *Cymbidium* expressing transient and stable transgenic GUS (**Fig. 1**) could be obtained when either PLBs or callus were used as the initial starting material. The bombardment conditions were optimized since the level of transient transgene expression will impact on stable transgene expression, as observed with ornamental chrysanthemums (Teixeira da Silva and Fukai 2002).

Fig. 1 Stable GUS expression (A) in pWI-GUS transformants (B) 8 months following particle bombardment growing on kanamycin (30-50 mg/l)-supplemented medium.

Two shots resulted in significantly higher GUS spots or GFPs per explant (both PLBs and callus) than one shot (**Table 1**) without impacting explant mortality. Gold was superior to tungsten as the microcarrier (**Table 1**). A 1100 p.s.i. rupture pressure gave significantly higher GFP values than either 900 or 1350 p.s.i. for both PLBs and callus but for callus explant mortality was higher at 1110 than at 900 p.s.i. (**Table 1**). A 6-cm target distance was ideal for both explant types (**Table 1**). Pre-culture period did not affect GFP formation in either explant type (**Table 1**). The trend was the same for both plasmid types (pBI121 and pWI-GUS) although the latter plasmid resulted in significant higher GFP values than the former, but with no difference in explant survival (data not shown).

The first reports of successful orchid transformation in the genera *Vanda* (Chia *et al*. 1990, 1994) 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).

Varying gas pressure had no substantial effect on transformation efficiency in *Dendrobium* (Nan and Kuehnle 1995), but did affect it significantly in other genotypes (Tee and Maziah 2005). 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 affect 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).

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

Table 1 Effect of different particle bombardment conditions on *Cymbidium* Twilight Moon 'Day Light' PLB and callus transient transformation efficiency.

Trial No.	Conditions	Explant survival (%)	No. GUS spots/PLB explant	No. GUS spots/callus cluster	Other notes
1: No. of shots					
	1	98 a	12.61 ± 1.08 b	4.68 ± 0.23 b	
	2	53 b	19.34 ± 0.98 a	7.18 ± 0.56 a	
2: Microcarrier choice					
	Gold	96 a	12.61 ± 1.08 a	4.68 ± 0.23 a	
	Tungsten	98 a	3.46 ± 0.61 b	1.23 ± 0.08 b	
3: Rupture disk pressure (p.s.i.)					
	900	100 a	1.09 ± 0.34 c	0.06 ± 0.02 c	
	1100	92 a	12.61 ± 1.08 a	4.68 ± 0.23 a	
	1350	46 b	9.86 ± 1.04 b	1.76 ± 0.38 b	Deformed shoots
4: Explant pre-culture period (days)					
	3	92 a	11.31 ± 1.68 a	3.98 ± 0.43 a	
	6	96 a	12.61 ± 1.08 a	4.68 ± 0.23 a	
	9	96 a	13.52 ± 0.88 a	4.26 ± 0.48 a	
5: Target distance (cm)					
	3	71 b	1.43 ± 0.61 b	0.28 ± 0.09 b	Deformed shoots
	6	92 a	12.61 ± 1.08 a	4.68 ± 0.23 a	
	9	96 a	2.63 ± 0.33 b	0.36 ± 0.12 b	

*= control; CV = culture vessel (see text for explanation of codes).
Data scored 72 h after particle bombardment and represent the mean ± SD (standard deviation) of at three replicates of $n = 20$ each. In each column and for any ONE trial, the values with different letters are significantly different ($P \leq 0.05$) according to DNMRT (Duncan's new multiple range test) or according to the χ^2 test ($P \leq 0.05$) for percentage values.
Shaded conditions indicate conditions that were assumed as standard while testing other conditions in different trials.

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'.

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.

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*. 2003).

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