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# Genetic transformation of *Cymbidium* orchid by particle bombardment

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**Abstract** A protocol is presented for genetically engineering *Cymbidium* orchid using particle bombardment. This protocol enabled the routine transformation of orchid plants that were previously difficult to transform. Liquid culture was used to generate a large number of protocorm-like bodies (PLBs) to be bombarded and to promote continued development of the bombarded meristematic tissue. Plasmid DNA (pKH200) carrying the GUS-INT and NPTII genes flanked by tobacco matrix attachment regions was introduced into the meristematic cells of PLBs by particle acceleration. The transformed PLBs were proliferated and selected for kanamycin resistance conferred by the introduced NPTII gene. Shoot regeneration was then induced from the kanamycin-resistant PLBs, and transgenic plantlets were produced. Both the kanamycin-resistant PLBs and regenerated shoots expressed the GUS-INT gene. The presence of the introduced gene in the transformed orchid plants was confirmed by PCR analysis, sequencing and Southern blot analysis of the PCR product. The recovered transgenic plants were established in soil and acclimatized in the greenhouse.

**Key words** Orchid · Particle bombardment · PLB · Transformation

**Abbreviations** *BA* Benzyladenine · *GUS-INT* $\beta$-glucuronidase gene with intron · *MS* Murashige and Skoog (1962) · *NAA* $\alpha$-naphthalene acetic acid · *NPTII* neomycin phosphotransferase gene · *PLB* protocorm-like body · *psi* pounds per square inches

**Key terminology** *Protocorm* A small storage organ formed from the germinating embryo, possessing an apical meristem and a leaf primordium · *protocorm-like body (PLB)* A somatic protocorm derived from in vitro culture of apical or axillary bud meristems · *primary PLBs* PLBs induced by culturing apical meristem-tips aseptically · *secondary PLBs* PLBs formed on the surface of a primary PLB in culture · *proliferate PLBs* PLBs proliferating on the surface of either primary or secondary PLBs

## Introduction

There is substantial interest in the genetic improvement of orchids. Orchids form the largest family of flowering plants with more than 800 genera and over 25,000 species that are commercially grown globally (Arditti 1992). Genetic modification of orchids for disease and stress resistance, precocious flowering, and the improvement of flower color and morphology is thus of major commercial importance. Orchid improvement by conventional plant breeding methods has been limited mainly due to the prohibitively long reproduction cycles (several years) and slow seed maturation (several months) of these plants. Therefore, the application of genetic engineering techniques to orchid improvement appears to be an attractive alternative.

Although transformation protocols are available for the members of genus *Dendrobium*, there is no report available on the transformation of *Cymbidium* orchid. Kuehnle and Sugii (1992) reported obtaining transgenic

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*Dendrobium* plants from the protocorm-like bodies (PLBs) using particle bombardment. Transformation was confirmed by kanamycin selection and polymerase chain reaction (PCR) analysis of the putative transgenic orchids. Chia et al. (1994) transformed *Dendrobium* orchid using particle bombardment. To overcome the inherent low sensitivity of the orchid to kanamycin (requiring more than 500 mg/l kanamycin for selection), they developed an alternative selection method that relied on the expression of the introduced firefly luciferase gene. In this system, light-emitting cell clumps were screened and isolated using a photon-counting video camera-photomultiplier system and a high-power dissecting microscope. The screening and isolation process was repeated until a pure transgenic cell line was isolated. The isolated cell line was then induced to regenerate into the whole plant. Because of the expensive equipment requirement and the time-consuming microscopic screening of transgenic cells, this technique seems to be unrealistic for the practical use of a genetic transformation approach for orchid improvement.

We conducted a number of studies to develop a stable gene transfer system for *Cymbidium* orchids using particle bombardment. We studied several factors involved in particle bombardment-mediated transformation. Those factors included bombardment conditions, pre- and post-bombardment culture conditions, and selection. Here, we report a reproducible method for the gene transfer and regeneration of transformed plants from the *Cymbidium* PLBs using particle bombardment. A key to the transformation success was to adopt the liquid culture system to stimulate active proliferation of the meristematic tissues pre- and post-bombardment and to develop a selection regime that minimizes the toxic influence of dying cells on neighboring transformed cells.

## Materials and methods

PLBs, subcultured monthly for 6 months, were purchased from a commercial orchid laboratory where apical meristems were isolated and induced to form PLBs. The PLBs were maintained at 15 °C until use on Knudson orchid medium (Duchefa Biochmie BV, Izaak Enchedeweg 40, 2031 CS Haarlem, The Netherlands) supplemented with 5% sucrose, 10.7 $\mu M$ $\alpha$-naphthaleneacetic acid (NAA), 0.1% charcoal, and 0.7% Bacto-agar (pH 5.4). All of the cultures except for the maintenance one at low temperature used MS medium supplemented with or without 0.7% Bacto-agar. The culture room was maintained at 25 °C with a 16-h photoperiod supplied by cool-white fluorescent bulbs following the general conditions described previously (Han et al. 1997a).

The plant gene expression vector pKH200 carries $\beta$-glucuronidase with intron (GUS-INT), NPTII (Han et al. 1997b); NPTII is under the control of the NOS promoter and GUS-INT is driven by a 35 S promoter. The NPTII and GUS-INT genes are both flanked by matrix attachment region fragments (MARs) derived from a tobacco genomic clone (Hall et al. 1991). Figure 1 schematically illustrates the T-DNA region of plasmid pKHZ200. We used kanamycin as the selective agent in all transformation experiments. The basic transformation protocol is as follows.

Steps in orchid transformation

1) Primary PLBs are cultured for 7 days prior to bombardment in a liquid MS medium without growth regulator with slow gyratory shaking (50–100 rpm), and then placed in a circular area 1–2 cm in diameter on MS medium supplemented with 3% sucrose and 0.7% agar in a 60-mm petri dish which served as the target surface.
2) Plasmid DNA is precipitated in the presence of gold particles as described by Klein et al. (1987).
3) The pre-cultured PLBs are bombarded using a Model Biolistic® PDS-1000/He (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, Calif.) following the manufacturer's instructions. The bombardment chamber was evacuated at a pressure of 28 inches of mercury. Each bombardment delivered approximately 500 μg of particles associated with 0.42 μg of plasmid DNA in a total slurry volume of 6 μl. All bombardments were carried out with pressurized helium in the range of 1,100–1,550 psi, at a range of approximately 60 mm from the stopping plate to the surface of the treated tissue.
4) The bombarded PLBs are cultured for a period of 10–45 days in a 250-ml Erlenmeyer flask containing 50 ml MS liquid medium, with shaking (50–100 rpm) to induce proliferate PLBs.
5) PLBs having numerous proliferate PLBs are then transferred to a 90-mm petri dish containing 25 ml MS medium supplemented with 0.7% agar, 11.3 $\mu M$ BA, 2.7 $\mu M$ NAA, and 100–200 mg/l kanamycin. The cultures were sub-cultured weekly to fresh medium.
6) Regenerated shoots are further screened by rooting on MS medium supplemented with 200 mg/l kanamycin but without growth regulator.

For factor optimization, three different sizes of gold particles (0.6, 1.0 and 1.6 μm) were coated with plasmid DNAs and bombarded into the PLB explants at different helium gas pressures (1,100, 1,350, and 1,550 psi). Five different concentrations (0, 50, 100, 200, 500 mg/l) of kanamycin were tested for their effectiveness in suppressing (or killing) the growth of non-transformed PLBs. We compared two culture methods, liquid versus solid, for their effectiveness in inducing proliferation of PLBs. For solid culture, 50 PLBs were plated in a 90-mm petri dish containing 25 ml of MS medium supplemented with 0.7% agar, 3% sucrose, but without growth regulator. Liquid culture was done using 40 PLBs in a 250-ml Erlenmeyer flask containing 50 ml MS liquid medium, with gyratory shaking (50–100 rpm). Experiments were repeated three times and the results pooled.

Subsets of bombarded PLBs were sampled at various times (1, 3, 5, 7 days and 3 months) to determine GUS activity. GUS expression in transgenic plants was assayed using either leaf segments or the whole plant 9 months after the bombardment. The GUS expression was assayed by histochemical staining according to Jefferson et al. (1987).

Genomic DNA for PCR was isolated using the method described by Junghans and Metzlaff (1990). Amplification of the NPTII gene was performed with *Taq* polymerase in a three-temperature program (94 C for 1 min, 55 °C for 1 min and, 72 C for 2 min) using the primers 5'ATTGCACG-CAGGTTCTCCGG3' and 5'AGAACTCGTCAA-GAAGCGGA3' (Franche et al. 1997). The PCR product was confirmed both by Southern blot analysis using the NPTII gene as a probe (Han et al. 1997a) and sequencing using an ALF automatic sequencer (Perkin Elmer Co.).

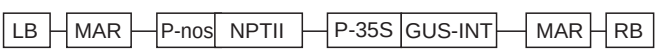


**Fig. 1** Schematic map of the T-DNA region of the plasmid pKH200. The relative position of the GUS-INT gene and the NPTII gene are flanked by the tobacco matrix regions (*MAR*s) and shown with respect to the left border (*LB*) and right border (*RB*) of the T-DNA. The T-DNA regions are not drawn to scale

## Results and discussion

### Pre-bombardment culture of PLBs

We compared two culture methods (liquid versus solid) for PLB proliferation. The liquid culture with slow shaking yielded significantly higher PLB proliferation than did solid culture (Table 1). The liquid culture technique was therefore adopted to stimulate meristemoid development from the PLBs prior to the bombardment. Actively dividing cells provided a window of competence for transformation. Tobacco cells bombarded at the M- and $G_2$-phases have 4–6 times higher transformation efficiencies than those at the S- and $G_1$-phases (Iida et al. 1991). T-DNA integrates preferentially into sequences that can potentially be transcribed (Herman et al. 1990; Kertubundit et al. 1991; Koncz et al. 1989). Studies on the integration of T-DNA into the plant genome strongly suggest that host DNA synthesis is required (Gheysen et al. 1987). Thus, the greater the number of actively dividing cells in the tissue being bombarded, the higher probability of obtaining stable expression in the bombarded tissue. In the present protocol it was critical that the PLBs were bombarded after the tissue was pre-cultured in liquid medium for a time at least sufficient to induce the formation of superficial meristematic tissues (i.e., secondary PLBs) on the target tissue.

Rapid proliferation of PLBs was observed after 3 days of culture and continued past 10 days (Table 1). The pre-cultured PLBs were subjected to bombardment after 7 days when the size of the newly forming secondary PLBs reached 1 mm in diameter. At this stage the newly forming PLBs were visible and presumed to be very active in cell division. Cells hit by the DNA-coated particles at this stage were likely to be the cells from which proliferate PLBs are derived. Although different sizes of PLBs were not tested, optimizing the pre-culture period for the right-sized PLBs may be an important factor in transformation success. A developmental window of competence for agroinfection has been observed in the apical meristems of immature maize embryos at different developmental stages (Schlappi and Hohn 1992). Ellis et al. (1993) observed that the percentage of spruce somatic embryos expressing the GUS gene after bombardment increased progressively with advanced developmental stages.

**Table 1** Comparison of the two different culture methods on *Cymbidium* PLB proliferation

| | Days | Mean number of PLBs[a] | |
|---|---|---|---|
| | | Liquid culture | Solid culture |
| Pre-bombardment | 0 | 50 (1.0) | 50 (1.0) |
| | 3 | 69 (1.38 ± 0.04) | 55 (1.10 ± 0.03)*[a] |
| | 5 | 110 (2.21 ± 0.23) | 75 (1.51 ± 0.03)* |
| | 7 | 160 (3.20 ± 0.22) | 101 (1.99 ± 0.09)* |
| | 10 | 194 (3.88 ± 0.10) | 121 (2.34 ± 0.20)* |
| Post-bombardment | 0 | 282 (1.0) | 308 (1.0) |
| | 30 | 2177 (7.72 ± 0.25) | 1294 (4.20 ± 0.08)* |

* Means separated by LSD (Steel and Torrie 1980) are significant at 0.05 probability level

[a] Mean number of PLBs proliferated at the end of each culture period. Number in parentheses is PLB multiplication rate ± SE. The multiplication rate is expressed as total number of PLBs resulting from multiplication divided by the number of initial PLBs

### Bombardment and subsequent culture of PLBs

The secondary PLBs developed on the surface of the primary PLB and remained associated during bombardment. Those PLB clusters containing both primary and secondary PLBs were bombarded. Among three different sizes of gold particles (0.6, 1.0, and 1.6 μm), the smallest particles were most efficient in delivering the genes when judged by GUS staining 3 days post-bombardment (Table 2). Different levels of helium gas pressures (1,100, 1,350, and 1,550 pounds per square inch) did not affect transformation efficiency (data not shown).

Bombarded PLBs were first induced to form proliferate PLBs by being incubated in a medium without kanamycin for 30-days. The use of the liquid culture with slow shaking produced significantly higher number of PLBs than did the solid culture over a 30 day culture period (7.7 vs. 4.2 times; Table 1). A longer culture period of 45 days in the liquid medium caused PLB swelling and tended to develop shoots from the PLBs (Yang and Han, unpublished observation). These shoots were likely to be chimeric, and most died off during subsequent selection. The initial 30 days of culture without kanamycin selection was intended to promote active proliferation of PLBs on the surface of the bombarded PLBs. The proliferate PLBs are then induced to regenerate shoots under kanamycin selection. This sequential manipulation was critical for transformation success as prior attempts to obtain transformants from PLBs transferred directly to solid medium containing kanamycin after bombardment failed.

**Table 2** Effect of particle size on the transformation of *Cymbidium* PLB. GUS activity was assayed 3 days after bombardment

| Particle size (μm) | Number of PLBs bombarded | Mean no. GUS foci[a] ± SE | Percentage PLBs expressing GUS[a] |
|---|---|---|---|
| 0.6 | 40 | 3.3 ± 0.3 a | 87 a |
| 1.0 | 40 | 2.3 ± 0.2 b | 71 ab |
| 1.6 | 40 | 1.2 ± 0.2 c | 55 b |

[a] Means with different letters are significantly different at the 0.01 probability level. Percentage data were arcsin-transformed (Steel and Torrie 1980) before analysis to improve normality

Orchid plants in culture produce large amounts of phenolics that are released by bombardment wounding. Use of the liquid culture reduces the toxicity of the phenolic oxidation products (Bonga 1981). Also, omitting kanamycin selection for 30 days after bombardment ensures prolific growth of the bombarded cells while avoiding the toxic influence of dying cells on the neighboring transformed cells during the initial selection.

The use of dominant selectable markers is an integral part of transformation strategies. The sensitivity of plant cells to a selectable marker depends on the genotype, the physiological condition, size and type of the explant, and tissue culture conditions. Therefore, the minimum level of a selection agent that can fully inhibit the growth of un-transformed cells should be determined for each transformation and regeneration system. Orchid is known to be resistant to commonly used aminoglycosides such as kanamycin and often requires more than 500 mg/l kanamycin to select transgenic cells (Chia et al. 1994). To evaluate the sensitivity of *Cymbidium* orchid cells to kanamycin, we subcultured a total of 846 PLBs from three different genotypes onto the solid medium supplemented with various levels of kanamycin (0, 50, 100, 200, 500 mg/l). All of the PLBs cultured on 100 mg/l or higher died 45 days after the subculture.

## Presence and expression of the introduced genes in orchid cells

GUS expression was observed in transient gene expression following particle-mediated transformation and in virtually all of the plant tissues after selective regeneration. Three days after the introduction of pKH200 (Fig. 2) by bombardment, all bombarded PLBs expressed GUS activity (Fig. 3A). High transient GUS expression was maintained (more than 100 GUS foci per PLB) for 3 days and then declined rapidly (less

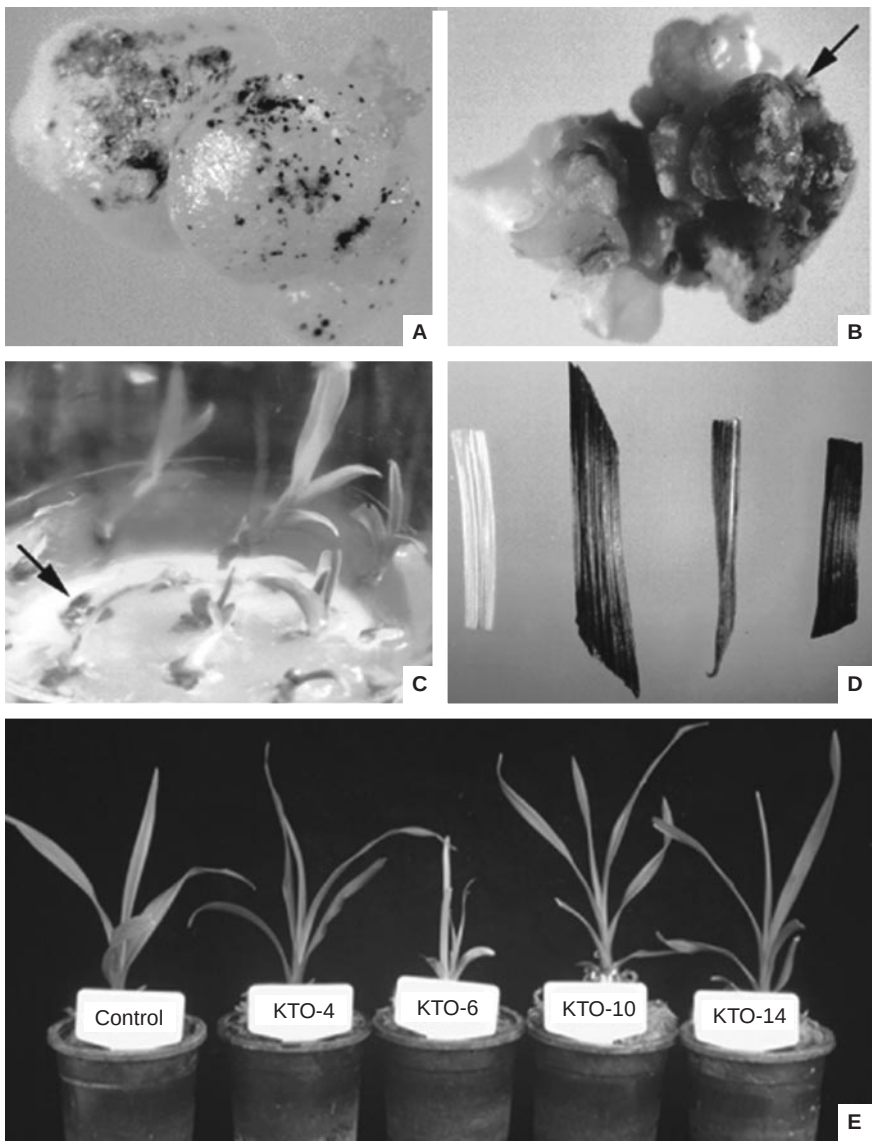


**Fig. 2A–E** Regeneration of transgenic orchids and histochemical detection of GUS-INT gene expression in different phases of transformation. **A** A PLB showing transient GUS-INT gene expression 24-h post-bombardment, **B** a PLB differentiating into a secondary PLB that is transformed as evidenced by the GUS-INT expression 3-months post-bombardment, **C** regeneration of transgenic orchid plants in the presence of 200 mg/l kanamycin; *arrow* indicates a dying PLB, **D** leaf samples from three transgenic orchid plants were assayed for GUS-INT gene expression; the *far left* sample is from a non-transgenic plant and all of the other three (stained *blue)* are from transgenic plants, **E** transgenic orchids established in soil

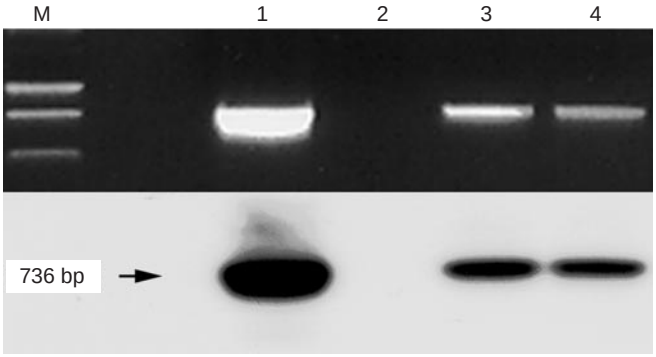


**Fig. 3** Upper panel PCR-amplified fragments of the NPTII gene from different transgenic orchid plants. *Lane M* 750-bp size marker, *lane 1* plasmid pKH200 DNA, *lane 2* untransformed orchid plant, *lanes 3*, *4* transgenic orchid lines KTO 3 and 8, respectively. Lower panel The PCR products were blotted and cross-linked to Hybond-N nylon membrane (Amersham) by UV radiation. The probe for both hybridizations was a [$^{32}$P]-labelled NPTII gene (PCR-amplified 736-bp fragment). Hybridization was for 18 h at 65 C in 0.7 *M* sodium chloride, 40 m*M* sodium phosphate (pH 7.6), 4 m*M* EDTA, 0.1% (w/v) SDS, and 200 μg salmon sperm DNA. The blot was washed at 65 C twice in 1×SSP, 0.5% SDS and twice in 0.2×SSP, 0.5% SDS. The membrane was exposed to an X-ray film for 2 h at –70 C

than 10 foci per PLB) by 5 days post-bombardment. This loss of expression may be a major obstacle to the selection of transformants in an orchid system that is characterized by slow growth in tissue culture. Thus, the culture regimes that stimulate post-bombardment cell division will provide a larger window for transformed cells to differentiate into PLBs. Long-term and stable expression of the introduced GUS gene was also achieved in differentiating PLBs and tissues of the regenerated kanamycin-resistant plantlets (Fig. 2B,C). The vector pKH200 carries both GUS-INT and NPTII flanked by the MAR (matrix attachment region) elements (Hall et al. 1991). Use of an intron-containing GUS gene is generally preferred as the gene with intron is expressed only in eukaryotic (i.e., plant) cell, not in any contaminating microorganisms. The use of MAR elements significantly increased the expression of flanked genes in poplar compared to the genes not flanked by the MARs (Han et al. 1997b). Any beneficial effect of the elements in orchid cells is unknown.

Kanamycin selection screens the relatively rare stable integration events from high transient activity levels. With poplar, the escape rate is below 3% (Han et al. 1997b). Transgenic PLBs survived the kanamycin selection whereas the un-transformed PLBs were effectively killed with as low as 50 mg/l kanamycin in 6 weeks. A high proportion (69.4%) of kanamycin-resistant shoots were GUS-positive. It is possible that the GUS negatives among the selected shoots may be escapes. However, we observed that some GUS-negative plants turned out to be positive in PCR analysis for NPTII gene. In particle bombardment, separate incorporation of the genes of a vector is possible.

Since it was not possible to obtain enough genomic DNA for Southern blot analysis due to the slow growth of transgenic orchid plants, we used the PCR technique combined with sequencing analysis. Figure 3 shows the PCR amplified products using NPTII-specific primers. The size of the PCR products matches the expected size on the map of the plasmid pKH200. Furthermore, sequencing and Southern blot analysis of the amplified DNA confirmed that the PCR was NPTII-specific (Fig. 3).

## Transformation protocol

The keys to the success of this transformation protocol are in the pre- and post-bombardment culture phases. Pre-bombardment cultures are intended to stimulate continued development of meristematic tissues (secondary PLBs) by culturing the PLBs in a liquid medium without growth regulator. The bombarded PLBs were cultured in a liquid medium without kanamycin for 30 days to promote the proliferation of PLBs rather than shoot regeneration, and then the proliferate PLBs were subjected to selection. This approach produced transgenic *Cymbidium* orchids. A total of 600 PLBs were initially bombarded and subsequently propagated for 30 days after bombardment. Approximately 4,600 proliferate PLBs have been produced from the post-bombardment PLB propagation. Of the initial 600 PLBs, 28% survived the kanamycin selection 10 months post-bombardment and culture. Ninety-two shoots were obtained from these kanamycin-resistant PLBs (Table 3). Of these kanamycin-resistant shoots, 52 were assayed histochemically for GUS expression, 69% of the shoots expressed GUS gene in their leaves.

Orchids are substantially different from other plants in their requirements for a transformation system. First, the orchid cells have a low rate of proliferation. Second, the orchid cells are recalcitrant to tissue cultural manipulations. Plant regeneration from de-differentiated cells has not been achieved for orchids. Third, the orchid cells in tissue culture exude a large quantity of phenolics that become toxic to the cells when oxidized. Finally, no *Agrobacterium*-mediated transformation has been reported for orchids. The particle bombardment of organized and easily regenerable tissue such as PLBs allowed us to regenerate transgenic orchids. The advantage in this approach is that shoots develop directly from the primary and secondary meristems without an intervening explant-organogenesis phase. This minimizes the necessity for treatment with phytohormones and thus the opportunity for somaclonal variation. Its limitations are a low transformation frequency and extensive chimerism as observed with soybean (Christou et al. 1989) and papaya (Fitch et al. 1990). However, recent reports suggest that non-chimeric transformants can be produced by the direct delivery of DNA into the cells

**Table 3** Transformation frequency using the protocol described in this report (*km*$^r$ kanamycin resistant)

| Experiments[a] | Number of PLBs bombarded | Number of km$^r$ shoots (%) | Number of km$^r$ PLBs (%)[b] | Number of GUS positive/ km$^r$-shoots (%) |
|---|---|---|---|---|
| 1 | 100 | 8 | 20 | 0/5 (0) |
| 2 | 100 | 39 | 24 | 12/18 (66.7) |
| 3 | 100 | 27 | 18 | 17/20 (85) |
| 4 | 100 | 7 | – | 2/3 (66.7) |
| 5 | 100 | 9 | 7 | 5/6 (83.3) |
| 6 | 100 | 2 | 6 | N/A |
| Total | 600 | 92 (15.3) | 75 (12.5) | 36/52 (69.4) |

[a] Each experiment was separated over time and used PLBs stored in a growth chamber maintained at 15 °C
[b] PLBs that survived the kanamycin selection but did not produce shoots during the culture period

of organized tissues (Christou et al. 1991; D'Halluin et al. 1992; Nehra et al. 1994). One approach to obtain homogeneously transformed individuals from bombarded meristematic tissues is by selfing the treated generation and selecting for the added trait. The other is to insert DNA into meristematic tissues in early stages of organization and then stimulate continued meristem development during antibiotic selection. The former approach is limited in orchid transformation mainly due to the long generation time and slow growth. However, the latter can be successfully applied to orchid transformation if proper culture regimes and transformation conditions can be devised.

Previous reports on the transformation of *Dendrobium* and *Vanda* orchids showed that the general approach of the particle-mediated transformation of plant tissues can be successfully applied to orchids (Kuehnle and Sugii 1992; Chia et al. 1994). However, despite its commercial value, *Cymbidium* orchids have been up to now recalcitrant to transformation. Through extensive evaluations of factors involved in the particle bombardment-mediated transformation, we developed the present protocol with which a large number of stably transformed *Cymbidium* orchid plants were generated with high efficiency (over 15%). This protocol, with slight modification if necessary, appears applicable to all orchids and other species that have been recalcitrant to transformation. Using this protocol, we are currently focussing on genetic engineering of the commercially important *Cymbidium* clones with genes conferring virus resistance, modification of flowering time, manipulation of flower morphology, and other ornamental qualities.

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