

# *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*

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**Abstract** Genetically transformed plants of *Cymbidium* were regenerated after cocultivating protocorm-like bodies (PLB) with *Agrobacterium tumefaciens* strain EHA101 (pIG121Hm) that harbored genes for  $\beta$ -glucuronidase (*gus*), hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase II (*nptII*). PLB of three genotypes maintained in liquid new Dogashima medium (NDM), were subjected to transformation experiments. The PLB inoculated with *Agrobacterium* produced secondary PLB, 4 weeks after transfer onto 2.5 g L<sup>-1</sup> gellan gum-solidified NDM containing 10 g L<sup>-1</sup> sucrose, 20 mg L<sup>-1</sup> hygromycin and 40 mg L<sup>-1</sup> meropenem. Transformation efficiency was affected by genotype and the presence of acetosyringone during cocultivation. The highest transformation efficiency was obtained when PLB from the genotype L4 were infected and cocultivated with *Agrobacterium* on medium containing 100  $\mu$ M acetosyringone. Transformation of the hygromycin-resistant plantlets regenerated from different sites of inoculated PLB was confirmed by histochemical GUS assay, PCR analysis and Southern blot hybridization.

**Keywords** *Agrobacterium tumefaciens* · *Cymbidium* · Orchid · Protocorm-like body · Transformation

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**Abbreviations** AS: Acetosyringone ·  
GUS:  $\beta$ -Glucuronidase · Hm: Hygromycin ·  
Km: Kanamycin · ND: New Dogashima ·  
PLB: Protocorm-like body

## Introduction

Orchidaceae is the largest family of flowering plants, comprising more than 800 genera and 25,000 species widely distributed around the world that includes a large number of important ornamental species belonging to diverse genera such as *Cymbidium*, *Dendrobium*, *Phalaenopsis*, *Cattleya* and *Oncidium*. Among these, cymbidium orchid is one of the most popular orchids grown for commercial production of cut flowers and pot plants. Since *Cymbidium* is one of the most improved genera in the family Orchidaceae, a large number of novel cultivars with new flower colors, shapes, plant length, disease and pest resistance have been released through interspecific hybridization since the late 19th century. As a supplement to these conventional breeding programs, improvement of *Cymbidium* by means of genetic engineering is now expected to confer new and desirable traits such as blue or purple flowers, disease resistance and stress tolerance.

To date, a few studies of genetic transformation have been reported in orchids by using *Agrobacterium*-mediated method (Belarmino and Mii 2000; Chai et al. 2002; Chen et al. 2002; Liao et al. 2003a; Men et al. 2003; Mishiba et al. 2005) and biolistic-mediated method (Kuehnle and Sugii 1992; Chia et al. 1994; Anzai et al. 1996; Yang et al. 1999; Yu et al. 1999; Knapp et al. 2000; Tee et al. 2003). Among these studies, Yang et al. (1999) first reported the production of transgenic *Cymbidium* by particle bombardment method and obtained transformed plants that

expressed *nptII* and *gus* genes from the bombarded PLB. Recently, Chen et al. (2002) described the *Agrobacterium*-mediated transfer of genes for *nptII* and *gus* into rhizomes of *C. niveo-marginatum*, which is oriental terrestrial species, using *Agrobacterium tumefaciens*. However, there has been no report on the successful *Agrobacterium*-mediated transformation of other *Cymbidium* species and cultivars derived from interspecific hybridization between epiphytic species that are more important for commercial production.

In recent years, introduction of disease resistance traits such as antimicrobial peptides and viral coat proteins into the genome of other orchids was achieved by genetic engineering (Liau et al. 2003b; Chan et al. 2005; Sjahril et al. 2006). Transgenic plants over-expressing those genes showed enhanced resistance to *Erwinia carotovora* and Cymbidium Mosaic Virus (CymMV). Hence, gene transfer approach may provide a powerful tool for improvement of disease resistance in orchid species and it may be possible to extend this approach to *Cymbidium* species.

In the present study, we developed a reproducible procedure for *Agrobacterium*-mediated transformation of cymbidium orchid by using PLB, which is derived from shoot-tip culture of a cultivar and PLB cultures derived from two seedlings, each derived from a different cross between elite clones. We examined the influences of genotypes, the presence of acetosyringone (AS) in the cocultivation medium and the concentration of sucrose in medium for maintaining PLB used on the transformation.

## Materials and methods

### Plant materials

PLB of the three different *Cymbidium* lines, RY, L4 and L23 were used in the transformation experiments. PLB of RY were obtained from a commercial orchid company, while PLB of L4 and L23 were obtained from single seedlings, each derived from a different cross combination between elite clones of *Cymbidium*. PLB were maintained by subculturing every month 0.7 g fresh weight of PLB after cutting transversely into ca. 0.5 mm thick segments into 70 mL liquid ND medium (Tokuhara and Mii 1993) containing 10 or 30 g L<sup>-1</sup> sucrose (10S or 30S) without any plant growth regulators in a 200 mL flask at pH 5.4. Cultures were incubated on a reciprocal shaker by agitation at 60 rpm at 25°C under constant illumination with cool-white-fluorescent lamps (National FL30SN, Osaka, Japan). One-month-old PLB after each subculture was used for transformation experiments.

### Influence of sucrose concentration and selective antibiotics on PLB proliferation

Number and fresh weight of PLB were recorded 1 month after subculture to compare the proliferation rates between the cultures with 10S and those with 30S for each genotype. Sensitivity of the PLB to kanamycin and hygromycin was also examined using these two kinds of cultures. For each culture, 0.5 g of 1-month-old PLB were inoculated onto a 2.5 g L<sup>-1</sup> gellan gum-solidified 10S ND medium containing either kanamycin at 100, 200, and 400 mg L<sup>-1</sup>, or hygromycin at 5, 20, and 40 mg L<sup>-1</sup>. Fresh weight of PLB on media containing these antibiotics was measured 1 month after the transfer.

### Plasmid vector and bacterial strain

*A. tumefaciens* strain EHA101 (Hood et al. 1986), which harbors a binary vector pIG121Hm (Ohta et al. 1990) that contains a neomycin phosphotransferase II gene (*nptII*), a hygromycin phosphotransferase gene (*hpt*) and an intron-*GUS* gene in the T-DNA region were used.

### Inoculation and cocultivation with *Agrobacterium*

*Agrobacterium* was grown overnight at 28°C in LB liquid medium containing 50 mg L<sup>-1</sup> hygromycin, 50 mg L<sup>-1</sup> kanamycin and 25 mg L<sup>-1</sup> chloramphenicol. Before inoculation with *Agrobacterium*, 4 g of 1-month-old PLB were transferred to 40 mL liquid 10S ND medium with or without 100 μM acetosyringone in a 100 mL flask. Four milliliters of *Agrobacterium* suspension culture were added to each flask and incubated for 7 h to give a density of OD<sub>550</sub> = 0.5. The PLB were blotted dry, and cocultivated on medium consisting of 2.5 g L<sup>-1</sup> gellan gum-solidified 10S ND medium supplemented with or without 100 μM AS in Petri plates at 20°C in the dark.

### Selection of transgenic PLB and plant regeneration

After 3 days of cocultivation, the PLB were washed with liquid 10S ND medium containing 10 mg L<sup>-1</sup> meropenem (Ogawa and Mii 2004, Meropen; Sumitomo Pharmaceuticals Co., Ltd., Osaka, JPN), and then transferred onto a 2.5 g L<sup>-1</sup> gellan gum-solidified 10S ND selection medium containing 20 mg L<sup>-1</sup> hygromycin and 10 or 40 mg L<sup>-1</sup> meropenem. PLB were subcultured every 10 days during the first month, and subsequently, every 2 weeks to a fresh medium with the same composition for 3 months. Secondary PLB showing green coloration produced on the selection medium were then proliferated by cutting transversely into several pieces and transferred to a medium either with or without 20 mg L<sup>-1</sup> hygromycin. Plantlets regenerated

from the pieces of secondary PLB were selected as putative transformants and transplanted in culture bottles containing 20 mg L<sup>-1</sup> hygromycin. Four months after the transplanting, some of the plants with five to six fully developed leaves and several roots were transferred into vermiculite-filled pots and grown in a growth chamber at 25°C under a 16-/8-h (day/night) photoperiod without any acclimatization treatment.

#### GUS assay

A histochemical assay to detect GUS activity was performed on hygromycin-resistant PLB, leaves and roots. These tissues were immersed in X-Gluc solution (Jefferson et al. 1987), placed under a mild vacuum for 10 min, and then incubated overnight at 37°C. The tissues were soaked in 70% ethanol for several hours to remove chlorophyll before observation.

#### DNA isolation and molecular analysis

Plants regenerated from selected PLB were subjected to DNA isolation. Total genomic DNA was extracted from leaf tissues (1.5 g FW) using the CTAB method (Murray and Thompson 1980). A set of primers specific to the regions of the CaMV35S promoter 5'-GATGTGATATCTCCACTGAC-3' and the NOS terminator 5'-CGCAAGACCGGCAACAGGAT-3', respectively, were used to amplify both 2.2-kb fragment of the *gus* gene and 1.7-kb fragment of the *hpt* gene simultaneously. PCR amplification was carried out by using the following conditions: 30 cycles of thermal treatment with 1 min at 94°C, 1 min at 62°C and 1.5 min at 72°C.

Ten micrograms of genomic DNA from untransformed and hygromycin-resistant plants was digested with *Xba*I, which cuts a single site within the T-DNA, and fractionated on 0.9% agarose gels. Southern blot hybridization and detection were carried out using digoxigenin-labeled *gus* probe following the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany).

## Results and discussion

#### Influence of sucrose concentration in the culture media and selective antibiotics on PLB growth

We initially induced two types of PLB from each genotype by using different sucrose concentrations (10S or 30S), i.e., relatively large PLB with dark green coloration in 10S and small-sized yellow ones in 30S, respectively. These PLB were maintained in liquid ND medium without any plant growth regulators and subcultured every month by transfer-

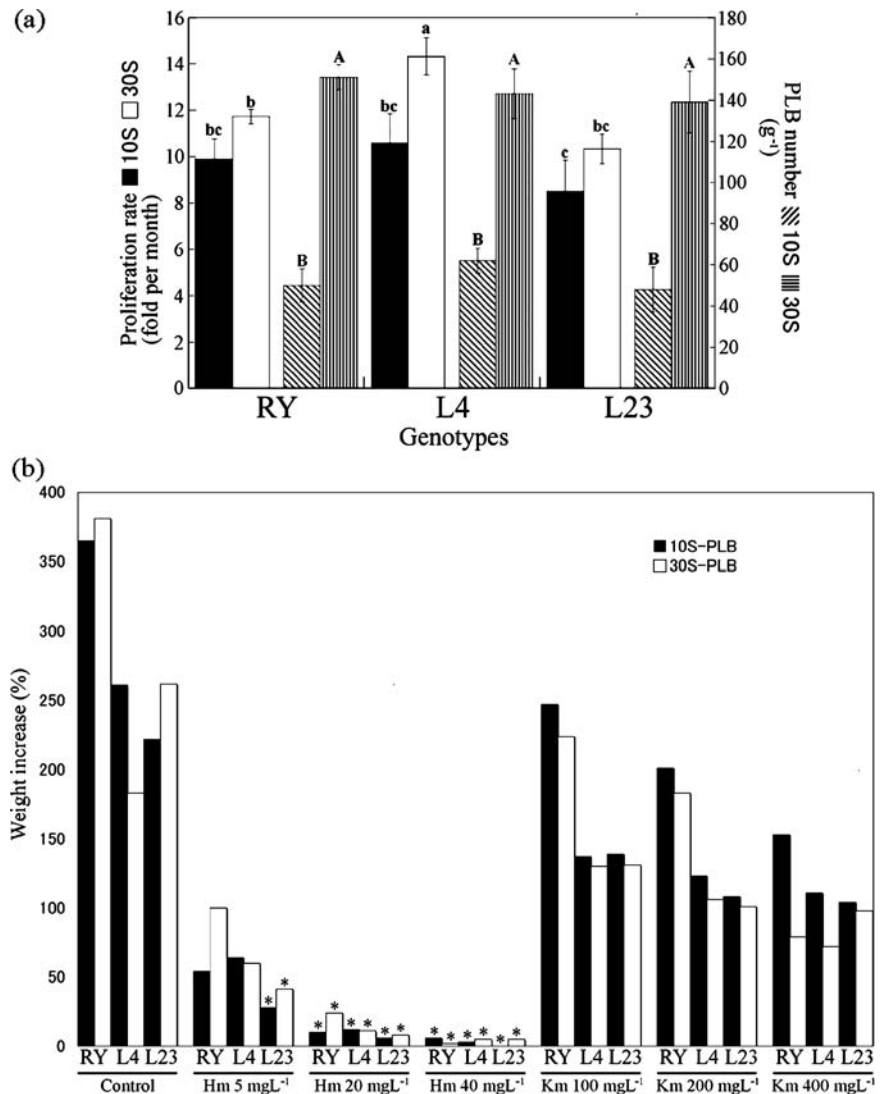
ring to fresh medium after cutting transversely into several pieces of approximately 5 mm in size. This process has allowed us to maintain the PLB without regeneration of plantlet under the same conditions for more than 3 years. The new PLB that formed from cut surfaces maintained their initial characteristics during the subcultures in these two media. As shown in Fig. 1a, PLB cultured on 30S ND medium showed higher numbers of PLB as well as proliferation rate (fresh weight) for all genotypes. However, proliferation of PLB was inhibited when the concentration of sucrose in the culture media was increased up to 6%, probably due to high osmotic stress (data not shown). The effect of carbon source on the growth of tissues and organogenesis has also been observed in other orchid species (Chia et al. 1988; Tokuhara and Mii 2003). Since transformation efficiency was influenced by the type of tissue used for both *Agrobacterium*-mediated transformation of rice (Hiei et al. 1994) and biolistic-mediated transformation of dendrobium orchid (Tee et al. 2003), we compared these two types of PLB for subsequent transformation experiments.

Since the plasmid used in this study (pIG121Hm) contains both *nptII* and *hpt* genes as selectable markers, the optimal concentrations of selection agents were determined by transferring untransformed PLB, which had been cultured in liquid medium, onto gellan gum-solidified 10S ND medium containing various concentrations of kanamycin or hygromycin. As shown in Fig. 1b, hygromycin completely inhibited the growth of PLB in all genotypes at 20 mg L<sup>-1</sup>, while kanamycin failed to inhibit the growth even at 400 mg L<sup>-1</sup> after 1 month of culture. These results indicate that kanamycin is not suitable as a selective agent for *Cymbidium*, as previously reported in *Dendrobium* (Chia et al. 1994) and other monocot plants (Hauptmann et al. 1988; Eady and Lister 1998). In the present study, therefore, hygromycin at 20 mg L<sup>-1</sup> was used for the selection medium.

#### Selection of hygromycin-resistant PLB and plant regeneration

After transfer of PLB onto selection medium, RY and L4 successfully produced secondary PLB within 4 weeks (Fig. 2a), while L23 showed overgrowth of *Agrobacterium* on and around PLB within 1 week and turned brown. In our previous study, meropenem exhibited the highest activity against *Agrobacterium* strain EHA101 among 12  $\beta$ -lactam antibiotics tested (Ogawa and Mii 2004). We also demonstrated that meropenem was suitable for eliminating *Agrobacterium* at a low concentration (5 mg L<sup>-1</sup>) for transformation of phalaenopsis orchid (Mishiba et al. 2005; Sjahril and Mii 2006). In the present study, however, 10 mg L<sup>-1</sup> meropenem failed to suppress the overgrowth of *Agrobacterium* in L23 during the early stages of selection. This may be due to the weak

**Fig. 1** Effect of sucrose concentration in preculture medium on proliferation of PLB (a) and sensitivity of PLB to selective agents (b) in three different *Cymbidium* genotypes. **a** Proliferation rate (fold per month) and number (per gram) of PLB 1 month after subculture of 0.7 g PLB into a flask containing 70 mL of 10S or 30S liquid ND medium. Each value represents a mean  $\pm$  SE of the three independent experiments. Bars with the same letter are not significantly different according to the Turkey HSD test at 0.05%. **b** Sensitivity of PLB to selective agents evaluated as the growth rates, 1 month after transfer of 1-month-old PLB to medium containing various concentrations of kanamycin or hygromycin. Each value represents a mean of the three independent experiments. The columns marked with an asterisk indicate that the cultures turned brown without further growth

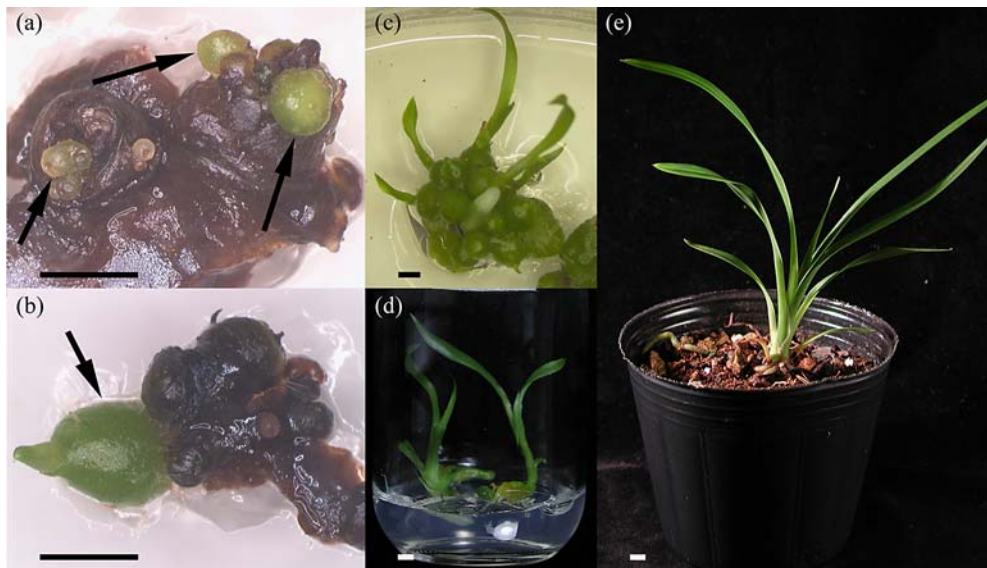


anti-bacterial activity of L23 compared to L4 and RY, and PLB of L23 died more rapidly by the harmful substances produced by overgrown bacteria. Since overgrowth of bacteria in L23 was not observed when PLB were exposed to meropenem at 40 mg L<sup>-1</sup> in the selection medium, a higher concentration of meropenem was required to suppress the growth of *Agrobacterium* in L23 genotype. Since the other two strains, RY and L4 showed comparably high transformation efficiency both at 10 and 40 mg L<sup>-1</sup> meropenem, bacterial elimination could be generally achieved at 40 mg L<sup>-1</sup> without exhibiting apparent cellular necrosis in *Cymbidium* (Fig. 3).

Three months after the transfer, the newly formed secondary PLB reached approximately 5 mm in diameter (Fig. 2b). These PLB were proliferated by cutting transversely into several pieces (Fig. 2c), which subsequently developed into plantlets within 4 months (Fig. 2d). When the plantlets had five to six leaves and several roots, a total of 32 plants

were transferred into pots containing vermiculite (Fig. 2e). All of the plants surviving and no difference in their morphology compared to nontransgenic plants was observed. Since regenerated plants were grown in culture bottles for more than 4 months before being transferred into pots, the plants were large in size and this contributed to their high rate of survival *ex vitro*. As shown in Fig. 3, no significant changes were observed in transformation efficiency between PLB maintained on 10S and those on 30S ND medium for each genotype. In general, transformation efficiency was increased in the presence of AS during the inoculation and cocultivation period (Drake et al. 1997; Song and Sink 2004). The effectiveness of AS in *Agrobacterium*-mediated transformation has also been reported previously in *Phalaenopsis* (Belarmino and Mii 2000), *Dendrobium* (Men et al. 2003), and *Oncidium* (Liau et al. 2003a). In this study, 100  $\mu$ M AS at inoculation and cocultivation increased the putative transformation efficiency. The highest number

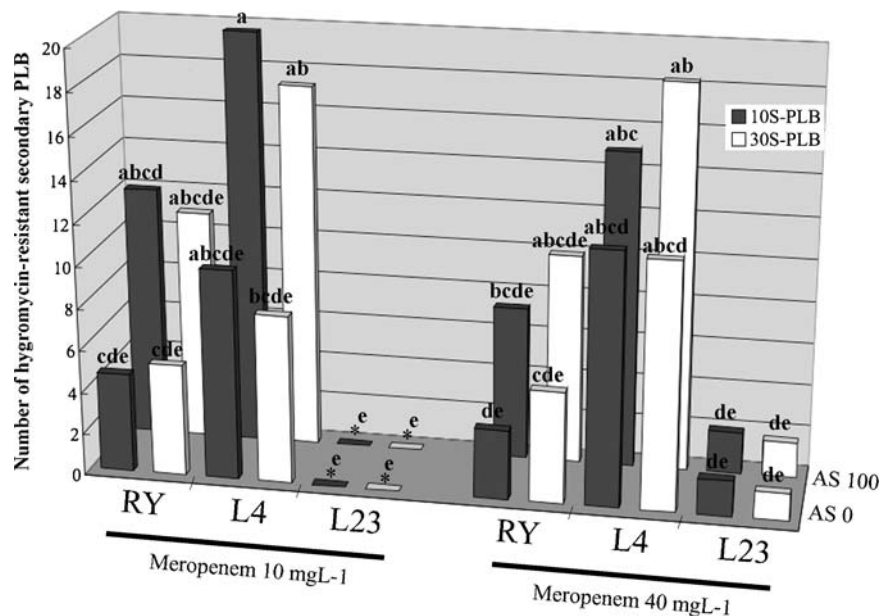




**Fig. 2** Selection and regeneration of putative transformants in *Agrobacterium*-mediated transformation of *Cymbidium*. **a** Hygromycin-resistant secondary PLB (arrow) of L4 proliferated on the selective medium 1 month after infection with *A. tumefaciens* EHA101 (pIG121Hm). **b** A hygromycin-resistant secondary growth of PLB of L4 (arrow) on hygromycin-containing medium 3 months after selection.

**c** Multiple PLB of RY derived from a hygromycin-resistant PLB after cutting transversely into several pieces. **d** RY plantlets with developing shoots regenerated from hygromycin-resistant PLB, 8 months after inoculation with *Agrobacterium* on hygromycin-containing medium. **e** Transgenic RY plant after acclimatization. Bars 5 mm

**Fig. 3** Effects of various factors on the efficiency of putative transformation in three *Cymbidium* genotypes by *A. tumefaciens* EHA101 (pIG121Hm). Each bar represents number of hygromycin-resistant secondary PLB derived from 4 g primary PLB after 2 months of culture on selection medium containing 20 mg L<sup>-1</sup> hygromycin. Each value represents a mean of the three independent experiments. Bars with the same letter are not significantly different according to the Turkey HSD test at 0.05%. The columns marked with an asterisk indicate that the cultures showed overgrowth of *Agrobacterium*



of hygromycin-resistant secondary PLB was obtained when PLB of L4 were treated with 100 μM AS during inoculation and cocultivation, which resulted in 19.7 independent hygromycin-resistant PLB per 4 g primary PLB following selection on hygromycin-containing medium. In contrast, the lowest number of hygromycin-resistant secondary PLB was obtained in L23, suggesting that genetic background played an important role in *Agrobacterium*-mediated transformation of *Cymbidium*.

GUS assay

Twenty hygromycin-resistant PLB from each genotype were selected at random and used for GUS assay. As shown in Table 1, more than 90% of hygromycin-resistant PLB showed blue staining of the tissues, which were not observed in the untransformed PLB. Although some of the PLB did not show GUS activity among the selected PLB, the presence of the transgenes was confirmed by PCR in these GUS

**Table 1** Percentage of GUS and PCR positive PLB in hygromycin-resistant PLB of 3 genotypes<sup>a</sup>

Genotypes	Number of samples	GUS positive PLB (%)	PCR positive PLB (%) <sup>b</sup>
RY	20	19 (95)	20 (100)
L4	20	18 (90)	20 (100)
L23	20	18 (90)	20 (100)

<sup>a</sup>Samples were selected at random from independent PLB after 4 months of culture on selection medium containing 20 mg L<sup>-1</sup> hygromycin.

<sup>b</sup>The presence of both *gus* and *hpt* genes as shown in Fig. 5a.

negative samples. The leaves and roots of twenty putative transformants (83%; 20/24) also showed GUS activity in the regenerated plants (Fig. 4a–c).

#### Molecular assays of transformants

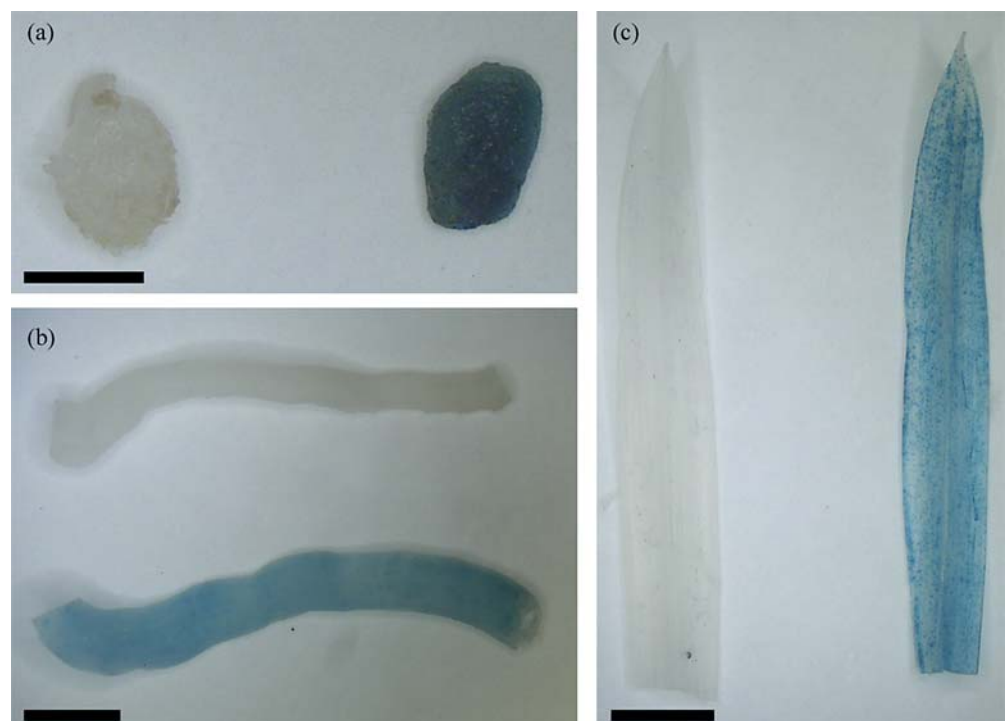
The presence of the *gus* and *hpt* genes in putatively transformed plants was confirmed by PCR amplification by producing the expected 2.2- and 1.7-kb amplification products, respectively (Fig. 5a). As shown in Fig. 5b, Southern blot analysis using the *gus* gene as the probe revealed that three transformed plants had a single copy, while others showed multiple copies of T-DNA integrated in the plant genomes.

In the present study, we have succeeded in the production of transgenic plants in an important orchid, *Cymbidium* through establishing a protocol using *A. tumefaciens*,

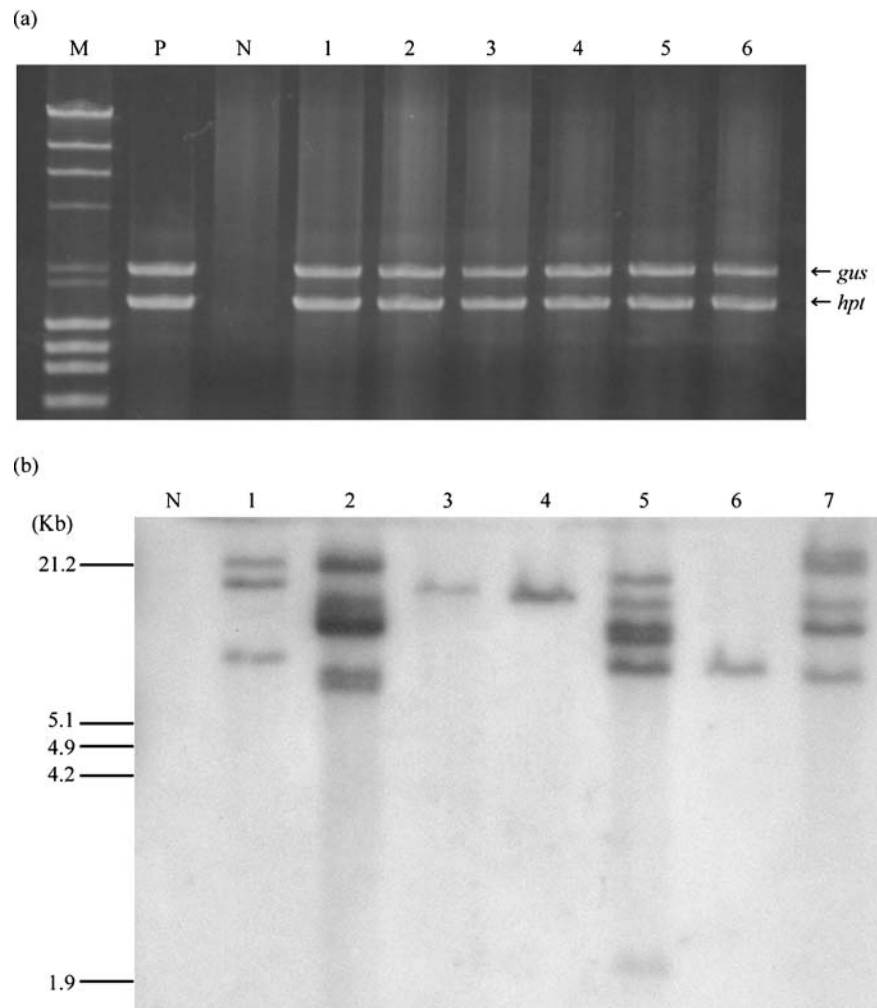
although some of the cultivars and/or genotypes may require optimization of the protocol for overcoming the low efficiency of transformation. Since *Agrobacterium*-mediated method has already been applied successfully for the transformation of several orchids such as *Phalaenopsis* (Belarmino and Mii 2000), *Dendrobium* and *Oncidium* (Yu et al. 2001; Liao et al. 2003a), it should be capable of transforming a wide range of species similar to the particle bombardment method.

In the previous study on the transformation of *Cymbidium* using the biolistic method, Yang et al. (1999) initiated the selection of transformants with kanamycin 1 month after bombardment. In the present study, however, we used hygromycin as a selective agent and performed the selection of transformants immediately after cocultivation of PLB with *Agrobacterium*. Although kanamycin is one of the most widely used antibiotics as a selective agent for transformation of plants, it was often ineffective for monocot plants (Hauptmann et al. 1988; Eady and Lister 1998). Our present results also showed that kanamycin was almost ineffective for inhibiting the continual PLB growth on all the three *Cymbidium* genotypes examined even at 400 mg L<sup>-1</sup> which gave a mild damage. In contrast, all of the genotypes we have tested showed a high sensitivity to hygromycin. At the concentration that we used in transformation experiments (20 mg L<sup>-1</sup>), whole tissues of untransformed plants turned brown within 1 month. These results suggest that the use of *hpt* gene will be much more suitable than *nptII* as a selection agent for transformants in *Cymbidium* and possibly for

**Fig. 4** Histochemical GUS assay of transgenic *Cymbidium* strain L4. **a** Stable GUS expression on hygromycin-resistant (*right*) and control (*left*) PLB. **b** Stable GUS expression on root of transformed (*bottom*) and control (*upper*) plants. **c** Stable GUS expression on leaf of transformed (*right*) and control (*left*) plants. Bars 5 mm



**Fig. 5** Molecular analysis of transgenic plants. **a** PCR analysis of transgenic plants for the *gus* (upper) and *hpt* (bottom) genes. Lane *M* molecular size marker ( $\lambda$ DNA/*Hind*III,  $\phi$ X174/*Hae*III). Lane *P* plasmid (pIG121Hm). Lane *N* nontransformed control plant. Lanes 1–6 transgenic plants. **b** Southern blot analysis of transgenic plants. Genomic DNA was digested with *Xba*I. Blot was hybridized with a digoxigenin-labeled *gus* gene probe. Lane *N* nontransformed control plant. Lane 1–7 transgenic plants



many other orchids, since most of them have considerable tolerance to kanamycin (unpublished results).

Although *Agrobacterium*-mediated transformation system has also been reported in *C. niveo-marginatum*, an oriental terrestrial species, by using rhizomes as a target for gene transfer (Chen et al. 2002), there has been no report of the successful production of transgenic plants in *Cymbidium* by using PLB or protocorms, which are the main target material available in commercially important cymbidium orchids as targets for the transformation. In the present study, therefore, we chose PLB as a target material for *Agrobacterium*-mediated transformation. Our results indicate that hygromycin-resistant secondary PLB were produced from meristems of PLB. This implies that the T-DNA transfer and integration are likely to occur in unwounded site (Escudero and Hohn 1997). Since PLB derived from 30S had more apical meristems than those from 10S, it is expected that 30S-derived PLB might have a higher transformation efficiency. However, no significant increase in the transformation efficiency was observed in PLB derived from 30S. In addition, PLB derived from 30S showed less overgrowth

of *Agrobacterium* than those derived from 10S in RY and L4 during selection of transformants, when meropenem was added to the medium at concentrations as low as 5 mg l<sup>-1</sup> (unpublished observation). Therefore, it can be speculated that PLB in 30S medium had higher expression of antimicrobial defense system associated with higher mitotic activity compared to those in 10S medium, although the mechanisms involved in this phenomenon are unknown.

In this study, inoculation of PLB with *Agrobacterium* was carried out in liquid medium for 7 h, which might be also the cause for overgrowth of *Agrobacterium* in L23. Although inoculation of many other plant tissues with *Agrobacterium* has most often been carried out in liquid medium for a short period (a few minutes), no hygromycin-resistant PLB were obtained in this study, when less than 1 h inoculation of PLB in liquid medium was performed (data not shown). In *Phalaenopsis*, optimum inoculation periods for obtaining transformed callus and protocorms were 10 and 7 h, respectively, in *Agrobacterium*-mediated transformation (Belarmino and Mii 2000; Mishiba et al. 2005). These results indicate that a relatively long period might be needed for



adhesion of *Agrobacterium* on the surface of target orchid tissues.

In *Agrobacterium*-mediated transformation, phenolic compounds such as AS serve as a potent inducer for the expression of the virulence (*vir*) genes located on the Ti plasmid of *A. tumefaciens* (Ashby et al. 1987). Since orchids are not natural hosts of *A. tumefaciens*, previous studies have shown that addition of AS to infection and coculture medium is effective in producing transgenic plants in several orchid species belonging to *Dendrobium* (Men et al. 2003), *Phalaenopsis* (Belarmino and Mii 2000) and *Oncidium* (Liau et al. 2003a). Our present results also indicate that the presence of AS during infection and cocultivation period improved the transformation efficiency in most of the genotypes used. Since hygromycin-resistant secondary PLB were also obtained even when AS was omitted from the infection and coculture medium, *Cymbidium* may have an activator for *vir* genes of *A. tumefaciens*, as reported in *Dendrobium* (Nan et al. 1997), which is an important factor for successful *Agrobacterium*-mediated transformation (Yu et al. 2001).

In conclusion, we have established a transformation procedure in *Cymbidium* using PLB as a material for *Agrobacterium* infection. The transformation system will be used efficiently for introducing genes that confer commercially important traits such as flower color and resistance to diseases into *Cymbidium*.

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