Plant Cell Reports (2000) 19:435–442

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Agrobacterium-mediated genetic transformation of a phalaenopsis orchid

Received: 10 November 1998 / Revision received: 4 June 1999 / Accepted: 22 June 1999

Abstract Genetically transformed plants of a phalaenopsis orchid [*Doritaenopsis* Coral Fantasy × *Phalaenopsis* (Baby Hat × Ann Jessica)] were regenerated after cocultivation of cell clumps with *Agrobacterium tumefaciens* strains LBA4404 (pTOK233) and EHA101 (pIG121Hm) that harbored genes for β-glucuronidase (GUS) and hygromycin resistance. The efficiency of transformation was markedly increased by 10 h cocultivation of cell clumps with *A. tumefaciens* that had been induced with 200 μM acetosyringone, and by inclusion of 500 μM acetosyringone in the cocultivation medium. Hygromycin-resistant cell clusters (0.5–3 mm in diameter) were selected from the infected cell clumps after 4–6 weeks of culture on agar (8 g/l)-solidified new Dogashima medium (NDM) containing 20 g/l sucrose, 0.1 mg/l naphthaleneacetic acid, 1.0 mg/l benzyladenine (BA), 50 mg/l hygromycin and 300 mg/l cefotaxime. The cell clusters proliferated 4 weeks after transfer onto the same medium. To induce callus greening, the carbon source was changed from sucrose to maltose. The green calli obtained produced protocorm-like bodies (PLBs) after 4 weeks of culture on phytohormone-free NDM medium. Regeneration of transgenic plantlets was enhanced by incubating PLBs on NDM medium supplemented with 0.1 mg/l abscisic acid, followed by partial desiccation for 10–30 min. Successful transformation was confirmed by histochemical GUS assay, PCR analysis and Southern hybridization of transformants. With this transformation system, more than 100 hygromycin-resistant phalaenopsis plantlets were produced about 7 months following infection of the cell aggregates.

Key words Acetosyringone · *Agrobacterium tumefaciens* · Genetic transformation · Plant regeneration · Orchid

Abbreviations *ABA*: Abscisic acid · *BAP*: Benzylaminopurine · *CaMV*: Cauliflower mosaic virus · *GUS*: β-Glucuronidase · *NAA*: Naphtaleneacetic acid · *NDM*: New Dogashima medium · *PLB*: Protocorm-like body

Introduction

Phalaenopsis orchid (*Phalaenopsis* and *Doritaenopsis*) is one of the most important orchids grown for commercial production of cut flowers and potted plants. Genetic improvement of phalaenopsis through sexual hybridization is, however, restricted by a long growth and reproductive cycle, and limited genetic variability within the germplasm. One effective alternative procedure is genetic transformation either by direct delivery of genes into plant cells or indirectly through the mediation of *Agrobacterium.* Transformation studies in orchids such as *Phalaenopsis* (Anzai et al. 1996), *Dendrobium* (Kuehnle and Sugii 1992; Chia et al. 1994), and *Vanda* (Chia et al. 1990) were conducted through particle bombardment of protocorms. Although particle bombardment was successfully applied to produce transgenic plants with a β-glucuronidase (GUS) gene in phalaenopsis, both transformation and regeneration frequency were relatively low (Anzai et al. 1996). To date, there has been no report of successful production of transgenic phalaenopsis using *Agrobacterium.*

In this paper, we report the successful production of transgenic plantlets through the transformation of cells in suspension cultures of phalaenopsis using *Agrobac-*

Communicated by J.M. Widholm

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terium tumefaciens strains LBA4404 (pTOK233) and EHA101 (pIG121Hm).

Materials and methods

Plant material

Cell clumps derived from friable calli from flower stalk cuttings of phalaenopsis [*Doritaenopsis* Coral Fantasy × *Phalaenopsis* (Baby Hat × Ann Jessica)] were used. The cultures were maintained by subculturing 0.3 g callus every 2 weeks in 40 ml of new Dogashima medium (NDM) (Tokuhara and Mii 1993) containing 20 g/l sucrose, 0.1 mg/l naphthaleneacetic acid (NAA) and 1.0 mg/l benzylaminopurine (BAP) at pH 5.4, which was designated NDM-20S. The cultures were incubated by agitation at 60 rpm at 25 °C under constant illumination provided by fluorescent lamps (Toshiba FLR40S W/M/36) at 33 μmol m^{-2} s^{-1}. Eight-day-old suspension cells that had been subcultured at least three times were used for transformation experiments.

Bacterial strains

The *A. tumefaciens* strains LBA4404 (pTOK233) and EHA101 (pIG121Hm) (Hiei et al. 1994) were used for transformation studies. The plasmid pTOK233 has inserted *virB*, *virC*, and *virG* genes derived from supervirulent Ti-plasmid pTiBo542 (Watson et al. 1975), which are known to enhance the virulence of *A. tumefaciens* (Jin et al. 1987). The T-DNA region of pTOK233 contained genes for hygromycin phosphotransferase (*hpt*) under the control of cauliflower mosaic virus (CaMV) 35S promoter, neomycin phosphotransferase (*npt*II) under the control of the nopaline synthase promoter, and GUS with an intron fused to the CaMV 35S promoter (Fig. 1A). This intron-GUS reporter gene expresses GUS activity in plant cells but not in *A. tumefaciens* cells.

The *A. tumefaciens* strain EHA101 (Hood et al. 1986) harbors a binary vector pIG121Hm that contains a kanamycin resistance gene (*npt*II), hygromycin resistance gene (*hpt*), and the intron-GUS in the T-DNA region (Fig. 1B) as pTOK233.

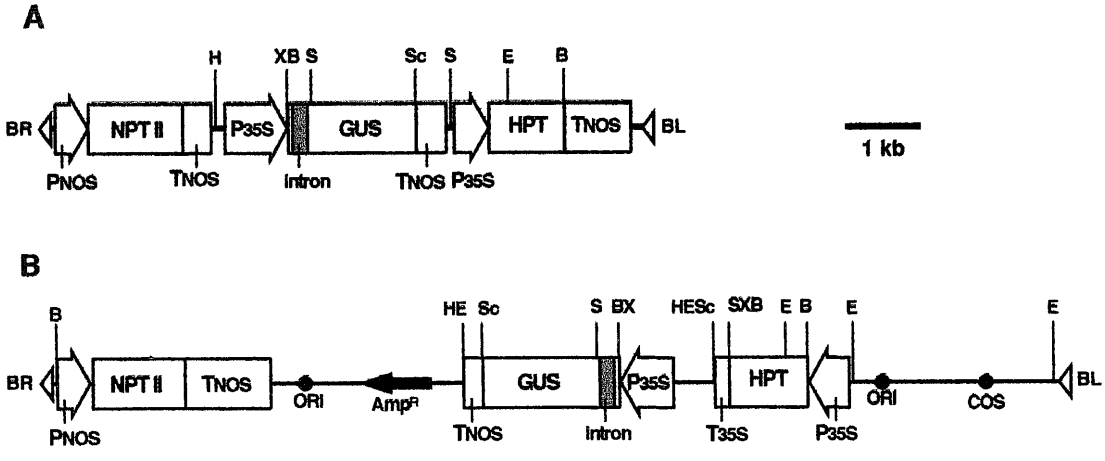
Fig. 1 Structure of T-DNA regions in pIG121Hm (**A**) and pTOK233 (**B**) (*BR* right border, *BL* left border, *NPTII* neomycin phosphotransferase, *GUS* β-glucuronidase, *HPT* hygromycin phosphotransferase, *NOS* nopaline synthase promoter, *35S* 35S promoter, *TNOS* 3′ signal of nopaline synthase, *T35S* 3′ signal of 35S RNA, *ORI* origin of replication of ColE1, *AmpR* ampicillin resistance gene, *B Bam*HI, *E Eco*RI, *H Hin*dIII, *S Sal*I, *Sc Sac*I, *X Xba*I)

Transformation

The general protocol for transformation after optimizing the conditions was as follows. *A. tumefaciens* strains LBA4404 (pTOK233) and EHA101 (pIG121Hm) were grown overnight at 28 °C in AB medium at pH 7.5 (Chilton et al. 1974) containing 5 g/l sucrose, 50 mg/l hygromycin, and 200 μM acetosyringone to activate the *vir* genes. Cocultivation of suspension cells with 200 μM acetosyringone-treated *A. tumefaciens* was carried out using a two-step cocultivation method. The first step involved the incubation of 1 g suspension cells in 20 ml solution consisting of 1:10 (vol/vol) *A. tumefaciens* suspension:NDM-10S medium in which the sucrose concentration was 10 g/l. After 10 h of 30 rpm agitation at 28 °C, all suspension cells were collected on a nylon mesh (20 μM pore size), washed with sucrose-free NDM and blotted dry with sterile filter paper. In the second step, the cells were spread on a piece of sterile filter paper placed on 20 ml of cocultivation medium consisting of NDM-20S supplemented with 500 μM acetosyringone and solidified with 0.8 g/l agar in 90 mm × 20 mm petri plates, and cocultivated for 3 days in the dark. The non-cocultivated suspension cells were plated on NDM-20S medium without hygromycin to serve as the control.

Selection and regeneration of transformants

Immediately after cocultivation, the suspension cells were washed with sucrose-free NDM containing 500 mg/l cefotaxime. The cultures were transferred to selective medium and incubated in the dark for 2 days prior to light exposure (24 h at 33 μmol m^{-2} s^{-1}). The selective medium comprised 20 ml of 0.8% agar-solidified NDM-20S supplemented with 50 mg/l hygromycin and 300 mg/l cefotaxime. Calli were subcultured to new selective medium every 2 weeks. Hygromycin was included in the medium throughout the transformation and culture phases, whereas cefotaxime was omitted after visually confirming the absence of *Agrobacterium*. Hygromycin-resistant cell clusters were plated for 4 weeks on NDM-20S to induce proliferation, then on phytohormone-free NDM supplemented with 10% maltose (NDM-10M) for 4 weeks to induce greening, and for a further 4 weeks on fresh NDM-10M for the formation of protocorm-like bodies (PLBs). The PLBs were separated from the mass of green calli and cultured on the same medium supplemented with 0.1 mg/l abscisic acid (ABA) to induce normal development of protocorms. The concentration of agar was increased to 12 g/l of medium to avoid vitrification. After 4 weeks of ABA treatment, PLBs were partially desiccated to induce shoot formation and to prevent vitrification by placing them on a sterile filter paper in empty open petri plates, which were kept in the laminar air flow bench for 10–30 min. Desiccated PLBs were finally cultured on half-strength NDM-10M lacking ABA, for shoot and root development. Plants regenerated from hygromycin-resistant cell lines and



control plants were transferred for 4 weeks to NDM-10M containing either 50, 100, or 200 mg/l hygromycin or G418 (geneticin).

GUS assay

A histochemical assay to detect GUS activity was performed on hygromycin-resistant calli and PLBs, and then on leaves and roots of hygromycin-resistant plants. Tissues were immersed in X-Gluc solution (Jefferson et al. 1987), placed under a mild vacuum for 15 min, and then incubated overnight at 37 °C. Transient GUS expression of callus, scored as number of blue spots per 100 mg callus, was examined after 3 days of cocultivation. For detecting GUS expression of regenerated plants, the materials were treated with 70% ethanol to remove chlorophyll prior to tissue sectioning (Microslicer DTK 1000, Dosaka EM Co., Japan).

DNA extraction and PCR analysis

Genomic DNAs were isolated from fresh leaf tissues of hygromycin-resistant plants and a control plant according to the cetyltrimethyl-ammonium bromide method (Murray and Thompson 1980). DNA amplification was carried out according to the method of Hoshino et al. (1998) in a programmed temperature control system (PC-700, Astec, Tokyo) using specific oligonucleotide primers that amplify the 1.2-kb fragment of the GUS gene (Hamill et al. 1991). Samples of PCR products were separated by electrophoresis on 1.0% (wt/vol) agarose gels using Tris-acetate as a running buffer at 50 V for 1 h, and then visualized with ethidium bromide.

PCR products were also confirmed by Southern blot hybridization. The 2.0-kb pBI 221 fragment was digested with *Sac*I and *Hin*dIII to produce the 1.2-kb GUS fragment that was used as a probe with PCR products. Southern blotting and hybridization were performed according to the Amersham protocol for the Hybond-N^+ charged nylon membranes. Chemiluminescence signal bands were visualized by exposure for 1 min to an X-ray film (HyperfilmTM -ECL, Amersham).

Southern hybridization

Genomic DNA from hygromycin-resistant plants transformed by EHA101 (pIG121Hm) was digested with *Sal*I to release the 2.26-kb fragment containing the GUS gene from the integrated T-DNA. The hygromycin-resistant plant obtained after transformation with LBA4404 (pTOK233) and the untransformed control plant were digested with *Hin*dIII which produces one 3.1-kb GUS DNA fragment. The digested DNA fragments were subjected to electrophoresis on 0.8% (wt/vol) agarose gels at 50 V for 2 h. The bands of DNA were transferred to a nylon membrane (Hybond-N^+) by standard methods (Southern 1975). The GUS DNA used as probe was the same as that used in PCR analysis. Labelling, hybridization, and washing were performed according to the instruction manual for the ECL direct nucleic acid labelling and detection systems (Amersham).

Results and discussion

Factors affecting transient GUS expression

We initially conducted two preliminary experiments to clarify the effects of three factors, namely (1) cocultivation of suspension cells in 200 μM acetosyringone-activated *A. tumefaciens* strain LBA4404 (pTOK233), (2) inclusion of acetosyringone in the cocultivation medium and (3) duration of the cocultivation period, on transient GUS expression in friable callus of phalaenopsis. The results showed that 200 μM acetosyringone-treated *Agrobacterium* and a two-step cocultivation method, i.e., 10 h cocultivation of suspension cells in a 1:10 (vol/vol) suspension of *A. tumefaciens*:NDM-10S (first step), immediately followed by 3 days cocultivation on 0.8% agar-solidified cocultivation medium containing 500 μM acetosyringone (second step), were effective for obtaining transient GUS expression of cells (Table 1). We therefore adopted these conditions for all subsequent transformation experiments.

GUS activity scored as number of GUS spots per 100 mg of cells was the highest in cells cocultivated with 200 μM acetosyringone-treated *A. tumefaciens* using the two-step cocultivation method (Table 1). Some of these cells proliferated on selective medium and stable GUS expression was also observed in these cells 4 weeks after infection. Acetosyringone is known to activate the virulence genes of the Ti plasmid at pH 5.0–5.5 and to initiate the transfer of the T-DNA (Stachel et al. 1985, 1986). The importance of pH for the infection and the presence of an inducer of *A. tumefaciens* virulence genes have also been confirmed in dendrobium orchids (Nan et al. 1997). In the present study, therefore, it appears that an inducer of the *vir* gene might also have been secreted from phalaenopsis orchids and that the pH of the NDM-10S medium (pH 5.4) favored the induction of the *A. tumefaciens vir* genes. Induction of *A. tumefaciens* with acetosyringone and a two-step cocultivation of suspension cells might act synergistically to increase the infection frequency in phalaenopsis.

Inclusion of acetosyringone in the cocultivation medium also increased the number of GUS spots, with the highest number, 42.4, obtained with 500 μM (Table 2). However, doubling the concentration reduced the GUS activity. Positive effects of including acetosyringone in the cocultivation medium for increasing transient GUS expression have also been

Table 1 Effect of cocultivation period in liquid medium [20 ml of 1:10 (vol/vol) solution of *Agrobacterium* suspension:NDM] with 200 μM acetosyringone-treated *Agrobacterium tumefaciens* strain LBA4404 (pTOK233) on GUS expression of cell clumps of phalaenopsis [*DTps.* Coral Fantasy × *Phal.* (Baby Hat × Ann Jessica)] after 3 days of further cocultivation on 0.8% agar-solidified NDM-10S containing 100 μM acetosyringone

Cocultivation period in liquid medium (h)	Number of GUS spots per 100 mg cell clumps		
	Experiment 1	Experiment 2	Average
0	2.0	2.0	2.0
0.5	2.5	1.5	2.0
1.0	3.5	2.0	2.8
2.0	4.0	3.0	3.5
6.0	7.5	6.0	6.8
10.0	11.4	9.6	10.5

Table 2 Effect of acetosyringone concentration in the 0.8% agar-solidified cocultivation medium (NDM-20S) on transient GUS expression of cell clumps of phalaenopsis after 3 days of cocultivation with 200 μM acetosyringone-treated *A. tumefaciens* strain LBA4404 (pTOK233). Before the cocultivation on agar-solidified medium, the cells were cocultivated for 10 h in 1/10-diluted bacterial suspension with NDM-10S

Acetosyringone (μM)	Number of GUS spots per 100 mg cell clumps		
	Experiment 1	Experiment 2	Average
0	10.8	10.1	10.4
100	12.0	9.1	10.6
200	13.1	12.8	12.9
300	12.6	16.5	14.5
400	24.7	18.0	21.3
500	35.2	49.5	42.4
1000	13.5	24.1	18.8

reported in other plants such as *Brassica rapa* L. (Takasaki et al. 1997), *Betula platyphylla* (Mohri et al. 1997), and rice (Aldemita and Hodges 1996; Rashid et al. 1996).

The cocultivation period also affected transient GUS expression of phalaenopsis cells: transient GUS expression was undetectable in cells cocultivated for 1 day but could be observed in cells cocultivated for 3 days in 0.8% agar-solidified cocultivation medium. Extending the cocultivation period to 5–7 days did not increase GUS activity but caused necrosis and death of cells on the selective medium. These results suggest that *vir* gene activity must be sustained at a high level during the 10-h infection and 3-day cocultivation periods by maintenance of a high acetosyringone level.

Sensitivity of suspension cells to hygromycin and cefotaxime

In preliminary tests, there was no significant difference between the mean growth increment (fresh weight) of suspension cultures obtained on NDM-20S containing 300 mg/l of cefotaxime and that on medium lacking the antibiotics (Student's *t*-test, $P = 0.393$ at the 0.01 confidence level). Both cefotaxime-treated and untreated cells formed PLBs after 12 weeks in NDM-10M. On the other hand, the addition of 500 mg/l cefotaxime decreased suspension cell growth (Student's *t*-test, $P = 0.000167$ at the 0.01 confidence level) and inhibited the formation of PLBs. On medium supplemented with 30 and 50 mg/l hygromycin, PLBs were not produced and necrosis occurred. From these results, 300 mg/l cefotaxime was added in NDM-20S (selective medium) to eliminate the bacteria, and 50 mg/l hygromycin was used to discriminate between transformed and non-transformed cells.

Selection of hygromycin-resistant callus and plant regeneration

Four weeks (two transfers at 2-week intervals) after bacterial infection, both cocultivated and non-cocultivated cells turned brown and died on selective medium containing 50 mg/l hygromycin. However, within the third transfer, some cocultivated cells initiated yellow cell clusters (0.5–3 mm in diameter: Fig. 2A), whereas non-cocultivated cells failed to form them on the same medium. Hygromycin-resistant cell clusters at least 1.0 mm in size were plated on fresh selective medium for 4 weeks to establish prolific callus cultures (Fig. 2B). After the transfer, only a few cell clusters survived and proliferated under hygromycin selection pressure. Using LBA4404 (pTOK233), 11 hygromycin-resistant calli were produced per gram fresh weight of cocultivated suspension cells, while EHA101 (pIG121Hm) produced 5 resistant calli (Table 3). A change of carbon source from sucrose to maltose in NDM (NDM-10M) is required to induce callus greening and subsequent formation of PLBs (Fig. 2C). Almost all hygromycin-resistant calli formed PLBs within 4–8 weeks of culture on this medium. The PLB-to-plant conversion, however, was initially poor compared to non-cocultivated wild-type cells (Table 3). In further experiments, the difficulty was overcome by culturing PLBs on half-strength NDM-10M medium supplemented with 0.1 mg/l ABA at least 4 weeks prior to the desiccation treatment of 10–30 min. Four to 8 weeks following desiccation, an average of 4–6 plantlets were obtained per gram fresh weight of PLBs (data not shown). The promotive effect of ABA and desiccation on conversion of PLBs to plantlets may be likened to the conversion of somatic embryos to plantlets reported in white spruce (Attree et al. 1991), alfalfa (Senaratna et al. 1989), and celery (Kim and Janick 1989). From our transformation experiments, a total of 24 and 10 hygromycin-resistant plants can be obtained per gram fresh weight of suspension cells cocultivated, with LBA4404 (pTOK233) and EHA101 (pIG121Hm), respectively, 4–8 weeks after desiccation treatment

Table 3 Transformation efficiency of phalaenopsis using *A. tumefaciens* strains LBA4404 (pTOK233) and EHA101 (pIG121Hm). One gram of cell clumps was used as starting material. In the non-infected control culture, about 2,000 plantlets were obtained from 0.1-g cell clumps

Agrobacterium strain	Number of hygromycin-resistant calli obtained	Number of hygromycin-resistant calli forming PLBs	Number of hygromycin-resistant regenerated plantlets
LBA4404 (pTOK233)	11	8	24
EHA101 (pIG121Hm)	5	4	10

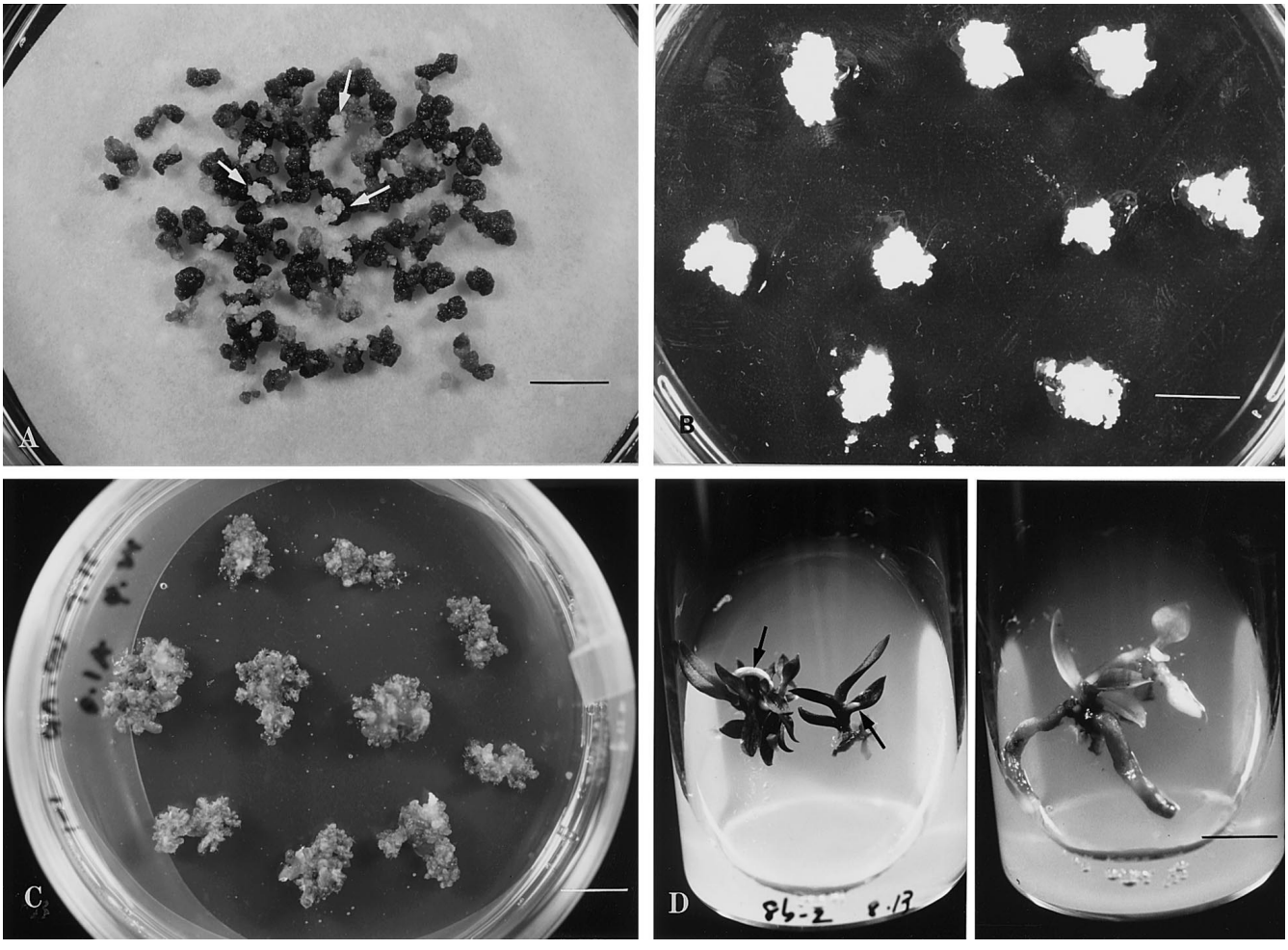


Fig. 2A–D Genetic transformation of phalaenopsis using *Agrobacterium tumefaciens (bar* 10 mm). **A** Hygromycin-resistant cell clusters (*arrows*) produced from *Agrobacterium*-infected cell clumps 6 weeks after cocultivation. **B** Calli proliferating on NDM-10S medium containing 50 mg/l hygromycin 8 weeks after cocultivation. **C** *ABA*-treated (0.1 mg/l) PLBs partially desiccated for 10–30 min. **D** Transgenic plantlets vigorously growing on NDM medium containing 200 mg/l hygromycin (*left*) and non-transformed plants, dead after 2 weeks of culture on the same medium (*right*). *Arrows* indicate the roots produced after transfer onto hygromycin-containing medium

(Table 3). The efficiency of normal plant regeneration from PLBs may be increased by determining the most suitable PLB stage for ABA treatment prior to desiccation. In total, more than 100 hygromycin-resistant plants were produced from six separate transformation experiments about 7 months after infection. The plantlets regenerated from hygromycin-resistant calli survived on medium containing extremely high concentrations (200 mg/l) of hygromycin or geneticin, whereas the plantlets obtained from non-cocultivated cells became yellow and died within 2 weeks in the presence of the antibiotics (Fig. 2D). The successful expression of the *npt*II gene shown as the resistance to geneticin in the plantlets derived from hygromycin-resistant calli may suggest that useful genes which will be cotransformed with a selectable marker gene may also be expressed in the transgenic phalaenopsis plants.

Histochemical GUS assay

Four putative transformants were selected at random from each transformation experiment and used in GUS assays. Hygromycin-resistant calli (Fig. 3A), PLBs (Fig. 3B), and PLB tissue (Fig. 3C) expressed GUS activity and showed intense blue staining in tissue sections. Similarly, whole leaves (Fig. 3D) and roots (Fig. 3F) of putative transformants, and their sectioned tissues (Fig. 3E,G) also showed GUS activity, suggesting that an integrated GUS gene was expressed at high levels under the control of the 35S promoter. The leaves, however, required wounding prior to the histochemical test to see expression (Fig. 3D). In contrast to these observations, callus, PLBs, leaf, and root samples from the non-cocultivated cells did not stain blue. Since the GUS construct in LBA4404 (pTOK233) and EHA101 (pIG121Hm) used in the

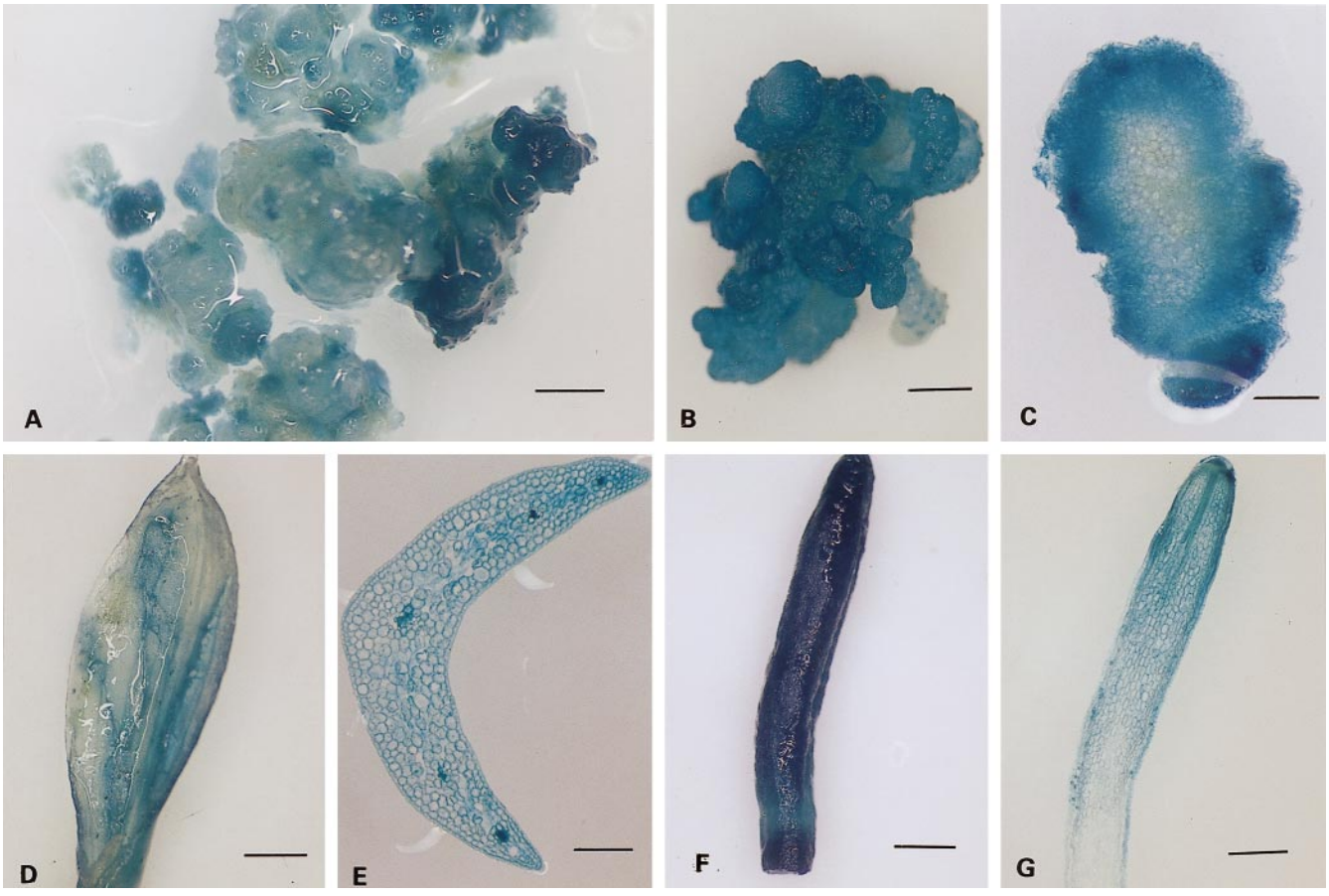


Fig. 3A–G Histochemical GUS assay of *Agrobacterium-i*nfected phalaenopsis. **A** Cell clumps showing stable GUS expression 6 weeks after cocultivation (*bar* 1.0 mm). **B** PLBs produced from hygromycin-resistant callus (*bar* 2.0 mm). **C** Sectioned transformed PLB showing blue-stained tissue (*bar* 1.0 mm). **D** Leaf of transformed plant showing blue-stained veins (*bar* 4 mm). **E** Cross-section of leaf tissue from transformed plant indicating darkly stained vascular bundles (*bar* 1.5 mm). **F** Root of transformed plant expressing GUS (*bar* 1.0 mm). **G** Longitudinal section of root tissue from transformed plant revealing darkly stained root cap (*bar* 1.0 mm).

present study contained introns, the observed expression must be coming from a GUS gene inserted into the plant genome (Janssen and Gardner 1990).

PCR analysis

The presence of the GUS gene was confirmed by PCR amplification of a 1.2-kb fragment in the DNA extracted from seven randomly chosen hygromycin-resistant plants. There was no amplification with the DNA from the control plant. Southern blot analysis of PCR products revealed that the 1.2-kb fragment hybridized with the GUS gene probe, confirming its presence in the seven transformants (Fig. 4A). No corresponding fragment was observed in the control plant.

Southern hybridization

Southern blot analysis of the genomic DNA from five hygromycin-resistant plants confirmed the presence of GUS gene sequences in the plant genomes. With the exception of the control, the plants tested had sequences that hybridized to the DNA fragment of the GUS gene (Fig. 4B). The plants transformed with EHA101 (pIG121Hm) showed the presence of the expected fragment of 2.26 kb corresponding to the GUS gene (Fig. 4B, lanes 2–5). Likewise, the plants transformed by LBA4404 (pTOK233) revealed the 3.14-kb GUS fragment (Fig. 4B, lane 6). Since the GUS DNA probe only hybridized to DNA from transgenic plants but not to the DNA from the untransformed control plant, the result suggests that the GUS DNA was integrated into the genome of phalaenopsis, although the possibility that *A. tumefaciens* still remained in the regenerated plants could not be totally excluded.

We have therefore established for the first time, a simple and reliable procedure for *A. tumefaciens*-mediated transformation of phalaenopsis. Several factors contributed to the success of the transformation process, and optimization of conditions for cocultivation was shown to be important. Cocultivation of suspension-culture-derived cells with 200 μM acetosyringone-preactivated *A. tumefaciens* and inclusion of a

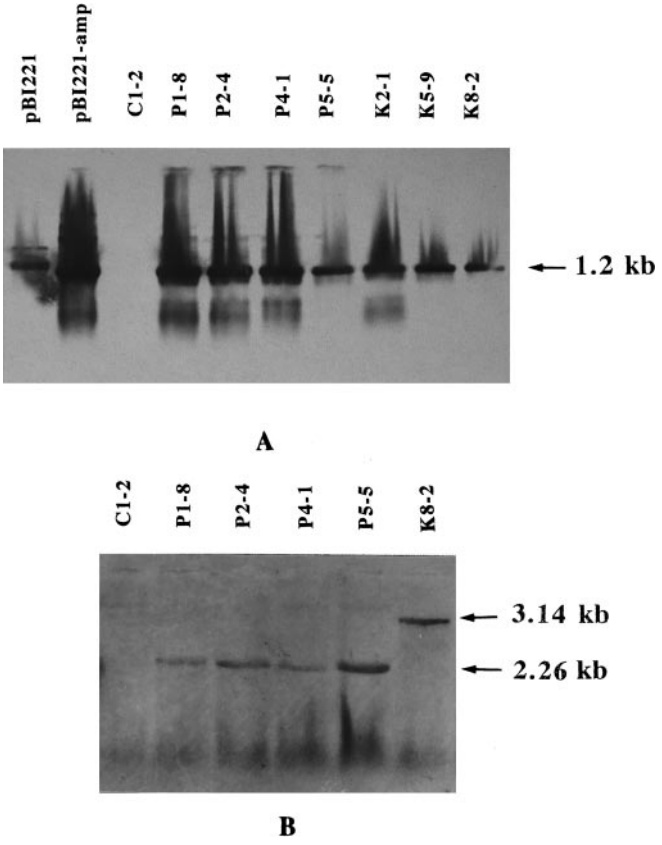


Fig. 4A, B Detection of the GUS gene in hygromycin-resistant phalaenopsis. **A** Southern blot analysis of PCR products indicating the specific 1.2-kb DNA fragment of the GUS gene in the transformed plants (P1-8, P2-4, P4-1, P5-5, K2-1, K5-9, and K8-2), pBI221 and PCR-amplified pBI221 (pBI 221-amp), which is absent in the control plant (C1-2). **B** Southern hybridization of genomic DNA of plants transformed with *A. tumefaciens* strains EHA101 (pIG121Hm) and LBA4404 (pTOK233). DNA of transformed plants (P1-8, P2-4, P4-1, and P5-5) was cut with *Sal*I, while transformed plant K8-2 was cut with *Hin*dIII, and then allowed to hybridize to the GUS probe. *Arrows* indicate the expected bands of 3.14 kb and 2.26 kb which are absent in the non-transformed plant, C1-2

high acetosyringone concentration (500 μM) in the agar-solidified cocultivation medium enhanced the infection rate. Equally important was hygromycin selection (50 mg/l) of transformed calli. We have also demonstrated efficient production of hygromycin-resistant plants, i.e., 10 and 24 hygromycin-resistant plants per gram fresh weight of suspension culture using *A. tumefaciens* strains EHA101 (pIG121Hm) and LBA4404 (pTOK233), respectively. The integration of the 1.2-kb fragment of the GUS gene in the hygromycin-resistant plants was revealed by histochemical GUS assay (Fig. 2), PCR analysis (Fig. 3A), and Southern hybridization (Fig. 3B). The use of fine cell clumps from actively dividing suspension cultures as starting material prevented the regeneration of chimeric or escape plants because infected cells were exposed immediately to the selection pressures (Finer and McMullen 1990; Gordon-Kamm et al. 1990). Chimerism may be a problem using orchid protocorms (Kuehnle and Sugii 1992) as target tissue because plant regeneration proceeds directly without an intervening callus or de novo embryogenesis phase during which non-transformed cells might be more effectively eliminated. Furthermore, we have not yet observed chimeric plants among the transformants in the present study.

Use of the transformation system for phalaenopsis established in the present study will permit the selective introduction of genes that regulate morphological and economically important traits such as flower color and size, and resistance to insects and diseases, which are otherwise difficult or impossible to achieve using conventional breeding techniques. More detailed studies of the transformation efficiency are now in progress using the protocol established in the present study.

Acknowledgements We wish to thank Dr. T. Komari of Japan Tobacco Inc. for the gift of *Agrobacterium* strains and Mr. K. Tokuhara for providing the *Phalaenopsis* suspension cultures. We are also grateful to Ms. Yoko Akasaka for her valuable assistance. This study was partially funded by the Japanese Ministry of Education, Culture and Science through a postdoctoral fellowship grant to M.M.B. from the Japanese Society for the Promotion of Science.

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