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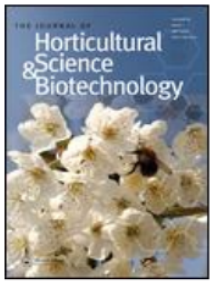
Submission date: 17-Aug-2021 09:27PM (UTC+0700)
Submission ID: 1632476638
File name: High_Efficiency_Agrobacterium-mediated.pdf (923.41K)
Word count: 6033
Character count: 32589



The Journal of Horticultural Science and Biotechnology

ISSN: 1462-0316 (Print) 2380-4084 (Online) Journal homepage: http://www.tandfonline.com/loi/thsb20

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To cite this article: Rinaldi Sjahril & Masahiro Mii (2006) High-efficiency Agrobacterium-mediated transformation of Phalaenopsis using meropenem, a novel antibiotic to eliminate Agrobacterium, The Journal of Horticultural Science and Biotechnology, 81:3, 458-464, DOI: 10.1080/14620316.2006.11512088

To link to this article: http://dx.doi.org/10.1080/14620316.2006.11512088

Journal of Horticultural Science & Biotechnology (2006) **81** (3) 458–464

High-efficiency *Agrobacterium*-mediated transformation of *Phalaenopsis* using meropenem, a novel antibiotic to eliminate *Agrobacterium*

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(Accepted 30 December 2005)

SUMMARY
The type and concentration of antibiotic used to eliminate *Agrobacterium* affected the efficiency of bacterial elimination from, and phytotoxicity to *Phalaenopsis* cells. To eliminate *Agrobacterium*, high concentrations (500 mg l^{-1}) of cefotaxime and carbenicillin were required, which also caused necrosis of cells when added to the culture medium for 2 weeks. In contrast, meropenem successfully suppressed growth of the bacteria at low concentrations (5 mg l^{-1}) and had no phytotoxic effect. A reproducible genetic transformation method for *Phalaenopsis* was established by co-cultivating cell suspension cultures of *Phalaenopsis* Wataboushi '#6.13' with *A. tumefaciens* strain 'EHA101' (pIG121Hm), harbouring genes for β-glucuronidase (GUS) and hygromycin resistance (*hpt*). The highest transformation efficiency was obtained when 5 d-old cells were infected for 2 h after sub-culture with *Agrobacterium*, as assessed by transient as well as stable GUS expression. Successful *GUS* gene expression in putative transgenic plantlets was confirmed by histochemical GUS assays on calli, protocorm-like bodies (PLBs), leaves and roots of plantlets selected on New Dogashima medium (NDM) containing 25 mg l^{-1} hygromycin. PCR and Southern blot analysis of genomic DNA confirmed successful incorporation and transformation of the *GUS* gene in regenerated plantlets. *Agrobacterium* was completely eliminated using 10 mg l^{-1} meropenem during a 10 min wash after 3 d co-cultivation followed by 5 mg l^{-1} for the first 3 months of culture in Gelrite-solidified medium. Ninety-two percent of transgenic PLBs obtained after 6 months, and 97% of transgenic plantlets after 12 months showed almost complete elimination of *Agrobacterium*.

The genus *Phalaenopsis* consists of about 50 species, which are distributed mainly in tropical Asia (Pridgeon, 1992). *Phalaenopsis* orchid is one of the most important and popular orchids grown commercially as cut or potted flowers, and is becoming important ornamental plant in the World. This orchid has a number of important characteristics, such as easy and clean potting, free flowering with inflorescences bearing a few to more than 100 flowers which come in a wide range of sizes, shapes, colours and patterns, and a long vase-life (2 – 3 weeks) making it ideal for global cut-flower markets. As well as having long-lasting flowers, *Phalaenopsis* plants have a tendency to re-flower from old nodes along an inflorescence that flowered previously (Pridgeon, 1992).

During commercial production of *Phalaenopsis* orchids, disease problems frequently cause enormous losses in both yield and quality. Consequently, breeding of disease resistant cultivars of this orchid has long been desired. However, genetic improvement of *Phalaenopsis* cultivars to confer disease resistance through conventional plant breeding (sexual hybridisation) is restricted by its long vegetative growth phase, and by the lack of available, useful genes in the genus *Phalaenopsis* and in related genera (Burnett, 1975; Simone and Burnett, 1995; Liau *et al*., 2003). As an alternative procedure to transfer genes conferring disease resistances, genetic transformation either by direct delivery of genes into plant cells (particle bombardment) or indirectly through the use of *Agrobacterium*-mediated procedures have been reported on orchids (Anzai *et al*., 1996; Belarmino and Mii, 2000; Chia *et al*., 1990).

Since *Phalaenopsis* orchids are monocots, *Agrobacterium*-mediated gene transfer has been considered to be difficult, although transgenic orchid plants have been reported in a cultivar of *Vanda* (Chia *et al*., 1990) and two cultivars of *Dendrobium* (Kuehnle and Sugii, 1992; Chia *et al*., 1994) through direct gene transfer into cells of proto-corms by particle bombardment. Anzai *et al*. (1996) also reported transformation by particle bombardment in a *Phalaenopsis* hybrid.

Belarmino and Mii (2000) reported the first successful protocol for producing transgenic plantlets by transformation of cell aggregates in suspension cultures of *Phalaenopsis* using *A. tumefaciens* strains LBA4404 (pTOK233) and EHA101 (pIG121Hm). Both *Agrobacterium* strains had the intron-containing *GUS* reporter gene (Hood *et al*., 1986; Belarmino and Mii, 2000), which is not expressed in bacterial cells, but is in plant cells. According to their protocol, about 24 and ten transgenic plantlets were regenerated from 1 g cells after

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10 h incubation in either bacterial strain, respectively. In spite of these results, we encountered a low efficiency of transformation, possibly due to the phytotoxic effect of a prolonged incubation period and necrosis caused by the antibiotics used to eliminate *Agrobacterium*.

Antibiotics are used widely in genetic transformation technology to select transgenic tissues and/or to eliminate *Agrobacterium* after infection and co-cultivation. Elimination of *Agrobacterium* from cell cultures is essential since its continued presence with the cultured explants may reduce cell proliferation, leading to the death of transgenic tissue (Cassells, 1991; Craig and Ebert, 1991; Demain and Elander, 1999). However, antibiotics also have negative effects on plant tissues such as inhibition of cell division and plant regeneration (Mathias and Boyd, 1986; Nauerby *et al*., 1997). Therefore, an appropriate choice of a suitable antibiotic at its optimal concentration is important not only for determining transformation efficiency, but also from an economic point of view.

Here, we report an improved protocol based on the method reported by Belarmino and Mii (2000), using meropenem, a novel antibiotic, to eliminate *Agrobacterium* (Ogawa and Mii, 2005). Meropenem is a carbapenem antibiotic, which has excellent bactericidal activity *in vitro* against most aerobes and anaerobes.

MATERIALS AND METHODS

Plant materials

An embryonic cell suspension culture of *Phalaenopsis* Wataboushi '#6.13', induced from a meristem culture of axillary bud tissue as described previously (Tokuhara and Mii, 1993), was used in the present study. Cells were maintained by sub-culturing 1 g cells every 2 weeks in 100 ml conical flasks each containing 40 ml New Dogashima medium (NDM; Tokuhara and Mii, 1993) with 20 g l^{-1} sucrose (NDM-20S), supplemented with 0.1 mg l^{-1} naphthaleneacetic acid (NAA) and 1.0 mg l^{-1} benzylaminopurine (BAP) at pH 5.4. Cultures were incubated on a reciprocal shaker by agitation at 70 rpm, at 25°C, under constant illumination provided by fluorescent lamps (Toshiba FLR40S W/M/36) at 60 µmoles m^{-2} s^{-1}. After each sub-culture, 5 d-old suspension cells were used for transformation experiments.

Agrobacterium tumefaciens strains and preparation for inoculation

Agrobacterium tumefaciens strain EHA101 (pIG121Hm) which harbours the binary vector pIG121Hm that contains a kanamycin resistance gene (*npt*II), a hygromycin resistance gene (*hpt*) and the intron-containing *GUS* gene in the T-DNA region (Hood *et al*., 1986) was used in the present study. Bacteria were grown overnight at 28°C in liquid LB medium at pH 7.2 containing 50 mg l^{-1} hygromycin, 50 mg l^{-1} kanamycin, 30 mg l^{-1} chloramphenicol and 200 µM acetosyringone.

Inoculation and co-cultivation of Phalaenopsis *cells with* A. tumefaciens

Phalaenopsis suspension cells were inoculated with *A. tumefaciens* by incubating 1 g cells in 30 ml bacterial suspension consisting of a 1:10 (v/v) ratio of an overnight culture of *A. tumefaciens* suspension in NDM-10S medium (Belarmino and Mii, 2000), containing 10 g l^{-1} sucrose. After 0.5–10 h of incubation at 50 rpm (rotary agitation) at room temperature (25°C), the cells were collected on a 42 µm pore-size nylon mesh, washed with sucrose-free NDM and blotted dry with sterile Kimwipes® tissue paper. The cells were spread over a sterile filter paper placed on 30 ml co-cultivation medium consisting of 2.5 g l^{-1} Gelrite-solidified NDM-20S medium supplemented with 500 µM acetosyringone in a 90 mm × 20 mm Petri dish. After sealing the dish with Parafilm®, the cells were co-cultivated in the dark for 3 d at 25°C.

Selection of optimum antibiotic treatment for eliminating Agrobacterium

The efficacy of antibiotics in eliminating *Agrobacterium* from plant cells after inoculation was evaluated using three β-lactam antibiotics: cefotaxime (Claforan®; Hoechst, Frankfurt, Germany), carbenicillin (Sigma Chemical Co., St. Louis, MO, USA) and meropenem (Meropen®; Sumitomo Pharmaceuticals, Osaka, Japan), each belonging to different classes of cephalosporins, penicillins and carbapenems, respectively.

In treatments with cefotaxime or carbenicillin, cells (1 g) were collected from each filter paper placed on the culture medium after 3 d co-cultivation in the dark, and washed by incubating, with 50 rpm agitation, in 50 ml 400 mg l^{-1} or 500 mg l^{-1} cefotaxime or carbenicillin for 10, 30 or 60 min, respectively. Each culture, with a different washing treatment, was then collected on a 42 µm nylon mesh, washed with sucrose-free NDM and divided into three portions, each of which was placed on separate solid NDM-20S medium containing 0, 200 or 300 mg l^{-1} cefotaxime, or 0, 400 or 500 mg l^{-1} carbenicillin. These cultures were placed in the dark for 2 d prior to exposure to light. The same procedure was performed for meropenem, except that the concentrations used for washing were 1, 3, 5 or 10 mg l^{-1}, and those used for culture were 0, 1, 3 or 5 mg l^{-1}. Calli were sub-cultured into the same fresh medium every 2 weeks and kept for approx. 4–6 weeks to confirm the elimination of bacteria by observing visual re-growth.

Evaluation of bacteria-eliminating antibiotics for their effects on callus growth

The effects of the three bacteria-eliminating antibiotics, meropenem, cefotaxime and carbenicillin on callus growth were examined. For this experiment, suspension cells were transferred initially on plates of 0.2% Gelrite-solidified NDM-20S medium, supplemented with 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BAP at pH 5.4 for 4 weeks, with 2-week sub-culturing to establish callus cultures. A 0.5 g sample of callus was placed at five points on the same fresh medium containing the different kinds and concentrations of antibiotics (i.e., meropenem at 3, 5, 10 or 20 mg l^{-1}, cefotaxime or carbenicillin at 100, 200, 300, 400 or 500 mg l^{-1}). Each treatment consisted of three replicated plates, and all were placed in the dark for 3 d before exposing them to light. Callus growth was measured at the end of every 2-week sub-culture period. The experiment was repeated four times.

Selection of transformants

After washing, cells were transferred onto selective medium composed of 40 ml 2.5 g l^{-1} Gelrite-solidified NDM-20S supplemented with 25 mg l^{-1} hygromycin and 5 mg l^{-1} meropenem. Calli that grew on the selective medium were sub-cultured into the same fresh selective medium every 2 weeks. Hygromycin was included in the medium as the selective agent after co-cultivation, whereas the bacteria-eliminating antibiotic was omitted after the fifth or sixth sub-culture by visually confirming the absence of *Agrobacterium*.

Putative transgenic calli were thus selected and transferred onto Gelrite-solidified NDM-20S medium containing 25 mg l^{-1} hygromycin for further proliferation and sub-cultured to Gelrite-solidified NDM containing 10 g l^{-1} maltose (NDM-10M) for 1 month, to allow callus greening, with the same concentration of hygromycin. Green calli were then sub-cultured onto fresh NDM-10S medium containing 30 mg l^{-1} hygromycin for 3 – 4 months, with sub-cultures at 1-month intervals, for protocorm-like body (PLB) and plantlet formation.

Hygromycin-resistant calli were checked for stable GUS expression 30 d after co-cultivation. GUS expression was also examined in the roots and leaves of regenerated plantlets 10 months after co-cultivation. The transgenic nature of these plantlets was confirmed by PCR and Southern blot analyses.

Detection of Agrobacterium *persisting in plant tissue*

To examine the antibacterial activities of meropenem, *in planta*, 0.5–1.0 g samples were collected from cell cultures before inoculation, from calli produced 6–8 weeks after inoculation, and from PLB and 12 month-old plantlets, and homogenised with a mortar and pestle in 1–2 ml phosphate-buffered saline (PBS; 0.43 g l^{-1} KH_2PO_4, 1.48 g l^{-1} Na_2HPO_4, 7.2 g l^{-1} NaCl) in Milli-Q water, pH 7.2, as described by Ogawa and Mii (2005). An homogenate of each sample (0.5 µl) was diluted 200-fold with PBS to give 47 µl total volume, which was spread evenly over LB agar plates (90 mm × 20 mm Petri dishes) containing 30 ml LB medium. Plates were incubated at 28°C for 3 d to allow colony formation from any bacteria persisting in the tissue. The numbers of *Agrobacterium* colonies formed 2–3 d after inoculation were defined as colony forming units g^{-1} fresh weight of explant (cfu g^{-1} FW). The persistence of *Agrobacterium* in plant tissues was evaluated as the percentage of samples showing re-growth of persistent *Agrobacterium* in the total number of samples.

GUS histochemical assay

Transient *GUS* gene expression was examined in cell cultures 10 d after co-cultivation by counting the number of blue foci per 50 mg cell culture. Stable *GUS* gene expression was also examined in the hygromycin-resistant calli, PLBs and plantlets obtained after culture on selection medium. Cells (approx. 10 mg) and excised leaf and root tissues were immersed in X-Gluc solution containing 50 mM phosphate buffer, 1 mg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Wako Pure Chemical Industries Ltd., Osaka, Japan) and 0.5% (v/v) Triton X-100 (Jefferson *et al.*, 1987), then placed under mild vacuum for 15 min and incubated overnight at 37°C. After the reaction, calli and sample tissues were washed with 70% (v/v) ethanol to remove the chlorophyll. Tissues that stained blue with the indigogenic dye were scored as those expressing the *GUS* gene.

DNA extraction and molecular analysis

Total genomic DNA was isolated from young leaves of putative transgenic plants and control plants following the cetyl-trimethyl-ammonium bromide (CTAB) 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, Japan) using specific oligonucleotide primers that amplified a 1.2 kb fragment of the *GUS* gene (Hamill *et al.*, 1991). PCR amplification was carried out under the following conditions: 35 cycles of 1 min at 92°C, 1 min at 55°C and 2 min at 72°C. Primers used to amplify the expected 1.2 kb fragment of the *GUS* gene were 5'-GGTGGGAAAGCGCGTTACAAG-3' and 5'-GAGGCTATTCGGCTATGACTG-3'. PCR products were separated by electrophoresis in 1.0% (w/v) agarose gels in Tris-acetate (1× TAE, pH 8.0) at 50 V for 1 h, then visualised by immersion in 100 µg ml^{-1} ethidium bromide before photographing.

Southern hybridisation to detect the *GUS* gene was carried out using a digoxigenin-labelled hygromycin resistance gene (*hpt*) probe, following the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Genomic DNA from hygromycin-resistant plantlets transformed by EHA101 (pIG121Hm) was digested by *Hin*dIII, which produced a single cut in the T-DNA fragment containing the *GUS* gene.

RESULTS AND DISCUSSION

Effect of meropenem, cefotaxime and carbenicillin on bacterial elimination and callus growth

The type and concentration of antibiotic affected the efficacy of elimination of *Agrobacterium* in *Phalaenopsis* cell cultures after 4–6 weeks of culture (Table I). Both cefotaxime and carbenicillin required high concentrations (300 mg l^{-1} and 500 mg l^{-1}, respectively) to suppress the re-growth of *Agrobacterium*, whereas meropenem required much lower concentrations (3–5 mg l^{-1}) to obtain the same result. The effectiveness of meropenem at lower concentrations, compared with the other two antibiotics, was shown in a previous study on tobacco (Ogawa and Mii, 2005) and was confirmed in the present study on *Phalaenopsis*. With all three antibiotics, the use of relatively high concentrations of the antibiotic in the washing solution, and the duration of the washing treatment, were shown to be effective at reducing the concentration of antibiotic required to suppress the growth of *Agrobacterium* in the post-co-cultivation medium.

For cefotaxime treatment, the use of 500 mg l^{-1} in the washing solution, in combination with 300 mg l^{-1} in the post-co-cultivation treatment, resulted in a reduced washing treatment of 30 min, compared to 400 mg l^{-1} which required 60 min to suppress the re-growth of *Agrobacterium*. With carbenicillin, bacterial growth was suppressed only when 500 mg l^{-1} was applied during both

TABLE I
Effect of three antibiotics used in the washing solution and culture medium on re-growth of Agrobacterium tumefaciens *strain EHA101 (pIG121Hm) 4 – 6 weeks after infection treatment*

		Cefotaxime						Carbenicillin						Meropenem									
Concentration for washing (mg l^{-1})		400			500			400			500			3				5				10		
Concentration in culture medium (mg l^{-1})		0	200	300	0	200	300	0	400	500	0	400	500	0	1	3	5	0	1	3	5	0	1	3	5
Washing time (min)	10	+++[a]	+	+	++	+	+	+++	+++	++	++	+	–	++	++	+	–	+	+	+	–	+	+	–	–
	30	++	+	+	+	+	–	+++	++	++	++	+	–	++	++	–	–	+	+	–	–	+	+	–	–
	60	++	+	–	+	–	–	+++	+	+	+	+	–	+	+	–	–	+	+	–	–	–	–	–	–

Cells were sub-cultured twice at 2 week-intervals.
[a](+++) re-growth over the surface of most of the *Phalaenopsis* cell clumps and on the filter paper; (++) re-growth on three or more cell clumps; (+) re-growth at one to two cell clumps; (–) no re-growth.

the washing treatment and post-co-cultivation medium, whereas washing treatments with 400 mg l^{-1} carbenicillin could not suppress the growth of bacteria even when 500 mg l^{-1} was applied in the post-co-cultivation medium.

Significant suppression of bacterial growth was observed in washing treatments using meropenem. Increased concentrations of meropenem in the washing solution resulted in the use of lower concentrations of the antibiotic in the Gelrite-solidified medium, and no meropenem was required in the post-co-cultivation medium to prevent bacterial growth when 10 mg l^{-1} meropenem was applied for 60 min after the *Agrobacterium*-infection treatment.

Both cefotaxime and carbenicillin inhibited callus growth and caused some cellular necrosis at the optimum concentrations required to suppress the growth of *Agrobacterium* in the post-co-cultivation medium, although callus growth was enhanced slightly at lower concentrations of both antibiotics (Figure 1). In contrast, meropenem, a new antibiotic effective against *Agrobacterium* (Ogawa and Mii, 2004), suppressed the growth of bacteria at quite low concentrations (3 – 5 mg l^{-1}) irrespective of its concentration and/or the duration of the washing treatment (Table I). Moreover, meropenem had no phytotoxic effect, expressed as an inhibition of callus growth, even at higher concentrations (20 mg l^{-1}; Figure 1). Similar results were reported by Ogawa and Mii (2005), who found that meropenem at 6.25 mg l^{-1} was optimal to eliminate *A. tumefaciens* strain EHA101 from infected tobacco leaf segments without phytotoxic effects. Based on these results, meropenem

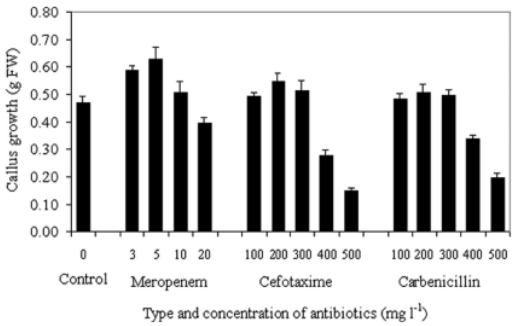

FIG. 1
Effect of bacteria-eliminating antibiotics incorporated into solidified NDM-20S medium on callus growth of *Phalaenopsis* Wataboushi '#6.13', 2 weeks after sub-culturing calli with initial weights of 0.5 g. Treatments were replicated three times, data were taken from average of three samples per plate of each replicated treatment. The experiment was repeated four times with similar results. Vertical lines (+ SE; n = 12).

was selected for subsequent transformation experiments using 10 mg l^{-1} for 10 min at the washing stage and 5 mg l^{-1} for the post-co-cultivation stage, respectively.

In planta *anti-bacterial activity of meropenem*

Many reports exist on the persistence of agrobacteria in transgenic plants when β-lactam antibiotics such as carbenicillin, ticarcillin or cefotaxime were applied as agents to eliminate them after the infection treatment (Van der Hoeven *et al*., 1991; Barret *et al*., 1997). The activities of these drugs against *A. tumefaciens* strains are known to exhibit "concentration-independent killing" or "time-dependent killing" (Craig and Ebert, 1991), and the same was also found for meropenem in tobacco tissue (Ogawa and Mii, 2004). In the present study, the persistence of agrobacteria, examined by inoculating homogenised tissues onto LB agar plates, was found in 31.8% of calli, with an average of $\leq 7.6 \times 10^7$ cfu g^{-1} FW when cultured on medium containing 5 mg l^{-1} meropenem for 8 weeks, with calli sub-cultured onto fresh medium at 2 week-intervals (Table II), although no agrobacterial re-growth was observed during this period. However, the percentage of explants showing bacterial re-growth was reduced to 7.6% in PLBs (with 2.3×10^6 cfu g^{-1} FW) and only 2.7% in regenerated plantlets (with 6.9×10^5 cfu g^{-1} FW) when examined after 12 months of culture without applying meropenem after the fifth or sixth sub-cultures (10–12 weeks; Table II). Apparent decreases in both the percentage of explants with persisting agrobacterial cells, and the average values (cfu g^{-1} FW) were observed during the regeneration process from calli to plantlets without the application of meropenem. *Agrobacteria* that persisted at the callus stage were most likely killed by antibacterial factors produced in *Phalaenopsis* tissue, particularly after regeneration of PLBs. Since *Phalaenopsis*, like other monocotyledonous plants, is not a natural host of

TABLE II
In planta *anti-bacterial activities of meropenem against* Agrobacterium tumefaciens *strain EHA101 (pIG121Hm)*

Tissue	Number of samples	Persistence of *Agrobacterium* Percentage[d]	cfu g^{-1} FW[e]
Callus[a]	44	31.8	$7.6 \pm 1.0 \times 10^7$
PLB[b]	35	7.6	$2.3 \pm 0.8 \times 10^6$
Plantlet[c]	70	2.7	$6.9 \pm 0.3 \times 10^5$

[a, b, c]8 weeks, 7 months and 12 months after co-cultivation of callus with *Agrobacterium*, respectively.
[d]Percentage of explants with visible re-growth of bacteria 2 – 3 d after inoculation of each homogenised sample with PBS on LB medium.
[e]Mean ± SE (n = 132, 105 and 210 for samples of callus, PLBs and plantlets, respectively). Each sample was tested three times by using small portions, and the data were expressed as the average number (± SE) of colony forming units per gram fresh weight of sample (cfu g^{-1} FW).

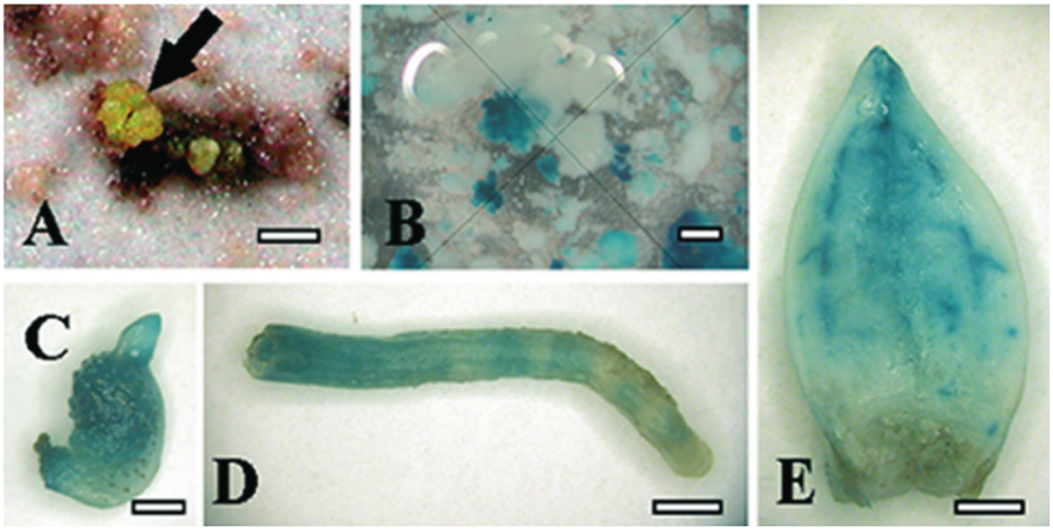

FIG. 2
GUS expression in calli and plant tissues after *Agrobacterium*-mediated transformation of *Phalaenopsis*. Panel A, emergence of greenish-yellow, hygromycin (Hm)-resistant calli from browned cell clumps 4 – 6 weeks after co-cultivation with *Agrobacterium*. Bar = 2.0 mm. Panels B–E, stable expression of *GUS* gene in Hm-resistant calli (Panel B), in PLB (Panel C) and in plantlet tissues (Panels D, E) of *Phalaenopsis* after histochemical GUS staining. Panel B, small calli 30 d after co-cultivation. Bar = 0.5 mm. Panel C, PLB 7 months after co-cultivation. Bar = 2 mm. Panels D and E, root and leaf of a plantlet 10 months after co-cultivation. Bars = 5 mm.

Agrobacterium (Hughes, 1996; Hooykaas and Schilperoort, 1992; Hooykaas-Van Slogteren, *et al*., 1984; Zupan and Zambryski, 1995), it is possible that it could produce antibacterial factors at the stage of regenerating plantlets (McClintock, 1984). It is also interesting to note that no bacterial growth was observed after 4 weeks of culture on medium containing no meropenem when *Phalaenopsis* cells were washed with 10 mg l^{-1} meropenem for 60 min after *Agrobacterium* infection (Table I). These results suggest that complete elimination of agrobacteria in regenerated transgenic *Phalaenopsis* plants could be achieved when the density of persisting agrobacteria is reduced to a low level (10^7 cfu g^{-1} FW) during an early stage after co-cultivation with *Agrobacterium*. A similar strategy, with a reduced period of antibiotic treatment, to eliminate *Agrobacterium* might be applied to many other plant species which are resistant to *Agrobacterium*-infection under natural conditions.

Selection of stable transformants

When 5 d-old sub-cultured *Phalaenopsis* cells were infected with *A. tumefaciens* strain EHA101 (pIG121Hm), co-cultivated under dark conditions for 3 d, and cultured on selection medium (i.e., NDM containing 5 mg l^{-1} meropenem and 25 mg l^{-1} hygromycin), most cell clumps turned brown and died after 4–6 weeks. After another month, small creamy white, hygromycin (Hm)-resistant cell colonies started to grow among the brown or dead cell populations and formed small greenish-yellow calli (Figure 2A). An average of 10.3 callus clones were selected g^{-1} of cells co-cultivated with *Agrobacterium* in three separate transformation experiments. From these calli, an average of 19.7 PLBs and 34.8 plantlets were regenerated, respectively (Table III). In comparison to a previous report (Belarmino and Mii, 2000), which used cell clumps or clusters, 1 g cells yielded only about five Hm-resistant calli, from which ten Hm-resistant plantlets were regenerated. The use of finer cell aggregates in the present study might have resulted in a higher yield, probably because of the increased chance of *Agrobacterium* cells making direct contact with *Phalaenopsis* cells. It is also possible that fine cell aggregates are exposed directly to the selective antibiotics after infection, resulting in fewer false transformants or escaped plants.

Putative transgenic calli, PLBs, leaves and roots of plantlets showing Hm-resistance were then subjected to GUS assays. All expressed GUS activity (blue staining;

TABLE III
Effect of infection period on transformation efficiency of Phalaenopsis orchid using Agrobacterium tumefaciens *strain EHA101 (pIG121Hm)*[#]

Infection period (h)	No. of GUS foci g^{-1} FW 3 d after co-cultivation	No. of stable Hm-resistant calli[*] g^{-1} FW	No. of Hm-resistant PLBs[*] g^{-1} FW	No. of Hm-resistant plantlets[*] g^{-1} FW
0.5	200.0 ± 14.5	0.3 ± 0.2	0.0	0.0
1	599.5 ± 22.8	1.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
2	1,439.2 ± 79.2	10.3 ± 0.9	19.7 ± 1.4	34.8 ± 0.8
4	840.8 ± 22.2	5.3 ± 0.9	7.3 ± 0.9	10.2 ± 2.3
6	839.8 ± 21.0	2.8 ± 0.5	3.2 ± 0.3	5.3 ± 1.2
10	503.8 ± 13.7	0.3 ± 0.1	0.0	0.0
12	0.0	0.0	0.0	0.0

[#]Data were taken from three separate experiments with two replicates for each treatment. Values are means ± SE (n = 6).
[*]Hygromycin concentration used was 25 mg l^{-1}.

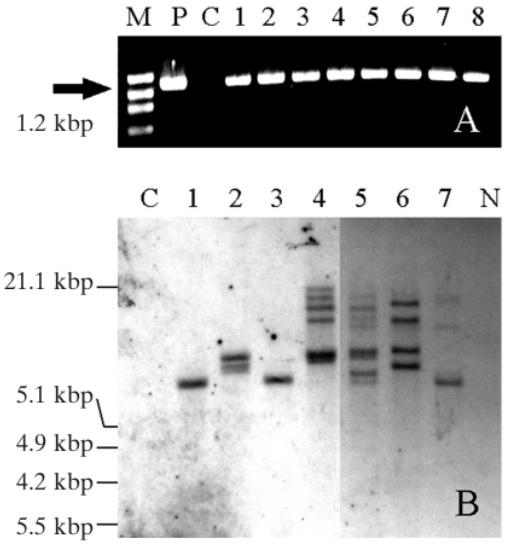

FIG. 3
Molecular analysis of putative transgenic plants. Panel A, PCR analysis of genomic DNA samples (lanes 1–8) from different transgenic *Phalaenopsis* plants to detect the GUS gene. Arrow indicates the specific 1.2 kbp DNA fragment. Lane M, molecular markers (ϕX174/*Hae*III). Lane P, plasmid pIG121Hm as a positive control. Lane C, untransformed plant as a control. Panel B, Southern blot analysis of genomic DNA from an untransformed plant (lane C), and seven transgenic plants (lane 1–7) transformed with *A. tumefaciens* strain A101 (pIG121Hm). Lanes 1–3 and 7 represent an infection period of 2 h; lane 4, 4 h; and lanes 5 and 6, 6 h. Lane N is a negative control. Genomic DNA was cut with *Hin*dIII and then hybridised with a digoxigenin (DIG)-labelled GUS probe.

Figure 2B-E), suggesting that an integrated *GUS*-gene was expressed in different organs in *Phalaenopsis* under the control of the CaMV 35S promoter.

PCR and Southern blot analysis

Putative transgenic plantlets, regenerated from Hm-resistant calli, were kept on NDM-10S medium containing 25 mg l^{-1} hygromycin. The presence of the *GUS* gene in putative transgenic plantlets was tested by PCR analysis of total genomic DNA extracted from leaves (Figure 3A). All eight Hm-resistant plantlets showed a positive amplification of the GUS gene, suggesting the presence of the transgene in the genomic DNA of the plantlets. Evidence for the integration of the transgenes into the *Phalaenopsis* genome was demonstrated by Southern blot hybridisation of genomic DNA from the putative transgenic plants using a probe that targeted the *GUS* gene (Figure 3B). Since *Hin*dIII cuts once within the T-DNA region, the number of bands obtained represented the number of insertion sites. Seven putative transgenic lines were analysed, and all gave hybridisation signals with the *gusA* gene probe, which were not detected in control plants. Figure 3B (lanes 1–3, 7) presents the results obtained from a 2 h-infection period, and show only one to two *GUS*/T-DNA insertion sites; Figure 3B (lanes 4–6) also shows multiple insertion sites obtained by 4 h and 6 h infection periods, respectively. These results suggest that 2 h of *A. tumefaciens* infection may be more desirable than other periods of infection to avoid inserting multiple T-DNA copies into the genome, which makes subsequent plant breeding programmes difficult and sometimes causes silencing of transgenes (Fagard and Vaucheret, 2000; Chandrakanth *et al.*, 2002; Maike *et al.*, 1997).

CONCLUSION

In the present study, we have improved the current procedure for *Agrobacterium*-mediated genetic transformation of *Phalaenopsis* orchids, resulting in higher efficiencies. A shorter period of inoculation with *Agrobacterium* (2 h) and the application of meropenem at 10 mg l^{-1}, a novel *Agrobacterium*-eliminating antibiotic during washing followed by 5 mg l^{-1} in the post-co-cultivation medium for 12 weeks, resulted in high numbers of transgenic *Phalaenopsis* plants. This procedure is simple and economically efficient as it requires lower concentrations and shorter times of application of meropenem.

Using this procedure, we have already succeeded in producing disease-resistant transgenic *Phalaenopsis* plantlets by transferring defensin and chitinase genes isolated from *Wasabia japonica* and *Streptomyces griseus*, respectively, within a very short period (7 months). These transgenic plants will greatly reduce the bacterial and fungal disease problems faced by mass production growers, especially in the tropics. Genetic transformation studies using other important transgenes, which could confer useful traits such as increased flower longevity, new flower colours and/or pest resistance to *Phalaenopsis* orchid are now in progress.

We wish to thank Dr. Kei-ichiro Mishiba (Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan) for his kind assistance during the course of this research and Dong Poh Chin, M.Agr. (Graduate School of Science and Technology, Chiba University, Chiba, Japan) for his assistance with the molecular analyses of the putative transgenic plants.

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