Introducing Genes into *Zygopetalum* by the Use of a Particle Gun

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Protocorms and small seedlings of *Zygopetalum* grown aseptically were bombarded with accelerated gold particles coated with plasmids containing a ß-glucuronidase (GUS) gene as the reporter. Blue transformed cells were detected by the enzymatic GUS assay. Neomycin phosphotransferase II (NPT II) gene was used as the selectable marker. Two kinds of plasmid were used, namely pBI 121 and pBI 221. Gun parameters influencing DNA delivery such as the number of bombardments and helium gas pressure as the particle propulsion were investigated. The optimal conditions for introducing foreign genes were two bombardments at 1300 psi helium gas pressure. Although there was a possibility of the escape from kanamycin, several transgenic plants were obtained after a 4-week selection culture.

INTRODUCTION

The details of the molecular biology of natural gene transfer systems of *Agrobacterium tumefaciens* and *A. rhizogenes* have been reviewed by Morrish et al. (1992). In orchids, difficulties have been encountered both in whole plant regeneration from protoplasts and in the general insusceptibility of monocotyledons to *Agrobacterium*-mediated transformation. Therefore, microprojectile bombardment is now one of the most efficient methods to induce transgenic plants in orchids. The advantage of this method is that physical penetration of the plant cell wall allows species-independent transfer of DNA into a wide range of target tissues. This microprojectile bombardment by a particle gun was developed by Sanford et al. (1984).

Because of its fragrance and good cold tolerance, *Zygopetalum* has been introduced to Japan as a pot plant. The purpose of the present study is to try to introduce GUS and NPT II genes into protocorms and small seedlings of *Zygopetalum* by the use of a particle gun. Few studies have been carried out on gene transfer by particle gun treatments in orchids.

MATERIAL AND METHODS

Plant Material. *Zygopetalum blackii* was used as the plant material. A protocorm (actually a mass of 3 to 4 intact protocorms, 5 to 10 mm in diameter) and small seedlings (with two leaves 1 to 2 cm in height obtained from aseptic seed culture) were grown in vials (Φ 6 cm × 12 cm high) on 100 ml of half-strength Murashige and Skoog (Murashige and Skoog,1962) culture medium containing 20 g liter^{-1} sucrose, 3 g liter^{-1} Hyponex, and 2.5 g liter^{-1} gelan gum. The medium was adjusted

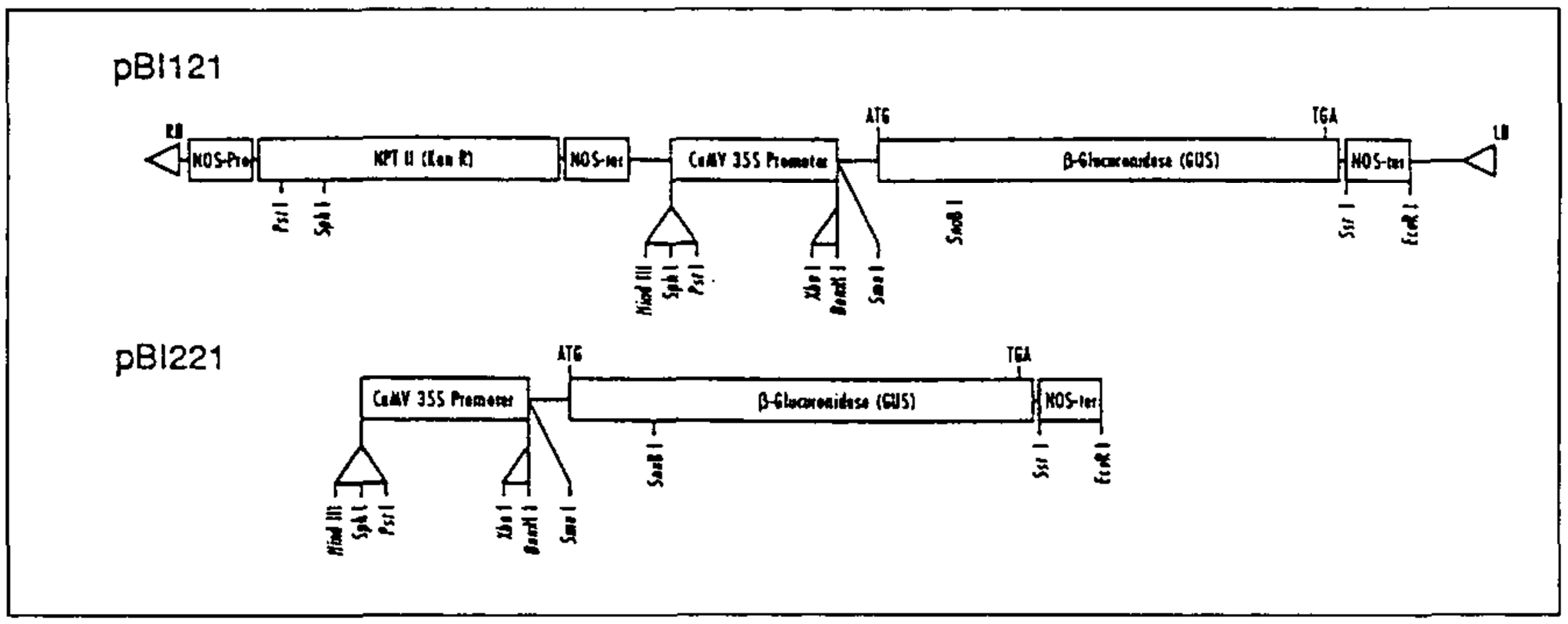

Figure 1. Two plasmids of pBI 121 and pBI 221 were used to introduce genes. Plasmid pBI 121 consists of NPTII (including kanamycin resistant gene) gene, GUS gene and CaMV 35S promoter. Plasmid pBI 221 contains GUS gene and CaMV 25S promoter.

to pH 5.8 before autoclaving. Cultures were incubated at 23C, under white fluorescent tubes for 16 h daily at 5000 lx. At 7 days before particle gun treatment, about 60 protocorms or 15 seedlings were transplanted in a petri dish (Φ 9 cm) containing 40 ml of the medium mentioned above.

Bombardment. Protocorms and seedlings of *Zygopetalum* were bombarded with accelerated gold particles (1 or 1.6μm) coated with pBI 121 plasmid or pBI 221 plasmid containing a GUS gene as the reporter with a CaMV 35S promoter. An NPT II gene with a nopaline synthase (nos) promoter, which was included in a pBI 121 plasmid, was used as the selectable marker. The two genes were bombarded by a BIORAD PDS-1000/He particle gun. Gun parameters influencing DNA delivery, such as the number of bombardments (1 or 2 shots), helium gas pressure as the particle propulsion, using 1100 psi or 1300 psi rupture disks, and the effectiveness of a 12-cm-target distance, were investigated, respectively. Two plasmids of pBI 121 and pBI 221 are shown in Figure 1.

GUS Transent Assay. Two days after bombardment blue transformed cells were detected by 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Jefferson, 1988). All protocorms and seedlings bombarded with plasmid 221 were examined for the GUS transent assay.

Selection of Transgenic Plantlets. After 1 week of culture under dark conditions, all protocorms and seedlings bombarded with plasmid 121 were transplanted into vials (Φ 6 cm × 12 cm) and cultured under dark conditions. Each vial contained 100 ml of ½ MS medium with a 500 mg liter^{-1} kanamycin for selection. After a 4-week selection culture, the surviving plantlets were transplanted onto the selection media and cultured under white fluorescent tubes for 16 h daily at 5000 lx.

RESULTS AND DISCUSSION

Gene transfer techniques for many dicotyledonous crops have been successfully developed by using *A. tumefaciens*-mediated gene transfer (Klee et al., 1987). Because of the difficulties involved in transformation and regeneration of monocotyledons using protoplasts (Morrish et al., 1992), microprojectile bombardment was tested to introduce foreign DNA into intact plant cells (Sanford, 1988).

Table 1. Expression of GUS gene introduced by a particle gun in protocorms of *Zygopetalum*.

Rupture disk		Protocorms with blue spots (%)	Average number of blue spot/protocorn	
1100 psi	1 shot	0%	0	(0)
	2 shots	3.3%	1.5	(0.7)
1300 psi	1 shot	4.5%	6.5	(3.5)
	2 shots	28.8%	3.9	(3.4)

Plasmid 221; gold particle size 1.6 μm; (SD); X-Gluc GUS assay; total number of protocorms was 60

Table 2. Expression of GUS gene introduced by a particle gun in seedlings of *Zygopetalum*.

Rupture disk		(%) of protocorm with blue spots	Average number of blue spot/seedling	
1100 psi	1 shot	0%	0	(0)
	2 shots	0%	0	(0)
1300 psi	1 shot	0%	0	(0)
	2 shots	40.0%	14.8	(14.4)

Plasmid 221; gold particle size 1.6 μm; (SD); X-Gluc GUS assay; total number of seedlings: 15

Table 3. Percentage of survival protocorms and seedlings (including transgenic plants) after selection culture with kanamycin.

Rupture disk		Survival percentages of protocorms	seedlings
1100 psi	1 shot	0	0
	2 shots	3.3	0
1300 psi	1 shot	3.3	0
	2 shots	10.7	0.05
	Control	0	0

Plasmid 121; gold particle size 1.0 μm; modified (½ MS) medium containing 500 mg liter^{-1} kanamycin.

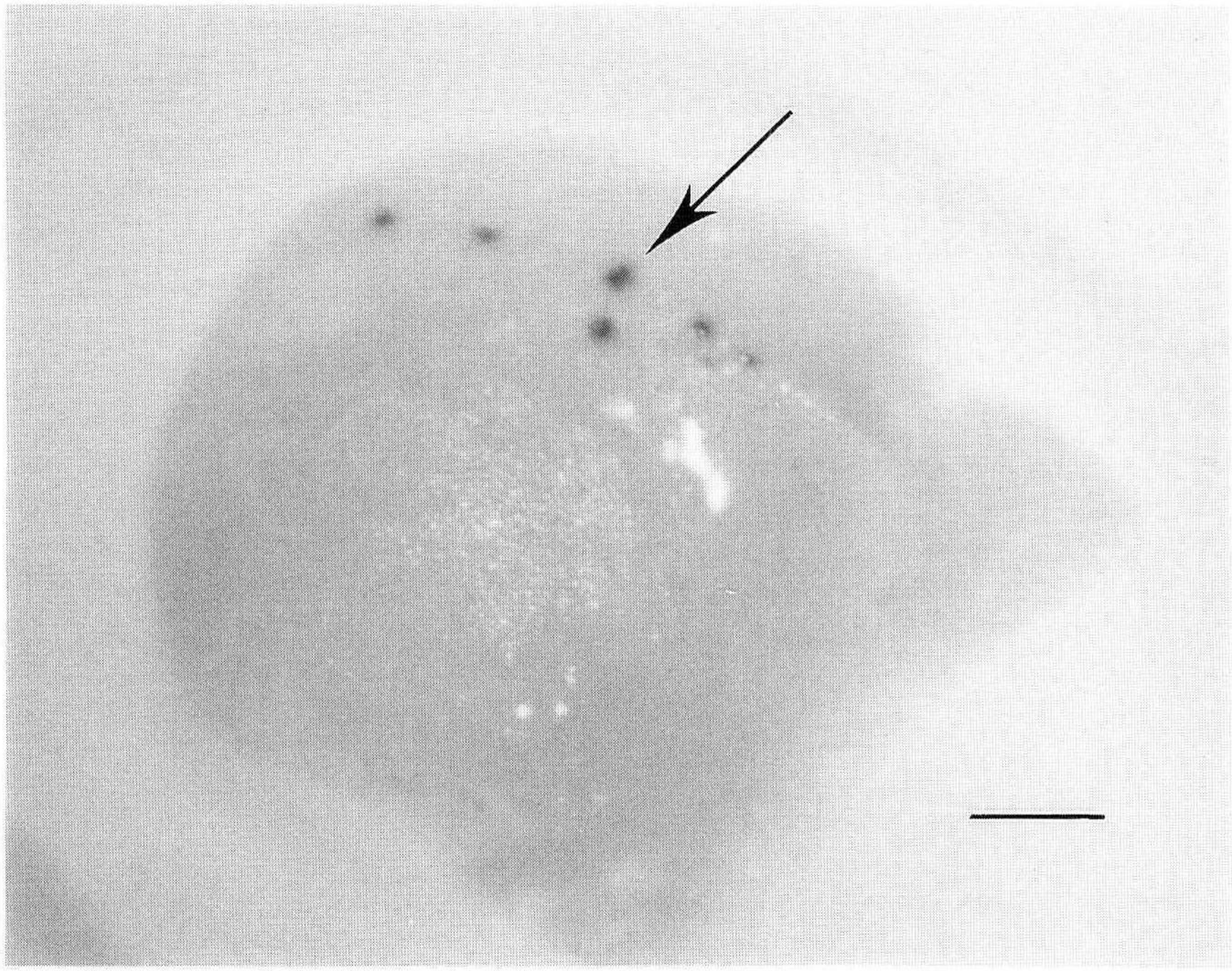

Figure 2. A protocorm with blue spots as the GUS gene expressions, which was treated with a 70% ethanol solution to remove chlorophyll. An arrow points to one of the blue spots. Scale bar = 1 mm.

GUS Transent Assay. In general, GUS (Jefferson, 1988), chloramphenicol acetyltransferase (CAT) (Fromm et al., 1985) and luciferase (Ow et al., 1987) have been used as reporter genes. Expression of the GUS gene introduced by particle gun into protocorms of *Zygopetalum* is shown in Table 1. After a one shot bombardment with 1100 psi rupture disks, no blue spot was observed, in other words, no GUS gene was expressed. However, after two shots, 1.5 blue spots on average were observed in 3.3% of protocorms. After one shot with 1300 psi rupture disks, 6.5 blue spots were observed in 4.5% of protocorms. In the case of 2 shots, 3.9 blue spots on average were observed in 28.8% of protocorms. GUS genes were expressed more when the bombardment was performed by using 1300 psi rupture disks rather than 1100 psi rupture disks, and by two shots rather than one shot, as indicated by the increase in blue spots. Therefore, it is best to bombard the protocorms twice with 1300 psi rupture disks. Figure 2 shows a protocorm with blue spots as the GUS gene expression. There is a possibility that a higher gas pressure than 1300 psi would result in better particle propulsion, because the number of blue spots was relatively small. The expression of the GUS gene introduced by a particle gun in seedlings of *Zygopetalum* is shown in Table 2. When seedlings were bombarded twice with 1300 psi rupture disks, 40% of seedlings exhibited 14.8 blue spots on average as the GUS gene expression. Therefore, it is also desirable to bombard seedlings twice with 1300 psi rupture disks. It seems that 1300 psi gas pressure at least is necessary

to introduce GUS genes into seedlings with a particle gun. It was shown that the target distance of 12 cm is enough to introduce foreign genes into protocorms and seedlings, because the damage to the materials was observed after both one and two shot bombardments. It was found possible to use both 1.6 μm and 1.0 μm diameter gold particles for introducing genes by bombardment.

Survival after selection culture. The survival percentages of both protocorms and seedlings, bombarded by the particle gun, after selection culture with Kanamycin for 3 weeks are shown in Table 3. Very few bombarded seedlings, and no protocorms or seedlings in the control survived after selection culture. If bombarded twice with 1100 psi rupture disks, 3.3% of protocorms survived and with 1300 psi, 10.7% of protocorms survived. Therefore, two shots of the bombardment with 1300 psi rupture disks might be the best way to introduce foreign genes into protocorms. The reason for the low seedling survival rate was because the cells were unable to recover and grow under a state of chimera introduced by a pBI 121 plasmid having NPT II- and kanamycin-resistant genes. On the other hand, 10.7% of bombarded protocorms survived, because if some parts of the cells in a protocorm obtained kanamycin-resistant genes, included in pBI 121 plasmids, by means of the bombardments, a protocorm could recover and grow even from a small surviving part of it in a selection medium containing Kanamycin. In general, a protocorm can grow and develop even from part of a divided protocorm.

Transgenic Plantlet. After 4 months of selection culture, seven plantlets were obtained, which might be transformants with GUS and NPT II genes. However, there is still a possibility that these plantlets escaped foreign gene introduction. It is therefore necessary to detect introduced GUS and NPT II genes by the PCR (polymerase chain reaction) method following the Southern analysis of transgenic plants. Although kanamycin selection using the NPT II gene has been widely used in the recovery of transformed dicotyledonous plants, monocotyledonous cells have been quite resistant (Morrish et al., 1992). It might be better to use geneticin instead of kanamycin for the selection medium. There is also the possibility of using a smaller plasmid in place of plasmid 121, because plasmid 121, containing the NPTII gene (0.8 kb) and the GUS gene (1.9 kb), is relatively large to introduce genes into the HB 101 host. The 35S promoter from cauliflower mosaic virus (CaMV) has been widely used in plant transformation studies including a study to get transgenic plants in maize (Fromm et al., 1990), but it is not regarded as the optimum promoter for use with all monocots, so T-DNA genes for opine synthase, nopaline synthase, or other promoters must be considered in order to increase transformation frequency.

CONCLUSIONS

It was found that GUS and NPT II genes could be introduced into protocorms and seedlings of *Zygopetalum* by microprojectile bombardment. The optimum conditions for introducing two genes were two shots of bombardment with 1300 psi of helium gas pressure. Although there was a possibility of escape from kanamycin, several transgenic plants were obtained after selection.

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