Optimization of *Cymbidium* Transformation System by the Particle Gun Techniques

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ABSTRACT

Process of particle bombardment for efficient transformation of *Cymbidium* virescence rhizome microcross sections was investigated using Biolistic particle delivery system with pBI121 harboring the β-glucuronidase(GUS) and the neomycin phosphotransferaseII(NPTII), and pBI221 containing GUS. The best result was obtained from the combination of 1.11 μm tungsten particles coated with pBI121, 77.33 kg/cm^2 helium pressure, 6.35 mm gap distance, and 3.8 cm target distance.

Transient expression of the reporter gene, GUS, bombarded into the rhizome microsections was observed by the histochemical assay. The selectable marker gene, NPT II, delivered by bombarding the tungsten particles coated with the plasmid DNA was identified using the polymerase chain reaction technique.

INTRODUCTION

Although mass propagation of *Cymbidium* virescence has been successfully carried out by the tissue culture techniques in the farms recently, breeding for new varieties mostly remains to be improved by introducing the transgenic plant techniques.

Electroporation and *Agrobacterium*-mediated transformations are the mostly used methods for gene transfer into plants but the former has a disadvantage of using protoplast which is difficult to regenerate to whole plant and the latter has difficulties of limited host range, low transformation efficiency and the need for *Agrobacterium* removal after transformation.

Since the efficient technique for delivering DNA into the cells and tissues of plant via high-velocity particles was firstly reported by Sanford, et al. in 1987(4), transformations of cells or subcellular organelles of various plants (2) have been demonstrated as well as in animals and microorganisms.

There are numerous factors affecting transformation efficiency but in this study the size and amount of microparticles, helium pressure, the distance between the rupture disk and macrocarrier, the distance between microprojectile and the stopping screen, and the distance between stopping screen and the biological target were considered.

The purpose of this study is to establish the optimum conditions of the particle gun system for the transformation of *Cymbidium* virescence rhizome and to demonstrate the transient expression of GUS and NPTII genes in the bombarded tissues.

MATERIALS AND METHODS

Plant materials

Rhizome of *Cymbidium* virescence was cultured in the MS media(1/4, 1/2, full strength) and 1/1000-diluted Hyponex medium. As biologically active compounds, arginine, aspartic acid and rutin were compared at the concentrations of 1, 2, 5, 10 and 20 mg/l. Different levels of NAA(0, 0.1, 1 and 2.5 mg/l) and Fulmet(0.1, 5, 10 and 20 μg/l) were treated as plant growth regulators.

The cultured rhizome was cut into small pieces: 400 μm,

600 μm, 2 mm and 5 mm, and bombarded with the tungsten particles coated with plasmid DNA under the different parameter conditions of particle gun system.

Preperation of plasmid DNA and tungsten pellets

The plasmid pBI121(obtained from Korea Univ, Professor S. Y. Lee) cloned in E.coli HB101 was isolated and purifided with the Wizard megaprep kit.

Plasmid pBI121 contains both GUS and NPTII genes but pBI121 purchased from Clontech has GUS only.

For DNA coating on the tungsten particles, 5 μl of plasmid DNA (1 μg/μl) was added to 25 μl of 60 mg/ml tungsten particles suspended in 50% (v/v) glycerol solution, followed by 25 μl of 2.5 M $CaCl_2$ and 10 μl of 0.1 M spermidine. The tungsten pellet obtained by gentle centrifugation was washed with absolute ethanol and finally added to 24 μl of 100% ethanol.

6 μl of the suspension was applied on the macrocarrier and used for bombardment.

Bombardment parameters

Microcarrier size(0.73 μm, 1.11 μm, 1.32 μm), helium pressure (63.27 kg/cm^2, 77.33 kg/cm^2, 91.39 kg/cm^2), gap distance (3.175 mm, 6.350 mm, 9.525 mm) and target distance (3.8 cm, 7.0 cm, 10.2 cm) were compared.

Histochemical GUS assay

Expression of the GUS gene in the bombarded *Cymbidium* rhizomes was observed histochemically. The tissue was incubated at 37℃ for 50hr in the mixed solution containing 1.9 mM X-gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.3% Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.0). The blue-colored GUS expression was observed under a binocular microscope.

Identification of NPTII gene by polymerase chain reaction(PCR)

Total DNA of cultured rhizome was isolated by the method Chee, et al. (1).

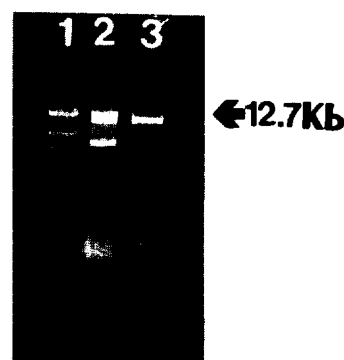
The length of primer pair used for PCR of NPTII was 20 mer and the size of DNA amplified was 0.795 kb. The reaction medium for PCR contained 1.5 mM-$MgCl_2$, 200 μm of each dNPT, 0.25 M of each primer, and 2.5 μl of Taq DNA polymerase(2.5 U/μl) per 50 μl reaction volume. The thermal cycle was [96℃/1min] - [58℃/30sec] - [72℃/1min] with total 30 cycles.

RESULTS AND DISCUSSION

Rhizome growth of *Cymbidium* virescence and bombardment parameters.

Proper growth of rhizome was found above 600 μm of section size in 1/4 strength MS media enriched with biological active compounds (2 mg/l arginine, 2 mg/l aspartic acid, and 10 mg/l rutin) and growth regulators (1 mg/l NAA and 5 μg/l Fulmet). All of the rhizome sections regardless of size became brownish when they were placed on the growth media after bombardment and began to produce new cells two weeks after cultivation.

The plasmid pBI121 was isolated from the E.coli culture suspension, purified by the Wizard megaprep kit, and electrophoresed as shown in Fig. 1.



1. Marker(λDNA/Hind III)
2. pBI121
3. pBI121 digested with EcoRI

Fig.1. Agarose gel electrophoresis of pBI121 isolated.

Table 1 shows the optimum conditions of bombardment. One of the most important things was how to load the DNA suspension uniformly just on the central point of macrocarrier to get good replication and distribution of tungsten particles coated with DNA. Higher helium pressure and shorter gap distance increased particle velocity but also increased variability and gas shock impact. Longer target distance brought about deacceleration and off-centered flight of microprojectiles.

Table 1. Bombardment conditions for *Cymbidium virescence* rhizome.

Tungsten size	He pressure	Gap distance	Target distance
11 μm	77.33 kg/cm^2	6.35 mm	77 mm

Selection for transgenic rhizomes, and transient expression of GUS and NPTII genes.

Two months after bombardment, rhizome sections which were resistant to kanamycin could be selected visually and separated for further culture(Photo 1).

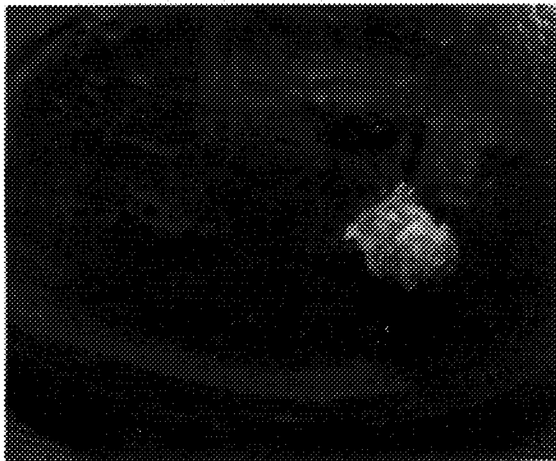


Photo 1. Direct organogenesis from the sliced rhizome section

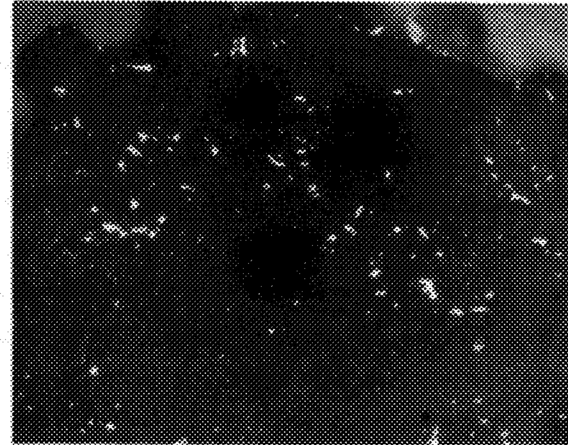
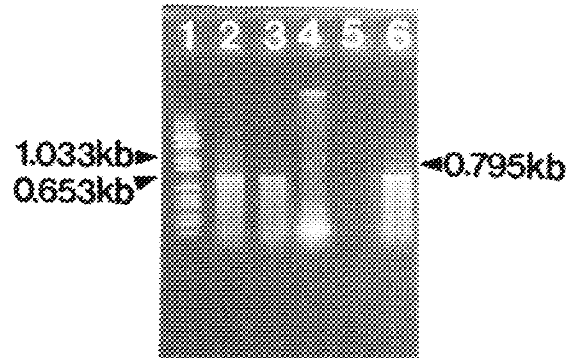


Photo 2. Transient GUS expression in the bombarded rhizomes

Photo 2 shows the blue color in the bombarded rhizome sections when they were incubated in the X-gluc solution, indicating transient expression of GUS. It took 50 hr incubation time at 37℃ to get full color development, which is much longer than the time used for lily pollen(3).



1. Marker (pBR328/Bgl I-Hinf I)
2. PCR of pBI121 with NPTII
3. PCR of pfad3 with NPTII
4. Total DNA from rhizome without PCR
5. PCR of DNA from nonbombarded rhizome
6. PCR of DNA from bombarded rhizome

Fig.2. PCR products of NPTII gene in pBI121

Since it takes long time to culture *Cymbidium* virescence from rhizome to flowering plant, the early confirmation of a target gene in transformed rhizomes is very necessary. So the plants contaning no target gene can be discarded early. For this purpose the PCR technique was introduced to identify NPTII gene which can be flanked by other useful genes. Total plant DNA was isolated from the bombarded and 4~week cultured rhizome, and NPTII gene was amplified by PCR to confirm its expression. The electrophoretic result is given in Fig. 2.

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