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Transformation of Phalaenopsis by Particle Bombardment

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Transgenic phalaenopsis plants have been obtained by particle bombardment with a pneumatic particle gun device. All promoters of cauliflower mosaic virus(CaMV)35S, maize ubiquitin and rice actin genes could be expressed in phalaenopsis using transient assay of β-glucuronidase(*gus*)gene as a reporter. Bialaphos resistance gene(*bar*)which confers tolerance to herbicide bialaphos, was used as a selectable marker. Protocorm-like bodies(PLBs)of phalaenopsis derived from the leaf segment culture were bombarded by gold particles coated with pMSP38 and pWI-GUS DNA containing the *bar* gene and the *gus* gene respectively driven by 35S promoter. Newly-formed PLBs were selected on the medium including bialaphos. Finally seven transgenic plants resistant to bialaphos were obtained, and one of them contained and expressed *gus* gene. PCR analysis confirmed their transgenic nature and western analysis and histochemical GUS assay showed the expression of *bar* and *gus* gene respectively.

Introduction

Breeding of phalaenopsis orchid by the conventional method of sexual hybridization takes a long time and requires much effort because of its long growth and reproductive cycle. Genetic engineering techniques have made possible the introduction of the gene encoding desirable traits into many plant species.

Phalaenopsis is a monocot plant and *Agrobacterium*-mediated gene transfer was thought to be difficult in such plants. Recently, transformation of *Vanda*[1] and *Dendrobium*[2,3] by particle bombardment have been reported. However, there was no report on phalaenopsis. In phalaenopsis, the scheme for *in vitro* propagation of PLBs obtained from cultured leaf segments has been established by Tanaka[4] and this would provide a good method of regeneration of transgenic plants. Since rice actin and maize ubiquitin promoters are effectively expressed in monocot plants[5], these are expected to express in a monocot phalaenopsis.

In this study we have tried to develop a transformation system of phalaenopsis by using a particle bombardment method, and examined the suitability of the *bar* gene as a selectable marker and the reliability of PLBs as target tissues.

Materials and Methods

1. *Preparation of plant materials*

A phalaenopsis hybrid(phalaenopsis Danse×*Doritaenopsis* Happy Valentine)was used in this

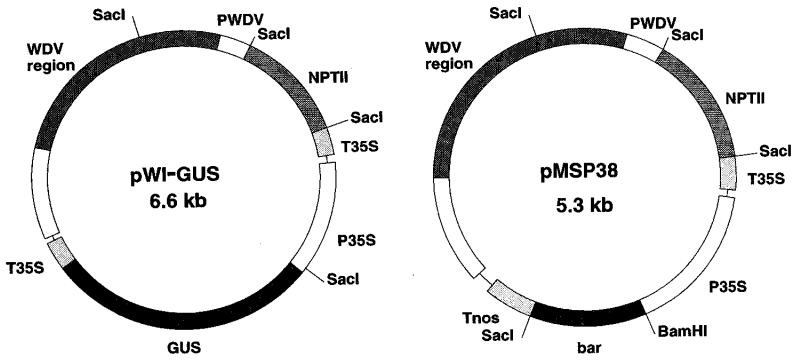

Fig. 1 Schematic representation of pWI-GUS[8] and pMSP38 plasmids.
P35S, cauliflower mosaic virus(CaMV)35S promoter; Tnos, nopaline synthase terminator; T35S, CaMV 35S terminator; *bar*, bialaphos resistance gene; NPTII, neomycin phosphotransferase gene; GUS, β-glucuronidase gene; WDV region, a portion of wheat dwarf virus.

study. PLBs formed in the leaf segments obtained from flower-stalk cuttings culture were bisected transversely to induce proliferation of new PLBs repeatedly[4].

For particle bombardment, these bisected PLB segments were placed cut-surface down in a circular area of 3 cm in diameter on modified Vacin and Went's agar medium(VW)[4] in petri dishes (approx. 50-60 segments per dish).

2. *Plasmid DNAs*

The plasmid pBI221[6] containing *gus* gene as a reporter was used to determine the effects of accelerating pressure on the transient GUS expression. The effect of the expression of promoters on the transient GUS expression was examined using three different plasmids with *gus* gene; pBI 221, pAHC27[7] and pActI-F8, including the 35S promoter from CaMV, the ubiquitin one from maize, and the actin one from rice, respectively.

Since a geminivirus shuttle plasmid(pWI-11)[9] derived from wheat dwarf virus(WDV)has been proved to replicate in monocot plants and to enhance transformation efficiency, pWI-GUS[9] and pMSP38 were used to obtain transformed plantlets(**Fig. 1**). The bialaphos resistant gene(*bar*)[10] expression cassette driven by 35S promoter and flanked by nopaline synthase(nos)terminator was inserted into wheat geminivirus shuttle plasmid pWI-11 to give a plasmid pMSP38.

3. *Particle bombardment*

Bombardment was carried out using an automatic pneumatic particle gun device(Rehbock model 260, Rehbock Co., Japan)[11]. Plasmid DNAs were coated onto gold particle(1.1 μm in diameter, Tokuriki Honten Co., Tokyo, Japan)as reported previously[12]. Other conditions for bombardment were essentially the same as described by Iida *et al.*[13].

4. *Histochemical GUS assay*

After bombardment, the samples were kept on the modified VW medium[4] for 24 hr at 25°C under 16 hr photoperiod. Transient expression of the *gus* gene was histochemically assayed by transferring the samples to GUS substrate mixture containing X-Gluc according to the method of Iida *et al.*[13] and incubating overnight at 37°C.

5. *Selection of bialaphos-resistant PLBs*

The sensitivity of phalaenopsis PLBs to bialaphos(Meiji Seika Kaisha, Ltd., Yokohama, Japan) was tested preliminarily by using untransformed PLBs. Levels of bialaphos tested ranged from 0.1 to 10 mg/*l*. Regenerated PLBs from bombarded segments were cultured in a 16 hr photoperiod at

25°C on the modified VW medium.

6. *DNA analysis using PCR*

Genomic DNA of phalaenopsis was isolated by CTAB method from root tissues according to Kuehnle and Sugii[2]. The DNA was subjected to the polymerase chain reaction(PCR). The primer pairs were designed to amplify an internal 412 bp *bar* fragment; BAR 5: 5′-CGAGACAAGCACGGTCAACTTC and BAR 6: 5′-AAACCCACGTCATGCCAGTTC and an internal 448 bp *gus* fragment; GUS 1: 5′-AATTGATCAGCGTTGGTGG and GUS 2: 5′-GGTGTAGAGCATTACGCTGC. Reaction Mixture(50 μl) contains 1×reaction buffer(Pharmacia Co.), 200 μM of dNTP, 1 μM of each primer, 1.25 unit of *Taq* DNA polymerase(Pharmacia Co.) and DNA. Amplification condition was 30 cycle of 1 min. at 94°C, 2 min. at 60°C and 2 min. at 72°C using a DNA Thermal Cycler(Atto, Co.) The amplified DNA was electrophoresed in a 1.5% agarose(FMC) gel.

7. *Western blot analysis*

phalaenopsis root tissues(100 mg) were homogenized in 50 μl of extraction buffer containing 50 mM Tris-HCl(pH 7.5), 0.15 mg/ml phenylmethanesulfonylfluoride(PMSF). The extracts were centrifuged and protein concentration was determined by the Bio-Rad protein assay. The samples were analyzed on 10-20% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon Transfer Membrane(Millipore) according to the manufacturer. The blots were incubated with rabbit anti-PAT antibodies and were then visualized by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG followed by color development with Immunostain HRP-1000 (Konika, Co.).

8. *Herbicide application*

Transgenic plants were grown in the greenhouse for 7 months. Control and transgenic plants were sprayed with a 1.5% solution of the commercial herbicide HERBIE™ containing 18% bialaphos(Meiji Seika Kaisha, Ltd., Japan) and evaluated for herbicide resistance 10 days later.

Results and Discussion

1. *Determination of the optimum condition of bombardment for the transient GUS expression*

Blue spots of transient GUS expression were observed after two bombardments were given to each PLB sample with pBI221-coated particles at the various accelerating pressures; 5, 10, 15, 20 and 25 times pumping(**Table 1, Fig. 2-A**). The significant expression of GUS were observed at over 15 times and maximum at 25 times pumping. It is considered that new PLBs are derived from epidermal cells of PLB segments cultured in phalaenopsis[14]. Therefore 15 times pumping was used for the accelerating pressure in the following experiments to introduce DNA into epidermal cells.

As shown in **Table 2**, blue spots of transient GUS expression were observed in three different

Table 1. Effect of accelerating pressure on the transient GUS expression in phalaenopsis PLBs.

Accelerating pressure (times pumping)	Number of blue spots*1
5	1.0± 0.8
10	9.0± 3.3
15	60.0±31.2
20	64.7± 3.3
25	107.7±43.4

Ten PLBs and pBI221 were used for each treatment.

*1 Average of three experiments.

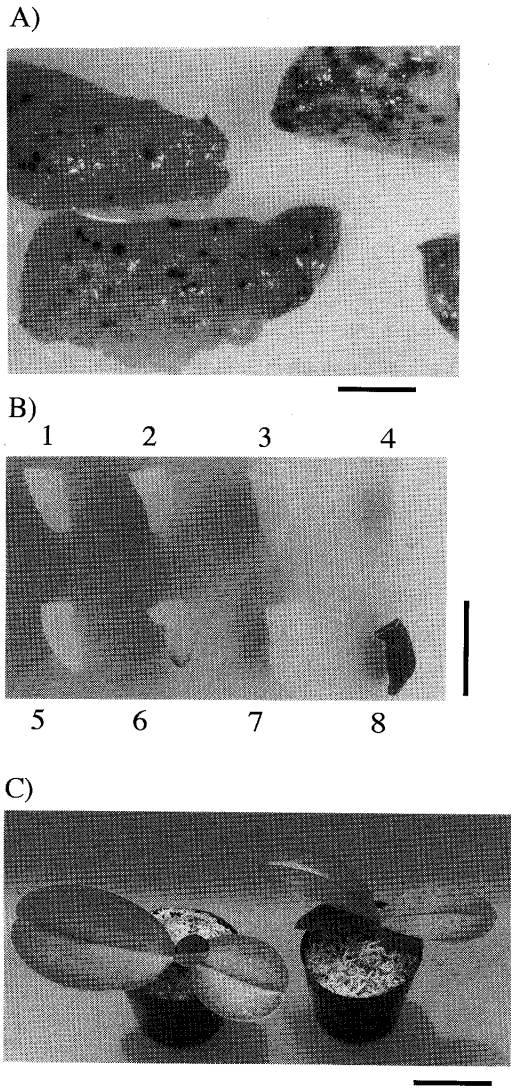

Fig. 2 Histochemical assay of GUS activity in phalaenopsis and herbicide application.
A : Transient GUS expression of PLB 24 hr after bombardment with pBI221 by 15 times pumping. Bar: 2 mm
B : Stable GUS expression in the root tips of transgenic plantlets. 1, untransformed plant; 2-8, transgenic plantlets. Bar: 10 mm
C : Untransformed control(left) and transgenic(right) plants were sprayed with HERBIE™. Bar: 5 cm

Table 2. Effect of promoter on the transient GUS expression in phalaenopsis PLBs.

Plasmid	Promoter	Number of blue spots*1
None		0.0
pBI221	CaMV 35S	98.0±42.2
pAHC27	maize ubiquitin	61.3±14.8
pActI-F	rice actin	13.7± 7.6

Ten PLBs and 15 times pumping were used for each treatment.
*1 Average of three experiments.

promoters compared. Though all promoters tested were functioned in phalaenopsis, the 35S promoter was the most effective among them. Therefore, plasmids containing 35S promoter were used in later experiments.

2. *Selection of transformants*

622 PLB segments were bombarded twice with gold particles coated with equal amounts of pMSP 38 and pWI-GUS at 15 times pumping. These bombarded segments were then cultured on the medium without bialaphos in order to induce new PLBs from all the segments, because the new PLBs were not formed in bombarded segments directly on the medium containing bialaphos due to the browning caused by oxidation of the phenolic compounds.

The growth of untransformed PLBs was almost completely inhibited at 1 mg/*l*, and at 5 mg/*l* bialaphos, it was lethal. Based on this result, about 6000 of newly-formed PLBs from bombarded segment were transferred to the modified VW medium[4] with 1 mg/*l* bialaphos approximately 2 months after bombardment. After another 1-2 months incubation, PLBs which survived in the initial selection were then re-transferred to the medium containing 5 mg/*l* bialaphos. Finally seven plantlets resistant to 5 mg/*l* bialaphos were obtained from the 622 PLB segments bombarded initially, after 3-4 months from start of selection at 1 mg/m*l* bialaphos. These 7 plantlets developed from bialaphos-resistant PLBs were used for further analysis.

3. *Characterization of transgenic plants*

One out of the seven plantlets resistant to bialaphos (5 mg/*l*) was positive to the assay for GUS activity. GUS expression is shown in the histochemical staining of root tip from bialaphos-resistant phalaenopsis (**Fig. 2-B**).

DNA analysis using PCR detected the amplified DNA in the expected size of 412 bp for *bar* or 448 bp for *gus* gene from potentially transgenic plantlets, while no product was observed from the untransformed control plantlet (**Fig. 3**). All of seven transgenic plants contained *bar* gene and one

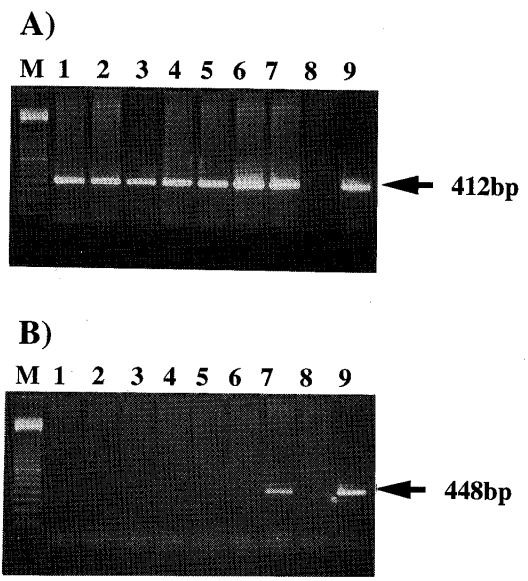

Fig. 3 PCR analysis of transgenic phalaenopsis.
A : Detection of the *bar* gene.
B : Detection of the *gus* gene.
Lane 1-7, DNAs from seven transgenic plantlets; lane 8, untransformed plant; lane 9, pMSP38 DNA in (A) and pWI-GUS DNA in (B) as positive control; lane M, 100 bp ladder as a molecular size marker.

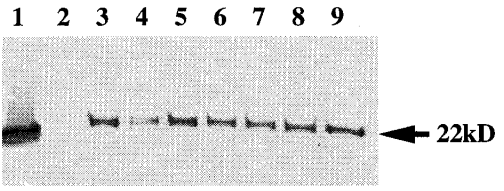

Fig. 4 Western blot analysis of the product of *bar* gene, PAT protein from transgenic phalaenopsis.
Protein (10 μg) extracted from roots was separated on 10-20% SDS-polyacrylamide gel and was analyzed by immunoblotting using anti PAT serum. Lane 1, 25 ng of purified PAT from *Streptomyces hygroscopicus*; lane 2, untransformed plant; lane 3-9, transgenic plantlets.

of them harbored both *bar* and *gus* gene. Southern analysis confirmed the integration of *bar* genes into the phalaenopsis genome and that pMSP38 did not exist extrachromosomally (data not shown). However we don't have any data whether gemini shuttle plasmid could replicate during the formation of transgenic PLBs and plantlets.

Spencer *et al.*[15] described that co-transformation frequency of two plasmids was 50% or greater in maize. In our experiment co-transfomation frequency was unexpectedly low, 14%. Because a number of transgenic plants is still few, we should obtain more of them to examine this further.

Western blot analysis of PAT, the product of *bar* gene, indicated that seven potentially transgenic plantlets produced a 22 kD protein that co-migrated with the authentic PAT and cross-reacted with antibody to PAT (**Fig. 4**).

These results indicated that the bialaphos resistant plantlets were transgenic for the introduced *bar* and/or *gus* gene. The *bar* gene, encoding PAT from *Streptomyces hygroscopicus*[10], has been shown to be a useful selective marker for stable transformation of various monocots[16] and dicots[17]. Bialaphos-resistant PLBs were successfully selected in the present study, which showed that the *bar* gene was suitable as a selective marker for phalaenopsis also.

We evaluated whether expression of *bar* gene confers resistance to commercial formulations of bialaphos. Control and transgenic plants were sprayed with 1.5 L/10 a HERBIE™ for which the field application rate is 0.5-1.0 L/10 a. Within 10 days, the control plant was almost killed, while the transgenic plant was completely resistant to high doses of the commercial formulations of bialaphos (**Fig. 2-C**).

Kuehnle and Sugii[2] have pointed out the possibility of the regeneration of chimeric plants following bombardment using protocorms as target tissues. In phalaenopsis, however, it was shown that PLBs were derived from single epidermal cells of cultured leaf segments[14]. Therefore, transgenic plantlets were expected not to be chimeric when new PLBs regenerated from a single cell of the bisected PLB segments using DNA-coated particles. Hence PLB segments, used in the present study, were recommended as targets in phalaenopsis.

In conclusion, a technique to transform phalaenopsis through particle bombardment was developed in this study. This is the first report of the transformation by particle bombardment in phalaenopsis. The frequency of transformation, however, was low, and needed to be improved. Further experiments which need to be done are (1) direct selection with bialaphos after bombardment to improve transformation frequency and (2) the introduction of a useful trait such as disease resistance, flower colors and cold tolerance into phalaenopsis.

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《和文要約》

パーティクルガン法によるファレノプシスの形質転換

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パーティクルガン法によりファレノプシス（コチョウラン）の形質転換体を作出した．β-グルクロニダ

ーゼ(*gus*)遺伝子をレポーターとしたトランジエントアッセイにより35Sプロモーターを用いる事とし，また，形質転換の選抜マーカーにはビアラホス耐性(*bar*)遺伝子を用いた．遺伝子を導入する組織としては，プロトコーム状球体(PLB)を用いた．本研究で用いたPLBは，花茎培養により得られるシュートから葉片を採取し，その培養によって形成されるラン特有の不定胚である．*bar*遺伝子を有するpMSP38と*gus*遺伝子を有するpWI-GUSプラスミドを金粒子にコートし，PLB切片にショットした．各切片より新たに形成された2次PLBをビアラホスにより選抜した結果，ビアラホス耐性の形質転換体を7個体得た．この内1個体には*gus*遺伝子も導入され発現していた．