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Production of Transgenic *Petunia hybrida* cv. Rosanpion Using *Agrobacterium*-mediated Transformation

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ABSTRACT

Transgenic *Petunia hybrida* cv. Rosanpion was produced by *Agrobacterium tumefaciens* LBA4404 harboring a binary vector pBI 121 containing *β-glucuronidase* (*gus*) and neomycin phosphotransferase (*npt*II). For genetic transformation, leaf discs were precultured on MS medium supplemented with 0.5 mg/L NAA and 1.0 mg/L BA (MNB) for 2 days and cocultured for 15 mins with *A. tumefaciens*. For selection of transformant, leaf discs were transferred to fresh MNB containing 50 mg/L kanamycin and 500 mg/L cefotaxime. Eighteen plants were regenerated and four were confirmed by PCR for detection of *gus* and *npt*II gene integrated into the nuclear genome of petunia 'Rosanpion'. Using this transformation system, we expect that transgenic petunia 'Rosanpion' incorporating a useful gene can be produced.

Key Words : Petunia, *Agrobacterium tumefaciens*, *β*-glucuronidase (gus), neomycin phosphotransferase (*npt*II), transformation, PCR.

INTRODUCTION

Petunia is a perennial herbaceous plant in South America, where it is treated as an annual due not to survive at low temperature in winter in Korea. As the color and shape of flower are so beautiful, it is planted on the flower box to decorate a veranda or a parkway. Recently petunia breeding was carried out widely for variety improvement (Horsch *et al.*, 1988), but there are few reports for culture condition (Daykin et al., 1976; Peak et al., 1984) and genetic manipulation using *Agrobacterium tumefaciens* (Aeom et al., 1996; Chung et al., 1992; Kim, 1998).

These facts prompt us to establish an efficient transformation system as a model for gene manipulation in petunia 'Rosanpion' with marker gene and to apply a useful gene for breeding in the future.

MATERIALS AND METHODS

Plant materials

The seeds of *Petunia hybrida* cv. Rosanpion were first sterilized in 70% ethyl alcohol for 10 sec, and then surface-sterilized in 7% calcium hypochlorite solution for 10 min. Finally, the seeds were rinsed three or four

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times with sterile distilled water. They were germinated at 25±1℃ on MS (Murashige and Skoog, 1962) medium without plant growth regulators under the light condition. After 7 days, leaf discs (5 x 5 mm) were cultured on MS medium supplemented with 2,4-D (0.1 to 1.0 mg/L) or NAA (0.1 to 1.0 mg/L) in combination with 1.0 mg/L BA. The culture was maintained at 25℃ in a 16h light / 8h dark photoperiod. All media were supplemented with 3 g/L phytagel and 30 g/L sucrose, adjusted to pH 5.8 before adding phytagel.

Agrobacterium-mediated transformation and selection

Agrobacterium tumefaciens strain LBA4404 containing a binary vector pBI 121 was used. The binary vector carries the β-glucuronidase (*gus*) gene fuses to a CaMV 35S promoter as well as the neomycin phosphotransferase (*npt*II) which specify kanamycin resistance. *Agrobacterium* were grown overnight in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 50 mg/L kanamycin in the dark at 28℃. For transformation, leaf discs were precultured on MS medium with 0.5 mg/L NAA and 1.0 mg/L BA (MNB) for two days and cocultured with the *Agrobacterium* suspension for 15 min. After cocultivation, the leaves were transferred to MNB containing 50 mg/L kanamycin and 500 mg/L cefotaxime for the selection of transgenic plants. Every three weeks the explants derived from leaves were transferred to fresh selective medium with antibiotics.

GUS assay and PCR analysis

Total genomic DNA was isolated from expanded leaves of transgenic and untransformed control *in vitro* according to Rogers and Bendich (1988). The primer for the gus gene fragment, forward primer (5' -TGG ACA AGG CAC TAG CGG-3') and reverse primer (5' -ACC GCC AAC GCG CAA TAT GC-3') were used for amplification. PCR mixture was contained 1 unit of Taq ploymerase, 2.5 mM dNTP mixture, 10 pM primer 200 ng genomic DNA. The PCR was performed with 30 cycles, each consisting of 1 min at 94℃, 1 min at 55℃ for 90sec. at 72℃. PCR of *npt*II gene (5' -GAG GCT ATC GGC TAT GAC TG-3' 5' -ATC GGG AGC GGC GAT ACC GTA-3') was also performed to the same condition as *gus* gene. PCR products were electrophoresed in 1% agarose gel and detected by ultraviolet light.

RESULTS AND DISCUSSION

Selection and plant regeneration

For preliminary test, we investigated the effect of plant growth regulators on MS medium with various concentrations of 2,4-D, NAA, and BA in petunia 'Rosanpion' leaves. After 10 days of culture, callus was easily induced from the cutting edges of leaf explants on the all MS media supplemented with tested plant growth regulators.

Out of many PGR combinations (not shown) in this paper, the best for direct plant regeneration was MS medium with 0.5 mg/L NAA and 1.0 mg/L BA (MNB)(Fig. 1A). Based on this result, we applied this medium to the following transformation experiment. For transformation, leaf discs were precultured on MNB medium and co-cultured with *A. tumefaciens* LBA 4404 containing the binary vector pBI 121 for 15mins. After this procedure, leaf discs were transferred to MNB contaning selection medium with 50 mg/L kanamycin and 500 mg/L cefotaxime for plant regeneration.

No callus or shoot formed from the non-transformed discs in kanamycin medium (Fig. 1B: left), while shoot was regenerated from the transformed (Fig. 1B: right). As it was reported (Colby and Meredith, 1990), kanamycin sensitivity affects the recovery of transformed plants and it should be determined in the initial stage of developing a plant transformation system. In addition, there are many reports that the

kanamycin concentration of transgenic plant was 20 to 800 mg/L in Arabidopsis (Scmidt and Willmitzer, 1985), alfalfa (Chabaud *et al.,* 1985), lettuce (Chung *et al.,* 1998), potato (Park *et al.,* 1996), tobacco (Rhim, 1998) and triticum (Hauptmann *et al.*, 1988).

Shoot primodia were differentiated on surface of yellowish and compact tissue after 30 days of culture. We regenerated (Fig. 1C) putative transformed eighteen plants from 140 leaf discs. Regeneration efficiency was not higher than that of Horsch et al. (1988). It may be attributed to different genotype of donor plant tested.

GUS assay and PCR analysis

Histochemical *gus* activity in regenerated plants was

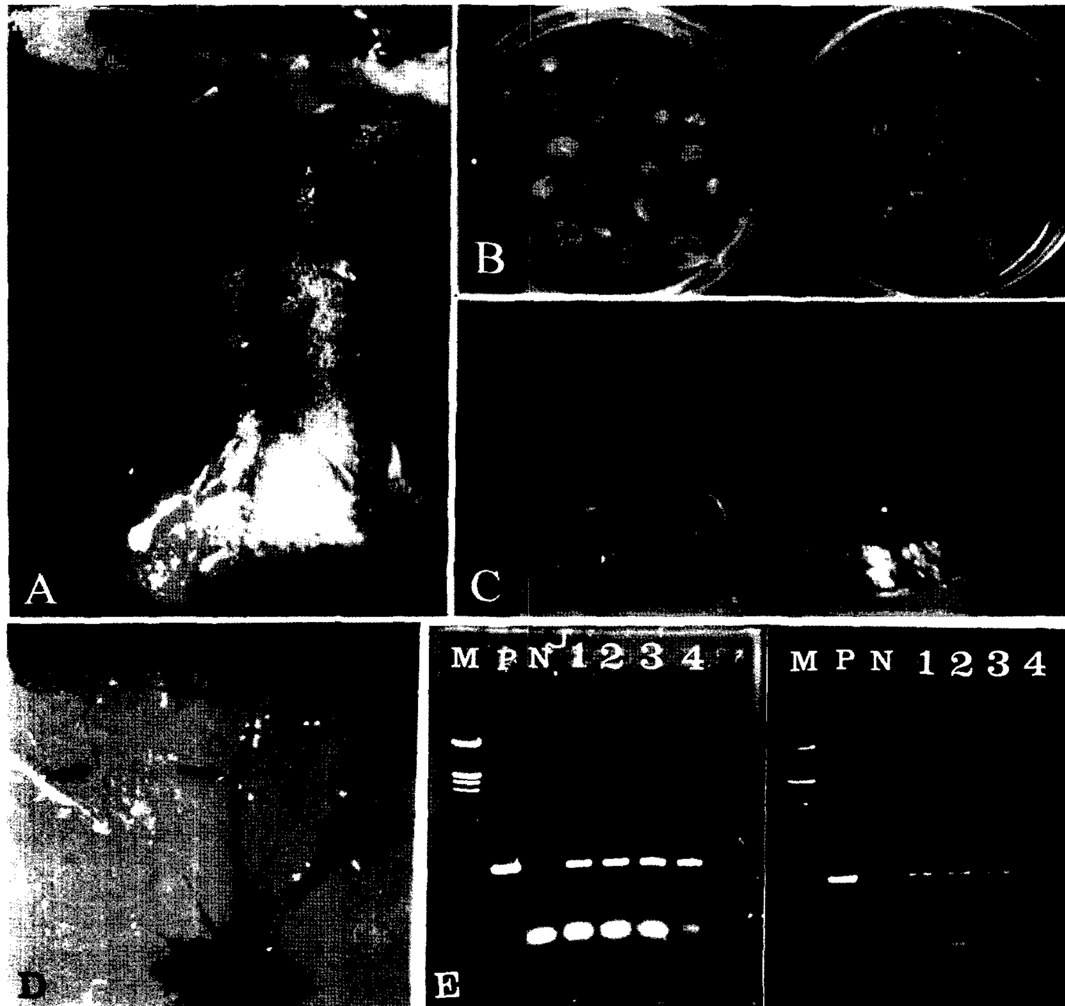

Fig. 1. Production of transgenic *Petunia hybrida* cv. Rosanpion plant using *Agrobacterium tumefaciens.* A : Direct shoot differenciated on MS medium supplemented with 0.5mg/l NAA and 1.0mg/ml BA (MNB). B : Kanamycin resistant response with MNB containing 50mg/l kanamycin and 500mg/l cefotoxime (left: non-transformed, right: transformed). C : Regenerated transgenic plant from leaf disc. D : The three arrows indicate *gus* gene expression (blue) in leaf veins. E : Agarose gel electrophoresis of PCR amplification products. The bands indicate approximately 1.0 kb of amplified *gus* product(left) and 0.7 kb of amplified *npt*II product(right). M: size maker (λ/HindⅢ + EcoR Ⅰ), P: positive control; lane N: Negative control (amplified product from genomic DNA of non-trasnformed plant); lane 1, 2, 3 and 4: amplified product from genomic DNA of transgenic plant.

examined in the leave by X-Gluc(5-bromo-4chloro-3-indoleyl glucuronidase) staining. The blue colored products of gus activity showed in leaf veins (Fig. 1D).

Out of putative transformed 18 plants, PCR analysis was accomplished to identify the transgenic petunia with *gus* and *npt*II gene specific primers, respectively. DNA amplified by PCR shows bands at the lane of 4 transgenic plants. The bands at 1.0 kb (*gus*)(Fig. 1E: lane 1, 2, 3, and 4 in left) and 0.7 kb site (*npt*II)(Fig. 1E: lane 1, 2, 3, and 4 in right) were observed from the transgenic petunia, but there was no band in control plant (Fig. 1E: lane N in left and right). These data indicate that gus and *npt*II gene were stably integrated in genomic DNA of petunia.

Recently, genetic manipulation using *Agrobacterium tumefaciens* was reported in various plants (Aeom et al., 1996; Chabaud et al., 1985; Chung et al., 1992; Park et al., 1996; Scmidt and Willmitzer, 1985). Regardless of the method of gene transfer, all investigation have been performed using so-called 'reporter' genes and molecular markers such as *npt*II and *gus* gene (Reinhard *et al.*, 1988). Reporter gene, GUS, has been described in great detail using as a model system in transformed tobacco plants (Hauptmann *et al.*, 1987), lettuce (Chung *et al.*, 1998), potato (Park *et al.*,1996). We identified the incorporation of *gus* and *npt*II gene in *Petunia hybrida* cv. Rosanpion by PCR analysis. Using this transformation system, we expect that transgenic petunia 'Rosanpion' which was incorporated a useful gene can be produced in the future.

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