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Production of Transgenic Grapevine (*Vitis vinifera* L. cv. Koshusanjaku) Plants by Co-cultivation of Embryogenic Calli with *Agrobacterium tumefaciens* and Selecting Secondary Embryos

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Abstract

Embryogenic calli induced from leaf segments of grapevine (*Vitis vinifera* L. cv. Koshusanjaku) were co-cultivated for 5 days with *Agrobacterium tumefaciens* strains EHA101 (pIG121Hm) or LBA4404 (pTOK233), both of which contained the plasmid carrying the neomycin phosphotransferase II (NPT II), hygromycin phosphotransferase (HPT) and the β-glucuronidase (GUS) genes. Putative transgenic calli were selected on 2 g/*l* gellan gum-solidified Nitsch's medium (1969) containing 50 mg/*l* kanamycin and 20 g/*l* sucrose after co-cultivation with *A. tumefaciens*. Transformation frequency of the embryogenic calli evaluated by GUS histochemical assay was increased by the addition of acetosyringone to co-culture medium. Complete transgenic plants were selected among secondary embryos formed on the surface of embryos in the presence of kanamycin. Finally, kanamycin-resistant plants expressing GUS gene were obtained. PCR analysis confirmed their transgenic nature by detecting GUS and NPT II genes.

1. Introduction

Grapevines (*Vitis* spp.) are one of the major fruit crops throughout the world. For the genetic transformation of grapevines, it is necessary to establish highly efficient system of plant regeneration from cell and tissue cultures. Recently, induction of somatic embryogenesis from leaves [1, 2], ovaries [3], ovules [4], immature zygotic embryos [5] and anthers [1, 3, 6] has been reported in several species of *Vitis*. These embryogenic cultures have been shown to be useful materials for *Agrobacterium*-mediated transformation in *Vitis* species [7, 8]. However, transformation efficiency of embryogenic callus in co-cultivation with *Agrobacterium* has not yet been studied in detail. In the present study, therefore, we tried to establish a protocol for genetic transformation of *Vitis* by using embryogenic calli and showed the recovery of transgenic plants with GUS activity among secondary embryos formed on somatic embryos in the presence of kanamycin.

2. Materials and Methods

2.1 Plant materials and induction of embryogenic calli

Embryogenic calli were initially induced from leaf segments excised from *in vitro*-subcultured plantlets of *V. vinifera* cv. Koshusanjaku according to the method of Matsuta and Hirabayashi [2] with several modifications [8]. The embryogenic calli were maintained by subculturing monthly on Nitsch's medium (1969) [9] lacking vitamins, inositol and glycine but supplemented with 1 μM 2,4-D and 30 g/*l* sucrose, and solidified with 2 g/*l* gellan gum (Wako Pure Chemical Industries, Ltd.). The friable callus cultures containing no appreciable embryos established after several subcultures were used as the material for co-cultivation with *Agrobacterium tumefaciens*.

2.2 Agrobacterium tumefaciens *strains*

The *A. tumefaciens* strain LBA4404 (pTOK233) which was provided by Japan Tobacco Inc. [10] was

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used for the transformation studies. The plasmid pTOK233 possessed the inserted *virB*, *virC* and *virG* genes derived from supervirulent Ti-plasmid pTiBo542 [11], which is known to retain an ability to enhance the virulence of *A. tumefaciens* against plant cells [12]. The T-DNA region of pTOK233 contained neomycin phosphotransferase II (NPT II) under the control of nopaline synthase (NOS) promoter, hygromycin phosphotransferase (HPT) under the control of cauliflower mosaic virus (CaMV) 35S promoter, and β-glucuronidase (GUS) with an intron fused to CaMV 35S promoter.

Another *A. tumefaciens* strain used was EHA101 [13] which was harboring plasmid pIG121Hm [14] containing NPT II, HPT and GUS in the T-DNA region as pTOK233.

2.3 *Co-cultivation of embryogenic calli with* Agrobacterium

Prior to co-cultivation, 30 m*l* liquid AB medium [15] containing 50 mg/*l* hygromycin was prepared in 100 m*l* Erlenmeyer flask and inoculated with *A. tumefaciens* strains LBA4404 (pTOK233) or EHA101 (pIG121Hm). The bacterial cultures were incubated for more than 30 hours at 28°C with reciprocal shaking (130 min^{-1}).

Two gram fresh weight of embryogenic calli 3 weeks after subculture were put on nylon membrane (40 μm pore size) and then exposed with 5 m*l* of freshly grown suspension culture of bacteria. Inoculated embryogenic calli were washed several times with sterilized distilled water and placed for co-cultivation on 2 g/*l* gellan gum-solidified Nitsch's medium (1969) containing 30 g/*l* sucrose and 1 μM 2, 4-D with or without 100 μM acetosyringone. After 5 days of co-cultivation in the dark, embryogenic calli were transferred for eliminating *Agrobacterium* to the same medium lacking acetosyringone but containing 500 mg/*l* cefotaxime (Claforan; Hoechst AG), which was supplemented after autoclaving. After 2 weeks, the cultures were transferred for inducing somatic embryogenesis to 2 g/*l* gellan gum-solidified Nitsch's medium (1969) lacking plant growth regulators but containing 20 g/*l* sucrose and 500 mg/*l* cefotaxime, and kept under 24 h illumination (35 μE $m^{-2}s^{-1}$) with fluorescent lamps (National FL30SN). Four weeks after the bacterial inoculation, the cultures were placed on the same medium but supplemented with 50 mg/*l* kanamycin which was filter-sterilized (Millipore, 0.45 μm pore size) and added after autoclaving the medium for selecting transformed cells. The embryogenic calli with somatic embryos were subcultured every 2 weeks on the same medium with kanamycin. The pH of the culture media used throughout this experiment was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All the calli and somatic embryos were cultured in 90 mm×20 mm plastic Petri dishes (Terumo SH-20S, Japan), each containing 40 m*l* of medium. All the dishes were sealed with Parafilm® and maintained at 26±1°C.

2.4 *GUS histochemical assay*

Histochemical localization of GUS gene expression was detected in kanamycin-resistant calli, somatic embryos and leaves of regenerated plants according the method of Jefferson *et al.* [16] with several modifications. For staining, tissues were incubated at 37°C for 3-5 hours in Na-phosphate buffer (50 mM, pH 7.0) which contained 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc : Wako Pure Chemical Industries, Ltd.). To remove chlorophyll, leaf tissues were washed several times with 70% ethanol after the incubation. The tissues developing indigogenic dye were evaluated as those expressing GUS gene.

2.5 *Plant regeneration*

Heart- to torpedo-shaped embryos derived from embryogenic calli were picked by forceps and placed on 2 g/*l* gellan gum-solidified Nitsch's medium (1969) containing 20 g/*l* sucrose and 50 mg/*l* kanamycin. The cultures were transferred monthly onto the same medium. Regenerated plantlets with well-expanded 5 to 6 leaves were washed carefully to remove the gellan gum and transferred to pots (9×9 cm) containing vermiculite. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 20±1°C under 24 h illumination (45 μE m^{-2} s^{-1}) with fluorescent lamps in the growth chambers.

2.6 *DNA extraction and PCR analysis*

Total genomic DNAs were isolated from leaves of putative transgenic plants following the method of Harding and Roubelakis-Angelakis [17]. Specific oligonucleotide primers for detecting GUS and NPT II gene sequences [18] were used to identify the presence of these genes in the genomic DNAs. Fifty nanogram of template DNA and 5 μM primers each at 1 μl were mixed with 2.5 μl of 10 × *Taq* DNA polymerase reaction buffer, 100 μM (final concentration) of a dNTP mixture (equimolar dATP, dCTP, dGTP and dTTP) and 1 unit of *Taq* DNA polymerase (Toyobo, Japan) in a total volume of 25 μl. Forty cycles of PCR were performed in a programmed temperature control system (PC-7000, Astec, Tokyo). A single cycle consisted of the following steps: denaturation of DNA at 92°C for 1 min, annealing at 55°C for 1 min, and DNA synthesis at 73°C for 1.5 min. Amplified DNAs were detected after running on 1.5% (w/v) agarose gel electrophoresis using TAE as a

Fig. 2 Eight categories of flower variants derived from shoot tip culture of flower stalk buds in 2 genotypes of *Phalaenopsis* Wedding Promenade, PM70 (A-C, E-H) and PM79 (D, I).
A: normal flower, B: flower with incomplete labellum-like petals (Type 1), C: flower with complete labellum-like petals (Type 2), D: flower with labellum-like lateral sepals (Type 3), E: flower with irregular morphology in all perianths (Type 4), F: flower with faded flower color (Type 5), G: dwarfed flower (Type 6), H: flower with petaloid column (Type 7), I: dwarfed flower with petaloid column (combination of Type 6 and Type 7 in **Table 3**).
Bar=1 cm.

running buffer at 100 V for 1 h.

3. Results and Discussion

Prior to co-cultivation experiments, sensitivity of embryogenic calli to kanamycin was investigated to select transformed cells. In our preliminary experiment, kanamycin at 50 mg/*l* showed toxicity to embryogenic calli of *V. vinifera* cv. Koshusanjaku and completely inhibited the growth of calli with browning 4 weeks after transfer [8]. As a result, we employed 50 mg/*l* kanamycin to carry out selection of transformed cells after co-cultivation with *A. tumefaciens*.

The calli used in this study (**Fig. 1-A**) were friable and brownish white, and had retained embryogenic potential with high plant regeneration ability for more than 4 years. These embryogenic calli were subjected to GUS assay 7 days after inoculation. GUS-positive cells were observed as shown in **Fig. 1-B**, whereas no endogenous GUS activity was detected in non-co-cultivated tissues. Frequency of blue spots obtained by GUS histochemical assay was different between the 2 bacterial strains examined and LBA4404 (pTOK233) always showed higher number of GUS spots than EHA101 (pIG121Hm) (**Table 1**). It has been reported that addition of acetosyringone to co-culture media or bacterial suspensions was effective to increase transformation efficiency in several plant species such as *Brassica* [19], apple [20] and tomato [21, 22]. In the present study, effectiveness of acetosyringone was also confirmed in grapevine. The number of GUS spots was drastically increased by adding acetosyringone to co-culture medium in both bacterial strains although the data fluctuated in the 2 repeated experiments (**Table 1**). The highest frequency of GUS spots was obtained when acetosyringone was used with *A. tumefaciens* stain LBA4404 (pTOK233). In the present study, it was revealed that *A. tumefaciens* strain LBA4404 (pTOK233) could transform *Vitis* at high transformation frequency compared to EHA101 (pIG121Hm) (**Table 1**). It has previously been reported that the former bacterial strain had an ability to introduce foreign genes to rice [10, 23], which was a recalcitrant plant for transformation by *Agrobacterium*. Predominance of LBA4404 (pTOK233) for the transformation of *Vitis* may be due to the plasmid pTOK233 which possessed *virB*, *virC* and *virG* derived from supervirulent Ti-plasmid pTiBo542 [10].

Embryogenic calli could produce embryos within 1 month after co-cultivation treatment. However, these embryos turned brown at some part of the tissues due to the damage caused by transferring to 50 mg/*l* kanamycin-containing medium. During the subsequent culture on kanamycin-containing medium, they initiated the production of secondary embryos on surviving tissues which had been expected to originate from transformed cells (**Fig. 1-C**). Four months after bacterial inoculation, approximately 20-30% of the secondary embryos showed GUS expression as shown in **Fig. 1-D** (**Table 2**). It is possible that the GUS-negative secondary embryos were transgenic ones with NPT II gene but lost or silenced GUS gene, or escaped ones which did not integrate any foreign genes. Therefore, we tried to recover transgenic plants from secondary embryos by the selection of kanamycin-containing medium.

Thus, secondary embryo-derived plants were successfully regenerated on kanamycin-containing medium after co-cultivation with LBA4404 (pTOK233) or EHA101 (pIG121Hm) of *A. tumefaciens*. GUS-positive plants were selected among regenerated plants showing GUS activity in excised leaves (**Fig. 1-E**). They were readily acclimatized after transfer to pots in soil where they grew into the plants with fully expanded 4-5 leaves, and were then successfully transferred to the growth chambers (**Fig. 1-F**).

GUS-positive plants were subjected to DNA analysis using PCR for detecting GUS and NPT II sequences. The reaction using total genomic DNAs extracted from leaves allowed the detection of 1.2 and 0.7 kbp bands which coincided with GUS and

Table 1. Effect of acetosyringone on GUS activity in embryogenic callus of *Vitis vinifera* cv. Koshusanjaku following co-cultivation with *Agrobacterium tumefaciens*.

Bacterial strains	Acetosyringone (μM) *1	Number of GUS spots / 100 mg callus*2	
		exp. 1	exp. 2
EHA101 (pIG121Hm)	0	5	2
	100	31	165
LBA4404 (pTOK233)	0	15	13
	100	56	695

*1 Acetosyringone was added to co-culture medium.

*2 Data were obtained 7 days after inoculation.

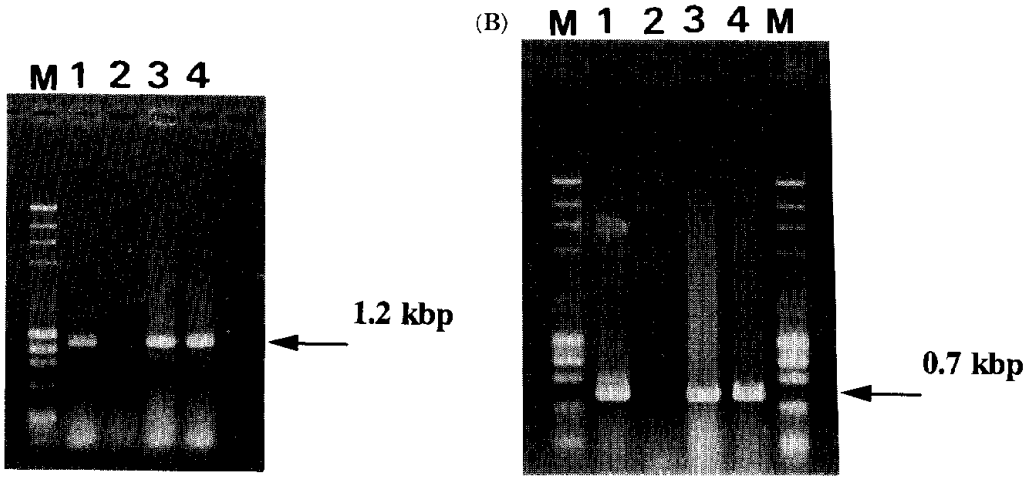

Fig. 2 PCR analysis of transgenic *Vitis* plants.
(A) Detection of GUS gene.
(B) Detection of NPTII gene.
Lane M, λ/*Hind* III and φX174/*Hae* III as molecular markers (23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60 kbp fragments from top to bottom); lane 1, plasmid pTOK233 as a positive control; lane 2, non-transformed plant; lane 3 and 4, transgenic plants. Arrows indicate the position of the expected 1.2 and 0.7 kbp fragments including GUS and NPT II genes, respectively.

Table 2. Effect of bacterial strains on transformation efficiency of embryogenic calli of *Vitis vinifera* cv. Koshusanjaku evaluated by GUS activity after co-cultivation with *Agrobacterium tumefaciens*.

Bacterial strain	No. of GUS-positive embryos/no. of embryos tested (%)*1	No. of GUS-positive plants/no. of regenerated plants examined (%)*2
EHA101 (pIG121Hm)	17/80 (21.3)	3/8 (37.5)
LBA4404 (pTOK233)	26/84 (31.0)	4/9 (44.4)

Acetosyringone was added to co-culture medium.
*1 Number of GUS-positive embryos was counted 4 months after bacterial inoculation.
*2 Number of GUS-positive plants was determined by examining GUS activity in leaves 6 months after bacterial inoculation.

NPT II genes, respectively (**Fig. 2-A** and **2-B**). As a result, we could confirm that they were transgenic plants with both GUS and NPT II genes.

In the previous study, we produced transgenic plants of grapevine via *A. rhizogenes*-mediated transformation of embryogenic calli [8]. However, it was needed to select transgenic plants which had no introduced undesirable Ri T-DNA. In the present study, we succeeded in introducing only the marker genes by using disarmed *A. tumefaciens*. Transformation of embryogenic calli with *A. tumefaciens* has previously been reported by Martinelli and Mandolino [7]. However, they did not show the transformation efficiency. In the present study, we developed the method for producing transgenic plants of *V. vinifera* L. by selecting the secondary embryos which were resistant to kanamycin after co-cultivation of embryogenic calli with *A. tumefaciens*. After selection with kanamycin, approximately 40% of the regenerated plants were transgenic with NPT II and GUS genes (**Table 2**). Relatively high transformation efficiency shown in the present study will encourage the introduction of some useful genes for grapevine breeding. Further studies will be concentrated to produce transgenic *Vitis* plants with useful genes such as those encoding disease resistance by using the method established in the present study.

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References

[1] Stamp, J. A., Meredith, C. P., 1988. Scientia Hortic., **35**: 235-250.

[2] Matsuta, N., Hirabayashi, T., 1989. Plant Cell Rep., **7**: 684-687.

[3] Gray, D. J., Mortensen, J. A., 1987. Plant Cell Tissue Organ Culture, **9**: 73-80.

[4] Rajasekaran, K., Mullins, M. G., 1985. Vitis, **24**: 151-157.

[5] Emershad, R. L., Ramming, D. W., 1994. Plant Cell Rep., **14**: 6-12.

[6] Mozsár, J., Sule, S., 1994. Vitis, **33**: 245-246.

[7] Martinelli, L., Mandolino, G., 1994. Theor. Appl. Genet., **88**: 621-628.

[8] Nakano, M., Hoshino, Y., Mii, M., 1994. J. Exp. Bot., **45**: 649-656.

[9] Nitsch, J. P., Nitsch, C., 1969. Science, **163**: 85-87.

[10] Hiei, Y., Ohta, S., Toshihiro, K., Komari, T., 1994. Plant J., **6**: 271-282.

[11] Watson, B., Currier, T. C., Gordon, M. P., Chilton, M.-D., Nester, E. W., 1975. J. Bacteriol., **123**: 255-264.

[12] Jin, S., Komari, T., Gordon, M. P., Nester, E. W., 1987. J. Bacteriol., **169**: 4417-4425.

[13] Hood, E. E, Helmer, G. L., Fraley, R. T., Chilton, M.-D., 1986. J. Bacteriol., **168**: 1291-1301.

[14] Ohta, S., Mita, S., Hattori, T., Nakamura, K., 1990. Plant Cell Physiol., **31**: 805-813.

[15] Chilton, M.-D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P., Nester, E. W., 1974. Proc. Natl. Acad. Sci. USA, **71**: 3672-3676.

[16] Jefferson, R. A., Kavanagh, T. A., Bevan, M., 1987. EMBO. J., **6**: 3901-3907.

[17] Harding, K., Roubelakis-Angelakis, K. A., 1994. Vitis, **33**: 247-248.

[18] Hamill, J. D., Rounsley, S., Spencer, A., Todd, G., Rhodes, M. J. C., 1991. Plant Cell Rep., **10**: 221-224.

[19] Charest, P. J., Lyer, V. N., Miki, B. L., 1989. Plant Cell Rep., 8: 303-306.

[20] James, D. J., Uratsu, S., Cheng, J., Negri Paola, N., Viss, P., Dandekar, A. M., 1993. Plant Cell Rep., **12**: 599-563.

[21] Davis, M. E., Miller, A. R., Lineberger, R. D., 1991. J. Exp. Bot., **42**: 359-364.

[22] Lipp João, K. H., Brown, T. A., 1993. Plant Cell Rep., **12**: 422-425.

[23] Aldemita, R. R., Hodges, T. K., 1996. Planta, **199** : 612-617.