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Agrobacterium – mediated Transformation and Transgenic Plant Regeneration from Embryogenic Calli Derived from an Immature Seed Produced from Miniature Rose Cultivar 'Shortcake'

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Abstract

The immature seeds were harvested from self – pollinated miniature rose 'Shortcake'. The pale yellow and friable embryogenic calli were induced from an immature seed. The calli showed high frequency (50-60%) of transient GUS expression in *Agrobacterium* – mediated transformation. The population density of *Agrobacterium* and the sonication assistance treatment had slight effects on the transformation frequency. Among 65 putative transgenic shoots grown *in vitro*, seven transgenic rose plants have been established and flowered in a contained greenhouse. The flowers on the primary shoots showed unstable and quite different characteristics from those on the 2nd or later emerged shoots, which were very similar to the flowers of non-transgenic regenerated plants. The integration of T-DNA fragment in transgenic plants was confirmed by both PCR-Southern blot analysis with a GUS probe and Southern blot analysis with a hygromycin phosphotransferase RNA probe.

Key words: embryogenic calli, Miniature rose 'Shortcake', PCR, Southern hybridization, transient GUS expression.

Abbreviations

BA, 6-benzyladenine; EC, embryogenic calli; GUS; β -glucronidase; HPT, hygromycine phosphotransferase; IBA, indolebutyric acid; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; NPT II, neomycine phosphotransferase II; PCR, polymerase chain reaction; SAAT, sonication-assisted *Agrobacterium*-mediated transformation.

Introduction

The rose flower is called the queen of flowers and one of the important ornamental plants cultivated world wide. The biotechnological technique is a powerful tool to improve genetic qualities of roses. However, only a few reports on transformed rose plants have been published (Firoozabady *et al.*, 1994; Souq *et al.*, 1996) mainly on *Rosa hybrida*. Also only a few papers on rose plant regeneration from embryogenic calli (EC) derived from immature seeds have been reported (Burger *et al.*, 1990; Kunitake *et al.*, 1993; Viesessuwan *et al.*, 1997). The present report describes the study on the *Agrobacterium*-mediated transformation and the transgenic plant regeneration of the EC derived from an immature seed produced from a miniature rose 'Shortcake'.

Materials and Methods

Plant materials

Immature seeds obtained by self-pollination of a miniature rose cultivar, *Rosa* x *hybrida* 'Shortcake' (Keisei Rose Nurseries, Chiba, Japan), which had been cultivated in a green house for 3 years, was used in this study.

Methods for EC induction and proliferation

Three to four weeks after self-pollination, fruits were harvested. Intact immature seeds excised from the fruits were sterilized with 1.5% NaClO solution for 5 min, rinsed twice with sterilized water, and then cut longitudinally by a scalpel into two pieces. Half-divided 60 immature seeds were cultured on a medium consisting of Murashige and Skoog's mineral salts and vitamins (Murashige and Skoog, 1962), 30 g 1^{-1} sucrose and 2.5 g 1^{-1} Gelrite (Merck) (pH 5.7) (hereafter referred as MS medium) without phytohormone, at 25 °C under 16 h light/8 h dark for



Fig. 1 Schematic T-DNA construction of *Agrobacterium* EHA101/ pHTS-35S-GUS (or pBI121-Hm).

RB: right border, NP: nopaline synthase promoter, NPT II: neomycine phosphotransferase II, NT: nopaline synthase terminator, 35S: 35S cauliflower mosaic virus promoter, GUS: β – glucronidase, HPT: hygromycin phosphotransferase, LB: left border. Cutting sites of restriction endonucleases are indicated as follows; B: *Bam*HI, P: *Pst*I, Sh: *Sph*I, H: *Hind*III, SI: *Sal*I, E: *Eco*RI.

3 months. Consequently two EC were induced, and one of them was used in the present study (Asano and Tanimoto, 2002).

Agrobacterium-mediated transformation of EC

The schematic T-DNA fragment construction of Agrobacterium tumefaciens EHA101/(pHTS-35S-GUS or pBI121Hm) carrying neomycine phosphotransferase II (NPT II), β -glucronidase (GUS) and hygromycin phosphotransferase (HPT) genes was shown in Fig. 1. The cells of A. tumefaciens were cultured in a 100-ml flask containing 40 ml YEB liquid medium (0.5% beef extract, 0.1% yeast extract, 0.5% pepton, 0.5% sucrose, 2 mM MgSO₄, pH 7.2) supplemented with $50 \text{ mg } 1^{-1}$ hygromycin (WAKO Pure Chemical, Japan) at 30 °C in a reciprocal water bath shaker, at 120 cycles per min for 2 days. Then the bacteria were collected by centrifugation, rinsed twice with the YEB liquid medium and incubated in a 100-ml Erlenmyer flask containing 30 ml YEB liquid medium. OD_{595} of the A. tumefaciens suspension in liquid medium was adjusted to 0.1, 0.3, 0.77, 1.36 and 1.7 respectively by a spectrometer (U 2000A, Hitachi) to study the effect of the bacterial density on the transient GUS gene expression. Twenty pieces of EC (each 0.1-0.2 g) were co-cultured with the A. tumefaciens in the YEB liquid medium for 15 min. In order to clarify the effect of the sonication duration on the transient GUS gene expression (Trick and Finer, 1997, 1998), 0, 3, 7, 15, or 30 s of ultrasound was applied to 20 pieces of EC for each treatment with a supersonic cleaner (EYELA, Japan) at the presence of the A. tumefaciens in the YEB liquid medium $(OD_{595} = 0.7)$. After removal of the excess liquid medium from the EC with filter paper, the EC pieces were transferred onto the filter paper soaked with the regeneration medium consisting of the MS medium with 30 g 1^{-1} sucrose, 2.5 g 1^{-1} Gelrite and $1 \text{ mg } l^{-1}$ 6-benzyladenine (BA), and incubated at 25 °C for 2 days under dark condition for further

cocultivation.

Elimination of Agrobacterium

After cocultivation, the EC pieces were transferred into a 100-ml flask containing 30 ml sterilized water and 500 mg l^{-1} carbenicillin (SIGMA), and incubated for 1 h on a reciprocal shaker. Then the supernatant was discarded and rinsed with sterilized water twice. This sterilization procedure was repeated twice.

Plant regeneration

The sterilized EC pieces were transferred onto the selection and regeneration medium (S & R medium) consisting of an MS medium with $30 \text{ g} \text{ } 1^{-1}$ sucrose, 2.5 g l^{-1} Gelrite, 1 mg l^{-1} BA and 0, 0.05, 0.1, 0.2, or 0.4 mg l^{-1} 1-naphthaleneacetic acid (NAA), 500 mg 1^{-1} carbenicillin and 35 mg 1^{-1} hypromycin, and cultured at 25 °C under 16 h light/8 h dark condition for 6 months with subcultures every 2-3 weeks. The concentration of carbenicillin was reduced to 0 after 2 months. The developed shoots were transplanted onto the rooting medium (Hasegawa, 1980) consisting of the half strength MS medium with $15 \text{ g } \text{l}^{-1}$ sucrose, 2.5 g 1^{-1} Gelrite, 0.25 mg 1^{-1} indolebutyric acid (IBA) and 35 mg 1^{-1} hygromycin, and cultured for 1 month. After acclimatization treatment, rooted plantlets were transplanted on the soil of a contained greenhouse.

GUS assay

The transient GUS expression in the EC tissues was assayed after 3 days of the co-culuture with *A*. *tumefaciens* by dipping the EC tissues in a GUS mix (Jefferson, 1987; Jefferson *et al.*, 1987).

Preparation of a GUS probe

In order to obtain 430 bp GUS DNA fragment, two primers; 5'-primer (5'-ACACCGATAC-CATCAGCGAT-3'), 3'-primer (5'-TCACCGA-AGTTCATGCCAGT-3') (Tsuchiya *et al.*, 1995; Rashid *et al.*, 1996), and pHTS-35S-GUS as a template, were used for the PCR. The other conditions of the PCR were the same as those previously reported (Asano and Tanimoto, 2002). Then the amplified DNA fragment was labeled according to the protocol of DIG DNA Labeling and Detection kit (BOEHRINGER MANNHEIM).

Hybridization

The genomic DNA was extracted from young leaves of 1 non-transformed plant and 7 transformed plants, and 0.2 μ g of genomic DNA was used for the PCR on the same condition as reported previously (Asano and Tanimoto, 2002). All amplified DNA fragments were separated by electrophoresis on the agarose gel and transferred to the membrane (Hybond-N⁺, Amersham Pharmacia Biotech, Ireland). After drying the blotted membrane, the hybridization with the GUS probe and detection were performed according to the protocol of DIG DNA Labeling and Detection kit.

Preparation of a HPT probe for Southern blot analysis

The RNA probe was preferred in the present paper due to its higher sensitivity than the DNA probe. In order to obtain the full length DNA fragment of the HPT gene (1,023 bp), the plasmid pCH carrying the HPT gene between two cutting sites of *Bam*HI was digested with *Bam*HI.

The DNA fragment was inserted into the multicloning sites of the plasmid pBluescript II SK(+) (TOYOBO), which was cut with the same restriction enzyme, BamHI. The competent E. coli HB101 (TOYOBO) was used for the subcloning of the DNA fragment. After the blue/white selection, the positive plaque of transformed HB101 was incubated overnight at 37 °C in a 300 ml Erlenmyer flask containing 100 ml of liquid LB medium (tryptone $10 \text{ g } 1^{-1}$, yeast extract 5 g 1^{-1} , NaCl 10 g 1^{-1} , pH 6.7) in a reciprocal water bath shaker at 100 cycles per min. The plasmid was extracted according to the protocol of large-scale preparation (Sambrook et al., 1989) and digested with XbaI. The linearized plasmid obtained was purified with the proteinase K (SIGMA) and used as the DNA template of the RNA probe. The RNA probe was labeled according to the protocol of DIG-RNA labeling kit SP6/T7, CDP-Star TM (BOEHRINGER MANNHEIM).

Hybridization with RNA probe and detection

Fifteen μ g of each genomic DNA (1 non-transformed plant and 5 transformed plants) were digested with *Eco*RI and separated by electrophoresis on 0.8% agarose gel with DNA ladder marker. The digested genomic DNA was transferred onto the

membrane. After drying the blotted membrane, hybridization with about 1 kb-long RNA HPT probe and detection for HPT gene were performed according to the protocol of DIG RNA Labeling and Detection Kit (BOEHRINGER MANNHEIM).

Ploidy Analysis

The ploidy level of the transgenic regenerated plants was compared with that of the 'Shortcake' original plant by the same method previously reported using a ploidy analyzer (type PA, Partec, Germany) (Asano and Tanimoto, 2002).

Results and Discussion

Generally the range of preferable population density of bacteria for the Agrobacterium-mediated transformation has been recognized to lie between the initial and exponential phases (OD₅₉₅ = 0.1-0.7), and the acetosyringone has been used to enhance the activity of the Agrobacterium infection. In the present study, the frequency of the transient GUS gene expression in the rose EC was not greatly affected by the growth phase or density of bacteria, i.e. 35-55 % at the range of bacterial density from $OD_{595}=0.1$, which corresponds to the density at initial phase, to 1.72 which corresponds to the saturated phase (Table 1). A technique, sonicationassisted Agrobacterium-mediated transformation (SAAT) was tremendously effective to increase the transient GUS expression in various recalcitrant plants such as soybean, cowpea, wheat and maize (Trick and Finer, 1997). However, as far as the EC used in the present study were concerned, the SAAT treatment seemed not so effective as expected; only 5% increase at 3 s treatment (Table 2). The reason is not clear but probably the physical and chemical strength of the cell wall of the EC tissue would be far weaker against both the attack of Agrobacterium and ultrasound cavitation than that of the other plants reported previously (Trick and Finer, 1997).

The sonication duration more than 15 s seemed to make serious damages to the EC tissue, which turned brown and eventually died. **Fig. 2A** shows the aspect of non-transformed EC derived from an immature seed after one month of culture on the callus proliferation medium which is MS medium containing 30 g l⁻¹ sucrose, 2.5 g l⁻¹ Gelrite, 0.25 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA. The EC had a pale yellow color and consisted of a mixed structure with globular, leafy and friable tissues. After the coculture with *Agrobacterium* and sterilization treatment, the EC were transplanted onto the S & R

OD _{596nm}	0.1	0.3	0.77	1.36	1.72
Total no. of calli (A)	20	20	20	20	20
No. of callus with blue spots (B)	10	11	7	8	8
(B)/(A)%	50	55	35	40	40

Table 1Effect of Agrobacterium concentration (OD595 value) on thefrequency of transient GUS expression in embryogenic calli.

Fresh weight of each callus was 0.1 - 0.2 g.

 Table 2
 Effect of the duration of sonication treatment on the frequency of transient GUS expression in embryogenic calli.

Sonication duration (s)	0	3	7	15	30
Total no. of calli (A)	20	20	20	20	20
No. of calli with blue loci (B)	11	12	8	2	0
(B)/(A)%	55	60	40	10	0

Concentration of Agrobacterium was adjusted to OD₅₉₅=0.7.

medium. Fig. 2B shows a strong transient GUS expression of transformed EC after culture on the S & R medium for 1 month. Fig. 2C shows the transformed EC with regenerated shoots after culture for 2 months on the S & R medium.

As shown in Fig. 3A, many green spots, which could develop into shoots with leaves, appeared on the EC after subculture for 4 months (2 months on the S & R medium and 2 months on the S & R medium lacking carbenicillin). The subculture on the S & R medium for the 1st two months was enough for the complete elimination of the Agrobacterium. Agrobacterium contamination was not observed even on the S & R medium lacking carbenicillin. Intact shoots were developed after subculture for 6 months, i.e., 2 months on the S & R medium and 4 months, on the carbenicillin-free S & R medium (Fig. 3B). Table 3 shows the effect of phytohormone on the plant regeneration of the transformed EC. The regeneration frequency of the transformed EC was less than about 20% of that of the non-transformed EC (Asano and Tanimoto, 2002) probably due to the hygromycin in the S & R medium and the integration of T-DNA fragment into the genome. The combination of $1 \text{ mg } 1^{-1} \text{ BA}$ and $0.1 \text{ mg } l^{-1}$ NAA was optimum for the regeneration of transformed EC. The shoots were transplanted onto the rooting medium and subcultured for 1 month. Among the 65 shoots emerged from 25 putative transgenic EC, 32 shoots (49%) were successfully developed roots. After acclimatization treatment, intact plantlets were transplanted on the soil in a contained greenhouse, but only 7 out of 32 plantlets (22%) survived. This is probably due to the poor development of their roots compared with those of the original plantlet, suggesting that the

Fable 3	Effect of pl	nytohormones	s on plan	t regenerat-
io	n frequency	of embryoge	nic trans	genic calli.

Phytohormone $(mg l^{-1})$		No. of	No. of	No. of shoots per	
BA	NAA	$- \operatorname{cam}(\mathbf{A})$	snoots(B)	callus(B)/(A)	
1	0	168	246	1.46	
1	0.05	168	351	2.09	
1	0.1	448	1415	3.16	
1	0.2	168	25	0.15	
1	0.4	168	0	0	

Callus weight : ~3 g/piece.

No. of shoots was counted 6 months after transplantation onto S & R medium.

transgenic plants would not be tolerant to drought. The 7 survived plantlets developed into flowering stage after about 1.5 years and flowered twice, mainly in spring and autumn, but did not set any seed.

The flower of the non-transgenic regenerated plant (Fig. 4B) originated from the seed of 'Shortcake' was somewhat different from that of the original plant (Fig. 4A) because of the heterozygous nature of the parental cultivar 'Shortcake'. Compared with the control flower (Fig. 4B), flowers produced on the primary shoots of transformed plants showed quite different shape and color (Fig. 4C-E). However, interestingly all the flowers on the 2nd or later emerged shoots of transformed plants (Fig. 4F) were almost the same as those of the non-transgenic regenerated plants (Fig. 4B). The reason is unknown why those unstable variant flowers appeared only on the primary shoots. In



Fig. 2 GUS assay on the transformed calli and shoots. Embryogenic non-transformed callus (A), GUS assayed transformed callus (B), shoots emerged on transformed callus (C). (Bar = 1 cm)



Fig. 3 Regeneration of transformed shoots from embryogenic callus.Transformed callus with green spots (A) and regenerated shoots (B). (Bar = 1 cm)



Fig. 4 Variations of flowers in transgenic plants regenerated from embryogenic calli.
Flower of original 'Shortcake' plant (A), flower on shoot of non-transgenic regenerated plant (B), 3 typical variant flowers on primary shoots of transgenic plants (C, D and E) and flower on the 2nd or later emerged shoot of transgenic regenerated plants (F). (Bar = 1 cm)



Fig. 5 Transient GUS expression in various organs of transgenic plant regenerated from the embryogenic calli. Flower (A), petals with calli cultured on callus inducing medium for 1 month (B), leaves (C), leaves with calli cultured on callus inducing medium for 1 month (D), transverse cross section of young stem (E, Bar = 3 mm), and young non-transformed (F, left) and transformed (F, right) roots. (Bar = 1 cm, except E)

order to study the variations of the primary flowers in detail, approximately 1300 putative transgenic shoots (about 900 EC were co-cultured with the Agrobacterium) are now on the rooting process in vitro. Fig. 5 shows the transient GUS expression in the various organs of the transformed plants. All stigma, some anthers and some filaments showed the GUS expression but no expression was observed in petals (Fig. 5A). However, all petal-derived calli (20 petals were tested) produced on the callus inducing medium, which was MS medium containg $30 \text{ g} \text{ l}^{-1}$ sucrose, 2.5 g l^{-1} Gelrite , 0.1 mg l^{-1} BA and $0.5 \text{ mg } 1^{-1}$ NAA, for 1 month under the dark condition, showed the GUS expression strongly (Fig. 5B). Leaves of transformed plants also showed diffused blue loci (Fig. 5C), whereas all leafderived calli (20 leaves were tested), formed on the same medium and conditions mentioned above, showed strong GUS expression (Fig. 5D) like the petal-derived calli. The calli induced from petals and leaves of non-transformed plants did not show any blue locus. Although the cause for the strong GUS expression in the calli compared with that of the original transgenic organs is uncertain, it might be supposed that the cell walls of petal and leaf tissues were physically and mechanically stronger than those of induced calli. Consequently the infiltration of X-Gluc into the cells might be blocked to some extent and the number of blue loci decreased in spite of GUS gene expression in those tissues. Transverse stem section of transformed young plant showed that the GUS expression was observed strongly in the phloem and xylem (Fig. 5E). Any blue locus was not observed in the young root of non-transformed original plant (Fig. 5F left), whereas that of transformed plant showed GUS gene expression (Fig. 5F right). The PCR-Southern blot analysis using the 430 bp DIG-labelled GUS DNA probe showed that all of the 7 transgenic plants were GUS (+) and the non-transgenic plant was GUS (-). Fig. 6 shows the result of Southern blot analysis of 5 survived transformed plants (2 out of 7 plants withered) using approximately 1 kblong DIG-labelled HPT RNA probe for the hybridization. The T-DNA fragment of the pHTS-35S-GUS has one cleavage site of EcoRI on the hygromycin resistance gene (Fig. 1). Therefore, if these 5 transgenic plants are derived from the cells with different transgenic events, different signals will be expected among them. However, all hybridization signals of transformed plants (lane 2-6) showed the same pattern consisting of 1 dominant band (4 kbp) and 2 minor bands (Fig. 6). This suggests that all 5 transgenic plants were regenerated from single transgenic cell and supposed to be one clone. The



Fig. 6 Southern blot analysis on non-transformed and transformed plants regenerated from the embryogenic calli. A HPT RNA probe was used for the present study, and 15 μ g genomic DNA digested with *Eco*RI was applied per lane. lane 1: non-transformed plant, lane 2-6: transformed plants.

ploidy level of the transgenic regenerated plants was compared with one of the original plants by the same method previously reported (Asano and Tanimoto, 2002) but no difference was observed: the transgenic regenerated plants were tetraploid.

Techniques in the present paper will be greatly helpful to improve qualities of rose plants by *Agrobacterium*-mediated transformation method. We are endeavoring to create prickleless miniature roses by using those techniques.

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References

Asano, G., Tanimoto, S., 2002. Plant regeneration from embryogenic calli derived from immature seeds in miniature rose cultivar 'Shortcake'; Somaclonal variation, cytological study and RAPD analysis. Plant Biotechnol., 19: 271-275.

- Burger, D. W., Liu, L., Zary, K. W., Lee, C. I., 1990. Organogenesis and plant regeneration from immature embryos of Rosa hybrida L. Plant Cell, Tissue Organ Cult., 21: 147-152.
- Firoozabady, E., Moy, Y., Courtney-Gutterson, N., Robinson, K., 1994. Regeneration of transformed rose (*Rosa hybrida*) plants from embryogenic tissue. Bio/Technology, **12**: 613-613.
- Hasegawa, P. M., 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. J. Am. Soc. Hort. Sci., 105: 215-220.
- Jefferson, R. A., 1987. Assaying chimeric genes in plants; the GUS gene fusion system. Plant Mol. Biol. Rep., 5: 387-405.
- Jefferson, R. A., Kawanagh, T. A., Bevan, M. W., 1987. GUS fusion: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6: 3901-3907.
- Kunitake, H., Imamizo, H., Mii, M., 1993. Somatic embryogenesis and plant regeneration from immature seedderived calli of rugosa rose (*Rosa rugosa* Thunb.). Plant Sci., **90**: 187-194.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., **15**: 473-497.

- Rashid, H., Toriyama, K., Hinata, K., 1996. Transformed plant production from leaf disc of *Moricandia arvensis* using *Agrobacterium tumefaciens*. Plant Cell Rep., 15: 799-803.
- Sambrook, J., Frisch, F. F., Mariatis, T., 1989. Molecular cloning 2nd. ed. Cold Spring Harbor Laboratory Press.
- Souq, F., Coutos-Thevenot, P., Yeam, H., Delbard, G., Maziere, Y., Barbe, J. P., Bouley, M., 1996. Genetic transformation of roses, 2 examples: one on morphogenesis, the other on anthocyanin biosynthetic pathway. Acta Hort., 424: 381-388.
- Trick, H. N., Finer, J. J., 1997. SAAT: sonication assisted Agrobacterium - mediated transformation. Transgenic Res., 6: 329-336.
- Trick, H. N., Finer, J. J., 1998. Sonication-associated Agrobacterium-mediated transformation of soybean [Glycine max (L.) Merrill] embryogenic suspension culture tissue. Plant Cell Rep., 17: 482-488.
- Tsuchiya, T., Toriyama, K., Yoshikawa, M., Ejiri, S., Hinata, K., 1995. Tapetum-specific expression of the gene for an endo- β -1,3- glucanase causes male sterility in transformed tobacco. Plant Cell Physiol., 36: 487-494.
- Visessuwan, R., Kawai, T., Mii, M., 1997. Plant regeneration from cell suspension culture derived from immature embryo of rose. Plant Biotechnol., 14: 29-33.