Original Papers

Plant Tissue Culture Letters, **13**(2), 161-167 (1996)

Agrobacterium-mediated Transformation of *Hibiscus syriacus* and Regeneration of Transgenic Plants

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(Received February 16, 1996)
(Accepted March 15, 1996)

Hibiscus syriacus leaf tissues were transformed and transgenic plants were regenerated. Leaf pieces from *in vitro* grown plantlets were inoculated with *Agrobacterium tumefaciens* strain LBA4404 harboring the binary plasmid pBI121 containing β-glucuronidase (GUS) reporter and neomycin phosphotransferase II (NPT II) selectable marker genes. Twenty eight percent of inoculated leaf pieces produced kanamycin-resistant calli when cultured on MS medium supplemented with 0. 2 mg/*l* 2, 4-dichlorophenoxyacetic acid (2, 4-D), 2. 0 mg/*l* benzyladenine (BA) and 50 mg/*l* kanamycin (Km). Shoot regeneration was achieved by transferring the calli to MS medium containing 2. 0 mg/*l* 2-isopentenyladenine (2ip), 0. 1 mg/*l* naphthylacetic acid (NAA) and 50 mg/*l* Km. Regenerated shoots were rooted in medium with 0. 5 mg/*l* indolebutyric acid (IBA) and 25 mg/*l* Km. GUS activity was detected in leaves, stems and roots of transgenic plants by both histochemical and fluorometric assays. Northern hybridization analysis provided molecular evidence for stable genetic transformation.

Introduction

The numerous cultivars of *Hibiscus syriacus* (Malvaceae) reflect its importance as an ornamental plant[1]. In addition, it is used in various other applications, including the production of fibre, as well as an additive in the beverage industry in several Asian countries[2,3]. The hardiest species in its genus, *H. syriacus*, is highly resistant to pests and diseases[4] and is frost-tolerant[5]. Therefore, this species may be suitable for the genetic improvement of other related species through somatic hybridization. For example, protoplast fusion with *H. rosa-sinensis* may confer frost tolerance to this cold-sensitive plant[6]. To accomplish this goal, we determined the proper conditions to culture recalcitrant protoplasts of *H. rosa-sinensis*[7], and also developed an *in vitro* plant regeneration system for *H. syriacus*[8]. The objective of this study was to develop an *Agrobacterium*-mediated genetic transformation system of *H. syriacus* and subsequently the regeneration of transgenic plants. Although transgenic calli were obtained from *H. cannabinus*[9] and *H. rosa-sinensis*[10], there have been no reports on transgenic plant regeneration from the genus *Hibiscus*. This is the first report on successful regeneration of transgenic *Hibiscus* plants by *Agrobacterium*-mediated transformation.

Materials and Methods

1. *Plant material and bacterial strain*

Leaf-derived *Hibiscus syriacus* cv. Heikeyama plantlets were used for transformation experiments. Plantlets were maintained on MS medium[11] supplemented with 0. 5 mg/*l* IBA as described previously[8].

Agrobacterium tumefaciens strain LBA4404[12] with or without a binary plasmid pBI121 was used for leaf transformation. The pBI121 contained *npt II* selectable marker and *gus* reporter genes, driven by nopaline synthase promoter (Nos) and CaMV35S promoter, respectively[13].

2. *Transformation and regeneration of transgenic plants*

Young leaves, about 2. 5 cm in size, were harvested from aseptically grown plantlets and cut into about 1 cm^2 pieces. Leaf pieces were soaked in an *A. tumefaciens* culture (O. $D._{650}$ = 0. 5) for 5 min. and blotted dry on sterile filter paper. Then the leaf pieces were plated on MS-agar (0. 8%) media containing various combinations of growth regulators as shown in **Table 1**, but without antibiotics, for two days in the dark at 25°C. Thereafter, the leaf pieces were transferred to fresh media containing 500 mg/*l* cefotaxime and 50 mg/*l* kanamycin, and incubated at 25°C, with a 14 h light (4000 lux, fluorescent lamp, Toshiba FL 40S. D) and a 10 h dark photoperiod. Developing kanamycin-resistant calli were subcultured on the same type of media. To confirm death of *A. tumefaciens*, portions of the calli were crushed and smeared onto LB plates that were incubated at 28°C. No colonies were observed. Shoots were induced by transferring the calli onto medium containing 2. 0 mg/*l* 2ip, 0. 1 mg/*l* NAA and 50 mg/*l* Km but lacking cefotaxime. Rooting of kanamycin-resistant shoots was achieved by transferring shoots to MS medium containing 0. 5 mg/*l* IBA with 25 mg/*l* Km. Acclimatization and maturation of transgenic plants was done as described in a previous study[8].

3. *Fluorometric and histochemical analysis of GUS activity*

GUS activity in the extract of regenerated plants was measured fluorometrically with a spectrofluorometer (FP-777, JASCO Corporation, Japan). *In situ* expression in leaf, stem and root segments was determined histochemically. Both assays were done according to Jefferson *et al*[13]. Total protein concentration in the extract was measured using a protein assay kit (Bio-Rad Laboratories, USA).

4. *Northern blot analysis of* gus *and* npt II *expression*

Total RNAs were isolated from leaves of both transformed and untransformed plants by the acid guanidinium thiocyanate-phenol-CsCl method[14]. Poly $(A)^+$ RNAs were isolated with a polyATtract mRNA isolation system IV kit (Promega Corporation, USA). 5 mg of each of poly $(A)^+$ RNA isolate was separated in 1. 2% denaturing agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Germany) as described by Chomczynski[15]. Northern hybridization was carried out following standard techniques[16]. ^{32}P-labelled GUS and NPT II probes were prepared from the *Eco*RI-*Bam*HI and *Pst*I fragments of pBI121, respectively, with a BcaBest radiolabeling kit (Takara, Japan). After hybridization with the GUS probe, the filter was washed with boiling water for 30 min. and rehybridized with the NPT II probe.

Results and Discussion

1. *Transformation and regeneration of transgenic plants*

Experiments were carried out to determine the minimal concentration of Km to inhibit growth of untransformed *H. syriacus* leaf tissues. Km at concentrations ranging from 0 to 100 mg/*l* were

Table 1. Percentage of kanamycin resistant calli produced from *Hibiscus syriacus* leaf explants on MS medium with various combinations of growth regulators (mg/*l*) and 50 mg/*l* Km.

Treatment	Number of leaf explants used	Number of explants producing Km^r calli	Km^r calli formation frequency (%)
control*[1]			
BA 2.0+2,4-D 0.2	54	0	0
transformation*[2]			
BA 2.0+2,4-D 0.2	75	21	28
BA 2.0+NAA 0.1	67	2	3
2ip 2.0+NAA 0.1	71	2	3

*[1] Leaf pieces were inoculated with *A. tumefaciens* LBA4404.
*[2] Leaf pieces were inoculated with *A. tumefaciens* LBA4404: pBI121.

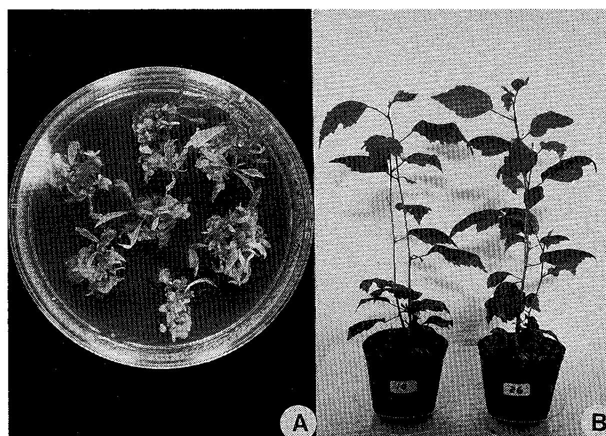

Fig. 1 Regeneration of transgenic *H. syriacus* plants.
(A) Development of transgenic shoots from calli on medium containing 50 mg/*l* kanamycin.
(B) Regenerated transgenic plants, T-10 and T-26, growing in pots.

tested. On the media containing Km at the concentration higher than 25 mg/*l*, the leaf pieces became white and semi-transparent in two to three weeks, which indicated that the leaf tissues were killed (data not shown). Therefore, we used 50 mg/*l* Km in all selection steps with the exception of the rooting step, for which 25 mg/*l* Km was used.

After six week culture on Km-containing media, yellowish friable calli became visible at the edges of the leaf pieces inoculated with *A. tumefaciens* LBA4404: pBI121, while no callus formation was observed in control leaf pieces treated with *A. tumefaciens* LBA4404 (**Table 1**). In the medium with the combination of 0.2 mg/*l* 2,4-D, 2.0 mg/*l* BA and 50 mg/*l* Km, up to 28% leaf pieces produced kanamycin resistant calli.

The kanamycin resistant calli were subcultured once on the same medium for further proliferation. Calli of about 5 mm in size were transferred to fresh medium with 50 mg/*l* Km, 2.0 mg/*l* 2ip and 0.1 mg/*l* NAA, where they grew into greenish compact structures. Shoots developed from all of these structures within 4 weeks (**Fig. 1-A**). Elongated shoots were rooted upon transfer to medium containing 0.5 mg/*l* IBA and 25 mg/*l* Km. Plantlets were hardened off in a growth chamber (**Fig. 1-B**) and later transferred to greenhouse conditions, where they developed into mature plants and produced flowers.

2. *Analysis of GUS activity in transformed plants*

Five transgenic plants T-1, T-4, T-10, T-20 and T-26 were chosen and tested for their GUS

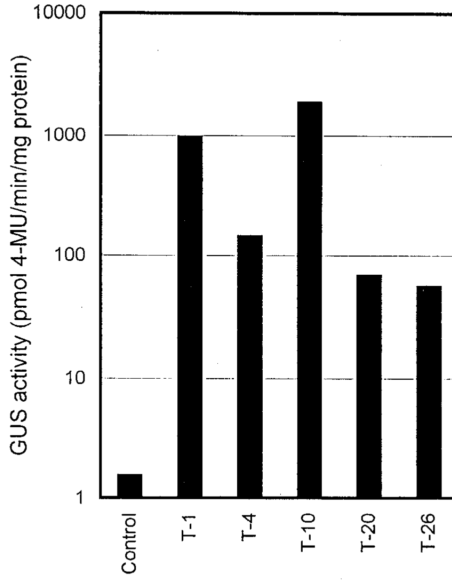

Fig. 2 Quantitative analysis of GUS activity in leaf extracts of *H. syriacus* transgenic plants. The data shown are averages from three independent assays.

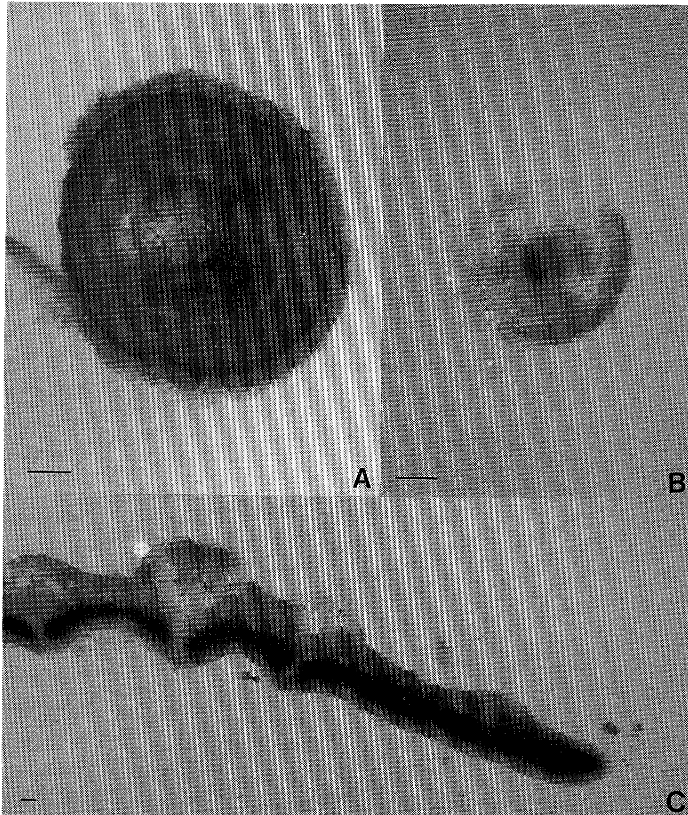

Fig. 3 Histochemical analysis of GUS activity in transgenic *H. syriacus* plant organs. (Bar = 250 μm)

(A) transverse stem section

(B) transverse root section

(C) transverse leaf section

activity in leaf extracts (**Fig. 2**). The GUS activity of the control untransformed leaf was less than 2 pmol 4-MU/min./mg protein, while leaf extracts of kanamycin resistant plants displayed various GUS activity. The highest GUS activity was found in the T-10 plant (1887 pmol 4-MU/min./mg protein), the lowest in T-26 (56 pmol 4-MU/min./mg protein). The variation in *gus* expression among the different transgenic plants tested may be attributed to different transcription and translation rates of chimeric genes.

In addition, GUS activity in leaf, stem and root sections of regenerated transgenic plants was analyzed histochemically as described by Jefferson *et al*[13]. GUS activity could be visualized in all organs tested (**Fig. 3-A, B** and **C**). In stem sections, GUS activity was observed in all cell types except cortex cells, which had no/or little GUS activity (**Fig. 3-A**). In roots, the expression in vascular tissue is stronger than that in parenchyma tissue and no expression in epidermis and epidermal hairs was found (**Fig. 3-B**). In leaf sections, GUS activity was visualized in vascular bundles and epidermis (**Fig. 3-C**).

By either fluorometric or histochemical methods, we also observed GUS expression in flowers of some transgenic plants, with extremely strong activity in calyces (data not shown).

Taken together, these results suggest that the shoots and plants were stably transformed and also indicate that the CaMV35S promoter acts constitutively in all organs of *H. syriacus*, though not in all types of cells.

3. *Northern blot analysis of stable* gus *and* npt II *expression in transgenic plants*

Northern hybridization analysis was performed to determine the expression of *gus* and *npt II* in the kanamycin resistant plants (**Fig. 4**). Poly(A)$^+$ RNAs, isolated from leaves of 25 cm height untransformed and transformed plants, were hybridized with GUS and NPT II probes. A single 2.1 kb *gus* transcript was detected in all 9 transgenic plant samples (**Fig. 4-A**, lanes 2 to 10), while no *gus* transcript was detected in untransformed leaves (**Fig. 4-A**, lane 1). *gus* expression in three transgenic plants, T-1, T-10 and T-35 (**Fig. 4-A**, lanes 2, 3 and 5, respectively), was stronger than the others.

For *npt II* analysis, a transcript of about 1.4 kb was detected in all samples from transgenic plants (**Fig. 4-B** lanes 2 to 7, 9 and 10) except in T-15 (**Fig. 4-B** lane 8). Since T-15 grew well on Km containing medium, we suppose that the expression of *npt II* in this plant was under the detection limit of northern hybridization. As expected, no band was detected in control untransformed plant (**Fig. 4-B**, lane 1). The bands of about 2.1 kb in transgenic plants T-1, T-10 and T-35 (**Fig. 4-B** lanes 2, 3 and 5) were the remaining signals of GUS probe. These data further demonstrated that the plants were transformed and expressed both genes constitutively.

In conclusion, the *Agrobacterium*-mediated transformation and transgenic plant regeneration of *H. syriacus* was achieved. This achievement opens the way for the improvement of *Hibiscus* spp. by genetic engineering techniques. The kanamycin resistant *H. syriacus* will be useful in future protoplast fusion experiments with transgenic *H. rosa-sinensis*[10] for increased chilling tolerance, allowing the selection of somatic hybrids by dual antibiotic resistance. The transgenic *H. syriacus* may also be useful for the improvement of economically important plants of the same family, such as cotton.

Acknowledgement

This work was carried out using the facilities of the Biotechnology Research Center, The University of Tokyo, Japan.

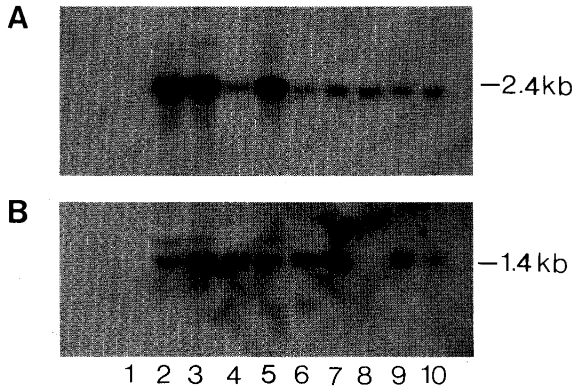

Fig. 4 Northern blot analysis of *gus* (A) and *npt II* (B) expressions in transgenic *H. syriacus* leaf tissues.
The positions of 1. 4 kb and 2. 4 kb of RNA ladder (GIBCO BRL, USA) are indicated on the right.
Poly(A)$^+$ RNAs from untransformed control leaves (lane 1) and from leaf tissues of 9 transformed plants (lanes 2 to 10) were analyzed with (A) GUS probe and (B) NPT II probe. Lanes 2 to 10 correspond to RNA samples from the transgenic plants T-1, T-10, T-26, T-35, T-4, T-30, T-15, T-20 and T-34, respectively.

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《和文要約》

Agrobacterium 法によるムクゲ形質転換体植物の作出

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ムクゲ(*Hibiscus syriacus*)の葉切片に binary vector pBI121(NPT II, GUS)を持つ *Agrobacterium tumefaciens* LBA4404 を感染させ形質転換植物体を得た. 0.2 mg/*l* 2, 4-D, 2.0 mg/*l* BA, 50 mg/*l* Km を含む MS 培地上で約 28% の葉切片から Km 耐性カルスが形成された. カルスを 2 mg/*l* 2ip, 0.1 mg/*l* NAA, 50 mg/*l* Km を含む MS 培地に移すことによりシュートが再生した. 0.5 mg/*l* IBA, 25 mg/*l* Km を含む培地上でシュートから根が発生した. 再生植物体の葉, 茎及び根から, 組織化学的染色法と蛍光測定法の両方法によって GUS 活性が検出された. GUS 及び NPT II プローブを用いた Northern hybridization により, 再生植物体が両遺伝子を発現する安定な形質転換体であることが示された.