Original Paper

Plant Biotechnology, 19 (2), 87-93 (2002)

Transformation of Statice (*Limonium sinuatum* Mill.) by *Agrobacterium* tumefaciens-Mediated Gene Transfer

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Received 30 October 2001; accepted 20 December 2001

Abstract

A successful transformation procedure using *Agrobacterium* has been established for the most important ornamental statice, *Limonium sinuatum* Mill. Cell suspension cultures derived from friable calli, which were originated from leaf segments, were infected with *Agrobacterium tumefaciens* strain EHA105 (pIG121Hm) and co-cultivated for 2 days. The concentration of acetosyringone in co-cultivation medium and the growth stage of host plant cell used for infection were important factors for obtaining high transient GUS expression. After selection on medium with 75 mg l^{-1} kanamycin, approximately 20 putative transgenic callus lines with kanamycin-resistance and GUS expression were obtained from 1 g F. W. of cell cultures infected. Adventitious shoots were induced from 5 different putative transgenic callus lines on the regeneration medium containing 75 mg l^{-1} kanamycin, and finally 16 transgenic plants which showed GUS expression in leaves were obtained. Presence of NPT II and GUS genes was confirmed by PCR analysis in the genomic DNAs of transgenic callus. Integration of GUS gene into the genome of transgenic plantlets derived from different transgenic callus lines was detected by Southern blot analysis.

Introduction

The genus *Limonium*, formerly called 'statice', distributes throughout the world and comprises over 300 species. Among them, 15-20 species have been horticulturally important as cut and dried flowers and they have been cultivated for commercial production in various regions of the world. In Japan, the planted area and the wholesale quantity of statice were ranked 8th among all cut flowers in 2000. Among horticultural statice including some hybrids, *L. sinuatum* is the most important species including many cultivars with wide variations of sepal colors.

In *L. sinuatum*, production of novel cultivars with important traits such as disease resistance and modified plant morphology has strongly been desired. However, conventional breeding of this species has been limited because of the cross-incompatibility with other species. Therefore, genetic transformation is expected to be an effective strategy for improving this crop.

There has been only one successful report on the production of transgenic plants in statice, which was produced by *Agrobacterium*-mediated gene transfer in hybrid statice (*L. otolepis* Kuntze × *L. latifolium* Kuntze) (Mercuri *et al.*, 2001). However, production of transgenic plants of *L. sinuatum*, which is the most important statice, has not been successful because plant regeneration procedure applicable for transformation has not been established. In the present study, we first report the successful transformation of *L. sinuatum* by using plant regeneration system from cell cultures of *L. sinuatum*, which has been established recently at our laboratory (Igawa *et al.*, in press).

Materials and Methods

Plant material

Cell suspension cultures with high proliferation and regeneration ability were induced from friable calli originated from leaf segments of *L. sinuatum* 'Early Rose' (**Fig. 1A**) (Igawa *et al.*, in press). The cell suspension cultures were maintained by subculturing 0.5 g fresh weight [F. W.] of cells in 30 ml of callus proliferation medium [CPM], which is 1/2 Murashige & Skoog [MS] medium (Murashige and Skoog, 1962) containing 20 g l^{-1} sucrose and 1.0 mg l^{-1} picloram at pH 5.8, every week. The suspension cultures were kept on a rotary shaker at 100 rpm at

25 ℃ under 24 h illumination (35 μmol $m^{-2}s^{-1}$) with cool white fluorescent lamps (National FL30SN, Osaka, Japan) and used for transformation.

Bacterial strain

A. tumefaciens strain EHA105 (Hood *et al.*, 1993) bearing chloramphenicol-resistant gene was used for transformation. EHA105 harbored plasmid pIG121Hm (Ohta *et al.*, 1990), carrying a kanamycin-resistant gene driven by the *nos* promoter, a hygromycin-resistant gene driven by the CaMV 35S promoter and a *uid* A gene with an intron of a catalase gene from caster bean under the CaMV 35S promoter.

Transformation

Bacteria were cultured overnight at 28 ℃ in Luria-Bertani medium [LB medium] containing 50 mg l^{-1} kanamycin, 20 mg l^{-1} hygromycin and 25 mg l^{-1} chloramphenicol at pH 7.2. *A. tumefaciens* suspension was centrifuged at 100 g for 15 min at room temperature, and the pellet was re-suspended to OD_{600}=0.5 with CPM. The cells in suspension cultures of *L. sinuatum* (4-5 g F. W.) were blotted on filter paper for 5 min, put into 30 ml of *A. tumefaciens* suspension and vacuum-infiltrated for 10 min. The cells were then collected on the nylon mesh (77 μm pore size) and blotted away the excess bacterial suspension. The cell cultures infected were spread over 2 g l^{-1} gellan gum-solidified CPM containing 0-400 μM of acetosyringone, and co-cultivated for 2 days at 25 ℃ under 24 h illumination (35 μmol $m^{-2}s^{-1}$) with cool white fluorescent lamps (National FL30SN). The stock of acetosyringone (100 mM) dissolved in dimethyl sulfoxide was filter-sterilized and added to autoclaved co-cultivation medium. For culture, plastic Petri plates (90 × 20 mm; IWAKI, Tokyo, Japan) containing 30 ml of medium were used and sealed with surgical tape (3M Health Care, Borken, Germany). After co-cultivation, cell cultures were washed twice with CPM, blotted to remove excess liquid, spread over 2 g l^{-1} gellan gum-solidified CPM containing 250 mg l^{-1} cefotaxime (Claforan, Hoechst), and cultured for 1 month for eliminating *Agrobacterium*.

Flow cytometry analysis

Relative nuclear DNA content of the cell suspension culture was measured at different stages after subculture by flow cytometry (Ploidy Analyzer; Partec, GmbH-Munster, Germany). To release nuclei, approximately 100 mg of cell suspension cultures were chopped with a razor blade in 0.2 ml of solution A of Plant High Resolution DNA kit type P (Partec, Germany) in a plastic Petri dish. After incubating the crude nuclei suspension for 1 min at room temperature, 2 ml of staining solution (0.1 M Tris-HCl, 2 mg l^{-1} DAPI [4', 6-diamidino-2-phenylindole dihydrochloride], 0.1% Triton X-100 and 2 mM $MgCl_2$, pH 7.5) was added to the nuclei suspension and filtered through a 40 μm nylon mesh. After 1 min staining, nuclei suspension was subjected to the measurement of the relative nuclear DNA content on a liner scale histogram. Measurement was replicated at least 3 times independently at different stages after subculture. Percentage of nuclei at 4C level in the total nuclei was calculated from histogram with FLOMAX software (Partec, Germany).

Selection and regeneration of transformants

After proliferation on the *Agrobacterium*-elimination medium, the cells were transferred onto 2 g l^{-1} gellan gum-solidified CPM containing 75 mg l^{-1} kanamycin and cultured for 1 month for selecting transformants. For elimination of possibly contaminating non-transformed cells from each callus line, each small callus (ca. 5 mm in diameter) surviving on the medium was transferred and spread onto 2 g l^{-1} gellan gum-solidified CPM containing 75 mg l^{-1} kanamycin, and separately subcultured every 2 weeks. For regeneration from each callus line, 0.5 g F. W. of callus was transferred to 30 ml of liquid CPM containing 75 mg l^{-1} kanamycin for dispersing friable calli into small cell colonies. After 1 week of culture, each callus line was collected on 77 μm mesh and inoculated for shoot regeneration in the test tube (25 × 80 mm) containing 10 ml of 1/2 MS medium supplemented with 20 g l^{-1} sucrose, 5 g l^{-1} gellan gum, 1.0 mg l^{-1} zeatin and 75 mg l^{-1} kanamycin.

GUS assay

Transient β-glucuronidase (GUS) expression of cell cultures was measured by counting blue foci per 20 mg of cell cultures immediately after 2 days of co-cultivation by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide [X-gluc] (Jefferson *et al.*, 1987). Counting of the GUS foci was conducted 10 times in each experiment and the experiment was replicated 3 times independently. For statistical analysis, Duncan's multiple range test was used to determine the significant differences in means of number of GUS foci. GUS activity was also detected on the kanamycin-resistant colonies and the leaves of regenerated putative transformants.

DNA extraction and PCR analysis

Total genomic DNA was isolated from cell cultures (ca. 5.0 g F. W.) of 5 different putative transgenic callus lines and non-transformed control cell cultures of *L. sinuatum* using the CTAB method (Murray and Tompson, 1980). Specific oligonucleotide primers for detecting NPT II and GUS gene sequences (Hamill *et al.*, 1991) were used to show the presence of these genes in the genomic DNAs. Twenty ng of template DNA and 1 μl of 5 μM primers were mixed with 2 μl of 10 × *Taq* DNA polymerase reaction buffer, 100 μM dNTP mixture and 1 unit of *Taq* DNA polymerase (TAKARA SHUZO CO., LTD., Shiga, Japan) in a total volume of 20 μl. The PCR was performed with PTC-200 (MJ RESEARCH INC., Massachusetts, USA). For amplification of 0.7 kb fragment of NPT II gene, following thermal cycle treatment was made : 1 cycle of 2 min at 92 ℃ , 1 min at 57 ℃ and 2 min at 72 ℃ followed by 35 cycles of 1 min at 92 ℃ , 1 min at 59 ℃ and 1 min at 72 ℃ . For amplification of 1.2 kb fragment of GUS gene, the PCR condition consisted of 5 min at 94 ℃ followed by 30 cycles of 1 min at 94 ℃ , 1 min at 55 ℃ and 1.5 min at 72 ℃.

Southern hybridization

Ten μg genomic DNA extracted from non-transgenic cell culture and 4 putative transgenic plantlets, which were derived from 4 different transgenic callus lines used in PCR analysis, was each digested with *Hind* III and fractionated through 8 g l^{-1} agarose gel by electrophoresis. The DNAs were then blotted onto Hybond N^+ membrane (Amersham Pharmacia Biotech, England) under alkaline conditions following the manufacturer's instructions. A 1.2 kb fragment of the GUS gene used as a probe was amplified by PCR using pIG121Hm. Labelling of probe, hybridization, and detection were carried out according to the instruction of the ECL [enhanced chemiluminescence] direct nucleic acid labelling and detection systems (Amersham Pharmacia Biotech).

Results and Discussion

Influence of acetosyringone on transient GUS expression in cell suspension culture

Acetosyringone is one of the phenolic compounds acting as a signal that allows *Agrobacterium* to recognize a plant cell susceptible to transformation (Stachel *et al.*, 1985). The phenolic compounds are directly sensed by Vir A protein, which activates other *vir* genes located on the Ti plasmid (Lee *et al.*, 1995). Increase in the transformation efficiency by adding acetosyringone to *Agrobacterium* suspension and/or co-cultivation medium has been reported in several plant species such as grapevine (Hoshino *et al.*, 1998), lavender (Mishiba *et al.*, 2000) and phalaenopsis orchid (Belarmino and Mii, 2000). In the present study, the effect of acetosyringone in co-cultivation medium on cultured cells of *L. sinuatum* was initially assessed by transient GUS expression (**Fig. 1B, Fig. 2**). The number of GUS foci increased as the increase in the concentration of acetosyringone, and the highest value was obtained at 200-300 μM acetosyringone, which almost coincide with the valued obtained in other plant species. Inclusion of acetosyringone in co-cultivation me-

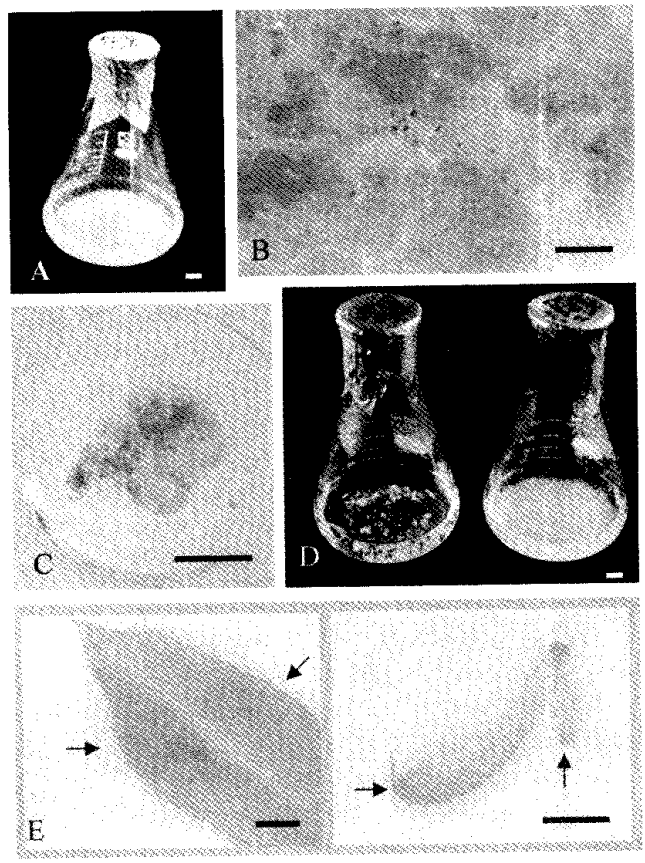

Fig. 1 Production of transgenic statice, *L. sinuatum* Mill.

(A) Cell suspension culture used for infection with *A. tumefaciens*. Bar=10 mm

(B) Transient GUS expression in cells after 2 days of co-cultivation with *A. tumefaciens* for on medium containing 200 μM acetosyringone. Bar=2 mm

(C) Stable GUS expression on kanamycin-resistant callus. Bar=5 mm

(D) Proliferation of cell suspension cultures with 75 mg l^{-1} kanamycin. Left; non-transformed cell culture, Right; transformed cell culture. Bar=10 mm

(E) GUS expression (arrows) on leaves of regenerated plants derived from individual transgenic callus lines. Left; Bar=5 mm, Right; Bar=2 mm

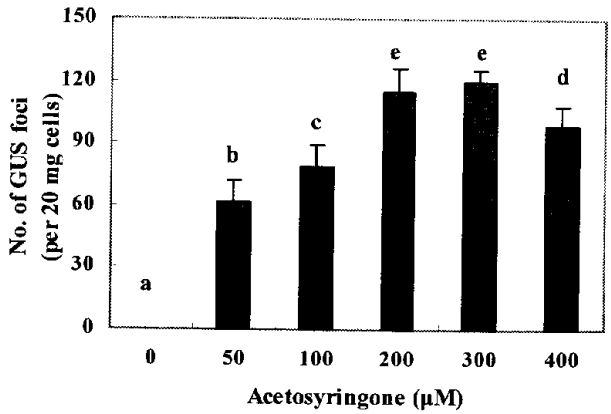

Fig. 2 Influence of acetosyringone concentration in co-cultivation medium on transient GUS expression of 5-day-old cell suspension cultures of *L. sinuatum*. Transient GUS expression is represented as number of blue foci in 20 mg of cell cultures. Vertical bars indicate the standard error from 3 experiments. Different letters indicate significant difference at 0.01 level.

dium was essential for transformation in this species because GUS staining had never been observed on the cells co-cultivated without acetosyringone. In our preliminary experiment using leaf segments as source explants, the same phenomenon was observed in 8 cultivars of *L. sinuatum* (data not shown). These results suggest that the amount of phenolic compounds produced by the tissues of *L. sinuatum* might not be sufficient for activating *vir* gene of *Agrobacterium* even if the tissues were wounded. Reduction of GUS staining in cell cultures co-cultivated with 400 μM acetosyringone may be due to the harmful effect of supra-optimal concentration of acetosyringone or the solvent used in this experiment.

Influence of days after subculture of cell suspension culture on transient GUS expression

The cell suspension culture used in the present study has been subcultured at one-week intervals with high proliferation rate and it showed 16 times increase in fresh weight during the one week of subculture (0.5 g at day 0 and ca. 8 g at day 7) (**Fig. 3**). Transient GUS expression of the cell suspension culture was also affected by the cell age after subculture (**Fig. 3**). Significantly higher GUS expression was observed in 3 to 5-day old cell cultures, which are in early log phase, and the highest GUS expression was observed in 4 to 5-day old cell cultures. Some studies have revealed that the dividing cells within the plant tissue are highly competent for *Agrobacterium* infection (An, 1985; Valvekens *et al.*, 1988; Sangwan *et al.*, 1991, 1992; Kathen and Jorgjacobsen, 1995). Moreover, Villemont *et al.* (1997) reported that cycling cells, which showed a high ratio of S-G_2/M phase cells, were highly competent for transformation. Therefore, 1 to 7-day old cell suspension cultures were analyzed by flow cytometry to monitor the cell division activity (**Fig. 4**). As the result, relatively high ratio of nuclei at 4C level, which indicates the presence of cycling cells, was detected in 3 to 5-day old cell suspension cultures. Therefore, high competence of the 3 to 5-day old cells might attribute to the high division activity of the cells at this subculture stage, although the reason for the slight difference between the peak day for GUS expression and that for G_2/M cells is still unclear.

PCR analysis of transgenic cell culture

Approximately 20 kanamycin-resistant callus lines were obtained from 1 g cell suspension cultures infected, and stable and uniform expression of GUS was observed in these calli (**Fig. 1C**). PCR analysis of 5 different transformed callus lines with kanamycin-resistance and uniform GUS expression revealed that the 0.7 kb and 1.2 kb fragments corresponded to the amplified products of NPT II and GUS genes, respectively, suggesting the presence of transgenes in the genomic DNAs of calli of *L. sinuatum* (**Fig. 5A, B**).

Regeneration of transgenic plants

Five different callus lines which showed the positive transformation response by PCR analysis were transferred into liquid medium containing 75 mg l^{-1} kanamycin, and cultured as cell suspension culture, individually. After 1 week of culture, each transgenic callus line proliferating vigorously (**Fig.**

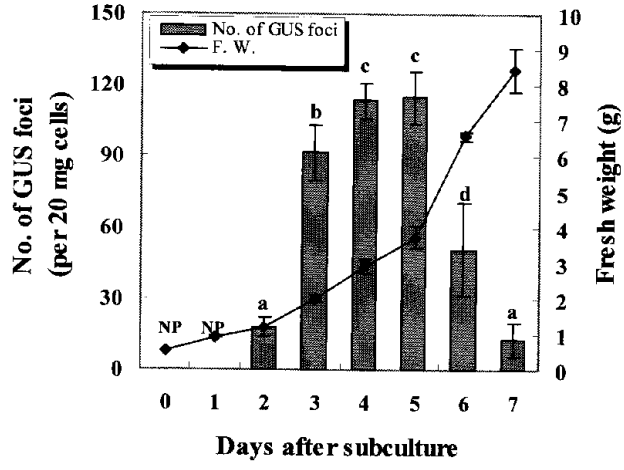

Fig. 3 Influence of days after subculture of cell suspension cultures of *L. sinuatum* on transient GUS expression. Vertical bars indicate the standard error from 3 experiments. Different letters on the histograms indicate significant difference at 0.01 level. NP; not performed.

1D) was transferred onto gellan gum-solidified regeneration medium containing 75 mg l^{-1} kanamycin. However, adventitious shoots did not differentiate efficiently from the transgenic calli on regeneration medium, whereas adventitious shoot formation from non-transformed cell culture was achieved by 2 months from inoculation (Igawa *et al.*, in press). It is possible that various factors, such as existence of antibiotics in the medium and positional effect of transgene, might inhibit the regeneration of transformants. Despite such difficulties, we finally succeeded in regenerating 16 shoots from 5 different transformed callus lines after 6 months from inoculation.

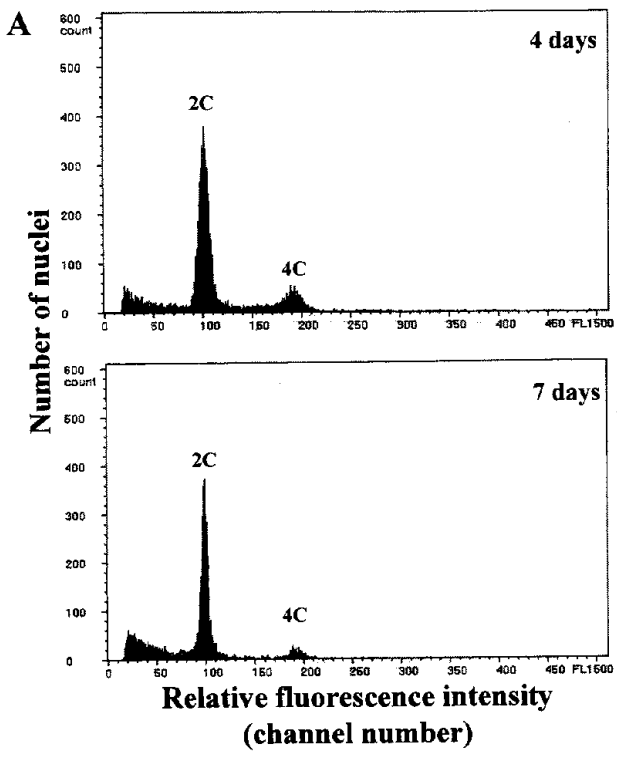

Fig. 4 Flow cytometric analysis of DNA contents on 1 to 7-day old cell suspension cultures of *L. sinuatum*.
(A) Nuclear DNA was stained with DAPI and the peaks corresponding to nuclear DNA C-values were indicated above each peak. 2C and 4C peaks reflect the G_0/G_1 and G_2/M nuclei, respectively.
(B) Percentage of nuclei at 4C level in the total nuclei. Values were indicated as mean ± SE calculated from at least 3 histograms detected by flow cytometry. *; Histograms were shown in A.

Southern blot analysis

To confirm the integration of GUS gene into host plant genome, and to exclude the possibility of the expression of GUS gene in putative transgenic plantlets by residual *Agrobacterium*, Southern blot analysis was conducted with the probe coding GUS gene. Genomic DNAs of non-transgenic cells and the leaves of putative transgenic plantlets were digested with *Hind*Ⅲ, since the plasmid used in the present study contained a unique *Hind*Ⅲ reaction site. Consequently, single hybridized bands ap-

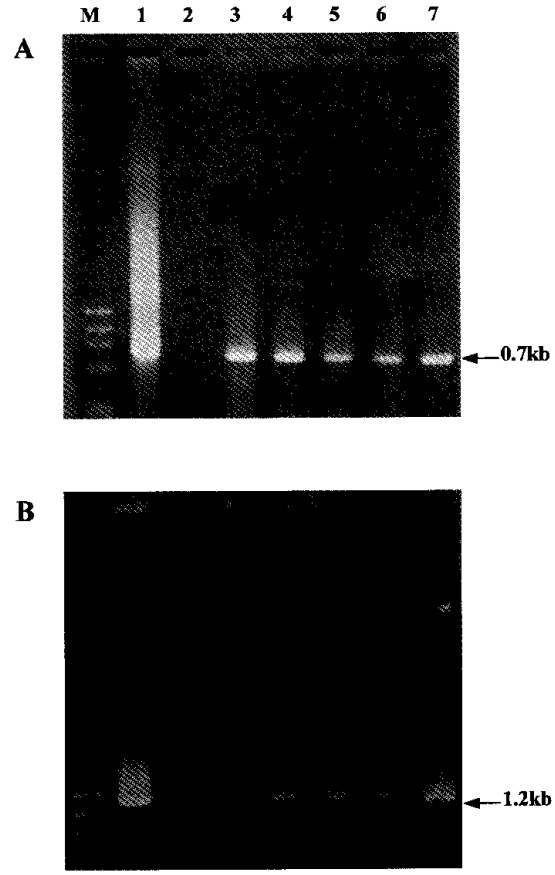

Fig. 5 PCR analysis of genomic DNA samples from transgenic calli to detect NPT Ⅱ (A) and GUS (B) genes. Arrows indicate 0.7 kb band for NPT Ⅱ (A) and 1.2 kb for GUS (B), respectively. Lane M, ϕ X174/*Hae*Ⅲ as size marker; lane 1, pIG121Hm as a positive control; lane 2, non-transformed control calli; lane 3-7, different transgenic callus lines.

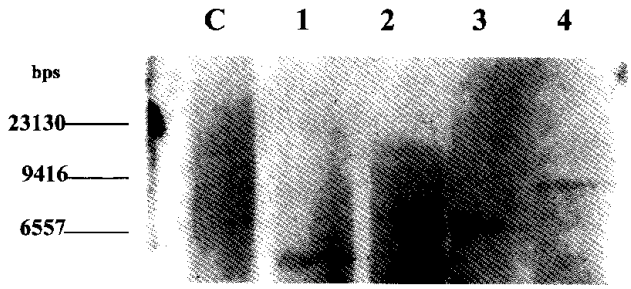

Fig. 6 Southern blot analysis of genomic DNA samples from transgenic plantlets.

Genomic DNA was digested with *Hind*Ⅲ and the blot was hybridized with a probe containing GUS coding region. Lane C, non-transformed control callus; lane 1-4, transgenic plantlets derived from different callus lines.

peared in all 4 putative transgenic plantlets, which indicate that T-DNA was integrated into the genome of all *L. sinuatum* transformants tested, at different positions each other (**Fig. 6**).

GUS expression in transgenic plants

Regenerated plantlets showed a similar morphology to that of wild type plants, and GUS expression was observed on a part of the leaves tested (**Fig. 1E**). Irregular GUS expression observed in these regenerants suggests that occurrence of 'gene silencing' (Matzke *et al.*, 1995) or 'rearrangement' (Weising, *et al.*, 1988) of the transgene in the specific tissue, although the possibility that these transgenic plants are chimera consisting of transformed and non-transformed cells can not be completely excluded.

In the present study, we have first established a transformation procedure in *L. sinuatum*. For further use of the protocol established in the present study for introducing useful genes into this important flower crop, it is necessary to increase the regeneration efficiency from transformed cell culture and to overcome the irregular expression of transgene in transgenic plants.

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