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Agrobacterium-mediated genetic transformation of *Dianthus* hybrid 'Telstar Scarlet' by using regenerable callus.

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Summary

Regenerable callus culture of *Dianthus* hybrid 'Telstar Scarlet' was successfully applied to *Agrobacterium*-mediated genetic transformation. Five days coculture with 5 mM glucose and 50 mM acetosyringone was suitable for *Agrobacterium*-infection. Transformed shoots were obtained from inoculated calluses selected on MS medium containing 1.0 mg/l thidiazuron, 0.1 mg/l α-naphthalene acetic acid, 20 g/l sucrose, 2 g/l gellan gum and 20-80 mg/l G-418 within 20 weeks from inoculation. Transformation efficiency based on the number of calluses that produced transformed plants, and based on the total number of transformed shoots were 6% and 27% respectively. About 10% of transformants were tetraploid. All diploid transformants grew, flowered normally and produced seeds when crossed with other *Dianthus* species.

Key word : *Agrobacterium*, callus culture, *Dianthus*, genetic transformation.

Introduction

The genus *Dianthus* includes important ornamental plants with carnation (*D. caryophyllus*) at the head of the list. Many *Dianthus* species including *D. barbatus, D. chinensis, D. plumarius, D. superbus* etc. and their hybrids are also widely used as cut flowers, pot plants and bedding plants.

Recent advances in molecular breeding have been applied to many ornamental crops including carnation. Attempts have been made to produce novel flower colour [1,2], disease resistance [3] and longer flower longevity [4,5,6,7] in carnation through genetic transformation. However, there are few reports on the genetic transformation of Dianthus [8].

Recently we established highly regenerable callus cultures of *Dianthus* hybrid 'Telstar Scarlet' derived from cotyledon tissue [9]. The aim of this work was to apply callus culture to Agrobacterium-mediated genetic transformation.

Materials and methods

Callus culture

Regenerable callus of *Dianthus* hybrids 'Telstar Scarlet' was established according to our previous report [9]. Callus was divided into small clumps (3-5 mm φ) and sub-cultured on MS medium containing 1.0 mg/l Thidiazuron (TDZ), 0.1 mg/l a-naphthalene acetic acid (NAA), 20 g/l sucrose and 2 g/l gellan gum (R medium) every 3 weeks. Plastic petri dishes (9cm φ) containing 25 ml medium were used as culture vessels throughout this experiment, except for shoot culture. All cultures were incubated at 25℃ under a 16 h photoperiod with a light level of 38 μmol・m^{-2}・sec^{-1} provided by fluorescent lamps for plant growth (40PG National).

In order to clearify antibiotic sensitivity of the callus, 20 callus clumps were cultured on R medium supplemented with 0, 20, 40, 80 or 120 mg/l G-418. Each treatment had 2 replications.

Agrobacterium inoculation

Agrobacterium tumefaciense AGL0 (pKT3) was used in this experiment. The binary plasmid pKT3 consists of a 35 S promoter-driven NPTII and a NOS promoter-driven GUS gene. In order to enhance gene expression both NPTII and GUS gene cassettes contained enhancers [10].

Two methods for bacteria inoculation were compared. First, 20 callus clumps were placed on filter paper overlaying R medium and then 10 μl of bacterial suspension (O. D.$_{600}$=0.5) containing 10 mM glucose and 100 mM acetosyringone (AS) was pipetted onto each callus clump. The cultures were co-cultured for 1, 2, 3, 5, 8 or 13 days. In the second method, 20 callus clumps were soaked in bacterial suspension for 10 min, then placed on

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three layers of filter paper moistened with 5 mM glucose and 50 mM AS in a vacant plastic petri dish. The callus lumps were cocultured for 2, 3 or 5 days. Each treatment had 2 replications.

After cocultivation all calluses were assayed with GUS medium containing 1 mM X-Gluc for 12 h at 37℃according to Jefferson [11]. The number of blue spots on callus clumps was counted after de-coloration and fixation by 70% ethanol.

Selection of transformed plants

One hundred inoculated callus clumps were cultured on R medium containing 20 to 80 mg/l G418 and 250 mg/l cefotaxime. All calluses were subcultured every 3 weeks. Regenerated shoots on the selection medium were harvested and transferred onto medium containing 3% Hyponex (complete soluble fertilizer, N : P : K=6.5-6-19, Japan Hyponex Co Ltd.) and 20 g/l sucrose (H medium) in a culture box (6.5 × 6.5 × 9.5 cm).

Genomic DNA of the harvested shoots was extracted by the CTAB method as modified by Aljanabi *et al.* [12]. Transformation was confirmed by PCR and Southern analysis as described in our previous report [13].

Growth and flowering of transformants

Transformed plants were grown on H medium. The top parts of vegetative shoots were acclimatized and grown in a greenhouse. The ploidy level of plants was determined by using Flow cytometry as described previously [9]. Pollen was collected from both diploid and tetraploid transformants. Viability of the pollen was estimated by pollen germination on a medium containing 80 g/l sucrose, 200 mg/l H_3BO_3 and 2 g/l agar. Several microscopic fields (total more than 1,000 pollen grain) were observed. Randomly chosen diploid and tetraploid transformants were crossed with *D. chinensis, D. barbatus and D. superbus var.longicalycinus.* About 100 flowers were used in each cross. Seed numbers in harvested pods were recorded. All seeds were sown in a soil mixture (Metromix 350) and grown in a greenhouse. A part of seedlings in each cross combination were subjected to GUS assay.

Results and Discussion

Sensitivity to G418

G418 suppressed callus growth and shoot regeneration considerably, even when at a low concentration. Only 32.5% and 10% of callus clumps produced adventitious shoots on R medium supplemented with 20 mg/l and 40 mg/l G418, respectively (Table 1). No shoot regeneration was observed in the medium containing more than 80 mg/l G418. The results showed that 40 to 80 mg/l G418 was a suitable selection pressure in this callus culture. The sensitivity of *Dianthus* callus to G418 is equivalent to carnation [13].

Table 1. Sensitivity of *Dianthus* callus to G418

G418 (mg/l)	No. callus cultured (A)	No. callus with shoot (B)	B/A (%)
0	40	40	100
20	40	13	32.5
40	40	4	10.0
80	40	0	0
120	40	0	0

Effect of co-culture conditions on transient GUS expression

No blue spots were observed when calluses were cocultured on R medium, even though cocultivation was prolonged to 13 days. On the other hand, callus clumps cocultured on filter paper moistened with glucose and AS showed clear blue spots (Fig. 1-A). A higher degree of transient GUS expression was observed with longer cocultivation periods (Fig. 2). Enhanced transient GUS expression under low nutrient conditions during cocultivation was reported in *Eustoma* [14]. We also observed high transient GUS expression in carnation node explants when cocultured on glucose and AS solution, resulting in efficient genetic transformation [13]. From the results of this study five days cocultivation with glucose and AS solution was applied in the following experiments.

Selection of transformed shoots

In the preliminary experiment, all *Agrobacterium*- inoculated callus clumps did not grow well when cultured on R medium supplemented with 40 mg/l G418 just after cocultivation. Therefore step-wise selection was applied. One hundred independent inoculated calluses were cultured on R medium supplemented with 20 mg/l G418 for the first 3 weeks. Ninety-three callus clumps out of 100 stayed green. These were transferred onto R medium supplemented with 40 mg/l G418 and kept on this medium for another 3 weeks. Eighty-three callus clumps maintained green and all could regenerate adventitious shoots. A small part of callus

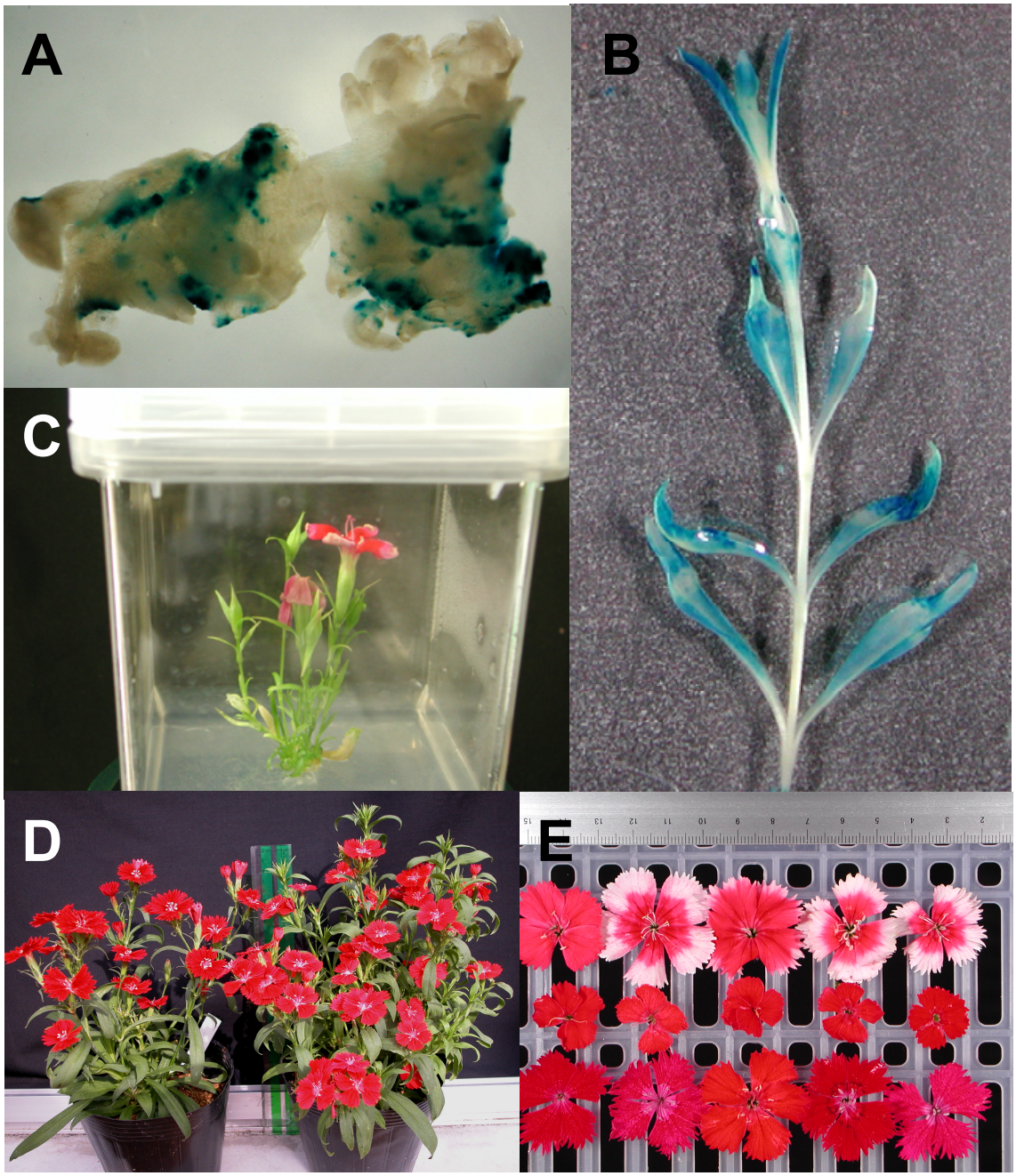

Fig. 1. A : Transient GUS expression in callus ; B : GUS expression in a plantlet ; C : precocious *in vitro* flowering ; D : flowering of diploid (right) and tetraploid (left) transformants ; E : flowers of progenies crossed with *D.chinensis* (upper), *D. barbatus* (middle) and *D. superbus* var *longicalycinus* (bottom).

was removed from all green callus clumps and subjected to GUS assay. Only 6 out of 83 had blue areas, suggesting that selection had not worked well yet. We assumed that the calluses consisted of transformed and non-transformed cells. Then 83 calluses were divided into 244 smaller pieces, and the origin of the divided pieces was recorded, and these were transferred onto R medium containing 80 mg/l G418. After 3 weeks of culture, 171 callus clumps survived. These showed a green and brown mosaic. Most early regenerated shoots became etiolated, while only green part of each callus (a total of 236 pieces) were removed and transferred onto fresh R medium containing 80 mg/l G 418. After 3 weeks of culture, 30 shoots from 16 callus clumps (from 7 initially inoculated callus clumps) were harvested. When multiple shoots developed from callus, only one shoot was harvested per regeneration locus on the

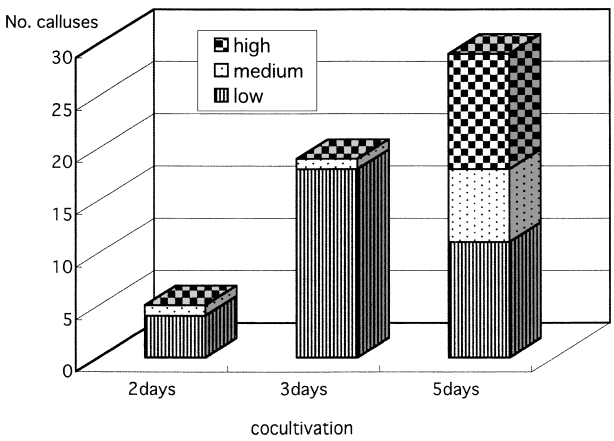

Fig. 2. Effect of cocultivation period on transient GUS expression.
low : 1 to 19 blue spots per callus clump,
medium : 20 to 40 blue spots per callus clump,
high : >50 blue spots per callus clump.

callus.

Twenty-three out of 30 shoots were clearly GUS positive and PCR showed 27 lines (from 6 initial inoculated calluses) were positive in both GUS and NPTII genes (Fig. 3). Southern analysis showed that the tested transformants had one to two copies (Fig. 4). Transformation efficiency based on the number of calluses that produced transformed plants, and based on the total number of transformed shoots were 6% and 27%, respectively. The results show that transformed plants can be obtained within 20 weeks from inoculation using this culture method.

All transformants were multiplied on H medium. Developed shoots expressed GUS in all parts of the shoot (Fig. 1-B). Eighteen clones flowered *in vitro* (Fig. 1-C). They produced vegetative shoots after cut back of flowering shoots. Precocious *in vitro* flowering was reported in carnation petal culture [15, 16, 17], *D. babatus* protoplast

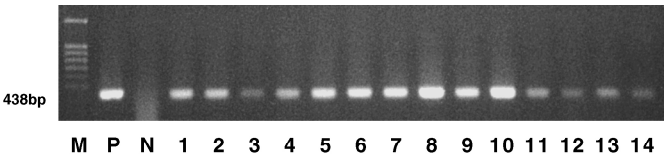

Fig. 3. PCR of analysis of regenerated shoots from *Dianthus* callus. A part of the NPT II gene (438 bp) was amplified. P : positive control (plasmid) ; N : negative control (control plant) ; 1~14 : putative transformants.

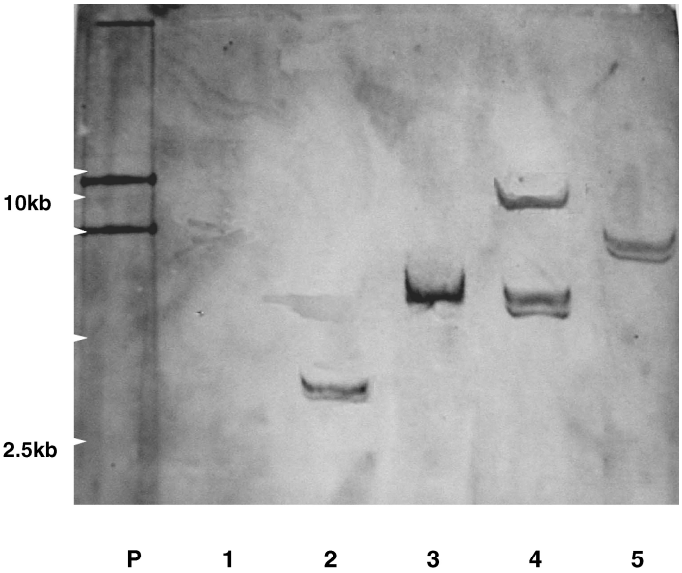

Fig. 4. Southern blot analysis of the transformed plants derived from *Dianthus* callus.
DNA was cut by *Xba*I restriction enzyme. Size marker used : 2.5 kb Molecular Ruler. P : plasmid ; 1 : negative control ; 2~5 : transformants.

culture [18] and callus culture via leaf segments in *D. chinensis* [19]. Factors affecting precocious *in vitro* flowering are not clear yet.

Flow cytometry analysis showed 3 plants derived from one callus clump were tetraploid (10%) and other 2 plants were chimera (7%), while all others were diploid. The frequency of polyploid plants in regenerants from this regenerable callus was about 10% in our previous report [9]. The results suggested that genetic transformation procedure did not affect the frequency of polypliod plant occurrence.

Growth and flowering of transformants

Vegetative shoots were acclimatized and grown in a greenhouse. All diploid transformants grew and flowered normally. Tetraploid plants showed a short plant height and had small and thick leaves (Fig. 1-D). Pollen fertility of diploids was low while high fertility was observed in tetraploids (Table 2). Low pollen fertility of diploid plants and the restored fertility of tetrapolid plants can be attributed to the genetic background of 'Telstar Scarlet' an interspecific hybrid between *D. chinensis* and *D. barbatus.*

Table 2. Pollen fertility of diploid and tetraploid transformants.

Plodiy level of plants	Pollen fertility (stained % ± SE)
Diploid	38.1±0.3
Tetraploid	96.0±0.2

Crossing with other Dianthus species

No seed set was observed in tetraploid transformants while diploid transformants produced seed even though the average number of obtained seeds per pod was low (Table 3). About 30 seedlings in each cross combination were subjected to GUS assay. 54~68% of seedlings showed GUS activity regardless of the cross combination. The result indicates that integrated T-DNA was transferred to the progenies in a Mendelian fashion, because most transformants had one to two T-DNA copies (Fig. 4). All progenies grew normally and flowered. Leaf and flower shape of progenies were intermediate of parents (Fig. 1-E).

In this study we demonstrated that regenerable callus culture of *Dianthus* 'Telstar Scarlet' can be successfully applied to *Agrobacterium*-mediated genetic transformation. The results also show the possibility for combinating genetic transformation and conventional cross breeding to improve *Dianthus* ornamentals.

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Table 3. Crossing of transformants with other *Dianthus* species.

Cross combination	Number of seed per pod	% GUS expression of progeny
T_0 x D. *superbus* var. *longicalycinus*	5.33±0.19	68.2±3.4
T_0 x *D. burbatus*	4.14±0.16	54.2±4.0
T_0 x *D. chinensis*	4.56±0.15	64.6±4.6

T_0 : diploid transformants.

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再生能力あるカルスを用いたダイアンサスの形質転換

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高い再生能力を有するダイアンサス‘テルスタースカーレット’のカルスを用いた形質転換系を検討した．カルスを5mMのグルコースと50mMのアセトシリンゴンだけで湿らせたろ紙上で共存培養することにより，アグロバクテリアの感染が向上した．接種したカルスをG-418濃度を段階的に20-80 mg/lに上げた再生培地上で選抜することにより形質転換体を獲得した．形質転換体の一部はin vitroで早期開花を示した．また約10%の転換体が四倍体であった．順化した植物は正常に生育・開花し，さらに他のナデシコ属植物と交配することにより形質転換した後代が得られた．