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Agrobacterium-mediated genetic transformation of carnation (*Dianthus caryophyllus* L.) using leaf explants derived from *in vitro* plants.

Chalermsri NONTASWATSRI* and Seiichi FUKAI

Abstract

Genetic transformation of leaf explants from *in vitro* plants of five carnation genotypes (Killer, Laurella, Master, Setono-Otome, and Tanga) was developed using *Agrobacterium tumefaciens* AGL0 with pKT3 plasmid consisting of GUS and NPTII genes. Preparation of explants was an important factor to obtain the transformed plants. The GUS-staining area was located only on the cut end of the leaf explants. Only those explants with a cut end close to the leaf base (connecting area between node and leaf) produced GUS-positive shoots. The transformation efficiency of leaf explants from *in vitro* plants varied, depending on the genotype and the position of the leaf on a shoot. Cultivar Master produced the highest numbers of GUS-positive shoots followed by Setono-Otome, Tanga, Killer, and Laurella. All the transformants grew and flowered normally. No visible variation was observed.

Key word : *Agrobacterium*, carnation, genetic transformation, leaf explants.

Abbreviations

AS, acetosyringone ; G418, geneticin disulfate ; GUS, β-glucronidase ; IBA, 3-indolebutyric acid ; MS, Murashige and Skoog ; NPT II, neomycin phosphotransferease II ; TDZ, thidiazuron.

Introduction

Several protocols for genetic transformation of carnation using node explants [1,2,3,4] and leaf explants [5] have been reported. In these protocols, explants were derived from greenhouse plants because such explants were more susceptible to *Agrobacterium* than *in vitro* explants [5]. Therefore, few protocols for genetic transformation using *in vitro* plants are proposed. In this report, we propose a protocol for genetic transformation of carnation using *in vitro* leaf explants with an emphasis on the importance of the cut end position of explants for successful genetic transformation.

Materials and methods

Five genotypes of carnation (Killer, Laurella, Master, Setono-Otome, and Tanga) were cultured on MS medium containing 1 mgl^{-1} BA, 0.1 mgl^{-1} NAA, 20 gl^{-1} sucrose, and 2 gl^{-1} Gellan Gum for a period of 1 month [6]. The leaves were detached from the first (the top, fully expanded leaf) to the sixth nodes of the *in vitro* mother plants. These leaves were then cut into 3 mm long explants containing the leaf base (connecting area between the leaf and the stem).

In order to verify the regeneration ability of each genotype, 100 explants were taken from each node position of five genotypes and cultured on MS medium supplemented with 0.5 mgl^{-1} IBA, 0.22 mgl^{-1} TDZ, 20 gl^{-1} sucrose, and 2 gl^{-1} Gellan Gum.

Subsequently 100 explants from each node position of five genotypes were used in the genetic transformation test. The *Agrobacterium tumefaciens* AGL0, containing a binary plasmid pKT3 vector consisting of NOS-LI-GUS and 35 S-L-NPT II [2] was used. Bacteria were cultured overnight in LB medium at 27℃ under dark conditions. The bacterial density was adjusted to 0.5 (OD 600) with the solution of 10 mM glucose and 100 μM AS. The explants were immersed in the *Agrobacterium* suspension, blotted briefly, transferred onto a filter paper moistened with 50 μM AS in a plastic dish, and then co-cultured for 5 days. The explants were transferred onto MS selection medium supplemented with 0.5 mgl^{-1} IBA, 0.22 mgl^{-1} TDZ, 20 gl^{-1}

*Department of Horticulture, Faculty of Agricultural Production, Maejo University, Sansai, Chiangmai, Thailand.

sucrose, 20 mgl^{-1} G418, 250 mgl^{-1} cefotaxime, and 400 mgl^{-1} vancomycin. The explants were subcultured every month onto a fresh selection medium in which G 418 concentration was gradually increased from 20 to 40 mgl^{-1}. All the cultures were incubated for a 16 h photoperiod at 36 $\mu molm^{-2}s^{-1}$ at 25℃.

Explants and regenerated shoots on the selection medium were subjected to a GUS assay according to the method of Jefferson [8]. The numbers of GUS-positive shoots per explant were recorded.

Genomic DNA was extracted from 10 g of young leaves of GUS-positive plants by the CTAB method, as modified by Aljanabi *et al.* [9] for removing polysaccharides in the extracted DNA. PCR and Southern blot analysis were performed as described in our previous paper [2].

Results and Discussion

First, the explants with base areas at various positions were inoculated with *A.tumefaciens* and subjected to the GUS assay. The results showed that the GUS-positive area was located only on the cut end of the explants. Although the leaf explants had large wound areas, the transformed cells were located only at the cut ends of the explants. When the cut ends of the explants were at a distance from the leaf base, the explants failed to produce GUS-positive shoots (Fig. 1-A), while the explants whose cut ends were close to the leaf base had GUS-positive cells at the leaf base (Fig. 1-B) and produced GUS-positive shoots (Fig. 1-C). The study previously conducted by us revealed that regenerable cells were located only at the leaf base [6]. Therefore, preparing explants in which the cut end is made close to the leaf base is the key to success in obtaining transformed

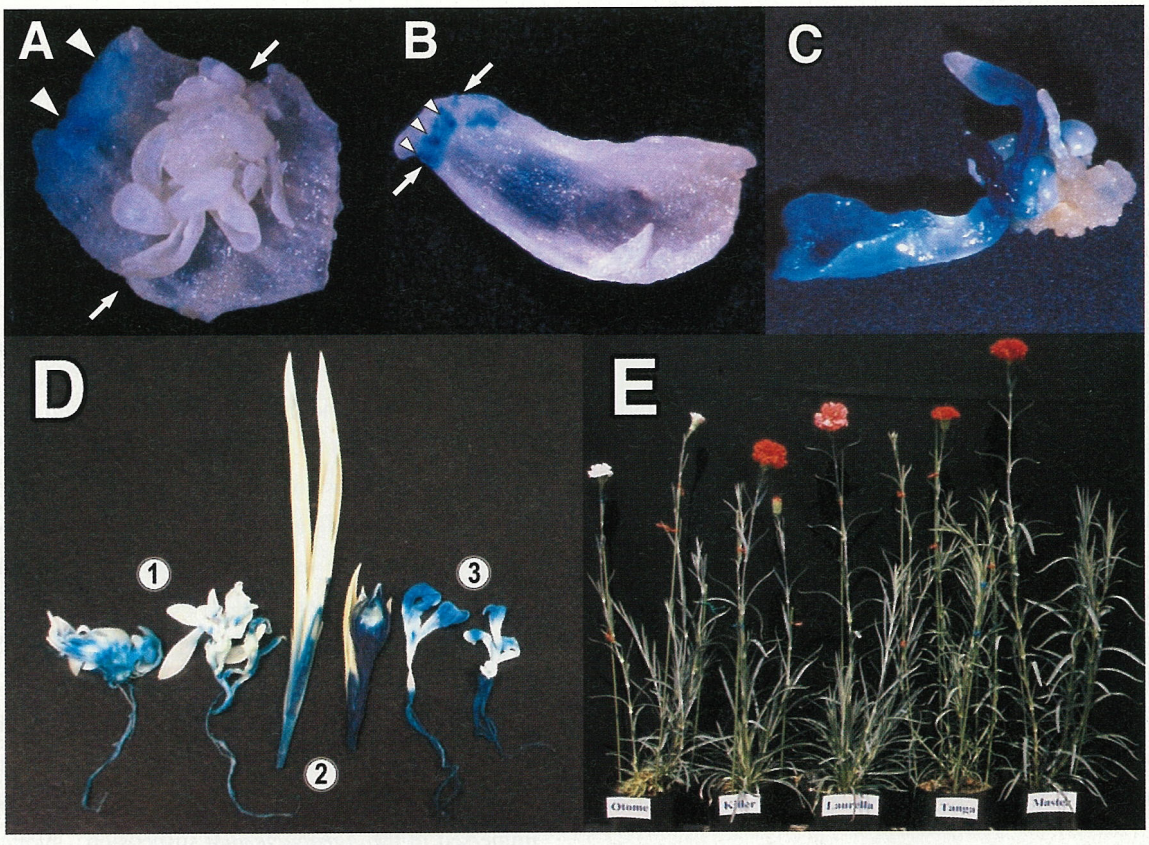

Fig. 1 GUS assay of explants, regenerated shoots and acclimatized plants, and flowering of transformed 5 genotypes carnations.
A : GUS assay of an explant with the cut end at a distance from the leaf base (arrows). The GUS staining area was located only on the cut end (arrow heads).
B, C : The leaf explant with the cut end close to the leaf base (arrows) had transform cells at the leaf base area (arrow heads) and produced GUS-positive shoots (C).
D : GUS assay of regenerated shoots in vitro (①), a shoot and a flower bud from acclimatized plants (②), and seedlings of the transformed plants (③).
F : Five genotypes of transformed carnations showed normal growth and were true to type.

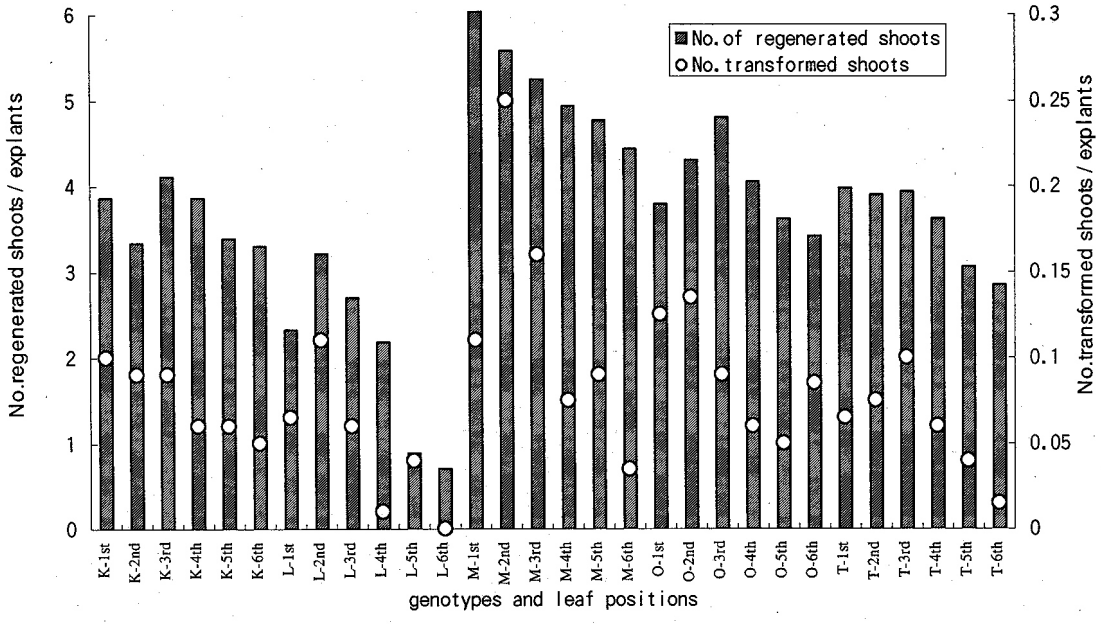

Fig. 2 Effects of leaf position and genotypes on adventitious shoot regeneration and GUS-positive shoot production.

shoots. In case of explants with the cut ends at a distance from the leaf base, although *A.tumefaciens* could transform the cells at the cut ends of the explants, those cells could not produce any shoots, resulting in failure to produce transformed shoots.

Adventitious shoot regeneration varied depending on the genotypes (two to five shoots per explant) and leaf positions on a shoot, as shown in our previous report [6]. Higher numbers of adventitious shoots were produced from explants derived from the upper position of a shoot (Fig. 2).

All the five genotypes produced GUS-positive shoots. Master and Setono-Otome produced higher numbers of GUS-positive shoots, followed by Killer and Tanga. Laurella was least productive. Explants derived from the upper position of a shoot produced higher numbers of GUS-positive shoots.

Due to hyper-hydricity of the initial regenerated shoots from explants, the shoots were subcultured on MS phytohormone free medium until normal healthy shoots developed. The top parts of the shoots were successfully acclimatized and grown in a greenhouse.

All the GUS-positive plants were PCR positive in both GUS and NPT II genes (Fig. 3-A, B). Some shoots, which were randomly chosen, were subjected to Southern blot analysis. They had one to three copies of both GUS and NPT II genes (Fig. 3-C, D).

Some in vitro shoots (Fig. 1-D ①), shoots and flower buds (Fig. 1-D ②) from acclimatized plants were subjected to GUS assay once again, and were observed to be GUS-positive. Seedlings raised from self-pollinated seeds of transformed plants were also observed to be GUS-positive (Fig. 1-D ③), thus indicating stable genetic transformation. All the acclimatized plants, from all the five genotypes, grew and flowered normally (Fig. 1-E). No visible genetic variation was observed.

A comparison of the genetic transformation efficiency between leaf explants from in vitro plants presented here and node explants from greenhouse plants in our previous report [2], using the same *A.tumefaciens* and plasmid, showed that node explants produced higher numbers of transformed shoots than leaf explants. The difference in transformation efficiency between the node and leaf explants may be due to the size of explants and their regeneration ability. Node explants from greenhouse plants produce ten times more adventitious shoots than *in vitro* leaf explants. Therefore, the node explants have a higher chance of producing transformed shoots. Even though rather low transformation efficiency, the *in vitro* materials have some advantages in genetic transformation study. Obtained transformants from *in vitro* starting materials are expected to be clean, and they can be evaluated their characteristics just after acclimatization. Some genetic transformation studies, such as an attempting to introduce virus-resistant genes into plants, also required clean materials.

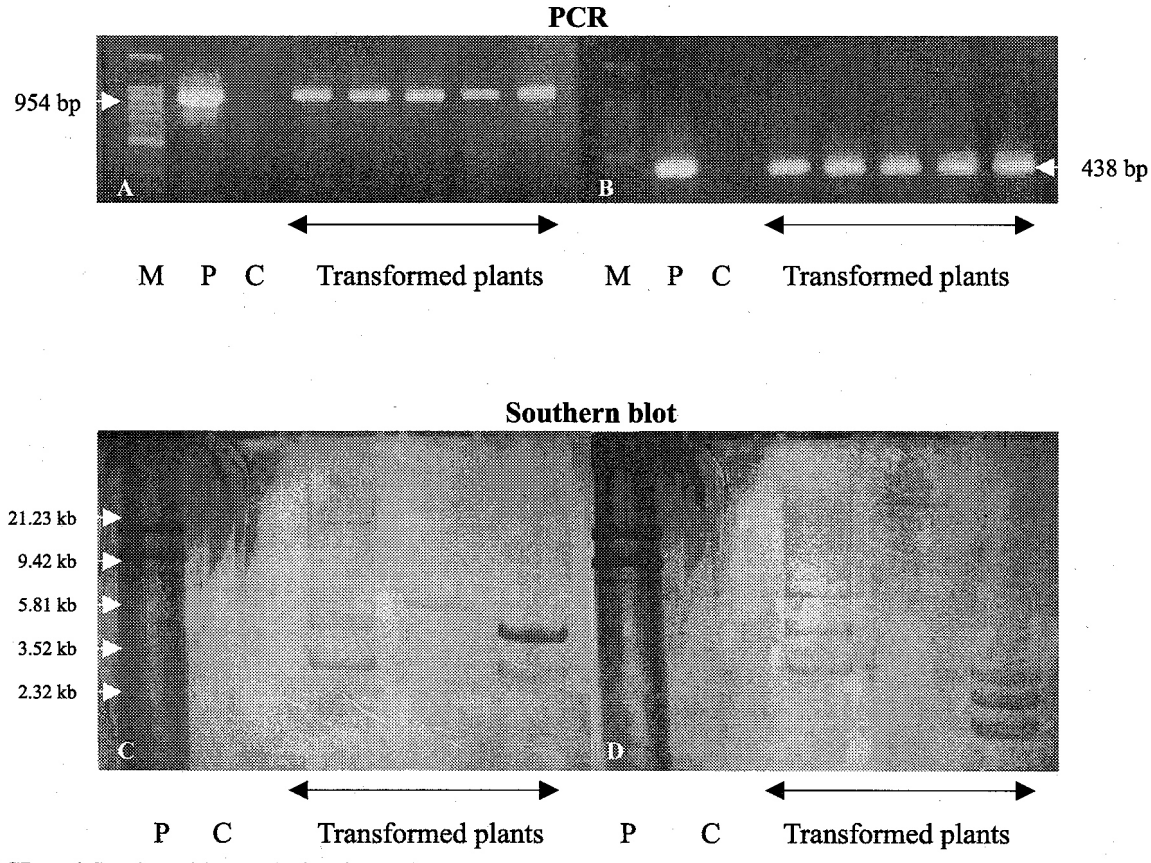

Fig. 3 PCR and Southern blot analysis of transformed carnation.
PCR amplification of a 954 bp fragment of GUS gene (A) and a 438 bp fragment of NPT II gene (B). Southern blot analysis using the NPT II (C) and GUS (D) probes. (C = negative control (non-transformed plant), M = size maker, P = positive control by pKT3 plasmid).

The protocol presented here is reproducible and applicable to other genotypes of carnation.

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In vitroの葉を用いたカーネーションの形質転換

チャルムシィ　ノンタサワッシィ・深井誠一

要　　約

5品種のカーネーション（キラー，ローレラ，マスター，セトノオトメ，タンガ）のin vitro植物の葉を用い，形質転換の方法を確立した．*Agrobacterium* AGL0およびプラスミドpKT3（GUSおよびNPT IIを含む）を用いた．GUSの一過的発現部位は外植体の切り口に限られた．外植体の調整方法が重要であり，葉の基部（葉が茎に付いていた部分）が外植体の下端にある場合のみGUSポジティブなシュートが得られた．上位節ほど再生能力が高く，形質転換効率も高い傾向にあった．形質転換効率は，マスター，セトノオトメ，タンガ，キラー，ローレラの順で高かった．得れらたカーネーションは正常に生育・開花した．