Original Paper

Plant Biotechnology, 19 (5), 335-343 (2002)

An Efficient Transformation System in Chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] for Stable and Non-chimeric Expression of Foreign Genes.

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Received 28 May 2002; accepted 25 August 2002

Abstract

We succeeded in establishing a stable and efficient transformation system of chrysanthemum (cv. Shuho-no-chikara) which could eliminate both the appearance of the chimeric regenerants consisting of transgenic and non-transgenic tissues and that of the transgene inactivated regenerants. We compared two transformation systems, callus induction (CI) system and adventitious shoot induction (SI) system. The transformation frequency in CI system (4.4%) were higher than that in SI system (0.3%). All regenerated plantlets obtained by CI system express *gus* gene stably even after vegetative propagation. While a few regenerants obtained by SI system have *gus* gene and express *gus* gene in chimeric manner. Then we applied the CI system in other famous and commercial cultivars of chrysanthemum and obtained transformed plants with high transformation frequency in 15 among 21 cultivars. Regarding the stable *gus* gene expression in all regenerants, CI system should eliminate gene inactivation in regenerants and is beneficial for the production of genetically engineered chrysanthemum.

Key words: callus, chimera, chrysanthemum cultivars, gene silencing, *gus* gene expression

Introduction

Chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] which was introduced into Japan from China during the Nara Era (A.D.710-794), become to be one of the important ornamental plants and is propagated vegetatively by stem cutting and *in vitro* adventitious shoot formation from various tissues and calli (Hill, 1968; Iizuka *et al.*, 1973; Earle and Langhans, 1974; Khalid *et al.*, 1989).

Many useful agronomical traits have been introduced into chrysanthemum by conventional cross breeding and selection, and more recently through mutation breeding (Broertjes *et al.*, 1976; Preil *et al.*, 1983; De Jong and Custers, 1986; Dalsou and Short, 1987; Huitema *et al.*, 1987). In conventional cross breeding, hereditary elements from same or other species are combined by sexual reproduction to create completely new gene combinations. However, in cross breeding, utilizable gene resources are limited to related species which are able to be cross pollinated, so genetic diversity is narrow in chrysanthemum. All of the color variants of elite genotypes were obtained by mutation breeding, either spontaneous or induced, but mutation breeding has only a limited potential, as modification of existing pathways.

The introduction of agronomically interesting traits by genetic engineering can be an alternative to such breeding methods, especially for vegetative crops. The genetic engineering has a potential to expand the range of genetic variation in chrysanthemum. However, in chrysanthemum, transformation frequency was still low (Renou *et al.*, 1993; De Jong *et al.*, 1994; Urban *et al.*, 1994), and chimeric plants (Pavingerova *et al.*, 1994; Benetka and Pavingerova, 1995) or the transformants showing inactivation of transgene were widely reported

(Pavingerova *et al.*, 1994; Benetka and Pavingerova, 1995; Takatsu *et al.* 2000). So the aim of this experiment is to establish a stable and efficient *Agrobacterium*-mediated transformation system of chrysanthemum (*D.* × *grandiflorum*) to solve such problems by using the neomycin phosphotransferase II (*npt II*) gene as a selective marker for G418 resistance and the β-D-glucuronidase (*gus*) gene as a reporter gene.

Materials and Methods

Plant materials

The chrysanthemum [*Dendranthema* × *grandiflorum* (Ramat.) Kitamura] cultivar 'Shuho-no-chikara' was used for establishment of the experimental protocol, and 21 cultivars were used for the application. Shoot tips of plants growing in the greenhouse were surface-sterilized by dipping briefly in 70% ethanol, and then in a 1% sodium hypochlorite solution for 15 min and rinsed 3 times in sterile distilled water. The shoot tip explants were cultivated *in vitro* (meristem culture) on Murashige and Skoog's (1962) basal medium (MS) (Murashige and Skoog, 1962) containing 3% sucrose and 0.3% Gellan Gum (Pure Chemical Co., Japan). The medium was adjusted to pH 5.8 prior to autoclaving at 120 ℃ for 15 min. The cultures were put at 25 ℃ under a 16 h photoperiod using cool-white fluorescent lamps or at 25 ℃ in darkness. The lamps provided a photosynthetic photon flux [PPF (400-700nm)]of 60 μmol m^{-2} s^{-1}.

Agrobacterium infection condition

Agrobacterium tumefaciens strain EHA101 (Hood *et al.*, 1986) harboring a binary vector pIG121-Hm (Ohta *et al.*, 1990; Hiei *et al.*, 1994) was supplied by Dr. K. Nakamura (University of Nagoya) (**Fig. 1**). EHA101 has a C58 chromosomal background and a disarmed pTi Bo542 (Sciaky *et al.*, 1978). *Intron-gus* gene and hygromycin-resistance gene (*hpt*) were driven by the CaMV 35S promoter and the nopaline synthase (Nos) terminator. Kanamysin-resistance gene (*npt II*) was driven by the Nos promoter and the Nos terminator.

The *Agrobacterium* had been cultured in a liquid LB medium on BIO-SHAKER BR-15 (TAITEC Co. Japan) at 28 ℃ for 5 h.

Transformation using callus induction (CI) system

The system reported previously (Shinoyama *et al.*, 1998) was used after minor modification. Leaf segments were cut from axenic plants by cork-borer (ϕ = 6 mm). They were immersed for 30 min at room temperature in MS liquid medium containing 5% Tween 20 and 50 μM acetosyringon with *Agrobacterium* (final OD_{660} = 0.1). After immersion, the leaf segments were placed onto callus induction (CI) medium[MS medium containing 1.0 mg l^{-1} 1-naphthylacetic acid (NAA), 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and 1.0 g l^{-1} casamino acid] and co-cultivated for 3 days at 25 ℃ in darkness. The leaf segments were transferred to bacteria elimination CI medium (CI medium containing 250 mg l^{-1} cefotaxime sodium salt) for elimination of *Agrobacterium*, and after 10 days, they were transferred to selection CI medium I (CI medium containing 250 mg l^{-1} cefotaxime sodium salt and 20 or 30 mg l^{-1} G418) for selection of putative transformed callus. After 3 subcultures on new selection CI medium I, the explants were transferred to selection CI medium II (CI medium containing 100 mg l^{-1} cefotaxime sodium salt and 20 or 30 mg l^{-1} G418) in which the concentration of cefotaxime sodium salt was reduced for promotion of callus proliferation. The leaf segments forming green calli were transferred to plantlet regeneration medium[MS medium containing 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 0.2 mg l^{-1} gibberelline A_3 (GA_3), and 100 mg l^{-1} cefotaxime sodium salt]for

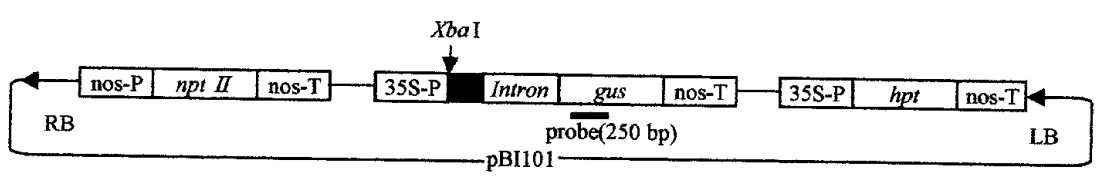

Fig. 1 Construct of T-DNA of pIG121-Hm.
pIG121-Hm was constructed from pBI101 vector (Jefferson *et al.* 1987), pIG221 (35S-P: *Intron gus*; Ohta *et al.* 1990) and pLAN101MHYG (*hpt* ; Dr. K. Shimamoto).
RB, Right border; LB, Left border; nos-P, nopalin synthase promoter; nos-T, nopalin synthase terminater; 35S-P, cauliflower mosaic virus 35S promoter; *npt II*, neomycin phosphotransferase gene; *Intron*, the intron of castor bean catalase gene within the N-terminal part of the *gus* gene coding sequence; *gus*, β-D-glucuronidase gene; *hpt*, hygromycin phosphotransferase gene; A prove used for Southern blot analysis of *Xba*I-digested DNAs (250-bp PCR product) was indicated bellow the *gus* gene.

Table 1. The time table for transformation of chrysanthemum.

1) Transformation using the callus induction (CI) system.

Day	Procedure
0	Culture *Agrobacterium* in liquid LB medium for 5 h
	Immerse leaf segments into MS liquid medium containing *Agrobacterium* for 30 min
	Cocultivate leaf segments with *Agrobacterium* on cocultivation CI medium
3	(End of cocultivation for 3 days)
	Transfer to bacteria elimination CI medium
10	Transfer to selection CI medium I (selection of putative transformed cells)
24	Transfer to fresh selection CI medium I
38	Transfer to fresh selection CI medium I
	(Callus induction on the edge of leaf segments)
52	Transfer to selection CI medium II
66	Transfer to fresh selection CI medium II
80	Transfer G418-resistant calli to plantlet regeneration medium
101	Transfer to fresh plantlet regeneration medium
122	Transfer to fresh plantlet regeneration medium (shoot regeneration)
	Collect elongating shoots (first collection) and transfer to rooting medium
143	Transfer remaining shoots and calli to fresh plantlet regeneration medium
	Collect elongating shoots (second collection) and transfer to rooting medium
143-180	Transfer rooted plantlets to green house
200 onwards	Plants available for testing

Medium construction
CI medium: MS+NAA 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, Sucrose (Suc.) 3%, Gellan Gum (Gel.) 0.3%
Cocultivation CI medium: MS+NAA 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, Casamino acids 1.0 g l^{-1}, Suc. 3%, Gel. 0.3%
Bacteria elimination CI medium: MS+NAA 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, Suc. 3%, Gel. 0.3%, Cefotaxime sodium salt (Cef.) 250 mg l^{-1}
Selection CI medium I: MS+NAA 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, Suc. 3%, Gel. 0.3%, Cef. 250 mg l^{-1}, G418 20 mg l^{-1}
Selection CI medium II: MS+NAA 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, Suc. 3%, Gel 0.3%, Cef. 100 mg l^{-1}, G418 20 mg l^{-1}
Plantlet regeneration medium: MS+BA 0.5 mg l^{-1}, GA_3 0.2 mg l^{-1}, Suc. 3%, Gel. 0.4%, Cef. 100 mg l^{-1}
Rooting medium: MS+ Suc. 3%, Gel. 0.4%, Cef. 100 mg l^{-1}

2) Transformation using adventitious shoot induction (SI) system.

Day	Activity
0	Culture *Agrobacterium* in liquid LB medium for 5 h
	Immerse leaf segments into MS liquid mrdium containing *Agrobacterium* for 30 min
	Cocultivate leaf segments with *Agrobacterium* on cocultivation SI medium
3	(End of cocultivation for 3 days)
	Transfer to bacteria elimination SI medium
10	Transfer to fresh selection SI medium I
24	Transfer to fresh selection SI medium I
38	Transfer to fresh selection SI medium I
52	Transfer to selection SI medium II
	Collect elongating shoots (first collection) and transfer to rooting medium
66	Transfer remaining shoots to fresh selection SI medium II
	Collect elongating shoots (second collection) and transfer to rooting medium
80-120	Transfer rooted plantlets to green house
140 onwards	Plants available for testing

Medium construction
SI medium : MS+NAA 2.0 mg l^{-1}, BA 1.0 mg l^{-1}, Sucrose (Suc.) 3%, Gellan Gum (Gel.) 0.3%
Cocultivation SI medium : MS+NAA 2.0 mg l^{-1}, BA 1.0 mg l^{-1}, Casamino acids 1.0 g l^{-1}, Suc. 3%, Gel. 0.3%
Bacteria elimination SI medium: MS+NAA 2.0 mg l^{-1}, BA 1.0 mg l^{-1}, Suc. 3%, Gel. 0.3%, Cefotaxime sodium salt (Cef.) 250 mg l^{-1}
Selection SI medium I: MS+NAA 2.0 mg l^{-1}, BA 1.0 mg l^{-1}, Suc. 3%, Gel 0.3%, Cef. 250 mg l^{-1}, G418 20 mg l^{-1}
Selection SI medium II: MS+NAA 2.0 mg l^{-1}, BA 1.0 mg l^{-1}, Suc. 3%, Gel. 0.3%, Cef. 100 mg l^{-1}, G418 20 mg l^{-1}
Rooting medium: MS+ Suc.3 %, Gel. 0.4%, Cef. 100 mg l^{-1}

obtaining of putative transformed plantlets. The shoots tips of the regenerated plantlets were cultured on the plantlet regeneration medium for GUS assay and the remaining parts were transferred to rooting medium (MS medium without phytohormones) for Southern blot analysis and GUS assay (**Table 1**).

Transformation using adventitious shoot induction (SI) system

The leaf segments which were immersed into *Agrobacterium* suspension as described above, were placed onto adventitious shoot induction (SI) medium (MS medium containing 2.0 mg l^{-1} NAA, 1.0 mg l^{-1} BA and 1.0 g l^{-1} casamino acid) and co-cultivated for 3 days at 25 ℃ in darkness. The leaf segments were transferred to bacteria elimination SI medium (SI medium containing 250 mg l^{-1} cefotaxime sodium salt) for elimination of *Agrobacterium*, and after 10 days, they were transferred to selection SI medium I (SI medium containing 250 mg l^{-1} cefotaxime sodium salt and 20 mg l^{-1} G418) for selection of putative transformed cells. After 2 subcultures on new selection SI medium I, the explants were transferred to selection SI medium II (SI medium containing 100 mg l^{-1} cefotaxime sodium salt and 20 mg l^{-1} G418) in which the concentration of cefotaxime sodium salt was reduced for promotion of adventitious shoot formation. The shoot tips of the regenerated plantlets were cultured on the plantlet regeneration medium for GUS assay and the remaining parts were transferred to rooting medium (MS medium without phytohormones) for Southern blot analysis and GUS assay (**Table 1**).

Southern blot analysiss

Total DNA was extracted from 100 mg of fresh young leaves of regenerated plantlets or non-transformed control plantlets by the method of Takagi *et al.* (1993). The leaves were homogenized in liquid nitrogen using a ceramic mortar and a pestle and suspended in 1 ml of HEPES buffer [0.1 M HEPES (pH 8.0), 0.1% polyvinylpyrrolidone (PVP) K-30, 4% 2-mercaptoethanol]. After centrifugation at 10,000*g* for 5 min at 4 ℃, the supernatant was discarded and the pellet was resuspended in new HEPES buffer. This procedure was repeated three times to remove polyphenols and polysaccharides. Total DNA was isolated from the pellet by sodium dodecyl sulfate (SDS) extraction method described earlier (Honda and Hirai, 1990).

The DNA digested with *Xba*I was subjected to gel-electrophoresis and blotted onto a positively charged Nylon membranes (Roche & Boehringer Mannheim, Germany). Southern analysis (Southern, 1975) was carried out using a *gus* gene fragment (250 bp) as a probe (see **Fig. 1**), with digoxigenin (DIG) labeling and CDP-star substrate detection systems (Roche & Boehringer Mannheim, Germany) according to the supplier's instruction.

GUS assay

The plantlets which were clonally propagated from a primary shoot by stem cuttings were assayed for expression of *gus* gene after incubation with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc.) (Jefferson *et al.*, 1987, Murakami and Ohashi, 1992). The shoots, roots (cut into 15 mm in length) and small plantlets (cut into 3 cm) were incubated in 50 mM phosphate buffer (pH 7.2) containing 1 mM X-gluc., 5 mM dithiothreitol (DTT), 0.3% TritonX-100, 5% methanol, 0.5 mM potassium ferrocyanide and 0.5 mM potassium ferricyanide for overnight at 37 ℃ . After staining, the shoots, roots and small plantlets were rinsed with 70% ethanol for overnight, then mounted for binocular.

Results

Transformation frequency

The regeneration processes in CI and SI systems were summarized in **Table 1** and **Fig. 2**. On CI system, callus induction stage is required at the first step. This caused the longer duration for obtaining of regenerated plantlets as compared to SI system. Regarding the regeneration frequency, plantlets were more easily obtained in SI system than in CI system.

Using CI system, 479 leaf segments among 3,513 formed G418 resistant calli, with an efficiency of 13.6%, and 153 plantlets were regenerated from the calli on the regeneration medium, corresponding to 4.4% regeneration frequency based on the initial leaf segments. They were obtained after 143 to 180 days of infection with *Agrobacterium*. On the other hand, using SI system, 979 adventitious shoots were finally obtained from 3,413 leaf segments, corresponding to 28.7% regeneration frequency based on the initial leaf segments. They were obtained after 80 to 120 days of infection with *Agrobacterium* (see **Table 2**).

Then, all regenerated plantlets which were obtained by using CI and SI systems were analyzed by Southern blot analysis to confirm transformation. The genomic DNA of all the plantlets was digested by *Xba*I, because only one *Xba*I site is present in the T-DNA region.

In the regenerants obtained by CI system, all the plantlets showed multiple unique bands with the

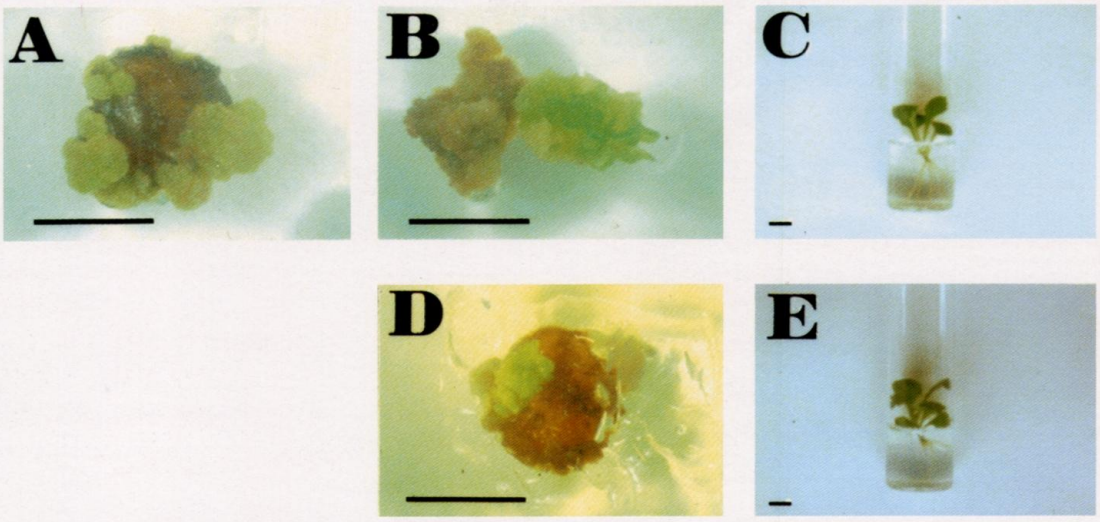

Fig. 2 Production of transgenic plants by two transformation systems.
1) Transformation using callus induction (CI) system.
(A). Callus induced on selection CI medium II.
(B). Shoots formed from G418 resistant callus on plantlet regeneration medium.
(C). A rooted plantlet on rooting medium.
2) Transformation using adventitious shoot induction (SI) system.
(D). Adventitious shoots induced directly on segment cultured in selection SI medium II.
(E). A rooted plantlet on rooting medium.
Scale bar indicates 5 mm.

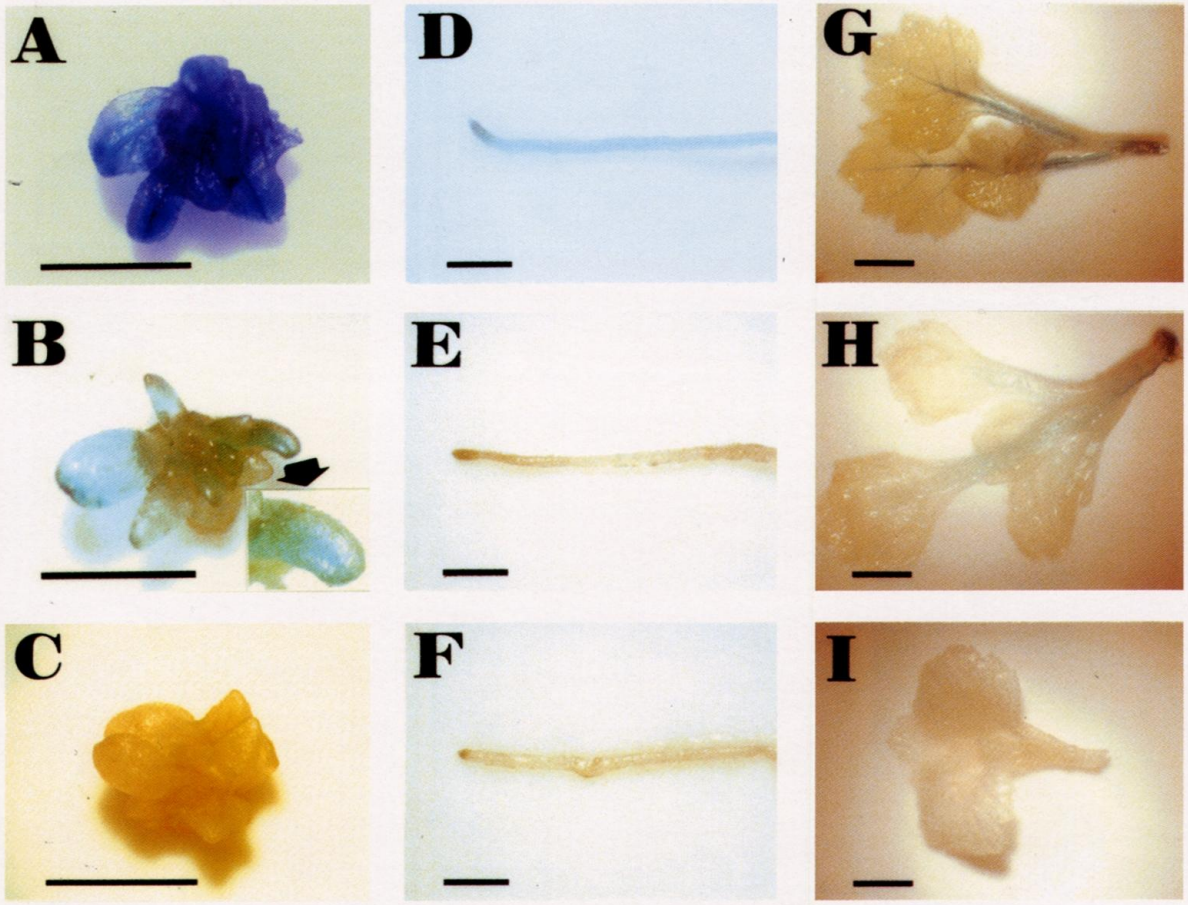

Fig. 4 Expression of *gus* gene in plants obtained by two transformation systems.
(A)-(C). Shoots of plants obtained by callus induction (CI) system (A) and by adventitious shoot induction (SI) system (B), and of non-transformed plants (C) were stained with X-gluc. Scale bar indicates 5 mm.
(D)-(F). Roots of plants obtained by callus induction (CI) system (D) and by adventitious shoot induction (SI) system (E), and of non-transformed plants (F) were stained with X-gluc. Scale bar indicates 10 mm.
(G)-(I). Above ground parts with leaves of plants obtained by callus induction (CI) system (G) and by adventitious shoot induction (SI) system (H), and of non-transformed plants (I) were stained with X-gluc. Scale bar indicates 10 mm.
In panel B, an arrow indicates the magnified photograph of a part of the leaf.

Table 2. Transformation frequency of chrysanthemum cultivar 'Shuho-no-chikara' in two transformation systems.

Transformation system	No. of leaf segments cultured (A)	No. of regenerated plantlet (B)	No. of transformed plantlets* (C)	Transformation frequency (C/A:%)
Callus induction (CI)	3,513	123	123(0)[1)]	4.4(0.0)[2)]
Adventitious shoot induction(SI)	3,413	979	45(36)[1)]	1.3(0.3)[2)]

[1)]: ( ) means No. of plantlets expressing *gus* gene in chimeric manner.

[2)]: ( ) means transformation frequency of plantlets expressing *gus* gene in chimeric manner.

*: Transformation was confirmed by Southern blot analysis.

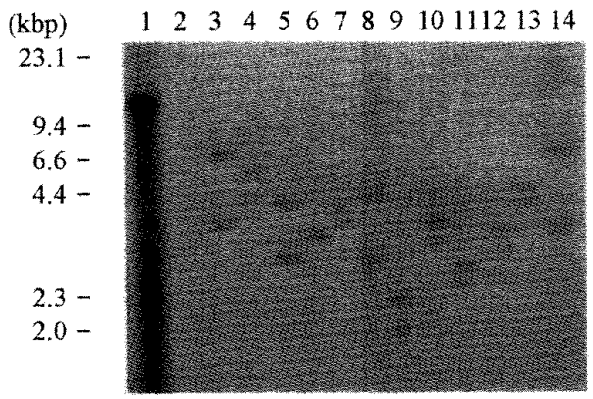

Fig. 3 Southern blot analysis of regenerated plantlets. DNA was digested with *Xba* I and hybridized with a probe of *gus* gene.
Lane 1: Plasmid DNA of pIG121-Hm, used as positive control.
Lane 2: DNA from a non-transformant, used as negative control.
Lane 3-6: DNA from plantlets expressing *gus* gene in chimeric manner. The plantlets were obtained by adventitious shoot induction (SI) system.
Lane 7-10: DNA from plantlets expressing *gus* gene in whole plant. The plantlets were obtained by adventitious shoot induction (SI) system.
Lane 11-14: DNA from plantlets expressing *gus* gene in whole plant. The plantlets were obtained by callus induction (CI) system.

gus-probe (**Fig. 3**). On the other hand, in the regenerants obtained by SI system, 45 among 979 plantlets showed multiple unique bands with the *gus*-probe (**Fig. 3**), but the remaining 934 plantlets did not show any bands (data not shown). No hybridization signal was detected in non-transformed control (**Fig. 3**). These results indicate that no escape plantlets by G418 selection were obtained in CI system and all the transformed plantlets had multiple copy of *gus* gene, but not single copy.

Analysis of chimerism

All regenerants showing *gus* gene positive band by Southern blot analysis were assayed by GUS staining for testing of chimerism. Three organs of the regenerated plants, shoots, roots and small plantlets, were used for this experiment.

In the case of CI system, blue staining was observed strongly in all the tested shoots and no chimeric shoot was found (**Fig. 4A**). In the case of SI system, blue staining was observed in 9 among 45 plantlets having *gus* gene. The remaining 36 shoots showed mosaic and weak blue staining in a part of the plant, for example, stomata, trichome or cells in the proximal part of petiole (**Fig. 4B**). Moreover, in the case of CI system, blue staining was observed in all small plantlets and roots, and chimeric or non-staining one was not found (**Fig. 4D, G**). This blue staining was observed especially in the vascular bundle. Whereas, in the case of SI system, blue staining without chimerism was observed in 9 among 45 small plantlets and their roots, but the remaining 36 plantlets whose shoots showed mosaic blue staining exhibited again mosaic and weak blue staining or no staining (**Fig. 4E, H**). Non-transformed control did not show blue staining (**Fig. 4C, F, I**)

All the plantlets obtained by CI system and 45 plantlets obtained by SI system were acclimatized, potted in soil and grown in greenhouse. After flowering (six months after acclimatization), the leaves, roots and petals of all the plants were assayed again by GUS staining. In the plants obtained by CI system, the *gus* gene expression was observed again in the leaf veins (vascular bundle) and roots, but not in the petals. Moreover, all these plants showed stable gus gene expression in leaf veins and roots during 3 generations of vegetative propagation by stem cuttings. On the other hand, 36 plants originated from plantlets which were obtained by SI system and showed mosaic *gus* gene expression in the previous assay on the plantlets exhibited again chimeric staining or no blue staining. The remaining 9 plants obtained by SI system

showed full blue staining in the entire leaves and roots. In all the plants obtained by SI system, no blue staining was observed in petals (**Table 2**).

These results indicate that CI system is a very efficient method to induce transformants which express stably foreign genes in non-chimeric manner even after vegetative propagation.

Transformation in various cultivars of chrysanthemum

To verify the applicability of CI system for other chrysanthemum cultivars, 21 famous and commercial cultivars were transformed by CI system (see **Table 3**).

At first, we tested the sensitivity to *Agrobacterium* infection on these cultivars. Five cultivars, 'Ohgon-jo', 'Monroe', 'Miss Betty', 'Utage' and 'Kin-fusha', were more sensitive to *Agrobacterium* than 'Shuho-no-chikara' (marked on '+++'), and 2 cultivars, 'Peach' and 'Seiun', were less sensitive to *Agrobacterium* (marked on '-'). The remaining 14 cultivars showed same sensitivity to 'Shuho-no-chikara' (marked on '++' or '+').

Secondary, we tested the regeneration ability. Six cultivars, 'Yamate-shiro', 'Hiroshima-beni', 'Kofuku-no-tori', 'Rosanna', 'Utage' and 'Kin-fusha', showed the ability higher than 'Shuho-no-chikara' (marked on '+++'), and 4 cultivars, 'Peach', 'Symbol', 'Bingo' and 'Swan', showed very low ability or no regeneration (marked on '-'). The remaining 11 cultivars showed same ability to 'Shuho-no-chikara' (marked on '++' or '+').

Then, we determined the optimum G418 concentration for selection of transformants. Optimum concentration was 30 mg l^{-1} in 4 cultivars, 'Yamate-shiro', 'Hiroshima-beni', 'Kosuzu', and 'Kin-fusha', and 20 mg l^{-1} in the remaining 17 cultivars. When we used 20 mg l^{-1} G418 for selection of transformants in the former 4 cultivars, many escape plantlets which had no *gus* gene were obtained. When we used 30 mg l^{-1} G418 for selection in the latter 17 cultivars, regeneration of transformed plantlets having *gus* gene was difficult.

Finally, we transformed these cultivars by CI system and the regenerated plantlets were obtained in 15 cultivars. The regenerated plantlets were

Table 3. Transformation frequency of some chrysanthemum cultivars using callus induction (CI) system.

Cultivars	No. of leaf segments cultured (A)	Sensitivity to *Agrobacterium* infection[1]	Regeneration ability[2]	G418 concentration[3] (mg l^{-1})	No. of regenerated prantlets (B)	No. of transformed plants (C)	Transformation frequency (C/A:%)
Seiun	180	-	+	20	0	0	0.0
Summer yellow	180	++	++	20	13	13	7.2
Yamate-shiro	180	++	+++	30	39	39	21.7
Hiroshima-beni	180	++	+++	30	43	43	23.9
Kosuzu	180	++	+	30	5	5	2.8
Kofuku-no-tori	180	++	+++	20	34	34	18.9
Rosanna	180	+	+++	20	5	5	2.8
Snow queen	180	++	-	20	0	0	0.0
Ohgon-jo	180	+++	++	20	25	25	13.9
Monroe	180	+++	++	20	25	25	13.7
Miss Betty	180	+++	++	20	2	2	1.1
Utage	180	+++	+++	20	32	32	17.8
Kin-fusha	180	+++	+++	30	36	36	20.0
Pinky	180	+	+	20	7	7	3.9
Peach	180	-	-	20	0	0	0.0
Symbol	180	+	-	20	0	0	0.0
Bingo	180	++	-	20	0	0	0.0
Rocky	180	++	+	20	7	7	3.9
Orange pinky	180	++	+	20	5	5	2.8
Swan	180	++	-	20	0	0	0.0
Susie	180	+	+	20	4	4	2.2
Shuho-no-chikara	180	++	++	20	10	10	5.6

[1] Judged by No. of GUS blue spots observed per leaf segment after Agrobacterium infection. -: no spot, +: 1 to 10, ++: 11 to 50, +++: 51 to 100.

[2] Judged by No. of plantlets formed per leaf segment. -: no plantlets, +: 1 to 5, ++: 6 to 10, +++: more than 11.

[3] At the concentration, leaf segments of non-transformed plants could not form any callus.

tested by GUS assay and Southern blot analysis, and all of them has *gus* gene and showed full blue staining. The transformation frequency was 1.1 to 23.9% based on the initial number of leaf segments and 8 cultivars showed higher frequency than 'Shuho-no-chikara'. The highest frequency of transformation was obtained in 'Hiroshima-beni'. In the remaining 6 cultivars which were less sensitive to *Agrobacterium* and showed low or no regeneration ability, no transformed plants was obtained.

Discussion

Successful transfer of a foreign gene to plants was first described in 1985 in tobacco (*Nicotiana tabacum*) using genetically manipulated strains of *Agrobacterium tumefaciens* (Horsch *et al.*, 1985). After that, *Agrobacterium* has extensively been used to transform a lot of plant species. However, susceptibility to *Agrobacterium* is different depending on plant species and cultivars and specific knowledge of *Agrobacterium*-host compatibility is required for successful transformation (Godwin *et al.*, 1992)

The susceptibility of chrysanthemum to *Agrobacterium tumefaciens* (Miller, 1975; De Cleene and De Ley, 1976; Hooykaas *et al.*, 1994) and the genetic variation in the susceptibility among different chrysanthemum cultivars on relation to various *Agrobacterium* strains have been demonstrated (Wordrangen *et al.*, 1991). Although the susceptibility of chrysanthemum to *A. tumefaciens* is widely reported, there are only limited reports indicating successful transformation of chrysanthemum in which the introduced foreign gene expressed (Renou *et al.*, 1993; De Jong *et al.*, 1994; Urban *et al.*, 1994), and the transformation frequency was still low (about 1 to 12%). Moreover, chimeric plants consisting of both transgenic and non-transgenic tissues were reported in chrysanthemum (Pavingerova *et al.*, 1994; Benetka *et al.*, 1995).

We previously reported two different transformation methods to obtain transgenic chrysanthemum by using *Agrobacterium*, the callus induction (CI) system and the adventitious shoot induction (SI) system. In this report, we examined whether the two systems for transformation are able to eliminate chimerism and to establish stable expression of foreign genes in the entire plants. As the results, CI system was very excellent to produce many transformants which show the stable expression of foreign genes in the entire plants and to eliminate chimerism.

The duration from infection with *Agrobacterium* to plantlet regeneration in SI system was about 60 days earlier than that in CI system. Then, we tested the transformation frequency by Southern blot analysis on all the regenerants. The transformation frequency in CI system was higher than that in SI system. This advantage of CI system overcomes the durational problem. In addition, all the regenerants obtained by CI system have gus gene, but many regenerants obtained by SI system have no gus gene.

Next, we analyzed the expression pattern of *gus* gene by GUS assay. All the regenerants obtained by CI system expressed *gus* gene stably in the entire plants. However, almost the plantlets obtained by SI system (934 among 979) did not have *gus* gene and 36 among the remaining 45 plantlets having *gus* gene showed *gus* gene expression in chimeric manner (**Fig. 3B**). Moreover, we analyzed the expression of *gus* gene by GUS assay on the plants which were grown in the green house. All the plants obtained by CI system expressed *gus* gene stably during 3 generations of vegetative propagation by stem cuttings. However, almost the plants obtained by SI system expressed *gus* gene in chimeric manner (**Fig. 3B**) or not expressed it. Regarding to the results, it is expected that the plants which are obtained by CI system should express *gus* gene stably even after sequential generations by vegetative propagation.

Inactivation of transgene (silencing) has been observed in chrysanthemum (Pavingerova *et al.*, 1994; Benetka and Pavingerova, 1995; Takatsu *et al.*, 2000). GUS activity level in transgenic chrysanthemum was 10-fold less than those of tobacco (Daub *et al.*, 1994) and 100-fold less than those of *Kalanchoe blossfeldiana* (Aida and Shibata, 1996). In addition, Wordrangen *et al.* (1992) reported that the expression of *gus* gene driven by CaMV35S promoter started slowly in chrysanthemum (5 days after infection) as compared to tobacco (2 days after infection). These facts indicate that CaMV35S promoter behaves weak in chrysanthemum than in tobacco. In our experiment, aged plantlets which were obtained by CI system showed *gus* staining only in the vascular bundle (**Fig. 3G**), but not in petals. This indicates that the use of chrysanthemum original promoter is necessary for establishing high expression of foreign genes in chrysanthemum. On the other hand, inactivation of transgene was observed in many regenerated plants obtained by SI system (**Fig. 3H**), but not in those obtained by CI system (**Fig. 3G**). There is a possibility that the plants showing chimeric gene expression are consisted of the tissues with activated and inactivated transgenes. However, the reason why only the plants obtained by SI system showed chimerism remaines to be clarilified. In either case, CI system

overcomes the problem of chimerism, because all the plants obtained by CI system did not show any chimerism.

Moreover, we showed that, using CI system, transformants showing full *gus* gene expression were successfully obtained in many cultivars of chrysanthemum at the frequency of 1.1 to 23.5%. Thus our CI system is useful and efficient to obtain transgenic chrysanthemum as compared to the previously reported ones.

We will create genetically engineered chrysanthemum with some agronomically important genes by using CI system, and modify this system to apply to the cultivars which we could not obtain any transformed plant in this experiment.

Acknowledgment

We would like to thank Dr. K. Tanaka (Kyoto Prefectural University), Dr. K. Wakasa (National Institute of Crop Science) and Dr. H. Kamada (University of Tsukuba) for valuable discussions and critical reading of the manuscript, and Mr. H. Yamada (Shizuoka Prefectural Agricultural Experiment Station) and Dr. K. Ueno (Kagoshima Biotechnology Institute) for valuable suggestions.

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