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Transgene inactivation in *Agrobacterium*-mediated chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) transformants

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Abstract

To compare transformation frequency and to investigate the developmental alterations in transgene expression, two cut-flower chrysanthemums 'Yamabiko' (spray-type) and 'New Summer Yellow' (standard-type) were transformed with three disarmed *Agrobacterium tumefaciens* strains C58C1, MP90 and LBA4404, all strains having the pBI121 plasmid. No marked difference was observed in transformation efficiency among cultivar/bacterial strain combinations. β-glucuronidase (GUS) activity levels in transformants were fairly low and varied among the different transgenic lines, ranging from 30 to 250 pmol min^{-1} (mg protein)$^{-1}$. GUS expression was not observed in the transgenic lines transformed with strain LBA4404. The alterations of GUS activity levels were examined for a long term, and it was observed that GUS activities reduced to zero level in most of transgenic lines 12 months after the inoculation of bacteria.

Chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) is a major cut flower in the world, and its various characteristics have been improved through conventional breeding programs. Molecular breeding is an alternative approach to introduce specific characteristics, and it has an especial advantage in 'one point' crop improvement. During the last decade, transgenic plants of chrysanthemum have been produced by using an *Agrobacterium*-mediated transformation technique (Ledger *et al.*, 1991; Wordragen *et al.*, 1991; Renou *et al.*, 1993; Urban *et al.*, 1994; De Jong *et al.*, 1994; Fukai *et al.*, 1995; Sherman *et al.*, 1998a), and transgenic chrysanthemums with practical characteristics have also been produced (Sherman *et al.*, 1998b; Takatsu *et al.*, 1999). However the transformation efficiency remains fairly low, and the chrysanthemum-cultivar/*Agrobacterium*-strain specificity (Bush and Pueppke, 1991; Urban *et al.*, 1994) remains to be investigated, especially in Japanese cultivars. Recently, the silencing of a transgene has been observed in transformants of several species (Finnegan and McElroy, 1994; Meyer, 1995; Vaucheret *et al.*, 1998), and it was also reported that the expression levels of a transgene varied among different transformants in chrysanthemum (Sherman *et al.*, 1998b). In this study, we examined the cultivar/bacterial strain specificity affecting the transformation efficiency and the developmental alteration of GUS activity in transformants to improve the transformation protocol for chrysanthemum as a practical breeding technique.

We selected two cut-flower chrysanthemums 'Yamabiko' (spray-type) and 'New Summer Yellow' (standard-type) because of their high regeneration ability (Takatsu *et al.*, 1998). Stem segments 3 mm long were excised from the shoots and cut into half sections vertically, which were used as explants in the transformation experiments. MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 3 g l^{-1} gelrite (Wako, Japan), 2.0 mg l^{-1} indoleacetic acid and 0.2 mg l^{-1} benzyladenine was used as a shoot induction medium (MIB medium). All cultures were incubated in a growth chamber maintained at 25 ℃ and illuminated at 50 μmol m^{-2} s^{-1} for 16 h per day.

Three disarmed *A. tumefaciens* strains C58C1 (C58C1Rif^R having the octopine-type helper plasmid pGV2260: Deblaere *et al.*, 1985), MP90 (C58C1Rif^R having the nopaline-type helper plasmid pMP90: Koncz and Schell, 1986) and LBA4404 (Clontech, USA) were used. All three strains harbored the binary plasmid pBI121, which had the neomycin phosphotransferase II (NPTII) and GUS genes in the T-DNA region. Bacteria were cultured overnight at 28 ℃ in Luria Broth medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, pH7.0) containing 50 mg l^{-1} kana-

mycin sulfate. *Agrobacterium* suspension was diluted to 1:20 in sterilized distilled water (about 5×10^7 cells/ m*l*) and was supplemented with 100 μM acetosyringone (3', 5'-Dimethoxy-4'-hydroxy-acetophenone) just before inoculating explants. Stem segments were dipped in the diluted suspension and placed on MIB medium. After two days of co-cultivation, these explants were transferred to fresh MIB medium containing 10 mg l^{-1} kanamycin sulfate and 250 mg l^{-1} cefotaxime (Kyowa, Japan) to select transformants. Forty-five days after inoculation, regenerated green healthy shoots were separated from the explants and were transferred to hormone-free MS medium containing 10 mg l^{-1} kanamycin sulfate. Rooted shoots were continuously maintained on the same medium as putative transformants.

Putative transformants were screened for the presence of GUS gene by the PCR-Southern analysis. Genomic DNA was extracted from leaf tissues using Nucleon™ Phytopure (Amersham LIFE SCIENCE). DNAs extracted from a non-transformant and purified pBI121 were used as negative and positive controls, respectively. The integrated GUS gene was detected by PCR using two oligonucleotide primers (GUS1 and GUS2) specific for the GUS gene. GUS1 (5'-CAGC-GAAGAGGCAGTCAACGGGGAA-3') and GUS2 (5'-CATTGTTTGCCTCCCTGCTGCGGTT-3') primers amplify a 684 bp fragment. The PCR mixture (50 μl) was composed of 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each of dATP, dGTP, dCTP and dTTP, 0.2 μM primers, 100 ng template DNA and 2.5 units AmpliTaq Gold™ DNA Polymerase (Perkin Elmer) in 10 mM Tris-HCl (pH8.5). The amplification conditions for the GUS fragment were: 95 ℃ for 9 min at preheating; then 94 ℃ denaturing for 1 min, 62 ℃ annealing for 2 min and 72 ℃ extension for 1 min for 40 cycles; and another 72 ℃ extension for 5 min. Amplified DNA fragment was separated on a 1.5 % agarose gel, transferred onto a nylon membrane (BIODYNE™B, Paul), and then hybridized with the labeled GUS gene prove prepared from the plasmid. Labeling of the GUS gene, Southern hybridization and visualization were performed according to the protocols given in the DIG DNA Labeling and Detection Kit (Roche & Boehringer Mannheim).

We used totally 1,071 stem segments of 'Yamabiko' as explants, and obtained 59 shoots as putative transformants (**Table 1**). The GUS gene was detected in 12 lines of different origin by PCR-Southern blot analysis (**Fig. 1**), and the transformation efficiencies (No. of transgenic lines/No. of explants) were 0.81 %, 1.64 % and 0.89 % for

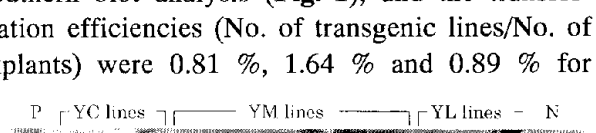

Fig. 1 PCR-Southern blot analysis of transgenic chrysanthemum 'Yamabiko'. Three YC lines, 6 YM lines and 3 YL lines were transformed by C58C1, MP90 and LBA4404, respectively.
P: positive control from pBI121, N: non-transformant, C58C1: C58C1Rif^R (pGV2260), MP90: C58C1Rif^R (pMP90).

Table 1. Cultivar/*Agrobacterium* strain combinations affecting the efficiencies in the transformation of chrysanthemum.

Chrysanthemum cultivar	*Agrobacterium* strain[1]	No. of inoculated explants (A)	No. of putative trans-formants	No. of transgenic lines[2] (B)	No. of GUS-positive lines[3] 2 m (C)	6 m (D)	12 m (E)	Efficiency (%) (B/A)	(C/A)	(C/B)	(D/C)	(E/D)
Yamabiko	C58C1	368	21	3	1	1	1	0.81	0.27	33.3	100.0	100.0
	MP90	366	30	6	3	1	1	1.64	0.82	50.0	33.3	100.0
	LBA4404	337	8	3	0	0	0	0.89	0.0	0.0	0.0	0.0
		1,071	59	12	4	2	2	1.12	0.37	33.3	50.0	100.0
New Summer Yellow	C58C1	291	13	9	5	2	1	3.09	1.72	55.6	40.0	50.0
	MP90	272	10	5	5	5	3	1.84	1.84	100.0	100.0	60.0
	LBA4404	274	7	2	0	0	0	0.73	0.0	0.0	0.0	0.0
		837	30	16	10	7	4	1.91	1.20	62.5	70.0	57.1

[1] C58C1: C58C1Rif^R (pGV2260), MP90: C58C1Rif^R (pMP90).
[2] β-glucuronidase (GUS) gene was detected by PCR-Southern analysis.
[3] Transgenic lines showing GUS ativity 2 months (2 m), 6 months (6 m) and 12 months (12 m) after inoculation.

strains C58C1, MP90 and LBA4404, respectively. With 837 stem segments of 'New Summer Yellow', we obtained 30 shoots as putative transformants. The GUS gene was detected in 16 lines, and the transformation efficiencies were 3.09 %, 1.84 % and 0.73 % for strains C58C1, MP90 and LBA4404, respectively. Wordragen *et al.* (1991) reported that the nopaline-type strain C58 (the parent strain of C58C1 and MP90) showed a higher virulence to chrysanthemum than the octopine-type strain Ach5 (the parent strain of LBA4404). However, in our study, no marked difference was observed in the transformation efficiency among bacterial strains, and between the octopine type (pGV2260) and nopaline type (pMP90) helper plasmids.

To investigate the developmental alterations in GUS expression, GUS assays were performed 2 months (just after regeneration) and 6 months (with 2 times of subculture) after inoculation using transgenic plants growing *in vitro*, and 12 months after inoculation using acclimatized plants growing in a closed greenhouse. GUS activity was assayed by measuring the conversion of 4-methylumbelliferyl β-D-glucuronide (MUG) to 4-methylumbelliferone (MU) by extracts from secondary leaves of transgenic plants as described by Jefferson (1987). GUS assay buffer contained 20 % methyl alcohol was used to eliminate endogenous GUS activity (Kosugi *et al.*, 1990). Protein concentration in the reaction mixtures was determined by the method of Bradford (1976) using a kit supplied by Bio-Rad Laboratories (USA). GUS activity was detected for 60 min at 37 ℃.

GUS activity levels 2 months after inoculation in the present study was found to be comparable to those of other studies of transgenic chrysanthemum (Pavingerova *et al.*, 1994; Urban *et al.*, 1994; Sherman *et al.*, 1998a), but varied greatly among different transgenic lines, ranging from 30 to 250 pmol min^{-1} (mg protein)$^{-1}$, as shown in **Fig. 2**. GUS expression was detected in 33.3 % (1/3), 50.0 % (3/6) and 0.0 % (0/3) of transgenic 'Yamabiko', and in 55.6 % (5/9), 100.0 % (5/5) and 0.0 % (0/2) of transgenic 'New Summer Yellow' transformed by strains C58C1, MP90 and LBA4404, respectively (**Table 1**). Transgenic lines expressing GUS activity 2 months after inoculation were defined as GUS-positive (2 months) lines. No marked difference was observed in the efficiency of GUS-positive (2 months) lines (No. of transgenic lines expressing GUS gene / No. of explants) between C58C1 and MP90 in 'Yamabiko' and 'New Summer Yellow' (**Table 1**). While, GUS expression was not detected in all lines transformed with LBA4404, although the presence of the GUS gene was confirmed by PCR-Southern analysis in these lines. Previous studies showed that some transformants of chrysanthemum did not express an introduced foreign gene (Renou *et al.*, 1993; De Jong *et al.*, 1994; Urban *et al.*, 1994). Although the failure to obtain transformants expressing GUS gene may be due to the inactivation of transgenes, these transformants that do not express GUS gene are able to eliminate in early step of experiments.

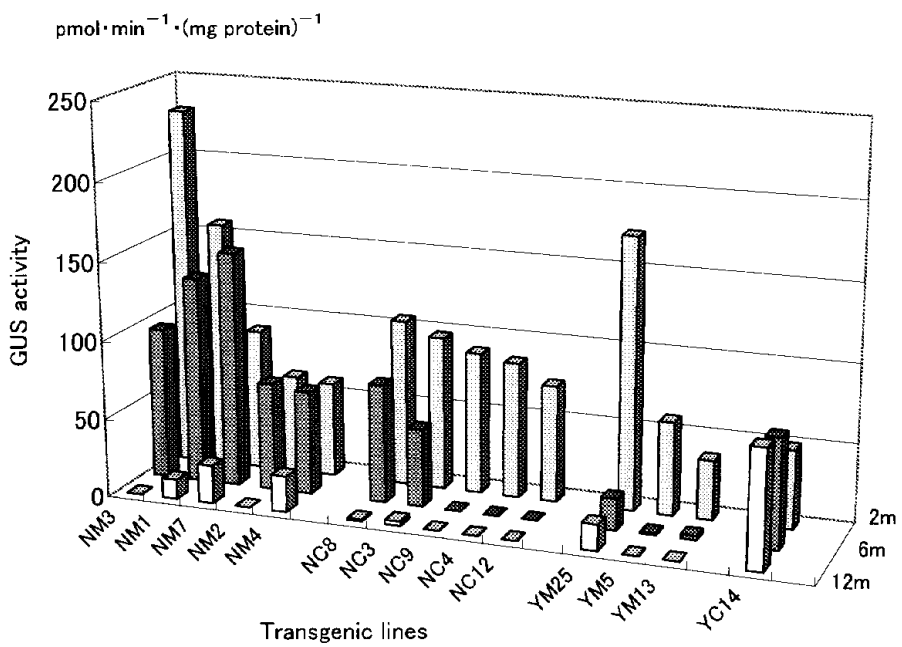

Fig. 2 GUS activities in different transgenic lines 2 months (2 m), 6 months (6 m) and 12 months (12 m) after inoculation of bacteria. Five NM lines and 5 NC lines are 'New Summer Yellow' transformed with strain MP90 and C58C1, and 3 YM lines and YC14 line are 'Yamabiko' transformed with strain MP90 and C58C1, respectively.

Stable expression of the transgene is necessary for plant molecular breeding, and thus the developmental alterations in GUS activity levels were examined 6 months and 12 months after inoculation. After 6 months, GUS expression was observed in 100.0 % (1/1) or 33.3 % (1/3) of GUS-positive (2 months) lines of 'Yamabiko', and in 40.0 % (2/5) or 100.0 % (5/5) of those of 'New Summer Yellow' transformed with strains C58C1 and MP90, respectively (**Table 1**). In the other lines, GUS activities were reduced nearly to zero level (**Fig. 2**). After 12 months, GUS expression was observed in 100.0 % (1/1) or 100.0 % (1/1) of GUS-positive (6 months) lines of 'Yamabiko', and in 50.0 % (1/2) or 60.0 % (3/5) of those of 'New Summer Yellow' transformed with strains C58C1 and MP90, respectively (**Table 1**). GUS activities were reduced to very low levels in most lines except for YC14 (**Fig. 2**).

Transgene inactivation (silencing) has been observed in several plant species and some models have been proposed for transcriptional and post-transcriptional silencing (Vaucheret *et al.*, 1998). Transgene can undergo transcriptional silencing when single or multiple copies are integrated into a locus located in next to silent hypermethylated genomic sequences (position effect), and it has also been suggested that strong discrepancy between the DNA composition of the transgene and that of the surrounding genomic sequences leads to the specific methylation and inactivation of foreign sequence. On the other hand, post-transcriptional silencing is defined as that RNA does not accumulate even though transcription occurs, and it has been suggested that it is mainly due to the overproduction and specific degradation of transgene RNA (RNA threshold model). Aida and Shibata (1998) observed developmental transgene silencing in *Torenia fournieri* and reported that initial levels of GUS activity correlated with the timing of silencing, and it occurred more rapidly in the homozygous plants compared with the hemizygous plants. They also suggested that transgene silencing was explained by the RNA threshold model in torenia. In the present study, GUS activity levels in transgenic chrysanthemums were 10-fold less than those of tobacco (Daub *et al.*, 1994) and 100-fold less than those of *Kalanchoe blossfeldiana* (Aida and Shibata, 1996) both transformed with pBI121. The initial levels (2 months after inoculation) of GUS activity were fairly low, and 50.0 % ('Yamabiko') and 70.0 % ('New Summer Yellow') of GUS-positive (2 months) plants still showed GUS activity 6 months after inoculation, and it was showed that silencing occurred slowly in chrysanthemum. According to the RNA threshold model, these observations imply that it needs a long term to produce the transgene RNA above a putative threshold level in chrysanthemum. However, as developmental regulation of transgene silencing by methylation (transcriptional silencing) is also reported in tobacco plant (Sonoda and Nishiguchi, in press), there is room for further investigation about developmental transgene inactivation in chrysanthemum.

GUS activity reduced to very low level in most of transformants 12 months after inoculation, and only spray-type chrysanthemum YC14 line showed stable GUS expression for a long term with a relatively low-activity level. Sherman *et al.* (1998a) reported that the stability of GUS expression varied according to cultivar. The difference in genetic background of cultivar may also affect the transgene inactivation in chrysanthemum. In addition, Wordragen *et al.* (1992) reported that expression of the GUS gene driven by the cauliflower mosaic virus 35S (CaMV35S) promoter started slowly in chrysanthemum (5 days after infection) compared to tobacco plants (2 days after infection). This observation implies that the CaMV35S promoter behaves differently in chrysanthemum than in tobacco plant. Although the mechanisms influencing transgene expression remains to be investigated, attention should be given to them in the application of transformation techniques in chrysanthemum breeding programs.

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References

Aida, R., Shibata, M., 1996. Transformation of *Kalanchoe blossfeldiana* mediated by *Agrobacterium tumefaciens* and transgene silencing. Plant Sci., **121:** 175-185.

Aida, R., Shibata, M., 1998. Developmentally Regulated Transgene Silencing in Torenia. Breed.Sci., **48:** 36-69.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem., **72:** 248-254.

Bush, A.L., Pueppke, S., 1991. Cultivar-strain specificity between *Chrysanthemum morifolium* and *Agrobacterium tumefaciens*. Physiol. Mol. Plant Pathol., **39:** 309-323.

Daub, M.E., Jenns, A.E., Urban, L.A., Brintle, S.C., 1994. Transformation frequency and foreign gene expression in burley and flue-cured cultivars of tobacco. Tob. Sci., **38:** 51-54.

Deblaere, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M., Leemans, J., 1985. Efficient octopine Ti plasmid-derived vectors for

Agrobacterium-mediated gene transfer to plants. Nucl. Acids. Res., **13:** 4777-4788.

De Jong, J., Mertens, M.M.J., Rademaker, W., 1994. Stable expression of the GUS reporter gene in chrysanthemum depends on binary plasmid T-DNA. Plant Cell Rep., **14:** 59-64.

Finnegan, J., McElroy, D., 1994. Transgene inactivation: Plants fight back! Bio/Technology, 12: 883-888.

Fukai, S., De Jong, J., Rademaker, W., 1995. Efficient genetic transformation of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) using stem segments. Breed. Sci., **45:** 179-184.

Jefferson, R.A., 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rpt., **5:** 387-405.

Koncz, C., Schell, J., 1986. The promoter of T_1-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet., **204:** 383-396.

Kosugi, S., Ohashi, Y., Nakajima, K., Arai, Y., 1990. An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci., **70:** 133-140.

Ledger, S.E., Deroles, S.C., Given, N.K., 1991. Regeneration and Agrobacterium-mediated transformation of chrysanthemum. Plant Cell Rep., **10:** 195-199.

Meyer, P., 1995. Variation of transgene expression in plants. Euphytica, **85:** 359-366.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.Plant., **15:** 473-497.

Pavingerova, D., Dostal, J., Biskova, R., Benetka, V., 1994. Somatic embryogenesis and Agrobacterium- mediated transformation of chrysanthemum. Plant Sci., **97:** 95-101.

Renou, J.P., Brochard, P., Jalouzot, R., 1993. Recovery of transgenic chrysanthemum (*Dendranthema grandiflora* Tzvelev) after hygromycin resistance selection. Plant Sci., **89:** 185-197.

Sherman, J.M., Moyer, J.W., Daub, M.E., 1998a. A regeneration and *Agrobacterium*-mediated transformation System for genetically diverse *Chrysanthemum* cultivars. J. Amer. Soc. Hort. Sci., **123**(2): 189-194.

Sherman, J.M., Moyer, J.W., Daub, M.E., 1998b. Tomato Spotted Wilt Virus Resistance in Chrysanthemum Expressing the Viral Nucleocapsid Gene. Plant Disease. **82**(4): 407-414.

Sonoda, S., Nishiguchi, M., in press. Delayed activation of post-transcriptional gene silencing and de novo transgene methylation in plants with the coat protein gene of sweet potato feathery mottle potyvirus. Plant Sci.

Takatsu, Y., Tomotsune, H., Kasumi, M., Sakuma, F., 1998. Differences in adventitious shoot regeneration capacity among Japanese chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) cultivars and the improved protocol for *Agrobacterium*-mediated genetic transformation. J. Japan. Soc. Hort. Sci., **67**(6): 958-964.

Takatsu, Y., Nishizawa, Y., Hibi, T., Akutsu, K., 1999. Transgenic chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) expressing a rice chitinase gene shows enhanced resistance to gray mold (*Botrytis cinerea*). Sci. Hort., **82:** 113-123.

Urban, L.A., Sherman, J.M., Moyer, J.W., Daub, M.E., 1994. High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). Plant Sci., **98:** 69-79.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, D., Morel, J.B., Mourrain, P., Palauqui, J.C., Vernhettes, S., 1998. Transgene-induced gene silencing in plants. Plant J., **16**(6): 651-659.

Wordragen, M.F., De Jong, J., Huitema, H.B.M., Dons, H.J.M., 1991. Genetic transformation of chrysanthemum using wild type *Agrobacterium* strains; strain and cultivar specificity. Plant Cell Rep., **9:** 505-508.

Wordragen, M.F., De Jong, J., Schornagel, M.J., Dons, H.J.M., 1992. Rapid screening for host-bacterium interactions in *Agrobacterium*-mediated gene transfer to chrysanthemum, by using the GUS-intron gene. Plant Sci., **81:** 207-214.