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Efficient Transgene Expression in Chrysanthemum, *Chrysanthemum morifolium* Ramat., with the Promoter of a Gene for Tobacco Elongation Factor 1 α Protein

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Abstract

We investigated the usefulness of the promoter of a gene for tobacco elongation factor 1α protein (*EF1 α*) for transgene expression in the chrysanthemum (*Chrysanthemum morifolium* Ramat.). The *EF1 α* promoter was fused to the *β*-glucuronidase gene (*gus*) and introduced into the chrysanthemum. We obtained 238 putative transformants and GUS assay of the leaves of the *in vitro* plants revealed that 29.0% (69/238) of the putative plants were GUS-positive. The plants in the greenhouse that were 20 months after regeneration still showed a GUS activity in their leaves and petals. The tobacco *EF1 α* promoter expressed the transgene more efficiently than the 35S promoter of Cauliflower mosaic virus and could be used for transgene expression in chrysanthemum.

Discipline: Biotechnology
Additional key words: *Agrobacterium*, tissue culture, transformation, ornamental plants

Introduction

Chrysanthemum is one of the most popular and important ornamental plants in the world. There have been many reports of genetic transformation in chrysanthemum[7,16]. Many researchers have used the *β*-glucuronidase gene (*gus*) driven by the 35S promoter of cauliflower mosaic virus (CaMV), abbreviated as 35S/*gus*, as a reporter gene for investigating transgene expression in chrysanthemum. The GUS activity levels recorded in transgenic chrysanthemum with the 35S/*gus* transgene have been low, ranging from 10 to 160[17], 30 to 240[13], and 30 to 250[15] pmol 4-MU mg^{-1} protein min^{-1}. The 35S or modified 35S promoter has been used to express practical transgenes for modifying characters such as resistance to diseases[9,15,17,19], resistance to insects[14], and flower color[5,6]. In some of these attempts, the mRNAs[5] or protein[17,19] of the transgene could not be detected, even when the transgenes were inserted in the genome. These results suggest that the 35S promoter does not function efficiently in chrysanthemum.

Recently, several reports have been published on efficient promoters for transgene expression in chrysanthemum with the *gus* as a reporter gene. The potato *Lhca3.St.1* (encoding the apoprotein 2 of the light-harvesting complex of photosystem I) promoter expressed high GUS activity in leaf, stem, pedicel and ray floret (mean activity in the leaves among GUS positive plants was about 25,000 pmol 4-MU mg^{-1} protein min^{-1})[2]. The chrysanthemum *UEP1* (encoding ubiquitin extension protein) promoter also expressed high GUS activity in ray florets (mean activity in the ray florets among total plants was 8,500 pmol 4-MU mg^{-1} protein min^{-1})[3]. The promoter-terminator of chrysanthemum *rbcS1* (encoding riburose-1, 5-bisphosphate carboxylase small-subunit) expressed high GUS activity in leaf (mean activity in the leaves among total plants was about 17,000 pmol 4-MU mg^{-1} protein min^{-1})[12]. These reports used an experimental line '1581' and adoptability of these promoters to other cultivars/lines was not mentioned in any report. We previously reported efficient *gus* expression in 8 chrysan-

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themum cultivars/lines with the *cab* (encoding chlorophyll-*a/b*-binding protein) promoter of chrysanthemum[1] (mean activity in the leaves was about 4,300 and 1,100 for GUS positive plants and total plants, respectively).

The translation elongation factor genes play a major role in protein synthesis. The elongation factor 1 subunit α (*EF1* α) gene is highly expressed in tobacco culture cells (K. Yoshida, data will be published elsewhere). There was a possibility that the tobacco *EF1* α promoter would also function in chrysanthemum, so we examined the promoter for expression of chrysanthemum transgenes.

Materials and methods

1. Plant materials for transformation

We used the chrysanthemum (*Chrysanthemum morifolium* Ramat.) cultivars 'Sei-Marine', 'Shuho-no-Takara', 'Seiko-Kogyoku', 'Cherry', and 'Hiroshima-Beni' and the laboratory lines '94-704', '94-750', '94-787' as plant materials for transformation. These lines were selected from preliminary experiments for their high regeneration potential (unpublished data). The plants were grown *in vitro* in Murashige–Skoog medium with half-strength minerals (1/2 MS)[11], solidified with 0.2% (w/v) gellan gum, at 25 ºC under a 16-h light: 8-h dark photoperiod regime with fluorescent light (photon flux density 70 μmol s^{-1} m^{-2}). Leaves of the plants were cut into about 5-mm squares and used as explants for the transformation experiments.

2. Vector plasmids and bacterial strain

A vector plasmid pBIEF1 α (Fig. 1) was used in this experiment. The promoter region of the tobacco *EF1* α gene with its starting 21 codons (DDBJ accession No. BD438335) was fused in frame to the *gus* coding region (EF1 α/*gus*), since addition of the 5'-untranslated region and/or starting codons might to be effective for transgene expression (K. Yoshida, data will be published elsewhere). The vector contained the neomycin phosphotransferase II gene (*npt II*) under the control of the nopaline synthase promoter. The *Agrobacterium tumefaciens* strain EHA105[8] was used for the experiments.

3. Transformation of chrysanthemum

Chrysanthemum was transformed as described previously[1]. We used paromomycin as a selective agent. To confirm the resistance to paromomycin, we performed a leaf test on the leaf segments of the elongated shoots. The leaf segment squares (about 5 mm) were placed on the selection medium and cultured for 1 week. The paromomycin-sensitive segments died within 1 week of culture, while the paromomycin-resistant ones remained green and began to form calli.

4. GUS assay and Southern blot analysis

A quantitative and histochemical GUS assay was performed as described previously[1] according to the procedure reported by Jefferson et al.[10]. GUS activity was expressed as picomoles of 4-methylumbelliferone (4-MU) produced at 37ºC per milligram of protein per minute (pmol 4-MU mg^{-1} protein min^{-1}). Plants showing GUS activities of more than 100 pmol 4MU mg^{-1} protein min^{-1} were considered to be GUS-positive, because in the untransformed wild-type plants GUS background activity of 20–30 pmol 4-MU mg^{-1} protein min^{-1} was sometimes observed. For histochemical GUS assay, the inner part of samples was cut vertically before assay for soaking well with the buffer.

Southern blot analysis was performed as described

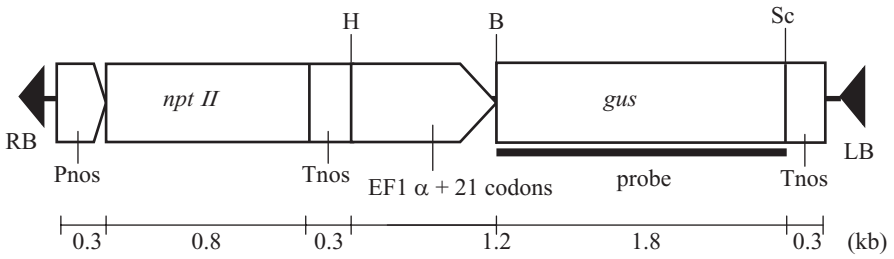


Fig. 1. Structure of the T-DNA regions of the binary vector pBIEF1 α

The chimeric genes were inserted between the right and left border sequences of T-DNA. The GUS probe was used for Southern blot analysis. RB & LB: Right and left border sequences of T-DNA, Pnos & Tnos: Promoter and terminator of nopaline synthase gene, EF1 α + 21 codons: Promoter and first 21 codons of tobacco elongation factor 1 subunit α gene, *npt II*: Coding region of neomycin phosphotransferase II gene, *gus*: Coding region of β-glucuronidase gene, H, B & Sc: Restriction sites of *Hin*d III, *Bam*H I and *Sac* I, respectively.

previously[1]. Total DNA was extracted from the leaf tissues and the DNA digested with *Hin*d III was used for analysis. *Hin*d III cuts the plasmid at a single site outside the coding region of the *gus* gene (Fig. 1). The coding region of the *gus* gene was used as a probe.

Results and discussion

1. Transformation

We obtained a total of 301 independent regenerants from 5,800 explants of the 8 chrysanthemum lines (Table 1). The leaf test on the selection medium clearly revealed the differences in resistance to paromomycin among the regenerants. Some of the leaf segments died within 1 week of culture (paromomycin-sensitive), and the others remained green and began to form calli (paromomycin-resistant) (data not shown). The leaf test showed that 238 regenerants out of 301 were paromomycin-resistant, i.e. putative transformants (Table 1).

2. Southern blot analysis

We selected three GUS-negative (≤100 pmol 4-MU mg^{-1} protein min^{-1}), three GUS-moderate (>100) and three GUS-strong (>1,000) paromomycin-resistant plants (cv. 'Sei-Marine') for Southern blot analysis. Analysis showed that one GUS-negative plant did not have the *gus* gene (Fig. 2). Because the inserted genes were linked as RB- *npt II- gus* -LB and T-DNA transfer was known to take place from the right to the left T-DNA border[18], it is possible that the T-DNA transfer was incomplete and the *gus* gene was lacking. The other 8 paromomycin-resistant plants examined – even the GUS-negative two plants – harbored the *gus* gene in their genomes (Fig. 2). Digestion of the vector with *Hin*d III cuts the plasmid at a single site outside the coding region of the *gus* gene (Fig. 1).

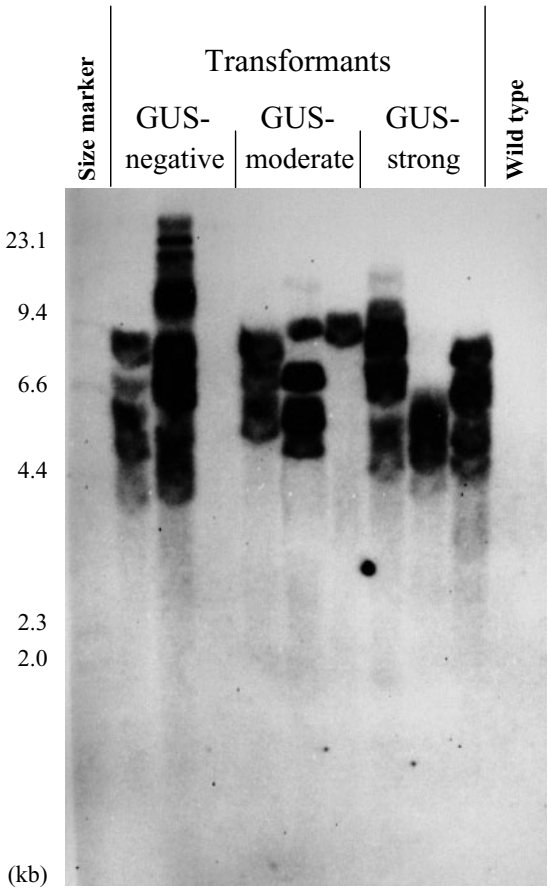


Fig. 2. Southern blot analysis of the putative transformants
Three GUS-negative (≤100 pmol 4-MU mg^{-1} protein min^{-1}), three GUS-moderate (>100) and three GUS-strong (>1,000) paromomycin-resistant plants (cv. 'Sei-Marine') were analyzed. The coding region of the GUS gene was used as a probe.

Table 1. GUS activity with *EF1 α* promoter among chrysanthemum lines

Chrysanthemum lines	No. of explants	No. of regenerants	No. of paromomycin-resistant plants	Paromomycin-resistant plants/ explant (%)	No. of GUS-positive plants[a)]	GUS-positive plants/ paromomycin-resistant plants (%)	No. of plants with GUS activity in leaves	
							> 100	> 1,000
Sei-Marine	1,000	61	53	5.3	14	26.4	4	10
Shuho-no-Takara	1,600	104	77	4.8	28	36.4	13	15
Seiko-Kogyoku	600	19	17	2.8	5	29.4	0	5
Cherry	600	4	3	0.5	1	33.3	1	0
Hiroshima-Beni	600	32	17	2.8	8	47.1	6	2
94-704	400	14	13	3.3	2	15.4	2	0
94-750	600	35	31	5.2	9	29.0	9	0
94-787	400	32	27	6.8	2	7.4	2	0

a): Plants with GUS activities above 100 pmol 4MU mg^{-1} protein min^{-1} were considered to be GUS-positive.

Contamination of the tissues by the plasmid should be detected by the presence of a single band at 13.4 kb. The 8 *gus* detected plants showed several bands with different sizes, indicating multiple-copy integration of the *gus* gene into the genome.

3. GUS assay in the leaves

We examined the 238 paromomycin-resistant plants for quantitative GUS activity in the leaves of 1 or 2 months after regeneration. The assay revealed that 29.0% (69/238) of the putative transformants with the EF1 α/*gus* were GUS-positive. We formerly reported, by using same cultivars/lines and same selection marker gene, that only 9.6% (11/115) of the putative transgenic plants to which 35S/*gus* had been introduced and 24.3% (45/185) of the putative transgenic plants to which Cab/*gus* had been introduced were GUS-positive[1]. The percentage of GUS-positive plants in the paromomycin-resistant population of each cultivar was 7.4–47.1% with the EF1 α/*gus* (Table1). The percentage was higher than that of 35S/*gus* (0–33.3%) and comparable to that of Cab/*gus* (15.4–50.0%)[1]. Thus, the *EF1* α promoter shows a higher ability to express the transgene in chrysanthemum leaves than the 35S promoter. The average GUS activity in the leaves with the EF1 α/*gus* was about 2,000 and 570 for GUS positive and total plants, respectively. The highest activity among the transformants was about 14,000.

Several efficient promoters for transgene expression in the leaf tissues of chrysanthemum have been proposed, such as potato *Lhca3.St.1*[2], chrysanthemum *UEP1*[3], chrysanthemum *rbcS1*[12] and chrysanthemum *cab*[1]. All the reports showed higher expression ability than the 35S promoter. The reported average/highest-GUS activities in the leaf tissues of chrysanthemum transformants were about 25,000/140,000 pmol mg^{-1} protein min^{-1} (potato *Lhca3.St.1*; average was calculated with only GUS-positive plants)[2], 900/1,400 (chrysanthemum *UEP1*)[3], 17,000/70,000 (chrysanthemum *rbcS1*)[12] and 1,100/27,000 (chrysanthemum *cab*). In the cases that very high expression would be needed in the leaves, the promoter of potato *Lhca3.St.1*or chrysanthemum *rbcS1* should be more suitable than the tobacco *EF1* α promoter. However, in the cases that moderate expression would be sufficient, the tobacco *EF1* α promoter might be useful for transgene expression in chrysanthemum leaves. Moreover, we showed efficiency of the tobacco *EF1* α promoter in 8 cultivars/lines, in opposition to the each promoter of the potato *Lhca3.St.1*[2], chrysanthemum *UEP1*[3] and chrysanthemum *rbcS1*[12] that high expression was showed only in an experimental line '1581'.

We transferred some of the transformants (cv. 'Sei-Marine') to a greenhouse and maintained them vegetatively for further investigation. Most of the transformants grew and flowered normally (data not shown). We further examined the quantitative GUS activity of these greenhouse plants (8 and 20 months after regeneration; Table 2) and the histochemical GUS activity (20 months after regeneration; Fig. 3). For histochemical GUS assay, the samples showed blue vertical lines that corresponded to the cuts for soaking with the buffer. The blue lines seemed to be results from well-soaking of the buffer and not to be results from induction by injury, because we observed the same phenomena with the 35S/*gus*-introduced plants (unpublished results). The chrysanthemum tissue from plants growing in greenhouse might be too hard to be soaked with the buffer uniformly. The plants of 20 months after regeneration still showed a GUS activity, suggesting that the transgene would be expressed stably even after vegetative propagation.

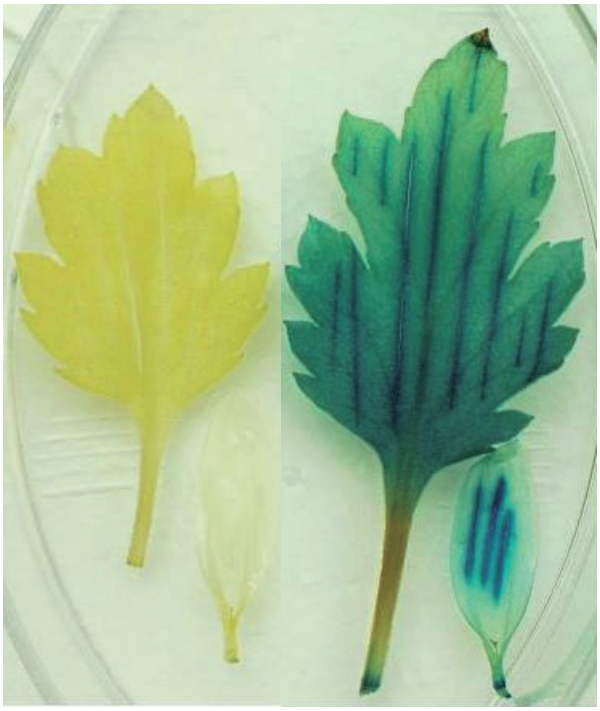
4. GUS assay in the petals

There have been many reports of modification of the flower color by genetic transformation[4]. In chrysanthemum, the development of new colors, such as blue, has been an important breeding target. The functional pro-

Table 2. GUS activities in leaves of *in vitro* plants and in leaves or petals of greenhouse plants (cultivar 'Sei-Marine')

Plant line	GUS activity (pmol 4MU mg^{-1} protein min^{-1})						
	In vitro leaf 1st time[a)]	*In vitro* leaf 2nd time[b)]	Greenhouse leaf[c)]	Greenhouse petal[c)]	Greenhouse leaf[d)]	Greenhouse leaf[e)]	Greenhouse petal[e)]
1	8,500	14,000	6,400	28,000	5,700	4,900	26,000
2	2,200	2,000	2,400	8,800	1,400	2,200	12,000
3	12,000	8,700	6,400	29,000	4,000	4,000	19,000
4	2,400	1,200	2,700	12,000	190	300	5,200
5	1,400	190	440	11,000	220	39	4,700

a): One to two months after regeneration. b): Four months after regeneration. c): Eight months after regeneration.
d): Sixteen months after regeneration. e): Twenty months after regeneration.



Wild type EF1 α/*gus*

Fig. 3. Histochemical GUS assay of a transformant
The leaves and ray florets of greenhouse plants 20 months after regeneration were used for assay. Blue precipitations represent the GUS activity. A chrysanthemum transformant harboring the EF1 *α*/*gus* showed blue precipitations both in the leaf and petal.

moter for transgenes in the petal tissues of chrysanthemum has been desired.

We examined the GUS activity in the petals of the several transformants (cvs. 'Sei-Marine' and 'Shuho-no-Takara') 8 and 20 months after regeneration (Table 2, Fig. 3). The average/highest GUS activity in the petals of the selected transformants having the EF1 *α*/*gus* was about 16,000/29,000. The petals still showed a GUS activity after 20 months from regeneration.

Among the recently reported promoters for transgene expression in the chrysanthemum, the chrysanthemum *rbcS1* promoter was not mentioned for expression in petals[12] and we reported that the chrysanthemum *cab* promoter hardly expressed in the petals[1]. The GUS activity in the petals was at a reduced level (about 17%) as compared with that in the leaves with the potato *Lhca3.St.1* promoter[2]. The average/highest-GUS activities in the petal tissues were about 8,500/16,500 pmol mg^{-1} protein min^{-1} in the chrysanthemum *UEP1* promoter[3]. Thus, when compared with these promoters, the tobacco *EF1 α* promoter has excellent ability for transgene expression in chrysanthemum petals.

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