Efficient Transgene Expression in Chrysanthemum, *Dendranthema grandiflorum* (Ramat.) Kitamura, by Using the Promoter of a Gene for Chrysanthemum Chlorophyll-a/b-binding Protein


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We investigated the usefulness of the promoter of a gene for chrysanthemum chlorophyll-a/b-binding protein (Cab) for transgene expression in the chrysanthemum *Dendranthema grandiflorum* (Ramat.) Kitamura. We used the promoter region of a *Cab* gene isolated from the chrysanthemum-wild species *D. japonicum* Makino. The 35S promoter of cauliflower mosaic virus (CaMV) or the Cab promoter was fused to the β-glucuronidase gene (gus) and introduced into the chrysanthemum. We obtained 300 putative transformants (115 with 35S/gus and 185 with Cab/gus). GUS assay of the leaves of the *in vitro* plants revealed that 9.6% (11/115) of the putative plants to which 35S/gus had been introduced and 24.3% (45/185) of the putative plants to which Cab/gus had been introduced were GUS-positive. Southern blot analysis showed that even the GUS-negative plants harbored the gus gene in their genomes. The Cab promoter expressed the transgene more efficiently than the 35S promoter and could be used for transgene expression in chrysanthemum leaf tissues.

Key Words: *Dendranthema grandiflorum*, chrysanthemum, transformation, chlorophyll a/b-binding protein gene, promoter.

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, as reviewed by Deroles *et al.* (2002). Many researchers have used the β-glucuronidase gene (gus) with 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 (Urban *et al.* 1994), 30 to 240 (Sherman *et al.* 1998), and 30 to 250 (Takatsu *et al.* 2000) pmol mg\(^{-1}\) protein min\(^{-1}\). Takatsu *et al.* (2000) pointed out that the GUS levels in chrysanthemum with 35S/gus were 90% lower than those in transgenic tobacco, and they also reported a reduction in GUS activity in most of the transformants 12 months after transformation. The 35S or modified 35S promoter has been used to express practical transgenes for modifying characters such as resistance to diseases (Urban *et al.* 1994, Yepes *et al.* 1995, Takatsu *et al.* 1999), resistance to insects (Shinoyama *et al.* 2002), and flower color (Courney-Guttemer *et al.* 1994, Boase *et al.* 1998). In some of these attempts, the mRNAs (Boase *et al.* 1998) or protein (Urban *et al.* 1994, Yepes *et al.* 1995) 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 operate efficiently in chrysanthemum. Therefore, it is necessary to use a promoter that is more efficient than 35S for transgene expression in chrysanthemum.

We assumed that the use of a proper chrysanthemum promoter of a highly expressed gene would also be suitable well for chrysanthemum transgenes. We therefore used the promoter region of a chrysanthemum chlorophyll-a/b-binding protein (Cab) gene that might be highly expressed in leaf tissues. Here we report the use of the chrysanthemum Cab promoter for transgene expression in chrysanthemum.

Materials and Methods

Plant materials for transformation

We used the chrysanthemum [*Dendranthema grandiflorum* (Ramat.) Kitamura] cultivars ‘Sei-Marine’, ‘Shuho-no-Takara’, ‘Seiokou-Kougyoku’, ‘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. The plants were grown *in vitro* in Murashige-Skoog medium with half-strength minerals (1/2...
MS) (Murashige and Skoog 1962), solidified with 0.2% (w/v) gellan gum, at 25°C 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.

Vector plasmids and bacterial strain
The promoter region (918 bp) of the Cab gene isolated from Dendranthema japonicum Makino (DDBJ accession no. AB110219) was used in this experiment. A vector plasmid, pIGcab1 (Fig. I), was constructed from pIG121Hm (Hiei et al. 1994) by replacing the CaMV 35S promoter with the chrysanthemum Cab promoter. The CaMV 35S promoter (pIG121Hm) or the chrysanthemum Cab promoter (pIGcab1) was followed by the gus gene with a modified intron from the castor bean catalase gene (Ohta et al. 1990). Both vectors contained the neomycin phosphotransferase II gene (nptII) and the hygromycin phosphotransferase gene (hpt) under the control of the nos promoter and the 35S promoter, respectively.

The Agrobacterium tumefaciens strains AGL0 (Lazo et al. 1991), harboring the vector plasmid pIG121Hm, and EHA105 (Hood et al. 1993), harboring the vector plasmid pIGcab1, were used for the experiments. AGL0 and EHA105 are kanamycin-sensitive derivatives of EHA101 (Hood et al. 1986), and the genetic background of AGL0 and EHA105 is almost the same. Agrobacterium cells harboring the plasmids were inoculated into liquid YEB medium (5 g/l sucrose, 1 g/l beef extract, 1 g/l yeast extract, 1 g/l peptone) containing 50 mg/l kanamycin and shaken for 48 h at 28°C. The bacterial cells were pelleted by centrifugation and re-suspended in a 10 mM magnesium sulfate solution containing 1% Tween 20 and 0.1 mM acetoxyrignon to a density of 1.0 × 10⁸ cells/ml for inoculation.

Transformation of chrysanthemum
The leaf explants were incubated in the Agrobacterium suspension for 30 min, then blotted dry on sterilized filter paper. Explants were placed on MS medium solidified with 0.2% (w/v) gellan gum containing 1.0 mg/l benzyladenine (BA), 2.0 mg/l naphthaleneacetic acid (NAA), 1.0 mg/l casamino acids, and 0.1 mM acetoxyrignon, and co-cultured for 2 days in the dark at 22°C.

After co-culture, the explants were washed in a 10 mM magnesium sulfate solution containing 300 mg/l carbenicillin and 1% Tween 20 and shaken (at about 150 rpm) for 1 h at 25°C. After washing, the leaf explants were blotted dry on a sterilized filter paper and placed on MS medium solidified with 0.8% (w/v) agar containing 1.0 mg/l BA, 2.0 mg/l NAA, 300 mg/l carbenicillin and 25 mg/l paromomycin (selection medium), then cultured under a low light intensity (photon flux density 7 μmol s⁻¹ m⁻²) at 20°C. Gellan gum could not be used as a gelling agent because paromomycin is insoluble in a medium containing gellan gum. The selection medium was changed every 2 weeks.

Shoots had been formed on the explants by 2 to 3 months after inoculation, but most of the shoots remained short (2–3 mm long) and barely elongated on the selection

![Diagram of vector plasmids](image-url)

**Fig. 1.** Structure of the T-DNA regions of the binary vectors pIG121Hm (Hiei et al. 1994) and pIGcab1. 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 and LB=right and left border sequences of T-DNA; Pnos and Tnos=promoter and terminator of nopaline synthase gene; 35S=promoter of CaMV 35S RNA gene; Cab=promoter of chrysanthemum chlorophyll a/b-binding protein gene; nptII=coding region of neomycin phosphotransferase II gene; Intron-gus=coding region of β-glucuronidase gene with an intron; hpt=coding region of hygromycin phosphotransferase gene; H, X, B, S and Sc=restriction sites of HindIII, XbaI, BamHI, SalI and SacI, respectively.
medium. A single regenerated shoot was excised from each explant and cultured on 1/2 MS medium solidified with 0.2% (w/v) gellan gum containing 300 mg/l carbenicillin (elongation medium), then cultured under a standard light intensity (photons flux density 70 µmol s⁻¹ m⁻²) at 25°C.

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.

**GUS assay**

A histochemical GUS assay was performed according to the procedure reported by Jefferson et al. (1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GLUC) as a substrate. The GUS assay buffer used in this experiment contained 20% methyl alcohol to eliminate the endogenous GUS activity, as reported by Kosugi et al. (1990). The samples were incubated at 37°C for 16 h.

Quantitative GUS activity in the leaves and petals was examined according to the procedure reported by Jefferson et al. (1987) using 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate. The GUS assay buffer also contained 20% methyl alcohol to eliminate the endogenous GUS activity. The protein concentration of the plant extracts was determined by the dye-binding method of Bradford (1976) with a protein mini kit supplied by Bio-Rad Laboratories (Hercules, CA, USA). GUS activity was expressed as picomoles of 4-methylumbelliferyl (4-MU) produced at 37°C per milligram of protein per minute (pmol 4-MU mg⁻¹ protein min⁻¹). Plants with 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 sometimes the levels of GUS background were 20–30 pmol 4MU mg⁻¹ protein min⁻¹.

**Southern blot analysis**

Total DNA was extracted from the leaf tissues using a Phytopure plant DNA extraction kit (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England), according to the manufacturer’s instructions. About 20 µg of DNA digested with HindIII was electrophoresed in a 0.6% agarose gel and transferred to the positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). HindIII cuts the plasmid at a single site outside the coding region of the gus gene (See Fig. 1). The coding region of the gus gene was used as a probe. Southern hybridization was performed using a DIG-High Prime and DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics, Mannheim, Germany). Blots were finally washed with 0.2×SSC, 0.1% SDS, at 68°C.

**Results and Discussion**

**Transformation experiments**

Some of the explants were used for the transient GUS assay 7 days after Agrobacterium inoculation. All of the explants examined showed a blue precipitation corresponding to the GUS activity (Fig. 2A), indicating that the gus gene had been transferred to the chrysanthemum cells and was successfully expressed. About 1 month after Agrobacterium inoculation, calli were formed on the explants (Fig. 2B). About 2 months after inoculation, adventitious buds emerged from the calli (Fig. 2C). After transfer to the elongation medium, the buds grew normally (Fig. 2D). Throughout the experiments, we obtained a total of 566 independent regenerants from 12,400 explants of the eight chrysanthemum lines (Table 1).

The leaf test on the elongation 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). The leaf test showed that 300 regenerants out of 566 were paromomycin-resistant, i.e. putative transformants (Table 1). In the experiments, the Agrobacterium strains AGL0 were used for 35S/gus and EHA105 for Cab/gus. The transformation efficiency (transformants/explants) was 0.8–3.5% for AGL0 and 0.5–6.5% for EHA105. de Jong (1994) reported that AGL0 showed a higher transformation efficiency in chrysanthemum than strain LBA4404, and Urban et al. (1994) reported that EHA105 showed a higher efficiency than LBA4404. We also demonstrated that both AGL0 and EHA105 strains could transform chrysanthemum successfully. AGL0 (Lazo et al. 1991) and EHA105 (Hood et al. 1993) have almost the same genetic background because both strains are kanamycin-sensitive derivatives of EHA101 (Hood et al. 1986). It appeared that AGL0 and EHA105 would display a similar transformation ability, and would give similar integration patterns of transgenes which should affect the expression levels.

**Southern blot analysis**

We selected three GUS-negative and three GUS-positive paromomycin-resistant plants from each of the regenerant groups (cv. ‘Sei-Marine’) to which 35S/gus- and Cab/gus had been introduced for Southern blot analysis. Analysis showed that all the paromomycin-resistant plants examined—even the GUS-negative ones—harbored the gus gene in their genomes (Fig. 3). Digestion of each vector with HindIII cuts the plasmid at a single site outside the coding region of the gus gene (Fig. 1). Contamination of the tissues by the plasmid should be detected by the presence of a single band at 15.6 kb (35S/gus) or 15.7 kb (Cab/gus). All the paromomycin-resistant plants examined showed one or more bands with different sizes, indicating single- or multiple-copy integration of the gus gene into the genome. We concluded that all the examined plants, even the GUS-negative ones, harbored transgene(s).
Fig. 2. Transformation of chrysanthemum. (A) Transient GUS assay 7 days after *Agrobacterium* inoculation. All the explants showed a blue precipitation indicating GUS activity. (B) About 1 month after *Agrobacterium* inoculation, calli were formed on the explants. (C) About 2 months after the inoculation, adventitious buds emerged from the calli. (D) After transfer to the elongation medium the buds grew normally.

Table 1. Comparison of promoters for transgene expression among chrysanthemum lines

<table>
<thead>
<tr>
<th>Chrysanthemum lines</th>
<th>Promoter</th>
<th>No. of explants</th>
<th>No. of regenerants</th>
<th>No. of paromomycin-resistant plants</th>
<th>Paromomycin-resistant plants/explant (%)</th>
<th>No. of GUS-positive plants</th>
<th>GUS-positive plants/paromomycin-resistant plants (%)</th>
<th>No. of plants with GUS activity in leaves&lt;sup&gt;2&lt;/sup&gt;</th>
<th>No. of plants with GUS activity in leaves&lt;sup&gt;2&lt;/sup&gt;</th>
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<td>2000</td>
<td>112</td>
<td>69</td>
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<td>0.5</td>
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<td>46.7</td>
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<td>6.5</td>
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<td>15.4</td>
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</table>

<sup>1</sup> Plants with GUS activities above 100 pmol 4MU mg<sup>−1</sup> protein min<sup>−1</sup> were considered to be GUS-positive.

<sup>2</sup> GUS activity is expressed as pmol 4MU mg<sup>−1</sup> protein min<sup>−1</sup>.
introduced were GUS-positive. The Cab promoter expressed 45/185 of the putative plants to which Cab/gus resistant population of each cultivar was 0–33.3% (Table 1). The assay revealed that 9.6% (11/115) of the putative plants with 35S/gus and Cab/gus had been introduced were GUS-positive. The Cab promoter expressed the gus gene more efficiently than the 35S promoter. The percentage of GUS-positive plants in the paromomycin-resistant population of each cultivar was 0–33.3% for 35S/gus and 15.4–50.0% for Cab/gus. GUS-positive plants with 35S/gus were observed only in the cultivars ‘Sei-Marine’, ‘Shuho-no-Takara’ and ‘Seikou-Kougyoku’. There were no GUS-positive plants in the remaining five lines. In contrast, we obtained at least one GUS-positive plant with Cab/gus in all the eight lines (Table 1).

The Cab promoter thus shows a higher ability to express the transgene in chrysanthemum than the 35S promoter. Although we have no direct evidence, it is possible that the native promoter might be less effective by the gene-silencing mechanism in chrysanthemum than the 35S promoter derived from cauliflower mosaic virus. Even with the Cab promoter, many paromomycin-resistant plants did not show the GUS activity. A still more efficient promoter than the Cab promoter for transgene expression in chrysanthemum should be developed in the future.

In earlier reports, GUS activity levels in transgenic chrysanthemum with 35S/gus were low, for example, 10 to 160 (Urban et al. 1994), 30 to 240 (Sherman et al. 1998) or 30 to 250 (Takatsu et al. 2000) pmol mg\(^{-1}\) protein min\(^{-1}\). We observed a GUS activity of more than 1,000 pmol mg\(^{-1}\) protein min\(^{-1}\) in six ‘Sei-Marine’ transformants and one ‘Seikou-Kougyoku’ transformant with 35S/gus. The higher GUS activity may be attributed to cultivar-dependent phenomena, for example, ‘Sei-Marine’ and ‘Seikou-Kougyoku’ might express transgenes with the 35S promoter more efficiently than other chrysanthemum lines. Another possibility is the position effect. Transgene expression is affected by the integration position in the genome (Bhattacharyya et al. 1994, Peach and Velten 1991). Transgenes might be methylated and silenced when integrated into a highly repetitive or highly methylated region (Meyer 1995). Of 115 putative transformants that harbored 35S/gus, only seven plants (6.1%) showed a high GUS activity (>1,000 pmol mg\(^{-1}\) protein min\(^{-1}\)). In earlier reports, the GUS activity was determined in only 34 (Urban et al. 1994), 15 (Sherman et al. 1998) and 28 transformants (Takatsu et al. 2000). If the position effect in chrysanthemum reveals a higher transgene expression only at around the 6% level for 35S/gus, we can speculate that in these earlier studies on small numbers of transformants, the highly expressed transformants were not included.

In contrast, in our study, 27 plants out of 185 putative transformants with Cab/gus (14.6%) showed a high GUS activity (>1,000 pmol mg\(^{-1}\) protein min\(^{-1}\)), suggesting that the Cab promoter is superior to the 35S promoter for inducing a high level of transgene expression in chrysanthemum.

Expression patterns of linked transgenes

The three inserted genes were linked as RB–nptII gene–gus gene–hpt gene–LB (Fig. 1), and T-DNA transfer is known to take place from the right to the left T-DNA border (Wang et al. 1984). It was, therefore, possible that the T-DNA transfer was incomplete and the intact gus gene was lacking. However, we confirmed by Southern blot analysis that several GUS-negative plants harbored transgene(s). We considered that most of the GUS-negative plants would have silenced gus gene(s). All the transformants expressed the nptII gene (resistant to paromomycin) and only 9.6% of the putative plants with 35S/gus and 24.3% of the putative plants with Cab/gus expressed the gus gene (GUS activity of >100 pmol mg\(^{-1}\) protein min\(^{-1}\)) that was linked to the nptII gene. It was observed that the levels of expression of different linked transgenes were not correlated with each other in tobacco (Mlynarova et al. 1995) and Kalanchoe (Aida and Shibata 1996). These reports indicate that the expression levels of the linked transgenes are sometimes independent of each other, even though the transgenes are located in nearly the same region of the genome. However, because a higher ratio of the plants with Cab/gus showed a GUS activity compared to the plants that carried 35S/gus, it is apparent that the Cab promoter expresses transgenes more efficiently than the 35S promoter.

![Fig. 3. Southern blot analysis of three GUS-negative and three GUS-positive paromomycin-resistant plants (putative transformants) from regenerants with 35S/gus and Cab/gus. The coding region of the gus gene was used as a probe. All the paromomycin-resistant plants examined—even the GUS-negative ones—harbored the gus gene in their genomes.](image-url)
GUS activity in flowering transformants

We transferred some of the transformants (cv. ‘Sei-Marine’) to a greenhouse for further investigation. Most of the transformants grew and flowered normally (Fig. 4). Eight and 20 months after regeneration, we further examined the GUS activity of the leaves and petals of these greenhouse plants. Table 2 shows the GUS activity of the leaves of the in vitro plants and the leaves and petals of the greenhouse plants. The plants in the greenhouse to which 35S/gus and Cab/gus had been introduced still showed a GUS activity in their leaves 20 months after regeneration. Takatsu et al. (2000) reported that the GUS activity of most transgenic chrysanthemums harboring 35S/gus was reduced 6 or 12 months after transformation ( cvs. ‘Yamabiko’ and ‘New Summer Yellow’). Our results showed that, in the cultivar ‘Sei-Marine’, both the 35S promoter and the Cab promoter could still express the gus gene 20 months after transformation.

Although plants with 35S/gus showed a GUS activity in their petals, we seldom observed a GUS activity in the petals of the plants with Cab/gus (Table 2). The wheat Cab promoter is light-inducible and acts predominantly in the leaves, but not in the roots or petals, of transgenic tobacco (Lamppa et al. 1985). The chrysanthemum Cab promoter also showed a tissue-specific expression, it operated well in the leaf tissues and poorly in the petal tissues. We showed that the chrysanthemum Cab promoter display a higher ability than the CaMV 35S promoter for transgene expression in the leaf tissues of chrysanthemum. It was observed that genetic transformation could be used as a practical breeding method. The Cab promoter could thus be suitable for the introduction of useful genes to improve chrysanthemum characteristics, such as resistance to diseases or insects. Takatsu et al. (1999) reported that transgenic chrysanthemum plants expressing a rice chitinase gene with a CaMV 35S promoter showed an increased resistance to gray

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

<table>
<thead>
<tr>
<th>Plant lines</th>
<th>GUS activity (pmol 4MU mg⁻¹ protein min⁻¹)</th>
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<tbody>
<tr>
<td>35S/GUS 1</td>
<td>2409</td>
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<tr>
<td>35S/GUS 2</td>
<td>2631</td>
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<tr>
<td>35S/GUS 10</td>
<td>26526</td>
</tr>
</tbody>
</table>

1) One to two months after regeneration
2) Four months after regeneration
3) Eight months after regeneration
4) Sixteen months after regeneration
5) Twenty months after regeneration
mold (Botrytis cinerea). They observed very mild symptoms in three transgenic plants out of 11. Shinoyama et al. (2002) reported that the introduction to chrysanthemum of a deltaprotease gene of Bacillus thuringiensis with a CaMV 35S promoter increased the resistance to the tobacco budworm (Helicoverpa armigera). They reported that, in five transgenic plants out of 20, the area of leaf fed on by the H. armigera larvae was significantly smaller than that in the non-transgenic control. They pointed out the needs for cloning original promoters of chrysanthemum to obtain transformants with high levels of expression. We showed that the chrysanthemum Cab promoter could be used, particularly for the kinds of experiments in which transgene expression is needed in leaf tissues. In addition to conferring resistance to diseases or insects, proper genes under the control of the Cab promoter could be used to modify ornamental characters such as plant shape.

There have been many reports of modification of the flower color by genetic transformation (Ben-Meir et al. 2002). In chrysanthemum, the development of new colors, such as blue, has been an important breeding target. Unfortunately, the Cab promoter cannot be used for changing the flower color, because it operates poorly in petal tissues. We therefore need to develop efficient promoters for chrysanthemum that would operate in the petals.

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Literature Cited


