

Agrobacterium-mediated Transformation of Torenia (Torenia fournieri)

Ryutaro Aida and Michio Shibata

National Research Institute of Vegetables, Ornamental Plants and Tea, Ano, Mie 514-23, Japan

Summary

A method for Agrobacterium-mediated transformation of torenia (Scrophulariaceae) is reported. Leaf segments of torenia plants grown in vitro were infected with the Agrobacterium strain LBA4404 harboring a binary vector containing the neomycin phosphotransferase II gene, β-glucuronidase (GUS) gene with an intron and hygromycin phosphotransferase gene. The infected explants were cultured on Murashige and Skoog medium supplemented with 1 mg/l benzyadenine, 100 mg/l carbenicillin and 300 mg/l kanamycin (selection medium) for regeneration. Twelve shoots out of 67 shoots which regenerated from green compact calli on the selection medium were GUS-positive. Polymerase chain reaction analysis confirmed the presence of the GUS gene in the GUS-positive plants. Mendelian inheritance of the GUS activity in the progenies of the transformed plants was demonstrated.

Key Words: Torenia fournieri, wishbone flower, Agrobacterium, transformation, β-glucuronidase gene.

Introduction

The transformation system for ornamental plants is important especially to modify ornamental characteristics such as flower color, flower shape and flower longevity. Several attempts have been made to modify these characteristics in ornamental plants by genetic engineering (Hutchinson et al. 1992; Robinson and Firoozabad 1993).

Torenia fournieri, commonly known as torenia or wishbone flower, is one of the bedding ornamental plants used during summer which belong to the family Scrophulariaceae. The culture conditions of torenia have been well-documented (Tanimoto and Harada 1990), and the induction system of adventitious buds from organs was established (Tanimoto and Harada 1981). However the transformation of torenia had not been reported until now.

In this report, we describe the Agrobacterium-mediated transformation system for torenia.

Materials and Methods

Plant materials

Torenia (Torenia fournieri) cultivar ‘Crown Mix’ was used as plant material. Seeds of ‘Crown Mix’ were soaked in 70 % ethyl alcohol for 15 seconds then surface-sterilized with 1 % sodium hypochlorite for 30 minutes, followed by 2 successive rinses with sterilized distilled water for 10 minutes. Seeds were germinated on half strength minerals of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 0.2 % (w/v) gellan gum. Cultures were maintained under a 16 h photoperiod regime with fluorescent light at 25 °C.

For transformation experiments, leaves were excised from torenia plants grown in vitro for one to two months after germination. The leaves were cut into 3 to 5 mm squares then used as explants.

Agrobacterium

Agrobacterium tumefaciens LBA 4404 (Clontech, USA) which harbors a binary vector pIG 121-Hm (Ohta et al., in preparation) containing the neomycin phosphotransferase II (NPT II) gene, β-glucuronidase (GUS) gene with a modified intron of the castor bean catalase gene (Ohta et al. 1990) and hygromycin phosphotransferase (HPT) gene (Fig. 1) was used in the transformation experiments.

Agrobacterium was inoculated into liquid YEB medium (sucrose 5 g/l, beef extract 1 g/l, yeast extract 1 g/l, peptone 1 g/l) containing 50 mg/l kanamycin and 50 mg/l hygromycin, and shaken for 48 hours at 28 °C. The culture was diluted to 1.0×10^8 cell/ml for infection.

Transformation

About 500 leaf explants were incubated in the Agrobacterium suspension for 5 minutes then blotted dry on sterilized filter paper. The explants were placed onto another sterilized filter paper on MS medium solidified with 0.2 % (w/v) gellan gum containing 1 mg/l benzyladenine (BA) and 20 μM acetosyringone for seven days. Acetosyringone was added to enhance the DNA transfer (Stachel et al. 1985). After co-cultivation, the explants were transferred to MS solid medium (0.2 % gellan gum) containing 1 mg/l BA, 100 mg/l carbenicillin and 300 mg/l kanamycin (selection medium) for regeneration. The selection medium was changed every two weeks.

Leaf test, GUS assay and PCR experiment

A leaf segment of each regenerated shoot was cultured on the selection medium to observe the resistance to kanamycin (leaf test).

GUS activity in leaves was examined by the procedure reported by Jefferson et al. (1987) with 4-methylumbelliferyl-β-D-glucuronide (MUG) as a sub
strate. 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). No GUS background activity was detected in the wild type control.

Total DNA was isolated from leaves according to the procedure reported by Edwards et al. (1991) for the PCR experiment. Two primers 5'-CCCTTATGTTACGTCGTAGAAACC-3' and 5'-CCAATCCAGTCATATGCGTGTCG-3' were used which amplify a 970 bp fragment of the GUS gene. DNA was amplified by 45 cycles for 0.5 minute at 94°C, 2 minutes at 60°C and 3 minutes at 72°C.

Inheritance of the GUS gene
Transformants were potted and grown in a closed greenhouse for transgenic plants. During the first seven days, the plants were covered with plastic cups for acclimatization. Two to 3 months after potting, bagging was performed on the flower buds just before flowering for artificial pollination. The flower buds to be crossed with wild type plants were emasculated at the time of bagging. Seeds were harvested about one month after pollination, followed by 4°C treatment for one month. Chilling-treated seeds were germinated in vitro, and used to determine the segregation ratio of introduced GUS gene. Four to 5 weeks after germination, leaf segments were excised to examine the GUS activity. PCR analysis was also conducted to confirm the inheritance of the GUS gene.

Results and Discussion

Two weeks after inoculation with Agrobacterium, light green adventitious buds appeared. However, they became gradually bleached and died on the selection medium. About 4 weeks after inoculation, dark green compact calli were formed and shoots were regenerated from these calli (Fig. 2-A). To confirm their resistance to kanamycin, a leaf segment of each regenerated shoot was cultured on the selection medium. Within three months after the Agrobacterium infection, 67 shoots were examined by the leaf test. The leaf segments from putative transformants formed shoots on the selection medium, although those from non-transformed escapes did not survive on the selection medium (Fig. 2-B). Fourteen shoots out of 67 shoots showed the resistance to kanamycin. These 14 shoots were assayed for the GUS activity. Twelve shoots out of 14 shoots showed the GUS activity. The shoots showing the GUS activity were further examined by polymerase chain reaction (PCR) analysis for the detection of the incorporated GUS gene. All the 12 shoots were found to harbor the GUS gene fragment by the PCR experiment (Fig. 2-C). These 12 shoots were cultured in vitro and after rooting they were potted for further culture in the closed greenhouse.

Transformants grown in the greenhouse flowered normally (Fig. 2-D) and seeds of the transformants were obtained by self-pollination or crossing with wild type plants. The offsprings of eight transformants were analyzed for the inheritance of the GUS activity. PCR analysis of the progenies confirmed the inheritance of the GUS gene (Fig. 2-C). Table 1 shows the segregation ratio of the GUS activity in the progeny of torenia transformants. Selled progeny of the primary transformants 1, 2, 3, 5, 6 and 8 segregated in the ratio of about 3 : 1 (GUS+ : GUS-) for the GUS activity, indicating that T-DNA was inserted at a single locus in each parental transformant. On the other hand, the selfed progeny of both transformants 4 and 7 showed a segregation ratio of about 15 : 1 (GUS+ : GUS-), indicating that T-DNA was inserted at two loci. Both progenies of the transformant 2 × wild type and transformant 8 × wild type showed a segregation ratio of about 1 : 1 (GUS+ : GUS-). These values agreed with the results obtained for the selfed progeny.

Thus, Mendelian inheritance of the introduced character from primary transformants to their progenies was demonstrated, as reported for other plants (Fang and Grunet 1990; Akama et al. 1992; Sarria et al. 1994). However, an aberrant segregation was also reported by other researchers in several plants (McCormick et al. 1985; Schmidt and Willmitzer 1988; Barfield and Pua 1991; Dong and McHughen 1993; Schmidt and Willmitzer 1988 and Dong and McHughen 1993) suggested that the irregular segregation resulted from the chimeric nature of the transformants, while McCormick et al. (1986) suggested that it was due to multiple inserts of T-DNA. Mendelian segregation of torenia transformants in this experiment indicates that these transformants were non-chimeric and harbored only a single or two T-DNA inserted loci.

As mentioned above, we developed an Agrobacterium-mediated transformation system for torenia. We have already obtained more than 200 primary transgenic torenias, and observed the promoter-dependent expression by using different kinds of promoters (in prepara-
tion). This transformation system will be used for torenia breeding by genetic engineering to modify characteristics such as flower color and flower longevity. Introduction of herbicide resistance by genetic engineering would be also useful for torenias because of their use as bedding plants.

Recently, a great deal of progress has been made on the mechanisms controlling floral development through the analysis of mutants in *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz 1991). Several homeotic genes have been cloned and analyzed (Schmidt et al. 1993). Advances in molecular biology studies on
Table 1. Segregation of GUS activity in progenies of the toremia transformants

<table>
<thead>
<tr>
<th>Progeny</th>
<th>GUS+ : GUS−</th>
<th>Expected ratio</th>
<th>$\mu^2$</th>
<th>Probability</th>
<th>Expected number of T-DNA inserted loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformant-1 self</td>
<td>38 : 10</td>
<td>3 : 1</td>
<td>0.222</td>
<td>0.5 - 0.7</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-1 x wt(1)</td>
<td>23 : 24</td>
<td>1 : 1</td>
<td>0.021</td>
<td>0.8 - 0.9</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-2 self</td>
<td>37 : 11</td>
<td>3 : 1</td>
<td>0.111</td>
<td>0.7 - 0.8</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-3 self</td>
<td>36 : 12</td>
<td>3 : 1</td>
<td>0</td>
<td>0.8 - 0.9</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-4 self</td>
<td>34 : 2</td>
<td>15 : 1</td>
<td>0.030</td>
<td>0.7 - 0.8</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-5 self</td>
<td>27 : 8</td>
<td>3 : 1</td>
<td>0.086</td>
<td>0.3 - 0.5</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-6 self</td>
<td>22 : 10</td>
<td>3 : 1</td>
<td>0.667</td>
<td>0.5 - 0.7</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-7 self</td>
<td>33 : 3</td>
<td>15 : 1</td>
<td>0.267</td>
<td>0.1 - 0.2</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-8 self</td>
<td>32 : 16</td>
<td>3 : 1</td>
<td>1.778</td>
<td>0.7 - 0.8</td>
<td>1</td>
</tr>
<tr>
<td>Transformant-8 x wt(1)</td>
<td>23 : 25</td>
<td>1 : 1</td>
<td>0.083</td>
<td>0.7 - 0.8</td>
<td>1</td>
</tr>
</tbody>
</table>

(1) wt : wild type
(2) Expected number of T-DNA inserted loci per genome in each parental transformant.

flowering should contribute to the progress of the flower industry in the future. We consider that toremia could also be useful as an experimental plant for molecular biology studies.

Acknowledgements

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Literature Cited


