Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation

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Abstract

We modified flower color in torenia (*Torenia fournieri* Lind.) by transferring the chalcone synthase (CHS) or the dihydroflavonol-4-reductase (DFR) gene in sense or antisense orientation by *Agrobacterium*-mediated gene transfer. The modification patterns of flower color among the transformants formed three groups: (1) same color as the wild-type plant; (2) whole corolla changed to a uniformly light color; and (3) with greater degree of lightening in the tube than in the lip. Transformants incorporating antisense transgene(s) tended to become group 2 types, with no plants becoming group 3 type. Transformants harboring sense transgene(s) tended to become group 3 types, rather than group 2 types. Sense genes and antisense genes seemed to have different potential for changing the flower color. We also produced transformants with new characters in torenia flower color; for example, lines with pastel flowers, wavy patterned flowers and parti-colored flowers. We regard this system to be useful for flower color breeding in torenia and for studying gene expression. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Anthocyanin; Gene silencing; *Torenia fournieri*; Transformation; Flower color

1. Introduction

Flower color is one of the most important characters for ornamental plants. Creation of new flower colors is one of the important targets for breeding. By controlling expression levels of genes related to the biosynthetic pathway of flower color pigments we can breed novel varieties with respect to flower color.

Genetic transformation can be done by either introducing and expressing a new gene or re-introducing an existing gene in sense or antisense orientation to inactivate an endogenous gene [1].

Flower color had been modified by transformation in several ornamental plants by suppressing the chalcone synthase (CHS) or the dihydroflavonol-4-reductase (DFR) gene that encode enzymes in the biosynthetic pathway of anthocyanins. The anthocyanins are important pigments for flower color. Introduction of the antisense CHS gene suppressed the formation of flower pigments in petunia and tobacco [2]. Introduction of the sense CHS gene [3] or sense DFR gene [4] caused the production of transgenic petunia plants with reduced flower color pigmentation. Introduction of the sense CHS gene in chrysanthemum caused the production of transgenic plants whose flowers changed from pink to white [5]. Introduction of the antisense CHS gene in gerbera caused the production of transgenic plants whose flowers changed from red to pink [6], and in lisianthus, produced transgenic plants whose flowers changed from purple to white [7].

*Torenia fournieri* Lind. (family Scrophulariaceae), commonly known as torenia, is the most important species in the genus for ornamental use. We introduced the DFR or CHS gene into torenia in sense or antisense orientation to modify flower color.
2. Materials and methods

2.1. Vectors

The full length cDNA encoding CHS (about 1.4 kb, AB012923), isolated from a torenia cv. 'summerwave' and fused with E12Ω promoter [8] in sense (pBETC6) or antisense (pBETC7) orientation, was inserted into a binary vector plasmid pBin9 [9] (Fig. 1(A)). A fragment of cDNA encoding DFR (about 1.1 kb; lacking 5' side of the coding region, AB012924), isolated from the 'summerwave' and fused with E12Ω promoter in sense (pBETD10) or antisense (pBETD11) orientation, was also inserted into binary vector plasmid pBin9 (Fig. 1(B)).

2.2. Plant materials and transformation

The experiments used 3 clonal laboratory lines: (1) a violet line selected from 'crown mix' (crown violet); (2) a reddish-purple line selected from 'crown mix' (crown reddish-purple); and (3) a violet line selected from 'common violet' (common violet). Transgenic plants were obtained by the Agrobacterium-mediated transformation system described previously [10]. Transformants were grown in vitro until flowering. Plants whose flower color was considered to have changed were transferred to a closed greenhouse for further investigation.

2.3. Southern blot analysis and northern blot analysis

Seven putative transgenic plants transformed with pBETC6 derived from the crown reddish-purple line were used for Southern blot analysis. Total DNA was isolated as follows: leaf tissue was homogenized in a homogenization buffer containing 15% sucrose, 50 mM EDTA, 250 mM NaCl, 50 mM Tris–HCl (pH 8.0) and 0.1% 2-mercaptoethanol. After centrifugation, DNA was extracted from the pellet with ISOPLANT (Nippon Gene, Toyama, Japan). About 10 μg of DNA digested with HindIII was electrophoresed in a 0.6% agarose gel and transferred to a nylon membrane. HindIII cuts the plasmid pBETC6 at a single site outside the coding region of the CHS gene. The cloned region of the CHS gene was used as a probe. Blots were finally washed with 0.5 × SSC, 0.1% SDS, at 68°C.

For northern blot analysis, we selected four transformants derived from the crown violet line as follows: a plant harboring sense CHS transgene(s): 400-29; a plant harboring antisense CHS transgene(s): 411-3; a plant harboring sense DFR transgene(s): 405-2; and a plant harboring antisense DFR transgene(s): 416-16. Each of those...
Table 1
Transformation efficiency in torenia with vectors harboring a transgene related to flavonoid biosynthesis

<table>
<thead>
<tr>
<th>Cultivar and transgene</th>
<th>Number of leaf explants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of regeneration&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Number of transformants&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Efficiency&lt;sup&gt;d&lt;/sup&gt; (transformants/explant) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crown mix: violet corolla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFR gene, sense</td>
<td>500</td>
<td>31</td>
<td>28</td>
<td>5.6</td>
</tr>
<tr>
<td>antisense</td>
<td>250</td>
<td>37</td>
<td>35</td>
<td>14.0</td>
</tr>
<tr>
<td>CHS gene, sense</td>
<td>500</td>
<td>62</td>
<td>62</td>
<td>12.4</td>
</tr>
<tr>
<td>antisense</td>
<td>250</td>
<td>27</td>
<td>26</td>
<td>10.4</td>
</tr>
<tr>
<td><strong>Crown mix: reddish purple corolla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFR gene, sense</td>
<td>500</td>
<td>44</td>
<td>40</td>
<td>8.0</td>
</tr>
<tr>
<td>antisense</td>
<td>250</td>
<td>52</td>
<td>45</td>
<td>18.0</td>
</tr>
<tr>
<td>CHS gene, sense</td>
<td>500</td>
<td>50</td>
<td>44</td>
<td>8.8</td>
</tr>
<tr>
<td>antisense</td>
<td>250</td>
<td>48</td>
<td>43</td>
<td>17.2</td>
</tr>
<tr>
<td><strong>Common violet: violet corolla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFR gene, sense</td>
<td>250</td>
<td>24</td>
<td>21</td>
<td>8.4</td>
</tr>
<tr>
<td>antisense</td>
<td>250</td>
<td>12</td>
<td>11</td>
<td>4.4</td>
</tr>
<tr>
<td>CHS gene, sense</td>
<td>250</td>
<td>27</td>
<td>25</td>
<td>10.0</td>
</tr>
<tr>
<td>antisense</td>
<td>250</td>
<td>18</td>
<td>16</td>
<td>6.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Leaf explants were infected with *Agrobacterium* and co-cultured for 7 days on a medium containing 0.5 mg/l BA, 0.1 mg/l IAA and 100 μM acetosyringone. After co-culturing, they were further cultured on a selection medium containing 1.0 mg/l BA, 100 mg/l carbenicillin and 300 mg/l kanamycin.<br>
<sup>b</sup> Regenerated shoots were collected 13 weeks after *Agrobacterium* infection. Only a single shoot was collected from each callus to obtain independent transformants.<br>
<sup>c</sup> Transformation was confirmed by leaf tests.<br>
<sup>d</sup> Number of transformants/explant.

2.4. Evaluation of flower color and measurement of anthocyanin contents

Flower color of all the transformants was observed by eye. We measured anthocyanin contents in the lips and tubes of corollas of some transformants. The experiments used plants derived from the crown violet line as follows: plants harboring sense CHS transgene(s) (CHS-S): 387-2, 387-15, 400-2, 400-26, 400-27; plants harboring antisense CHS transgene(s) (CHS-A): 411-3, 411-9, 411-10, 411-18, 411-22; plants harboring sense DFR transgene(s) (DFR-S): 405-2, 405-14; and plants harboring antisense DFR transgene(s) (DFR-A): 416-8, 416-16, 416-18, 416-20, 416-34. The corolla was divided into lip and tube parts, and anthocyanins were extracted from each part with 1% hydrochloric acid in methanol. Concentrations were determined by absorbance measurements at 530 nm.

3. Results

Results of transformation experiments are shown in Table 1. Transformation efficiency ranged from 4.4 to 18.0%. Putative transformants showed 1 or 2 extra bands not present in the wild-type plant on the Southern blot analysis (Fig. 2). Digestion of pBETC6 DNA with *HindIII* cuts the plasmid at a single site outside the coding region of the CHS gene (Fig. 1). The presence of
contaminating plasmid in the tissues should be detected by the presence of a single 14.1 kb band. The plants showed 1 or 2 extra bands of differing sizes, indicating single- or multiple-copy integration of the CHS transgene into the genome. We considered that the extra bands represent the existence of transgene(s). The intensity of the extra bands was varied even in a same line. It is not clear why the intensity is not equal. Some transgenes might be tandem-repeat structures that make strong signal, or some transgene might be partial structures because of incomplete integration that makes a weak signal.

Photographs of corollas from crown violet transformants are shown in Fig. 3. Some transformants had a pastel flower color (e.g. 411-1, 411-3, 411-9, 416-18 and 416-20); one had a wavy pattern on the flower lip (411-7); another had particolored flowers (403-20, Fig. 4(A)). These patterns do not exist in normal cultivars.

Flower color of the transformants was classified by eye into three groups: (1) same color as the wild-type plant; (2) whole corolla changed to a uniformly light color (e.g. 411-1, 411-3 and 416-20); and (3) greater degree of lightening of color in the tube than in the lip, creating strong contrast between colored and white parts of the corolla (e.g. 387-2, 400-26 and 405-2). Relationships between the plant lines/transgenes and type of flower color change are shown on Table 2. The color changed in most combinations at a rate of 18–89%. Transformants harboring antisense transgene(s) tended to become group 2 types (0–39% of plants harboring DFR transgene(s), and 56–89% of plants harboring CHS transgene(s); no plants became group 3 type. However, transformants harboring sense transgene(s) tended to become group 3 types (18–33% of plants harboring DFR transgene(s), and 32–46% of plants harboring CHS transgene(s); fewer became group 2 types (from 0 to 18% of plants harboring DFR transgene(s), and 2–20% of plants harboring CHS transgene(s). These results show that each sense and antisense transgene has a unique potential for changing flower color.

Northern blot analysis revealed that transformants harboring the CHS transgene showed reduced CHS mRNA level and normal DFR mRNA level; transformants harboring the DFR transgene showed reduced DFR mRNA level and normal CHS mRNA level (Fig. 5). Thus, the CHS gene and the DFR gene were independently inactivated in the torenia transformants. The manner of reduction of the mRNA level was different between a plant harboring sense transgene and a plant harboring antisense transgene. The transformants harboring sense transgenes had less mRNA at the stage for coloring tube (stage III; about 3 days before flowering) than at the stage for coloring lip (stage I; about 9 days before flowering). The mRNA was hardly detected at the stage III on transformants harboring sense transgenes. On the contrary, the transformants harboring antisense CHS transgenes had little mRNA at both stages and the transformants harboring antisense DFR transgenes had some amount of mRNA at both stages. There were additional DFR mRNA bands at a lower molecular weight than the control on the transformants harboring DFR transgenes. The additional bands might be produced from transgenes because the DFR transgene lacked 5′ side of the coding region.

Anthocyanin contents in lip and tube parts of corollas of the transformants derived from the crown violet line are shown in Fig. 6. In plants harboring either DFR or CHS sense transgene(s),
Fig. 3. Flower color patterns within transformants derived from the Crown violet line.
Fig. 4. (A) Parti-colored flowers on a transformant (403-20) harboring the sense CHS transgene derived from the Common violet line. (B–D) Uniformly colored flowers on transformants 411-3, 411-7 (harboring the antisense CHS transgene derived from the Crown violet), and 405-2 (harboring the sense DFR transgene derived from the Crown violet), respectively.

Fig. 7. Developmentally regulated coloring pattern of the torenia flower. The lip, anther and tube become colored about 9, 6 and 3 days before flowering, respectively.
Table 2
Patterns of flower color observed within transgenic torenia plants

<table>
<thead>
<tr>
<th>Cultivar and transgene</th>
<th>Number of transgenic plants</th>
<th>Patterns of flower color (number of plants %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Crown mix: violet corolla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFR gene, sense</td>
<td>24</td>
<td>17(71)</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>28   (71)</td>
</tr>
<tr>
<td>CHS gene, sense</td>
<td>47</td>
<td>31(61)</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>17</td>
</tr>
<tr>
<td>Crown mix: reddish purple corolla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFR gene, sense</td>
<td>33</td>
<td>16(48)</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>36</td>
</tr>
<tr>
<td>CHS gene, sense</td>
<td>41</td>
<td>14(34)</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>41</td>
</tr>
<tr>
<td>Common violet: violet corolla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFR gene, sense</td>
<td>11</td>
<td>9(82)</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>4</td>
</tr>
<tr>
<td>CHS gene, sense</td>
<td>15</td>
<td>8(53)</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>9</td>
</tr>
</tbody>
</table>

\[a\] Number of transgenic plants in which flower color has been evaluated.

\[b\] (1) Same color as the wild-type plant, (2) whole corolla changed to a uniformly light color, (3) greater degree of lightening of color in the tube than in the lip, creating strong contrast between colored and white parts of the corolla.

\[c\] Percentages rounded off to whole numbers.

There was greater reduction of anthocyanin content in the tube than in the lip. In plants harboring antisense CHS transgene(s), the degree of reduction was almost the same in the tube and lip. In plants harboring antisense DFR transgene(s), there was greater reduction in the lip than in the tube. Thus, flower color change was associated with reduction of anthocyanin content.

4. Discussion

The inactivating mechanisms are thought to be different for the two gene types. Antisense genes produce complementary RNA to the target RNA, and binding of the two RNAs inhibits protein production [11]. While there are many examples of gene suppression by sense genes (i.e. sense suppression or homology-dependent gene silencing), the mechanism of the suppression is not yet clear [12–14].

There are many reports on modification of flower color using these techniques. The antisense CHS gene has been used to reduce flower pigments in petunia [3] and chrysanthemum [5], as has the sense DFR gene to reduce flower pigments in petunia [4]. Many transformants have been obtained and examined in petunia, but few in other plant species. We obtained 76 phenotypically altered sense-introduced plants and 64 phenotypically altered antisense-introduced plants of torenia, matching the number of petunia transformations.

In this report, all the torenia transformants that were considered to have modified flower color had lighter color than that of wild-type plants. The mRNA levels of the CHS or DFR gene were reduced (Fig. 5), and anthocyanin contents of the corolla were also reduced (Fig. 6). These results suggest that the modification of flower color was caused by gene suppression and resultant reduction of flower pigments. Further investigations would be clarify the reason for the flower color modification in the aspect of actual pigment composition.

Of the three modification patterns of flower color in torenia (Table 2), transformants harboring antisense transgene(s) tended to become group 2 types (no plants changed to group 3 type), while transformants harboring sense transgene(s) tended
Fig. 5. Northern blot analysis of the CHS and DFR gene in the torenia transformants. Total RNA was extracted from the flowers excluding sepals during the period of lip coloring (stage I; about 9 days before flowering) or during the period of tube coloring (stage III; about 3 days before flowering). The RNA was electrophoresed and transferred to a nylon membrane. The cloned region of the CHS or DFR gene was used as a probe. An actin probe was used to estimate the load of RNA. Blots were finally washed with 0.1 \( \times \) SSC, 0.1% SDS at 68°C. Transformants harboring the CHS transgene showed reduced CHS mRNA level and normal DFR mRNA level while transformants harboring the DFR transgene showed reduced DFR mRNA level and normal CHS mRNA level. The transformants harboring sense transgenes had less mRNA in stage III than in stage I.

Fig. 6. Anthocyanin content of lip and tube of the corolla in torenia transformants derived from Crown violet. CHS-S: plants harboring sense CHS transgene(s), CHS-A: plants harboring antisense CHS transgene(s), DFR-S: plants harboring sense DFR transgene(s), DFR-A: plants harboring antisense DFR transgene(s).

Jorgensen et al. [15] compared the modification patterns of flower color between sense- and antisense-gene-introduced petunia plants in transformants harboring the CHS transgene. They reported that the sense gene produced several patterns (e.g. junction and Cossack dancer) that the antisense gene did not, and that the tube was not affected in antisense-gene-introduced plants. Our results on torenia demonstrate similar phenotypic changes: torenia transformants harboring the sense gene produced high contrast corollas (group 3) that were not observed in antisense-gene-introduced plants, and the tube had a greater tendency to become lighter in color with the sense transgene than with the antisense transgene. However, some torenia transformants had high contrast corollas and there was no petunia-like white sector pattern (junction or Cossack dancer type). Nevertheless, it seems that transgene-mediated modification of flower color in both torenia and petunia are caused, at least in part, by the same gene-silencing mechanisms.

Petunia plants that harbor CHS transgene(s) often show clearly differently-colored flowers on
Fig. 8. Model for gene regulation through flower development in torenia transformants. I: the lip coloring period; II: the anther coloring period; III: the tube coloring period.

We have produced novel flower colors in torenia, for example, pastel, wavy patterned and parti-colored flowers. Genetic transformation is a useful method for breeding torenia flower color. In the future, breeding flower color by genetic transformation will be even more effective through progress in studies on construction of and synthetic pathways of flower pigments.

We expect that this experimental system will be a good model for analysis of gene expression, because examination can be performed by eye. There are many studies on gene expression in petunia plants harboring CHS or DFR transgenes because of the advantages mentioned above [15,18–22]. Torenia would also useful as an experimental plant for such studies, because it is easy to transform and it is taxonomically different from petunia. Another advantage of torenia is that it can easily flower in vitro [23], making experimentation more efficient.

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References


