Genetic Engineering of Novel Bluer-Colored Chrysanthemums Produced by Accumulation of Delphinidin-Based Anthocyanins

Naonobu Noda1,*, Ryutaro Aida1, Sanae Kishimoto1, Kanako Ishiguro2, Masako Fukuchi-Mizutani3, Yoshikazu Tanaka2 and Akemi Ohmiya1

1National Agriculture and Food Research Organization, Institute of Floricultural Science (NIFS), 2-1, Fujimoto, Tsukuba, Ibaraki, 305-8519 Japan
2Research Institute, Suntory Global Innovation Center Ltd., 1-1-1 Wakayamadai, Shimamoto, Mishima, Osaka, 618-8503 Japan
3Suntory Holdings Ltd, 1-1-1 Wakayamadai, Shimamoto, Mishima, Osaka, 618-8503 Japan
*Corresponding author: E-mail, naonobun@affrc.go.jp; Fax, +81-29-838-6841.

(Received May 16, 2013; Accepted July 29, 2013)

Chrysanthemums (Chrysanthemum morifolium Ramat.) have no purple-, violet- or blue-flowered cultivars because they lack delphinidin-based anthocyanins. This deficiency is due to the absence of the flavonoid 3',5'-hydroxylase gene (F3'5'H), which encodes the key enzyme for delphinidin biosynthesis. In F3'5'H-transformed chrysanthemums, unpredictable and unstable expression levels have hampered successful production of delphinidin and reduced desired changes in flower color. With the aim of achieving delphinidin production in chrysanthemum petals, we found that anthocyanin biosynthetic gene promoters combined with a CmF3H production in chrysanthemum petals, we found that anthocyanin biosynthetic gene promoters combined with a translational enhancer increased expression of some F3'5'H genes and accompanying delphinidin-based anthocyanin accumulation in transgenic chrysanthemums. Dramatic accumulation of delphinidin (up to 95%) was achieved by simple overexpression of Campanula F3'5'H controlled by a petal-specific flavanone 3-hydroxylase promoter from chrysanthemum combined with the 5'-untranslated region of the alcohol dehydrogenase gene as a translational enhancer. The flower colors of transgenic lines producing delphinidin-based anthocyanins changed from a red–purple to a blue hue in the Royal Horticultural Society Colour Charts. This result represents a promising step toward molecular breeding of blue chrysanthemums.

Keywords: Anthocyanin • Chrysanthemum • Delphinidin • Flower color • Metabolic engineering.

Abbreviations: ADH, alcohol dehydrogenase gene; CaMV, Cauliflower mosaic virus; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; CmF3H, chrysanthemum flavanone 3-hydroxylase gene; EF-1α, elongation factor 1α; F3'5'H, flavonoid 3',5'-hydroxylase; F3'H, flavonoid 3'-hydroxylase; GUS, β-glucuronidase; LC-FTICR MS, liquid chromatography–Fourier transform ion cyclotron resonance mass spectrometry; MS medium, Murashige and Skoog medium; RHSCC, Royal Horticultural Society Colour Chart; RT–qPCR, real-time quantitative reverse transcription–PCR; 5'-UTR, 5'-untranslated region.

Introduction

Flower color is a major factor in pollination by insect or bird attraction, and is also a major selection trait of flower breeders. Anthocyanin pigments play an important role in flower color development. The color of anthocyanins shifts towards blue hues as the number of hydroxyl groups on the B-ring of anthocyanidins, which is the chromophore of anthocyanins, increases. Most blue-hued (purple, violet or blue) flowers contain delphinidin-based anthocyanins, with 3',4',5'-trihydroxy substituents in the B-ring, whereas red and magenta flowers contain pelargonidin- or cyanidin-based anthocyanins, with 4'-monohydroxy or 3',4'-dihydroxy substituents. The enzymes that determine hydroxylation are flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H), which are Cyt P450 enzymes (Holton and Cornish 1995; Fig. 1). cDNA cloning of F3'5'H has been reported from Petunia hybrida (petunia) and other plants (Holton et al. 1993, Tanaka 2006). These achievements have provided the molecular tools for molecular breeding of blue-hued flowers. Extending the anthocyanin pathway to delphinidin-based anthocyanins in ornamental plants lacking blue flower color is also of industrial interest. In recent years, expression of F3'5'H and accompanying delphinidin production in the petals of Dianthus caryophyllus (carnation) and Rosa hybrida (rose) has been successful in yielding flowers with a blue/violet hue that are now available in the world cut-flower market (Katsumoto et al. 2007, Chandler and Sanchez 2012, Tanaka and Brugliera 2013). Chrysanthemum morifolium (chrysanthemum), a member of the Asteraceae family, is among the most important plants in the world floriculture industry. Chrysanthemums are the...
most popular cut flower in Japan. Their production and sale account for >30% of the Japanese domestic flower market. Many varieties of chrysanthemums have been developed by both extensive cross-breeding and mutation breeding, resulting in a variety of flower colors, including white, pink, magenta, red, orange and yellow. These colors are mainly derived from red malonylated cyanidin glucosides and/or yellow carotenoids (Nakayama et al. 1997, Kishimoto et al. 2004). 7-Glycosylflavones in the ray florets (Chen et al. 2012) may also influence chrysanthemum flower color as co-pigments. However, no purple-, violet- or blue-flowered chrysanthemums have been developed to date because the species lacks delphinidin-based anthocyanins that are dominant in most purple, violet and blue flowers. Therefore, a new range of chrysanthemum varieties having purple, violet or blue flowers will be of great commercial value.

\( F3'5'H \) in the genera Pericallis, Osteospermum and Callistephus of the Asteraceae family evolved from the \( F3'H \) that was grouped into the CYP75B, a Cyt P450 playing a critical role in the flavonoid biosynthetic pathway (Seitz et al. 2006). However, \( F3'5'H \) is not present in species of the genus Chrysanthemum. The absence of production and accumulation of delphinidin-based anthocyanins is due to the absence of \( F3'5'H \). Therefore, it is difficult to produce purple, violet or blue chrysanthemums by conventional breeding methods. Accordingly, genetic engineering techniques have been applied toward this goal. However, unpredictable and unstable heterologous \( F3'5'H \) gene expression and/or its protein expression have prevented delphinidin production in chrysanthemums, and therefore breeding of blue-hued chrysanthemums has not been achieved by genetic engineering (Kim et al. 1998, Seo et al. 2007).

In transgenic roses with violet flowers, the constitutive Cauliflower mosaic virus (CaMV) 35S promoter induced the expression of a \( F3'5'H \) transgene, leading to successful production of delphinidin-based anthocyanins in petals (Katsumoto et al. 2007). In chrysanthemums transformed with GUS (β-glucuronidase) under the control of the CaMV 35S promoter, the activity of GUS has been reported to decrease in nearly all
transgenic plants after 1 year following transformation (Takatsu et al. 2000). With the aim of achieving stable gene expression in chrysanthemums, we found that the Nicotiana tabacum elongation factor 1α (EF-1α) gene promoter efficiently promoted transgene expression in chrysanthemums (Aida et al. 2005, Ohmiya et al. 2006). However, introduction of F3′S′H cDNAs from various plants, including P. hybrida (petunia), Lavandula sp. (lavender) or Clitoria ternatea (butterfly pea), under the control of the EF-1α promoter failed to yield chrysanthemums with delphinidin-accumulating flowers (unpublished data). Expression of a foreign gene in transgenic plants is often unpredictable, and it is not always easy to achieve the proper level of expression of the gene in the target species. Genes encoding the same enzymatic activity in different plant species may perform differently in transgenic plants, depending on the target species (Togami et al. 2006).

In addition to increasing transcription, up-regulating translational efficiency per transcript is important (Sugio et al. 2008). The 5′-untranslated region (5′-UTR) of the Nicotiana alcohol dehydrogenase gene (NtADH-5′-UTR) enhances the translational efficiency of GUS in chrysanthemums. NtADH-5′-UTR enhances the translational efficiency of GUS to 100 times that without NtADH-5′-UTR in chrysanthemums (Aida et al. 2008).

In the present study, we investigated the effective combination of a promoter, translational enhancer and F3′S′H gene to achieve delphinidin production and accompanying changes in flower color from magenta or pink to purple or violet in the ray florets of chrysanthemums.

Results

A suitable promoter for delphinidin production in chrysanthemum

To identify a suitable promoter for delphinidin production and accumulation in ray florets of the chrysanthemum line 94-765, we generated transgenic chrysanthemum lines using Viola witrockiana ‘Black Pansy’ F3′S′H cDNA [clone BP18 (FWS6950) or clone BP40 (AB332097)] driven by various promoters. We chose the pansy F3′S′H because it worked well for producing novel violet-colored transgenic roses and carnations (Katsumoto et al. 2007, Tanaka and Brugliera 2013). The promoters tested were the constitutive promoter CaMV 35S and six anthocyanin biosynthetic gene promoters. Each of these promoters was fused to the pansy F3′S′H cDNAs with or without NtADH-5′-UTR and transferred to chrysanthemum plants. Transformations using each of the binary vector constructs generated paromomycin-resistant lines. Ray florets from individual lines were analyzed for aglycone anthocyanins by HPLC. The delphinidin aglycone in the acid hydrolysate of petal extracts was found in six lines transformed with F3′S′H constructs driven by five different promoters (Table 1).

The binary vector pB247, containing chrysanthemum flavanone 3-hydroxylase (CmF3H) proc: NtADH-5′-UTR:pansy F3′S′H, showed the best results, with maximum delphinidin contents of 26.8% in total petal anthocyanidins with an average of 14.9%. Six other promoters, Gerbera hybrida (gerbera) chalcone synthase gene (CHS), rose CHS, Rosa rugosa (rugosa rose) dihydrolavandulol 4-reductase gene (DFR), rugosa rose flavanone 3-hydroxylase gene (F3H), pansy F3′S′H BP40 and the CaMV 35S promoter showed trace amounts of up to 5.4% of delphinidin in total anthocyanidins of ray florets (Table 1). Petal color change was hardly observed in the transgenic plants transformed with these vectors. The results indicated that CmF3H promoter-driven F3′S′H is the most effective for producing delphinidin-based anthocyanins in chrysanthemum petals. Accordingly, we used the CmF3H promoter in further experiments.

A suitable F3′S′H cDNA clone for delphinidin accumulation

To evaluate the activity of various F3′S′H cDNAs in the chrysanthemum line 94-765, the delphinidin content in the ray florets of transgenic lines was analyzed. An additional eight F3′S′H cDNAs from various plant species tested with the CmF3H promoter were combined with a translational enhancer. Results of anthocyanidin analyses of the generated transgenic flowers are summarized in Table 2. F3′S′H derived from Pericallis hybrida (cineraria; maximum 35.9%, average 8.6%) and Verbena hybrida (verbena; maximum 28.4%, average 8.9%) showed moderate delphinidin contents, with almost the same levels as that of the pansy F3′S′H (maximum 26.8%, average 14.9%). F3′S′H cDNA from Campanula showed the highest delphinidin content of 88.0% in total anthocyanidins with an average of 37.5%, and the accumulation of delphinidin was confirmed in 32 of 37 pigment-analyzed plants (Table 2). In contrast, Lobelia erinus (lobelia) #1, lobelia #4 and Gentiana triflora (gentian) F3′S′H overexpressing transgenic lines had no delphinidin-based anthocyanins in their petals. The flower color of chrysanthemum line 94-765 producing delphinidin was altered from red-purple group 61A to purple group 77C (around 50% delphinidin content) or violet group 83B (>75% delphinidin content) on the Royal Horticultural Society Colour Chart (RHSCC).

Effect of translational enhancer

We also tested the effect of the ADH-5′UTR from tobacco, Arabidopsis thaliana and Oryza sativa (rice), reported as translational enhancers (Satoh et al. 2004, Aida et al. 2008, Sugio et al. 2008), on delphinidin production. In addition to the CmF3H promoter, rose CHS and Perilla anthocyanin 3-acyltransferase gene (3AT) promoters were used to drive Campanula F3′S′H fused with the ADH-5′UTR in chrysanthemums. Unexpectedly, the average delphinidin content in each transgenic group, having a translational enhancer with the CmF3H promoter, ranged from 37.5% to 54.0%, similar to the average (49.4%) of plants without a translational enhancer (Table 3).

In contrast, the highest delphinidin content in each transgenic group with a translational enhancer ranged from 80.5% to 95.1%, which were higher than the value of plants without a
Table 1 Chrysanthemum $F3H$ promoter-driven $F3'S'H$ highly directed delphinidin-type anthocyanin production in transgenic chrysanthemum

<table>
<thead>
<tr>
<th>Binary vector</th>
<th>Transgene</th>
<th>Pro: $F3'H$ Enhancer</th>
<th>$NtADH5'$ $F3'S'H$ Terminator</th>
<th>No. of transgenic plants</th>
<th>No. of delphinidin-producing plants</th>
<th>Best (%)</th>
<th>Ave (%)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB247</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>T NOS</td>
<td>5</td>
<td>4</td>
<td>26.8</td>
<td>14.9</td>
<td>6.9</td>
</tr>
<tr>
<td>pSPB3341</td>
<td>CaMV 35S</td>
<td></td>
<td>T NOS</td>
<td>6</td>
<td>3</td>
<td>0.7</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>pBBBP18</td>
<td>Rose $F3H$</td>
<td></td>
<td>T NOS</td>
<td>21</td>
<td>7</td>
<td>5.4</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>pBBBP18-ADH</td>
<td>Rose $F3H$</td>
<td></td>
<td>T NOS</td>
<td>29</td>
<td>5</td>
<td>1.9</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>pSPB3323</td>
<td>Pansy $F3'S'H$ BP40</td>
<td></td>
<td>T NOS</td>
<td>6</td>
<td>4</td>
<td>1.4</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>pSLF724</td>
<td>Rugosa rose $DFR$</td>
<td></td>
<td>T NOS</td>
<td>3</td>
<td>1</td>
<td>0.6</td>
<td>0.6</td>
<td>–</td>
</tr>
<tr>
<td>pSLF814</td>
<td>Rugosa rose $F3H$</td>
<td></td>
<td>T NOS</td>
<td>3</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pSPB3380</td>
<td>Gerbera $F3H$</td>
<td></td>
<td>T NOS</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$ Percentage of delphinidin-based anthocyanins in total anthocyanins of ray florets.

Table 2 Campanula $F3'S'H$ showed effective delphinidin accumulation in transgenic chrysanthemum ray florets

<table>
<thead>
<tr>
<th>Binary vector</th>
<th>Transgene</th>
<th>Pro: $F3'H$ Enhancer</th>
<th>$NtADH5'$ $F3'S'H$ Terminator</th>
<th>No. of transgenic plants</th>
<th>No. of delphinidin-producing plants</th>
<th>Best (%)</th>
<th>Ave (%)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB244</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>37</td>
<td>32</td>
<td>88.0</td>
<td>37.5</td>
<td>4.7</td>
</tr>
<tr>
<td>pB239</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>44</td>
<td>38</td>
<td>35.9</td>
<td>8.6</td>
<td>1.1</td>
</tr>
<tr>
<td>pB243</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>12</td>
<td>11</td>
<td>28.4</td>
<td>8.9</td>
<td>2.5</td>
</tr>
<tr>
<td>pB247</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>5</td>
<td>4</td>
<td>26.8</td>
<td>14.9</td>
<td>6.9</td>
</tr>
<tr>
<td>pB237</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>5</td>
<td>1</td>
<td>4.4</td>
<td>4.4</td>
<td>–</td>
</tr>
<tr>
<td>pB238</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>1</td>
<td>1</td>
<td>2.9</td>
<td>2.9</td>
<td>–</td>
</tr>
<tr>
<td>pB242</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>19</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pB245</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>11</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pB246</td>
<td>Chrysanthemum $F3H$</td>
<td></td>
<td>TNOS</td>
<td>20</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$ Percentage of delphinidin-based anthocyanins in total anthocyanins of ray florets.

Table 3 Suitable combination of different promoters and $ADHS'$ translational enhancers for delphinidin accumulation in transgenic chrysanthemum

<table>
<thead>
<tr>
<th>Binary vector</th>
<th>Campanula $F3'S'H$ gene cassette</th>
<th>$ADHS'$ translational enhancer$^a$</th>
<th>Terminator</th>
<th>Host</th>
<th>No. of transgenic plants</th>
<th>No. of delphinidin-producing plants</th>
<th>Delphinidin content$^b$</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB315</td>
<td>Chrysanthemum $F3H$</td>
<td>Tobacco</td>
<td>NOS</td>
<td>Taihei</td>
<td>42</td>
<td>22</td>
<td>95.1</td>
<td>50.0</td>
</tr>
<tr>
<td>pB317</td>
<td>Chrysanthemum $F3H$</td>
<td>Arabidopsis</td>
<td>94-76S</td>
<td>36</td>
<td>33</td>
<td>92.1</td>
<td>54.0</td>
<td>4.4</td>
</tr>
<tr>
<td>pB315</td>
<td>Chrysanthemum $F3H$</td>
<td>Tobacco</td>
<td>94-76S</td>
<td>37</td>
<td>32</td>
<td>88.0</td>
<td>37.5</td>
<td>4.7</td>
</tr>
<tr>
<td>pB316</td>
<td>Chrysanthemum $F3H$</td>
<td>Rice</td>
<td>94-76S</td>
<td>7</td>
<td>7</td>
<td>80.5</td>
<td>41.0</td>
<td>9.2</td>
</tr>
<tr>
<td>pB304</td>
<td>Chrysanthemum $F3H$</td>
<td>No</td>
<td>94-76S</td>
<td>11</td>
<td>10</td>
<td>76.3</td>
<td>49.4</td>
<td>6.1</td>
</tr>
<tr>
<td>pB300</td>
<td>Perilla $3AT$</td>
<td>Tobacco</td>
<td>3AT</td>
<td>Taihei</td>
<td>23</td>
<td>6</td>
<td>54.8</td>
<td>36.6</td>
</tr>
<tr>
<td>pB307</td>
<td>Perilla $3AT$</td>
<td>No</td>
<td>94-76S</td>
<td>38</td>
<td>22</td>
<td>55.8</td>
<td>21.3</td>
<td>3.1</td>
</tr>
<tr>
<td>pB302</td>
<td>Rose $CH5$</td>
<td>Tobacco</td>
<td>NOS</td>
<td>94-76S</td>
<td>17</td>
<td>9</td>
<td>49.7</td>
<td>23.0</td>
</tr>
</tbody>
</table>

$^a$ Translational enhancer ($ADH5$ UTR) was directly fused to the start codon of Campanula $F3'S'H$.

$^b$ Percentage of delphinidin-based anthocyanins in total anthocyanins of ray florets.
translational enhancer (76.3%). It appeared that a suitable translational enhancer was required for a high level (>80%) of delphinidin content. Transgenic chrysanthemums, carrying the Perilla 3AT promoter-driven Campanula F3′S′H, showed the highest delphinidin content of 54.8% or 55.8%, and averages of 36.6% or 21.3% with or without a translational enhancer, respectively. Thus, our results suggested that a translational enhancer was not necessary to enhance the accumulation of delphinidin in transgenic chrysanthemums expressing CmF3H or Perilla 3AT promoter-driven F3′S′H.

**Flavonoids in transgenic chrysanthemums**

Anthocyanins in the transgenic flowers were revealed to be delphinidin 3-(6″-malonyl)glucoside [m/z, 551.1029 (actual mass: 551.10350); MS/MS m/z, 303.10, 304.20, 465.04] and delphinidin 3-(3″,6″-dimalonyl)glucoside [MS m/z, 637.103281 (actual mass: 637.103807; MS/MS m/z, 303.10, 304.20, 550.99] by liquid chromatography–Fourier transform ion cyclotron resonance (LC-FTICR) mass spectrometry analyses. These anthocyanins are delphinidin derivatives analogous to natural chrysanthemum cyanidin-based anthocyanins. The delphinidin aglycone was also identified with the authentic anthocyanidin by HPLC analyses of acid hydrolysates from transgenic chrysanthemum lines (Fig. 2A).

F3′S′H is thought to act in flavone and anthocyanin biosynthesis (Fig. 1). Luteolin- and apigenin-based flavones accumulated in ray florets of the transgenic chrysanthemums, whereas only trace amounts of the tricetin, 3′,4′,5′-trihydroxyflavone, were detected in higher delphinidin-accumulating transgenic lines (Fig. 2B). No change of B-ring hydroxylation of the flavone that accompanied the expression of the F3′S′H gene was observed. This result indicated that the CmF3H promoter-driven F3′S′H was not expressed at the synthesis stage or location of flavones.

**Relationship between delphinidin content and flower color**

Petals containing approximately 30% of delphinidin-based anthocyanins such as pansy F3′S′H expression lines (Fig. 3B right, 27% content) or cineraria F3′S′H expression lines (Fig. 3C right, 36% content) showed a visible color change toward purple. Marked color changes were observed in flowers containing >50% of delphinidin-based anthocyanins (Fig. 3D, E). As shown in Fig. 3, transgenic chrysanthemums expressing Campanula F3′S′H contained up to 92% or 95% delphinidin in chrysanthemum line 94-765 (Fig. 3F) or ‘Taihei’ (Fig. 3H), and showed dramatic alterations in flower color from magenta (RHSCC red–purple group 61A) to purple (violet group 83B) or pink (red–purple group 65C) to violet (violet group 88C), respectively.

Our data indicated that changes in color hue from magenta/pink to purple/violet were accompanied by elevation of the percentage delphinidin content (Fig. 4A). The component a* value gradually decreased with an increase of delphinidin content. This negative correlation between the a* value and the percentage delphinidin content in petals clearly indicated that B-ring hydroxylation of anthocyanin, changing cyanidin to delphinidin, directly led to a reduction in redness of flower color in transgenic chrysanthemums (Fig. 4B). There was also a negative correlation between the b* value and the delphinidin content within the range of 0% to approximately 50% (corresponding to an increase in bluish color). However, at higher delphinidin contents, of over about 50%, no further reduction in the b* value was observed (Fig. 4C).

**Expression of Campanula F3′S′H in transgenic chrysanthemums**

Relative transcript levels of the Campanula F3′S′H transgene in petals at the flowering stage and in leaves and stems were evaluated using the actin gene as a reference by real-time quantitative reverse transcription–PCR (RT–qPCR). The normalized
F3’SH expression level of transgenic line 1352-18, which contained delphinidin-based anthocyanins as 50% of total anthocyanins in ray florets, was used as a calibrator, with relative expression 1.0. The increase in delphinidin content in Campanula F3’SH transformants may be due to transcriptional up-regulation of the new pathway resulting from the genetically engineered formation of delphinidin-based anthocyanins. RT–qPCR analysis revealed that the transcript levels of F3’SH in transformants that contained 80–95% delphinidin-based anthocyanins were 1.5- to 2.5-fold higher than those in the 50% delphinidin-containing transformant line 1352-18 (Fig. 5). No expression of the Campanula F3’SH transgene was observed in the petals of wild-type plants or of transgenic line 1429-30, which did not contain delphinidin-based anthocyanins. No expression of the F3’SH transgene was also observed in leaves and stems of transformants showing >90% delphinidin content in petals (data not shown).

**Discussion**

**Suitable promoters**

Introduction of F3’SH, isolated from various plants, under the control of the tobacco EF-1α promoter (Aida et al. 2005) failed to yield delphinidin in transgenic chrysanthemums. Furthermore, the chrysanthemum actin promoter and the CaMV 35S
In most plants, anthocyanin biosynthetic genes are expressed only in specific tissues or organs, or are expressed time specifically (Koes et al. 2005). It is suggested that a tissue- or organ-specific or a time-specific promoter is required for suitable expression of transgenes in transgenic plants. In molecular breeding for violet-colored carnation, the Antirrhinum majus CHS promoter was successfully used to express a F3'S'H transgene (Tanaka and Brugliera 2013). In rose (R. hybrida), expression of the F3'S'H transgene was achieved by an enhanced CaMV 35S or rose CHS promoter (Katsumoto et al. 2007). These results indicated that an anthocyanin biosynthetic gene promoter is suitable for promoting F3'S'H expression. Expression of pansy F3'S'H under the control of the rose CHS promoter in the daisy-type chrysanthemum 'Improved Reagan' and decorative-type chrysanthemum Sei 050-0382 line resulted in 37% and 50% delphinidin content, respectively (Brugliera et al. 2013). In our study, pansy F3'S'H under the control of the rose CHS promoter has not successfully produced delphinidin-based anthocyanins in decorative-type transgenic chrysanthemums. We found in this study that the CmF3H promoter had superior potential to that of other promoters, such as gerbera CHS, rose CHS, rugosa rose DFR, rugosa rose F3H and pansy F3'S'H BP40 for modifying chrysanthemum anthocyanin biosynthesis (Tables 1, 2). The Perilla 3AT promoter and rose CHS promoter were also used to express Campanula F3'S'H fused with the ADH-5'UTR in chrysanthemums. The delphinidin contents in the ray florets of chrysanthemums transformed with the Campanula F3'S'H driven by the two promoters were as high as 54.8% (average 36.6%) and 49.7% (average 23.0%), respectively (Table 3). However, these promoters were insufficient for delphinidin production relative to the CmF3H promoter. Although various promoters such as nopaline synthase (nos) and manopine synthase (mas) had been used for introduction of agronomic traits to chrysanthemum by genetic engineering (Shinozaki et al. 2012), for the engineering of the anthocyanin biosynthetic pathway in ray florets, the CmF3H promoter seemed to be the most suitable in this study. To date, the purple/violet transgenic chrysanthemums expressing Campanula F3'S'H under the control of the CmF3H promoter stably maintained their delphinidin contents and flower color phenotypes throughout vegetative propagation and cultivation in the greenhouse for 4 years (data not shown).

**Suitable F3’S’H gene for delphinidin production**

Selection of a suitable plant species as a gene source is important for successful redirection of the biosynthetic pathway; expression of gerbera DFR cDNA produced more pelargonidin than rose DFR cDNA in torenia (Nakamura et al. 2010). Introduction of petunia or pansy F3’S’H cDNA resulted in the accumulation of delphinidin-based anthocyanins in carnations, roses (Tanaka and Brugliera 2013) and chrysanthemums (Brugliera et al. 2013). In particular, pansy F3’S’H was the most effective among the tested F3’S’H cDNAs for producing
transgenic line 1433 carried the \textit{CamF3} gene and delphinidin-based anthocyanin content in ray florets among transformants. The levels of \textit{F3} correlated with up-regulation of \textit{F3}, which contained approximately 50% of delphinidin-based anthocyanins in the ray florets. An increase in delphinidin content appeared to be transformant of ‘Taihei’. Transgenic lines 1340, 1348, 1352 and 1408 carried the chrysanthemum flavanone 3-hydroxylase gene (\textit{CmF3H}) promoter-driven \textit{Nicotiana} \textit{F3} with RNA interference-mediated endogenous \textit{F3} (Tanaka and Brugliera 2013). Whereas overexpression of pansy delphinidin-based anthocyanins in rose (Katsumoto et al. 2007, Fig. 5), \textit{Campanula} flavonoid 3’,5’-hydroxylase (\textit{F3’S’H}) transgene and delphinidin-based anthocyanin content in ray florets of transgenic chrysanthemums was more efficient than the petunia or \textit{Eustoma grandiflorum} (lisianthus) \textit{F3’S’H} in tobacco (Okinaka et al. 2003). These results suggest that \textit{Campanula} \textit{F3’S’H} may be compatible with the enzyme complex, called a metabolon, of the anthocyanin biosynthetic pathway in various host plants. Furthermore, \textit{Campanula} \textit{F3’S’H} may have kinetic properties suitable for dihydrokaempferol (DHK) catalysis such as a higher affinity for DHK than endogenous F3’H, DFR, flavonol synthase (FLS) and other transgenic \textit{F3’S’H}. Alternatively, the gene and enzyme were more efficiently transcribed and translated, or the enzyme was more stable than endogenous enzymes and other transgenic \textit{F3’S’H}. The reason for stable \textit{Campanula} \textit{F3’S’H} expression in some host plants remains to be investigated. We have demonstrated that the most suitable combination of promoter and \textit{F3’S’H} gene for delphinidin production in chrysanthemum petals is of the \textit{Chrysanthemum} \textit{F3H} promoter and \textit{Campanula} \textit{F3’S’H}.
Effect of ADH translational enhancer

We used the ADH translational enhancer in this study in view of its potential to induce efficient translation in chrysanthemums (Aida et al. 2008). We examined the effect of three ADH-5′ UTRs on the accumulation of delphinidin in petals of transformants by introducing constructs of Campanula F3′5′H fused with the ADH-5′ UTR (Table 3). The best lines of delphinidin-accumulating chrysanthemums were transformants into which Campanula F3′5′H fused with a tobacco or Arabidopsis translational enhancer had been introduced. However, no significant differences in the average delphinidin contents of transgenic lines with or without various ADH translational enhancers were found. Although we expected that the effect would be lower in dicotyledonous plants, purple-colored transformants were also obtained with a rice translational enhancer (Sugio et al. 2008; Table 3; Supplementary Fig. S1). We speculated that the original cis-element that functions as a translational enhancer is present in the Chrysanthemum F3H and Perilla 3AT promoter; therefore, an additional translational enhancer did not have a significant positive or negative effect on the translational efficiency of F3′5′H.

Expression of exogenous F3′5′H and endogenous chrysanthemum DFR is sufficient for delphinidin-based anthocyanin accumulation

A high delphinidin content in ray florets was shown to be associated with up-regulation of the Campanula F3′5′H transgene (Fig. 5). It is clear that high levels of F3′5′H transgene expression are necessary for accumulation of delphinidin-based anthocyanins in chrysanthemums. However, several transformants such as lines 1433-3 and 1340-10 showed a low delphinidin content relative to their high F3′5′H expression levels. Given that the translational enhancer ADH-5′ UTR was combined with F3′5′H, we concluded that the translation level was not always proportional to the number of transcripts. Moreover, translational efficiency of the F3′5′H mRNA and/or other factors may determine the final delphinidin content.

On the molecular breeding of transgenic carnation with bluer flowers, a line lacking endogenous DFR was used as a host plant, and an exogenous Petunia DFR gene, which has high potential to catalyze dihydromyricetin to leucodelphinidin, precursor of delphinidin, was additionally introduced with F3′5′H (Tanaka and Brugliera 2013). To generate a transgenic rose with bluer flowers, exogenous Iris hollandica DFR was also introduced for efficient delphinidin accumulation (Katsumoto et al. 2007, Tanaka and Brugliera 2013). In contrast, in this study, we demonstrated that endogenous Chrysanthemum DFR could catalyze the reaction leading to delphinidin, and no exogenous DFR gene was a prerequisite for the accumulation of delphinidin in transgenic chrysanthemums.

Future prospects

In the present study, the amount of delphinidin in the petals of the transformants ranged as high as 95% of total anthocyanins. Substitution of anthocyanin aglycone from cyanidin to delphinidin dramatically changes chrysanthemum ray floret color from magenta/pink to purple/violet.

To develop bluer coloration, a further decrease in the a* value (decrease of redness) and b* value (increase of blueness) is required. To approach 100% delphinidin accumulations, we propose to engineer the suppression of the endogenous F3′H gene and/or the overexpression of the exogenous DFR gene in addition to overexpressing Campanula F3′5′H. In addition to delphinidin production, manipulation of blue color development by polyacylation, co-pigmentation, metal complexation and vacuolar pH conditions appeared to be necessary to obtain true blue chrysanthemum flowers (Tanaka et al. 2008, Yoshida et al. 2009).

Conclusions

In this study, we modified the anthocyanin biosynthesis pathway in chrysanthemum to produce delphinidin-based anthocyanins instead of producing cyanidin-based anthocyanins by metabolic engineering. We established a method for molecular breeding of delphinidin-producing chrysanthemums. Furthermore, we successfully obtained transgenic chrysanthemum plants producing high levels of delphinidin-based anthocyanins in ray florets and exhibiting color changes toward the purple/violet group of the RHSCC. A Chrysanthemum F3H promoter-driven ADH translational enhancer-fused Campanula F3′5′H efficiently induced delphinidin production in chrysanthemum ray florets, leading to high accumulation of delphinidin (up to 92% in chrysanthemum line 94-765 and up to 95% in ‘Taihei’).

Materials and Methods

Plant materials

Chrysanthemum morifolium Ramat (chrysanthemum) strain 94-765 (kindly provided by Seikoen Co. Ltd.) and ‘Taihei’ were sterilized, maintained in vitro, and subjected to Agrobacterium-mediated transformation as described previously (Aida et al. 2004, Aida et al. 2005), using Agrobacterium, tumefaciens strain EHA105 harboring a binary vector. Tissue cultured plants were vegetatively maintained on half-strength Murashige and Skoog (MS) salt medium without hormone containing 3% sucrose and 0.2% gellan gum at 25°C. Paromomycin-resistant regenerated plants were potted after habituation for 1 month and chilling treatment at 4°C for 1 month. Transgenic chrysanthemum plants were grown in a glasshouse for genetically modified plants and compared with control host plant. The day-length was adjusted to 12 h to induce short-day flowering.

Binary vector construction containing the promoter region of CmF3H

The promoter region of CmF3H was amplified by PCR using as template genomic clone DGF3H (DDBJ/GenBank/EMBL...
accession No. FWS70861), which was obtained by subcloning a DNA fragment containing an F3H promoter region obtained by screening a genomic DNA library using a partial cDNA fragment of chrysanthemum F3H (Y. Kanno, S. Kimura, K. Kazuma, H. Tsugawa, S. Kiyokawa and M. Suzuki, personal communication). The CmF3H promoter DNA fragment (FWS70860) amplified by PCR using HANS-F3Hpro1k-Fd and NSM-F3Hpro-Rv primers (Supplementary Table S1) was cloned into pCR2.1 (Invitrogen) to obtain pCR HANS-CmF3Hp-SN. Next, a binary vector fragment obtained by digesting pBI121 ADHNF (Satoh et al. 2004) with HindIII and Xbal, and a Chrysanthemum F3H promoter DNA fragment obtained by digesting pCR HANS-CmF3Hp-SN with HindIII and Nhel were ligated to obtain pBI121 HANS-CmF3Hp1k-S.

**Transformation of chrysanthemum with F3’S’H transgenes**

The Perilla anthocyanin 3AT promoter (FWS70911) was derived from a Perilla frutescens var. crispa genomic clone. The rose CHS promoter (FWS56964) was selected based on the report from Katsumoto et al. (1997). Viola wittrockiana ‘Black Pansy’ F3’S’H BP40 (FWS70840), R. rugosa (rugosa rose) DFR (AB811950), rugosa rose F3H (AB811949), and G. hybrida CHS (AB811951) promoters were also used to transcribe F3’S’H in transgenic chrysanthemums.

F3’S’H cDNAs from Campanula medium (FWS70877), E. grandiflora (AB078957, Noda et al. 2004), L. erinus (Jobelila #1 (AB221078) and #4 (AB221078)), C. ternatea (butterfly pea) (AB185900), V. wittrockiana ‘Black Pansy’ BP18 (FWS56950, Katsumoto et al. 2007) and BP40 (AB332097, Katsumoto et al. 2007)], Antirrhinium kelloggii (climbing snapdragon; AB547159, Ishiguro et al. 2012), Pericallis hybrida (cineraria; FWS70902), G. triflora (gentian; DS5184), and V. hybrida (verbena; AB234898, Togami et al. 2006), were mainly used to induce overexpression in chrysanthemums.

ADH-S’UTR:F3’S’H cDNAs were mainly ligated into pBI121 HANS-CmF3Hp1k-S. The binary vectors pB239, pB244, pB243, pB247, pB238, pB242, pB315, pB316, pB317, pB304, pB300, pB307 and pB302 were constructed from pBI121, pG121-Hm and pBinPLUS binary vectors were also used. pG121-Hm was used for vectors pB237, pB245 and pB246. pBinPLUS was used for vectors pSPB3341, pSPB3380, pSPB3323, pBRBP18, pBRBP14-ADH, pSLF724 and pSL814. The AtADH-S’UTR and OsADH-S’UTR were synthesized according to the method by Sugio et al. (2008). The F3’S’H cDNAs were driven by various promoters, and terminators of the Agrobacterium nopaline synthase gene (NOS) or Perilla 3AT were used. The constructs were confirmed by DNA sequencing using an ABI PRISM sequencer 3100 or 3110x and Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Binary vectors were transformed into A. tumefaciens strain EHA105 by electroporation using a Micropulser (Bio-Rad). Chrysanthemum line 94-765 and ‘Taihei’, which accumulate cyanidin 3-malonylglucosides, were transformed using constructed binary vectors and the Agrobacterium-mediated method (Aida et al. 2004).

**Construction of binary vector for Campanula F3’S’H expression**

A binary vector for tobacco ADH-S’UTR-fused Campanula F3’S’H expression was constructed as follows. Total RNA was extracted from the flower petals of commercially available Campanula flowers using the RNasy Mini Plant Kit (Qiagen), and first-strand cDNA was synthesized using the SuperScript One-Step RT-PCR System (Invitrogen). PCR was performed using this first-strand cDNA as a template under the following conditions: 25 cycles of PCR at 95°C for denaturation, 55°C for annealing and 72°C for extension. The specific primers CamF1 and CamR1 (Supplementary Table S1) were synthesized on the basis of F3’S’H cDNA of C. medium (FWS70877). The amplified fragment was cloned into pCR-TOPO II (Invitrogen) and designated as pSPB2561. Next, a vector obtained by fusing NtADH-S’UTR and Campanula F3’S’H cDNA was constructed. Two types of DNA fragments consisting of a DNA fragment amplified by PCR using pSPB2561 as a template and ADH-Campa-Fd and Hpal-Campa-Rv as primers (Supplementary Table S1) and a DNA fragment amplified by PCR using pBI121 ADH-221 (Satoh et al. 2004) as template and using Xbal-ADH-Fd and Campa-ADH-Rv as primers (Supplementary Table S1) were synthesized. A DNA fragment in which NtADH-S’UTR was directly coupled to the start codon of Campanula F3’S’H was obtained by PCR using these two types of DNA fragments as templates and using Xbal-ADH-Fd and Hpal-Campa-Rv as primers. This DNA fragment was then cloned into the pCR2.1-TA vector (Invitrogen) followed by digestion with Xbal and Hpal, and the resulting 650 bp fragment was ligated with a vector fragment obtained by digesting pSPB2561 with Xbal and Hpal to obtain a pCR vector containing NtADH-S’UTR-fused Campanula F3’S’H (pCR ADHNF-CamF3’S’H).

Next, pCR ADHNF-CamF3’S’H was digested with KpnI, followed by blunting with Blunting High (Toyobo), and digesting with Xbal; the resulting DNA fragment was ligated with pBI121 HANS-CmF3Hp-S digested with SpeI and EcoCR1 to obtain pB244: pBI121 CmF3Hpro-NtADH-S’UTR:Campanula F3’S’H:NOSter.

**Total RNA extraction and cDNA synthesis**

Total RNA was extracted from frozen petals, leaves and stems of chrysanthemum transformants. Total RNA was isolated using TRIzol reagent and a PureLink RNA Mini kit (Invitrogen) treated with PureLink DNase I (Invitrogen). cDNAs were synthesized from total RNA (2.5 μg) using the SuperScript III first-strand synthesis system (Invitrogen) with random hexamers according to the manufacturer’s instructions.

**Quantitative RT–PCR analysis**

The transcript levels of the F3’S’H transgene were analyzed by RT–qPCR with the SYBR Premix Ex Taq II polymerase (Takara Bio Inc.) and Thermal Cycler Dice TP800 (Takara Bio Inc.) according to the manufacturer's instructions. RT–qPCR mixtures were heated to 95°C for 20 s, followed by 40 cycles at 95°C
for 5 s and 60°C for 20 s. Campanula F3′5′H-specific primers Cam461_RTf1 and Cam547_RTR1 (Supplementary Table S1) were designed using the Primer3 program (Rozen and Skaletsky 1999). Expression levels of actin were used to normalize the transcript levels of each sample (Ohmiya et al. 2006). The primer pairs used for actin transcripts are listed in Supplementary Table S1.

Flavonoid pigment preparation and analyses
The aglycones of anthocyanins and flavones were extracted with 1 mL of methanol containing 1% HCl from frozen and pulverized ray florets (100–200 mg) of chrysanthemum. After centrifugation at 13,000 × g for 15 min, the extracts were added to an equal volume of 4 M HCl aqueous solution and acid-hydrolyzed by 100°C for 1 h. The hydrolysate was applied to a Sep-Pak C18 Plus Light cartridge (Waters) and then washed with 0.05 M trifluoroacetic acid (TFA) in water; the flavonoid fraction was eluted with 300 μL of 80% acetonitrile containing 0.05 M TFA. The eluate was analyzed with an Agilent 1100 Series HPLC system (Agilent Technologies).
HPLC was performed using an Intersil ODS-2 (particle size: 5 μm, 4.6 mm × 250 mm, GL Science) column and a solvent flow rate of 0.8 mL min⁻¹. The solvent program used was as follows: after a 20 min linear gradient elution from 6.25% to 25% acetonitrile and 5% to 20% acetic acid containing 15% phosphoric acid in distilled water, 5 min of isocratic elution with 25% MeCN containing 20% acetic acid and 1.5% phosphoric acid in H₂O was performed, and was detected with an Agilent 1100 photodiode array detector (Agilent Technologies), measuring absorbance at 250–600 nm. Detection was performed at 530 nm for anthocyanin(di)ns and 320 nm for flavones. A calibration curve was constructed with anthocyanidin (Extrasynthese) and flavone (Funakoshi) standards. Flavanoids were also analyzed by LC-FTICR MS for detection of high resolution mass and MS/MS number (Nakamura et al. 2010).

Flower color analysis
The colors of chrysanthemum ray florets were quantified by three replicated measurements of each transgenic line with a CD100 spectrophotometer (Yokogawa Meters & Instruments Corporation), set on the CIE L*a*b* system. The L* value indicates lightness of the color and represents the proportion of total incident light that is reflected. The axes in the L*a*b* color space are red–green a* and yellow–blue b*. Positive a* values indicate redness and negative a* values indicate greenness. Positive b* values indicate yellowness and negative b* indicate blueness. Hue angle (H° = arctan b*/a*) is derived from the L*a*b* color-space wheel with values stepped counterclockwise from red at 0° or 360°, yellow at 90°, green at 180° and blue at 270°. Floral colors were also identified according to RHSCC.

Funding
This work was supported by the National Agriculture and Food Research Organization (NARO) [a grant under the project ‘Development of innovative crops through the molecular analysis of useful genes’].

Acknowledgments
We are grateful to Dr. Ko Kato (Nara Institute of Science and Technology, NAIST) for providing the translational enhancer tobacco ADH-5'UTR, Professor Kazuki Saito (Chiba University) for providing the genomic DNA library of Perilla frutescens var. crispa, Aomori Green Biocenter, Aomori Prefectural Agriculture and Forestry Research Center (reorganized as Agriculture Research Institute, Aomori Prefectural Industrial Technology Research Center) for providing a genomic DNA clone containing the chrysanthemum F3H promoter and cDNA clones encoding F3′5′H from Citlaria, Eustoma and Lobelia, and Seikoen Co. Ltd. for providing chrysanthemum line 94-765. We also thank Ms. Yumi Ohtsuka, Ms. Kiyomi Shimizu, Ms. Misako Takahashi and Ms. Mayumi Amaki for technical assistance in the NIFS laboratory.

Disclosures
The authors have no conflicts of interest to declare.

References


