The identification of flavonoids and the expression of genes of anthocyanin biosynthesis in the chrysanthemum flowers

S.-M. CHEN1, C.-H. LI2,3, X.-R. ZHU1, Y.-M. DENG1, W. SUN4,5, L.-S. WANG2, F.-D. CHEN1* and Z. ZHANG1

College of Horticulture, Nanjing Agricultural University, Nanjing 210095, P.R. China
Beijing Botanical Garden, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, P.R. China
Graduate University of Chinese Academy of Sciences, Beijing 100049, P.R. China
College of Landscape Architecture, Beijing Forestry University, Beijing 100083, P.R. China
Urumqi Botanical Garden, Urumqi 830011, P.R. China

Abstract

In order to provide additional information on the coloration of chrysanthemum flowers, the flavonoid composition and the expression of six structural genes involved in anthocyanin pathway in the ray florets of a pink flowering (cv. H5) and two white flowering (cvs. Keikai and Jinba) Chrysanthemum grandiflorum cultivars were examined. HPLC-DAD/ESI-MSn analysis showed that cyanidin 3-O-(6″-O-malonylglucoside) and cyanidin 3-O-(3″,6″-O-dimalonylglucoside) were the two major flavonoids presented in H5, while white flowering cultivars contained flavones instead of anthocyanins. Nine flavone derivatives were detected in the three cultivars, the amount of each flavone varied upon cultivars, and seven of these were identified as luteolin 7-O-arabinosylglucuronide, apigenin 7-O-glucoside, luteolin 7-O-malonylglucoside, apigenin 7-O-malonylglucoside, chrysoeriol 7-O-malonylglucoside, acacetin 7-O-rutinoside and acacetin 7-O-malonylglucoside. The two white flowering cultivars showed similar total flavonoid content, which was about two fold higher than that in H5. A high expression of the genes encoding dihydroflavonol 4-reductase and 3-O-glucosyltransferase was detected only in H5 but not in Keikai or Jinba. Chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, and flavonoid 3′-hydroxylase were expressed in all flowers, suggesting that the lack of anthocyanin in white flowering cultivars cannot be due to any blockage of their expression.

Additional key words: Chrysanthemum grandiflorum, cyanidin, flavone, flower coloration, structural gene.

Introduction

The flavonoids, a class of secondary plant metabolites, are involved in many physiological processes, including flower coloration and protection against UV irradiation pathogens and pests. They have antioxidative properties (reviewed by Forkmann and Stefan 2001, Ahmed et al. 2009), regulate the auxin response or distribution (Rusak et al. 2010) and response to P-deficiency (Ismael and Mohamed 2010). The flavonoids have been sub-classified into anthocyanins, flavones and flavonols, etc. Anthocyanins are responsible for the purple, blue and red pigmentation of a number of plant tissues (Schwinn and Davies 2004), while the flavones and flavonols are presented in white carnation flowers (Mato et al. 2000). The flavonoid composition has been previously

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Abbreviations: Ac-7-MalGlc - acacetin 7-O-malonylglucoside; Ac-7-Rut - acacetin 7-O-rutinoside; ANS - anthocyanidin synthase; ANR - anthocyanidin reductase; Ap-7-G - apigenin 7-O-glucoside; Ap-7-MalGle - apigenin 7-O-malonylglucoside; Ch-7-MalGlc - chrysoeriol 7-O-malonylglucoside; CHI - chalcone isomerase; CHS - chalcone synthase; Cy-3-MalGlc - cyanidin 3-O-(6″-O-malonylglucoside); Cy-3-MalMalGlc - cyanidin 3-O-(3″, 6″-O-dimalonylglucoside); DFR - dihydroflavonol 4-reductase; F3H - flavanone 3-hydroxylase; F3′H - flavonoid 3′-hydroxylase; FNS - flavone synthase; 3GT - 3-O-glucosyltransferase; Lu-7-AraGluc - luteolin 7-O-arabinosylglucuronide; Lu-7-MalGlc - luteolin 7-O-malonylglucoside; OMT - O-methyltransferase.

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* Corresponding author; fax: (+86) 25 84395266, e-mail: chenfd@njau.edu.cn

458
investigated in flowers of several chrysanthemum cultivars. Saito et al. (1988) identified cyanidin using thin layer chromatography (TLC). Nakayama et al. (1997) used high performance liquid chromatography (HPLC), fast atom bombardment-mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) analysis to identify the cyanidin 3-O-(3′,6′-O-dimalonyl-ß-glucopyranoside). The known major flavones in chrysanthemum are apigenin (Ap), acacetin (Ac), luteolin (Lu), diosmetin and eriodictyol, presented in the form of either 7-O-glucosides, 7-O-malonylglucosides, 7-O-glucuronides or 7-O-rutinosides (Schwinn et al. 1993). In the Chrysanthemum morifolium flower, flavonoids also occur as aglycones and/or glucosides (Glc), glucuronides (Gluc), acetyl glucosides (AylGlc), neopesperidosides (Neo) or rutinosides (Rut) (Lai et al. 2007). However, the flavonoid and phenolic composition of C. morifolium, Artemisia annua and C. coronarium is quite variable, even though all three species belong to the same family (Lai et al. 2007). Flavonoid compositions can even vary between cultivars with the same flower colour in carnation (Mato et al. 2000). Since anthocyanins are sensitive to both pH (Castañeda et al. 2009) and heat (Prasain et al. 2004), it has been suggested that their correct identification can be dependent on the methods for extraction, separation and detection.

A number of enzymes are involved in the flavonoids biosynthesis (Fig. 1), genes encoding these enzymes have been isolated from many plants (Nesi et al. 2001). In the grape, flavonoid 3-O-glucosyltransferase (UGFT) is responsible for skin colour (Kereamy et al. 2002). In the apple, chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UGFT are all involved in the formation of the red pigment of the skin (Honda et al. 2002). In chrysanthemum, Lee et al. (2008) have compared the expression profiles of CHS, F3H, chalcone isomerase (CHI), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′,5′H), DFR, leucoanthocyanidin oxygenase (LDOX) and anthocyanidin synthase (ANS) transcripts in flowers of wild type and γ-ray induced mutant, but no correlation was found between the level of any of these and flower colour.

Here we attempted to characterize the flavonoid composition in the ray florets of one pink and two white-flowering chrysanthemums, and to determine the expression levels of a number of structural genes in the flavonoid pathway. These findings can enrich the understanding of flower pigmentation in chrysanthemum.

Fig. 1. Simplified biosynthesis pathway of flavonoids. Enzyme names are abbreviated as follows: CHS - chalcone synthase, CHI - chalcone isomerase, F3H - flavanone 3-hydroxylase, F3′H - flavonoid 3′-hydroxylase, F3′,5′H - flavonoid 3′,5′-hydroxylase, DFR - dihydroflavonol 4-reductase, ANS - anthocyanidin synthase, UGFT - UDP-glucose:flavonoid 3-O-glucosyltransferase.

Materials and methods

The chrysanthemum [Chrysanthemum grandiflorum (Ramat.) Kitam] cultivars H5 (pink flowering), Keikai and Jinba (white flowering) are maintained at the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Inflorescences were harvested at stage I (tight buds, only the tips of the H5 ray florets showing as pink), stage II (enlarged closed bud, upper 2/3 of the H5 ray florets pigmented), stage III.
(half-open flower with upright outer ray florets, fully pigmented in H5), and stage IV (fully open flower). The ray florets from stage I - IV inflorescences were used for transcript analysis, while flavonoid identification and quantification were performed on stage IV ray florets.

The extraction procedure followed Zhang et al. (2007) and Yang et al. (2009), with only minor modifications. Briefly, 0.5 g of fresh material was added to 3 cm³ of methanol: water: formic acid (70:27:2, v/v/v) mixture at 4 °C in the dark for 24 h, with vortexing every 6 h. The material was filtered through sheets of filter paper (Millipore, Billerica, MA, USA). Ultra-pure water was obtained from a Mill-Q system (Millipore, Billerica, MA, USA). Rutin (quercetin 3-O-rutinoside) as a standard was used for quantification of total flavonoid (TF) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The extraction procedure followed Zhang et al. (2007) and Yang et al. (2009), with only minor modifications. Briefly, 0.5 g of fresh material was extracted in 3 cm³ of methanol: water: formic acid (70:27:2, v/v/v) mixture at 4 °C in the dark for 24 h, with vortexing every 6 h. The material was filtered through sheets of filter paper (Hangzhou Special Paper Industry, Zhejiang, China), and the filtrate passed through a 0.22 μm reinforced nylon membrane filter (Shanghai ANPEL, Shanghai, China) before submission to HPLC-DAD/ESI-MSn analysis. Three replicate extractions were made from each biological sample.

HPLC system Dionex (Sunnyvale, CA, USA) equipped with P680 HPLC pump, UltiMate 3000 autosampler, TCC-100 column and PDA100 photodiode array detector was used to separate the constituents of the ray floret extracts. The column used was a C18 ODS-80Ts QA (150 × 4.6 mm i.d., Tosoh, Tokyo, Japan) protected with a CARB Sep Coregel 87C guard cartridge (Transgenomic, Omaha, NE, USA). An aqueous solvent of formic acid and acetonitrile represented the mobile phase (Lin et al. 2007, Li et al. 2009). A 10 mm³ aliquot was injected, and the resulting chromatograms were read at 515 nm for anthocyanins and 350 nm for flavonoids and flavonol.

Anthocyanins were initially identified on the basis of their HPLC retention time, elution order, UV-VIS spectrum, MS fragmentation pattern and by comparison with published data (Saito et al. 1988, Nakayama et al. 1997). The HPLC-ESI-MS² analysis was performed using an Agilent-1100 HPLC system coupled to a DAD system and an LC/MS Trap VL electrospray ion mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation conditions were as mentioned above. The MS conditions were: positive ion mode; gas (N₂) temperature 325 °C; flow rate 8 dm³ min⁻¹; nebulizer pressure 241.3 kPa; HV voltage 3.5 kV; octopole RF amplitude 150 Vpp; skim 1 voltage 37.5 V; skim 2 voltage 6.0 V; capillary exit 111.8 V; cap exit offset, 74.3 V and scan range, m/z 100 - 1000 u. The percentages of each flavonoid glycoside were obtained directly from the peak areas in chromatograms by adding the areas of flavonoid glycoside peaks. Flavonoid glycoside content was measured semi-quantitatively by linear regression of rutin and quantified at 350 nm as rutin equivalent (Zhang et al. 2007, Yang et al. 2009). For preparation of standard solution, rutin was accurately weighted, dissolved in methanol, and then diluted to appropriate concentrations. Total flavonoid (TF) content was calculated in the form of mg g⁻¹ rutin per ray floret fresh mass (f.m.) and the volume of samples injected into the HPLC is 10 mm³, equal for all samples. Each sample was run by HPLC repeated three times with the same conditions.

Real time quantitative PCR followed Yang et al. (2005). Total RNA was extracted from 0.2 g ray floret f.m. at each inflorescence stage using RNAiso reagent (Takara, Tokyo, Japan). Putative contaminating DNA was removed by DNasel (Takara) treatment. The first cDNA strand was synthesized from 2 μg total RNA using the SuperScript III first-strand synthesis system (Invitrogen, CA, USA) according to the manufacturer’s instruction. Real time quantitative PCR (RT-qPCR) of CHS, CHI, F3H, F3’H, DFR and 3GT was performed on a Bio-Rad (Hercules, USA) iQ5 PCR platform using SYBR GreenER qPCR SuperMix (Invitrogen) with the following amplification regime: incubation at 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 15 s. Chrysanthemum EF1α was used as a constitutively expressed control. Primer sequences (Table 1) were designed using Primer 5 software. The identities of the amplicons and the specificity of the reaction were verified by agarose gel electrophoresis and melting curve analyses. Three replicates of each RT-qPCR were performed, and the data were analysed using Bio-Rad iQ5 optical system software v.1.0. The level of gene expression was given by 2^ΔΔCt.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’)</th>
<th>Acc. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHS</td>
<td>F CAGGCTTTTCTCCATTAGTT</td>
<td>DQ521272</td>
</tr>
<tr>
<td></td>
<td>R GAGGACCGGTTTCCGAC</td>
<td></td>
</tr>
<tr>
<td>CHI</td>
<td>F TGGTGCAACCATGGACAGT</td>
<td>EU286277</td>
</tr>
<tr>
<td></td>
<td>R AAAATTTGCTTCGACATCGTAGT</td>
<td></td>
</tr>
<tr>
<td>F3H</td>
<td>F CATATTATTGGAGCAACGGGAAGA</td>
<td>U86837</td>
</tr>
<tr>
<td></td>
<td>R TGCCAATCTCTAAGGCAAGAT</td>
<td></td>
</tr>
<tr>
<td>F3’H</td>
<td>F CTTGACGGTGAGGAGGGAA</td>
<td>EU286276</td>
</tr>
<tr>
<td></td>
<td>R TGGGCTTGAAGGTTGAG</td>
<td></td>
</tr>
<tr>
<td>DFR</td>
<td>F TGGCCAGAGATCTAATGACCA</td>
<td>EF094936</td>
</tr>
<tr>
<td></td>
<td>R CGCCTTTGGCTAGGGTGCT</td>
<td></td>
</tr>
<tr>
<td>3GT</td>
<td>F ATCACAGGGACATCAACC</td>
<td>AB523845</td>
</tr>
<tr>
<td></td>
<td>R TCCACACCCAGACACTA</td>
<td></td>
</tr>
<tr>
<td>EF1α</td>
<td>F AGACCCCAAGACTACTGCGC</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>R CCACAAATCTTGTACACATCC</td>
<td></td>
</tr>
</tbody>
</table>
Results

Extracts of H5 generated two HPLC peaks at 515 nm (A1, A2, Table 2), in contrast, no peaks were detected in the ray florets extracts of Keikai or Jinba, showing that these cultivars produce no anthocyanin in their flowers. Their UV-VIS absorption spectra were identical to one another, with neither showing any absorption between 290 and 340 nm, indicating the absence of acylation (Fossen et al. 1998). The MS² analysis showed that the fractionations of the A1 and A2 peaks both included a fragment of m/z 287 u, typical for a [cyanidin]⁺ ion. Based on Saito et al. (1988) and Nakayama et al. (1997), peaks A1 and A2 were assigned as cyanidin 3-O-(6″-O-malonylglucoside) (Cy-3-MalGlc) and cyanidin 3-O-(3″, 6″-O-dimalonylglucoside) (Cy-3-MalMalGlc).

During analysis of flavones and flavonols, nine peaks were identified, seven of which could be assigned as flavonoid glycosides, with the remaining two left unassigned (Table 2). Flavonol glucosides were not detected at present. MS² and UV absorption spectrometry analysis identified four flavone aglycones, namely luteolin (peaks 1 and 5), apigenin (peaks 4 and 6), chrysoeriol (Ch) (peak 7), and acacetin (peaks 8 and 9). The molecular ion of peak 1 produced the fragment m/z 463 u by the loss of the m/z 132 u (a pentose substituent) was assigned as luteolin 7-O-arabinosylglucuronide (Lu-7-AraGluc) on the basis of Schwinn et al. (1993). Peaks 5, 6, 7, and 9 lost m/z 248 u (162 u + 86 u) in MS² spectra, peak 5 was assigned to contain luteolin 7-O-malonylglucoside (Lu-7-MalGlc), peak 6 apigenin 7-O-malonylglucoside (Ap-7-MalGlc), peak 7 chrysoeriol 7-O-malonylglucoside (Ch-7-MalGlc) and peak 9 acacetin 7-O-malonylglucoside (Ac-7-MalGlc), while peaks 4 and 8 included, respectively, apigenin 7-O-glucoside (Ap-7G) and acacetin 7-O-rutinoside (Ac-7-Rut) (Schwinn et al. 1993, Lai et al. 2007). The major assigned flavonoids in pink flowering H5 were peaks 1, 4, 5, 6, 7, and 9 (Table 2). Keikai contained peaks 1, 4, 5, 6, 7, 8 and 9 (Table 2) and flavonoids detected in Jinba were similar to those in Keikai, except for lack of peak 9 (Table 2).

The flavonoid content of the three cultivars was 2.17 (H5), 4.05 (Keikai) and 4.14 (Jinba) mg (rutin) g⁻¹ (f.m.), respectively, showing that the two white flowering cultivars have two fold higher flavonoid content than the pink flowering H5. Lu-7-MalGlc accounted for 28.7 % of the total flavonoids of H5, Ap-7-MalGlc represented 19.32 %, Ch-7-MalGlc 16.39 % and Ac-7-MalGlc 21.71 %. In Keikai, Ap-7-MalGlc (33.75 %) and Ac-7-MalGlc (17.41 %) were the two major flavonoids, while in Jinba, Ap-7-MalGlc accounted for 54.65 % of the flavonoids presented (Table 3).

Table 2. UV-VIS and MS spectra characteristics of the flavonoids present in the ray florets of chrysanthemum (t_R - retention time).

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>t_R</th>
<th>λ_max [nm]</th>
<th>Fragments (m/z)</th>
<th>[M⁺] (m/z)</th>
<th>Tentative identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>20.9</td>
<td>281, 518</td>
<td>287</td>
<td>535</td>
<td>cyanidin 3-O-(6″-O-malonylglucoside)</td>
</tr>
<tr>
<td>A2</td>
<td>25.6</td>
<td>281, 518</td>
<td>287</td>
<td>621</td>
<td>cyanidin 3-O-(3″, 6″-O-dimalonylglucoside)</td>
</tr>
<tr>
<td>1</td>
<td>18.0</td>
<td>255, 348</td>
<td>287, 463</td>
<td>595</td>
<td>luteolin 7-O-arabinosylglucuronide</td>
</tr>
<tr>
<td>2</td>
<td>18.4</td>
<td>250, 327</td>
<td>-</td>
<td>539</td>
<td>unknown</td>
</tr>
<tr>
<td>3</td>
<td>21.3</td>
<td>268, 336</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
</tr>
<tr>
<td>4</td>
<td>22.4</td>
<td>268, 336</td>
<td>271</td>
<td>433</td>
<td>apigenin 7-O-glucoside</td>
</tr>
<tr>
<td>5</td>
<td>23.8</td>
<td>254, 349</td>
<td>287</td>
<td>535</td>
<td>luteolin 7-O-malonylglucoside</td>
</tr>
<tr>
<td>6</td>
<td>33.3</td>
<td>268, 337</td>
<td>271</td>
<td>519</td>
<td>apigenin 7-O-malonylglucoside</td>
</tr>
<tr>
<td>7</td>
<td>39.2</td>
<td>254, 347</td>
<td>301</td>
<td>549</td>
<td>chrysoeriol 7-O-malonylglucoside</td>
</tr>
<tr>
<td>8</td>
<td>42.6</td>
<td>267, 334</td>
<td>285</td>
<td>593</td>
<td>acacetin 7-O-rutinoside</td>
</tr>
<tr>
<td>9</td>
<td>50.8</td>
<td>268, 334</td>
<td>285</td>
<td>533</td>
<td>acacetin 7-O-malonylglucoside</td>
</tr>
</tbody>
</table>

Table 3. Total flavonoid content [mg g⁻¹ (f.m.))] and the percentage of each specific compound present. 1 - luteolin 7-O-arabinosylglucuronide, 2 - unknown, 3 - unknown, 4 - apigenin 7-O-glucoside, 5 - luteolin 7-O-malonylglucoside, 6 - apigenin 7-O-malonylglucoside, 7 - chrysoeriol 7-O-malonylglucoside, 8 - acacetin 7-O-rutinoside, 9 - acacetin 7-O-malonylglucoside. Mean ± SD, n = 3.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5</td>
<td>2.17±0.08</td>
<td>1.32±0.06</td>
<td>0.28±0.12</td>
<td>0.28±0.01</td>
<td>0.21±0.02</td>
<td>28.70±0.85</td>
<td>19.32±0.40</td>
<td>16.39±0.04</td>
<td>0.00</td>
<td>21.71±0.52</td>
</tr>
<tr>
<td>Keikai</td>
<td>4.05±0.47</td>
<td>3.62±0.04</td>
<td>6.15±0.94</td>
<td>0.94±0.17</td>
<td>9.56±0.16</td>
<td>9.75±0.07</td>
<td>33.75±0.68</td>
<td>1.51±0.08</td>
<td>0.12±0.03</td>
<td>17.41±0.29</td>
</tr>
<tr>
<td>Jinba</td>
<td>4.14±0.54</td>
<td>1.19±0.05</td>
<td>3.83±1.25</td>
<td>3.77±0.28</td>
<td>8.06±0.41</td>
<td>6.32±0.56</td>
<td>54.65±0.57</td>
<td>0.19±0.01</td>
<td>0.08±0.01</td>
<td>0.00</td>
</tr>
</tbody>
</table>

In pink flowering H5, low CHS, CHI, F3H and F3’H expression was observed throughout four stages. DFR and 3GT transcripts, however, accumulated to significantly higher levels. The expression of DFR
peaked at stage III, and 3GT peaked at stage IV. The expression of DFR and 3GT in H5 were overall higher than in two white flowering cultivars. Low CHS transcription level was also detected in Keikai, but CHI expression was greater than that in Jinba and H5 throughout four stages, and was higher than any of the other tested genes. F3H, F3’H, DFR and 3GT were only slightly expressed in Keikai. Patterns of gene expression in Jinba were similar to those of Keikai. Low CHS expression was observed during stage I and II, while CHI was the most prominently expressed one of the six tested genes. Expression of CHI increased from stage I to III, and maintaining a relatively high level at stage IV. Only trace expression of the remaining four genes was observed in Jinba.

Table 4. Expression profiles of structural genes involved in anthocyanin biosynthesis in the ray florets of pink and white flowering chrysanthemum cultivars. I, II, III, IV means different flowering stages. Genes encoding for: CHS - chalcone synthase, CHI - chalcone isomerase, F3H - flavanone 3-hydroxylase, F3’H - flavonoid 3’-hydroxylase, DFR - dihydroflavonol 4-reductase, 3GT - 3-O-glucosyltransferase. Three replicates of each RT-qPCR were performed. Means ± SE; different letters show the significant differences among cultivars at each time point at P < 0.01 level according to Duncan’s test.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cultivar</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHS</td>
<td>H5</td>
<td>2.36 ±0.0684A</td>
<td>0.89 ±0.0513A</td>
<td>2.82 ±0.0466A</td>
<td>0.35 ±0.0047A</td>
</tr>
<tr>
<td>Keikai</td>
<td>0.08 ±0.0030B</td>
<td>1.06 ±0.0347B</td>
<td>5.68 ±0.0502B</td>
<td>0.01 ±0.0023B</td>
<td></td>
</tr>
<tr>
<td>Jinba</td>
<td>1.00 ±0.1073C</td>
<td>1.59 ±0.0628B</td>
<td>5.68 ±0.0502B</td>
<td>0.19 ±0.0049C</td>
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<tr>
<td>CHI</td>
<td>H5</td>
<td>3.98 ±0.2641A</td>
<td>0.37 ±0.0329A</td>
<td>0.80 ±0.0462A</td>
<td>1.86 ±0.0315A</td>
</tr>
<tr>
<td>Keikai</td>
<td>59.58 ±0.6664B</td>
<td>65.65 ±0.5140B</td>
<td>51.74 ±0.5032B</td>
<td>441.62 ±0.7075B</td>
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<tr>
<td>Jinba</td>
<td>11.31 ±0.2902C</td>
<td>17.11 ±0.5026C</td>
<td>20.92 ±0.9324C</td>
<td>15.07 ±1.0270C</td>
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</tr>
<tr>
<td>F3H</td>
<td>H5</td>
<td>0.012 ±0.0005A</td>
<td>0.008 ±0.0001A</td>
<td>0.06 ±0.0016A</td>
<td>0.079 ±0.0033A</td>
</tr>
<tr>
<td>Keikai</td>
<td>0.18 ±0.0045B</td>
<td>0.237 ±0.0040B</td>
<td>0.10 ±0.0009B</td>
<td>1.25 ±0.0281B</td>
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<tr>
<td>Jinba</td>
<td>0.02 ±0.0009B</td>
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<td>0.05 ±0.0002C</td>
<td>0.04 ±0.0017B</td>
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<td>H5</td>
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<tr>
<td>DFR</td>
<td>H5</td>
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**Discussion**

The most frequent anthocyanins found in higher plants are derivatives of pelargonidin, cyanidin and delphinidin, which differ in the number of hydroxyl groups at the B-ring. Cyanidin is believed to be the simplest of these anthocyanidins (Schwinn and Davies 2004). Saito *et al.* (1988) and Nakayama *et al.* (1997) reported the identification of derivatives of cyanidin in chrysanthemum. Cyanidin derivatives have also been identified as being the major anthocyanins presented in purplish-red, bronze and pink chrysanthemum inflorescences (Schwinn *et al.* 1993) and in azalea (Nakatsuka *et al.* 2008), and also in the skin of red apples (Honda *et al.* 2002). In present study, cyanidin derivatives were also presented in the pink flowering H5; but not in two white flowering cultivars. No evidence for any involvement of either delphinidin or pelargonid was detected in tested three cultivars.

In addition to cyanidin derivatives, the H5 ray florets also contains several flavonoid glucosides. These flavonoids are either colourless or slightly yellow. Since they are also present in the white flowering colour cultivars, it is unlikely that they play any role in the pigmentation of H5 flowers. Schwinn *et al.* (1993) suggested that Ap, Ac, Lu, diosmetin and eriodictyol derivatives are the major flavonoids found in the chrysanthemum, and here we were able to confirm the presence of Ap, Ac and Lu. However, we have also been able to detect the presence of Ch. Flavonoid composition varied between the two white flowering cultivars, as it does between white-flowering carnations (Mato *et al.* 2000), inferring that flavonoid biosynthesis in two white chrysanthemum cultivars is differently regulated. The difference in Ch and Ac between pink and white flowering and Ac presence between two white flowering cultivars suggested that these flavonoids could be important pigments for chrysanthemum flower coloration.

Genes CHS, CHI, F3H and F3’H are all expressed
both in pigmented and non-pigmented flowers, it is clear that the lack of anthocyanin in white flowering types cannot be due to any blockage of their expression. CHI expression was higher in Keikai compared to the other two cultivars. A similar content of total flavonoids but different expression profiles of CHI inferred that other genes may be still involved in the probably feed-forward regulation of the flavonoid biosynthesis in different chrysanthemum cultivars. The DFR gene of blood orange (CsDFR-bo) was proved to be a single-copy gene and localized on chromosome 3p (Lu et al. 2010). DFR uses either dihydroxymercuricin, and/or dihydrokaempferol or dihydroquercetin as substrates, and catalyzes the synthesis of leucoanthocyanidin. Suppression of DFR transcription in carnation or low expression in Arabidopsis hampered their pigmentation (Stich et al. 1992, Feyissa et al. 2009). DFR expression was abundant in H5, but only a trace of expression was observed in the two white flowering ones, suggesting that DFR may be important for anthocyanin biosynthesis and pigmentation probably via a quantitative regulatory system rather than absolute blockage of their expression. 3GT (namely UFGT) contributes to anthocyanidin glycosylation. UFGT plays an important role in the accumulation of anthocyanins in red grape cultivars (Kereamy et al. 2002) and anthocyanin synthesis during azalea flower pigmentation (Nakatsu et al. 2008). Here, 3GT expression was higher in H5 than in other cultivars, and it increased along with the extent of inflorescence pigmentation. We suggest therefore that a high 3GT expression is important for determining the pigmentation of the chrysanthemum inflorescence, and that DFR, 3GT expression is likely quantitatively regulated. However, we can’t conclude which flower stage is critical for flavonoid accumulation based on the change of gene expression, and temporal gene expression patterns also varied between cultivars.

Recently, a bHLH like regulatory gene has been shown to suppress the expression of DFR and thereby prevent the synthesis of anthocyanin in A. thaliana (Feyissa et al. 2009). A mutation of the regulatory gene suppressed the expression of UFGT, rather than a mutation of UFGT itself, caused a white to red grape skin colour (Kereamy et al. 2002). Whether the DFR and/or 3GT alleles themselves differ between white and pink flowering chrysanthemums, or whether their differential expression is caused by a regulatory gene(s) remains to be investigated in our ongoing research. Besides the expression profiles of genes investigated here, a tentative survey on the expression profiles of genes such as anthocyanin synthase (ANS), anthocyanin reductase (ANR), flavone synthase (FNS) are necessary for better understanding of difference in flavonoid composition between the two white flowering cultivars and between pigmented from non-pigmented flowers.

References

Nakatsu, A., Mizuta, D., Kii, Y., Miyajima, I., Kobayashi, N.: Isolation and expression analysis of flavonoid biosynthesis