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

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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-MS^n 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 antioxidant 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 (Ismail 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-MalGlc - 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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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-β-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 (UFGT) 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 UFGT 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,* leucocyanidin 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.

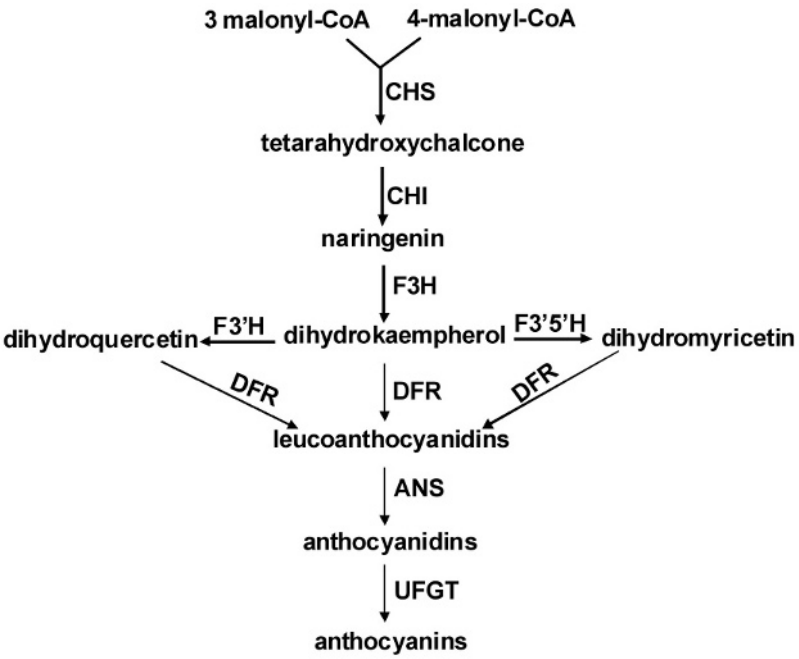

Fig. 1. Simplified biosynthesis pathway of flavonoids. Enzyme names are abbreviated as follows: CHS - chalcone synthase, CHI - chalcone isomerase, F3H - flavanone 3-hydroxylase, F3'H - flavonoid 3'-hydroxylase, F3'5'H - flavonoid 3',5'-hydroxylase, DFR - dihydroflavonol 4-reductase, ANS - anthocyanidin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

Chromatographic grade methanol and acetonitrile used for HPLC-DAD/ESI-MS^n analysis were purchased from *Alltech Scientific* (Beijing, China). Trifluoroacetic acid (TFA) was obtained from *Merck* (Darmstadt, Germany). Analytical grade methanol and formic acid were purchased from *Beijing Chemical Works* (Beijing, China). Ultra-pure water was obtained from a *Milli-Q* system (*Millipore*, Billerica, MA, USA). Rutin (quercetin 3-*O*-rutinoside) as a standard 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 cm^3 of methanol : water : formic acid : TFA (70:27:2:1, 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 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 C_{18} *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 mm^3 aliquot was injected, and the resulting chromatograms were read at 515 nm for anthocyanins and 350 nm for flavones and flavonols.

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-MS^n analysis was performed using an *Agilent-1100* HPLC system coupled to a DAD system and an LC/MSD *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 (N_2) temperature 325 °C; flow rate 8 dm^3 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 mm^3, 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 DNaseI (*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 $2^{-\Delta Ct}$.

Table 1. Primers used in this study. F - forward, R - reverse.

Gene		Sequence (5'→3')	Acc. No.
CHS	F	CAAGCCTTTTCTCCATTAGGT	DQ521272
	R	GAGGACCACGGTTTCGAC	
CHI	F	TGGTGCAACCATTGACAAGT	EU286277
	R	AAATTTGGTTCAGCATCTGTAGTT	
F3H	F	CATTATTTGAGCAACGGAAGA	U86837
	R	TGCCAATCTCTAAGCCAAGAT	
F3'H	F	CTGACGGTGAGGGAGGGAA	EU286276
	R	TGGCGTTGAAGGGTGGAG	
DFR	F	TGGCCAGAGTATCAAATTCCA	EF094936
	R	CGCTCTTTGCTAGAGGTGCT	
3GT	F	ATCACAGGGACTATCAACC	AB523845
	R	TCCACCACCAGACAACTA	
EF1α	F	AGACCACCAAGTACTACTGCAC	this study
	R	CCACCAATCTTGTACACATCC	

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 MS^2 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. MS^2 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 MS^2 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).

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

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.

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

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.

Gene	Cultivar	I	II	III	IV
CHS	H5	2.36 ±0.0684A	0.89 ±0.0513A	2.82 ±0.0466A	0.35 ±0.0047A
	Keikai	0.08 ±0.0030B	1.06 ±0.0347B	5.68 ±0.0502B	0.01 ±0.0023B
	Jinba	1.00 ±0.1073C	1.59 ±0.0628B	0.10 ±0.0012C	0.19 ±0.0049C
CHI	H5	3.98 ±0.2641A	0.37 ±0.0329A	0.80 ±0.0462A	1.86 ±0.0315A
	Keikai	59.58 ±0.6664B	65.65 ±0.5140B	51.74 ±0.5032B	441.62 ±0.7075B
	Jinba	11.31 ±0.2902C	17.11 ±0.5026C	20.92 ±0.9324C	15.07 ±1.0207C
F3H	H5	0.012±0.0005A	0.008±0.0001A	0.06 ±0.0016A	0.079±0.0033A
	Keikai	0.18 ±0.0045B	0.237±0.0040B	0.10 ±0.0009B	1.25 ±0.0281B
	Jinba	0.02 ±0.0009B	0.038±0.0005C	0.05 ±0.0002C	0.04 ±0.0017B
F3'H	H5	0.001±0.0000A	0.001±0.0000A	0.007±0.0002A	0.02 ±0.0005A
	Keikai	0.007±0.0000B	0.014±0.0012B	0.017±0.0006B	0.12 ±0.0006B
	Jinba	0.001±0.0002C	0.002±0.0002B	0.002±0.0000C	0.005±0.0002C
DFR	H5	106.15 ±0.2129A	53.94 ±0.5365A	155.06 ±0.5364A	36.67 ±0.3319A
	Keikai	0.005±0.0003B	0.004±0.0005B	0.025±0.0028B	0.012±0.0007B
	Jinba	0.011±0.0006B	0.017±0.0005B	0.002±0.0001B	0.038±0.0043B
3GT	H5	26.48 ±0.0372A	31.34 ±0.1077A	22.01 ±0.5638A	56.62 ±0.3458A
	Keikai	0.017±0.0004B	0.04 ±0.0057B	8.04 ±0.0384B	0.051±0.0048B
	Jinba	0.005±0.0000B	0.02 ±0.0012B	0.002±0.0000C	0.013±0.0002B

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 pelargonidin 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 dihydromyricetin, 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 (Nakatsuka *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.

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