Transcriptional control of anthocyanin biosynthesis genes and transcription factors associated with flower coloration patterns in *Gerbera hybrida*

Aung Htay Naing¹ · Ji Hye Lee¹ · Kyeong Il Park² · Kyoung-ook Kim¹ · Mi Young Chung³ · Chang Kil Kim¹

Received: 21 September 2017 / Accepted: 5 January 2018 / Published online: 12 January 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

We analyzed the expression of anthocyanin biosynthesis genes and transcription factors (TFs) in the *Gerbera hybrida* cultivars ‘Bintang’ and ‘Alliance’ that exhibit different coloration patterns. Differential expression of biosynthesis genes and TFs was associated with variable anthocyanin content at different flower developmental stages (S1–S3) in both cultivars; higher anthocyanin content was correlated with higher levels of gene expression. Exposure to different temperatures (6 and 22 °C) also resulted in different anthocyanin content levels: the lower temperature (6 °C) enhanced anthocyanin content compared to the higher temperature (22 °C). However, the increased anthocyanin content of ‘Bintang’ compared to ‘Alliance’ was the result of higher levels of expression of all detected genes, regardless of flower stage and temperature conditions. Therefore, we conclude that transcriptional control of the detected genes is associated with the mechanisms of anthocyanin biosynthesis and coloration patterns in gerberas; however, further studies of the key genes are needed.

Keywords Anthocyanin · Flower stages · Gerberas · Gene expression · Temperature

Introduction

Anthocyanins are major pigments produced by the flavonoid biosynthesis pathway, and they are responsible for the formation of a range of colors from red to blue in horticultural plants, particularly in flowering plants (Chandler and Tanaka 2007; Wessinger and Rausher 2014). The regulation of anthocyanin biosynthesis has been studied in a number of flowering plants, and the involvement of biosynthesis genes such as those encoding phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), flavanone-3-hydroxylase (*F3H*), dihydroflavonol-4-reductase (*DFR*), anthocyanidin synthase

(*ANS*), and UDP-glycosyl transferase (*UGT*) have been well-characterized (Quattrocchio et al. 1998; Grotewold 2006; Yamagishi et al. 2010; Deng et al. 2014; Bashandy et al. 2015; Ai et al. 2016). The anthocyanin biosynthesis genes involved in the anthocyanin pathway are mostly regulated by a complex of R2R3-MYB and basic helix–loop–helix (bHLH) transcription factors (TFs) and WD40 proteins (Broun 2005; Koes et al. 2005; Petroni and Tonelli 2011), suggesting that transcriptional regulation of biosynthesis genes and TFs mainly controls flower coloration patterns in flowering plants.

Gerbera hybrida is an economically important cut flower that is commonly obtained by crossing two wild species, namely *Gerbera jamesonii* and *G. viridifolia* (Hansen 1999). Because of the diverse flower colors in the hybrids, *G. hybrida* has been proposed as a model for anthocyanin biosynthesis research. Although the relationship between the regulation of anthocyanin biosynthesis mechanisms and flower color has been investigated in gerbera, the underlying causes of flower color variation remain unclear. Previous studies have shown that the functional roles of individual genes involved in anthocyanin synthesis differ. For example, *CHS1* and *CHS4* are responsible for anthocyanin accumulation in the vegetative and floral organs of gerbera (Helariutta

Aung Htay Naing and Ji Hye Lee: Equally contributed to this work.

✉ Chang Kil Kim
ckkim@knu.ac.kr

¹ Department of Horticultural Science, Kyungpook National University, Daegu 4165122, South Korea

² Department of Horticulture & Life Science, Yeungnam University, Gyeongsan 712-749, South Korea

³ Department of Agricultural Education, Suncheon National University, Suncheon, South Korea

et al. 1993; Deng et al. 2014), while the loss of one of the two enzymatically functional *DFR* genes causes white-colored petals in gerbera 'Ivory' (Bashandy et al. 2015). The roles of *CHS1*, *F3'H*, and *DFR* in anthocyanin accumulation in gerbera have been investigated (Martens et al. 2002; Meng et al. 2004; Seitz et al. 2006; Deng et al. 2014; Bashandy et al. 2015). Additionally, the involvement of the R2R3-MYB TF GMYB10 alone or in combination with the bHLH TF GMYC1 in the regulation of anthocyanin biosynthetic genes in gerbera has been reported (Elomaa et al. 1998, 2003; Laitinen et al. 2008). However, more work is required to elucidate the relationships between gene expression patterns and gerbera flower color.

Accumulation of anthocyanin in the flowers of some species is sensitive to temperature: low temperatures elevate anthocyanin accumulation in petunias and roses (Biran et al. 1973; Biran and Halevy 1974; Shvarts et al. 1997). However, to date, there is little information on the effect of low temperature on anthocyanin accumulation in gerbera. Moreover, the regulatory mechanisms through which low temperatures affect anthocyanin biosynthesis in flowers remain uncertain.

In this study, we isolated anthocyanin biosynthesis genes and TFs from two gerbera cultivars with distinctly different flower colors and investigated the expression patterns of the genes during flower developmental stages. In addition, the mechanism by which temperature influences anthocyanin biosynthesis in gerbera was also investigated by determining the expression patterns of the biosynthesis genes and TFs.

Materials and methods

Plant materials

Two *G. hybrida* cultivars ('Bintang' and 'Alliance') with different flower colors were used in this experiment. The flowers were grown at a commercial flower production farm in Youngju, Korea. Flowers were collected at different developmental stages (S1 to S3), including the early flower stage to the fully open stage (Fig. 1). The anthocyanin content at each stage was then determined, and expression of related biosynthesis genes and TFs was analyzed.

Effect of temperature on anthocyanin regulation

To determine the effect of temperature on anthocyanin synthesis, flower stems at the middle flower stage (S2) were cut from the two cultivars and placed in 2-L plastic beakers containing distilled water. They were then placed in a growth chamber setting up the different temperatures (6 and 22 °C) with a 16-h photoperiod (30 mmol m⁻² s⁻¹; cool white fluorescent lamps) for 3 days. Anthocyanin content was then measured from

Fig. 1 Different petal developmental stages (S1–S3) of the gerbera cultivars 'Bintang' and 'Alliance'

each cultivar, and expression of related biosynthesis genes was analyzed.

Analysis of total anthocyanin content

Total anthocyanin content during petal developmental stages and following petal exposure to different temperatures was analyzed using the methods described in Ai et al. (2016) with some modifications. Briefly, ~ 500 mg of petals per sample was collected and ground in liquid nitrogen into a fine powder. To extract anthocyanin, the fine powder was transferred to an extraction solution containing 45% hydrochloric acid and methanol (1:99). The extracted solution was then incubated at 4 °C for 24 h, and it was then centrifuged at 13,000 rpm at 4 °C for 20 min. The supernatant containing anthocyanin was analyzed using a spectrophotometer (Shimadzu, Kyoto, Japan), in which quantification of anthocyanins was performed using the following equation:

$$Q_{\text{anthocyanins}} = (A_{530} - 0.25 \times A_{657}),$$

where $Q_{\text{anthocyanins}}$ is the amount of anthocyanins. Data for each sample are reported as the mean of three replicates.

RNA extraction

Total RNA was isolated from 100 mg of petals from each flower developmental stage as well from flowers exposed to different temperatures (from the two cultivars) using TRI Reagent™ Solution (Ambion, USA). RNA integrity was assessed using 1% agarose gel electrophoresis, and the concentration of extracted RNA was determined using a NanoDrop spectrophotometer (Thermo, USA).

Analysis of expression of anthocyanin biosynthesis and TF genes using qRT-PCR

To measure expression levels of anthocyanin biosynthesis and TF genes, cDNA was synthesized from 1 µg of total

RNA, and an oligo dT₂₀ primer was used following the manufacturer's protocol (ReverTra Ace-á, Toyobo, Japan). Transcript levels of the investigated genes (Table 1) were measured using a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Waltham, USA) (Ai et al. 2016), and gene expression levels were normalized to the *2-pyrone synthase* (*2PS*) genes to minimize variation in the cDNA template. The accession numbers, primers and PCR condition for the examined genes are listed in Table 1. Data for each sample are reported as the mean of three replicate experiments.

Statistical analysis

Data were analyzed using SPSS v. 11.09 (IBM Corporation, Armonk, NY, USA), and are presented as the mean of three replicate experiments. Least significant difference (LSD) tests and *t* tests were used to identify the differences between means. The significance level was set at $P < 0.05$.

Results

Variation in anthocyanin content in gerbera cultivars during petal developmental stages

Variation in anthocyanin content during different petal developmental stages was observed in the 'Bintang' and 'Alliance' cultivars. One notable difference between cultivars was the linear increase from S1 to S3 in 'Bintang', whereas 'Alliance' showed an increase from S1 to S2. Hence, the highest anthocyanin content in 'Bintang' was observed at S3

($2.94 \pm 0.6 \text{ mg g}^{-1}$), while that of 'Alliance' was observed at S2 ($0.40 \pm 0.1 \text{ mg g}^{-1}$) (Fig. 2a, b). Although anthocyanin content varied during the developmental stages, they did not appear to be related to changes in flower color within cultivars. However, the differences in anthocyanin content between cultivars were strongly related to the differences in flower color.

Influence of anthocyanin biosynthesis and TF gene expression patterns on flower coloration

The variation in anthocyanin content during petal development and between cultivars is shown in Fig. 2a, b. We investigated the expression patterns of anthocyanin biosynthesis genes (*CHS1*, *CHS4*, *F3H*, *F3'H*, *DFR*, and *ANS*) and transcription factor genes (*MYB10* and *MYC1*) using qRT-PCR, to determine if these controlled the different anthocyanin contents during development and in the two cultivars. Although gerbera possesses different alleles encoding *DFR*, we detected the expression of the *DFR* (*DFR1-3*), which was assumed to be specific to cyanidin accumulation in certain gerberas, in the cultivars that appeared to contain cyanidin pigment. We found that all of the examined anthocyanin biosynthesis genes were expressed in both cultivars in all three developmental stages. However, the levels of expression differed markedly, particularly between the two cultivars (Figs. 3, 4). In 'Bintang', the levels of expression of all biosynthesis genes during petal development were similar, with the exception of *DFR*; this finding was consistent with the linear increase in anthocyanins observed from S1 to S3. However, *DFR* expression was highest at the S1 stage

Table 1 Primer sequences and qRT-PCR condition used to detect genes related to anthocyanin biosynthesis

Accession number	Gene	Primer sequence (5'–3')	PCR conditions
Z38096	<i>CHS1</i>	F-CGG CCG TTG TTT GAA ATG GT R-ATC AAC CCA GGC ACG TCT TT	95 °C (10 min) → [95 °C (15 s) → 57 °C (1 min) → 72 °C (35 s)] × 40 cycles → 95 °C (15 s) → 59.3 °C (1 min) → 95 °C (15 s)
AM906210	<i>CHS4</i>	F-AAA CTA CGT GCC ACA CGA CA R-AAA CCC AAA CAA AAC GCC CC	
FJ605513	<i>F3H</i>	F-TCA CAT TGG GTC TGA AGC GG R-AAT CCA ACT CTT GCC ACC GT	95 °C (10 min) → [95 °C (15 s) → 57 °C (1 min) → 72 °C (35 s)] × 40 cycles → 95 °C (15 s) → 59.3 °C (1 min) → 95 °C (15 s)
DQ218417	<i>F3'H</i>	F-GTC GGA AAC CTA CCG CAT CT R-TCG CGT CGT GAG TCT TCA AA	
KF734593	<i>DFR</i>	F-ACT TTG GCT GAA AAA GCC GC R-GCC CGT GAT CAA AGA AAG CG	95 °C (10 min) → [95 °C (15 s) → 57 °C (1 min) → 72 °C (35 s)] × 40 cycles → 95 °C (15 s) → 59.3 °C (1 min) → 95 °C (15 s)
AY997841	<i>ANS</i>	F-TTA TCA ACG AGC GGC ATC CA R-TTT TTC CGG GTC GTC GGA TT	
AJ554700	<i>MYB10</i>	F-TGG AAC ACC CAT CTT CGC TC R-GCT TTG CCT GTT ACC GAA CC	95 °C (10 min) → [95 °C (15 s) → 57 °C (1 min) → 72 °C (35 s)] × 40 cycles → 95 °C (15 s) → 59.3 °C (1 min) → 95 °C (15 s)
AJ007709	<i>MYC1</i>	F-CCG CAT TAT CCC CCG AAG AT R-GAC GCG CTT TTT GCT AGG AG	
Z38097	<i>2-pyrone synthase (2PS)</i>	F-CAA AGA AGC CGC AGT CAA GG R-AGC GTT TGA CTG AAG GGG AG	95 °C (10 min) → [95 °C (15 s) → 57 °C (1 min) → 72 °C (35 s)] × 40 cycles → 95 °C (15 s) → 59.3 °C (1 min) → 95 °C (15 s)

Fig. 2 Detection of anthocyanin content from the different petal development stages (S1–S3) of two gerbera cultivars, **a** ‘Bintang’ and **b** ‘Alliance’. Data represent the mean of three replicates, and the bar

indicates the standard deviation. Means with different letters are significantly different (LSD test, $P < 0.05$)

(Fig. 3a–f). Interestingly, the expression level of *CHS4* was distinctly higher than that of *CHS1*, and was the highest among the biosynthetic genes, as well. In ‘Alliance’, the levels of expression of *CHS4*, *F3H*, *DFR*, and *ANS* were generally similar with highest transcription in S2, followed by the S3 and S1 stages. However, *CHS1* and *F3'H* levels were highest at the S1 stage (Fig. 4a–f). These results indicated that the variation in anthocyanin content during petal development in this cultivar was likely to be more closely associated with *CHS4*, *F3H*, *DFR*, and *ANS* expression than that of *CHS1* and *F3'H* as these exhibited higher expression at the S1 stage when the lowest anthocyanin content

was present. Our expression analysis indicated that *CHS4*, *F3H*, *DFR*, and *ANS* were highly expressed at the middle petal developmental stage (S2), while high expression levels of *CHS1* and *F3'H* were observed at an early stage (S1). As observed in Bintang, the expression level of *CHS4* was relatively higher than that of *CHS1* in Alliance, which was observed to probably be the highest among the detected genes. Despite the association of anthocyanin contents and expression of *CHS4*, *F3H*, *DFR*, and *ANS*, a potential gene that influenced anthocyanin biosynthesis could not be identified. The relative levels of expression of the genes differed at every stage between the cultivars, with higher expression

Fig. 3 Characterization of expression profiles of anthocyanin biosynthesis genes (A, *CHS1*, B, *CHS4*, C, *F3H*, D, *F3'H*, E, *DFR*, F, *ANS*) during different flower developmental stages (S1–S3) of gerbera cul-

tivar ‘Bintang’. Data represent the mean of three replicates, and the bar indicates the standard deviation. Means with different letters are significantly different (LSD test, $P < 0.05$)

Fig. 4 Characterization of expression profiles of anthocyanin biosynthesis genes (A, *CHS1*, B, *CHS4*, C, *F3H*, D, *F3'H*, E, *DFR*, F, *ANS*) during different flower developmental stages (S1–S3) of gerbera cul-

tivar ‘Alliance’. Data represent the mean of three replicates, and the bar indicates the standard deviation. Means with different letters are significantly different (LSD test, $P < 0.05$)

levels of all genes at all stages in ‘Bintang’ than in ‘Alliance’; these differences were associated with differences in anthocyanin levels observed between the two cultivars at the different developmental stages.

The genes for the TFs MYB10 and MYC1 also showed differential expression at all developmental stages in both cultivars (Fig. 5). In ‘Bintang,’ both TFs increased from S1 to S3, and these increases were consistent with the observed anthocyanin contents in both cultivars. However, the expression level of MYB10 was higher than that of MYC at all developmental stages. Similarly, in ‘Alliance,’ the TFs were expressed at all stages; however, their expression levels were found to be highest at S2 followed by S3 and S1, and these changes were associated with the corresponding changes in anthocyanin content. In addition, comparison of the transcript levels of the TFs between the two cultivars indicated that both TFs were higher in ‘Bintang’ than in ‘Alliance’, revealing that TF transcript levels were positively associated with high anthocyanin content.

Enhancement of anthocyanin content in gerberas under low temperature condition

Exposure of flowers (S2) to two different temperatures (6 and 22 °C) for 3 days resulted in differential accumulation of anthocyanin in both cultivars. The anthocyanin contents from flowers exposed to 6 °C were distinctly greater than those exposed to 22 °C (Fig. 6a, b). Therefore, low

temperature affected the regulation of anthocyanin biosynthesis in both cultivars.

Transcriptional regulation of anthocyanin biosynthesis genes and TFs under low-temperature conditions

We measured transcript levels of anthocyanin biosynthesis genes in both cultivars after exposure to 6 and 22 °C, using qRT-PCR. As expected, differential expression of *CHS1*, *CHS4*, *F3H*, *F3'H*, *DFR*, and *ANS* at the transcriptional level was observed in both cultivars. Furthermore, gene expression was found to be more regulated at 6 °C than at 22 °C based on the contents induced in both cultivars. We found that *CHS1* and *DFR* were more regulated at 6 °C than other genes in ‘Bintang’, while greater regulation of *CHS1*, *F3H*, and *ANS* at 6 °C was observed in ‘Alliance’ samples (Figs. 7, 8). Although the low temperature treatment (6 °C) affected expression of all biosynthesis genes, exception of *F3'H* in ‘Bintang’, compared to the 22 °C treatment in both cultivars, the roles of specific genes associated with anthocyanin biosynthesis were unclear, despite the greater potential for control by *CHS1* and *DFR* in ‘Bintang’ sample and *CHS1*, *F3H*, and *ANS* control in ‘Alliance’ samples.

Figure 9 shows the effects of temperature on transcriptional activation of the TFs, and low temperature (6 °C) could induce the synthesis of MYB10 and MYC transcripts at higher levels in both Bintang and Alliance than normal

Fig. 5 Characterization of expression profiles of MYB10 and MYC1 during different flower developmental stages of gerbera cultivars (a, b) 'Bintang' and (c, d) 'Alliance'. Data represent the mean of three

replicates, and the bar indicates the standard deviation. Means with different letters are significantly different (LSD test, $P < 0.05$)

Fig. 6 Detection of anthocyanin content from the S2 petal developmental stage of two gerbera cultivars, a 'Bintang' and b 'Alliance', under two different temperatures. Data represent the mean of three replicates, and the bar indicates the standard deviation. Means with asterisk(s) are statistically significant (t test, $*P < 0.05$)

temperature (22 °C). However, the level of TF expression was higher in Bintang than in Alliance. In this study, the induction of high TF expression at 6 °C was related to high transcript levels of the biosynthetic genes, resulting in increased anthocyanin accumulation at 6 °C. Additionally, anthocyanin accumulation at higher levels in Bintang than in Alliance was consistent with the higher expression levels of their TFs. Overall, low temperature played a distinct role in the regulation of anthocyanin biosynthesis in both cultivars.

Discussion

Differential expression of biosynthesis genes and TFs that determines the formation of diverse flower colors has been reported in many ornamental plants (Quattrocchio et al. 1998; Elomaa et al. 2003; Nakatsuka et al. 2005; Zhao et al. 2012). Analysis of gene expression patterns is a common approach to elucidate the mechanisms underlying coloration in various plants. Because of the existence of diverse flower colors in gerberas, the plants are considered as a potential model for flower coloration in the Asteraceae (Helariutta et al. 1996; Elomaa et al. 1998; 2003; Laitinen et al. 2005; Deng et al. 2014; Bashandy et al. 2015). Despite

Fig. 7 Characterization of expression profiles of anthocyanin biosynthesis genes (A, *CHS1*, B, *CHS4*, C, *F3H*, D, *F3'H*, E, *DFR*, F, *ANS*) (at S2) of gerbera cultivar 'Bintang' under two different temperatures.

Data represent the mean of three replicates, and the bar indicates the standard deviation. Means with asterisk(s) are statistically significant (*t* test; ** $P < 0.01$; ns non-significant)

Fig. 8 Characterization of expression profiles of anthocyanin biosynthesis genes (A, *CHS1*, B, *CHS4*, C, *F3H*, D, *F3'H*, E, *DFR*, F, *ANS*) (at S2) of gerbera cultivar 'Alliance' under two different temperatures.

Data represent the mean of three replicates, and the bar indicates the standard deviation. Means with asterisk(s) are statistically significant (*t* test, ** $P < 0.01$, * $P < 0.05$)

the increased information on anthocyanin pathways in gerberas, the currently available information does not adequately describe the complex mechanisms controlling flower

coloration. For this reason, we examined expression profiles of biosynthesis genes and TFs in 'Bintang' and 'Alliance' cultivars to determine whether coloration patterns

Fig. 9 Characterization of expression profiles of MYB10 and MYC1 (at S2) of gerbera cultivars (a, b) 'Bintang' and (c, d) 'Alliance' under two different temperatures. Data represent the mean of three

replicates, and the bar indicates the standard deviation. Means with asterisk are statistically significant (*t* test, **P* < 0.05)

are controlled by expression profiles. We also investigated whether temperature conditions (6 and 22 °C) influenced flower pigmentation in both cultivars via effects on gene expression levels.

Anthocyanin content increased linearly from S1 to S3 in 'Bintang', whereas the highest content was observed at S2 in 'Alliance'. Generally, the anthocyanin contents of both cultivars mirrored the expression profiles of the biosynthesis genes, indicating increased expression of genes from S1 to S3 in 'Bintang' and highest expression at S2 for 'Alliance'. In 'Bintang', it was difficult to determine the mechanisms by which genes influenced anthocyanin production even though the transcript levels of *CHS4*, *DFR*, and *ANS* were increased, because the expression of almost all biosynthesis genes was elevated along with higher anthocyanin content. However, in general, transcript levels of *CHS1* and *F3'H* were relatively low at all stages. Although *CHS4* expression was the highest among the biosynthetic genes, it would not predominantly control anthocyanin accumulation, because Deng et al. (2014) confirmed that higher expression of *CHS4* in gerbera petals than *CHS1* is not primarily responsible for flower pigmentation. Moreover, *DFR* expression levels would also not involve in the anthocyanin biosynthesis because its expression level did not reflect the result of S1. Furthermore, Bashandy et al. (2015) recently claimed that *DFR* expression could not result in pelargonidin-anthocyanin biosynthesis in cultivars Ivory and Estelle; however, it

exhibited specificity for cyanidin-anthocyanin biosynthesis. Probably, the anthocyanin present in the cultivars would be pelargonidin. Therefore, it is likely that *F3H* and *ANS* are potential candidates for the primary control of anthocyanin synthesis in this cultivar. The potential functional roles of all biosynthesis genes (*CHS1*, *F3'H*, *ANS*, and *DFR*), with the exception of *F3H*, in anthocyanin accumulation in gerberas has been reported in earlier studies (Meng et al. 2004; Seitz et al. 2006; Deng et al. 2014; Bashandy et al. 2015), and the results suggested that these individual genes likely differ in their role depending on the cultivars or pigment (cyaniding, pelargonidin, and delphinidin) compounds induced by the cultivars. In 'Bintang', *F3H* and *ANS* were more likely to be critical to anthocyanin biosynthesis.

In 'Alliance', variable anthocyanin content during petal development was likely associated with *CHS4*, *F3H*, *DFR*, and *ANS* expression patterns. However, as mentioned with regard to 'Bintang', *CHS4* would not be primarily responsible for flower pigmentation in gerberas (Deng et al. 2014); thus, *F3H*, *DFR*, and *ANS* are candidate genes for primary influence on anthocyanin synthesis. The functional involvement of *DFR* in regulating anthocyanin synthesis has been reported in President gerberas (Bashandy et al. 2015) and was consistent with the observations in 'Alliance'. Overall, *F3H* and *ANS* are more likely to be key biosynthesis genes in 'Bintang'; *F3H*, *DFR*, and *ANS* may be important in 'Alliance'. The presence of higher anthocyanin levels in the

former could have resulted from the existence of increased expression levels of all biosynthesis genes compared to the latter.

The expression patterns of genes for the TFs MYB10 and MYC1 were consistent with anthocyanin content during the three developmental stages. Thus, it is likely that co-expression of the TFs regulates anthocyanin biosynthesis genes involved in anthocyanin pathways, because the transcript levels of most of the biosynthetic genes (*CHS1*, *CHS4*, *F3H*, *F3'H*, and *ANS*) correlated with those of the TFs. Elomaa et al. (1998, 2003) demonstrated that the co-expression of MYB10 and MYC1 regulates anthocyanin biosynthetic genes in gerbera. Deng et al. (2014) claimed that elevation in MYB10 TF expression strongly increases the level of *CHS* transcript, which supports our finding. Similarly, the strong induction of *ANS* expression by homologous GMYB10 expression in gerbera has been reported (Laitinen et al. 2008).

The effect of temperature on anthocyanin synthesis has been documented for some species; for example, high temperatures cause a decrease in anthocyanin pigmentation in chrysanthemums (Nozaki et al. 2006), lilies (Lai et al. 2011), and roses (Dela et al. 2003), while low temperatures enhance pigmentation in petunias (Shvarts et al. 1997) and roses (Biran et al. 1973; Biran and Halevy 1974). However, the mechanisms by which temperature regulates the biosynthesis genes that control flower pigmentation remain unknown. The effect of temperature on pigmentation in gerbera flowers has not been studied in detail. Therefore, we investigated the influence of two different temperature conditions (6 and 22 °C) on anthocyanin pigmentation in 'Bintang' and 'Alliance' cultivars and determined the expression profiles of biosynthesis genes. Exposure of flowers to the low temperature treatment (6 °C) resulted in higher anthocyanin contents compared to plants exposed to 22 °C; this effect could be explained by the higher induction of all biosynthesis genes at 6 °C than at 22 °C. However, in 'Bintang' samples, *CHS1* and *DFR* were more highly regulated at 6 °C than the other genes, so the elevation of pigmentation content at 6 °C could be due to the higher induction of these two genes. Strong induction of *CHS* and *DFR* under low temperature conditions has been reported in *Arabidopsis*, citrus species, blood oranges, purple kale, and red oranges (Leyva et al. 1995; Lo Piero et al. 2005; Zhang et al. 2010; Crifò et al. 2012; Zhang et al. 2012; Carmona et al. 2017). However, based on above results in 'Bintang', *DFR* was assumed not to be primarily responsible for anthocyanin accumulation; it is likely that *CHS1* is potentially primary genes during exposure to a low temperature. The functional roles of *CHS1* in anthocyanin accumulation in gerberas have been documented (Meng et al. 2004; Deng et al. 2014). In addition, upregulation of *CHS* in petunia flowers under low temperature conditions has also been reported (Shvarts et al. 1997). In 'Alliance'

samples, anthocyanin content at 6 °C was higher than that observed at 22 °C, and this increase could be attributed to the increased expression levels of *CHS1*, *F3H*, and *ANS*. Higher induction of *F3H* and *ANS* expression under low temperature conditions has been reported in citrus species, *Arabidopsis*, and blood oranges (Zhang et al. 2010, 2012; Carmona et al. 2017).

In both the cultivars, the induction of higher biosynthetic gene expression at 6 °C could be explained by the upregulation of TF expression, because those expressed at 6 °C coincided with high TF expression, and the positive role of the TFs in the regulation of the anthocyanin biosynthetic genes has been discussed above. It can be suggested that the TFs (MYB10 and MYC) were observed to be highly expressed at 6 °C than at 22 °C; this supports the results of previous studies reporting the upregulation of MYB and MYC TF expression controlled by temperature in certain crops (Ban et al. 2007; Rowan et al. 2009; Lin-Wang et al. 2011). Overall, in both cultivars, it was likely that low temperature enhanced anthocyanin biosynthesis mainly through the increased expression of the TFs activating the expression of the detected genes, particularly *CHS1* in Bintang and *CHS1*, *F3H*, and *ANS* in Alliance, despite the increased expression of the other biosynthetic genes.

Conclusions

Flower pigmentation was initiated at an early stage (S1) in gerberas, but the maximum pigmentation obtained for each cultivar differed. The highest anthocyanin contents occurred at the S3 stage in 'Bintang' and at the S2 stage in 'Alliance'. Pigmentation patterns during petal development were generally associated with expression patterns of biosynthesis genes and TFs. However, the presence of higher anthocyanin content in 'Bintang' compared to 'Alliance' resulted from the cumulative expression levels of the detected genes. Low temperature treatment enhanced anthocyanin content via the strong induction of TFs and biosynthesis genes, particularly *CHS1* expression in 'Bintang' and *CHS1*, *F3H*, and *ANS* in 'Alliance'. This study demonstrated that pigmentation patterns, which varied during petal development and/or between the two cultivars, were associated with the expression profiles of biosynthesis genes and TFs as well as the transcriptional regulation of TFs and biosynthesis genes that are responsive to temperature changes.

Acknowledgements This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the Agri-Bio industry Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (Grant #: 315002-5).

Authors' contributions AHN designed the study, conducted the experiments, and wrote the manuscript. CKK supervised experiments at all stages and performed critical revisions of the manuscript. JHL, KIP, MYC, and KOK were involved in the experimentation and data analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

- Ai TN, Naing AH, Arun M, Kim CK (2016) Sucrose-induced anthocyanin accumulation in vegetative tissue of *Petunia* plants requires anthocyanin regulatory transcription factor genes. *Plant Sci* 252:144–150
- Ban Y, Honda C, Hatsuyama Y, Igarashi M, Hideo Bessho H, Moriguchi T (2007) Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red colouration in apple skin. *Plant Cell Physiol* 48:958–970
- Bashandy H, Pietiäinen M, Carvalho E, Lim K-J, Elomaa P, Martens S, Teeri TH (2015) Anthocyanin biosynthesis in gerbera cultivar 'Estelle' and its acyanic sport 'Ivory'. *Planta* 242:601–611
- Biran I, Halevy AH (1974) Effect of short-term heat and shade treatments on petal colour of 'baccara' roses. *Physiol Plant* 31:180–185
- Biran I, Enoch H, Zieslinand N, Helevy A (1973) The influence of light intensity, temperature and carbon dioxide concentration on anthocyanin content and bluing of 'baccara' roses. *Sci Hortic* 1:157–164
- Broun P (2005) Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in *Arabidopsis*. *Curr Opin Plant Biol* 8:272–279
- Carmona L, Alquézar B, Marques VV, Peña L (2017) Anthocyanin biosynthesis and accumulation in blood oranges during postharvest storage at different low temperatures. *Food Chem* 237:7–14
- Chandler S, Tanaka Y (2007) Genetic modification in floriculture. *Crit Rev Plant Sci* 26:169–197
- Crifò T, Petrone G, Lo Cicero L, Lo Piero A (2012) Short cold storage enhances the anthocyanin contents and level of transcripts related to their biosynthesis in blood oranges. *J Agric Food Chem* 60:476–481
- Dela G, Or E, Ovadia A, Nissim-Levi A, Weiss D, Oren-Shamir M (2003) Changes in anthocyanin concentration and composition in 'jaguar' rose flowers due to transient high-temperature conditions. *Plant Sci* 164:333–340
- Deng X, Bashandy H, Ainasoja M, Kontturi J, Pietiäinen M, Laitinen R, Albert VA, Valkonen JP, Elomaa P, Teeri TH (2014) Functional diversification of duplicated chalcone synthase genes in anthocyanin biosynthesis of *Gerbera hybrida*. *N Phytol* 201:1469–1483
- Elomaa P, Mehto M, Kotilainen M, Helariutta Y, Nevalainen L, Teeri TH (1998) A bHLH transcription factor mediates organ, region and flower type specific signals on dihydroflavonol-4-reductase (dfr) gene expression in the inflorescence of *Gerbera hybrida* (Asteraceae). *Plant J* 16:93–99
- Elomaa P, Uimari A, Mehto M, Albert VA, Laitinen RAE, Teeri TH (2003) Activation of anthocyanin biosynthesis in *Gerbera hybrida* (Asteraceae) suggests conserved protein-protein and protein-promoter interactions between the anciently diverged monocots and eudicots. *Plant Physiol* 133:1831–1842
- Grotewold E (2006) The genetics and biochemistry of floral pigments. *Annu Rev Plant Biol* 57:761–780
- Hansen HV (1999) A story of the cultivated *Gerbera*. *N Plantsman* 6:85–95
- Helariutta Y, Elomaa P, Kotilainen M, Seppänen P, Teeri TH (1993) Cloning of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterization of dfr expression in the corollas of *Gerbera hybrida* var *Regina* (Compositae). *Plant Mol Biol* 22:183–193
- Helariutta Y, Kotilainen M, Elomaa P, Kalkkinen N, Bremer K, Teeri TH, Albert V (1996) Duplication and functional divergence in the chalcone synthase gene family of Asteraceae: evolution with substrate change and catalytic simplification. *Proc Natl Acad Sci USA* 93:9033–9038
- Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci* 10:236–242
- Lai YS, Yamagishi M, Suzuki T (2011) Elevated temperature inhibits anthocyanin biosynthesis in the tepals of an Oriental hybrid lily via the suppression of LhMYB12 transcription. *Sci Hortic* 132:59–65
- Laitinen RAE, Immanen J, Auvinen P, Rudd S, Alatalo E, Paulin L, Ainasoja M, Kotilainen M, Koskela S, Teeri TH (2005) Analysis of the floral transcriptome uncovers new regulators of organ determination and gene families related to flower organ differentiation in *Gerbera hybrida* (Asteraceae). *Genome Res* 15:475–486
- Laitinen RA, Ainasoja M, Broholm SK, Teeri TH, Elomaa P (2008) Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*. *J Exp Bot* 59:3691–3703
- Leyva A, Jarillo JA, Salinas J, Martinez-Zapater JM (1995) Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis thaliana* a light-dependent manner. *Plant Physiol* 108:39–46
- Lin-Wang K, Micheletti D, Palmer J (2011) High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. *Plant Cell Environ* 34:1176–1190
- Lo Piero AR, Puglisi I, Rapisarda P, Petrone G (2005) Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage. *J Agric Food Chem* 53:9083–9088
- Martens S, Teeri T, Forkmann G (2002) Heterologous expression of dihydroflavonol 4-reductases from various plants. *FEBS Lett* 531:453–458
- Meng XC, Xing T, Wang X (2004) The role of light in the regulation of anthocyanin accumulation in *Gerbera* hybrid. *Plant Growth Regul* 44:243–250
- Nakatsuka T, Nishihara M, Mishiba K, Yamamura S (2005) Temporal expression of flavonoid biosynthesis related genes regulates flower pigmentation in gentian plants. *Plant Sci* 168:1309–1318
- Nozaki K, Takamura T, Fukai S (2006) Effects of high temperature on flower colour and anthocyanin content in pink flower genotypes of greenhouse chrysanthemum (*Chrysanthemum morifolium* Ramat.). *J Hortic Sci Biotechnol* 81:728–734
- Petroni K, Tonelli C (2011) Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Sci* 181:219–229
- Quattrocchio F, Wing JF, van der Woude K, Mol JNM, Koes R (1998) Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. *Plant J* 13:475–488
- Rowan DD, Cao M, Lin-Wang K (2009) Environmental regulation of leaf colour in red *35S:PAP1 Arabidopsis thaliana*. *N Phytol* 182:102–115
- Seitz C, Eder C, Deiml B, Kellner S, Martens S, Forkmann G (2006) Cloning, functional identification and sequence analysis of flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase cDNAs reveals independent evolution of flavonoid 3',5'-hydroxylase in the Asteraceae family. *Plant Mol Biol* 61:365–381

- Shvarts M, Borochoy A, Weiss D (1997) Low temperature enhances petunia flower pigmentation and induces chalcone synthase gene expression. *Physiol Plant* 99:67–72
- Wessinger CA, Rausher MD (2014) Predictability and irreversibility of genetic changes associated with flower color evolution in *Penstemon barbatus*. *Evolution* 68:1058–1070
- Yamagishi M, Shimoyamada Y, Nakatsuka T, Masuda K (2010) Two R2R3-MYB genes, homologs of *Petunia* AN2, regulate anthocyanin biosyntheses in flower tepals, tepal spots and leaves of Asiatic hybrid lily. *Plant Cell Physiol* 51:463–474
- Zhang Y, Zheng S, Liu Z, Wang L, Bi Y (2010) Both HY5 and HYH are necessary regulators for low temperature-induced anthocyanin accumulation in *Arabidopsis* seedlings. *J Plant Physiol* 167:934–945
- Zhang B, Hu Z, Zhang Y, Li Y, Zhou S, Chen G (2012) A putative functional MYB transcription factor induced by low temperature regulates anthocyanin biosynthesis in purple kale (*Brassica Oleracea* var. *acephala* f. *tricolor*). *Plant Cell Rep* 31:281–289
- Zhao DQ, Tao J, Han CX, Ge JT (2012) Flower color diversity revealed by differential expression of flavonoid biosynthetic genes and flavonoid accumulation in herbaceous peony (*Paeonia lactiflora* Pall.). *Mol Biol Rep* 39:11263–11275