Flower color is one of the most important traits in ornamental plants. Poor coloration of pink flower chrysanthemum during the high temperature season is a serious problem. To determine the effect of high temperature on the pigmentation, inflorescence development was divided into five stages. Plants were exposed to both 20 and 30°C during various developmental stages of inflorescence. HPLC analysis showed the main anthocyanins of pink flower chrysanthemum (cv. Pelican) were cyanidin 3-O-(6'-O-monomalonyl-β-glucopyranoside) and cyanidin 3-O-(3',6'-O-dimalonyl-β-glucopyranoside). The content of the two anthocyanins at 20°C was much higher than that at 30°C. In the inflorescence exposed to 30°C during bud break to vertical stage, pigmentation was not enhanced, even though the plants were subjected to 20°C from the vertical stage to 1-week-old. On the other hand, when the plants were exposed to 30°C during vertical stage to 1-week-old, pigment content decreased drastically, even though the inflorescence was kept at 20°C from the bud break to vertical stage. The results indicate that the petal extension to vertical stage is the most temperature sensitive and important for pigmentation. Expression of the anthocyanin biosynthesis-related genes (CmplCHS1, CmplCHS2, CmplCHI, CmplF3H2, CmplC3'H, CmplDFR1, CmplDFR2, and CmplANS) was depressed at 30°C compared with those at 20°C.

Keywords: anthocyanins, cyanidin, flower, developmental stages

INTRODUCTION

Chrysanthemum (Chrysanthemum morifolium Ramat.) is one of the most popular ornamental plants and is used as cut flowers, pot plants, landscape plants, and hobby plants all over the world. Chrysanthemum is the second largest cut flower in the world flower market (Royal Flora Holland, 2016). Year-round availability with stable quality is the key to success as a commercial floriculture item. The discovery of photoperiodism and its practical use (night break) has enabled year-round cut-flower production of chrysanthemum (Kahar, 2008). Recently, the importance of chrysanthemums has increased in Asia, and the expansion of cultural regions of cut chrysanthemum flowers has brought about new technical challenges to produce high quality cut flowers under various climates (Fukai, 2014). High temperature is one of the most serious problems for high quality horticultural production under cover cultivation conditions. When chrysanthemum is grown under high temperature conditions, the delay in flowering and insufficient petal coloration are major problems for stable flower production.

The inflorescences of pink flower chrysanthemum cultivars grown under high temperature conditions have shorter petals of ray florets with a pale color (Nozaki and Fukai, 2008). The degree of change in flower color differs depending on genotype (Nozaki et al., 2006). The main anthocyanins in red and pink flower chrysanthemum cultivars are cyanidin 3-O-(6'-O-monomalonyl-β-glucopyranoside) (Cy 3-6'-MMG) and cyanidin 3-O-(3', 6'-O-dimalonyl-β-glucopyranoside) (Cy 3-3',6'-DMG) (Nakayama et al., 1997; Nozaki et al., 2006). The contents of these two anthocyanins decrease under high temperature conditions, resulting in poor petal coloration of chrysanthemum (Nozaki et al., 2006).

Temperature is an important factor affecting anthocyanin biosynthesis. Anthocyanin, a major flower pigment, is the largest subclass of flavonoids widely found in plant species and is responsible for the orange and red to blue color in many plant tissues. Accumulation of flavonoids and anthocyanins plays important roles in attracting pollinators and protecting young plant tissues from damage caused by UV radiation and active oxygen species. Anthocyanin accumulation is sensitive to environmental conditions such as light and temperature. Reduced anthocyanin accumulation under high temperature conditions has been reported in many flowers including chrysanthemum (Nozaki et al., 2005), lily (Lai et al., 2011), rose (Dela et al., 2003), and fruits including grape (Mori et al., 2005) and apple (Ubi et al., 2006).

The anthocyanin biosynthetic pathway has been elucidated in detail, and most of the genes involved in the pathway have been isolated in many plants (Holton and Cornish, 1995). Lower anthocyanin accumulation in some fruits at elevated temperatures is brought about by a decreased rate of anthocyanin biosynthesis (Mori et al., 2005; Piero et al., 2005; Ubi et al., 2006; Li et al., 2012).
increased anthocyanin biosynthesis should be related to the expression level of anthocyanin biosynthesis-related genes. Cool temperatures have been shown to increase the transcription levels of genes that produce key enzymes, such as PAL, CHS, CHI, and DFR, in several plants (Mori et al., 2005; Wei et al., 2011). The anthocyanin biosynthesis-related genes in chrysanthemum have been reported (Puangkrit et al., in press). Lower gene expression of DFR and ANS in relation to high temperature has been reported (Huh et al., 2008).

Accumulation of anthocyanin is known to occur with petal development in various flowers (Justesen et al., 1997; Nakatsuka et al., 2005; Abe et al., 2008; Yamagishi et al., 2010; Zhou et al., 2011). However, it is not clear which developmental stage of inflorescence in chrysanthemum is most sensitive to high temperatures. Knowing the temperature-sensitive developmental stage is important in considering practical measures in commercial cut-chrysanthemum production under high temperature conditions.

The aim of this study is to reveal the effect of high temperatures on petal coloration, pigmentation, and gene expression of the flavonoid biosynthesis genes in chrysanthemum by the developmental stage of inflorescence.

MATERIALS AND METHODS

Plant materials

Pink chrysanthemum (*Chrysanthemum morifolium* cv. Pelican) was used in this study. Plants were grown in a growth chamber controlled at 25°C with natural radiation for two to three weeks and then subjected to short-day conditions (8:00–17:00) (SD) until flower buds developed to the BB stage. The plants were then transferred to growth chambers controlled at 20 or 30°C. The plants were transferred again to the growth chambers controlled at 20 or 30°C when the inflorescence developed a specific stage described in the experimental design.

Petal coloration

Five ray florets were sampled at different temperatures in each developmental stage of inflorescence. A colorimeter (Handy Colorimeter, NR-3000; Nippon Denshoku Industries Co., Ltd., Japan) was used to measure the color value of L* which represents the degree of dark and bright, a* which represents the degree of green and red, and b* which represents the degree of blue and yellow. In the CIELAB, the color coordinates C* and H* were calculated based on the following equations: C* = (a* ² + b* ²) ½ and H* = tan⁻¹ (b*/a*).

Pigment analysis

The anthocyanins were identified and their contents determined by using high-performance liquid chromatography (HPLC) controlled by the LC solution program (Shimadzu Co., Japan). Five ray florets were harvested and dried at 40°C for 24 h and then preserved under dry conditions at −20°C until use. Dry samples were immersed in acidified methanol (5% (v/v) formic acid : 100% methanol, v/v) and kept at 4°C for 24 h. These extracts were evaporated to dryness under reduced pressure and re-dissolved in 2 ml of the acidified methanol. The filtered extracts were injected into the HPLC. The chromatography system comprised two LC-20AT pumps (Shimadzu Co., Japan) equipped with two Cosmosil 5C18AR-II columns (4.6 mm i.d.×50 mm and 4.6 mm i.d.×250 mm; Nacalai Tesque Inc., Kyoto, Japan) and a SPD-M10A detector (Shimadzu Co., Japan). The column temperature was maintained at 40°C using a CTO-20A column oven (Shimadzu Co., Japan). Linear gradient elution for 40 min from 25 to 85% solvent B (1.5% H₃PO₄, 20% CH₃COOH, and 25% CH₃CN in H₂O, v/v) in solvent A (1.5% H₃PO₄ in H₂O, v/v) was used as the solvent system, and a flow rate of 0.8 ml min⁻¹ was maintained. The two main anthocyanins were identified by comparing their retention times at 520 nm with those of standard anthocyanins. The content of the anthocyanins was defined as the area within each anthocyanin pigment peak. The peaks by flavonoid pigments and their area in the chromatograms at 330 nm and 360 nm were also investigated.

Gene expression analysis

Total RNA was extracted from about 100 mg of petals by using an RNasy Mini Kit (Qiagen) and an RNase-Free DNase Set (Qiagen) to remove genomic DNA. The cDNA was synthesized from 1 μg total RNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Japan) according to the manufacturer’s instructions. The cDNA derived from 50 ng total RNA was used for quantitative real-time PCR (qRT-PCR). The qRT-PCR was carried out with the Thermal Cycler Dice Real-time System (Takara Bio Inc., Japan) using a KOD SYBR qPCR Mix (Toyobo). Gene specific primers to detect *CmplCHS1, CmplCHS2, CmplCHI, CmplF3H1, CmplF3H2, CmplF3H, CmplDFR1, CmplDFR2*, and *CmplANS* were designed between the open reading frame (ORF) and 3' untranslated region (3' UTR) of the sequences using the Primer 3 program (https://primer3.ut.ee). *Chrysanthemum actin* (*CmACT, AB205087*) was used as an internal control. The expression level of each gene was normalized using the internal control. Each qRT-PCR analysis was performed with three biological replicates.

Experiment design

Exp.1 Effect of high temperature on anthocyanin accumulation

Development of inflorescence was divided into five stages: bud break (BB), top of the inflorescence opened and transparent, membrane break; petal extension start (PE), petals become visible; vertical (VE), vertically elongated petals; 1 week old (1W), one week after petals vertically elongated; and 2 weeks old (2W), two weeks after petals vertically elongated. When the inflorescences were in the BB stage, the temperature treatments were started. First, plants were separated into two groups and each group was transferred to the growth chambers controlled at 20 or 30°C with a natural day length (Table 1). Petal coloration and anthocyanin content were determined at each developmental stage.

Exp.2 Effect of high temperature exposure during specific developmental stages of inflorescence on anthocyanin accumulation

To expose the inflorescence to different temperatures
during different developmental stages, exposure to specific temperatures was defined as (1) BB to PE, (2) PE to VR, (3) VR to 1W, and (4) 1W to 2W (Table 1). First, plants were separated into two groups and each group was transferred to the growth chambers controlled at 20 or 30 °C. Then, the plants were again separated into two groups and transferred to the growth chambers controlled at 20 or 30 °C when the inflorescence developed a specific stage. When the inflorescences reached the VE and 1W stage, petal coloration and anthocyanin content were determined to evaluate developmental stage-specific temperature effects.

**Exp.3 Effect of temperature on gene expression of key enzymes**

To reveal developmental stage-specific temperature effects on pigmentation and gene expression of flavonoid biosynthetic-related genes, the PE to 1W stage was subdivided into early vertical stage and full open stage. The plants were transferred to the growth chambers controlled at 20 or 30 °C from the BB stage. Samples for gene expression and flavonoid analysis were taken at the PE, early vertical, and petal full open stages (Table 2).

**RESULTS AND DISCUSSION**

**Exp.1 Effect of high temperature on anthocyanin accumulation**

Petal coloration and pigmentation were determined by using a colorimeter and HPLC. Petals showed distinct color differences when the inflorescences developed under 20 or 30 °C conditions (Fig. 1). Flowers developed at 30 °C displayed a pale petal color, especially in the later developmental stages of inflorescence. An increase in L*, and decreases in a* and C* were observed from the VE to 2W stage, regardless of the temperature conditions (Table 3). The a* and C* values at 30 °C were significantly lower than those at 20 °C.

HPLC analysis showed that the main anthocyanins of cv. Pelican were Cy 3-6≤-MMG and Cy 3-3≤,6≤-DMG, which were the same as other red-pink flower chrysanthemum genotypes (Nakayama et al., 1997; Nozaki et al., …)

**Table 1** Temperature (°C) treatment scheme in experiments 1 and 2.

| Experiment | Vertical to 1-week-old stage | 1-week-old to 2-week-old stage | Bud break to Petal extension stage |
|------------|-----------------------------|-------------------------------|----------------------------------|
| Exp.1      | 30                          | 30                            | 30                               |
| Exp.2      | 20                          | 30                            | 30                               |
| Exp.2      | 20                          | 30                            | 30                               |
| Exp.2      | 20                          | 30                            | 30                               |

**Table 2** Temperature (°C) treatment scheme in experiment 3.

| Experiment | Early vertical to Full open |
|------------|-----------------------------|
| Exp.1      | 20 (1)                      |
| Exp.2      | 20 (2)                      |
| Exp.2      | 20 (3)                      |

Numbers in parentheses indicate sampling time in Fig. 6.

**Table 3** Effect of temperature on flower color at each stage of chrysanthemum.

| Temperature | Stage       | CIE value measurement | Mean values followed by different letters in column indicate significant differences by Tukey’s multiple range test. |
|-------------|-------------|-----------------------|----------------------------------------------------------------------------------------------------------------|
|             |             | **L**                 | **a**                 | **b**                 | **C**                  | **h**                  |
| 20 °C       | Vertical    | 61.96                  | 40.80                 | −6.82                 | 41.39                  | 350.36                  |
|             | 1-week-old  | 66.89                  | 34.27                 | −8.70                 | 35.42                  | 345.66                  |
|             | 2-week-old  | 75.92                  | 18.80                 | −3.80                 | 19.23                  | 350.72                  |
| 30 °C       | Vertical    | 80.38                  | 3.89                  | 18.49                 | 17.19                  | 72.06                   |
|             | 1-week-old  | 87.11                  | 2.21                  | 8.58                  | 9.71                   | 72.99                   |
|             | 2-week-old  | 87.74                  | −0.54                 | 7.83                  | 7.87                   | 93.46                   |
Temperature conditions did not affect the component of the main anthocyanin, but the total amount of anthocyanin at 20°C was considerably higher than those at 30°C (Fig. 2) as reported by Nozaki et al. (2006). Anthocyanin content almost doubled from the VE to 1W stage, then decreased rapidly until the 2W stage at 20°C (Fig. 3). On the other hand, a constant decrease in both anthocyanins was observed at 30°C after the VE stage. The inconsistency of decreased a+ and increased anthocyanin content at 1W at 20°C might be explained by the surface structure of young petals in which wrinkles change the reflecting property.

Temperature is one of the major environmental factors influencing anthocyanin accumulation in plant organs. High temperature conditions have been shown to cause a decrease in anthocyanin accumulation in red apples (Tomana and Yamada, 1988), asters (Shaked-Sachray et al., 2002), roses (Dela et al., 2003), and chrysanthemums (Shibata et al., 1988; Nozaki et al., 2006). Poor coloration caused by lower pigmentation in chrysanthemum cv. Pelican petals at 30°C was confirmed in this study. The results show that coloration and pigmentation change depending on petal development at both 20 and 30°C.

Visible petal coloration started in the early developmental stage and accumulation increased according to petal development at 20°C (Figs.1 and 2). Developmental stage-dependent petal coloration and anthocyanin accumulation have been reported in campanula (Justesen et al., 1997), carnation (Mato et al., 2000; Abe et al., 2008), peony (Zhou et al., 2011), eustoma (Noda et al., 2004), gentian (Nakatsuka et al., 2005), and lily (Yamagishi et al., 2010). The results show that anthocyanin content decreases during petal senescence under both temperature conditions, especially at 30°C. The results also suggest that anthocyanin biosynthesis occurs during petal development, and decomposition is much higher than production after the flower fully opens.

Pigment content at a certain point is determined by subtracting decomposition from the production of the pigments. The present result suggests that high temperature accelerates decomposition of anthocyanins. It is thought that the disappearance of anthocyanin is due to both controlled catabolism and lower chemical stability of pigments (Oren-Shamir, 2009). Presented processes that anthocyanins are hydrolyzed by b-glucosidase, resulting in a change to chemically unstable anthocyanidins. The anthocyanidins are oxidized by polyphenol oxidase. Treatment with the reducing reagents dithiothreital and glutathione prevents anthocyanin degradation in Brunfelsia calycina petals (Vaknin et al., 2005), suggesting oxidation plays an important role in degradation of anthocyanins in petals. The oxidation is, in general, accelerated at elevated temperatures. This finding might be one reason for poor coloration and a quick change to a pale color in petals at 30°C.

Exp.2 Effect of high temperature exposure during specific developmental stages of inflorescence on anthocyanin accumulation

The inflorescence subjected to 30°C during the PE to VR stage showed low anthocyanin accumulation in the pet-
als, regardless of temperature conditions during the BB to PE stage (Fig. 4). Although the inflorescence was subjected to 30°C during the BB to PE stage, sufficient accumulation of anthocyanins occurred if the inflorescence was kept at 20°C during the PE to VE stage. The inflorescence exposed to 30°C from the BB to VE stage did not show sufficient pigmentation, even though the inflorescence was exposed to 20°C after the VE stage (Fig. 5). The results indicate that pigmentation in the petals occurs mainly in the PE to VE stage. The inflorescence exposed to 30°C after the VE stage also showed poor pigmentation, even though the inflorescence was kept at 20°C until the VE stage. The results suggest that quick decomposition of anthocyanin occurred at 30°C.

The present results showed clear developmental stage-dependent temperature effects on the pigmentation of chrysanthemum petals. The temperature conditions at the early petal developmental stage, i.e., the BB to PE stage, did not affect anthocyanin accumulation in the later developmental stage of the petals. On the other hand, the PE to VE stage was the most temperature-sensitive developmental stage of the chrysanthemum petals. The importance of temperature conditions during petal development for sufficient pigmentation is consistent with the findings of Huh et al. (2008) and Donzono et al. (2014). When inflorescence was exposed to 30°C from the BB to VE stage and then moved to 20°C, there was no further increase in pigmentation. This finding indicates that the ability of petal pigmentation is determined before completion of petal development. The pigment that was supposed to have accumulated sufficiently until the VE stage at 20°C disappeared because of the high temperature exposure after the VE stage (20°C-20°C-30°C). The result indicated that decomposition of anthocyanin is accelerated at 30°C as shown in Exp.1.

**Exp.3 Effect of temperature on gene expression of key enzymes**

In this experiment, to focus on the early developmental stage of inflorescence, pigmentation and anthocyanin biosynthesis-related gene expression was compared at the PE, early vertical, and petal full open stages. Anthocyanin content increased constantly from the PE to petal full open stage at 20°C, but only a small amount of anthocyanin was detected at 30°C (Fig. 6B). The flavonoid biosynthetic pathway produces not only anthocyanins but also flavonols and flavones. The two main peaks at λ<sub>max</sub> 330 nm and λ<sub>max</sub> 360 nm were considered to represent flavones and flavonols, respectively. No difference in flavone and flavonol accumulation was observed in petals developed at 20 and 30°C. This result is consistent with the result of Nozaki et al. (2005) in that the amount of flavonol did not change at high temperature.

Only CmplCHS was expressed considerably at the PE stage as an early gene in the anthocyanin biosynthesis pathway (Puangkrit et al., in press). According to petal development and anthocyanin accumulation, other gene expressions were up-regulated at 20°C. On the other hand, all
gene expression was depressed at 30°C. Similar lower expression of anthocyanin biosynthesis-related genes at elevated temperatures was reported in roses (Dela et al., 2003), grape (Mori et al., 2005), chrysanthemum (Huh et al., 2008), lily (Lai et al., 2011), and kiwifruit (Li et al., 2003), grape (Mori et al., 2005), chrysanthemum (Huh et al., 2008) reported a difference in temperature conditions because of treatment 3 was defined as 100%. C, relative content of flavones (\( \lambda_{\text{max}} \), 330 nm); the value of treatment 4 was defined as 100%. D, relative content of flavonols (\( \lambda_{\text{max}} \), 360 nm); the value of treatment 6 was defined as 100%. Vertical bars indicate SD (n=3).

Numbers in figure indicate the following:
1. Sampled at petal extension stage from inflorescence kept at 20°C.
2. Sampled at early vertical stage from inflorescence kept at 20°C.
3. Sampled at petal full open stage from inflorescence kept at 20°C.
4. Sampled at petal extension stage from inflorescence kept at 30°C.
5. Sampled at early vertical stage from inflorescence kept at 30°C.
6. Sampled at petal full open stage from inflorescence kept at 30°C.

Fig. 6 Effect of temperature on gene expression and flavonol accumulation during the petal development stages under difference temperature treatments. A, the relative expression levels of anthocyanin-biosynthetic genes. B, relative content of total anthocyanin; the value of treatment 4 was defined as 100%.

Depression of all flavonoid biosynthesis-related gene expression suggests the existence of temperature-dependent transcription factors. High temperature-dependent transcription factors were reported in pomegranate (Borochov-Neori et al., 2011) and oriental hybrid lily (Lai et al., 2011), but there is no information about chrysanthemum. Identifying high temperature-dependent transcription factor(s) in chrysanthemum would allow a better understanding of poor coloration of chrysanthemum petals under high temperature conditions.

Genotype difference in poor coloration of chrysanthemum petals has been reported (Nozaki et al., 2005; Huh et al., 2008). Huh et al. (2008) reported a difference in F3H expression level was observed in the genotype difference of petal coloration. If the F3H expression level was related to poor anthocyanin production under high temperature conditions, accumulation of flavones would change considerably. The present result shows no difference in flavone accumulation, suggesting that F3H is not key to poor anthocyanin production under high temperature conditions. Chen et al. (2009) reported that lower expression of DFR and GT is characterized in white flower chrysanthemum genotypes in which flavonol accumulation occurs instead
of anthocyanin. The present results showed no difference in flavonol accumulation by temperature conditions, suggesting that poor anthocyanin accumulation in pink flower chrysanthemums under high temperature conditions and white flower chrysanthemums are produced via different mechanisms.

ACKNOWLEDGMENTS

This study was supported by KAKENHI (26292021).

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