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Research article

Comparative analyses of light-induced anthocyanin accumulation and gene expression between the ray florets and leaves in chrysanthemum

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ABSTRACT

Light is one of the key environmental factors that affect anthocyanin biosynthesis. However, the underlying molecular mechanism remains unclear, and many problems regarding phenotypic change and corresponding gene regulation have not been solved. In the present study, comparative analyses of light-induced anthocyanin accumulation and gene expression between the ray florets and leaves were performed in *Chrysanthemum* × *morifolium* 'Purple Reagan'. After contrasting the variations in the flower color phenotype and relative pigment content, as well as expression patterns of structural and regulator genes responsible for anthocyanin biosynthesis and photoreceptor between different plant organs under light and dark conditions, we concluded that (1) both the capitulum and foliage are key organs responding to light for chrysanthemum coloration; (2) compared with flavones, shading makes a greater decrease on the anthocyanins accumulation; (3) most of the structural and regulatory genes in the light-induced anthocyanin pathway specifically express in the ray florets; and (4) *CmCHS*, *CmF3H*, *CmF3′H*, *CmANS*, *CmDFR*, *Cm3GT*, *CmMYB5-1*, *CmMYB6*, *CmMYB7-1*, *CmbHLH24*, *CmCOP1* and *CmHY5* are key genes for light-induced anthocyanin biosynthesis in chrysanthemum ray florets, while on the transcriptional level, the expressions of *CmPHYA*, *CmPHYB*, *CmCRY1a*, *CmCRY1b* and *CmCRY2* are insignificantly changed. Moreover, the inferred comprehensive effect of multiple signals on the accumulation of anthocyanins and transmission channel of light signal that exist between the leaves and ray florets were further discussed. These results further our understanding of the relationship between the gene expression and light-induced anthocyanin biosynthesis, and lay foundations for the promotion of the molecular breeding of novel flower colors in chrysanthemums.

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1. Introduction

The accumulation of anthocyanins, a class of plant flavonoid metabolites, governs flower color pigmentation in many plant families (Weiss, 1995). The genetics and biochemistry of the anthocyanin biosynthetic pathway have been well characterized in many model plants, such as *Arabidopsis* (*Arabidopsis thaliana*), snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*) (Winkel-Shirley, 2001). Light is one of the key environmental factors that affect anthocyanin biosynthesis, particularly light intensity (Albert et al., 2009). The exclusion of fruits or flowers from light has in many cases been demonstrated to suppress the expression of anthocyanin pathway genes, resulting in decreased amounts of anthocyanin compounds. For example, in the pericarp of the non-climacteric litchi (*Litchi chinensis*), fruit bagging treatments have been shown to inhibit the accumulation of anthocyanins and the expression of six structural genes in the anthocyanin biosynthetic pathway that were again up-regulated and accompanied by elevated anthocyanin accumulation after debagging and exposing the fruits to sunlight (Wei et al., 2011). Similar results were also obtained in grape (*Vitis vinifera*) (Matus et al., 2009; Azuma et al., 2012; Koyama et al., 2012), apple (*Malus domestica*) (Feng et al., 2013), pear (*Pyrus pyrifolia*) (Yu et al., 2012), and bayberry (*Myrica rubra*) (Niu et al., 2010). However, considering species- or even cultivar-specific anthocyanin biosynthesis (Feng

Abbreviations: 3GT, UDP-glucose: flavonoid-3-*O*-glucosyltransferase; ANS, anthocyanin synthase; bHLH, basic helix loop helix; CHS, chalcone synthase; CIRG, color index for red grapes; COP1, constitutive photomorphogenic 1; CRY, cryptochrome; DFR, dihydroflavonol-4-reductase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3'-hydroxylase; GA, gibberellic acid; HPLC, high performance liquid chromatography; HY5, elongated hypocotyl 5; PAP, purple anthocyanin pigment; PHOT, phototropin; PHY, phytochrome; RT-qPCR, real-time quantitative reverse transcription-PCR; SPA, Suppressor of PHYA; TFs, transcription factors.

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et al., 2013; Qian et al., 2013; Zheng et al., 2013), the underlying molecular mechanism of light-induced anthocyanin biosynthesis in higher plants remains unclear.

The ability to perceive and transduce light signal is important for optimal growth and development of higher plants (Zoratti et al., 2014), which is a precondition for investigating the deep molecular mechanism of light-induced anthocyanin biosynthesis. Plants employ a complex array of photoreceptors to coordinate their response to the ambient light environment (Wagner et al., 2005; Möglich et al., 2010; Rizzini et al., 2011). Principal among these photoreceptors is the phytochrome superfamily, including photoreceptors (PHYA, PHYB, PHYC, PHYD and PHYE) absorbing red/far-red light, cryptochromes (CRY1, CRY2 and CRY3), phototropins (PHOT1 and PHOT2) sensing UV-A/blue light, and UV-B photoreceptor UV RESISTENCE LOCUS 8 that has been recently identified (Favory et al., 2009; Rizzini et al., 2011; Casal, 2013). Upon light absorption, these photoreceptors activate various signal transduction cascades to regulate light-dependent responses and related gene expression in plants, mainly via combining with target transcription factors (TFs) (Zoratti et al., 2014). Although it has been reported that some photoreceptor genes also participate in the regulation of anthocyanin biosynthesis, such as in *Arabidopsis* (Fox et al., 2012) and grape (González et al., 2015), we know little about the relationship between the expression of photoreceptor genes and light-induced anthocyanin biosynthesis in ornamental plants.

The RING-finger type ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) acts as a negative regulator of light signaling directly at the downstream of the photoreceptors and control different light-regulated plant development processes by adjustment of its subcellular localization (Lau and Deng, 2012). ELONGATED HYPOCOTYL 5 (HY5), a bZIP TF promoting photomorphogenesis (Lee et al., 2007), is a direct target of COP1. HY5 becomes stabilized in light when the COP1 protein is removed from nucleus (Zoratti et al., 2014). HY5 has also been linked to activation of the R2R3-MYBs and key structural genes in the anthocyanin pathway as well as the accumulation of anthocyanins in response to light in *Arabidopsis* and apple (Hardtke et al., 2000; Stracke et al., 2010; Maier et al., 2013; Peng et al., 2013; Shin et al., 2013). Whether HY5 is also important for the regulation of light-induced anthocyanin biosynthesis in ornamental crops, however, has been rarely reported.

Chrysanthemums (*Chrysanthemum* × *morifolium*) are widespread ornamental crops with rich germplasms, whose yield and output values make them a leader in the global flower industry (Teixeira da Silva et al., 2013). Compared with other ornamental traits, the phenotypes of chrysanthemum flowers are particularly rich in color variation (Hong et al., 2012), which directly reflects their ornamental quality and commercial value. During the process of practice, we have found that the generation of chrysanthemum colors is significantly affected by light intensity. Therefore, investigating how the light regulates anthocyanin biosynthesis in chrysanthemums is attractive and significant.

In our previous study, a hypothetic anthocyanin biosynthetic pathway in the chrysanthemum cultivar 'Purple Reagan' was inferred using transcriptomic analyses (Hong et al., 2015b). We also inferred that six structural genes (*CmCHS*, *CmF3H*, *CmF3′H*, *CmANS*, *CmDFR* and *Cm3GT*) and four TFs (*CmMYB5-1*, *CmMYB6*, *CmMYB7-1*, and *CmbHLH24*) might be involved in light-induced anthocyanin biosynthesis. However, no key genes and related TFs that involve in the light signaling pathway were found. Moreover, previous studies mainly focused on the expression of anthocyanin genes in flowers or photoreceptor genes in leaves, the comparative analyses of these two gene groups among different plant organs have not been systematically performed. In the present study, the expression of five genes regarded as photoreceptors, including *CmPHYA*, *CmPHYB*, *CmCRY1a*, *CmCRY1b* and *CmCRY2*, as well as *CmCOP1* and its direct target *CmHY5*, were further contrasted in different plant organs (ray florets and leaves) of 'Purple Reagan' under light and shading conditions, together with the 10 genes we screened previously. The present study aims at furthering our understanding of the relationship between the expression of these two gene groups and light-induced anthocyanin biosynthesis in chrysanthemum, and laying foundations for the promotion of the molecular breeding of novel flower colors in chrysanthemums.

2. Materials and methods

2.1. Plant materials and shading treatments

The chrysanthemum cultivar 'Purple Reagan' was selected as the experimental material. This cultivar has floral competence at the 14-leaf stage and a limited inductive photoperiod of 43 days under short daylight conditions (12 h light/12 h dark) (Hong et al., 2015a). According to Sun et al. (2010), a total of five stages have been defined during capitulum development, named S1 through S5. In the present study, the capitulum (SC), all leaves (SL) and the whole plant (SWP) were respectively 100% shaded with silver paper during S1, after which the ray florets were collected, photographed and weighed in three replicates of 0.25 g every other day at 13:00 throughout the capitulum developmental stages from S2 to S5. All samples were collected in June 2015 from an artificial chamber (intensity of illumination = 50 ± 5 μmol m^{-2} s^{-1}, photoperiod = 16 h day/8 h night, and temperature = 20 °C) located at the Beijing Forestry University, Beijing, China. The samples were subsequently rapidly frozen in liquid nitrogen and kept at −80 °C for RNA extraction and pigment measurement. Samples treated with a fluorescent lamp (FL) were used as the control group (Fig. 1).

2.2. Phenotypic measurements

Ray floret color throughout the capitulum developmental stages was measured using a Hunter Lab Mini Scan XE Plus colorimeter (Hunter Associates Laboratory Inc., Tucson, Arizona, USA). The Commission Internationale del'Eclairage $L^*a^*b^*$ color scale was adopted (Hong et al., 2012), and raw data such as L^*, a^* and b^* were obtained. To better integrate the information of the raw data, the color index for red grapes (CIRG) was calculated according to CIRG = $(180\text{-}H)/(L^* + C)$, where $C = (a^{*2} + b^{*2})^{0.5}$ and $H = \arctan(b^*/a^*)$ (Carreño et al., 1995). Three random measurements were performed for each sample; the mean value was used for analysis.

2.3. Pigment determination

Anthocyanins were extracted as described previously (Hong et al., 2015b). The quantitative analysis was based on the method described by Lin and Harnly (2010). Each sample run for the high performance liquid chromatography (HPLC) was repeated three times under the same conditions.

2.4. Real-time quantitative reverse transcription-PCR analysis

For expression analysis using real-time quantitative reverse transcription-PCR (RT-qPCR), RNA was first isolated and used to generate cDNA from the ray florets of the plants subjected to the four shading treatments and sampled at the five different developmental stages. Then, RNA was also isolated from the leaves to contrast the gene expression trends between the ray florets and leaves. PCR reactions were performed using a Mini Opticon Real-time PCR System (Bio-Rad Laboratories Inc., Hercules, California, USA) with SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan) in

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