Molecular analysis of anthocyanin-related genes in ornamental cabbage

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Complete List of Authors:	Jin, Si-Won ; Suncheon National University, Department of Horticulture Rahim, Md Abdur; Suncheon National University, Department of Horticulture Kim, Hoy-Taek ; Suncheon National University, Department of Horticulture Park, Jong-In; Suncheon National University, Department of Horticulture Kang, Jong-Goo; Suncheon National University, Department of Horticulture Nou, Ill-Sup; Suncheon National University, Department of Horticulture
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1 **Molecular analysis of anthocyanin-related genes in ornamental cabbage**

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3 **Si-Won Jin†, Md Abdur Rahim†, Hoy-Taek Kim, Jong-In Park, Jong-Goo Kang and Ill-Sup Nou***

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6 Department of Horticulture, Sunchon National University, Suncheon 57922, Republic of Korea;

7
8
9
10
11 *Corresponding author: Ill-Sup Nou

12 Tel: +82-61-750-3249

13 E-mail: nis@sunchon.ac.kr

14 †These authors contributed equally to this work

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Draft

17 Abstract

18 Ornamental cabbage (*Brassica oleracea* var. *acephala*) is a winter-grown and important decorative plant
19 of the *Brassicaceae* family, which displays an exceptional coloration in the central leaves of the rosette.
20 Anthocyanins are the key determinant of the red, purple and blue colors of vegetative and reproductive
21 parts of many plant species including ornamental cabbage. Total anthocyanin content was measured
22 spectrophotometrically and the highest anthocyanin content detected in the red followed by light-red and
23 white ornamental cabbage lines. Anthocyanin biosynthesis is controlled by members of three different
24 transcription factor (TF) families such as MYB, basic helix-loop-helix (bHLH) and WD40 repeats (WDR)
25 which function as a 'MBW' complex. We identified three MYBs, six bHLHs, and one WDR that regulate
26 of anthocyanin biosynthesis in ornamental cabbage. The expression of the regulatory and the biosynthetic
27 genes for anthocyanin synthesis was determined by qPCR. The tested structural genes of anthocyanin
28 pathway were shown to be up-regulated in the red followed by light-red ornamental cabbage, however,
29 the expression levels of the late biosynthetic genes were barely detected in the white ornamental cabbage.
30 Among the regulatory genes, *BoPAP2* as *MYB*, *BoTT8*, *BoEGL3.1* and *BoMYC1.2* as *bHLH* and *BoTTG1*
31 as *WDR* were identified as candidates for the regulation of anthocyanin biosynthesis. This work could be
32 useful for the breeding of novel colorful ornamental cabbage cultivars.

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35 **Keywords:** ornamental cabbage; anthocyanin biosynthesis; structural genes; MYB; bHLH

36

37 **Introduction**

38 Ornamental cabbage (*Brassica oleracea* var. *acephala*) is an important fall-grown popular ornamental
39 plants belonging to the *Brassicaceae* family. The smooth leaves of this plant show striking coloration in
40 the center of the plants (Gibson and Whipker 2000). The demand of ornamental cabbage as a decorative
41 plant is increasing gradually due to its exceptional coloration in the central part of the plant and
42 availability during harsh winter when there is a scarcity of winter plants for gardeners. Therefore, the
43 ornamental cabbage provides an opportunity to the gardeners to grow it during winter.

44 Red, purple and blue color in foliage, flowers, fruits, cereal grains, and other plant parts are primarily
45 due to the accumulation of anthocyanin pigments (Abdel-Aal et al. 2006; Lin-Wang et al. 2010; Rahim et
46 al. 2014). In the flowers and ornamental plants, anthocyanins play an important role through providing
47 diverse brilliant colors. Anthocyanins are water soluble bioactive phenolic compounds (Eiro and
48 Heinonen 2002; Miguel 2011). They are belonging to the flavonoid class of plant pigments (Wang et al.
49 1997). They synthesized in the cytosol and transported into the cell vacuoles where they are more stable
50 (Mathews et al. 2003). The color variations of the anthocyanins are dependent on the vacuolar pH, co-
51 pigments (flavonols and flavones) and metal ions (Goto and Kondo 1991; Mol et al. 1998; Yoshida et al.
52 2003; Rahim et al. 2014). Anthocyanins play a significant role in pollination and seed dissemination
53 through painting flowers and fruits (Ryan et al. 2002; Whitney and Lister 2004; Lin-Wang et al. 2010;
54 Petroni and Tonelli 2011), however, they also defend plants from environmental stresses (Pourcel et al.
55 2007).

56 In plants, anthocyanin is synthesized via enzymes encoded for the structural genes and regulatory
57 genes (Quattrocchio et al. 1993). The structural genes are characterized in various plant species, including
58 flowers and ornamental plants. Anthocyanins are biosynthesized through the phenylpropanoid skeleton

59 where several enzymes involved in each step (Borevitz et al. 2000). The structural genes are regulated by
60 members of three different transcription factors (TFs) such as R2R3-MYB, basic helix-loop-helix (bHLH)
61 as well as WD40 repeats (WDR) families (Gonzalez et al. 2008; Lin-Wang et al. 2010). The MYB TFs
62 interact with the bHLH TFs to play a key regulatory role in anthocyanin biosynthesis (Allan et al. 2008;
63 Yuan et al. 2009; Feng et al. 2010; Lin-Wang et al. 2010; Albert et al. 2011). Moreover, the WDR is also
64 known to involve in the regulation process particularly in vegetative tissues (Albert et al. 2011). These
65 previous reports revealed that members of these three TF families coordinately control anthocyanin
66 biosynthesis through the formation 'MBW' (MYB, bHLH and WDR) transcriptional complexes. The
67 regulatory roles of these TFs have been demonstrated in many plant systems, for example, R2R3-MYBs
68 such as AtMYB75/PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1; AT1G56650),
69 AtMYB90/PAP2 (AT1G66390), AtMYB113 (AT1G66370) and AtMYB114 (AT1G66380) are involved
70 in anthocyanin biosynthesis in *Arabidopsis thaliana* (Borevitz et al. 2000; Ramsay and Glover 2005;
71 Stracke et al. 2007), while other MYBs regulating this process have been identified in various ornamental
72 species like petunia (PhAN2), gerbera (GMYB10), orchid (OgMYB1), lily (LhMYB12), gentian
73 (GtMYB3), lotus (NnMYB5) (Quattrocchio et al. 1999; Chiou and Yeh 2008; Laitinen et al. 2008;
74 Nakatsuka et al. 2008; Yamagishi et al. 2012; Sun et al. 2016). In red cabbage, MYB2 (an ortholog of
75 AtPAP1) plays a major regulatory role in anthocyanin biosynthesis (Yuan et al. 2009). In purple kales,
76 *BoPAP1* has shown to be up-regulated at low temperature condition (Zhang et al. 2012). In the curd of
77 purple cauliflower, MYB2 (an ortholog of AtPAP2 or AtMYB113) shown to regulate anthocyanin
78 production (Chiu and Li 2012). Heim et al. (2003) categorized 133 *Arabidopsis* bHLH TFs into 12-
79 subfamilies based on their numbers of introns, protein lengths, and the position of their bHLH domains.
80 The authors found that, only members of subgroup III had the plant-specific well-conserved amino acid
81 residues at the N-terminus, which are involved in flavonoid biosynthesis in *Arabidopsis*. In particular,

subgroup IIIf TRANSPARENT TESTA 8 (AtTT8; At4g09820), GLABRA 3 (AtGL3; At5G41315), ENHANCER of GLABRA 3 (EGL3; AtEGL3/At1G63650; also called AtEGL1) and AtMYC1 (At4G00480) were involved in flavonoid/anthocyanin biosynthesis (Bailey et al. 2003; Heim et al. 2003). In red cabbage, TT8 together with MYB2 up-regulates the structural genes of the anthocyanin pathway (Yuan et al. 2009). Likewise, Chiu et al. (2012) demonstrated that *BobHHLH1* (an orthologue of *AtTT8*) acts as a linker between the MYB and WDR in the 'MBW' complex in purple cauliflower. When expressed in *Arabidopsis bHLH* mutants, all three of these *bHLH* (*TT8*, *EGL3*, and *GL3*) genes were found to regulate anthocyanin biosynthesis when the cauliflower *MYB2* gene was constitutively overexpressed (Chiu et al. 2012). Furthermore, the WDR TRANSPARENT TESTA GLABRA 1 (AtTTG1; AT5G24520) was reported to contribute to anthocyanin biosynthesis in *Arabidopsis* (Walker et al. 1999). Conversely, a single repeat MYB (R3-MYB) TF called AtMYBL2 negatively regulates the anthocyanin biosynthesis in *Arabidopsis* (Matsui et al. 2008). Up to now, very little is known about the regulation of anthocyanin biosynthesis in ornamental cabbage, therefore, this work focused on the molecular analysis of anthocyanin-related genes in this plant.

96

97 **Results and Discussion**

98 **Total anthocyanin content in ornamental cabbage lines**

Ornamental cabbage displays a color variation in the central part of the leaves from red to light-red and whitish. In this study, total anthocyanin contents in the central leaves of red, light-red and white ornamental cabbage lines were analyzed (Fig. 1). The highest anthocyanin content was found in the red, with moderate levels in the light-red and extremely low levels of anthocyanins in the white ornamental cabbage (Fig. 2). Similar results were previously described for differently colored kale (Zhang et al. 2012) and bok choy (Zhang et al. 2014).

Identification of regulatory genes for anthocyanin biosynthesis in ornamental cabbage

Next, we performed a phylogenetic analysis using Mega 6.0 (Tamura et al. 2013) to determine the potential candidate MYB, bHLH and WDR TFs involved in anthocyanin regulation in ornamental cabbage. At the time of writing, no genome database for ornamental cabbage was available. Heading cabbage and ornamental cabbage are both sub-species of *Brassica oleracea* and very closely related, therefore, we used the heading cabbage database, ‘The *Brassica oleracea* Genome Database’ (Bolbase, <http://www.ocri-genomics.org/bolbase/>) as the genome reference for this study. To identify MYB TFs involved in anthocyanin biosynthesis, we compared several known anthocyanin regulating MYB TFs from different plant species and presented in a circular tree (Fig. 3). The analysis showed that there were three MYB-like sequences (BoPAP1, BoPAP2 and BoMYB114) in ornamental cabbage when compared with *Arabidopsis* anthocyanin promoting MYB TFs (AtPAP1, AtPAP2, AtMYB113 and AtMYB114) (Stracke et al. 2007; Heppel et al. 2013) in *Arabidopsis* (Fig. 3). Recently, Rahim et al. (2017) and Guo et al. (2014) also reported three candidates of MYB TFs in kholrabi (*Brassica oleracea*) and Chinese cabbage (*Brassica rapa*), respectively. Four MYB TFs were previously reported in both red cabbage (Yuan et al. 2009) and purple cauliflower (Chiu et al. 2010) when there was no reference genome database available for *Brassica oleracea*. However, the other regulators of anthocyanins are the bHLH and WDR. The *Arabidopsis* bHLH TFs like AtTT8, AtGL3, AtEGL3/EGL1 and AtMYC are known to contribute to flavonoid biosynthesis including anthocyanins (Heim et al. 2003). We analyzed ornamental cabbage together with several previously reported bHLH TFs from a different species and identified six bHLH-like sequences were identified in ornamental cabbage (Fig. 4). We followed similar nomenclature to the *Arabidopsis* for ornamental cabbage bHLH TFs (one TT8-like, BoTT8; one GL3-like, BoGL3; two EGL3/EGL1-like, BoEGL3.1 and BoEGL3.2, and two MYBC1-like, BoMYC1.1 and BoMYC1.2).

Several recent studies have suggested that shown TT8 is the best bHLH candidate for anthocyanin regulation in purple cultivars of *B. oleracea* (Yuan et al. 2009; Zhang et al. 2015; Lim et al. 2016). To date, four bHLH TFs (BrTT8, BrGL3, BrEGL3.1 and BrEGL3.2) have been reported in Chinese cabbage (*B. rapa*) by Guo et al. (2014) and three (BoTT8, BoGL3 and BoEGL3) have been reported in Kohlrabi (*Brassica oleracea* var. *gongylodes* L.) by Rahim et al. (2017). But for the first time we reported a total of six bHLH TFs in *B. oleracea* in this study. Likewise, we also analyzed WDR and identified just one putative candidate, BoTTG1 in ornamental cabbage (Fig. 5). Several studies are also in agreement with our result for *TTG1* gene in *B. oleracea* (Yuan et al. 2009; Zhang et al. 2015; Lim et al. 2016). Albeit, two *TTG1*-like (*BrTTG1.1* and *BrTTG1.2*) sequences have been reported but only *BrTTG1.1* is functional in *B. rapa* (Guo et al. 2014).

Expression profiles of anthocyanin biosynthetic genes in ornamental cabbage

Anthocyanins are produced via phenylpropanoid biosynthetic pathway, which is one of the most characterized secondary metabolite pathways in plants (Winkel-Shirley 2001; Li et al. 2012) including cabbage, cauliflower and Chinese cabbage (Yuan et al. 2009; Chiu and Li 2012; Zhang et al. 2012; Guo et al. 2014). We analyzed the transcript levels of several structural genes of the anthocyanin biosynthetic pathway by quantitative real time PCR (qPCR) in ornamental cabbage and the outcomes are presented in Fig. 6. The expression of ‘early biosynthetic genes (EBGs)’ like *BoPAL*, *BoCHS*, *BoCHI*, *BoF3H* were relatively higher in the red and light-red but were also expressed in the white ornamental cabbage. On the other hand, the ‘late biosynthetic genes (LBGs)’ such as *BoF3’H*, *BoDFR*, *BoLDOX* and *BoUFGT* were up-regulated in the red and decreased in the light-red but barely detected in the white ornamental cabbage. This indicates that these two sets of structural genes are differentially regulated in ornamental cabbage. Anthocyanins are biosynthesized in the cytoplasm where they are very unstable and need to be

transported to the cell vacuole for the stabilization (Mathews et al. 2003). We therefore considered the expression of *glutathione S-transferase (GST)* which is known to act as a vacuolar transporter of anthocyanins (Kitamura et al. 2004), and found that *BoGST* had an identical the expression pattern to the LBGs. The structural genes included in the study were followed the similar expression pattern as total anthocyanin contents (Fig. 2), as always relatively higher in the red, moderate levels in the light-red, very low in the white ornamental cabbage. These findings are in agreement with previous work reported by Zhang et al. (2012) in purple kale. Yuan et al. (2009) also showed that all of the structural genes (except *CHI*) of the anthocyanin pathway were highly expressed in red cabbage. Besides, the structural genes also positively correlated with the total anthocyanins in ornamental cabbage (Table 1).

Expression profiles of the regulatory genes of anthocyanin biosynthesis in ornamental cabbage

In this study, among the identified MYB TF genes, only *BoPAP2* shown to be up-regulated in the red and light-red ornamental cabbage (Fig. 7). In contrast, the remaining two namely, *BoPAP1* and *BoMYB114* had high expression levels in the white, with moderate expression in the light-red and low expression in the red ornamental cabbage genotypes. The expression pattern of *BoPAP2* was strikingly similar to the ‘LBGs’ and the anthocyanin contents suggesting that *BoPAP2* might play a central role in the activation of the structural genes specifically ‘LBG’ in red and light red ornamental cabbages. Some previous reports have highlighted the MYBs as the main regulator of anthocyanin biosynthesis in a variety of species including red radish (Lim et al. 2016), red cabbage (Yuan et al. 2009), purple cauliflower (Chiu et al. 2010), peach (Rahim et al. 2014), apple (Espley et al. 2007), sweet potato (Mano et al. 2007), lotus (Sun et al. 2016), petunia (Albert et al. 2011), gentian flower (Nakatsuka et al. 2008), and *Arabidopsis* (Borevitz et al. 2000). Moreover, previous reports on *Brassica* species have revealed that *PAP2/MYB2*, ortholog of *AtPAP2/AtMYB90*, was the key regulatory gene for anthocyanin biosynthesis in

174 this genus (Yuan et al. 2009; Chiu and Li 2012). In contrast, Zhang et al. (2012) reported that *PAP1* (an
175 ortholog of *AtPAP1/AtMYB75* and *BoMYB1*) is the key regulator for anthocyanin pigmentation in purple
176 kales. In monocotyledonous species, R2R2-MYB TFs regulate anthocyanin biosynthesis without forming
177 a complex with the bHLHs, however, these ‘MBW’ complexes are required to regulate anthocyanin
178 biosynthetic genes, specifically the LBG (*DFR*, *LDOX/ANS* and *UFGT*) in dicotyledonous species
179 (Petroni and Tonelli 2011). In this work, we identified six bHLH TFs in ornamental cabbage. Among
180 these, *BoTT8*, *BoEGL3.1* and *BoMYC1.2* exhibited a similar expression pattern to *BoPAP2* (Fig. 8). The
181 expression of the remaining three bHLH genes, *BoGL3*, *BoEGL3.2* and *BoMYC1.1* was found to be
182 highest in the red ornamental cabbage and the lowest in the light-red and white genotypes. There were no
183 distinguishable expression differences between the bHLHs in the light-red and white genotypes. These
184 results reveal that *BoTT8*, *BoEGL3.1* and *BoMYC1.2* might be the bHLH candidates for anthocyanin
185 regulation in ornamental cabbage. The bHLH gene called *TT8* shown to be up-regulated in purple
186 cultivars of *Brassica* crops including cabbage, kohlrabi Chinese cabbage, radish (Yuan et al. 2009; Ahmed
187 et al. 2014; Zhang et al. 2015; Lim et al. 2016). *BoTTG1* encoding a WD40 repeat protein was expressed
188 at a similar magnitude in all three ornamental cabbage lines, which did not match the expression pattern
189 of *BoPAP2* and LBGs. Certain MYBs also play a negative impact on anthocyanin biosynthesis (Dubos et
190 al. 2008; Matsui et al. 2008). One such negative regulator is *AtMYBL2*, a short protein that contains single
191 MYB (R3-MYB) domain that competes with the R2R3-MYBs for the opportunity to interact with the
192 bHLHs and regulate the flavonoid biosynthesis genes (Chiu and Li 2012). In ornamental cabbage, we
193 identified two *MYBL2*-like sequences (*BoMYBL2.1* and *BoMYBL2.2*). The expression of *BoMYBL2.1* was
194 dramatically increased in the light-red and white genotypes, while it was hardly detected in red
195 ornamental cabbage (Fig. 9). In contrast, the expression levels of *BoMYBL2.2* were roughly similar in all
196 three ornamental cabbage lines. In addition, *BoMYBL2.1* expression was negatively correlated with the

expression of *BoPAP2* (Table S2) and the total anthocyanin content (Table 1).

Our data show that *BoPAP2* expression had a highly significant positive correlation with anthocyanin content (Table 1) and the expression of the *bHLH* genes except *BoGL3* (Table S2). It also revealed a negative correlation between *BoPAP2* expression and that of *BoPAP1* and *BoMYB114*. Furthermore, the expression levels of all the anthocyanin biosynthetic genes investigated in this study were significantly correlated with *BoPAP2* (Table 2). These findings suggest that *BoPAP2* might be a vital regulatory gene for anthocyanin biosynthesis in ornamental cabbage.

Materials and Methods

Experimental plant materials

The advanced lines ornamental cabbage with distinct color phenotypes such as red (SCNU-OC-41-4, SCNU-OC-41-5 and SCNU-OC-41-6), light-red (SCNU-OC-41-30, SCNU-OC-41-31 and SCNU-OC-41-32) and white (SCNU-OC-41-43, SCNU-OC-41-50 and SCNU-OC-41-51) were used in this study (Fig. 1). These advanced lines were developed in the Department of Horticulture, Sunchon National University, Republic of Korea. The seeds of selected lines were sown in a nursery soil mixture and the seedlings were grown at plant growth chamber. After four weeks, the young seedlings were transferred to the glasshouse. The samples were taken from the 10th leaves from each line for anthocyanin quantification.

Extraction of total RNA and cDNA synthesis

Total RNA was isolated from the 10th leaves of the red, light-red and white advanced lines of ornamental cabbage. The leaves were collected in liquid nitrogen and stored at -80°C. The frozen leaves were ground into a fine powder in liquid nitrogen. After that, RNA was extraction from 100 mg finely grounded powder with an RNeasy mini kit (Qiagen, USA). The quantity and purity were ensured by a

220 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). A total of 1 µg
221 total RNA was converted into cDNA with SuperScript® III commercial kit (Invitrogen, Gaithersburg,
222 MD).

223

224 **Expression profiling using qPCR**

225 The gene expression analysis was accomplished by quantitative real time PCR (qPCR) using
226 ‘LightCycler®96’ instrument (Roche, Mannheim, Germany). The reactions were done using ‘2x SyGreen
227 Mix Lo-ROX (qPCRBIO)’ (PCR Biosystems, London, UK) with gene-specific oligonucleotide primers
228 (Table S1) and 50 ng/µl cDNA as templates. The sequences were retrieved from Bolbase database
229 (<http://ocri-genomics.org/bolbase/>) for designing primers. The PCR conditions were 95°C for 5 min for
230 enzymatic activation followed by 95°C for 10 s, 60°C for 10 s, 72°C (15 s) for 50 cycles. After
231 completion of PCR cycles, the Cq values were analyzed using LightCycler® 96 software (Roche,
232 Germany). Furthermore, the efficiency of each primer pair was checked using the standard curves, which
233 were generated using pooled mixture of all individual cDNA samples of ornamental cabbages and four
234 series dilution of pooled mix (Table S1). The expression analyses for all the genes mentioned in this study
235 were performed with three independent biological and three technical replicates. Their relative expression
236 levels were analyzed with the comparative $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) using *ACTIN* as
237 the house-keeping gene.

238

239 **Extraction and quantification of total anthocyanins**

240 Total anthocyanins were taken out from the leaves (similar area of leaves used for RNA extraction) of
241 ornamental cabbage by methanol with 1% HCl (Mehrtens et al. 2005). The samples were collected from
242 the center of the leaf where the red pigmentation was visible. The leaves were powdered in liquid nitrogen

and transferred into a tube containing methanol (1% HCl) and kept overnight at room temperature. Later, the solution was centrifuged at 14,000 rpm for 10 min and optical density was determined spectrophotometrically (wavelength 530 and 657 nm). Finally, total anthocyanin was stated as $Q = (A_{530} - 0.25 \times A_{657}) \times FW^{-1}$, where, Q = total anthocyanins; A_{530} = absorption at 530 nm; A_{657} = absorption at 657 nm; FW = fresh weight of leaves (g).

Statistical analysis

A Pearson's correlation analysis was performed using Cq values for each gene and the total anthocyanin content by MINITAB17 (Minitab Inc., USA).

Conclusions

The red ornamental cabbage lines had the greatest total anthocyanin contents in the center of their leaves. A total of 21 genes related to anthocyanins biosynthesis including structural and regulatory genes were analyzed by qPCR. Each of the structural genes was up-regulated in the red ornamental cabbage and in the light-red genotypes to a lesser extent. Of the three MYB regulatory genes identified, only *BoPAP2* was up-regulated in the red and light-red ornamental cabbage genotypes. All the *bHLHs*, were up-regulated in the red ornamental cabbage, but only *BoTT8*, *BoEGL3.1* and *BoMYC1.2* had a similar expression pattern to *BoPAP2*. Furthermore, only one gene, *BoTTG1*, was identified for the WDR family, which was found to be expressed similarly in all three ornamental cabbage genotypes. Conversely, the negative regulator *BoMYBL2.1* was highly expressed in the light-red and white genotypes while its expression was hardly detectable in the red ornamental cabbage genotypes. These findings could be valuable for the development of colorful ornamental cabbage cultivars, particularly using marker assisted breeding.

266

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List of Tables

Table 1. Correlation (r) of total anthocyanin contents with the expression of anthocyanin related genes in ornamental cabbage.

	Gene name	Anthocyanin content (r)
MYB genes	<i>BoPAP1</i>	-0.778
	<i>BoPAP2</i>	0.972***
	<i>BoMYB114</i>	-0.875*
bHLH genes	<i>BoTT8</i>	0.965**
	<i>BoGL3</i>	0.820*
	<i>BoEGL3.1</i>	0.976***
	<i>BoEGL3.2</i>	0.913**
	<i>BoMYC1.1</i>	0.913**
	<i>BoMYC1.2</i>	0.958**
WDR gene	<i>BoTTG1</i>	0.690
MYBL2	<i>BoMYBL2.1</i>	-0.799*
	<i>BoMYBL2.2</i>	0.559
Biosynthetic genes	<i>BoPAL</i>	0.957**
	<i>BoCHS</i>	0.981***
	<i>BoCHI</i>	0.924**
	<i>BoF3H</i>	0.911**
	<i>BoF3'H</i>	0.919**
	<i>BoDFR</i>	0.964**
	<i>BoLDOX</i>	0.972***
	<i>BoUFGT</i>	0.963**
	<i>BoGST</i>	0.933**

[*: $p \leq 0.05$; **: $p \leq 0.01$ and ***: $p \leq 0.001$]

413 **Table 2.** Relationship between the expression levels of the MYBs and the structural genes of anthocyanin biosynthesis in
414 ornamental cabbage.

Gene name	<i>BoPAP1</i>	<i>BoPAP2</i>	<i>BoMYB114</i>
<i>BoPAL</i>	-0.041	0.942**	-0.067
<i>BoCHS</i>	-0.254	0.989***	-0.194
<i>BoCHI</i>	-0.217	0.954**	-0.219
<i>BoF3H</i>	-0.135	0.945**	-0.136
<i>BoF3'H</i>	-0.362	0.909**	-0.146
<i>BoDFR</i>	-0.407	0.911**	-0.177
<i>BoLDOX</i>	-0.412	0.913**	-0.177
<i>BoUFGT</i>	-0.238	0.980***	-0.125
<i>BoGST</i>	-0.338	0.947**	-0.115

415

416 [*: $p \leq 0.05$; **: $p \leq 0.01$ and ***: $p \leq 0.001$]

418 List of Figures

419 **Fig. 1.** Ornamental cabbage lines used in this study.

420
421 **Fig. 2.** Total anthocyanin content in the ornamental cabbage lines.

422
423 **Fig. 3.** A circular phylogenetic analysis of anthocyanin regulating MYB transcription factors in different species. Trees were
424 constructed by UPGMA method considering bootstrap values as 1000 by Mega 6.0 (Tamura et al. 2013). The green and red
425 solid circles represent anthocyanin promoting MYB TFs of *Arabidopsis* and ornamental cabbage, respectively. The deduced
426 amino acid sequences were retrieved from <http://www.ocri-genomics.org>, <http://plants.ensembl.org>,
427 <https://www.arabidopsis.org> and GenBank databases. The accessions are Bol042409 (BoPAP1), Bo6g100940 (BoPAP2),
428 Bol012531 (BoMYB114), AT1G56650 (AtPAP1), AT1G66390 (AtPAP2), AT1G66370 (AtMYB113), AT1G66380
429 (AtMYB114), ABX71488.1 (PpyMYB10), ABB84755.1 (MdMYB10a), ABK58136.1 (MdMYB1), AAQ55181.1 (SIANT1),
430 ABB83828.1 (AmVENOSA), ABB83826.1 (AmROSEA1), XP_018444376.1 (RsMYB1), CAE75745.1 (CaA), AAF66727.1
431 (PhAN2), AAA19819.1 (ZmP1), AAA33482.1 (ZmC1), BAD04024.1 (OsC), ADP76649.1 (MYB1_cauliflower),
432 ADP76651.1 (MYB2_cauliflower), ADP76652.1 (MYB3_cauliflower), ADP76653.1 (MYB4_cauliflower), Bra001917
433 (BrMYB1), Bra004162 (BrMYB2), Bra039763 (BrMYB114), ALU11263.1 (NnMYB5), BAF96933.1 (GtMYB3),
434 BAO04193.1 (LhMYB12) and ABS58501.1 (OgMYB1).

435
436 **Fig. 4.** Phylogenetic tree (circular) showing anthocyanin promoting bHLH TFs of different plant species. Tree was constructed
437 by UPGMA method considering bootstrap values as 1000 using Mega 6.0 (Tamura et al. 2013). The deduced amino acid
438 sequences were retrieved from <http://www.ocri-genomics.org>, <https://www.arabidopsis.org> and GenBank databases. The green
439 and red solid circles indicate anthocyanin regulating bHLH TFs of *Arabidopsis*, ornamental cabbage, respectively. The
440 sequence accessions are Bol010751 (BoMYC1.1), Bol011507 (BoMYC1.2), Bol004077 (BoTT8), Bol014556 (BoGL3),
441 Bol022614 (BoEGL3.1), Bol029662 (BoEGL3.2), Bra037887 (BrTT8), Bra025508 (BrGL3), Bra027796 (BrEGL3.1),
442 Bra027653 (BrEGL3.2), AAA32663 (AmDEL), ADL36597 (MdbHLH3), ABB84474 (MdbHLH33), BAE94393 (InDEL),
443 BAE94394 (InIVS), BAJ33515 (DvIVS), BAJ33516 (DvDEL), AAC39455 (PhJAF13), AAG25928 (PhAN1), AT4G00480
444 (MYC1), AT5G41315 (GL3), AT1G63650 (EGL3) and AT4G09820 (TT8).

445 **Fig. 5.** A phylogenetic tree showing WD40 repeats (WDR) in different plant species. Tree was constructed by UPGMA method
446 with 1000 bootstrap values by Mega 6.0 (Tamura et al. 2013). The deduced amino acid sequences were retrieved from
447 <http://www.ocri-genomics.org>, <https://www.arabidopsis.org> and GenBank databases. The solid green and solid red circles
448 denote anthocyanin regulating WDR of *Arabidopsis* and ornamental cabbage, respectively. The accessions are Bol022420
449 (BoTTG1), Bol031394 (BoWD40 repeat-like, as outgroup), XP_009150978.1 (BrTTG1), XP_013643416.1 (BnTTG1),
450 XP_018484504.1 (RsTTG1), AAC18914.1 (PhAN11), XP_010251384.1 (NnTTG1), AAM76742.1 (ZmPAC1), AT5G24520
451 (AtTTG1), ADI58760.1 (MdTTG1), BAE94398.1 (InWDR1).

452
453 **Fig. 6.** Relative expression of anthocyanin biosynthetic genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the
454 means of triplicates.

455
456 **Fig. 7.** Relative expression of MYB genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the means of three
457 replications.

458
459 **Fig. 8.** Relative expression of bHLH genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the means of three
460 replications.

461
462 **Fig. 9.** Relative expression of *TTG1* (WD40 repeats) gene and *MYBL2* genes in ornamental cabbage lines. Error bar denotes $\pm$
463 SE of the means of three replications.

Fig. 1. Ornamental cabbage lines used in this study.

207x96mm (300 x 300 DPI)

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Fig. 2. Total anthocyanin content in the ornamental cabbage lines.

90x81mm (300 x 300 DPI)

Fig. 3. A circular phylogenetic analysis of anthocyanin regulating MYB transcription factors in different species. Trees were constructed by UPGMA method considering bootstrap values as 1000 by Mega 6.0 (Tamura et al. 2013). The green and red solid circles represent anthocyanin promoting MYB TFs of Arabidopsis and ornamental cabbage, respectively. The deduced amino acid sequences were retrieved from <http://www.ocri-genomics.org>, <http://plants.ensembl.org>, <https://www.arabidopsis.org> and GenBank databases. The accessions are Bol042409 (BoPAP1), Bo6g100940 (BoPAP2), Bol012531 (BoMYB114), AT1G56650 (AtPAP1), AT1G66390 (AtPAP2), AT1G66370 (AtMYB113), AT1G66380 (AtMYB114), ABX71488.1 (PpyMYB10), ABB84755.1 (MdMYB10a), ABK58136.1 (MdMYB1), AAQ55181.1 (SIANT1), ABB83828.1 (AmVENOSA), ABB83826.1 (AmROSEA1), XP_018444376.1 (RsMYB1), CAE75745.1 (CaA), AAF66727.1 (PhAN2), AAA19819.1 (ZmP1), AAA33482.1 (ZmC1), BAD04024.1 (OsC), ADP76649.1 (MYB1_cauliflower), ADP76651.1 (MYB2_cauliflower), ADP76652.1 (MYB3_cauliflower), ADP76653.1 (MYB4_cauliflower), Bra001917 (BrMYB1), Bra004162 (BrMYB2), Bra039763 (BrMYB114), ALU11263.1 (NnMYB5), BAF96933.1 (GtMYB3), BAO04193.1 (LhMYB12) and ABS58501.1 (OgMYB1).

207x214mm (300 x 300 DPI)

Fig. 4. Phylogenetic tree (circular) showing anthocyanin promoting bHLH TFs of different plant species. Tree was constructed by UPGMA method considering bootstrap values as 1000 using Mega 6.0 (Tamura et al. 2013). The deduced amino acid sequences were retrieved from <http://www.ocri-genomics.org>, <https://www.arabidopsis.org> and GenBank databases. The green and red solid circles indicate anthocyanin regulating bHLH TFs of Arabidopsis, ornamental cabbage, respectively. The sequence accessions are Bol010751 (BoMYC1.1), Bol011507 (BoMYC1.2), Bol004077 (BoTT8), Bol014556 (BoGL3), Bol022614 (BoEGL3.1), Bol029662 (BoEGL3.2), Bra037887 (BrTT8), Bra025508 (BrGL3), Bra027796 (BrEGL3.1), Bra027653 (BrEGL3.2), AAA32663 (AmDEL), ADL36597 (MdbHLH33), ABB84474 (MdbHLH33), BAE94393 (InDEL), BAE94394 (InIVS), BAJ33515 (DvIVS), BAJ33516 (DvDEL), AAC39455 (PhJAF13), AAG25928 (PhAN1), AT4G00480 (MYC1), AT5G41315 (GL3), AT1G63650 (EGL3) and AT4G09820 (TT8).

201x191mm (300 x 300 DPI)

Fig. 5. A phylogenetic tree showing WD40 repeats (WDR) in different plant species. Tree was constructed by UPGMA method with 1000 bootstrap values by Mega 6.0 (Tamura et al. 2013). The deduced amino acid sequences were retrieved from <http://www.ocri-genomics.org>, <https://www.arabidopsis.org> and GenBank databases. The solid green and solid red circles denote anthocyanin regulating WDR of Arabidopsis and ornamental cabbage, respectively. The accessions are Bol022420 (*BoTTG1*), Bol031394 (*BoWD40 repeat-like*, as outgroup), XP_009150978.1 (*BrTTG1*), XP_013643416.1 (*BnTTG1*), XP_018484504.1 (*RsTTG1*), AAC18914.1 (*PhAN11*), XP_010251384.1 (*NnTTG1*), AAM76742.1 (*ZmPAC1*), AT5G24520 (*AtTTG1*), ADI58760.1 (*MdTTG1*), BAE94398.1 (*InWDR1*).

176x97mm (300 x 300 DPI)

Fig. 6. Relative expression of anthocyanin biosynthetic genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the means of triplicates.

191x290mm (300 x 300 DPI)

Fig. 7. Relative expression of MYB genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the means of three replications.

89x173mm (300 x 300 DPI)

Fig. 8. Relative expression of bHLH genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the means of three replications.

190x174mm (300 x 300 DPI)

Fig. 9. Relative expression of TTG1 (WD40 repeats) gene and MYBL2 genes in ornamental cabbage lines. Error bar denotes $\pm$ SE of the means of three replications.

190x116mm (300 x 300 DPI)

Table S1. . List of gene-specific primers used in this study for qPCR, their amplification efficiencies and correlation coefficients.

Genes		Primer sequences (5'-3')	E (%)	r ²	Bolbase Accessions
<i>BoPAP1*</i>	F	TCTTCTACCACACCCGCCCA	95.1	0.999	Bol042409
	R	CCTCTAGCAAACCTCTCCACCACA			
<i>BoPAP2*</i>	F	TCTTATACCACACCAGCCCAAAAA	94.8	0.996	Bol012528
	R	TTCCACCACCTATTCTGACCATC			
<i>BoMYB114*</i>	F	AGACCCAAATGAAAAAGAGAAACC	99.2	0.970	Bol012531
	R	CCAAGGCATAGGGGAACAAAT			
<i>BoTT8*</i>	F	TCATGTCACAACCCACCAGTCTTC	98.52	0.999	Bol004077
	R	TCGCGGCAGCCTCTTATTTT			
<i>BoGL3*</i>	F	CATCTGGAACCGCATCAATCGTAG	90.9	0.995	Bol014556
	R	TCTCCCGCGTTTCTTCTCTGA			
<i>BoEGL3.1*</i>	F	ACCGGGAACCATGCCTTGTC	99.9	0.999	Bol022614
	R	TGCTCTCTCATCTTCATCTCCG			
<i>BoEGL3.2</i>	F	ACGCGGTGGAGGAGGTCACTA	105.9	0.999	Bol029662
	R	CGTGGTTCGCGTTTCATCTC			
<i>BoMYC1.1</i>	F	CTTGGGCGGTGTGATTGAGC	100.6	0.999	Bol010751
	R	GGGTTTCTTGAGTGCCGAGT			
<i>BoMYC1.2</i>	F	GAGCTTTGGTGACGGGTGAGAC	98.9	0.999	Bol011507
	R	TGGCGAGCAGAGAAGTTGATGA			
<i>BoTTG1*</i>	F	GCGGAGCTTGAAAGGCACCAG	94.9	0.999	Bol022420
	R	CAATCAGGCAGCGAAGAAGACCAC			
<i>BoMYBL2.1</i>	F	GCCACGGCGCTCTATCTCG	93.3	0.997	Bol016164
	R	GTCGGTTCGTCCAGGCAATCT			
<i>BoMYBL2.2*</i>	F	CCTTAGGCTTCATGCACCTCTTG	100.2	0.999	Bol034966
	R	ACGATGGTTGGTTGGGTCTATTC			
<i>BoPAL*</i>	F	AAGGCGAGCAGTGATTGGGTGAT	93.6	0.999	Bol025522
	R	CTTGTGGCGGAGTGTGGCAGTGT			
<i>BoCHS*</i>	F	GCCAGCCCAAGTCCAAGATTACC	100.1	0.999	Bol043396
	R	CCGCCGCGAAGCAACCT			
<i>BoCHI*</i>	F	ACCGGCTTCCTCCAACCTCTC	100.0	0.999	Bol044343
	R	CGGCAGCTTCATCGTCACTTG			
<i>BoF3H*</i>	F	TCGTACCCGGTGAGAAACAGAGA	98.4	0.999	Bol010585
	R	TATCGACGCATGCATTGGTGA			
<i>BoF3'H*</i>	F	GAATCTGCGCCGGGTGAG	95.9	0.998	Bol043829
	R	CCGATCCGAGTCCGTAAGCA			
<i>BoDFR*</i>	F	CCGGCGCATCAGGATTCATT	99.8	0.998	Bol035269
	R	CGCCGTCGCATCCGTTTAT			
<i>BoLDOX*</i>	F	AGCAAAAAGCGGAATCAACTCAA	93.0	0.999	Bol014986
	R	CCAATCCATAGCCGCTTCTT			
<i>BoUFGT*</i>	F	CCTCTCGCGTTGTTATCATCTGC	98.9	0.994	Bol021317
	R	GACTCCAACCTTCCGCTATCG			
<i>BoGST*</i>	F	GGGCTGATGTTGAGGTGGGTAC	96.9	0.986	Bol019821
	R	CCGGCATATGCGTCAAATCAG			
<i>BoActin*</i>	F	CTGAGGAGCACCCGTTCTTCTTA	99.2	0.999	Bol003004
	R	CGGAGGATGGCGTGTGGTAGA			

E, qRT-PCR efficiencies (%); r², correlation coefficient of standard curves; standard curve was calculated with 3 points; *, *Brassica oleracea* primers reported from our laboratory by Rahim et al. (2017).

Table S2. Relationship among the expression levels of the anthocyanin regulatory and biosynthetic genes in ornamental cabbage.

Gene name	<i>BoPAP1</i>	<i>BoPAP2</i>	<i>BoMYB114</i>	<i>BoTT8</i>	<i>BoGL3</i>	<i>BoEGL3.1</i>	<i>BoEGL3.2</i>	<i>BoMYC1.1</i>	<i>BoMYC1.2</i>	<i>BoBoTTG1</i>	<i>BoMYBL2.1</i>
<i>BoPAP2</i>	-0.309										
<i>BoMYB114</i>	0.743	-0.232									
<i>BoTT8</i>	-0.320	0.975***	-0.158								
<i>BoGL3</i>	0.066	0.898	-0.088	0.837*							
<i>BoEGL3.1</i>	-0.255	0.947**	-0.195	0.970***	0.885						
<i>BoEGL3.2</i>	-0.125	0.912**	-0.201	0.874**	0.960	0.944**					
<i>BoMYC1.1</i>	-0.154	0.912**	-0.223	0.888*	0.945	0.961**	0.997***				
<i>BoMYC1.2</i>	-0.282	0.988***	-0.162	0.988***	0.893	0.978***	0.931**	0.936**			
<i>BoTTG1</i>	-0.010	0.902**	0.099	0.905**	0.918	0.931**	0.940**	0.934**	0.943**		
<i>MYBL2.1</i>	0.195	-0.842*	0.449	-0.716	-0.899	-0.760	-0.885*	-0.864*	-0.790	-0.726	
<i>MYBL2.2</i>	0.090	0.827*	0.201	0.881*	0.855	0.917**	0.868*	0.879*	0.890**	0.961**	-0.579

[*: $p \leq 0.05$; **: $p \leq 0.01$ and ***: $p \leq 0.001$]