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# 冬性植物红菜薹在不同温度处理下花青素积累的分子机制

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**摘 要**: 芸薹属植物红菜薹(*Brassica rapa*)是一种常见的蔬菜, 它的花茎和叶柄表皮中均积累有花青素。为了解红菜薹中花青素合成的分子机制, 进行了花青素含量的测定和花青素合成相关基因的表达分析。研究结果表明, 叶柄表皮中的花青素含量显著高于叶片(去主脉)的花青素含量。同时, 叶柄表皮花青素合成相关基因的表达水平高于叶柄(去表皮)和叶片(去主脉)的表达水平, 这表明红菜薹中花青素的合成调控发生在转录水平。*BrMYBA1* 仅在叶柄表皮中表达, 但 *BrbHLH1* 和 *BrWD40* 在叶片和叶柄表皮中均能检测到表达。因此, *BrMYBA1* 的转录激活可能与红菜薹的花青素合成相关。连续低温处理时, 红菜薹叶柄表皮中的花青素含量逐渐增加, 而该组织中花青素合成的结构基因表达水平逐渐降低。

**关键词**: 红菜薹; 花青素; *BrMYBA*; 转录因子

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# Molecular Characterization of Anthocyanin Accumulation under Different Temperatures in Winter Plant Hongcaitai (*Brassica rapa* L.)

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**Abstract**: Hongcaitai (*Brassica rapa*) is a vegetable that accumulates anthocyanins in both floral stems and leaf petioles. To understand the mechanism underlying the regulation of anthocyanin biosynthesis in *B. rapa*, anthocyanin accumulation and expression patterns of anthocyanin biosynthesis genes in seedlings of Hongcaitai were investigated. Anthocyanin content in epidermal tissues of petioles were significantly higher than those in leaves with excised mid-veins. Expression levels of all anthocyanin biosynthesis pathway genes were significantly higher in epidermal tissues of petioles than those detected in either endodermal tissues of petioles or in leaves, suggesting that anthocyanin biosynthesis was regulated at the transcriptional level. Transcripts of *BrMYBA1* were exclusively expressed in the petiole epidermis; whereas, transcripts of *BrbHLH1* and *BrWD40* were detected in both leaves and petiole epidermal tissues. This suggests that activation of *BrMYBA1* was likely responsible for anthocyanin pigmentation in Hongcaitai. Following cold treatment, seedlings demonstrated increased accumulation of anthocyanins in petiole epidermal tissues, while the transcription of anthocyanin pathway genes was reduced in petiole epidermal tissues.

**Key words**: *Brassica rapa*; Anthocyanin; *BrMYBA*; Transcription factors

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Hongcaitai (*Brassica rapa* L. ssp. *chinensis* L. var. *purpurea* Bailey), one of the most popular Chinese vegetables and a specialty crop in Wuhan, China, produces anthocyanin pigments in its floral stems and leaf petioles. *Brassica rapa* (syn. *B. campestris*) is a diploid with 2n = 20.

Anthocyanins are not only responsible for red coloration in various plant species, but they also provide benefits for human health due to their antioxidant activities[1]. Despite the functional value of anthocyanin pigments, however, few studies have been reported on the mechanism regulating anthocyanin biosynthesis in *B. rapa*. The anthocyanin biosynthetic pathway is the most well-known secondary metabolite pathway in plants[2]. The synthesis of anthocyanins is complex and involves coordinated activities of different classes of enzymes, including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanone-3′-hydroxylase (F3′H), flavanone 3′5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), flavonol synthase (FLS), and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT). Several genes involved in anthocyanin biosynthesis have been isolated and functionally characterized in many plants. These can be divided into two classes, structural genes encoding anthocyanin pathway enzymes and regulatory elements[3]. The regulatory elements consist of three groups of transcription factors (TFs), including MYB, basic helix-loop-helix (bHLH), and WD40. The structural genes are regulated at the transcriptional level by combinatorial actions of MYB, bHLH, and WD40 TFs. Therefore, anthocyanin pigmentation is primarily controlled by regulatory genes[4].

Besides internal genetic factors, external environmental factors such as temperature, light, and nutrition also influence anthocyanin biosynthesis[5-7]. Among these environmental factors, temperature exerts a major influence on anthocyanin accumulation in a wide variety of plants. For example, high temperature reduces anthocyanin concentration, resulting in reduced red color intensity of fruit skin of grapes and apples[8,9]. Two mechanisms have been proposed for the observed lower anthocyanin levels in plants exposed to high temperature; specifically, high temperature inhibits transcription of anthocyanin biosynthetic genes, leading to a reduced rate of anthocyanin synthesis[7,10,11], and high temperature results in both reduced synthesis and increased degradation of anthocyanins[12]. In contrast, low temperature can induce the expression of anthocyanin biosynthetic genes, resulting in increased anthocyanin pigmentation in plants such as maize[13], *Arabidopsis*[14], petunia[15], red orange[16], grape[11], and apple[17]. However, few studies have been reported on anthocyanin pigmentation in annual plants that germinate in fall or winter, survive the winter, and then bloom in late winter or spring.

Hongcaitai is a winter annual vegetable, and its leaves, flower buds, and stems are all edible. Its commercial importance is highly related to anthocyanin pigmentation in flower stems and petioles. The red coloration of flower stems and petioles increases as temperature drops, and shows high pigmentation following the first frost. To understand the mechanisms that underlie anthocyanin pigmentation and its response to low temperature in *B. rapa*, we investigated anthocyanin accumulation and expression of anthocyanin biosynthesis genes in petioles of Hongcaitai grown under low temperature. Our results revealed that *BrMYBA1* plays an important role in regulation of anthocyanin accumulation, and that expression levels of anthocyanin structural genes decrease at cold temperatures. These findings have impli-

cations for the management of *B. rapa* vegetables and selection of cultivars with enhanced red coloration during cold winter weather.

## 1 Materials and Methods

### 1.1 Materials

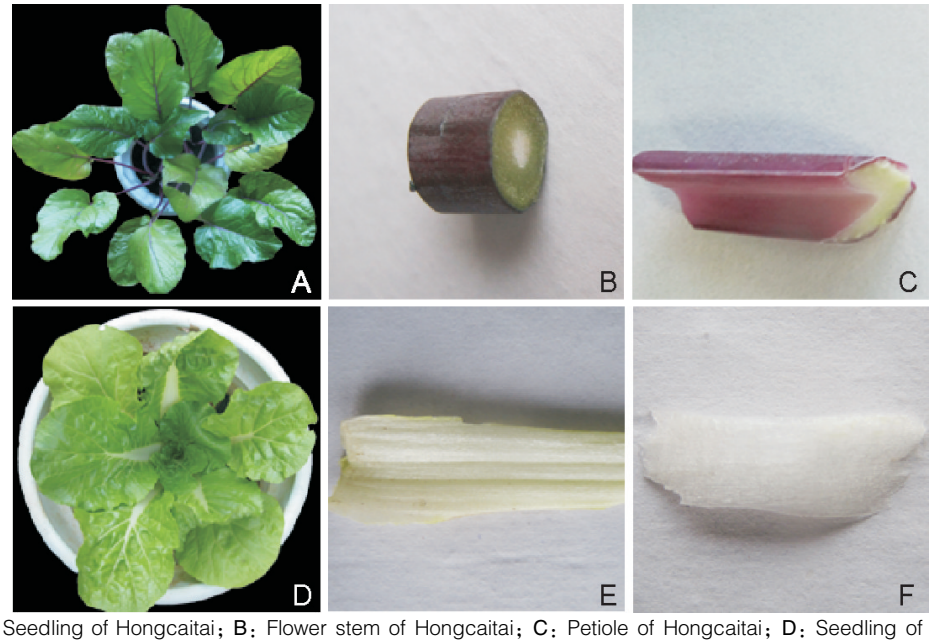
Two subspecies of *B. rapa*, Hongcaitai and Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* (Lour.) Makino), were used in the study. Hongcaitai produces purple-colored stems and petioles with green and/or white-colored flesh along with green-colored leaves, but with red mid-veins, while Chinese cabbage accumulates no anthocyanins and has green leaves and white petioles (Fig. 1).

Seeds were sown on 20 August, 2012, and seedlings were transplanted into small pots on 28 September, 2012. Each pot contained a single seedling. All seedlings were grown outdoors for a period of one month at Wuhan Botanical Garden (Chinese Academy of Sciences, Hubei, China). Seedlings were then transferred to a growth chamber and grown under three consecutive environmental conditions: 20℃ and 60% relative humidity (RH) for 7 d, 10℃ and 60% RH for 7 d, and 2℃ and 60% RH for 7 d. Leaves and petioles were collected, with three biological replicates, after 7 days of growth at each of the temperature treatments. Subsequently, mid-rib veins were excised from the leaves, and epidermal and endodermal tissues of petioles were separated. All tissues were immediately frozen in liquid nitrogen, and stored at −75℃ until use.

### 1.2 Methods

#### 1.2.1 Extraction and determination of total anthocyanins in leaves and petiolar tissues

Anthocyanin content was assayed as previously described by Niu *et al.* (2010)[18]. Briefly, 0.1 g of sample was ground into powder in liquid nitrogen, then added to 1 mL of extraction solution (0.1% HCl in methanol), and incubated at 4℃ for 20 h. The mixture was centrifuged at 12 000 r/min for 20 min, and the supernatant was transferred to a clean tube. The pellet was dissolved in 1 mL of extraction solution, and the mixture was extracted again. The supernatants were combined and diluted in 3 mL of extraction solution. Then, 200 μL of supernatant was mixed




A: Seedling of Hongcaitai; B: Flower stem of Hongcaitai; C: Petiole of Hongcaitai; D: Seedling of Chinese cabbage; E: Petiole of Chinese cabbage; F: Petiole endodermis of Chinese cabbage.

Fig. 1 Diagram of coloration in *B. rapa*

with 3 mL of either buffer A (0.05 mol/L KCl and 0.15 mol/L HCl, pH 1.0) or buffer B (0.2 mol/L NaAc with pH 4.5) and stored at 4℃ for 2 h. The absorbance of buffer A and B was measured at 520 and 700 nm, respectively. The anthocyanin content was calculated according to the following formula[19]: TA = [(A × MW × DF × V)/(ε × L × Wt)] × 100, where TA stands for total anthocyanin content (mg/100 g, as cyanidin-3-O-glucose equivalent), A = [A520 nm (pH 1.0) − A700 nm (pH 1.0)] − [A520 nm (pH 4.5) − A700 nm (pH 4.5)], DF for dilution factor, V for final volume (mL), L for optical path (1 cm), and Wt for sample weight (g). Anthocyanin concentration was calculated as cyanidin-3-glucoside following the method of Wrolstad *et al.* (1982)[20], with a molar absorptivity (ε) of 26 900 and a molecular weight (MW) of 449.2. Three measurements for each biological replicate sample were performed.

### 1.2.2 Isolation of anthocyanin biosynthetic genes

Sequences of anthocyanin biosynthetic genes in *Arabidopsis* were BLASTed against the genome sequence of *B. rapa* (http://brassicadb.org/brad/blastPage.php). Homologous sequences were recovered and used to design primers to amplify cDNA derived from the petiole epidermis of Hongcaitai. The PCR products were purified and cloned into *pEASY*-T1 vector using TA cloning kit (TransGen Biotech). Plasmid DNAs were extracted using a Plasmid Mini kit (Zoman) and then subjected to sequencing.

### 1.2.3 RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA was extracted using a GREENspin plant RNA extraction kit (Zomanbio, Beijing, China) according to the manufacturer's manual. The RNA samples were treated with DNaseI (TaKaRa, Kyoto, Japan) to remove DNA contamination. Approximately 1 μg of total RNA was subjected to first-strand cDNA synthesis using an oligo-dT primer (Takara) and PrimeScript® Reverse Transcriptase (Takara).

Real-time PCR analysis was conducted using the 7500 Fast Real-Time PCR System (Applied Biosystems). All reactions were performed using SYBR® *Premix Ex Taq*™ Ⅱ (Takara) according to the manufacturer's instructions. Reactions were carried out in a total volume of 20 μL reaction mixture containing 10.0 μL of 2× SYBR Green Ⅰ Master Mix (Takara), 0.2 μmol/L of each primer, and 100 ng of template cDNA. PCR amplification was performed using two-step cycling conditions of 95℃ for 30 s, followed by 40 cycles of 95℃ for 3 s and 60℃ for 30 s. A *BrEF1A* gene in *B. rapa* was used as a constitutive control[21]. Melting curve analysis was performed at the end of 40 cycles to ensure proper amplification of target fragments. Fluorescence readings were consecutively collected during the melting process from 60℃ to 90℃ at a heating rate of 0.5℃/s. Reaction mixtures without cDNA templates were also run as a negative control. The relative quantities of the transcripts were calculated using the comparative Ct method. All analyses were repeated with three biological replicates.

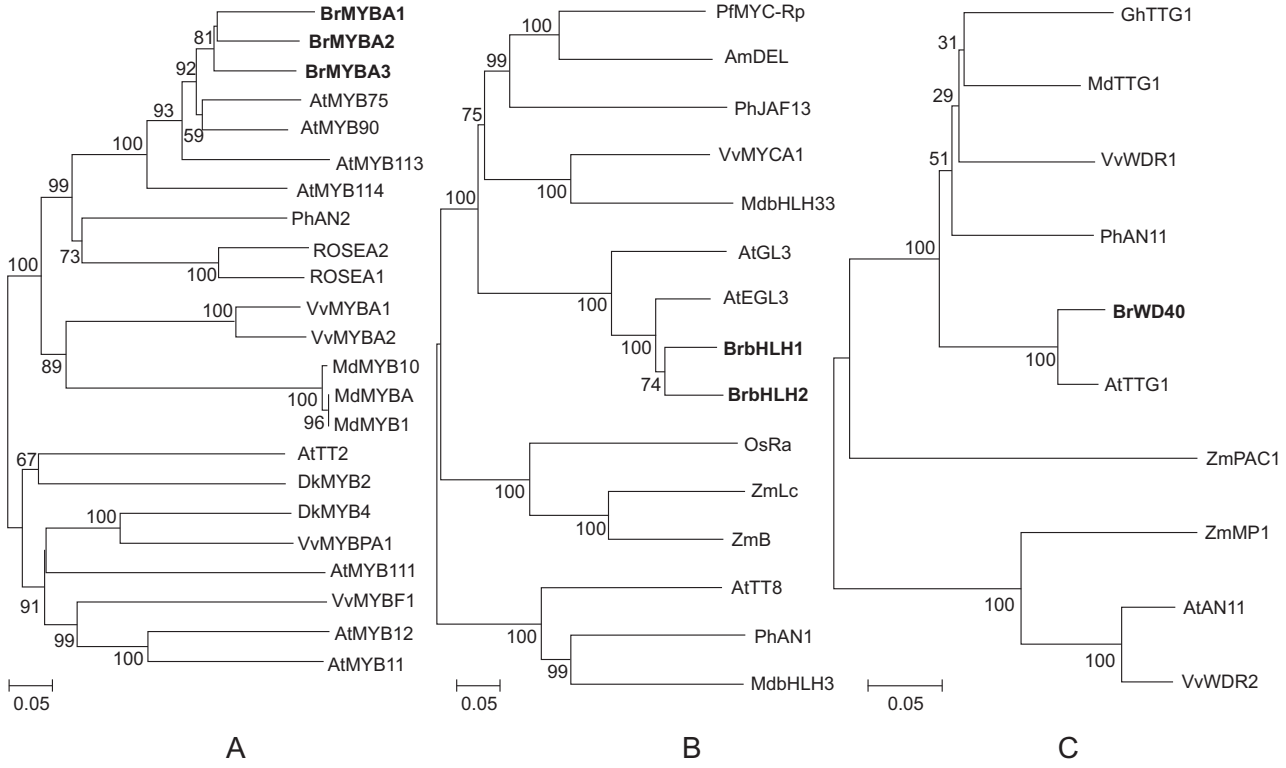
## 2 Results

### 2.1 Genes involved in anthocyanin biosynthesis in *B. rapa*

Sequences of genes involved in *Arabidopsis* anthocyanin biosynthesis were compared against the genome sequence of *B. rapa* line Chiifu-401. Four, two, one, two, one, two, and one copy of genes encoding CHS, CHI, F3′H, F3H, DFR, LDOX, and UFGT, respectively, were identified. The different copies of *CHS*, *CHI*, *F3H*, and *LDOX* gene families shared 90%, 89%, 92%, and 93% identities in coding DNA sequences, respectively. In addition, three, one, and two homo-

logues of *Arabidopsis AtMYB75*, *AtTTG1* and *AtEGL3*, respectively, were also identified in the genome of Chiifu-401. The three *MYBA* genes, designated *BrMYBA1*, *BrMYBA2*, *BrMYBA3*, shared 87% identity in coding DNA sequences. The two *bHLH* genes, designated *BrbHLH1* and *BrbHLH2*, showed 90% identity in coding DNA sequences.

To reveal the level of coding DNA sequence identities between Hongcaitai and Chiifu-401, conserved sequences of each gene family were used to design primers to amplify cDNAs from petiole epidermal tissues. As a result, 10 cDNA fragments of anthocyanin biosynthetic genes were cloned and sequenced. The cDNA fragments ranged from 572 to 945 bp in size. Overall, anthocyanin biosynthetic genes in Hongcaitai showed over 98% identities in coding DNA sequences with those in Chiifu-401. The sequence differences all arose from single nucleotide polymorphisms (SNPs). This indicated that Hongcaitai was closely related to Chiifu-401, and the reference genome of Chiifu-401 could be used to design primers to analyze expression profiles of genes in Hongcaitai. In addition, phylogenetic analysis indicated that *BrMYBA*, *BrbHLH*, and *BrWD40* genes in *B. rapa* were closely related to anthocyanin regulatory genes found in other plant species such as apple, grapevine, and petunia (Fig. 2).




A, B, and C represent MYB, bHLH, and WD40, respectively, and the genes in *B. rapa* are highlighted in black bold. GenBank accession numbers of the sequences are as follows: AtMYB113 (NM105308), AtMYB75 (NM_104541), AtMYB90 (NP176813), MdMYBA (AB279598), MdMYB1 (ABK58136), MdMYB10 (ABB84754), VvMYBA1 (AB242302), VvMYBA2 (AB097924), PhAN2 (AAF66727), ROSEA1 (ABB83826), ROSEA2 (ABB83827), AtTT2 (NM_122946), DkMYB4 (AB503701), DkMYB2 (AB503699), VvMYBPA1 (AM259485), VvMYBF1 (FJ948477), AtMYB11 (NP_191820), AtMYB12 (NM_130314), AtMYB111 (NM_124310), AtEGL3 (NP_176552), AtGL3 (NP_680372), VvMYCA1 (ABM92332), PhAN1 (AAG25928), AtTT8 (CAC14865), ZmB (CAA40544), ZmLc (ABD72707), OsRa (AAC49219), AmDEL (AAA32663), PfMYC-Rp (BAA75513), PhJAF13 (AAC39455), MdbHLH3 (ADL36597), MdbHLH33 (DQ266451), AtTTG1 (NP_851070), ZmMP1 (AAR01949), GhTTG1 (AAM95641), AtAN11 (AAC18912), ZmPAC1 (AAM76742), MdTTG1 (AAF27919), PhAN11 (AAC18914), VvWDR1 (ABF66625), VvWDR2 (ABF66626).

Fig. 2 Phylogenetic trees derived from amino acid sequences of anthocyanin regulatory genes in plants

### 2.2 Anthocyanin content in leaves and petioles of *B. rapa*

Anthocyanin accumulation was detected in leaves and petiole epidermal tissues of Hongcaitai seedlings grown at 20℃ (Table 1). The petiole endodermis was green in color and did not accumulate anthocyanin. The anthocyanin level in petiole epidermis was almost 7-fold higher than that in leaf tissues (with excised mid-rib veins). In contrast, anthocyanins were not detectable in the leaf, petiole epidermis, and petiole endodermis of Chinese cabbage seedlings grown at 20℃.

To assess patterns of anthocyanin accumulation during the winter growing season, seedlings of Hongcaitai and Chinese cabbage were further subjected to two consecutive treatments of cold stress. The anthocyanin concentration increased up to 2.78-fold in the petiole epidermis of Hongcaitai after a 7 d cold stress at 10℃, and increased slightly (up to 1.24-fold) after an additional cold stress at 2℃ for 7 d. Leaves of Hongcaitai showed a slight increase in anthocyanin content (up to 1.57-fold) after a period of 7 d cold stress at 10℃, but without an increase in anthocyanin accumulation after an additional 7 d period of cold stress at 2℃. Cold stress treatments at 10℃ and 2℃ did not induce anthocyanin accumulation in the leaves or petioles of Chinese cabbage.

### 2.3 Expression profiling of anthocyanin biosynthetic genes in *B. rapa*

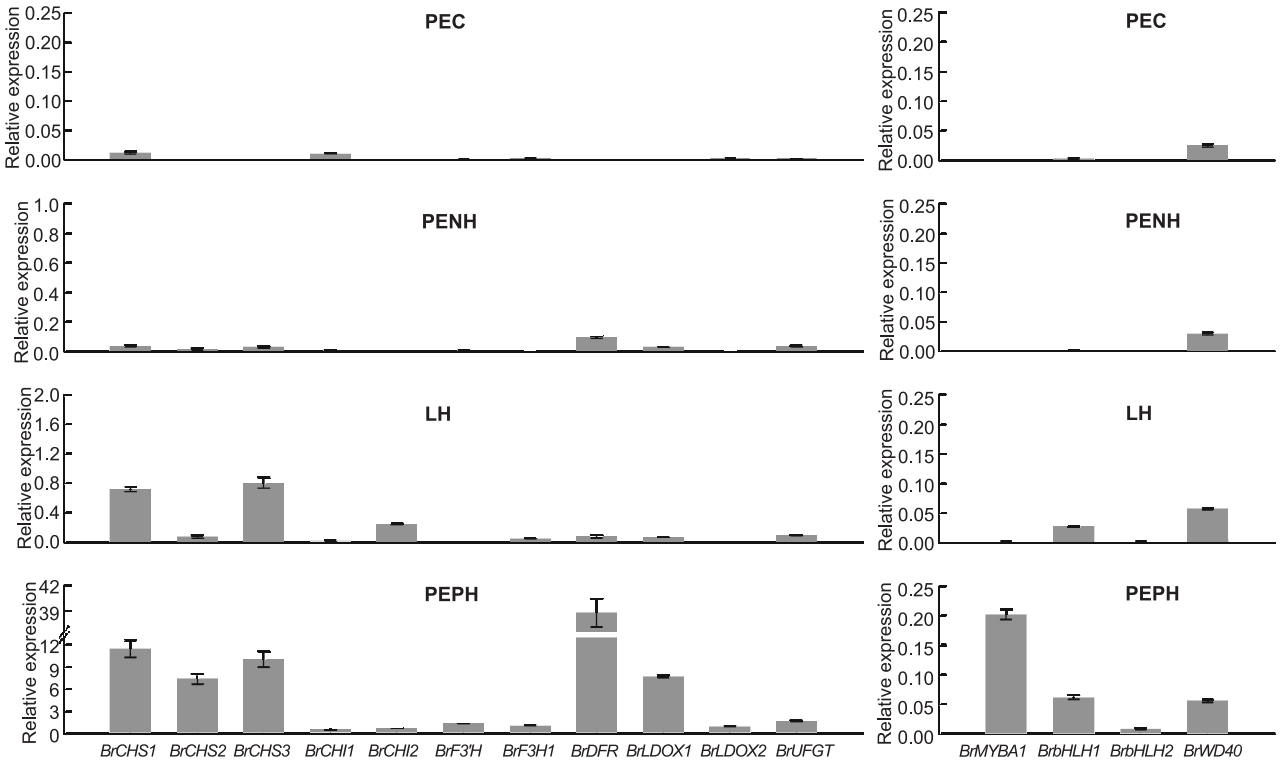
Initially, expression profiles of anthocyanin biosynthetic genes were investigated in seedlings of Hongcaitai grown at 20℃. RT-PCR analysis revealed that transcripts of four genes, *BrCHS4*, *BrF3H2*, *BrMYBA2*, and *BrMYBA3*, were not detected in petiole epidermal tissues, and thus these genes were not included in further analysis. Subsequently, qRT-PCR analysis revealed that transcripts of anthocyanin biosynthetic genes were mainly accumulated in leaf and petiole epidermal tissues (Fig. 3). Expression levels of anthocyanin biosynthetic genes were significantly higher in petiole epidermal tissues than in leaves. For example, transcript levels of *BrDFR*, *BrLDOX1*, and *BrUFGT* in petiole epidermal tissues were 481-, 120-, and 18-fold higher than those in leaves, respectively. *BrF3′H* and *BrLDOX2* were exclusively expressed in petiole epidermal tissues, though the expression level of *BrLDOX2* was very low. For anthocyanin regulatory genes, *BrMYBA1* was exclusively expressed in petiole epidermal tissues, *BrbHLH1* was expressed in both leaf and petiole epidermis, and *BrbHLH2* was expressed at very low levels in petiole epidermal tissues, but was not detectable in leaf and petiole endodermal tissues. *BrWD40* transcripts accumulated in all analyzed tissues, including leaf and petiole epidermal and endodermal tissues.

As mentioned above, anthocyanins were not detected in leaves and petioles of Chinese cabbage; therefore, the petiole epidermis of Chinese cabbage was selected for gene expression analysis, and no transcripts of anthocyanin pathway

Table 1　Anthocyanin concentration (mg/100 g) in leaf and petiole in *B. rapa* vegetables

| Treatment | Chinese cabbage | | | Hongcaitai | | |
|---|---|---|---|---|---|---|
| | Leaf | Petiole epidermis | Petiole endodermis | Leaf | Petiole epidermis | Petiole endodermis |
| T1 | N/D | N/D | N/D | 5.26 ± 0.53 | 34.79 ± 0.53 | N/D |
| T2 | N/D | N/D | N/D | 8.27 ± 1.06 | 96.58 ± 0.53 | N/D |
| T3 | N/D | N/D | N/D | 8.27 ± 1.06 | 119.4 ± 0.00 | N/D |

Note: **T1**: A period of 7 d growth at 20℃; **T2**: A period of 7 d cold stress at 10℃ after T1 treatment; **T3**: An additional 7 d period of cold stress at 2℃ after T2 treatment. Values represent average of three replicates. N/D: not detectable.




PEC: Petiole epidermal tissues of Chinese cabbage; PENH: Petiole endodermal tissues of Hongcaitai; LH: Leaves (with excised mid-rib veins) of Hongcaitai; PEPH: Petiole epidermal tissues of Hongcaitai.

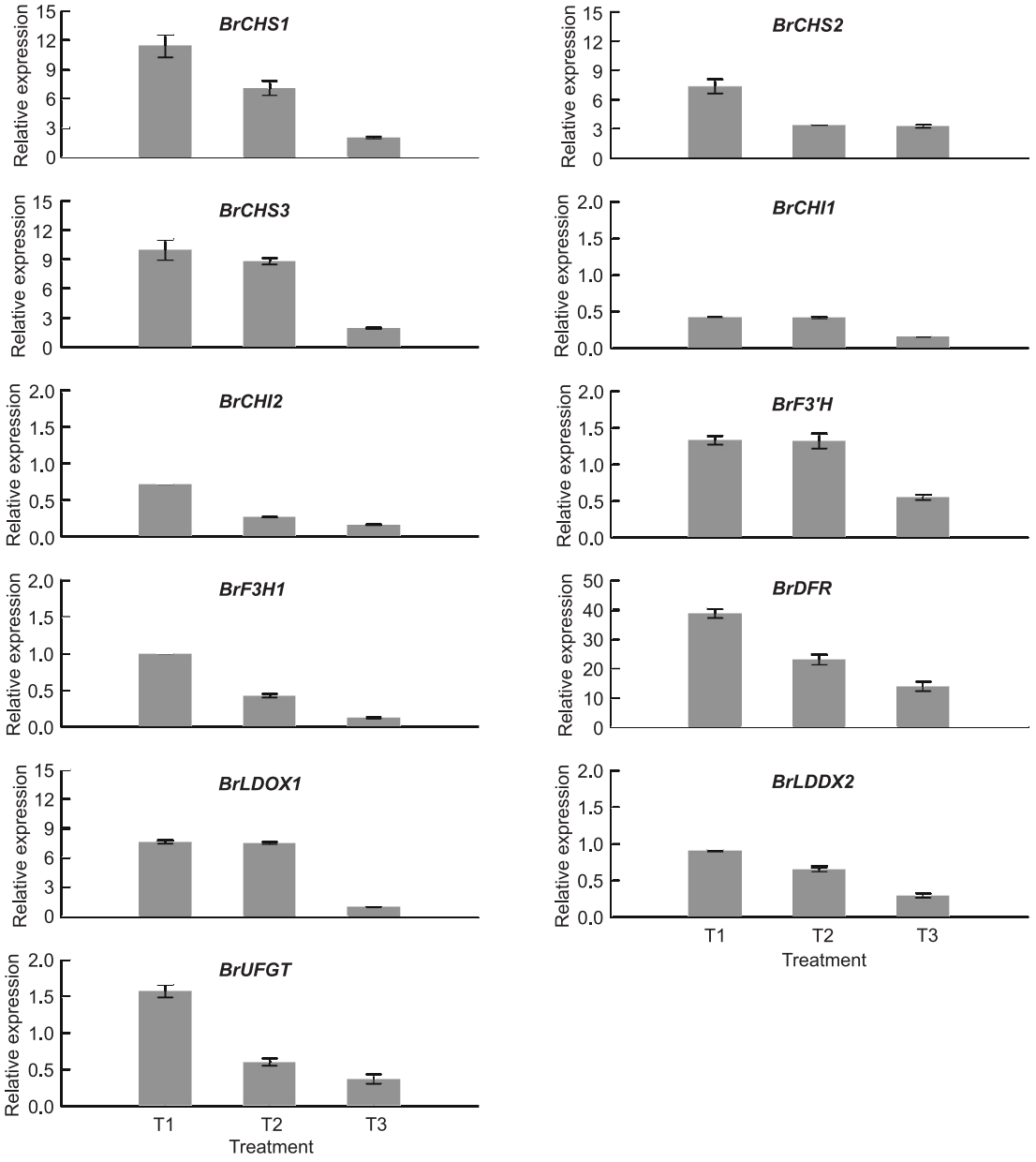
Fig. 3 qRT-PCR analysis of anthocyanin biosynthetic genes in Hongcaitai and Chinese cabbage seedlings grown at 20℃

genes were detected. *BrWD40* was expressed at a low level in petiole epidermis, while transcripts of *BrMYBA1*, *BrbHLH1*, and *BrbHLH2* were not detected in petiole epidermis.

### 2.4 Effect of cold temperatures on the expression of anthocyanin biosynthetic genes in petiole epidermis of Hongcaitai

As seedlings of Hongcaitai accumulated anthocyanins in the petiole epidermis, expression of anthocyanin biosynthetic genes in response to cold temperatures was only conducted in this tissue. Structural genes of the anthocyanin pathway showed reduced transcript levels following cold treatment (Fig. 4). Transcription levels of *BrCHS1*, *BrCHS2*, *BrCHS3*, *BrCHI1*, *BrCHI2*, *BrF3′H*, *BrF3H1*, *BrDFR*, *BrLDOX1*, *BrLDOX2*, and *BrUFGT* in petiole epidermal tissues after 7 d growth at 10℃ decreased by 38%, 54%, 12%, 3%, 63%, 1%, 58%, 41%, 2%, 28%, and 62%, respectively, compared to those in tissues grown at 20℃ for 7 d. Similarly, transcript levels of *BrCHS1*, *BrCHS2*, *BrCHS3*, *BrCHI1*, *BrCHI2*, *BrF3′H*, *BrF3H1*, *BrDFR*, *BrLDOX1*, *BrLDOX2*, and *BrUFGT* in petiole epidermal tissues after an additional treatment at 2℃ for 7 d decreased significantly by 83%, 56%, 81%, 64%, 77%, 59%, 88%, 64%, 88%, 68%, and 77%, respectively, compared to those in tissues grown at 20℃ for 7 d. On average, transcript levels of anthocyanin structural genes decreased by 33% and 73% after cold treatment at 10℃ for 7 d and subsequent-cold treatment at 2℃ for 7 d, respectively, compared to those in tissues grown at 20℃ for 7 d.

Transcript levels of *BrMYBA1* and *BrWD40* in the petiole epidermis after cold treatment at 10℃ for 7 d increased by 85% and 87%, respectively, compared to those in the petiole epidermis grown at 20℃ for 7 d. However, transcript levels of




**T1**: A period of 7 d growth at 20℃; **T2**: A period of 7 d cold stress at 10℃ after T1 treatment; **T3**: An additional 7 d period of cold stress at 2℃ after T2 treatment.

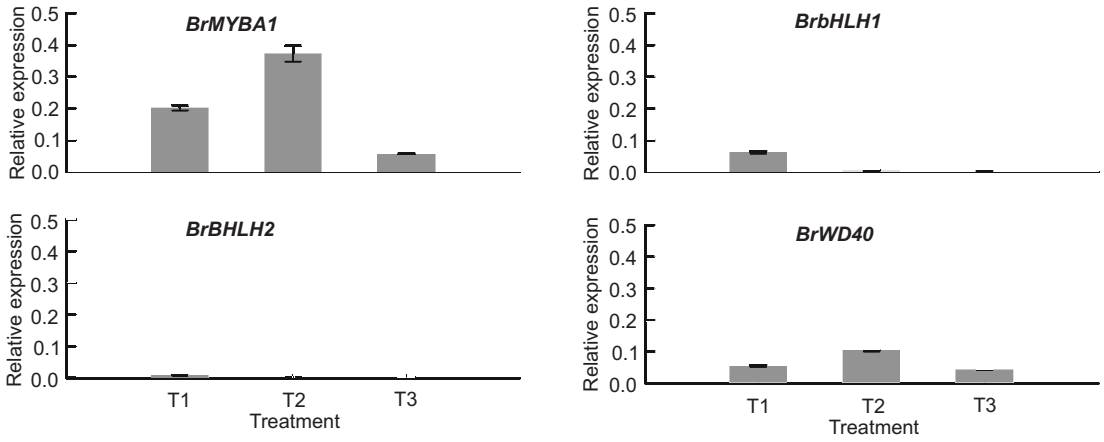
Fig. 4　qRT-PCR analysis of anthocyanin structural genes in petiole epidermis of Hongcaitai seedlings under cold treatment

*BrbHLH1* and *BrbHLH2* in petiole epidermal tissues after 7 d growth at 10℃ decreased significantly by 93% and 92%, respectively, compared to those in tissues grown at 20℃ for 7 d. Transcript levels of *BrMYBA1*, *BrbHLH1*, *BrbHLH2*, and *BrWD40* in petiole epidermal tissues after subsequent cold treatment at 2℃ for 7 d decreased by 72%, 96%, 80%, and 25%, respectively, compared to those in tissues grown at 20℃ for 7 d (Fig. 5).

## 3　Discussion

### 3.1　Mechanism underlying the regulation of anthocyanin pigmentation in Hongcaitai

In this study, we report for the first time on the mechanism underlying anthocyanin pigmentation in




T1: A period of 7 d growth at 20℃; T2: A period of 7 d cold stress at 10℃ after T1 treatment; T3: An additional 7 d period of cold stress at 2℃ after T2 treatment.

Fig. 5 qRT-PCR analysis of anthocyanin regulatory genes in petiole epidermal tissues of Hongcaitai seedlings under cold treatment

Chinese vegetable Hongcaitai. Seedlings of Hongcaitai grown at 20℃ accumulated anthocyanins in leaf and petiole epidermal tissues, and anthocyanin content was about 7-fold higher in petiole epidermis than in leaf tissues. All structural genes showed higher levels of expression in petiole epidermis than in both leaf and petiole endodermal tissues. These findings clearly suggest that anthocyanin accumulation in Hongcaitai is controlled at the transcriptional level. Subsequently, expression of anthocyanin regulatory genes *BrMYBA*, *BrbHLH*, and *BrWD40* were determined. Transcript levels of *BrbHLH* and *BrWD40* in petiole epidermal tissues were similar to those in leaf tissues, suggesting that *BrbHLH* and *BrWD40* were not likely responsible for anthocyanin pigmentation in Hongcaitai. In contrast, the transcript level of *BrMYBA1* in petiole epidermal tissues was more than 300-fold higher than that detected in leaf tissues. Therefore, *BrMYBA1* is a likely candidate gene responsible for regulation of anthocyanin pigmentation in Hongcaitai.

It is worth noting that *BrMYBA1* and three structural genes, *BrF3'H*, *BrDFR*, and *BrLDOX1*, were expressed at extremely low levels in leaf tissues, but were highly expressed in petiole epidermal tissues. In purple-colored cauliflower (*Brassica oleracea* var. *botrytis*), the MYB TF *BoMYB2* specifically activates *BobHLH1* along with a subset of anthocyanin structural genes, including *BoF3'H*, *BoDFR*, and *BoLDOX*[22]. *BrMYBA1* and *BoMYB2* shared 98% identity in coding DNA sequences. Interestingly, Hongcaitai and purple cauliflower have the same ancestor and belong to the same genus, *Brassica*. Thus, it is reasonable to speculate that *BrMYBA1*, like *BoMYB2*, may also have the ability to activate the regulatory gene *BrbHLH* as well as such structural genes as *BrF3'H*, *BrDFR*, and *BrLDOX1*. This comparative analysis of anthocyanin biosynthesis genes between *B. rapa* and *B. oleracea* further supports our proposal that *BrMYBA1* is responsible for the regulation of anthocyanin accumulation in Hongcaitai.

If anthocyanin biosynthesis in Hongcaitai is indeed controlled by *BrMYBA1*, then the question arises as to whether anthocyanin accumulation occurs in the leaves of Hongcaitai. In this study, transcripts of *BrMYBA1* were almost undetectable in leaf tissues; however, leaves had low levels of anthocyanin accumulation. Transcript levels of upstream genes in the anthocyanin biosynthesis

pathway, including *BrCHS1*, *BrCHS3*, and *BrCHI2*, were relatively highly expressed in leaf tissues. In *Arabidopsis*, three closely-related MYB TFs, including *AtMYB11*, *AtMYB12*, and *AtMYB111*, regulate transcription of genes *AtCHS* and *AtCHI*[23]. Thus, we cannot exclude the possibility that other regulatory genes may also be involved in the regulation of anthocyanin biosynthesis in Hongcaitai. Moreover, transcripts of *BrF3'H* were almost undetectable in leaf tissues. It is known that the *F3'H* gene plays an important role in the hydroxylation of anthocyanins, and it is involved in the production of cyanidin-based pigments[24,25]. In addition to *F3'H*, *F3H* and *F3'5'H* are also involved in hydroxylation of anthocyanins, leading to the generation of pelargonidin- and delphinidin-based pigments, respectively[2]. Because Hongcaitai mainly accumulates cyanidin-based anthocyanins[26], there is no *F3'5'H* gene present in the *B. rapa* genome[21]. *BrF3H* was expressed at low levels in leaves of Hongcaitai. Thus, it seems likely that leaves accumulated only pelargonidin-based anthocyanins, while petiole epidermis contained both cyanidin-and pelargonidin-based anthocyanins in Hongcaitai. In addition, it is important to note that the mid-rib vein was red in color, and thus contained anthocyanins. The possibility cannot be excluded that anthocyanin accumulation in leaves of Hongcaitai is partially attributed to movement of anthocyanins from mid-rib veins to the mesophyll.

RBr (rapid-cycling *B. rapa*) are anthocyaninless (*anl*) mutants of *B. rapa* that do not produce anthocyanin pigments. The *anl* locus has been mapped to *B. rapa* linkage group 9[27]. In this study *BrMYBA1*, located on linkage group 7, was likely responsible for the regulation of anthocyanins in Hongcaitai. The other two *BrMYBA* genes, *BrMYBA2* and *BrMYBA3*, were located on linkage groups 3 and 2, respectively. These results suggest that genes other than *BrMYBA* may also play important roles in the regulation of anthocyanin biosynthesis in *B. rapa*. *BrbHLH1*, *BrbHLH2* and *BrDFR* were all located on linkage group 9, and may be candidate genes for the *anl* locus.

### 3.2 Effect of cold temperatures on anthocyanin pigmentation in Hongcaitai seedlings

It has been widely reported that low temperatures induce transcription of anthocyanin biosynthetic genes, leading to increased accumulation of anthocyanins in plants[14,17]. However, the effect of low temperatures on anthocyanin accumulation has not been reported in any winter plants. In this study, we investigated the effects of cold temperatures on anthocyanin accumulation in seedlings of a winter vegetable, Hongcaitai. The anthocyanin content in petiole epidermis increased after one or two weeks of growth at cold temperature. This finding was consistent with the fact that red pigmentation in Hongcaitai increases as temperature drops in the winter growing season. However, transcript levels of anthocyanin structural genes decreased in the petiole epidermis after one or two weeks of growth at cold temperatures. This was similar to the finding that high temperatures inhibit the transcription of anthocyanin biosynthetic genes[7]. High temperatures increase the degradation of anthocyanins[12], while anthocyanins are stable at low temperatures[28]. Therefore, the stability of anthocyanins is likely responsible for the increased accumulation of anthocyanins in the petiole epidermis of Hongcaitai grown under cold temperatures.

Unlike anthocyanin structural genes, the regulatory genes *BrMYBA1* and *BrWD40* were expressed at higher levels in the petiole epidermis of Hongcaitai at 10℃ than in the petiole epidermis grown at 2℃. However, transcript levels of

*BrbHLH1* in the petiole epidermis at 10℃ decreased significantly compared to that of *BrbHLH1* in the petiole epidermis at 20℃. While overexpression of *Myb113* or *Myb114* upregulated the anthocyanin pathway and is bHLH-dependent in *Arabidopsis*, it seems likely that the decreased transcript levels of *BrbHLH1* reduced the transcript levels of *BrMYBA1*[29]. This finding was in contrast to previous reports indicating that low temperature (17℃) induced the expression of *bHLH* in apple fruits[17]. This may be attributed to differences in both temperature and plant species. Transcription of anthocyanin structural genes are regulated via the combinatorial actions of MYB, bHLH, and WD40 proteins[2]. Thus, it is clear that inhibition of the transcription of anthocyanin structural genes in the petiole epidermis at 10℃ was probably caused by lower transcript levels of *BrbHLH1*. Moreover, the transcription of all regulatory genes, including *BrMYBA1*, *BrbHLH1*, and *BrWD40*, were significantly inhibited in the petiole epidermis of Hongcaitai grown at 2℃, leading to a significant decrease in the expression of anthocyanin structural genes. This was consistent with our finding that anthocyanin content in the petiole epidermis of Hongcaitai showed a slight increase after a 7 d growth at 2℃.

In brief, this study reports for the first time on the mechanism underlying the regulation of anthocyanin accumulation in Hongcaitai. Anthocyanin pigmentation in Hongcaitai appeared to be controlled by *BrMYBA1*. The petiole epidermis of Hongcaitai grown at cold temperatures showed increased accumulation of anthocyanin, although the transcription of anthocyanin biosynthetic genes was inhibited.

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