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SNP in *DFR1* Coding Sequence Is Tightly Associated with Anthocyanin Accumulation in Cabbage (*B. oleracea* var. *capitata* f. *alba*) at Low Temperature

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Abstract: Keeping green leaf color at the time of harvest is one of the important traits for breeding of *Brassica oleracea* var. *capitata* f. *alba*, and this trait is related to low anthocyanin contents. To understand the differential accumulation of anthocyanins in cabbage, we selected high anthocyanin accumulators (HAAs) and low anthocyanin accumulator (LAAs) of cabbages and examined the anthocyanin content and the expression of anthocyanin biosynthesis-related genes. Among many genes investigated, *BoDFR1* was found to be closely related to anthocyanin accumulation, even under low temperature (LT) conditions. *BoDFR1* sequence analyses between HAAs and LAAs revealed that there is a single nucleotide polymorphism (SNP) (1118T/A) in the coding sequence, which substitutes one amino acid from Leu261 to His261; we named *BoDFR1* with His261 substitution as *BoDFR1v*. This amino acid substitution did not affect dihydroflavonol 4-reductase (DFR) activity and substrate specificity, but the polymorphism showed tight association to the *BoDFR1* expression, i.e., high level expression of *BoDFR1* and low level expression of *BoDFR1v* under LT conditions. The high levels of *BoDFR1* expression were due to the high levels of *BoMYB114* and *BobHLH* expressions combined with low level expression of *BoMYBL2*, a repressor MYB. On the other hand, low levels of *BoDFR1v* expression seemed to be related to very low level expressions of *BoMYB114* and *BobHLH* combined with a high level expression of *BoMYBL2*. It seems that different expression levels of these regulatory genes for MBW (MYB-bHLH-WD40) complex between HAAs and LAAs regulate *BoDFR* expression and anthocyanin accumulation. Using a single nucleotide polymorphism (SNP) between *BoDFR1* and *BoDFR1v*, molecular markers for PCR and high resolution melt analyses were developed and validated to distinguish between HAAs and LAAs. Combined use of the *BoDFR1* SNP marker with other stress markers, such as a cold tolerant marker, will greatly improve cabbage breeding.

Keywords: dihydroflavonol 4-reductase (DFR); anthocyanin content; allelic variation; single nucleotide polymorphism (SNP); cabbage

1. Introduction

Anthocyanins are water-soluble plant pigments that range from blue to red in color. Anthocyanins impart color to leaves, flowers, and fruits, thus attracting pollinators and seed distributors [1–4],

and protect plants from abiotic stresses, such as ultraviolet (UV) radiation, cold, and drought, and biotic stresses such as microbial infection [5–13]. Anthocyanins exhibit potent antioxidant activity [14–16] and therefore act as functional compounds that promote human health by lowering the incidence of cardiovascular diseases and chronic and degenerative diseases [17–26].

In plants, anthocyanin biosynthesis is controlled by several genes encoding structural proteins and transcription factors. Genes encoding structural proteins are divided into two groups: early biosynthetic genes (EBGs), which are involved in the initial stages of anthocyanin biosynthesis such as chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and flavanone 3-hydroxylase (*F3H*) [27], and late biosynthetic genes (LBGs), which are involved in the late stages of anthocyanin biosynthesis such as dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*) (or leucoanthocyanidin oxygenase; *LDOX*) [28–30]. The expression of LBGs is tightly regulated by MYB, basic helix-loop-helix (*bHLH*), and WD-repeat proteins [31,32], which together form the MBW complex and regulate the spatiotemporal expression pattern of LBGs in response to developmental and environmental factors [30,32,33].

DFR (EC1.1.1.219) catalyzes a key step late in the biosynthesis of anthocyanins, condensed tannins (proanthocyanidins), and other flavonoids important for plant survival and human nutrition [34]. *DFR* reduces three dihydroflavonols, including dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM), to leucoanthocyanidins, which are common precursors of anthocyanins and condensed tannins [35,36]. Thus, *DFR* activity regulates the level of anthocyanins, and its substrate preference results in the accumulation of different types of anthocyanins. It has been reported that anthocyanin accumulation and purple (or red) coloration are related to the high levels of *DFR* expression in purple kale (*B. oleracea* var. *acephala* f. *tricolor*) [33], red (or purple) cabbage (*B. oleracea* var. *capitata* f. *rubra*) [37–39], purple cauliflower (*B. oleracea* var. *botrytis*) [38], purple mustard (*Brassica juncea*) [40], purple bok choy (*Brassica rapa* subsp. *chinensis*) [41], purple kohlrabi (*B. oleracea* var. *gongylodes*) [42,43], red lettuce (*Lactuca sativa*) [44], and apple (*Malus domestica*) [45]. Increase in anthocyanin accumulation upon exposure to low temperature (LT) is a common phenomenon in various plant species including *Arabidopsis thaliana* [46], grape (*Vitis vinifera*) [47], orange (*Citrus sinensis*) [48], kale [33], Chinese cabbage (*B. rapa*) [49], and apple [50]. Because of the antioxidant activity of anthocyanins, their accumulation confers cold tolerance [9,51,52] and is concomitant with the increase in *DFR* expression under LT conditions [12,33,49]. Recently, An et al. reported that an MYB transcription factor regulates both cold tolerance and anthocyanin accumulation in apple by binding to the promoters of C-repeat binding factor (*CBF*) and *DFR* genes, thus activating their expression [13].

Genetic and molecular characterization of *DFR* genes have been conducted in various plant species including alfalfa (*Medicago truncatula*) [36], trembling aspen (*Populus tremuloides*) [53], lotus (*Lotus japonicus*) [54], *Saussurea medusa* [55], Chinese cabbage [49], and *Freesia hybrida* [56]. In plants, the *DFR* gene generally exists as two or more copies, for example, two copies in maize (*Zea mays*) [57], alfalfa [36], poplar (*Populus spp.*) [58], and yam (*Dioscorea alata*) [27], three copies in morning glory (*Ipomoea batatas*) [59], wheat (*Triticum aestivum*) [60], onion (*Allium cepa*) [61], and ginkgo (*Ginkgo biloba*) [62], six copies in lotus [63], eight copies in *Freesia hybrida* [56], and twelve copies in Chinese cabbage [49]. Multiple copies of the *DFR* genes imply the functional diversification of *DFR* proteins for color determination, stress response, and other horticultural traits. In addition to the multiple copies of *DFRs*, allelic polymorphisms are also associated with bulb color in onion [61,64], flesh color in potato (*Solanum tuberosum*) [65], and grain quality in barley (*Hordeum vulgare*) [66]. Polymorphisms in the untranslated region (UTR) and the intron of the *DFR* gene have been used to develop molecular markers in common bean (*Phaseolus vulgaris*) [67] and onion [61,64] to distinguish among haplotypes or genotypes with different bulb colors.

Despite the positive effects of anthocyanins on plant and human health, one of the goals of cabbage breeding programs, especially in Korea and China, is to maintain the deep green color of leaves at the time of harvest regardless of the harvest season. Therefore, a dilemma in cabbage breeding is the development of stress tolerant varieties with low anthocyanin content at harvest when LT conditions may occur. To resolve the dilemma, we identified two contrasting cabbage types, high anthocyanin

accumulators (HAAs) and low anthocyanin accumulators (LAAs), under LT conditions, and selected *BoDFR1* in both cabbage types as the target gene that regulates anthocyanin accumulation. *BoDFR1* expression levels were closely related to a single nucleotide polymorphism (SNP) identified in the gene (*BoDFR1* in HAAs vs. *BoDFR1v* in LAAs) as well as to the level of anthocyanins. This SNP showed perfect association with the expression of upstream regulatory genes and was used to discriminate between HAAs and LAAs.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Cabbage (*Brassica oleracea* var. *capitata*) varieties used in this study were divided into two groups: one used for sequence and expression analyses (Table 1) and the other used for marker validation (Table S1). Seed and leaf samples of cabbage varieties were kindly provided by Korean Seed Companies or the GSP Horticultural Center of Suncheon National University, Chonnam, Korea. Initially, eight inbred lines, including two purple cabbage lines, were obtained from the Biotechnology and Breeding Institute of Asia Seed Co., Icheon-si, Gyeonggi-do, Republic of Korea (Table 1). Then, additional lines were selected for detailed analysis, including Speed King, Golden Cross, CT-415, T-523, and T-530, from more than 40 inbred lines and cultivars grown in the field under LT conditions. Seeds of these additional lines were kindly provided by Joen Seed Co., Goesan-gun, Chungbuk, Korea, and Asia Seed Co. These lines were usually classified into two groups under LT conditions, HAAs and LAAs.

Table 1. Cabbage genotypes used for *DFR1* sequence and expression analyses.

Genotype ID or Name	Characteristic [†]	Genetic Status	Use [§]	Seed Source
337	HAA	Inbred line	NTS, RT-PCR	Asia Seed Co.
2437	HAA	..	NTS, RT-PCR	Asia Seed Co.
154	HAA	..	NTS, RT-PCR, qRT-PCR	Asia Seed Co.
09WH45	LAA	..	NTS, qRT-PCR	Asia Seed Co.
2409	LAA	..	NTS, qRT-PCR	Asia Seed Co.
842	LAA	..	NTS	Asia Seed Co.
Speed King	LAA	F ₁ cultivar	NTS, qRT-PCR	Takii Seed Co.
BN106	Cold tolerance	Inbred line	qRT-PCR	Asia Seed Co.
7S4-63	Purple cabbage	..	NTS	Asia Seed Co.
7S4-51	Purple cabbage	..	NTS	Asia Seed Co.
CT-415	HAA	F ₁ cultivar	NTS, qRT-PCR	Asia Seed Co.
T-523	HAA	F ₁ cultivar	NTS, qRT-PCR	Takii Seed Co.
T-530	HAA	..	NTS, qRT-PCR	Takii Seed Co.
Golden Cross	LAA	..	NTS, qRT-PCR	Takii Seed Co.

[†] HAA, high anthocyanin accumulator; LAA, low anthocyanin accumulator. [§] NTS, nucleotide sequence analysis; RT-PCR, reverse transcription PCR; qRT-PCR, quantitative real-time PCR.

Seeds were germinated, and plants were grown for 3 weeks in a growth chamber under controlled conditions (23 °C, 16 h light/8 h dark photoperiod and 140 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity). Then, plants were grown in a growth chamber under LT (<10 °C) or normal conditions (control) for 1 month. To conduct experiments under natural conditions, plants were cultivated inside and outside the greenhouse of Chungnam National University, Daejeon, Korea. LT treatment (<10 °C) under natural conditions (outside) was conducted for 30 days, whereas plants grown under normal conditions (inside) were maintained in the greenhouse at set minimum (20 °C) and maximum (30 °C) temperatures. Seedlings of the inbred line BN106 were grown in the growth room for 3 weeks and exposed to LT (10 °C, 4 °C, or 0 °C) for 2 h.

2.2. Analysis of *BoDFR1* Gene and Promoter Sequences

Genomic DNAs were extracted from the leaves of cabbage genotypes listed in Table 1, except BN106, using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The promoter and the genomic DNA

(from ATG to TGA), including the coding sequence (CDS) of the *BoDFR1* gene, were cloned by PCR using Ex Taq (Takara, Kyoto, Japan) and sequence-specific primers designed on the basis of *BoDFR1* (Bol035269) sequence obtained from the *Brassica* database (<http://brassicadb.org/brad/index.php>); primers DFR-F (5'-ATGGTAGCTCACAAAGAGACCGT-3') and DFR-R (5'-CTAAGCACAGATCTGCTGTGCCG) were used to amplify 1.6 kb *BoDFR1* genomic DNA from ATG to TGA, and primers pDFR-F1 (5'-CCTCTCACTTCGAAGCCTTCCT) and pDFR-R (5'-CACGGTCTCTTTGTGAGCTACCAT) were used to amplify the *BoDFR1* promoter region. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and synthesis at 72 °C for 1 min and a final extension at 72 °C for 7 min. PCR products were electrophoresed on 1% agarose gels and purified using LaBoPass Gel Extraction Kit (COSMOGENETECH, Seoul, Korea). The purified DNA fragments were cloned into the T&A cloning vector (RBC, Banqiao, Taiwan) and transformed into *Escherichia coli* strain DH5 α . Plasmid DNA was isolated from the transformed bacteria using LaBoPass Plasmid Mini Kit (COSMOGENETECH, Seoul, Korea). To avoid PCR and sequencing errors and to identify different *BoDFR1* alleles, at least ten clones were sequenced for each genotype. The obtained sequences were compared and analyzed using the multiple sequence alignment program CLUSTAL Omega (EMBL-EBI, UK), and cis-elements in the promoter region were identified using web-based promoter analysis tools, PLACE (<http://www.dna.affrc.go.jp/htdocs/PLACE/>) and PlantPAN (<http://PlantPAN2.itsps.ncku.edu.tw>). To validate T/A SNP, 1177 bp fragment spanning exons 1–5 was amplified using the forward primer, 5'-TGCCACTGTTCGCGATCCTG-3', and reverse primer, 5'-CATCCACTCCTTCAAACCTGTTCCA-3', and ca. 30 bp were used for an alignment.

2.3. Expression Analysis of *BoDFR1*

Total RNA was extracted from leaf samples with TRIzol Reagent (Thermo Scientific, Waltham, USA), and 1 μ g of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, USA) at 65 °C for 10 min. First-strand cDNA was synthesized using oligo dT primers and Revertra Ace Kit (TOYOBO, Osaka, Japan). Primers used to analyze *BoDFR* expression are listed in Table S2. Semi-quantitative RT-PCR was performed as follows: initial denaturation at 94 °C for 5 min, followed by 27 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. DNA fragments amplified by semi-quantitative RT-PCR were separated on 1.5% agarose gels and stained with ethidium bromide. Quantitative real-time PCR (qRT-PCR) was performed on the MiniOpticon system (Bio-Rad, Hercules, California, USA) using SYBR Green Realtime Master Mix (TOYOBO, Osaka, Japan). Primers for *BoDFR1* were designed to amplify 275 nucleotides between exon 4 and exon 6: forward primer, 5'-CTCTCTCCTATCACTCGTAACGAGGC and reverse primer, 5'-TCCCATATCAATCAGCTTCTTGGAAGT. PCR conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Fluorescence values were measured at the last step of each cycle. All analyses were performed with three biological replicates. Transcript levels of target genes were normalized relative to the *BoActin2* gene (internal reference) and analyzed using the $2^{-\Delta\Delta C_T}$ method [68].

2.4. Expression Analysis of Anthocyanin Biosynthesis-Related Genes

Total RNA extraction and cDNA synthesis were performed as described above. Expression analysis of anthocyanin biosynthesis-related genes (two representative genes for EBGs and LBGs and eight regulatory genes) was performed by qRT-PCR using primers listed in Table S3. Relative transcript levels were quantified as described above.

2.5. Anthocyanin Extraction and Quantification

Anthocyanin content was measured as described previously [69]. Briefly, 1 mL of 1% (*v/v*) HCl-methanol was added to 0.3 g of the powdered sample. The sample was shaken gently in darkness at room temperature for 24 h and then centrifuged at $13,000 \times g$. The upper aqueous phase was collected, and its absorbance was measured at 530, 620, and 650 nm. Relative anthocyanin content was calculated according to the following equation:

$$\text{Relative anthocyanin content} = (A_{530} - A_{620}) - 0.1 \times (A_{650} - A_{620}) \quad (1)$$

where A_{530} , A_{620} , and A_{650} represent the absorbance of the aqueous layer at 530, 620, and 650 nm, respectively.

Total anthocyanin content (mg anthocyanin/100 g fresh weight) was then calculated on the basis of the reference, cyanidin-3-glucoside [70], according to the following equation:

$$\text{Total anthocyanin content} = \text{Relative anthocyanin content} \times \text{MW} \times \text{DF} \times 1000 \times \vartheta \quad (2)$$

where MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol); DF is the dilution factor; and ϑ is the cuvette optical path length (1 cm).

2.6. Establishment of PCR-Based Assay

To discriminate between *BoDFR1* and *BoDFR1v*, an allele-dependent PCR assay was developed, which utilized a common reverse primer and two forward allele-dependent primers in a single reaction. Forward primers possessed different tails containing a destabilizing mismatch (or artificial mismatch) (Table 2) [71,72], while the reverse primer was complementary to a common reference sequence located 200–300 bp away from the forward primer-binding site. Several PCRs were conducted to select primer combinations with high SNP discriminating power and to optimize template concentration, annealing temperature, and amplification cycle number. The selected primer combinations and PCR reaction conditions are summarized in Table 2. PCR reactions contained 5–10 ng of genomic DNA template, 4 μL of *Taq* Premix (Elpisbiotech, Daejeon, Korea), and 1 μL (10 pmol) each of forward and reverse primer in a total volume of 20 μL .

Table 2. Primers and conditions used for PCR and high resolution melt (HRM) analyses of three SNPs in cabbage genotypes. Gothic underlined nucleotide indicated a destabilizing mismatch in forward primer.

Use	SNP Position (bp)	Primer Orientation	Target SNP	Primer Name	Primer Sequence (5'→3')	PCR Reaction Conditions			Product Size (bp)
						Template DNA (ng)	Annealing Temperature (°C)	No. of Cycles	
PCR Analysis	423	Forward	C	BoDFR 423C-F1	GTTATACTATGAACCTTTTCTTATTACAC	5	60	27	266
		Reverse	T	BoDFR 423T-F1	GTTATACTATGAACCTTTTCTTATTACAT	5	60.5	27	266
				BoDFR 423CT-R1	CTGTCACTCTCTTGGACATGATAAAGT				
	628	Forward	C	BoDFR 628C-F2	ATGATGAAAACGATTGGAGTGATATC	5	60	27	295
		Reverse	T	BoDFR 628T-F2	ATGATGAAAACGATTGGAGTGATATT	5	61	27	295
				BoDFR 628CT-R1	GCGTAATAAAGGCTAGG				
	1118	Forward	T	BoDFR1-1118T-F2	TTTGTTCTCTCACGATGCAACGATACT	7.5	62.7	27	197
		Reverse	A	BoDFR1-1118A-F1	GTTTGTTCCTCTCACGATGCAACGATTTA	7.5	62.7	27	198
HRM	1118			BoDFR1-1118TA-R	CTCTTTAGATTCTCATCCACTCCTTCAAA				
				BoDFR-HRM-aF12	TCCACTTGGACGACTTATGCAATGCT		66.3		
				BoDFR-HRM-aR1	ATCTTACGTTGAAGGCACGTTATATTCTG		66.2		
				BoDFR-HRM-pF1	GCAACGATTCATACTATCTC		54.3		

2.7. High Resolution Melt (HRM) Analysis

To detect SNPs in *BoDFR1* rapidly and efficiently, HRM analysis was carried out on a LightCycler 96 (Roche, Basel, Switzerland) instrument post-PCR in a volume of 20 μ L containing 10 ng of template DNA, 1 μ L (10 pmol) each of forward, reverse, and probe primer, and 10 μ L of the LightCycler 480 High Resolution Melting Master Mix (Roche, Basel, Switzerland). The reaction was pre-incubated for 10 min at 95 °C, followed by 50 cycles of amplification including denaturation for 10 s at 95 °C, annealing for 20 s between 63 and 55 °C under touchdown command, and synthesis for 20 s at 72 °C. The amplified PCR products were then subjected to the following HRM cycle: 60 s at 95 °C, 120 s at 40 °C and 1 s at 83 °C. The fluorescence value ($-dF/dT$) was recorded during the final step with six readings per °C. The LightCycler 96 software (Roche, Basel, Switzerland) was used to analyze the HRM curves. Each of the samples was appropriately labeled in sample editor. Both delta T_m discrimination and curve shape discrimination were set at 75%, where a 0.2 positive/negative threshold level was set to obtain the final output. Primers used for HRM analysis are listed in Table 2.

2.8. Recombinant DFR1 Protein Expression

To generate N-terminal His-tagged fusions of BoDFR1 and BoDFR1v proteins, the *BoDFR1* gene was amplified from the cDNA of HAA and LAA inbred lines 154 and 09WH45, respectively, by PCR using Ex Taq (Takara, Kyoto, Japan), forward primer BoDFR-poxF (5'-ACAGAATTCATGGTAG CTCACAAAGAG-3') containing *Eco*RI restriction site (underlined), and reverse primer BoDFR-poxR (5'-GCAAGCTTGAGCACAGATCTGCTGTG-3') containing *Hind*III restriction site (underlined). PCR products were digested with *Eco*RI and *Hind*III restriction enzymes and cloned into the pET28a expression vector (Sigma-Aldrich, St. Louis, Missouri, USA) previously digested with the same enzymes. The resulting plasmids were transformed into *E. coli* DH5 α cells, purified and sequenced. The sequence confirmed plasmids were transformed into *E. coli* strain BL21 cells (RBC, Banquiao, Taiwan), and protein expression was induced by isopropyl-thio- β -D-galactoside (IPTG; 0.3 mM) treatment at 23 °C for 16 h. Cells were harvested, resuspended in phosphate-buffered saline (PBS), and lysed by sonication. The His-tagged BoDFR1 and BoDFR1v proteins were purified by Ni Sepharose High Performance His-tagged protein purification resin (GE Healthcare, Chicago, Illinois, USA), and the purified proteins were dialyzed using MOPS (3-(N-morpholino)propanesulfonic acid) buffer. Protein concentrations were measured by the Bradford assay and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels.

2.9. Enzyme Activity Assay

Enzyme activity of BoDFR1 and BoDFR1v was examined as described previously [73] using 10 mg/ml of DHK (AdipoGen, San Diego, California, USA), DHQ (Sigma-Aldrich, St. Louis, Missouri, USA), and DHM (Sigma-Aldrich, St. Louis, Missouri, USA) substrates. A reaction (500 μ L total volume) containing 100 mM Tris-HCl buffer (pH 7.0), 35 μ g of enzyme extract, 100 μ g of substrate, and 2 mM NADPH (Reduced nicotinamide adenine dinucleotide phosphate) was incubated at 30 °C for 1 h. The activity of DFR1 was determined by measuring the rate of NADPH oxidation. To determine the content of NADPH, absorbance at 340 nm was measured using a spectrophotometer and calculated according to Beer's law:

$$A = \epsilon \times \varnothing \times C \quad (3)$$

where A is the absorbance of a 1 M solution of NADPH at 340 nm; ϵ is the extinction coefficient of NADPH (6.22 mM⁻¹ cm⁻¹); $\varnothing$ is the path length of the cuvette in cm; and C is the molar concentration.

2.10. Statistical Analysis

Anthocyanin contents, BoDFR1 activity, temperature, and gene expression levels were analyzed using one-way analysis of variance (ANOVA) and Tukey's pair-wise comparisons in Minitab v.17

(Minitab Inc., State College, PA, USA). The data were presented as the mean of three biological replicates $\pm$ standard deviation ($\pm$ SD).

3. Results

3.1. Identification of *BoDFR1* as the Key Gene Responsible for Anthocyanin Accumulation under LT Conditions

To identify the gene that regulates anthocyanin accumulation under LT conditions, we examined the expression profiles of two early anthocyanin biosynthetic genes (*BoCHS* and *BoF3H*) and two late anthocyanin biosynthetic genes (*BoDFR1* and *BoLDOX*) encoding structural proteins in the LT tolerant inbred line BN106. The temperature was decreased to 0 °C, and gene expression was analyzed by qRT-PCR (Figure 1A). The expression of *BoF3H* and *BoDFR1* increased in response to the decrease in temperature, whereas the expression of *BoCHS* and *BoLDOX* decreased or remained constant. Since the increase in *BoDFR1* expression in response to LT was more notable than that of *BoF3H*, we selected *BoDFR1* for further analysis.

Figure 1. Expression analysis of anthocyanin biosynthesis genes in cabbage genotypes. (A) Expression analysis of two early and two late anthocyanin biosynthetic genes in BN106, a cold tolerant inbred line, by quantitative real-time PCR (qRT-PCR). Plants were grown under normal growth conditions (23 °C) for 3 weeks and then subjected to warm or cold temperatures for 2 h. Three replicates were performed for each sample. Data represent mean $\pm$ standard deviation (SD). (B) Expression analysis of putative *DFR* genes in cabbage genotypes under low temperature (LT) by reverse transcription PCR (RT-PCR). Plants were grown in the greenhouse for 4 weeks and then placed either in the greenhouse (G) or in the field (L) (under LT) for 4 weeks. Average temperature varied from 20 °C (low) to 30 °C (high) in the greenhouse, and from 6 °C (low) to 18 °C (high) in the field. Putative *BoDFRs* were selected on the basis of *B. rapa* *DFR* sequences [49].

The presence of several *DFR* genes with differential expression under different abiotic stresses is suggested to have functional implications [49]. Based on the *DFR* sequences of Chinese cabbage, we identified eight putative *DFR* genes in cabbage (<http://brassicadb.org/brad/>) (Table S4) and investigated their expression by semi-quantitative RT-PCR in three HAA genotypes (337, 154, and 2437) under LT (Figure 1B). In these genotypes, three putative *BoDFR* genes (*BoI006592*, *BoI015141*, and *BoI024018*) were expressed constitutively, whereas four genes showed no expression; only one gene (*BoI035269*) showed LT-dependent expression (Figure 1B). An ortholog of *BoI035269* (*Bo9g058630*) was identified in *B. oleracea* var. *oleracea* (http://plants.ensembl.org/Brassica_oleracea/Info/Index), which is hereafter referred to as *BoDFR1*. The expression of *BoDFR1* was then studied in various cabbage genotypes exhibiting differential anthocyanin accumulation under LT.

Initially, we focused on six inbred lines, three of which accumulated anthocyanins to high levels under LT (HAAs: 337, 154, and 2437) but three to show low levels (LAAs: 09WH45, 842, and 2409). Based on RT-PCR results (data not shown), we selected four inbred lines, including two HAAs (337 and 154) and two LAAs (09WH45 and 2409), for further analyses. First, we examined the level of anthocyanins and *BoDFR1* expression in these lines grown at LT (<10 °C) for at least 30 days in a growth chamber. Upon LT exposure, anthocyanin content increased 4–10-fold in both HAA genotypes, and this

increase was proportional to the increase in *BoDFR1* expression (5.5–10-fold) (Figure 2). However, anthocyanin accumulation and *BoDFR1* expression levels were not remarkable in the LAA genotypes.

Figure 2. Anthocyanin content (A) and *BoDFR1* expression (B) in four inbred lines of cabbage grown under normal (23 °C) or low temperature (10 °C) treatment for 30 days. Plants were grown in a growth chamber for 3 weeks before treatments. Data represent mean $\pm$ SD ($n = 3$).

Next, to examine the relationship between anthocyanin accumulation and *BoDFR1* expression under natural environmental conditions, we grew more than 40 cabbage genotypes, including previously examined inbred lines, either in the greenhouse or field at LT (<10 °C) for 1 month. Four HAAs (154, T-523, T-530, and CT415) and four LAAs (09WH45, 2409, Golden Cross, and Speed King) were selected from field grown genotypes (Table 1, Figure 3) for further expression analyses and marker development and validation. Under LT, the anthocyanin content of HAAs varied from 30–53 mg/100 g fresh weight (FW), whereas that of LAAs varied from 4–16 mg/100 g FW (Figure 4A). The expression level of *BoDFR1* was more than 5-fold higher in HAAs than in LAAs (Figure 4B), indicating that *BoDFR1* expression is closely associated with anthocyanin content.

Figure 3. Variation in temperature and anthocyanin content under the natural environment. (A) Temperature profile expressed as weekly average temperature (low, high and mean) from 7 September to 16 November under the natural environment. The daily low temperature in the field was below 10 °C during the last one month. Data represented mean $\pm$ SD ($n = 7$). (B) Variation in anthocyanin content in cabbage genotypes harvested on Nov. 16. The top four samples represent high anthocyanin accumulators (HAAs), and the bottom four samples represent low anthocyanin accumulators (LAAs).

Figure 4. Analysis of anthocyanin content (A) and *DFR1* expression (B) in cabbage genotypes shown in Figure 3. 154, T-523, T-530, and CT-415 are cabbages belonging to the genotype of HAAs and 09WH45, 2409, Golden Cross, and Speed King are cabbages belonging to the genotype of LAAs. Plants were grown in the greenhouse at temperatures ranging from 20 °C to 30 °C (control, CK) or at low temperature (LT) for one month. *DFR* transcript levels were measured by qRT-PCR. Data represent mean $\pm$ SD ($n = 3$).

3.2. Analysis of the *BoDFR1* Gene Sequence Isolated from HAAs and LAAs

To investigate any relationship between genomic sequences and the expression levels of *BoDFR1* in HAAs and LAAs, we isolated the *BoDFR1* gene, including its promoter, from six HAAs (337, 154, 2437, T-523, T-530, and CT-415) and five LAAs (09WH45, 842, 2409, Golden Cross, and Speed King) (Table 1) using primers designed against Bol035269 (<http://brassicadb.org/brad/>) and Bo9g058630 (http://plants.ensembl.org/Brassica_oleracea/Info/Index) sequences and sequence information obtained from the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table S2). To avoid any errors due to PCR and sequencing, we analyzed at least ten clones for each sample. The promoter region of *BoDFR1* (1380 bp upstream of the start codon) and 1580 bp genomic DNA, including CDS, were sequenced. Sequences of *BoDFR1* isolated from cabbage genotypes 337, 154, 2437, 09WH45, 2409, 842, T-523-1, T-523-2, T-530-1, T-530-2, CT415-1, CT415-2, Speed King, and Golden Cross were deposited in GenBank under the following accession numbers: MN537957, MN537958, MN537959, MN537960, MN537961, MN537962, MN537963, MN537964, MN537965, MN537966, MN537967, MN537968, MN537969, and MN537970, respectively (Figure S1). All genes (six genes from HAAs and five genes from LAAs) comprised six exons and five introns (1580 bp) and 1380 bp promoter regions (Figure 5). Only three SNPs were identified in 1580 bp genomic DNA of *BoDFR1* between HAAs and LAAs at nucleotide positions 423 (second intron), 628 (third exon), and 1118 (fifth exon). SNPs at nucleotide positions 423 and 628 occurred together in only three F1 cultivars (T-523, T-530, and T-415), whereas the remaining genotypes harbored only one of these SNPs. At nucleotide position 1118, HAAs harbored a T, whereas LAAs harbored an A. This T/A SNP was predicted to cause a substitution at amino acid position 261 from leucine (Leu; L) to His (H). Since most of the *DFR* sequences available in nucleotide databases harbored a T at nucleotide position 1118, we hereafter refer to the *BoDFR1* allele in LAAs as the variant (*BoDFR1v*) and that in HAAs as *BoDFR1*.

Next, analysis of the number and the distribution of cis-acting elements revealed two MYB-binding sites (MBS1 or MYB-core; CNGTTR) [74], three bHLH-binding sites (E/G-BX; CANN TG/CACGTG) [75,76], and two MYB recognition sites (MYB1AT; A/TAACCA) [77] in the *BoDFR1* promoter region, and their sequences were highly similar between HAAs and LAAs (Figure 5). By contrast, the MYBD-binding site (MYBST1; GGATA) [78] at −581 bp was present in reverse orientation in *BoDFR1* promoter. However, the presence of MYBST1 might not have affected *BoDFR1* expression and anthocyanin accumulation because the *BoDFR1* gene in red (or purple) cabbage genotypes 7S4-63 and 7S4-51 (GenBank accession numbers: MN537971 and MN537972, respectively), which exhibited high anthocyanin accumulation and high *BoDFR1* expression, does not contain MYBST1 in its promoter.

Figure 5. Schematic representation of the *BoDFR1* and the *BoDFR1v* alleles identified in cabbage. The 1580 bp genomic DNA from ATG to TAG codon consists of six exons (black rectangles) and five introns (solid line). Locations of SNPs in the genomic DNA and putative transcription factor binding sites in the promoter are indicated. Analysis of cis-regulatory elements in the promoter region was performed using PlantPAN and PLACE databases. MYB-core, MYB-binding site; E/G-BX, bHLH-binding site; MYB1AT, MYB recognition site; MYBST1, *Solanum tuberosum* MYB-binding site 1.

3.3. In Vitro Enzyme Activity Assay of *BoDFR1* and *BoDFR1v*

Previously, Johnson et al. (2001) [79] reported that minor changes in the *DFR* gene affect the activity and the substrate specificity of the encoded protein. Additionally, we identified one amino acid substitution in *BoDFR1v* (L261H). Therefore, we examined the enzyme activity of *BoDFR1* and *BoDFR1v* by expressing His-tagged recombinant proteins in *E. coli*. Based on the crystal structures of both these proteins (Figure 6A), we hypothesized no differences in their enzyme activity or substrate specificity. The L261H substitution in *BoDFR1v* is located in the α_{10} helix of the small C-terminal domain, distant from the ball-and-stick structure, which is important for substrate- and NADP⁺-binding [80]. We generated recombinant *BoDFR1* and *BoDFR1v* constructs (Figure 6B) and purified the His-tagged recombinant DFR proteins (Figure 6C). Enzyme activity, expressed as the decline in the oxidation rate of NADPH, differed among the substrates DHK, DHQ, and DHM but not between *BoDFR1* and *BoDFR1v* (Figure 6D). These results suggest that the L261H amino acid substitution does not affect the activity or substrate specificity of the DFR protein.

Figure 6. Production and activity of recombinant *BoDFR1* and *BoDFR1v* proteins. (A) Predicted structure of *BoDFR1* based on grape DFR crystal structure (PDB (protein data bank) ID: 2C29) [76]. The L261H amino acid substitution identified in Bo035269 is located in the α_{10} helix of the small C-terminal domain (green arrow). (B) Schematic representation of recombinant expression vectors. (C) SDS-PAGE analysis of recombinant proteins. Protein samples were separated on 10% polyacrylamide gels. M, protein marker. Lane 1, total lysate of pET-28a-*BoDFR1*; lane 2, total lysate of pET-28a-*BoDFR1v*; lane 3, purified His-tagged *BoDFR1* protein; lane 4, purified His-tagged *BoDFR1v* protein. The molecular weight of *BoDFR1* and *BoDFR1v* is 43 kDa. (D) Activity of *BoDFR1* based on the oxidation of NADPH (Reduced nicotinamide adenine dinucleotide phosphate). The oxidation of NADPH was measured as the decrease in absorbance at 340 nm. DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin.

3.4. Relationship between *BoDFR1* and Other Anthocyanin Biosynthesis-Related Genes

BoDFR1 showed allele-dependent expression (Figure 4), and anthocyanin content was proportional to the expression level of *BoDFR1* under LT treatment. However, the sequence of the promoter, which controls gene expression levels, was highly similar among HAAs (337, 154, 2437, T-523, T-530, and CT-415) and LAAs (09WH45, 842, 2409, Golden Cross, and Speed King) (Figure 5, Figure S1). Additionally, both *BoDFR1* and *BoDFR1v* showed the same substrate specificity and enzyme activity (Figure 6). These results led us to hypothesize that the function of upstream regulatory gene(s) is associated with the *DFR1* expression level. To test this hypothesis, we examined the expression levels of anthocyanin biosynthetic genes (encoding structural and regulatory proteins) under normal and LT conditions in the same samples as those used in Figure 4, and *BoDFR1* expression data served as a reference (Figure 7, Figure 8). The expression of EBGs, *BoCHS* and *BoF3H*, was similar between HAAs and LAAs; however, the expression of LBGs, *BoLDOX* and *BoDFR1*, was lower in LAAs than in HAAs (Figure 7). This similar expression pattern of *BoLDOX* and *BoDFR1* may reflect that *BoLDOX* levels were dependent to DFR activity in directly.

Figure 7. Expression analysis of anthocyanin biosynthesis genes encoding structural proteins in cabbage genotypes. Plants were grown either under normal growth temperature (CK; 20–30 °C) or at LT (see Figure 3). Gene expression was analyzed by qRT-PCR. Expression level of *BoActin2* was set to one and used for data normalization. Data represent mean $\pm$ SD ($n = 3$).

We also examined the expression of anthocyanin biosynthesis regulatory genes (Figure 8). Expression of *BoHY5* and *BoMYBH1* (a homolog of *MYBD*) was slightly increased by LT, but no significant difference was detected between HAAs and LAAs. The expression of *BoMYBH2* showed no significant differences between HAAs and LAAs or between normal and LT conditions. However, the expression of genes comprising the MBW complex, *BoMYB114* (representative *MYB* in cabbage), *BoHLH*, and *BoTTG1* (WD-repeat gene), seemed to provide some clues about the expression of *BoDFR1*. Expression levels of *BoMYB114* and *BoHLH* were higher in HAAs than LAAs, regardless of the temperature (normal vs. LT). However, the expression of the genes did not show a significant increase upon exposure to LT; instead, the expression of *BoHLH* in LAAs was decreased by LT treatment. Although the expression of *BoTTG1* showed no significant difference between HAAs and LAAs, it increased significantly upon LT treatment. These results reveal a novel phenomenon in cabbage, which explains why LAAs exhibit low anthocyanin content and low *BoDFR1v* expression under LT condition. Moreover, expression of the repressor of MBW complex, *BoMYBL2*, was low in HAAs and greatly increased in LAAs in response to LT. The expression of *BoMYBL2* might be negatively associated with *BoMYBD* expression suggested by a previous study [81]. Thus, the expression pattern of *BoMYBL2* may also contribute to *BoDFR1* expression and anthocyanin accumulation in cabbage.

Figure 8. Expression analysis of anthocyanin biosynthesis genes encoding regulatory proteins in cabbage genotypes. Plants were grown either under normal growth temperature (CK; 20–30 °C) or under LT (see Figure 3). Gene expression was analyzed by qRT-PCR. Expression level of *BoActin2* was set to one and used for data normalization. Data represent mean $\pm$ SD ($n = 3$). *BoDFR1* data were same as that in Figure 4B and used as a reference.

In *Arabidopsis*, anthocyanin accumulation is associated with freezing tolerance, and freezing tolerant accessions show higher *DFR* expression levels than freezing sensitive accessions [8,9,82]. In apple, a MYB transcription factor binds to both the *CBF* and the *DFR* promoters, thereby activating their expression [13]. However, cabbage genotypes (HAAs and LAAs) used in this study seemed to be freezing tolerant, as some of them survived the Korean winter (data not shown). Therefore, any association of *BoDFR1* or *BoDFR1v* expression with cold tolerance (or anthocyanin accumulation and cold tolerance) must be elusive. In cabbage genotypes used in this study, all four *CBF* genes were upregulated upon LT treatment with no difference in expression between HAAs and LAAs (Figure 9). This suggests that anthocyanin accumulation is not related to cold tolerance at least in the genotypes used in this study.

Figure 9. Expression analysis of four *CBF* genes by qRT-PCR. Cabbage plants were grown either under normal growth temperature (CK; 20–30 °C) or under LT condition (see Figure 3). Expression level of *BoActin2* was set to one and used for data normalization. Data represent mean $\pm$ SD ($n = 3$).

3.5. Development of a Molecular Marker to Distinguish between *BoDFR1* and *BoDFR1v*

We designed PCR primers to distinguish between *BoDFR1* and *BoDFR1v* based on cytosine (C)/adenine (A), cytosine (C)/thymine (T), and thymine (T)/adenine (A) SNPs at nucleotide positions 423, 628, and 1118, respectively (Figure 5, Table 2). First, PCR was performed using these primers and genomic DNA extracted from six inbred lines provided by Asian Seed Co. (Figure 10A) and eight inbred lines and cultivars selected in Figure 3 for further analysis (Figure 10A,C, Table 2). All primer pairs could discriminate the six inbred lines based on the three SNPs (Figure 10A), but only the primer pair specific to the T/A SNP at position 1118 correctly discriminated among the eight inbred lines and cultivars (Figure 10C). Cabbage cultivars T-523, T-530, and CT-415 were heterozygous for SNPs at nucleotide positions 423 and 628 (Figure S1, Figure 5). Then, based on these PCR results, we designed HRM primers targeting the T/A SNP at nucleotide position 1,118. The results showed that this SNP distinguished between *BoDFR1* and *BoDFR1v* (Figure 10B,D). Therefore, we conclude that the T/A SNP could be used to discriminate between HAAs and LAAs under LT conditions.

Figure 10. Validation of *BoDFR* SNP marker designed to distinguish between HAAs and LAAs. (A,C) PCR analysis. Optimal annealing temperatures are indicated on the right side. (B,D) HRM analysis. Cabbage genotypes used for the analysis are indicated on the right side.

To validate this marker, we used the HRM primer set to genotype 120 cabbage inbred lines and cultivars (Figure 11A,B). Among these 120 samples, 70 (58%) were homozygous for the TT allele (HAA homozygote), 32 (27%) were homozygous for the AA allele (LAA homozygote), and 18 (15%) were heterozygous (TA allele). Interestingly, all Chinese cultivars tested in this study carried the AA type (*BoDFR1v*) (Table S1). Some of the heterozygotes were confirmed by sequencing (Figure S2), and association of the T/A SNP with anthocyanin accumulation was confirmed by examining the level of anthocyanins and *BoDFR1* expression (Figure 11C,D). The phenotype of heterozygotes (T/A allele) resembled that of HAA homozygotes (TT allele), indicating dominance of the T allele (HAA genotype was TT or TA). These results indicate that the HRM primer pair developed in this study is useful for distinguishing between *BoDFR1* and *BoDFR1v* genotypes. Notably, HAAs were more abundant than LAAs or heterozygotes, and *BoDFR1* was dominant to the *BoDFR1v* allele.

We also investigated the association between anthocyanin accumulation and *BoDFR1* SNP in *B. oleracea*, Chinese cabbage, and mustard genotypes, which showed high anthocyanin accumulation and very low accumulation. As shown in Figure S3, the *BoDFR1v* allele was found in cabbage and ornamental kale (*B. oleracea* var. *acephala*) but not in cauliflower (*B. oleracea* var. *botrytis*), kohlrabi, Chinese cabbage, and mustard. Interestingly, F1 cultivars of ornamental or flower kale including M23 (Benitaka-red) and M24 (Shirotaka-white) were heterozygous and homozygous for the *BoDFR1v* allele, respectively. Recently, Guo et al. (2019) [83] reported that *BoDFR1* expression is associated with anthocyanin accumulation under LT, but the authors did not discuss allele-dependent expression. Overall, we conclude that genotypes heterozygous at the 1,118 SNP show HAA phenotype.

Figure 11. HRM analysis of 120 cabbage genotypes (inbred lines and cultivars). (A) HRM graph, (B) genotype of the SNP in 120 cabbage genotypes. Anthocyanin content (C) and *BoDFR1* expression (D) in selected cabbage genotypes to confirm the utility of the marker. Data represent mean $\pm$ SD ($n = 3$).

4. Discussion

Anthocyanin accumulation in plants is closely related to abiotic stress tolerance, particularly LT tolerance, in plants and is proportional to *DFR* gene expression. Despite the positive effects of anthocyanins on human health, one of the breeding objectives of green cabbage is to maintain the deep green color of leaves at harvest, regardless of the season, especially under LT conditions. In this study, we investigated cabbage cultivars and inbred lines that show low anthocyanin accumulation under LT but retain their LT tolerance. Our results showed that *BoDFR1* expression is related to anthocyanin accumulation in cabbage. Analysis of the *BoDFR1* gene in contrasting cabbage genotypes (HAAs vs. LAAs) revealed that an SNP at nucleotide position 1118 in 1580 bp *BoDFR1* genomic DNA is closely related to its expression levels. We used this SNP to develop a PCR-based marker as well as HRM marker for the identification of HAAs and LAAs under LT conditions.

4.1. Selection of HAA and LAA Lines

Analysis of six inbred lines (three HAAs and three LAAs) and five cultivars (three HAAs and two LAAs) of cabbage revealed that *BoDFR1* expression was associated with anthocyanin accumulation under LT conditions (Figures 1–4). This association study was performed using plants grown either in an environmentally controlled growth chamber or in the field, where light intensities vary from 140 to 1300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Since light intensity influences anthocyanin biosynthesis [84], we examined gene expression under both conditions. Previous reports imply that the *DFR* gene is a good candidate for anthocyanin accumulation. The *BoPr* gene, which confers purple leaf color in ornamental kale (*Brassica oleracea* L. var. *acephala*), has been mapped and identified as Bo9g058630 (orthologous to Bol035269 in cabbage) and shows high homology to the gene encoding dihydroflavonol reductase in *Arabidopsis* (AT5G42800) [85]. In crabapple (*Malus crabapple*) leaves and apple peels, upregulation of *McDFR* or *MdDFR* expression, respectively, is accompanied by a proportional increase in the expression of other genes involved in anthocyanin biosynthesis [45]. This result and our data together imply that the *BoDFR* gene is a good candidate for the investigation of anthocyanin accumulation in cabbage.

4.2. *BoDFR1* Sequence Analysis and SNP Detection

The genomic sequence of *BoDFR1* was analyzed in 13 cabbage genotypes including six HAAs, five LAAs, and two red (or purple) cabbage cultivars (Table 1). However, sequences of *BoDFR1* from two red cabbage cultivars were excluded from the alignment shown in Figure S1. The genomic DNA of *BoDFR1* analyzed in this study comprised the promoter region (1380 bp upstream of the ATG start codon) and the 1580 bp genomic DNA from ATG to TAG stop codon, which includes six exons and five introns (Figure 5). Sequences of *BoDFR1* in 13 cabbage genotypes were highly similar. Only three SNPs were identified in the 1580 bp genomic DNA. Among these, only the T/A SNP at the 1118 nt was predicted to cause an amino acid substitution (Leu to His), and this SNP could be used to discriminate between HAA-type *DFR1* (*BoDFR1*) and LAA-type *DFR1* (*BoDFR1v*).

In the promoter region, the number and the distribution of cis-acting elements, such as MYB1AT, MYB-core (or MYBS1), and E/G-BX, were highly similar between HAAs and LAAs (Figure 5); only MYBST1 was found in reverse orientation at -581 bp in *BoDFR1* promoter compared with *BoDFR1v* promoter. HY5-induced MYBD binds to MYBST1 and the MYB-binding motif (A/TAACCA) in the promoter of the repressor *MYBL2*, resulting in the inhibition of *MYBL2* expression [81]. Thus, the presence of the MYBST1 motif in the *BoDFR1* promoter affects gene expression. However, the MYBST1 motif was not detected in the *BoDFR1* gene isolated from purple (or red) cabbage, which is highly rich in anthocyanins. Moreover, the expression pattern of *BoMYBD* was not similar to *BoMYBL2* expression (Figure 8). These data suggest the possibility that the MYBST1 motif found in the *BoDFR1* promoter does not affect the difference in anthocyanin levels between HAAs and LAAs.

A mutation in *DFR* promoter in onion [61] and two insertion/deletion mutations (InDels) in *DFR* CDS in kale [85] is associated with anthocyanin accumulation. Rearrangement of a 23 bp sequence in the promoter regions of *MdMYB10* and *McMYB10* is responsible for the difference in gene expression between white- and red-fleshed apples and red-leaf crabapples [86]. The bHLH transcriptional regulator binds to the CANNTG site (E/G-BX) present in the *DFR* promoter [50]. Additionally, a MYB transcription factor recognizes the MYBS1, thus regulating the expression of *DFR* [87]. Together, these data suggest that differential expression of the *DFR* allele results from differences in promoter sequences or cis-acting sequences. However, promoter sequences of *BoDFR1* and *BoDFR1v* were highly similar, implying that differential expression of *BoDFR1* between HAAs and LAAs probably resulted from differences in upstream regulatory proteins.

4.3. Regulation of *BoDFR1* Expression

DFR regulates the essential step of anthocyanin biosynthesis and possesses a preference for a precursor of the three different types of anthocyanins [35,36]. However, *BoDFR1* and *BoDFR1v*, which differed by one amino acid substitution, showed identical enzyme activity and substrate specificity (Figure 6) because the substituted amino acid was distant from the active site of *DFR* [79]. This suggested that the difference in anthocyanin accumulation between HAAs and LAAs could be associated with the difference in *BoDFR1* expression levels. However, no significant differences were detected in the promoter and the intron sequences between HAAs and LAAs (Figure S1), which could control gene expression, suggesting the possibility that upstream regulatory transcription factors are involved in the regulation of *BoDFR1* expression.

Data shown in Figure 8 provided hints as to why LAAs accumulate less anthocyanins than HAAs under LT conditions. The expression of *BoTTG1* increased upon exposure to LT, but that of *BoMYB114* and *BobHLH* did not, especially in LAAs. Instead, *BoMYB114* and *BobHLH* expression levels were high in HAAs. These expression patterns represent a novel characteristic of cabbage plants because the expression of *MYB* and *bHLH* genes increases in purple kale in response to LT, whereas that of *TT1* shows no change [33]. Similar results have been reported in *Arabidopsis* [88]. This difference in gene expression levels might explain why prolonged LT treatment (lasting at least one month) is required for increasing anthocyanin accumulation or inducing *DFR* expression in cabbage (data not shown). In addition, the expression of *BoMYBL2* (a repressor) might also affect the formation of the MBW complex. *BoMYBL2*

expression was low in LAAs but greatly increased upon LT treatment (Figure 8). The expression of *BoMYBL2* might be slightly repressed by *HY5*-dependent *MYBD* (*BoMYBD*), as mentioned above [81], although this would not be critical. Taken together, these data suggest that the level of WD-repeat proteins would be sufficient in all cabbage types under LT, but the levels of *BoMYB114* and *BobHLH* were high in HAAs and low in LAAs, regardless of the temperature. The level of *BoMYBL2* increased in LAAs upon LT exposure, resulting in the inhibition of MBW complex formation. The MBW complex regulates the expression of LBGs, including *DFR*, at the transcriptional level [28,89,90]. Moreover, the MBW complex regulates *bHLH* (*TT8*) expression in a positive feedback loop, which ensures high level accumulation of proanthocyanins [91]. Our results suggest that formation of the MBW complex, which induced *BoDFR1* expression, would be greatly reduced in LAAs but unaffected in HAAs, leading to differential anthocyanin accumulation. However, how the *BoDFR1* SNP is associated with the expression of upstream regulatory components remains elusive.

CBF transcription factors play a key role in the cold acclimation of plants by regulating the expression of cold-regulated (*COR*) genes [92,93]. High level of *DFR* expression is associated with high anthocyanin accumulation, which confers cold (or freezing) tolerance in *Arabidopsis* [8,9,82], Chinese cabbage [49], tobacco (*Nicotiana tabacum*) [12], and purple kale [33,83]. In apple, a MYB transcription factor regulates both cold tolerance and anthocyanin accumulation by binding to the promoters of *CBF* and *DFR* genes, thus activating their expression [13]. However, four LAAs used in this study were freezing tolerant (data not shown). Additionally, we did not observe differences in the induction of *BoCBFs* between HAAs and LAAs under LT treatment (Figure 9), indicating that the mechanisms of anthocyanin accumulation and cold tolerance in cabbage were independent.

4.4. Molecular Marker Development and Application

In cabbage breeding programs, one of the desired horticultural traits is the green color of leaves at the harvest time, regardless of the season. To maintain green leaf color, anthocyanin accumulation should be repressed, which can be achieved by inhibiting *BoDFR1* expression. Fortunately, cabbage genotypes harboring the *BoDFR1v* allele accumulate anthocyanins to a very low level not only under normal conditions but also under LT. The *BoDFR1v* allele harbors an A at nucleotide position 1118, whereas the *BoDFR1* allele harbors a T. This SNP has also been detected in ornamental kale genotypes, M23 (Benitaka–red) and M24 (Shirotaka–white), which are heterozygous and homozygous for *BoDFR1v*, respectively (Figure S3). Based on this T/A SNP, we developed and validated a PCR-based and an HRM marker, which perfectly discriminated between LAAs and HAAs (Figures 10 and 11). Although we could not explain why LAAs express *BoDFR1v* at low levels while maintaining cold tolerance, both PCR-based and HRM markers will be very useful for cabbage breeding.

5. Conclusions

Cabbage and some ornamental kale genotypes carried the *BoDFR1v* allele, which contains a single amino acid substitution (Leu to His) due to a single nucleotide change (T to A). However, both *BoDFR1v* and *BoDFR1* showed identical enzyme activity and substrate specificity. *BoDFR1v* homozygotes exhibited green leaf color, regardless of the temperature, and a very low level of *BoDFR1* expression. Using the T/A SNP identified between *BoDFR1* and *BoDFR1v*, molecular markers were developed and validated to distinguish between HAAs and LAAs. Further investigation is needed to identify upstream elements or key regulators that control the expression of anthocyanin-related genes in LAAs, which could be further utilized in cabbage breeding programs.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/4/602/s1>. Figure S1: Nucleotide sequence alignment of *BoDFR1* promoter and genomic DNA from ATG to TGA isolated from 11 cabbage genotypes, Figure S2: Validation of the T/A SNP at nucleotide position 1,118 in *BoDFR1* genomic DNA, Figure S3: Genotyping various *B. oleracea*, *B. rapa*, *B. napus* and *B. juncea* accessions using the 1118T/A SNP marker, Table S1: List of cabbage genotypes used in HRM analysis, Table S2: List of primers used for expression analysis and cloning of *BoDFR* genes, Table S3: List of primers used for qRT-PCR experiments, Table S4: Putative *BoDRF* genes identified in *B. oleracea* using *B. rapa* *DFR* (*BrDFR*) genes [49].

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