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A 43 bp-deletion in the F3'H gene reducing anthocyanins is responsible for buds-keeping-green at low temperature in broccoli

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Abstract

Most broccoli cultivars or accessions have green buds at an appropriate growth environment. They would develop red or purple buds at cold temperature, while some have green buds both at normal growth or cold temperature. In this study, through BSA-seq, fine mapping and transcriptome analyses, we identified a candidate gene (*F3'H*) responsible for reducing the accumulation of anthocyanins in a mutant GS and HX-16 broccoli (DH line) (*Brassica oleracea* L. var. *italica*), which would have caused green buds even at low temperature. A 43-bp deletion was detected in the CDS of the *F3'H* gene of HX-16 and mutant GS, which largely reduced the expression of *F3'H* and the accumulation of cyanidin and delphinidin in the mutant GS. The expression of *F3'H* was up-regulated at low temperature in the wild line PS.The detection of 43-bp deletion in the broccoli germplasm resources and other varietas in *Brassica oleracea* showed that there could be other factors for green buds at low temperature in *Brassica oleracea*. This study provides the critical genetic and molecular information for the molecular breeding of *B. oleracea* and illuminates the molecular basis of the low-temperature effect on buds' color in broccoli.

Introduction

Anthocyanins, which endow plants with orange, red, purple, or blue, belong to a kind of second metabolites called flavonoids widely distributed in plants (Springob *et al* ., 2003; Lepiniec *et al* ., 2006; Saito *et al* ., 2013). Besides, anthocyanins are regarded as stress protectants protecting plants against abiotic/biotic stress such as cold, strong sunshine, and microbe infection (Tohge and Fernie, 2017). As anthocyanins are prevalent but not indispensable in plants, the biosynthesis of anthocyanins, which is a part of the flavonoid biosynthesis pathway, is studied widely by relevant mutants, especially in the model plant *Arabidopsis thaliana*(Winkel-Shirley, 2001; Grotewold, 2006; Saito *et al* ., 2013). In*Arabidopsis* , many genes associated with anthocyanins biosynthesis were identified by the mutant lines whose seed coat has*transparent testa* (*tt*) (Li *et al* ., 2017). The biosynthesis pathway of anthocyanins starts with phenylalanine which converts into 4-coumaroyl CoA by a series of enzymes: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL), that's the general phenylpropanoid pathway (Winkel-Shirley, 2001; Zhang *et al* ., 2014). Malonyl-CoA and 4-coumaroyl CoA were catalyzed by a series of enzymes such as chalcone synthase (CHS), chalcone isomerase (CHI), and flavone 3-hydroxylase (F3H) to produce dihydroflavonols, which would then convert into anthocyanidins by another series of enzymes, such as F3'H (flavonoid 3'-hydroxylase), DFR (Dihydroflavonol 4-Reductase), ANS (Anthocyanidin Synthase)/LDOX (leucoanthocyanidin dioxygenase). Dihydroflavonols could also be oxidized to flavonols by FLS (flavonol synthase). The anthocyanidins are unstable and usually become stable by glycosylation, methylation and/or acylation (Winkel-Shirley, 2001; Zhang *et al* ., 2014). The biosynthesis of anthocyanins is also regulated by many factors, including transcription factors (R2R3MYB and bHLH), WD Repeat Proteins (WDR), epigenetic modification, and environmental factors (light, temperature, sugar, hormones, fertilizer, drought, infection of pathogens, etc.) (Espley *et al*

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., 2007; Spelt *et al.* , 2000; Winkel-Shirley, 2001; Lorenc-Kukula *et al.* , 2005; Zhang *et al* ., 2014; Wang *et al* ., 2020; Yu *et al* ., 2022).

Environmental factors regulate anthocyanin biosynthesis by modifying the expression of its structural or regulatory genes. Low temperature and strong sunshine could together induce the accumulation of anthocyanins by promoting the expression of *CHS, ANS* and *UFGT* in apple skin (Ubi *et al* ., 2006), while light promotes anthocyanin accumulation in apple fruit skin by MdSIZ1 modifying MdMYB1 (Takos*et al.,* 2006; Zhou *et al.* , 2017). Anthocyanin rhamnosyl transferases, UGT79B2 and UGT79B3, were regulated by CBF1 (CRT/DRE-binding factor1, also named DREB1B) and increase low temperatures tolerance via modulating anthocyanin accumulation in*Arabidopsis* (Li *et al.* , 2017). Structure genes of synthesis of anthocyanins, such as *CHS, F3'H* , *F3H* , and*UFGT* , and transcript factor *MYB* , were regulated by low temperature (Zhang *et al* ., 2010 and 2012). Low temperature has different influences on anthocyanin biosynthesis in different species (Mao *et al.* , 2022; Jiang *et al.* , 2022). The low temperature usually induces the accumulation of anthocyanins in plants, such as *Arabidopsis* (Li *et al* ., 2017), apple (Ubi *et al* ., 2006; Jiang *et al* .,2022), and kale (Zhang *et al* ., 2012), while exerts converse function to accumulation of anthocyanins in peach and strawberry (Zhu *et al.* , 2020; Mao *et al* ., 2022). Anthocyanin decoration was also important in increasing environmental stress tolerance (Saigo *et al* ., 2020).

There are several cultivars or accessions with purple leaves, bulbs or curds at normal growth environment in *Brassica* (Chiu *et al.* , 2010; Yan *et al* ., 2019). A Harbinger DNA transposon insertion in the regulatory region of *R2R3 MYB* transcription factor activated the gene and some anthocyanins structural genes such as*F3'H, DFR* , and *ANS* to promote the accumulation of the pigments in the purple cauliflower (Chiu *et al.* , 2010). A 7600 bp CACTA-like transposon and point mutation and 1-bp insertion in*BoMYB2* promoter region were found in purple kohlrabi and purple cabbage, respectively (Yan *et al* ., 2019). Most broccoli cultivars or accessions show no purple or red bud trait in optimum growth temperature but would turn red or purple in abiotic stressed environments, such as low temperature and strong sunshine. In contrast, some broccoli plants would keep green buds even at cold temperature. In this study, we have investigated the genetics of controlling the keeping-green trait at low temperature (KGLT) and the turning-purple at low temperature (TPLT) of the buds in broccoli (*Brassicaoleracea* L. var. *italica*). Segregating population with about 1000 individuals was constructed, the KGLT trait was fine-mapped, and the candidate gene was cloned. Another segregating population with the same trait and other accessions in *Brassica* were investigated. This study provides some important information for the genetic breeding of *B. oleracea* and illuminates the genetic mechanism of the low-temperature effect on buds' color in broccoli.

Materials and methods

Plant materials

Development of F_2 and Backcross populations

The broccoli DH lines HX-16 with KGLT and HX-11 with TPLT were selected as paternal and maternal lines, respectively, and were crossed to develop the F_1 hybrid. The BC_1 was obtained by pollinating the F_1 hybrid with HX-16, and the individuals with TPLT were selected to cross with HX-16 again to produce the BC_2 population. In the BC_2population, the individuals with TPLT were selected and selfed to construct the BC_2F_2 segregation population. An F_2 segregation population was obtained by selfing the hybrid F_1 derived from a mutant GS with green buds and the wild line PS with purple buds at low temperature. Plug-seedlings were obtained and seedlings were transplanted in a tunnel greenhouse at Yangdu experimental farm of Zhejiang academy of agricultural sciences in the autumn of 2017 (HX-11, HX-16 and their F_1, the mutant GS and wild DH line PS), 2018 (HX-11, HX-16 and their F_1, BC_1, the mutant GS and the wild DH line PS), 2019 (HX-11, HX-16 and their F_1, BC_1 and BC_2, the hybrid F_1 of the mutant GS and the wild lines PS and germplasm materials in *Brassica oleracea* (Table 1), 2020 (HX-11, HX-16 and their F_1 and BC_2F_2, the mutant GS and the wild lines PS and their F_2 segregation population,) and 2021 (HX-11, HX-16 and their F_1, the mutant GS and the wild lines PS, purple broccoli (PB)). Meanwhile, the wild line PS plants were cultured in an artificial chamber with 20/25 night/day temperature and a 14-hour photoperiod for the

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experiment of the temperature effect on the bud color in 2021 in order to check the effect of temperature on anthocyanin. Phenotyping of the buds in the segregation populations, respective parents, and the germplasm materials was conducted after experiencing cold air (0-5^0 C) for 10 days as the temperature of this range could make the buds of the maternal line HX-11 turn purple completely.

Table 1 Germplasm materials in *Brassica oleracea*

Material codes	Species	Material codes	Species	Material codes	Species
1015	*B. oleracea* L. var. *italica*	SG1921	*B. oleracea* L. var. *italica*	SG1911	*B. oleracea*
1403	*B. oleracea* L. var. *italica*	SG1614	*B. oleracea* L. var. *botrytis*	SG1912-1	*B. oleracea*
1405-1	*B. oleracea* L. var. *italica*	51932-2	*B. oleracea* L. var. *italica*	SG1920	*B. oleracea*
1409	*B. oleracea* L. var. *italica*	51936-1	*B. oleracea* L. var. *italica*	SG2009-2	*Brassica o*
1502-1	*B. oleracea* L. var. *italica*	51938-13	*B. oleracea* L. var. *italica*	SG2021	*B. oleracea*
1610-4B	*B. oleracea* L. var. *italica*	PR2003	*B. oleracea* L. var. *botrytis*	UC001	*B. oleracea*
1910-1	*B. oleracea* L. var. *italica*	2035	*B. oleracea* L. var. *italica*	UC002	*B. oleracea*
2035	*B. oleracea* L. var. *italica*	1947	*B. oleracea* L. var. *italica*	UC003	*B. oleracea*
2057-11	*B. oleracea* L. var. *italica*	VR2003	*B. oleracea* L. var. *botrytis*	UC012	*B. oleracea*
5815-6A	*B. oleracea* L. var. *italica*	SG1401-1	*B. oleracea* L. var. *italica*	UC018	*B. oleracea*
K2145	*B. oleracea* L. var. *acephala*	SG1913	*B. oleracea* L. var. *italica*	UC044	*B. oleracea*
K2150	*B. oleracea* L. var. *caulorapa*	SG1914-1	*B. oleracea* L. var. *italica*	UC019	*B. oleracea*
2018K1M3	*B. oleracea* L. var. *acephala*	SG1917-2	*B. oleracea* L. var. *italica*	UC020	*B. oleracea*
2018K1M5	*B. oleracea* L. var. *acephala*	SG1908	*B. oleracea* L. var. *italica*	UC030	*B. oleracea*

Anthocyanin extraction and measurement

The buds of the mutant GS, the wild line PS, purple broccoli PB at low temperature (Figure 1C, D, E), were taken for this experiment. Total anthocyanin from buds of different broccolis were extracted with 0.1mol/L ethanol hydrochloride solution, and detected by UV/VIS spectrophotometer. The anthocyanidins were obtained after the anthocyanins were hydrolyzed by 37% HCl and boiling. The anthocyanidin content and constituent were determined by HPLC-MS as described by Yin*et al* ., 2015 and Zhang *et al.* , 2004.

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Figure1. Phenotype of the seedlings, seeds and buds heads in different broccoli materials. (A) and (B), seedlings and seeds of the wild line PS (left) and the mutant GS (right), respectively. (C-H), bud heads from the wild line PS, the mutant GS, purple broccoli PB, the maternal line HX-11 and the paternal line HX-16, respectively. (F), the buds of representative individuals in the BC_2F_2population.

Genomic DNA extraction and library construction

The whole genomic DNA was extracted from frozen young leaves according to the CTAB method (Yu *et al.* , 2019). The p-pool and g-pool representing buds turning purple and buds keeping green samples, respectively, were constructed by collecting 30 BC_2F_2 individuals in each pool. DNA quality was detected, and their concentration was quantified by electrophoresis on 0.8% agarose gel and a spectrophotometer (NanoDrop, United States), respectively. Sequencing libraries were constructed according to the instruction of the TruSeq DNA PCR-free prep kit (Illumina). The quality of libraries was tested using Agilent High Sensitive DNA kit by Agilent Bioanalyzer, and the qualified libraries were sequenced by the Illumina NovaSeq platform. The raw sequencing data were filtered using fastp (v0.20.0) to discard those reads with low quality.

QTL mapping

The high-quality reads were aligned to the reference genomic sequences (HDEM (*http://www.genoscope.cns.fr/externe/plants/chromosomes.html*) by BWA (0.7.12-r1039) (Li and Durbin, 2009). The reads near InDel variant sites were realigned by Indel Realigner of GATK to improve the precision of SNP acquiring. Precise and reliable SNPs were obtained by Unified Genotyper and filtering atypical SNPs. Then, SNP-index was calculated and mapped after SNP calling. The SNP-index in the p-pool and g-pool and their subtraction were mapped to show their distribution on the chromosome. The candidate region for the target trait was selected based on the subtraction value of the p-pool and g-pool. Precise phenotyping is a fundamental requirement for accurate QTL mapping, so the bud color trait was investigated after experiencing cold for ten days.

KASP primers designing and genotyping

KASP primers were designed according to the flanking sequences of the SNPs/InDels (Table S6). Genotyping of the BC_2F_2 and other population were performed using the KASP platform (IntelliQube, LGC, Biosearch Technologies) according to the KASP genotyping protocol. Total 1.6 μL of the KASP reaction mixture included 5~10 ng DNA sample, 1.74 μM common reverse primer, 0.69μM each of Fam and Hex labelled forward primer, and 1×KASP Master Mix. The KASP protocol was performed by touchdown PCR as follows: step 1, 94 for 15 min; step 2 (touchdown), 94 for 20 sec, 61 for 60 sec (decrease 0.6 per cycle), 10 cycles; step 3, 94 for 20sec, 55 for 60 sec, 26 cycles. The fluorescence signal of amplification products was detected and classified.

Transcriptome analysis

The buds were collected and frozen in liquid nitrogen and preserved in -80 refrigerator. The RNA was extracted from the samples using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, USA). The quality of extracted RNA was tested by a NanoDrop spectrophotometer (Thermo Scientific, MA, USA). Sequencing libraries were constructed using the TruSeq mRNA Sample Prep Kit (Illumina, San Diego, CA, USA). The mRNA was isolated from total RNA and broken into fragments with the size of about 200-300 bp. The cDNA was synthesized from mRNA, and the cDNA with the fragments size of about 300-400 bp were chosen to construct the banks. DNA was assayed on a Bioanalyzer 2100 system (Agilent, Santa Clara, CA, USA). The sequencing library was then sequenced on a Hiseq platform (Illumina, San Diego, CA, USA). Taking HDEM genome sequence as a reference, clean and high-quality sequences were aligned to the reference genome. According to the alignment result, the expression level of each gene was calculated and the aligned reads were assembled into the transcript sequences.

The CDS sequences and a phylogenetic analysis of *F3'H*

The buds of HX-11 and HX-16 were collected 10 days after experiencing cold air ($0\text{-}5^0$ C) and frozen in

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liquid nitrogen, and preserved in a -80 refrigerator. The RNA was extracted from the samples using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, USA). The cDNA was synthesized from mRNA using TIANScript Kit (KR104) (Tiangen Biotech Co., LTD, Beijing) according to the instruction. The primers were designed based on the sequence of *F3'H* CDS in the HDEM reference genome to amplify the CDS sequences of HX-11 and HX-16 (Table S7). The amino acid sequence of *F3'H* in *Arabidopsis thaliana* was aligned in NCBI. Then the highly identical amino acid sequences were downloaded and used for phylogenetic analysis by MEGA X.

Results

Anthocyanins contents

Besides the color difference of buds at low temperature (Figure 1C, D), the mutant GS and the wild DH line PS have a different color of seeds and seedlings hypocotyl (Figure 1A, B). Although more than 600 anthocyanins have been reported, the core structures of anthocyanins, which could be absorbed in both visible lights and ultraviolet, have only six types that's cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Saigo *et al.* , 2020). Total anthocyanin and six types of anthocyanidins were tested to check the content of anthocyanins and anthocyanidins in buds between GS and PS in cold winter (between 0 and 5^0 C). In this test experiment, a purple broccoli accession (PB) was used as a check to compare. As expected, the total anthocyanin content of the buds in PB was the highest among these three materials, while the total anthocyanin content in the buds of GS is about 66% of PS (Table 2). Cyanidin and delphinidin contents in GS were 45% and 26% lesser than in PS, respectively. Malvidin content was very low even in the purple broccoli sample and similar in PS and GS. In all broccoli samples, there were no pelargonidin, peoidin and petunidin (Table 2).

Table 2 Total anthocyanins and six types of anthocyanidins contents

	Total anthocyanins C μg/g	C C μg/g	D C μg/g	Mv C μg/g	PelC μg/g	PeoC μg/g	PtC μg/g
GS	12.123±0.770	3.830±0.420	4.190±0.210	0.002±0.011	0	0	0
PS	20.712±0.378	6.980±0.280	5.690±0.090	0.002±0.012	0	0	0
PB	103.138±4.399	87.890±4.480	9.110±1.360	0.881±0.323	0	0	0

Note: Cyanidin C (CC), Delphinidin C (DC), Malvidin C (Mv C), pelargonidin (PelC), peonidin (PeoC), petunidin (Pt).

Mapping of the keeping-green trait at cold temperature (KGLT) of the buds in broccoli

HX-16 and HX-11, whose buds would keep green and turn purple in cold temperature, respectively, were chosen to construct a BC_2F_2 segregating population to map the trait (Figure 1F, G, H). Bulked segregant analysis (BSA) was used to map the target region of the trait. In the BC_2F_2 segregating population with 987 individuals, 252 and 735 plants exhibited green buds and turned purple at low temperature, respectively, which confirmed the segregation ratio of 1:3. It indicated that a single locus controls KGLT.

From the BC_2F_2 segregating population, 30 KGLT and 30 TPLT individuals were utilized to analyze the locus linked to KGLT/TPLT buds by BSA-seq after experiencing low temperature (ranging from 0-5^0C). Genomic DNA was sequenced by the Illumina NovaSeq platform, and 63.86 G of clean data were obtained (Table S1). Low N rates (0) and high scores of Q20 (96.66-98.13) and Q30 (91.06-94.51) in table S1 showed that the reads obtained were of high quality. High-quality data were produced by filtering raw data, and more than 99.34%-99.59% of the data were mapped to the reference genome HDEM (*http://www.genoscope.cns.fr/externe/plants/chromosomes.html*) (Table S2). The average sequencing depths of maternal, paternal, keeping-green, and turning-purple pools were 22.58, 22.87, 27.30, and 27.82, respectively, and the mapping coverage ranged from 86.50%-89.03% (Table S3). In the parental lines,

2334791 homozygous SNPs same as the reference genome were called (ref), and 3120872 homozygous SNPs were different from the reference genome (alt) (Table S4). Similarly, 322714 homozygous InDels, same as the reference genome and 615872 homozygous InDels, different from the reference genome were called in the parental lines (Table S5). The SNP-index distribution in the genome of the G-pool and P-pool was analyzed and shown in Figure 2A and 2B. The subtraction of the two SNP indexes produced Delta (Δ) SNP index (Figure 2C). According to the Δ SNP index value, the peak area above the 99% confidence coefficient was chosen as the candidate region associated with the target trait. Therefore, the 4.6Mb of area between 58,700,000 and 63,300,000 bp on chromosome 9 was identified as the target region for the KGLT/TPLT at cold temperature.

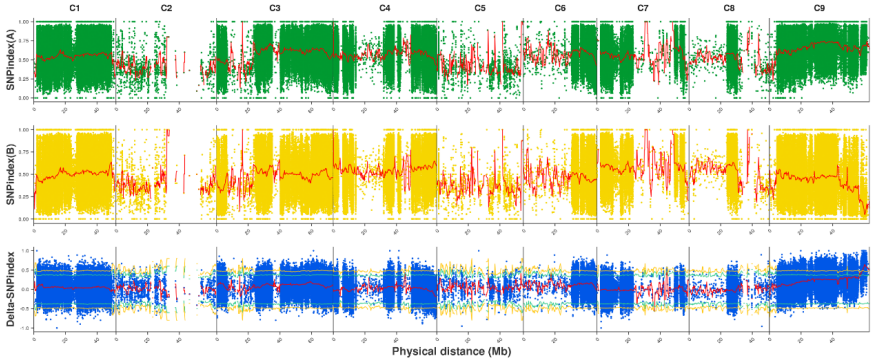

Figure 2 The SNP index distribution in the genome of buds-keeping-green pool (A) and buds-turning-purple pool (B). (C) ΔSNP index value.

Fine mapping of the target bud-keeping-green trait gene

Thirty-one sets of KASP primers were designed according to the SNP information in the target region between C9: 58,700,000 - 63,300,000 of the parent lines. Initially, the parental lines and random 20 plants out of 987 BC_2F_2 individuals were used to screen the primers, and the resultant 30 sets of KASP markers could be used for further testing of the remaining individuals of the segregating population. The 987 individual plants in the BC_2F_2 population were phenotyped and genotyped with the 4 sets of KASP primers which were at both side of the target region to find the recombined individuals, and finally, 96 individuals with recombined chromosome segment in the target region were used for fine mapping. These recombined individuals were genotyped by the left 26 sets of KASP markers. Phenotyping and genotyping (with 30 KASP markers) results of the recombined individuals are depicted in Figure 3A. The genotypic data were arranged from top to bottom according to ascending order of 30 KASP markers lined from left to right by physical location in the reference genome as described in the materials and methods. The data strongly supported the mapping result that the target area was closely associated with bud color at low temperature.

According to the genotypic and phenotypic data of the recombinants with chromosome segment exchange in the target region, the trait was fine mapped in the region of about 271 Kb between bropK17 (C9:59974705) and bropK19 (C9:60246054). In the range of C9:59974705-60246054, another 6 groups of KASP primers were developed according to the relative SNP information, and 19 individual plants with recombined chromosome segment in the above range were genotyped with these 6 KASP markers. The phenotyping and genotyping analysis of the 19 recombinants reduced the target area into 241Kb range between bropK41 (C9:59998177) and bropK43 (C9: 60239356) (Figure 3B). Then, according to the InDel information in region of C9: 59998177-60239356 of the parental lines, another new 6 sets of KASP primers were developed and used to genotype the 17 individuals with the recombined genome in this area (Figure 3C). Finally, the target gene was fine mapped to a region of about 240 kb between bropK47 and bropK43, located between C9:59999594 and 60239356 (Figure 3C).

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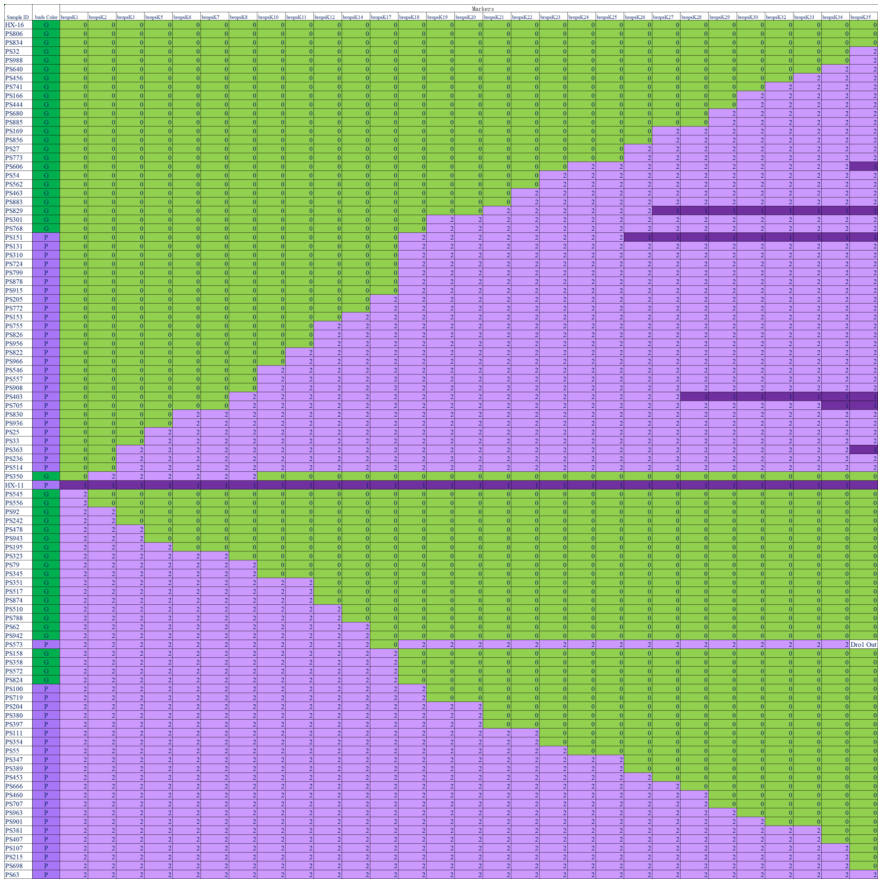

Sample	buds color	Markers						
		bropsK39	bropsK40	bropsK41	bropsK18	bropsK42	bropsK43	bropsK44
HX-16	G	0	0	0	0	0	0	0
PS301	G	0	0	0	0	0	2	2
PS748	G	0	0	0	0	0	2	2
PS131	P	0	0	0	2	2	2	2
PS151	P	0	0	0	2	2	2	2
PS310	P	0	0	0	2	2	2	2
PS573	P	0	0	0	2	2	2	2
PS617	P	0	0	0	2	2	2	2
PS724	P	0	0	0	2	2	2	2
PS799	P	0	0	0	2	2	2	2
PS878	P	0	0	0	2	2	2	2
PS915	P	0	0	0	2	2	2	2
HX-11	P	1	1	1	1	1	1	1
PS572	G	2	0	0	0	0	0	0
PS158	G	2	2	1	0	0	0	0
PS358	G	2	2	1	0	0	0	0
PS824	G	2	2	1	0	0	0	0
PS713	P	2	2	1	2	2	0	0
PS719	P	2	2	1	2	2	0	0
PS935	P	2	2	1	2	2	0	0
PS100	P	2	2	1	2	2	2	0

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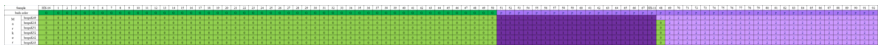
Sample	buds color	Markers								
		bropsK45	bropsK46	bropsK47	bropsK49	bropsK18	bropsK51	bropsK52	bropsK42	bropsK43
HX-16	G	0	0	0	0	0	0	0	0	0
PS131	p	0	0	0	2	2	2	2	2	2
PS151	p	0	0	0	2	2	2	2	2	2
PS310	p	0	0	0	2	2	2	2	2	2
PS573	p	0	0	0	2	2	2	2	2	2
PS617	p	0	0	0	2	2	2	2	2	2
PS724	p	0	0	0	2	2	2	2	2	2
PS799	p	0	0	0	2	2	2	2	2	2
PS878	p	0	0	0	2	2	2	2	2	2
PS915	p	0	0	0	2	2	2	2	2	2
HX-11	P	1	1	1	1	1	1	1	1	1
PS158	G	2	2	2	0	0	0	0	0	0
PS358	G	2	2	2	0	0	0	0	0	0
PS824	G	2	2	2	0	0	0	0	0	0



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`image14.emf` available at https://authorea.com/users/516720/articles/591470-a-43-bp-deletion-in-the-f3-h-gene-reducing-anthocyanins-is-responsible-for-buds-keeping-green-at-low-temperature-in-broccoli

Figure 3. Phenotyping and genotyping data of the recombined individuals in the (A-C) BC_2F_2 population, (D) F_2 segregation population derived from the mutant and the wild line and (E) germplasm materials in *Brassica oleracea* .

Note: (A) indicates phenotyping and genotyping of 96 individual plants with recombined chromosome segment in the target. (B) and (C) depict further genotyping of the recombined individuals based on (A) and B with new KASP markers, respectively. (D) and (E) show the application of the relative KASP markers. 'G' with green background and 'P' with purple background indicate buds-keeping-green trait (KGLT) and buds-turning-purple trait at low temperature (TPLT), respectively. '0' (light green background) and '1' (dark violet background) represent the homozygous genotypes same as the paternal and maternal line, respectively, and '2' with light violet background is for heterozygous genotype.

The application of the KASP markers

The fine mapping results showed 6 KASP markers (bropsK49, bropsK18, bropsK51, bropsK52, bropsK42, bropsK43) were closely associated with the target gene and, possibly any one of them could be in the target gene region. These 6 markers were used to screen 92 individuals of the F_2 segregation population derived from the mutant GS and the wild line PS. The genotyping results of 5 KASP markers (bropsK18, bropsK51, bropsK52, bropsK42 and bropsK43) were in agreement with the phenotypic data by more than 98%, while the genotyping result of bropsK49 was in 100% accordance with the phenotypic data (Figure 3D). It showed that the six KASP markers based on C9: 59999594 to 60239356 could perform well in genotyping for each plant in the new population (Figure 3D). Based on the genotypic and phenotypic data of the F_2 individuals, the range of genomic location of bud-color at low temperature was further narrowed down to 229.649Kb, located between bropK47 and bropK18, i.e., C9:59999594 ~ 60229243, and the gene where bropsK49 locates could probably be the target gene.

Also, the 6 KASP markers were used to screen forty-two germplasm materials in *Brassica oleracea* L. (Figure 3E). Out of the 6 KASP markers, the genotyping data of two KASP markers (bropsK18 and bropsK49) matched more than 95% with the phenotyping data and were effective in predicting whether the buds would turn purple or keep green in cold temperature in *Brassica oleracea*L. Within the 42 germplasm lines, 40 lines showed consistent results for both bropsK18 and bropsK49 with the genotypic data in conformity with the phenotyping, whereas 2 lines (1947 and K2145), which would keep green buds at low temperature, lack

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a 43-bp deletion in bropsK49 where there was a 43-bp InDel, and exhibited inconsistent results between genotyping and phenotyping.

Expression profile of anthocyanins relative genes in the mutant and the wild line

A total of 1729 DEGs (Differentially Expressed Genes) were detected between the green buds of GS and the purple buds of PS. The broccoli reference genome contained 53 genes in the 229.6 Kb range between 59999594 and 60229243bp of the C9 chromosome. Out of the 53 genes, only one gene, *BolC9t59639H (F3'H)* , was differentially expressed between the mutant and the wild line. The expression of*BolC9t59639H (F3'H)* in the buds of the mutant GS was one-third of the wild line PS at low temperature. Except for the *BolC9t59639H (F3'H)* , no other genes were differentially expressed between the mutant and the wild line within the target range on chromosome 9. According to KEGG database, 43 genes relative with anthocyanin biosynthesis and regulation pathway were found to express in the buds of broccoli PS and GS at low temperature. Besides *BolC9t59639H (F3'H)* , the expression level of *BolC3t18539H* (*FLS*) was different in both buds, which had almost 1.5 times in the buds of the mutant GS. The KEGG Pathway enrichment analysis showed that BolC9t59639H (F3'H, EC: 1.14.14.82) participated in the biosynthesis of cyanidins and delphinidins, while BolC3t18539H (FLS, EC: 1.14.20.6) took part in the biosynthesis of flavone and flavonol biosynthesis (Figure 4A).

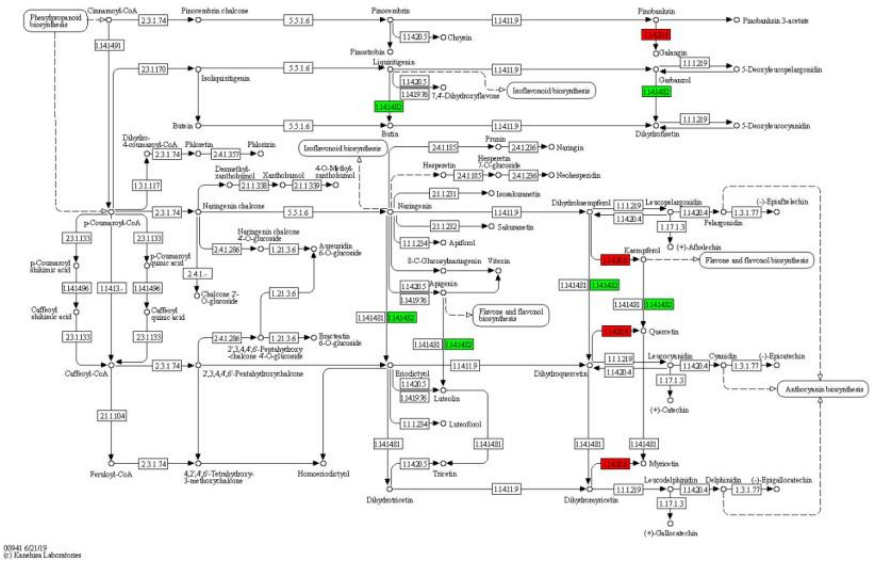

Figure 4. The KEGG pathways showing the DEGs related to flavonoid biosynthesis in the mutant and the wild line participate. The protein in the green background was down-regulated in the mutant, and the one in the red background up-regulated.

Expression profile of anthocyanins relative gene in the wild line at different temperature

43 genes relative with anthocyanin biosynthesis were found to express in the buds of broccoli PS, which was accordant with the above result. The expression of most of the anthocyanins relative genes was regulated by low temperature. Among 43 genes, the expression of 29 genes were regulated by low temperature, out of which 20 genes were up-regulated, and 9 down-regulated at low temperature (Figure 5). One of six*PALs* , four of five *C4Hs* , one *F3H* , one *F3'H* , and one of two *FLS* showed higher expression at low temperature than at room temperature, whereas both *ANS* and one of three*CHI* were down-regulated. And out of ten *4CLs* , four*4CLs* were up-regulated at low temperature, and four showed lower expression. Out of four *CHSs* , three *CHSs* expressed higher at low temperature and one lower. There were 6 regulating factors that were differentially expressed, five of which were up- regulated by low temperature and one was down-regulated

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(Table S6).

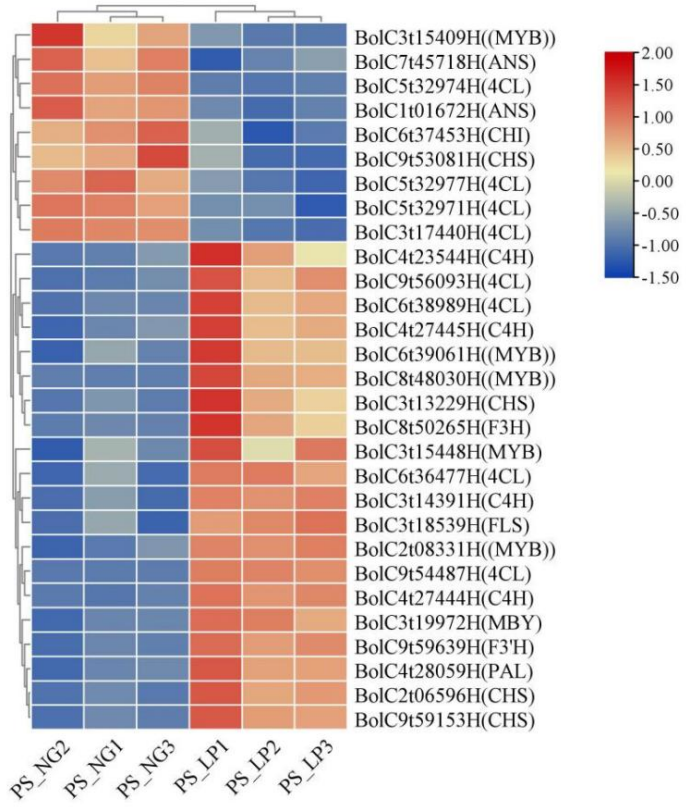

Figure 5 The relevant genes of flavonoid biosynthesis and regulation modified by low temperature in the buds of broccoli PS.

The CDS sequences and a phylogenetic analysis of F3'H

A 43-bp deletion was detected in the CDS sequence of HX-16, whose buds would keep green in cold temperature, compared with the maternal line, HX-11, whose buds turn purple in cold temperature. The CDS sequence of HX-11 had 2 SNPs compared with the reference genome HDEM. A phylogenetic analysis was conducted using the amino acid sequences of the *F3'H* gene deduced from some plants of *Brassica, Raphanus, Arabidopsis, Sinapis, Isatis, Eutrema, Camelina, Capsella and Matthiola incana* . The phylogenetic analysis showed that the *F3'H* gene with a deletion was grouped into the same clade as the *F3'H* from the reference genome HDEM, and the *F3'H* s from *Brassica, Raphanus, Arabidopsis, Camelina* and *Matthiola* were classified into a different group, indicating that the amino acid sequences of *F3'H* owned genus-specific conservation (Figure 6).

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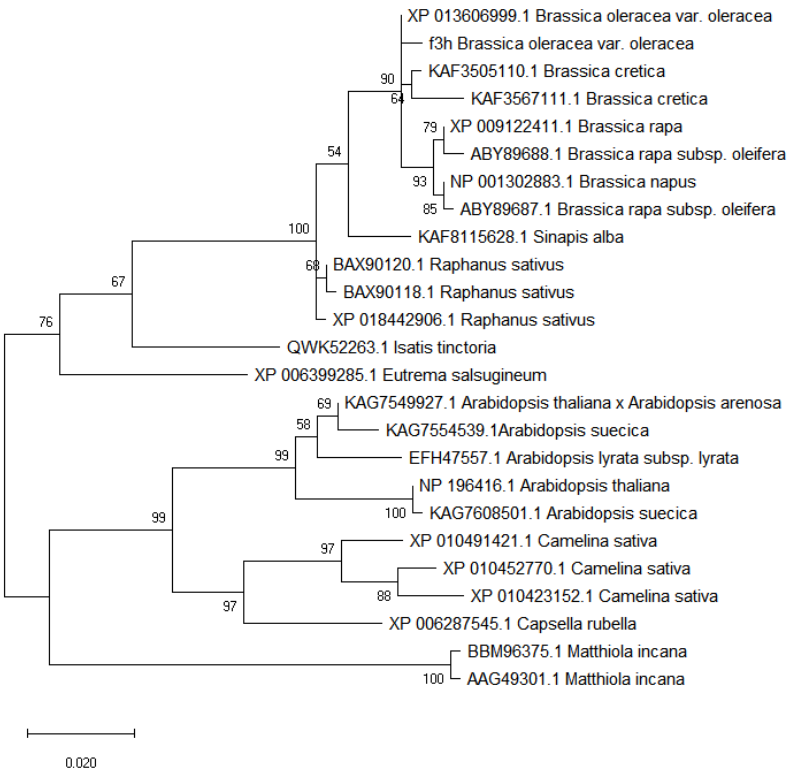

Figure 6. The phylogenetic tree according to the amino acid sequences of genes encoding flavonoid 3'-hydroxylase in Cruciferae. The phylogenetic analysis was conducted by the Maximum Likelihood method with 500 numbers of bootstrap replications in MEGA X.

Discussion:

Genotyping in the BC_2F_2 segregating population with 987 individuals with the marker bropsK18 was perfectly consistent with the phenotyping data. However, genotyping in the F_2 segregating population derived from PS and GS with the marker bropsK18 was not completely consistent with the phenotyping. On the contrary, phenotyping concurred entirely with the genotyping of marker bropsK49, whose primers were designed according to the DNA sequences of the *BolC9t59639H* (*F3'H*) gene on the target region between HX-11 and HX-16, where a 43-bp deletion in the second exon of *BolC9t59639H* has occurred. These results showed that the 43-bp deletion in *BolC9t59639H* (*F3'H*) should be responsible for the buds keeping-green at low temperature (KGLT) in HX-16, the mutant GS and most broccoli materials with this trait.

The core structures of anthocyanins have only six types, cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Saigo *et al.* , 2020). In the biosynthetic pathway of anthocyanins, the substrate specificity of some key enzymes and the competition among them on the branch nodes results in the genus- and -species specificity of anthocyanins (Saigo *et al* ., 2020). In this study, no pelargonidin, peonidin or petunidin was detected, and only cyanidin, delphinidin and malvidin were detected in three broccoli materials. Cyanidin was absolutely dominant in the purple broccoli, had much the same content as delphinidin in PS, and had less content than delphinidin in GS. The result was almost consistent with the previous study (Moreno *et al* ., 2010) and demonstrated that species characteristics of anthocyanins were prominent in broccoli. It appeared that the vivid purple color of buds was due to the accumulation of cyanidins in purple broccoli, and low temperature mainly promoted the accumulation of cyanidins in PS, as the cyanidin content

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was decreased the most in GS compared to PS. In*Arabidopsis* , many genes associated with anthocyanins biosynthesis were identified by corresponding mutants whose seed coat has*transparent testa* (*tt*) due to the lack of a particular step of anthocyanin biosynthesis (Li *et al* ., 2017). Arabidopsis*tt7* mutant, lacking a flavonoid 3'-hydroxylase lacks anthocyanins accumulation. Transgenic Arabidopsis *tt7* seedlings expressing apple *MdF3'H* regained red color pigmentation in seed coats, and the seedlings increased the accumulation of both pelargonidin and cyanidin under nitrogen-deficient conditions (Han *et al.* , 2010). This illuminates that *MdF3'H* affect the accumulation of pelargonidin and cyanidin in *Arabidopsis* . According to the biosynthetic pathway of anthocyanins, *F3'H* , which encodes flavonoid 3'-hydroylase that catalyzes the conversion of kaempferol into quercetin and dihydrokaempferol into dihydroquercetin, is the key gene for cyanidins and delphinidins biosynthesis (Seitz *et al.* , 2007; Han *et al.* , 2010; ko00941 in https://www.kegg.jp). Therefore, in this study, KGLT in GS and HX-16 could result from the 43-bp deletion in the *F3'H* CDS, decreasing the accumulation of cyanidins and delphinidins.

In the mutant GS and wild line PS, there were only two genes relative to anthocyanins biosynthesis, which expressed differently in the buds; *F3'H* expressing lower and *FLS* expressing higher in the mutant. Although *F3'H* and*FLS* are involved in the biosynthesis pathway of anthocyanin, flavone and flavonol, respectively, a part of the substrates that F3'H catalyzes, i.e., dihydroflavonol is also the substrate for *FLS*(Wang *et al* ., 2021; Guo *et al* ., 2019). *FLS* could catalyze dihydroflavonol to flavonol, while *F3'H* could hydroxylate the 3'-position of B-ring in flavanone, flavonol and dihydroflavonol (Guo *et al* ., 2019; Jia *et al* ., 2019). Therefore, *FLS* could express higher if *F3'H* expresses lower due to the competitive relationship of the substrate. In the mutant, the expression profile of *F3'H* and *FLS* should result from their competitive relationship for the same substrate.

The accumulation of anthocyanins is affected by ambient temperature in plants, and usually, low temperature can stimulate the expression of the relevant genes. Low-temperature treatment would stimulate the gene expression of anthocyanin biosynthesis, such as *PAL, C4H, 4CL, CHS, CHI, F3H, F3'H, DFR, ANS* and *UGT75C1* , and regulating factors *MYB* (Dai *et al* ., 2022). In this study, cold temperature also could promote the expression levels of *PAL, C4H, FLS, F3H, F3'H,* some of *4CL, CHS* and *MYB* but would reduce the expression of *ANS, CHI,* and some of *4CL, CHS* and*MBY* . The expression of *ANS* reduces at low temperature, which seems contradictory to the pigment accumulation because *ANS*is an important enzyme that can catalyze the production of anthocyanins monomers from leucoanthocyanidins (Saito *et al* ., 1999; Wang*et al* ., 2021). However, *ANS* not only catalyzes the synthesis of anthocyanins, but also has the same activity as *FL* S (Turnbull *et al* ., 2000; Wellmann *et al* ., 2006; Wilmouth*et al* ., 2002). Meanwhile, in *Arabidopsis, AtFLS1*and/or *AtANS* would have partial activity of the *F3H* enzyme (Owens *et al.* , 2008). Additionally, *FLS* could partially complement *ANS* in Arabidopsis *tt6* mutants (Owens *et al.* , 2008). *FLS*and *ANS* shared highly similar polypeptides, and both could react with the leucoanthocyanidins (Turnbull *et al* ., 2004).

Consequently, due to the species-specificity and complexity of anthocyanins, low temperature primarily induces the accumulation of anthocyanins in most plants (Li *et al* ., 2017; Ubi *et al* ., 2006; Jiang *et al* .,2022; Zhang *et al* ., 2012), while in some plants, it would reduce the accumulation of anthocyanins (Zhu*et al.* , 2020; Mao *et al* ., 2022). Hence, although the biosynthesis pathway of anthocyanins in *Arabidopsis* has been well explained, the specificity of anthocyanins biosynthesis and regulation in broccoli is yet to be discovered. In the present investigation, the 43-bp deletion in the *F3'H* CDS decreased the expression of the gene and ultimately reduced the accumulation of cyanidin and delphinidins in some broccoli cultivars and accessions, which would keep green buds at cold temperature. However, the application accuracy of the marker bropsK49 in the germplasm accessions of *Brassica* was 96 percent. This showed that besides the 43-bp deletion in F3'H, there might be different mutations occurring in F3'H or other relative factors affecting the accumulation of anthocyanins in Brassica at low temperature. This has been illustrated by other reports, where a 68-bp deletion in the DNA sequences of F3'H and a 1-bp insert in the exon of DFR are responsible for green head and green leaves in broccoli and kale, respectively (Liu *et al.* , 2021; Tang *et al.* , 2017).

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Conflicts of Interest: The authors declare no conflict of interest.

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