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Full Length Article

Molecular Characterization of *BrMYB73*: A Candidate Gene for the Purple-Leaf Trait in *Brassica rapa*

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Abstract

We used SNP and InDel markers to construct a genetic linkage map from a segregating BC_1 population in order to study the genetics of anthocyanin accumulation in *Brassica rapa*. A dominant locus, *Bra-Pur*, for the purple-leaf trait was mapped within a 833 kb region on one end of chromosome A03. *BrCHI3*, *BrMYB73* and *BrLBD39.2*, are three potential anthocyanin biosynthesis candidate genes. We found that the expression of *BrMYB73* was significantly increased in the leaves and flowers and was induced by cold stress. Sequence analysis of *BrMYB73* identifed one SNP and one single-nucleotide deletion in the coding region that caused a deletion of 75 amino acids in the C-terminus of the protein in the purple-leaved parent but not in the green-leaved parent. We used the single-nucleotide deletion in the *BrMYB73* allele from the purple-leaved parent to develop a kompetitive allele-specific polymerase chain reaction (KASP) marker for this gene. The KASP marker was co-dominant, and it also co-segregated with the purple trait in an additional F_2 population. These results suggest that *BrMYB73* is the most promising candidate gene involved in anthocyanin accumulation and the information obtained here will have a positive impact on the process of molecular breeding for improved nutritional quality in Chinese cabbage. © 2019 Friends Science Publishers

Keywords: *Brassica rapa*; Purple trait; Genetic linkage mapping; Anthocyanin biosynthesis

Introduction

Anthocyanins are a diverse subclass of flavonoids that confer red, blue and purple colors to the fruits, flowers, leaves and other organs of higher plants. Anthocyanins are water soluble pigments that play important ecological roles in plant reproduction and dispersal by attracting insects, birds and other animals that pollinate the flowers and eat the fruits. In addition, anthocyanins provide protection against damage from UV radiation, act as scavengers of reactive oxygen species, and also protect against some forms of cancer and disease in human health (Harborne and Williams, 1992; Kong *et al.*, 2003; Schijlen *et al.*, 2004; Jana *et al.*, 2017; Tong *et al.*, 2017). Anthocyanin biosynthesis involves two classes of genes; those that encode the catalytic enzymes in the anthocyanin pathway, and regulatory genes that encode transcription factor (TF) proteins that regulate transcription of the structural genes through protein complexes such as the MBW complex, which consists of MYB and bHLH TFs associated with a WD40 repeat (Broun, 2005). In addition, it is well known that some environmental conditions can affect anthocyanin biosynthesis in some species; for example, anthocyanin production is elevated upon exposure to low temperatures in grape, maize, red orange and apple (Christie *et al.*, 1994; Mori *et al.*, 2005; Piero *et al.*, 2005; Ubi *et al.*, 2006).

Chinese cabbage (*Brassica. rapa* L. ssp. *pekinensis*) varieties with purple leaves are mainly derived from purple pakchoi, *B. campestris* var. *purpuraria*, and turnip. Early studies mapped several genes associated with anthocyanin accumulation in purple *Brassica* varieties, such as the dominant *Anp* locus on linkage group A07 in the purple turnip *B. rapa* cv. 'Iyo-hikabu' (Hayashi *et al.*, 2010), the *anl* locus on linkage group A09 in *B. campestris var. purpuraria* (Burdzinski and Wendell, 2007), the *BjPl1* locus on linkage group B2 of *B. juncea* (Zhao *et al.*, 2017), the *BoPr* locus on chromosome C09 in *B. oleracea L. var. acephala*. (Liu *et al.*, 2017) and the *Anm* locus on chromosome A02 in *B.rapa* (Zhang, 2014). Liu *et al.* (2013) and Wang *et al.* (2014) mapped the *BrPur* locus to one end of linkage group A03 in segregating F_2 and BC_1 populations derived from crossing purple pakchoi inbred line 09N-742 with Chinese cabbage inbred 09-680 (green leaves). Guo *et al.* (2014) mapped the purple trait in a purple pakchoi X 'Caixin' F_2 population to a 1.5-Mb DNA region, also located on the distal end of chromosome A03 using a high throughput DNA sequencing method. The results of previous studies show that the candidate

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genes that control anthocyanin accumulation in the different purple-leaved varieties of *B. rapa* are not identical, which suggests that genetic mechanisms controlling anthocyanin biosynthesis are intricate.

In this study, we mapped a purple trait locus to an 833 kb region on chromosome A03. Three candidate genes, *BrCHI3*, *BrMYB73* and *BrLBD39.2* are expected to control anthocyanin accumulation. *BrCHI3* is a homolog of *AtCHI*, an Arabidopsis gene that encodes chalcone isomerase, an enzyme that catalyzes the isomerization of chalcone to naringenin, a key reaction in the biosynthesis of flavonoids. *BrMYB73* is a homolog of *AtMYB73* that encodes a transcription factor in the R2R3-MYB family. *BrLBD39.2* is a homolog of *AtLBD39*, which is one of three related *LBD* genes that encode proteins that function to negatively regulate anthocyanin biosynthesis in arabidopsis. We characterized the *BrCHI3*, *BrMYB73*, and *BrLBD39.2* genes by sequencing and expression analysis to determine whether they are involved in anthocyanin biosynthesis in *B. rapa*.

Materials and Methods

Plant Material

The spring Chinese cabbage inbred cutivar 'Chunyuehuang' (CYH: green leaf) (Fig. 1a) was the female parental line, and an isogenic line 'ZiChunyuehuang' (ZCYH: dark purple leaf) (Fig. 1b) was the male parent in an F_1 hybrid that was used to construct a BC_1 population by backcrossing to CYH for one generation. The BC_1 population consisted of 901 plants. The line 'ZCYH' is derived from the purple-leaved pakchoi inbred line 'Te3X10010' and is a novel source of the purple-leaf locus in breeding. KASP markers developed for mapping the *Bra-Pur* locus were tested on another F_2 population (560 individuals) constructed from a cross of the pakchoi lines Te3X10010 (the *Bra-Pur* donor) and 'Changtongbai'. We scored leaf color in all F_2 progeny visually at the 3-leaf stage. The leaves were sampled from 3-week-old seedlings of the parental inbreds and the segregating population were then freeze-dried immediately after harvest for future DNA isolation.

Cold Stress Treatments

Seeds of CYH and ZCYH were sown in a mixture of consisting of 1 part soil and 2 parts peat and cultivated under long-day conditions (16 h light, 8 h dark) at 25°C/20°C with a photon flux density of 140 μmol m^{-2} s^{-1} in the greenhouse. The seedings were moved to 4°C when they had reached the 4-leaf stage and were kept there for 72 h. Leaf tissues were collected into liquid nitrogen after 0, 12, 24, 36, 48 and 72 h of cold treatment. RNA was then extracted for gene expression analysis.

PH Differential Spectrophotometry

Total anthocyanin content (TAC) was determined in the parental lines using pH differential spectrophotometry (Lee *et al.*, 2005). Leaf tissues were collected after 0, 36 and 72 h of cold treatment. Measurements of TAC were replicated three times in all samples.

Molecular Marker Analysis

Genomic DNA extracted from the parental lines was re-sequenced by BioMarker Technologies Co. (Beijing, China) and the results were compared with *B. rapa* reference genome sequence downloaded from BRAD (the *Brassica* database; http://brassicadb.org/brad/). Genome-wide differences between the parental line sequences were used to develop InDel and SNP markers. The InDel markers used here were designed as described by Liu *et al.* (2013). The PCR amplifications contained 2 μL PCR buffer, 0.8 μL of each dNTP (2.5 m*M*), 1.0 μL of the forward and reverse primers (10 μM), 0.5 U Taq DNA polymerase and 100 ng DNA template (2 μL) in a final volume of 20 μL. Amplification reactions were performed in an automated thermocycler (model PTC-200; Bio-Rad Laboratories, Hercules, CA) programed for an initial DNA denaturation a 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 5 min. The amplified DNA fragments were visualized by electrophoresis on 3% agarose gels.

SNP marker primers were designed and synthesized in the UK by LGC Ltd. for genotyping using the Kompetitive Allele Specific PCR (KASP) method as described as Su *et al.* (2018). KASPar assays were performed in 1536-well plates (KBioscience; catalog number KBS-0751-001), reaction volumes were 1 μL and each reaction contained 1X KASP reaction mix (KBioscience; catalog number KBS-1016-011), 4 ng of genomic DNA, the allele-specific forward primer at a concentration of 12 nM and reverse primer at 30 nM.

Linkage Analysis and Map Construction

We constructed a genetic linkage map using JoinMap 4.0 software with a minimum LOD (logarithm of the odds) score of 4.0 (VanOoijen, 2006). The recombination values were converted into genetic linkage distances (in cM) using the Kosambi mapping function (Kosambi, 1943).

RNA Extraction

We used the plant RNeasy kit (TIANGEN, China) to isolate total RNA from four tissues: roots, stems, leaves and flowers. First-strand cDNA was synthesized from the RNA samples by reverse transcriptase using a PrimeScript™RT reagent Kit (TaKaRa, Japan).

Real-time PCR Analysis

Real-time PCR (RT-PCR) assays were performed using the SYBR Green I Master Mix and were quantified with the Light Cycler 480 II (Roche, USA). RT-PCR amplification conditions were: an initial denaturation step of 95°C for 3 min, 40 cycles of 95°C for 15s (denaturation) and 60°C for 30 s (annealing), with a final extension step of 72°C for 45 s. Amplification was followed by heating for 1 min at 60–95°C for melting curve analysis. Each reaction was performed in a volume of 10 μL with three replicates and contained 5 μL Master Mix, 0.25 μM of each primer, 1 μL diluted cDNA template, and DNase-free water. The amplified DNA fragments were sent to a DNA sequencing company to confirm the identities of the gene-specific amplifications. The primers used for RT-PCR were designed using Primer 3 (http://frodo.wi.mitedu/primer3/).

Gene Cloning and DNA Sequence Analysis

The gene-specific primers used to amplify *BrCHI3*, *BrMYB73* and *BrLBD39.2* were designed using reference sequences from the *Brassica* database (http://brassicadb.org/brad/index.php). We then cloned the PCR products were into the pCR™8/GM/TOPO entry vector (Invitrogen, USA) and sequenced the inserts. Sequence similarities were calculated with DNAMAN software (ver. 5.2.2).

Results

Genetic Mapping of *Bra-Pur*

To locate the *Bra-Pur* locus involved in anthocyanin accumulation in leaves of *B. rapa*, 25 markers that anchor 10 *B. rapa* chromesomes and that are polymorphic between the two parental lines were used to screen the green and purple bulks (consisting of 20 individuals each) by bulked segregant analysis. A single Indel marker, BrID10399, was determined to be linked to *Bra-Pur* and it mapped to a locus at the end of linkage group A03.

In addition, we developed 50 new Indel markers on A03 in an effort to find additional marker loci tightly linked to *Bra-Pur*. Six of these Indel markers (Table 1) detected polymorphisms between the two parental lines 'CYH' and 'ZCYH' and were then used to screen the BC_1 individuals to identify recombinants. The *Bra-Pur* locus was found to be located between marker loci Indel-5 and Indel-6. We then developed 40 SNP markers that mapped to loci in the region between the Indel-5 and Indel-6 loci, but only one marker, SNP1, detected recombinants in the BC_1 mapping population of 901 individuals. Finally, we mapped *Bra-Pur* to a region between the Indel-5 and SNP-1 marker loci on chromosome A03 (Fig. 2b). Moreover, we determined the sequences of the seven markers that are closely linked to *Bra-Pur*, and found that the marker order was consistent with that of their homologs on *B. rapa* ssp. *pekinensis* cv. 'Chiifu-401-42' linkage group 3 (Fig. 2a).

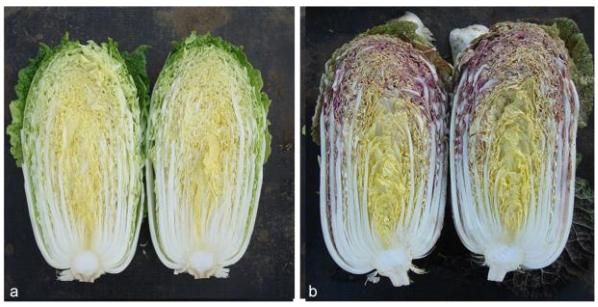

Fig. 1: Two parental lines of Chinese cabbage. 'Chunyuehuang' (CYH) (a) and 'ZiChunyuehuang' (ZCYH) (b)

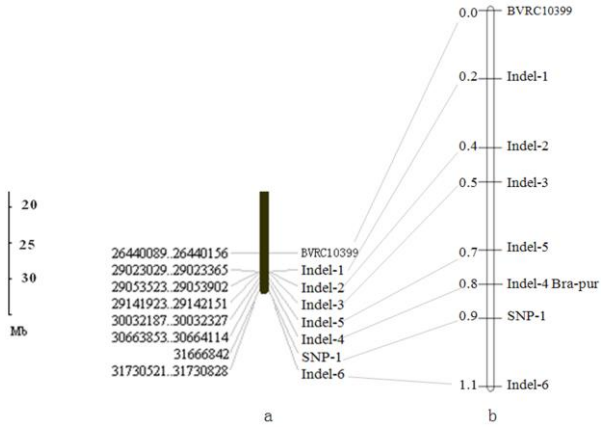

Fig. 2: Physical mapping of the *Bra-Pur* gene. Physical distances are shown on the left, and the marker locus names are indicated to the right of the figure (a). Genetic linkage map showing the *Bra-Pur* locus. Marker locus names are shown to the right, and recombination distances (in cM) to the left on linkage group (b)

Candidate Gene Identification

The physical distance between the Indel-5 and SNP-1 loci was 833 Kb (Fig. 2a). We found that 117 annotated genes were predicted to be present in this region (Table 2). We analyzed 117 predicted genes to identify any gene that could be involved in anthocyanin biosynthesis. We found that three candidate genes, *BrCHI3*, *BrMYB73*, and *BrLBD39.2*, which are syntenic orthologs of *AtCHI*, *AtMYB73* and *AtLBD39*, are located within this region (Table 3).

BrCHI3, *BrLBD39.2* and *BrMYB3* were cloned from the two lines CYH and ZCYH and sequenced. We then compared the nucleotide sequences of these three genes to reveal sequence variations between the parental lines. For *BrCHI3*, we found one single-nucleotide nonsynonymous mutation that led to a predicted amino acid substitution (Trp75Gly) in which a lysine is replaced by arginine in the ZCYH allele (Fig. 3a). No sequence variations were detected in the coding regions

Table 1: Oligonucleotide primers used for mapping the *Bra-Pur* locus and the cloning of candidate genes

Primer name	Primer sequence (5'-3')	Marker type
BrID10399	GTGCATCAGTGAGGGTATCT ACACAGACGTGGTTAGTGTG	InDel
Indel-1	TTATTTGGATCGGGTCTGG GTGATTAGTAGTTCGGTCTC	InDel
Indel-2	CAATGCAGCACTGAAATACG CTGAAACGAACCAAACCCT	InDel
Indel-3	ATTACCTCCTGAAGAACCT AAATCTCATCTACCGTGTC	InDel
Indel-4	ATTTACTTCCAGTGCCCTTTC AAATCAAGCCGTGGACCTA	InDel
Indel-5	TACTGGCTTGCTGCTGATT TATCGTGCCTTTACCTTTCC	InDel
Indel-6	TGCAAAGGCTGATAGGTGT CATCAATGGAGCAAGAAAGT	InDel
SNP-1	GAAGGTGACCAAGTTCATGCTCTGCAAAGC TCAAAGGGAAGAATG GAAGGTCGGAGTCAACGGATTCTGCAAAG CTCAAAGGGAAGAATA ACCTTGGCATGGTAAATATGGAAGC	SNP
Br-M-SNP	GAAGGTGACCAAGTTCATGCTGAGGAAAG AGTGGCCCTTATCG GAAGGTCGGAGTCAACGGATTGAGGAAAG AGTGGCCCTTATCC CTATGCCAATAGCTGCCACCAATGT	SNP
BrCHI3	ATGTTTTCTTCCGGCTCTCAG TCAAGAACTGGCCTCTGTCAAC	gene
BrMYB73	ATGTCAGGTCCGTCCCGAAA CTACTCCATCTTCCCGATTTGG	gene
BrLBD39.2	ATGAGTTGCAATGGATGTAGAG TTAAACAAAAAGGTTTAACAACTTTCTCTC	gene

(exons) of the *BrLBD39.2* gene between the two parental lines (Fig. 3b). The sequence variations present in *BrMYB73* between the two parental lines included one SNP and one single-nucleotide deletion located in the coding sequences; neither caused amino acid substitutions in the R2 or R3 domains, but lead to a 75 amino acid deletion at the carboxy-terminal end (C-ter) of the ZCYH allele (Fig. 3c).

Expression Analysis of Candidate Genes

To investigate the relationship between the candidate genes and pigment accumulation in the purple cultivar, We used qRT-PCR to quantify the expression of anthocyanin biosynthesis structural and regulatory genes. Transcription of *BrCHI3*, *BrMYB73*, and *BrLBD39.2* in different organs of the parental inbreds 'ZCYH' (purple) and 'CYH' (green) is shown in Fig. 4. We found that the relative expression of *BrMYB73* was significantly lower (P = 0.05) in the roots and stems and significantly higher (P = 0.05) in the leaves and flowers of the purple cultivar compared to the green cultivar, indicating that *BrMYB73* may be responsible for the leaf coloration in ZCYH. *BrCHI3* and *BrLBD39.2* specific mRNA accumulated to roughly equal amounts in the purple and green parental lines in the roots, stems, leaves, and floral organs.

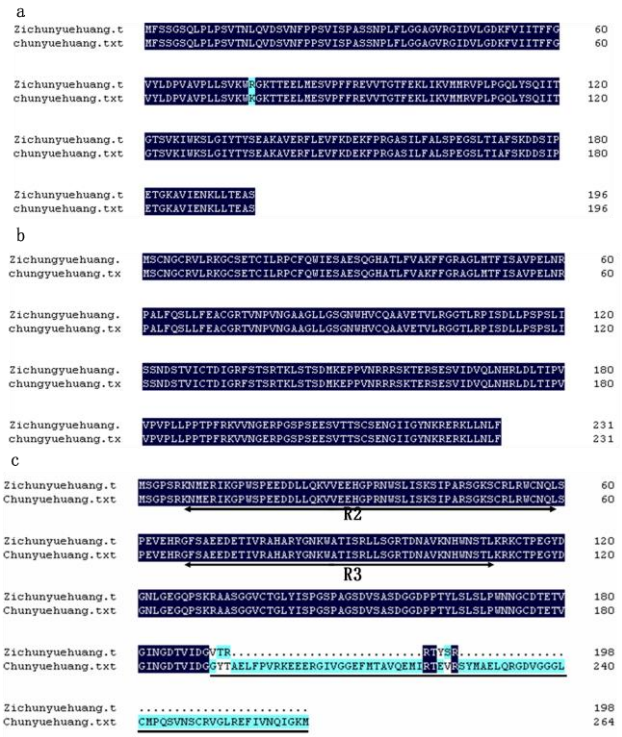

Fig. 3: Alignments of protein sequences predicted for *B. rapa* genes *BrCHI3*, *BrLBD39.2*, and *BrMYB73*. The single amino acid substitution in *BrCHI3*, in which lysine (K) in the CYH allele is replaced by arginine (R) in the ZCYH allele (a). No amino acid differences in the predicted BrLBD39.2 protein sequence were detected between the two parental lines (b). The predicted deletion of 75 amino acids in the C-terminus of the BrMYB73 protein is shown underlined. The predicted R2 and R3 domains are indicated by double-ended arrows underneath the sequence (c)

After exposing seedings at 4-leaf stage to 4°C for 72 h, the purple color of the leaves was intensified (Fig. 5a). The total anthocyanin content of the parental line ZCYH increased significantly under low temperature stress, but that of CYH showed no change (Fig. 5b). To further evaluate the effects of exposure to cold temperature on anthocyanin accumulation in *B. rapa*, we examined expression of *BrCHI3*, *BrMYB73* and *BrLBD39.2* in response to cold treatment over time from 0 to 72 h. Compared with expression at 0 h, the relative expression of *BrMYB73* increased significantly at 24, 36, 48 and 72 h of cold stress in the purple-leaved line (ZCYH). But in the green-leaved line (CYH), *BrMYB73* showed no significant changes in expression between each time period (Fig. 5c). The expression of both *BrCHI3* and *BrLBD39.2* decreased significantly throughout the treatment period in both parental lines (Fig. 5c). Thus, anthocyanin accumulation showed a correlation with the expression profile of only one gene, *BrMYB73*, in seedlings of the two parental lines, indicating a strong relationship between the *BrMYB73* gene and the response to cold stress.

Table 2: The 117 annotated genes in the 833 Kb region on chromosome A03

B. rapa gene	Chromosome	Physical position	*A. thaliana* gene	*A. thaliana* annotations
Bra017720	A03	30031149-30032217	AT4G35550	HB-4, WOX13, ATWOX13; WOX13 (WUSCHEL-RELATED HOMEOBOX 13); DNA binding/transcription factor
Bra017721	A03	30041083-30041385	AT1G65580	FRA3; FRA3 (FRAGILE FIBER3); inositol or phosphatidylinositol phosphatase
Bra017722	A03	30044508-30049934	AT4G35560	FUNCTIONS IN: molecular_function unknown; LOCATED IN: CUL4 RING ubiquitin ligase complex
Bra017723	A03	30050450-30052285	AT2G17560	HMGB4, NFD4, NFD04; HMGB4 (HIGH MOBILITY GROUP B 4); DNA binding / chromatin binding / structural constituent of chromatin / transcription factor
Bra017724	A03	30058308-30060574	AT4G35600	CONNEXIN 32; CONNEXIN 32; ATP binding / kinase/ protein kinase/ protein serine/threonine kinase
Bra017725	A03	30061324-30063511	AT4G35600	CONNEXIN 32; CONNEXIN 32; ATP binding / kinase/ protein kinase/ protein serine/threonine kinase
Bra017726	A03	30079433-30081433	AT4G35620	CYCB2;2; CYCB2;2 (Cyclin B2;2); cyclin-dependent protein kinase regulator
Bra017727	A03	30086331-30087176	AT4G35660	unknown protein
Bra017728	A03	30108206-30109397	AT3G55120	TT5, A11, CFI; TT5 (TRANSPARENT TESTA 5); chalcone isomerase
Bra017729	A03	30111826-30114904	AT4G35720	unknown protein
Bra017730	A03	30130817-30134389	AT4G35790	ATPLDDELTA, PLDDELTA; ATPLDDELTA; phospholipase D
Bra017731	A03	30138161-30139312	AT4G32700	DNA-directed DNA polymerase family protein
Bra017732	A03	30154182-30155101	AT4G35840	zinc finger (C3HC4-type RING finger) family protein
Bra017733	A03	30155767-30156940	AT4G35860	ATRABB1B, ATGB2, ATRAB2C; ATGB2 (GTP-BINDING 2); GTP binding
Bra017734	A03	30159853-30162513	AT4G35890	La domain-containing protein
Bra017735	A03	30168716-30169744	AT4G35900	FD, FD-1, atbzip14; FD; DNA binding / protein binding / transcription activator/ transcription factor
Bra017736	A03	30170993-30171785	AT4G35905	unknown protein
Bra017737	A03	30172476-30174843	AT4G35920	MCA1; MCA1 (mid1-complementing activity 1)
Bra017738	A03	30178527-30179743	AT4G35930	Cyclin-like F-box (InterPro:IPR001810); BEST Arabidopsis thaliana protein match is: F-box family protein (TAIR:AT1G61340.1)
Bra017739	A03	30180652-30182101	AT4G35940	unknown protein
Bra017740	A03	30182514-30183623	AT2G17800	ARAC1, ATGP2, ATRAC1, ROP3, ATROP3; ARAC1; GTP binding
Bra017741	A03	30189932-30192402	AT4G35985	senescence/dehydration-associated protein-related
Bra017742	A03	30194324-30195115	AT4G36020	CSDP1; CSDP1 (cold shock domain protein 1); RNA binding / double-stranded DNA binding / nucleic acid binding / single-stranded DNA binding
Bra017743	A03	30200242-30202197	AT4G36030	ARO3; ARO3 (ARMADILLO REPEAT ONLY 3); binding
Bra017744	A03	30219214-30219699	AT4G36040	DNAJ heat shock N-terminal domain-containing protein (J11)
Bra017745	A03	30222753-30223849	AT4G36060	basic helix-loop-helix (bHLH) family protein
Bra017746	A03	30224277-30226826	AT4G36070	CPK18; CPK18; ATP binding / calcium ion binding / calmodulin-dependent protein kinase/ protein kinase/ protein serine/threonine kinase
Bra017747	A03	30231005-30231304	AT4G36110	auxin-responsive protein, putative
Bra017748	A03	30237245-30238021	AT5G41640	unknown protein
Bra017749	A03	30238501-30239804	AT4G36130	60S ribosomal protein L8 (RPL8C)
Bra017750	A03	30246033-30247965	AT4G36160	ANAC076, VND2; ANAC076 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 76); transcription factor
Bra017751	A03	30254416-30257187	AT4G36195	serine carboxypeptidase S28 family protein
Bra017752	A03	30270972-30273006	AT4G36220	FAH1, CYP84A1; FAH1 (FERULIC ACID 5-HYDROXYLASE 1); ferulate 5-hydroxylase/ monooxygenase
Bra017753	A03	30292563-30294896	AT4G36250	ALDH3F1; ALDH3F1 (Aldehyde Dehydrogenase 3F1); 3-chloroallyl aldehyde dehydrogenase/ aldehyde dehydrogenase (NAD)
Bra017754	A03	30298230-30299569	AT4G36260	STY2, SRS2; STY2 (STYLISH 2); transcription factor
Bra017755	A03	30302749-30311600	AT1G56360	PAP6, ATPAP6; PAP6 (PURPLE ACID PHOSPHATASE 6); acid phosphatase/protein serine/threonine phosphatase
Bra017756	A03	30315036-30323697	AT4G36360	BGAL3; BGAL3 (beta-galactosidase 3); beta-galactosidase/ catalytic/ cation binding / sugar binding
Bra017757	A03	30331821-30336096	AT4G36380	ROT3; ROT3 (ROTUNDIFOLIA 3); oxidoreductase, acting on paired donors, with incorporation or reduction of molecular oxygen, NADH or NADPH as one donor, and incorporation of one atom of oxygen / oxygen binding / steroid hydroxylase
Bra017758	A03	30341536-30344470	AT4G36400	FAD linked oxidase family protein
Bra017759	A03	30345285-30346147	AT4G36410	UBC17; UBC17 (UBIQUITIN-CONJUGATING ENZYME 17); small conjugating protein ligase/ ubiquitin-protein ligase
Bra017760	A03	30347202-30347717	AT4G36420	ribosomal protein L12 family protein
Bra017761	A03	30348508-30349789	AT4G36430	peroxidase, putative
Bra017762	A03	30351713-30354515	AT4G36480	ATLCB1, LCB1, EMB2779, FBR11; ATLCB1 (LONG-CHAIN BASE1); protein binding / serine C-palmitoyltransferase
Bra017763	A03	30355458-30357091	AT4G36470	S-adenosyl-L-methionine:carboxyl methyltransferase family protein
Bra017764	A03	30357511-30357879	AT4G36500	unknown protein
Bra017765	A03	30366233-30367530	AT4G36540	BEE2; BEE2 (BR Enhanced Expression 2); DNA binding / transcription factor
Bra017766	A03	30372137-30372819	AT4G36620	zinc finger (GATA type) family protein
Bra017767	A03	30381981-30386176	AT4G36630	EMB2754; EMB2754 (EMBRYO DEFECTIVE 2754); binding / small GTPase regulator
Bra017768	A03	30386951-30388159	AT4G36640	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein
Bra017769	A03	30392382-30394232	AT4G36650	ATPBRP; ATPBRP (PLANT-SPECIFIC TFIIB-RELATED PROTEIN); RNA polymerase II transcription factor/ rDNA binding
Bra017770	A03	30394468-30395537	AT4G36660	unknown protein
Bra017771	A03	30395749-30397447	AT4G36670	mannitol transporter, putative
Bra017772	A03	30403843-30405484	AT4G36700	cupin family protein
Bra017773	A03	30405897-30409478	AT4G23160	protein kinase family protein
Bra017774	A03	30411882-30412715	AT3G48010	ATCNGC16, CNGC16; ATCNGC16; calmodulin binding / cyclic nucleotide binding / ion channel
Bra017775	A03	30427930-30429991	AT4G36710	transcription factor
Bra017776	A03	30431706-30432133	AT1G69980	unknown protein
Bra017777	A03	30432703-30433032	AT1G59590	ZCF37; ZCF37
Bra017778	A03	30442558-30448058	AT2G16920	UBC23, PFU2; UBC23 (UBIQUITIN-CONJUGATING ENZYME 23); small conjugating protein ligase/ ubiquitin-protein ligase
Bra017779	A03	30454177-30455379	AT4G35120	kelch repeat-containing F-box family protein
Bra017780	A03	30470161-30471721	AT4G36740	HB-5, ATHB40; ATHB40 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 40); DNA binding / transcription factor

Table 2: Continued

Table 2: Continued

Bra017781	A03	30490394-30491688	AT4G36750	quinone reductase family protein
Bra017782	A03	30492719-30494026	AT4G36780	transcription regulator
Bra017783	A03	30494738-30495056	AT4G11810	SPX (SYG1/Pho81/XPR1) domain-containing protein
Bra017784	A03	30503619-30504517	AT4G36800	RCE1; RCE1 (RUB1 CONJUGATING ENZYME 1); NEDD8 ligase/ small conjugating protein ligase
Bra017785	A03	30508053-30508361	AT4G36810	GGPS1; GGPS1 (GERANYLGERANYL PYROPHOSPHATE SYNTHASE 1); farnesyltranstransferase
Bra017786	A03	30512369-30513831	AT4G36820	unknown protein
Bra017787	A03	30515168-30517778	AT4G36820	unknown protein
Bra017788	A03	30520567-30521466	AT4G36830	GNS1/SUR4 membrane family protein
Bra017789	A03	30545095-30545610	AT4G37240	unknown protein
Bra017790	A03	30547347-30548655	AT5G66570	PSBO-1, OEE1, OEE33, OE33, PSBO1, MSP-1; PSBO1 (PS II OXYGEN-EVOLVING COMPLEX 1); oxygen evolving/ poly(U) binding
Bra017791	A03	30551736-30554486	AT1G51520	nucleic acid binding / nucleotide binding
Bra017792	A03	30565059-30567270	AT4G37190	Beta tubulin, autoregulation binding site (InterPro:IPR013838), Tubulin/FtsZ, GTPase (InterPro:IPR003008)
Bra017793	A03	30570133-30571602	AT1G52950	replication protein-related
Bra017794	A03	30572661-30573350	AT1G67623	F-box family protein
Bra017795	A03	30574376-30575602	AT4G37180	myb family transcription factor
Bra017796	A03	30581108-30583361	AT4G37160	sks15; sks15 (SKU5 Similar 15); copper ion binding / oxidoreductase
Bra017797	A03	30585277-30590470	AT4G37150	ATMES9, MES9; MES9 (METHYL ESTERASE 9); hydrolase, acting on ester bonds / methyl indole-3-acetate esterase/ methyl jasmonate esterase/ methyl salicylate esterase
Bra017798	A03	30592362-30595459	AT4G37070	PLP1, PLA IVA; patatin, putative
Bra017799	A03	30597986-30600158	AT4G37050	PLP4, PLA V; PLP4 (PATATIN-LIKE PROTEIN 4); nutrient reservoir
Bra017800	A03	30606774-30607682	AT4G36990	HSF4, HSFB1, AT-HSFB1, ATHSF4; HSF4 (HEAT SHOCK FACTOR 4); DNA binding / transcription factor/ transcription repressor
Bra017801	A03	30610425-30611832	AT4G36970	remorin family protein
Bra017802	A03	30612033-30617149	AT4G36960	RNA recognition motif (RRM)-containing protein
Bra017803	A03	30629212-30631626	AT4G36945	phospholipase C/ phosphoric diester hydrolase
Bra017804	A03	30632957-30634303	AT3G12470	nucleic acid binding
Bra017805	A03	30637315-30638027	AT5G34940	AtGUS3; AtGUS3 (Arabidopsis thaliana glucuronidase 3); beta-glucuronidase
Bra017806	A03	30639236-30639901	AT1G10170	ATNFXL1; ATNFXL1 (ARABIDOPSIS THALIANA NF-X-LIKE 1); protein binding / transcription factor/ zinc ion binding
Bra017807	A03	30641273-30645533	AT2G14080	disease resistance protein (TIR-NBS-LRR class), putative
Bra017808	A03	30646781-30649609	AT4G36940	NAPRT1; NAPRT1 (NICOTINATE PHOSPHORIBOSYLTRANSFERASE 1); nicotinate phosphoribosyltransferase
Bra017809	A03	30661490-30663651	AT4G36920	AP2, FLO2, FL1; AP2 (APETALA 2); transcription factor
Bra017810	A03	30673597-30675655	AT4G36910	LEJ2, CDCP2; LEJ2 (LOSS OF THE TIMING OF ET AND JA BIOSYNTHESIS 2)
Bra017811	A03	30676488-30677066	AT4G36900	RAP2.10; RAP2.10 (related to AP2 10); DNA binding / transcription factor
Bra017812	A03	30689155-30689920	AT3G47680	DNA binding
Bra017813	A03	30713601-30714398	AT4G37260	MYB73, ATMYB73; MYB73 (MYB DOMAIN PROTEIN 73); DNA binding / transcription factor
Bra017814	A03	30716884-30717105	AT4G37290	unknown protein
Bra017815	A03	30727701-30727955	AT4G37295	unknown protein
Bra017816	A03	30728842-30729533	AT4G37300	MEE59; MEE59 (maternal effect embryo arrest 59)
Bra017817	A03	30731260-30733183	AT4G37320	CYP81D5; CYP81D5; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
Bra017818	A03	30741799-30743869	AT4G37330	CYP81D4; CYP81D4; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
Bra017819	A03	30744522-30746728	AT4G37370	CYP81D8; CYP81D8; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
Bra017820	A03	30747469-30747939	AT4G37445	BEST Arabidopsis thaliana protein match is: calcium-binding EF hand family protein (TAIR:AT1G64850.1)
Bra017822	A03	30751647-30752117	AT4G37445	BEST Arabidopsis thaliana protein match is: calcium-binding EF hand family protein (TAIR:AT1G64850.1)
Bra017823	A03	30757964-30758812	AT4G37450	AGP18, ATAGP18; AGP18 (ARABINOGALACTAN PROTEIN 18)
Bra017824	A03	30759675-30760655	AT2G47750	GH3.9; GH3.9 (PUTATIVE INDOLE-3-ACETIC ACID-AMIDO SYNTHETASE GH3.9)
Bra017825	A03	30761694-30763869	AT4G37460	SRFR1; SRFR1 (SUPPRESSOR OF RPS4-RLD 1); protein complex scaffold
Bra017826	A03	30765247-30766246	AT4G37470	hydrolase, alpha/beta fold family protein
Bra017827	A03	30770412-30770873	AT1G52950	replication protein-related
Bra017828	A03	30775121-30775456	AT3G08880	unknown protein
Bra017829	A03	30788356-30788556	AT4G37510	ribonuclease III family protein
Bra017830	A03	30793243-30794512	AT4G37520	peroxidase 50 (PER50) (P50) (PRXR2)
Bra017831	A03	30822548-30823423	AT4G37540	LBD39; LBD39 (LOB DOMAIN-CONTAINING PROTEIN 39)
Bra017832	A03	30848442-30849556	AT4G12040	zinc finger (AN1-like) family protein
Bra017833	A03	30848442-30848594	AT4G12040	zinc finger (AN1-like) family protein
Bra017835	A03	30854211-30855900	AT4G37580	HLS1, COP3, UNS2; HLS1 (HOOKLESS 1); N-acetyltransferase
Bra017836	A03	30861729-30862352	AT2G07505	unknown protein

Table 3: Candidate gene identification in the corresponding region on the end of chromosome A03

B. rapa gene	*A. thaliana*	*A. thaliana* annotations	E-value	GO ID
BrCHI(Bra017728)	AT3G55120	"TT5, A11, CFI; TT5 (TRANSPARENT TESTA 5); chalcone isomerase	5.00E-73	GO:0010224
BrMYB(Bra017813)	AT4G37260	MYB73, ATMYB73; MYB73 (MYB DOMAIN PROTEIN 73); DNA binding/transcription factor"	2.00E-114	GO:0010200
BrLBD(Bra017831)	AT4G37540	LBD39; LBD39 (LOB DOMAIN-CONTAINING PROTEIN 39)	9.00E-75	GO:0008150

Development of a Co-dominant KASP Marker for the Candidate Gene *BrMYB73*

DNA sequencing analysis revealed that a single-nucleotide deletion in the coding region of the *BrMYB73* allele from 'ZiChunyuehuang' (ZCYH, purple leaf) caused a 75 amino acid deletion in the BrMYB73 protein. This single-nucleotide deletion was converted into the allele-specific KASP marker Br-M-SNP (Table 1) and tested against the parental inbred lines to determine the reliability of scoring. The BC_1 and an additional F_2 population also segregating for the *Bra-Pur* locus were used to demonstrate co-

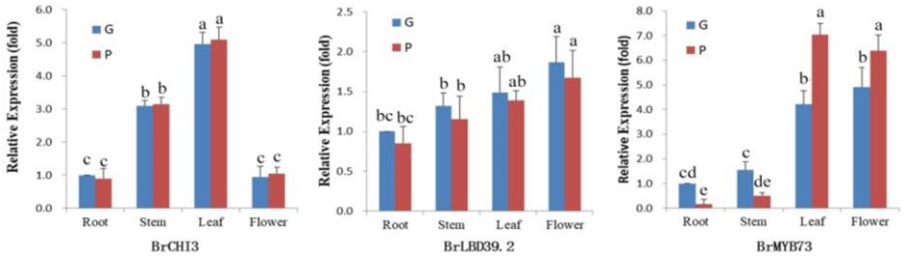

Fig. 4: Quantitative real-time PCR (qRT-PCR) expression analysis of three anthocyanin biosynthesis genes in four different plant tissues (G; 'Chunyuehuang', P; 'ZiChunyuehuang'). Error bars represent the standard error of the means of three independent replicates. Values denoted by the same letter did not differ significantly at $P<0.05$ according to Duncan'smultipler ange tests

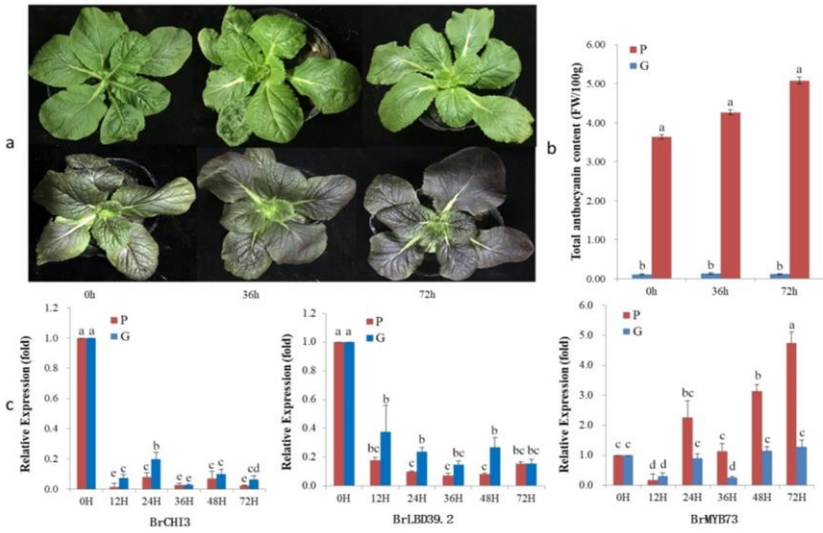

Fig. 5: Representative plants of the two parental lines 'Chunyuehuang' (upper) and 'ZiChunyuehuang' (lower) grown for three different times under the low temperature treatment (a). Total anthocyanin content in 'Chunyuehuang'(G) and 'ZiChunyuehuang' (P) at 0, 36, and 72 h of low temperature treatment (b). Gene expression analysis of *BrCHI3*, *BrMYB73*, and *BrLBD39.2* over 72 h of low temperature treatment in the parental lines of the BC_1 population, 'Chunyuehuang'(G) and 'ZiChunyuehuang'(P) (c). Error bars represent the standard error of the means of three independent replicates. Values denoted by the same letter did not differ significantly at $P<0.05$ according to Duncan'smultipler ange tests

segregation of Br-M-SNP. Genotyping analysis showed that the alleles of the newly-developed marker, Br-M-SNP, co-segregated perfectly with the *Bra-Pur* locus in both populations (Fig. 6).

Discussion

In our study, we mapped the *Bra-Pur* locus to an 833 kb region on the end of chromosome A03 in a segregating BC_1 population of Chinese cabbage. We found that *Bra-Pur* is tightly linked to the two flanking marker loci, Indel-5 and SNP-1, at a genetic distance of 0.1 cM each, although the physical distances varied considerably (Li *et al.*, 2016), which may imply the presence of a sequence deletion on the distal end of linkage group A03 in the purple-leaved lines that could make it difficult to fine map the *Bra-Pur* gene. We found that the *Bra-Pur* locus on the end of chromosome A03 is similar to the *BrPur* gene that was mapped in 2013, but the purple-leaved parental lines differed between the two studies; this implied that ZCYH could share a common origin with 09N-742, the purple-leaved line used by Liu *et al.* (2013) and Wang *et al.* (2014). However, no candidate genes in this physical DNA region were investigated in the earlier study, and we initially characterized these genes to study their relationship with anthocyanin biosynthesis.

Chalcone isomerase (*CHI*) is one of the structural genes of anthocyanin biosynthesis isolated from various plant species (Guo *et al.*, 2014). Some studies have found that the *CHI* is relatively more significantly up-regulated in purple-leaved materials (Deng and Davis, 2001; Zhang *et al.*, 2014). Unlike the results from previous reports, we found no significant difference in the expression of *BraCHI3* between the purple and non-purple cultivars of *B. rapa* used in our study, and it decreased markedly in

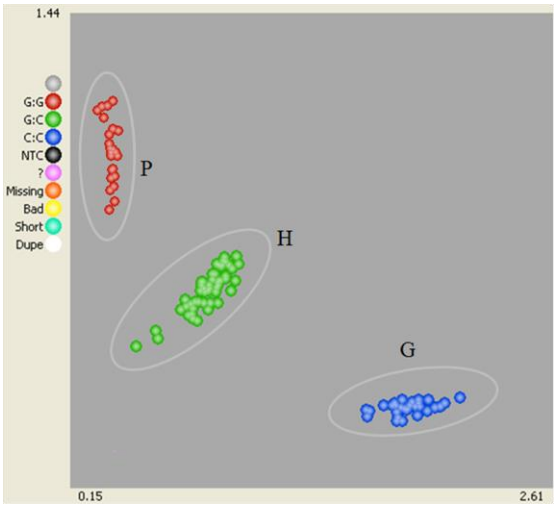

Fig. 6: Genotyping of the co-segregating marker Br-M-SNP in the parents and F_2 segregating population. P; purple-leaved parent (ZCYH) and homozygous purple-leaved individuals, H; heterozygous purple-leaved individuals, G; green-leaved parent (CYH) and homozygous green-leaved individuals

response to cold temperature treatment, although some studies have shown that low temperature stress can increase anthocyanin production in many crops (Christie *et al.*, 1994; Mori *et al.*, 2005; Piero *et al.*, 2005; Ubi *et al.*, 2006). This suggests that *BrCHI3* with a single amino acid substitution (Fig. 3) may not play a major role in anthocyanin accumulation in ZCYH, the *B. rapa* cultivar with purple leaves studied here.

In *A. thaliana*, there are two groups of anthocyanin biosynthesis regulatory genes: positive and negative. Rubin *et al.* (2009) characterized *LBD39*, a gene that functions to negatively regulate anthocyanin biosynthesis in *A. thaliana*. In our study, we found no significant differences in the relative expression of *BraLBD39.2*, the *Brassica* ortholog, between the purple and non-purple cultivars of *B. rapa*, which strongly indicated that it was not the candidate *Bra-Pur* gene.

MYB, bHLH, and WD repeat proteins are classes of TFs that positively regulate gene expression in plants (Lepiniec *et al.*, 2006; Gonzalez *et al.*, 2008; Gao *et al.*, 2018). R2R3-MYB are generally positive regulatory TFs in the MYB family that regulate transcription of the anthocyanin biosynthesis pathway structural genes. In *A. thaliana*, eight R2R3-MYB proteins (PAP1, PAP2, MYB113, MYB114, MYB11, MYB12 and MYB111) regulate anthocyanin biosynthesis structural genes in the flavonoid pathway (Borevitz *et al.*, 2000; Tohge *et al.*, 2005; Gonzalez *et al.*, 2008). In other crops, such as maize, apple, and wheat, the upregulation of R2R3-MYB gene expression is also required to activate anthocyanin biosynthesis (Grotewold *et al.*, 1994; Ban *et al.*, 2007; Wang *et al.*, 2015). The *BrMYB73* gene, with predicted R2 and R3 domains (Fig. 3), was identified in this study. Consistent with the previous conclusion, the expression of *BrMYB73*, as determined by qPCR, was up-regulated significantly in the purple-leaved cultivar ZCYH and showed an increasing trend under low temperature conditions. Through DNA sequence analysis, we identified a 1-bp deletion in the *BrMYB73* coding region that results in a predicted deletion of 75 amino acid residues at the C-terminus of the *BrMYB73* protein. The C-terminal end is considered to be important for transcriptional activation in the R2R3-MYB protein family. In *A. thaliana*, amino acid substitutions and deletions that occur in this region always affect transcriptional activation (Kranz *et al.*, 1998). The results presented here indicate that *BrMYB73* is an important gene that regulates anthocyanin biosynthesis in *B. rapa*.

Conclusion

In this study, we mapped the purple-leaf trait to an 833 kb region of DNA on chromosome A03. One candidate gene in this region (*BrMYB73*) was found to be strongly associated with anthocyanin accumulation and is most likely the candidate gene for the *Bra-Pur* locus. In addition, a co-dominant KASP marker, Br-M-SNP, was developed that co-segregates with *Bra-Pur*. These results will be important in further studies of the genetic mechanisms that control anthocyanin biosynthesis in purple Chinese cabbage.

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