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Mapping and expression profiling reveal an inserted fragment from purple mustard involved anthocyanin accumulation in Chinese cabbage

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Abstract Anthocyanins are the major pigments responsible for purple coloration in flowers, fruits and leaves, and the genes involved in their biosynthetic pathway have been identified in many plants. A purple-leaf Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) was bred by interspecies crossing between Chinese cabbage and purple-leaf mustard [*Brassica juncea* (L.) Coss. var. *foliosa* L. H. Bailey]. In this study, high-performance liquid chromatographic analysis indicated purple coloration in Chinese cabbage is due to the accumulation of the same kind of cyaninin as in purple mustard. To elucidate the genetic factors controlling anthocyanin accumulation in this purple-leaf Chinese cabbage, we mapped the anthocyanin gene from the mustard (*Anm*) locus in an F_2 population and performed expression profiling of anthocyanin-related genes. A genetic analysis revealed that the purple-leaf phenotype is a qualitative trait and that its inheritance is unstable in purple-leaf Chinese cabbage. Mapping insertion/deletion markers from 288 individuals of the F_2 population located the *Anm* locus within a 2.5-cM interval on *B. rapa* chromosome A02. The sequencing and alignment of the amplified fragments demonstrated that purple Chinese cabbage contains fragments of purple mustard on chromosome A02. We evaluated the expression profiles of 12 anthocyanin-related genes on A02 by reverse-transcription and quantitative real-time PCR methods, which revealed that the expression levels of five genes were higher in purple Chinese cabbage than in the non-purple variety. These results offer insights into the molecular mechanism of anthocyanin biosynthesis and improve the knowledge on molecular breeding of purple-type Chinese cabbage.

Keywords Mapping · Expression analysis · HPLC-MS · Purple mustard fragment · Chinese cabbage

Shujiang Zhang and Peirong Li have equally contributed to this work and are regarded as joint first authors.

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Introduction

Anthocyanin pigments are a group of flavonoid compounds that fulfill important biological functions by protecting plants against various biotic and abiotic stresses (Andersen and Markham 2005; Harborne and Baxter 1993). Anthocyanins can make vegetables more colorful and have beneficial roles in human health, such as acting as potentially protective factors against cancer

and heart disease (Lam et al. 2009; van Poppel et al. 1999). These beneficial health properties have been partially attributed to the strong antioxidant capacity of these compounds (Yamasaki et al. 1997), which provides an incentive for the breeding of purple vegetables to further improve their nutritional quality and available commercial value.

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is an important vegetable crop cultivated in Asia. Chinese cabbage characterized by colored leaves has increasingly been the focus of interest as a functional food because of its high levels of anthocyanin and carotenoid pigments, which have important nutritional effects (Li et al. 2015b; Zhang et al. 2011). To date, the purple trait associated with anthocyanin accumulation has been found in many *Brassica* varieties, such as zicaitai (*B. rapa* L. ssp. *chinensis* var. *purpurea*), purple turnips (*B. rapa* var. *rapa*), purple cabbage (*B. oleracea* var. *capitata*), purple kale (*B. oleracea* var. *acephala* f. tricolor), purple cauliflower (*B. oleracea* var. *botrytis*) and purple mustard. Although some intermediate purple Chinese cabbage varieties have been created by the interspecific hybridization of Chinese cabbage with purple pak choi (Zhang et al. 2008a) or zicaitai (Zhang et al. 2008b), the outer leaves of these materials are green. A fully purple Chinese cabbage has been created by the interspecific hybridization of Chinese cabbage ($2n = 2x = 20$, AA) and purple mustard ($2n = 4x = 36$, AABB) (Sun et al. 2006).

Advances in molecular biology have aided our understanding of the details of the mechanisms of anthocyanin pigmentation and the genes involved in biosynthetic pathway and transcription factors in regulating anthocyanin pigmentation in plants (Holton and Cornish 1995). Studies on anthocyanin biosynthetic pathway genes and numerous regulatory factors have been carried out in Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*), petunia (*Petunia hybrida*), snapdragon (*Antirrhinum majus*) and other plant species (Broun 2005; Dixon et al. 2005; Grotewold 2006; Koes et al. 2005). In addition, many anthocyanin-related genes have been mapped and cloned in *Brassica* species. In a study with zicaitai, 17 anthocyanin compounds were identified as cyanidin glycosides, with candidate genes *BrEGL3.1* and *BrEGL3.2* encoding basic helix-loop-helix transcription factors mapped on chromosome A09 (Guo et al. 2015). Another study revealed that purple coloration in purple turnip (*B. rapa* ssp. *rapa*) is under the control of a single dominant gene, and a doubled haploid population derived from a cross between purple turnip and Chinese cabbage was used to map the *Anp* locus regulating anthocyanin pigmentation on chromosome A07 (Hayashi et al. 2010). A *Pr* gene encoding a R2R3 MYB transcription factor was isolated via a combination of candidate gene analysis and fine mapping in mutant purple cauliflower (Chiu et al. 2010). A genome-wide comparative analysis of *A. thaliana* and *B. rapa* was useful in elucidating biosynthetic pathway genes (Li et al. 2015a). Using this approach, 73 genes in *B. rapa* have been identified as orthologs of 41 anthocyanin biosynthetic genes in *A. thaliana* (Guo et al. 2014). This strategy can also assist the search for key genes controlling the purple trait in Chinese cabbage. Although purple-heading Chinese cabbage was generated from the distant hybridization between purple mustard and Chinese cabbage (Sun et al. 2006), the genetic and molecular basis of the purple-color trait derived from purple mustard in this new improved germplasm resource has not been reported.

The present study was conducted to map the *Anm* locus and to comprehensively analyze anthocyanin accumulation at the molecular level. In this study, the HPLC-MS method with positive detective mode was used to identify individual anthocyanins in different varieties. An F_2-segregating population for the purple-color trait was used for mapping. In addition, reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) were used to analyze the expression levels of anthocyanin-related genes in different varieties. Our results lay a foundation for the elucidation of molecular mechanisms of anthocyanin biosynthesis in heading Chinese cabbage with purple leaves.

Materials and methods

Plant materials

We obtained F_1 individuals from the distant hybridization and embryo rescue of purple leaf mustard 'M58' (*Brassica juncea*) crossed with Chinese cabbage self-incompatible line 'Charming Yellow'. The hybrid F_1 plants were then backcrossed with self-incompatible line 'BP058'. Purple Chinese cabbage 'B90335-5' was

obtained by multiple generational self-crossings of purple individuals selected from a separated population (Sun et al. 2006). An F_2 mapping population with 288 individuals, 'A12608,' was developed from a single purple F_1 individual from a cross between purple-type 'B90335-5' and green-type 'H165A'.

Purple mustard M58, the parent purple Chinese cabbage B90335-5, three purple Chinese cabbages (Z-1, Z-2, Z-3) and three green Chinese cabbages of F_2 population were used in anthocyanin analysis. All plant materials were provided by the Chinese Cabbage Research Group of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences.

Anthocyanin extraction and total anthocyanin content

The acidified ethanol method was used for anthocyanin extraction of each sample (Liu et al. 2004). The total anthocyanin contents in selected cultivars of purple mustard, purple Chinese cabbage and green Chinese cabbage were detected using the pH differential method (Giusti and Wrolstad 2001). The appropriate dilution factor for each sample was determined by dilution with 0.025 M aqueous potassium chloride (KCl) buffer, pH 1.0. Two dilutions of the sample were prepared, one with 0.025 M KCl buffer, pH 1.0, and the other with 0.4 M sodium acetate buffer, pH 4.5 (You et al. 2011). An UV1800 spectrophotometer (Shimadzu, Japan) was used for spectral measurements at 530 and 700 nm.

HPLC-MS analysis

The chromatographic system consisted of a high-pressure chromatography system (Agilent 1200, USA) equipped with an ultraviolet detector. Anthocyanins were eluted with a gradient mobile phase formed by a 5 % (v/v) formic acid water solution (phase A) and acetonitrile (phase B) at a flow rate of 0.8 ml/min. The linear gradient conditions of phase B were as follows: 10–13 % B from 0 to 15 min, 13–20 % B from 15 to 45 min; 20–23 % B from 45 to 50 min, 23–100 % B from 50 to 55 min. The wavelengths of the UV-visible detector were set at 530 nm.

The analytes were identified using a triple quadrupole linear ion trap mass spectrometer (Agilent, 6310 Ion Trap) equipped with a turbo-spray ion source. Mass spectra were acquired in a positive ion mode because anthocyanins are subject to electron loss and are stable in the positive form because of their structures. Ions were scanned from 100 to 1500 m/z with a scan speed of 1000 amu/s. Nitrogen was used as the nebulizing gas in a flow rate of 12 l/min. The drying gas pressure was 45 psi, and the temperature was 350 °C.

Statistical analysis

The purple and green phenotypes were measured by visual inspection and also with spectrophotometric analysis. Segregation data (green and purple) for the F_2 generation were analyzed by the chi-square test for goodness of fit to expected segregation ratios.

Genetic mapping and anthocyanin-related gene screening of the *Anm* locus

Genomic DNA was extracted from fresh leaves using the conventional cetyltrimethylammonium bromide method (Chen and Ronald 1999) and stored at −20 °C. After the extraction of genomic DNA from individual plants, green and purple DNA bulks were made (10 green or 10 purple inner leaves of F_2 individuals/bulk). Insertion/deletion (InDel) markers covering the *Anm* locus were developed based on the *B. rapa* genomic sequence and are shown in Supplementary Table 1. InDel marker amplification reactions were performed in 15-μl reaction mixtures containing 0.3 μl forward primer, 0.3 μl reverse primer, 50 ng DNA, 1.5 μl 10 × PCR buffer (containing $MgCl_2$), 1.2 μl 0.8 mM dNTPs and 0.5 U Ampli Taq Gold. InDel amplification conditions were 10 min at 95 °C, followed by 36 cycles of 40 s at 95 °C, 40 s at 57 °C, 40 s at 72 °C and finally 10 min at 72 °C. Genetic linkage map construction was carried out using Joinmap 4.0 software (Van Ooijen 2006). The Kosambi function was applied to convert recombination fractions into map distances (Kosambi 1943).

To screen for related anthocyanin genes, we used *B. rapa* genome information available in the *Brassica* database (BRAD; http://brassicadb.org/brad/), which includes the complete *Brassica* A genome sequence, predicted genes and associated annotations, *B. rapa* genes orthologous to those in *A. thaliana*, and genetic markers and maps of *B. rapa*. Anthocyanin pathway genes were priorities for screening.

Cloning and sequencing of DNA fragments

InDel fragments were recovered and purified from polyacrylamide gels as follows. After excising target bands from a polyacrylamide gel, 50 µl ddH_2O was added followed by overnight soaking. The gel extract was incubated in a water bath for 30 min at 95 °C and then centrifuged for 10 min at 10,000 rpm. Using 5 µl of the resulting supernatant as a template and corresponding primers, amplifications were performed as described above for InDel markers. PCR products were purified with 75 % ethanol and dissolved in 10 µl ddH_2O. The PCR products were cloned into a p*EASY*-T1 vector (TransGen, Beijing, China), and the resulting plasmid was used to transform *Escherichia coli* TransT1 (TransGen). Sequences of the inserts were obtained using an ABI 3730xl DNA Analyzer (Applied Biosystems, Beijing, China), and multiple sequence alignments were performed using MEGA and GENEDOC software.

RT-PCR and qRT-PCR

Total RNA was extracted separately from the fourth leaf of two green individuals (G-1 and G-2) and two purple individuals (P-1 anad P-2) selected from 'A12608' at the heading stage. Single-strand cDNA synthesis was performed using a TransGen kit (TransGen) according to the manufacturer's protocol. A 25-fold dilution of the resulting cDNA was used for RT-PCR and qRT-PCR. Primers of anthocyanin-related genes were designed using Primer 5 software (Supplementary Table 2). Primer specificity was first confirmed by performing a BLAST algorithm-based search querying each primer sequence against the *B. rapa* genome. We then performed a series of melting curve analyses to verify that each curve yielded a single sharp peak.

The *GAPDH* housekeeping gene was used as the internal control for normalization of gene expression (Supplementary Table 2). RT-PCR and qRT-PCR amplification conditions were as described previously (Li et al. 2015b). To ensure reproducibility and reliability, at least three independent biological replicates and three technical replicates of each biological replicate were analyzed for each sample by qRT-PCR. The $2^{-\Delta\Delta CT}$ method was used to analyze relative changes in gene expression (Livak and Schmittgen 2001).

Results

Anthocyanin profile analysis

The total anthocyanin content of purple mustard was 84.77 ± 3.60 mg/100 g fresh weight (FW). The total amounts of anthocyanins in purple Chinese cabbage B90335-5, Z-1, Z-2 and Z-3 were 68.27 ± 0.09 mg/100 g FW, 67.73 ± 3.63 mg/100 g FW, 76.63 ± 1.32 mg/100 g FW and 69.36 ± 1.17 mg/100 g FW, respectively. The results indicate that the purple individuals in the F_2 were not significantly difference from the parental purple Chinese cabbage. Furthermore, we did not detect any anthocyanins in three green individuals.

The high-performance liquid chromatography-mass spectrometry method (HPLC-MS) with positive detective mode was used to identify individual anthocyanins in purple leaf mustard and purple Chinese cabbage. In total, 20 anthocyanin compounds were detected in both purple mustard and purple Chinese cabbage (Fig. 1). Identification of anthocyanins in extracts was based on chromatographic behavior, accurate molecular masses, characteristic MS/MS fragmentation product ions, UV spectra and comparisons with scientific publications (Mazza and Miniati 1993; Wu and Prior 2005; Lin et al. 2011). In purple mustard, canidin and delphinidin were detected, accounting for 99.95 and 0.05 %, respectively. Anthocyanins such as cyanidin 3-feruloylmalonylsophoroside-5-glucoside (peak 12), cyanidin 3-sinapoylferuloylsophoroside-5-malonylglucoside (peak 14) and cyanidin 3-sinapoylferuloylsophoroside-5-malonylglucoside (peak 16) were highly accumulated in mustard. The major anthocyanin compound in purple Chinese cabbage is cyanidin, and the minor compounds are delphinidin and petunidin, accounting for 99.35, 0.59 and 0.06 %, respectively. Purple coloration in Chinese cabbage is mainly due to the accumulation of cyanidin 3-feruloylmalonylsophoroside-5-glucoside, cyanidin 3-sinapoylferuloylsophoroside-5-malonylglucoside and cyanidin 3-diferuloylsophoroside-5-malonylglucoside, accounting for 180.17 ± 1.79, 134.71 ± 1.12 and 126.89 ± 1.30 µg/g·FW, respectively (Supplementary Table 3).

Genetic analysis of the purple trait

Parent B90335-5 was an inbred purple Chinese cabbage line, and parent H165A was a green type

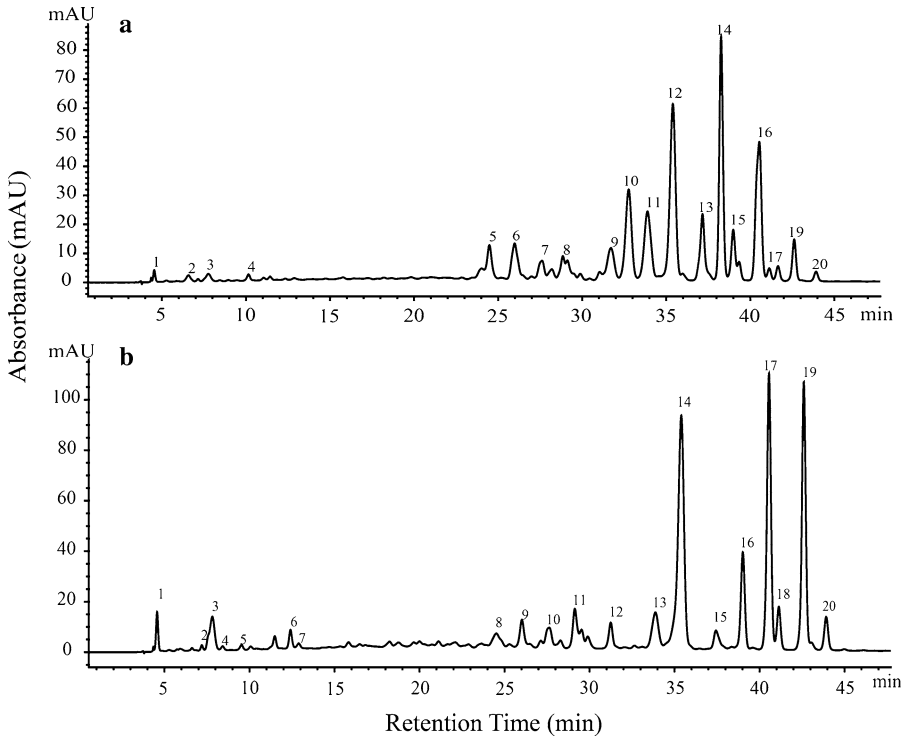

Fig. 1 Chromatograms of HPLC analysis for individual anthocyanin compounds of **a** purple mustard M58 and **b** parent purple Chinese cabbage B90335-5. *Horizontal axis* shows retention time; *vertical axis* gives the strength of the chromatographic peak response. Labels correspond to compounds shown in Supplementary Table 3

(Fig. 2). Of the 20 F_1 individuals, 19 were purple and 1 was green. One purple F_1 individual was used to generate an F_2 population. The F_2 individuals could be separated into two groups based on color (purple or non-purple) with no intermediate types, which indicates that purple leaf color is a qualitative trait. The segregation data for the F_2 generation (116 purple and 172 green) did not fit the expected ratio for a Mendelian model based on the action of a single dominant allele ($\chi_c^2 = 185.18 > \chi_{0.05}^2 = 3.84$).

Marker screening and mapping of the *Anm* locus in Chinese cabbage

Screening of 1000 InDel primer pairs revealed nine pairs that were polymorphic between the purple and green bulks: BrID10199, BrID101151, BrID101125, BrID11131, BrID10207, BrID11737, BrID11893, BrID111333 and BrID101589. All of these markers were located on chromosome A02 except for BrID101589, which was located on the unassembled Scaffold000241. Furthermore, all of the linked markers were dominant, yielding nonspecific bands in both green and purple individuals and specific bands in purple individuals (Fig. 3a).

We used the nine InDel markers to genotype 288 individuals from the F_2 generation. Linkage between the DNA markers and the *Anm* locus was established using Joinmap 4.0 software on the basis of the genotyping data from these 288 individuals. All nine markers were found to map to a 2.5-cM region around the *Anm* gene. Five of these markers were located on one end of the *Anm* gene, and two were on the other end. In addition, two markers (BrID101125, BrID11131) were found to cosegregate with the *Anm* gene within this F2 population (Fig. 3b).

Sequencing and alignment of marker-amplified fragments

The linked markers were amplified in purple mustard 'M58' and also found to yield the same bands as purple Chinese cabbage, including both nonspecific and specific bands. Nonspecific and specific amplified fragments were cloned and sequenced in green- and purple-type F_2 and mustard 'M58' individuals. Sequence analysis demonstrated that the sequences of fragments specific to purple Chinese cabbage and purple mustard were identical, which was different with the nonspecific sequence in purple Chinese

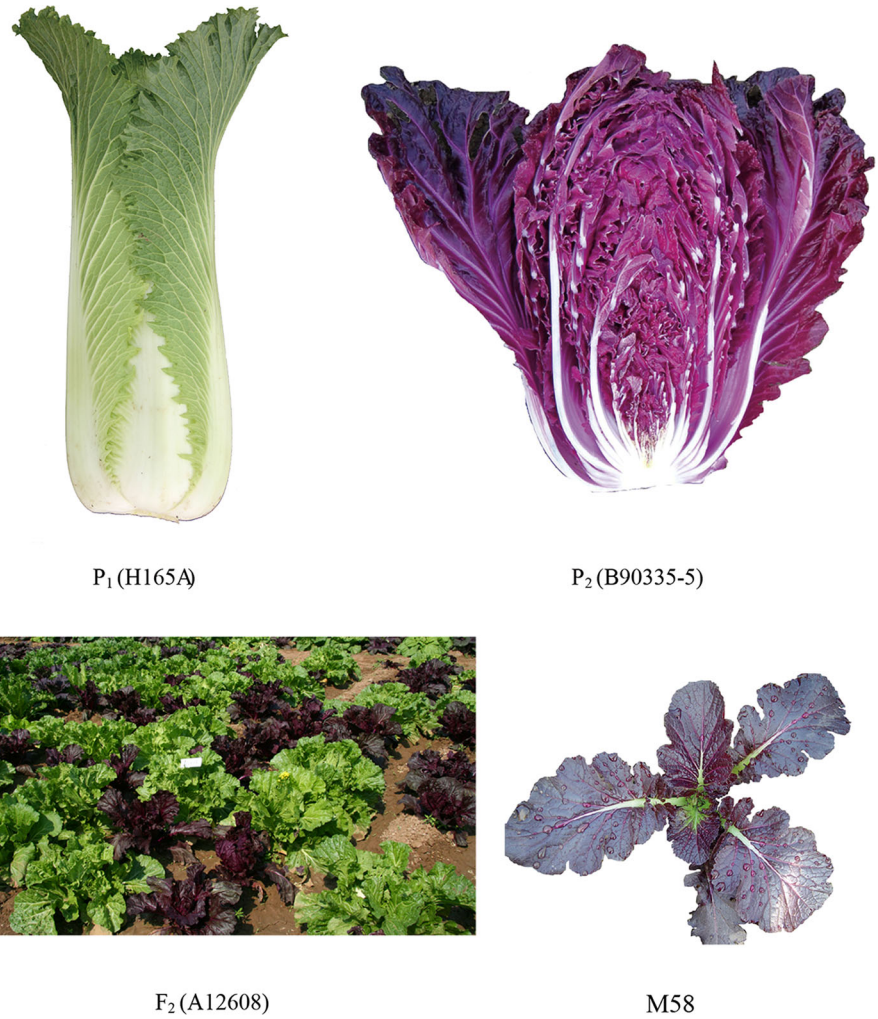

Fig. 2 Phenotypes of purple-leaf mustard 'M58,' purple Chinese cabbage 'B90335-5,' green Chinese cabbage 'H165A' and the F_2 mapping population 'A12608'

cabbage (Fig. 4). Thus, we speculated that the sequences associated with bands specific to purple Chinese cabbage were derived from the introgression or insertion of purple mustard fragments.

A BLASTN analysis revealed that the nonspecific sequences amplified by the nine markers (except for BrID101125) in the purple individuals were the same as those in the *B. rapa* genome sequence (version 1.5, http://brassicadb.org/brad/), indicating their derivation from the Chinese cabbage genome. Specific band sequences in purple Chinese cabbage were queried using the BLAST algorithm against the *B. rapa* genome, which revealed sequence similarities ranging from 85 to 94 %. Furthermore, the sequences associated with bands specific to purple Chinese cabbage and mustard that were amplified by BrID11737 could not be aligned with the *B. rapa* genome (Table 1). Therefore, purple Chinese cabbage contained fragments of purple mustard on chromosome A02, which we initially speculated were due to fragment insertions or substitutions between purple mustard 'M58' and Chinese cabbage.

Expression analysis of anthocyanin-related genes on chromosome A02

We used the anthocyanin biosynthetic genes of *A. thaliana* to align with the genome and protein sequences of *B. rapa* using BLASTN and BLASTP algorithms. In total, we identified 12 syntenic orthologs between *A. thaliana* and *B. rapa* on chromosome A02. These anthocyanin-related genes were comprised of six structural genes, five regulatory genes and one transport gene: chalcone synthase genes

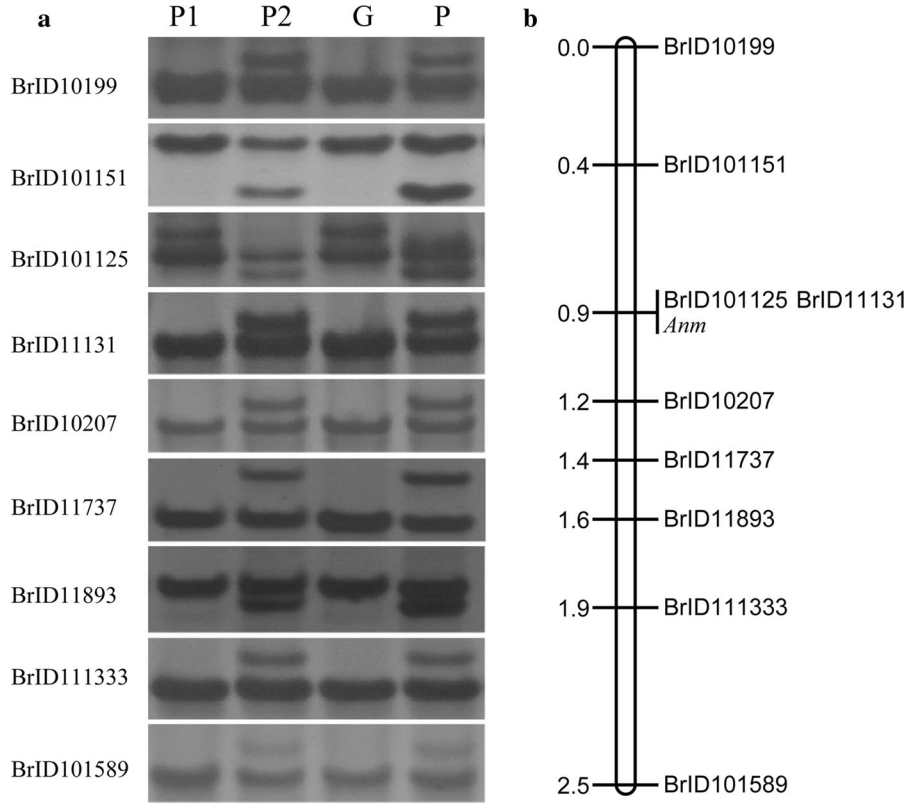

Fig. 3 Marker screening and genetic mapping of the *Anm* locus. **a** Polymorphism in the P_1 plant (H165A), the P_2 plant (B90335-5), the purple bulk (P) and the green bulk (G). **b** Genetic linkage map of the *Anm* locus on chromosome A02 of Chinese cabbage, which was generated from the F_2 population (288 individuals) derived from a 'B90335-5'/'H165A' cross

(Bra023441 and Bra020688), anthocyanidin 3-O-glucosyltransferase gene (Bra023594), MYB domain protein 75 gene (Bra039763), MYB-Like 2 gene (Bra007957), transparent testa 19 gene (Bra023602), flavonol synthase gene (Bra029211), phenylalanine ammonia-lyase gene (Bra028793), myricetin 3'-O-methyltransferase gene (Bra022700), transparent testa glabrous gene (Bra029411), MYB domain protein111 gene (Bra020647) and LOB domain-containing protein gene (Bra031833). To study the influence of inserted fragments of purple mustard in the ChrA02 of Chinese cabbage, we monitored the expression of 12 anthocyanin-related genes on chromosome A02 by RT-PCR and qRT-PCR.

Primers designed to amplify genes Bra039763, Bra028793, Bra029411, Bra020647 and Bra029211 yielded no bands, indicating that those genes were not expressed at the monitored stage in either green or purple plants. Bra023441, Bra020688, Bra023594, Bra007957 and Bra023602 produced stronger amplification bands in purple than in green plants, while the expression of Bra022700 was not significantly different between the two types. The expression of Bra031833 was upregulated in green plants (Fig. 5a). We next used qRT-PCR to further examine expression patterns in green- and purple-type Chinese cabbage cultivars. Bra023441, Bra020688 and Bra007957 were 20 times more highly expressed in the purple than in the green plants. Bra023594 and Bra023602 showed two- to fourfold higher expression levels in purple individuals, whereas the expression of Bra031833 was upregulated in green ones (Fig. 5b). Consequently, the expression patterns of the 12 genes assessed by RT-PCR and qRT-PCR may indicate the mediation of mustard fragments, and the upregulated genes may be responsible for accumulation of anthocyanins in purple Chinese cabbage.

Discussion

Role of the mustard fragment in instability of purple trait inheritance

Alien addition and translocation lines can be derived from interspecific hybridization and backcrossing of

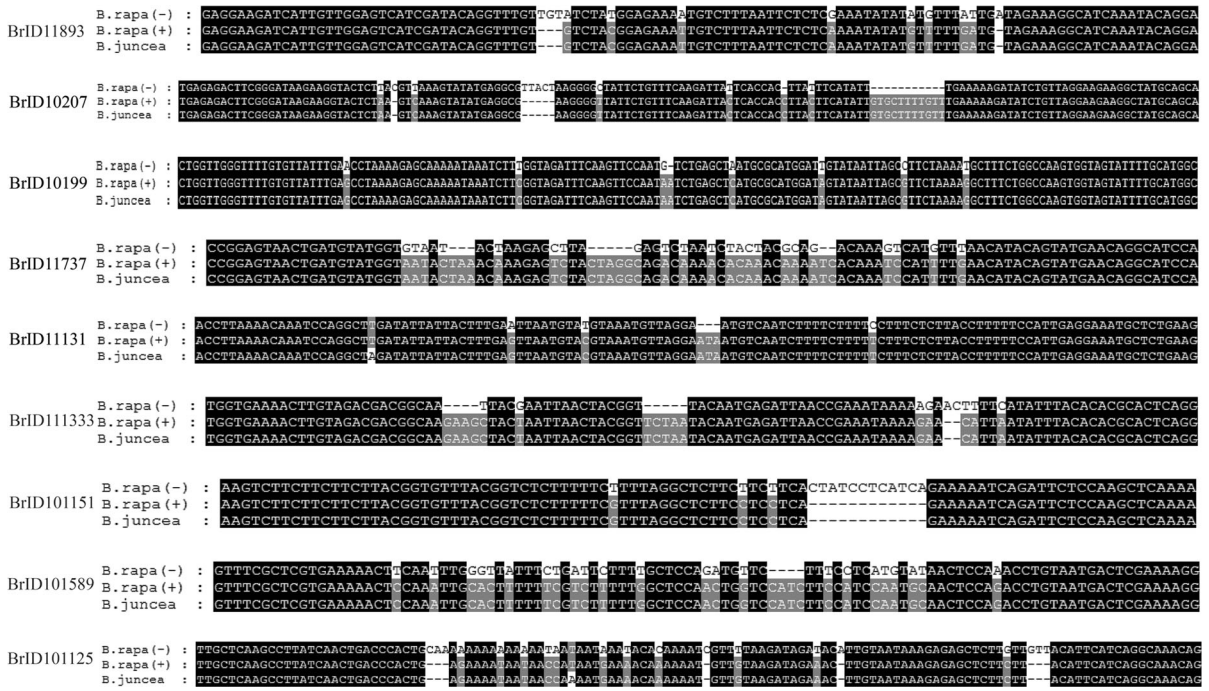

Fig. 4 Sequencing and alignment of amplified markers in purple Chinese cabbage and purple-leaf mustard. *B. rapa* (−) refers to sequences from the nonspecific band in purple Chinese cabbage; *B. rapa* (+) represents sequences of the specific band in purple Chinese cabbage; *B. juncea* indicates sequences of the band in purple mustard

Table 1 Identities of amplified sequences of linked markers BLASTed against the *B. rapa* genome

Marker	Nonspecific band sequence in purple Chinese cabbage blast with *B. rapa* genome		Specific band sequence in purple Chinese cabbage blast with *B. rapa* genome		Band sequence in purple mustard blast with *B. rapa* genome	
	Identity	Ratio (%)	Identity	Ratio (%)	Identity	Ratio (%)
BrID11893	112/113	99	102/113	90	102/113	90
BrID111333	102/102	100	94/109	86	94/109	86
BrID10207	137/137	100	89/101	88	89/101	88
BrID10199	147/147	100	140/148	94	140/148	94
BrID11737	97/97	100	Not aligned	0	Not aligned	0
BrID101151	98/98	100	86/98	88	86/98	88
BrID11131	119/119	100	61/64	95	60/63	95
BrID101125	113/127	89	108/127	85	108/127	85
BrID101589	101/101	100	86/95	90	86/95	90

Brassica, thereby opening new horizons for the study of the genetics and evolution of this complex genus. In this study, purple Chinese cabbage was generated by distant hybridization and backcrossing of purple mustard and Chinese cabbage (Sun et al. 2006). Our cytological examination uncovered no additional chromosomes in this new cultivar (data not shown), suggesting that purple Chinese cabbage is a translocation line instead of a chromosome addition line. In mustard, we found that the purple leaf trait is controlled by a single dominant locus, as inferred by the expected Mendelian segregation ratio (data not

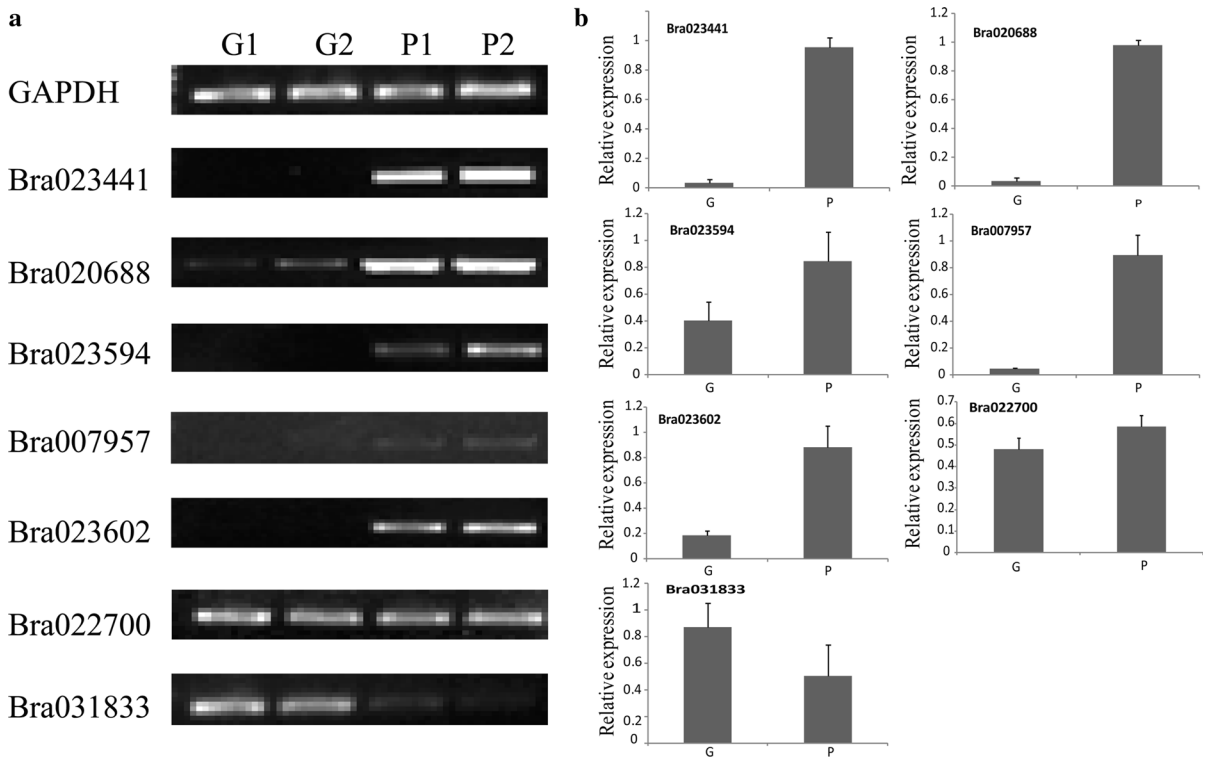

Fig. 5 Expression pattern of anthocyanin-related genes on chromosome A02 of Chinese cabbage. **a** Expression of anthocyanin-related genes in different tissues based on reverse-transcription PCR. **b** Expression of anthocyanin-related genes in different tissues based on quantitative real-time PCR

shown). In Chinese cabbage, purple leaves are also a qualitative character, but the inheritance of this trait does not follow Mendelian patterns because of the unstable characteristics of the extra chromosome fragment. Compared with traditional mapping populations, offspring of the heterozygous individuals in this F_2 population can quickly revert to green because the purple mustard fragment is easily lost and does not readily undergo recombination (Yan et al. 2015).

Many studies have been conducted with the aim of transferring and mapping alien genes among distantly related species (Jiang et al. 1993). In an attempt to transfer genes from barley to wheat, Islam and Shepherd (1992) isolated wheat-barley recombinant chromosomes induced by homologous pairing in the absence of the *Ph1b* gene. A primary goal of the present study was to map the *Anm* locus responsible for anthocyanin accumulation in Chinese cabbage. We mapped a candidate gene between markers BrID101151 and BrID10207 using a genetic mapping approach.

After aligning amplification band sequences with the *B. rapa* genome, we are certain that the polymorphic fragments were from purple mustard. We have therefore concluded that purple Chinese cabbage contains a genomic fragment from mustard 'M58' on chromosome A02, possibly caused by the insertion of the mustard fragment into Chinese cabbage (Fig. 6). Although translocation lines have the same chromosome number as the recipient parent and are expected to be more stable (Banga 1988), we nonetheless found a non-purple F_1 individual. Based on the current work, we speculate that the fragment is derived from the B genome of mustard because of its unstable inheritance. The B genome of mustard shares low homology with the A genome of Chinese cabbage, causing the chromosomal fragment to be easily lost and hindering recombination. Pending the release of the complete *B. juncea* genome sequence, however, we cannot confirm whether the mustard fragment is from the A or B genome. We can, nevertheless, assert that the gene controlling the purple leaf trait in Chinese cabbage is

from purple-leaf mustard and forms a translocation system between the different types.

Mediation by the *Anm* locus of the upregulation of anthocyanin biosynthetic genes in chromosome A02 of purple Chinese cabbage

The anthocyanin biosynthetic pathway is an intensively studied metabolic system in plants, with much effort directed toward the elucidation of anthocyanin biosynthetic genes at the molecular level (Winkel-Shirley 2001a). In *B. rapa*, 73 genes have been identified as orthologs of 41 anthocyanin biosynthetic genes in *A. thaliana* based on comparative genomic analysis between those two species (Guo et al. 2014). In the present study, we focused our attention on 12 anthocyanin biosynthetic genes of chromosome A02. Because purple Chinese cabbage contains a genomic fragment from purple mustard, the *Anm* locus on this fragment mediates the upregulation of five anthocyanin biosynthetic genes in Chinese cabbage, *CHS* (Bra023441), *UFGT* (Bra023594), *CHS* (Bra020688), *MYB-Like 2* (Bra007957) and *TT19* (Bra023602).

Bra023441 is an ortholog of AT5G13930 encoding chalcone synthase in *A. thaliana*. Chalcone synthase, a key enzyme involved in flavonoid biosynthesis, catalyzes the first committed step of the branch of the phenylpropanoid pathway that leads to flavonoid synthesis (Winkel-Shirley 2001b). The product of this reaction is required for the accumulation of purple anthocyanins in plant leaves and stems (De Jong et al. 2004; Sun et al. 2015). Yildiz et al. (2013) have proven that *chalcone synthase 1* transcripts accumulate at high levels in solid purple carrots, with low or no transcription in orange carrots.

Bra023594 is an ortholog of AT5G17050 encoding UDP-glycosyltransferase (*UFGT*) in *A. thaliana*. We found that transcripts of *UFGT* were highly abundant in purple Chinese cabbage. Considering the complexity of anthocyanin biosynthesis, the association of anthocyanin pigmentation with *UFGT* expression is not surprising. For example, *UFGT* is required for the synthesis of anthocyanin and 3-O-glucosylated flavonol (Tohge et al. 2005). Furthermore, the ectopic expression of *UFGT* produces purple-colored seed coats owing to the accumulation of anthocyanins in *Arabidopsis* (Lee et al. 2005). Boss et al. (1996) have reported that *UFGT* is expressed in red grapes but is undetectable in white grapes.

Another important gene, Bra023602, encodes a glutathione transferase belonging to the phi class of glutathione S-transferases (GSTs). Previous studies on model and ornamental plants have indicated that a member of the GST gene family is involved in vacuolar accumulation of anthocyanins (Marrs et al. 1995). In their most well-characterized role, GSTs catalyze the addition of a glutathione molecule to a heterocyclic organic anion (Edwards and Dixon 2000).

Although the molecular biology of anthocyanin biosynthesis has been studied in many plants, few studies evaluating anthocyanin expression or accumulation in Chinese cabbage have been previously reported. The expression of anthocyanin biosynthesis genes reported here improves the knowledge for studying this complex pathway and offers insights into the molecular mechanism of anthocyanin biosynthesis in Chinese cabbage.

The *Anm* locus as a novel gene for breeding purple *Brassica* species

Brassica crops have rich genetic resources for breeding new varieties by interspecific hybridization (Quiros et al. 1987). *B. rapa* ($2n = 2x = 20$, AA), *B. nigra* ($2n = 2x = 16$, BB) and *B. oleracea* ($2n = 2x = 18$, CC) are diploid species, whereas *B. juncea* ($2n = 4x = 36$, AABB), *B. carinata* ($2n = 4x = 34$, BBCC) and *B. napus* ($2n = 4x = 38$, AACC) are allotetraploid (Nagaharu 1935). Prior to our study, some purple trait loci had been investigated at the molecular level in *Brassica* varieties, such as zicaitai, purple cabbage, purple kale and purple cauliflower (Chiu et al. 2010; Guo et al. 2015; Hayashi et al. 2010; Zhang et al. 2012), but not purple mustard.

Based on the present analysis, we speculate that the *Anm* locus is derived from purple-leaf mustard and is located on chromosome A02 of Chinese cabbage, which has not been studied previously. As confirmed by the identification of distinct linkage groups representing the A and B genomes, mustard is an amphidiploid species originating from hybridization between the diploid species *B. rapa* and *B. nigra* (Axelsson et al. 2000). In this work, we used the new germplasm resource of Chinese cabbage with all purple leaves created by the interspecific hybridization of Chinese cabbage and purple mustard (Sun et al. 2006). The anthocyanin contents in this new cultivar

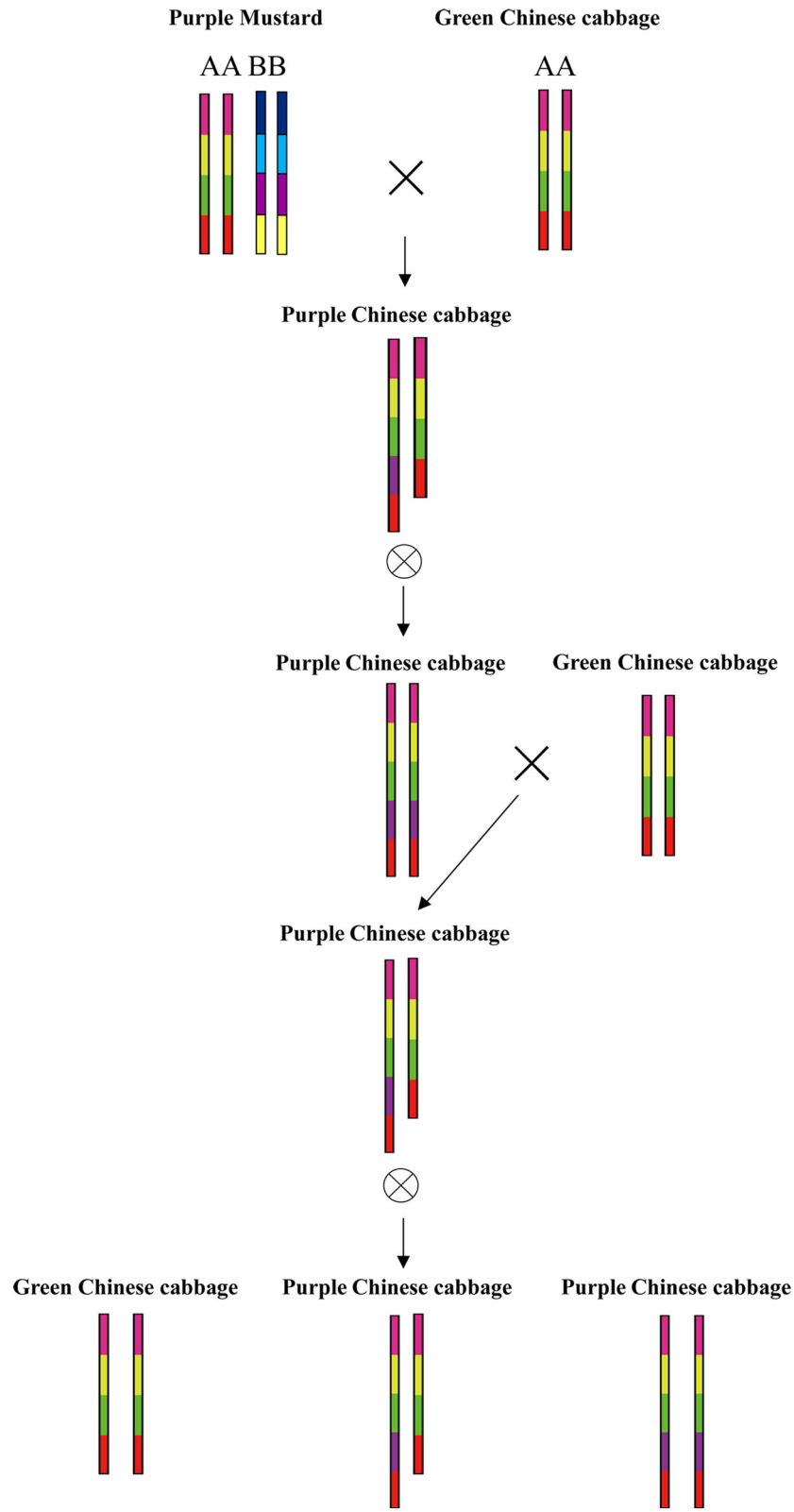

Fig. 6 Genetic model of purple leaf trait inheritance in Chinese cabbage. The *purple box* in the mustard chromosome depicts the mustard fragment being inserted into Chinese cabbage. The heterozygote is unstable, and the inserted fragment is easily lost. (Color figure online)

were highly accumulated, primarily because of regulation by the *Anm* locus, and can be used to breed new varieties. Above all, a better understanding of the *Anm* locus will contribute to the future development of conventional and transgenic *Brassica* cultivars with enriched anthocyanins levels.

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Author contribution statement SZ constructed the mapping populations and performed the genetic analysis and anthocyanin profile. PL performed mapping and expression analyses and wrote the paper. WQ extracted the DNA of the F_2 population and provided advice on the manuscript. SFZ, FL, HZ and XW provided advice on experiments. RS designed and supervised the work. All the authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that this study complies with the current laws of the countries in which the experiments were performed.

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