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Anthocyanin profile characterization and quantitative trait locus mapping in zicaitai (*Brassica rapa* L. ssp. *chinensis* var. *purpurea*)

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Abstract Anthocyanins have several biological functions in plants and are beneficial to human health. To elucidate the metabolic profile of anthocyanins and determine the genetic basis controlling anthocyanin accumulation in zicaitai (*Brassica rapa* L. ssp. *chinensis* var. *purpurea*), we conducted anthocyanin profile characterization and quantitative trait locus (QTL) analysis. Seventeen anthocyanin compounds were identified as cyanidin glycosides in zicaitai. A genetic linkage map based on 200 F2 lines was constructed using 161 insertion/deletion markers. Total anthocyanin content (TAC) was determined by pH differential spectrophotometry for the F2 lines. Using the map and phenotypic data, a major QTL which explained 56.7 % of phenotypic variation was identified for TAC on chromosome A09. Two genes, *BrEGL3.1* and *BrEGL3.2*, as syntenic orthologs of *AtEGL3* encoding basic helix–loop–helix transcription factors in this QTL region, are candidate genes for a key role in the control of anthocyanin accumulation in zicaitai.

Keywords Anthocyanin profile · QTL · *EGL3* · *Brassica rapa*

Introduction

Anthocyanin pigments are an important type of flavonoid compound and exhibit a wide range of biological functions in plants. These include serving as

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attractants for pollinators and seed dispersers, and protecting plants against abiotic and biotic stresses (Harborne et al. 1999; Andersen and Markham 2006). Anthocyanins have beneficial roles in human health, and are potentially protective factors against cancer and heart disease (Lam et al. 2009; van Poppel et al. 1999). These beneficial health properties are partially attributed to their strong antioxidant capacity (Yamasaki et al. 1997). Their bioavailability and activity varies widely depending on chemical structure and content (Prior and Wu 2006). Zicaitai (*Brassica rapa* L. ssp. *chinensis* var. *purpurea*) has purple petioles and flower stalks, and is a representative purple variety of *B. rapa* vegetables. The purple pigments of zicaitai have been identified as anthocyanins (Podsędek 2007). Although several studies have characterized anthocyanins in different *Brassica* species, they have only investigated either *B. oleracea*, including cauliflower and cabbage (Lo Scalzo et al. 2008), or *B. juncea*, including mustard (Mobin and Khan 2007) and red mustard greens (Lin et al. 2011).

Studies on the genetic or molecular mechanisms of anthocyanin biosynthesis and accumulation have been performed in many kinds of plants (Holton and Cornish 1995). The biosynthetic pathways of anthocyanins are well characterized, and corresponding genes have been isolated from various plants (Broun 2005; Koes et al. 2005; Grotewold 2006). In *B. rapa*, Burdzinski and Wendell (2007) mapped the *anthocyaninless* (*anl*) locus to chromosome A09 in a rapid-cycling *B. rapa* line using an F2 population. Hayashi et al. (2010) reported that anthocyanin pigmentation in purple turnip (*B. rapa* ssp. *rapa*) is controlled by a single dominant gene, and mapped a novel locus (*Anp*) regulating anthocyanin pigmentation on chromosome A07, based on a doubled haploid (DH) population from a cross between purple turnip and Chinese cabbage (*B. rapa* L. ssp. *pekinensis*). Microarray analysis on green and red Chinese cabbage identified anthocyanin-specific genes that likely play regulatory roles in anthocyanin production, and anthocyanin-related transcription factors that may be active in the pigmentation signaling pathway (Kim et al. 2010, 2011). Liu et al. (2013b) mapped the *pur* gene, controlling purple leaf color, to the end of chromosome A03 using an F2 population constructed from a Chinese cabbage line and a purple pakchoi (*B. rapa* L. ssp. *chinensis*) line and proposed that this phenotype was controlled by a dominant gene with an additive effect. In these studies, qualify distances of the closest flanking markers were too far from the target genes to determine candidate genes. Results from previous research indicate that the genetic or molecular mechanisms of anthocyanin biosynthesis and accumulation are complicated, and candidate genes are not identical in different purple varieties of *B. rapa*.

In this study, the anthocyanin profile was determined in zicaitai using a liquid chromatography–mass spectrometry (LC–MS) technique. We analyzed the total anthocyanin content (TAC) in zicaitai leaves using an F2 population derived from a cross between zicaitai and caixin (*B. rapa* L. ssp. *parachinensis*). We mapped this trait to a 1.9-Mb region on chromosome A09. Two syntenic orthologs of *AtEGL3*, *BrEGL3.1* and *BrEGL3.2*, encoding basic helix–loop–helix transcription factors, are candidate genes for controlling anthocyanin accumulation in zicaitai.

Materials and methods

Plant materials for UFLC-PDA-ESI/MS/MS and mapping population

Zicaitai DH line ZCT095 was used as the female parent and Caixin DH line L58DH as the male parent for construction of an F2 population. ZCT095 accumulates purple pigments in the petioles (Fig. 1a), whereas the leaves and petioles of L58DH are green (Fig. 1b). An F2 population consisting of 200 plants was constructed from a cross between these two parental lines. Leaves from parental and F2 lines were harvested 50 days after sowing, and tissues immediately freeze-dried for future anthocyanin extraction and DNA isolation.

Sample treatment and anthocyanin extraction

Anthocyanin extraction was performed in accordance with the method described by Lin and Harnly (2007), with slight modifications. Freeze-dried leaf material ($\sim$0.2 g) was extracted in 30 ml of acidified methanol (0.1 % HCl, v/v) by ultrasonication with an FS30 Ultrasonic sonicator (40 kHz, 100 W) (Fisher Scientific, Pittsburgh, PA, USA) for 30 min at room temperature (<35 °C). The acidified extraction solvent was necessary to prevent degradation of the anthocyanins (Longo and Vasapollo 2006). The slurry mixture was centrifuged (Centrifuge 5810R,

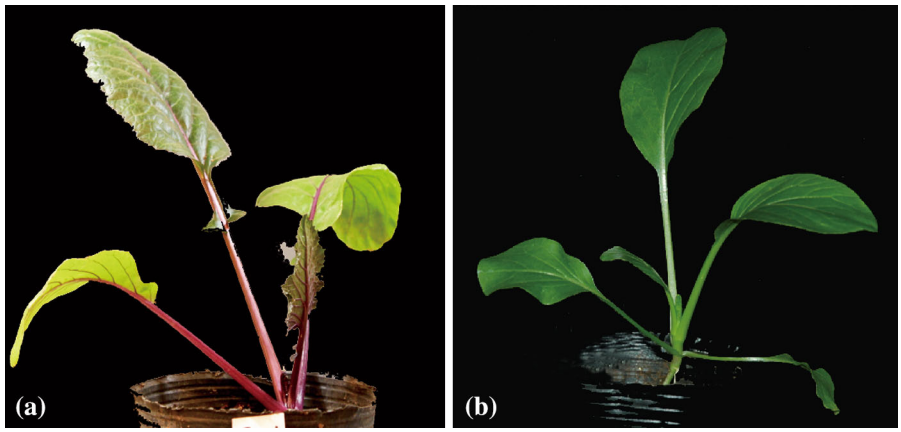

Fig. 1 **a** ZTC095 and **b** L58DH parental lines

Eppendorf, Germany) at 5,000 rpm for 15 min at 4 °C. The supernatant was collected and concentrated using a rotary evaporator (RV10, IKA, Germany) at 35 °C. The residue was reconstituted in 2 ml or 10 ml acidified water (0.1 % formic acid, v/v), and filtered through 0.22-µm filters for ultra-fast liquid chromatography (UFLC)–ultraviolet (UV) triple quadrupole linear ion trap mass spectrometry (Q-Trip), time-of-flight (TOF) mass spectrometry, or pH differential spectrophotometry analysis.

Anthocyanin profile characterization

The chromatographic system, which was set up in accordance with the method described by Lin et al. (2011) and Ferreres et al. (2008) with modifications, consisted of an UFLC system (Prominence^{TM} UFLC, Shimadzu, Japan) equipped with an ultraviolet detector. Chromatographic separation was achieved using a Shim-pack XR-ODS column (75 mm × 2.0 mm i.d., 2.2 µm particle size; Shimadzu, Japan) at a flow rate of 0.25 ml/min. The column oven temperature was set at 40 °C. The mobile phase was a binary solvent system consisting of solvent A (formic acid 0.1 % v/v in water) and solvent B (acetonitrile). The gradient conditions were as follows: 5–15 % B from 0 to 25 min, maintained at 15 % B for 20 min; 15–18 % B from 45 to 60 min; 18–95 % B from 60 to 70 min, maintained at 95 % B for 2 min; and 5 % B from 72 to 75 min for equilibration of the column for the next run. The sample injection volume was 5 µl. Chromatograms were recorded at 520 nm for anthocyanins. The analytes were identified using a triple quadrupole linear ion trap mass spectrometer (AB SCIEX 5500 Qtrap^{TM}, USA) equipped with a turbo-spray ion source. Product ion spectra (MS2 and MS3) were acquired using enhanced product ion (EPI) and MS3 profile scans in positive ionization modes. High-resolution accurate mass measurements were made using a 6540 Agilent quadrupole/time-of-flight mass spectrometer (Q-TOF, Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source. Mass spectrometer acquisition was operated in the positive ion mode with accurate mass ranging from 100 to 1,700 *m*/*z* at resolution 100,000.

pH differential spectrophotometry

The pH differential spectrophotometry method was performed in accordance to that reported by (Lee et al. 2005), with slight modifications, to determine total anthocyanin content (TAC) in parental and F2 lines. Absorbance was measured at 520 and 700 nm. The TAC was expressed as cyanidin-3-glucoside (cyd-glu, molar extinction coefficient 26,900 l/cm mol, molecular weight 449.2 g/mol). The units for TAC were mg/g dry weight (DW) of detected samples. Measurements of TAC in all samples were replicated three times.

Molecular marker assays

DNA from parents and F2 individuals was isolated from freeze-dried mature leaves, as described by Wang et al. (2005).

The two parental lines were re-sequenced to a depth of 5× using the Illumina sequencing platform service provided by the BGI Tech Solutions Co., Ltd. The genome sequence of *B. rapa* was retrieved from BRAD (http://brassicadb.org/brad/) and used as the reference sequence. Genome-wide detection of short insertion/deletion length polymorphisms (InDels, <5 bp) and the development of InDel markers were performed as described by Liu et al. (2013a). In total, 300 pairs of InDel primers uniformly distributed (~2,000 kb) across the whole genome were designed to construct the linkage map. Each 20-μl polymerase chain reaction (PCR) mixture contained 2 μl PCR buffer, 1.6 μl dNTPs (2.5 mM each), 0.4 μl *Taq* DNA polymerase (2.5 U/μl), 2 μl DNA template (50 ng/μl), 0.6 μl forward primer (10 μM), 0.6 μl reverse primer (10 μM), and 12.8 μl ddH_2O. The reaction mixture was incubated in a thermal cycler (9700, ABI, USA) at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis on 8 % polyacrylamide gel running at 160 V for 1.5 h, and then subjected to silver staining for band statistics (Bassam et al. 1991).

Construction of linkage map

The linkage map was constructed with JoinMap 4.0 (http://www.kyazma.nl/) using a minimum logarithm of odds (LOD) score of 4.0 (Van Ooijen 2006). The linkage groups (LGs) were determined using a LOD threshold ≥4.0 and a maximum recombination fraction of 0.4. The Kosambi mapping function was used to obtain genetic distances between markers (Kosambi 1944).

Statistical analysis and quantitative trait locus (QTL) mapping

The software MapQTL 5.0 (Van Ooijen 2004) was used to detect QTLs by both interval mapping and multiple QTL mapping (MQM) methods. In regions containing putative QTLs, markers with the highest LOD values were taken as co-factors. The LOD thresholds for QTL significance were determined by permutation tests (1,000 replications) in MapQTL with a genome-wide significance level of $P = 0.05$ for significant linkages; LOD = 3.0 was used as the significance threshold based on the permutation test. Frequency distribution and ANOVA analysis of TAC was performed using the SPSS version 12.0.1 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Anthocyanin profile analysis in zicaitai

Several anthocyanin groups (e.g., cyanidin, pelargonidin, and delphinidin) exist in the plant kingdom (Tanaka et al. 2008). To characterize the anthocyanin profile in zicaitai and caixin, we performed UFLC-UV-Q-trip/TOF MS analysis on the parental lines. In total, 17 anthocyanin compounds were detected in zicaitai ZCT095 (Fig. 2a; Table 1), while no anthocyanin was detected in caixin L58DH (Fig. 2b). Identification of anthocyanins in extracts was based on chromatographic behavior, accurate molecular masses obtained by Q-TOF mass spectrometry, characteristic MS/MS fragmentation product ions obtained by Q-Trip mass spectrometry, UV spectra, and comparison with scientific publications (Mazza and Miniati 1993; Wu and Prior 2005; Lin et al. 2011). All 17 anthocyanins were different forms of cyanidin glycosides, with glycosyl groups at both the 3- and 5-positions. The glycosyl groups at the 3-position were di/triglucosides; these were acylated by coumaroyl, sinapoyl, caffeoyl, and feruloyl. Glucoside was the only glycosylated modification at the 5-position, and some of the anthocyanins were acylated by malonyl. Therefore, all 17 anthocyanins were different acylated cyanidin 3-di/triglucosides-5-glucosides. The use of UFLC-UV-Q-Trip and UFLC-Q-TOF analysis generated a comprehensive metabolic profile of anthocyanins in zicaitai (Table 1).

Quantitative variation in total anthocyanin content

The TAC of the parents and F2 lines was determined by pH differential spectrophotometry. The TAC of the female parental line ZCT095 and the male parental line L58DH was 0.8 mg/g DW and 0.0 mg/g DW, respectively. The purpleness of plants and plant extracts from the F2 population varied widely in comparison to the purple or green seen in the parental lines (Fig. 3a); this indicated that anthocyanin accumulation was under multigenic control in zicaitai leaves. The TAC of the F2 population ranged from 0.0

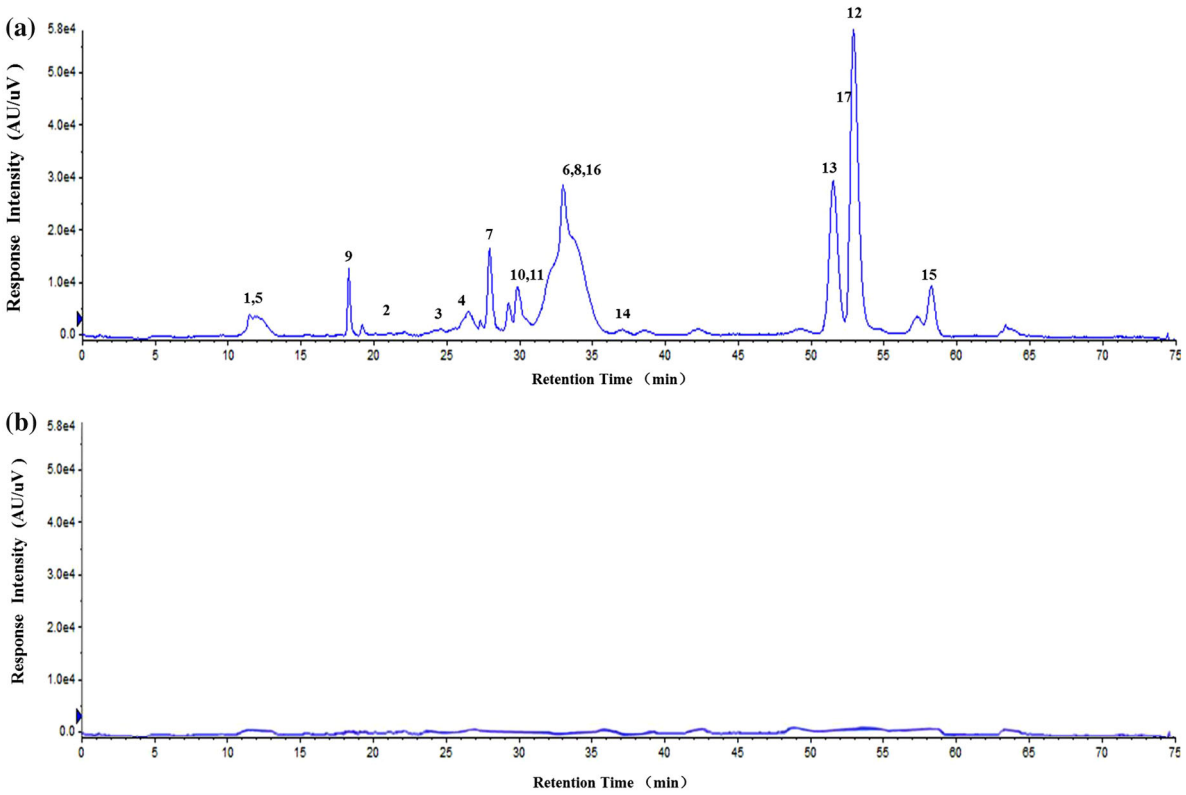

Fig. 2 Liquid chromatography chromatograms (520 nm) of **a** Zicaitai ZCT095 and **b** Caixin L58DH anthocyanin extracts. *Horizontal axis* shows retention time (min); *vertical axis* gives the strength of the chromatographic peak response (AU/uV). *Labels* correspond to compounds shown in Table 1

to 0.78 mg/g DW, and the average TAC was 0.19 mg/g DW. As the variation among F2 lines for TAC was between that of the parental lines, it indicated that this trait showed inter-parent variations and that alleles with positive effects for anthocyanin accumulation might only exist in the female parental line. The TAC of the F2 population showed a bias distribution lower than the parent, at approximately 67 % lower than the average level.

Construction of the genetic linkage map

A total of 1,228 InDel primer pairs, comprising 928 previously designed (Wang et al. 2011; Liu et al. 2013a) and 300 newly developed (Supplementary Table 1) for this study, were screened for polymorphisms between the parental lines. Seventy-two of the previously developed InDel primer pairs and 107 newly developed ones showed polymorphisms between parents. The 179 polymorphic InDel markers were relatively evenly distributed on the *B. rapa* genome, with 12, 14, 17, 18, 19, 18, 15, 11, 35, and 16 on chromosomes A01–A10 respectively, and a further four located on unassigned scaffolds. All polymorphic primers returned reliable PCR products, and could distinguish parental and heterologous genotypes in the F2 population. These markers were then used to genotype the F2 mapping population. A total of 161 InDel markers were assigned to 10 linkage groups (LGs); these covered a genetic distance of 1,314.3 cM, with an average internal length of 8.16 cM between two adjacent markers (Fig. 4). The linkage groups were anchored to their corresponding reference chromosomes in accordance with the physical positions of the markers.

Identification of a QTL controlling total anthocyanin concentration in zicaitai

In total, two QTLs associated with this trait were identified that satisfied the threshold LOD of >3. A major QTL was identified on chromosome A09 with a

Table 1 UFLC-PDA-Q-Trip/TOF data and putative identification of anthocyanins in zicaitai ZCT095

Peak no.	t_R (min)[a]	$[M]^+$ wt[b]	Major and important productions (*m*/*z*)	Anthocyanins
1	12.35	859.2131	611, 535, 491, 287	Cyanidin 3-diglucoside-5-malonlyglucoside
2	21.00	935.2453	773, 449, 287	Cyanidin 3-caffeoyldiglucoside-5-glucoside
3	24.27	919.2502	757, 449, 287	Cyanidin 3-coumaroyldiglucoside-5-glucoside
4	25.94	949.2614	787, 449, 287	Cyanidin 3-feruloyldiglucoside-5-glucoside
5	11.49	979.2747	817, 449, 287	Cyanidin 3-sinapoyldiglucoside-5-glucoside
6	32.97	1,005.2514	757, 535, 491, 287	Cyanidin 3-coumaroyldiglucoside-5-malonylglucoside
7	26.95	1,021.2411	773, 535, 491, 287	Cyanidin 3-caffeoyldiglucoside-5-malonylglucoside
8	34.77	1,035.2629	787, 535, 491, 287	Cyanidin 3-feruloyldiglucoside-5-malonylglucoside
9	18.28	1,065.2685	817, 535, 287	Cyanidin 3-sinapoyldiglucoside-5-malonylglucoside
10	30.48	1,125.3081	963, 449, 287	Cyanidin 3-diferuloyldiglucoside-5-glucoside
11	31.9	1,155.3224	993, 449, 287	Cyanidin 3-sinapoylferuloyldiglucoside-5-glucoside
12	53.42	1,167.31	919, 535, 287	Cyanidin 3-coumaroyltriglucoside-5-malonylglucoside
13	51.57	1,211.3077	963, 535, 287	Cyanidin 3-diferuloyldiglucoside-5-malonylglucoside
14	37.39	1,197.2923	949, 535, 287	Cyanidin 3-feruloylcaffeoyldiglucoside-5-malonylglucoside
15	58.32	1,211.3097	963, 535, 287	Cyanidin 3-coumaroylsinapoyldiglucoside-5-malonylglucoside
16	33.02	1,227.3191	979, 535, 287	Cyanidin 3-caffeoylsinapoyldiglucoside-5-malonylglucoside
17	52.94	1,241.3186	1,197, 993, 535, 491	Cyanidin 3-feruloylsinapoyldiglucoside-5-malonylglucoside

[a] t_R: retention time

[b] $[M]^+$: high-resolution mass spectrometry (HRMS) mass

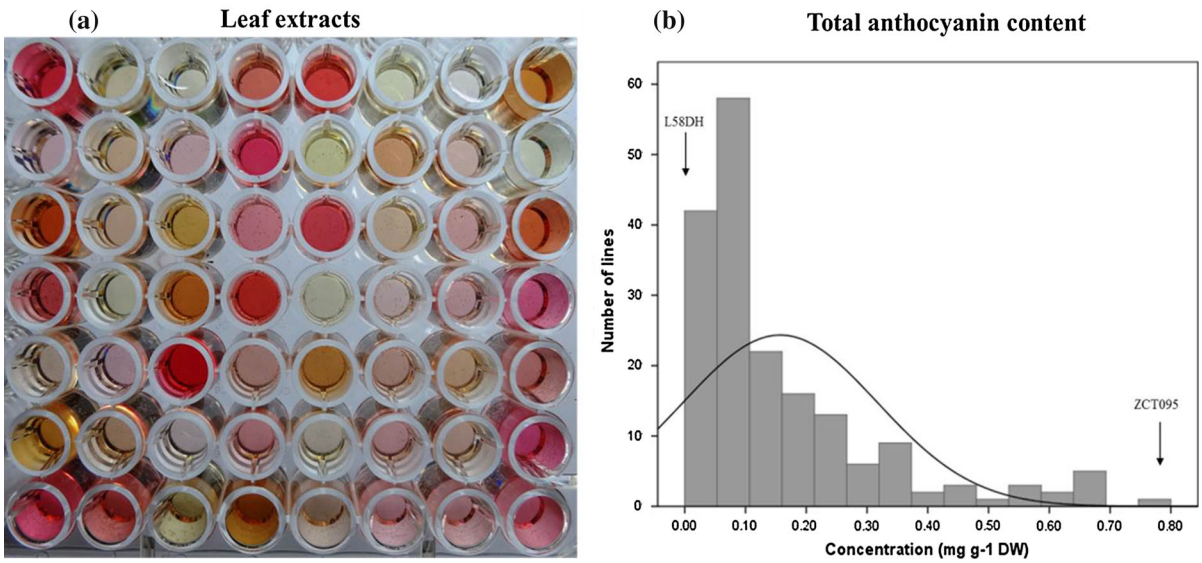

Fig. 3 **a** Leaf extracts and **b** frequency distribution of total anthocyanin content in the Zicaitai × Caixin F2 population. The parental values are indicated by *black arrows*

LOD value of 22.95, and explained 56.7 % of TAC variation in the F2 population (Fig. 5). The flanking markers of this major QTL were BrID11259 and BrID11861. This major QTL showed a positive additive effect, indicating that the purple parent ZCT095 at this locus mostly increased the

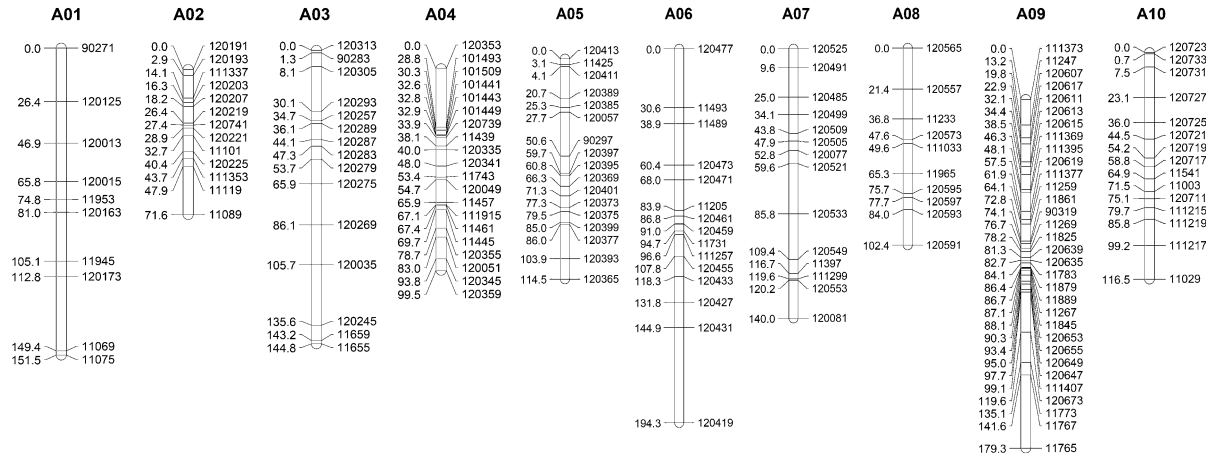

Fig. 4 Genetic linkage map based on a population of 200 F2 lines of *B. rapa*. Marker loci are listed to the *right*, and recombination distances (cM) to the *left* of each linkage group. Marker IDs listed in this linkage map omit "BrID" in front of the ID number; for example, "120013" and "90271" represent "BrID120013" and "BrID90271", respectively, in Supplemental Table S1 and previous studies (Wang et al. 2011)

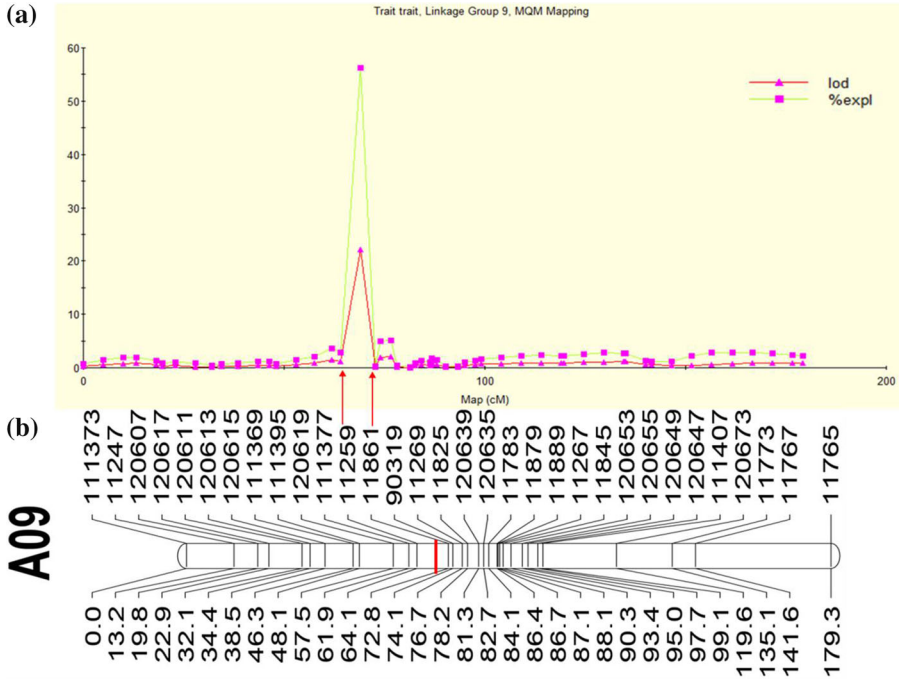

Fig. 5 The major QTL on chromosome A9 for total anthocyanin content in zicaitai. **a** *Red* and *green lines* indicate the LOD value and contribution rate of the QTL, respectively. **b** Position of flanking markers surrounding the major QTL on chromosome A09; the *red line* indicates the peak position of the QTL. (Color figure online)

anthocyanin accumulation. A minor QTL was observed on chromosome A07 between InDel markers BrID120533 and BrID120549 with a LOD value of 6.10, and 16.3 % of TAC variation was explained by this QTL which showed a negative additive effort opposite to the major one. Taken together, the major QTL on chromosome A09 might play a key role in the anthocyanin accumulation of zicaitai.

Identification of candidate genes

The genetic distance between the two flanking markers of the major QTL identified on chromosome A09 was 8.7 cM; this corresponds to a physical distance of 1.9 Mb. There are 274 annotated genes distributed in this region. As *B. rapa* is closely related to *Arabidopsis thaliana*, comparative genomic analysis between *B.*

rapa and *A. thaliana* allowed genes involved in the anthocyanin biosynthesis pathway of *B. rapa* to be identified on a genome-wide level (Guo et al. 2014). We evaluated the 274 predicted genes within the QTL interval to identify potential genes involved in the anthocyanin biosynthesis pathway. Two candidate genes, *BrEGL3.1* (Bra027796) and *BrEGL3.2* (Bra027653), which are syntenic orthologs of *AtEGL3*, were located in this region.

We compared re-sequencing data from the parental lines to the Chiifu-401-42 reference genome to reveal sequence variations in these two genes between the parents. For *BrEGL3.1*, we found two InDels and 34 single-nucleotide polymorphisms (SNPs). The two InDels, and 18 of the 34 SNPs, were located in non-coding region (introns). The 16 SNPs located in the coding region (exons) comprised six synonymous and 10 nonsynonymous mutations, resulting in eight amino acid substitutions (Supplementary Fig. 1a). Of the eight amino acid substitutions, one (Leu180Phe) was located in the MYB interacting region (MIR), five (Ser226Lys, Ala342Thr, Ser379Asn, Lys394Thr, Ile409Val) were located in the domain of interaction with WD40, and two (Asp490Glu and Ser529Arg) were located in the carboxy-terminal end (C-ter), while there were no variations in the basic helix–loop–helix (bHLH) domain. Three substitutions (Ser226Lys, from polarity without charge to polarity with positive; Ala 342Thr, from nonpolarity to polarity without charge; Lys394Thr, from polarity with positive to polarity without positive) in the domain of interaction with WD40 and one (Ser529Arg, from polarity without charge to polarity with positive) located in the carboxy-terminal end (C-ter) caused changes in the chemicophysical properties of amino acid residues. The sequence variations of *BrEGL3.2* between the parental lines were relatively less than those of *BrEGL3.1*, and included two InDels and 10 SNPs. The two InDels were located in non-coding regions, while the 10 SNPs were all located in coding sequences, leading to two amino acid substitutions (Gly233Glu and Leu352Phe) which were both in the domain of interaction with WD40 (Supplementary Fig. 1b), and the substitution of Gly233Glu changed the chemicophysical property of the amino acid residue from polarity without charge to polarity with negative. The amino acid substitutions of *BrEGL3.1* and *BrEGL3.2* between the parents were mainly located in the domain of interaction with WD40 proteins, while there were no amino acid substitutions in the bHLH domain. For *BrEGL3.1*, there was one amino acid substitution in the MYB-interacting region, five in the domain of interaction with WD40 proteins, and two in the C-ter region. Of these amino acid substitutions, three in the domain of interaction with WD40 proteins and one in the C-ter region showed different chemicophysical properties. The two amino acid substitutions between parents of *BrEGL3.2*, one of which had identical characteristics, were both in the domain of interaction with WD40.

Discussion

Cyanidin glucosides are the major anthocyanins in zicaitai

Anthocyanin compounds are the sugar-conjugated forms of anthocyanidins, and are widely dispersed throughout the plant kingdom. The chemical structure of the anthocyanin determines its stability, color intensity, and potential biological activity. The commonest anthocyanins are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, with cyanidin being the most common in *Brassica* crop species (Tatsuzawa et al. 2006; Scalzo et al. 2008; Lin et al. 2011). In *Brassica*, anthocyanins are complex, glycosylated with one to five sugar moieties, and acylated with different hydroxycinnamic acids (Podsędek 2007; Moreno et al. 2010). Until now, no study has comprehensively analyzed anthocyanin composition in zicaitai, a species with high accumulation of anthocyanin in the petiole.

In this study, despite the lack of commercial available standards, the accuracy of the mass measurement of MS^n fragmentation analysis by Q-trap and TOF MS, together with UV/visible absorption spectra, allowed identification of the anthocyanin composition with high reliability. A total of 17 cyanidin glycosides were identified. The glycosylated and acylated properties revealed the chemical structural characteristics of these anthocyanins in zicaitai (Table 1), and lay a foundation for further investigations into the genetic mechanisms and genes that control anthocyanin pigmentation in *B. rapa*.

Purple pigmentation is a quantitative trait in zicaitai

The genetic mechanism controlling purple pigmentation in *Brassica* is complex. Previous studies revealed

that the purple characteristics in rapid-cycling *B. rapa*, purple turnip, purple pakchoi, and purple cauliflower (*B. oleracea* var. *botrytis*) were qualitative traits controlled by different single genes, and that purple is dominant over green (Burdzinski and Wendell 2007; Hayashi et al. 2010; Chiu et al. 2010). In this study, we investigated the purple characteristics of an F2 population derived from crossing zicaitai and caixin. TAC showed continuous variation in the F2 population, indicating that the genetic mechanism of anthocyanin accumulation in zicaitai was under multigenic control. This is in contrast to the previously reported single gene control observed in purple turnip, purple pak choi, rapid-cycling *B. rapa*, and purple cauliflower. This indicates that anthocyanin accumulation in plants is complicated. The different anthocyanin compounds may involve specific gene control, and for some compounds it is necessary to activate a whole branch of the biosynthesis pathway. Future studies of different *B. rapa* purple crops will helpfully clarify gene function in each step of the anthocyanin biosynthesis pathway.

The major QTL is a novel locus regulating anthocyanin pigmentation in *B. rapa*

A major QTL controlling anthocyanin accumulation in zicaitai was mapped to chromosome A09. In previous studies, three loci were reported to control anthocyanin pigmentation in different *B. rapa* varieties, namely *anl* on chromosome A09 in rapid-cycling *B. rapa* (Burdzinski and Wendell 2007), *Anp* on chromosome A07 in purple turnip (Hayashi et al. 2010), and *Pur* on the end of chromosome A03 in purple pak choi (Liu et al. 2013a, b). Using flanking simple sequence repeat markers of *anl*, we compared the position of locus *anl* with the major QTL identified in this study. The *anl* locus was located within the 20,696,245–23,679,772 region of chromosome A09, while the newly identified major QTL was located in the 6,068,769–8,028,904 interval on A09, a physical distance far from *anl*. We therefore assume that this QTL is a novel locus regulating anthocyanin accumulation in *B. rapa*.

BrEGL3.1 and *BrEGL3.2* are candidate genes controlling anthocyanin accumulation in zicaitai

Transcriptional regulation of structural genes is a major mechanism by which anthocyanin biosynthesis is regulated in plants. R2R3-MYB and bHLH transcription factors and WD40 proteins represent the three major families of anthocyanin regulatory proteins (Paz-Ares et al. 1987; Chandler et al. 1989; Ludwig and Wessler 1990; de Vetten et al. 1997; Quattrocchio et al. 1999). They form regulatory complexes that activate expression of anthocyanin structural genes (Goff et al. 1992; Grotewold et al. 2000). In *Arabidopsis thaliana*, bHLH transcription factors playing important roles in anthocyanin biosynthesis regulation include TT8 (TRANSPARENT TESTA 8), GL3 (GLABROUS 3), and EGL3 (ENHANCER OF GLABRA 3) (Nesi et al. 2000; Zhang et al. 2003). Of these, EGL3 has a major role in the regulation of anthocyanin biosynthesis in *A. thaliana*, with activation of *DFR* and *ANS*/*LDOX* predominantly due to *EGL3* (Gonzalez et al. 2008). Alteration of the bHLH transcription factor *In1* causes an increase in red pigment production in maize aleurone (Burr et al. 1996). Knockout of bHLH *Rc* function through frame-shifting changes the color of seed pericarps from red to white in rice (Sweeney et al. 2006). An insertion of a DNA transposon into the bHLH regulatory gene *bHLH2* alters the color of common morning glory (*Ipomoea tricolor*) flowers to pale pigmented flowers (Park et al. 2007). Two syntenic orthologs of *AtEGL3*, *BrEGL3.1* and *BrEGL3.2*, were located within the QTL region identified in this study. The re-sequencing data revealed that there were eight non-synonymous substitutions of *BrEGL3.1* in the purple parent ZCT095. The protein sequence of *BrEGL3.1* from the male line L58DH was the same as that from Chiifu-401-42, the Chinese cabbage inbred line used for whole-genome sequencing. None of the non-synonymous substitutions of *BrEGL3.1* and *BrEGL3.2* was in the bHLH domain, while three amino acid substitutions with variant chemicophysical properties of *BrEGL3.1* in the domain of interaction with WD40 proteins may influence the interaction between BrEGL3.1 and WD40 as well as the stability of the ternary transcriptional complex for activation of anthocyanin biosynthetic structure genes. These results indicate that *BrEGL3.1* may be responsible for enhanced anthocyanin biosynthesis in ZCT095. As there is another syntenic ortholog within the QTL interval, it will be interesting to know whether the two copies are functionally diversified. Studies combining gene expression profiling with anthocyanin metabolic

profiling of accessions with different colors, and fine mapping based on a larger F2 population, will be conducted to clarify the genetic mechanisms underlying anthocyanin accumulation in zicaitai.

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