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REVIEW

Carotenoid identification and molecular analysis of carotenoid isomerase-encoding *BrCRTISO*, the candidate gene for inner leaf orange coloration in Chinese cabbage

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Abstract In Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*), the locus associated with orange inner leaves has been mapped. The mechanism responsible for carotenoid pigment synthesis in orange inner leaves of Chinese cabbage is still unclear, however, and limited molecular research has been conducted on the candidate gene. In this study, high-performance liquid chromatographic analysis indicated that orange leaf coloration in Chinese cabbage is due to accumulation of prolycopene and other carotenoid pigments. Mapping of simple sequence repeat and insertion/deletion markers from 269 individuals of an F_2 population pinpointed the *Bror* locus within a 1.0-cM interval, with the physical distance of 65.555 kb, on *B. rapa* chromosome A09. Using *B. rapa* genome annotation information, the candidate gene *BrCRTISO* (*Bra031539*) was predicted to reside within the mapped *Bror* locus. Coding and promoter regions were sequenced in white- and orange-type homozygous lines. A 6-bp deletion and 53 single nucleotide polymorphisms (SNPs), corresponding to two glutamic acid deletions and 12 amino acid mutations, were identified in the coding sequence of the orange-type cultivar. The orange-type promoter was approximately 141 bp shorter than that of the white type, and many SNPs were located in the promoter region. Two functional markers were designed and found to cosegregate with the *Bror* locus in F_2 individuals. Expression analysis demonstrated that *BrCRTISO* transcription levels were higher in white-type Chinese cabbage than in the orange-type cultivar. These results offer insights into the molecular mechanism of

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carotenoid biosynthesis and lay the foundation for molecular breeding of orange-type Chinese cabbage.

Keywords Chinese cabbage · Orange inner leaves · Carotenoids · *Bror* candidate gene · *BrCRTISO* · Sequence analysis · Expression analysis

Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) originated in China, and is an important vegetable crop worldwide. The inner leaves are found in various colors, such as white, yellow and, rarely, orange. Orange-type Chinese cabbage varieties, which were first developed by hybrid mutation in Japan (Yu et al. 2005), contain significant amounts of carotenoids compared with white-type cultivars (Watanabe et al. 2011). Using C_{18} high-performance liquid chromatography (HPLC), Li (2011) detected lutein, β-carotene, α-carotene and lycopene in orange-type Chinese cabbage, and reported that the orange coloration of the inner leaves is due to β-carotene accumulation. Conversely, Lee et al. (2014) attributed this orange coloration to the accumulation of lycopene compounds.

The orange color of the inner leaves of Chinese cabbage is controlled by a single recessive locus, *Bror* (Feng et al. 2012; Zhang et al. 2011). A wide variety of molecular markers have been linked to the *Bror* locus, including those based on random amplified polymorphic DNA (RAPD), sequence-characterized amplified regions (SCARs), simple sequence repeats (SSRs) and insertions/deletions (InDels) (Feng et al. 2012; Su et al. 2014; Zhang et al. 2008, 2013), and the *Bror* locus has been delimited to a 9.47-kb genomic region (Su et al. 2014). By comparing orange- and yellow-type cultivars, Lee et al. (2014) identified a candidate gene, *BrCRTISO1*, and found many sequence variations within the orange-type cultivar.

Carotenoids are yellow, orange and red pigments that exhibit a variety of functions in all photosynthetic organisms (Sandmann et al. 2006). These pigments are vital for collecting light energy and protecting the photosynthetic apparatus against harmful reactive oxygen species produced by over excitation of chlorophyll (Grotewold 2006; Isaacson et al. 2002). Our understanding of carotenoid biosynthesis has changed considerably in recent years (Hirschberg 2001). The carotenoid synthetic pathway, which begins with the formation of phytoene, involves a series of reactions including desaturations, cyclizations, hydroxylations and epoxidations (Park et al. 2002). In cyanobacteria and plants, four desaturation reactions are involved in the conversion of phytoene to lycopene (Supplementary Fig. 1). To complete the synthesis of lycopene from phytoene, plants require three enzymes: phytoene desaturase, ζ-carotene desaturase and carotenoid isomerase (CRTISO) (Bartley et al. 1999).

In the carotenoid biosynthetic pathway, CRTISO plays an important role by converting prolycopene (7Z,9Z,7′Z,9′Z-tetra-*cis*-lycopene) to all-*trans*-lycopene via *cis*-configuration intermediates in non-green tissue and via light in green tissue (Breitenbach et al. 2001; Isaacson et al. 2002; Masamoto et al. 2001; Park et al. 2002). The existence of a potential carotenoid isomerase enzyme has been inferred from phenotypes of recessive mutations in *Lycopersicon esculentum* (Tomes et al. 1953) and *Scenedesmus* (Ernst and Sandmann 1988). In tangerine tomato, loss of function of *CRTISO* causes prolycopene to accumulate instead of all-*trans*-lycopene (Isaacson et al. 2002). Similarly, deletions in an allele at the *CRTISO* locus in *Arabidopsis thaliana* causes accumulation of poly-*cis*-carotene in dark-grown *ccr2* plants and a reduction in lutein in light-grown ones (Park et al. 2002). In *Oryza sativa*, mutation of the *ZEBRA2* (*CRTISO*) gene results in the accumulation of the all-*trans*-lycopene precursor and prolycopene in dark-grown *zebra2* tissues (Chai et al. 2011).

The current study was conducted to comprehensively and fully identify the carotenoid components responsible for orange leaf color in Chinese cabbage. We also aimed to determine whether this coloration is controlled by the same gene in different orange-type cultivars and to analyze the candidate gene at the molecular level. In this study, HPLC was used to identify carotenoid pigments in the leaves of different Chinese cabbage lines, and an F_2 segregating population for the orange-color trait was used for fine mapping. We also analyzed promoter and coding sequences of *Bror* candidate genes. Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) were used to analyze the expression of *BrCRTISO* in different tissues. Two functional markers that were found to cosegregate with the *Bror* locus in F_2 individuals should be useful for marker-assisted

selection breeding. Our results lay a foundation for the elucidation of molecular mechanisms of carotenoid biosynthesis in orange inner leaves of Chinese cabbage.

Materials and methods

Plant materials

Chinese cabbage homozygous lines A21530, A30110, JiHong No. 82 and A14316 with orange inner leaves, as well as white-type A21445, were used in the present study. An F_2 mapping population was developed from a cross between orange-type A21530 and white-type A21445. All plant materials were provided by the Chinese Cabbage Research Group of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences.

Carotenoid analysis by HPLC

Extraction of carotenoids from Chinese cabbage inner leaves followed unpublished Chinese national standards. To extract leaf pigments, 0.500-g portions of dry samples were placed in a mortar with quartz sand and covered with acetone–petroleum ether solution (1:1, v/v) containing 0.1 % 2,6-di-*tert*-butyl-cresol. After grinding, fully milled samples were transferred to a core funnel for vacuum filtration, and the filtrates were collected in test tubes. The above steps were repeated until sample filtrates were colorless. All filtrates from a given sample were then transferred to a round bottom flask in a 35 °C water bath and concentrated on a rotary evaporator to near dryness. The residues were dried under a nitrogen stream and dissolved in 3.00 mL of 1:1 (v/v) acetone–acetonitrile solution. A C_{30} column (250 × 4.6 mm, 5 μm) coupled to a HPLC device (Waters Alliance 2695, Sigma-Aldrich, USA) and a photodiode array detector (Waters 2998 PDA) was used in the subsequent HPLC analysis. Solutions were eluted by HPLC with an acetonitrile (A) and acetone gradient at a constant flow rate of 1.5 mL/min as follows: 100 to 95 % A for 10 min, 95 to 40 % A for 40 min, 40 to 30 % A for 60 min, 30 to 10 % A for 80 min, and then 0 % A.

Absorption spectra of carotenoids were detected at a wavelength of 450 nm. A mixed standard composed of capsanthin, lutein, zeaxanthin, cryptoxanthin and β-carotene was prepared by dilution of a stock solution in methyl t-butyl ether–methanol (1:1, v/v) to 1 mg/L. For quantification of lutein, a lutein standard was purchased from Sigma-Aldrich. Semi-quantification of prolycopene, lycopene and other carotenoids was performed based on a calibration curve prepared using the lutein standard, with the *y*-axis equal to the peak area divided by the molar absorption coefficient of lutein at 450 nm and the *x*-axis corresponding to the injection concentration. The peak area was obtained using Millennium chromatography software (Waters). Carotenoids were identified by their characteristic absorption spectra, distinctive retention times and, in some cases, comparison with authentic standards and literature data (Isaacson et al. 2002; Park et al. 2002; Rodriguez-Amaya 2001; Taylor et al. 2006).

Genetic mapping of the orange inner leaf gene in Chinese cabbage

Genomic DNA was extracted from dried leaves by the conventional cetyltrimethylammonium bromide method (Chen and Ronald 1999) and stored at −20 °C. After extraction of genomic DNA from individual plants, white and orange DNA bulks were made (10 white or 10 orange inner leaves of F_2 individuals/bulk). 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). InDel markers were developed based on the *B. rapa* genomic sequence, and a sequence-based genetic linkage map was constructed (Wang et al. 2011). SSR and InDel markers covering the *Bror* locus are shown in Supplementary Table 1.

Candidate gene screening and sequence analysis

To screen for candidate 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 *Arabidopsis thaliana,* and genetic markers and maps of *B. rapa*. Carotenoid pathway genes were priorities for screening.

Coding and promoter sequence primers were designed according to the BRAD database, with functional markers designed from polymorphic

regions of coding and promoter sequences. Primer sequences are provided in Supplementary Table 2. For sequence alignment, we used MEGA 4 software (Tamura et al. 2007). *Cis*- and *trans*-acting elements of the *BrCRTISO* promoter region were predicted using PLACE (http://www.dna.affrc.go.jp/PLACE/index.html) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) online analysis software programs.

Expression analysis by RT-PCR and qRT-PCR

Total RNA was extracted separately from each Chinese cabbage sample. Single-strand cDNA was synthesized using a TransGen kit (TransGen, China) according to the manufacturer's protocol. A 25-fold dilution of the resulting cDNA was used for RT-PCR and qRT-PCR. Chinese cabbage *CRTISO*-specific primers were designed using Primer 5 software. Primer specificity was first confirmed by BLASTing 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. *BrCRTISO*-specific primers were 5′-GGAAAGGCTG TGGGTGTAAG-3′ (forward) and 5′-GGTTGGGAT GCTGAGGAAGATA-3′ (reverse). The *GAPDH* housekeeping gene was used as an internal control (forward primer: 5′-CCACTTGCCAAGGTTATCAA CGAC-3′; reverse primer: 5′-CAACTGAAACAT CAACGGTGGG-3′). RT-PCR amplifications were performed in 15-μL volumes containing 0.4 μL of each primer, 2 μL DNA, 7 μL HiFi Mix (TransGen, China) and 5.2 μL ddH_2O. The RT-PCR conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, and a final step of 72 °C for 10 min. The resulting PCR products were separated by electrophoresis on 2 % agarose gels and stained with ethidium bromide. qRT-PCRs were carried out in 15-μL reaction volumes containing 0.5 μL of each primer, 7.5 μL SYBR Green Real Master Mix (Thermo), 2 μL DNA and 4.5 μL ddH_2O. qRT-PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s. The $2^{-\Delta\Delta C_T}$ method was used to analyze relative changes in gene expression (Livak and Schmittgen 2001). RT-PCR and qRT-PCR amplifications were repeated independently three times.

Phylogenetic analysis

A phylogenetic tree of BrCRTISO and representative CRTISO protein sequences was constructed by neighbor-joining using Poisson-corrected distances in MEGA 4 with subsequent visualization in TreeView. Support for nodes in the resulting tree was assessed by the bootstrap method with 1,000 replicates. GenBank accession numbers of the analyzed sequences (and source organisms) are AAF63149 (*Arabidopsis thaliana*), Q8S4R4 (*L. esculentum*), AFU10971.1 (*Nicotiana tabacum*), ACO71189 (*Zea mays CRTISO 1*), NP_001148055 (*Z. mays CRTISO 2*), XP_002449729 (*Sorghum bicolor*), ABO27804.1 (*O. sativa*), YP_291830 (*Prochlorococcus*), BAA10798 (*Synechocystis*), YP_171014 (*Synechococcus*), YP_323615 (*Anabaena* FAD-dependent oxidoreductase), YP_003522456.1 (*Pantoea ananatis crtI*), AAA24820 (*Erwinia crtI*), AAC44850 (*Flavobacterium crtI*), AAA50313 (*Rhodobacter crtI*) and AAC84034 (*Heliobacillus crtN*). The aligned sequences were visualized using GeneDoc (http://www.nrbsc.org/gfx/genedoc/) as presented in Figs. 3 and 4.

Results

Carotenoid composition of white- and orange-type cultivars

Accumulated carotenoids in inner leaves of white- and orange-type cultivars were extracted and analyzed by HPLC. In the white-type cultivar, we only detected violaxanthin and zeaxanthin. We identified nine carotenoids in the inner leaves of the orange-type cultivars: violaxanthin, lutein, prolycopene, proneurosporene (7,9,9′-tri-*cis*-neurosporene), pro-ζ-carotene, ζ-carotene, 9-*cis*-β-carotene, β-carotene and neurosporene (Fig. 1 and Supplementary Table 3).

The white cultivar was found to possess more violaxanthin than orange varieties, and was the only cultivar in which zeaxanthin was detected. The inner leaves of the three orange-type cultivars analyzed (A21530, A14316 and JiHong No. 82) accumulated large quantities of prolycopene, pro-ζ-carotene, 9-*cis*-β-carotene, proneurosporene and other carotenoid pigments that were not present in the white type (Fig. 1). The identification of isomers of orange-leaf

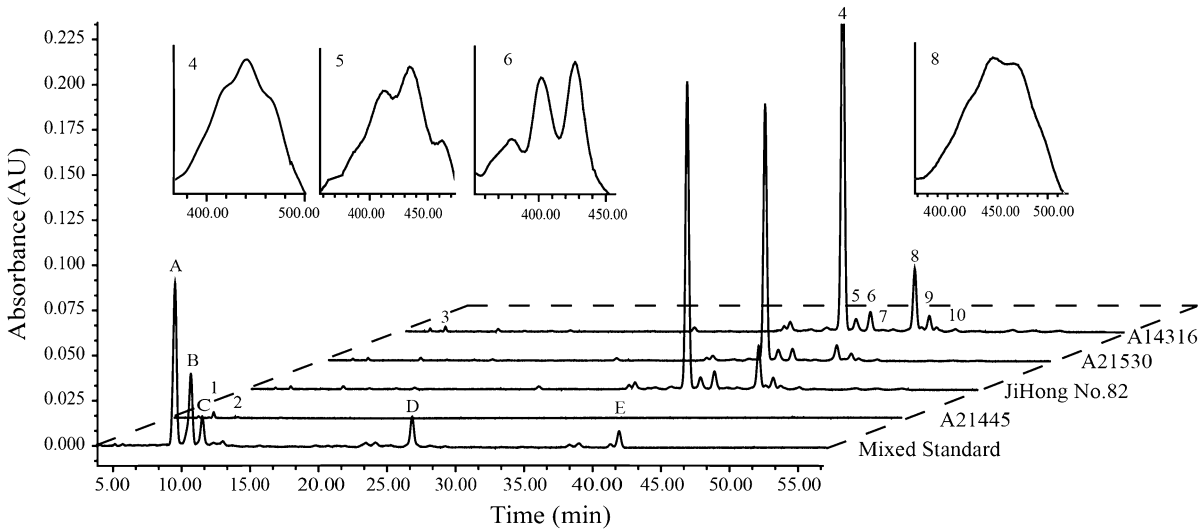

Fig. 1 Results of HPLC analysis of carotenoid pigments in one white- and three orange-type Chinese cabbage cultivars. *Peak 1* violaxanthin, *peak 2* lutein, *peak 3* zeaxanthin, *peak 4* prolycopene, *peak 5* proneurosporene, *peak 6* pro-ζ-carotene, *peak 7* ζ-carotene, *peak 8* 9-*cis*-β-carotene, *peak 9* β-carotene, *peak 10* neurosporene. *A*–*E* are the components of a mixed standard: *A* capsanthin, *B* lutein, *C* zeaxanthin, *D* cryptoxanthin, *E* β-carotene. *Insets* show absorption spectra of some major peaks

carotenoids was based on a comparison between the HPLC data and the results of previously published analyses. All of the data described above support the involvement of the *Bror* locus in the accumulation of prolycopene essential for orange coloration of Chinese cabbage.

Mapping and *Bror* candidate gene screening

Screening of 1,000 InDel and 200 SSR primer pairs revealed 227 pairs that were polymorphic between parents A21530 and A21445 of an F_2 mapping population. Fourteen InDel and six SSR markers exhibiting polymorphisms were detected between the white bulk and orange bulk. We then used the 14 InDel markers to genotype 269 individuals of the F_2 generation. Linkage between the DNA markers and the *Bror* locus was established using Joinmap 4.0 software on the basis of the genotyping data from the 269 F_2 individuals. Finally, we constructed a genetic map based on the 20 InDel and SSR markers (Supplementary Table 1). The *Bror* candidate gene was mapped to an interval on chromosome A09 between markers SB13049 (0.5 cM) and SB14001 (0.5 cM). SB14001 and SB13049 were located at positions 37,625,037 and 37,690,592, respectively, of chromosome A09, corresponding to a physical separation distance of 65.555 kb. Seventeen genes were located in this region (Fig. 2).

We analyzed the genes in the identified region using *B. rapa* genome annotation information in the BRAD database. By BLASTn analysis, homologous genes including transcription factors, structural genes and some unknown genes were found in *A. thaliana*. Because orange inner leaf color is caused by the accumulation of prolycopene, proneurosporene, pro-ζ-carotene and other lycopene precursors, we focused our search on carotenoid pathway genes. Finally, a gene encoding an authentic carotenoid isomerase, designated *CRTISO*, was identified in the positional region. The orange gene ID in *B. rapa* is *Bra031539*, and its ortholog is *AT1G06820* in *A. thaliana*. We therefore tentatively concluded that *Bra031539*, which encodes an authentic carotenoid isomerase, is the candidate *Bror* gene.

Analyses of the *BrCRTISO* coding sequence in white-type Chinese cabbage and *Bror* alleles

To characterize the transcribed sequence of the candidate gene *BrCRTISO*, the primers shown in Supplementary Table 2 were designed to sequence the full length of the candidate *Bror* gene. The nucleotide and protein sequences were then used as queries in

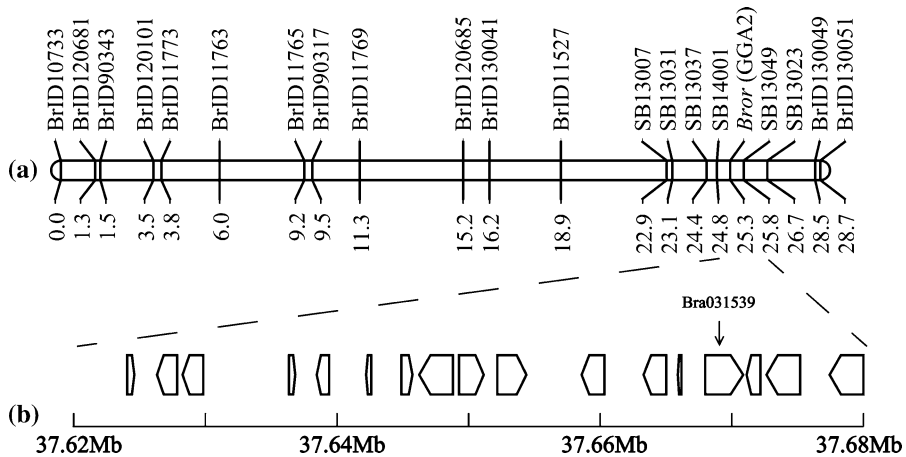

Fig. 2 Genetic mapping and candidate gene screening of *Bror*. **a** Genetic map of the *Bror* locus on chromosome A09 of Chinese cabbage. The chromosomal segment that overlaps the *Bror* locus is shown at the *top* of the figure, with the *numbers* below the segment corresponding to centimorgans and the *labels* above it representing the mapped markers (GAA2 is a functional marker). **b** Annotated genes located between the two closely linked markers SB14001 and SB13049. The distance between genes is scaled in accordance with the physical distance

BLAST searches. Comparison between genomic and cDNA sequences revealed that the gene is composed of 13 exons and 12 introns (Fig. 3a). The BLASTn analysis indicated that *BrCRTISO* exists as a single copy in *B. rapa.* This gene structure is consistent with *CRTISO* genes in *O. sativa*, *L. esculentum* and *A. thaliana*.

Analysis of cDNA and genomic sequences of *BrCRTISO* from the parents of the mapping population indicated that this allele contains a 6-bp deletion in exon 1 (Fig. 3c) and 53 SNPs in the entire coding sequence. Protein sequence analysis demonstrated that *BrCRTISO* has two glutamic acid deletions and 10 amino acid mutations (Fig. 3b). To validate our findings, we examined two additional cultivars: Chuifu, a white-type cabbage used as the model material in the *B. rapa* Genome Sequencing Program, and A30110, an orange-type Chinese cabbage. Cloning and analysis of the coding sequences of these additional materials indicated that the two orange-type cultivars (A21530 and A30110) have the same variations compared with the varieties with white interiors.

Based on the unique 6-bp sequence deletion between parents, a pair of InDel primers (Bio130275/Bio130276) were designed to amplify a functional marker (named GAA2). Analysis of the GAA2 marker confirmed the polymorphism between parents and the cosegregation of this marker with the *BrCRTISO* locus in F_2 individuals (Fig. 3d). This result suggests that the GAA2 marker can be used for marker-assisted selection in orange inner leaf Chinese cabbage hybrid breeding programs.

Analysis of the *BrCRTISO* promoter sequence

To investigate variations within the *BrCRTISO* promoter, the 1,342-bp upstream regulatory region of *BrCRTISO* was amplified by PCR in orange- and white-type Chinese cabbage, and the amplification products were tested on a 1 % agarose gel. This region was consistently amplified in white-type cabbage, and the amplification bands were significantly stronger than those amplified from the orange-type cultivars. The *BrCRTISO* promoters of white- and orange-type Chinese cabbage were named Procro-W and Procro-O, respectively.

We cloned the *BrCRTISO* promoter from orange-type A21530 and white-type A21445 to confirm the variation between these types. Procro-O was found to be 141 bp shorter than Procro-W, with the deletion located approximately 394–600 bp upstream of the gene start codon. Many SNPs were also located in the sequence (Fig. 4b). To develop DNA molecular markers to distinguish the orange-type phenotype, we used the InDel regions of the *BrCRTISO* promoter sequence identified in this study. A primer set, Bio140286 and Bio140287, was designed to amplify

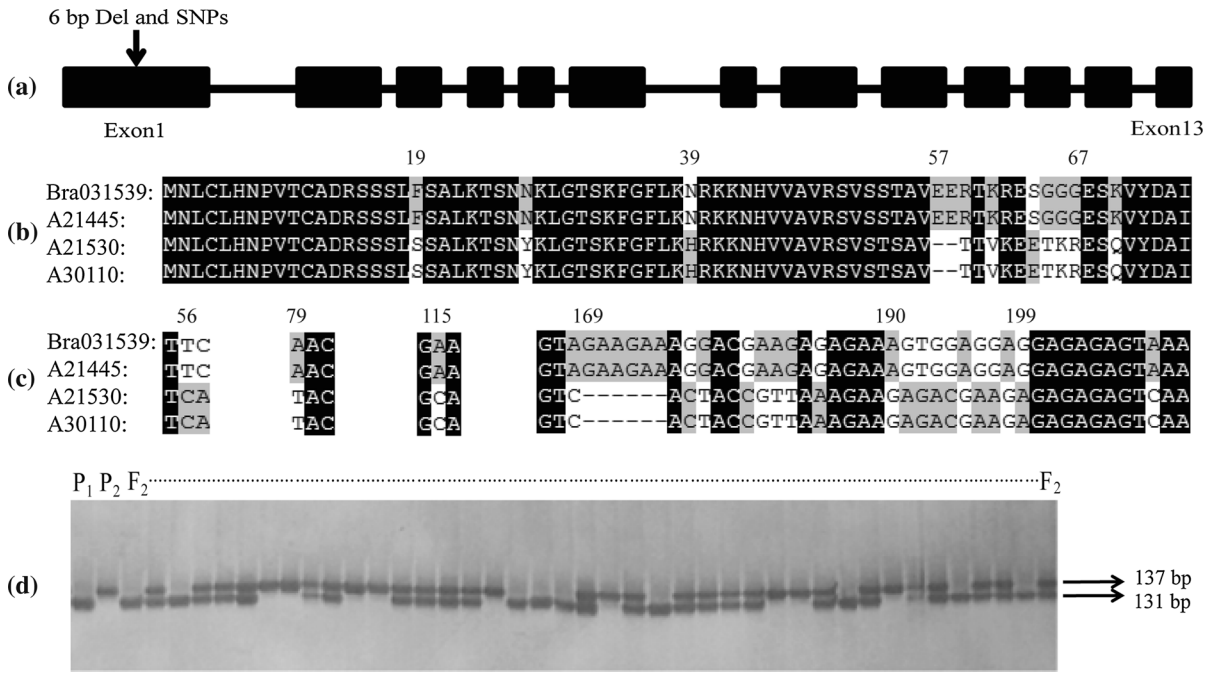

Fig. 3 *BrCRTISO* structure and mutation positions. **a** *Solid black boxes* represent exons. Deletions found in *BrCRTISO* orange-type alleles are indicated. **b** Variable amino acid sites. *Numbers* represent the specific position of the variable site. **c** Nucleotide substitutions and deletions causing missense mutations. **d** Polymorphism of functional marker GAA2 (Bio130275/Bio130276) in a P_1 plant (A21530), a P_2 plant (A21445) and F_2 individuals

a co-dominant marker for the orange phenotype. When the co-dominant marker was used to analyze the genotypes of parents and other homozygous lines, the orange-type and the white-type parents generated DNA fragments of about 341 and 480 bp, respectively (Fig. 4a).

Cis- and *trans*-acting elements of the *BrCRTISO* promoter region were predicted using PLACE and PlantCARE online analysis tools. As a result, additional transcription factor binding sites were identified in the promoter region. In addition, we uncovered one copy each of *cis*-acting elements Box I, the GCN4_ motif, HSE, the Skn-1_motif and Sp1 as well as five AAT-box and 12 TATA-box elements in the variable regions of the *BrCRTISO* promoter, typical of this complex regulatory region (Supplementary Table 4).

Analysis of *BrCRTISO* expression in different tissues

To investigate *BrCRTISO* expression in Chinese cabbage, RT-PCR was used to analyze *BrCRTISO* transcript levels in different tissues (outer leaves, inner leaves and roots) of white- and orange-type Chinese cabbage cultivars. *BrCRTISO* mRNA was detected in all tissues examined, with the strongest amplification bands observed in all three tissues of the white-type cabbage (Fig. 5a).

We used qRT-PCR to further examine *BrCRTISO* expression patterns in white- and orange-type Chinese cabbage cultivars. In white-type Chinese cabbage, expression levels in the inner leaves were slightly higher than those in the outer leaves, with the lowest expression in roots. In orange-type cabbage, however, the outer leaves showed higher expression than that in the inner leaves (Fig. 5b). In general, *BrCRTISO* transcription levels were higher in white-type Chinese cabbage than in orange-type cultivars.

Phylogenetic analysis of *BrCRTISO* and its orthologs

Because Chinese cabbage (*B. rapa*) experienced a whole-genome triplication event prior to the origin of the diploid *Brassica* species, it possesses three nuclear subgenomes (Freeling and Thomas 2006).

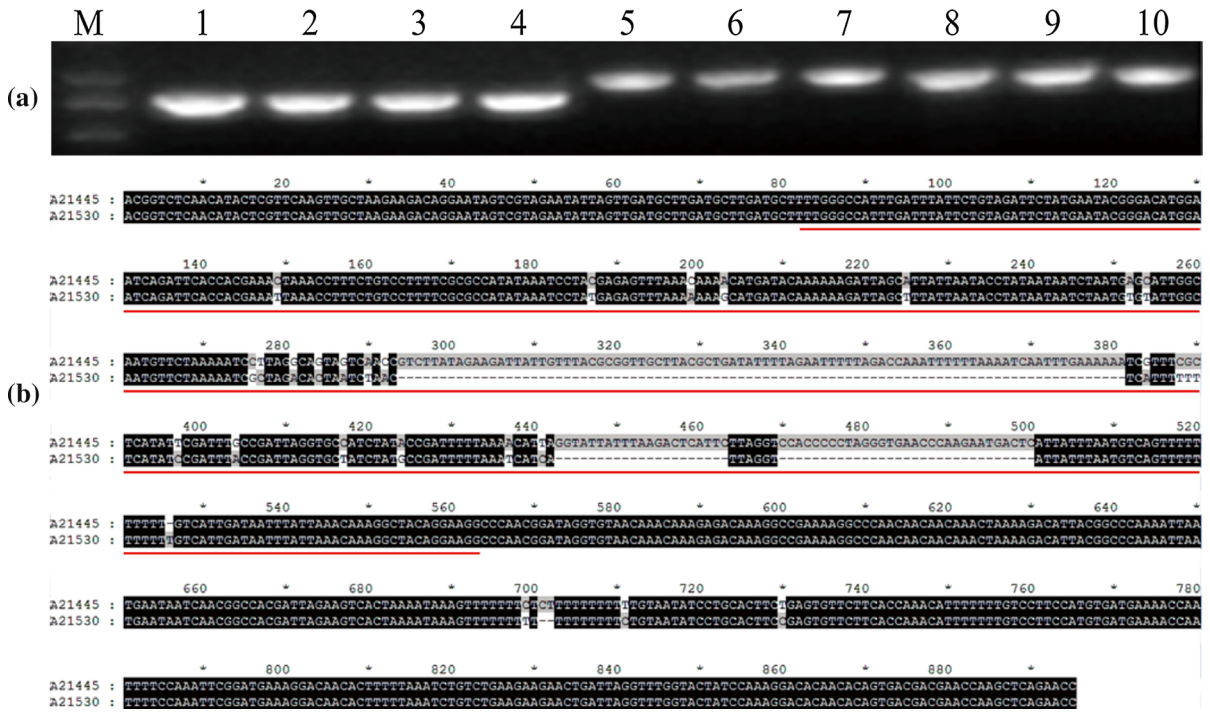

Fig. 4 Sequence variation in the *BrCRTISO* promoter of Chinese cabbage. **a** Results of functional marker amplification of different homozygous lines of Chinese cabbage. *Lanes 1–4* orange-type Chinese cabbage, *lane 5* yellow-type cultivar, *lanes 6–10* white-type cultivars. **b** Comparison of aligned *BrCRTISO* promoter sequences from white-type (A21445) and orange-type (A21530) Chinese cabbage cultivars. InDel markers were designed based on the *red-underlined* sequence region. (Color figure online)

Following gene fractionation, however, only one *CRTISO* was retained in Chinese cabbage. To determine the evolutionary relationship between *BrCRTISO* and *CRTISO* orthologs from other organisms, an unrooted phylogenetic tree was constructed by neighbor-joining based on amino acid sequence similarity (Supplementary Fig. 2). The phylogenetic analysis of *CRTISO* from *B. rapa*, other plant species and bacteria separated the genes into three main clades: eukaryotes, prokaryotes and bacterial carotenoid desaturases (*crtN* and *crtI*). The eukaryotic clade includes both dicotyledonous and monocotyledonous species, reflecting its evolutionary history. *CRTISO* of Chinese cabbage was most closely related to that of *A. thaliana*, consistent with the position of the two plant species in the Brassicaceae family. *CRTISO* showed 20–30 % identity to bacterial carotenoid desaturases (*crtI* and *crtN*) and shared several conserved motifs such as the dinucleotide binding domain. This similarity suggests the possibility that *CRTISO* evolved from *crtI*.

Discussion

Orange inner leaves of Chinese cabbage due to prolycopene

As a new variety, Chinese cabbage with orange inner leaves is of significant research value. Although considerable effort has been previously devoted to analyses of carotenoid pigments in Chinese cabbage (Tuan et al. 2012), little information is available regarding the source of the inner-leaf orange coloration. Feng et al. (2012) have reported that orange inner leaves are due to lycopene or β-carotene accumulation, whereas Lee et al. (2014) have claimed that lycopene-like compounds are responsible because more lutein-related pigments are found in Chinese cabbage with yellow and orange inner leaves. In our study, very little lutein was detected in orange-type cultivars. Although inner leaves of orange-type Chinese cabbage were enriched in prolycopene, 9-*cis*-β-carotene and proneurosporene, no lycopene was detected (Fig. 1). This result suggests that orange

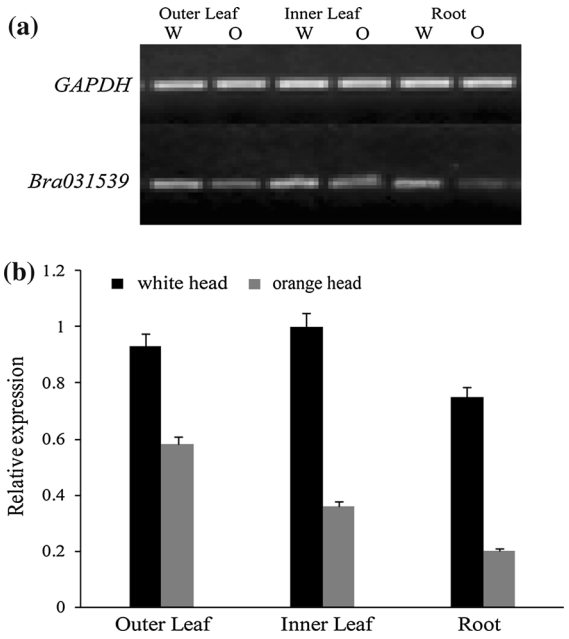

Fig. 5 Expression pattern of *BrCRTISO* in different Chinese cabbage plant tissues. **a** *BrCRTISO* expression in different tissues based on reverse transcription PCR. **b** *BrCRTISO* expression in different tissues based on quantitative real-time PCR

inner leaves are mainly the result of prolycopene accumulation.

A prior study related to carotenoids in Chinese cabbage, which concluded that lycopene only exists in orange varieties, used a C_{18} HPLC column (Feng et al. 2012). Although a C_{18} column can effectively separate different kinds of carotenoids, it cannot easily separate isomers and analyze carotenoids accurately (Sander et al. 1994). The newer multi-silane C_{30} column has obvious advantages with respect to isomer separation (Emenhiser et al. 1995; Lessin et al. 1997). We used a C_{30} column (250 × 4.6 mm, 5 μm) coupled to a HPLC device and a photodiode array detector for the HPLC analysis in our study. Using this approach, we were able to isolate multiple components. We confirmed 10 carotenoid components, which is the largest number determined to date.

Variation within *BrCRTISO* coding and promoter sequences

Genetic and physical mapping is required for gene isolation and map-based cloning (Tanksley et al. 1995; Vrebalov et al. 2002). Several genetic mapping studies involving the *Bror* locus have been conducted in Chinese cabbage (Feng et al. 2012; Su et al. 2014; Zhang et al. 2008, 2013). In one such study, Su et al. (2014) mapped the *Bror* locus to a 9.47-kb region and hypothesized that the candidate gene encoded carotenoid isomerase. SSR and InDel markers were used for screening and mapping in our study. Mapping of PCR-specific markers using 269 individuals of an F_2 population narrowed the location of the *Bror* locus to a 1.0-cM interval corresponding to a physical distance of 65.555 kb on chromosome A09 of the *B. rapa* genome (Fig. 2). This position corresponds to the same area identified by Su et al. (2014), indicating that the orange inner-leaf trait is controlled by the same candidate gene in different orange-type varieties.

We detected different polymorphic variations in the *BrCRTISO* genomic sequence than those uncovered in previous studies. Although Lee et al. (2014) determined that the first exon of *BrCRTISO1* in an orange-type cultivar had a 9-bp insertion and a 15-bp deletion relative to a yellow-type cultivar, they were unable to amplify the 3′ end of the *BrCRTISO1* sequence and thus could not obtain complete *BrCRTISO1* transcripts. In this study, we not only cloned and sequenced the coding region, but also obtained the promoter sequence of *BrCRTISO* in both orange- and white-type cultivars. However, mutations were not detected in the 3′ end as described by Lee et al. (2014). By examining several other orange- and white-type cultivars, we confirmed that wide variation (53 SNPs and a 6-bp deletion) exists between white and orange inner-leaf cultivars, with most variations found in the first exon (Fig. 3c).

Sequence analysis revealed that the orange-type promoter was shorter than the white type by about 141 bp, and many SNPs were present in the promoter region (Fig. 4b). However, the observed variations were different from those identified by Zhang et al. 2013. The *BrCRTISO* promoter region in which these mutations are present contain many *cis*-acting elements, including one copy each of Box I, the GCN4_motif, HSE, the Skn-1_motif and Sp1, five AAT-box elements and 12 TATA-box elements. In addition, *BrCRTISO* expression in white-type Chinese cabbage was higher than that in orange-type cultivars (Fig. 5), but the expression pattern is different from that described by Lee et al. (2014) between orange and yellow cultivars. These differences suggest that sequence variations in the promoter region may interfere with normal *BrCRTISO* mRNA transcription.

Molecular markers are being used to accelerate plant selection gains through marker-assisted selection (Collard and Mackill 2008). In addition, genes of scientific and agronomic importance can be isolated solely on the basis of their position on a genetic map (Tanksley et al. 1995). In this study, two functional markers were developed based on polymorphic regions within *BrCRTISO* coding and promoter sequences (Figs. 3d, 4a). Both of these markers can be used for molecular breeding of orange-type Chinese cabbage.

Carotenoid isomerase

The plant carotenoid synthetic pathway is very complicated, with a much more complex set of carotene isomers involved in the phytoene to lycopene conversion in plants than in bacteria (Beyer et al. 1989; Britton 1998). CRTISO is a *cis–trans*-isomerase. In many CRTISO loss-of-function mutants, prolycopene, poly-*cis*-carotene and lycopene precursors accumulate instead of all-*trans*-lycopene in dark-grown plants (Chai et al. 2011; Isaacson et al. 2002; Park et al. 2002). We also have demonstrated that orange inner leaves accumulate a large proportion of prolycopene, 9-*cis*-β-carotene, pro-ζ-carotene and proneurosporene, whereas white inner leaves mainly contain violaxanthin and zeaxanthin. Consistent with the fact that CRTISO enzyme activity appears partially redundant in the presence of light (Park et al. 2002), no difference has been previously observed in β-carotene and lutein contents of outer leaves between white- and orange-type Chinese cabbage (Li 2011).

The catalytic activity of *cis*-to-*trans* isomerization requires redox-active ingredients, suggesting that isomerization is achieved by redox reactions acting at specific double bonds (Isaacson et al. 2004). The dinucleotide-binding domain is highly conserved among most carotenoid biosynthetic enzymes. In addition, reduced flavin adenine dinucleotide (FAD) involved in the stabilization of a transition state is needed in lycopene cyclization; a specific glutamate is also required in acid/base catalysis (Mialoundama et al. 2010; Yu et al. 2010). The CRTISO enzyme has a conserved dinucleotide-binding domain at its N terminus (Isaacson et al. 2004). Yu et al. (2011) have reported that CRTISO is a member of the FAD_{red}-dependent class of flavoproteins catalyzing non-redox reactions. Our phylogenetic analysis indicated that *CRTISO* may have evolved from bacterial carotenoid desaturases (*crtI* and *crtN*). In this study, the orange inner leaf candidate gene *BrCRTISO* was sequenced, revealing the presence of 12 amino acid mutations and two amino acid deletions (Fig. 3b). Relative to the white-type Chinese cabbage sequence, the 19th hydrophobic nonpolar phenylalanine has mutated into a polar hydrophilic serine, the 39th aspartic acid has mutated into alkaline histidine, two acidic glutamates have been deleted at the 57th amino acid position, and the 67th glycine has been replaced by alkaline arginine. On the basis of the above information, we believe that changes in the polarity and acidity of the amino acids may affect enzyme activity. The mutations in exon 1 may be responsible for the decrease or loss of activity of carotenoid isomerase (*BrCRTISO*) in Chinese cabbage.

As stated above, we have completely identified the carotenoid pigments that cause orange coloration of inner leaves in Chinese cabbage. The candidate orange inner leaf gene *BrCRTISO* (*Bra031539*) was mapped and analyzed at the molecular level. This gene can serve as a novel genetic tool for improving nutritional value in vegetable crops, and the functional markers developed in this study can be applied during marker-assisted selection in orange inner leaf Chinese cabbage hybrid breeding programs. On the basis of the existing experimental data, transgenic technology should allow functional identification in the future.

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