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ORIGINAL PAPER

Association of molecular markers derived from the *BrCRISTO1* gene with prolycopene-enriched orange-colored leaves in *Brassica rapa*

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Abstract

Key message **Sequence polymorphism in *BrCRTISO1*, encoding carotenoid isomerase, is identified in orange-colored *B. rapa*, and three resulting gene-based markers will be useful for marker-assisted breeding of OC cultivars.**

Abstract Carotenoids are color pigments that are important for protection against excess light in plants and essential sources of retinols and vitamin A for animals. We identified a single recessive gene that might cause orange-colored (OC) inner leaves in *Brassica rapa*. The inner leaves of the OC cultivar were enriched in lycopene-like compounds, specifically prolycopene and its isomers, which can be a useful functional trait for Kimchi cabbage. We used a candidate gene approach based on the 21 genes in the carotenoid pathway to identify a candidate gene responsible for the orange color. Among them, we focused on two *carotenoid isomerase* (*CRTISO*) genes, *BrCRTISO1* and *BrCRTISO2*. The expression of *BrCRTISO1* was higher than that of *BrCRTISO2* in a normal yellow-colored (YE) cultivar, but full-length *BrCRTISO1* transcripts were not detected in the OC cultivar. Genomic sequence analysis revealed that *BrCRTISO1* of the OC cultivar had many sequence variations, including single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels), compared to that of the YE cultivar. We developed molecular makers for the identification of OC phenotype based on the polymorphic regions within *BrCRTISO1* in *B. rapa* breeding. The *BrCRTISO1* gene and its markers identified in this study are novel genetic resources and will be useful for studying the carotenoid biosynthesis pathway as well as developing new cultivars with unique carotenoid contents in *Brassica* species.

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S. Lee and S.-C. Lee contributed equally to this work.

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Introduction

Carotenoids are a large group of isoprenoid-derived compounds generated in plants and some algae and are the pigments responsible for yellow to red colors of fruits and flowers. There are hundreds of naturally occurring carotenoid structures, which can be divided in two main groups: carotene and xanthophyll (Ruiz-Sola and Rodríguez-Concepción 2012). In photosynthesis, carotenoids fulfill an important role in light harvesting and in protection of the photosynthetic apparatus against excess light. In addition, some carotenoids are precursors of phytohormones such as abscisic acid (Schwartz et al. 1997) and strigolactones (Gomez-Roldan et al. 2008; Umehara et al. 2008). Animals cannot synthesize carotenoids and need to obtain them in

their food as essential precursors of retinols and vitamin A. In humans, carotenoids serve as health-promoting and antioxidant phytonutrients (Ruiz-Sola and Rodríguez-Concepción 2012).

Carotenoids have been of interest to organic chemists since the early nineteenth century and large-scale genetic and molecular studies of carotenoids began in the early twentieth century (Bartley and Scolnik 1995). Since the identification of *Psy1*, which converts geranylgeranyl diphosphate (GGPD) into phytoene in the first step of the carotenoid biosynthesis pathway in tomato (Ray et al. 1987; Bird et al. 1991; Bartley et al. 1992), almost all genes involved in the pathway have been identified in various plant species. Using this genetic information, a number of studies have been performed to increase carotenoid contents and alter carotenoid composition in different plant species (Shewmaker et al. 1999; Römer et al. 2000; Ye et al. 2000; Ducreux et al. 2005; Fujisawa et al. 2009). Among these, some have reported mutant plants with different colored fruits and leaves, caused by aberrant accumulation of specific carotenoids (Li et al. 2001, 2006; Isaacson et al. 2002; Park et al. 2002; Lu et al. 2006; Lopez et al. 2008). These color variations are particularly interesting because they provide not only opportunities to study the functions of genes in carotenoid biosynthesis, but also potentially unique nutritional and agricultural commodities for farmer and consumer.

Brassica species belong to the Brassicaceae family and include a number of important crop vegetables such as *Brassica rapa* (Kimchi cabbage, pak choi and turnip) and *Brassica oleracea* (broccoli, cabbage and cauliflower) as well as oilseed crops such as *Brassica napus*. These *Brassica* crops are widely cultivated for human nutrition. Among them, *B. rapa* is the top leafy vegetable crop in China and Korea. Chinese cabbage, also called Kimchi cabbage, is particularly important in Korea because it is used to make the Korean national dish "Kimchi" (Schmidt and Bancroft 2011).

In cauliflower (*B. oleracea* var. *botrytis*), the semi-dominant *Orange (Or)* mutation was found to cause abnormal accumulation of β-carotene, resulting in orange-colored curds (Crisp et al. 1975; Dickson et al. 1988; Li et al. 2001). Map-based cloning of the *Or* gene revealed that it encodes a Dna-J cysteine-rich domain containing protein required for the formation of chromoplasts where carotenoids accumulate (Lu et al. 2006). Similar to the phenotype of the cauliflower *Or* mutant, orange-colored (OC) inner leaves were reported in a *B. rapa* cultivar, Orange queen, which was developed by crossing and subsequent backcrossing of Chinese cabbage and turnip, and this trait was found to be inherited under the control of single recessive gene (Matsumoto et al. 1998; Zhang et al. 2008; Feng et al. 2012). Thereafter, several studies developed molecular markers linked to the trait and tried to identify the gene responsible for the OC phenotype in *B. rapa* (Zhang et al. 2008; Feng et al. 2012); however, none of the studies successfully identified the responsible gene. Meanwhile, investigation of carotenoid composition and content revealed that the Orange queen cultivar accumulates prolycopene and phytoene as major carotenoids (Watanabe et al. 2011) instead of lutein and β-carotene, which accumulate in the conventionally cultivated normal cultivar with yellow-colored (YE) inner leaves (Wills and Rangga 1996). In addition, the total carotenoid content of the Orange queen cultivar is twofold higher than that of the YE cultivar (Watanabe et al. 2011).

The current study was conducted to characterize a candidate gene that might cause OC characteristics as well as to develop molecular markers for the trait in *B. rapa*. We identified mutations of a gene encoding carotenoid isomerase (CRTISO) in an OC cultivar and validated several molecular markers distinguishing the OC genotype. To our best knowledge, this is the first report identifying the gene for CRTISO as a candidate to contribute to OC inner leaves in *B. rapa* plants. This study will be valuable for basic research aimed at understanding *B. rapa* carotenoid biosynthesis and for development of new functional cultivars unique in agricultural and nutritional characteristics.

Materials and methods

Plant materials and color phenotype assessment

Inbred parental lines with the OC phenotype were bred from Orange queen cultivar (Matsumoto et al. 1998). Inbred lines with the YE phenotype were breeding lines. F_1 and F_2 progenies established by crossing between OC (female parent) and YE (male parent) inbred lines were grown in the greenhouse and in the field. Leaves of plants were harvested and then stored at −70 °C until use for DNA and RNA isolation. For phenotyping, the color of the inner leaves, which were defined as the inner leafy tissues that lacked chlorophyll, and flowers was observed at the stage of heading and flowering, respectively. Additionally, F_3 progenies of 66 F_2 individuals ($F_{2:3}$) were further inspected for segregation of OC phenotype to judge the genotype of each F_2 plant. To investigate color change of inner leaves under various abiotic stresses, inner leaves of OC and YE cultivars were treated with sunlight (average light intensity of 1,400 μmol m^{-2} s^{-1}), fluorescent light (average light intensity of 5 μmol m^{-2} s^{-1}), or UV (365 nm, UV-A). Alternatively, the inner leaves were treated with cold (4 °C) or heat (60 °C) stress in the dark. For drought stress, the segments of inner leaves were dried on the paper towel in

a growth chamber in the dark. All treatments were continued until color change of leaves was clearly observed for at least 2 days. Inner leaves of the Orange queen cultivar were also treated with sunlight to confirm the same color phenotype as the OC cultivar.

Carotenoid analysis by HPLC

All of the inner leafy tissues of OC and YE cultivars except for the external leaves (two or three layers) with chlorophyll were ground using an electric blender, freeze-dried, and then stored at −70 °C until use. The method to extract the carotenoids was based on the procedure established by Bohoyo-Gil et al. (2012) with minor modification. Briefly, samples of 0.5 g were suspended in 40 mL acetone:*n*-hexane (1:1, v/v) and extracted for 5 min by vortexing. Distilled water (10 mL) was added, and the sample was extracted again by vortexing. Afterward, the extracted solution was centrifuged at 1,400×*g* for 5 min. The organic phase at the upper layer was collected, concentrated by evaporation using nitrogen, and dissolved in 1.5 mL methyl-*t*-butyl-ether (MTBE):methanol (1:1, v/v). The extract was filtered through a 0.2-μm membrane filter (regenerated cellulose, Sartorius Stedim Biotech, Germany) and subjected to HPLC. The carotenoids were separated on a Dionex 680 HPLC system with a C30 column (150 × 4.6 mm, 3 μm, YMC, Wilmington, NC) and detected with a photodiode array (PDA) detector at a wavelength of 437 nm. Mobile phase solvents A consisted of 83 % methanol, 15 % MTBE and 0.03 % ammonium acetate, and mobile phase solvents B were 8 % methanol, 90 % MTBE and 0.03 % ammonium acetate. The flow rate was maintained at 1.0 mL min^{-1} and samples were eluted with the following gradient: 0 min 90 % A/10 % B, 5 min 90 % A/10 % B, 34 min 20 % A/80 % B, 34.1 min 5 % A/95 % B, 36 min 5 % A/95 % B, 36.1 min 90 % A/10 % B, and 40 min 90 % A/10 % B. The column was maintained at 30 °C. Identification and peak assignment of carotenoids were primarily based on comparison of their retention time and chromatogram data with those of standards previously reported (Hengartner et al. 1992; Melendez-Martinez et al. 2013; Sun et al. 2009). For quantification, all-*trans*-lycopene standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of all-*trans*-lycopene was prepared in MTBE:methanol (1:1, v/v). Quantification of prolycopene, lycopene, and its isomers was performed based on a calibration curve prepared using the all-*trans*-lycopene standard (*Y*-axis, peak area divided by molar absorption coefficient of all-*trans*-lycopene at 470 nm; *X*-axis, corresponding injection concentration). The peak area of prolycopene obtained was normalized using its molar absorption coefficients (Glenn et al. 1999). The carotenoid analyses were performed with technical duplicates of two independent biological samples.

Identification of genes involved in carotenoid biosynthesis from the *B. rapa* genome database

Candidate genes involved in carotenoid biosynthesis of *B. rapa* were identified in the *B. rapa* genome database (http://brassicadb.org/brad/; BRAD; Cheng et al. 2011; Wang et al. 2011) using as queries protein sequences of 16 Arabidopsis genes encoding enzymes functioning in the carotenoid biosynthesis pathway (Ruiz-Sola and Rodríguez-Concepción 2012). These searches for candidate genes were performed using both the BlastP algorithm and the synteny paralog search tool provided in BRAD. Among these genes, those encoding CRTISO were further analyzed to identify their locations on chromosomes using the information in BRAD. The expressed sequence tags (ESTs) for *CRTISO* genes in *B. rapa* were also searched in EST databases at NCBI (http://blast.ncbi.nlm.nih.gov/) and RIKEN (http://www.brc.riken.go.jp/lab/epd/Eng/species/brassica.shtml).

Expression analysis of *BrCRTISO* genes

The expression of *BrCRTISO* genes was investigated through RT-PCR analysis. Total RNAs from leaves of OC and YE cultivars were isolated using the RNeasy plant mini kit (Qiagen, Germany) and treated with RNase-free DNaseI (Qiagen, Germany) to remove genomic DNA contamination, according to the manufacturer's instructions. The quality and quantity of the total RNAs were monitored by spectrophotometric analysis using the Nano-drop instrument (Thermo Fisher Scientific Inc, USA). Five μg total RNA was reverse transcribed into first stand cDNAs using RNA to cDNA EcoDry Premix with oligo $(dT)_{18}$ primers (Clontech, USA), according to the manufacturer's instructions. The synthesized cDNAs were diluted ninefold with distilled water and then used as templates for PCR amplification. Gene-specific primers of *BrCRTISO* genes were used for PCR analysis. Specific primers were designed based on coding sequences and EST sequences (Supplementary Table 1). As a control, we used primers specific to the *B. rapa* actin gene, *BrActin* (GenBank accession no. EX087730; Lee et al. 2008). PCR conditions were as follows: 94 °C for 5 min; followed by cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and finally extension at 72 °C for 7 min. The number of PCR cycles used ranged between 22 and 35 for specific amplification of the genes. Betaine to a final concentration of 1.0–1.5 M was added to PCR mixtures to increase the specificity of PCR primers and the efficiency of amplification.

Genomic DNA PCR analysis and nucleotide sequencing of *BrCRTISO1*

Genomic DNAs were isolated from leaves of OC and YE cultivars and their hybrid F_1 and F_2 progenies using

the cetyltrimethylammonium bromide (CTAB) method, as described in Allen et al. (2006). Specific primers were designed to cover the genomic sequence of *BrCRTISO1* (BRAD gene ID: Bra031539) from the putative promoter region to the 500 bp downstream region from the stop codon (Supplementary Table 1). PCR conditions were as follows: 94 °C for 5 min; followed by cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 7 min. The number of PCR cycles used ranged between 22 and 35 for specific amplification of the genes. *Pfu* DNA polymerase (Speed-Pfu, HelixAmp, Korea) was used to prevent possible sequence alteration during PCR amplification. After electrophoresis in 1.0–1.3 % agarose gels, the genomic amplicons were extracted using a gel-extraction kit (Solgent, Korea) and their nucleotide sequences were determined using sequencing service provide by NICEM (http://nature.snu.ac.kr/kr.php). The nucleotide sequences of genomic amplicons were assembled using SeqMan pro (DNASTAR, USA) with default parameters, and compared between OC and YE cultivars. The sequences of *BrCRTISO1* genes from YE and OC cultivars were deposited in NCBI GenBank with accession no. KC907717 and KC907718, respectively.

Design and validation of DNA molecular markers

Primer sets that could amplify specific regions were designed based on sequence variations found in the *BrCRTISO1* genomic sequences of OC and YE cultivars using Primer-Blast tools (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were first examined with genomic DNA templates isolated from parental and F_1 hybrid lines of OC and YE cultivars, and then further validated using the F_2 population and 30 various *B. rapa* breeding lines. For rapid and accurate genotyping, amplified DNA fragments were separated using capillary electrophoresis and their separation patterns were analyzed by using Fragment analyzer (Advanced Analytical Technologies Inc., USA), according to manufacturer's instructions.

Results

Comparison of inner leaf color between OC and YE cultivars

The OC cultivar showed a more deep yellow (orange) color of the inner leaves (Fig. 1d) compared to the YE cultivar (Fig. 1a). Interestingly, when directly exposed to sunlight, the inner leaves of the OC cultivar became more orange or reddish within 5 min of exposure (Fig. 1e), whereas no significant color change was observed in the inner leaves of the YE cultivar (Fig. 1b). However, exposure to sunlight for more than 1 day induced chlorophyll accumulation in the inner leaves of both cultivars, and therefore any difference of inner leaf color was obscured. In addition, inner leaves of the Orange queen cultivar also showed the same color phenotype in inner leaves as the OC cultivar before (Fig. 1g) and after (Fig. 1h) sunlight exposure.

To test for abiotic factors affecting color change in the OC cultivar, inner leaves were subjected to various abiotic stresses such as cold (4 °C), heat (60 °C), drought (air-drying), UV (365 nm, UV-A), and fluorescent light, as described in the "Materials and methods". Of those, only the UV treatment for 30 min caused conversion of inner leaf color to orange in the OC cultivars.

Flower color was also different between the cultivars. OC cultivars had pale-yellow petals (Fig. 1f), whereas those of YE cultivars were bright yellow (Fig. 1c). Three F_2 populations were established by crossing two OC (inbred line no. S510-G6 and 32S-G7) and two YE (NS-G6 and S955-G8) inbred lines: S510-G6 × NS-G6, 32S-G7 × S955-G8, 32S-G7 × NS-G6. A total of 736 F_2 plants were inspected. Among them, 176 showed pale-yellow flowers and the remaining 560 had yellow flowers, which fits well to the 1:3 ratio expected for Mendelian single recessive gene segregation. Flower color co-segregated with the color of the inner leaves, indicating that both traits were pleiotropically controlled by single gene. Collectively these data confirmed that the OC phenotype was controlled by a single recessive gene, as reported in previous studies of OC cultivars (Matsumoto et al. 1998; Zhang et al. 2008; Feng et al. 2012).

Carotenoid analysis of the OC cultivar

We compared the carotenoid composition in the inner leaves of OC with those of YE cultivars using HPLC analysis (Fig. 2). A lutein-related peak (peak no. 1) was present in both OC and YE inner leaves, although OC inner leaves showed a peak area of less than half that found in YE inner leaves. The other lutein-related peak (no. 2) was detected only in YE inner leaves. Except for these peaks, other peaks of carotenoids were detected only in OC inner leaves, of which four peaks, no. 3, 6, 7, and 8, corresponded to prolycopene, all-*trans*-lycopene (lycopene), and its isomers and accounted for large proportion compared to lutein. In particular, the contents of prolycopene and lycopene were 38.8 ± 0.9 and 14.1 ± 0.8 μg per 1 g dry weight of OC inner leaves, respectively. A peak at the same retention time as that of prolycopene was also detected in YE inner leaves but was so tiny that it could not be quantified. Overall, the total amount of lycopene-like compounds including prolycopene, lycopene and its isomers was 76.9 ± 1.8 μg per 1 g dry weight of OC inner leaves, but not quantifiable in YE inner leaves. This indicates that OC inner leaves had

Fig. 1 Representative phenotypes of YE and OC cultivars. The YE cultivar (**a**, **b**, and **c**) has yellow inner leaves (**a**) and flowers (**c**), whereas the OC cultivar (**d**, **e**, and **f**) has deep yellow (orange) inner leaves (**d**) and pale-yellow flowers (**f**). After sunlight exposure for 5 min (**b**, **e**, and **h**), inner leaf color of the OC cultivar changed to orange (**e**) but no change was observed in the YE cultivar (**b**). The Orange queen cultivar showed the same color phenotypes as the OC cultivar both before (**g**) and after (**h**) sunlight exposure

lycopene-like compounds as the major carotenoid in contrast to YE inner leaves, in which lutein was the major carotenoid. Lutein was previously reported to be predominantly accumulated in normal cultivars with yellow inner leaves (Wills and Rangga 1996; Watanabe et al. 2011). Recently, a carotenoid study of the Orange queen cultivar revealed that it contains a large amount of prolycopene (Watanabe et al. 2011). Considering that the OC cultivar was derived by breeding Orange queen with the traditional Korean cultivar, our result confirmed again that carotenoid profiles of the OC cultivar were different from those of YE cultivar.

Identification of *B. rapa CRTISO* genes

On the basis of the results of carotenoid analysis in this study and in Watanabe et al. (2011), we hypothesized that the different color of the OC cultivar was due to different carotenoid profiles and therefore that OC cultivar had an alternation in the carotenoid biosynthesis pathway compared to YE cultivars. To test this, we began by identifying the entire set of *B. rapa* genes involved in carotenoid biosynthesis. We searched the *B. rapa* genome database (BRAD) using 16 Arabidopsis genes encoding enzymes in the carotenoid biosynthesis pathway (Ruiz-Sola and Rodríguez-Concepción 2012) as queries, finding 21 *B. rapa* genes highly similar to Arabidopsis counterparts. Among the 16 Arabidopsis genes, *CRTISO1* (At1g06820) was reported to encode a functional CRTISO and its knock-out mutant accumulates lycopene isomers including prolycopene (Park et al. 2002). Similarly, mutation of a gene encoding CRTISO was identified in the tomato *tangerine* mutant, which accumulates prolycopene (Isaacson et al. 2002). Based on these reports, two *B. rapa* homologues of Arabidopsis *CRTISO* genes, Bra031539 and Bra027908, were chosen as candidates whose mutation might cause alteration of carotenoid biosynthesis in the OC cultivar. We designated the two genes *BrCRTISO1* and *BrCRTISO2* based on the names of their Arabidopsis counterparts.

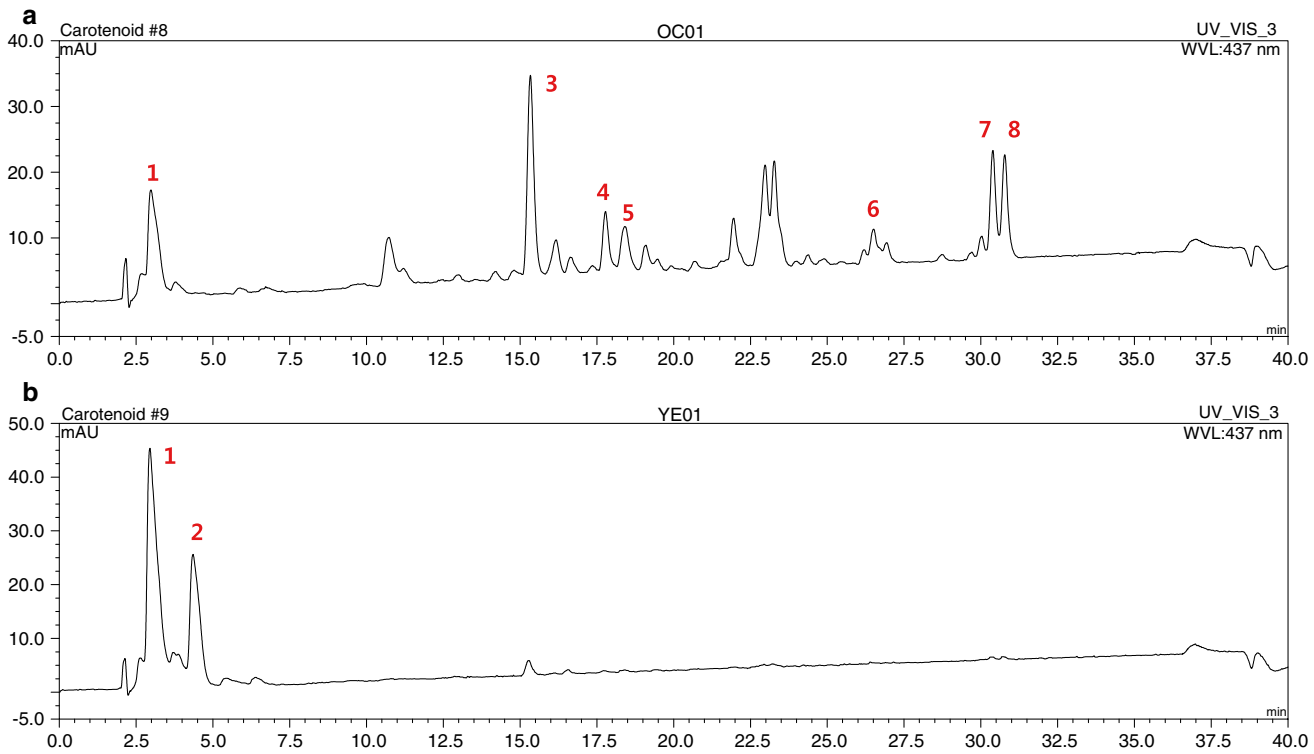

Fig. 2 HPLC chromatogram of carotenoid extracts from inner leavers. Carotenoids extracted from inner leaves of OC (**a**) and YE (**b**) cultivars were analyzed using HPLC and each carotenoid pigment was detected at the wavelength of 437 nm. The peaks correspond to the following: *1* 13 or 15Z-lutein, *2* all-*trans*-lutein, *3* prolycopene, *4* and *5* 1′-OH-γ-carotene and its isomer, *6* 5Z, 9Z- or 5Z, 9′Z-lycopene, *7* all-*trans*-lycopene (lycopene), *8* 5Z-lycopene. Other peaks without number correspond to unknown compounds

To investigate the chromosomal location of *BrCRTISO1* and *BrCRTISO2*, we performed *in silico* mapping of the genes on chromosomes using genomic information in BRAD. Two genes were located on *B. rapa* chromosome A09, of which *BrCRTISO1* was mapped between two simple sequence repeat (SSR) markers, syau15 and syau19, co-segregated with the orange inner leaf trait in the OC cultivar (Feng et al. 2012). Collectively these data suggested that *BrCRTISO1* might be one of candidates for causing the OC phenotype.

Expression of *BrCRTISO* genes

To investigate the expression of the two *BrCRTISO* genes in the reference cultivar, Inbred line Chiifu, of *B. rapa*, we searched the microarray database of *B. rapa* (http://nabic.rda.go.kr/; Lee et al. 2008) for expression values of the genes during development from seed to flowering stage. Both genes were expressed throughout the entire growth period. However, *BrCRTISO1* expression was much higher than that of *BrCRTISO2*, indicating that BrCRTISO1 likely plays a main role as an enzyme converting prolycopene into lycopene in *B. rapa* plants. The expression of *BrCRTISO1* was also investigated in leaves of OC and YE cultivars using RT-PCR analysis with primers Bra031539-3eF1 and Bra031539-3eR1, respectively, specific to the ninth exon sequence and 3′ untranslated region (UTR) of the gene. As shown in Fig. 3a, all YE plants tested generated about 500 bp bands as expected, but none of the OC plants did. Meanwhile, *BrCRTISO2* transcript was detected at a similar level in all YE and OC plants tested. In addition, other primer sets specific to the 1st–13th exon sequence of *BrCRTISO1* were also used for RT-PCR analysis (Fig. 3b). Of those, three primer sets specific to the 1st–9th exon sequence generated almost similar cDNA amplification between OC and YE plants. However, when a reverse primer, Bra031539_TeR4, specific to sequence near stop codon in the last 13th exon of *BrCRTISO1* was used together with Bra031539-3eF1 as a forward primer, none of OC plants tested generated a PCR amplicon, similar to in Fig. 3a. These results indicated that a lack of normal *BrCRTISO1* transcript, probably due to sequence variation at the 3′ end, could be one of the causes responsible for the OC phenotype.

Genomic sequence of the *BrCRTISO1* gene

To further confirm that the 3′ sequence of *BrCRTISO1* could not be amplified from the OC cultivar, F_2 plants

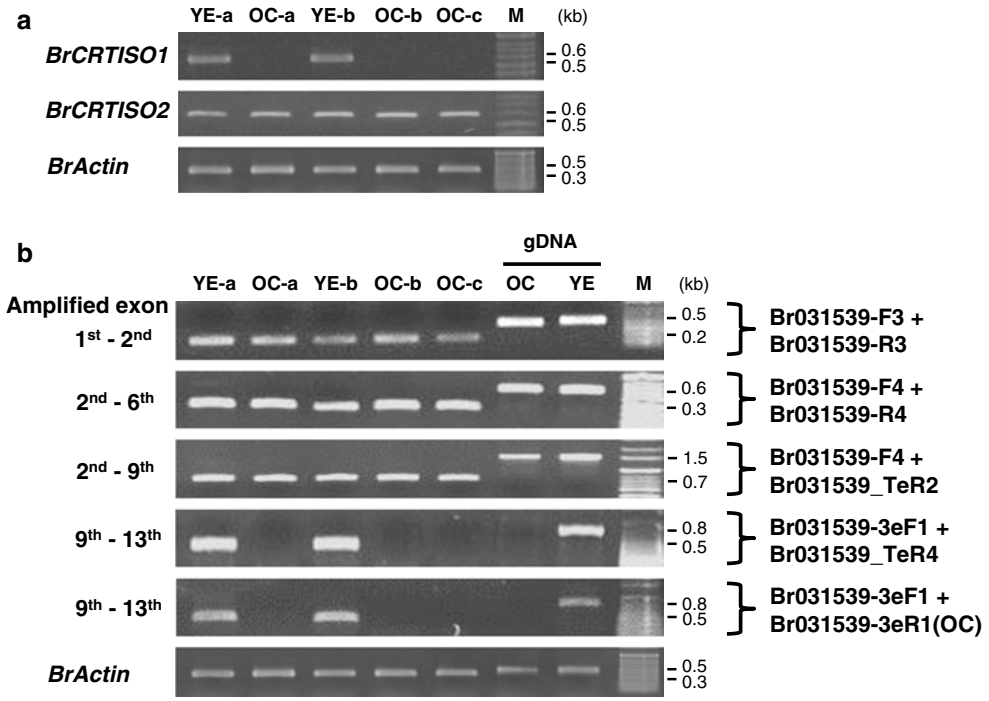

Fig. 3 RT-PCR analyses of the *BrCRTISO* genes in OC and YE cultivars. Among the F_2 progeny established by crossing between parental YE and OC inbred lines, two YE plants, *YE-a* and *YE-b*, and three OC plants, *OC-a*, *OC-b*, and *OC-c*, were used for this RT-PCR analysis. Total RNAs were isolated from leaves and their synthesized cDNA templates were used for RT-PCR analysis. **a** RT-PCR analysis of the *BrCRTISO1* and *BrCRTISO2* genes using primers specific to the 3′ end of each gene. **b** RT-PCR analysis of *BrCRTISO1* using primers specific to each exon sequence. *Numbers* on the *left* side indicate exon regions amplified by PCR. Three primer sets, Bra031539-F3 and Bra031539-R3, Bra031539-F4 and Bra031539-R4, and Bra031539-F4 and Bra031539_TeR2 (Supplementary Table 1), common to both *BrCRTISO1* sequences of OC and YE were used for amplification of the *1st–9th* exons. For the *9th–13th* exons, two primer sets, Bra031539-F4 and Bra031539_TeR4 specific to the YE *BrCRTISO1* sequence, and Bra031539-F4 and Bra031539-3eR1(OC) specific to the OC *BrCRTISO1* sequence, were used. PCR results using a primer set, Bra031539-F4 and Bra031539-3eR1 specific to YE *BrCRTISO1,* were omitted because the set generated the same results as those of the last two primer sets and **a**. Genomic DNAs from parental YE and OC inbred lines were also used as genomic DNA templates. The observed size of cDNA and genomic DNA amplicons in agarose gels matched the expected sizes based on the *BrCRTISO1* genomic sequence. *BrActin* was used as a PCR control. *M* indicates 100 bp DNA size marker, and sizes (kb) of marker bands close to amplicons are shown on the *right* side of the gel

generated by crossing OC and YE lines were analyzed by PCR from genomic DNA with the same primer set used in RT-PCR analysis. Among 60 F_2 plants, an amplicon was detected only in the 30 YE F_2 plants, but not in the 30 OC F_2 plants. Accordingly, the results confirmed that the OC cultivar has a genomic sequence variation in *BrCRTISO1*.

To investigate this variation, we amplified genomic DNA fragments of *BrCRTISO1* with various primers designed based on BRAD genome information (Supplementary Table 1) and then determined their nucleotide sequences. After assembly of the fragment sequences, two genomic sequences of about 3.5 kb covering the coding sequence of *BrCRTISO1* were obtained independently from OC (inbred line no. 32S-G7) and YE (inbred line no. S955-G8) cultivars (Fig. 4; Supplementary Fig. 1). The nucleotide sequence of the YE cultivar showed a perfect match with the *BrCRTISO1* sequence (Bra031539) deposited in BRAD, except for two SNPs in the intron between the sixth and seventh exon, where two Gs were changed to As. However, many SNPs and InDels were identified in the OC cultivar (Fig. 4). In particular, the first exon had two large InDels, of which one was a 9-bp insertion and the other a 15-bp deletion in the OC cultivar. All other InDels were found in intronic regions. SNPs were also found in some exons including the first exon, but were mainly present in introns. These sequence variations resulted in modification of 19 amino acid residues and deletion of two residues in the deduced protein encoded by the OC *BrCRTISO1* gene, compared with the 589 amino acid residues encoded by *BrCRTISO1* of the YE cultivar. Except for three amino acid residues, the residues modified were located near or within the putative transit peptide for chloroplast targeting at the N-terminus of the protein.

The reverse primer Bra031539-3eR1 (5′-CGCTCTCATGGACTTGAGCAACA-3′) used for RT-PCR analysis of the 3′ UTR of the gene was located in a region harboring two SNPs (Supplementary Fig. 1), indicating that the lack of PCR amplification of the 3′ end in the OC cultivar

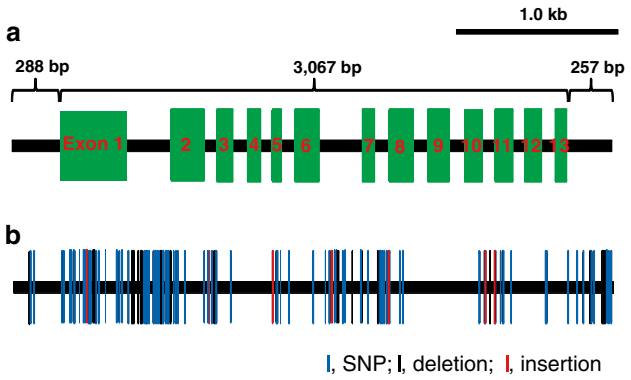

Fig. 4 Sequence variation of the *BrCRTISO1* sequence in the OC cultivar. **a** Structure of the 3.5 kb *BrCRTISO1* sequence obtained from the OC cultivar. After sequencing and assembly of genomic DNA fragments covering *BrCRTISO1* genes, exons and introns were predicted based on BRAD information. **b** SNPs and InDels found in *BrCRTISO1* of the OC cultivar. Sequence variation was determined by comparison with *BrCRTISO1* of the YE cultivar

might be due to those SNPs in the primer site. Another reverse primer, Bra031539-3eR1(OC) (5′-CGCTC***CT***ATGGACTTGAGCAACA-3′; OC-specific bases underlined), specific to the putative 3′ UTR of OC *BrCRTISO1* was designed based on the SNP sequence and used for PCR analysis (Fig. 3b). However, again no amplicon was generated from cDNA or genomic DNA in the OC cultivar. This implies that unknown factors such as methylation and secondary structure may preclude transcription as well as PCR amplification in the 3′ end of *BrCRTISO1* in the OC cultivar.

Development of DNA molecular markers for the OC cultivar

To develop DNA molecular markers that can distinguish the OC phenotype, we used polymorphic regions of *BrCRTISO1* genomic sequence identified in this study. A primer set, Bra031539-OC_MF1 and Bra031539-OC_MR1, was designed as a dominant marker for OC phenotype, based on unique sequences in the first exon and first intron of *BrCRTISO1* in the OC cultivar (Table 1). Genomic DNA PCR with the primers amplified an about 480-bp fragment specific to the OC phenotype (Fig. 5). As a YE-specific dominant marker, a primer set, Bra031539-3eF1 and Bra031539-3eR1, amplified an about 900-bp YE-specific band. As a co-dominant marker, a primer set, Bra031539-F3 and Bra031539-R3, was developed to amplify InDel regions between the first exon and second exon of *BrCRTISO1* and therefore generate two amplicons with more than 30 bp difference in size between the OC and YE cultivars. When the co-dominant marker was used to analyze the genotypes of parents and their F_1 hybrids, OC (inbred line no. 32S-G7 and S510-G6) lines consistently generated a DNA fragment of about 510 bp, whereas two YE (inbred line no. S955-G8 and NS-G6) lines generated DNA fragments of about 550 and 490 bp, respectively (Fig. 5), which was confirmed by further sequencing. Even though the NS-G6 line has the same YE phenotype as the S955-G8, its product is smaller due to additional deletions of nucleotides compared to the YE 550-bp fragment (Fig. 6). This indicates that more than two YE-type *BrCRTISO1* alleles are present in *B. rapa*.

Despite the two unique alleles in YE cultivars, the OC alleles were uniformly 510 bp in both OC parental lines (Fig. 5). For further validation, all three markers, one co-dominant and two dominant (OC-specific and YE-specific), were utilized to determine the genotypes of F_2 populations. A total of 66 $F_{2:3}$ progenies were inspected for flower color to judge the genotypes of each F_2 plant and the genotyping results showed an almost 1:2:1 Mendelian ratio of YE-homozygous: YE- and OC-heterozygous: OC-homozygous. The OC genotypes inferred from the $F_{2:3}$ progenies coincided with the genotypes of the three markers in all three cross combinations (Fig. 5).

Finally, the three markers were applied to determine the genotypes of 30 Chinese cabbage breeding lines developed from crossing OC and YE inbred lines with other Korean *B. rapa* cultivars (Fig. 7; Supplementary Table 2). Among 30 breeding lines, 15 and 15 were revealed as OC-homozygotes and YE-homozygotes by Markers ID M1, M2 and M3 that is perfectly coincided with the colors of inner leaves.

Taken together, these results strongly indicate that the markers developed in this study can successfully distinguish genotype differences based on *BrCRTISO1* allele variations among OC, YE, and their hybrid cultivars.

Table 1 List of primers used as molecular markers

Primer name	Sequence (5′→3′)	Specificity	Marker ID
Bra031539-OC_MF1	GAGGTCTGTTTCTACGAGTACGG	OC unique	M1
Bra031539-OC_MR1	CTCATTAGTCCATCTCCGACCA		
Bra031539-3eF1	ATCCATCCTTGGCTCCAGATGGTCG	YE unique	M2
Bra031539-3eR1	CGCTCTCATGGACTTGAGCAACA		
Bra031539-F3	TCCCTGGTGGGAGCTCCGGT	Co-dominant	M3
Bra031539-R3	TCCCTTCCTTCTCGTGCGGAAAC		

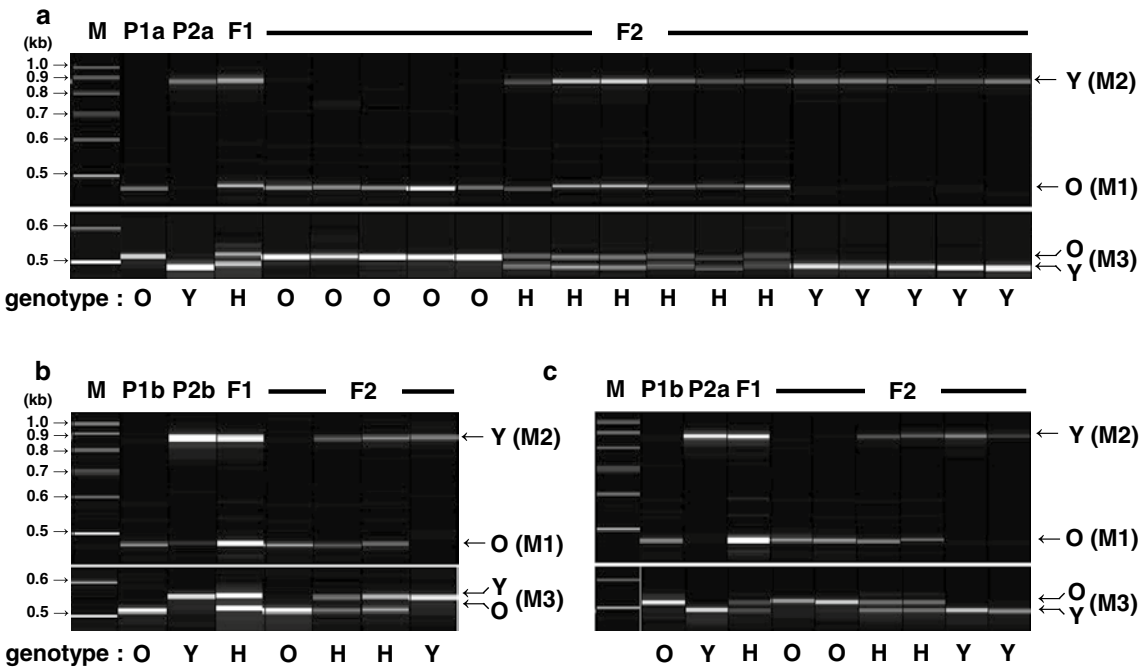

Fig. 5 Validation of molecular markers in F_2 populations. Three F_2 populations (**a**, **b**, and **c**) established from three cross combinations (S510-G6 × NS-G6, 32S-G7 × S955-G8, 32S-G7 × NS-G6, respectively) were analyzed using three markers (*M1*, *M2*, and *M3*). Amplified DNA fragments were separated and analyzed using Fragment analyzer, as described in the "Materials and methods". *Y* or *O* before the marker ID indicates specificity of the marker to *BrCRTISO1* of the YE or OC cultivar, respectively. *M1* and *M2* markers are dominant markers for YE and OC phenotypes, respectively, and they are on the same gel after independent PCRs because they show clear band size differences. *M3* is a co-dominant marker showing both genotypes. 'Genotype' shown at the *bottom* indicates genotyping results of the OC and YE traits for each F_2 plant judged by inspection of flower color in each F_2 plant and their $F_{2:3}$ progenies. Genotype is denoted as *O*, *Y*, and *H* for OC homozygote, YE homozygote, and their hybrid heterozygote genotype, respectively. Among 66 F_2 plants, representative samples from 26 plants are shown. *P1a* and *P1b* indicate parental OC inbred lines, S510-G6 and 32S-G7, respectively; *P2a* and *P2b* indicate parental YE inbred lines, NS-G6 and S955-G8, respectively; *P1b* (32S-G7) and *P2b* (S955-G8) were used to identify *BrCRTISO1* genomic sequences and develop molecular markers. *M* indicates 100 bp DNA ladder

Discussion

Current health trends have made qualitative aspects of crops more important for consumers as well as plant researchers. A number of studies have been performed to understand biological pathways in order to develop crops with unique nutritional values. Carotenoids are important pigments for both plants and animals, and changes of carotenoid content and composition in crops are considered to be one of the best choices to enhance nutritional value as well as to meet the demands of consumers. Therefore, researchers have aimed to develop new crops with high contents and/or unique compositions of carotenoids. A representative successful example is 'Golden Rice' with high β-carotene content (Beyer et al. 2002; Paine et al. 2005).

In this study, we analyzed a *B. rapa* cultivar that has OC inner leaves. The OC cultivar was originally developed in Japan by crossing and subsequent backcrossing between Chinese cabbage and turnip several decades ago (Matsumoto et al. 1998) and is currently being grown as the Orange queen cultivar. In addition, new cultivars have been developed by crossing Orange queen with other breeding lines to enhance their agricultural traits and meet consumers' demand. In this study, we used OC breeding lines originated from crossing YE cultivars with the Orange queen cultivar. The colors of the inner leaves and flowers of the OC cultivar used in this study were almost the same as those reported for other *B. rapa* OC lines (Zhang et al. 2008; Feng et al. 2012).

A few DNA molecular markers linked to the OC phenotype were only recently reported and the gene responsible for the OC phenotype has not been identified until now, even though the OC phenotype is known to be controlled by single recessive gene (Matsumoto et al. 1998; Zhang et al. 2008; Feng et al. 2012), Recently, Watanabe et al. (2011) reported that the Orange queen cultivar contains more prolycopene and total carotenoids than normal cultivars. Those results were confirmed in this study using HPLC analysis (Fig. 2). The accumulation of prolycopene was also reported in *CRTISO* mutants of Arabidopsis and tomato, in which the *CRTISO* gene encodes an enzyme converting prolycopene into lycopene and its knock-out

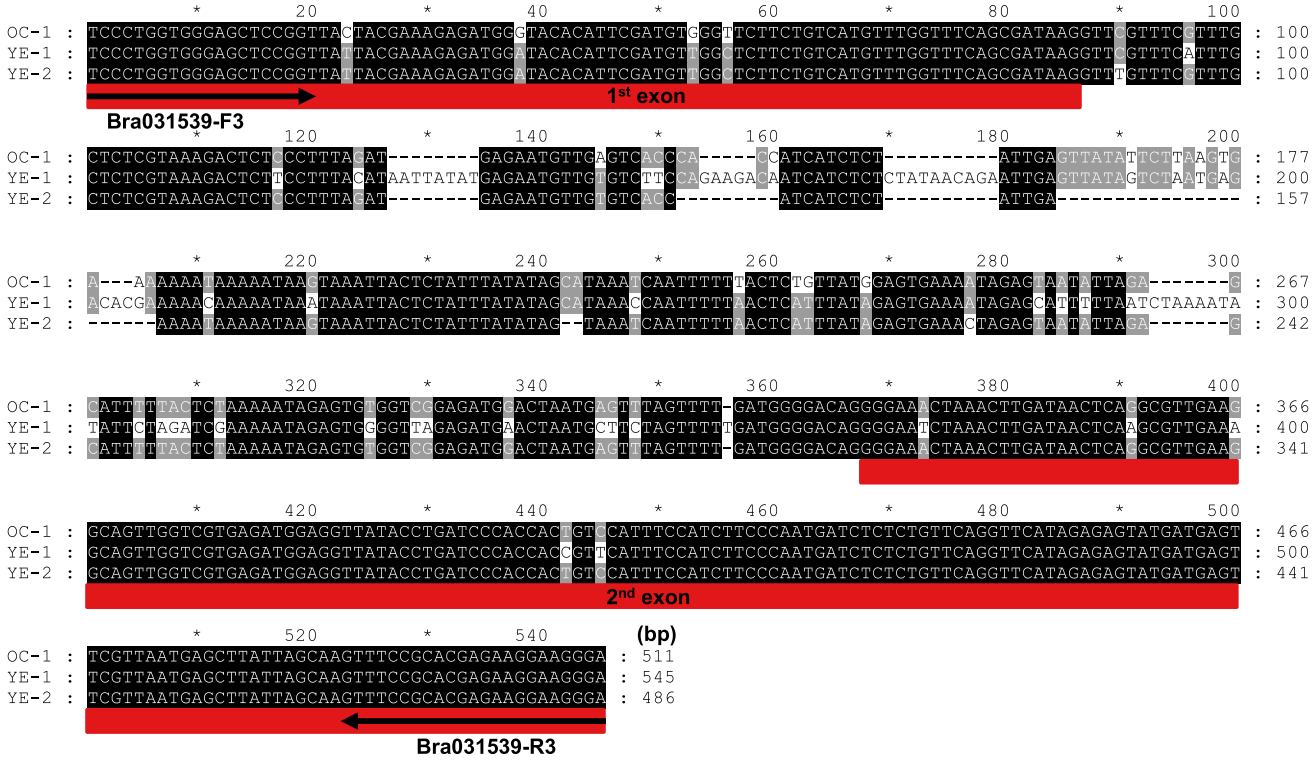

Fig. 6 Sequence validation of DNA fragments amplified by a co-dominant marker, marker ID M3. Genomic DNA sequences amplified by the M3 marker were determined by direct sequencing with primers at both ends and compared with one another. *OC-1*, *YE-1* and *YE-1* indicate DNA sequences from OC inbred lines (*P1a* and *P1b*), YE inbred line (*P2b*), and YE inbred line (*P2a*), respectively, as analyzed in Fig. 5. Sizes (bp) of sequences are shown at the end of alignment. The two primers, Bra031539-F3 and Bra031539-R3, for the M3 marker are shown by *black arrows*. *Red boxes* indicate exons (1st and 2nd exons) predicted based on BRAD information. The alignment was generated using ClustalW (http://www.genome.jp/tools/clustalw/) and GeneDoc software

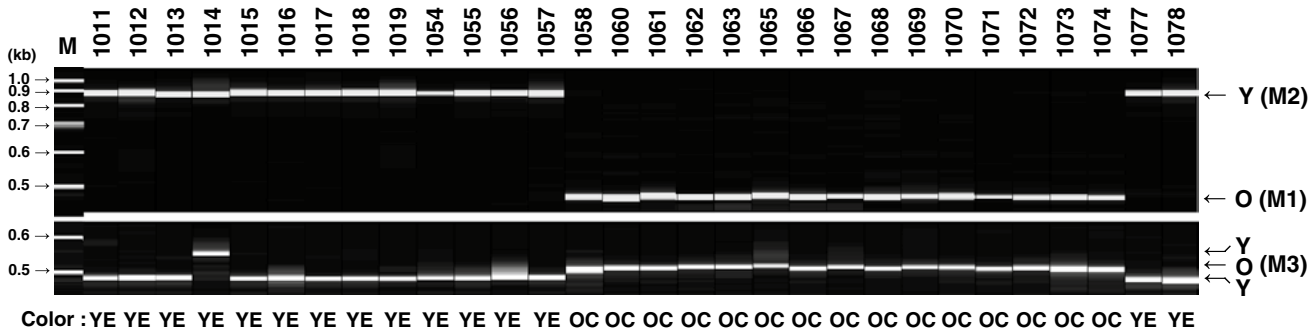

Fig. 7 Application of molecular markers to Chinese cabbage breeding lines. Thirty cultivars (no. 1011–1078 on the *top*) developed from crossing OC and YE inbred lines with other Korean *B. rapa* cultivars were analyzed using three markers (*M1*, *M2*, and *M3*). Amplified DNA fragments were separated and analyzed using Fragment analyzer, as described in the "Materials and methods". *Y* or *O* before the marker ID indicate specificity of the marker to *BrCRTISO1* of the YE or OC cultivar, respectively. *M1* and *M2* markers are dominant markers for YE and OC phenotypes, respectively, and they were run on the same gel after independent PCRs because they show clear band size differences. M3 is a co-dominant marker showing both genotypes. 'Color' shown at the *bottom* indicates color of inner leaves observed at the heading stage. *M* indicates 100 bp DNA ladder

causes accumulation of prolycopene and other lycopene isomers in cotyledons and fruits (Isaacson et al. 2002; Park et al. 2002).

In the light of these reports, we hypothesized that the OC phenotype may result from mutation of *CRTISO*. Thus, we focused on two *B. rapa CRTISO* genes,

BrCRTISO1 (Bra031539) and *BrCRTISO2* (Bra027908), and based on comparative analysis with Arabidopsis counterpart genes and expression profiling during growth of YE lines, *BrCRTISO1* is likely to play the main role in carotenoid biosynthesis. Similarly, in Arabidopsis only *CRTISO1* (AT1G06820) encodes a functional carotenoid isomerase and causes prolycopene accumulation when it is mutated (Park et al. 2002). As expected, *BrCRTISO1* was not normally expressed and had sequence variations in the OC cultivar, compared to the counterpart gene in the YE cultivar (Figs. 3, 4). Moreover, PCR analysis with F_2 progenies revealed that sequence mutation of *BrCRTISO1* co-segregated with OC phenotype (Fig. 5). Together, our current data and those in the literature imply that mutation of *BrCRTISO1* might be one explanation for the OC phenotype by causing accumulation of prolycopene.

Among 13 exons in *BrCRTISO1*, the 3′ sequences covering the 9th–13th exon were not amplified in the OC cultivar (Fig. 3). Even with OC-specific primers, the region could not be amplified. This suggests that sequence variations present in those exons could also interfere with normal *BrCRTISO1* mRNA transcription. However, no nucleotide variations that could cause abnormal transcription or frame-shift mutations could be identified in the OC *BrCRTISO1* sequence. In addition, the deduced protein sequence of the *BrCRTISO1* sequence of the OC cultivar was quite similar to that of *BrCRTISO1* of the YE cultivar, except for deletions and substitutions of some amino acid residues.

In Arabidopsis, *CRTISO1*, a syntenic homolog of *BrCRTISO1,* is regulated by epigenetic mechanisms involving CCR1/SDG8 (AT1G77300), a chromatin-modifying histone methyltransferase (Cazzonelli et al. 2009). The *B. rapa* syntenic ortholog of *CCR1*/SDG8, Bra015678, was identified on chromosome A07. Even though the *B. rapa* CCR1/SDG8-homolog is located at a chromosomal locus far from the possible chromosomal position responsible for the OC phenotype identified in both this study and previous reports (Zhang et al. 2008; Feng et al. 2012), epigenetic regulation of the OC phenotype by the homolog cannot be ruled out. To elucidate why *BrCRTISO1* of the OC cultivar is not normally expressed, further molecular studies including epigenetic analysis will be needed.

In this study, the inner leaves of the OC cultivar became deep-orange or reddish after sunlight and UV exposure (Fig. 1). Most genes involved in carotenoid biosynthesis are strongly up-regulated during light exposure in Arabidopsis (Ruiz-Sola and Rodríguez-Concepción, 2012). In addition, all Arabidopsis genes involved in carotenoid biosynthesis are highly up-regulated by UV treatment according to the Arabidopsis microarray database (http://www.weigelworld.org/resources/microarray/AtGenExpress/). This means that carotenoid biosynthesis is highly activated and thus more prolycopene can be generated under sunlight including UV in both YE and OC cultivars, whereas the prolycopene will be converted into lycopene and other downstream carotenoids such as lutein only in YE cultivars. This nicely explains why OC cultivars contain less lutein and more prolycopene than YE cultivars (Fig. 2). The presence of lycopene in the OC cultivar is likely due to another CRTISO enzyme, encoded by *BrCRTISO2*; however, why the lycopene was accumulated without conversion into downstream compounds is difficult to explain and thus needs to be addressed in further study. Considering the constitutive expression of *BrCRTISO1* and the report of Watanabe et al. (2011), we assume that prolycopene is accumulated in other parts of the OC cultivar, including green leaves, but that the color variation is hidden by chlorophyll.

One of the initial objectives of this study was development of reliable molecular markers linked to the OC phenotype. Of the three molecular markers we developed (Table 1), two could dominantly detect *BrCRTISO1* sequence variations specific to the OC or YE phenotype, and the other could co-dominantly detect sequence variations for both phenotypes through generating two DNA fragments of different size. The combination of three markers worked perfectly in determining three genotypes: OC- and YE-homozygotes and their hybrid heterozygote (Fig. 5). The markers developed in this study are gene (exon)-based markers and thus will provide highly reliable genotyping data without the loss of marker site that can result from recombination during breeding, as proven by application of the markers to Kimchi cabbage OC cultivars (Fig. 7).

In conclusion, this study characterized the *BrCRTISO1* gene encoding carotenoid isomerase and its sequence variation in a *B. rapa* OC cultivar. Moreover, the findings reported here suggest that mutation of *BrCRTISO1* might be one of the causes for the orange-color trait, and allowed the development of reliable gene-based markers for the OC trait. The results in this study will be helpful for subsequent research on genes involved in carotenoid biosynthesis of *B. rapa* as well as for development of new functional cultivars with unique carotenoid contents.

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Ethical standards The experiments comply with the current laws of the country in which they were performed.

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

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