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Development of Molecular Markers and Identification of Gene Responsible for Prolycopene Enriched Orange-Colored Leaves in Kimchi-Cabbage, *Brassica rapa*

BY

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THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Development of Molecular Markers and Identification of Gene Responsible for Prolycopene Enriched Orange-Colored Leaves in Kimchi-Cabbage, *Brassica rapa*

UNDER THE DIRECTION OF DR. TAE-JIN YANG

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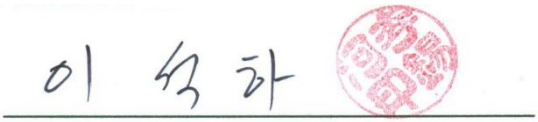

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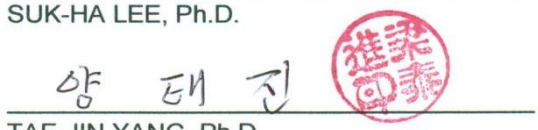

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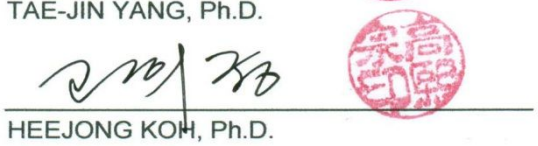

Development of Molecular Markers and Identification of Gene Responsible for Prolycopene Enriched Orange-Colored Leaves in Kimchi-Cabbage, *Brassica rapa*

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ABSTRACT

Carotenoids are essential components for photosynthesis process and photoprotection in plant. Furthermore, it is known to promote health, such as prevention of cancers and aging in human. However, most animals including human can't biosynthesize carotenoids by themselves and thus must take them from diets as essential nutrients.

Due to this reason, carotenoid itself and its biosynthesis genes have long been a subject of researches. In this study, I analyzed orange-colored (OC) Kimchi cabbage, a cultivar of Chinese cabbage (*Brassica rapa*) in Korea specialized for Korea traditional food, Kimchi, and identified a gene causing OC phenotype. OC cultivar had inner leaves of deep yellow color, which was changed into orange or reddish color under sunlight, whereas generally cultivated Chinese cabbage had yellow-colored (YE) inner leaves, regardless of sunlight exposure. In addition, OC cultivar showed pale-yellow flower color co-segregated with OC inner leaf trait and the flower color was segregated with 1:3 ratio of OC:YE in F2 progenies of crossing OC and YE inbred lines. This indicated that OC phenotype is controlled by single recessive gene. Through HPLC analysis and comparison with previous studies, OC cultivar was identified to contain high amounts of all-*trans*-lycopene (lycopene) and 7Z,9Z,7'Z,9'Z-tetra-*cis*-lycopene (prolycopene), compared to YE with high β-carotene and none of lycopene and prolycopene, implying that OC phenotype might be due to a mutation in carotenoid isomerase (CRTISO) converting prolycopene into lycopene. I found two genes, *BrCRTISO1* and *BrCRTISO2*, by searching of *B. rapa* genomic database and determined their expression. RT-PCR analysis revealed that *BrCRTISO1* was not normally expressed in OC cultivar whereas *BrCRTISO2* was constitutively expressed in both cultivars. In addition, genomic DNA PCR analysis

confirmed that *BrCRTISO1* of OC cultivar had many sequence alternations such as single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels), compared to that of YE cultivar. Taken together, this result strongly suggests that *BrCRTISO1* is a gene responsible for OC phenotype. *BrCRTISO1* is a novel *B. rapa* gene for carotenoid isomerase and will be very useful to study carotenoid biosynthesis pathway as well as to develop new cultivar with unique carotenoid contents in *Brassica* species. Currently, to assist marker-associated breeding for OC cultivar, DNA molecular markers based on sequence polymorphism found in *BrCRTISO1* gene are being examined.

Key words: carotenoid, *Brassica rapa*, orange color, prolycopene, carotenoid isomerase, *BrCRTISO1*

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LIST OF ABBREVIATIONS

PCR : Polymerase Chain Reaction

RT-PCR : Reverse Transcriptase PCR

HPLC : High performance Liquid Chromatography

EST : Expressed Sequence Tags

UTR : Untranslated Region

SNP : Single Nucleotide Polymorphism

SSR : Simple Sequence Repeat

InDel : Insertion or Deletion

INTRODUCTION

Carotenoids are a large group of isoprenoid-derived compound generated in plants and some algae and regarded as pigments responsible for yellow to red colors of fruits and flowers. Hundreds of carotenoid structures exist in nature, 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 the protection of photosynthetic apparatus against excessive light. In addition, some carotenoids are precursors of phytohormons such as abscisic acid (Schwartz et al., 1997) and strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008). Animal should take carotenoids in their food as essential source of retinols and vitamin A. It is very important because animal cannot synthesize carotenoids. In addition, ingested carotenoids are also used as pigments for characteristic colors of many birds, fish and invertebrates. In humans, carotenoids have been known to act as health-prompting and antioxidant phytonutrients (Ruiz-Sola and Rodríguez-Concepción, 2012).

Carotenoid attracted the attention of organic chemists in early 19th century and full-scale genetic and molecular studies have started from early 20th century. (Bartley and Scolnik, 1995) Since isolation of *Psy1*

in tomato converting geranylgeranyl diphosphate (GGPD) into phytoene in the first step of the carotenoid biosynthesis pathway (Ray et al., 1987; Bird et al., 1991; Bartley et al., 1992), almost genes involved in the biosynthesis pathway have been identified in various plant species (Fig. 1).

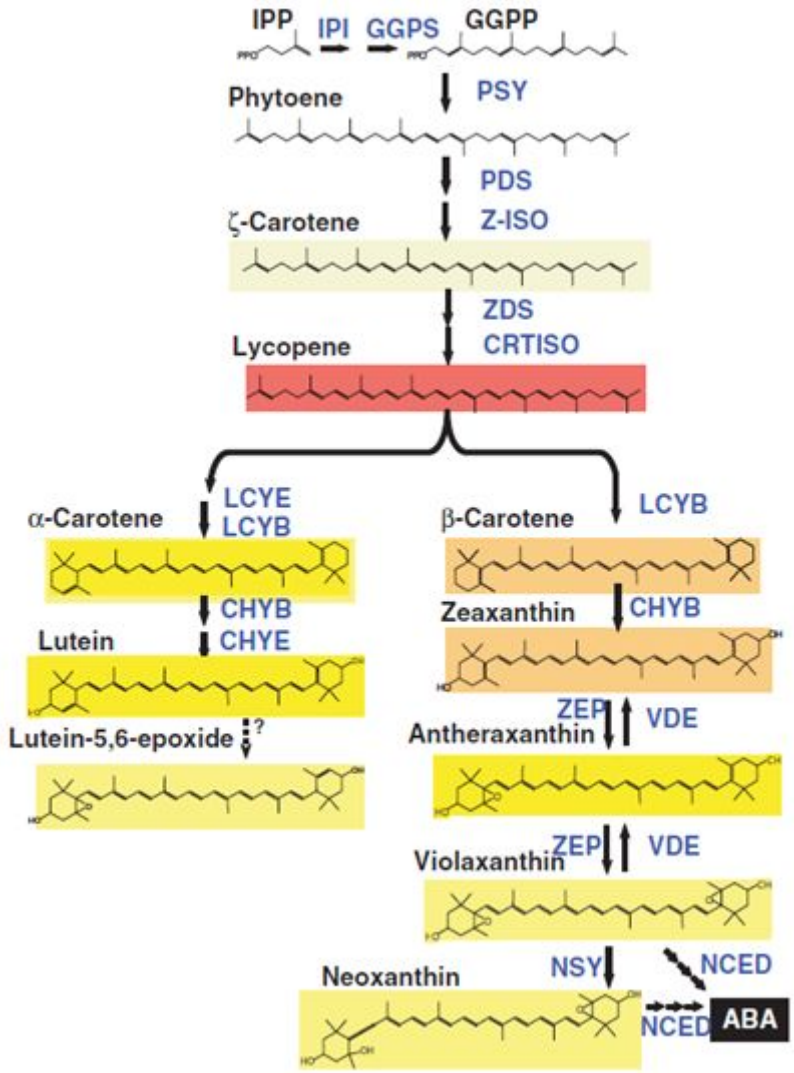


Fig. 1. Carotenoid biosynthesis pathway in plant. For the sake of simplicity only all-*trans*-configuration are shown. DXPS, 1-deoxy-Dxylulose-5phosphate synthase; DXR; 1-deoxy-D-xylulose-5phosphate reductoisomerase; IPI, isopentenyl pyrophosphate isomerase; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYB, lycopene ß-cyclase; LCYE, lycopene ε-cyclase; CHYB, ß-ring hydroxylase; CHYE, ε-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE; violaxanthin de-epoxidase; CRTISO, carotenoid isomerase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid deoxygenase (Zhu et al., 2010).

So far, a number of studies have been performed to increase carotenoid contents and to alter carotenoid composition in various plant species (Shewmark et al., 1999; Römer et al., 2000; Ye et al., 2000; Ducreux et al., 2005; Masaki et al., 2009). Among these studies, some reported mutant plants with different color of fruits and leaves, caused by aberrant accumulation of specific carotenoid (Li et al., 2001; Isaacson et al., 2002; Park et al., 2002; Li et al., 2006; Lu et al., 2006; Lopez et al., 2008). The color variations are very interesting phenomena because these provide not only opportunities to study function of gene in carotenoid biosynthesis but also unique nutritional

and agricultural values different from conventional cultivars for farmer and consumer.

Brassica species belong to the Brassicaceae family and include a number of important crop vegetables such as *Brassica rapa* (Chinese cabbage, pak choi and turnip) and *Brassica oleracea* (broccoli, cabbage and cauliflower) as well as oil seed crops such as *Brassica napus*. These *Brassica* crops are widely cultivated for human nutrition and also intensively analyzed as a plant species for polyploidy study to elucidate genome evolution. Among the species, *B. rapa* ssp. *pekinensis* (Chinese cabbage) is the number one leafy vegetable crop in China and Korea, which supplies vitamins and dietary fibers for human health. Especially, in Korea, Chinese cabbage is the most important vegetable crop to make a Korean national food "Kimchi (Schmidt and Bancroft, 2011).

In cauliflower (*B. oleracea* var. *botrytis*), semidominant *Orange (Or)* mutation was found to cause abnormal accumulation of β-carotene resulting in orange color curds (Crisp et al., 1975; Dickson et al., 1988; Li et al., 2001). The *Or* gene was map-based cloned and identified to encode a Dna-J cysteine-rich domain containing protein controlling the formation of chromoplasts where carotenoid was accumulated (Lu et al., 2006). Similarly to phenotype shown in *Or* mutant of cauliflower, orange-colored (OC) inner leaves has also reported in a cultivar, Orange queen, of Chinese cabbage which was produced by crossing

and subsequent backcrossing of Chinese cabbage and turnip, and furthermore this trait was identified to be inherited under the control of single recessive gene (Matsumoto et al. 1998; Zhang et al., 2003; Feng et al., 2010). Thereafter, quantitative trait loci (QTL) analysis and development of molecular markers linked to the trait were performed to identify a kind of *Or* gene in *B. rapa* (Zhang et al., 2008; Feng et al., 2010; Li et al., 2012), but none of study could not elucidate identity of the gene yet. Meanwhile, investigation of carotenoid composition and contents revealed that OC Chinese cabbage accumulated prolycopene and phytoene as the major carotenoid (Watanabe et al., 2011), instead of lutein and β-carotene which was mainly accumulated in conventionally cultivated normal cultivar with yellow-colored (YE) inner leaves (Wills and Rangga, 1996). In addition, OC Chinese cabbage also contained two-fold higher total carotenoid than that of YE cultivar (Watanabe et al., 2011).

This study was conducted to characterize a gene causing abnormal carotenoid composition in OC *B. rapa,* through carotenoid contents and molecular biology analyses. As a result, we identified mutation of a gene encoding carotenoid isomerase leading to orange-colored inner leaves. To our best knowledge, this study is the first report identifying gene for carotenoid isomerase contributing orange-colored inner leaves in *B. rapa* plants. This study will be valuable as a basic research of *BrCRTISO1* involved in *B. rapa* carotenoid biosynthesis and helpful for

development of new cultivar unique in agricultural and nutritional aspects.

MATERIAL AND METHODES

1. Plant materials

Seeds of inbred parent lines for orange-colored (OC) and yellow-colored (YE) Chinese cabbage and seeds of the F1 and F2 seeds from crossing between OC (female parent) and YE (male parent) were obtained from a Chinese cabbage breeding company titled “Chinese Cabbage and Breeding Company” in Korea. The seeds of inbred parent and F1 lines were planted in a pot with soil for general horticulture and then grown in a growth chamber for one month. For F2 lines, three F2 populations were established and maintained in field for 5 months. Leaves of plants were harvested and then stored in deep freezer until use for DNA and RNA isolation. For phenotyping, color of inner leaves was observed after longitudinal section of a head and flower color was observed when flower of vernalization-treated F2 plant was completely opened.

2. Quantification of carotenoid by HPLC analysis

Inner leaves of OC and YE cultivar were ground using a blender. Two g of the sample were suspended 20 mL of hexane:acetone:ethanol (2:1:1, v/v/v) and extracted for 10 min at 250 rpm. Distilled water of 10 mL was added and then extracted again for 5 min at 250 rpm. Afterward, the extract solution was centrifuged at 2,900 rpm for 5 min.

The organic phase at upper layer was separated, concentrated by using a rotary evaporator at 40℃, and dissolved with 10 mL of methyl-t-butyl-ether (MTBE). The extract was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) membrane filter and evaporated using nitrogen. The concentrated extract was redissolved in 2 mL MTBE. Then, this solution was injected into the HPLC. The HPLC analysis was serviced by Molecular and Analytical Service Center for Quality Improvement of Crops (Seoul National University, Korea, http://pgbi.snu.ac.kr/). The carotenoids were separated on a Shimadzu-10A HPLC system with a C18 column (250 × 4.6 mm, 5 micron; Shiseido UG120) and detected with a photodiode array (PDA) detector at 476 nm. Solvent A and B consisted of 100% methanol and 100% MTBE. The flow rate was maintained at 1.2 ml/min and samples were eluted with the following gradient: 0 min, 100% A/0% B; 7 min, 100% A/0% B; 14min, 82% A/18% B; 18 min, 82% A/18% B; 20 min, 100% A/0% B; and 30 min, 100% A/0% B. Identification and peak assignment of carotenoids were primarily based on comparison of their retention time and chromatogram data with those of standards, and with guidelines previously reported (Fraser et al., 2000; Howe and Tanumihardjo, 2006). The carotenoid analyses were technically triplicated with three independent samples.

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

Candidate genes involved in carotenoid biosynthesis of *B. rapa* were investigated in *B. rapa* genome database (http://brassicadb.org/brad/; BRAD; Cheng et al.,2011; Wang et al., 2011) using queries of protein sequences of Arabidopsis 16 genes reported to encode enzymes functioning in carotenoid biosynthesis (Ruiz-Sola and Rodríguez-Concepción, 2012). These searches for candidate genes were performed by using both BlastP algorithm and synteny paralog finding tool provided in BRAD. Among these genes found, *CRTISO* genes encoding carotenoid isomerase were further analyzed to identify their locations on chromosomes and synteny genome blocks using the information in BRAD and TAIR (http://www.arabidopsis.org/index.jsp) database. 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).

4. Expression analysis of *BrCRTISO* genes

The expression of *BrCRTISO* genes was investigated through microarray database searches and RT-PCR analysis. For microarray database analysis, probe sequences and IDs in the *B. rapa* 24K oligo microarray database (http://www.brassica-

rapa.org/BrEMD/microarray_ overview.jsp; Lee et al., 2008) were searched using nucleotide sequences of *BrCRTISO* genes as queries. The perfect match (PM, a measure of signal intensity) values of probes for *BrCRTISO*s were retrieved and processed to determine expression patterns, as described in Lee et al (2008). For RT-PCR analysis, total RNAs from leaves of OC and YE cultivars were isolated using RNeasy plant mini kit (Qiagen, Germany) and then 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 confirmed by spectrophotometric analysis using Nano-drop instrument (Nanodrop, USA). Five ug of total RNA was reverse transcribed into first stand cDNAs using oligo $(dT)_{18}$ primers and RNA to cDNA EcoDry Premix (Clontech, USA), according to the manufacturer's instructions. The synthesized cDNAs was 9 fold diluted 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 the coding sequence and EST sequences (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℃ for 5 min; followed by cycles of 94℃ for 1 min, 58℃ for 1 min, and 72℃ for 1 min; and finally extension at 72℃ for 7 min. The number of PCR cycles used ranged between 22 and 35 for specific amplification of the genes.

Genomic DNAs of 100ng from OC and YE cultivars were also used as templates to examine if RT-PCR primers can generate genomic amplicons. Sometimes, betaine of final 1.0 - 1.5 M was added to PCR mixture to increase specificity of PCR primer or efficiency of amplification.

5. Genome DNA PCR analysis and nucleotide sequencing of *BrCRTISO1*

Genomic DNAs were isolated from leaves of OC and YE cultivars and their hybrid F1 and F2 progenies using CTAB method, as mentioned in Allen et al (2006). Specific primers were designed to be covered genomic sequence of *BrCRTISO1* (BRAD gene ID: Bra031539) from putative promoter region to 3' UTR (Table 1). PCR conditions were as follows: 94℃ for 5 min; followed by cycles of 94℃ for 1 min, 58℃ for 1 min, and 72℃ for 1 min; and finally extension at 72℃ for 7 min. The number of PCR cycles used ranged between 22 and 35 for specific amplification of the genes. After electrophoresis with 1.0 ~ 1.3% agarose gel, the genomic amplicons were extracted using gel-extraction kit (Solgent, Korea) and then determined their nucleotide sequence 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 then compared between OC and YE cultivars.

Table 1. List of primers used in this study

Primer name	Used for	Sequences (5'→3')
Bra031539-3eF1	*BrCRTISO1* RT-PCR	ATCCATCCTTGGCTCCAGATGGTCG
Bra031539-3eR1	*BrCRTISO1* RT-PCR	CGCTCTCATGGACTTGAGCAACA
Bra027908(CRTISO2)(RT)-3eF1	*BrCRTISO2* RT-PCR	AGGAACAAGGAATAGCAGATGACCTTGA
Bra027908(CRTISO2)(RT)-3eR1	*BrCRTISO2* RT-PCR	CAAGCCTATAGCATCAAGAAGCTCTGAA
Bra031539-F1	*BrCRTISO1* genomic DNA-PCR	TCCACCCCCTAGGGTGAACCTCA
Bra031539-R1	*BrCRTISO1* genomic DNA-PCR	TCCGTTGGGCCTTCCTGTAGCC
Bra031539-F2	*BrCRTISO1* genomic DNA-PCR	GGCTACAGGAAGGCCCAACGG
Bra031539-R2	*BrCRTISO1* genomic DNA-PCR	AACCGGAGCTCCCACCAGGG
Bra031539-F3	*BrCRTISO1* genomic DNA-PCR, RT-PCR	TCCCTGGTGGGAGCTCCGGT
Bra031539-R3	*BrCRTISO1* genomic DNA-PCR, RT-PCR	TCCCTTCCTTCTCGTGCGGAAAC
Bra031539-F4	*BrCRTISO1* genomic DNA-PCR, RT-PCR	TCCGCACGAGAAGGAAGGGA
Bra031539-R4	*BrCRTISO1* genomic DNA-PCR, RT-PCR	CCCACCAACACCACCAACAGGG
Bra031539_TeF1	*BrCRTISO1* genomic DNA-PCR	GGGCATTTTGGTGTCTACCCCATGT
Bra031539_TeR1	*BrCRTISO1* genomic DNA-PCR	TGCTAGCCTTACACCCACCTGA
Bra031539_TeF2	*BrCRTISO1* genomic DNA-PCR	ACCGAGTTCTTGAACATTGATTCTTGCT
Bra031539_TeR2	*BrCRTISO1* genomic DNA-PCR, RT-PCR	TGGGATGCTGAGGAAGATACTGCCA
Bra031539_TeF3	*BrCRTISO1* genomic DNA-PCR	GTGGTTATGTTCTTTTGTTGCAACCTGT

Bra031539_TeR3	*BrCRTISO1* genomic DNA-PCR	TTGGTGTGCCCACCTGCCTC
Bra031539_TeF4	*BrCRTISO1* genomic DNA-PCR	TGTTCCTGCCTAGATTTGCCAATTCAC
Bra031539_TeR4	*BrCRTISO1* genomic DNA-PCR	TGCGAGTGTCCTTAACCAACCAAGT
Bra031539_TeF5	*BrCRTISO1* genomic DNA-PCR	AGGTACTTGATGCTGGTCTTCTTGGT
Bra031539_TeR5	*BrCRTISO1* genomic DNA-PCR	GCAAGCAGAGGACGCAAAGCC
Bra031539_TeF6	*BrCRTISO1* genomic DNA-PCR	AGTCCCCTGCACATTTCACACCAA
Bra031539_TeR6	*BrCRTISO1* genomic DNA-PCR	CCCTGTGGCAGTGACTCTTGGA
Bra031539_TeF7	*BrCRTISO1* genomic DNA-PCR	GGGTCAGGCTCACAGCGGTT
Bra031539_TeR7	*BrCRTISO1* genomic DNA-PCR	ACCTCCCACTCGAAGTTGCCA
Bra031539_TeF8	*BrCRTISO1* genomic DNA-PCR	ACACGCAGAGGCCAATTGACAG
Bra031539_TeR8	*BrCRTISO1* genomic DNA-PCR	CGTTTGTGTGTTGACTTTGAGCGT
Bra031539_TeF9	*BrCRTISO1* genomic DNA-PCR	GGGAGGTTAAGGAGATCCGGAGGC
Bra031539_TeR9	*BrCRTISO1* genomic DNA-PCR	TCCCTTACTATCACTCCTACTTGCAACA
BrActin-forward	RT-PCR	CCCTAAGGCTAACAGGGAGAA
BrActin-reverse	RT-PCR	AGCTCCGATGGTGATGACTT

RESULTS

1. Comparison of inner leaves color between OC and YE cultivars

OC cultivar showed more yellow or deep yellow color of inner leaves than that of YE cultivar, immediately after cut their heads (Fig. 2). Interestingly, when directly exposed to sunlight (~ 1,400 umol/m^2/s), inner leaves color of OC cultivar was rapidly changed into orange or reddish color at least within 5 min exposure, whereas any clear color change was not observed in inner leaves of YE cultivar. However, exposure to sunlight for more than one day made chlorophyll accumulated in inner leaves of both cultivars, and therefore difference of inner leaves color did not become obvious. To find an abiotic factor affecting color change in OC cultivar, various abiotic stresses such as cold (4℃), heat (50 - 60℃), drought (air-drying), UV (365 nm, UV-A), fluorescence light (~ 5 umol/m^2/s) were treated to inner leaves. Of them, the only UV treatment for 30 min could convert inner leaves color into orange color in OC cultivars. In addition, flower color was also different between two cultivars. OC cultivars had pale-yellow petals, whereas YE cultivars had yellow ones (Fig. 2). The flower color in OC cultivar was co-segregated with orange color of inner leaves.

Fig. 2. Representative phenotypes of YE (A, B, and C) and OC (D, E, and F) cultivars. YE cultivar had inner leaves (A) and flower in yellow (C), whereas OC cultivar had inner leaves in deep yellow (D) and flower in pale-yellow (F). After sunlight exposure for 5 min (B, E, and H), inner leaves color of OC cultivar was changed into orange (E) but not in YE cultivar (B). Orange queen cultivar also showed the same color phenotypes as OC cultivar before and after sunlight exposure (G and H).

2. Segregation of OC phenotype in F2 population of OC x YE cultivar

I investigated segregation ratio of phenotype in F2 population using petal color as a marker phenotype for OC cultivar, because the petal color was co-segregated with orange color of inner leaves in OC cultivar. The F2 population was established by selfing of three F1 populations from crossing two OC (inbred line no. S510-G6 and 32S-G7) and two YE (NS-G6 and S955-G8) inbred lines. Among 736 F2 lines, 176 had flower in pale-yellow and the remaining 560 in yellow, showing approximately 1:3 ratio. This indicated that OC phenotype is controlled by single recessive gene.

Table 2. Segregation of flower color in F2 population

Flower color	**Pale yellow(OC)**	**Yellow (YE)**	**Total F2 plants**
No. of Plants	176	560	736
Ratio	1	3.1	-

3. Carotenoid contents of OC cultivar

I investigated carotenoid contents and compositions of inner leaves using HPLC analysis (Fig. 3). When compared carotenoid composition of OC and YE cultivars, β-carotene peaks were detected in inner leaves of YE cultivar but were weakly or not detected in inner leaves of OC cultivar. On the other hand, peaks representing all-*trans*-lycopene (lycopene) were present only in inner leaves of OC cultivar. In addition, unknown peaks near lycopene were also detected only in OC cultivar. Except for these peaks, other peaks were also detected but showed similar pattern between two cultivars. This

indicated OC inner leaves had lycopene together with unknown substance as a major carotenoid, which differs from YE inner leaves with β-carotene as a major carotenoid. β-carotene and lutein were already reported to be mainly accumulated in normal cultivars with yellow inner leaves (Wills and Rangga, 1996; Watanabe et al., 2011). I couldn't identify which carotenoid was represented by unknown peak near lycopene in this study, because various standards of carotenoid were not used in this study. Recently, carotenoid study of cultivar 'Orange queen' revealed that this cultivar contained a large amount of 7Z,9Z,7'Z,9'Z-tetra-*cis*-lycopene (prolycopene)(Watanabe et al., 2011). Considering breeding history that OC cultivar was developed from crossing of cultivar 'Orange queen' and Korean traditional cultivar, the unknown peak found in OC inner leaves could be considered to be due to accumulation of prolycopene, as detected in cultivar 'Orange queen' (Watanabe et al., 2011).

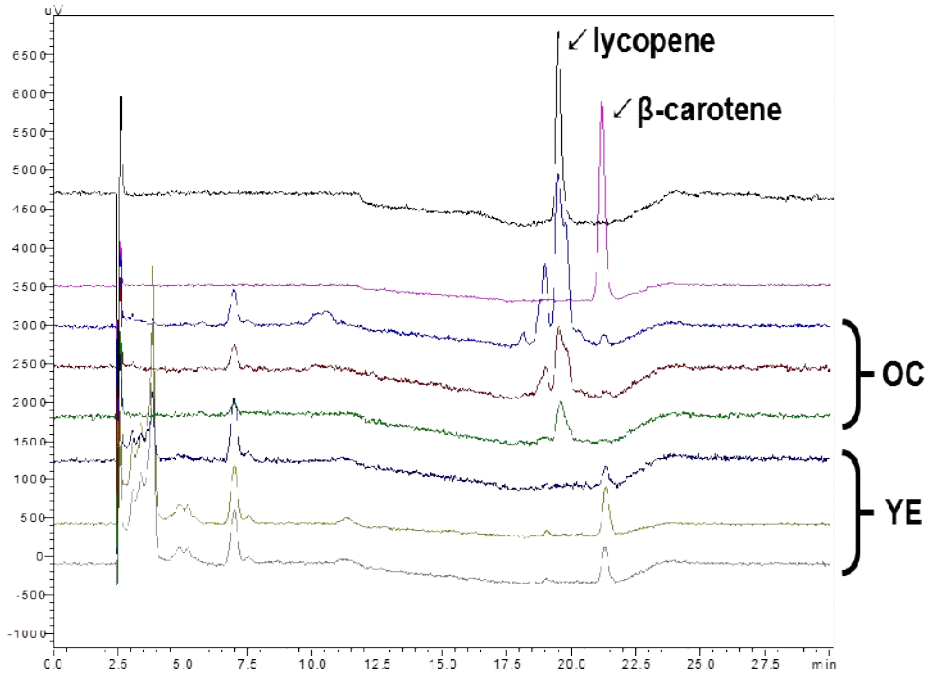


Fig. 3. HPLC chromatogram comparing carotenoid contents and composition in inner leavers of OC and YE cultivars. Carotenoid extracts of inner leaves were analyzed using HPLC and each carotenoid pigment was detected at 476nm. The upper two lines in black and pink are for standard of lycopene and β-carotene, respectively.

4. Identification of *Brassica rapa CRTISO* genes by comparative genomics

On the basis of results of carotenoid analysis in this study and in Watanabe et al (2011), I hypothesized that OC cultivar has an alternation in carotenoid biosynthesis pathway compared to YE cultivar. To prove this

hypothesis, whole set of *B. rapa* genes involved in carotenoid biosynthesis pathway was searched in *B. rapa* genome database (BRAD) using 16 Arabidopsis genes encoding enzymes involved in carotenoid biosynthesis (Ruiz-Sola and Rodríguez-Concepcióna, 2012) as quires. Finally, genome-wide searches identified 21 *B. rapa* gene highly similar to Arabidopsis counterpart genes (Table 3).

Table 3. Identification of *B. rapa* genes involved in carotenoid biosynthesis by comparative genomics

Brassica rapa (based on BRAD)			*Arabidopsis thaliana* (based on TAIR10)			
LF	MF1	MF2	Accession	Gene	Protein	Macro-synteny block
Bra008569	Bra006391	Bra023603	At5g17230	*PSY*	PSY	R
-	Bra032770	Bra010751	At4g14210	*PDS3*	PDS	T
-	Bra040411	-	At3g04870	*ZDS*	ZDS	F
Bra019899	-	-	At1g10830	*ZIC1*	Z-ISO	A
-	Bra031539	-	At1g06820	*CCR2/CRTISO*	CRTISO1	A
Bra027908	-	-	At1g57770	*CRTISO2*	CRTISO2	D
Bra029825	-	-	At3g10230	*LYC*	LCYB	F
Bra002769	-	Bra006838	At5g57030	*LUT2*	LCYE	W
Bra013912	Bra019145	-	At4g25700	*BCH1/CHY1*	BCH1	U
Bra003121	-	-	At5g52570	*BCH2/CHY2*	BCH2	W
-	-	Bra038437	At1g31800	*LUT5*	CYP97A3	B
-	-	Bra038092	At4g15110	*CYP97B3*	CYP97B3	T
-	-	-	At3g53130	*LUT1*	CYP97C1	N
Bra012127	Bra037130	-	At5g67030	*ABA1/NPQ2*	ZEP	X
Bra018616	-	-	At1g08550	*NPQ1*	VDE	A
-	Bra034026	-	At1g67080	*ABA4*	NSY	E
21 genes			16 genes			

Among the 16 Arabidopsis genes, *CCR2/CRTISO* (At1g06820) was reported to encode a functional carotenoid isomerase (CRTISO) and its knock-out mutant was reported to accumulate lycopene isomers including prolycopene (Park et al., 2002). Similarly, mutation of gene encoding CRTISO was also identified in tomato *tangerine* mutant with accumulation of prolycopene (Isaacson et al., 2002). Based on these reports, two *B. rapa* genes, Bra031539 and Bra027908, similar to Arabidopsis CRTISOs were chosen as candidates that can cause alternation of carotenoid biosynthesis in OC cultivar. I designated the two genes as *BrCRTISO1* and *BrCRTISO2*, respectively, based on the names of their Arabidopsis counterparts.

In order to investigate syntenic relationships and genomic organization, I performed *in silico* mapping of the *CRTISO* genes on chromosomes of *B. rapa* and *A. thaliana* (Fig. 4). Two genes were located on two syntenic blocks in chromosome I of the Arabidopsis genome: *CCR2/CRTISO* (At1g06820) and *CRTISO2 (*At1g57770) on the A and D blocks, respectively. These two chromosome blocks appeared to be triplicated in the *B. rapa* genome, although A block was found on several chromosomes, according to Wang et al (2011). Among the macrosyntenic blocks, the only two blocks A and D on chromosome A09 contained *BrCRTISO1* and *BrCRTISO2* genes. In particular, *BrCRTISO1* was present between two simple sequence repeat (SSR) markers, syau15 and syau19, reported to be co-segregated with orange inner leaf trait in OC cultivar (Feng et al., 2010). Taken together, *BrCRTISO1* was considered to be a strong candidate causing OC phenotype.

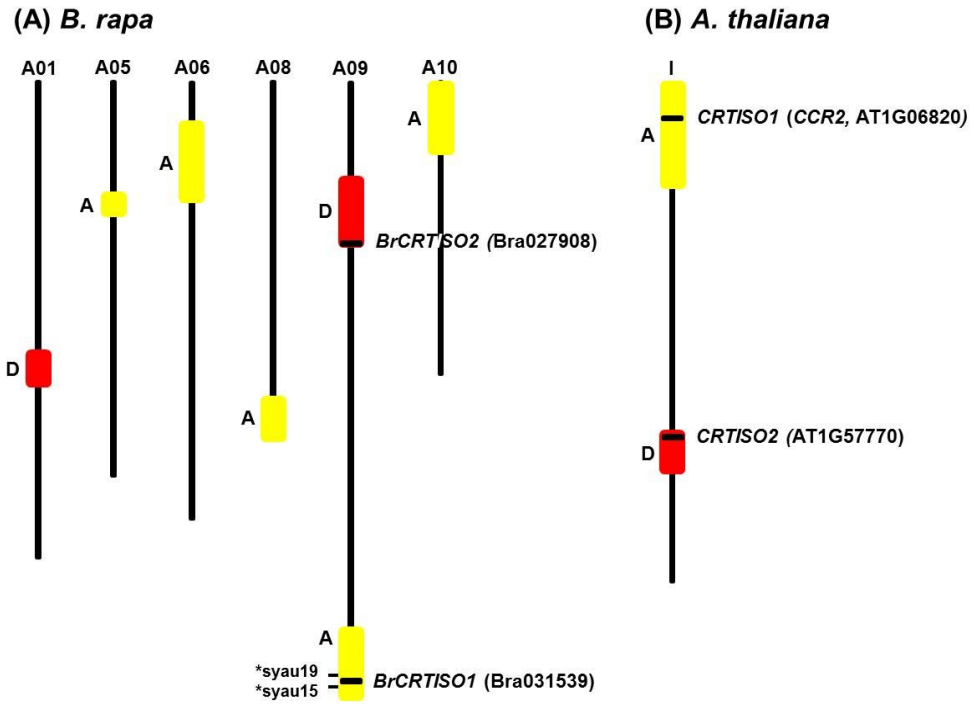


Fig. 4. Chromosomal location of *CRTISO* genes in *B. rapa* (A) and *A. thaliana* (B). The location of genes in both species was determined in BRAD and TAIR database and marked with black line with gene name on chromosome depicted as vertical line. Genome block showing macrosynteny between genomes of both species is drawn with vertical bar in same color, based on the report of Wang et al (2011). Block D on chromosome A09 is considered to be duplicated internally.

5. Expression of *BrCRTISO* genes

To investigate the expression of *BrCRTISO* genes in general cultivar of *B. rapa*, I searched the microarray database of *B. rapa* (Lee et al., 2008) and found expression values of the genes during development from seed through flowering (Fig. 5). Both genes were expressed actively during whole growth period tested. Besides, *BrCRTISO1* expression was higher than that of *BrCRTISO2*, indicating that *BrCRTISO1* of both genes plays a main role in carotenoid biosynthesis in *B. rapa.*

The expression of both genes was also investigated in leaves of OC and YE cultivars, using RT-PCR analysis with primers specific to 3' termini of the open reading frames (ORFs) of the genes. As shown in Fig. 6. *BrCRTISO1* transcript was detected in most YE cultivar but not in OC cultivar, although the first cDNA sample of YE cultivar did not generate *BrCRTISO1* amplicon. In addition, genomic amplicon of *BrCRTISO1* was also not generated in genomic DNA template of OC cultivar. On the other hand, *BrCRTISO2* transcript was detected with similar level in both cultivars. I also examined other primers specific to internal exon sequences to investigate sequence changes occurred in other regions of *BrCRTISO1*. RT-PCR analyses using primer sets covering the first to the ninth exon generated cDNA and genomic amplicons with expected size in both cultivars (Fig. 7). However, primer sets covering the ninth to the 13th exon did not generate any amplicon in OC cultivar, which was the same as RT-PCR results shown in Fig. 6. This indicated that *BrCRTISO1* was not normally expressed probably due to its sequence change at 3' terminus in OC cultivar.

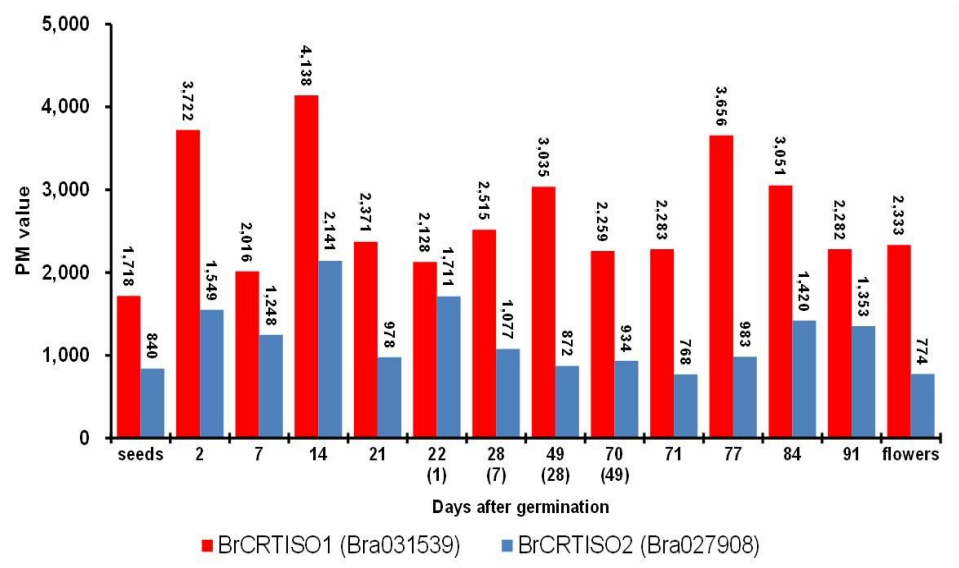


Fig. 5. The expressions level of *BrCRTISO1* and *BrCRTISO2* during development from seed through flowering according to 24K microarray analysis. The perfect match (PM) values for *BrCRTISO* genes were retrieved from the *B. rapa* microarray database and used to determine expression patterns. Numbers at the bottom indicate days after germination. Numbers in parentheses indicate days after cold (4℃) treatment for vernalization. PM values are shown at the tops of the bars. Microarray probe IDs for *BrCRTISO1* and *BrCRTISO2* are BRAS0001S00018473 and BRAS0001S00014836, respectively. The *B. rapa* 24K oligo microarray database provides only averaged PM values of repeated microarray experiments after statistical analysis (Lee et al., 2008), and therefore standard error or deviation was not calculated in this study.

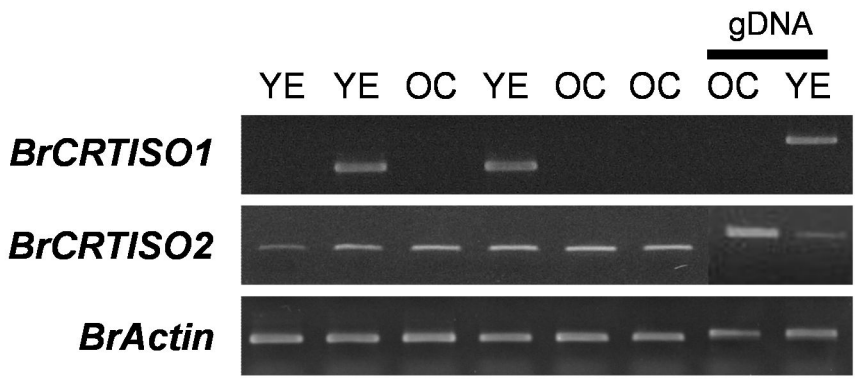


Fig. 6. RT-PCR analyses of the *BrCRTISO* genes in OC and YE cultivar. Total RNAs prepared from leaves of OC and YE cultivars were used for RT-PCR analysis with primers specific to 3' termini of open reading frames. *BrActin* was used as a PCR control. Genomic DNAs of both cultivars were also used.

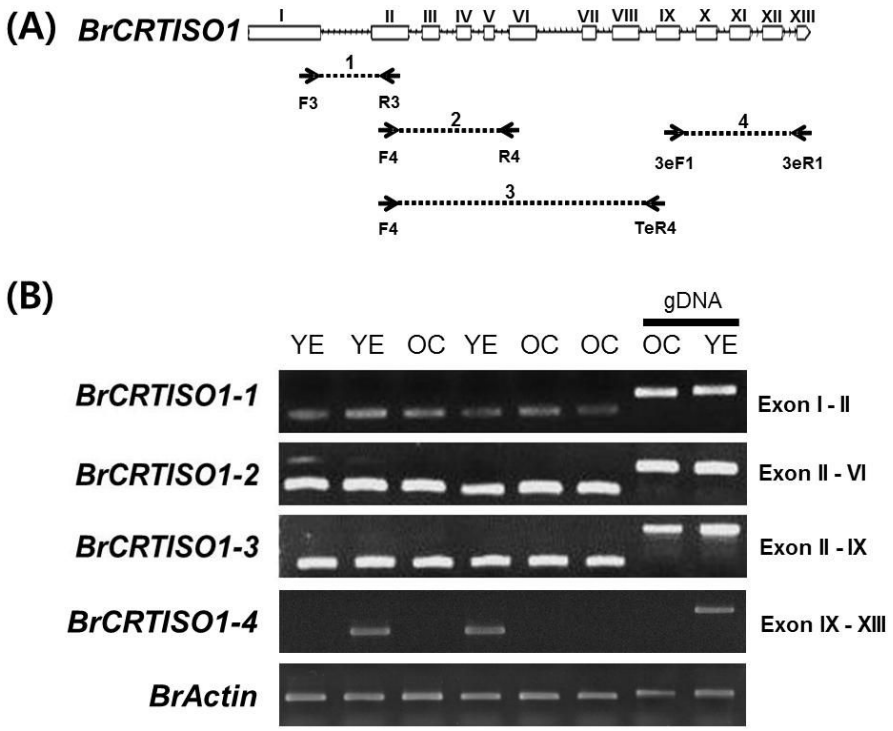


Fig. 7. RT-PCR analysis of *BrCRTISO1* expression using primers specific to each exon sequence. (A) Structure of *BrCRTISO1* gene and position of primers. White boxes and roman letters indicate exons and their no.; lines between white boxes indicate introns. Arrows and dotted lines indicate primers and regions to be amplified. Numbers above dotted lines and letters below arrows indicate primer set no. and primer names, respectively. (B) RT-PCR analysis for amplification of cDNA fragments covering exon sequences. *BrCRTISO1-1* to *-4* indicate amplicons generated using primer set no. 1 to 4, respectively. *BrActin* was used as a PCR control. Genomic DNAs of both cultivars were also used.

6. Genomic DNA PCR analysis of *BrCRTISO1* gene

To further confirm that 3'-terminus of *BrCRTISO1* that was not amplified in OC cultivar, F2 plants generated by crossing OC and YE cultivar were analyzed by genomic DNA PCR method using primer set no. 4. As shown in Fig. 8, amplicon of primer set no. 4 was detected only in YE F2 plants, but not in OC F2 plant even though amplification cycles were increase more than 40 cycles. This indicated again that OC cultivar has a defect in *BrCRTISO1* expression, probably due to mutation on genomic sequence of *BrCRTISO1*.

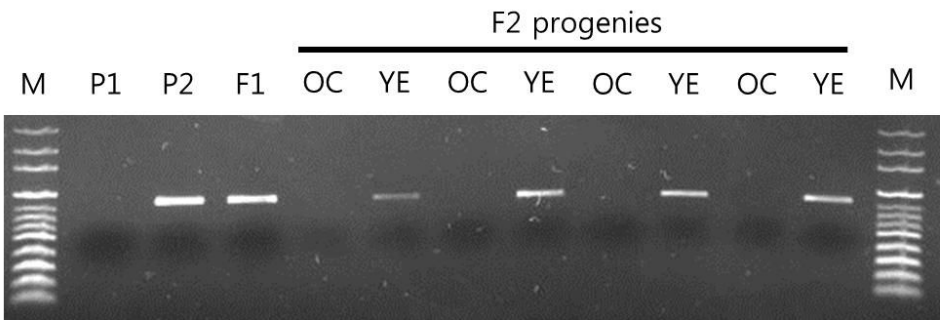
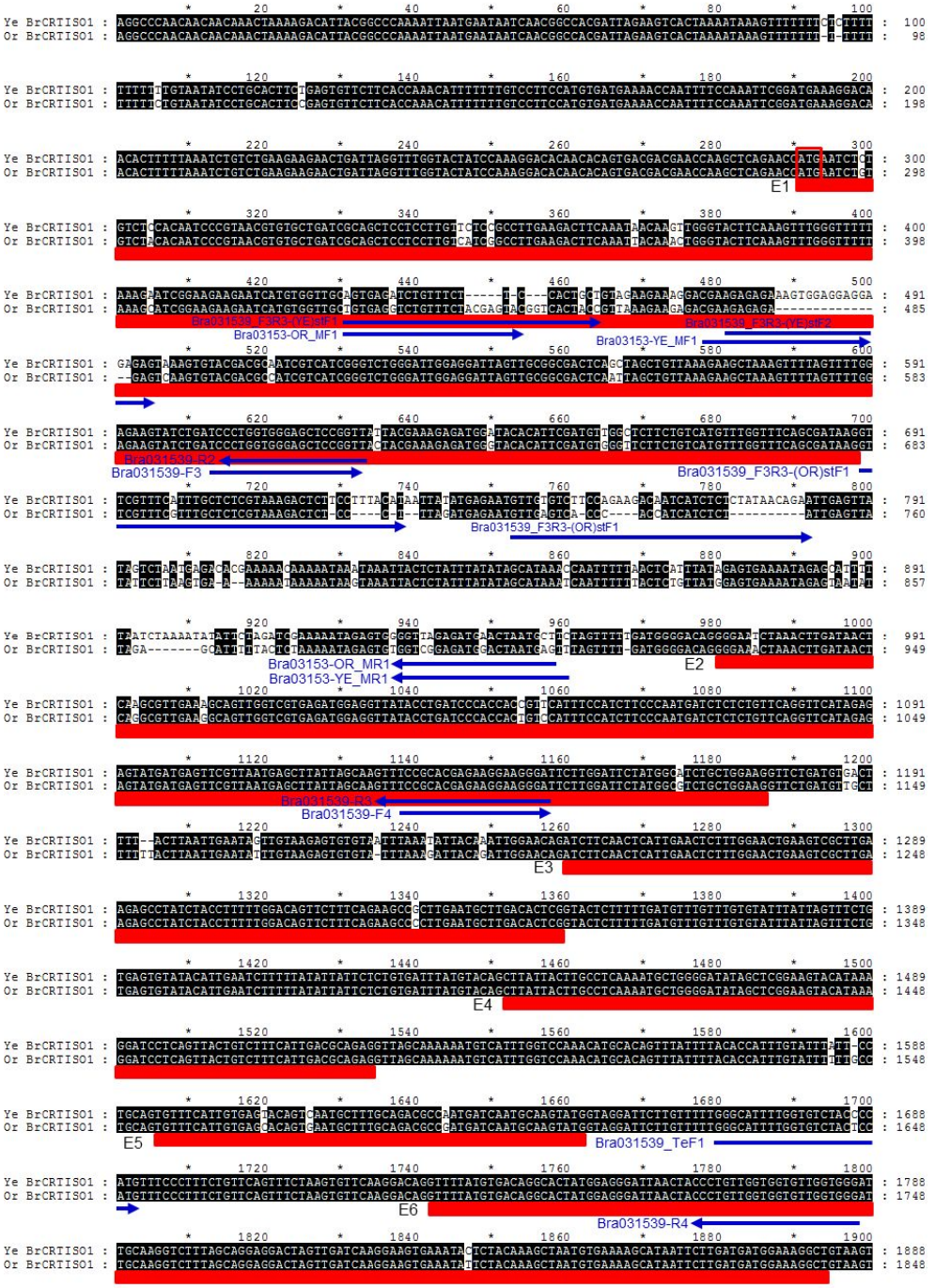


Fig. 8. Genomic DNA PCR analysis of *BrCRTISO1* in parents, F1 and F2 plants. F2 plants were generated by selfing of F1 plants from crossing OC and YE inbred lines, as mentioned in Materials and Methods. Phenotype of the F2 plants was determined based on flower color and 8 plants were randomly selected and used for genomic DNA PCR analysis with primer set no. 4 specific to 3' terminus of *BrCRTISO1*. M indicates 100 bp DNA ladder. Electrophoresis was performed with PCR reaction of 30 cycles.

7. Sequence variations of *BrCRTISO1* between OC and YE cultivars

To investigate sequence change of *BrCRTISO1* gene in OC cultivar, I amplified genomic DNA fragments of *BrCRTISO1* gene using PCR amplification with various primers designed based on BRAD genome information (Table 1) and then determined their nucleotide sequences. After assembly of the fragment sequences, about 3.5kb genomic DNA sequences covering whole ORF of *BrCRTISO1* were obtained respectively from OC and YE cultivar (Fig. 9 and 10). The nucleotide sequence of YE cultivar was almost the same as *BrCRTISO1* sequence present in BRAD, except for two bases in intron between exon VI and VII, where two Gs were changed into As in YE cultivar. When compared the sequence between both cultivars, a number of single nucleotide polymorphism (SNP) and insertion and deletion (InDel) were found (Fig. 10). In particular, the 1st exon has two large InDel regions, of which one has 9 bp insertion and the other 15 bp deletion in OC cultivar. Except for case of the 1st exon, other InDels were found only in intron regions. In case of SNP, it was also found in the some exon exons including the 1st exon, but mainly present in intron regions. These sequence changes resulted in modification of 19 residues and deletion of 2 residues compared with 589 amino acid residues encoded by *BrCRTISO1* of YE cultivar. Except for 3 amino acid residues, other residues modified were located near or within signal peptide region for chloroplast targeting at N-terminal. Among primers used in this study, a reverse primer, Bra031539-

3eR1 (5’- CGCTCTCATGGACTTGAGCAACA-3’), for RT-PCR analysis of 3’-terminal was located in the region harboring two SNPs (Fig. 9). The imperfect binding of the primer was thought to be a cause resulting in no PCR amplification in OC cultivar.


Ye BrCRTISO1
Or BrCRTISO1
E1
E2
E3
E4
E5
Bra03153-OR_MF1
Bra03153-YE_MF1
Bra031539_F3R3-(YE)stF1
Bra031539_F3R3-(OR)stF1
Bra031539_TeF1
Bra031539-R2
Bra031539-F3
Bra03153-OR_MR1
Bra03153-YE_MR1
Bra031539-R3
Bra031539-F4
Bra031539-R4

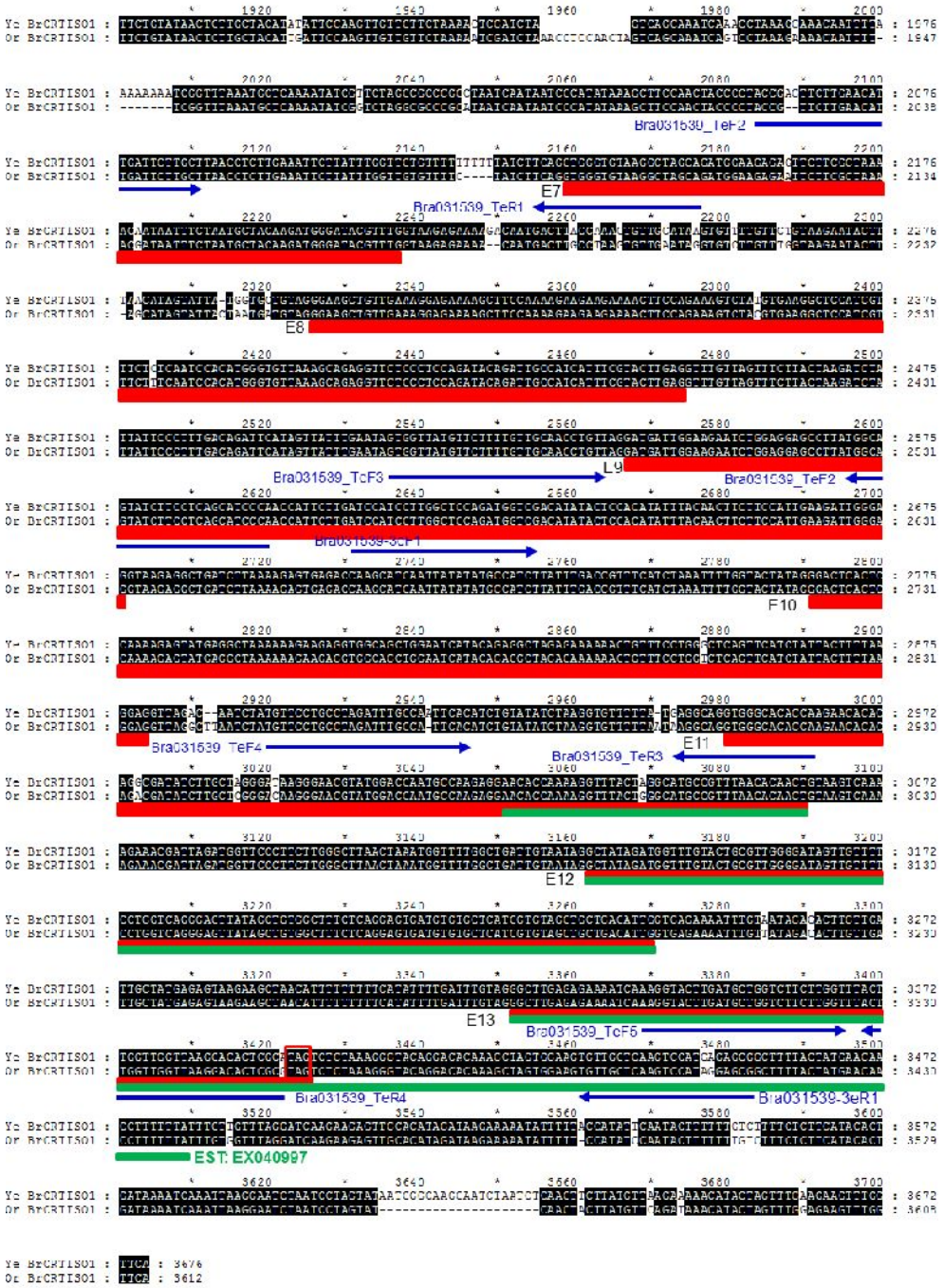


Fig. 9. Sequence alignment of the *BrCRTISO1* from OC and YE cultivars. Red and green boxes indicate exons (E1 to E13) predicted based on BRAD

information and 3'terminal region matching EST sequence (EX040997), respectively. Arrows indicate the location of primers used for RT-PCR and genomic DNA PCR analysis. The start codon and stop codon are marked by rectangles with red line. The alignment was made using ClustalW (http://www.genome.jp/tools/clustalw/) and GeneDoc software.

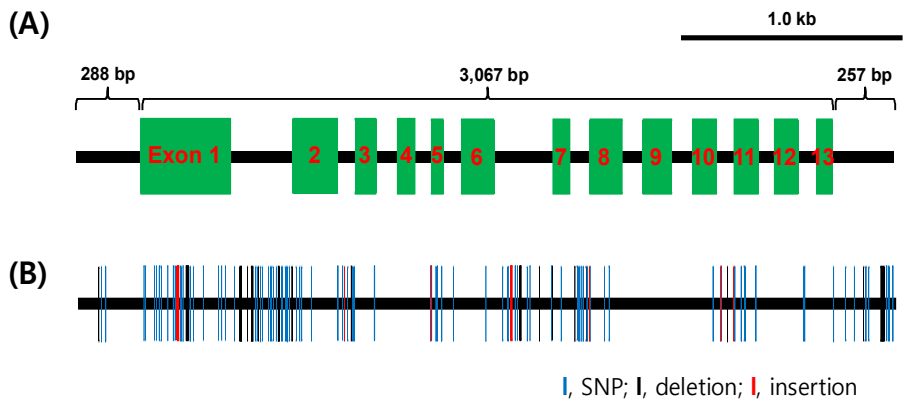


Fig. 10. Sequence variation of *BrCRTISO1* sequence in OC cultivar. (A) Structure of 3.5 kb *BrCRTISO1* sequence obtained from OC cultivar. After sequencing and assembly of genomic DNA fragments covering *BrCRTISO1* genes, its exon and intron were predicted based on BRAD information. (B) SNPs and InDels found in *BrCRTISO1* sequence of OC cultivar. Sequence variation was determined by comparison of sequence of OC cultivar with that of YE cultivar.

8. Development of DNA molecular marker for OC cultivar

To develop a DNA molecular marker that can distinguish OC phenotype, I designed primers based on InDel region of *BrCRTISO* genomic sequence identified in this study (Table 3). Provisionally, four forward primers were designed to be mapped to InDel sequence in Exon I and intron between Exon I and II. Among four primers, two, Bra03153_F3R3-(Or)stF1 and Bra03153_F3R3-(Or)stF2, are for OC and the others, Bra03153_F3R3-(Ye)stF1 and Bra03153_F3R3-(Ye)stF2, for YE cultivar. Bra03153_F3R3-(com)stRev primer in Exon II, the same as Bra031539-R3 (Table 1), was used as a common reverse primer. Additionally, I designed four primers, Bra03153-Or_MF1 and Bra03153-OrMR1 for OC cultivar and Bra03153-Ye_MF1 and Bra03153-Ye_MR1 for YE cultivar. These primers were examined through PCR analysis with genomic DNA templates of OC and YE cultivars. Among six combination of primers, the only two sets, Bra031539-F3R3_(YE)stF1 and Bra03153_F3R3-(com)stRev, Bra03153-Or_MF1 and Bra03153-Or_MR1, amplified DNA fragment specifically in YE and OC cultivars respectively. The other combinations did not amplified DNA bands specific to OC or YE cultivars. Now, the two specific primer sets are being further examined using F2 plants.

Table 4. List of primers used for DNA molecular marker

Primer name	Sequence (5'→3')
Bra03153_F3R3-(Or)stF1	GTTTGCTCTCGTAAAGACTCTCCCT
Bra03153_F3R3-(Or)stF2	GTTGAGTCACCCACCATCATCTCTA
Bra03153_F3R3-(Ye)stF1	AGTGAGATCTGTTTCTTCCACTGCT
Bra03153_F3R3-(Ye)stF2	AGAGAGAAAGTGGAGGAGGAGAGAG
Bra03153_F3R3-(com)stRev	TCCCTTCCTTCTCGTGCGGAAAC
Bra03153-Or_MF1	GAGGTCTGTTTCTACGAGTACGG
Bra03153-Or_MR1	CTCATTAGTCCATCTCCGACCA
Bra03153-Ye_MF1	CGAAGAGAGAAAGTGGAGGAGG
Bra03153-Ye_MR1	GAAGCATTAGTTCATCTCTAACCC

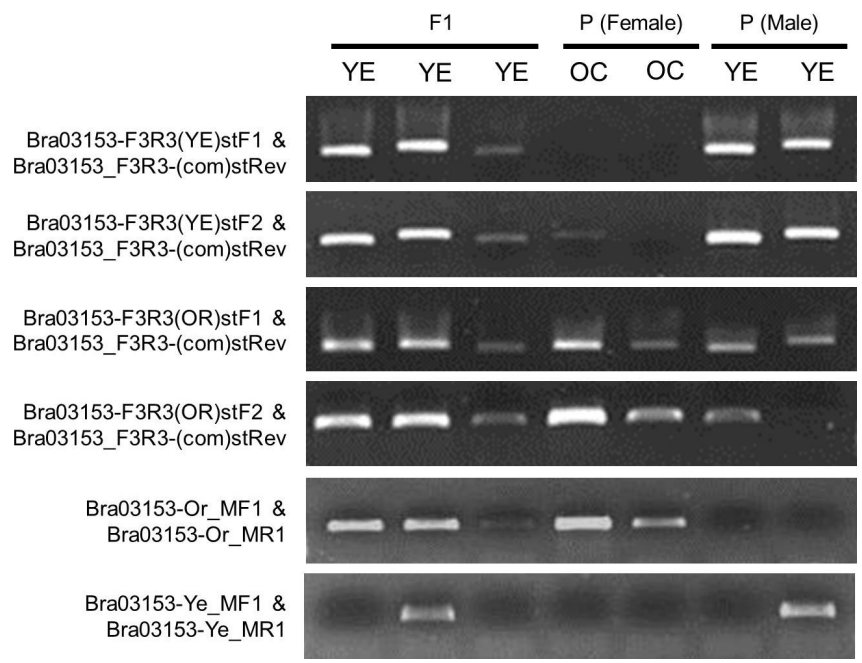


Fig. 11. Genomic DNA PCR analysis of primers to develop DNA molecular marker for OC phenotype. The names of six primer sets are indicated in left side of gel photos. Genomic DNAs of parents (P) and their crossed F1 progenies (F1) were used as PCR templates.

DISCUSSION

Carotenoids are important pigments in colors, light harvesting, and protection against excessive light in plants, and are essential sources of retinols and vitamin A in animals. In addition, current trend for health, so-called well-being, make qualitative aspect of crop more important for most people. To meet this trend, crops with unique nutrition values are being studied. Among traits, increase of carotenoid contents in crop is considered to be one of the best choices to enhance essential nutritive substance as well as to meet demand of market. Therefore, many researches have long been conducted for developing new crop 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, I analyzed a *B. rapa* cultivar that have orange-colored (OC) inner leaves. Originally, the OC cultivar was developed in Japan by crossing and subsequent backcrossing between Chinese cabbage and turnip a long time ago (Matsumoto et al., 1998), and currently is being cultivated as 'Orange queen' cultivar. In addition, new cultivars have been developed by crossing between conventionally cultivated cultivars and 'Orange queen' in East Asian countries including China and Korea, in order to meet their agricultural environmental condition and consumer's demand. In this study, I also used a cultivar originated from crossing between Korean cultivar with

yellow-colored (YE) inner leaves and ‘Orange queen’. The color of inner leaves and flowers of OC cultivar used in this study was almost same as that mentioned in other studies (Zhang et al., 2008; Feng et al., 2010).

Until now, a number of molecular biological studies of OC cultivar have been performed. However, some DNA molecular markers linked to the OC phenotype were just reported and the gene causing the phenotype is still not identified, even though OC phenotype is controlled by single recessive gene (Zhang et al., 2008; Feng et al., 2010; Li et al., 2012; Table 2), Recently, Watanabe et al (2012) reported that ‘Orange queen’ cultivar had more prolycopene and total carotenoid contents than normal cultivars. This result was confirmed again in this study using HPLC analysis (Fig. 3.). Although the peaks near lycopene was not identified as carotenoids in this study, I thought the peaks is due to accumulation of lycopene isomers including prolycopene, considering that the peaks appeared uniquely in OC cultivar originated from ‘Orange queen’ analyzed in Watanabe et al (2012).

The accumulation of prolycopene was also found in Arabidopsis and tomato mutants with knock-out mutation of gene encoding carotenoid isomerase (CRTISO), which is an enzyme converting prolycopene into lycopene, and its knock-out expression caused 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, I could hypothesize that OC phenotype may result from mutation of *CRTISO* gene. To prove the hypothesis, two *B. rapa CRTISO* genes, *BrCRTISO1* (Bra031539) and *BrCRTISO2* (Bra027908),

were identified by genome-wide searches (Table 3). Through comparative analysis with Arabidopsis counterpart genes and expression profiling during growth of normal Chinese cabbage cultivar, *BrCRTISO1* was expected to play a main role in carotenoid biosynthesis. Similarly in Arabidopsis, only *CCR2/CRTISO* (AT1G06820) of two *CRTISO* genes was reported to encode a functional carotenoid isomerase and be responsible for prolycopene accumulation (Park et al., 2002).

To investigate expression and mutation of *BrCRTISO* genes, RT-PCR and genomic DNA PCR analysis were conducted in OC and YE cultivars. As expected, *BrCRTISO1* was not normally expressed in OC cultivar and had many sequence variations, compared to results of YE cultivar (Fig. 6 and Fig. 9.). Moreover, PCR analysis with F2 progenies confirmed that sequence mutation of *BrCRTISO1* was co-segregated with OC phenotype (Fig. 7. and Fig. 8). This strongly indicated that mutation of *BrCRTISO1* is responsible for OC phenotype, as hypothesized. Abnormal expression of *BrCRTISO1* was thought to result in accumulation of prolycopene, which altered color of inner leaves and flowers. To find which sequence change affects expression of *BrCRTISO1,* RT-PCR and genomic DNA PCR analysis with several primers covering internal region of ORF were performed. Among 13 exons, 3'-terminal covering exon IX to XIII was not amplified in both analyses (Fig. 7.). Even though primers specific to mutated *BrCRTISO1* of OC cultivar were used, amplicon for Exon IX to XIII couldn't be detected (data not shown). This implied that sequence variations present in those exons probably interfere with normal

BrCRTISO1 mRNA transcription and thus its truncated form of mRNA can be expressed. However, any nucleotide variation that can change spliced form or frame shift couldn't be found in the exons. In addition, mutated *BrCRTISO1* sequence of OC cultivar could also be deduced into amino acid sequence, which was most similar to that of *BrCRTISO1* of YE cultivar, except for deletion of amino acid residues in N-terminal and substitutions of some amino acid residues in whole sequence. In Arabidopsis, *CRTISO1,* syntenic paralog of *BrCRTISO1,* was identified to be regulated by epigenetic mechanisms involving CCR1/SDG8, a chromatin-modifying histone methyltransferase (Cazzonelli et al., 2009). I could find *B. rapa* syntenic paralog of *CCR1*/SDG8 (AT1G77300), Bra015678, on Chromosome A07 by genome-wide searches. However, the gene was likely not to be related to OC phenotype, because QTL and DNA markers for OC phenotype were located on chromosome A09 together with *BrCRTISO1* (Zhang et al., 2008; Feng et al., 2010; Li et al., 2012; Fig. 4.). To elucidate a reason why *BrCRTISO1* of OC cultivar was expressed differently from that YE cultivar, further studies will be needed.

In this study, color of inner leaves became deep-orange or reddish in OC cultivar after sunlight exposure (Fig. 2.). Most genes involved in carotenoid biosynthesis are known to be strongly up-regulated during light exposure in Arabidopsis (Ruiz-Sola and Rodríguez-Concepción, 2012). In addition, all of Arabidopsis genes (Table 3) were highly up-regulated by UV treatment when Arabidopsis microarray database (http://www.weigelworld.org/resources/microarray/AtGenExpress/) was

searched. This means that carotenoid biosynthesis is accelerated and thus prolycopene can be more generated by sunlight including UV. Afterward, prolycopene will be converted into lycopene and other downstream carotenoids in YE cultivar. However, due to CRTISO mutation, OC cultivar is thought not to convert prolycopene into other carotenoids and accumulate it at high level, finally affecting color of inner leaves and flowers. As in inner leaves, prolycopene will be accumulated in green leaves of OC cultivar although the color variation is hidden by chlorophyll, considering Watanabe et al (2012)' study reporting prolycopene accumulation in whole heads of OC cultivar.

In conclusion, this study firstly characterized *BrCRTISO1* gene encoding carotene isomerase and its function in carotenoid biosynthesis through forward genetics using OC *B. rapa* cultivar. Moreover, this study confirmed that orange-color trait caused by prolycopene accumulation was due to mutation of *BrCRTISO1*. The results in this study will be helpful for subsequent researches on gene involved in carotenoid biosynthesis of *B. rapa* as well as for development of new cultivar with unique carotenoid contents.

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ABSTRACT IN KOREAN

카로티노이드는 식물에서 광합성과 광으로부터의 보호에 필수적인 요소이며 더불어 인간의 항암효과와 노화방지와 같은 건강증진의 효과가 있다고 알려져 있는 물질이다. 그러나 인간을 포함한 대부분의 동물은 스스로 카로티노이드를 합성하지 못하기 때문에 필수적인 영양소로서 식품을 통해 섭취해야 한다. 이러한 이유로 카로티노이드와 생합성 유전자는 오랫동안 연구 주제로 선택되어 왔다. 본 연구에서는 한국의 전통음식 '김치'의 재료인 배추의 품종 중 하나인 오렌지 배추(OC)와 해당 형질을 나타내게 하는 원인 유전자를 분석하였다.

오렌지 배추는 진한 노란색 내엽을 가지며 이 내엽색은 햇볕에 노출되었을 때 오렌지 색 또는 붉은 색으로 변화하는 특징이 있다. 반면 일반적으로 길러지는 일반 배추(YE)는 빛의 노출과는 상관없이 노란색 내엽을 갖는다. 또한 오렌지 배추는 이는 오렌지 색 내엽 형질과 co-segregation 하는 pale-yellow 의 꽃을 가지며 이 화색은 오렌지 배추와 일반 배추의 inbred line 의

교배로 만들어진 F2 집단에서 1:3 (OC:YE)의 분리비를 보인다. 이는 오렌지 배추의 형질이 단일 열성 유전자에 의해 조절됨을 시사한다. HPLC 분석과 선행연구와의 비교를 통해, 일반배추는 주된 카로티노이드로 β-carotene 을 함유하며 prolycopene 과 all-trance-lycopene 은 전혀 갖지 않는 반면, 오렌지 배추는 prolycopene 과 all-trance-lycopene 을 많이 함유하고 있음을 알 수 있었다. 이는 오렌지 배추의 형질이 prolycopene 을 lycopene 으로 전환하는 carotenoid isomerase (CRTISO) 유전자의 변이에 의해 나타나는 것임을 암시한다.

배추 지놈 데이터베이스 분석을 통해서 찾은 배추 CRTISO 유전자인 *BrCRTISO1* 과 *BrCRTISO2* 의 발현을 분석한 결과, *BrCRTISO1* 은 오렌지 배추에서 정상적으로 발현하지 않는 반면 *BRCRTISO2* 는 오렌지 배추와 일반 배추 모두에서 동일하게 발현함을 알 수 있었다. 또한 본 유전자 염기서열을 genomic DNA PCR 분석 및 염기서열 분석으로 확인한 결과, 오렌지 배추 *BrCRTISO1* 유전자상에 SNP 나 InDel 같은 염기서열 변이가 다수 존재함을 알 수 있었다. 종합하면, 본 연구 결과는 *BrCRTISO1* 유전자가 오렌지 배추 형질의 원인

유전자임을 강하게 시사하고 있다. *BrCRTISO1* 은 배추에서 전혀 연구되지 않은 새로운 유전자이며 배추과 식물에서 특정 카로티노이드를 축적하는 새로운 품종 개발에서뿐만 아니라 카로티노이드의 생합성 경로를 연구하는데도 매우 유용할 것이다. 현재 *BrCRTISO1* genomic sequence 상의 변이지역 염기서열을 바탕으로 오렌지배추 품종의 분자육종을 위한 분자마커를 설계하여 검정 중에 있다.