



Fine mapping and characterization of the *or* gene in Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*)

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ABSTRACT. Orange inner leaves/heads is a qualitative trait in Chinese cabbage that is controlled by a single recessive gene. Orange head Chinese cabbage contain more carotenoids than its white head counterpart; hence, this trait is of interest to both researchers and consumers. In this study, we selected the orange head Chinese cabbage line 07A163 and the white head Chinese cabbage line Chiifu as test materials. We localized the target gene controlling the orange head trait to the A09 linkage group, with a physical distance of approximately 19.9 kb between the two markers, *syau26* and *syau28*. This region contains six candidate genes, including *Bra031539*, which was predicted to encode CRTISO, a carotenoid isomerase specifically required for carotenoid biosynthesis. A comparison of the nucleic acid sequences of the two test materials revealed 88 and 7-bp deletions and 88 SNPs in the promoter region of *Bra031539* in line 07A163, along with a 6-bp deletion in the first exon and early termination at the 3' end

of this gene. BLAST analysis revealed that 22 amino acids were altered and 17 amino acids were lost in *Bra031539* in the orange head line 07A163. We developed the *BrPro1* molecular marker in the promoter region of *Bra031539* that can be used for early identification of orange head materials, thereby accelerating the breeding process of orange head Chinese cabbage.

Key words: Chinese cabbage; Fine mapping; CRTISO; Molecular marker; Orange head trait; Sequence blasting

INTRODUCTION

In recent years, orange head Chinese cabbage has become a popular focus of study for vegetable breeders. The inner leaves of Chinese cabbage are usually white or yellow and rarely orange. Much research has focused on the orange inner leaf trait in this important crop. More carotenoids accumulate in orange head Chinese cabbage than in white head Chinese cabbage (Watanabe et al., 2011).

An important role of carotenoids in plants is providing flowers and fruits with distinctive colors. The carotenoid biosynthetic pathway begins with the production of two 20-carbon geranylgeranyl diphosphate (GGPP) molecules that are catalyzed by geranylgeranyl diphosphate synthase (GGPS). Two GGPPs then form 15-*cis* phytoene via phytoene synthase (PSY), the first rate-limiting enzyme in the carotenoid biosynthesis pathway (Bramley et al., 1992; Fraser et al., 1994). Next, 15-*cis* phytoene is converted to tetra-*cis*-lycopene by two phylogenetically related enzymes: phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS) (Nisar et al., 2015). Carotenoid isomerase (CRTISO) and ζ -carotene isomerase (Z-ISO) function as carotenoid isomerases in plants during the conversion of tetra-*cis*-lycopene to all-*trans*-lycopene (Li et al., 2007; Chen et al., 2010). The cyclization of lycopene is an important step in carotenoid metabolism; the substrate for cyclases is all-*trans*-lycopene. There are two branches in this step: α -carotene is formed via lycopene ϵ -cyclase (ϵ -LCY), followed by lycopene β -cyclase (β -LCY), whereas β -carotene is formed via β -LCY only (Cunningham et al., 1993; Cunningham et al., 1996; Pecker et al., 1996; Ronen et al., 1999). Using these carotenes, a series of enzymes catalyze the production of abscisic acid, strigolactones, carlactone, β -citraurin, volatiles, and scents. Much progress has been made in elucidating the carotenoid biosynthesis mechanism in *Arabidopsis* (Ruiz-Sola and Rodríguez- Concepción, 2012) and tomato (Fraser et al., 1994), but less is known about this process in other, non-model plants.

The abnormal accumulation of carotene in the orange head Chinese cabbage is controlled by a single recessive gene (Feng et al., 2012). To analyze the reason behind this trait in Chinese cabbage, extensive research has been carried out to investigate the composition of carotenoids, develop molecular markers, complete fine mapping of the target gene and so on. The total carotenoid content in orange head Chinese cabbage is much higher than that in its white head counterpart. Moreover, lycopene is only present in orange head Chinese cabbage (Qing and Cheng, 2000; Chen, 2008; Watanabe et al., 2011; Tuan et al., 2011). Previous studies have led to the development of many molecular markers, such as SSR markers (Feng et al., 2012; Zhang et al., 2013), SCAR markers (Zhang et al., 2008), RAPD markers and so on, which are located near the *or* gene on linkage group A09. *Bra031539* is one such gene that is

in the target region considered to harbor the predicted candidate gene that encodes carotenoid isomerase (Zhang et al., 2013). In the current study, we analyzed the nucleic acid sequence structure and protein sequence characteristics of this target gene utilizing fine mapping. A comparison of wild-type Chinese cabbage (white head) and orange head Chinese cabbage revealed some differences between these materials. We developed a gene-specific marker that can be used to accelerate the breeding process of orange head Chinese cabbage.

MATERIAL AND METHODS

Plant materials and mapping population

The *B. rapa* inbred lines 07A163 and Chiifu were used as materials in the present study. Line 07A163 (which has orange inner leaves) and Chiifu (which has white inner leaves) were provided by Shenyang Agricultural University, China. We previously established a large F₂ population of 2,200 individuals with the recessive orange-colored inner leaf trait from the two parents and localized the *or* gene to a target region between BAC clones KBrH010M08 and KBrB017P15 on chromosome A09 (Feng et al., 2012).

Germinated seeds of the F₂ population were vernalized at 4°C for approximately 20 days and sown in a seedling plate. All plants were grown in a solar greenhouse under natural conditions. After flowering, recessive individuals in the fine mapping population were identified based on the presence of orange petals.

PCR amplification and SSR marker development

Genomic DNA was extracted from fresh leaves of the parents and F₂ individuals as described by Murray and Thompson (1980) with minor modifications. The concentration of genomic DNA was estimated by examination on agarose gels and adjusted to 50 ng/μL.

Polymerase chain reaction (PCR) was carried out in a volume of 10 μL: each reaction contained 1.0 μL 10X *Taq* Reaction Buffer (containing Mg²⁺), 1 U *Taq* DNA Polymerase, 0.8 μL of 2.5 mM dNTP, 1 μL of 0.5 μM primer and 50 ng template DNA. The PCR program used on the Bio-Rad (USA) iCycler Thermal Cycler was as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were separated on a 6% denaturing polyacrylamide gel in 1X TBE buffer, and the gel was stained with 2 g/L silver nitrate after electrophoresis.

Two SSR markers, *syau19* and *syau15*, were used to screen 2,200 F₂ recessive individuals. After confirming the target region containing the candidate *or* gene, the program Primer Premier 5.0 was used to design primers for markers more closely linked to the *or* gene candidate. Several new markers were designed to reduce the distance from the candidate gene using the 2,200 F₂ recessive individuals to calculate the recombination frequency for fine mapping. Linkage relationships were determined with DNAMAN 5.2, and the map distances were calculated in cM.

Candidate gene prediction and gene cloning

Gene sequences of *B. rapa* deposited in the *Brassica* database (<http://brassicadb.org/brad/index.php>) were aligned using the BLAST program from the National Center for

Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All genes located within the interval covering the target gene were identified and the candidate gene was predicted to be *Bra031539*.

Based on the *Bra031539* nucleotide sequence, 11 pairs of PCR primers were designed for cloning of the full-length gene (Table 1). PCR products from both parental lines were purified using a PCR purification kit for TA cloning and sequenced by SinoGenoMax Co., Ltd. (Beijing, China). The sequences were analyzed using ClustalX1.83 software.

Analysis of the protein sequences of *Bra031539*

DNASTar software was used to deduce the amino acid sequence of *Bra031539*, its subcellular localization was predicted using TargetP (<http://www.cbs.dtu.dk/services/TargetP>), SignalP (<http://www.cbs.dtu.dk/services/SignalP>) was used to predict the signal peptide region, the modification sites were identified using the Prosite program (<http://www.expasy.ch/prosite>), and the predicted product of *Bra031539* was characterized via comparison to the conserved domain database using the SMART program (<http://smart.embl-heidelberg.de>).

Development of the molecular marker based on differences in the promoter region

Three primers were designed for the promoter region of *Bra031539* based on the differences in nucleotide sequences between Chiifu and 07A163 (Table 2). White, yellow and orange head Chinese cabbage were used to verify these primers. F1 populations were established by crossing the orange line 07A163 with white lines (Chiifu, 14A014 and 14A065) and yellow lines (14A036, 14A038 and 14A029), respectively. Members of the F2 segregating population were used to verify the markers.

Table 1. Eleven primers used for *Bra031539* sequence amplification.

Primers	Forward (5'-3')	Reverse (5'-3')	Product length
Br1	GATTAGGATTAGGGTTAGTG	GATTCTTCTCCGATTCTTT	750
Br2	GCCCAACGGATAGGTGTAAC	CATCTCACGACCAACTGCTT	1064
Br3	AGCGATAAGGTTTCGTTTCAT	TCCATAGTGCCTGTACATA	1073
Br4	AGCGATAAGGTTTCGTTTCAT	TACAGCCTTCCATCATCAA	1205
Br5	GCTCGGAAGTACATAAAGGA	TTGGGATGCTGAGGAAGATA	1124
Br6	CCCTTCTGTTTCAGTTTCTA	CCTCTTACCTCCAATCTTC	990
Br7	TACAGATTGCCATCATTTCG	AGCCACAGCTATAACTCCCT	778
Br8	TACAGATTGCCATCATTTCG	GATTAGATTCTTGCCCAT	1205
Br9	CTATGTTCTCCTAGATTT	GTTGAGATTAGATTCCTTGC	742
Br10	CTATGTTCTCCTAGATTT	GATGATACATGAACCAAACCT	796
Br11	CACAGTGACGACGAACCAAG	TCTCAGACCAACTGCTTTC	758

Table 2. Molecular marker primers.

Primers	Forward (5'-3')	Reverse (5'-3')	Product length
BR1	TAGATTTTATGAATACGGGACA	CGAATTGGAAAATGGTTT	642
BR2	TTATGAATACGGGACATG	TTGGATAGTACCAAACCT	700
BR3	TTATGAATACGGGACATG	ACACTCAGAAGTGCAGGA	578

RESULTS

Fine mapping of the *or* gene and candidate gene analysis

In the 2,200 F₂ recessive individuals used to fine map the *or* gene, recombination occurred between SSR markers *syau19* and *syau15* and the target gene in 77 and 115 individuals, respectively. To obtain markers more closely linked to the target gene, we designed five SSR markers in the interval between *syau19* and *syau15* (Table 3). SSR markers *syau26* and *syau28* were ultimately localized to a 19.9 kb region, with a mapping distance between the markers and the candidate gene of 0.11 cM and 0.09 cM, respectively (Figure 1).

DNA sequence analysis of the 19.9-kb region based on the Brassica database (<http://brassicadb.org/brad/index.php>) revealed that it contains six genes (Table 4). Of these genes, *Bra031539* encodes the carotenoid isomerase CRTISO, an isomerase enzyme specific to the carotenoid biosynthetic pathway.

Table 3. Sequences, number of recombinants and map distances of SSR marker primers used for fine mapping.

Markers	Forward (5'-3')	Reverse (5'-3')	Recombinant	Map distance (cM)
<i>syau19</i>	CCGAAGCCAGATTAGGAG	GCCCATGCCTACGAATGATAT	77	1.75
<i>syau4</i>	TTTAAGGACTAATTAACGAGGTTAAT	ACGGAGTGATTTATTACATG	12	0.27
<i>syau26</i>	TTTTGGTTTCACCTGATGTTTCTT	TCCAAGTCTTCCTTCTTCTATTT	5	0.11
<i>syau28</i>	ATCATCCACTGGTCTTATCCTCC	AGTTTACAGAGCGAACGAAGC	4	0.09
<i>syau30</i>	TGAATTACGATGATCTAACTGGTT	GATTGTAAAGATTCTACGTCAAAGG	33	0.75
<i>syau7</i>	TCAAAATGCTTACAACCTCAATCTC	CATCAAGCGFATCATTTCCTGT	34	0.77
<i>syau15</i>	CGTGATTAACGCCTTCCAATAT	TGTGACAGCGGCAGAAAATAG	115	2.61

Table 4. Summary of predicted genes in the genomic target region on *B. rapa* LG A09.

Gene ID	Start	End	Best hits in GenBank [accession/species]	E value
<i>Bra031536</i>	37686812	37689589	P-loop containing nucleoside triphosphate hydrolases superfamily protein [NP_001184924/ <i>Arabidopsis thaliana</i>]	0.00E+00
<i>Bra031537</i>	37681637	37684412	Probable galacturonosyltransferase 6 [NP_563771/ <i>Arabidopsis thaliana</i>]	0.00E+00
<i>Bra031538</i>	37680010	37681151	RNA polymerase Rpb7 N-terminal domain-containing protein [NP_172164/ <i>Arabidopsis thaliana</i>]	2.00E-105
<i>Bra031539</i>	37676627	37679733	Carotenoid isomerase [NP_172167/ <i>Arabidopsis thaliana</i>]	0.00E+00
<i>Bra031540</i>	37674379	37674678	Glutaredoxin family protein [XP_002889620/ <i>Arabidopsis lyrata</i> subsp. <i>lyrata</i>]	3.00E-62
<i>Bra031541</i>	37671570	37673430	Similar to bZIP transcription factors [AAF63137/ <i>Arabidopsis thaliana</i>]	4.00E-142

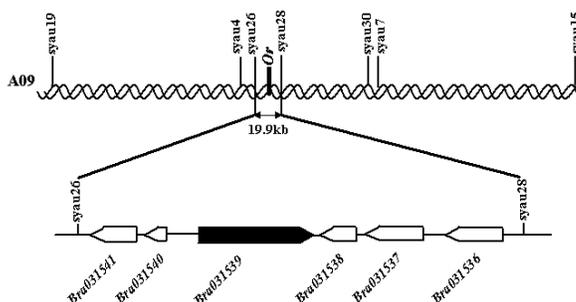


Figure 1. Genetic and physical maps of the *or* gene locus and candidate gene analysis in Chinese cabbage. Using the molecular markers, *syau19* and *syau15*, *or* was previously localized to chromosome A09 between KBrH010M08 and KBrB017P15 (Feng et al., 2012), two BAC clones. Using 2,200 F₂ homozygous recessive individuals, *syau26* and *syau28*, *or* was fine mapped to a 19.9 kb region containing six genes, including *Bra031539*, the predicted candidate gene.

Cloning of *Bra031539* and BLAST analysis of sequences in the two materials

BLAST analysis of *Bra031539* between Chiifu and 07A163 revealed some differences in the nucleic acid and protein sequences between lines, including the following: 88 SNPs were found in the promoter region and two positions contain base deletions, including an 88-bp deletion at the 751-838-bp position and a 7-bp deletion at 937-943 bp (Figure 2a). We identified 13 exons in *Bra031539* containing 100 SNPs. A 6-bp deletion was detected in the first exon, which is situated in the 1,477-1,482-bp region in orange head Chinese cabbage line 07A163 (Figure 2b). Early termination of protein translation occurs at the 3' end of *Bra031539* in line 07A163 compared to white head Chinese cabbage Chiifu, and the orange head line contains a 252-bp deletion (Figure 2c).

We compared the deduced protein sequences of the two materials, finding that 22 amino acids were altered in the orange head line and 17 amino acids were lost. The 43 SNPs present in the first exon resulted in 15 amino acid changes and the 6-bp deletion led to the loss of two amino acids. The early termination of protein translation occurs due to the 3'-end DNA deletion in the orange head material (Figure 3).

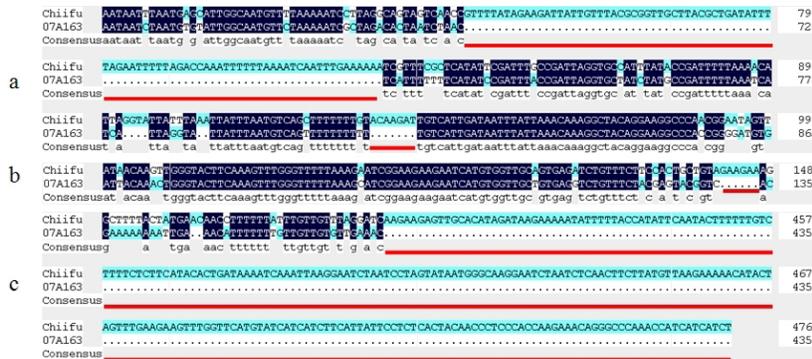


Figure 2. Comparison of *Bra031539* nucleic acid sequences in Chiifu and 07A163. **a** Sequence alignment of the promoter regions between Chiifu and 07A163; red lines indicate 88-bp and 7-bp deletions; **b** 6-bp deletion in CDS1; **c** due to early termination at the 3'-end, 252-bp of DNA were deleted in 07A163.

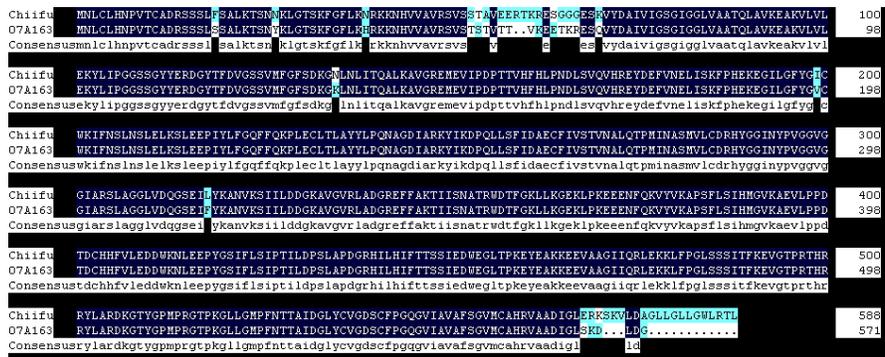


Figure 3. Comparison of *Bra031539* protein sequences in Chiifu and 07A163. 22 amino acids were altered in the orange head line and 17 amino acids were lost. The early termination of protein translation occurs due to the 3'-end DNA deletion in the orange head material.

Protein sequence analysis of Bra031539

The full-length sequence of *Bra031539* is 3402 bp long. Its 13 coding sequences (CDS) encode 589 amino acid sequences. TargetP analysis showed that Bra031539 is likely a chloroplast transport peptide, while signalP3.0 prediction also revealed that Bra031539 lacks an N-terminal signal peptide.

Protein sequence pattern analysis by Prosite showed that Bra031539 contains 12 casein kinase II phosphorylation sites, eight protein kinase C phosphorylation sites, one cAMP- and cGMP-dependent protein kinase phosphorylation site, 13 N-myristoylation sites, two tyrosine kinase phosphorylation sites, three N-glycosylation sites, and a leucine zipper pattern (Figure 4). SMART analysis revealed that Bra031539 contains four domains: a Pfam:DAO domain, an FAD binding-2 domain, a NAD binding-8 domain and an amino oxidase domain.

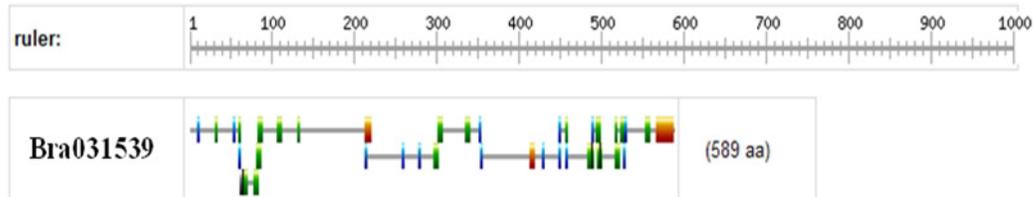


Figure 4. Sites with a high probability of occurrence in *Bra031539*, as revealed using Scan Prosite. The full-length sequence of *Bra031539* is 3,402 bp long encoding 589 amino acid sequences and protein sequence pattern analysis by Prosite showed that Bra031539 contains forty sites.

Development of a molecular marker based on the differences between the two parents

Three primer pairs were designed based on the promoter region of *Bra031539* and were verified through analysis of the white, yellow, and orange Chinese cabbage materials. The results showed that primer pair BR1 amplified different PCR products in the orange head materials than from those of the white and yellow head materials (Figure 5). We performed dominance analysis of all F1 populations (Figure 6). F2 homozygous recessive orange head individuals performed the same as orange head line 07A163. However, in F2 heterozygous individuals, the white head character co-segregated with BR1 (Figure 7). Therefore, the primer pair BR1, which amplifies marker *BrPro1* (*Bra031539*Promoter1), can be used to distinguish orange head materials from white and yellow head materials.

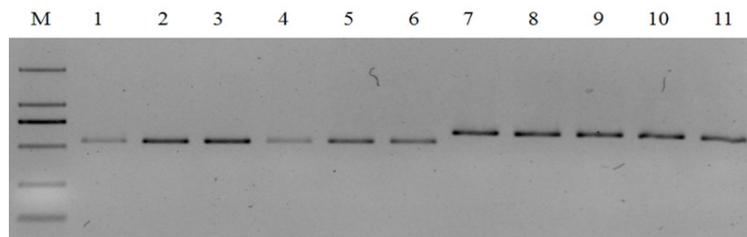


Figure 5. Results of PCR amplification of *BrPro1* in the three test materials. Lane M: DL2000 Markers; lanes 1-6 are orange head Chinese cabbages lines 07A163, Ju65, Ju62, Zhengcai1, Jihong308 and Jinzi-1, respectively; lanes 7 and 8 are white head Chinese cabbage lines Chiifu and Futian50, respectively; lanes 9-11 are yellow head Chinese cabbage lines Jin04-02, Dongbao and 76-F-2, respectively.

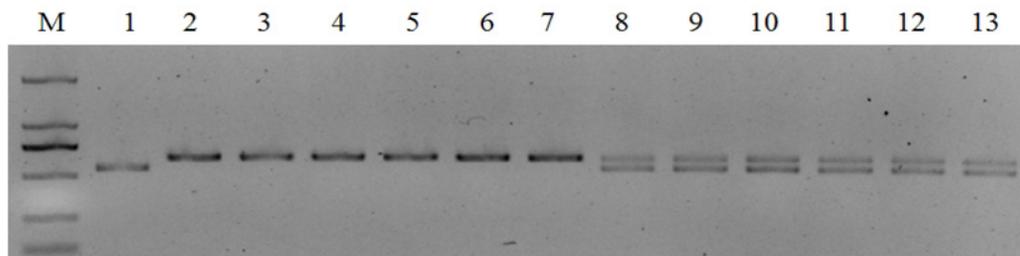


Figure 6. Results of PCR amplification of *BrPro1* in the F1 population. *Lane M*: DL2000 Markers; *lane 1* is orange head Chinese cabbage 07A163; *lanes 2-4* are white head Chinese cabbage lines Chiifu, 14A014 and 14A065, respectively; *lanes 5-7* are yellow head Chinese cabbage lines 14A036, 14A038 and 14A029, respectively; *lanes 8-10* are F1 lines from a cross between white head Chinese cabbage and orange head Chinese cabbage, i.e., Chiifu x 07A163, 14A014 x 07A163 and 14A065 x 07A163, respectively; *lanes 11-13* are F1 lines from a cross between yellow head Chinese cabbage and orange head Chinese cabbage, i.e., 14A036 x 07A163, 14A038 x 07A163 and 14A029 x 07A163, respectively.

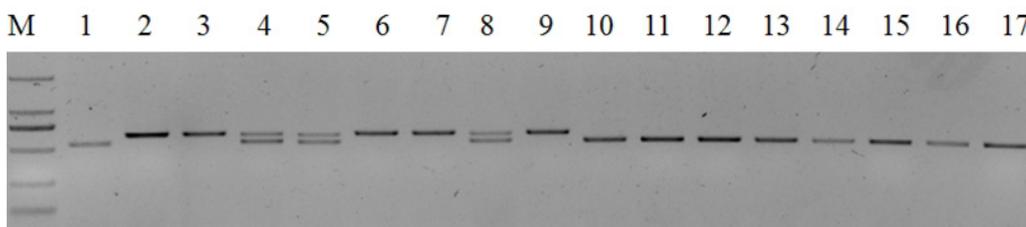


Figure 7. Results of PCR amplification of *BrPro1* in the F2 population. *Lane M*: DL2000 Markers; *lane 1* is orange head Chinese cabbage; *lane 2* is white head Chinese cabbage; *lanes 3-9* are white head Chinese cabbage in F2; *lanes 10-17* are orange head Chinese cabbage in the F2.

DISCUSSION

Based on the preliminary mapping of the *or* gene in Chinese cabbage (Feng et al., 2012), we constructed a fine mapping of a population containing 2200 F2 homozygous recessive individuals. Using two SSR markers, *syau26* and *syau28*, we localized the *or* gene, which controls the orange head character, to linkage group A09. The physical distance between these two markers is 19.9 kb. Using an F2S4 population, a single recessive gene, *Br-or*, was previously localized to a 16.7-kb region, with three ORFs (open reading frames) predicted in the target area (Zhang et al., 2013). *Br-or* was then localized to a 9.47 kb region containing one predicted gene (Su et al., 2014). Using two insertion-deletion (InDel) markers, *SB14001* and *SB13049*, the target gene was subsequently localized to a 65.555 kb region containing 17 candidate genes (Li et al., 2015). In summary, our results confirm that the target gene that controls the orange head character in Chinese cabbage is located at the end of linkage group A09.

Analysis based on information in the *Brassica* database (<http://brassicadb.org/brad/index.php>) and the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) confirmed the presence of six candidate genes in the target region. *Bra031539* encodes the carotenoid isomerase CRTISO, which plays an important role in the carotenoid biosynthetic pathway. CRTISO is the enzyme that converts tetra-*cis*-lycopene to all-*trans*-

lycopene. The Arabidopsis *CRTISO* locus was identified through analysis of *crtiso* mutants exhibiting partial inhibition of lutein biosynthesis in light-grown tissue and the accumulation of poly-*cis*-carotene precursors in dark-grown tissue (Park et al., 2002). The *tangerine* mutant in tomato has a deletion in *CRTISO* and accumulates prolycopene instead of all-*trans*-lycopene, resulting in orange fruit (Isaacson et al., 2002). The mutation of *BrCRTISO1* in Chinese cabbage was predicted to cause the orange-color trait (Lee et al., 2014). Based on the above result, we also predict that *Bra031539* is the target gene that controls the orange head character in Chinese cabbage.

Recent studies by several groups have involved sequence alignments between white head Chinese cabbage and orange head Chinese cabbage. Comparison of the OC (orange-colored) and YE (yellow-colored) cultivars revealed that *Bra031539* contains two large InDels and many SNPs in the first exon in the OC cultivar. The sequence variations result in 19 amino acid modifications. Lee et al. (2014) proposed that methylation and secondary structures might exist to preclude transcription at the 3' end of *BrCRTISO1* in the OC cultivar. A BAC library was developed to clone *BrCRTISO* in the orange head material. A 90-bp DNA deletion in the promoter and a 501-bp DNA insertion prior to the stop codon are the distinctive features revealed between the two experimental materials (Su et al., 2014). Li et al. (2015) found that an orange-type A21530 mutant also lacks the 3' end sequence. However, a 141-bp deletion in the promoter region and 6-bp deletion, as well as 53 SNPs in the first exon, were found in orange-type cultivars. Zhang et al. (2015) compared the genomic sequences of two Chinese cabbage materials and found that their differences included an 88-bp deletion in the promoter region, a 501-bp insertion at the 3' end, and two SNPs. The two SNPs cause two amino acid substitutions and a 501-bp insertion, resulting in the addition of 143 amino acids at the end of BrCRTISO.

In summary, the results obtained with these different cabbage materials vary. Due to the longer sequence of *Bra031539*, we did not obtain the sequence of the 3' end of orange head Chinese cabbage line 07A163 by gene amplification and clone sequencing. The amplified fragment and sequence assembly were used to obtain the full-length *Bra031539* in white head Chinese cabbage Chiifu and orange head line 07A163. Based on BLAST analysis by DNAMAN, two deletions of 88 and 7 bp were detected in the promoter region of *Bra031539* in 07A163, which also contains 88 SNPs. A deletion of 6 bp in the first exon results in the failed translation of two amino acids, and 43 of the SNPs lead to 15 amino acid substitutions. The nucleic acid sequence at the 3' end exhibits frameshift mutations in 07A163 that result in premature termination of protein translation. This might explain why the isomerase activity of CRTISO was lost in this orange head line. Some of our results differ from those of previous studies. Future studies will focus on identifying which mutation results in the inactivation of CRTISO in different orange head cabbage lines.

We designed gene-specific markers based on the nucleotide deletion in the promoter region and verified them in white, yellow, and orange parental lines and in the F1 and F2 generations. Marker *BrPro1* was codominant in the F1 generation and co-segregated in the F2 generation. This marker can therefore be used to distinguish orange materials from white and yellow materials. The use of this marker may enable identification of hybrid orange head materials in the early stage of breeding, which could speed up the breeding process of orange head Chinese cabbage. Compared with the markers developed in fine mapping studies, such as those closely linked to *or* (Zhang et al., 2008; Feng et al., 2012; Zhang et al., 2013; Su et al., 2014), functional markers are developed in mutated genes, thereby allowing genotypes to be distinguished accurately. At the same time, an allele-specific marker (*Br-Pro-Indel*) was also

developed to identify allelic variations in Chinese cabbage cultivars (Zhang et al., 2015). In the current study, six different orange lines, six different yellow lines, four different white lines, and F1 and F2 generation hybrids were used to verify *BrPro1*. This marker can distinguish orange head Chinese cabbage from yellow and white in both homozygous from heterozygous plants, suggesting that it will be used extensively for marker-assisted selection breeding.

Conflicts of interest

The authors declare no conflict of interest.

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