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ORIGINAL ARTICLE

Molecular characterization and transcriptome analysis of orange head Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Abstract

Main conclusion **The orange head phenotype of *Br-or* resulted from a large insertion in carotenoid isomerase (*BrCRTISO*) . Comparative transcriptome analysis revealed that the mutation affected the expression of abundant transcription factor genes. A new orange trait-specific marker was developed for marker-assisted breeding.**

Orange head leaves are a desirable quality trait for Chinese cabbage. Our previous fine mapping identified *BrCRTISO* as the *Br-or* candidate gene for the orange Chinese cabbage mutant. Here, we examined the *BrCRTISO* gene from white and orange head Chinese cabbage. While *BrCRTISO* from the white control plant was able to complement the *Arabidopsis Atcrtiso* mutant phenotype, *Brcrtiso* with a large insertion from the orange head Chinese cabbage failed to rescue the *Arabidopsis* mutant phenotype. The results show that *Brcrtiso* was non-functional, concomitant with the accumulation of prolycopene in *Br-or* to yield orange head. Comparative transcriptome analysis by RNA-seq identified 372 differentially expressed genes between the control and *Br-or* mutant using two near-isogenic lines with white and orange inner leaves. The mutation in *BrCRTISO* specifically affected many genes in the functional groups involved in RNA, protein, transport, and signaling. Particularly, expressions of many transcription factor genes were dramatically altered in *Br-or*, suggesting a potential role of *BrCRTISO* or carotenoid metabolites in affecting transcription. A novel co-dominant gene-specific marker was developed that co-segregated with orange color phenotype and would be useful for marker-assisted selection with enhanced selection efficiency. Our study provides new insights into understanding of the molecular basis of *Br-or* in mediating head leaf color and depicts a global view of the effect of *BrCRTISO* on cellular processes in plant. It also provides a molecular tool to accelerate breeding new Chinese cabbage cultivars with unique health quality and visual appearance.

Keywords Chinese cabbage · Orange head · *BrCRTISO* · Transcriptome · Gene-specific marker

Abbreviations

MAS	Marker-assisted selection
NILs	Near-isogenic lines
GGPP	Geranylgeranyl pyrophosphate

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PSY	Phytoene synthase
PDS	Phytoene desaturase
ZDS	ζ-Carotene desaturase
CRTISO	Carotenoid isomerase
LCYB	Lycopene β-cyclase
LCYE	Lycopene ε-cyclase
CHYB	β-Carotene hydroxylase
CHYE	ε-Carotene hydroxylase
ZEP	Zeaxanthin epoxidase
CCD	Carotenoid cleavage dioxygenase
NCED	9-*Cis*-epoxycarotenoid dioxygenase

Introduction

Carotenoids are a major group of pigments naturally existing in plants and microbes. Carotenoids play significant roles in plant development, such as in light harvesting, photoprotection against excess light, synthesis of plant hormones, and bringing color to flowers and fruits (Nisar et al. 2015; Shumskaya and Wurtzel 2013). Apart from their essential functions in plants, carotenoids are vitamin A precursors and possess high antioxidant activity, which are beneficial for human nutrition and health (Cazzonelli and Pogson 2010). The pathway of carotenoid biosynthesis in plants has been well elucidated (Fig. 1). Phytoene synthase (PSY) is the first rate-limiting enzyme in carotenoid biosynthesis pathway, which catalyzes two geranylgeranyl diphosphate molecules (GGPP) to phytoene. Phytoene is desaturated by a series of desaturation reactions. All-trans lycopene can be finally produced by carotenoid isomerase (*CRTISO*) and light in non-green and green tissue, respectively (Farré et al. 2010). SET DOMAIN GROUP 8 (SDG8) has been shown to change the methylation of chromatin associated with the *CRTISO* gene, which in turn affects the expression of *CRTISO* (Cazzonelli et al. 2009). The produced lycopene is then cyclized by lycopene β-cyclase (LYCB) and/or lycopene ε-cyclase (LYCE) to generate α- and β-carotene. These carotenes are further hydroxylated and oxygenated by a series of enzymes to produce xanthophylls (lutein, zeaxanthin, etc.). Carotenoids are cleaved to produce apocarotenoids including abscisic acid, strigolactones, and aroma compounds (Ruiz-Sola and Rodríguez-Concepción 2012). In addition, sequestration and increase in storage capacity also play significant roles in carotenoid accumulation (Li and Yuan 2013), as shown in the cauliflower *OR* mutant (Lu et al. 2006) and tomato *HIGH PIGMENT* 1 and 2 (Kolotilin et al. 2007) and *HIGH PIGMENT* 3 mutant (Galpaz et al. 2008). In spite of great progresses achieved in uncovering the mechanism of carotenoid biosynthesis and accumulation in model plants (*Arabidopsis* and tomato), the molecular basis in many non-model species is still poorly understood.

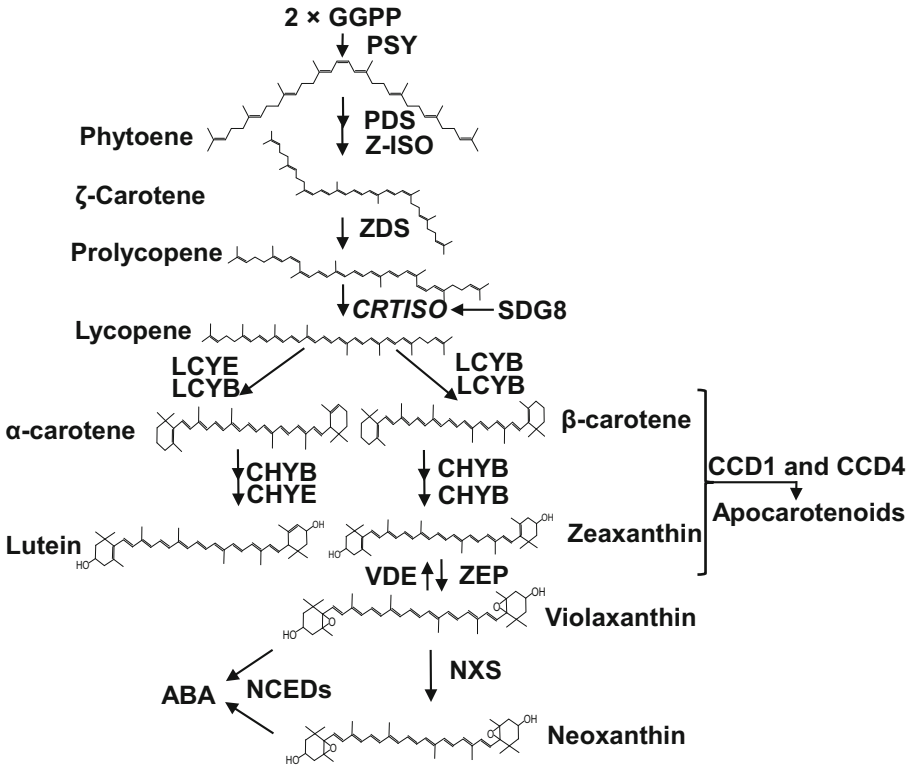

Fig. 1 Carotenoid metabolic pathway in plant. The gene responsible for orange head phenotype is shown in *italic*. *GGPP* geranylgeranyl pyrophosphate, *PSY* phytoene synthase, *PDS* phytoene desaturase, *Z-ISO* ζ-carotene isomerase, *ZDS* ζ-carotene desaturase, *CRTISO* carotenoid isomerase, *LCYB* lycopene β-cyclase, *LCYE* lycopene ε-cyclase, *CHYB* β-carotene hydroxylase, *CHYE* ε-carotene hydroxylase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *NXS* neoxanthin synthase, *CCD* carotenoid cleavage dioxygenase, *NCED* 9-*cis*-epoxycarotenoid dioxygenase, *SDG8* histone methyltransferase (SET DOMAIN GROUP 8)

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is widely consumed in Asia. Orange head Chinese cabbage, a novel mutant that is controlled by a single recessive gene *Br-or* (Zhang et al. 2013), confers carotenoid accumulation. The mutant produces striking inner yellow leaf phenotype that turns to orange following exposure to sunlight. In our previous research, we first reported the identification of a *CRTISO* (*BrCRTISO*) as the candidate gene for orange head by high-resolution genetic mapping using F_2S_4 population (Zhang et al. 2013). *BrCRTISO* shares the same identity as *CRTISO* identified in *Arabidopsis ccr2* mutant (Park et al. 2002) and tomato *tangerine* mutant (Isaacson 2002). Recently, our finding was further confirmed by two groups using a BC1 population (Su et al. 2014) and candidate gene approach (Lee et al. 2014). However, the effects of *Br-or* on carotenoid accumulation and on global cellular processes remain to be elucidated. Here, we examined the *BrCRTISO* gene and analyzed the carotenoid composition difference between white and orange head Chinese cabbage. Comparative transcriptome analysis was performed to identify cellular processes specifically affected in *Br-or*. A new genetic marker was developed for accelerating breeding of new orange head Chinese cabbage cultivars with unique health quality and visual appearance.

Materials and methods

Plant materials and growth conditions

White (WT) and orange (*Br-or*) head Chinese cabbages used in this study were grown in the field of Northwest A&F University, Yangling, China, for phenotype testing. The near-isogenic lines (NILs) were produced following the methods detailed previously (Zhang et al. 2013). The inner white and *Br-or* head leaves were harvested, frozen immediately in liquid nitrogen, and stored at −80 °C until use for DNA, RNA, and protein isolation as well as for carotenoid extraction.

Arabidopsis wild-type and *crtiso* mutant seeds were grown on soil under controlled chamber conditions with 16 h light/8 h dark photoperiod at 24 °C. For seedlings grown on agar plates, seeds were surface sterilized and germinated on Murashige and Skoog (MS) agar medium containing 4.3 g L^{-1} MS salts, 10 g L^{-1} sucrose, and 6 g L^{-1} agar.

Carotenoid extraction and analysis

Frozen-dried mature head leaves were used to extract carotenoids as described previously (Park et al. 2002). The leaves were powdered in liquid nitrogen and immediately frozen-dried by a commercial lyophilizer. All the processes of extraction were carried out under dim light. Retention time and spectra were used to compare with commercial standards or published information to identify the peaks.

Multiple sequence alignment and phylogenetic analysis

The alignment of the CRTISO proteins and phylogenetic analysis were conducted using ClustalX (http://www.clustal.org/; Larkin et al. 2007) and MEGA 6.0 software (http://www.megasoftware.net/; Tamura et al. 2013). Neighbor-joining (NJ) method was used for constructing phylogenetic tree with the following parameters: Poisson correction, pairwise deletion, and bootstrap analysis (1,000 replicates; random seeds). The CRTISO proteins used for the phylogenetic tree analysis were from Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*), cucumber (*Cucumis sativus*), carrot (*Daucus carota* subsp. *sativus*), strawberry (*Fragaria vesca* subsp. *vesca*), soybean (*Glycine max*), grape (*Vitis vinifera*), chrysanthemum (*Chrysanthemum morifolium*), ipomoea (*Ipomoea* sp. *Kenyan*), marigold (*Calendula officinalis*), oncidium (*Oncidium*), paperwhite (*Narcissus tazetta*), populus (*Populus trichocarpa*), ricinus (*Ricinus communis*), foxtail millet (*Setaria italic*), coccomyxa (*Coccomyxa subellipsoidea*), crtN (*Heliobacillus mobilis*), and crtI (*Pantoea agglomerans*).

Plasmid construction and plant transformation

For phenotypic complementation of the *Arabidopsis Atcrtiso* mutant, full-length cDNAs of *BrCRTISO* and *Brcrtiso* without and with the large insertion were amplified by PCR and cloned into pCAMBIA1300s (Zhou et al. 2011) to generate the overexpression plasmids *35S::BrCRTISO*, *35S::Brcrtisoꭤ,* and *35S::Brcrtiso*, respectively. For β-glucuronidase (GUS) expression, the promoter regions of WT and mutant genes were amplified by PCR and introduced into pCAMBIA1305.1 to produce the plasmids $BrCRTISO_{pro}$*::GUS* and $Brcrtiso_{pro}$*::GUS*. The primer sets used for PCR amplification are shown in Table S1. The constructs used in this study were confirmed by sequencing.

All the plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and introduced into 5-week-old *Arabidopsis* plants using the floral dip method. Positives were selected on MS plates containing 30 mg L^{-1} carbenicillin and 50 mg L^{-1} hygromycin or 10 mg L^{-1} Basta and further confirmed by PCR analysis of gene expression.

RNA isolation and transcript analysis

Total RNA from mature WT and *Br-or* inner head leaves was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using the Superscript III First-stand kit (Invitrogen) with Oligo(dT) primer. Real-time qRT-PCR was conducted using iTaqTM Universal SYBR Green Supermix (Bio-Rad) with gene-specific primer sets (Table S1) as described by Zhang et al. (2014). Relative expression levels were calculated using the $^{\Delta\Delta}C_t$ method (Lyi et al. 2007) and normalized first with the level of the β-actin internal transcript control and then with the expression of WT control. Each sample was quantified in triplicate with three biological replicates.

Immunoblot analysis

Total proteins were extracted from mature WT and *Br-or* head leaves using phenol (Yang et al. 2007). Proteins (30 μg) were resolved by 12 % SDS-PAGE gel and transferred onto nitrocellulose membranes. PSY and ZEP proteins were detected with rabbit polyclonal anti-PSY and anti-ZEP antibodies, respectively. Signals were detected with enhanced chemiluminescence (ECL) western blotting analysis system (GE Healthcare) and then exposed to X-ray film.

GUS assay

Five-day-old *Arabidopsis* seedlings germinated on MS medium were put into 1.5 ml microfuge tube with GUS staining solution (50 mM sodium phosphate buffer pH 7.2, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.2 % triton X-100, and 2 mM X-gluc) and incubated at 37 °C for 6 h. Seedlings were rinsed by a series of ethanol (30, 50, 75, and 95 %) at room temperature for 30 min each. GUS expression was recorded with a color CCD camera under Olympus SZX-12 Stereo Microscope. To compare the GUS staining between *BrCRTISO*$_{pro}$*::GUS* and *Brcrtiso*$_{pro}$*::GUS,* at least five T_2 seedlings from five independent transgenic lines were tested.

RNA-seq analysis

Total RNA from Chinese cabbage inner head leaves of WT or *Br-or* NILs with three biological replicates was isolated and purified using RNeasy Plant Mini kit following the manufacturer's instruction (Qiagen, USA). RNA concentration was quantified by NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). Strand-specific RNA-Seq libraries were constructed following the protocol described by Zhong et al. (2011) and sequenced on an Illumina HiSeq 2000 system at the Cornell University Life Sciences Core Laboratories Center. Sequence reads were aligned to the *Brassica rapa* genome (http://brassicadb.org/brad/) using Tophat (Trapnell et al. 2009). Following alignments, the number of reads mapped to each *Brassica rapa* gene model was derived and then normalized reads per kilobase of exon model per million mapped reads (RPKM). Differentially expressed genes between WT and *Br-or* were identified with the DESeq package (Anders and Huber 2010). The functional classification of significantly differentially expressed genes was carried out based on the bincodes of MapMan (http://mapman.gabipd.org/web/guest/mapman).

Result

Phenotype and carotenoid composition in WT and *Br-or* head leaves

While mature inner head leaves of WT were white/light green, those of *Br-or* were yellow (Fig. 2a) but turned

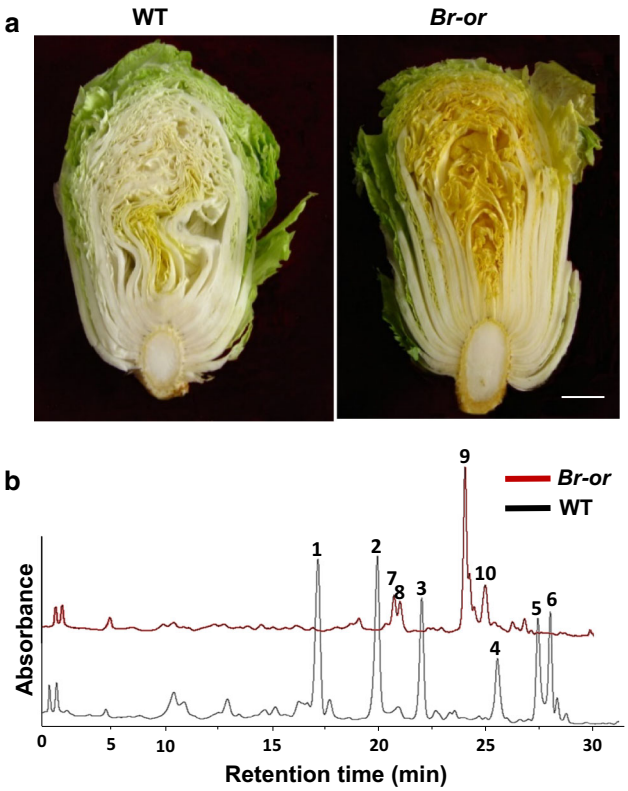

Fig. 2 Head leaf phenotypes and pigment composition of WT (*white*) and *Br-or* (*orange*) Chinese cabbage. **a** Representative head leaf phenotypes of WT and *Br-or*. *Bars* 4 cm. **b** HPLC analysis of mature inner head leaves from WT and *Br-or* Chinese cabbage. *Peak 1* lutein, *peak 2* chlorophyll *a*, *peak 3* chlorophyll *b*, *peak 4* chlorophyllide, *peak 5* pheophytin, *peak 6* β-carotene, *peak 7* γ-carotene, *peak 8* γ-carotene isomer, *peak 9* lycopene, *peak 10* prolycopene

orange under sunlight as showed in other reports (Lee et al. 2014; Su et al. 2014). Interestingly, the color of *Br-or* leafstalk and rootstalk was also yellow/orange (Fig. S1a and S1b). The inner leaf color was only visually at the mature stage when the leaves were tightly wrapped in the head. As shown in Fig. S1c, there was no great difference on the phenotypes of 2-month-old WT and *Br-or*, which might show that *Br-or* mutant affected carotenoid biosynthesis and accumulation at mature stage or suggest that dark environment was essential for the mutant phenotype.

To examine the pigment difference between WT and *Br-or*, we conducted HPLC analysis to evaluate the composition of representative inner head leaves from WT and *Br-or*. As shown in Fig. 2b, the *Br-or* mutation significantly altered the pigment composition. There were six major peaks in WT. They were two carotenoids, i.e., lutein and β-carotene, and chlorophyll *a*, chlorophyll *b*, chlorophyllide, and pheophytin. However, only four main peaks of carotenoids were detected in *Br-or*. Instead of lutein and β-carotene, *Br-or* accumulated γ-carotene, γ-carotene isomer, lycopene, and 7,9,9′,7′-tetra-cis-lycopene (prolycopene). The spectral properties of the carotenoid peaks from WT and *Br-or* are shown in Fig. S2. The orange leaves resulted mainly from the accumulation of lycopene and pro-lycopene. WT was of significance for the accumulation of cyclized carotenoids, showing total different carotenoid profiles from *Br-or*.

Br-Or encodes CRTISO and complementation analysis in an *Atcrtiso* mutant

Our previous fine mapping identified *BrCRTISO* as the candidate gene mediating Chinese cabbage head color (Zhang et al. 2013). *BrCRTISO* was isolated from white and orange head leaves using gene-specific primers (Table S1) based on *B. rapa* genome sequence (Wang et al. 2011). *BrCRTISO* consisted of 13 exons and 12 introns (Fig. 3a). It shared similar gene structure as *CRTISOs* in tomato and *Arabidopsis* (Isaacson et al. 2002; Park et al. 2002). The full-length *BrCRTISO* cDNA had an open reading frame that encoded a protein of 587 amino acids with a calculated molecular mass of 64.6 kDa. In comparison with the genomic sequence of WT, *Br-or* contained a deletion of 88 bp in the promoter region (−453 → −540), a large insertion of 501 bp at the 3′-end, and two SNPs (Fig. 3a). The two SNPs caused two amino acid substitutions (Phe to Ser and Leu to Phe, respectively) of the BrCRTISO (Fig. 3a). The insertion of 501 bp resulted in an addition of 143 amino acids in the end of BrCRTISO, as shown in a previous report (Su et al. 2014).

The sequence variation of *Br-or* might lead to the orange leaf phenotype. To see whether the substitution of two amino acids or the large insertion of 143 amino acids in

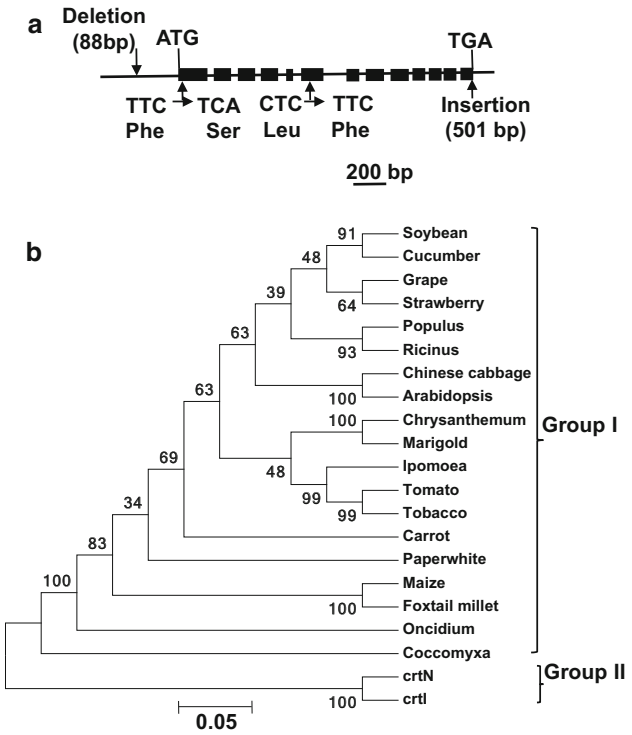

Fig. 3 Gene structure and phylogenetic tree of BrCRTISO. **a** Gene structure of *BrCRTISO*. Exons and introns are shown with *solid boxes* and *lines*, respectively. The alterations in *Brcrtiso* are indicated along with the gene structure. **b** Phylogenetic tree of CRTISO proteins from various species. The full-length sequences of CRTISO proteins from different organisms were aligned by clustalx, and the phylogenetic tree was constructed using the neighbor-joining method with MEGA6.0 software. Multiple sequence alignment of CRTISO proteins from Arabidopsis (*Arabidopsis thaliana*), Tomato (*Solanum lycopersicum*), Maize (*Zea mays*), Tobacco (*Nicotiana tabacum*), Cucumber (*Cucumis sativus*), Carrot (*Daucus carota* subsp. *sativus*), Strawberry (*Fragaria vesca* subsp. *vesca*), Soybean (*Glycine max*), Grape (*Vitis vinifera*), Chrysanthemum (*Chrysanthemum morifolium*), Ipomoea (*Ipomoea* sp. *Kenyan*), Marigold (*Calendula officinalis*), Oncidium (*Oncidium*), Paperwhite (*Narcissus tazetta*), Populus (*Populus trichocarpa*), Ricinus (*Ricinus communis*), Foxtail millet (*Setaria italic*), Coccomyxa (*Coccomyxa subellipsoidea*), crtN (*Heliobacillus mobilis*), and crtI (*Pantoea agglomerans*)

Br-or was responsible for the mutant phenotype, we performed phenotypic complementation tests in a newly isolated *Atcrtiso* mutant. In contrast to *Arabidopsis ccr2* mutants reported (Park et al. 2002), the *Atcrtiso* mutant showed pale green leaves under normal growth condition due to a point mutation that resulted in the induction of premature stop codon to produce a truncated CRTISO protein (Fig. 4a). The whole-length cDNA of WT (*BrCRTISO*) or *Br-or* that contained the two SNPs but without the large insertion of 501 bp (*BrcrtisoΔ*) was introduced into the *Atcrtiso* mutant. Over 10 positive transgenic lines overexpressing *BrCRTISO* or *BrcrtisoΔ*, respectively, were obtained by kanamycin-resistant screening and gene expression analysis. As expected,

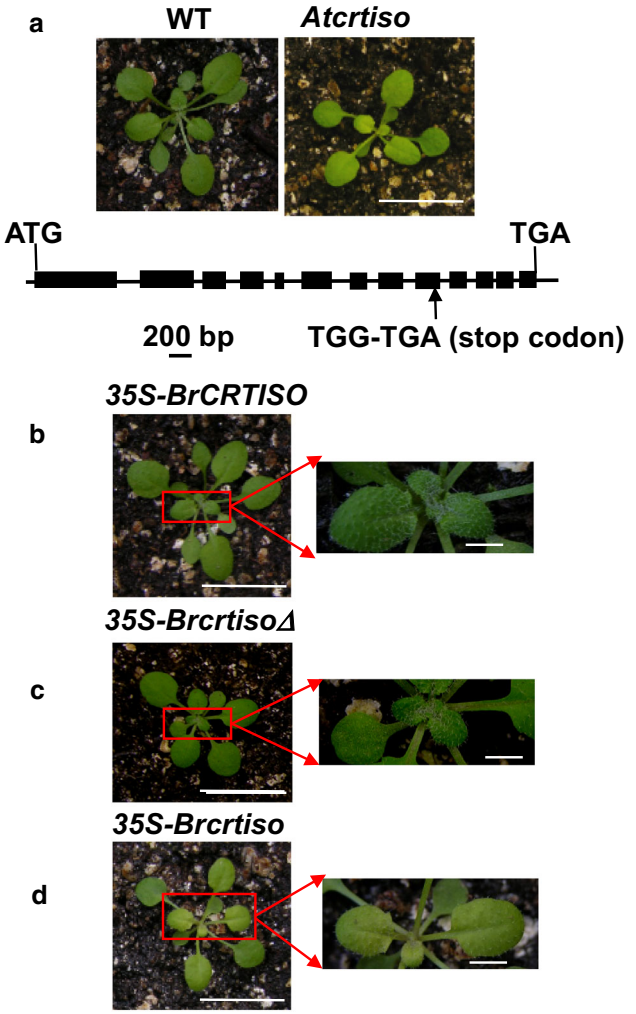

Fig. 4 Functional complementation in *Arabidopsis Atcrtiso* mutant. **a** The phenotype and gene structure of *Atcrtiso*. *Scale bar* 1 cm. **b** *Atcrtiso* mutant was complemented with whole-length cDNA of *BrCRTISO*. **c** *Atcrtiso* mutant was complemented by the cDNA of *BrcrtisoΔ* containing the two SNPs but without the large insertion of 501 bp. **d** Complementation test using the whole-length cDNA of *Brcrtiso*

BrCRTISO complemented the *Atcrtiso* mutant phenotype, and the transgenic lines exhibited wild-type green leaves (Fig. 4b). Similarly, the transgenic lines overexpressing *BrcrtisoΔ* also restored the *Atcrtiso* mutant phenotype (Fig. 4c), indicating that the two amino acid substitutions of BrCRTISO did not affect the gene/protein function. A complementation test was performed using the whole-length cDNA of *Br-or*. All of the positive transgenic lines overexpressing *Brcrtiso* did not recover the *Atcrtiso* mutant phenotype (Fig. 4d). These results indicated that a large insertion in the C-terminal end of BrCRTISO might produce a malfunction protein, which resulted in orange head leaves in *Br-or*.

To examine the evolutionary relationship between BrCRTISO and CRTISO orthologs from various species, we conducted multiple sequence alignment using the full-length amino acid sequences of CRTISO from different species. A phylogenetic tree was constructed employing neighbor-joining analysis (Fig. 3b). Phylogenetic analysis revealed that the CRTISO orthologs were clearly separated into two large groups. Group I and group II were from eukaryotic and prokaryotic organisms, respectively. BrCRTISO showed closer evolutionary relationship to CRTISO from *Arabidopsis* than others to grass family and algae organisms in Group I. crtN and crtI are bacterial phytoene desaturases, which catalyze the entire desaturation and isomerization process (Farré et al. 2010; Ruiz-Sola and Rodríguez-Concepción 2012). The phylogenetic analysis indicated that CRTISO was likely to originate from bacterial desaturase gene family. *CRTISO* is reported to be more closely to the bacterial phytoene desaturase than to the plant desaturases (Isaacson et al. 2002; Park et al. 2002).

Br-or exhibits a limited effect on the expression of *BrCRTISO* and other carotenoid metabolic genes and proteins

Transcriptional regulation is an important mechanism underlying carotenoid accumulation in plants (Nisar et al. 2015; Lu and Li 2008). It was of interest, therefore, to examine the effect of *Br-or* on the expression of *BrCRTISO* and other carotenoid metabolic genes in mature head leaves. No great difference in the transcriptional levels of *BrCRTISO* was observed between WT and *Br-or* by qRT-PCR and semi-quantitative RT-PCR (Fig. 5a). We also investigated the promoter activity of *BrCRTISO* and *Brcrtiso* fused to *GUS* gene in five independent *Arabidopsis* transgenic lines. As shown in Fig. 5b, GUS activity in the $BrCRTISO_{pro}$*::GUS* and $Brcrtiso_{pro}$*::GUS* transformants shared similar activity. The results suggested that the allelic alteration in the promoter region of *Br-or* did not greatly change the promoter activity of *BrCRTISO*.

We then compared carotenoid metabolic gene expression between WT and *Br-or*. The genes tested included *PSY*, *PDS, ZDS, LCYB, LCYE, CHYB, CHYE, ZEP, CCD4,* and *Orange* (*Or*). All of these test genes showed similar transcript levels between WT and *Br-or* by qRT-PCR and semi-quantitative RT-PCR (Fig. 5c, d).

PSY, an enzyme located in the upstream of carotenoid pathway, is the rate-limiting enzyme in carotenoid biosynthesis pathway (Cazzonelli and Pogson 2010). ZEP, a down-stream enzyme, is of importance in the xanthophyll

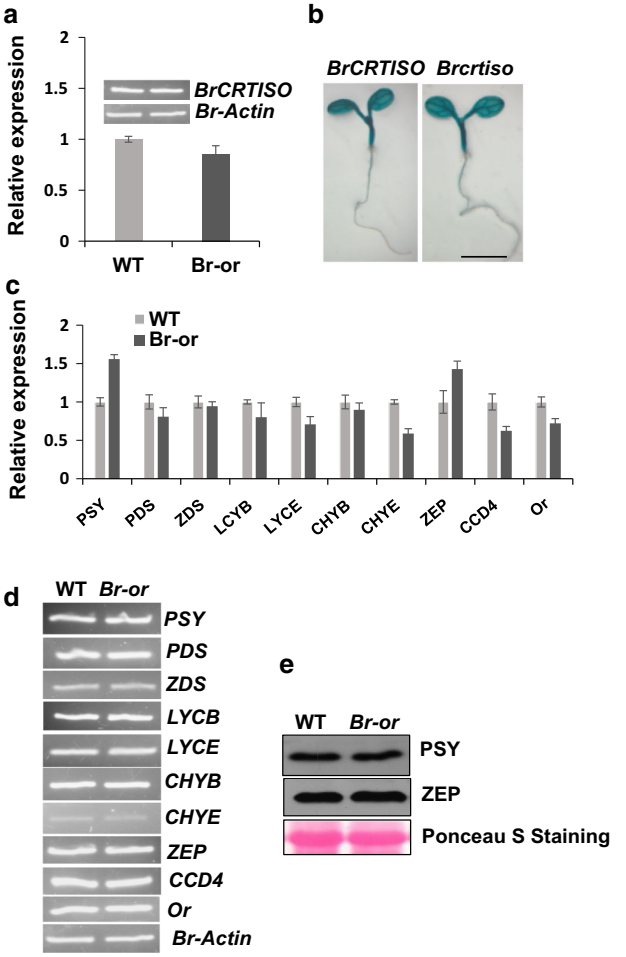

Fig. 5 Expression of carotenoid-related genes and proteins. **a** Gene expression of *BrCRTISO* in WT and *Br-or* Chinese cabbage by qRT-PCR and semi-quantitative RT-PCR (*inset*). **b** GUS staining of 5-day-old $BrCRTISO_{pro}$*::GUS* and $Brcrtiso_{pro}$*::GUS* transgenic plants. **c** qRT-PCR analysis of transcript levels of carotenoid-related genes in WT and *Br-or* Chinese cabbage. **d** Semi-quantitative RT-PCR analysis of transcript levels of carotenoid-related genes in WT and *Br-or* Chinese cabbage. **e** Western blot analysis of PSY and ZEP protein levels in WT and *Br-or* Chinese cabbage. Ponceau S staining was used as loading controls. qRT-PCR data represent mean ± SD from three biological replicates with three technical repeats

cycle (Ruiz-Sola and Rodríguez-Concepción 2012). To determine whether expression of these two proteins was altered in *Br-or,* the protein levels of PSY and ZEP were examined by immunoblot analysis. No dramatic difference in the amounts of these two proteins was observed (Fig. 5d), confirming that the mutation in *BrCRTISO* in general did not sharply affect carotenogenic enzyme expression.

Comparative transcriptome analysis of mature inner head leaves between WT and *Br-or*

To examine the global effect of the *BrCRTISO* mutation on gene expression in Chinese cabbage, RNA-seq analysis was employed to profile gene expression difference in the near-isogenic lines (NILs). The detail production of the two NILs with white and orange head leaf phenotypes for the RNA-seq analysis is shown in Fig. 6.

Approximately, 80 % of the sequenced reads (28.9 million mapped reads) were successfully aligned to the *Brassica rapa* reference genome (Wang et al. 2011; http://brassicadb.org/brad/). Statistical analysis identified 372 differentially expressed genes with at least twofold changes between WT control and *Br-or* mutant from the three biological replicates (adjusted P values <0.05). Among them, 210 genes were down-expressed (Table S2) and 162 genes were up-expressed in the *Br-or* mutant (Table S3). None of carotenoid metabolism genes showed significant difference in *Br-or* compared with WT, which was consistent with our qRT-PCR and RT-PCR tested results (Fig. 5c, d).

To validate the expression data obtained from RNA-seq, qRT-PCR was carried out to test the expression level of 15 selected genes, which included ten down-regulated and five up-regulated genes in *Br-or.* They were genes involved in development, photosynthesis, lipid metabolism, hormone metabolism, cell wall, and transport. As shown in Table 1, all 15 chosen genes showed the same trends of mRNA accumulation patterns as identified in the RNA-seq data.

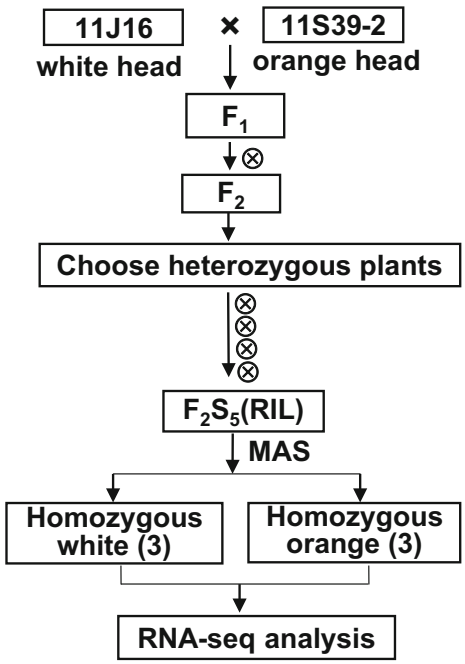

Fig. 6 The generation of near-isogenic lines (NILs) for RNA-seq analysis. A population of recombinant inbred lines (RILs) was derived from continuous selfing of single heterozygous individuals. Homozygous white and orange individuals for RNA-seq analysis were selected by marker-assisted selection

The significantly differentially expressed genes between WT and *Br-or* were catagorized into functional groups using MapMan (Thimm et al. 2004). Apart from the not assigned and misc group, the functional groups of genes associated with RNA, protein metabolism and process, cell wall, and signaling represented the most abundant groups of genes down-regulated in *Br-or* (Fig. 7a). The functional groups of genes involved in protein metabolism and process, transport, stress response, and RNA were present in high abundance among the up-regulated genes in *Br-or* (Fig. 7b).

Down-regulated genes in *Br-or*

While genes involved in multiple functional classes were among the down-regulated genes in *Br-or*, the functional group of RNA was over-represented. Notably, mutation of *Brcrtiso* down-regulated the expression of a large number of transcription factors such as GOLDEN2-like, MYB, TCP, AP2/EREBP, WRKY, bHLH, and Zn-finger families (Table S2). The transcript abundances of many of them were dramatically reduced. These transcription factors are known to regulate multiple processes of plant growth and development, ranging from fruit chloroplast development to stress responses (Dietz et al. 2010; Dubos et al. 2010; Rushton et al. 2010; Danisman et al. 2012; Powell et al. 2012). The results indicate that *BrCRTISO* might exert great influence on transcription regulation.

A large number of genes whose products are potentially involved in protein transcription, translation, post-translational modifications, and storage were also down-regulated in *Br-or* (Table S2). They included genes that encode RNA ligase, ribosomal proteins, eukaryotic translation initiation factors, protein kinases, peptidases, chaperones, ubiquitin enzymes, and latex-abundant family protein.

Genes involved in signaling were also over-representative among the down-regulated genes in *Br-or* (Table S2). Genes in the calcium signaling pathway were differentially expressed, which included calmodulin and calcineurin B-like protein. Two elements of largest subgroups of receptor-like kinases and cysteine-rich RLK were detected. Some of them are suggested to be involved in ROS signaling (Idänheimo et al. 2014).

Interestingly, high numbers of peroxidase genes were down-regulated but not detected among up-regulated genes in *Br-or* (Table S2). Peroxidases catalyze a number of oxidative reactions between hydrogen peroxide and various reductants and exert multiple functions varying from removal of H_2O_2 from plastids, ethylene biosynthesis, to defense responses (Hiraga et al. 2001). The specific down-regulation of many of them by the *BrCRTISO* mutation

Table 1 Verification of RNA-seq gene expression by qRT-PCR

Accession	Description	Ratio	qRT-PCR Ratio
Down-regulated genes			
Bra019297	WRKY family transcription factor	0.1	0.32
Bra005234	MYB transcription factor	0.43	0.76
Bra001710	TCP family transcription factor	0.14	0.34
Bra003883	Ethylene-responsive transcription factor ERF073	0.2	0.11
Bra001640	Ethylene-responsive transcription factor RAP2-3	0.29	0.5
Bra032947	bHLH DNA-binding superfamily protein	0.21	0.66
Bra011939	Ethylene-responsive transcription factor RAP2-7	0.3	0.35
Bra010713	B-box zinc finger family protein	0.24	0.35
Bra006195	Ethylene-responsive transcription factor ERF113	0.4	0.35
Bra026461	Putative GATA transcription factor 22	0.17	0.19
Bra027219	Late embryogenesis-abundant protein (LEA)	0.14	0.13
Bra020195	Fatty acid reductase 1	0.35	0.1
Bra037743	NAC domain containing protein 42	0.14	0.48
Bra009416	HSP20-like chaperones superfamily protein	0.24	0.54
Bra027957	Late embryogenesis-abundant (LEA) protein-related	0.12	0.39
Up-regulated genes			
Bra031728	F-box family protein	49.8	4.26
Bra034235	Cytochrome P450, family 71	7.06	2.69
Bra039113	Carbonic anhydrase 1	3.27	1.72
Bra008915	Elongation factor 1-beta 1	9.19	17.5
Bra006882	Sulfate transporter 3;1	4.16	2.38

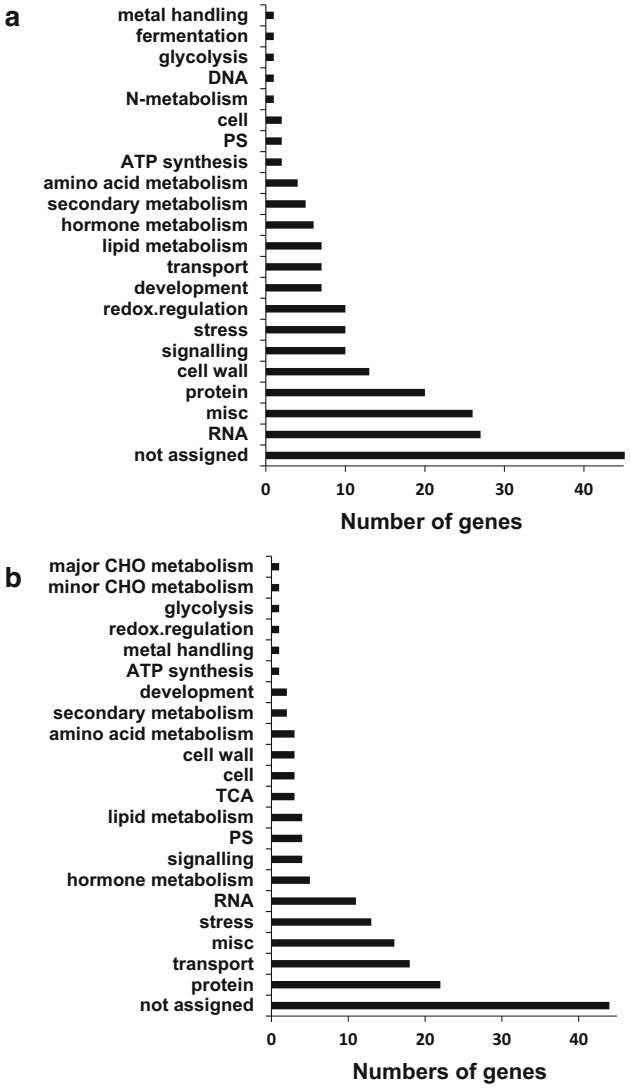

Fig. 7 Transcriptome analysis of the differentially expressed genes in *Br-or*. **a** Functional classification of down-regulated genes in *Br-or*. **b** Functional classification of up-regulated genes in *Br-or*. The functional classification of identified genes was analyzed according to the bincodes of Mapman (http://mapman.gabipd.org/web/guest/mapman)

might suggest a potential role of BrCRTISO in regulating the oxidative reactions.

Up-regulated genes in *Br-or*

The most abundant functional group of the up-regulated genes was the one associated with protein-related processes (Fig. 7b). Approximately, equal numbers of genes involved in protein metabolism were up- and down-regulated in *Br-or* (Table S3). The up-regulated genes included those encoded for several ribosomal proteins, protein kinase family proteins, and F-box proteins. In contrast with down-regulated ones, the up-regulated ribosomal protein genes encode plastid localized ribosomal proteins, suggesting a possible activity to alleviate the impairment of carotenoid biosynthesis pathway in the plastids. Dramatic up-regulations of a protein kinase gene and several F-box protein genes for protein modification and turnover were observed in *Br-or*. The large number of up-regulated along with a large number of down-regulated components in protein process suggests a potential effect of *BrCRTISO* in influencing protein metabolism.

Noticeably, a large number of transporter genes were up-regulated in the *Br-or* mutant (Fig. 7b). They included a number of genes encoding mineral nutrient transporters, multidrug resistance-associated proteins, and MATE transporters (Table S3). Some of the MATE transporters are known to be involved in trafficking of secondary metabolites and small metabolites (Lepiniec et al. 2006).

Another relatively over-representative group of genes was stress-related one in the *Br-or* mutant (Fig. 7b). Interestingly, most of the functional group genes were disease-resistant genes. The abundance of hydrophobic protein LTI6A gene was greatly induced, which is shown to be cold-inducible (Morsy et al. 2005). The increased expression of these stress-related genes might suggest a better adaption of the orange Chinese cabbage to the environment.

Different classes of transcription factors in comparison to the down-regulated genes represented another relative abundant functional group among the up-regulated genes in the *Br-or* mutant (Fig. 7b). They include several transcriptional factor B3 family proteins and in response to IAA and cytokinin treatment (Table S3). Among them, the response regulator 15 was dramatically up-regulated, which is known to be critical for the antagonistic interaction between auxin and cytokinin (Muller and Sheen 2008).

In addition, a few additional genes were dramatically up-regulated in the *Br-or* mutant (Table S3). They included a terpene synthase gene that is among a mid-size family of genes responsible for the synthesis of various terpene molecules including sterols and carotenes in plants (Chen et al. 2011), a UDP-glucosyltransferase 74E2 gene that affects auxin homeostasis and is strongly induced by H_2O_2 (Tognetti et al. 2010), and a FRIGIDA interacting protein 2 gene that affects flowering time (Geraldo et al. 2009). Clearly, the *Br-or* mutation affected not only carotenoid accumulation to give the unique orange phenotype, but also some specific processes in the plant.

Development of gene-specific marker for breeding orange head varieties

The orange phenotype was only visually observed after harvesting. Therefore, development of the trait-specific markers would facilitate the selection at seedling stage.

Based on the polymorphic sequences with a 88-bp deletion in the promoter region of *Brcrtiso*, an InDel co-dominant marker, Br-Pro-Indel, was developed (Fig. 8a). This primer set could amplify 299 bp DNA fragment in WT and 211 bp PCR product in *Br-or*, which could be easily detected by 2 % ethidium bromide-stained agarose gel (Fig. 8b).This marker was also able to discriminate the F_1 hybrid from the cross between WT and *Br-or*. In addition, this co-dominant marker produced clear polymorphism in F_2 population, which can be used to determine the genotypes of F_2 individuals (Fig. 8c). The genotypes and phenotypes of F_2 progenies were investigated based on marker analysis and colors of mature head leaves, respectively. As expected, the phenotypes from the F_2 individuals were totally consistent with the genotypes showed by the marker (Fig. 8c). Moreover, this marker was used to analyze the genotypes of 20 Chinese cabbage cultivars and lines. Genotype tested by the Br-Pro-Indel marker was completely consistent with the color phenotypes of head leaves in all these cultivars and lines (data not shown). Interestingly, we also found two sites of nucleotide substitutions in the coding region of *BrCRTISO* that were also co-segregated with the head color (Fig. 8d). Taken together, these data showed the development of an allele-specific marker, Br-Pro-Indel, that could successfully identify allele variations for WT and *Br-or* in Chinese cabbage cultivars.

Discussion

Our previous fine mapping indicated that *BrCRTISO* was the candidate gene for orange head phenotype in *Br-or* (Zhang et al. 2013). In this study, we demonstrated that a natural large insertion in *BrCRTISO* resulted in the orange

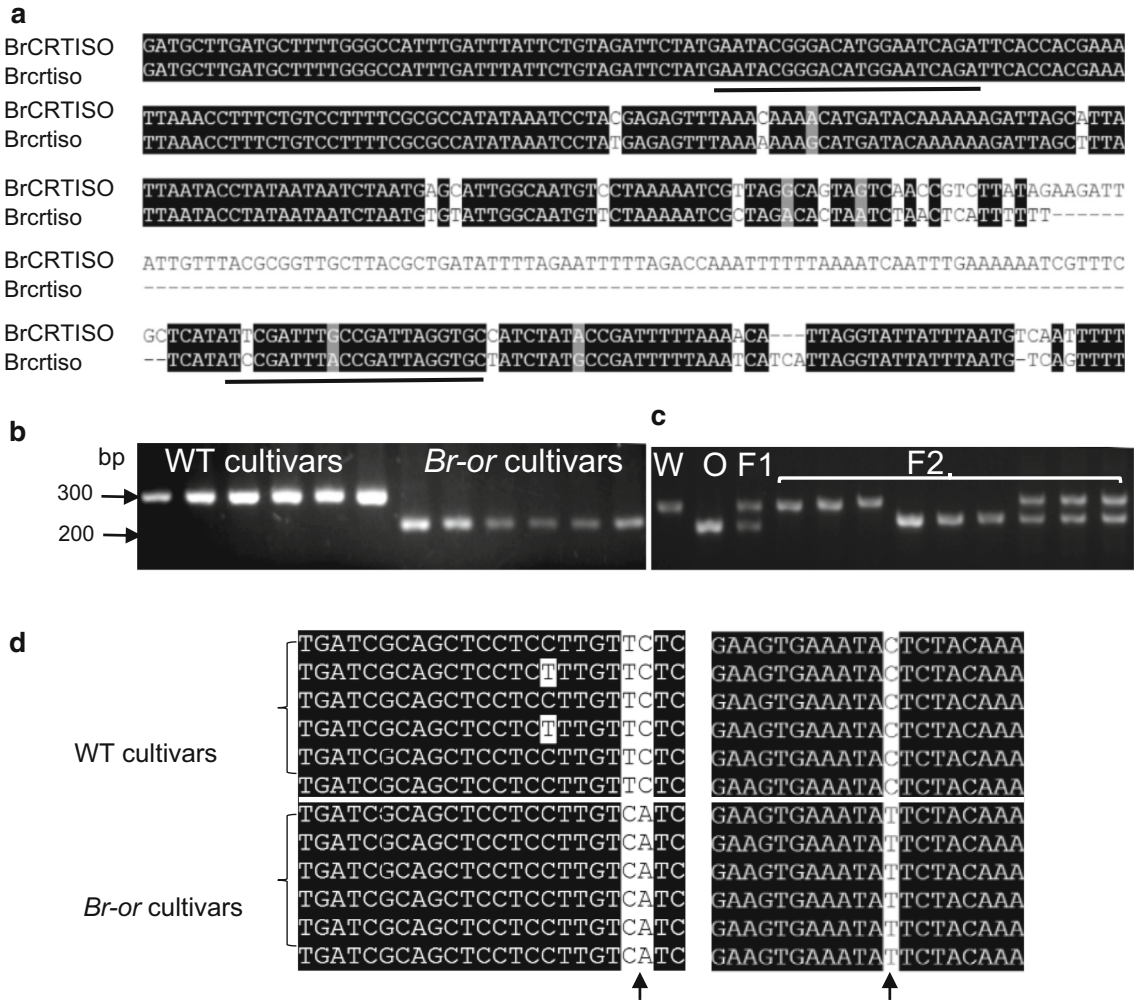

Fig. 8 Development of gene-specific marker for orange head phenotype. **a** Polymorphic sequences of *BrCRTISO* from WT and *Br-or* for marker development. The *underlines* represent the sites for primer design. **b** Validation of gene-specific markers in different white and orange head Chinese cabbage cultivars and lines. **c** Validation of gene-specific markers in F_2 populations. **d** The sites of two SNPs linked with head leaf color

head phenotype. In contrast to WT *BrCRTISO*, the mutated gene with the large insertion could not complement the *Atcrtiso* mutant phenotype and produced a malfunctional *BrCRTISO*. Comparative transcriptome analysis showed that the mutation in *BrCRTISO* resulted in alteration of large numbers of specific functional group genes involved in RNA, protein, transport, and signaling. In addition, a new *BrCRTISO* gene-specific marker was developed, which will be helpful for MAS to breed cultivars with improved nutritional quality and visual appearance of Chinese cabbage.

A natural large insertion in *BrCRTISO* is responsible for orange head phenotype

Orange head Chinese cabbage not only contains unique health benefit compounds, but also provides a valuable new agronomic trait and germplasm resource. *BrCRTISO* was identified as the candidate gene for orange phenotype of *Br-or* in our high-resolution genetic mapping (Zhang et al. 2013) and in a candidate gene analysis (Lee et al. 2014). We isolated the whole length of the *Br-or* gene. *Brcrtiso* contained a large insertion of 501 bp in the end of the coding region, two SNPs to cause missense mutations, and an 88-bp deletion in the promoter, showing the same gene sequence as reported (Su et al. 2014).

In contrast to the *tangerine* mutant in tomato where no *CRTISO* transcript is present in the fruits (Isaacson et al. 2002), *BrCRTISO* expression was found comparable in inner head leaves of Chinese cabbage between control and *Br-or* mutant. Similarly, the mutation appeared not to affect the expression of carotenoid-related genes participated in biosynthesis, degradation, and storage, different from a recent report (Su et al. 2014). The discrepancy could be due to the sampling leaf position differences. The transcript levels of all the genes except ZDS in the inner leaf of *Br-or* also show to exhibit similar expression in the recent report (Su et al. 2014). Consistent with gene expression, corresponding unchanged PSY and ZEP protein levels were also observed.

In contrast with *BrCRTISO* and *BrcrtisoΔ* that contains only two SNPs causing missense mutations, *Brcrtiso* with a large insertion could not complement the *Atcrtiso* mutant phenotype. Insertion of a large DNA fragment frequently perturbs gene functions as shown in many studied (Krysan et al. 1999). The results demonstrated that such a natural large insertion in *BrCRTISO* led to a malfunctional gene product. The mutation in BrCRTISO resulted in the accumulation of prolycopene, which was then partially photoisomerized to lycopene in the leaves of the *Br-or* mutant following expose to sunlight.

BrCRTISO mutation affects specific sets of genes

Comparative transcriptome analysis provides a more quantitative picture of global gene expression changes in *Br-or*. It is well established that change in carotenoid production or defect in carotenoid biosynthesis affects multiple processes in plants. Microarray analysis of *Arabidopsis pds3* mutant reveals that more than 20 different metabolic pathways including those involved in photosynthesis, the Calvin cycle, and GA biosynthesis are changed in mutant plants (Qin et al. 2007). Comparative transcript profiling in a sweet orange red-flesh mutant shows that 26 metabolic pathways are changed in the mutant, especially those functional groups involved in carotenoid biosynthesis, photosynthesis, and citrate cycle (Xu et al. 2009). A recent study shows that mutation in ζ-carotene desaturase alters expression of a large set of both plastid- and nucleus-encoded genes (Avendano-Vazquez et al. 2014). Similarly, the *BrCRTISO* mutation in *Br-or* also affected the expression of genes involved in multiple processes such as RNA, protein, transport, stress, signaling, development, lipid metabolism, hormone metabolism, and secondary metabolism (Fig. 7a, b). The mutation appeared to specifically affect a large number of genes associated with RNA and protein processes.

It is interesting that abundant genes of transcription factors such as AP2/EREBP, TCP, WRKY, GOLDEN2-like, and transcriptional factor B3 family proteins were significantly down- or up-regulated in *Br-or* (Table S2 and S3). Transcription factors activate or repress the transcription activity. The down-regulated AP2/EREBP transcription factors include five ethylene-responsive regulators. Transcription factor RAP2 subfamily belongs to AP2/EREBP transcription factor family that integrates metabolic, hormonal, and environmental signals in stress responses and retrograde signaling (Dietz et al. 2010). A member AtRAP2.2 was found to be able to bind to the upstream regulatory regions of *PSY* and *PDS* in carotenogenesis to regulate their expression in root-derived calli (Welsch et al. 2007). Three TCP and four WRKY families of transcription factors were also dramatically down-regulated in *Br-or* compared with WT. These transcription factors exert important functions in both activation and repression of plant processes involved in stress responses, development, and senescence (Rushton et al. 2010; Danisman et al. 2012). GOLDEN2-like regulates chloroplast development and photosynthesis as well as general metabolic processes in tomato fruits (Powell et al. 2012; Nguyen et al. 2014). Several up-regulated transcription factor B3 family proteins and those responsive to

phytohormones are involved in plant growth and development (Muller and Sheen 2008). It appears that *Br-or* affected expression of transcription factor genes participated in a range of processes from stress response to development.

Consistent with the alteration of many transcription factors, a large number of genes whose products are involved in protein metabolism including transcription, translation, post-translational modifications, turnover, and storage were also up- or down-regulated in *Br-or*. Although how the *BrCRTISO* mutation specifically influences transcription activity and protein metabolism remains to be discovered, the observation of alteration of high abundant genes in the specific functional groups in *Br-or* suggests a potential importance of either *BrCRTISO* or the specific carotenoid metabolites in mediating these processes. Indeed, recent studies indicate an important role of carotenoid-derived compounds as signals in mediating gene expression. The transcriptional repression of many plastid- and nucleus-encoded genes is demonstrated to be due to an uncharacterized carotenoid-derived signal in ZDS mutants (Avendano-Vazquez et al. 2014). Similarly, another uncharacterized β-carotene-derived molecule is required to specifically regulate oscillatory transcriptional mechanism to establish periodic root branching in *Arabidopsis* (Van Norman et al. 2014). An alternative explanation could be that BrCRTISO interacts with these transcription factors or mutation in BrCRTISO affects carotenoid oxidation products or exists feedback regulation, which in turn affects the expression of the transcription factors. In *Arabidopsis*, light stress causes the oxidation of β-carotene, and the carotenoid oxidation products have been shown to act as stress signals to induce expression of a large number of genes (Ramel et al. 2012). In tomato color mutations, cis-carotenoid metabolites are found to feedback regulate the expression of *PSY1* to affect carotenoid production in tomato color mutations (Kachanovsky et al. 2012).

A new marker for MAS breeding with high health benefit value

There is an increasing interest for plant breeder to develop vegetable cultivars with unique nutritional values and visual appearance. Orange Chinese cabbage, a new cultivar, is mainly composed of prolycopene and lycopene, which are totally different from white Chinese cabbage. However, the orange phenotype is only revealed at harvesting stage, and phenotyping is costly, time-consuming, and laborious (Zhang et al. 2013). Functional markers that are developed based on polymorphic sites within genes and co-segregated with phenotypic variations are considered as the ideal molecular markers for MAS in plant breeding (Andersen and Lübberstedt 2003).

We developed a new functional maker based on the deletion in the upstream regulatory region, which enables to identify the orange head Chinese cabbage at seedling stage. The genotypes of orange head, white head (homozygotes), and hybrid (heterozygote) can be easily determined by this co-dominant marker on ethidium bromide-stained agarose gels (Fig. 8b, c). In addition, we also found that two SNP sites in the coding region of *BrCRTISO* were linked with head color trait (Fig. 8d). The PCR-based markers are superior to other random DNA markers (RFLPs, SCAR, and SSRs) linked with orange phenotype (Matsumoto et al. 1998; Zhang et al. 2008, 2013; Feng et al. 2012) and also different from the markers described by Lee et al. (2014). Therefore, this gene-specific marker is convenient, useful, and efficient to distinguish the head color at early stage and will greatly accelerate breeding of Chinese cabbage with improved quality trait and visual appearance.

Accession numbers

Sequence of *BrCRTISO* (Bra031539) can be found in the *Brassica* database (http://brassicadb.org/brad/). Protein sequences for phylogenetic analysis in Fig. 3b can be found in GenBank database under the following accession numbers: *Arabidopsis* (NP_172167), *Solanum lycopersicum* (AAL91366), *Zea mays* (NP_001147881), *Nicotiana tabacum* (AFU10971), *Cucumis sativus* (XP_004163999), *Daucus carota* subsp. *sativus* (ABB52069), *Fragaria vesca* subsp. *vesca* (XP_004303382), *Chrysanthemum morifolium* (BAE79546), *Ipomoea* sp. *Kenyan* (BAI47575), *Calendula officinalis* (BAL27714), *Oncidium* (AAX84688), *Narcissus tazetta* (AGG18253), *Vitis vinifera* (XP_002269590), *Populus trichocarpa* (XP_002308337), *Ricinus communis* (EEF38309), *Glycine max* (XP_006575265), *Setaria italic* (XP_004981106), *Coccomyxa subellipsoidea* (XP_005650388), *Heliobacillus mobilis* (AAC84034), and *Pantoea agglomerans* (AAA24820).

Author contribution JZ, LL, and LZ conceived and designed the experiments. JZ conducted experiments. HY, JZ, and ZF did RNA-seq analysis. BJP performed HPLC analysis. JZ, LL, and LZ wrote and modified the manuscript. All authors in this study read and approved the manuscript.

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