Fine mapping and candidate gene analysis of the white flower gene \( Brwf \) in Chinese cabbage (\( Brassica rapa \) L.)

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Flower color can be applied to landscaping and identification of the purity of seeds in hybrid production. However, the molecular basis of white flower trait remains largely unknown in \( Brassica rapa \). In this study, an F\(_2\) population was constructed from the cross between 15S1040 (white flower) and 92S105 (yellow flower) for fine mapping of white flower genes in \( B. rapa \). Genetic analysis indicated that white flower trait is controlled by two recessive loci, \( Brwf1 \) and \( Brwf2 \). Using InDel and SNP markers, \( Brwf1 \) was mapped to a 49.6-kb region on chromosome A01 containing 9 annotated genes, and among them, \( Bra013602 \) encodes a plastid-lipid associated protein (PAP); \( Brwf2 \) was located in a 59.3-kb interval on chromosome A09 harboring 12 annotated genes, in which \( Bra031539 \) was annotated as a \textit{carotenoid isomerase} gene (\textit{CRTISO}). The amino acid sequences of BrPAP and BrCRTISO were compared between two yellow-flowered and three white-flowered lines and critical amino acid mutations of BrPAP and BrCRTISO were identified between yellow-flowered and white-flowered lines. Therefore, \( Bra013602 \) and \( Bra031539 \) were predicted as potential candidates for white flower trait. Our results provide a foundation for further identification of \( Brwf \) and increase understanding of the molecular mechanisms underlying white flower formation in Chinese cabbage.

In nature, flower color was used to attract insect for pollination in plants\(^1\). There are three chemically distinct pigments, carotenoids, flavonoids, and betalains, responsible for flower color, and among them, carotenoids accumulating in petals can generate yellow, orange, and red flower colors\(^2,3\). The most common carotenoids in petals are xanthophylls, which show high specificity in composition and quantity among plant species or varieties\(^4\).

Carotenoid accumulation was modulated by its biosynthesis, degradation, and sequestration\(^5,6\). The mutation of key genes involved in the above three processes could result in the conversion of flower and fruit colors. For example, a single-nucleotide mutation in \( \beta \)-carotene hydroxylase 2 (\( CHYB2 \)) caused orange fruit phenotype in pepper\(^9\). In \textit{Chrysanthemum morifolium}, \textit{Brassica napus}, and \textit{B. oleracea}, the loss-of-function mutation of \textit{carotene cleavage dioxygenase 4} (\( CCD4 \)) led to change in flower color from white to yellow\(^7,10–14\). The mutation of \textit{pale yellow petal} (\( PYP1 \)) that was involved in xanthophyll ester production was responsible for pale yellow petal phenotype in tomato\(^8\).

The \textit{Brassica} genus includes important oil crops and vegetables\(^15\) with yellow flower color as the most common form, while there are other colors, such as pale yellow, white, orange, and tangerine\(^16–25\). Compared with the studies of other traits in \textit{Brassica} genus, such as yield\(^23–28\), fertility\(^29–32\), disease resistance\(^33–38\), the genetic studies of flower colors have been conducted earlier\(^16,17,18\). However, only the molecular mechanisms of white flower formation were understood\(^11–14,37,38\). Recently, a few genes controlling flower colors have been reported. For example, a \textit{carotenoid isomerase} gene (\textit{CRTISO}) related to pale yellow flower in \( B. rapa \)\(^22\) and a \textit{carotenoid cleavage dioxygenase 4} gene (\( CCD4 \)) associated with white flower in \( B. napus \)\(^11\) and \( B. oleracea \)\(^12–14\) were cloned, respectively. In \( B. napus \) and \( B. oleracea \), a CACTA-like transposable element insertion caused disruption of \( CCD4 \)\(^13,32,34\) and \( CCD4 \) from white-flowered line could rescue the petal color of yellow-flowered line\(^11,12\). In addition, Zhang et al.\(^37,38\) reported that the \textit{BjuA008406} and \textit{BjuB027334} genes, which might be involved in carotenoid esterification,
were predicted as the potential candidates for white flower in *B. juncea*. However, none of white flower genes has been cloned yet, and the molecular mechanism of white flower formation remains poorly understood in *B. rapa*.

In this study, the inheritance pattern of white flower trait was analyzed using an F2 segregating population developed from the crossing of white flower line 15S1040 and yellow flower line 92S105. Molecular markers designed based on the genome re-sequencing data of 15S1040 and 92S105 were used to map white flower genes, and then the prediction of the candidate genes was performed; the coding sequences of two candidate genes (*BrPAP* and *BrCRTISO*) were compared between three white-flowered and two yellow-flowered lines; the expression levels of two candidate genes were tested in different tissues. Our findings provide insights in molecular mechanisms controlling flower color variation in *B. rapa*.

**Results**

**Genetic analysis of the white flower trait in *B. rapa***. The flower colors of F1 plants derived from the cross between white parent 15S1040 and yellow parent 92S105 were all yellow (Fig. 1a–c). Among 1282 F2 individuals, 718 individuals were yellow flower, 257 individuals were milky yellow flower, 227 individuals were pale yellow flower, and 80 individuals were white flower (Fig. 1d–g). The F2 segregation ratio was fitted into an expected ratio of 9:3:3:1 (χ² = 1.908, df = 3, P > 0.05) using χ² test (Table 1). These results indicated that yellow flower trait was dominant over white flower and the white flower trait was controlled by two recessive genes (*Brwf1* and *Brwf2*), therefore the genotypes of four flower color plants may be yellow flower (*BrWF1BrWF1BrWF2BrWF2*, *BrWF1BrWF1BrWF2Brwf2*), milky yellow flower (*Brwf1Brwf1BrWF2BrWF2* or *Brwf1Brwf1BrWF2Brwf2*), pale yellow flower (*BrWF1BrWF1Brwf2Brwf2* or *BrWF1BrWF1Brwf2Brwf2*), and white flower (*Brwf1Brwf1Brwf2Brwf2* and *BrWF1BrWF1Brwf2Brwf2*), respectively.

| Cross combination  | Generation | Total plants | Yellow flower plants | Milky yellow flower plants | Pale yellow flower plants | White flower plants | Mendelian expectation | χ² value (df = 3, P > 0.05) |
|--------------------|------------|--------------|----------------------|---------------------------|--------------------------|---------------------|-----------------------|--------------------------|
| 15S1040 × 92S105   | F1         | 20           | 20                   |                           |                          |                     |                       |                          |
|                    | F2         | 1282         | 718                  | 257                       | 227                      | 80                  | 9:3:3:1               | 1.908                    |

Table 1. The segregation of flower colors in the F1 and F2 population. *χ² > χ²*(0.05, 3) = 7.815 is considered significant.

**Carotenoid accumulation and ultrastructural analysis of chromoplasts in yellow and white petals**. Carotenoid composition and content in yellow and white petals at the flowering stage were analyzed using high performance liquid chromatography (HPLC). The results showed that the major carotenoids in yellow and white petals were both violaxanthin and lutein, however, the total carotenoid contents of yellow and white petals were 211.69 ± 21.70 μg/g and 10.49 ± 1.21 μg/g (Fig. 2a), respectively, which may result in the difference in color between yellow and white petals.

To study whether there were differences in chromoplast structures between yellow and white petals, the ultrastructural analysis of chromoplasts in the two parents was performed using transmission electron microscopy (TEM). The results indicated that yellow-flowered individuals had normal chromoplasts with numerous fully developed plastoglobules (PGs), however, white-flowered individuals showed abnormal chromoplasts with few PGs (Fig. 2b,c).
Preliminary mapping of the Brwf genes. To determine the locations of genes controlling white flower trait, 81 insertion/deletion (InDel) markers distributed on 10 chromosomes were developed based on the re-sequencing data of the two parents, and 34 InDel markers (W1-W11, W101-W288) exhibited polymorphism between 15S1040 and 92S105 (Supplementary Table S1). These polymorphic markers were used for bulk segregant analysis (BSA) of flower color trait. As a result, six markers (W101, W105, W107, W112, W114, and W116) on chromosome A01 and three markers (W1, W5, and W11) on chromosome A09 were linked with Brwf genes. Among them, W105 and W112 markers, W5 and W11 markers were randomly chosen to assay 30 milky yellow-flowered and 30 pale yellow-flowered plants from F2 population. The results showed that W105 and W112 markers were linked with the Brwf1 gene controlling milky yellow flower and W5 and W11 markers were linked with the Brwf2 gene controlling pale yellow flower, which indicated that Brwf1 and Brwf2 were located on chromosomes A01 and A09, respectively.

For preliminary mapping of the Brwf1 and Brwf2 genes, newly designed 36 InDel markers on chromosome A01 and 35 InDel markers on chromosome A09 were screened between the two parental lines, and 14 (W310-W339) and 12 (W23-W60, W67) markers showed polymorphism, respectively (Supplementary Table S1). These polymorphic markers were used for BSA of flower color trait and 14 markers on chromosome A01 and 5 markers on chromosome A09 were linked with the Brwf genes. To preliminarily map the Brwf1 and Brwf2 genes separately, A and B groups that were segregated in BrWF1/Brwf1 and BrWF2/Brwf2 loci, respectively, were selected from F2 population and A group included 108 yellow-flowered and 36 milky yellow-flowered individuals and B group included 108 yellow-flowered and 36 pale yellow-flowered individuals. Then obtained 19 linkage markers from chromosomes A01 and A09 were used to detect A and B groups, respectively. In A group, the Brwf1 gene, co-segregating with W323 marker, was localized to a region between W322 and W331 markers on chromosome A01, and the genetic and physical distances were 0.74 cM and 186.1 kb, respectively (Fig. 3a). In B group, the Brwf2 gene was mapped to a 0.71 cM interval flanked by W5 and W67 markers with the corresponding physical distance of 216.3 kb on chromosome A09, and one marker W11 co-segregated with Brwf2 (Fig. 3b).

Fine mapping of the Brwf genes. For fine mapping of the Brwf1 gene, the two markers W322 and W331 were used to detect recombination events in all F2 plants, and a total of 18 recombinants including 5 recombination events with W322 marker and 13 recombination events with W331 marker were obtained. Using the re-sequencing data of the two parents, 10 new InDel markers were developed from the preliminary mapping region and seven of them (W341-W352) exhibited polymorphism in the two parents (Supplementary Table S1).
These polymorphic markers were unceasingly used to screen all the 18 recombinants. The results indicated that the Brwf1 gene was delimited to a shortened interval between W323 and W351 markers with one recombinant and four recombinants, respectively (Fig. 4a–c and Supplementary Fig. S1). To further narrow down the mapping interval, two single-nucleotide polymorphism (SNP) markers (S361 and S371) were developed and used to test recombination events. As a result, one recombination event with S361 marker and two recombination events with S371 marker were found, and then a developed SNP marker S363 on the side of S371 was also used to detect two recombination events (Supplementary Table S1 and Fig. S2). The two SNP markers S361 and S363 further narrowed the Brwf1 gene to an interval of 0.11 cM with the corresponding physical distance of 49.6 kb. Finally, two markers, W348 and W350, co-segregating with Brwf1 were obtained (Fig. 3c).

To fine map the Brwf2 gene, a total of nine recombinants were identified using W5 and W67 markers, which included three recombination events occurring between W5 marker and Brwf2 and six recombination events occurring between W67 marker and Brwf2. Among 15 InDel markers developed from the preliminary mapping interval, five markers (W61, W72–W79) were polymorphic between 15S1040 and 92S105 (Supplementary Table S1). The nine recombinants were screened by five new polymorphic markers. As a result, the Brwf2 gene was restricted to a region between W11 and W78 markers and there were one recombinant with W11 marker and two recombinants with W78 marker (Fig. 4d–f and Supplementary Fig. S1). For further narrow down the mapping region, two more SNP markers (S82 and S83) were developed for detecting recombination events (Supplementary Table S1).
The result showed that one recombination event was found between each of the two SNP markers and \textit{Brwf2} (Supplementary Fig. S2) respectively, so the \textit{Brwf2} gene was delimited to a 0.08 cM region flanked by S82 and S83 markers, and the corresponding physical distance was 59.3 kb. Finally, two markers, W61 and W74, co-segregating with \textit{Brwf2} were obtained (Fig. 3d).

Identification and sequence analysis of the candidate genes. According to the \textit{B. rapa} reference genome in BRAD (\textit{Brassica} database, http://brassicadb.org/brad), 9 and 12 genes were annotated within the two final mapping intervals of \textit{Brwf1} and \textit{Brwf2} genes, respectively (Fig. 3e,f). Among 9 annotated genes in the \textit{Brwf1} interval on chromosome A01, \textit{Bra013602} encodes a plastid-lipid associated protein (PAP) that was previously reported to regulate carotenoid accumulation\textsuperscript{39,40} (Table 2). Out of 12 annotated genes in the \textit{Brwf2} interval on chromosome A09, \textit{Bra031539} was predicted to encode a carotenoid isomerase (CRTISO) that was involved in carotenoid biosynthesis\textsuperscript{41–44} (Table 2). Therefore, \textit{Bra013602} and \textit{Bra031539} were predicted as the two candidates for \textit{Brwf1} and \textit{Brwf2} genes, respectively.

The specific primers WY503 was designed for cloning and sequencing of the cDNA sequences of \textit{BrPAP} (Supplementary Table S1). The gene sequence comparison showed that there were 15 SNPs in the coding region of \textit{BrPAP} between 92S105 and 15S1040 (Supplementary Fig. S3a), which resulted in four amino acid residue mutations (Supplementary Fig. S4a). Based on previous studies\textsuperscript{42,44}, two designed primers, WY571 and WY572, were used to clone the cDNA sequences of \textit{BrCRTISO} in 92S105 and 15S1040, respectively (Supplementary Table S1). The sequence alignment indicated that there were many SNPs, one small deletion, and one large insertion in the coding region of \textit{BrCRTISO} in 15S1040. This large insertion had 943 bp that was located at the 3' end of \textit{BrCRTISO} (Supplementary Fig. S3b). After the amino acid sequence alignment, 17 amino acid residue changes and the deletion of two amino acid residues were found in \textit{BrCRTISO} of 15S1040, however, at the 3' end, the large insertion resulted in mutations of 15 amino acid residues, one amino acid residue insertion, and three amino acid residue deletions in \textit{BrCRTISO} of 15S1040 (Supplementary Fig. S4b).

To identify the key mutations of the two candidate genes between white-flowered and yellow-flowered lines, the genomic sequences of two candidate genes from one yellow-flowered line (09Q5) and two white-flowered lines (15S1001 and 17S690) were cloned using designed specific primers, which included WY503 for \textit{BrPAP} of yellow-flowered and white-flowered lines, and WY561, WY562, WY563 for the \textit{BrCRTISO} of yellow-flowered line and WY561, WY562, WY566 for the \textit{BrCRTISO} of white-flowered lines according to previous studies\textsuperscript{42,44} (Supplementary Table S1). The deduced amino acid sequences of \textit{BrPAP} and \textit{BrCRTISO} from three yellow-/white-flowered lines were compared with that from the two parental lines. The results indicated that the deduced amino acid sequence of \textit{BrPAP} in 09Q5 was same as that in 92S105, while there are seven amino acid residue mutations among 15S1040, 15S1001, and 17S690, but only one mutant amino acid residue (Leu → Pro) was found between two yellow-flowered and three white-flowered lines and it was located in the conserved domain of \textit{BrPAP} (Fig. 5a; Supplementary Fig. S4a); the deduced amino acid sequence of \textit{BrCRTISO} in 09Q5 had 17 amino acid residue mutations and one deletion of two amino acid residues compared with 92S105, while the sequences from 15S1001 and 17S690 were identical to that from 15S1040, however, two amino acid residue mutations (Ile → Val, Leu → Phe) and many amino acid residue changes at the end of sequences were consistent with the flower color and the two amino acid residues were located in the conserved domain of \textit{BrCRTISO} (Fig. 5b; Supplementary Fig. S4b).

Figure 4. Genotyping of screened recombinants for fine mapping of \textit{Brwf1} (a-c) and \textit{Brwf2} (d-f) using closely linked markers. (a) W323, (b) W348, (c) W351; (d) W11, (e) W61, (f) W78. M: DL50 marker; P1: 92S105; P2: 15S1040; F1: F1 individual; R: screened recombinants from the F1 population using markers W322 and W331, W5 and W67; H: heterozygous genotype plant; B: recessive homozygous genotype plant.
Expression analysis of the candidate genes and carotenoid metabolic genes.  Expression pattern analysis of BrPAP and BrCRTISO was conducted using Quantitative real-time PCR (qPCR) in different tissues (roots, stems, cauline leaves, and petals) from the two parental lines. BrPAP expressed mainly in petals and could hardly be detected in other tissues with expression level of BrPAP in petals of 92S105 being twofold higher than that in 15S1040 (Fig. 6a); BrCRTISO had relatively higher expression levels in cauline leaves and petals than in roots and stems, however, BrCRTISO did not exhibit significant difference in expression between the petals of the two parental lines (Fig. 6b). Moreover, the expression levels of genes related to carotenoid metabolism in BrPAP were detected. The results indicated that CRTISO and Lycopersicon esculentum (LCYE) had no significant differences in expression between the petals of the two parental lines, but expression of other seven genes showed down-regulated in petals of 15S1040 compared with 92S105 (Supplementary Fig. S5).

Table 2. Annotated genes within the mapping intervals of Brwfl and Brwf2 on chromosomes A01 and A09.

| Chr. | B. rapa | Gene position* | Gene function* | Arabidopsis thaliana homolog |
|------|---------|----------------|----------------|-----------------------------|
| A01  | Br013596| 6555476…6558338 | Protein kinase | AT4G23280 |
|     | Br013597| 6559518…6559832 | F-box family protein | AT4G22170 |
|     | Br013598| 6561855…6562856 | Unknown protein | AT4G22190 |
|     | Br013599| 6567555…6572888 | AKT2/3: cyclic nucleotide binding/inward rectifier potassium channel/protein binding | AT4G22200 |
|     | Br013600| 6577601…6578014 | PRAL.H: prenylated PAB acceptor 1.H | AT4G27540 |
|     | Br013601| 6597194…6597946 | ISU1: structural molecule | AT4G22220 |
|     | Br013602| 6598836…6599884 | PAP: plastid-lipid associated protein | AT4G22240 |
|     | Br013603| 6600885…6601496 | Zinc finger (CHC4-type RING finger) family protein | AT4G22250 |
|     | Br013604| 6602225…6604058 | Alternative oxidase | AT4G22260 |
| A09  | Br031532| 37708145…37710811 | 2-oxoglutarate-dependent dioxygenase | AT1G06650 |
|     | Br031533| 37705347…37707116 | 2-oxoglutarate-dependent dioxygenase | AT1G06650 |
|     | Br031534| 37703357…37704748 | PSBP-1: Photo system II subunit-1 | AT1G06680 |
|     | Br031535| 37693586…37703026 | Ribosome biogenesis | AT1G06720 |
|     | Br031536| 37686812…37689589 | Unknown protein | AT1G06750 |
|     | Br031537| 37681637…37684412 | GAUT6: Galacturonosyltransferase 6 | AT1G06780 |
|     | Br031538| 37680010…37681151 | RNA polymerase Rpb7 N-terminal domain-containing protein | AT1G06790 |
|     | Br031539| 37676627…37679733 | Carotenoid isomerase | AT1G06820 |
|     | Br031540| 37674379…37674678 | Glutaredoxin family protein | AT1G06830 |
|     | Br032412| 37717578…37717784 | 2-oxoglutarate-dependent dioxygenase | AT1G06640 |
|     | Br032413| 37718500…37719120 | 2-oxoglutarate-dependent dioxygenase | AT1G06650 |
|     | Br032414| 37722562…37728738 | Unknown protein | AT1G06590 |

Discussion

In Brassica species, genetic analysis of flower color traits has been carried out early16,17,18,46,47. However, several studies have reported that white flower trait was a recessive trait controlled by two major genes20,37,38,48. In this study, genetic analysis of white flower trait in B. rapa was conducted with F2 population derived from a cross between white-flowered line 15S1040 and yellow-flowered line 92S105. Our results showed that white flower trait was controlled by two separate loci and the white flower trait is recessive to yellow flower, consistent with previous reports20,37,38,48.

Multiple studies have reported recently on gene mapping of white flower trait in Brassica species. In B. napus, a white flower gene was mapped to a 0.39 cM region on chromosome C0311. Ashutosh et al.46 and Han et al.47 also mapped a white flower gene on chromosome C03 using populations derived from the crosses between broccoli and Chinese kale, cabbage and Chinese kale, respectively. In Chinese kale, a white flower gene was also delimited to chromosome C0313,14. The above results indicated that a single gene controlling white flower trait might be the same gene in B. napus and B. oleracea. In B. juncea, two recessive genes that controlled white flower trait were restricted to chromosomes A02 and B04 and the genetic distances were 0.13 cM and 0.25 cM, respectively37,38. In this study, we found that white flower trait in B. rapa was also controlled by two genes (Brwfl and Brwf2), which were mapped to intervals of 0.11 cM and 0.08 cM on chromosomes A01 and A09, respectively.

PAP, also called fibrillin, found in the pepper fruit chromoplasts and its homologous protein in chromoplasts of cucumber flower, was named as chromoplast-specific carotenoid-associated protein (CHRC)48. In chromoplast, fibrillin and CHRC were positively associated with carotenoid accumulation28,49. The suppression of the expression of CHRC gene in tomato flowers resulted in decreased carotenoids48, which indicated that CHRC plays a role in mediating carotenoid storage in chromoplasts of flowers. Over-expression of the pepper fibrillin gene in tomato increased the levels of carotenoids in fruit48. In this study, the Bra013602 gene encoding PAP was...
located in the final mapping region of Brwf1, which deduced amino acid sequence has four amino acid residue mutations between the two parents and one of the mutations (Leu → Pro) occurred in the conserved domain of BrPAP between yellow-flowered lines (92S105 and 09Q5) and white-flowered lines (15S1040, 15S1001, and 17S690), which might affect the function of BrPAP in white-flowered lines. In addition, fibrillin was involved in plastoglobule formation based on previous investigations. Over-expression of the fibrillin gene from pepper in tobacco resulted in the increased number of PGs in plastids of leaves and petals. In this study, ultrastructural analysis of chromoplasts in the two parents revealed that the number of PGs in yellow petal chromoplasts was more than that in white petal chromoplasts. Expression pattern analysis of BrPAP indicated that the expression level of BrPAP in petals was much higher than that in other tissues. These results indicated that BrPAP was the most possible candidate for white flower trait.

It was known that the role of CRTISO is the control of the conversion of prolycopene to lycopene. The functional disruption of BrCRTISO gene resulted in the orange head leaf formation in Chinese cabbage. However, Lee et al. reported that 19 amino acid residue changes and deletion of two amino acid residues were found in the amino acid sequence of BrCRTISO from pale-yellow flower cultivar compared with that in yellow

Figure 5. Gene structures and amino acid sequence analyses of BrPAP and BrCRTISO. (a) The coding region of BrPAP includes two exons and one intron. The nonsynonymous SNP mutation (T → C) in exon 2 results in the amino acid residue conversion (Leu → Pro) between yellow-flowered and white-flowered lines. (b) The coding region of BrCRTISO contains 13 exons and 12 introns. The nonsynonymous SNP mutation (A → G) in exon 2 and (C → T) in exon 6 and a large insertion in exon 13 cause the conversion of Ile to Val and Leu to Phe, and many amino acid residue changes, respectively, between yellow-flowered and white-flowered lines. The above amino acid residue mutations are consistent with flower color phenotypes. Black backgrounds indicate mutant amino acid residues. 92S105 and 09Q5 are yellow-flowered lines; 15S1040, 15S1001, and 17S690 are white-flowered lines.

Figure 6. Expression pattern analysis of BrPAP (a) and BrCRTISO (b) in different tissues of 92S105 and 15S1040. Error bars represent the SD, and asterisks indicate significant difference (t-test, P < 0.05) between 92S105 and 15S1040. The expression of the two genes in petals of 92S105 was considered as the standard of ‘relative’ expression.
flower cultivar. In this study, *Bra031539* encoding *BrCRTISO* was located in the final delimited genomic region of *Brw2*. The amino acid sequence analysis of *BrCRTISO* indicated that two amino acid residue mutations (Ile → Val, Leu → Phe) that were located in the conserved domain of *BrCRTISO* and many amino acid residue changes at the end of sequences were found between two yellow-flowered lines (92S105 and 09Q5) and three white-flowered lines (15S1040, 15S1001, and 17S690). In addition, although Zhang et al. reported that the amino acid residue mutation (Leu → Phe) of *BrCRTISO* could not affect the protein function in leaves, this mutation which was found in petals might affect *BrCRTISO* function in this study. Taken together, two amino acid residue mutations (Ile → Val, Leu → Phe) and many amino acid residue changes in the C-terminal end of *BrCRTISO* might affect its function, which suggested that *BrCRTISO* was the most promising candidate for white flower trait.

In *B. napus* and *B. juncea*, the major carotenoid in yellow and white petals was violaxanthin, but the total carotenoid contents in petals were forty-twofold and eightfold higher than that in white petals, respectively. In the present study, carotenoid analysis of yellow and white petals showed that violaxanthin and lutein were mainly accumulated in yellow and white petals of Chinese cabbage, however, the total carotenoid content was twenty times higher in yellow petals than in white petals, which were consistent with the previous studies. Moreover, because light could partially replace CRTISO activity, which combined with the phenotypic observation of F2 plants and the results of amino acid sequence comparison of *BrPAP* and *BrCRTISO*, we hypothesized that the mutations of *BrPAP* and *BrCRTISO* and light might jointly affect the prolycopene accumulation and resulted in barely detecting it in 15S1040. In *B. napus*, a single dominant gene, *BnaCCD4*, controls the white flower trait and associated with carotenoid degradation. In *B. juncea*, the white flower trait was jointly controlled by two recessive genes, *Bjpc1* and *Bjpc2* which encode esterase/lipase/thioesterase family protein and phytyl ester synthase 2, respectively, and were involved in carotenoid esterification. In this study, the potential candidate genes for the white flower trait in Chinese cabbage were *BrPAP* and *BrCRTISO* that were associated with carotenoid storage and biosynthesis, respectively. The results of TEM analysis and amino acid sequence alignment of *BrPAP* indicated that the mutation of *BrPAP* resulted in decrease of carotenoid accumulation by blocking PG formation. The mutant types of *BrCRTISO* in the present study were incompletely consistent with the previous investigations, which indicated that the function of *BrCRTISO* in 15S1040 might not be complete disruption. In addition, expression analysis of genes associated with carotenoid metabolism showed that the majority of carotenoid biosynthesis pathway genes were down-regulated expression in petals of 15S1040 compared with 92S105. Hence, the mutation of *BrCRTISO* might decrease the flux of carotenoid biosynthesis pathway. Taken together, we inferred that both mutations of *BrPAP* and *BrCRTISO* maybe lead to the white flower formation by decreasing total carotenoid content in 15S1040 (Fig. 7).

**Methods**

**Plant materials.** The five Chinese cabbage lines, the white-flowered 15S1040, 15S1001, 17S690, and the yellow-flowered 92S105 and 09Q5, were used in this study. 15S1040 and 92S105 (Fig. 1a,b) were selected as parents for constructing F2 population. To study the inheritance pattern of white flower trait and fine map the *Brwf* genes, a cross between the two parental lines, 15S1040 and 92S105, was used to produce the hybrid F1, then one F1 plant was self-pollinated to generate the F2 population with 1282 individuals. Other white-flowered and yellow-flowered lines were used for the identification of the candidate genes. All materials were bred and provided by the Chinese cabbage research group at the Northwest A&F University, Yangling, China.

All plants used in the present study were grown and naturally vernalized at the experimental field of the Northwest A&F University in 2018. During the flowering stage, the observations of at least ten flowers per plant were performed twice to evaluate the flower color of each individual with an 8-day interval.

**Carotenoid extraction and analysis.** Carotenoid were extracted from fresh petals at the flowering stage and detected following the methods of Cao et al. Carotenoid analysis was performed using LC-2010AHT HPLC (Shimadzu, Kyoto, Japan) with C30 column (YMC, Kyoto, Japan). Carotenoids were identified by the typical retention time of the standard compounds, including violaxanthin (Sigma-Aldrich, Saint Louis, America), lutein (Solarbio, Beijing, China), α-carotene and β-carotene (Wako, Osaka, Japan). The identification of prolycopene was performed based on reported the typical retention time and relative order of carotenoid compound peaks. Carotenoid content was quantified according to Morris’ method. The total carotenoid content was the sum of all the detected carotenoid compound contents. Three biological replicates were used for all analyses and the calculation of means and standard deviations were conducted. The significant difference between 92S105 and 15S1040 was analyzed by t-test.

**Transmission electron microscopy analysis.** Petals from 92S105 and 15S1040 flowers at the flowering stage were cut into 0.3 × 0.6 cm sections, fixed with 2.5% glutaraldehyde. The preparation of observation samples of petals and TEM analysis were performed according to Yi et al. described methods.

**DNA and RNA extraction, first-strand cDNA synthesis, and gel electrophoresis.** Total genomic DNA was isolated from fresh leaves using the cetyl trimethylammonium bromide (CTAB) method described by Porebski et al. Using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China), total RNA was extracted from petals of open flowers, roots, stems, and cauline leaves from the two parental lines, and first-strand cDNA, which was used for quantitative real-time PCR (qPCR), was synthesized by PRIMESCRIPT 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). To clone the cDNA sequences of the candidate genes, first-strand cDNA synthesis was performed with PRIMESCRIPT II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).
Two (yellow and white flowers) and four (yellow, milky yellow, pale yellow, and white flowers) kinds of F₂ individuals were used for BSA and fine mapping, respectively. Two DNA pools, yellow-flowered pool and white-flowered pool, were created by mixing equal amounts of DNA from 8 individuals with yellow flower and 8 individuals with white flower, respectively, which were randomly selected from F₂ population. The PCR reaction and separation of its products were performed as described by Zhang et al.31.

**Development of InDel and SNP markers.** To develop InDel and SNP markers, the two parental lines, 15S1040 and 92S105, were re-sequenced with HiSeq X Ten (Gene Denovo, Guangzhou, China) at 30- and 91-fold sequencing depths. The re-sequencing data of 15S1040 and 92S105 were mapped to the B. rapa reference genome in BRAD, the genomic variants were found using Genome Analysis Toolkit (GATK), and the annotation of the physical location of each genomic variant was carried out. The insertions/deletions > 3 bp and single-nucleotide polymorphism loci were used to develop InDel and SNP markers, respectively, with the Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/) software based on the corresponding flanking sequences in the B. rapa reference genome. The primers used in the present study were synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

**Identification of recombination events.** To obtain the DNA fragments that contained SNP loci in the recombinants, the specific primers were designed according to the reference genome of B. rapa. The purification of PCR products and sequencing were conducted using our previous method55. The nucleotide sequences were analyzed using the DNASTAR Lasergene 7.1 (http://www.dnastar.com) and Chromas 2.4.1 (http://technelysium.com.au/wp/chromas/) softwares.

**Fine mapping of the Brwf genes and identification of the candidate genes.** The polymorphic molecular markers were utilized to assay genotype of plants in the F₂ populations. The linkage analyses were conducted using the genotypic data of the polymorphic markers and phenotypic data of each individual in F₂ segregating population. The linkage map was then constructed using the JoinMap 4.0 (https://www.kyazma.nl/index.php/JoinMap/) software based on a LOD threshold score of 6.0. The candidate genes in the final delimited region were analyzed based on the annotation data of the B. rapa reference genome in BRAD.

**Cloning and sequence analysis of the candidate genes.** To clone the DNA and cDNA sequences of the putative candidate genes, the primers were designed according to the B. rapa reference genome. The cloning of putative candidate genes and sequencing were performed according to our previous method55. The complete coding sequences of two candidate genes from two yellow-flowered and three white-flowered lines were submitted to GenBank, the accession numbers: BrPAP: MN338556 (92S105), MN338557 (09Q5), MN338558

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**Figure 7.** Proposed molecular mechanism diagram of white flower formation in Chinese cabbage. PSY: phytoene synthase, PDS: phytoene desaturase, Z-ISO: ζ-carotene isomerase, ZDS: ζ-Carotene desaturase, CRTISO: carotenoid isomerase, LCYE: Lycopene ε-cyclase, LCYB: Lycopene β-cyclase, CHYB: β-carotene hydroxylase, CYP97: cytochrome P450-type monoxygenase 97, ZEP: zeaxanthin epoxidase, PAP: plastid-lipid associated protein. Enzymes with green represent the genes that encode these enzymes were down-regulated expression in white flower. Gray frames represent mutated genes.
Expression analysis of the candidate genes and carotenoid metabolic genes. qPCR was used to investigate the expression pattern of the candidate genes in different tissues and the expression levels of carotenoid metabolic genes in petals of the two parental lines, and carotenoid metabolic genes included phytoene synthase (PSY), phytoene desaturase (PDS), δ-Carotein desaturase (ZDS), carotenoid isomerase (CRTISO), Lycopene ε-cyclase (LCYE), Lycopene β-cyclase (LCYB), β-carotene hydroxylase (CHYB), zeaxanthin epoxidase (ZEP), and carotenoid cleavage dioxygenases 4 (CCD4). The specific primers were designed for qPCR using the Primer Premier 5.0 software (Supplementary Table S2), and Chinese cabbage elongation-factor 1α (EF1α) gene was selected as the internal reference. The qPCR tests were performed following the method described by Ren et al. All gene expression analyses were repeated three times with independent samples. The calculation of relative expression level was performed using the 2−ΔΔCT method. The significant difference analysis of expression data between 92S105 and 15S1040 was performed using t-test.

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Author contributions
L.Z. and N.Z. conceived and designed the experiments. N.Z. performed phenotypic observation, HPLC and TEM analysis, the genetic linkage map construction, and cloning of the candidate genes in the mapped region, and wrote this paper. L.C. participated in the genetic linkage map construction and cloning of the candidate genes. S.M., R.W., and M.T. participated in phenotypic observation, DNA and RNA extraction, and cDNA synthesis. Q.H. helped the sequence analysis. L.Z. provided the *B. rapa* materials, revised this paper, and supervised the research.

Competing interests
The authors declare no competing interests.

Additional information
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