RESEARCH ARTICLE

Genome-wide identification and expression profiling of the *carotenoid cleavage dioxygenase* (*CCD*) gene family in *Brassica napus* L

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Abstract

Carotenoid cleavage dioxygenase (*CCD*), a key enzyme in carotenoid metabolism, cleaves carotenoids to form apo-carotenoids, which play a major role in plant growth and stress responses. *CCD* genes had not previously been systematically characterized in *Brassica napus* (rapeseed), an important oil crop worldwide. In this study, we identified 30 *BnCCD* genes and classified them into nine subgroups based on a phylogenetic analysis. We identified the chromosomal locations, gene structures, and *cis*-promoter elements of each of these genes and performed a selection pressure analysis to identify residues under selection. Furthermore, we determined the subcellular localization, physicochemical properties, and conserved protein motifs of the encoded proteins. All the *CCD* proteins contained a retinal pigment epithelial membrane protein (RPE65) domain. qRT-PCR analysis of expression of 20 representative *BnCCD* genes in 16 tissues of the *B. napus* cultivar Zhong Shuang 11 (‘ZS11’) revealed that members of the *BnCCD* gene family possess a broad range of expression patterns. This work lays the foundation for functional studies of the *BnCCD* gene family.

Introduction

“Carotenoids” is the general term for the class of natural pigments widely present in animals, plants, and microorganisms. Carotenoids are lipid-soluble isoprene-like compounds that contain 40 carbon molecules and comprise more than 750 pigments with different structures [1]. These pigments have numerous important biological functions; for example, they are photoprotective and are indispensable components of photosynthesis [2, 3]. They also can scavenge free radicals and have antioxidant properties [4]. Furthermore, carotenoids are components of cell membranes, potential anti-cancer agents, and can interact with proteins [5]. Carotenoids are exploited as coloring agents in flowers and fruits to attract pollinators and agents of seed dispersal [6–8]. In addition, carotenoids are precursors of important phytohormones, such as
abscisic acid and strigolactones, which regulate plant development and plant–environment interactions [9–11].

The carotenoid biosynthesis pathway in plants has been fully elucidated; the catalytic oxidative cracking of carotenoids is a key process in this pathway. Multiple conjugated double bonds exist within the carotenoid center chain, which can be specifically cleaved by carotenoid cleavage dioxygenases (CCDs) to form a variety of apo-carotenoids, and some apo-carotenoids can be further degraded into small biologically active molecules [12]. In plants, the addition of two oxygen atoms to the cleavage product means that CCDs possess the characteristics of a dioxygenase [13]. Moreover, CCDs are also a class of non-heme oxygenases, whose catalytic activity requires Fe²⁺ as a cofactor [14, 15]. CCD proteins contain four highly conserved histidine residues bound to Fe²⁺ and all contain a retinal pigment epithelial membrane protein (RPE65) domain that is characteristic of enzymes involved in carotenoid cleavage [16, 17].

In plants, CCD proteins are encoded by an ancient gene family. CCD gene family consists of two subfamilies: carotenoid cleavage dioxygenases (CCDs) and 9-cis epoxycarotenoid dioxygenases (NCEDs) [12]. Through analysis of a novel ABA-deficient mutant of maize, the first protein found to specifically cleave carotenoids, viviparous14 (VP14), was identified by Schwartz et al [14]. Vallabhaneni et al. described the characteristics of the CCD gene family in the grass species maize (Zea mays), rice (Oryza sativa), and sorghum (Sorghum bicolor) [18]. During water stress and seed dormancy, TaNCED of Triticum aestivum might play a primary role in regulation of ABA content [19]. Besides, drought stress can induce expression of NCED3 and accumulation of endogenous ABA in Nicotiana tabacum [20]. Wei et al. found at least seven CCD genes in Solanum lycopersicum genome sequence and analyzed their expression patterns [21]. CCD genes have also been identified or functionally expressed in a variety of other plant species, such as Glycine max [22], Gossypium hirsutum, Solanum tuberosum [23], Cucurbita pepo [24], Saccharum officinarum [25], Crocus sativus, Osmanthus fragrans [26], Vitis vinifera [27], Mangifera indica [28] and Amygdalus persica [29].

In Arabidopsis thaliana, the CCD gene family consists of nine members: four CCD genes (AtCCD1, 4, 7, and 8) and five NCED genes (AtNCED2, 3, 5, 6 and 9) [30]. Carotenoid cleavage dioxygenase homologs in other plant species are named according to the system used for Arabidopsis CCD family members.

The CCD1 and CCD4 enzymes catalyze a variety of carotenoids and produce volatile apo-carotenoids, which are important for the biosynthesis of aromas and flavors of flowers and fruits, respectively [31, 32]. The carotenoid content of mature seeds of AtCCD1 mutants was higher than that in the wild type, indicating a role for CCD1 in carotenoid catabolism [33]. CCD4 regulates carotenoid homeostasis and CmCCD4a is specifically expressed in white petals of Chrysanthemum morifolium [34]. RNAi-mediated inhibition of CmCCD4a expression in white-petaled plants results in the production of yellow flowers [35]. In line with this observation, loss or downregulation of CmCCD4a function led to an increase in carotenoid content in petals [12]. CCD7 and CCD8 function in strigolactone biosynthesis, regulate axillary bud growth, and inhibit branching [36, 37]. Using RNAi to reduce Actinidia chinensis CCD8 expression increases in branch development and delays leaf senescence [38].

The reaction catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) is a rate-limiting step in ABA biosynthesis, and thus influences plant tolerance to diverse abiotic stresses [39]. AtNCED5, AtNCED6 and AtNCED9 are the dominant contributors to developmentally regulated ABA synthesis in seeds and thereby regulate seed embryo maturation and dormancy [30]. Overexpression of OsNCED3 increased drought resistance in rice and caused an increased ABA level [40]. AtNCED2 and AtNCED3 transcripts are abundant in Arabidopsis roots and their encoded proteins function in abscisic acid biosynthesis and thereby regulate lateral root growth [30]. Furthermore, heterologous expression of Brassica napus NCED3 led
to ABA accumulation and NO and ROS generation in transgenic Arabidopsis plants, thereby enhancing abiotic stress tolerance [41].

*B. napus*, an important oilseed crop globally, is an allopolyploid derived from a natural interspecific cross between *Brassica rapa* (turnip; 2n = 2x = 20) and *Brassica oleracea* (kohlrabi; 2n = 2x = 18). The important physiological functions of carotenoid cleavage products in plants, such as abscisic acid and strigolactones, have prompted studies of the lyases involved in carotenoid metabolism. Little is known about the *BnCCD* gene family. The availability of the *B. napus* genome sequence would enable the identification and analysis of members of this family [42].

In this study, we identified 30 *BnCCD* genes. In addition to analyzing their gene evolution and structure, chromosomal localization, conserved motifs, and *cis*-acting promoter elements, we determined their tissue- and organ-specific expression and examined the physicochemical properties of their encoded proteins. The results form a solid basis for further studies on the biological functions of the *BnCCD* gene family.

**Materials and methods**

**Plant materials**

The *B. napus* cultivar Zhong Shuang 11 (‘ZS11’) was planted in Chongqing, China (29˚45′ N, 106˚22′ E). To analyze the expression patterns of *BnCCD* genes, four different tissues of ‘ZS11’ were harvested when plants were in full flower: mature leaves (Le), sepals (Se), flowers (F), and stems (St). The seeds (S) and silique pericarps (Sp) were harvested at different timepoints following the termination of flowering (25, 30, 35, 40, 45, and 50 days after flowering). Samples were immediately frozen in liquid nitrogen and stored at –80˚C for further use.

**Identification of CCD genes in Brassica napus, Brassica rapa, and Brassica oleracea**

The coding sequences of *AtCCD* genes were downloaded from TAIR (https://www.arabidopsis.org/) and used as reference sequences. The *BnCCD*, *BrCCD*, and *BoCCD* genes were identified through the BLASTN analysis [43] of *AtCCD* genes against the Brassica Database (BRAD, http://brassicadb.org/brad/index.php) [44]. The local database was established using Geneious 4.8.5 software (http://www.geneious.com/; Biomatters, Auckland, New Zealand). The genome-wide alignment was verified by the MEGABLAST program [45], and the following screening standards were used: the consistency of aligned sequences with the reference sequence was ≥80% and gene sequences <700 bp were discarded. Because enzymes involved in carotenoid cleavage contain a retinal pigment epithelial membrane protein (RPE65) domain [16, 17], gene sequences from the Pfam database [46] that did not contain this domain were excluded.

**Multiple sequence alignment and phylogenetic analysis**

CCD amino acid sequences were subjected to multiple sequence alignment using MUSCLE [47] with default parameters. The evolutionary relationships of *B. napus* CCD proteins with those of *A. thaliana*, *B. oleracea*, and *B. rapa* were analyzed using MEGA7 [45]. To identify the conserved blocks of all predicted sequences, the Gblocks program was used [48]. The substitution saturation was detected using DAMBE [49]. A phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA7. The number of bootstrap replications was 1,000, and paired deletion was performed. The phylogenetic tree was visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).
Protein properties, sequence analysis, and duplication time inference

The chromosomal location of BnCCD genes, which was queried from the Brassica napus genome browser, and the genes were mapped onto chromosomal linkage groups by Mapchart software [50]. The Gene Structure Display Server (GSD 2.0) (http://gsds.cbi.pku.edu.cn/index.php) was used to portray the exon–intron structures of the BnCCD genes. The ExPASy proteomics server database (http://expasy.org/) [51] was used to predict the relative molecular weight, theoretical isoelectric point, protein stability, and aliphatic amino acid content of BnCCD proteins. The conserved motifs were identified using the MEME Version 5.0.5 online tool (http://meme-suite.org/tools/meme) [52] and the maximum motif retrieval value was set to 20; other parameters used the default settings. Annotations of the identified motifs were obtained from InterProScan (www.ebi.ac.uk/Tools/InterProScan/) [53].

To determine whether the CCD protein-coding sequences are under selective pressure, the ratios of synonymous substitution rate (ks) and non-synonymous substitution rate (ka) of homologous gene pairs were calculated using TBtools software [54]. The approximate date of duplication events was inferred by substituting Ks values into the formula

\[ T = \frac{Ks}{2 \times 1.5 \times 10^{-8}} \times 10^{6} \text{ million years ago (MYA)} \] [55].

Predicted subcellular localization of BnCCD proteins and promoter analysis of BnCCD genes

The subcellular localization of BnCCD proteins were predicted using WoLF PSORT (http://www.genscript.com/tools/wolf-psort) [56]. The SOPMA secondary structure prediction method (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) was used to predict the secondary structure of BnCCD proteins and the presence of transmembrane helices within BnCCD proteins were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The promoter sequences (2.0-kb region immediately upstream of the translation start sites) of the BnCCD genes were obtained from the B. napus genome database (http://www.genoscope.cns.fr/brassicanapus/) [57]. PlantCARE (Error! Hyperlink reference not valid.webtools/plantcare/html/) [58] was used to predict the presence of cis-acting sequences within each promoter.

RNA-seq analysis

To analyze the tissue-specific expression of the BnCCD genes, publically available B. napus RNA-sequencing (RNA-seq) data PRJNA358784 (BioProject) were downloaded from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) [59]. All BnCCD genes expression levels were quantified in terms of FPKM (fragments per kilobase of exon per million mapped fragments) using Cufflinks with default parameters [60], and then extracted from RNA-seq data according to their B. napus code, including expression data from thirteen different organs (roots, stems, leaves, buds, anthocauli, calyxes, petals, pistils, stamens, anthers, capillaments, seeds and silique pericarps) at different developmental stages in B. napus cultivar ZS11. The heatmap for BnCCD genes was constructed using TBtools software.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using the RNeasy Extraction Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Contaminating genomic DNA was removed with DNase I. Total RNA (1 μg) was used to synthesize cDNA by reverse transcriptase (TaKaRa). The gene-specific primer pairs used to analyze BnCCD genes expression by
qRT-PCR were designed using Primer Premier 5 [61] and the ACTIN 7 gene was used as an endogenous reference gene. All the primers were listed in S1 Table.

Quantitative RT-PCR was carried out using TB Green Premix Ex Taq on a Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The reaction mixture included 10 μL TB Green Premix Ex Taq, 0.5 μL forward primer, 0.5 μL reverse primer, 2 μL cDNA template, and 7 μL nuclease-free H2O in a total volume of 20 μL. The PCR cycling conditions were as follows: 95˚C for 30s, followed by 40 cycles of 95˚C for 5 s and 60˚C for 30 s. Following PCR amplification, the dissolution curve was analyzed to ensure the specificity of the amplified products. Three biological replicates and three technical replicates were performed for each reaction. The relative gene expression levels were calculated using the 2^−ΔΔCt method [61]. All the results were plotted as the mean ± standard error of mean (SEM) from three independent biological replicates using Graph Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results
Identification and characterization of BnCCD genes
Using the nine AtCCD coding sequences as a BLASTN query, we identified 30 B. napus CCD genes (BnCCDs), 16 originating from B. rapa (BrCCDs) and 14 from B. oleracea (BoCCDs)(S2 Table). All the genes contained sequences encoding the RPE65 domain. RPE65 belongs to a family of carotenoid oxygenases in plant, bacterial, and animal systems that typically oxidatively cleave conjugated double bonds in the polyene backbone of carotenoids [16, 17]. In addition to light related functions, the function and mechanism of RPE65 in plants have not been elucidated. To determine the evolutionary relationships among 60 CCD proteins, we constructed a phylogenetic tree by the neighbor-joining method. Based on the topology of the phylogenetic tree, CCD proteins could be grouped into nine distinct subgroups (I, II, III, IV, V, VI, VII, VIII and IX), each of which contained one of the AtCCD proteins and the corresponding subgroup members of B. rapa, B. oleracea, and B. napus (Fig 1).

The physical and chemical properties of the BnCCD proteins are summarized in Table 1. The length of BnCCD proteins ranged from 255 amino acids (aa) (BnNCED3f) to 668 aa (BnCCD7a), with a mean length of 551 aa. The predicted molecular weights varied from 28.65 kDa (BnNCED3f) to 74.79 kDa (BnCCD7a) and the theoretical isoelectric point (pI) ranged from 4.75 (BnNCED3f) to 8.83 (BnNCED3e). Out of the 30 BnCCD proteins, 27 had pI values of less than 7, and the values of the remaining three BnCCD proteins were greater than 7. Based on the hydrophilicity index of amphoteric proteins between –0.5 ~ +0.5 (a negative GRAVY value indicates hydrophilicity and a positive value indicates hydrophobicity), all BnCCD proteins are amphiphilic proteins.

Chromosomal localization and gene structure
By Mapchart, an analysis of the chromosomal distribution of the BnCCD loci showed that these genes were not evenly distributed across the chromosomes (Fig 2). The 30 BnCCD loci are distributed on 13 chromosomes in B. napus; the 14 BnCCDs are located on the A subgenome and the 16 BnCCDs are located on the C subgenome. Chromosomes A01 and C01 possess the most BnCCD loci, each containing four BnCCDs. Chromosomes A03, A04, A05, A07, A08, C06, A03-random, A07-random, and Ann-random each contain only one BnCCD locus, whereas chromosomes A09, C04, C07, C08, and Cnn-random each contain two BnCCD loci. BnNCED3e, BnNCED3f, and BnNCED3g (subgroup III) are located on chromosome C05. The positions of exons and introns in members of a gene family might have played crucial roles during evolution [62]. Therefore, we next analyzed the gene structure (exon–intron organization) of the 30 BnCCDs (Fig 3). Genes with similar structures were closely related.
BnNCED2, BnNCED5, BnNCED6, and BnNCED9 subgroups were intron-less. The BnCCD1 subgroup contained 13–15 exons; two genes contained 13 exons, two genes contained 14 exons, and only BnCCD1e possessed 15 exons. All BnCCD4 subgroup members contained one exon, except for BnCCD4b, which contained three. BnCCD8b contained 6 introns, one more than BnCCD8a.

Conserved motifs and protein profiles of BnCCD proteins

Twenty putative BnCCD protein motifs were predicted by the MEME program (Fig 4). The BnNCED2, NCED5, NCED6, and NCED9 proteins all contain motifs 1–17. Except for BnNCED3e, all other BnNCED3 proteins contain motifs 2, 6, 7, 10, 12, 16, and 17. Motifs 1, 2,
Table 1. The genes and encoded protein features of the 30 BnCCDs identified in this study.

| Gene name       | Gene ID          | Subgroup | Chromosome | Gene length (bp) | Gene position | Number of exons | Protein length (aa) | Molecular wt. (kDa) | pI | GRAVY | Subcellular location |
|-----------------|------------------|----------|------------|-----------------|---------------|-----------------|---------------------|---------------------|----|-------|---------------------|
| BnCCD1a         | BnaA09g41150D    | I        | A09        | 4466            | 28783045      | 13              | 483                 | 55.24182            | 6.36| −0.175| chlo: 5, cyto: 2, vacu: 2, E.R.: 2, nucl: 1, pero: 1 |
| BnCCD1b         | BnaA09g12520D    | I        | Ann_random | 3193            | 13541287      | 13              | 525                 | 59.31523            | 5.70| −0.256| cyto: 7, chlo: 3, nucl: 1, mito: 1, plas: 1 |
| BnCCD1c         | BnaC04g20610D    | I        | C04        | 3267            | 21697508      | 14              | 525                 | 59.35633            | 5.94| −0.257| cyto: 10, chlo: 2, plas: 1 |
| BnCCD1d         | BnaC08g33680D    | I        | C08        | 8770            | 32058916      | 14              | 612                 | 69.62388            | 6.95| −0.367| chlo: 2, mito: 3, nucl: 1 |
| BnCCD1e         | BnaC08g33690D    | I        | C08        | 7652            | 32077226      | 15              | 611                 | 69.52093            | 6.41| −0.289| chlo: 14 |
| BnCCD2a         | BnaA01g09090D    | II       | A01        | 1746            | 4447853       | 1               | 581                 | 64.52248            | 5.33| −0.224| chlo: 6, cyto: 5, plas: 1, cyk_plas: 1.5 |
| BnCCD2b         | BnaA03g58230D    | II       | A03_random | 1752            | 1600131       | 1               | 583                 | 64.95409            | 5.70| −0.237| chlo: 6, cyto: 4 |
| BnCCD2c         | BnaA03g58270D    | II       | C01        | 1746            | 6588592       | 1               | 581                 | 64.51950            | 5.31| −0.214| chlo: 6, cyto: 4 |
| BnCCD2d         | BnaC07g35240D    | II       | C07        | 1752            | 37711814      | 1               | 583                 | 64.82201            | 5.55| −0.243| chlo: 7, cyto: 4, plas: 1.5, cyk_plas: 1.5 |
| BnCCD2e         | BnaA01g29390D    | III      | A01        | 1662            | 20329204      | 2               | 553                 | 60.99077            | 5.28| −0.287| mito: 10, chlo: 4 |
| BnCCD2f         | BnaA03g33390D    | III      | A03        | 1194            | 16162114      | 2               | 396                 | 44.24510            | 4.99| −0.242| cyto: 6, cyk: 6, mito: 1 |
| BnNCED2a        | BnaA05g25030D    | III      | A05        | 580            | 18611263      | 1               | 597                 | 65.80847            | 5.94| −0.297| chlo: 12, mito: 1.5, cyk_mito: 1.5 |
| BnNCED2b        | BnaA08g09110D    | III      | C01        | 1797            | 36059468      | 1               | 598                 | 65.72044            | 5.89| −0.276| chlo: 8, cyto: 6 |
| BnNCED2c        | BnaC07g35210D    | III      | C07        | 1752            | 37775359     | 1               | 315                 | 34.06851            | 8.83| −0.291| mito: 11, chlo: 3 |
| BnNCED3a        | BnaA01g29390D    | III      | A01        | 1662            | 20329204      | 2               | 553                 | 60.99077            | 5.28| −0.287| mito: 10, chlo: 4 |
| BnNCED3b        | BnaA03g58230D    | III      | A03        | 1194            | 16162114      | 2               | 396                 | 44.24510            | 4.99| −0.242| cyto: 6, cyk: 6, mito: 1 |
| BnNCED3c        | BnaC07g35210D    | III      | A05        | 580            | 18611263      | 1               | 597                 | 65.80847            | 5.94| −0.297| chlo: 8, cyto: 6 |
| BnNCED3d        | BnaA08g09110D    | III      | C01        | 1797            | 36059468      | 1               | 598                 | 65.72044            | 5.89| −0.276| chlo: 8, cyto: 6 |
| BnNCED3e        | BnaC07g35210D    | III      | C07        | 1752            | 37775359     | 1               | 315                 | 34.06851            | 8.83| −0.291| mito: 11, chlo: 3 |
| BnNCED3f        | BnaA01g29390D    | III      | A01        | 1662            | 20329204      | 2               | 553                 | 60.99077            | 5.28| −0.287| mito: 10, chlo: 4 |
| BnNCED5a        | BnaA09g26450D    | V        | A09        | 1170            | 19605914      | 1               | 589                 | 65.33387            | 5.37| −0.324| chlo: 11, nucl: 2 |
| BnNCED5b        | BnaA04g26000D    | V        | Cnn_random | 1170            | 37143600      | 1               | 589                 | 65.45993            | 5.42| −0.320| chlo: 11, nucl: 1, mito: 1 |
| BnNCED6a        | BnaB07g06050D    | VI       | A07        | 1758            | 6379220       | 1               | 585                 | 65.00222            | 6.07| −0.321| chlo: 7.5, chlo_mito: 6.5, mito: 4.5 |
| BnNCED6b        | BnaC07g07580D    | VI       | C07        | 1758            | 11978406      | 1               | 585                 | 65.06919            | 6.00| −0.329| pero: 8, chlo: 3, cyto: 2 |
| BnCCD7a         | BnaB04g26000D    | VII      | A04        | 5803            | 18550548      | 10              | 668                 | 74.78965            | 5.77| −0.381| chlo: 9, plas: 3, mito: 1 |
| BnCCD7b         | BnaC04g50070D    | VII      | C04        | 3912            | 47874505      | 9               | 648                 | 72.69485            | 6.19| −0.303| chlo: 8, plas: 3, mito: 1, vacu: 1 |

(Continued)
7, and 15 are present in all BnCCD1, BnCCD4, and BnCCD8 proteins and all BnCCD7 members contain motifs 1, 4, 10, 12, 15, 18, and 19. Using the InterProScan program, we searched

### Table 1. (Continued)

| Gene name   | Gene ID                | Subgroup | Chromosome | Gene length (bp) | Gene position Start | Gene position End | Number of exons | Protein length (aa) | Molecular wt. (kDa) | pI | GRAVY | Subcellular location |
|-------------|------------------------|----------|------------|------------------|---------------------|-------------------|-------------------|---------------------|---------------------|----|-------|---------------------|
| BnCCD8a     | BnaA01g04140D          | VIII     | A01        | 3287             | 1908477            | 1911763           | 6                 | 569                 | 63.98902            | 7.13 | −0.341 | chlo: 10, mito: 3   |
| BnCCD8b     | BnaC01g05600D          | VIII     | C01        | 12036            | 2963042            | 2975077           | 7                 | 574                 | 64.98737            | 8.50 | −0.452 | chlo: 7, cyto: 4,   |
|             |                        |          |            |                  |                     |                   |                   |                     |                     |     |        | nucl: 2             |
| BnNCED9a    | BnaA07g39250D          | IX       | A07_random | 1809             | 2059180            | 2060988           | 1                 | 602                 | 66.75458            | 5.90 | −0.299 | chlo: 14             |
| BnNCED9b    | BnaC06g38870D          | IX       | C06        | 1809             | 36264799           | 36266607          | 1                 | 602                 | 66.79169            | 6.11 | −0.281 | chlo: 14             |

chlo: chloroplast; cyto: cytoplasmic; vacu: vacuolar; E.R: Endoplasmic Reticulum; pero: peroxisomal; mito: mitochondria; nucl: nucleus; plas: plasma membrane.

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Fig 2. Genomic distribution of BnCCD genes. The chromosomal location of each BnCCD locus was mapped to the *B. napus* genome: the chromosome number is indicated above each chromosome. The scale is in megabases (Mb). Ann and Cnn are pseudo-molecule chromosomes. Random means that the specific location of the gene is unknown.

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for annotations for the conserved motifs. Motifs 1–9 and 19 were associated with carotenoid oxygenase (IPR004294) and the remaining motifs had not been annotated.

Prediction of secondary structure using SOPMA showed that the main structure of the BnCCD proteins was a random coil. In addition to BnNCED3e, the alpha helix was more than the extension chain. The alpha helix was equal to the extension chain in BnCCD7a. The secondary structure characteristics of other BnCCD proteins ranged from more to less: random curl, extended strand, alpha helix, beta turn. Analysis using the TMHMM Server v. 2.0 showed that no BnCCD proteins possessed a transmembrane domain.

Nineteen of the 30 BnCCD proteins were predicted to be localized to the chloroplast, five to the cytoplasm, and five to the mitochondrion. BnNCED6b was predicted to be localized to the peroxisome.

**Ka and Ks calculation for orthologous CCD genes between Brassica napus and Arabidopsis thaliana**

To determine whether the CCD protein-coding genes in *B. napus* and *A. thaliana* are under selective pressure, the Ka/Ks ratios for 30 pairs of orthologous genes were calculated using TBtools software (Table 2). The Ka/Ks ratios of all the gene pairs were considerably lower than...
1, indicating that the CCD gene family has undergone purifying selection. The $K_s$ values for $B. napus$ relative to $A. thaliana$ ranged from 0.28852 to 0.65433, suggesting that gene duplications occurred approximately 9.62–21.81 million years ago (MYA).

The *cis*-acting elements predicted to be present in *BnCCD* promoters

The *cis*-acting elements present in the promoters are essential for transcriptional gene regulation. In total, 87 types of *cis*-acting elements were predicted to be present in 30 *BnCCD* promoters (S3 Table), using the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). In addition to CAAT and TATA boxes, each promoter contained more than 10 *cis*-acting elements. Among these, 26 different elements are associated with responses to light, with 206 occurrences across all promoters. The remaining elements are associated with hormonal and stress responses and tissue-specific expression. Hormone-responsive *cis*-elements account for a large proportion of the total and include ABRE (abscisic acid response element), AuxRR-core and TGA-elements (auxin response), ERE (ethylene-response element), TCA-element (salicylic acid response), P-box and GARE-motif (gibberel-lin-response), and the CGTCA and TGACG motifs (MeJA-response). Notably, all *BnCCDs* contain ABRE, CGTCA, and TGACG motifs, suggesting that this gene family functions in hormone response pathways. Additional *cis*-elements related to stress response were identified; for example, LTR (low-temperature responsiveness) is present in all *BnCCD* promoters except
for BnNCED3f and BnCCD8a. Additional elements include the drought-inducible MBS element, TC-rich repeats, a cis-acting element involved in defense and stress responsiveness, and the WUN-motif, a wound-responsive element.

Some cis-acting elements in BnCCD promoters might determine the tissue-specific expression pattern, such as the CAT-box, which is associated with meristematic expression; GCN4 motif, involved in endosperm expression; HD-Zip 1, involved in differentiation of the palisade mesophyll cells; and RY-element, which confers seed-specific expression.

**RNA-seq analysis**

We analyzed the expression of 30 BnCCDs at different developmental stages and in various tissues from the B. napus cultivar ZS11 and constructed an expression heat map (Fig 5). Whereas no BnNCED3f and BnNCED3g transcripts were detected in any tissue, the remaining 28 BnCCDs showed tissue- and development-specific expression levels. BnCCD1b and BnCCD1c were highly expressed in leaves, stems, buds, flowers, seeds, and siliques pericarp, but expression was relatively low in roots. BnNCED9a and BnNCED9b had similar expression patterns.

**Table 2. The non-synonymous (Ka) and synonymous substitution rate (Ks) for orthologous CCD gene pairs between B. napus and A. thaliana.**

| Orthologous gene pairs | Ka    | Ks    | Ka/Ks | Duplication date (MYA) |
|------------------------|-------|-------|-------|------------------------|
| AtCCD1 BnCCD1a         | 0.08151 | 0.36209 | 0.22510 | 12.07                  |
| AtCCD1 BnCCD1b         | 0.04122 | 0.32012 | 0.12875 | 10.67                  |
| AtCCD1 BnCCD1c         | 0.03384 | 0.28852 | 0.11730 | 9.62                   |
| AtCCD1 BnCCD1d         | 0.05913 | 0.38774 | 0.15250 | 12.92                  |
| AtCCD1 BnCCD1e         | 0.07611 | 0.35530 | 0.21422 | 11.84                  |
| AtNCED2 BnNCED2a       | 0.06843 | 0.46502 | 0.14716 | 15.50                  |
| AtNCED2 BnNCED2b       | 0.04285 | 0.41304 | 0.10374 | 13.77                  |
| AtNCED2 BnNCED2c       | 0.06108 | 0.47780 | 0.12784 | 15.93                  |
| AtNCED2 BnNCED2d       | 0.04606 | 0.43371 | 0.10620 | 14.46                  |
| AtNCED3 BnNCED3a       | 0.04945 | 0.56001 | 0.08829 | 18.67                  |
| AtNCED3 BnNCED3b       | 0.05042 | 0.55742 | 0.09045 | 18.58                  |
| AtNCED3 BnNCED3c       | 0.05052 | 0.51718 | 0.09768 | 17.24                  |
| AtNCED3 BnNCED3d       | 0.04962 | 0.52187 | 0.09509 | 17.40                  |
| AtNCED3 BnNCED3e       | 0.05472 | 0.45159 | 0.12116 | 15.05                  |
| AtNCED3 BnNCED3f       | 0.05271 | 0.58288 | 0.09043 | 19.43                  |
| AtNCED3 BnNCED3g       | 0.04581 | 0.51353 | 0.08921 | 17.12                  |
| AtNCED3 BnNCED3h       | 0.05693 | 0.55439 | 0.10268 | 18.48                  |
| AtCCD4 BnCCD4a         | 0.07819 | 0.59005 | 0.13252 | 19.67                  |
| AtCCD4 BnCCD4b         | 0.06912 | 0.50381 | 0.13720 | 16.79                  |
| AtCCD4 BnCCD4c         | 0.07461 | 0.60924 | 0.12246 | 20.31                  |
| AtNCED5 BnNCED5a       | 0.04001 | 0.38163 | 0.10484 | 12.72                  |
| AtNCED5 BnNCED5b       | 0.03847 | 0.40056 | 0.09605 | 13.35                  |
| AtNCED6 BnNCED6a       | 0.08570 | 0.57248 | 0.14970 | 19.08                  |
| AtNCED6 BnNCED6b       | 0.09078 | 0.57371 | 0.15824 | 19.12                  |
| AtCCD7 BnCCD7a         | 0.07106 | 0.45309 | 0.15683 | 15.10                  |
| AtCCD7 BnCCD7b         | 0.07166 | 0.42570 | 0.16834 | 14.19                  |
| AtCCD8 BnCCD8a         | 0.05711 | 0.42701 | 0.13374 | 14.23                  |
| AtCCD8 BnCCD8b         | 0.09405 | 0.49007 | 0.19191 | 16.34                  |
| AtNCED9 BnNCED9a       | 0.07262 | 0.61567 | 0.11796 | 20.52                  |
| AtNCED9 BnNCED9b       | 0.06799 | 0.65433 | 0.10391 | 21.81                  |

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and were highly expressed in seeds. Genes belonging to the same subgroup had a wide range of expression patterns: *BnCCD4a* and *BnCCD4c* were highly expressed in leaves, stems, and pericarps; however, *BnCCD4b*, which belongs to the *BnCCD4* subgroup, had a low expression level in leaves, stems, and pericarps but was highly expressed in anthers, suggesting that it might function in stamen growth and development.

Expression profiling of *BnCCDs* in different organs

To gain insight into the biological functions of *BnCCDs*, we analyzed their expression patterns in flowers, leaves, sepals, stems, seeds, and siliques pericarps, and for seeds and siliques pericarps also at different developmental stages (25, 30, 35, 40, 45, and 50 days after flowering) by qRT-PCR. Several *BnCCDs*, including *BnCCD1b*, *BnCCD1c*, *BnCCD1d*, *BnNCED2a*, *BnNCED2c*, *BnNCED3a*, *BnNCED3c*, *BnNCED3d*, *BnCCD4a*, *BnCCD4c*, *BnNCED5a*, *BnNCED5b*, *BnNCED6a*, *BnNCED6b*, *BnCCD7a*, *BnCCD7b*, *BnCCD8a*, *BnCCD8b*, *BnNCED9a*, and *BnNCED9b*, had diverse expression patterns in different tissues (Fig 6).

Except for *BnCCD8b*, the expression level of *CCD* genes was low in the stem. *BnNCED5b* and *BnCCD8* transcripts were abundant in flowers, *BnNCED5a* and *BnNCED5b* transcripts were abundant in sepals, and *BnNCED9a* and *BnNCED9b* transcripts were abundant in seeds. However, the expression patterns of some genes within the same family differed: the transcript levels of *BnCCD1b* and *BnCCD1c* in leaves were higher than those in other tissues, whereas *BnCCD1d* was most highly expressed in sepals. In addition, *BnNCED2a* and *BnNCED2c* transcript levels were higher in seeds at 40–50 days after flowering (40S, 45S, and 50S) than at 25–35 days after flowering (25S, 30S and 35S). *BnCCD4a* and *BnCCD4c* transcript levels were

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**Fig 5. Heatmap of the expression patterns of selected *BnCCD* genes in different tissues and at different developmental stages.** The expression data were obtained from publically available RNA-seq data. Ro: Roots; St: Stems; Le: Leaves; LeY: Young leaves; LeO: Old leaves; Bu: Buds; Ao: Anthocauli; Ca: Calyxes; Pe: Petals; Pi: Pistils; Sta: Stamens; At: Anthers; Cap: Capillaments; Se: Seeds; Sp: Siliques; _s: At seedling stage; _b: In the bud stage; _f: At the initial flowering stage; _3d: At the flourishing flowering stage; _3d: 3 days after flowering; _7,10,13,21,24,27,30,35,43d: 7, 10, 13, 21, 24, 27, 30, 35, 43 days after flowering; Ro_s: Roots at seedling stage.

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abundant in silique pericarps collected at the 25 days after flowering (25Sp), 35Sp, and 45Sp stages, but were present at low levels at the 30Sp, 40Sp, 45Sp and 50Sp stages.

**Discussion**

Arabidopsis contains nine CCD genes. Brassicaceae, including Arabidopsis, underwent an ancient α, β and γ whole-genome duplication polyploidization events, and also experienced an
additional recent genome-wide tripling (WGT) event [63]. Because it is an allotetraploid formed by interspecific hybridization between B. rapa and B. oleracea, B. napus would be expected to contain six copies of each Arabidopsis thaliana gene (i.e., 54) [64]. Because of genome shrinkage and gene loss, the number of BnCCDs identified in this study was a little lower (i.e., 30), with only 2–6 copies of each Arabidopsis gene present. The B. rapa and B. oleracea genomes contain 16 BrCCD and 14 BoCCD genes, respectively, and the number of BnCCDs is the sum of these. However, only two members of the BnNCED9 subgroup and three of the BnCCD4 subgroup were identified in this study, whereas two members were found in the corresponding subgroups of both B. rapa and B. oleracea, indicating that gene loss might have occurred among subgroups BnCCD4 and BnNCED9.

The molecular characteristics of all BnCCD protein members were found to differ, but proteins in the same subgroup had similar molecular weights and isoelectric points. The position of exon–intron boundaries reflects the evolution of these genes [62]. Subcellular localization predictions suggested that BnCCD proteins are localized to the chloroplast, mitochondrion, cytoplasm, and peroxisome. BnCCD1b, BnCCD1c, BnNCED3b, BnNCED3f, and BnNCED3h were predicted to be cytoplasmic, suggesting that BnCCD1 and BnNCED3 might interact in the cytoplasm. It also showed that these genes may not participate in chlorophyll photosynthesis, which was consistent with the research by Zhang et al. [28]. BnNCED2 subgroup is located in the chloroplast. Studies have shown that histidine residues in NCED amino acids can bind Fe $^{2+}$ to make NCED proteins function [65], and there is a chloroplast transit peptide structure in them [66]. Wang et al. [67] found that NCED2 protein in Camellia sinensis is localized in chloroplast and has N-terminal chloroplast targeting signal peptide sequences, which further proves that GsNCED2 has the biological activity of cleaving epoxy carotenoids to generate ABA precursors in plastids. Besides, in both sugarcane and rice, CCD8 are proteins located in chloroplast [25, 68]. Through online analysis we found that BnCCD8 is also localized in the chloroplast.

Seven cis-acting elements associated with various stress responses were predicted among the promoters of the 30 BnCCDs; LTR, MBS, TC-rich repeats, and nine cis-acting elements were associated with responses to abscisic acid, MeJA, salicylic acid, and auxin. CCD1, 4, and 8 and NCED2 and 9 are transcriptionally upregulated following treatment of B. rapa with the phytohormones ABA and SL [16]. Furthermore, Nicotiana tabacum plants that heterologously expressed NCED1 of Styllosanthes guianensis had increased tolerance to light, oxidative, drought, salt, and cold stress, indicating that CCD might regulate plant tolerance against these abiotic stresses [69]. The G-Box, GT1, and ACE motifs, which are transcriptionally responsive to light, are present in the BnCCD gene promoters. In addition, BnCCD promoters contain circadian response elements, RY-elements, and MSA-like and MBSI elements, indicating that most CCD genes function in plant growth and development and stress responses.

Based on RNA-seq data (S4 Table), we constructed an expression heatmap, which demonstrated that the 30 BnCCDs were differentially expressed in different B. napus tissues. Members of the same gene subgroup occasionally exhibited different expression patterns, such as BnCCD1 and BnCCD4, potentially reflecting new functionalization among the gene family during evolution. Genes of the CCD1 and CCD4 subgroups function in the formation of a variety of apo-carotenoids, which confer unique colors, tastes, and aromas [70–72]. A CCD1 loss-of-function mutant showed a decreased level of β-ionone in tomato fruit (Solanum lycopersicum) [31] and petunia flowers (Petunia hybrida) [73]. Carotenoid homeostasis is regulated by CCD4 in different tissues, such as Arabidopsis seeds [74] and potato tubers [23]. BnCCD4b was mainly expressed in stamens, suggesting that it might function in stamen growth and development. Species such as Solanum lycopersicum, Prunus persica, and Crocus, were also reported to preferentially express CCD4 in floral organs, indicating that the evolution of CCD4 genes...
might have been adaptive and have enhanced specific physiological traits unique to flowering plants [21, 75, 76]. CCD4 with normal function or lack of function will change the color of fruit and flower organs. Previous studies have suggested CCD4 gene can fade the yellow petals of Rhododendron japonicum and Eustoma grandiflorum [77, 78], because its ability of cleaving carotenoids. Inactivation of CCD4 will change the color of B.napus and Chrysanthemum morifolium petals from white to yellow [79, 80]. Controlling the expression of CCD gene family provides a way to change the color of plant petals.

**Conclusion**

In conclusion, we performed a comprehensive study of CCD gene family in B.napus. We identified 30 putative BnCCDs that were classed into nine subgroups (I-IX) on the basis of their phylogenetic relationships. The length of the BnCCD proteins ranged from 255 to 668 aa, and the Ks values for B. napus relative to A. thaliana ranged from 0.28852 to 0.65433. In addition, RNA-seq data and qRT-PCR analysis revealed that BnCCDs were differentially expressed in different tissues and organs, and had tissue/organ specificity and expression preference, suggesting that BnCCDs had clear function differentiation. Our results will help lay the foundation for the functional characterization of the CCD gene family and better understand the structural and functional relationships among these family members.

**Supporting information**

S1 Table. qRT-PCR primers used to analyze BnCCDs expression.
(XLSX)

S2 Table. List of identified CCD genes in B.napus, B.rapa, B.oleracea and A. thaliana.
(XLSX)

S3 Table. Cis-acting elements in the promoter regions of BnCCDs.
(XLSX)

S4 Table. RNA-seq data of 30 BnCCDs.
(XLSX)

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