

Article

Cloning and Analysis of Expression of Genes Related to Carotenoid Metabolism in Different Fruit Color Mutants of Pepper (*Capsicum annuum* L.)

Penglong Feng [†], Yayi Wang [†], Junqin Wen, Yanjing Ren , Qiwen Zhong ^{*} and Quanhui Li

Academy of Agricultural and Forestry Sciences, Qinghai University/Laboratory for Research and Utilization of Qinghai Tibet Plateau Germplasm Resources, Xining 810016, China; fengpenglong@126.com (P.F.); 2010990020@qhu.edu.cn (Y.W.); 2021990055@qhu.edu.cn (J.W.); 2018990082@qhu.edu.cn (Y.R.); liquanhui_2008@163.com (Q.L.)

^{*} Correspondence: 13997135755@163.com

[†] These authors contributed equally to this work.

Abstract: The formation of fruit color in pepper is closely related to the processes of carotenoid metabolism. In this study, red wild-type pepper XHB, SP01, PC01 and their corresponding mutants H0809 (orange), SP02 (yellow), and PC02 (orange) were used as research materials. The *Ggps*, *Psy*, *Lcyb*, *Crtz*, *Zep*, and *Ccs* genes involved in carotenoid biosynthesis were cloned, and bioinformatics and expression analyses were carried out. The results showed that the full lengths of the six genes were 1110 bp, 2844 bp, 1497 bp, 2025 bp, 510 bp, and 1497 bp, and they encoded 369, 419, 498, 315, 169, and 498 amino acids, respectively. Except for the full-length *Ccs* gene, which could not be amplified in the yellow mutant SP02 and the orange mutant PC02, the complete full-length sequences of the other genes could be amplified in different materials, indicating that the formation of fruit color in the SP02 and PC02 mutants could be closely related to the deletion or mutation of the *Ccs* gene. The analytical results of real-time quantitative reverse transcription PCR (qRT-PCR) showed that the *Ggps*, *Psy*, *Lcyb*, *Crtz*, and *Zep* genes were expressed at different developmental stages of three pairs of mature-fruit-colored materials, but their patterns of expression were not consistent. The orange mutant H0809 could be amplified to the full *Ccs* gene sequence, but its expression was maintained at a lower level. It showed a significant difference in expression compared with the wild-type XHB, indicating that the formation of orange mutant H0809 fruit color could be closely related to the different regulatory pattern of *Ccs* expression. The results provide a theoretical basis for in-depth understanding of the molecular regulatory mechanism of the formation of color in pepper fruit.

Keywords: pepper; ripening; carotenoids; gene cloning; expression analyses



Citation: Feng, P.; Wang, Y.; Wen, J.; Ren, Y.; Zhong, Q.; Li, Q. Cloning and Analysis of Expression of Genes Related to Carotenoid Metabolism in Different Fruit Color Mutants of Pepper (*Capsicum annuum* L.). *Genes* **2024**, *15*, 315. <https://doi.org/10.3390/genes15030315>

Academic Editors: Georgia Ntatsi, Umesh K. Reddy and Padma Nimmakayala

Received: 2 February 2024

Revised: 21 February 2024

Accepted: 26 February 2024

Published: 28 February 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Pepper (*Capsicum annuum* L.) is one of the most widely cultivated vegetable crops worldwide. It is popular among many consumers because its fruit is colorful and has many nutrients, as well as special flavor substances. The pepper fruit ranges in color from immature to mature, including a series of rich fruit colors, such as green, milky white, purple, yellow, and red, among others, which is one of the important commodity traits of pepper. The color of pepper fruit is determined by the types and levels of chlorophyll, carotenoids, and anthocyanins in the pulp cells [1,2]. Anthocyanins and chlorophyll are the primary pigments in immature pepper fruits [3], while carotenoids are the primary pigments in mature pepper fruits [4]. With the development of pepper fruit to physiological maturity, the orange and red carotenoids continue to accumulate, and the chlorophyll and anthocyanins gradually degrade [5]. Red pepper fruit typically contains capsanthin, β -cryptoflavin, β -carotene, capsorubin, zeaxanthin, antheraxanthin, and α -carotene [4,6]. Yellow pepper fruit contains violaxanthin, lutein, α -carotene, β -carotene, zeaxanthin, antheraxanthin,

and β -cryptoxanthin, while orange pepper fruit contains capsorubin, β -cryptoflavin, zeaxanthin, violaxanthin, α -carotene, β -carotene, and zeaxanthin [5,7]. The total carotenoid content of red pepper is higher than that of yellow and orange peppers [8,9].

The formation of pepper fruit color is regulated by a series of loci. In immature fruit, purple pepper is controlled by a single dominant gene *A* (Anthocyanin) [10]. The *A* gene is homologous with the *Petunia An2* (Anthocyanin2) gene, which is the R2R3 MYB transcription factor gene involved in regulating the biosynthesis of anthocyanin [11]. Milk white and green pepper fruits are controlled by three loci, including *sw1*, *sw2*, and *sw3* [12]. Currently, *APRR2-Like* [13], *CaGLK2* [14], and *CcLOL1* [15] have been identified as genes, which control the color changes in green pepper fruit. In mature pepper fruit, three independent genetic loci (*y*, *c1*, and *c2*) determine the red, yellow, orange, and white color of pepper fruit, respectively [16]. Brown pepper fruit is determined by the *cl* gene [3]. Popovsky [17] confirmed that the color of red fruit is related to the *Ccs* gene, and the *y* locus and *Ccs* gene co-segregate, indicating the unity of the two genes. However, owing to the fact that the red fruit contains capsanthin and capsorubin, the *y* locus is considered to be the gene locus, which controls its synthesis [18,19]. The gene of phytoene synthetase (*Psy*) was found to be co-segregated from the F₂ population phenotype. It was hypothesized that the *c2* locus controlling the formation of red pepper fruit could be the *Psy* gene, and the gene was located on chromosome 4 [19,20]. The *cl* locus has been determined to be related to the *CaSGR* gene. Typically, the chlorophyll content decreases sharply during the ripening process of pepper fruit with the increases in carotenoid synthesis owing to the transformation of chloroplasts into chromatosomes. However, it has been observed that mutations in the *CaSGR* gene can inhibit the degradation of chlorophyll during the fruit ripening process. In summary, the pepper fruit will be brown (or green) owing to the levels of chlorophyll and carotenoids in the various components [3,21].

Previously, the regulation of biosynthesis of pepper carotenoids has been examined primarily from the viewpoints of structures, the level of expression, and protein products of crucial genes in the carotenoid metabolism pathway. The goal has been to accurately reveal the genetic regulatory mechanism of color changes in mature pepper fruit. Many previous studies have shown that genes such as *Psy* [22], *Crtz* (β -carotene hydroxylase) [23], and *Ccs* (capsaicin synthase) [18] have richly varied structures [24,25]. These variations include gene deletions and mutations in pepper materials with different fruit colors, which lead to the formation of differently colored fruits. Tian [26] used a virus-induced gene silencing (VIGS) method to silence the *Ccs*, *Psy*, *Lcyb* (Lycopene β cyclase), and *Crtz* genes in pepper. The results revealed that the fruit changed from red to yellow or orange. In addition, fruit coloring was observed to be abnormal when multiple genes were silenced simultaneously. After single gene or multiple gene silencing, the levels of capsanthin decreased significantly. In addition, its synthetic substrates, such as β -carotene, β -cryptoxanthin, and zeaxanthin, were also reduced to varying degrees. Kilcrease et al. [25] reported that the increases in total carotenoid content correlated with increases in β -carotene and violaxanthin. The levels of expression of *Psy* and *Crtz-2* were determined to be associated with specific carotenoid contents.

In summary, it has been found that the formation of pepper fruit color has a complex regulatory process. In this study, to further supplement and improve the role of critical structural genes in pepper fruit type carotenoid metabolism pathways during the formation of color, three pairs of red peppers and their mutants were selected as the experimental material. This study cloned, sequenced, and analyzed the expression of critical genes *Ggps* (Geranylgeranyl Pyrophosphate Synthase), *Psy*, *Lcyb*, *Crtz*, *Zep* (Zeaxanthin epoxidase), and *Ccs* in the carotenoid metabolism pathways of samples to provide a theoretical basis for the formation and regulation of different colors in pepper fruit.

2. Materials and Methods

2.1. Materials

The test materials were red advanced inbred lines XHB (red), SP01, and PC01 and their mutants H0809 (orange), SP02 (yellow), and PC02 (orange) (Figure 1), which were

provided by the Institute of Horticulture, Qinghai Academy of Agriculture and Forestry Sciences (Xining, China). The test materials were sown in 72-hole trays for trial planting in early December 2019 and replanted in a solar greenhouse in March 2020. Based on the different time after anthesis, fruit color changes were divided into five different fruit ripening periods for sampling, including Stage I—green fruit stage, fruit color is green (20 days after anthesis); Stage II—early stage of color change (30 days after anthesis), area of color change accounts for approximately 25%; Stage III—middle stage of color change (40 days after anthesis), area of color change accounts for approximately 50%; Stage IV—late stage of color conversion (50 days after anthesis), area of color conversion accounts for approximately 75%; Stage V—mature stage (60 days after anthesis), area of color conversion is 100%. Uninjured and disease-free fruits were selected when fruit samples were collected. Seeds and placentas were removed, and the pulp was stored at -80°C after quick freezing with liquid nitrogen. There were three biological repeats for each sample. Healthy leaves were selected to extract the genomic DNA for cloning and an analysis of expression of genes related to carotenoid metabolism.



Figure 1. Experimental material: (A,B,E,F,I,J) are the field photos of PC01, PC02, SP01, SP02, XHB, and H0809 at the mature stage, respectively. PC01, SP01, and XHB are the wild-type pepper fruits, while PC02, SP02, and H0809 are the corresponding mutants, respectively. (C,D,G,H,K,L) are the images of fruit color at different developmental stages of PC01, PC02, SP01, SP02, XHB, and H0809, respectively.

2.2. Extraction and Reverse Transcription of DNA and RNA

The genomic DNA of leaves was extracted using a modified CTAB method [27]. The DNA absorbance values of A260/A280 and A260/A230 were 1.8–2.0 and 2.1–2.3 according to Nanodrop. The RNA of fruits at different periods was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China), and reverse transcription was performed using the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa) according to the manufacturer's instructions. RNA concentration and quality were determined, and the RNA values of A260/A280 and A260/A230 were between 1.9~2.1 and 2.1~2.3, respectively, which could be used for cDNA synthesis.

2.3. Cloning of Carotenoid-Related Genes

Pepper CM334 (<http://peppergenome.snu.ac.kr/>; accessed on 20 April 2023) was used as the reference genome sequence, and the carotenoid-related genes were cloned via homologous cloning. Primer Premier 5.0 was utilized to design the gene cloning primers (Table 1). With the use of gDNA as a template, the total volume of the PCR reaction system was 50 µL, which included 25 µL of 2 × Prime STAR Max DNA polymerase (TaKaRa), and 0.5 µL of 10 µmol·L⁻¹ upstream and downstream primers, respectively. A total of 100 ng of the DNA template and ddH₂O was added to 50 µL of total volume. The PCR reaction procedure was as follows: 98 °C denaturation for 10 s; 55 °C annealing for 15 s; 72 °C extension for 10 s; and 35 cycles. The PCR product was detected via 1% agarose gel electrophoresis, and the target fragment was gelled and recovered using a centrifuge column agarose gel DNA recovery kit (TIANGEN Biotech Co. Ltd., Beijing, China). Poly A was added to the recovered PCR products using a DNA A-tail Kit (TaKaRa). The total reaction system was 10 µL, which included 10 × A-tail buffer 1 µL; dNTP mixture 0.8 µL; A-tailase 0.1 µL; DNA fragment 100–500 ng; and ddH₂O added to 10 µL. The reaction procedure was as follows: 72 °C for 15 min and 4 °C heat preservation. The target fragment added with poly A was linked with the sequencing vector pMD18-T (TaKaRa). *Escherichia coli* DH5α competent cells were transformed, and positive mono-clones were selected. The sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Table 1. Sequence information of the related primers required for the experimental processes.

Primer Name	Primer Sequence (5'-3')	Purpose	Product Size (bp)	T _m (°C)
Ggps-F/R	GAACCTTGTTGATTTATGGGC CCAACATAAGCACACTGAAAG	PCR amplification	1110	57
Psy-F/R	ATGTCGTGCTTGCCTTGTTATGG CCTGATTCATGTTCTGTAGAAAG	PCR amplification	2844	58
Lcyb-F/R	ATGGATACGCTCTTGAGAACCCCAAAC TCATTCTTTATCCTGTAACAAATTGTT	PCR amplification	1497	58
CRTY-F/R	ATGGCTGCTGAAATTTCAATCTCCG CATAATCTCTTCGAACTTTTAATTC	PCR amplification	2024	58
Zep-F/R	ATGAAGCAATTTGTGCTAAGTTTGG TCACCTTTGACATAATTTTCCGCAA	PCR amplification	510	57.5
Ccs-F/R	TCTCTAATGGAAACCCTTCTAAAGCC TCAAAGGCTCTCTATTGCTAGATTGC	PCR amplification	1497	58
RT-Ggps-F/R	CATTGTCAACTCCACGGC CCGTAGATTTGTGGTTGGT	qRT-PCR	158	60
RT-Psy-F/R	ACAGGCAGGTCTATCCGACGAAG ACAACAGCAGAGATGCCAACACAG	qRT-PCR	164	60
RT-Lcyb-F/R	GTTGTTGGAATTGGTGGCACAGC ATGGCATTGGCAACGACAGGAG	qRT-PCR	182	60

Table 1. Cont.

Primer Name	Primer Sequence (5'-3')	Purpose	Product Size (bp)	T _m (°C)
RT-CRTY-F/R	GCACGAGTCACACCATAGACCAAG CGTGAACGAACATGTAGGCCATCC	qRT-PCR	155	60
RT-Zep-F/R	TGCACTTCATCCAATGACACCT GCCTCTGAAATGCACCTTGC	qRT-PCR	164	60
RT-Ccs-F/R	AGCACCCACATCAAAGCCAG GTGGTGAAGGGTCAACGCAA	qRT-PCR	145	60
Ubi3-F/R	TGTCCATCTGCTCTCTGTG CACCCCAAGCACAATAAGAC	Internal reference	155	60

Note: qRT-PCR, real-time quantitative reverse transcription PCR; F, forward primer; R, reverse primer.

2.4. Bioinformatics Analysis Process

The BLAST tool in NCBI was used to conduct homologous comparisons and conservative domain analyses of the obtained gene and its encoding protein. The ProtParam tool was used to predict the protein's molecular weight and theoretical isoelectric point, and DNAMAN 6.0 was used to compare the multiple sequences of amino acids. In addition, MEGA7.0 was adopted to construct a phylogenetic tree, and ProtScale (<http://web.expasy.org/cgi-bin/protscale/protscale.pl>; accessed on 15 July 2023) was used to analyze the hydrophilicity and hydrophobicity of amino acids. This study also utilized the NetPhos 3.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>; accessed on 15 July 2023) to predict the protein phosphorylation loci. TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html; accessed on 15 July 2023) was used to predict the transmembrane structure, and the SignalP (<http://www.cbs.dtu.dk/services/SignalP/>; accessed on 15 July 2023) online site was utilized to predict the signal peptide. In addition, WoLF PSORT (<https://wolfpsort.org/>; accessed on 15 July 2023) was used to predict subcellular localization, and SWISS-MODEL (<https://www.swissmodel.expasy.org/>; accessed on 15 July 2023) was used to predict the three-dimensional structures of the encoding protein.

2.5. qRT-PCR Analysis Process

Quantitative primers were designed according to the cloned gene sequences (Table 1), and the *Ubi3* gene of pepper was used as an internal reference gene [28]. qRT-PCR analysis was performed using the SYBR[®] Premix Ex Taq II (TaKaRa). A fluorescence quantitative PCR reaction process was performed using an IQ5 Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as follows: 95 °C for 3 min; 95 °C for 30 s; 60 °C for 90 s for 40 cycles; and 60 °C for 30 s for 71 cycles, with increases of 0.5 °C for each cycle. Three technical replicates were established for each sample, and the results were analyzed using the $2^{-\Delta\Delta CT}$ method [29]. SPSS 22.0 (IBM, Inc., Armonk, NY, USA) was adopted for statistical data analysis, and Duncan's new complex range method was used to complete multiple comparisons.

3. Results and Analyses

3.1. Cloning and Sequence Analysis of Genes Related to Carotenoid Metabolism

A homologous cloning strategy was used, which relied on the genome sequence of pepper CM334 and genomic DNA as a template to clone the full-length genes of *Ggps*, *Psy*, *Lcyb*, *Crtz*, *Zep*, and *Ccs* in different mature fruit color materials (Figure 2). The results showed that the full lengths of *Ggps*, *Psy*, *Lcyb*, *Crtz*, *Zep*, and *Ccs* genes were 1110 bp, 2844 bp, 1497 bp, 2025 bp, 510 bp, and 1497 bp, and they encoded 369, 419, 498, 315, 169, and 498 amino acids, respectively. An analysis of the gene structure showed that *Ggps*, *Lcyb*, *Zep*, and *Ccs* had no intron sequence; *Psy* contained six exons and five introns; and *Crtz* contained seven exons and six introns. A sequence comparison with related genes in the reference genome CM334 showed that there was one base difference (G/A) at 1437 bp of

the *Crtz* gene, but in our selected experimental materials, the sequence of the *Crtz* gene was completely consistent.

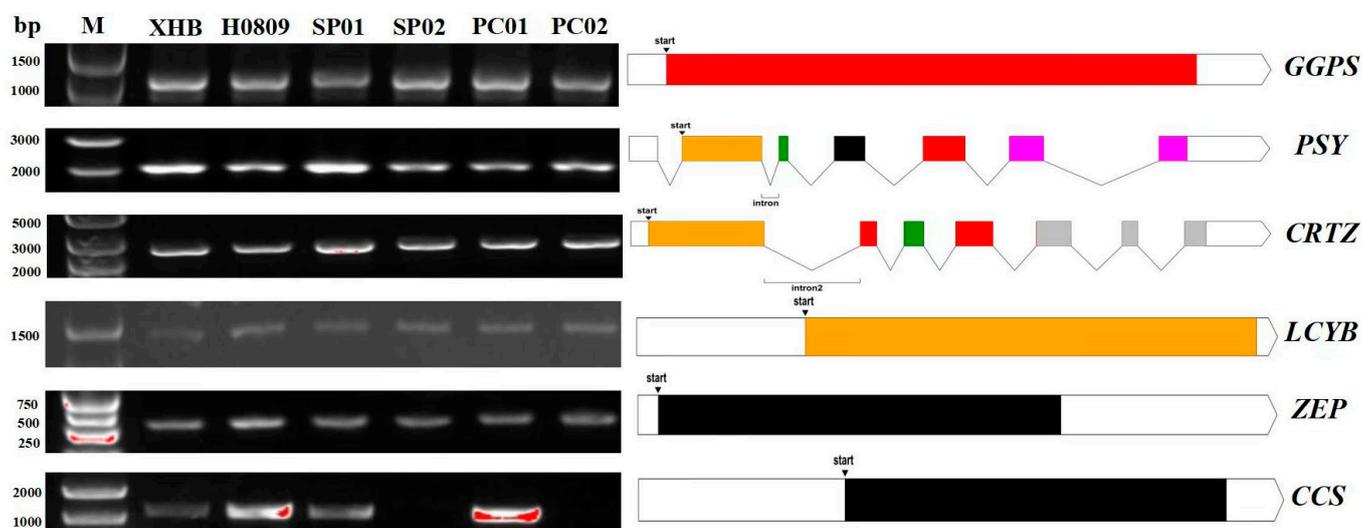


Figure 2. Structures of the key genes involved in the synthesis of carotenoids in pepper.

The results of sequence alignment among different materials showed that the sequences of other genes were completely consistent among the different cultivars, except that *Ccs* exhibited sequence differences among the different mutant materials. *Ccs* could be amplified in the three red wild-type pepper materials. Among the three mutants, only the complete *Ccs* full-length sequence could be amplified in H0809 but not in the other two mutants, indicating that the formation of fruit color in SP01 (yellow) and PC02 (orange) could be closely related to the deletion or mutation of *Ccs*.

3.2. Bioinformatics Analysis of Genes Related to Carotenoid Biosynthesis

The physical and chemical properties of Ggps, Psy, Lcyb, Crtz, Zep, and Ccs proteins were analyzed using ExpASy ProtParam (Table 2). The pIs of most proteins exceeded 7.0, with the exception of Ggps and Lcyb. All proteins but Zep had at least 315 total amino acids, and Crtz was the only protein, which had a positive hydrophilicity value.

Table 2. Physicochemical properties of proteins related to the synthesis of pepper carotenoids.

Gene Name	Total Amino Acids	Molecular Weight of the Protein	Molecular Formula	Isoelectric Point	Coefficient of Instability	Positively Charged Amino Acids	Negatively Charged Amino Acids	Total Hydrophilicity Value
Ggps	369	40,173.41	C ₁₇₈₀ H ₂₈₈₉ N ₄₈₅ O ₅₃₆ S ₁₆	6.12	37.01	43	47	−0.012
Psy	419	47,066.05	C ₂₀₈₃ H ₃₃₂₀ N ₅₈₈ O ₆₁₄ S ₂₀	9.01	53.5	60	52	−0.242
Lcyb	498	55,626.23	C ₂₄₉₈ H ₃₉₃₀ N ₆₆₈ O ₇₁₉ S ₂₅	6.34	37.73	54	58	−0.073
Crtz	315	35,317.91	C ₁₆₁₂ H ₂₄₇₅ N ₄₃₁ O ₄₃₄ S ₁₅	8.52	54.43	35	32	0.012
Zep	169	18,995.24	C ₈₄₂ H ₁₃₇₁ N ₂₂₇ O ₂₄₆ S ₁₂	8.95	38.15	24	18	−0.073
Ccs	498	56,658.66	C ₂₅₄₉ H ₄₀₀₇ N ₆₈₉ O ₇₁₈ S ₂₇	8.77	44.15	66	58	−0.216

The PSIPRED online software was used to predict the secondary structures of proteins in this study (Table 3, Figure 3). α -Helix and random coils were the primary structural elements constituting the secondary structures of proteins in the Ggps, Psy, Lcyb, Crtz, Zep, and Ccs proteins, and they accounted for more than 70%, as shown in Table 3. The signal peptide encoded by the SignalP3.0 online software predicted that Ggps, Lcyb, Crtz, Zep, and Ccs had no signal peptides. However, Psy contained a signal peptide with

20 amino acid residues, and its score was 0.342. Therefore, based on the results of an analysis using Plant-mPLOC software, the Zep, Lcyb, Ccs, and Psy proteins were localized in the chloroplasts. Ggps was localized in the chloroplasts or plastids, and Crtz proteins were localized in the vacuoles.

Table 3. Predictions of the secondary structures of gene proteins related to carotenoid synthesis in pepper.

Protein Name	α -Helix	Extension	B-Turn	Random Coil
Ggps	54.74%	6.5%	5.15%	33.6%
Psy	51.79%	12.17%	3.82%	32.22%
Lcyb	39.56%	16.87%	6.02%	37.55%
Crtz	45.08%	15.24%	5.4%	34.29%
Zep	56.21%	5.92%	4.73%	33.14%
Ccs	34.54%	17.27%	4.02%	44.18%

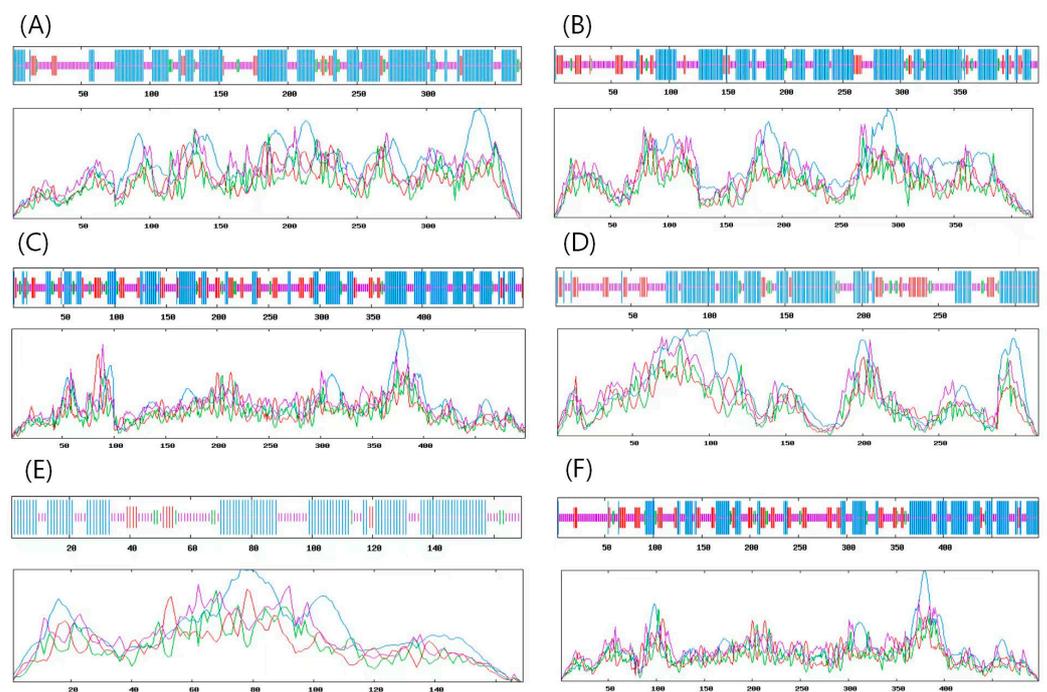


Figure 3. Prediction of proteins' secondary structures: (A–F) represent the secondary structures of Ggps, Psy, Crtz, Lcyb, Zep, and Ccs proteins, respectively. Blue indicates an α -helix; red denotes the extended strand; green is a β -fold; and purple indicates a random coil.

The possible configurations of protein tertiary structures were predicted using the SWISS-MODEL online software and were found to be consistent with the predicted protein secondary structures (Figure 4).

3.3. Evolutionary Analysis

In order to study the evolutionary relationship of genes related to carotenoid synthesis in pepper, a phylogenetic tree was constructed using the neighbor-joining method in MEGA7.0 (Figure 5). The results showed that *Ggps* of the different pepper varieties belonged to the same branch as that of tomato (*Solanum lycopersicon*), which was distantly related to the evolution of periwinkle (*Vinca minor*) and coffee (*Coffea* spp.), among others. *Psy* was found to have a close evolutionary relationship with pepper, tomato, and wolfberry (*Lycium barbarum*). *Lcyb*, *Crtz*, and *Ccs* of pepper were observed to have a close evolutionary relationship with tomato but a distant evolutionary relationship with coffee and rose (*Rosa* spp.). The *Zep* gene of pepper was not only closely related to the evolution of different varieties of pepper and tomato but also closely related to the evolution of different varieties of coffee. In general, the evolution of genes related to carotenoid synthesis in

pepper is in the same branch as that of other solanaceous crops, such as tomato and potato (*Solanum tuberosum*).

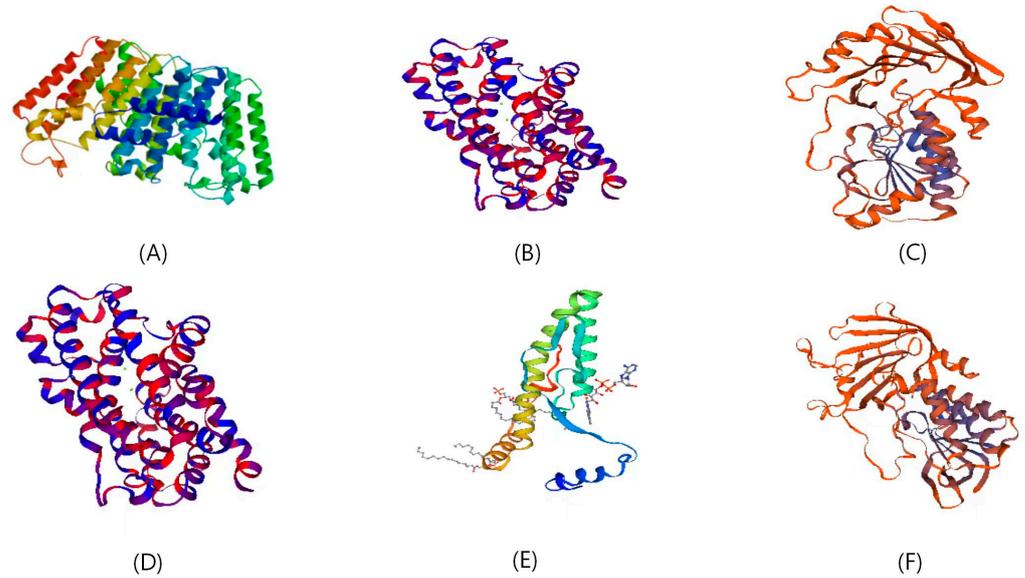


Figure 4. Tertiary structures of protein products encoded by genes related to carotenoid synthesis in pepper. (A–F) represent the tertiary structures of Ggps, Psy, Crtz, Lcyb, Zep, and Ccs proteins, respectively.

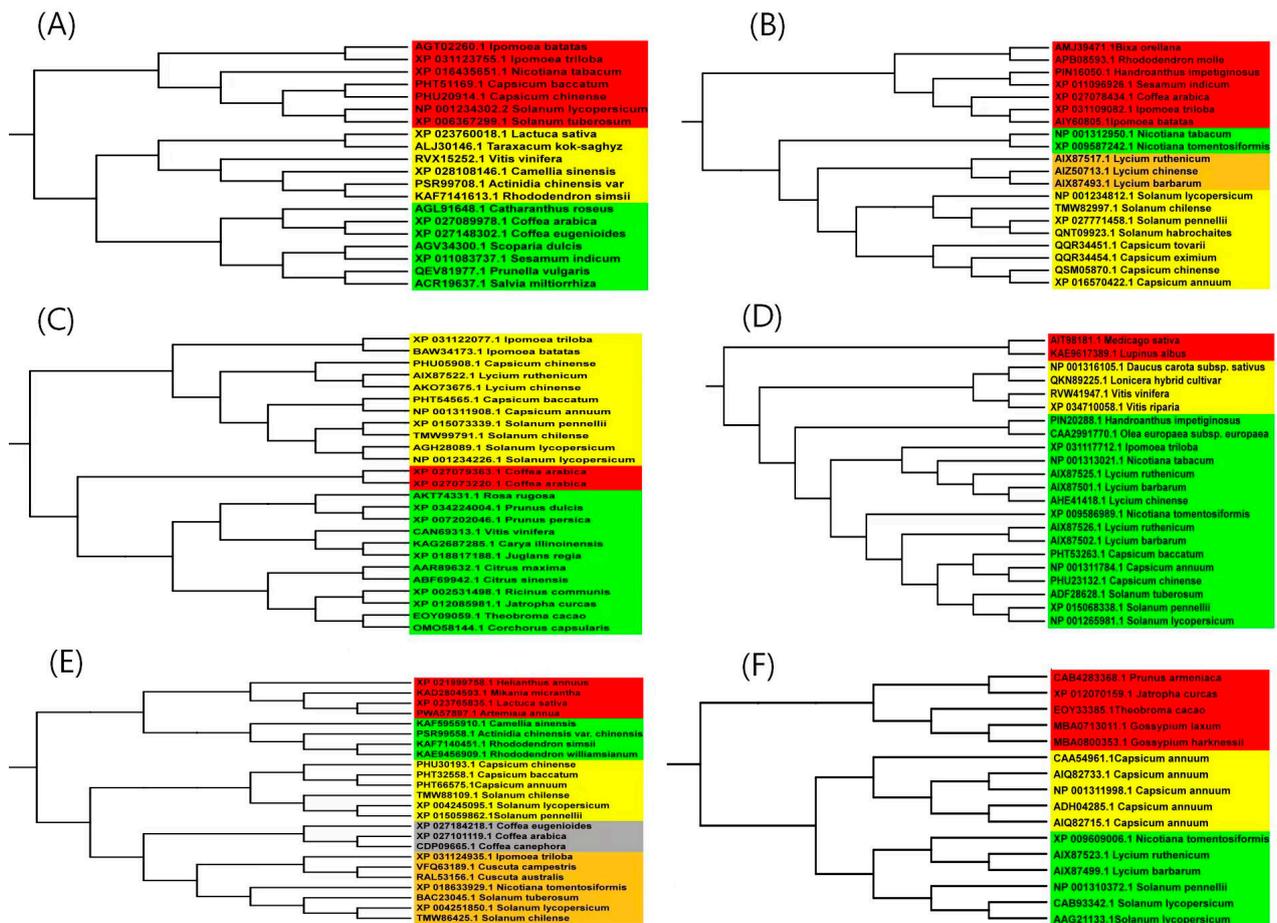


Figure 5. Evolutionary tree analysis of the amino acid sequences of genes related to carotenoid synthesis in pepper. (A–F) represent the evolutionary trees of Ggps, Psy, Crtz, Lcyb, Zep, and Ccs genes, respectively.

3.4. Homology Analysis

The MEME online software and TBtools software were used to visualize conservative motifs. The results showed that the closer the relatives are in the phylogenetic tree, the more similar or identical the conserved motifs contained in the Ggps, Psy, Lcyb, Crtz, Zep, and Ccs proteins are (Figure 6). There were 13 identical conserved motifs (Motifs 1–13) in the Ggps proteins in Solanaceae crops with close relatives (*Capsicum chinense* Jacq: PHU20914.1, *Capsicum annuum*: PHT51169.1, potato: XP_0063672991.1, and tomato: NP_001234302.2). The Psy protein also had 13 identical conserved motifs (Motifs 1–13) in different varieties of pepper (*Capsicum annuum*: QQR34451.1, *Capsicum eximium* Hunz.: QQR34454.1, *Capsicum annuum*: XP_016570422.1, and *Capsicum chinense* Jacq: QSM05870.1). The Zep, Ccs, Lcyb, and Crtz proteins also only had 1–2 differences in the conserved motifs among different species, which indicated that the sequences of these genes were relatively conserved in both pepper and other species.

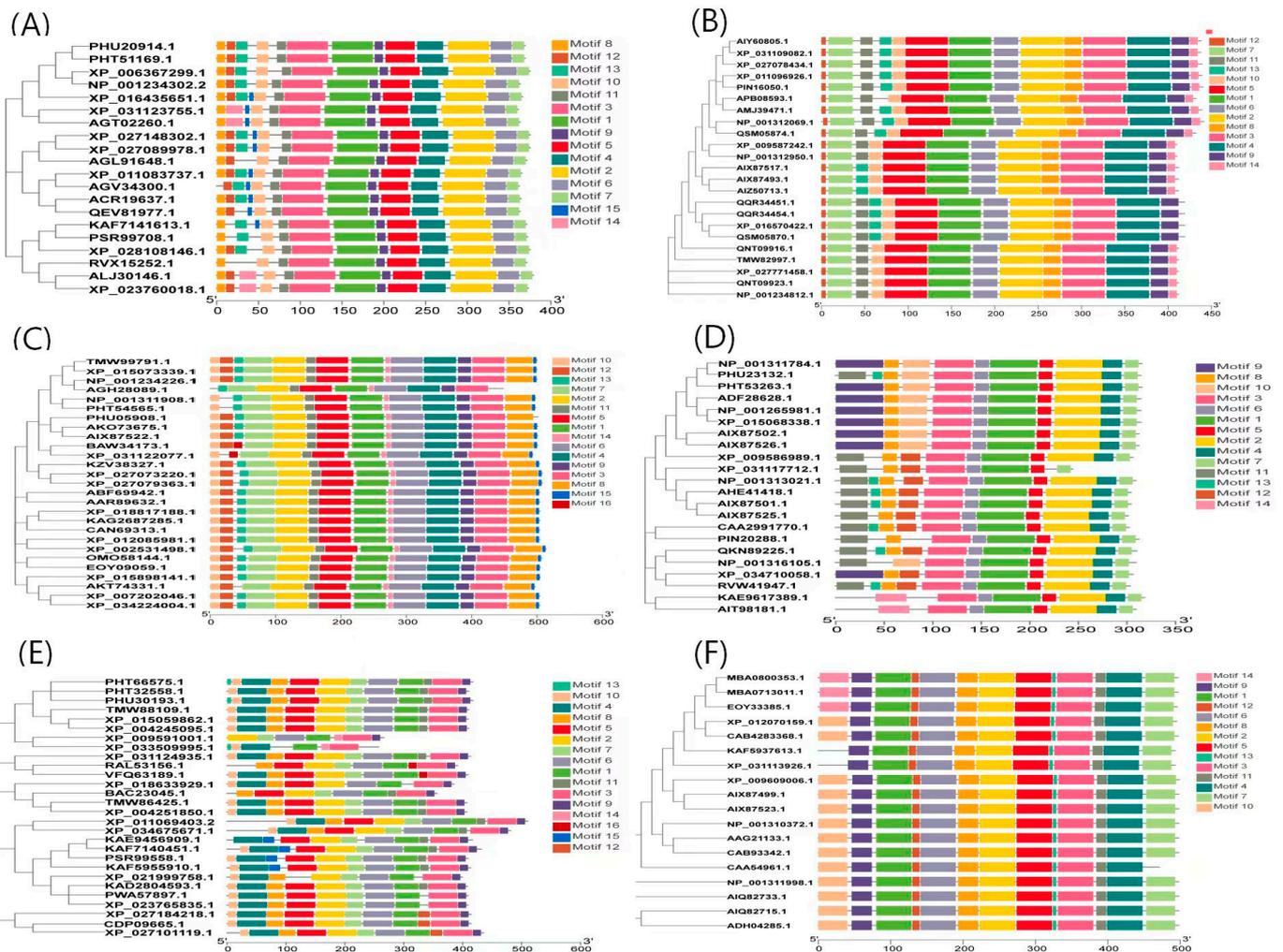


Figure 6. Conservative motifs: (A–F) represent the conserved motifs of Ggps, Psy, Crtz, Lcyb, Zep, and Ccs proteins, respectively.

3.5. qRT-PCR Analysis

The expression patterns of *Ggps*, *Psy*, *Lcyb*, *Crtz*, *Zep*, and *Ccs* genes in different fruit color materials and different fruit ripening stages were analyzed using qRT-PCR (Figure 7). The results showed that with the increase in fruit maturity, the expression of *Ggps* in XHB decreased gradually, and the expression was highest at Stage II. In the yellow wild-type H0809, the amount of expression first increased and then decreased, and the amount of

expression was highest at Stage III. In SP02 and PC02, *Ggps* was more highly expressed than in its red wild type.

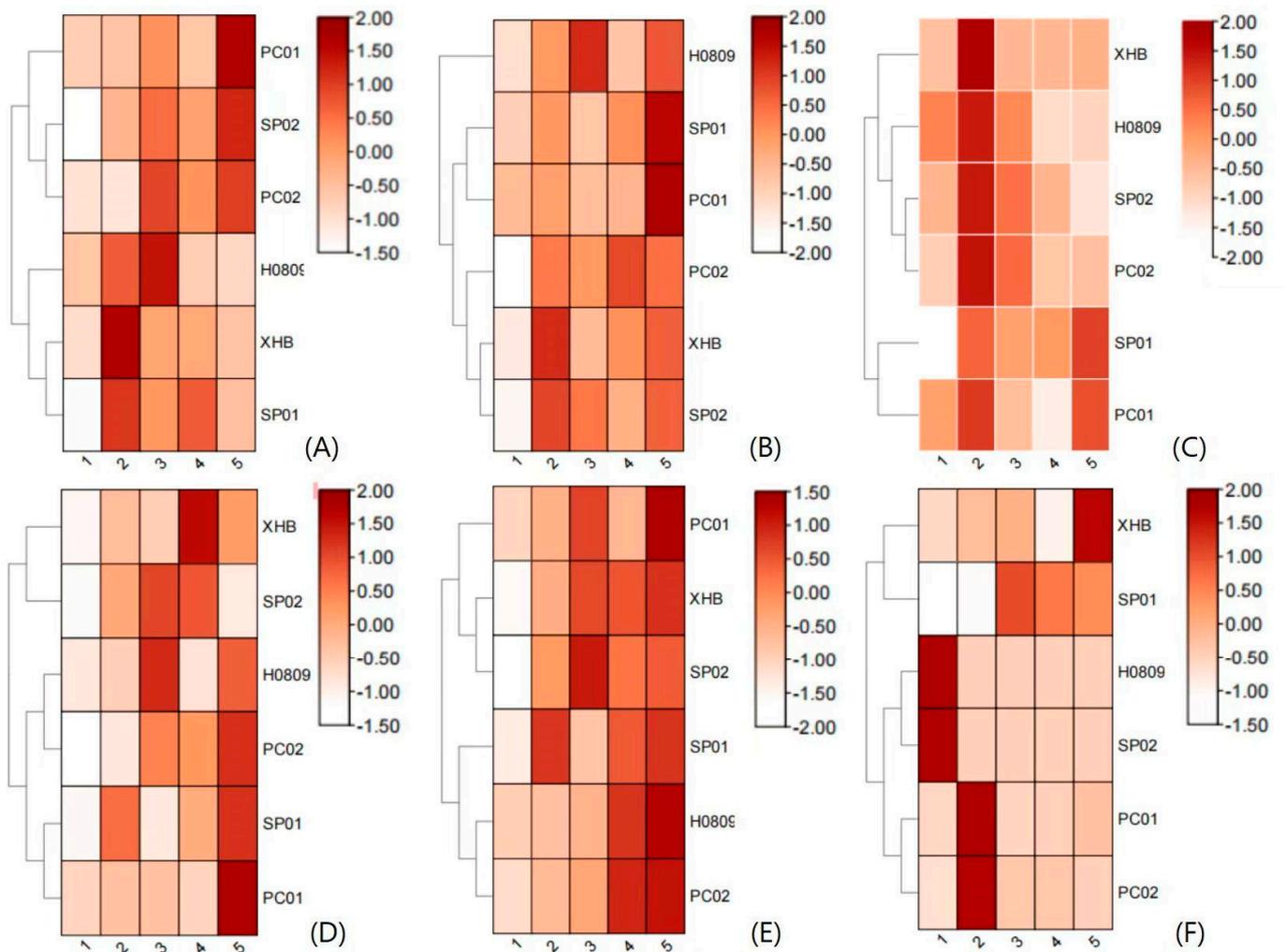


Figure 7. Analysis of the expression of genes related to carotenoid synthesis in pepper. (A–F) exhibit the expression heatmaps of genes representing *Ggps*, *Psy*, *Crtz*, *Lcyb*, *Zep*, and *Ccs*, respectively.

In XHB, the level of expression of *Psy* was highest at Stage II and lowest at Stage III. In H0809, the expression of *Psy* was higher than that of the wild-type XHB and reached its highest level at Stage III. The level of expression of *Psy* in SP01 was higher than that in its yellow mutant SP02. In SP01, the amount of expression was highest in Stage V. In SP02, the level of expression of *Psy* gradually decreased, while its level of expression increased slightly at Stage V. In PC01, *Psy* was primarily expressed at Stage V, and in PC02, except for Stage V, the level of expression of *Psy* was significantly higher than that of the wild-type PC01.

Except for the higher level of expression of the *Crtz* gene at Stage IV of XHB and Stage V of SP01, the level of expression of the *Crtz* gene in mutants was generally higher than that of the red wild type. The expression of model gene *Lcyb* in all the materials was basically the same. Except for SP02, the amount of expression reached its maximum at Stage II. In addition to the higher level of expression of XHB at Stage II, the level of expression of *Zep* in mutant materials was higher than that in the red wild type. The pattern of expression of *Zep* in all the other materials increased overall and reached its maximum at fruit maturity.

In terms of different materials, the *Ggps*, *Lcyb*, and *Ccs* genes were highly expressed at each stage of red pepper XHB, and the *Psy*, *Crtz*, and *Zep* genes were highly expressed in H0809. In sweet pepper SP01, the *Psy*, *Lcyb*, and *Ccs* had higher levels of expression, while

in the yellow mutant SP02, *Ggps*, *Crtz*, and *Zep* had higher levels of expression. In PC01, only the level of expression of the *Ccs* gene was higher, and the overall expression of *Ggps*, *Psy*, *Crtz*, *Lcyb*, and *Zep* genes in PC02 was higher than that in PC01. It can be deduced that the patterns of expression of genes related to carotenoid metabolism are not consistent in pepper materials with different fruit colors.

The expression of the *Ccs* gene was relatively high in the red wild-type materials and reached its maximum at Stages IV and V. The *Ccs* gene was not expressed in SP02. In the orange mutant H0809, the expression of the *Ccs* gene was maintained at a low level at different developmental stages of fruit color, which was significantly lower than that of the wild-type XHB, indicating that the formation of fruit color of orange mutant H0809 and yellow SP02 could be closely related to different levels of expression and regulatory modes of *Ccs*.

4. Discussion

Carotenoids are the primary pigments in the flowers and fruits of most plants. During the development of pepper fruits, the types and contents of carotenoids in different types of colored fruits vary. The composition and content of carotenoids affect the color of pepper fruits [30–33]. Currently, the regulation of carotenoid synthesis in pepper has been primarily focused on the structures, levels of expression, and protein products of *Psy*, *Lcyb*, *Lcye*, *Crtz*, and *Ccs* genes, with the goals of revealing the genetic regulatory mechanisms of changes in the ripening fruit color of pepper [4,34,35]. The results of this study showed that among the three pairs of different mutant materials, only the *Ccs* gene was diverse in different materials, and the sequences of other genes were completely consistent. The results of a qRT-PCR analysis showed that the patterns of expression of the six genes described above in different materials were not consistent, indicating that there could be complex regulatory mechanisms in the process of fruit color formation.

Currently, there are few reports on the role of *Ggps* in the formation of pepper fruit color. Geranylgeranyl pyrophosphate (GGPP) is the most direct precursor of carotenoids [36]. *Ggps* has been found to play an essential role in carotenoid synthesis and accumulation in some plants [37,38]. It has been observed that overexpression of the *Ggps* gene in brewer's yeast will significantly increase the content of β -carotene [39]. Overexpression of *Ggps* also significantly increased carotenoid production in yeast *Phaffia rhodozyma* [40,41]. Therefore, in plants, *Ggps* is often a polygene family compound, and its proteins are expressed in different subcellular structures [42]. Previous studies on *Ggps* in pepper have localized it in the plastid septum, and induced levels of expression were observed during the fruit ripening stages [43]. This study cloned the full-length *Ggps* gene, which encoded 291 amino acids from three groups of mutant materials with different fruit colors. There was no difference in the sequence of *Ggps* gene between the different materials. Subcellular localization predicted that *Ggps* was located in the chloroplast or plastid, which was consistent with previous research.

Psy is the primary regulatory enzyme in the carotenoid synthesis pathway, which also makes the *Psy* gene one of the first-choice genes for improved plant breeding [20]. There are two *Psy* genes in pepper [19,25]. Recent research results have shown that *Psy1* has the highest levels of expression in the fruit pericarp and petals, and the *Psy2* gene was detected in all organs analyzed. Its level of expression was highest in cotyledons and lowest in the pericarp. It was detected not only in the leaves and immature fruit but also in mature fruit [44]. Some studies also found that *Psy2* could compensate for the loss of *Psy1* in pepper fruit [45]. In this study, only the *Psy1* gene was cloned, and the patterns of expression were analyzed at different stages of fruit ripening. The results showed that there was no difference in the sequence of *Psy1* among different pepper materials, but its pattern of expression was very different. Typically, the gene was expressed more highly during the fruit color transformation and ripening stages, which was basically consistent with the conclusions of previous research.

In pepper, mutations at base 709 of the β -CHY2 (β -carotene hydroxylase 2) gene in the ethane methanesulfonate (EMS)-induced orange fruit mutants resulted in the content of β -carotene in orange fruit being 3.2-fold higher than that in red fruit, while the total amount of carotenoids was reduced by 58.3% [23]. Overexpression of the β -CHY gene in other crops contributes to the accumulation of zeaxanthin in cells, thereby increasing the content of carotenoids in cells [46]. The *Arabidopsis* mutants, which inhibit the expression of β -CHY via antisense suppression and T-DNA mutation, have a weakened ability to synthesize cryptoxanthin and other carotenoids, and their resistance to stress significantly decreases [47,48].

There are two genes encoding the *Lcyb* gene in pepper plants. *Lcyb1* plays a role in the β - ϵ -carotene branch, while *Lcyb2* plays a role in the β -carotene branch [49,50]. A single-base mutation in the coding region of *Lcyb* in papaya (*Carica papaya*) affects enzyme activity, resulting in red-fleshed papayas, which accumulate lycopene in the fruit [51]. In addition, *Lcyb2* in saffron (*Crocus sativa*) plays a decisive role in its stigma color [52]. In this study, there was no difference in the sequence of *Lcyb* in different materials, but the patterns of expression were inconsistent. *Zep* catalyzes the conversion of zeaxanthin to antherxanthin, which in turn produces vioxanthin [53]. Studies on sweet potato (*Ipomoea batatas*) found that the *ZEP* paralog *g1103.t1* could be involved in the accumulation of carotenoids owing to the epoxidation of β -carotene and β -cryptoxanthin [54]. In this study, there was no difference in the sequence of *Lcyb* and *Zep* in different materials, but the patterns of expression were inconsistent.

Ccs encodes capsanthin/capsanthin synthetase, which has a rich structural diversity. Deletion of *Ccs* or loss of function owing to its sequence variation results in the formation of yellow- or orange-fruited peppers [24,25,55]. Several studies confirmed the deletion of 211 and 220 bp at the 3' end of the *Ccs* gene in yellow-fruited peppers [17,56]. It was also reported that a frameshift mutation in the *Ccs* coding region of orange-fruited annual pepper 'Fogo' caused premature termination of transcription, and its fruit primarily contained β -carotene and zeaxanthin but did not contain capsanthin [5]. Similar reports showed that in the yellow fruit pepper 'CK7', a single-base (C/G) mutation occurred in the *Ccs* gene at 1095 bp downstream of the transcription start site, resulting in formation of a premature stop codon [57]. In yellow-fruited pepper 'Y2,' the *Ccs* gene was inserted 8 bp at 1431 bp, resulting in a frameshift mutation and premature termination of transcription. 'Y3' had a single-base (C/A) mutation at 599 bp. Therefore, it was assumed that variation in the *Ccs* gene was one of the reasons for the formation of yellow and orange mutants.

The results of this study also confirmed that the *Ccs* genes are also diverse in the different fruit-colored pepper materials used in this study. In the two mutant materials SP02 (yellow) and PC02 (orange), we were unable to amplify the wild-type *Ccs*. However, we were able to amplify the red XHB, SP01, and PC01 and orange mutant H0809 to the full-length sequence of the *Ccs* without sequence differences. Expression of the *Ccs* in H0809 only remained at a low level, and the difference was not significant at different developmental stages. Thus, we can hypothesize that the formation of orange pepper H0809 fruit color may not be due to *Ccs*. In addition to the mutation of genes, their differential regulatory patterns could lead to the formation of orange mutant fruit color, and its precise regulatory mechanism merits further study. No transcription of *Ccs* was detected in SP02, but in PC02, it was expressed to varying degrees. It was hypothesized that the formation of fruit color in SP02 could be caused by deletion of the *Ccs* gene, and there could be homologous sequences of the *Ccs* gene in PC02, which merits further study.

5. Conclusions

In this study, three pairs of different mature fruit color mutants of peppers were used as research materials to perform cloning, bioinformatics, and expression analyses of the *Ggps*, *Psy*, *Lcyb*, *Crtz*, *Zep*, and *Ccs* genes related to carotenoid biosynthesis. The results of this study showed the following:

- (1) In both wild-type XHB and its orange mutant H0809, we were able to amplify the complete *Ccs* gene. However, the level of *Ccs* expression remained at a low level in mutant H0809, and the difference in expression was significant compared with that of the wild-type XHB, indicating that the formation of fruit color of the orange mutant H0809 could be closely related to the different regulatory patterns of expression of *Ccs*.
- (2) Compared with H0809, we could not amplify the complete *Ccs* gene in mutants SP02 and PC02, and at the same time, we could not detect the transcript product of the *Ccs* gene in SP02, but the transcript product could be detected in PC02, indicating that the formation of mutant SP02 may be related to deletion of the *Ccs* gene, and there may be a homolog of the *Ccs* gene in PC02.
- (3) We were able to amplify the complete *Ggps*, *Psy*, *Lcyb*, *Crtz*, and *Zep* genes in three different pairs of wild-type and mutant materials, but their expression patterns were inconsistent across the different developmental stages of different materials. Overall, the expression levels of *Psy* and *Crtz* genes in different materials were relatively high, and the expression levels of *Ccs* genes in red wild type were generally higher than those in mutant materials.

Author Contributions: Q.L. and Q.Z. conceived and designed these experiments, wrote or reviewed the drafts and papers, supervised project management, fund acquisition, approval, and finalization of these experiments; Y.W. and P.F. conceived and designed the experiments, conducted the experiments, analyzed the data, prepared the charts and/or tables, prepared or reviewed the draft report, and approved the final draft; Y.W. and J.W. analyzed the data, wrote or reviewed the draft paper, investigated and approved the final draft; Y.R. analyzed the data, wrote or reviewed the draft paper, supervised, evaluated, and approved the final draft. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Qinghai Provincial Science and Technology Plan Project (2021-ZJ-763), National key research and development program (2022YFD1602400).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships, which could appear to influence the work reported in this paper.

References

1. Lightbourn, G.J.; Griesbach, R.J.; Novotny, J.A.; Clevidence, B.A.; Rao, D.D.; Stommel, J.R. Effects of anthocyanin and carotenoid combinations on foliage and immature fruit color of *Capsicum annuum* L. *J. Hered.* **2008**, *99*, 105–111. [[CrossRef](#)]
2. Paran, I.; Van Der Knaap, E. Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. *J. Exp. Bot.* **2007**, *58*, 3841–3852. [[CrossRef](#)]
3. Borovsky, Y.; Paran, I. Chlorophyll breakdown during pepper fruit ripening in the chlorophyll retainer mutation is impaired at the homolog of the senescence-inducible stay-green gene. *Theor. Appl. Genet.* **2008**, *117*, 235–240. [[CrossRef](#)]
4. Ha, S.H.; Kim, J.B.; Park, J.S.; Lee, S.W.; Cho, K.J. A comparison of the carotenoid accumulation in *Capsicum* varieties that show different ripening colours: Deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. *J. Exp. Bot.* **2007**, *58*, 3135–3144. [[CrossRef](#)]
5. Guzman, I.; Hamby, S.; Romero, J.; Bosland, P.W.; O’Connell, M.A. Variability of Carotenoid Biosynthesis in Orange Colored *Capsicum* spp. *Plant Sci.* **2010**, *179*, 49–59. [[CrossRef](#)]
6. Marín, A.; Ferreres, F.; Tomás-Barberán, F.A.; Gil, M.I. Characterization and quantitation of antioxidant constituents of sweet pepper (*Capsicum annuum* L.). *J. Agric. Food Chem.* **2004**, *52*, 3861–3869. [[CrossRef](#)]
7. Wahyuni, Y.; Ballester, A.R.; Sudarmonowati, E.; Bino, R.J.; Bovy, A.G. Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse accessions: Variation in health-related compounds and implications for breeding. *Phytochemistry* **2011**, *72*, 1358–1370. [[CrossRef](#)] [[PubMed](#)]
8. Kim, J.S.; An, C.G.; Park, J.S.; Lim, Y.P.; Kim, S. Carotenoid profiling from 27 types of paprika (*Capsicum annuum* L.) with different colors, shapes, and cultivation methods. *Food Chem.* **2016**, *201*, 64–71. [[CrossRef](#)] [[PubMed](#)]
9. Peterson, P.A. Linkage of Fruit Shape and Color Genes in *Capsicum*. *Genetics* **1959**, *44*, 407–419. [[CrossRef](#)]

10. Silveira Agostini-Costa, T.; da Silva Gomes, I.; de Melo, L.A.M.P.; Reifschneider, F.J.B.; da Costa Ribeiro, C.S. Carotenoid and total vitamin C content of peppers from selected Brazilian cultivars. *J. Food Compos. Anal.* **2017**, *57*, 73–79. [[CrossRef](#)]
11. Borovsky, Y.; Oren-Shamir, M.; Ovadia, R.; Walter, D.J.; Paran, I. The *A* locus that controls anthocyanin accumulation in pepper encodes a *MYB* transcription factor homologous to *Anthocyanin2* of *Petunia*. *Theor. Appl. Genet.* **2004**, *109*, 23–29. [[CrossRef](#)]
12. Wang, D.Y.; Bosland, P. The Genes of Capsicum. *HortScience* **2006**, *41*, 1169–1187. [[CrossRef](#)]
13. Pan, Y.; Bradley, G.; Pyke, K.; Ball, G.; Lu, C.G.; Fray, R.; Marshall, A.; Jayasuta, S.; Baxter, C.; Wijk, R.V.; et al. Network inference analysis identifies an *APRR2*-like gene linked to pigment accumulation in tomato and pepper fruits. *Plant Physiol.* **2013**, *161*, 1476–1485. [[CrossRef](#)] [[PubMed](#)]
14. Brand, A.; Borovsky, Y.; Hill, T.; Rahman, K.A.A.; Bellalou, A.; Deynze, A.V.; Paran, I. *CaGLK2* regulates natural variation of chlorophyll content and fruit color in pepper fruit. *Theor. Appl. Genet.* **2014**, *127*, 2139–2148. [[CrossRef](#)] [[PubMed](#)]
15. Borovsky, Y.; Monsonego, N.; Mohan, V.; Shabtai, S.; Kamara, I.; Faigenboim, A.; Hill, T.; Chen, S.Y.; Stoffel, K.; Van Deynze, A.; et al. The zinc-finger transcription factor *CcLLO1* controls chloroplast development and immature pepper fruit color in *Capsicum chinense* and its function is conserved in tomato. *Plant J.* **2019**, *99*, 41–55. [[CrossRef](#)] [[PubMed](#)]
16. Hurtado-Hernandez, H.; Smith, P.G. Inheritance of mature fruit color in *Capsicum annuum* L. *J. Hered.* **1985**, *76*, 211–213. [[CrossRef](#)]
17. Popovsky, S.; Paran, I. Molecular genetics of the *y* locus in pepper: Its relation to capsanthin-capsorubin synthase and to fruit color. *Theor. Appl. Genet.* **2000**, *101*, 86–89. [[CrossRef](#)]
18. Lefebvre, V.; Kuntz, M.; Camara, B.; Palloix, A. The capsanthin-capsorubin synthase gene: A candidate gene for the *y* locus controlling the red fruit colour in pepper. *Plant Mol. Biol.* **1998**, *36*, 785–789. [[CrossRef](#)]
19. Thorup, T.A.; Tanyolac, B.; Livingstone, K.D.; Popovsky, S.; Paran, I.; Jahn, M. Candidate gene analysis of organ pigmentation loci in the Solanaceae. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 11192–11197. [[CrossRef](#)]
20. Huh, J.H.; Kang, B.C.; Nahm, S.H.; Kim, S.; Ha, K.S.; Lee, M.H.; Kim, B.D. A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). *Theor. Appl. Genet.* **2001**, *102*, 524–530. [[CrossRef](#)]
21. Barry, C.S.; McQuinn, R.P.; Chung, M.Y.; Besuden, A.; Giovannoni, J.J. Amino acid substitutions in homologs of the STAY-GREEN protein are responsible for the green-flesh and chlorophyll retainer mutations of tomato and pepper. *Plant Physiol.* **2008**, *147*, 179–187. [[CrossRef](#)]
22. Kim, O.R.; Cho, M.C.; Kim, B.D.; Huh, J.H. A splicing mutation in the gene encoding phytoene synthase causes orange coloration in Habanero pepper fruits. *Mol. Cells* **2010**, *30*, 569–574. [[CrossRef](#)] [[PubMed](#)]
23. Borovsky, Y.; Tadmor, Y.; Bar, E.; Meir, A.; Lewinsohn, E.; Paran, I. Induced mutation in β -CAROTENE HYDROXYLASE results in accumulation of β -carotene and conversion of red to orange color in pepper fruit. *Theor. Appl. Genet.* **2013**, *126*, 557–565. [[CrossRef](#)]
24. Ha, S.H. Competitive activity as a constitutive promoter in the 5'-proximal regulatory region of the *Capsicum* capsanthin-capsorubin synthase gene. *Plant Biotechnol. Rep.* **2015**, *9*, 259–267. [[CrossRef](#)]
25. Kilcrease, J.; Rodriguez-Uribe, L.; Richins, R.D.; Arcos, J.M.; Victorino, J.; O'Connell, M.A. Correlations of carotenoid content and transcript abundances for fibrillin and carotenogenic enzymes in *Capsicum annuum* fruit pericarp. *Plant Sci.* **2015**, *232*, 57–66. [[CrossRef](#)]
26. Tian, S.L.; Li, L.; Chai, W.G.; Shah SN, M.; Gong, Z.H. Effects of silencing key genes in the capsanthin biosynthetic pathway on fruit color of detached pepper fruits. *BMC Plant Biol.* **2014**, *14*, 314. [[CrossRef](#)] [[PubMed](#)]
27. Murray, M.G.; Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325. [[CrossRef](#)] [[PubMed](#)]
28. Wan, H.J.; Yuan, W.; Ruan, M.Y.; Ye, Q.J.; Wang, R.Q.; Li, Z.M.; Zhou, G.Z.; Yao, Z.P.; Zhao, J.; Liu, S.J.; et al. Identification of reference genes for reverse transcription quantitative real-time PCR normalization in pepper (*Capsicum annuum* L.). *Biochem. Biophys. Res. Commun.* **2011**, *416*, 24–30. [[CrossRef](#)]
29. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
30. Breithaupt, D.E.; Schwack, W. Determination of free and bound carotenoids in paprika (*Capsicum annuum* L.) by LC/MS. *Eur. Food Res. Technol.* **2000**, *211*, 52–55. [[CrossRef](#)]
31. Delgado-Vargas, F.; Paredes-López, O. Natural Colorants for Food and Nutraceutical Uses. In *Natural Colorants for Food & Nutraceutical Uses*; CRC Press: Boca Raton, FL, USA, 2002. [[CrossRef](#)]
32. Cervantes-Paz, B.; Yahia, E.M.; de Jesús Ornelas-Paz, J.; Victoria-Campos, C.I.; Ibarra-Junquera, V.; Pérez-Martínez, J.D.; Escalante-Minakata, P. Antioxidant activity and content of chlorophylls and carotenoids in raw and heat-processed Jalapeño peppers at intermediate stages of ripening. *Food Chem.* **2014**, *146*, 188–196. [[CrossRef](#)] [[PubMed](#)]
33. Giuffrida, D.; Dugo, P.; Torre, G.; Bignardi, C.; Cavazza, A.; Corradini, C.; Dugo, G. Characterization of 12 *Capsicum* varieties by evaluation of their carotenoid profile and pungency determination. *Food Chem.* **2013**, *140*, 794–802. [[CrossRef](#)] [[PubMed](#)]
34. Moehs, C.P.; Tian, L.; Osteryoung, K.W.; Dellapenna, D. Analysis of carotenoid biosynthetic gene expression during marigold petal development. *Plant Mol. Biol.* **2001**, *45*, 281–293. [[CrossRef](#)] [[PubMed](#)]
35. Ronen, G.; Carmel-Goren, L.; Zamir, D.; Hirschberg, J. An alternative pathway to β -carotene formation in plant chromoplasts discovered by map-based cloning of β and *old-gold* color mutations in tomato. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 11102–11107. [[CrossRef](#)]

36. Cazzonelli, C.I.; Pogson, B.J. Source to sink: Regulation of carotenoid biosynthesis in plants. *Trends Plant Sci.* **2010**, *15*, 266–274. [[CrossRef](#)] [[PubMed](#)]
37. Burkhardt, P.K.; Beyer, P.; Wünn, J.; Klöti, A.; Armstrong, G.A.; Schledz, M.; Lintig, J.V.; Potrykus, I. Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J.* **1997**, *11*, 1071–1078. [[CrossRef](#)] [[PubMed](#)]
38. Zhao, W.E.; Lv, P.; Gu, H.H. Studies on carotenoids in watermelon flesh. *Agric. Sci.* **2013**, *4*, 13–20. [[CrossRef](#)]
39. Ukibe, K.; Hashida, K.; Yoshida, N.; Takagi, H. Metabolic engineering of *Saccharomyces cerevisiae* for astaxanthin production and oxidative stress tolerance. *Appl. Environ. Microbiol.* **2009**, *75*, 7205–7211. [[CrossRef](#)]
40. Breitenbach, J.; Visser, H.; Verdoes, J.C.; Ooyen AJ, J.V.; Sandmann, G. Engineering of geranylgeranyl pyrophosphate synthase levels and physiological conditions for enhanced carotenoid and astaxanthin synthesis in *Xanthophyllomyces dendrorhous*. *Biotechnol. Lett.* **2011**, *33*, 755–761. [[CrossRef](#)]
41. Csernetics, A.; Nagy, G.; Iturriaga, E.A.; Szekeres, A.; Eslava, A.P.; Vágvölgyi, C.; Papp, T. Expression of three isoprenoid biosynthesis genes and their effects on the carotenoid production of the zygomycete *Mucor circinelloides*. *Fungal Genet. Biol.* **2011**, *48*, 696–703. [[CrossRef](#)]
42. Lückner, J.; Bouwmeester, H.J.; Schwab, W.; Blaas, J.; Van Der Plas, L.H.; Verhoeven, H.A. Expression of Clarkia S-linalool synthase in transgenic petunia plants results in the accumulation of S-linalyl- β -D-glucopyranoside. *Plant J.* **2001**, *27*, 315–324. [[CrossRef](#)] [[PubMed](#)]
43. Kuntz, M.; Römer, S.; Suire, C.; Hugueney, P.; Weil, J.H.; Schantz, R.; Camara, B. Identification of a cDNA for the plastid-located geranylgeranyl pyrophosphate synthase from *Capsicum annuum*: Correlative increase in enzyme activity and transcript level during fruit ripening. *Plant J.* **1992**, *2*, 25–34. [[CrossRef](#)]
44. Dyachenko, E.A.; Filyushin, M.A.; Efremov, G.I.; Dzhos, E.A.; Shchennikova, A.V.; Kochieva, E.Z. Structural and functional features of phytoene synthase isoforms *PSY1* and *PSY2* in pepper *Capsicum annuum* L. cultivars. *Vavilovskii Zhurnal Genet Selektii.* **2020**, *24*, 687–696. [[CrossRef](#)]
45. Jang, S.J.; Jeong, H.B.; Jung, A.; Kang, M.Y.; Kim, S.; Ha, S.H.; Kwon, J.K.; Kang, B.C. *Phytoene synthase 2* can compensate for the absence of *PSY1* in the control of color in *Capsicum* fruit. *J. Exp. Bot.* **2020**, *71*, 3417–3427. [[CrossRef](#)] [[PubMed](#)]
46. Peguero-Pina, J.J.; Gil-Pelegrín, E.; Morales, F. Three pools of zeaxanthin in *Quercus coccifera* leaves during light transitions with different roles in rapidly reversible photoprotective energy dissipation and photoprotection. *J. Exp. Bot.* **2013**, *64*, 1649–1661. [[CrossRef](#)] [[PubMed](#)]
47. Pogson, B.J.; Rissler, H.M. Genetic manipulation of carotenoid biosynthesis and photoprotection. *Philos. Trans. R. Soc. B Biol. Sci.* **2000**, *355*, 1395–1403. [[CrossRef](#)]
48. Tian, L.; Magallanes-Lundback, M.; Musetti, V.; DellaPenna, D. Functional analysis of β - and epsilon-ring carotenoid hydroxylases in *Arabidopsis*. *Plant Cell* **2003**, *15*, 1320–1332. [[CrossRef](#)]
49. Liu, Y.H.; Lv, J.H.; Liu, Z.B.; Wang, J.; Yang, B.Z.; Chen, W.C.; Ou, L.J.; Dai, X.Z.; Zhang, Z.Q.; Zou, X.X. Integrative analysis of metabolome and transcriptome reveals the mechanism of color formation in pepper fruit (*Capsicum annuum* L.). *Food Chem.* **2020**, *306*, 125629. [[CrossRef](#)]
50. Wang, Q.; Cao, T.J.; Zheng, H.; Zhou, C.F.; Wang, Z.; Wang, R.; Lu, S. Manipulation of Carotenoid Metabolic Flux by Lycopene Cyclization in Ripening Red Pepper (*Capsicum annuum* var. *conoides*) Fruits. *J. Agric. Food Chem.* **2019**, *67*, 4300–4310. [[CrossRef](#)]
51. Blas, A.L.; Ming, R.; Liu, Z.Y.; Veatch, O.J.; Paull, R.E.; Moore, P.H.; Yu, Q.Y. Cloning of the papaya chromoplast-specific lycopene β -cyclase, *Cpcyc-b*, controlling fruit flesh color reveals conserved microsynteny and a recombination hot spot. *Plant Physiol.* **2010**, *152*, 2013–2022. [[CrossRef](#)]
52. Ahrazem, O.; Rubio-Moraga, A.; López, R.C.; Gómez-Gómez, L. The expression of a chromoplast-specific lycopene beta cyclase gene is involved in the high production of saffron's apocarotenoid precursors. *J. Exp. Bot.* **2010**, *61*, 105–119. [[CrossRef](#)]
53. Bouvier, F.; Hugueney, P.; d'Harlingue, A.; Kuntz, M.; Camara, B. Xanthophyll biosynthesis in chromoplasts: Isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid. *Plant J.* **1994**, *6*, 45–54. [[CrossRef](#)]
54. Suematsu, K.; Tanaka, M.; Kurata, R.; Kai, Y.M. Comparative transcriptome analysis implied a *ZEP* paralog was a key gene involved in carotenoid accumulation in yellow-fleshed sweetpotato. *Sci. Rep.* **2020**, *10*, 20607. [[CrossRef](#)]
55. Tian, S.L.; Li, Z.; Li, L.; Shah SN, M.; Gong, Z.H. Analysis of tandem repeat units of the promoter of Capsanthin/capsorubin synthase (*Ccs*) gene in pepper fruit. *Physiol. Mol. Biol. Plants* **2017**, *23*, 685–691. [[CrossRef](#)] [[PubMed](#)]
56. Lang, Y.Q.; Yanagawa, S.; Sasanuma, T.; Sasakuma, T. Orange Fruit Color in *Capsicum* due to deletion of capsanthin-capsorubin synthesis gene. *Breed. Sci.* **2004**, *54*, 33–39. [[CrossRef](#)]
57. Li, Z.; Wang, S.; Gui, X.L.; Chang, X.B.; Gong, Z.H. A further analysis of the relationship between yellow ripe-fruit color and the capsanthin-capsorubin synthase gene in pepper (*Capsicum* sp.) indicated a new mutant variant in *C. annuum* and a tandem repeat structure in promoter region. *PLoS ONE* **2013**, *8*, e61996. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.