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Transcript Analysis Reveals Positive Regulation of CA12g04950 on Carotenoids of Pigment Pepper Fruit under Nitrogen Reduction

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Abstract: This study investigates the relationship between nitrogen fertilization and pepper fruit color by employing five different nitrogen treatments (N1: 750 kg/hm^2, N2: 562.5 kg/hm^2, N3: 375 kg/hm^2, N4: 187.5, and N0: 0 kg/hm^2). Fruits were harvested at 30 (S1: green ripening stage), 45 (S2: color transition stage), and 60 days (S3: red ripening stage) after flowering. Subsequently, pigment content, carotenoid component content, carotenoid enzyme activity, and transcriptome sequence were analyzed, and CA12g04950 function was validated through virus-induced gene silencing (VIGS). The results indicate that a reduction in nitrogen application led to an earlier onset of fruit color breakdown, and increased the contents of total carotenoid, capsanthin, phytoene and PSY (phytoene synthase) activity, LCYB (lycopene β-cyclase) activity and CCS (capsanthin/capsorubin synthase) activity. The analysis of different expression genes indicated that the most differently expressed genes were enriched in the N1 vs. N4 comparison, with 18 genes involved in carotenoid metabolism and 16 genes involved in nitrogen metabolism. Most DE genes were enriched in the pathways of photosynthesis, porphyrin, carotenoid biosynthesis, seleno-compounds, and nitrogen metabolism. There were numerous differential transcription factor families, including ERF, bHLH, MYB, C2H2, and NAC. Pearson correlation analysis revealed a significant positive correlation between CA12g04950 expression and 11 carotenoid genes in the N4 treatment. Subsequent silencing of CA12g04950 using VIGS resulted in delayed color ripening while a significant decrease in total carotenoid content and the expression levels of carotenoid genes. In conclusion, nitrogen reduction led to an increase in carotenoid content in pigment pepper fruits. Furthermore, under nitrogen reduction, CA12g04950 positively influenced the redness of the fruits.

Keywords: pigment pepper; nitrogen fertilization; carotenoid; transcription factor

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1. Introduction

Pepper, one of the most extensively cultivated and highly demanded vegetables worldwide, accounted for approximately 40 million tons of global production and consumption in 2019 [1]. Xinjiang Province, China, serves as the foremost production hub for pigment peppers in China, with the current cultivation area of pigment peppers exceeding 33,000 hectares. Pepper fruits are widely utilized as natural colorants in both the food and cosmetics industries due to their richness in flavonoids and carotenoids [2,3].

Carotenoids are a class of tetraterpene pigments that exhibit yellow, orange-red, or red hues and contain eight double bonds [4]. The distinctive capsanthin found in chili peppers is a significant contributor to the red coloration of chili fruits, constituting approximately

46% of the total carotenoid content. In orange pepper fruits, α-carotene, β-carotene, and zeaxanthin play crucial roles in imparting the orange-yellow color, with zeaxanthin constituting 16% of the total carotenoid content [2,5]. Benefiting from the potent antioxidant properties of carotenoids, they are now extensively utilized in pharmaceuticals, food, and cosmetics industries [2], with capsanthin exhibiting the highest market demand [1].

Previous investigations have focused on elucidating the biochemical properties and physiological significance of carotenoids and flavonoids, as well as singular constituents within diverse cultivars of chili fruits. Several studies have undertaken research on the phenotypic manifestations, quantitative compositions, and chromosomal allocations of carotenoids and flavonoids within botanical specimens [6–10]. Feng [11] used three wild-type red peppers and their corresponding mutants (yellow and orange) as research materials to clone the genes involved in carotenoid biosynthesis, including GGPS, PSY, LCYB, CRTZ, ZEP, and CCS. It was discovered that in two mutant types, the complete CCS gene could not be cloned. In another mutant type (orange), although the complete CCS gene could be cloned, its gene expression level was significantly lower than that of the wild type. Therefore, it is believed that the color variation in this mutant fruit is related to the regulatory mechanism of the CCS gene. Through the integration of transcriptomics and metabolomics approaches, it was discerned that the genes CCS, NCED2, AAO4, VDE1, and CYP97C1 serve as pivotal regulators in the synthesis of carotenoids [12].

Modulating the expression levels of key genes involved in the carotenoid biosynthesis pathway significantly influences the biosynthesis of carotenoids. Using gene editing techniques to induce specific overexpression of the PSY gene has been demonstrated as an effective strategy to enhance the content of carotenoids in plant tissues [13,14]. Concomitant overexpression of the DXS, PSY, and CRTI genes leads to a substantial augmentation in carotenoid content in rice endosperm [15]. After silencing the PSY, CCS, LCYB, and CRTZ genes in peppers, there are changes in the levels of precursor compounds essential for capsanthin synthesis. Consequently, this disrupts the normal coloration of chili fruit, causing a transition from red to yellow or orange [16,17]. Transcription factors, as a crucial class of signaling molecules, can directly or indirectly regulate the synthesis and accumulation of carotenoids in plants [18]. In citrus, CsMADS5 positively regulates carotenoid synthesis in fruits by directly binding to the promoters of PSY, PDS, and CYB1 and by initiating their transcription [19]. In tomato, a model plant of the Solanaceae family, numerous studies have identified the involvement of transcription factors in the regulation of carotenoid biosynthesis [20]. Overexpression of SlWRKY35 can increase the content of lutein in tomato fruit, consequently enhancing the accumulation of carotenoids in the fruit [21]. Overexpression of SlNAC1 reduces ethylene synthesis in tomato fruit, leading to a decrease in carotenoid content and resulting in fruit appearing yellow or orange [22]. Recent studies in peppers have revealed that a specific R-R-type MYB transcription factor can promote carotenoid biosynthesis, thereby facilitating the accumulation of capsanthin in peppers [23].

In addition to the influence of genes on the composition and content of carotenoids, research has found that cultivation measures such as fertilization and pruning can change the content and type of plant pigments [24,25]. Nitrogen (N) is one of the significant influencing factors affecting plant growth and development, as well as fruit quality and yield. Studies have demonstrated that within a specific range of nitrogen application, an increase in the amount of urea applied leads to an increase in capsanthin content in pepper fruits [26,27]. Research indicates that when 750 kg/ha of nitrogen is applied to *Lycium chinense* Miller, the content of flavonoids and carotenoids in the fruits reaches its highest level [28]. There are also studies indicating that the content of carotenoids in plants does not continuously increase with nitrogen fertilizer concentration. Research indicates that increasing nitrogen fertilization decreases the content of lycopene and total carotenoids in tomato fruit [29]. Currently, research on carotenoids in peppers primarily focuses on the variations in carotenoid components and total carotenoid content across different varieties. However, there is relatively scant investigation into the impacts of nitrogen fertilizer application on metabolites related to chili pigment and gene regulation.

The relationship between cultivation factors like nitrogen fertilizer and fruit color, as well as carotenoid formation, remains ambiguous [30].

To elucidate the effects of nitrogen fertilizer on pepper fruit color, this study analyzed changes in carotenoid components' content, enzyme activities, and gene expression of pigment pepper fruits under different urea concentrations. The transcription mechanism of nitrogen reduction on fruit carotenoids is explored through the analysis of DE gene (DEG) and metabolic pathways. Then, virus-induced gene silencing (VIGS) is employed to confirm the function of the carotenoid DE gene that is significantly expressed under the N4 treatment. This study aims to provide a theoretical guide for optimizing fertilization to enhance the color and commercial quality of pepper fruits.

2. Materials and Methods

2.1. Materials and Design

The experiment was conducted in Shihezi City, Xinjiang Province, China. The selected variety was the pigment pepper named 'Honglong 23', developed by Xinjiang Tianjiaohongan Company (Shihezi, China). Pepper seedlings, aged 60 days according to the calendar, were transplanted into plots measuring 12 m in length and 1 m in width. Each plot comprised two rows, with a spacing of 50 cm between the rows, and 30 cm between the plants. Drip irrigation under mulch was utilized for water and fertilizer supply. Nitrogen fertilizer, in the form of urea (N $\geq$ 46%), was applied 10 days after transplanting.

The urea dosages was set as follows: 750 kg/hm^2 (N1, widely used in the fields and serving as the control for this study), 562.5 kg/hm^2 (N2, a 25% reduction in urea compared to N1), 375 kg/hm^2 (N3, a 50% reduction in urea compared to N1), 187.5 kg/hm^2 (N4, a 75% reduction in urea compared to N1), and N0 without urea application. Urea was applied once every 10 days for a total of 8 times after 10 days of planting (Table 1). The soil's organic matter, alkaline-dissolved nitrogen, available phosphorus, and available potassium contents were 11.8, 43.7, 18.9, and 155 mg/kg, respectively. Three replicate plots were established for each urea dosage. Fruits were sampled at 30 (S1: green ripening period), 45 (S2: color transition period), and 60 (S3: red ripening period) days after blooming. Each parameter was measured with three biological repeats.

Table 1. Fertilizer dosage was applied on the pigment pepper.

Treatment	Urea (kg/hm^2)	KH_2PO_4 (kg/hm^2)	Calcium, Magnesium, and Sulfur Nutrients (kg/hm^2)	Micronutrient Fertilizer on Leaf (%)
N1	750	200	22.5	0.5%
N2	562.5	200	22.5	0.5%
N3	375	200	22.5	0.5%
N4	187.5	200	22.5	0.5%
N0	0	200	22.5	0.5%

2.2. Determination of Fruit Pigment Content and Enzyme Activity

Three pepper plants from each treatment were selected, and three fruits were sampled, totaling 9 fruits to serve as experimental samples. Approximately 0.1 g of mixed fruit was frozen and ground into powder using liquid nitrogen. The resulting sample underwent extraction with a 10 mL mixture (2:1, v/v) of 100% ethanol and acetone using an ultrasonic instrument for 30 min. Subsequently, the mixture was centrifuged at 12,000 rpm for 20 min, and the supernatant was collected. This extraction process was repeated 2 to 3 times, and the collected supernatant was adjusted to a constant volume of 25 mL. The solution was then stored in a brown glass bottle for the measurement of carotenoid contents using a spectrophotometer (UV-2600i) (Shimadzu, Shanghai, China) as described by Burgos et al. [31].

Enzyme activities of phytoene synthase (PSY), capsanthin/capsorubin synthase (CCS), and lycopene β-cyclase (LCYB) were analyzed using ELISA kits sourced from Shanghai

Enzyme-linked Biotechnology Co., Ltd., Shanghai, China [32]. The samples were sent to Wuhan Maiwei Metabolism Company (Wuhan, China) for carotenoid friction determination using HPLC-MS, as described in [30,33].

2.3. RNA Extraction

Total RNA was isolated from N1, N3, and N4 fruits at stage S1 using Trizol (Invitrgen, Beijing, China) according to the manufacturer's protocol. The extracted RNAs were cleaned using an RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA).

2.4. RNA Sequencing

RNA was examined by Nanodrop spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA) and a Nano 6000 Assay Kit from the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). A total of 1 μg RNA per sample was used to generate a cDNA library by NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs Inc.; Ipswich, MA, USA). Messenger RNA (mRNA) was enriched by oligo beads, and synthesized cDNA. Used AMPure XP (Beckman Coulter, Brea, CA, USA) beads to select cDNA of approximately 400–500 bp in size, followed by PCR amplification, The PCR products were then purified again using AMPure XP beads to obtain 9 libraries, and the Sequel™ Sequencing Kit 2.0 was used to sequence 9 cDNA libraries. Then, 9 RNA-seq libraries were sequenced by the Illumina HiSeq 6000 (San Diego, CA, USA). cDNA library construction and RNA-seq were performed by Personal Bioinformatics Technology Co. (Shanghai, China). Low-quality reads were removed using Cutadapt 1.9.1 software, and the clean reads were mapped to the Pepper_1.55 reference genome (from the Sol Genomics Network) with the help of HISAT2 [34]. Based on the established reference genome, a mapping-based assembly approach was employed using the software StringTie 2.1.4 for assembling and splicing the mapped reads. The fragments per kilobase of transcript per million mapped reads (FPKM) for each gene was computed by considering the gene length and the count of reads mapped to it [35]. The raw transcriptome data underwent principal components analysis (PCA) and Pearson correlation based on gene expression profiles obtained from fragments per kilobase of exon per million fragments mapped (FPKM) among the nine samples using the package R 4.1.1. The identified transcripts underwent functional annotation against seven public databases, namely NR [36], KEGG [37], Swissprot [38], GO [39], and eggNOG [40], utilizing predefined parameters. GO enrichment analysis was carried out using GO-seq, and KEGG enrichment was performed with the hypergeometric test [41,42]. Differential expression analysis was performed using the DESeq R package [43]. Differentially expressed genes were identified with $|\log2FoldChange| > 1$ and a significance p-value < 0.05. Transcription factor families were analyzed using the PlanTFDB database. The raw transcriptome data were deposited into the NCBI SRA (BioProject ID: PRJNA1087067).

2.5. Quantitative Real-Time PCR

To validate the RNA-seq data, 15 genes from carotenoid pathways detected by KEGG functional analysis were selected for quantitative real-time PCR (qRT-PCR). RNA extraction was performed using the RNAprep Pure Plant Kit (DP432; Tiangen Technologies, Beijing, China), and cDNA was synthesized with the HyperScript III RT Super Mix for qPCR with gDNA Remover kit (EnzyArtisan Biotech Co., Ltd., Eveleigh, NSW, Australia). The qRT-PCR was conducted on a real-time PCR detection system (CFX96) using 2 × S6 Universal SYBR qPCR Mix (EnzyArtisan Biotech Co., Ltd., Eveleigh, NSW, Australia), and each sample had three replications. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expressions of target genes [44]. To standardize the relative expression, the actin gene (Actin Actin-depolymerizing factor 7 [*T. aestivum*]) was employed as the housekeeping gene [12]. The qRT-PCR primers were designed using Primer 5.0 software [45], and all primer sequences are provided in Table 2.

Table 2. qRT-PCR and VIGS primers.

Gene	Forward Primer	Reverse Primer
qRT-PCR Primers		
CA01g13640 (Indole-3-acetaldehyde oxidase-like)	CAGATGGATTGGTTGTTTCA	TGTCCGCTGTTTAGCACTT
CA04g18710 (Abscisic acid 8′-hydroxylase)	AAGGGAAAGGAAAGAGAAGAAG	CAAGAAACAACTCAACAACCC
CA11g15430 (Aldehyde oxidase 4)	ACGAACCTTACCAGCAAATC	ATGACAGAGTCCACCTCCA
CA03g25820 (BCH)	CCGCCACTTCTCTTCTTCT	CGCTTTGTTTTCCACTTTG
CA01g09070 (Abscisic acid 8′-hydroxylase CYP707A1)	CCAACTTTACTCACAAAACCC	TTCCCCAACATTCTCTCTTT
CA12g20770 (Z-ISO)	TTTACCATTTCCCACCTCTC	TTCACCCACCAAGATTTCA
CA08g10750 (ZDS)	CATCAGTTGTGAAGATTGGG	TGAGCCAGCAAGAAAGAAA
CA10g15950 (P450)	GAAGTTGACAGAGTTTTGGGA	GACAGGTGGATGTGGATAAAG
CA12g11380 (VDE)	CTAATCCCAGTGTTCTTGTCC	CAGGTGTCCGTATTCTCCA
CA01g33030 (Abscisic acid 8′-hydroxylase CYP707A2)	TGTCATTGGTGTCATATTTGC	TATTGCCTCTTGTTCCTCTGT
CA03g36860 (PDS)	AGATGGTTGCTCGCAAAGGA	CGCGGAGAAGATCGGAATGA
CA02g10990 (ZEP)	TGCCAAACAAGCCAGGAGAA	ACACCTCATCCGTCACCCTA
CA06g22860 (CCS)	AGTGGCCTGTGAGTTGTGTT	GAGCCACCATGTACCCAGAC
CA04g04080 (PSY)	CAAAGGCAACAACGGAGAGC	CGCTCAATTCGGTCACTCCT
CA05g00080 (Lcyb)	ACGTGGAGCTCAAGGAGAGA	ACGAGCCACCATTCGTTCTT
CA02g23040 (GGPS)	ATTGAAGCAGCACAGACGGA	GAGTATTGCGCCGAGTACGA
CA03g25820 (CrtZ)	ACGAGTCACACCACAAACCA	TTTCCAACTCTTCCAGCCCC
CA12g04950	GCAATGGTGATGAAGCGCAA	AACAAACGTCAGAGGTCGGG
VIGS Primers (the underlined part represent the enzyme-cleaved homologous arm)		
TRV-CA12g04950	AAGGTTACCGAATT TGCCGATGAAGAATTGGACAG	CTCGGTACCGGATC CTCTTGCAAACAAACGTCAGAG

2.6. VIGS-Induced Transcription Factor CA12g04950 Silencing

VIGS silencing was conducted following the method described by Shi et al. [46]. CA12g04950 was amplified using P505 high-fidelity enzyme (Vazyme Biotech Co., Ltd., Nanjing, China), and the target bands were purified using gel recovery kit (DP204, Tiangen, Beijing, China). The pTRV vector was digested with EcoR1 and BamH1, and then transformed into *E. coli* DH5α (Beijing TransGen Biotech Co., Ltd., Beijing, China) by ligating the TRV2 vector with c112 ligase (Vazyme). The recombinant bacterial solution was spread on LB solid medium (KanR, Yuanye, Shanghai, China), and a single colony was selected after 24 h of incubation for PCR identification and verification.

After successful identification, the viral plasmid was extracted and transformed into the Agrobacterium strain GV3101 to obtain the recombinant vector. Monoclonal Agrobacterium was confirmed by PCR, and subsequently, the infection solution was prepared (10 mM $MgCl_2$, 10 mM MES, 200 μM AS).

The pTRV1 was mixed with TRV2 in a 1:1 volume ratio, with an empty TRV2 vector serving as the negative control (TRV), and no TRV as the blank control (CK). Four points at the base of green maturing pepper (S1 stage) fruits were selectively marked, and 150 μL of bacterial solution was injected into each point, with three fruits serving as replicates. Fruits treated with TRV were placed in a box with agar half filled. The box was then transferred to an artificial climate chamber set at 23 °C, 70% relative humidity, and a light cycle of 16/8 h (200 $\mu mol/m^2/s$).

Fresh samples were collected in a circular pattern around the infected area at 3, 6, 9, 12, and 15 days after silencing. The fresh samples were immediately frozen in liquid nitrogen and stored at −80 °C for the determination of carotenoid content, friction, enzyme activity, and gene expression.

2.7. Statistical Analysis

Dates were analyzed with SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and Graphpad Prims 9.5 through one-way analysis of variance (ANOVA) followed by Dunnett's test. Data are shown as the mean ± standard deviation (SD), and p values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of Nitrogen Reduction on Pigment Content of Pepper Fruit

Figure 1A illustrates a noticeable change in fruit color 30 days after urea application (S1), reaching a vibrant red hue at 45 days (S2). Fruits subjected to nitrogen reduction exhibited a consistently darker red color across stages S1 to S3, with the order of redness being N0 > N4 > N3 > N2 > N1 (Figure 1A). Analysis of total carotenoids (Figure 1B) revealed that at stages S1 and S2, compared to N1, the total carotenoids of N0 increased by 4813.71% and 2446.23%, respectively. In contrast, at the S3 stage, the total carotenoids of N3 increased by 40.12%.

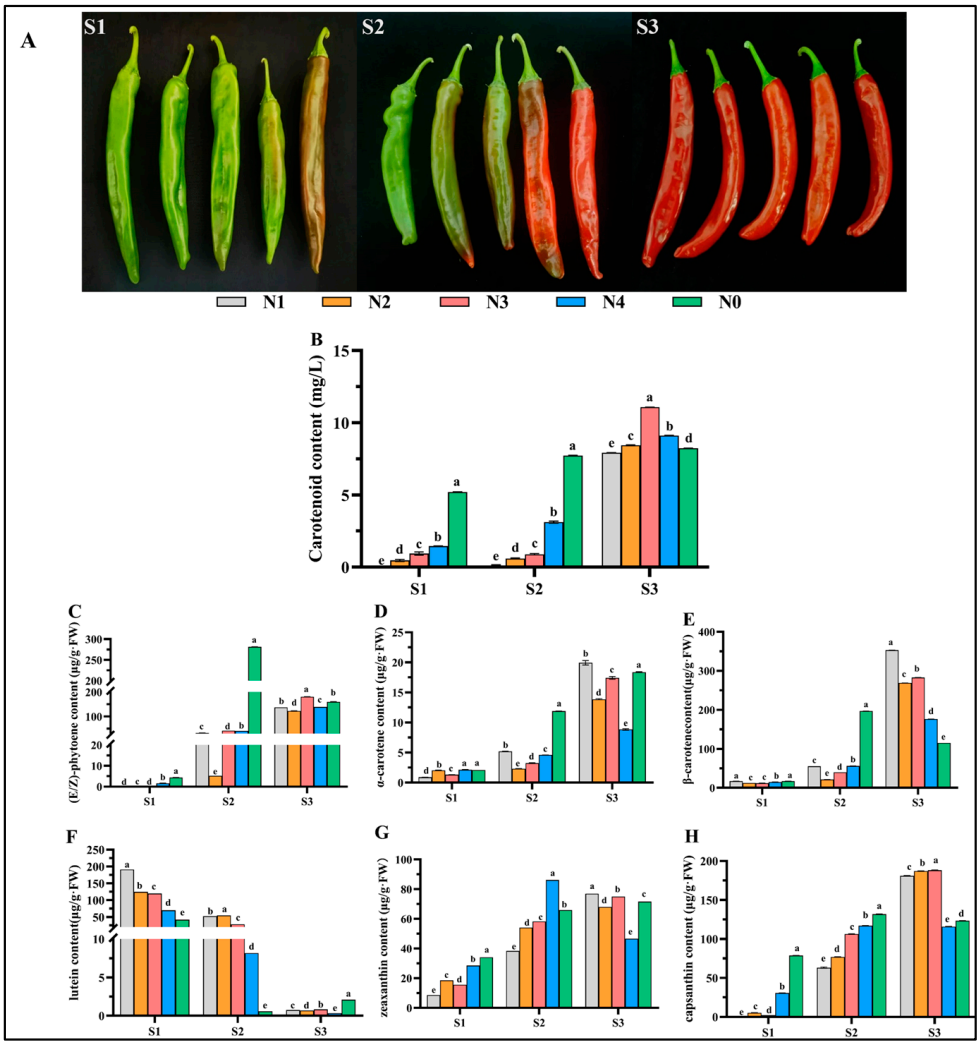


Figure 1. Phenotypes and pigment content of pepper fruits at different stages with nitrogen reduction. (**A**) Phenotypes of pigment pepper fruits. (**B**) Total carotenoids of pigment pepper fruits. (**C**–**H**) Contents of six carotenoids fractions of pigment pepper fruits. Data are presented as mean ± SD. Different letters (a, b, c, d, and e) indicate the significance of differences between nitrogen treatments ($p \leq 0.05$).

Examining the fractions of carotenoids in fruits (Figure 1C–H) unveiled that the contents of phytoene (Figure 1C), α-carotene (Figure 1D), β-carotene (Figure 1E), zeaxanthin (Figure 1G), and capsanthin (Figure 1H) were notably higher at the S3 stage, except for lutein (Figure 1F). Overall, except for N0, the contents of α-carotene and β-carotene in fruits with nitrogen reduction decreased at stages S2 and S3 compared to N1. Simultaneously, the contents of zeaxanthin and capsanthin increased. At the S2 stage, the contents of zeaxanthin and capsanthin in N4 were 86.17 μg/g-FW and 116.90 μg/g-FW, respectively, representing increases of 124.94% and 85.08% compared to N1.

3.2. Effect of Nitrogen Reduction on Carotenoid Enzyme Activity and Gene Expression in Pigment Pepper Fruit

In general, enzyme activities of PSY, LCYB, and CCS increased gradually with fruit development. Figure 2A–C illustrates that urea reduction led to an increase in PSY activity at the S3 stage and CCS activity at the S2 stage. qRT-PCR analysis (Figure 2D–I) revealed that the expressions of LCYB, CRTZ, and CCS genes increased with fruit development. There were slight decreases in GGPS and lutein gene expression with fruit development. At the S2 stage, fruits treated with urea reduction exhibited higher expression levels of GGPS, PSY, LCYB, CRTZ, and CCS genes compared to N1, and the expression of PSY, LCYB, CRTZ, and CCS in N4 increased by 5 to 25 times.

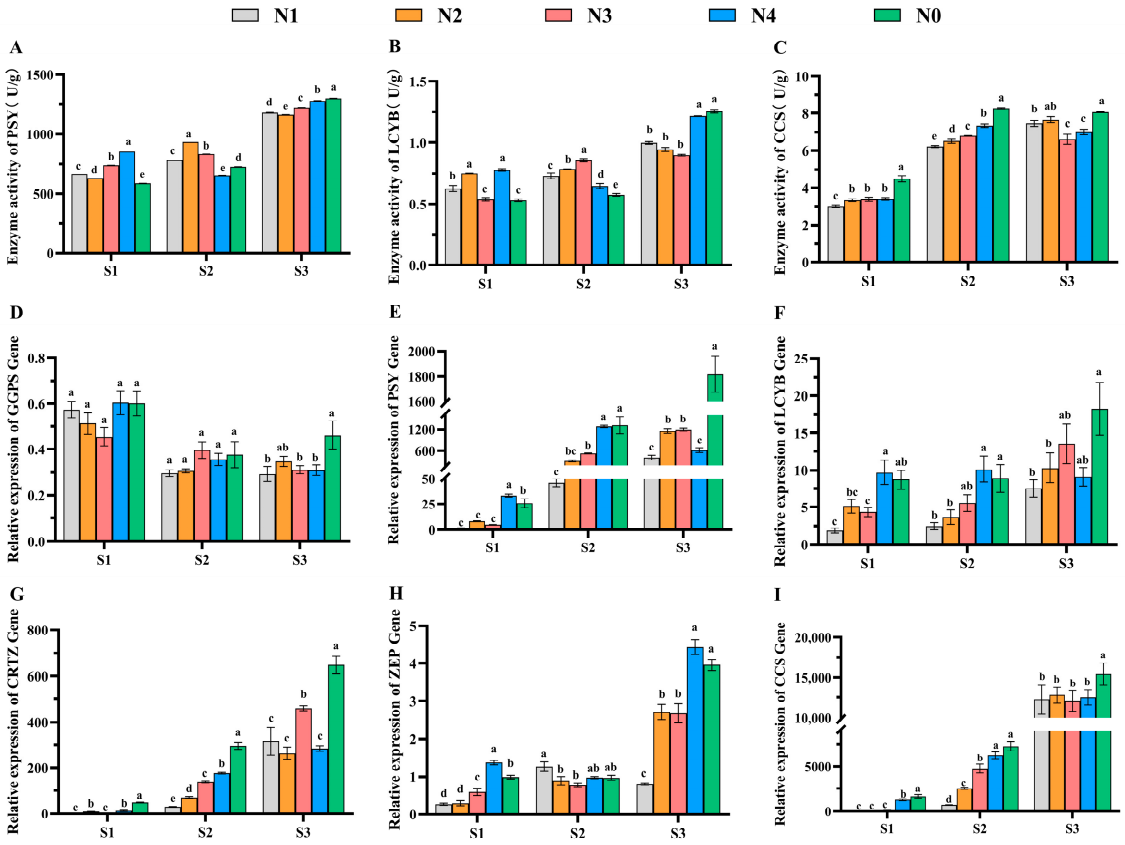


Figure 2. Enzyme activities of PSY (**A**), LCYB (**B**), CCS (**C**), and carotenoid gene expression (**D**–**I**) of pigment pepper fruits at different developmental stages under varied urea dosage. Different letters (a, b, c, d, and e) indicate the significance of differences between nitrogen treatments ($p \leq 0.05$).

3.3. Transcription and Metabolic Analysis of Pigment Pepper Fruits Treated with Nitrogen Reduction

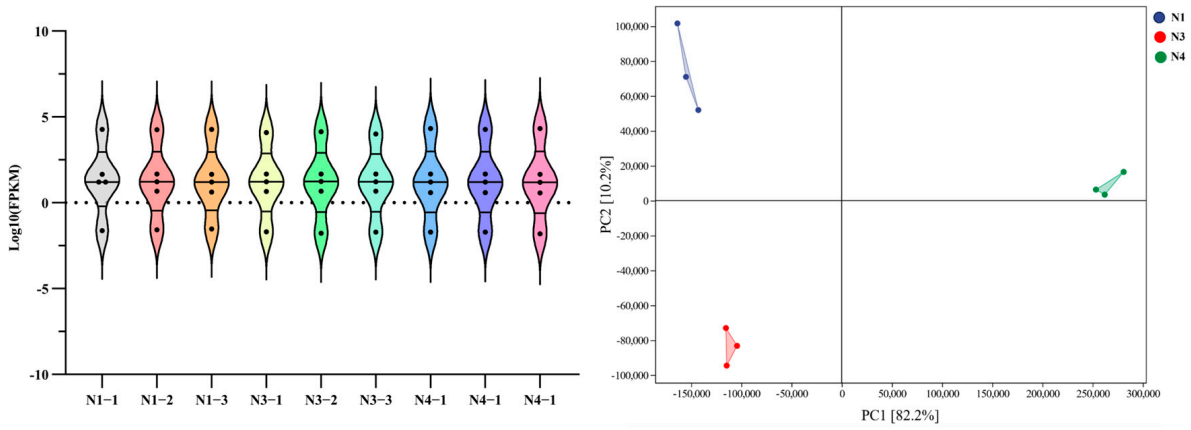
3.3.1. RNA Sequence Quality

Table 3 shows that a minimum of 84.01% of the reads aligned with the pepper reference genome, with Q30 scores ranging from 93.16% to 94.05%. These results indicate that the sequences are of high quality.

Table 3. RNA sequence qualities of pigment pepper fruits at S2 stage under nitrogen reduction.

Sample	Raw Read Number	Clean Reads	Total Mapped	% of Clean Reads	Raw Q30 Rate
N1_1	39,442,000	36,904,792	31,243,116 (84.66%)	93.57	93.61
N1_2	37,259,702	34,983,966	29,631,058 (84.70%)	93.89	93.54
N1_3	45,200,930	42,473,750	36,031,689 (84.83%)	93.97	93.9
N3_1	38,890,422	36,556,266	31,022,737 (84.86%)	94.00	93.32
N3_2	43,611,226	40,987,508	34,803,261 (84.91%)	93.98	93.82
N3_3	48,362,080	45,251,532	38,094,608 (84.18%)	93.57	94.05
N4_1	46,759,740	43,958,724	37,229,386 (84.69%)	94.01	93.7
N4_2	48,074,918	44,964,778	37,776,074 (84.01%)	93.53	93.6
N4_3	46,333,098	43,253,842	36,702,783 (84.85%)	93.35	93.16

FPKM analysis indicated high quality and repeatability in the nine samples (Figure 3A). Principal component analysis (PCA) was used to analyze the gene expression levels of multiple samples from the same period. It was found that the differences in gene expression between N1 and N4 treatments were most significant in pepper fruits (Figure 3B). In the PCA analysis, PCA1 accounted for 79.65% and PCA2 accounted for 10.2%. The grouping in PCA suggests that the differences between N1 and N4 treatment fruits were most pronounced during the same developmental stages.



(**A**) (**B**)

Figure 3. Distribution of kilobase per million exon kilobase fragment mapping (FPKM) in rNA-seq of pigment pepper fruits under urea reduction. (**A**) Violin plot; (**B**) FPKM-based principal component analysis.

Differential genes of transcription metabolism (DEGs) were calculated based on log2 fold change (FC) ≥ 1 and false discovery rate (FDR)-corrected p-value ≤ 0.05. In the comparison of N1 vs. N4 and N3 vs. N4, there were 5321 and 4590 differentially expressed genes, respectively, which were significantly higher than that of N1 vs. N3 (Figure 4A). The Venn plot (Figure 4B) showed that there were 1522 co-expressed genes, while 778 and 3799 genes were specifically expressed in the comparison of N1 vs. N3 and N1 vs. N4. Among DEGs in the two pairwise groups, 8134–17,192 genes were successfully annotated to one of the five public databases (Figure S1).

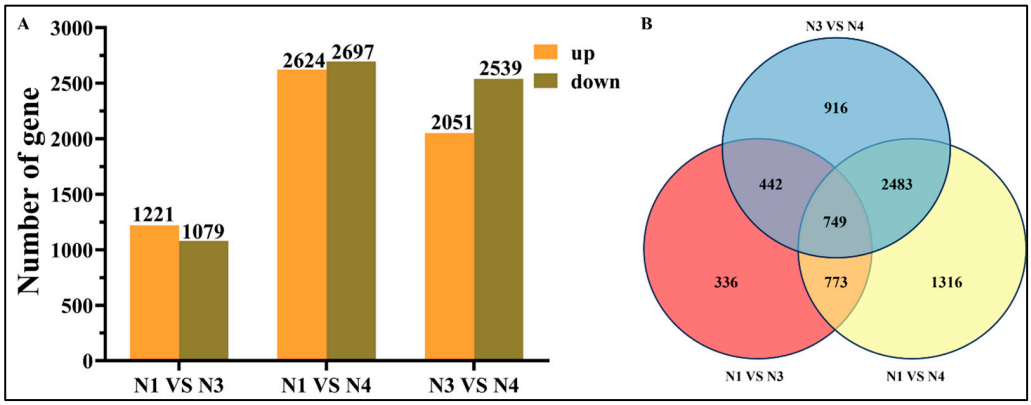


Figure 4. Analysis of differentially expressed genes (DEGs) in RNA-Seq of pigment pepper fruits under urea reduction. (**A**) Number of upregulated and downregulated DEGs; (**B**) Venn diagrams representing the intersection of DEGs between different nitrogen treatments.

3.3.2. Analysis of GO and KEGG Enrichment in Pigment Pepper Fruits under Nitrogen Reduction

Differentially expressed genes (DEGs) can be categorized into various metabolic pathways based on biological processes, molecular functions, and cellular components. Figure 5A presents the top 10 enriched metabolic pathways of DEGs, including porphyrin-containing compound metabolic processes, tetrapyrrole metabolic processes, pigment metabolic processes, chloroplast envelope, cell wall, external encapsulating structure, hydrolyzing O-glycosyl compounds, catalytic activity, and acting on glycosyl bonds. Notably, the most differently expressed genes were enriched in the N1 vs. N4 comparison (Table S1; Figure 5A, right column). In the pigment metabolic processes and pigment biosynthesis processes, −log10 (p-value) of DEGs were 7.61 and 6.96 in N1 vs. N4, which were much higher than those of N1 vs. N3 (0.96, 0.94) and N3 vs. N4 (4.61, 4.29). There was a similar trend in the chloroplast envelope, where carotenoid was produced (Figure 5A, right column).

In the KEGG analysis (Figure 5B), the DEGs from N1 vs. N3, N1 vs. N4, and N3 vs. N4 were annotated to 124, 124, and 126 metabolic pathways, respectively (Table S2), and the top five enriched pathways were photosynthesis-antenna proteins, porphyrin metabolism, carotenoid biosynthesis, seleno-compound metabolism, and nitrogen metabolism. log10(p-value) of porphyrin metabolism and carotenoid biosynthesis were 8.27 and 4.10 in N1 vs. N4 which were higher than that of N1 vs. N3 (2.69, 2.02) (Figure 5B, right column). Specifically, the most differently expressed genes were enriched in N1 vs. N4, with 18 genes involved in carotenoid metabolism and 16 genes involved in nitrogen metabolism. Furthermore, carotenoid content genes Z-ISO and AO 4 were upregulated, while the SDRs were downregulated under both the N3 and N4 treatments. Nitrogen metabolism genes NRT 2.5 and NRT 2.7 were upregulated in N3 and N4 treatments. Z-ISO participated in regulating the biosynthesis of strigolactone (SL) [47], and SL is an important mediator involved in nitrogen regulation within the plant [48]. The NRTs were involved in the absorption of NO^{3-} under low nitrogen conditions [49].

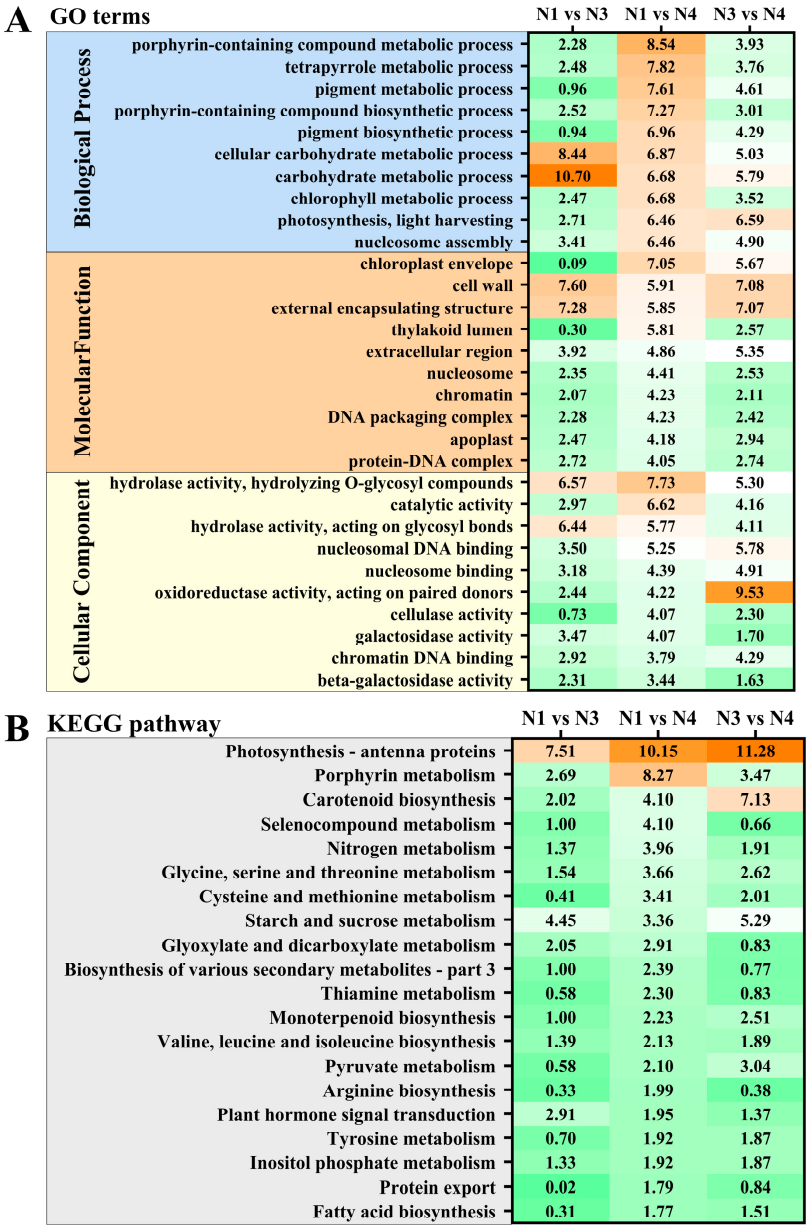


Figure 5. Enrichment analysis of DEGs metabolic pathways of pigment pepper fruits under urea reduction. (**A**) GO enrichment analysis; (**B**) KEGG enrichment analysis. The numbers in the figure represent the −log(*p*-value). Different colors represent the abundance of enriched genes, with orange indicating a higher number of genes, green indicating a lower number of genes, and white representing an intermediate level between the two.

3.3.3. Analysis of Differentially Expressed Genes (DEGs) in Carotenoid Metabolism of Pigment Pepper Fruits under Nitrogen Reduction

To validate the precision of the RNA-seq data, the expression of nine carotenoid genes was measured using qRT–PCR (Figure 6). The results demonstrated that the transcription levels of these nine carotenoid genes were consistent with the RNA-seq expression, indicating the reliability of the transcription data in this study.

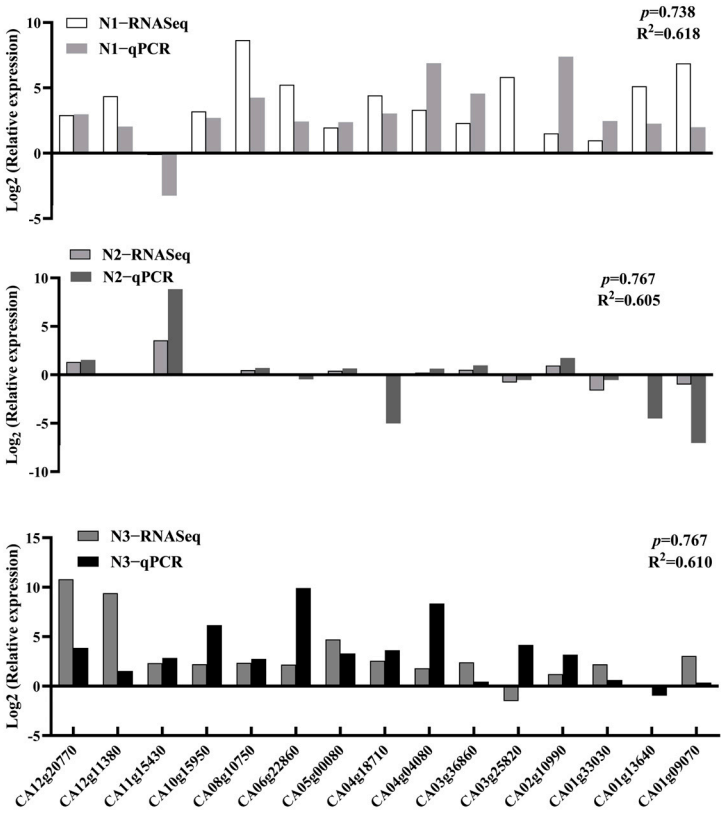


Figure 6. Correlation analysis between RNA-seq and qRT−PCR for carotenoid genes in pepper fruits under urea reduction.

3.3.4. Analysis of Transcription Factors in Pepper Fruits under Nitrogen Reduction

The analysis revealed the presence of numerous differential transcription factor families, including ERF, bHLH, MYB, C2H2, and NAC (Figure 7), under different urea dosages. The NAC family, crucially related to pigment metabolism [50,51], was selected as the focus of subsequent research. The analysis of NAC transcription factors in N1 vs. N4 showed that 12 NAC members, with the names CA02g22730, CA03g21000, CA04g14740, CA05g04410, CA06g11310, CA06g21590, CA07g21320, and CA08g15460, exhibited significant changes in expression levels. Correlation analysis (Figure 7B,C) demonstrated a high correlation between the expression of carotenoid genes such as ABA 8′, BCH, PSY, SDRS, CCS, and VDE and NAC transcription factors. Among these genes, the expression of CA12g04950 in N4 was significantly higher than that in N1.

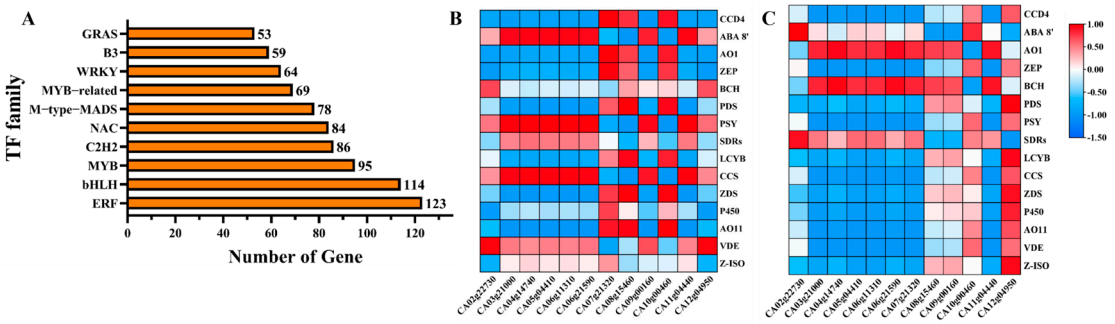


Figure 7. Analysis of differential transcription factors in pigment pepper fruits under urea reduction. (**A**) Top 10 transcription factor families; (**B**,**C**) heatmap of Pearson correlation analysis between NAC family and carotenoid genes of N1 (**B**) and N4 (**C**).

3.4. Regulation of CA12g04950 on Pepper Fruits' Carotenoids

CA12g04950 was silenced using the VIGS technique (Figure 8). As shown in Figure 8A, the fruit color of the control group (CK) and the empty vector (TRV) began to turn red at 9 days after silencing, while the fruits of TRV-CA12g04950 began to change color at 12 days. By the 15th day of silencing, the fruits of CK and TRV were fully red, whereas only part of the fruit surface of TRV-CA12g04950 became red. At day 6, the total chlorophyll content of TRV-CA12g04950 fruits (Figure 8C,D) increased by 13.03% and 24.75% compared to CK and TRV. At day 12, compared to CK and TRV, the chlorophyll content of TRV−CA12g04950 increased by 74.67% and 42.33%, respectively, while the total carotenoid content decreased by 87.31% and 69.15%, respectively. qRT−PCR indicated that the expression of the CA12g04950 gene in CK and TRV increased initially and then decreased (Figure 8E). In contrast, the expression level of CA12g04950 in silenced fruit was significantly lower than that in CK and TRV from day 3 to day 12. Specifically, at day 9, the CA12g04950 expression in silenced fruits decreased by 58.17% and 42.39%, respectively, compared to CK and TRV, but by day 15, the CA12g04950 expression increased by 51.3% and 39.2%, respectively. These results suggest that silencing CA12g04950 can inhibit the carotenoid content within a certain time.

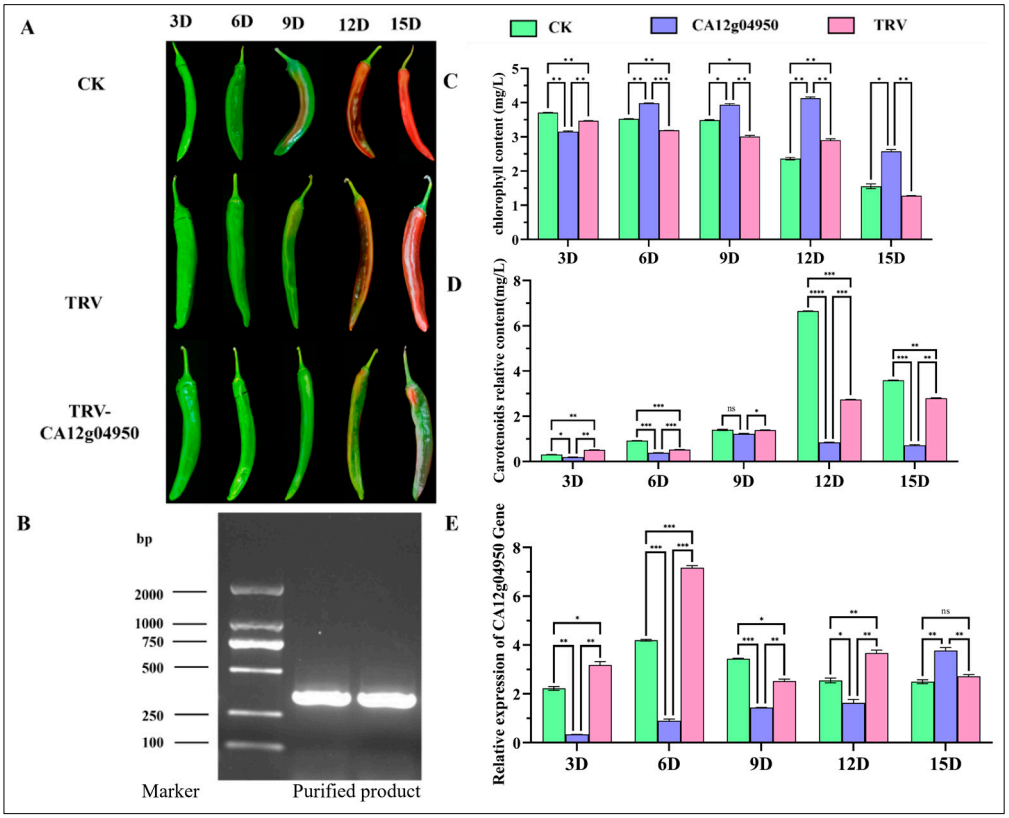


Figure 8. Silencing of the CA12g04950 gene in pigment pepper fruits using VIGS. (**A**) Fruits' colors after silencing; (**B**) electropherogram of silenced CA12g04950 gene (left: DL2000 marker; middle and right: purified product); (**C**) total chlorophyll content of silenced fruits; (**D**) total carotenoid content of silenced fruits; (**E**) expression level of CA12g04950 in silenced fruits; * indicates significant differences between different treatments (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$), ns indicates no significant differences between different treatments.

Determining the content of carotenoid components in the fruit after silencing (Figure 9A–D) revealed significant differences. The content of capsaicinoids in the sixth-day silenced fruit was 2.02 mg/kg·FW, which marked a decrease of 96.6% and 83.3% compared to the CK and TRV groups (Figure 9D), respectively. Similar trends were observed in the content of other carotenoids compared to the control and empty vector groups. At 12D,

the content of capsaicinoids was 93.08% and 85.44% lower than that of the CK and TRV groups, respectively (Figure 9D), consistent with the phenotypic results. These results were consistent with the fruit phenotype. After 6 days of silencing, the enzyme activities of PSY, LCYB, and CCS in TRV-CA12g04950 were lower than that in CK and TRV; however, the CCS enzyme activity increased rapidly after 12 days.

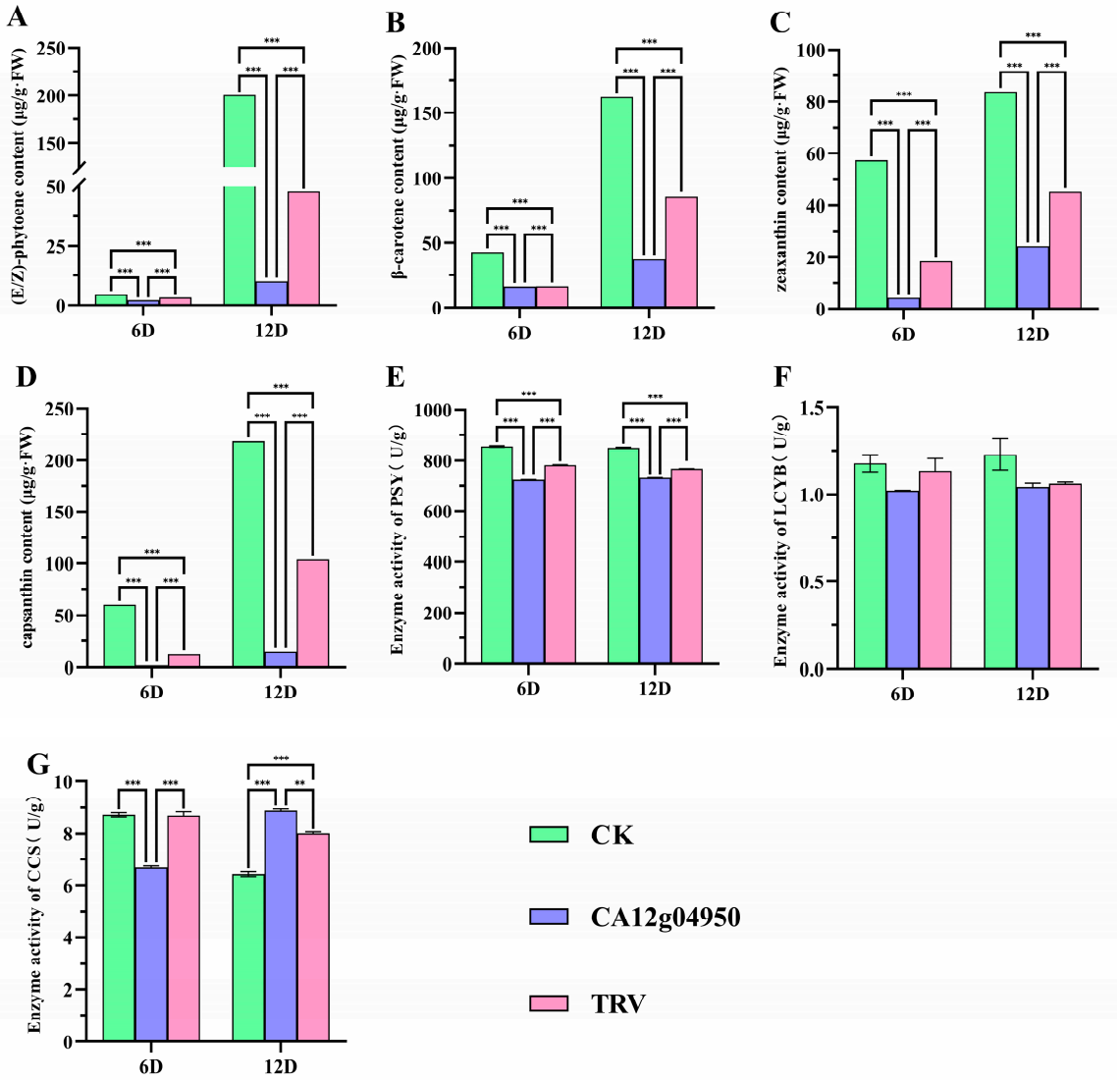


Figure 9. (**A**–**D**) Carotenoid Component Contents and (**E**–**G**) Metabolic Enzyme Activities of Pigment Pepper Fruits after Silencing the CA12g04950 Gene. (** $p \leq 0.01$, *** $p \leq 0.001$).

qRT-PCR analysis indicated that within 9 days of silencing, the transcription levels of PSY, LCYB, CRTZ, ZEP, and CCS genes were significantly suppressed (Figure 10A–F). After 6 days of silencing, PSY expression in TRV-CA12g04950 fruits decreased by 93.74% and 85.67% compared to CK and TRV, and by 41.38% and 62.49% after 12 days. Within 6 days, the transcription levels of ZEP decreased by 94.29% and 93.51% compared to CK and TRV. Throughout the silencing period, the expression level of CCS was significantly reduced. From days 6 to 15, the CCS expression in TRV-CA12g04950 was reduced by 97.29% to 24.18% compared to CK and by 97.68% to 25.57% compared to TRV.

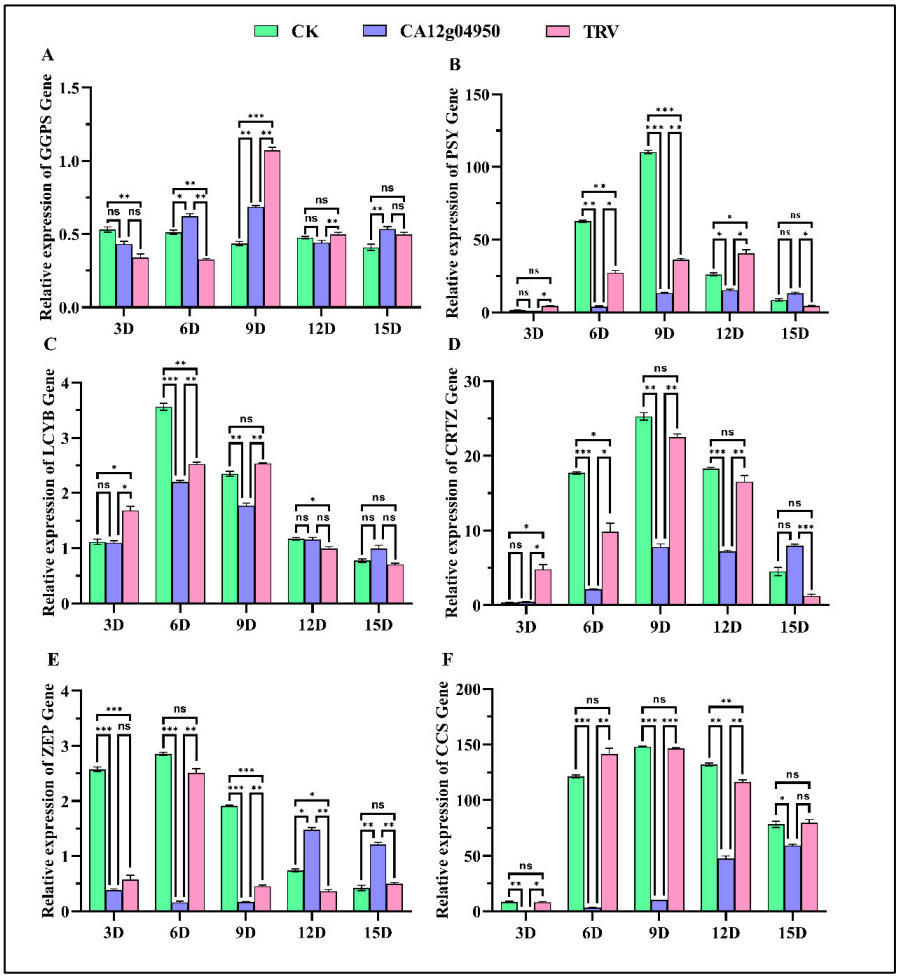


Figure 10. (**A**–**F**) Expression level of carotenoids' genes of pigment pepper fruits after silencing the CA12g0495 (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), ns indicates no significant differences between different treatments.

4. Discussion

4.1. Nitrogen Fertilizer and Pigment

Color serves as a critical quality indicator for crops, with plant color being primarily determined by flavonoids, chlorophyll, and carotenoids [52]. Nitrogen emerges as a crucial factor influencing plant color, as its concentration affects metabolic processes and chlorophyll content [53]. Notably, the contents of chlorophyll, lutein, β-carotene, neoxanthin, purple xanthin, and anther xanthin in sugar beets increased significantly as the ratio of NH_4^+ to NO_3^- decreased [54,55].

Chang [56] observed that when nitrogen fertilizer application decreased from 51 kg/hm^2 to 38 kg/hm^2, the carotenoid content in pepper fruits increased. However, when the nitrogen application rate decreased to 25 kg/hm^2, the carotenoid content decreased. In this experiment, it was observed that during stages S1 and S2, as the urea application rate decreased from 750 kg/hm^2 to 0 kg/hm^2, the carotenoid content in pepper fruits gradually increased. Remarkably, during the red ripening period (S3), N3 exhibited the highest carotenoid content (Figure 1B). This aligns with the findings of Kopsell [54] in kale, where carotenoid content increased with reduced ammonium nitrogen application. However, some discrepancies exist with the study by Francisco M. del Amor and T. Casey Barickman [55,57], who found that carotenoid content increased when ammonium nitrogen application was reduced early in development but increased at the peak of reproductive growth [58].

Barickman's [55] results demonstrated that as the ammonium nitrogen usage decreased from 100% to 50%, the carotenoid content increased in Swiss chard leaf tissue. Furthermore, carotenoid content rose with decreasing nitrogen application during the full reproductive stage. Enzyme activities of PSY, LCYB, and CCS in this study generally exhibited a trend of gradual increase with decreasing nitrogen application. Specifically, the enzyme activity of PSY increased significantly during the S3 period, and the expression of PSY also displayed significant upregulation during the same period. Zhang et al.'s [59] study demonstrates that N250 (250 kg N/hm^2) is beneficial for capsanthin accumulation and secondary metabolites in pepper fruits actively respond to nitrogen supply, leading to changes in the composition, content, and enzyme activity of pigment-related substances in the fruits. This study's conclusion is similar to Zhang's [59]. According to GO and KEGG (Figure 5), the differential expression of genes related to secondary metabolites at the same developmental stage, N1 vs. N4, is significantly higher compared to N1 vs. N3h and N3 vs. N4. Except for GGPSS, other key genes of carotenoid content exhibited an increase in expression levels with the reduction in nitrogen application. GGPP, a precursor for the synthesis of numerous plant olefins and belonging to the upstream carotenoid metabolic pathway [12], did not show a significant effect of nitrogen. This further confirms that plants respond positively to environmental changes by regulating transcript levels to adapt to varying nutrient levels [60].

4.2. Transcription Factors and Carotenoid

Transcription factors have garnered significant attention in recent years. In addition to NAC and ERF, a notable change in urea dosage led to significant differences in the expression of transcription factor families such as bHLH, MYB, and C2H2 (Figure 7A). Recent research has emphasized the regulatory role of transcription factors in determining the color of plant fruits. Jiali Song [61] conducted an analysis of the ERF family in peppers and identified CaERF82, CaERF97, CaERF66, CaERF107, and CaERF101 as participants in the carotenoid synthesis of pepper peel. Kang et al. [62] indicated that the early synthesis of anthocyanins in tomato fruit peels is regulated by the complex of MYB, bHLH, and WD40. Further findings revealed that bHLH and MYB regulated the expression of flavonoid biosynthesis genes, subsequently influencing fruit color [63].

Pearson correlation analysis between the expressions of upregulated transcription factors and carotenoid genes in N1 and N4 treatments revealed that NAC transcription factors significantly correlated with carotenoid gene expression in N4. Among them, CA12g04950, belonging to the NAC TF family, exhibited a significant positive correlation with 11 carotenoid genes. In this study, the expressions of CA12g04950 increased significantly in N4 (control) and decreased in the silenced fruits of N4. On the sixth day, the expression level of PSY decreased by 93.74% in silenced fruits compared to the control, while the expression level of CCS decreased by 97.29% compared to the control. It can be inferred that CA12g04950 is involved in the regulation of pepper carotenoid synthesis under low nitrogen conditions.

The NAC family, as the most abundant and functionally comprehensive transcription factors in plants, plays a crucial role in regulating immune signal transmission. NAC proteins have been shown to modulate plant hormones, including GA, ABA, and IAA, to respond to various stresses [51,64–66]. Zhang et al. [67] discovered positive regulations of abiotic stresses such as salt, cold, and heat on the expression level of CaNAC035.

Gao et al. [50] silenced Solyc07g063420, a member of the NAC family, and found that the maturation of tomato fruit was delayed, with fruit softening and chlorophyll degradation being delayed, leading to a decrease in lycopene accumulation. Knocking out NOR-like1 by CRISPR/Cas9 led to a decrease in ethylene content in tomato fruit, subsequently changing the expressions of carotenoid genes such as DXS, GGPP2, PSY1, and PSY, thereby affecting fruit color [68,69]. Research by Feng et al. [70] demonstrated that silencing the SINACs of tomato led to a decrease in ethylene content and lycopene content, resulting in yellow fruit. The expressions of carotenoid genes (PSY1, DXS2, SGR1,

CrtR-b2) in the SINAC9 mutant changed significantly, affecting the transformation process of chloroplasts and chromosomes. Many NAC family members are thus involved in the regulation of carotenoid genes.

PSY, as the upstream gene of the carotenoid metabolic pathway, is extensively researched. Liu et al. [30] found that the most important function of PSY1 in peppers was to provide precursors for downstream carotenoid synthesis by regulating phytoene accumulation, rather than directly participating in pigment synthesis. Yeast one-hybrid experiments demonstrated that SINAC9 interacted with PSY1 at the protein level, affecting the chlorophyll degradation and carotenoid synthesis of tomatoes [21,70]. This study similarly found that silencing CA12g04950 had markedly different effects on PSY and CCS. Silencing 78 resulted in a significant decrease in the expression level of CCS compared to PSY, similar to the results of the study mentioned above. This suggests that PSY may indirectly regulate carotenoid synthesis in peppers.

5. Conclusions

This study utilized transcriptomic analysis to investigate the differential expression of carotenoid-related genes and transcription factors in pepper fruits under varying nitrogen conditions. Pearson correlation analysis revealed a positive correlation between CA12g04950 and differentially expressed carotenoid genes under the N4 treatment. Then, silencing CA12g04950 under N4 treatment significantly decreased the contents of phytoene, β-carotenoids, zeaxanthin, and capsanthin, as well as the activity of carotenoid enzymes. These findings suggest that under nitrogen deficiency conditions, CA12g04950 may directly activate the biosynthetic genes of carotenoids in peppers, thereby increasing the levels of carotenoids. In this process, CA12g04950 simultaneously acts on multiple carotenoid biosynthetic genes, with the downstream CCS showing a more pronounced effect (Figure 11). Future research will focus on verifying the function of CA12g04950 in the coloration of pepper fruits through protein interaction, overexpression, and silencing techniques.

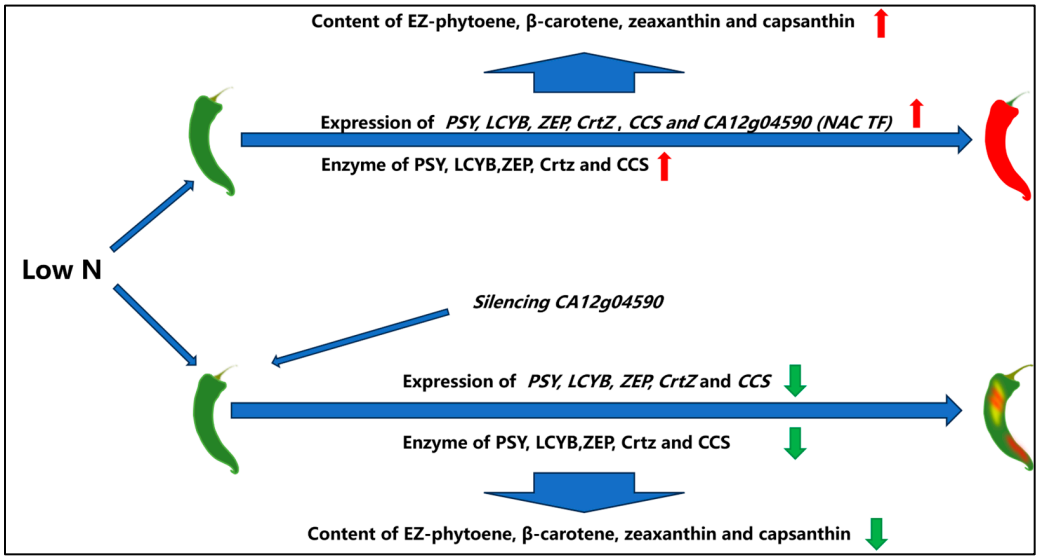


Figure 11. Working model of CA12g049501 in the synthesis of carotenoids in pigment pepper fruits under different nitrogen treatments and the expression of related genes. Red arrows represent up−regulation, green arrows represent down−regulation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14040521/s1, Figure S1: Functional annotations of transcripts to public databases; Table S1: Analysis of GO enrichment for DEGs; Table S2: KEGG pathways of differentially expressed genes.

Author Contributions: All authors contributed to the discussion. L.S. and X.J. conceived and designed the experiment; L.S. performed experiments and data analyses and wrote the manuscript; X.J. and S.U. were involved in revising the manuscript; L.S. and X.J. collected the literature and revised the manuscript; X.J. and S.U. checked the language. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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