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Integrated metabolome and transcriptome analysis provides clues to fruit color formation of yellow, orange, and red bell pepper

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Fruit color is a crucial trait for bell pepper. To investigate the mechanism of color formation, three bell pepper lines with different color (yellow, orange and red) were used as materials to conduct comprehensive targeted metabolomic and transcriptomic analyses. During the process of fruit development, 54 carotenoids metabolites were discovered, exhibiting unique accumulation patterns and notable variety specificity. The types and content of carotenoids in orange fruit (OM) were notably greater compared to the other two varieties. Red pigment (capsanthin and capsorubin) was specifically enriched in red fruit (RM), and yellow pigment (lutein and zeaxanthin) is the highest in yellow fruit (YM) and OM. Five modules positively correlated with carotenoid accumulation and one negative module was determined by weighted gene co-expression network analysis (WGCNA). Additionally, transcription factors (TFs) and hub genes related to carotenoid synthesis were predicted. By elucidating the regulation of 7 key carotenoid metabolites by 14 critical genes and 5 key TFs, we constructed a comprehensive carotenoid biosynthesis metabolic network that comprehensively explains the pigment changes observed in green and mature pepper fruit. Overall, the results not only provide important insights into carotenoid synthesis pathway, but also lay a solid base for revealing the mechanism of bell pepper color transformation.

Keywords Fruit color, Bell pepper, Metabolome analysis, Transcriptome analysis, Carotenoids

Capsicum annuum L., commonly known as bell pepper, is a valuable vegetable crop known for its high nutritional content and health benefits. One of the essential attributes is their fruit color, which plays a crucial role in influencing consumer preferences and breeder selection[1]. Mature bell peppers exhibit a diverse range of colors, including red, yellow and orange, arises from the diverse types and relative contents of carotenoids present in fruits[2–6].

Carotenoids are 40-carbon isoprenoids polymers widely found in higher plants, algae, fungi and bacteria[7,8]. At present, more than 700 kinds of carotenoids have been discovered, mainly divided into two categories based on their chemical structure. One is type carotenes composed of carbon and hydrogen, such as α-carotene, β-carotene and (E/Z)-phytoene etc., and the other is luteins, which also contains oxygen in addition to carbon and hydrogen, such as capsanthin, neoxanthin, lutein, zeaxanthin, etc[9]. In green photosynthetic tissues of plants, carotenoids are involved in photosystem assembly, light-harvesting and photoprotection[10–12]. In non-green organs, carotenoids bring vibrant colors to plants, attracting insects and animals to participate in plant pollination and seed dissemination[13]. Additionally, abscisic acid (ABA) and strigolactones (SLs) is synthesized from carotenoids as precursors in plants[14,15]. Carotenoids are dietary sources of provitamin A, and serve as a natural antioxidant that scavenges peroxidizing free radicals and prevents the accumulation of harmful oxygen, playing a positive role in human health by reducing the incidence of many diseases[9,16–19]. Besides, some carotenoids are widely used in food colorants as well as cosmetic and pharmaceutical industries[20,21].

In plant, isopentenyl diphosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), converting to each other through IPP isomerase (IPI), are products of methylerythritol phosphate (MEP) pathway and the initial precursors of carotenoids[22]. The direct precursor of carotenoids is geranylgeranyl pyrophosphate (GGPP),

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produced by IPP and DMAPP under the catalytic action of geranylgeranyl diphosphate synthetase (GGPS)[23,24]. When GGPP enters the carotenoid biosynthesis pathway, the first carotenoid product, colorless 15-cis-phytoene, is produced by the rate-limiting enzyme phytoene synthase (PSY)[25]. Under the successive isomerization and desaturation of phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO), colorless phytoene is converted into red lycopene[23]. Due to the existence of LCYB and LCYE in plants, lycopene downstream products synthesis divided into two branches, including α- and β-carotene. Lutein is the final product of α-branch, which is transformed from α-carotene under the catalysis of P450 carotene hydroxylase CYP97A and CYP97C. As to β-branch, β-cryptoxanthin, zeaxanthin, antheraxanthin and violaxanthin are formed, respectively[26,27]. Under the action of neoxanthin synthase (NSY), violaxanthin is converted to neoxanthin, and it is usually the last step for the core biosynthesis pathway of carotenoids[28]. But in red pepper fruits, capsanthin and capsorubin are the final products, produced by capsaicin/capsorubin synthetase (CCS) catalyzing antheraxanthin and violaxanthin[29].

Earlier studies perceive that three independent genes, including *y*, *c1* and *c2*, control the peel color of mature pepper[30], and genes encoding *CCS*, *PRR2* and *PSY1* were subsequently identified by genetic mapping techniques[31–33]. However, the three gene models were insufficient to explain the color variation of pepper fruits. Variation and expression of other genes in carotenoid metabolic pathway can also lead to different colors, such as *PSY2*, *DXS*, *CaGLK2*, *BCH2*, etc[34–39]. In higher plants, many transcription factors (TFs) also control fruit coloration by regulating carotenoid biosynthesis pathway[29,40–42]. In numerous plant species, *MYB* are known to control the synthesis and metabolism of carotenoids. For instance, in kiwifruit, *MYB7* activates the promoter of *AdLCY-β*, which is essential for the carotenoid biosynthesis pathway[43]. Similarly, in the petals of Medicago truncatula, the anthocyanin-related R2R3-MYB protein WHITE PETAL1 (WP1) directly regulates the genes *MtLYCe* and *MtLYCb* expression, resulting in high accumulation of lutein and yellow petals manifestation[44]. In contrast, in tomatoes, SlMYB72 interacts with SlZHD17, a member of zinc-finger homeodomain TF family, to inhibit the genes *SlPSY1* and *SlZISO* expression, thereby suppressing carotenoid synthesis[45]. Expanding on this theme, the phytochrome-interacting factor (PIF), a member of the *bHLH* TF family, downregulates carotenoid accumulation by inhibiting the expression of the rate-limiting enzyme gene *PSY*[46]. In addition, *CpbHLH1* and *CpbHLH2* play significant roles in regulating the transcription of *CpCYC-B* and *CpLCY-B* during the maturation of papaya fruit, thus influencing carotenoid biosynthesis[47]. Furthermore, the TF *MADS-box* also plays an important role in the carotenoid synthesis pathway. In citrus, *CsMADS3* enhances carotenoid biosynthesis by interacting with the promoters of *CsPSY1* and *CsLCYb2*[48]. *CsMADS6* also promotes the expression of *PSY*, *PDS*, and *LCYb1*, increasing carotenoid levels[49]. *CsMADS5* activates carotenoid biosynthetic genes by binding to *PSY*, *PDS*, and *LCYb1* promoters and interacts with *CsMADS6* to form an enhancer complex[50]. In addition, both *bZIP*[51] and *WRKY*[52] have been shown to play significant roles in regulating the expression of genes within the carotenoid biosynthesis pathway, further highlighting the complexity of regulatory mechanisms governing carotenoid accumulation in plants.

In short, as a member of genus *Capsicum*, fruit color of bell pepper is an important breeding trait and has always been valued[53]. However, the complete network of bell pepper fruit color formation remain incompletely elucidated. Therefore, in the present study, taking bell peppers with yellow, orange and red fruit colors as materials, we used transcriptomics and metabolomics to detect the difference between green and mature stages, and screen metabolites and regulatory genes that cause fruit color variation. The results will reveal the genetic mechanism of fruit color change in yellow, orange and red bell peppers, and provide theoretical basis for breeding new characteristic bell pepper varieties.

Results

Metabolome analysis of different colored bell peppers

Three pepper inbred lines were used in the study, IL-Y, IL-O and IL-R (Fig. 1A), and fruit color of the three lines was green at 50 days after planting (50 DAP), the corresponding samples named YG, OG and RG. The color transformed to yellow, orange and red at 65 DAP, and the samples named YM, OM and RM, respectively. The results from metabolome analysis on samples YG, OG, RG, YM, OM and RM showed that 68 carotenoid compounds were detected, including 61 lutein (yellow pigment) and 7 carotenes (orange pigment), and among the examined carotenoids, 54 were identified in at least one sample (Table S1).

At 50 DAP, the carotenoids accumulated in the green fruits of IL-Y (YG), IL-O (OG) and IL-R (RG) were mainly lutein, β-carotene and neoxanthin, but other compounds were extremely low (Fig. 1B, C and D). With the ripening of fruits, total carotenoid contents in mature fruits of IL-Y (YM), IL-O (OM) and IL-R (RM) were 571.29 μg/g, 2098.27 μg/g and 575.62 μg/g, and it was 6.06, 1.38 and 1.73 times higher than that in YG, OG and RG, respectively. The species were also increased from 20 (YG), 18 (OG) and 17 (RG) to 39 (YM), 49 (OM) and 27 (RM) (Table S2). In YM, the main carotenoids were lutein (31.36%), (E/Z) phytoene (27.89%), violaxanthin (10.96%), violaxanthin myristate (4.55%) and zeaxanthin (3.42%) (Fig. 1E). While in OM, zeaxanthin (37.93%) was the highest, followed by (E/Z) phytoene (12.69%), zeaxanthin dimyristate (11.97%), lutein (8.02%) and lutein dimyristate (4.42%) (Fig. 1F). The accumulation of (E/Z) phytoene were both high in YM and OM, but in RM, red capsanthin was the main component of carotenoids, reaching 71.02%, which is different from YM and OM (Fig. 1G). Other carotenoids compounds varied from 0 to 5.9%. In addition, the content of lutein in RM was extremely low (0.43 μg/g), which was also different from the high content of lutein in YM and OM (Fig. 1E, F and G, Table S2).

The metabolites heatmap showed significant differences among green fruits samples YG, OG and RG, and mature fruits of YM, OM and RM (Fig. 2A). Under the selection of Fold_Change (FC) ≥ 2 or ≤ 0.5, differential metabolites of three bell pepper varieties at the green ripening stage were less, ranging from 5 to 8 (Fig. 2B). At the mature stage, OM showed a higher accumulation of carotenoids compared to YM and RM. Then the differentially accumulated carotenoids (DACs) among samples were also screened (Table S3). In YM_vs_OM

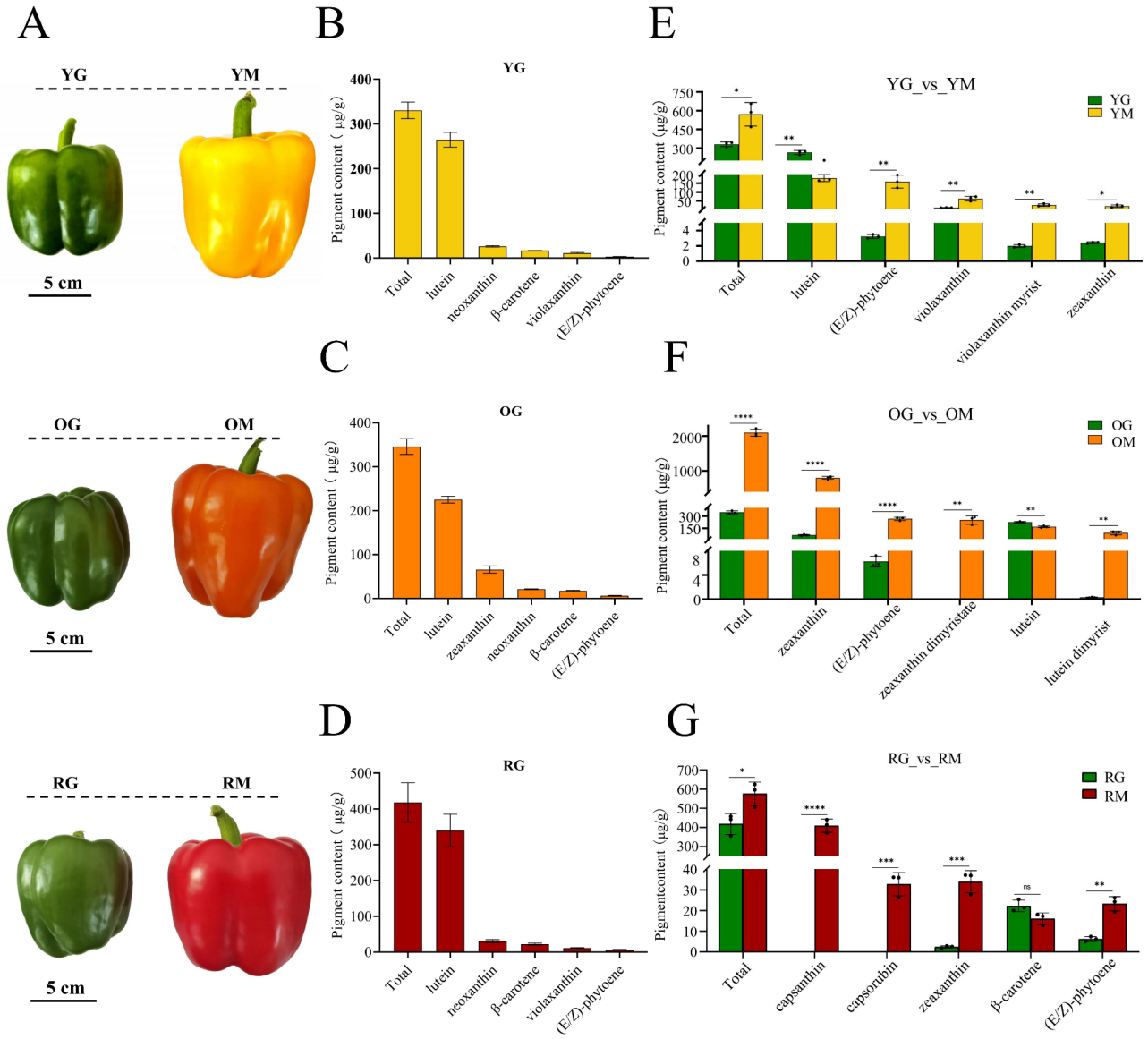

Fig. 1. The phenotypes and carotenoid contents of three different colored bell pepper fruits. (**A**) The phenotype of bell pepper fruits at 50 DAP (YG, OG, and RG, green) and 65 DAP (YM, OM, and RM; yellow, orange and red). (**B**–**D**) Detection of total carotenoids and top five carotenoid pigments of IL-Y, IL-O and IL-R at green ripening stage, and the samples named YG, OG, and RG, respectively. (**E**–**G**) Detection of total carotenoids and top five carotenoid pigments of IL-Y, IL-O and IL-R at mature stage, and the samples named YM, OM, and RM, respectively, and their changes compared to green samples.

group, 40 DACs were identified, including 33 up-regulated and 7 down-regulated. Among the DACs, zeaxanthin and zeaxanthin-related compounds were significantly up-regulated, and the same as to orange carotenes, α-carotene and β-carotene, while violaxanthin and violaxanthin-related compounds were significantly down-regulated. Similarly in RM_vs_OM group (44 up-regulated and 3 down-regulated), zeaxanthin, zeaxanthin-related compounds, lutein-related compounds, and carotenes were also highly up-regulated, while the red pigments capsanthin and capsorubin were down-regulated. In YM_vs_RM comparison group, 41 DACs were identified, among which capsanthin and capsorubin were significantly up-regulated, while lutein and related compounds were conversely down-regulated. A total of 47, 29 and 40 DACs were identified between green and mature stages for groups OG_vs_OM, RG_vs_RM and YG_vs_YM, respectively (Fig. 2B, Table S3). Venn diagram showed 24 DACs common to the three comparison groups (Fig. 2C), detail information of DACs was showed in Table S3. The above results indicated that the unique accumulation pattern of carotenoid-related metabolites was the main reason for the color difference in bell pepper fruits.

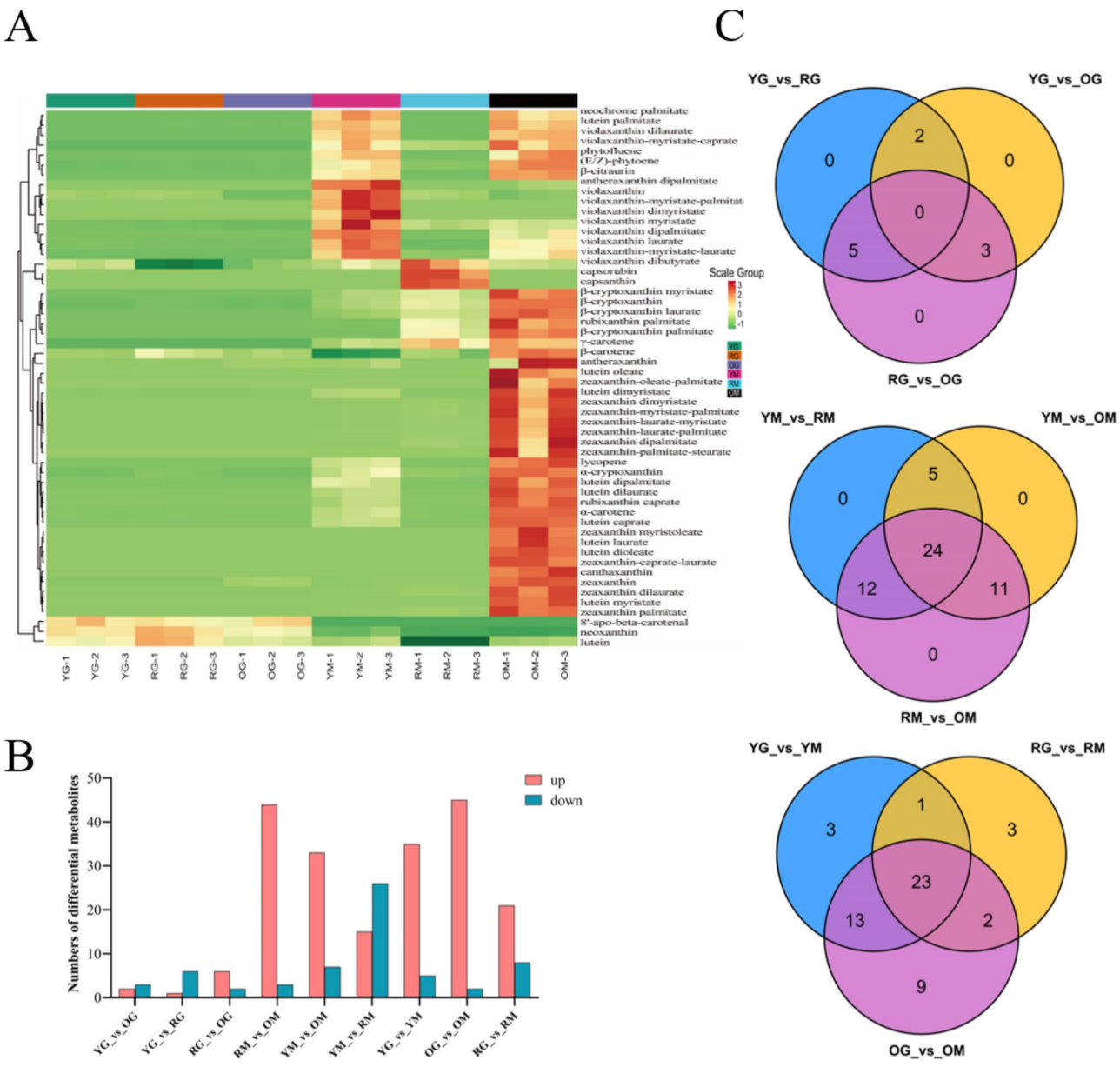

Fig. 2. The metabolome data analysis of three bell peppers lines. (**A**) Heatmap constructed based on 54 carotenoids metabolite profiles of IL-Y, IL-O and IL-R, three repeats (-1, -2 and −3) for sample YG, OG, RG, YM, OM and RM. (**B**) The DACs numbers of different comparison groups. (**C**) The universal and specific DACs numbers of different comparison groups.

RNA-seq detection and analysis of different colored bell peppers

To understand the molecular regulatory network on fruit color formation of yellow, orange and red bell pepper, we performed transcriptome sequencing analysis on YG, YM, OG, OM, RG and RM with three biological replicates per sample (-1, -2, and −3). Data analysis showed 124.82 Gb of total clean data was obtained, and clean reads per sample ranged from 41,358,224 to 52,919,370 (Table 1). The Q20 of all samples was above 97.3%, and Q30 was above 92.37%, and mean GC content was 42.53% (Table 1). The principal component analysis (PCA) score plot showed PC1, PC2 and PC3 accounted for 40.38%, 11.96% and 9.18% of the variance, respectively. Samples YG, YM, OG, OM, RG and RM were clearly separated in the analysis chart, and their three replicates were closely clustered (Fig. 3A).

Under the thresholds of log2 (FC) ≥ 1 and FDR ≤ 0.05, 14,961 differentially expressed genes (DEGs) were screened out in three bell pepper lines between green and mature stages. DEGs distribution showed in volcano plots suggested significant differences in samples YG, YM, OG, OM, RG and RM (Fig. S1). At the green ripening stage, we identified 1854, 2467 and 3059 DEGs in YG_vs_OG, YG_vs_RG and RG_vs_OG, respectively. Among them, 1099, 1452 and 1435 genes were up-regulated, while 755, 1015 and 1624 genes were conversely down-regulated. (Fig. 3B). In total, 240 common DEGs were identified at green ripening stages of yellow, orange and

Sample	Raw Reads	Clean Reads	Clean Base(G)	Q20(%)	Q30(%)	GC Content(%)
OG-1	46,530,998	44,655,450	6.7	97.62	93.15	42.65
OG-2	43,121,018	41,358,224	6.2	97.59	93.08	42.71
OG-3	47,223,748	45,014,448	6.75	97.53	92.97	42.55
OM-1	49,292,006	46,178,988	6.93	97.72	93.43	42.57
OM-2	48,808,226	45,865,292	6.88	97.61	93.2	42.47
OM-3	53,698,840	52,919,370	7.94	97.86	93.54	42.56
RG-1	48,982,534	46,584,348	6.99	97.3	92.37	42.73
RG-2	47,078,894	44,053,318	6.61	97.76	93.5	42.57
RG-3	47,272,994	45,078,648	6.76	97.64	93.22	42.9
RM-1	47,140,830	45,427,904	6.81	97.32	92.43	42.28
RM-2	49,236,310	46,708,260	7.01	97.54	92.96	42.46
RM-3	48,691,662	46,481,656	6.97	97.4	92.6	42.35
YG-1	51,603,776	49,636,440	7.45	97.41	92.65	42.66
YG-2	47,528,914	45,475,804	6.82	97.44	92.76	42.71
YG-3	52,411,018	50,004,026	7.5	97.58	93.05	42.59
YM-1	47,862,660	45,657,656	6.85	97.53	92.99	42.33
YM-2	45,830,776	43,821,608	6.57	97.39	92.65	42.23
YM-3	49,055,178	47,171,162	7.08	97.44	92.8	42.15

Table 1. Transcriptome sequencing data quality index.

red fruits (Fig. 3C). At the mature stage, 2468 (1034 up- and 1434 down-regulated), 3091 (1397 up- and 1694 down-regulated) and 2930 (1500 up- and 1430 down-regulated) DEGs were screened in comparison groups RM_vs_OM, YM_vs_OM and YM_vs_RM, respectively. Venn diagrams showed that 273 DEGs were common, indicating that these DEGs may be responsible for the key differences in fruit color among the three bell peppers at mature stage(Fig. 3B and C). For two different stages of the same cultivar, we detected 3205 up- and 5923 down-regulated DEGs in OG_vs_OM, 3479 up- and 6474 down- in RG_vs_RM, and 3166 up- and 5998 down- in YG_vs_YM. Venn diagram showed 5735 common DEGs detected in all comparison groups, indicating that these DEGs might be important reasons for the color change of three bell peppers lines (Fig. 3B and C).

DEGs functional prediction

DEGs functional prediction of GO and KEGG enrichment analysis was performed to elucidate their roles in fruit color change of bell pepper (Fig. S2 and S3). All the DEGs were enriched in molecular functions, cellular components and biological processes. KEGG enrichment analysis showed the highest abundance pathway was metabolic (ko01100) and secondary metabolites biosynthesis (ko01110). Most importantly, pathway of carotenoid biosynthesis (ko00906) was significantly enriched in groups YG_vs_YM, OG_vs_OM, RG_vs_RM, RG_vs_OG, YG_vs_RG, RM_vs_OM and YM_vs_RM (Fig. S3), providing a powerful clue on genes expression in fruit coloration of bell pepper. The DEGs of group YG_vs_OG was enriched in biosynthesis pathways of phenylpropanoid (ko00940), anthocyanin (ko00942) and flavonoid (ko00941). For group RG_vs_OG, DEGs was enriched in biosynthesis of phenylpropanoid (ko00940) and anthocyanin (ko00942), and DEGs of YG_vs_RG was also significantly enriched in anthocyanin biosynthesis pathway (ko00942). The results are consistent with previous study that flavonoids are mainly synthesized in the early stage of fruit[54], suggesting DEGs involved in these three pathways may be the reason for the early discoloration in bell pepper.

DEGs related to carotenoid biosynthesis

A total of 48 DEGs were screened to be related to carotenoid metabolism and assigned to pathway ko00906 (Table S4). After excluding 25 uncertain genes and low-expression genes, 23 DEGs were detected in at least one comparison group. Based on gene functional annotation, homology of these 23 genes was identified, which included 2 *PSY* (*gene-LOC107859651*, *gene-LOC107868281*), 1 *PDS* (*gene-LOC107861625*), 1 *Z-ISO* (*gene-LOC107850257*), 1 *ZDS* (*gene-LOC107839468*), 2 *LCY-E* (*gene-LOC107840923*, *gene-LOC107852092*), 1 *LCYB* (*gene-LOC107869983*), 2 *BCH* (*gene-LOC107873401*, *gene-LOC107863219*), 1 *ZEP* (*gene-LOC107872926*), 1 *CRTISO* (*gene-LOC107854534*), 1 *CYP97* (*gene-LOC107850957*), 1 *CCS* (*gene-LOC107875664*), 1 *NCEDs* (*gene-LOC107852027*), 1 *CCDs* (*gene-LOC107870081*), 3 *SDRS* (*gene-LOC107843702*, *gene-LOC107862477*, *gene-LOC107855067*), 2 *AAO* (*gene-LOC107847514*, *gene-LOC107847367*) and 2 *D27s* (*gene-LOC107863814*, *gene-LOC107875230*).

In plants, phytoene is the first product of carotenoids biosynthesis pathway, which is formed by PSY catalyzing condensation of two GGPP molecules[25]. In our study, compared with the green ripening stage, expression of *PSY1* (*gene-loc107868281*) was significantly increased in three bell pepper lines at coloring stage, which was corresponding to the higher content of phytoene in OM, RM and YM. The *CCS* gene (*gene-LOC107875664*) was only expressed in RM, consistent with capsaicin and capsorubin were detected only in RM. The expression level of *BCH1* gene (*gene-LOC107863219*) increased most significantly in OG_vs_OM, RG_vs_RM, YG_vs_YM comparison groups, while the expression of *BCH2* gene (*gene-LOC107873401*) decreased significantly, showing

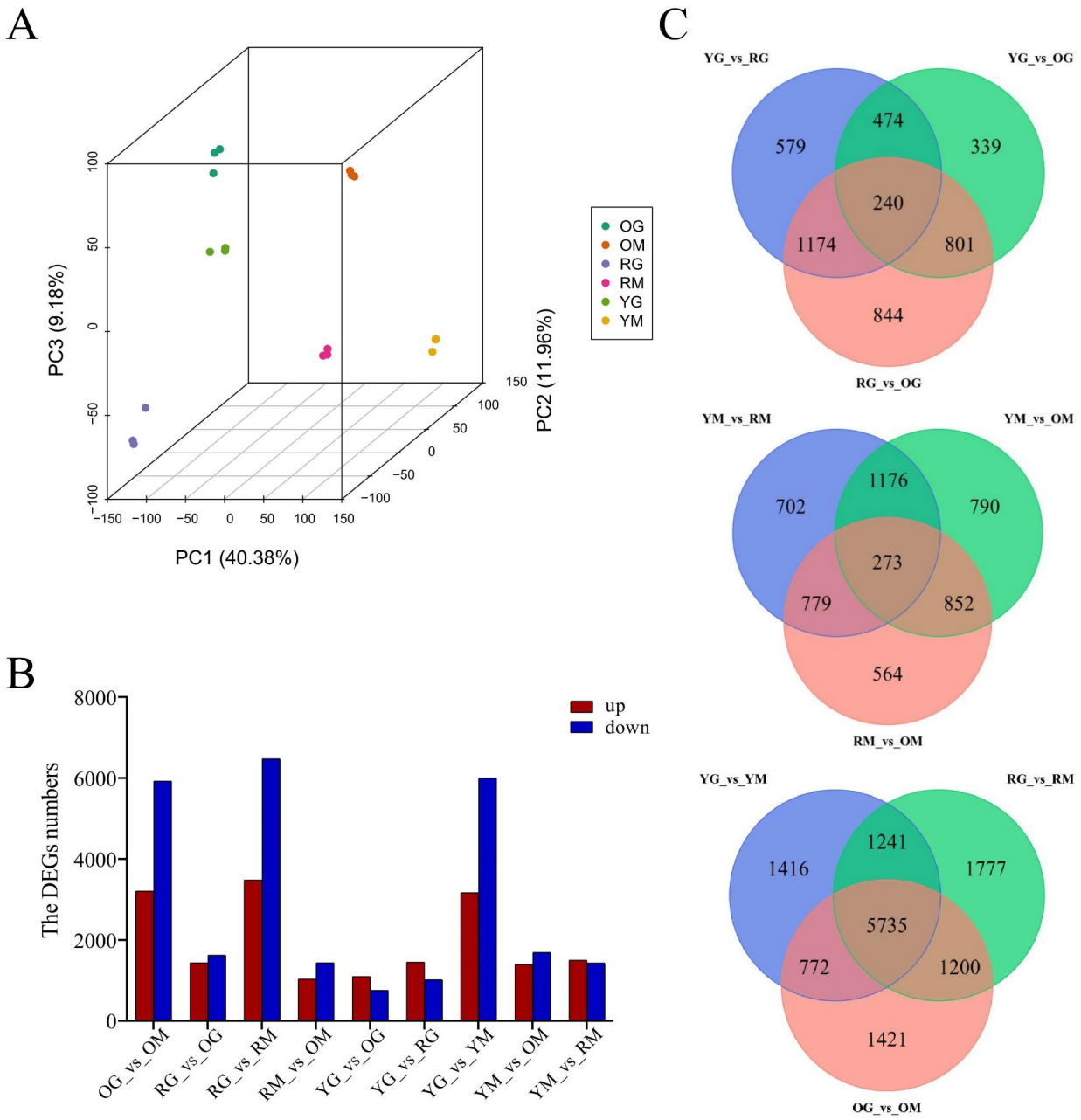

Fig. 3. Transcriptome sequencing analyses of three bell peppers. (**A**) PCA score plots for all samples. (**B**) Numbers of up- and down-regulated DEGs detected in comparison groups. (**C**) Venn diagram of DEGs among different comparison groups.

different expression patterns. Compared with the green ripening stage, other key genes including *PDS* (*gene-LOC107861625*), *ZDS* (*gene-LOC107839468*), *Z-ISO* (*gene-LOC107850257*), *LCYB* (*gene-LOC107869983*) and *ZEP* (*gene-LOC107872926*) also increased significantly. However, cyclase genes *LCYE* (*gene-Loc107852092*, *gene-LOC107840923*) and *CYP97* (*gene-Loc107850957*) involved in α-branch synthesis decreased during maturation. In addition, downstream products of carotenoids produces precursors for abscisic acid (ABA) and strigolactone (SL)[14,15]. One *NCED*, three *SDR*, two *AAO* (related to ABA synthesis) and two *D27* (related to SLs synthesis) were significantly differentially expressed in bell pepper lines at different periods (Table S4).

The direct carotenoids precursor, GGPP, is synthesized under the catalysis of GGPPS[22–24]. Two *GGPPS* (*gene-LOC107843186*, *gene-LOC107867046*) with high expression in YM, OM and RM were identified in our study, providing sufficient precursor compounds for carotenoid synthesis, which may be one of the reasons for the high carotenoid content of the three bell pepper lines at mature stage (Table S4).

DEGs analysis related to carbon and lipid metabolism

Previous studies showed positive correlation between sugar and pigment accumulation[55–57]. We found DEGs in groups YG_vs_YM, OG_vs_OM and RG_vs_RM were significantly enriched in carbon metabolism (ko01200) (Fig. S3). At least 80 DEGs were detected in one comparison group (Table S5). In groups YG_vs_YM, OG_vs_OM and RG_vs_RM, 32, 54 and 45 DEGs were up-regulated, while 17, 12 and 11 DEGs were down-regulated, respectively (Table S5).

In plant cells, carotenoids usually accumulate in chromoplasts, a kind of specialized storage organelles (plastids)[58]. In chromoplast differentiation, internal membrane remodeling is a prominent feature, and lipid substances synthesis plays an important role during the process[59]. In the pathway of fatty acid metabolism (ko01212), 44, 52 and 56 DEGs were detected in groups YG_vs_YM, OG_vs_OM and RG_vs_RM, respectively. Among them, 17, 22 and 25 DEGs were up-regulated, and 27, 30 and 31 were down-regulated, respectively (Table S6). In the fatty acid degradation pathway (ko00071), 17, 15, 21 DEGs were up-regulated, while 19, 15 and 22 were down-regulated in YG_vs_YM, OG_vs_OM and RG_vs_RM, respectively (Table S6). Besides, in the fatty acid biosynthesis pathway (ko00061), 7, 17 and 13 genes of YG_vs_YM, OG_vs_OM and RG_vs_RM were up-regulated, while 12, 12 and 13 genes were down-regulated (Table S6).

DEGs co-expression analysis

We performed weighted gene co-expression network analysis (WGCNA) using DEGs identified, and obtained 13 main branches (Fig. 4A, Table S7). Modules blue, red and magenta had a positive correlation with carotenoid metabolites, while turquoise had a negative correlation. The greenyellow module had the highest correlation with capsaicin and capsorubin, which was characteristic in RM, indicating genes in this module play more important roles in red pigment formation (Fig. 4B). Expression heat map of co-expressed genes of blue module in OM, YM and RM were much higher than OG, YG and RG, while genes of turquoise module was just the opposite, indicating that genes of the two modules played opposite roles in carotenoid synthesis (Fig. 4C). DEGs in yellow module was highly correlated with YM, while genes of magenta and greenyellow module were only highly expressed in OM and RM, showing obvious variety specificity (Fig. 4C and D). These results indicated DEGs in different modules may play different roles in carotenoid metabolites synthesis, and subsequent analysis also focuses on these six modules.

Transcription factors (TFs) prediction for carotenoid metabolism regulation

Transcription factors (TFs) are also key factors in plant carotenoid biosynthesis. MYB, MAD-box, bHLH, WRKY, NAC and NY-F play important roles in regulating transcription of carotenoid metabolism related genes[40–42]. In blue module, 210 TFs were identified, including 16 MYB, 10 NAC, 4 WRKY, 8 MAD, 2 BHLH, 11 AP2/ERF, 9 NF-Y, and 3 HD-ZIP (Table S8). Expression abundance of 4 *MYB* (*gene-LOC107850892*, *gene-LOC107867188*, *gene-LOC107868015* and *gene-LOC107871103*), 3 *HD-ZIP* (*gene-LOC107843791*, *gene-LOC107862495* and *gene-LOC107863056*), 4 *NAC* (*gene-LOC107842449*, *gene-LOC107845296*, *gene-LOC107847606* and *gene-LOC107867776*), 4 *AP2/ERF* (*gene-LOC107855040*, *gene-LOC107862115*, *gene-LOC107867626* and *gene-LOC107870958*), 3 *MAD* (*gene-LOC107847473*, *gene-LOC107855404* and *gene-LOC107878477*), 3 *WRKY* (*gene-LOC107840352*, *gene-LOC107859607* and *gene-LOC107872867*) and 1 *NF-Y* (*gene-LOC107861703*) was higher in the colored mature fruit, indicating they may contribute more in fruit color transformation of bell pepper (Table S8). TFs such as MYB, MADS, and bZIP have been reported to regulate key genes in the carotenoid synthesis pathway, including *LYC*, *PSY*, *PDS*, and *BCH*, controlling carotenoid accumulation and influencing fruit color[43–51]. Notably, one *MAD* family member (*gene-LOC107847473*) was almost not expressed at the green ripening stage, but showed high expression at the mature stage with the fold change ranging from 9.18 to 15.38, indicating its pivotal role in regulating carotenoids biosynthesis in bell pepper. In the turquoise module, 269 TFs genes were identified including 25 *AP2/ERF*, 20 *bHLH*, 8 *MAD*, 12 *MYB*, 4 *NAC*, 8 *HD-ZIP*, 6 *WRKY* and 4 *NF-Y*, and detected *HD-ZIP* and *WRKY* family members were identified as negative regulators associated with carotenoid metabolism (Table S8). In the yellow module, 56 TFs genes were identified, and expression of 3 *bHLH* (*gene-LOC107838913*, *gene-LOC107840824* and *gene-LOC107862125*) and 1 *AP2/ERF* (*gene-LOC107864060*) in YM were much higher than other samples (Table S8). The TF bHLH regulates the transcription of *CYC-B* and *LCY-B*[46], promoting the yellowing of chili pepper fruit. Besides, 15, 4 and 4 TFs were predicted in red, magenta and greenyellow modules, respectively. Interestingly, one *GRAS* (*gene-LOC107853465*) was highly expressed only in greenyellow module, but no GRAS family members have been reported in carotenoids synthesis regulation, so their functions need to be further studied (Table S8).

Candidate hub genes for carotenoid synthesis in different colored bell pepper

Correlation networks were constructed between genes within six key modules. In blue module, a total of 333 DEGs showed high co-expression with edge weight over 0.5, including 8 TFs, 6 carotenoid synthesis genes, 13 carbon metabolism pathway genes, and 13 genes involved in fatty acid synthesis and metabolism (Table S9), and mitochondrial carrier protein (*gene-LOC107839282*) and Zeta-carotene desaturase (ZDS, *gene-LOC107839468*) were hub genes (highly connected genes) in this module (Fig. 5A), and mitochondrial carrier protein (*gene-LOC107839282*) had high correlation with carotenoid synthesis genes *ZDS* (*gene-LOC107839468*), *PDS* (*gene-LOC107861625*), *BCH* (*gene-LOC107863219*), and *GGPS* (*gene-LOC107843186*), and TFs MAD (*gene-LOC107879769*), NAC (*gene-LOC107842449* and *gene-LOC107850240*), indicating its key function in carotenoids synthesis. The turquoise module contained 284 co-expressed genes, including 22 TFs, 1 carotenoid decomposition gene, 3 carbon metabolism pathway genes, and 2 genes related to fatty acid synthesis and metabolism (Table S9). Hypothetical protein (*gene-LOC107840002*) and Ras-related protein (*gene-LOC107839339*) had the highest connectivity (Fig. 5B), and were both highly correlated with the carotenoid-

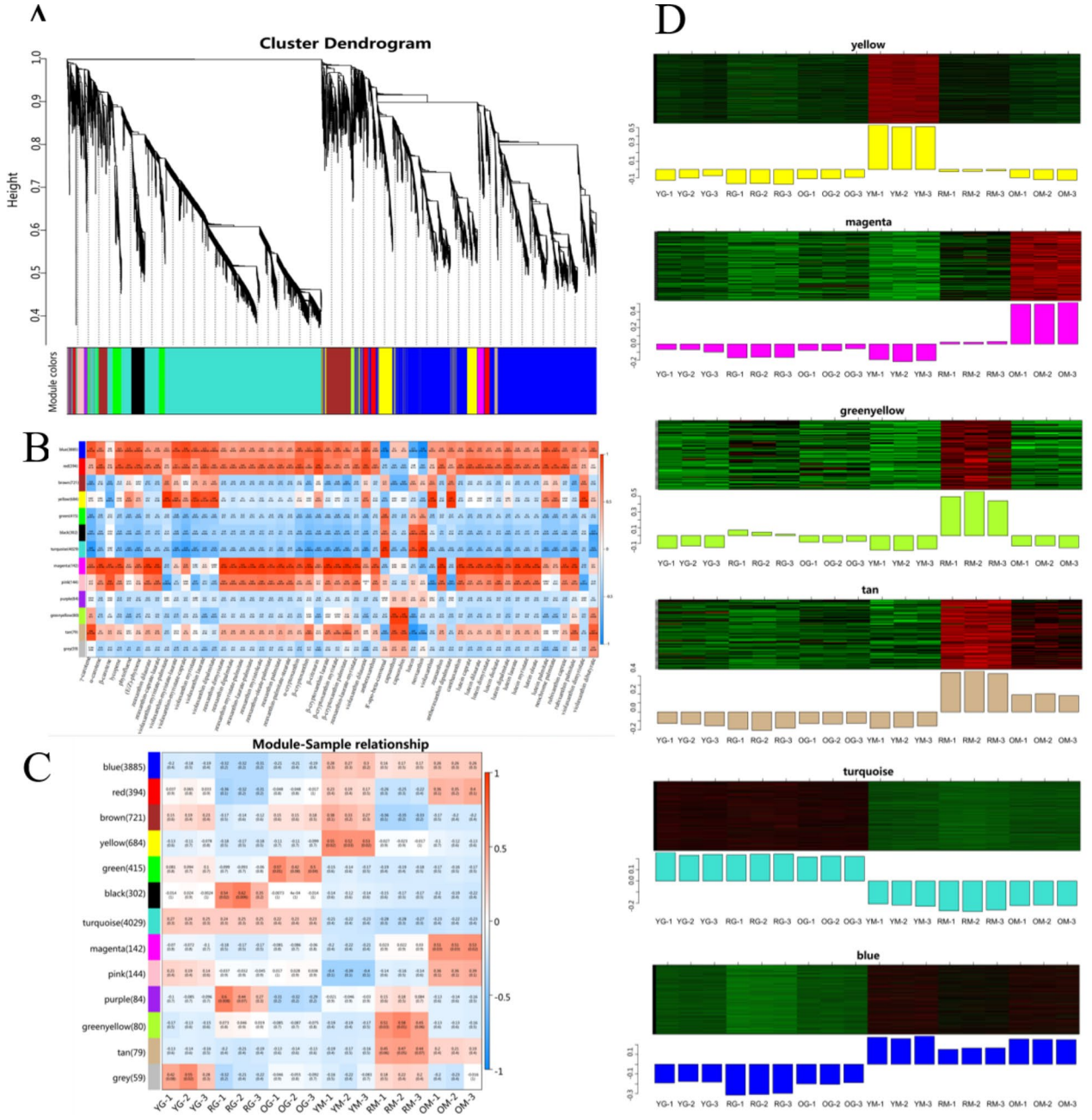

Fig. 4. WGCNA of DEGs. (**A**) Hierarchical cluster tree. (**B**) Relationship between modules and carotenoid metabolites. (**C**) Relationship between modules and samples YG, OG, RG, YM, OM and RM. (**D**) Gene expression pattern of six modules.

breakdown gene *CCD* (*gene-LOC107870081*), which was negatively related to carotenoid metabolism. The yellow module contained 79 co-expressed genes, and *gene-LOC107839932*, putative L-ascorbate peroxidase (*gene-LOC107838875*) and excision repair protein (*gene-LOC107870806*) had the highest connectivity (Fig. 5C, Table S9). In magenta module, genes *novel.3368*, Chaperonin-like RBCX protein (*gene-LOC107861269*) and isocitrate dehydrogenase (*gene-LOC107854067*) had the highest connectivity (Fig. 5D, Table S9). In the red module envoloved of 63 genes, of which obg-like ATPase 1 (*gene-LOC107853317*) and methionine-tRNA ligase (*gene-LOC107873333*) showed the highest connectivity (Fig. 5E, Table S9). There were 52 genes in greenyellow module, and *novel.3256* and *gene-LOC107869054* were the hub genes (Fig. 5F, Table S9).

Metabolite and gene associations underlying color formation in bell pepper fruit

To further analyze the relationship between metabolites and related genes in carotenoid biosynthesis pathway during yellow, orange and red pepper coloration, we have established a network based on key metabolites and related genes (Fig. 6). In different pepper fruit samples undergoing color changes (YG, YM, OG, OM, RG and

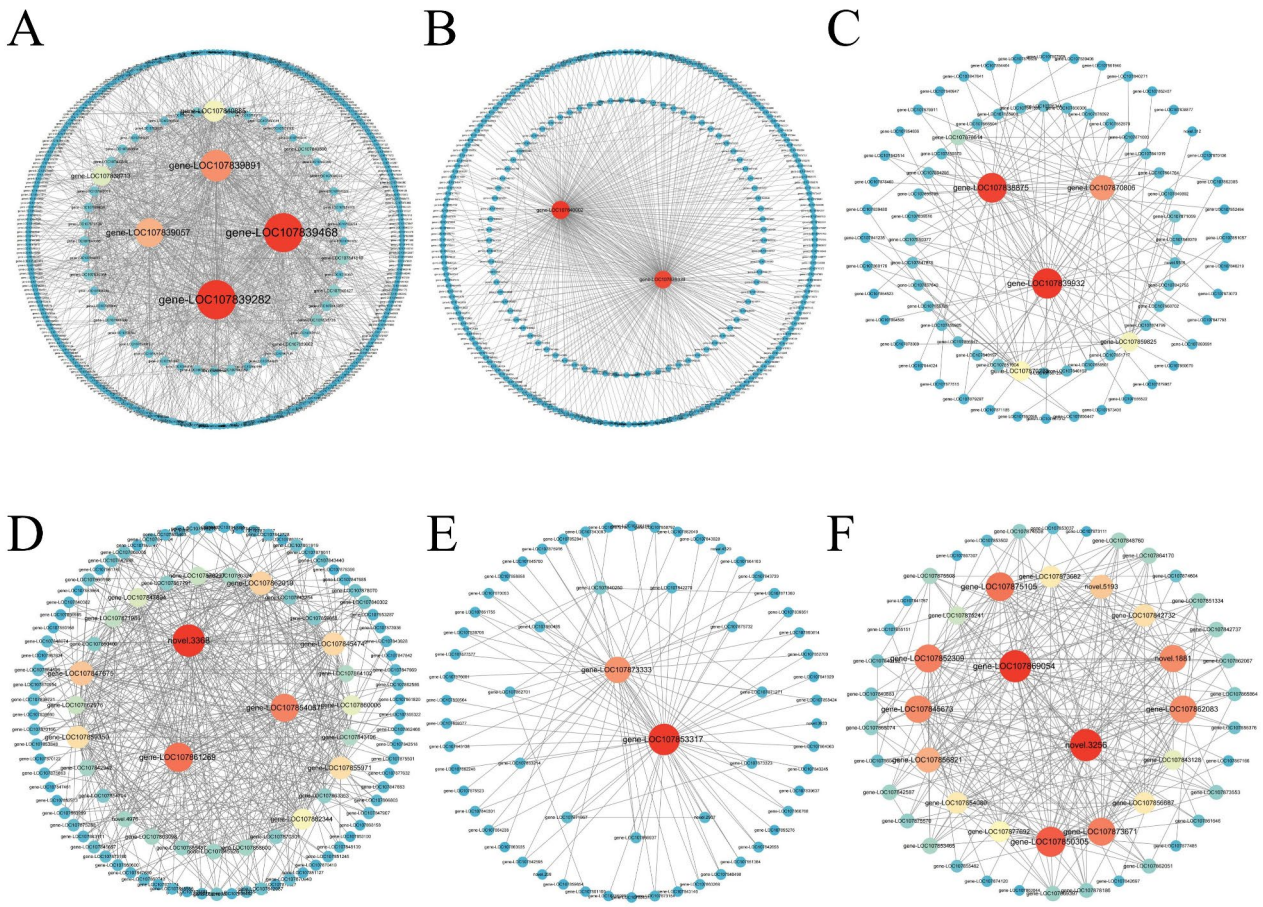

Fig. 5. The DEGs co-expressed networks. (**A**) Blue module. (**B**) Turquoise module. (**C**) Yellow module. (**D**) Magenta module. (**E**) Red module. (**F**) Greenyellow module. DEGs with edges weight above 0.5 were displayed. The color ranges from blue to red, and the dots from small to large represent the degree of connectivity from low to high.

RM), 7 key carotenoid metabolites and 14 key biosynthetic genes were involved in the regulation. The 14 key genes are: two *GGPPS* genes (*gene-LOC107843186* and *gene-LOC107867046*), *PSY1* (*gene-LOC107868281*) and *PSY2* (*gene-LOC107859651*), *PDS* (*gene-LOC107861625*), *Z-ISO* (*gene-LOC107850257*), *ZDS* (*gene-LOC107839468*), *CRTISO* (*gene-LOC107854534*), *LCYB* (*gene-LOC107869983*), *BCH1* (*gene-LOC107863219*) and *BCH2* (*gene-LOC107873401*), *LCYE* (*gene-LOC107840923* and *gene-LOC107852092*), *CCS* (*gene-LOC107875664*). Among them, *GGPPS*, *PSY1*, *PDS*, *Z-ISO*, *ZDS*, *LCYB*, *BCH1*, and *LCYE* were highly expressed at mature stage (65 DAP), positively regulating pigment accumulation. In contrast, *PSY2*, *CRTISO*, and *BCH2* showed higher expression in the green ripening fruit stage (50 DAP), which may be related to pigment accumulation in the green fruit.

The 7 key carotenoid metabolites are: α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin, lutein, capsanthin, and capsorubin. In YM and OM samples, α-carotene content was higher, related to high expression of *LCYB* gene. Genes *BCH2* and *BCH1* promoted lutein accumulation at green ripening and mature stages, respectively, laying the foundation for pepper fruit color changes. The significantly higher accumulation of zeaxanthin in OM samples was a key factor in color transition from green to orange. Furthermore, the *CCS* gene was only expressed in RM samples, and it determined capsanthin and capsorubin accumulatio, leading to red color in mature RM. The distinct accumulation patterns of different metabolites are closely related to specific DEGs during fruit development, which collectively regulate the color changes in pepper fruits.

qRT-PCR analysis

In order to confirm the accuracy of RNA-Seq data, we performed qRT-PCR experiments on the carotenoid biosynthesis pathway genes *PSY1*, *PSY2*, *PDS*, *Z-ISO*, *ZDS*, *CRTISO*, *LCYB*, *LCY-E*, *CYP97*, *BCH*, and *CCS* (Fig. 7). The expression levels of 12 genes were basically consistent with FPKM results, which supports the reliability of RNA-seq data in our study.

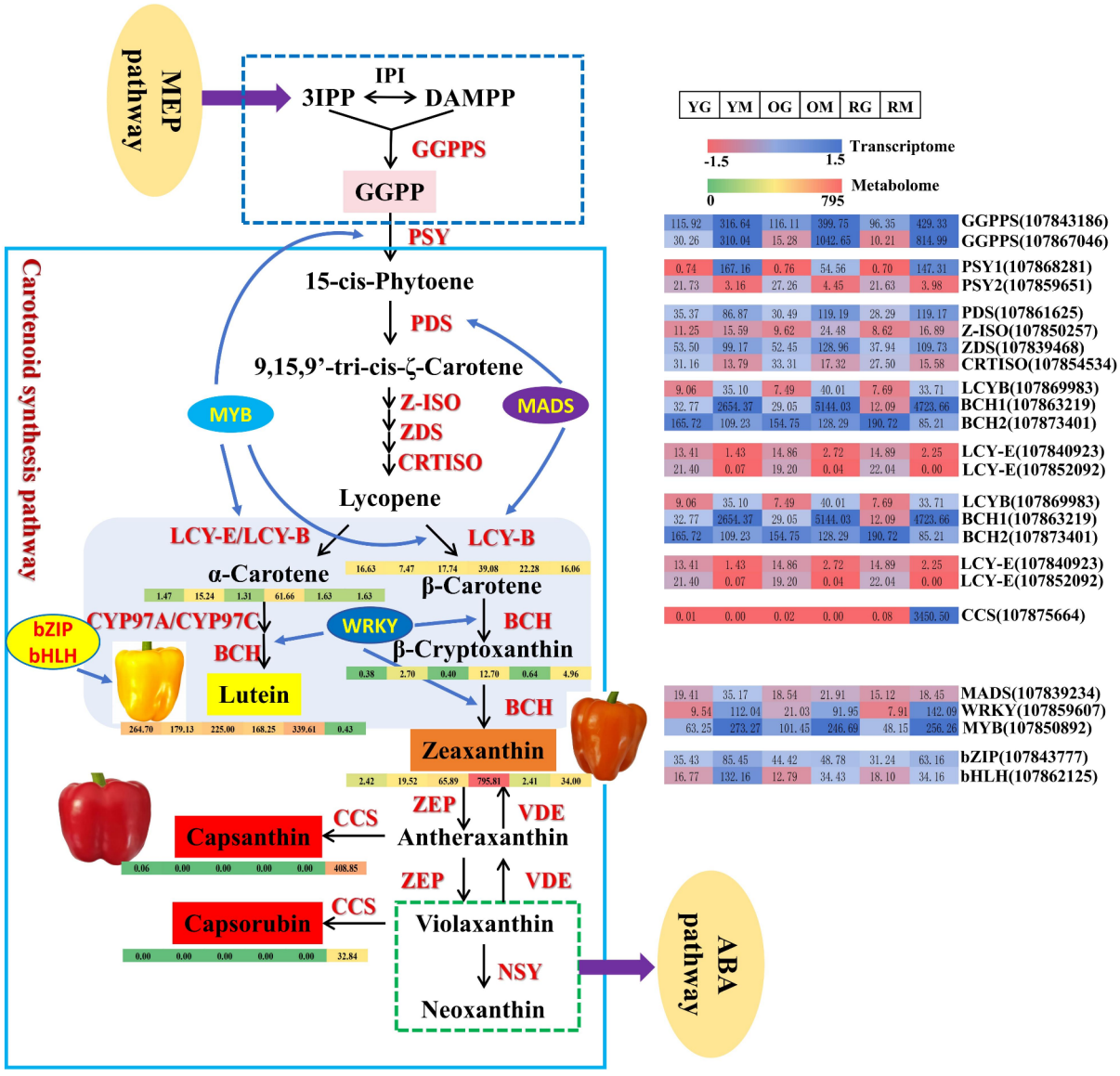

Fig. 6. Carotenoid synthesis pathway and key genes and metabolites regulating fruit color formation in bell pepper lines IL-Y, IL-O and IL-R. Expression of key genes and TFs (Transcriptome) and metabolites (Metabolome) were showed on scaled level among samples YG, YM, OG, OM, RG and RM.

Discussion

Carotenoids are natural pigments and essential fruits colorants of most horticultural crops[60–64]. Bell pepper contains many kinds of carotenoids with diverse fruit colors, and it is an excellent material for studying the regulation mechanism of carotenoid biosynthesis. In this study, three bell pepper lines with different fruit colors were analyzed by transcriptome and metabolome to explore the molecular mechanisms underlying the fruit color formation.

The main reason for the diversity of pepper fruit color is the variety of pigment types and contents accumulated, such as yellow, orange and red pepper, which is mainly due to the accumulation of various carotenoids[4–6,65]. In our study, 54 carotenoid metabolites were detected with various types enriched at different developmental stages (Table S1). Although carotenoids accumulates in fully expanded immature bell pepper fruits (50DAP), their types and content were very limited, and total carotenoid was mainly composed of lutein, β-carotene, etc. (Fig. 1B), consistent with previous study[66]. This suggests various carotenoids are generated mainly in late or middle developmental stages. However, in immature orange pepper OG, the content of zeaxanthin was also higher, which is worth our further exploration. With fruit ripening, (E/Z) phytoene accumulated more in OM and YM (Fig. 1F and G). As the first carotenoid product of carotenoid synthesis, (E/Z) phytoene provides precursors for the downstream carotenoid compounds synthesis. In YM and OM, yellow pigments lutein and

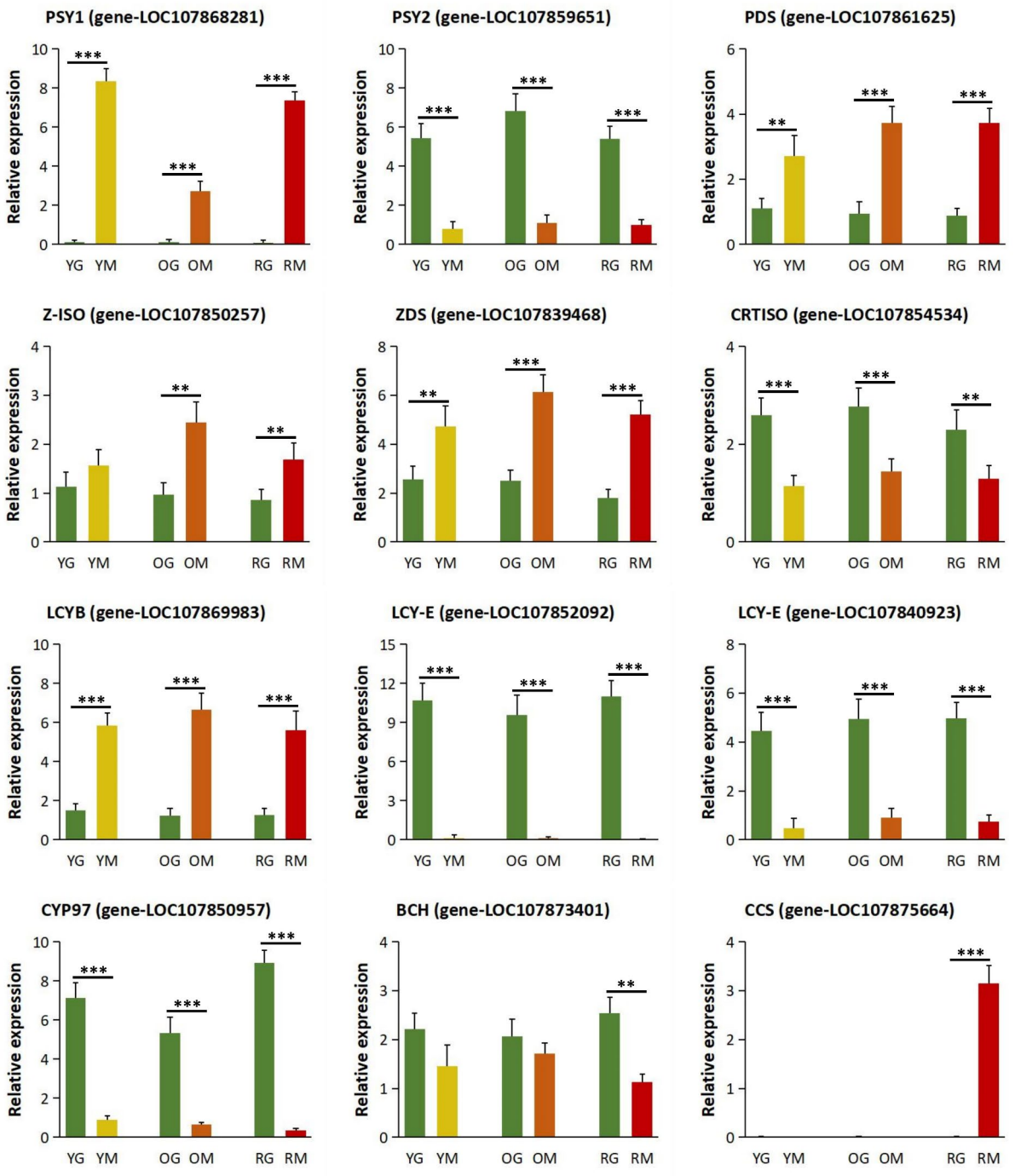

Fig. 7. Relative expression level of 12 genes related to carotenoid biosynthesis pathway in bell pepper lines IL-Y, IL-O and IL-R. Data were presented as the average of three biological replicates of samples YG, YM, OG, OM, RG and RM, respectively. ** and *** indicated significant differences between the green and mature stage at $p < 0.01$ and $p < 0.001$ level, respectively.

zeaxanthin was higher, while orange pigments α-carotene and β-carotene accumulated more in OM (61.567 μg/g and 39.081 μg/g), but lower in YM (15.24 μg/g and 7.47 μg/g). The differential accumulation of α-carotene and β-carotene may be the reason for color difference between orange OM and yellow YM fruits (Table S2). Carotenoids of red RM was obviously different from that in non-red pepper fruit YM and OM. RM contains 6

carotenoids, capsanthin, capsorubin, β-carotene, zeaxanthin, (E/Z)-phytoene and violaxanthin, among which capsanthin was the highest, accounting for 71.02% of the total carotenoids (Fig. 1C), but it was not detected in OM, YM and RG, indicating that capsanthin is a unique pigment type of mature red bell pepper. In addition, the lutein content in RM was extremely low (0.43184 μg/g), which was 0.07% of the total carotenoid content (Fig. 1C, Table S2). These results suggests that different patterns of carotenoid accumulation is responsible for color differences among varieties.

At present, carotenoids biosynthetic pathway was clear. With IPP and DMAPP as precursors, various carotenoids are generated through IPI, GGPPS, PSY, PDS, ZDS, LCYB, LCYE and other enzymes (Fig. 6)[23]. By RNA-seq analysis, 14,961 DEGs were identified, and 48 genes were identified within carotenoid metabolic pathway (ko00906) in bell pepper genome according to KEGG enrichment analysis (Table S4). In plant plastid, three IPP and one DMAPP molecule synthesized from MEP pathway produce one GGPP, an important precursor of carotenoid biosynthesis, catalyzing by GGPPS[67,68]. In this study, two *GGPPS* genes (*gene-LOC107843186* and *gene-LOC107867046*) were identified, with significantly increased expression at mature pepper (Table S4), thus increasing the proportion of GGPP in bell pepper fruits and changing the synthetic direction of penoids (Fig. 6). Phytoene is the first carotenoid product generated from rate-limiting reaction in carotenoid biosynthesis, GGPP condensation catalyzed by PSY[25]. Horticultural crops usually contain two or three *PSY* genes[69–72]. Two *PSY* genes were identified in our study, *PSY1* (*gene-LOC107868281*) and *PSY2* (*gene-LOC107859651*). Compared with the green ripening stage, *PSY1* (*gene-loc107868281*) expression was significantly higher in mature bell pepper fruits, which was corresponding to the higher content of phytoene in YM, OM and RM (Fig. 6). The expression of *PSY2* (*gene-LOC107859651*) decreased at maturity, indicating tissue expression preferences of *PSY1* and *PSY2*.

Generally, PDS, ZDS, Z-ISO and CRTISO are involved in the conversion of colorless phytoene into colored carotenoids[73]. In our study, *PDS* (*gene-LOC107861625*) and *ZDS* (*gene-LOC107839468*) expression was significantly induced at maturity stage. Previous studies showed that photo-isomerization appears to replace CRTISO in chloroplasts[74], may be responsible for why expression level of *CRTISO* (*gene-LOC107854534*) was not significantly different in comparison groups OG_vs_OM and RG_vs_RM. In plants, BCH and CYP play important roles in carotene hydroxylation[75]. BCH catalyzes the β-ring hydroxylation of β-carotene, and overexpression of *BCH1* increased lutein content, but decreased β-carotene in *Arabidopsis* and kiwifruit [76–78]. We found expression of *BCH1* (*gene-LOC107863219*) was extremely high in YM and OM, consistent with high accumulation of lutein and zeaxanthin in YM and OM (Figs. 1C and 6). As to *BCH2* (*gene-LOC107873401*), its expression decreased significantly at the mature stage. Similar results was obtained in sweet orange pulp, and *CsBCH2* gene expression increased at early stage then decreased after color change[39]. The different expression patterns of *BCH1* and *BCH2* suggested their different roles in carotenoids accumulation in bell pepper fruits. In RM, *BCH1* had high expression, but zeaxanthin was not accumulated much, and the possible reason may be that its downstream products were catalyzed by CCS to form capsanthin and capsorubin (Figs. 1C and 6). Previous study identified three loci controlling pepper fruit color, and *CCS* gene is the y loci[79]. Its structural variation is always the reason for color change during pepper ripening process[80,81]. *CCS* gene (*gene-loc107875664*) detected in our study showed high expression only in RM, consistent with the result that capsanthin and capsorubin were detected only in RM. Therefore, high expression of *CCS* caused red pigment accumulation and decrease of zeaxanthin, violaxanthin and antheraxanthin (Fig. 6, Table S1). Previous study showed inhibition of *LCYE* led to strong induction of *LCYB* and *CCS* with fruit maturity[82], the same as our study, with *LCYE* (*gene-LOC107840923* and *gene-LOC107852092*) expression decreasing, *LCYB* and *CCS* expression increased in mature bell pepper fruits. Therefore, antagonism between α and β branches, and decreased expression of *LCYE* may be the reason for lutein content decrease at mature stage (Table S1).

Taken together, we conclude that the structural genes *PSY1* (*gene-LOC107868281*), *PDS* (*gene-LOC107861625*), *ZDS* (*gene-LOC107839468*), *BCH1* (*gene-LOC107863219*), *LCYB* (*gene-LOC107869983*), and *CCS* (*gene-LOC107875664*) are key genes related to fruit color change in bell pepper.

TFs in plants, including *MYB*, *bHLH*, *MADS*, *bZIP*, *AP2/ERF*, and *WRKY*, play crucial roles in the regulation of carotenoid metabolism. An R-R-type *MYB* can directly bind to carotenoid biosynthetic genes promoter, and positively regulate capsanthin content[29]. In citrus, carotene genes expression was positively regulated by *MADS*[49,50]. In papaya, *CpNAC1* is a positive regulator for carotenoid by interaction with *CpPDS2/4* promoter[83]. The capsicum *bHLH* family may contribute to capsicum carotenoids and capsaicin biosynthesis[84]. In apple, *MdAP2-34*, a type of *AP2/ERF*, regulates phytoene and β-carotene accumulation[85]. By WCCNA analysis, blue and turquoise module were positively and negatively correlated with detected carotenoid metabolites, respectively (Fig. 5B). In blue module, 210 TFs were identified (Table S8), and 1 *MADs* (*gene-LOC107839234*), 2 *AP2/ERF*, 3 *HD-ZIP*, 1 *MYB* (*gene-LOC107850892*), 2 *NAC*, 1 *WRKY* (*gene-LOC107859607*) and 1 *NF-Y* were significantly up-regulated in YM, OM and RM (Table S8), and these TFs may be the positive regulator for carotenoid biosynthesis. In the blue module, structural genes such as *ZDS* (*gene-LOC107839468*), *PDS* (*gene-LOC107861625*), *BCH* (*gene-LOC107863219*), *GGPS* (*gene-LOC107843186*), and *LCYB* (*gene-LOC107869983*) involved in the carotenoid biosynthesis pathway were also identified (Fig. 6). These TFs and structural genes exhibit similar expression trends, jointly regulating carotenoid accumulation. It has been reported that *MADS* positively regulates the expression of *PDS*, *BCH*, and *LCYB* genes[48–50], promoting carotenoid accumulation, which is consistent with our experimental results (Fig. 6). Additionally, *WRKY* also regulates *BCH*[52], which aligns with our findings (Fig. 6). In turquoise module, 269 TFs were identified (Table S8), including 2 *MYBs*, 5 *AP2/ERF*, 3 *HD-ZIP*, 2 *BHLH*, 2 *MAD* and 1 *NAC*, which were significantly down-regulated, and they may be negative regulators for carotenoid biosynthesis. These results provide powerful clues for further study of carotenoid biosynthesis network.

Materials and methods

Plant materials

Three bell pepper lines, IL-Y, IL-O and IL-R, with yellow, orange and red fruit colors were used as materials (Fig. 1A). They were planted in the breeding field of Weifang University of Science and Technology (36.53°N, 118.46°E). Bell pepper fruits of IL-Y, IL-O and IL-R were sampled at 50 and 65 DAP with three replicates. After frozen immediately in liquid nitrogen, all samples were stored at -70 °C for subsequent transcriptome and metabolome analyses. Samples at 50DAP (green ripening stage) of IL-Y, IL-O and IL-R were named YG, OG and RG, and samples at 65DAP (mature period) were named YM, OM and RM.

Metabolome analysis of carotenoids

Analysis of sample preparation, metabolite extraction and identification were entrusted to Metware Biotechnology Co., Ltd. (Wuhan, China). Preparation and metabolite extraction were performed as described previously[86,87]. Carotenoids composition and content was detected by the ultra performance liquid chromatography (UPLC, ExionLC™ AD, https://sciex.com.cn/) system and tandem mass spectrometry (MS/MS, Applied Biosystems 6500 Triple Quadrupole, https://sciex.com.cn/) system. The multiple reaction monitoring (MRM) mode of triple quadrupole mass spectrometry was used for the quantification of carotenoid metabolite. Data acquisitions was processed by analyst 1.6.3 software (Sciex). The spectrometry data were analyzed by MultiQuant 3.0.3 software, in which the chromatographic peaks detected in different samples were integrated and corrected by referring to the retention times and peak shapes of standard compounds, ensuring the reliability of qualitative and quantitative analysis. The area of chromatographic peak represents the relative content of metabolites. Qualitative and quantitative results of tested substances was obtained using the linear equation and calculation formula. The DACs were screened under the thresholds of FC ≥ 2 or ≤ 0.5 and P-value ≤ 1.

Transcriptome analysis

Using methods described by Cao et al.[88], total RNA of samples OG, OM, RG, RM, YG and YM was extracted. A total of 18 libraries were constructed with three biological replicates for each sample. RNA quality was verified by Qubit2.0 and Agilent 2100 Bioanalyzer. After qualified library inspection, Illumina HiSeq platform HiSeq 4000 (Illumina, San Diego, CA, USA) was used for sequencing. The high-quality clean reads were aligned to the reference genome (https://solgenomics.net/ftp/genomes/Capsicum_annuum/C.annuum_zunla/) using HISAT2. DEGs were screened by DESeq2 [25] with a threshold of |log2(FC)| >= 1 and FDR < 0.05. FPKM was used to evaluate gene relative expression. Gene function annotation was performed using multiple databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Clusters of Orthologous Groups (COG), NCBI, the non-redundant protein sequence (Nr) database, the manually annotated and reviewed protein sequence database Swiss-Prot, and the TrEMBL database which contains all the translations of EMBL nucleotide sequence entries.

WGCNA analysis

We performed WGCNA using the R package[89]. All DEGs and key DACs detected were selected for combined analysis. The co-expression network was visualized by free software Cytoscape.

qRT-PCR verification

Total RNA of samples YG, YM, OG, OM, RG and RM was extracted using method previously described[88], respectively. The first-strand cDNA was reverse-transcribed by TIANScript IIcDNA Kit (Tiangen, China), and qRT-PCR was performed using SuperReal SYBR Green Premix kit (Tiangen, China) with three replications for each sample. *Actin* was used for reference, and relative gene expression was calculated by $2-\Delta\Delta Ct$ method. All primers used were listed in Table S10.

Conclusions

In this study, three bell pepper lines with different color (yellow, orange and red) were used as materials to conduct targeted metabolome and transcriptome analysis. The results showed that carotenoids showed different accumulation patterns during fruit development and showed significant variety specificity. The species and content of carotenoids in orange OM were significantly higher than YM and RM. Red pigment (capsanthin and capsorubin) was specifically enriched in RM, and yellow pigment (lutein and zeaxanthin) is the highest in YM and OM, and high content of orange pigment (α-carotene and β-carotene, etc.) was also accumulated in OM. Further analysis identified 27 genes involved in carotenoid biosynthesis, 9 of which expressed significantly higher at the mature stage (65DAP) than green ripening stage (50DAP), indicating their important roles in the color formation of bell pepper. Five modules positively correlated with carotenoid accumulation and one negative module were identified through WGCNA analysis, and key TFs and hub genes that may affect carotenoid content were predicted. By integrating the key DEGs and DACs, the study constructed a regulatory network for pepper fruit coloration, elucidating how the interplay between 14 key carotenoid biosynthesis genes, 5 key TFs, and 7 key metabolites collectively controlled the color changes of pepper fruits during different developmental stages. To sum up, the results not only provides important insights into carotenoid synthesis pathway, but also lays a solid data base for revealing color formation mechanism in bell pepper.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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