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Original article

Evaluation of the effect of high temperature on the full-length transcriptome and biochemical parameters of chili pepper fruit (*Capsicum annuum* L.) at different developmental stages

Rongyun Liu[1,2], Bozhi Yang[1], Lianzhen Mao[1], Yu Huang[1], Xiongze Dai[1], Wu Miao[2], Zhuqing Zhang[3], Wenchao Chen[3], Lijun Ou[1] and Zhoubin Liu[1]

[1] College of Horticulture, Hunan Agricultural University, Changsha, Hunan, China
[2] Hunan Xiangyan Seed Industry Co. Ltd., Changsha, China
[3] Vegetable Institution of Hunan Academy of Agricultural Science, Changsha, China

Summary

Background – **Chili pepper is one of the most important vegetable crops in the world, and the phenomenon of decreasing fruit yield and quality caused by high temperature stress seriously affects the production of pepper. However, there is little research on the molecular mechanism of high temperature stress on the fruit of chili pepper.** *Results* – **In this study, the results of biochemical analysis showed that high temperature stress significantly increased the contents of capsanthin and tocopherol in immature breaker (IMB) fruits, but did not change the length, weight, soluble sugar and soluble protein contents of IMB fruits. And through full-length transcriptome sequencing, a total of 23,609 genes, including 3,463 new genes were identified from immature green (IMG) fruit, IMB fruit and breaker (Br) fruits. And 1,720 differentially expressed genes (DEGs) were identified from the comparison of Br/IMG, Br/IMB and IMB/IMG groups. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that the DEGs are mainly concentrated in biosynthesis of amino acids, carbon metabolism pathway, starch and sucrose metabolism, DNA replication pathway and plant hormone transduction pathway.** *Conclusion* – **High temperature stress at fruit-set period inhibited the expression of fruit enlargement and expansion-related genes *auxin-responsive protein 27*, *auxin-responsive protein 16*, *auxin-induced protein 22-like*, *auxin-induced protein 22C-like*, *auxin-binding protein T92*, *polygalacturonase*, *xyloglucan endotransglucosylase/hydrolase protein 7*, *xyloglucan endotransglucosylase/hydrolase protein 9*, *expansin A10*, *expansin A13* and *glucomannan 4-beta-mannosyltransferase* in immature fruits, and increased the expression of *geranylgeranyl diphosphate synthase 1*, *phytoene synthase*, *beta-carotene 3-hydroxylase* and *capsanthin/capsorubin synthase 1* genes in the synthesis pathway of capsanthin, as well as *tocopherol cyclase* genes in the synthesis pathway of tocopherol, which led to the inhibition of enlargement and expansion in immature fruits and accelerated the fruit reddening.**

Keywords
capsanthin, fruit-set period, gene, immature, tocopherol

Significance of this study

What is already known on this subject?

- High temperature stress affects the pepper fruit yield and quality, but there is little research on the molecular mechanism of high temperature stress on the pepper fruits.

What are the new findings?

- Some key genes that restricted the growth of fruit expansion under high temperature stress were screened out, as well as key genes in the synthesis pathway of capsanthin with the immature fruit turning red in advance induced by high temperature stress.

What is the expected impact on horticulture?

- This study provided a new insight into the reasons why the expansion of pepper fruit was inhibited and the fruit turned red accelerated under high temperature stress, which lays a foundation for further research on the mechanism of fruit resistance to high temperature.

Introduction

In recent years, global temperature fluctuations have intensified, and the frequency of extreme temperature weather has increased. A large number of studies have found that low temperature stress can have adverse effects on the growth and development of many crops such as cucumber, tomatoes and sweet cherry (Miao et al., 2007; Liu et al., 2012; Vosnjak et al., 2021). In addition to low temperature stress, high temperature stress has also become one of the key environmental factors affecting the growth and development of vegetables (Mittler et al., 2012). Under high temperature stress, plant photosynthetic organs are directly damaged, resulting in a significant decrease in photosynthetic rate and chlorophyll content, which has become an important factor restricting plant production (Djanaguiraman et al., 2018). Studies have shown that the high temperature above 35°C leads to the accumulation of reactive oxygen species in plants and the destruction of cell components, the degradation of protein, cytoskeleton structure and biofilm stability, the reduction of enzymatic reaction efficiency, metabolic disorders, and the promotion of physiological aging (Suzuki et al., 2012; Han et al., 2013).

The effect of high temperature stress on fruit development has been studied. However, as the fruit is more sensitive to temperature changes as a reproductive organ, in addition to some common response mechanisms of plants to high temperature, compared with other crops or other non-fruit organs, the effect of high temperature stress on fruit has its own particularity. In terms of fruit quality formation, Fan et al. (2011) found that the titratable acid of apple fruit decreased after 8–12 h high temperature treatment at 46°C, while the soluble solid had no effect. In grapes, high temperature stress led to difficulty in sugar accumulation, lower PH value and anthocyanin content, as well as the reduction of nitrogen-containing compounds (Krasnow et al., 2010). In terms of fruit size, when subjected to high temperature stress, the average shoot biomass (including root) of mung beans was reduced by 41%, and the grain yield by 50% compared to normal condition (Patriyawaty et al., 2018). Exposure to high temperature stress hastens the development of reproductive parts and reduces the time to fruit ripening in strawberry regardless of flower position (Le et al., 1998). Ledesma and Kawabata (2016) also found that short but severe high temperature stress significantly reduces fruit set and fruit size in strawberry cultivars 'Nyoho' and 'Toyonoka'.

With the development of sequence technology, third-generation sequencing platforms can now be used to sequence full-length transcripts, which increase the accuracy of transcriptome characterisation compared with the transcript tags assembled from second-generation sequencing platforms. At present, full-length transcriptome sequencing (Iso-seq) technology has been widely used in plants. Zhu et al. (2017) used Iso-seq and other methods studied the alternative splicing mechanism of *Arabidopsis thaliana* after abscisic acid treatment, and found that about 83.4% of intron genes had variable splicing phenomenon. Wang et al. (2016) obtained 111,151 transcripts from 6 tissues of maize using Iso-seq technology, and captured about 70% of the annotated genes in the maize RefGen_v3 genome, among which 62,547 (57%) transcripts were completely new, and some were tissue-specific transcription isomers of some known genes. Dong et al. (2015), using Iso-seq technology, improved the gene annotation of wheat, found 3,026 new genes and 9,591 new homologous isomers, and also found the transcript of 72 different wheat gluten genes. However, there are few studies on the application of full-length transcriptome sequencing technology in *Solanaceae* crops, especially peppers.

Pepper (*Capsicum annuum* L.) is one of the most important vegetable crops worldwide, which originated in North America and belongs to the warmth-loving and heat-sensitive crops (Liu et al., 2017). The optimum temperature for chili pepper cultivation is 21–30°C, low and high temperature condition affect the size of fruit and seed germination ability (Korkmaz et al., 2010). When the temperature is higher than 35°C, a series of abnormal developmental phenomena will occur, such as delayed flower bud differentiation, abnormal flower organ development, decreased pollen viability, disturbance of fertilization process and influenced fruit size (Saha et al., 2010). At the same time, with the development of pepper cultivation facilities, a large number of greenhouse promotion, greenhouse high temperature, sub-high temperature phenomenon is more obvious in summer. In facility cultivation, it is difficult for early pepper to grow over summer, and the phenomenon of fruit size reduction and early reddening in medium and late ripening varieties is more common, leading to a sharp decrease in yield and affecting economic benefits. Therefore, through the full-length transcriptome sequencing (Iso-Seq) technology to analyze the molecular mechanism of high temperature stress affecting the fruit size development of capsicum, which was of great theoretical and practical significance for the mitigation of high temperature damage and promotion of commercial production of chili pepper.

Materials and methods

Plant growth and sampling

The pepper inbred line 'SJ11-3' was provided by Vegetable Research Institute, Hunan, China. In the middle of April 2019, the seeds were planted to the seedling cave plate and then placed in the Light incubator (CU-41L4, Percival Scientific Inc., U.S.A.) for unified management. In middle of May 2019, a total of 60 stronger seedlings were selected after the seedlings reached the five-leaf stage. Then 30 seedlings were transplanted to the solar greenhouse at the Vegetable demonstration base (113°09'E, 28°20'N), and the other 30 seedlings were transplanted to the open lands near to the solar greenhouse. The soil conditions for pepper planting in the two places were consistent with the fertilizer and water management. In late June, when the climate entered the high temperature period, the peppers planted in solar greenhouses and open lands were self-pollinated and the fruit development time was recorded. After self-pollinationed 20 days, immature green (IMG) fruits (average daily temperature of growing period 35 ± 3°C/22 ± 3°C) in open lands and immature breaker (IMB) fruits (daily temperature of growing period 43 ± 3°C/30 ± 3°C) in solar greenhouse were collected, and the breaker (Br) fruits (daily temperature of growing period 36 ± 3°C/22 ± 3°C) in open lands were also collected 40 days after self-pollinationed. During the whole treatment period, there was no abnormal climate such as abrupt temperature drop and continuous rainfall. All fruits were collected from three individual plants and measured for fruit length and weight. Then the fruits pericarp tissue was divided into two parts, one frozen in liquid nitrogen, stored at -80°C for transcriptome analyses, and one used for biochemical measurement. Three biological replicates in each treatment were performed.

Biochemical analysis and phenotypic data determination

All biochemical analyses were determined by spectrophotometer (UV1780, Shimadzu, Japan). The content of total soluble sugar was determined using the anthrone method (Min et al., 2014). Soluble proteins content was measured using a previously established method (Chu et al., 2010). The content of tocopherol was determined using the previously established method (Baydar and Erbas, 2005). Capsanthin were determined using our previously established method (Liu et al., 2019). The fruit weight was measured by weighing balance (BSA2245, Olabo, China), and the fruit length was measured by vernier caliper (DL92300, Deli, China).

RNA extraction and library preparation

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocols. RNA quality was detected by Rnase-free agar-gel electrophoresis, concentration and purity of total RNA samples were detected by NanoDrop 2000, and total RNA samples were detected by Agilent 2100 (Agilent Technologies, Santa Clara, CA, U.S.A.) for 28S/18S and RIN values. mRNA enrichment was carried out using the mRNA capture Beads (Novizan) to purify Poly(A) mRNA

from total RNA. The reverse transcription primers were used to bind to the Poly A tail of mRNA by annealing, and then the first strand was synthesized by adding the RT reversal rate enzyme. The primers were amplified to double strand cDNA by PCR amplification. The treatment of nucleic acid fragments with NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair/dA-Tailing Module was used to complete the damage repair and end repair plus A. Finally, the connection of the sequencing connector was completed with SQK-LSK109 (ONT) kit, and sequencing was conducted on the PromethION 48 platform of Oxford Nanopore Technologies. Nine RNA-seq libraries were constructed and sequenced after three replications in each sample.

Full-length transcriptome sequencing and sequence analysis

Oxford Nanopore Technologies Long Read Processing and Remove redundant: Raw reads were first filtered with minimum average read quality score = 7 and minimum read length = 500 bp. Ribosomal RNA were discarded after mapping to rRNA database. Next, full-length, non-chimeric (FLNC) transcripts were determined by searching for primer at both ends of reads. Clusters of FLNC transcripts were obtained after mapping to reference genome with minimap2, and consensus isoforms were obtained after polishing within each cluster by pinfish. Consensus sequences were mapped to reference genome using minimap2. Mapped reads were further collapsed by cDNA Cupcake package with min-coverage = 85% and min-identity = 90%. 5' difference was not considered when collapsing redundant transcripts.

Find fusion transcript: Fusion candidate genes should be screened according to the criteria that a single transcript must be mapped to two or more loci, the minimum coverage for each loci is 5% and the minimum coverage in bp is ≥1 bp, the total coverage is ≥95%, and the distance between the loci is at least 10 kb.

Functional enrichment analysis: Gene Ontology (GO) annotations of differentially expressed genes (DEGs) were obtained through the Blast2GO program. GO enrichment analysis was performed by the GOseq R packages based Wallenius non-central hyper-genometric distribution. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation was carried out with a BLAST search against the KEGG database (Ogata et al., 1999) and pathway enrichment analysis was performed by KOBAS 2.0 (Xie et al., 2011). GO terms and KEGG pathways with a corrected $P < 0.05$ were considered significantly enriched.

Quantitative real time PCR

qRT-PCR was conducted as described by Osorio et al. (2012). Three replicates were performed for each sample. *CpActin* gene was used as the internal control. Primers used in the study are listed in Supplemental Table S1. The relative gene expression levels were normalized using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Data analysis

The experimental results were expressed as mean ± standard error and analyzed using Excel 2010 and SPSS 22.0. Significance of differences among different data sets was analyzed using Duncan's multiple range test at a significance level of $p < 0.05$.

Accession numbers

RNA-Seq data generated in this study is available from the SRA-Archive (http://www.ncbi.nlm.nih.gov/sra) with accession number PRJNA669076.

Results

Phenotype and biochemical response of chili pepper fruit

Through phenotypic investigation, it was found that the fruit lengths and weights of immature breaker (IMB) fruit was the same as that of immature green (IMG) fruit, while the fruit color was the same as that of breaker (Br) fruit

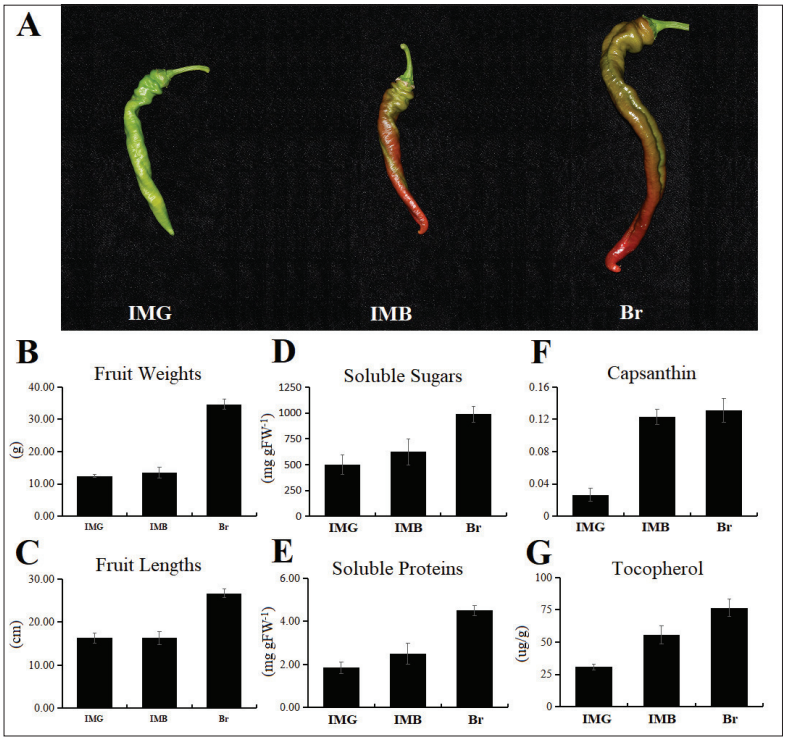


Figure 1. Phenotypic and physiological response of pepper fruit. IMG: immature green; IMB: immature breaker; Br: breaker.

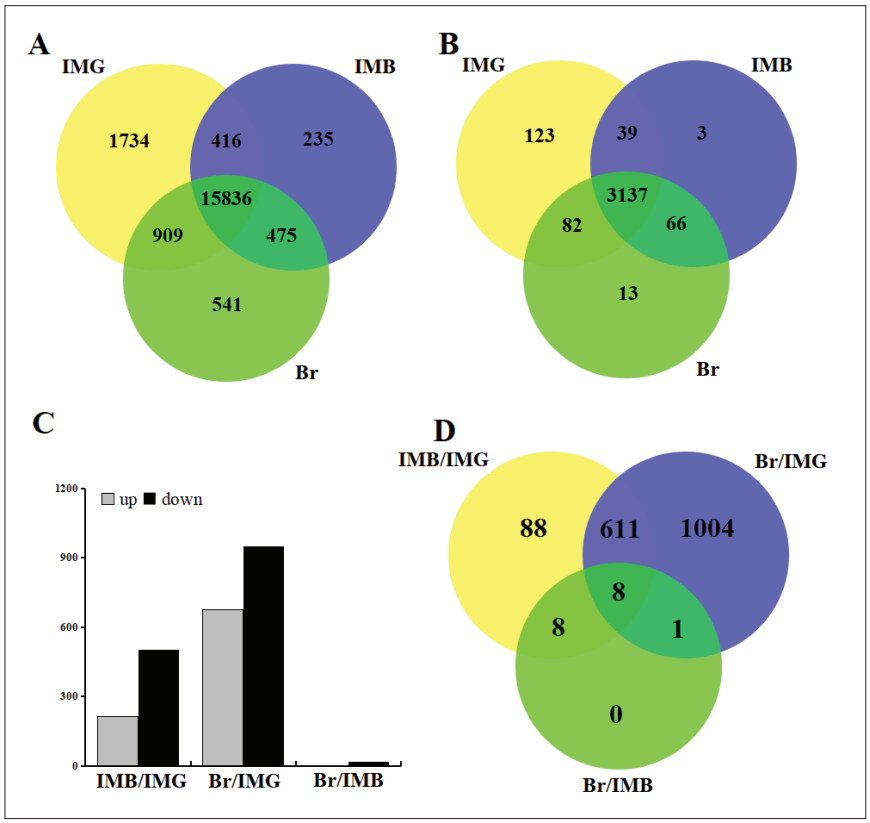


FIGURE 2. Statistics of identified genes. A: Venn diagram indicating known genes in each sample class; B: Venn diagram indicating novel genes coverage in each sample class; C: numbers of DEGs in different comparison groups; D: Venn diagram indicating DEGs coverage in each comparison group.

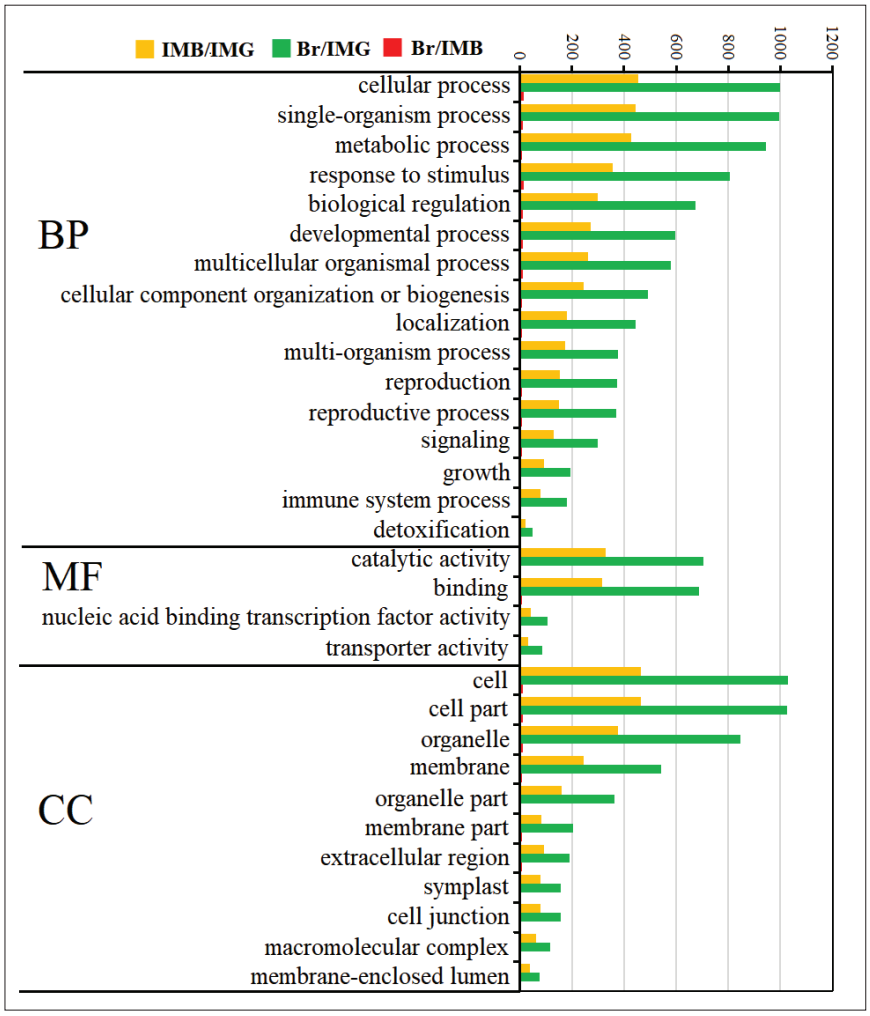


FIGURE 3. GO analysis of differentially expressed genes. BP: biological process; MF: molecular function; CC: cellular component.

(Figure 1A–C). In addition to phenotypic changes, the quality of IMB fruits also changed. It was found that the contents of soluble sugars and soluble proteins in IMB fruits were not significantly different from those in IMG fruits, but were significantly lower than those in Br fruits ($p \leq 0.05$) (Figure 1D, E). However, the content of capsanthin was significantly higher than that of IMG fruit, and there was no significant difference from that of Br fruit (Figure 1F). And the content of tocopherol in IMB fruit was significantly higher than that of IMG fruit and lower than that of Br fruit ($p \leq 0.05$) (Figure 1G).

Specificity of gene accumulation at different stages of pepper fruit

A total of 25,265,089 raw reads were obtained from nine pepper samples by Nanopore PromethION sequencing. After removing rRNA, adaptor and low-quality sequence, a total of 25,150,825 clean high-quality sequences were obtained, among which the number of full-length reads was 19,835,973, and the proportion of full-length reads in each sample was between 74.27% and 81.52%. After mapping to the *C. annuum* L_Zunla-1 Database (Qin et al., 2014), 38,835 transcripts were detected in whole samples. After

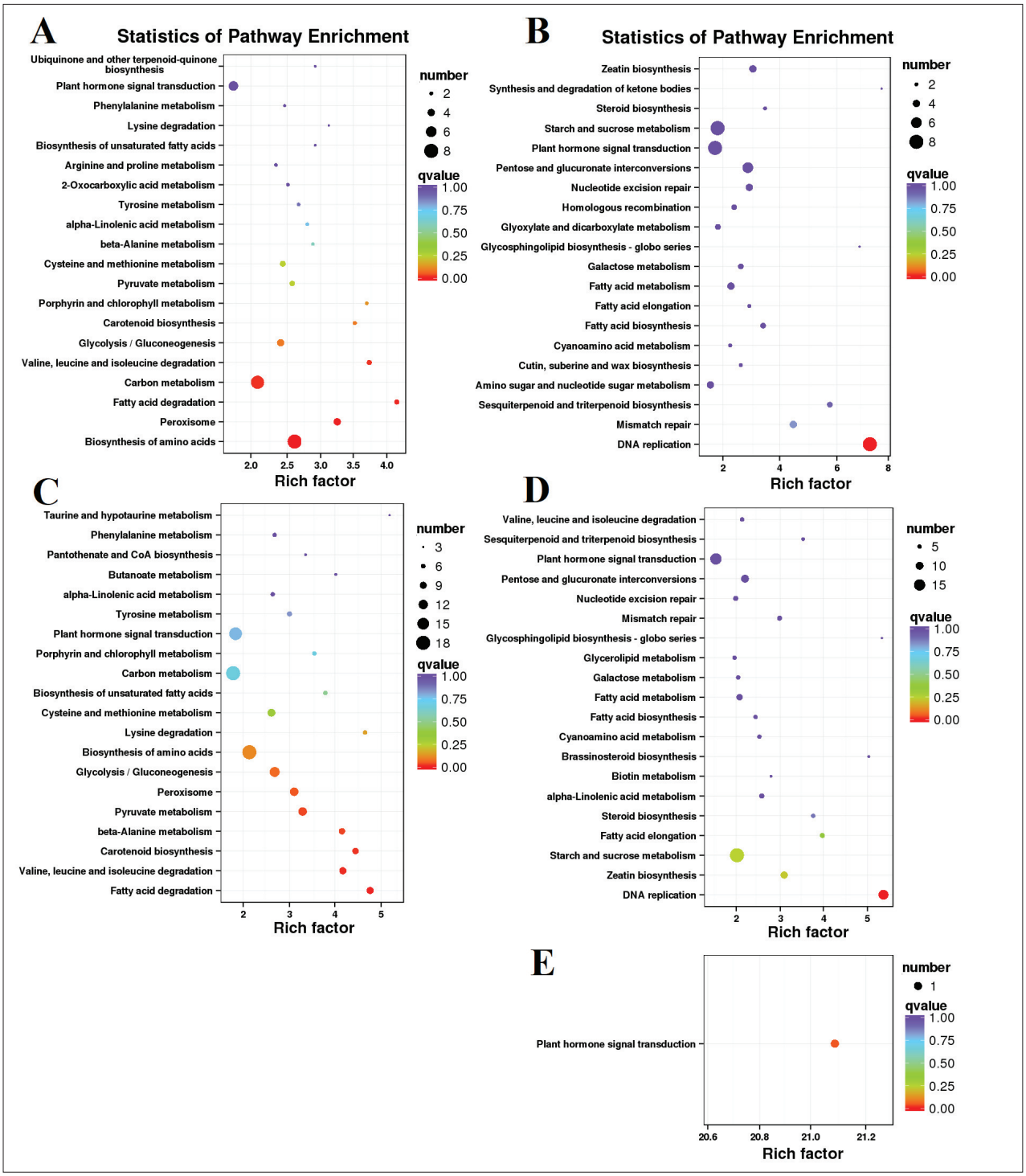


Figure 4. KEGG enrichment analysis of differentially expressed genes. A and B represent up-regulated and down-regulated genes in IMB/IMG fruit, respectively. C and D represent up-regulated and down-regulated genes in Br/IMG fruit, respectively. E represents down-regulated genes in Br/IMB fruit, respectively.

identification, a total of 23,609 genes, including 3,463 new genes, were identified. Among these known genes, 15,836 of 20,146 (78.61%) identified genes were found in all samples, and 1,734, 235 and 541 known genes were identified only in IMG, IMB and Br fruit, respectively (Figure 2A). Among those identified novel genes, 3,137 of 3,463 (90.59%) novel genes were found in all samples, and 123, 3 and 13 novel genes were identified only in IMG, IMB and Br fruits, respectively (Figure 2B).

Identification of differentially expressed genes (DEGs)

A total of 1,720 DEGs were identified in the comparison with each groups (Supplemental Table S2). Among which, 715 (212 up-regulated, 503 down-regulated), 1,624 (675 up-regulated, 949 down-regulated) and 17 (17 down-regulated) DEGs were obtained in IMB/IMG, Br/IMG and Br/IMB group, respectively (Figure 2C). Further analysis of DEGs revealed that eight genes, including *mitogen-activated protein kinase kinase kinase a-like* (Capana00g002533), *eid1-like F-box protein 3* (Capana06g000067) and *L-ascorbate oxidase homolog* (Capana08g002791), etc., were all significantly expressed in the three comparison groups (Figure 2D). In these comparison groups, in addition to the DEGs identified in other groups, there were 88 and 1,004 DEGs only identified in IMB/IMG and Br/IMG, respectively, while there were no specific DEGs identified in Br/IMB. This indicated that among the three fruit samples, the difference in fruit characters between IMG and Br was the largest, while the difference in fruit characters between Br and IMB was the smallest.

GO analysis

Gene Ontology (GO) database was used to categorize all of the identified DEGs (Figure 3). Results showed that DEGs cover a wide range of biological process, molecular function, and cellular component, which can be classified into 16, 4, and 11 categories, respectively. In terms of biological process, the largest group is cellular process, followed by single-organism process and metabolic process. Catalytic activity and binding were predominant in the category of molecular function. Then the cellular components of these DEGs mainly belong to cell, cell part, and organelle. Although there were significant differences in the number of DEGs in IMB/IMG and Br/IMG, but the functional classes in this two comparison groups maintained the largest DEGs number and the proportion was almost stable. At the same time, it was found that although there were only 17 DEGs in Br/IMB, these DEGs were mainly concentrated in cellular process and response to stimulus in biological process, as well as cells and cell parts in cell component.

KEGG analysis

KEGG pathways were performed to reveal the functions of DEGs that might be related to the process of pepper development. Pathway annotation results showed that 153, 325 and 1 DEGs were classified into 85, 105 and 1 pathways annotated in the IMB/IMG, Br/IMG and Br/IMB, respectively. Enrichment analysis was then performed on the metabolic pathways annotated by up-regulated and down-regulated DEGs. It was found that in IMB/IMG and Br/IMG fruit, the up-regulated DEGs were mainly enrichment in the biosynthesis of amino acids, carbon metabolism and plant hormone signal transduction pathways (Figure 4A, C), while down-regulated DEGs were mainly enrichment in the starch and sucrose metabolism, DNA replication and plant hormone signal transduction pathway (Figure 4B, D). And Br/IMB showed only one DEG enrichment in the plant hormone signal transduction pathway (Figure 4E). It was found that in these comparison groups, up- and down-regulated DEGs were both obviously enrichment in the plant hormone transduction pathway. This suggests that the Br and IMB fruits may be regulated by the genes related to biosynthesis, carbon metabolism pathway, and negative feedback regulation by genes re-

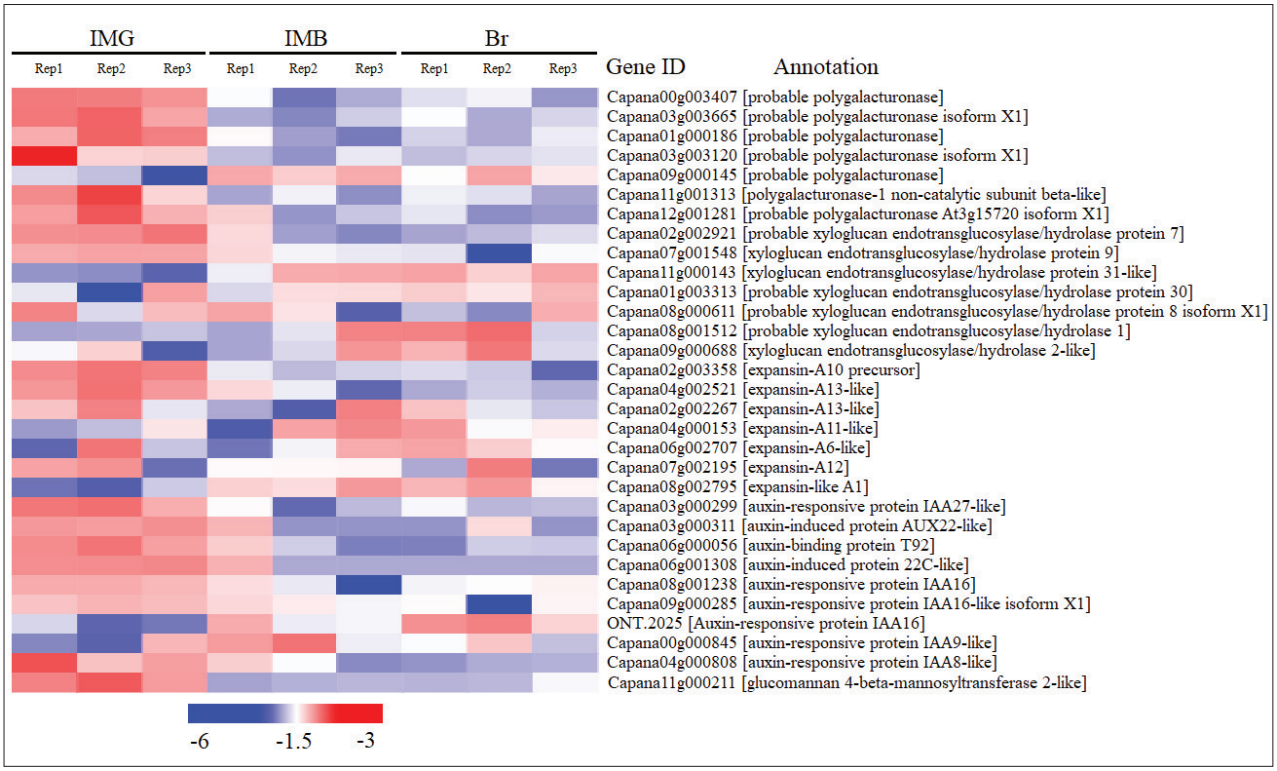


Figure 5. Heat map analysis of fruit enlargement and ripening related genes.

lated to starch and sucrose metabolism, and DNA Replication pathway. And plant hormone transduction pathway related gene regulation has both positive effect, also have negative feedback regulation effect.

Expression changes of fruit enlargement and ripening related genes

During the development of pepper fruit, a large number of gene changes related to fruit enlargement and ripening were involved. With the gene expression level Count Per Million (CPM) > 10 identified in at least one fruit as the screening condition, 31 fruit enlargement and ripening related genes, including *polygalacturonase* (*PG*), *xyloglucan endotransglucosylase/hydrolase protein* (*XTH*), *expansin* (*EXP*), *auxin-responsive protein* (*IAA*) and *glucomannan 4-beta-mannosyltransferase* (*CSLA*), etc., were quantified in the three pepper fruits (Figure 5). Among these genes, *PG* (Capana00g003407, Capana03g003665), *XTH7* (Capana02g002921), *XTH9* (Capana07g001548), *EXPA10* (Capana02g003358), *EXPA13* (Capana04g002521), *IAA27* (Capana03g000299), *IAA16* (Capana08g001238, Capana09g000285), *AUX22* (Capana03g000311), *AUX22C* (Capana06g001308), *AUXT92* (Capana06g000056), and *CSLA2* (Capana11g000211) had a high expression level in IMG fruit, and then decreased significantly in the Br fruit with the fruit development. Interestingly, the expression level of these genes in IMB fruit was also significantly lower than that in IMG fruit, while there was no significant difference from that in Br fruit ($p \leq 0.05$). *XTH31* (Capana11g000143), *EXPA1* (Capana11g000143) and *IAA16* (ONT.2025) had lower expression levels in the IMG fruit, and significantly increased in the Br fruit ($p \leq 0.05$). However, in general, the expression levels of these growth and expansion related genes were basically the same at the IMB and Br stages, but significantly different from those at the IMG stage ($p \leq 0.05$).

Genetic changes involved in the biosynthesis of capsanthin and tocopherol

Due to the phenotypic investigation found that the immatured pepper fruit turning red, the gene expression changes in the synthesis pathway of capsanthin were further analyzed (Figure 6). The results showed that the expression level of phytoene desaturase (*PDS*, Capana03g000054), lycopene beta-cyclase (*LcyB*, Capana05g000023), *beta-carotene 3-hydroxylase* (*CrtZ-2*, Capana03g002170), *capsanthin/capsorubin synthase* (*CCS1*, Capana06g000615), *phytoene synthase* (*PSY*, Capana04g002519) and *geranylgeranyl diphosphate synthase 1* (*GGPS1*, Capana04g000412) in the Br fruits were 1.86, 1.85, 7.16, 10.51, 5.31 and 3.74-fold higher than those of IMG fruits, respectively. At the same time, in IMB fruit, the expression levels of *LcyB*, *CrtZ-2*, *CCS1*, *PSY* and *GGPS1* were also 1.56, 7.09, 10.20, 5.75 and 3.47-fold higher than those of IMG fruits ($p \leq 0.05$), respectively. However, there was no significant difference in the genes related to capsanthin biosynthesis between IMB fruits and Br fruits. This indicates that the molecular mechanism of immatured pepper fruit turning red caused by high temperature is basically the same as that of normal color turning of capsanthin, which is caused by the induction of high expression of *LcyB*, *CRTZ-2*, *CCS1*, *PSY* and *GGPS1*, thus leading to the initiation of synthesis of capsanthin.

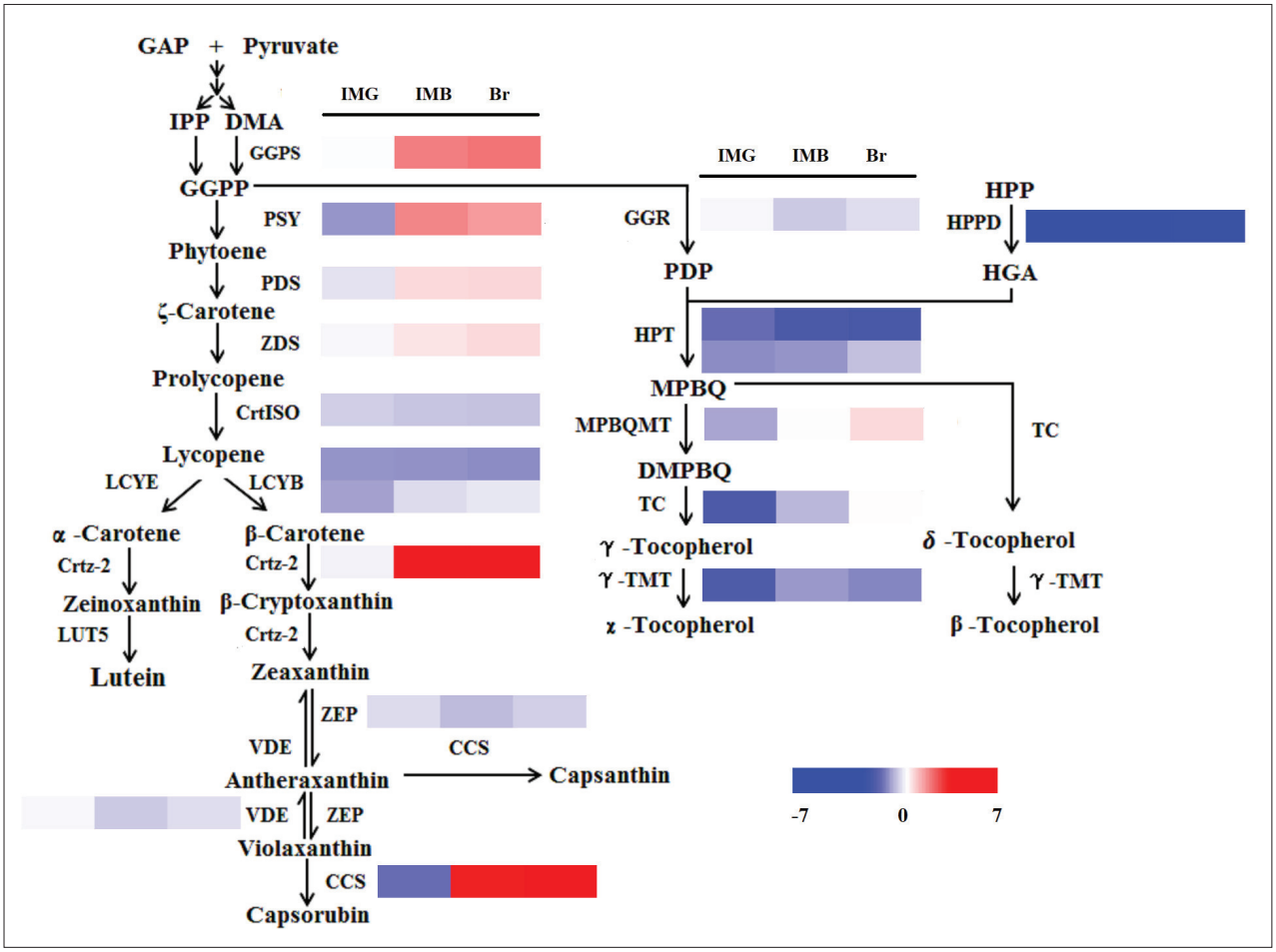


Figure 6. Comparative analysis of carotenoid and tocopherol metabolic genes.

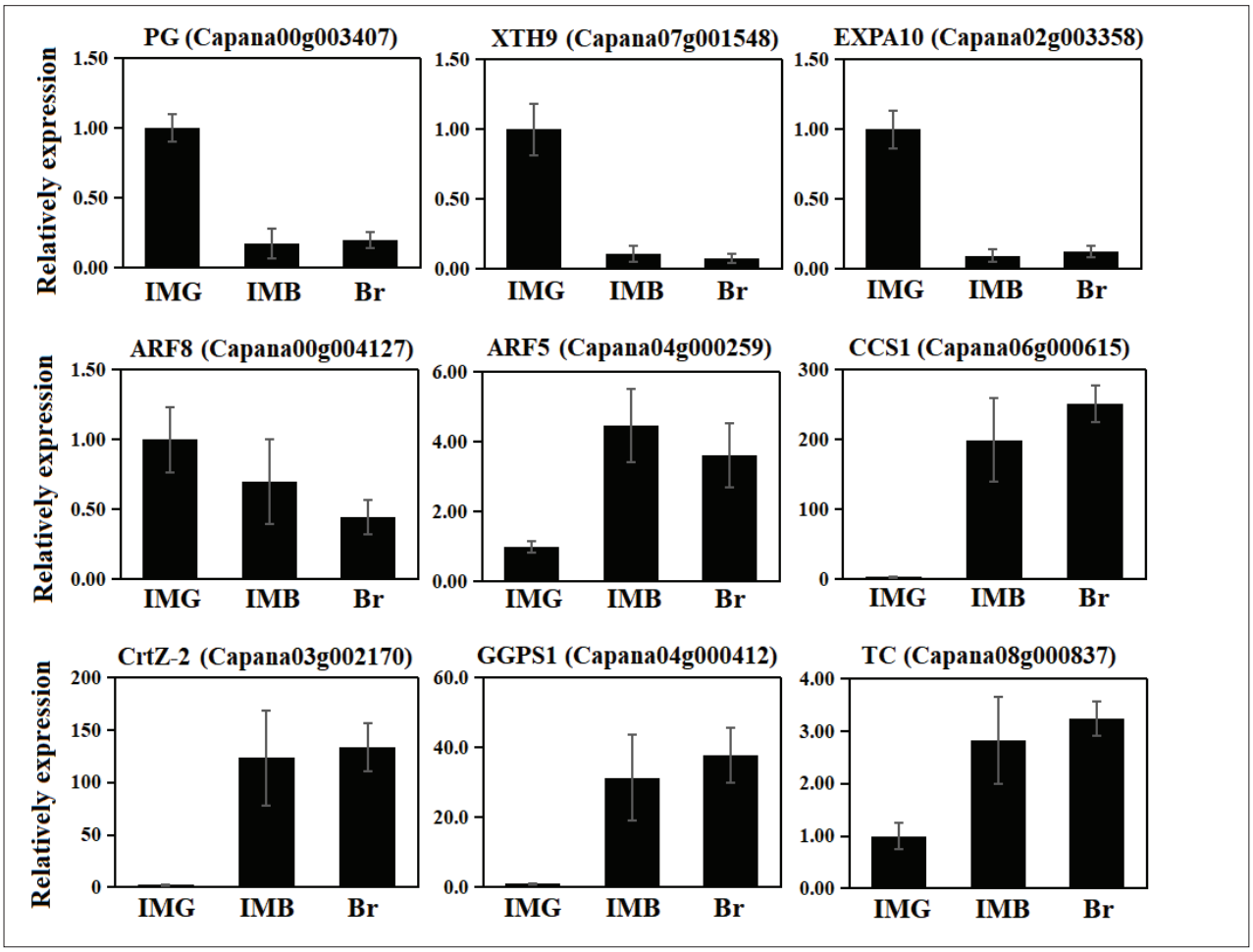


Figure 7. Validation and expression analysis of selected genes using qRT-PCR ($p \leq 0.05$).

In the biosynthesis pathway of tocopherol, compared with IMG fruits, the expression of *tocopherol cyclase* (*TC*, Capana08g000837) in IMB and Br fruits was significantly increased by 1.67- and 2.02-fold ($p \leq 0.05$), respectively. The expression levels of other genes, even though 2-methyl-6-phytylbenzoquinine methyltransferase (*MPBQMT,* Capana04g002867) and γ-tocopherol methyltransferase (*TMT*, Capana01g001103) were increased, *GGR* (Capana03g000791) was decreased, but there were no significant difference compared with IMG fruits. At the same time, there was no significant difference in the genes of tocopherol biosynthesis pathway between IMB and Br fruit.

qRT-PCR validation of gene expression patterns

In order to validate the results of Iso-Seq, qRT-PCR analysis were performed on 9 selected genes (*PG*, *XTH9*, *EXPA10*, *ARF8*, *ARF5*, *CCS1*, *Crtz-2*, *GGPS1*, and *TC*) using gene-specific primers. Transcript abundance patterns were calculated over the three pepper fruits. qRT-PCR analysis showed a similar trend of transcript abundance when assessed by real-time RT-PCR (Figure 7), thus confirming the CPM valve of each transcript determined by Iso-Seq was reliable in this study.

Discussion

Soluble sugar and soluble protein, as the basic nutrients of fruits, gradually increased with the development of fruits, which was consistent with the results of previous studies (Faurobert et al., 2007; Kahlau and Bock, 2008). However, high temperature stress did not cause significant changes in fruit length, weight, soluble sugar and soluble protein contents, indicating that high temperature stress may have little effect on these biochemical and phenotypic traits. In this study, the contents of capsanthin and tocopherol were significantly increased under high temperature stress, probably due to the accelerated process of turning color to red, and the formation of red color in fruits was caused by the synthesis and accumulation of capsanthin. The increase of tocopherol was due to the fact that tocopherol and capsanthin had the same metabolic intermediate substrate in the synthesis pathway, the increase of capsanthin promoted the increase of tocopherol content, which was consistent with the change of tocopherol content in soybeans under high temperature stress (Chennupati et al., 2011).

Auxin is the first plant hormone discovered, which can promote the expansion of plant cells, cause plant tropism growth, and participate in the formation of plant tissues and organs as well as the development and maturation of fruits (Kazan and Manners, 2009). During fruit development, the expression levels of *FaIAA1* and *FaIAA2* were higher in the small green stage and ginkgo stage, while the expression levels of *FaIAA1* and *FaIAA2* were lowest in the big green stage (Liu et al., 2011). Silencing of *SlIAA27* gene not only causes tomato parthenocarpy, but also causes fruit to be smaller (Bassa et al., 2012). While after the tomato *SlIAA17* gene was silenced, the fruit cells of the transgenic plants were signifi-

cantly increased, the skin was thickened, and the fruit was enlarged compared with the wild type (Su et al., 2014, 2015). This indicated that changes in *IAA*/*AUX* gene expression level could lead to changes in fruit size. In this study, the expression levels of *IAA27*, *IAA16*, *AUX22*, *AUX22C* and *AUXT92* were significantly decreased at the Br fruit, which was consistent with our previous results (Liu et al., 2019), suggesting that these genes may play an important regulatory role in promoting fruit expansion in the early stage of fruit development. Compared with IMG fruit, its expression in IMB fruit decreased significantly, but there was no significant difference compared with Br fruit. These results indicated that the expression of *IAA27*, *IAA16*, *AUX22*, *AUX22C* and *AUXT92* in the immatured fruit of pepper was affected by high temperature stress, and the expression in immatured fruit was inhibited, thus the growth of the fruit was inhibited.

Normally, the expansion of pepper fruit is basically completed before the breaking stage. In the early stage, the fruit growth is mainly the result of cell division, but when the cell division stops, the fruit continues to grow through cell expansion. During the process of plant growth, the increase of cell number and the change of cell volume are accompanied by the change of xyloglucan and the reconstruction of cell wall, which is a complex network structure, and its components include cellulose, hemicellulose, pectin and a small amount of structural proteins (Cosgrove, 2016). Cellulose is synthesized by cellulose synthase located in the plasma membrane. The mutation of *KOB1*, a gene related to cellulose synthesis, will affect the synthesis of cellulose in *Arabidopsis* root cells, thus stunting plants (Pagant et al., 2002). As the most important hemicellulose in the cell wall, the ratio of xyloglucan to XTH determines the elongation rate of the cells. When the ratio of XTH to xyloglucan is high, XTH will enter the cell wall as a free enzyme and directly cut and recut xyloglucan, thereby relaxing the cell wall and promoting cell elongation (Nishikubo et al., 2011). Expansin family are proteins that facilitate expansion of cell walls can directly induce cell growth by breaking the hydrogen bonds between cellulose microfilaments in the walls and cross-coupled polysaccharides (Cosgrove et al., 1997). In this study, *PG*, *XTH7*, *XTH9*, *EXPA10*, *EXPA13*, *CSLA2* and other growth-related genes were largely expressed in the IMG fruit and significantly decreased in the Br fruit, indicating that these genes were all involved in the growth and expansion of pepper fruits at the early stage of development, which was consistent with our previous research results (Liu et al., 2019). But in the IMB fruit, due to subjected the high temperature stress, although the fruit has not been completely expanded, but the expression level of these genes related to expansion has been significantly lower than that of IMG fruit with the same incomplete expansion, and there is no significant difference between the expression level of the fruit with the Br fruit which expansion growth has been completed. This indicated that the high temperature stress inhibited the expression of genes related to growth expansion in the immature green fruit, which led to the stop of growth expansion in the immature green fruit and the fruit became smaller and entered the mature stage. This is consistent with the results of the study that the fruit of strawberry becomes smaller after being subjected to high temperature stress (Ledesma and Kawabata, 2016). It is believed that the high temperature may enhance the strength of plant "library", promote the rapid operation of photosynthetic products (Walker and Ho, 1977), accelerate the development of fruit cells, accumulate more dry matter in the fruit than the control group, and thus advance the development of fruit. However, due to the harmful effect of high temperature on the plants, the photosynthesis is weakened and respiration is enhanced, and finally the supply of the necessary anabolic substances for the full development of fruit is insufficient, which leads to the early ripening period of fruit and the decrease of the weight of fruit.

The change of pepper fruit color to red is mainly caused by the large increase of capsanthin content in the carotenoid pathway (Tian et al., 2014). *PSY* is a key enzyme in plant carotenoid biosynthesis pathway, which plays an important role in controlling the total accumulation level of carotenoids (Ampomah-Dwamena et al., 2015) and its expression is up-regulated during fruit coloration (Tao et al., 2007). *Crtz-2* is one of the two isomers of the downstream gene *Crtz* in the synthesis pathway of capsanthin, which is highly expressed in the late stage of fruit ripening and plays a normal function in fruit coloration (Thorup et al., 2000). *CCS* gene exists on the membrane of the peel of peppers (Bouvier et al., 1994), and through high expression of synthetic capsanthin in the red pepper fruit instead of chlorophyll, the fruit turns red. Ha et al. (2007) found that CCS gene silencing would lead to the failure of normal synthesis of capsanthin, resulting in the yellow color of mature fruits. And the up-regulated expression of GGPS protein in sweet orange would promote the increase of substrate content used for lycopene accumulation (Pan et al., 2012). In this study, *GGPS1*, *PSY*, *Crtz-2* and *CCS1*, the key genes in the synthesis pathway of capsanthin, showed no significant difference in expression levels in IMB and Br fruits, and they were all significantly higher than IMG fruits. This indicated that the high temperature stress at fruit setting stage promoted the high expression of *PSY*, *Crtz-2* and *CCS1* in the synthesis pathway of capsanthin in the fruit of peppers, and made the expression level of the fruits in the immature fruit reach that of the fruits in the normal breaking fruit, so as to promote the rapid redness of the fruits.

The GGDP substrate catalyzed and regulated by *GGPS* gene is not only involved in the synthesis of capsanthin, but also in the biosynthesis of tocopherol (Liu et al., 2019). In this study, in addition to *GGPS* gene, the expression of *TC* in tocopherol biosynthesis pathway was also significantly higher in IMB and Br fruits than in IMG fruits. Previous studies have suggested that the deletion of *TC*, which encoding *VTE1* gene, would result in the absence of tocopherol synthesis in *Arabidopsis*, while overexpression of *VTE1* gene would lead to the increase of total tocopherol content in leaves (Kanwischer et al., 2005). Therefore, with the development of fruit, the expression of *GGPS* and *TC* genes is the key to promote the synthesis of tocopherol in pepper fruit. High temperature stress will also accelerate the high expression of *TC* gene in immature fruit, thus accelerating the synthesis of tocopherol.

Conclusion

In this study, it was found that high temperature stress significantly increased the contents of capsanthin and tocopherol in IMB fruits, but did not change the length, weight, soluble sugar and soluble protein contents of immature fruits. A total of 1,720 DEGs were identified from the comparison of Br/IMG, Br/IMB and IMB/IMG groups, and the proportion of down-regulated genes was the highest in each comparison group. KEGG enrichment analysis suggested that normal mature coloration and high temperature stress accelerated coloration of pepper fruits may be regulated by the genes related to biosynthesis, carbon metabolism pathway and negative feedback regulation by genes related to starch

and sucrose metabolism and DNA Replication pathway. And plant hormone transduction pathway related gene regulation has both positive effect and negative feedback regulation effect. Embodied in gene levels, high temperature stress at fruit-set period inhibited the expression of fruit enlargement and expansion-related genes *IAA27*, *IAA16*, *AUX22*, *AUX22C*, *AUXT92*, *PG*, *XTH7*, *XTH9*, *EXPA10*, *EXPA13* and *CSLA2* in immature fruits, and increased the expression of *GGPS1*, *PSY*, *CrTZ-2* and *CCS1* genes in the synthesis pathway of capsanthin, as well as *TC* genes in the synthesis pathway of tocopherol, which led to the inhibition of enlargement and expansion in immature fruits and accelerated the fruit reddening. This study provided a new insight into the reasons why the expansion of pepper fruit was inhibited and the fruit turned red accelerated under high temperature stress, which lays a foundation for further research on the mechanism of fruit resistance to high temperature.

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Addresses of authors:
Rongyun Liu[1,2], Bozhi Yang[1], Lianzhen Mao[1], Yu Huang[1], Xiongze Dai[1], Wu Miao[2], Zhuqing Zhang[3], Wenchao Chen[3], Lijun Ou[1,*] and Zhoubin Liu[1,**]

[1] College of Horticulture, Hunan Agricultural University, Changsha 410128, Hunan, China

[2] Hunan Xiangyan Seed Industry Co, Ltd., Changsha 410100, China

[3] Vegetable Institution of Hunan Academy of Agricultural Science, Changsha 410125, China

Corresponding authors; E-mail:

* ou9572@hunau.edu.cn

** hnlzb2020@hunau.edu.cn

Supplemental Information

Supplemental Information – Table S1. All the primers used for RT-qPCR in this study.

Primer name	Sequence (5'–3')
Actin-F	CCACCTCTTCACTCTCTGCTCT
Actin-RT	ACTAGGAAAAACAGCCCTTGGT
Capana00g003407-F	GGAATATCCACCCTGTTT
Capana00g003407-RT	TTACGGCTATTAGGTCATC
Capana07g001548-F	GGGCTTGGTTAGTTCTTC
Capana07g001548-RT	AAGGCTGTCAAGTTTCAT
Capana02g003358-F	GTGGATACGGAACAAACA
Capana02g003358-RT	CGCAAGAAGATAGGCTGA
Capana00g004127-F	ATTGAGCCTTTGACTACTT
Capana00g004127-RT	GTATTCCCTCTTAACCATG
Capana04g000259-F	GATAATACTTGGACATTTCGTC
Capana04g000259-RT	GGTTAGCTCGCCTCACTC
Capana06g000615-F	GGTAGGAAGATAAGCGGTAG
Capana06g000615-RT	CCTCCAATCCATAAGCATCA
Capana03g002170-F	CCGCCACTTCTCTTCTTC
Capana03g002170-RT	GTCTCTACTTCAATCTCATCAC
Capana04g000412-F	AGAATTGAAGCAGCACAGA
Capana04g000412-RT	CGTGAATGAGTGACATAGTG
Capana08g000837-F	TGACCACCACTGATTATGC
Capana08g000837-RT	ACGAAGAGGACGATTAGAAG

Supplemental Information – Table S2.
For Supplemental Table S2 see www.eJHS.org.