

Transcriptome and Metabolome-based Analysis of Anthocyanidin Biosynthesis in Purple Pepper

Yaning Meng

The institute of cash crops, hebei academy of agriculture and forestry sciences

Hongxiao Zhang

The institute of cash crops, hebei academy of agriculture and forestry sciences

Yanqin Fan

The institute of cash crops, hebei academy of agriculture and forestry sciences

Libin Yan (✉ tianjiaoshi@126.com)

The institute of cash crops, hebei academy of agriculture and forestry sciences

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Abstract

In order to clarify the profile of gene expression and metabolites for color formation and the molecular mechanism of anthocyanidin accumulation in purple pepper fruits, we analyzed the anthocyanidin metabolome data of the fruits of 2 purple pepper lines and 1 green pepper line and detected a total of 5 anthocyanidin-like metabolites, of which delphin chloride was unique to purple pepper fruits and 3 other anthocyanidin-like substances shared the metabolic pathway ko00942 and were up-regulated. Based on the transcriptome data, three pathways (ko00360, ko00400, and ko00941) related to anthocyanidin metabolism were identified through KEGG analysis. Three enzymes (DFR, ANS, and UFGT) and three transcription factors (MYB, BHLH, and WD40) in the purple pepper anthocyanidin biosynthetic pathway were up-regulated. We proposed a model to explain the regulation of pepper anthocyanidin biosynthesis: MYB, BHLH, and WD40 formed a ternary complex and bound to the specific *cis*-acting elements in the promoter region of the structural genes related to anthocyanidin biosynthesis to directly regulate their transcription, which resulted in the accumulation of a large amount of anthocyanidin metabolites including delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, and delphin chloride, giving color to pepper fruits. This study clarified the metabolic pathways and key genes affecting the color of purple pepper fruits and provided new insights into the synthesis and accumulation of anthocyanidins in pepper fruits.

Introduction

As living standards rise, more and more people begin to pay attention to their nutrition and healthcare. The development and utilization of anthocyanidin compounds have become hot spots in the fields of phytochemistry, medicine, and healthcare. Anthocyanidins, an important type of water-soluble pigment in plants, belong to the flavonoids and are widely distributed in plant organs, resulting in many colors including red, blue, and purple in plants¹⁻². Anthocyanidins have biological functions that improve health including anti-oxidation, anti-cancer, and anti-aging functions, protecting eyesight, preventing cardiovascular diseases, and improving memory³⁻⁵. In addition, anthocyanidins can reduce the photoinhibition of photosynthesis and photobleaching of chlorophyll under strong light. The accumulation of anthocyanidins can increase the photostability of the photosynthetic system in seedlings, reduce plant tissue damage caused by high levels of UV light, and improve stress resistance in plants⁶.

Pepper (*Capsicum annuum* L.) is a plant that belongs to the genus *Capsicum* of the Solanaceae family. There are many types of pepper germplasm resources, and purple pepper is one of the rarer types. Purple pepper is rich in anthocyanidins and has good physiological tolerance to high temperature and drought stresses. Generally, plant fruit skin has the highest anthocyanidin content. However, the fruit skin of most plants such as eggplant and purple sweet potato is inedible, whereas the edible part of purple pepper is the brightly-colored pericarp, which is valuable for human health and directly determines its economic value and popularity in the market.

In recent years, progress has been made in the study of the regulation of anthocyanidin biosynthesis and metabolism using genetics, genetic engineering, and molecular biology approaches. The biosynthesis of anthocyanins includes a series of metabolic reactions involving 20 different organic molecules and 12 different catalytic enzymes encoded by multiple homologous genes⁷. The biosynthesis of anthocyanidin can be divided into 5 steps⁸. In the first step, 4-coumoyl CoA is formed from phenylalanine, which is regulated by the activity of phenylalanine lyase (PAL) and cinnamic acid 4-hydroxylase (C4H) and shared by many secondary metabolic pathways. The second step is the synthesis of naringenin with 4-coumoyl CoA under the catalysis of chalcone synthase (CHS) and chalcone isomerase (CHI). Naringenin can be converted to dihydrokaempferol (DHK) by the catalysis of flavanone 3-hydroxylase (F3H), converted to dihydroquercetin (DHQ) by the catalysis of flavonoid 3'-hydroxylase (F3'H), or converted to dihydromyricetin (DHM) by the catalysis of flavonoid 3'5'-hydroxylase (F3'5'H). The third step is the synthesis of various unmodified anthocyanidins, which is regulated by dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS)⁹⁻¹⁰. In the fourth step, the colorless dihydroflavonol undergoes methylation, acylation, hydroxylation, and glycosylation modifications at different sites to form colored pelargonidin, cyanidin, and delphinidin, which finally form stable anthocyanidins. Most of the glycosylations of anthocyanidins are achieved with the help of uridine diphosphate-glucose-flavonoid-3-glucosyltransferase (UFGT) of the glucose transferase class^{2,11}. The fifth step is the transportation to and accumulation of anthocyanin in vacuoles.

In higher plants, the biosynthesis of most anthocyanidins is often regulated in different spatial temporal patterns by combinations of multiple regulatory factors such as R2R3-MYB transcription factor, MADS-box, bHLH transcription factor, and WD40 protein¹²⁻¹³. To date, several *R2R3-MYB* genes associated with either positive or negative regulation of the key genes (*DFR*, *ANS*, and *UFGT*) in anthocyanidin biosynthesis have been identified. However, their contributions to the color of fruits and other organs vary¹⁴⁻¹⁶. bHLH transcription factor is also a key regulator of anthocyanidin biosynthesis, usually independently regulating CHS, DFR, and UFGT¹⁷. WD40 proteins do not bind to the promoters of the anthocyanidin biosynthetic genes; instead, they interact with bHLH and MYB to regulate anthocyanin biosynthesis¹⁸. The R2R3MYB, bHLH, and WD40 proteins usually form a transcriptional activation complex (MYBbHLH-WD40, MBW) to regulate the transcription of late anthocyanin biosynthetic genes in most plants¹⁹⁻²¹.

At present, the molecular mechanism regulating anthocyanidin biosynthesis in pepper is rarely studied. In our study, 2 purple lines and 1 green pepper line (control) were analyzed by means of targeted metabolome and transcriptome technologies to clarify the differential profiles of structural genes and transcription factors associated with anthocyanidin synthesis between the pepper lines, which would be helpful for the revelation of the molecular mechanism regulating anthocyanidin synthesis in pepper pericarp and may provide a theoretical basis for improving pepper through breeding.

Results

Analysis of pepper anthocyanidin metabolome

Anthocyanidins are the most important flavonoid pigments in plants. In order to compare the composition of anthocyanidin metabolites between three pepper lines, L66, L29, and L9 (CK), the anthocyanidin-like substances in pepper fruits were examined. Principal component analysis (PCA) showed that the samples between the groups scattered and the samples within the groups clustered, indicating that the anthocyanidin metabolome data was reliable. On the principal component PC1 and PC2, L9 (CK) was clearly separated from L29 and L66 and the difference was significant (Fig. 1). A total of 5 anthocyanidin-like compounds were detected in the fruits of three pepper lines. The contents of delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, and delphin chloride in lines L66 and L29 were significantly higher than that in line L9 (CK), showing that they were significantly up-regulated. Delphin chloride was found in the fruits of purple pepper lines L66 and L29, but not in L9 (CK), suggesting that it is a unique metabolite in purple pepper fruits. The cyanidin 3-O-galactoside content in the fruits of the green pepper line was higher than that in the purple pepper line L29 fruits, indicating that the green pepper fruit also accumulated anthocyanidins to a certain extent (Table 1). The KEGG database was used to annotate the differential metabolites. No annotation result was obtained for cyanidin 3-O-galactoside. The rest of the detected metabolites were found to share the metabolic pathway ko00942. It can be seen from the KEGG pathway map that one of the 4 annotated metabolites remained at an unchanged level while the rest were delphinidin-derived products and up-regulated.

Table1

Type and content of anthocyanins in two purple and one green pepper cultivars.

Compound	Molecular weight	L29	L66	L9 (CK)	Kegg map
Cyanidin 3-O-glucoside	449.38	N/A	1.10E+05	N/A	Ko00942
Delphinidin 3-O-glucoside	500.84	4.55E+06	3.33E+06	3.45E+04	Ko00942
Delphinidin 3-O-rutinoside	611.53	6.24E+06	8.26E+06	5.00E+04	Ko00942
Cyanidin 3-O-galactoside	484.838	1.04E+04	2.65E+06	7.22E+04	—
Delphin chloride	627.52	4.83E+04	9.77E+04	N/A	Ko00942

Statistics of purple and green pepper transcriptome sequencing data

We further investigated the differences in gene expression among the three samples. With three biological replicates, the transcriptome sequencing of the 9 samples yielded a total of 63.26 Gb clean data with 94.14% of bases scoring Q30 (Table S1). The transcriptome sequencing reads were aligned with the reference genome with efficiencies ranged from 86.14% to 95.04% (Table S2), which showed a normal

rate of data utilization, suggesting that the selected reference genome was suitable for subsequent analysis.

DEGs between purple and green peppers and KEGG enrichment analysis

In order to clarify DEGs and their biological pathways between purple and green peppers, we analyzed the DEGs between the fruits of 2 purple pepper lines (L66 and L29) and 1 green pepper line (L9) and performed KEGG enrichment. The number of DEGs were 6567 (L66 vs. L9) and 5091 (L29 vs. L9). A total of 2224 DEGs were common in both of the purple pepper lines (Fig S1). The 2224 DEGs were annotated and 111 KEGG pathways were found. Eight of the top 20 KEGG pathways were shared by the two purple pepper lines, of which 3 pathways (ko00360, ko00400, and ko00941) were anthocyanidin-related (Fig.2).

Regulation of anthocyanidin biosynthetic pathway genes in purple and green pepper

Anthocyanins are one of the natural products synthesized through the metabolic pathways of phenylpropanes and flavonoids. A number of studies have revealed that the biosynthesis of anthocyanins is completed under the co-catalysis of different enzymes. Through metabolome and transcriptome analysis, we obtained 4 pathway diagrams. We mapped these biosynthesis pathway diagrams of pepper and found that three enzymes, namely DFR, ANS, and UFGT, in the anthocyanidin biosynthetic pathway were up-regulated in the fruits of purple pepper lines. DFR can selectively catalyze the formation of colorless leucopelargonidin and leucocyanidin from dihydrokaempferol and dihydroquercetin, respectively. ANS catalyzes the formation of colored pelargonidin, delphinidin, and cyanidin from leucopelargonidin and leucocyanidin. After that, mediated by UFGT, the metabolites delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, and delphin chloride were all up-regulated (Fig3). In this process, as the three enzymes DFR, ANS, and UFGT in fruits of purple pepper lines were up-regulated, those metabolites were also increased, resulting in an increase in the content of colored anthocyanidins. As a result, the degree of the purple color in the two purple pepper lines varied.

qRT-PCR validation

The transcriptome sequencing results showed that three enzymes, DFR, ANS, and UFGT, were up-regulated during anthocyanin formation in fruits of purple pepper lines. The expression of DFR coding genes *LOC107850726* and *LOC107860031*, ANS coding gene *LOC107866341*, and UFGT coding genes *LOC107843659*, *LOC107861697*, and *LOC107860695* were significantly up-regulated. In order to verify the results obtained from transcriptome sequencing, those 6 genes that were significantly up-regulated in the fruits of purple peppers (Line L29 and L66) compared with the fruits of green pepper (Line L9) were

further analyzed using qRT-PCR. The results showed that the expression levels of the 6 genes were significantly higher in fruits of purple pepper lines than that in fruits of the green pepper line, and their expression levels in line L66 fruits were higher than in line L29 fruits, which may be due to the difference in anthocyanin content in the fruits of different pepper lines. Line L66 fruits had a higher colored anthocyanin content and deeper purple color than line L29 fruits. The qRT-PCR results of the expression of 6 genes were highly consistent with the results obtained based on transcriptome sequencing (Fig. 4), indicating that DEG analysis was highly reliable.

Transcription factors regulating anthocyanidin biosynthesis in pepper fruits

Previous studies have shown that in most plant species a protein complex formed by three types of transcription factors from the MYB family, BHLH family, and WD40 family can bind to the promoter of a structural gene or genes involved in the anthocyanidin biosynthesis pathway to activate or inhibit expression²². In our study, analysis of the key genes for anthocyanidin biosynthesis in pepper fruits showed that the transcription factors MYB, BHLH, and WD40 were up-regulated in purple fruits (lines L29 and L66) compared with green fruits (line L9) (Fig. 5A), which was consistent with the increase of the expression level of the structural genes involved in anthocyanidin biosynthesis, indicating that MYB, BHLH, and WD40 positively regulated anthocyanidin biosynthetic genes. We proposed a model of anthocyanidin biosynthesis in pepper fruits: MYB, BHLH, and WD40 formed a ternary protein complex and bound to the specific *cis*-acting elements in the promoter of the structural genes related to anthocyanidin biosynthesis to regulate their expression, which altered the metabolites and resulted in color change in pepper fruits (Fig. 5B).

Discussion

Anthocyanidin biosynthesis belongs to a branch pathway of flavonoids that starts from phenylalanine. After carboxylation, glycosylation, methylation, and acylation, the anthocyanidins are transported to and accumulated in vacuoles. A variety of enzymes participate in the biosynthesis of anthocyanidins in higher plants²³⁻²⁴. Zhang *et al.*²⁵ cloned the *StANS* gene from potato tuber PA99P202 and demonstrated that potato anthocyanidin synthase *StANS* promoted the synthesis of anthocyanidin in potato tubers. In jujube, Zhang *et al.*²⁶ found that at the late stage of ripening, UFGT was activated to accelerate the glycosylation reaction and transfer unstable anthocyanidins to vacuoles to synthesize colored anthocyanidins that were stable.

In peppers, Borovsky *et al.*²⁷ found that CHS and CHI expressed in pigmented and nonpigmented pepper tissues, whereas DFR and ANS only expressed in pigmented tissues. Similarly, Zhang *et al.*¹⁰ reported that there was no significant difference in the expression levels of CHS and CHI between purple and green leaves, while the expression levels of F3H, F3'5'H, DFR, ANS, and UFGT were all significantly higher in

purple pepper plant leaves than in green leaves. Jung *et al.*²⁸ detected high levels of expression of F3H, DFR, ANS, and UFGT only in purple pepper leaves, and there was no significant difference in the expression levels of PAL, C4H, 4CL, and CHS between purple and green leaves. In our study, the enzymes required for anthocyanidin synthesis in pepper fruits were slightly different from that in leaves. Through analysis of the anthocyanidin metabolome and transcriptome, we found that three enzymes, DFR, ANS, and UFGT, were involved in anthocyanidin biosynthesis in pepper fruits. The expression level of the three enzymes was significantly higher in fruits of the purple pepper lines than that of the green pepper line and higher in fruits with deeper purple color (line L166) than that in fruits with lighter purple color (line L29), which was consistent with the previously reported conclusion that "the accumulation of anthocyanidins in different plant organs and tissues is usually related to the content and activity of enzymes involved in their biosynthesis"²⁹.

In addition to structural genes, transcription factors are also the key factors in the synthesis of anthocyanidins in plants. Transcription factor MYB can activate the expression of EBGs (early biosynthetic gene), and the ternary complex composed of MYB, bHLH, and WD40 transcription factors played a key role in regulating LBGs (late biosynthetic gene)³⁰. Through yeast two-hybrid screening and analysis of transcriptome and metabolome data, Jan *et al.*³¹ found that the regulation of anthocyanidin biosynthesis in strawberry fruits was related to the MYB-bHLH-WD40 regulatory complex. Lloyd *et al.*³² also confirmed that the MYB, bHLH, and WD40 ternary complex regulated the biosynthetic pathway of anthocyanidins.

Through qRT-PCR and RNA-Seq analysis, Tang *et al.*³⁰ found that 2 MYBs (CaANT1 and CaANT2), 1 bHLH (CaAN1), and 1 WD40 (CaTTG1) transcription factor participated in the accumulation of anthocyanidins in pepper flowers, and might activate anthocyanidin accumulation by forming a new MYB, bHLH, and WD40 complex. In our study, based on the analysis of the transcriptome data of purple and green peppers, we found that the gene expression of transcription factors MYB, BHLH, and WD40 in purple pepper fruits were all up-regulated, which was consistent with the increase of the expression level of structural genes related to anthocyanidin biosynthesis, indicating that MYB, BHLH, and WD40 positively regulated anthocyanidin biosynthetic genes. The result was consistent with that previously reported by other authors. In addition, we proposed a model to explain the regulation of anthocyanidin biosynthesis in peppers: MYB, BHLH, and WD40 formed a ternary protein complex and bound to specific *cis*-acting elements in the promoter of the structural genes *DFR*, *ANS*, and *UFGT* to directly regulate their transcription, which led to the change in the accumulation of anthocyanidin metabolites, making pepper fruits exhibit purple color of varying deepness.

This study clarified the metabolic pathways and key genes for the color of purple pepper fruits, which provided new insights into the synthesis and accumulation of anthocyanidins in pepper fruits and laid a basis for the effective improvement of purple peppers. Nevertheless, the biosynthesis of pepper anthocyanidins is not only affected by the key enzyme genes and transcription factors but also by

various enzymes, biochemical reactions, and environmental conditions, and more in-depth research is needed in the future.

Materials And Methods

Materials

Three pepper excellent Strains (L66, L29 and L9) were bred by the institute of cash crops of hebei academy of agriculture and forestry sciences after years of self-breeding. Among them, L66 and L29 were purple peppers, and L9 was green pepper. Purple pepper line L66 fruits have deeper purple color than L29 fruits (Fig. 6). Pericarp tissues of the matured fruits collected from different plants were sampled (each line had three repeats), stored at -80°C for subsequent experiment.

Determination of metabolites

Multiple reaction monitoring (MRM) was performed by Metware Biotechnology (Wuhan, China). Freeze-dried leaves were crushed and isolated extracts. The sample extracts were analyzed using an LC-MS/MS system³³⁻³⁴ (UPLC, Ultra Performance Liquid Chromatography, MS, Tandem mass spectrometry).

RNA extraction and Illumina sequencing

Total RNAs for constructing nine cDNA libraries of the three pepper lines, each with three replicates, were isolated from frozen pericarp tissues using the modified CTAB method³⁵. The qualified libraries were sequenced. HISAT2 software was used to quickly and accurately align the clean reads with the reference genome (<https://www.ncbi.nlm.nih.gov/genome/term=Capsicum%20annuum>) to locate the reads on it. FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) was used to correct the sequencing depth and gene length³⁶. DESeq software³⁷ was employed to analyze and screen the genes differentially expressed between sample groups. The differentially expressed genes (DEGs) were then analyzed through GO enrichment and KEGG functional annotation of metabolic pathways.

Verification of DEGs by qRT-PCR

Real-time fluorescence qRT-PCR was used to verify the results obtained based on transcriptome sequencing. Primer Premier 5.0 software (Primer, Canada) was used to design specific primers for DEGs. The PCR program was as follows: 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The PCR was repeated 3 times for each sample. Actin (serial number: GQ339766) was used as the internal reference gene to determine the relative expression level of DEGs.

Declarations

Statement

The pepper materials used in the experiment were developed by the Cash Crops Research Institute of Hebei Academy of Agriculture and Forestry Sciences, which were in compliance with the planting resources protection regulations in the Seed Law of China.

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files or are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Libin Yan and Yanqin Fan designed the project. Yaning Meng analyzed the data, carried out the experiments, and wrote the manuscript. Hongxiao Zhang participated in the design of experiments. All authors contributed to the revision of this manuscript and approved the final manuscript.

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Authors' Information

a. The Institute of Cash Crops, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050051, Hebei, China. b. Hebei Vegetable Engineering Technology Research Center, Shijiazhuang 050051, Hebei, China.

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Figures

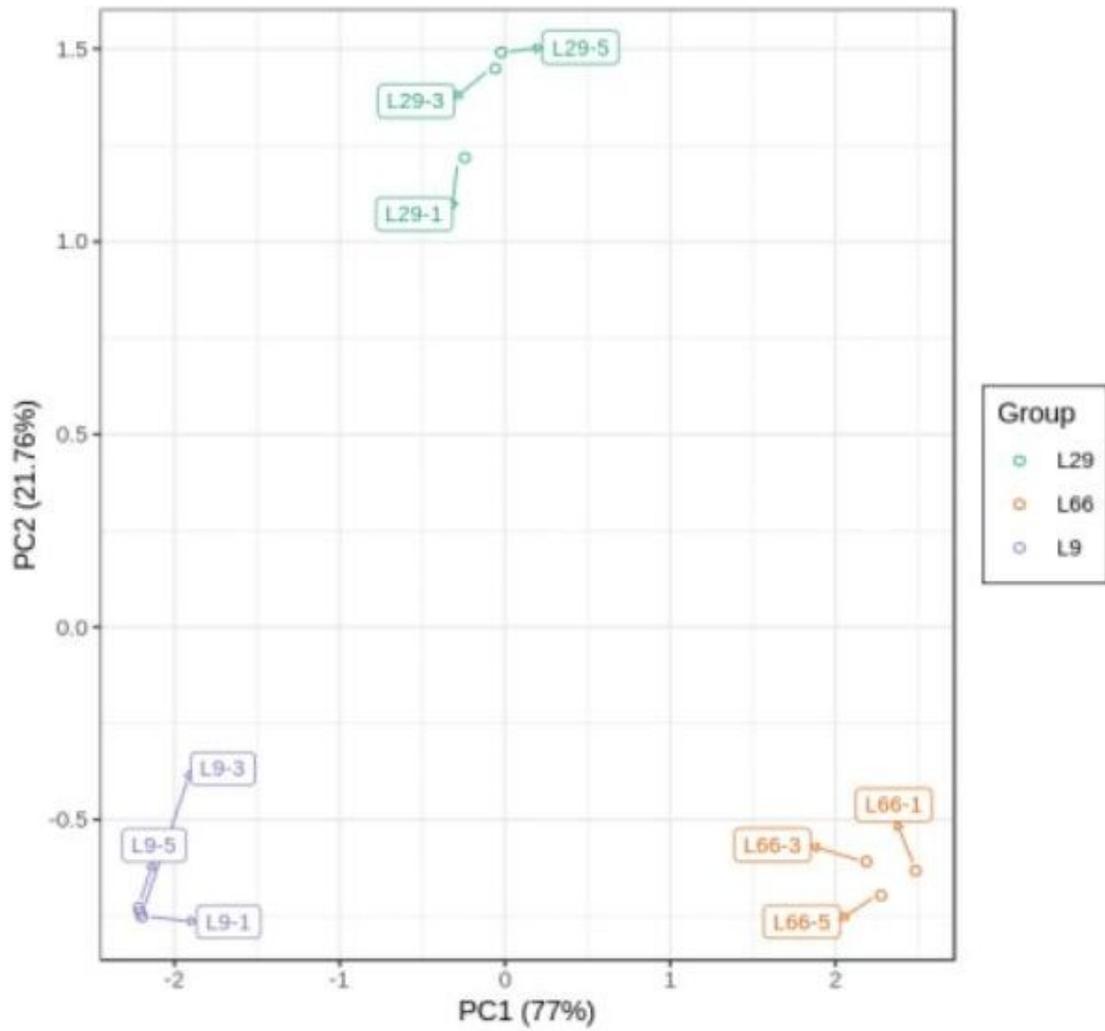


Figure 1

Principal component analysis (PCA) of the pepper lines.

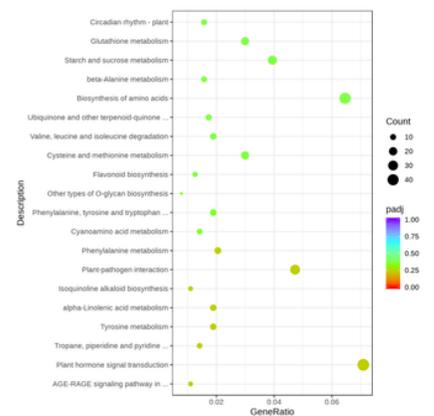
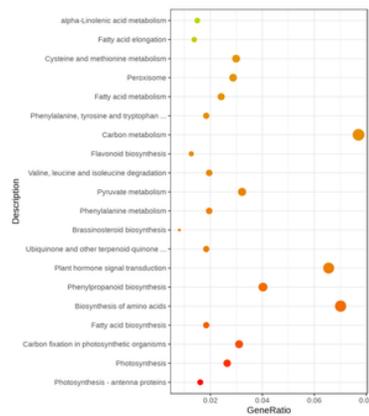
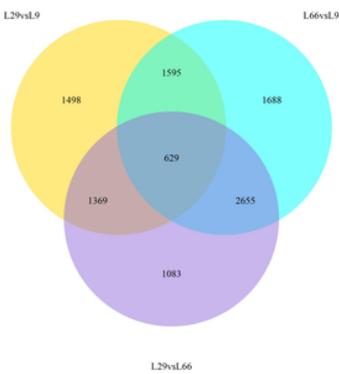


Figure 2

Venn diagram of differentially expressed genes (DEGs) and scatter plots of KEGG enrichment in 3 pepper lines. (A) Venn diagram of DEGs in 3 pepper lines. (B) KEGG enrichment scatter plot of L66 vs. L9. (C) KEGG enrichment scatter plot of L29 vs. L9. Red arrows indicate the three KEGG pathways related to anthocyanidin. Abscissa is the ratio of the number of DEGs annotated to the KEGG pathway to the total number of DEGs, and the ordinate is the KEGG pathways.

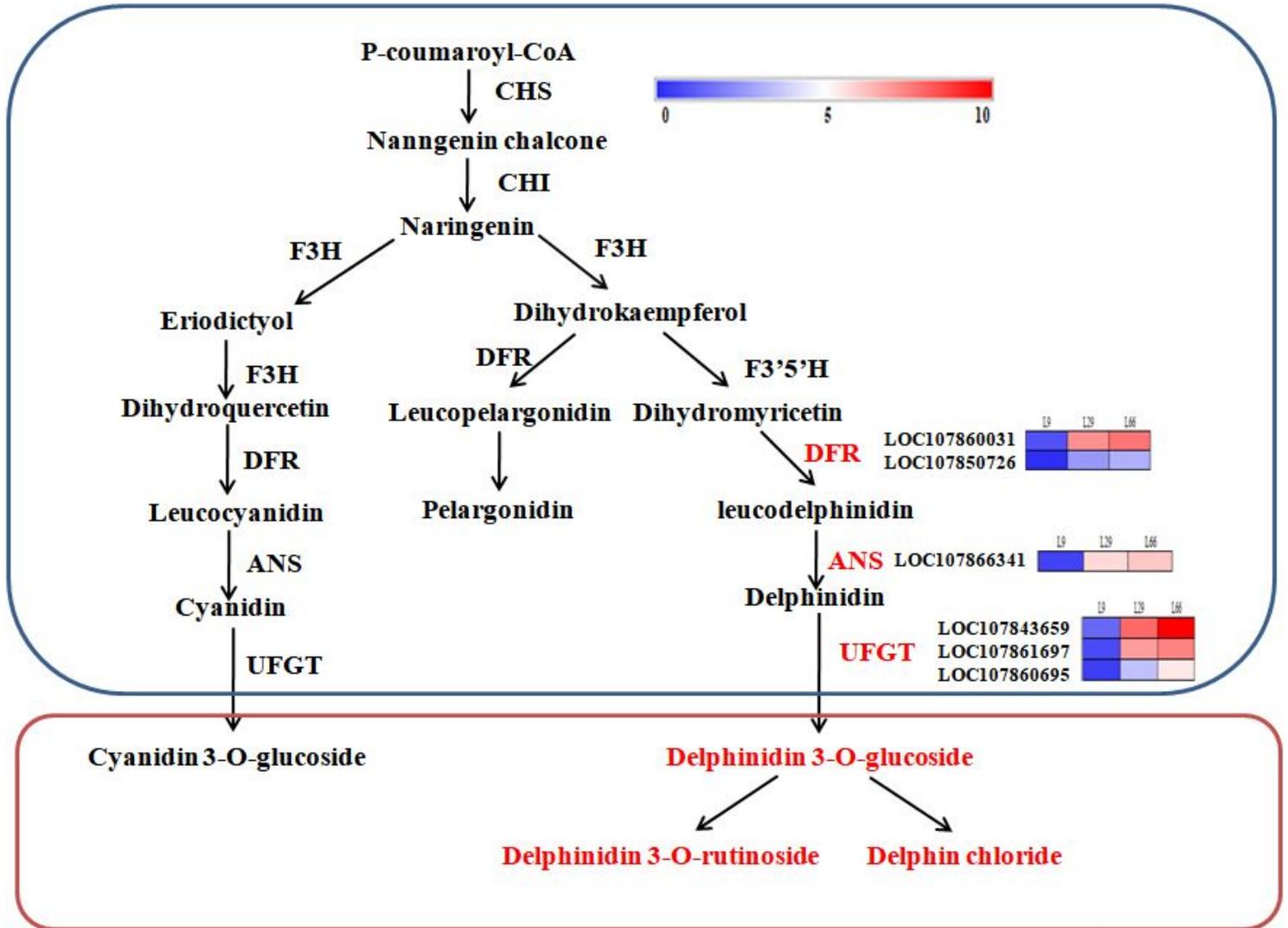


Figure 3

Heat map of pepper anthocyanidin biosynthetic pathway. Red color denotes a significant up-regulation. Chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3-hydroxylase (F3H), Dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-flavonoid glucosyltransferase, (UFGT), and flavonoid 3'5'-hydroxylase (F3'5'H).

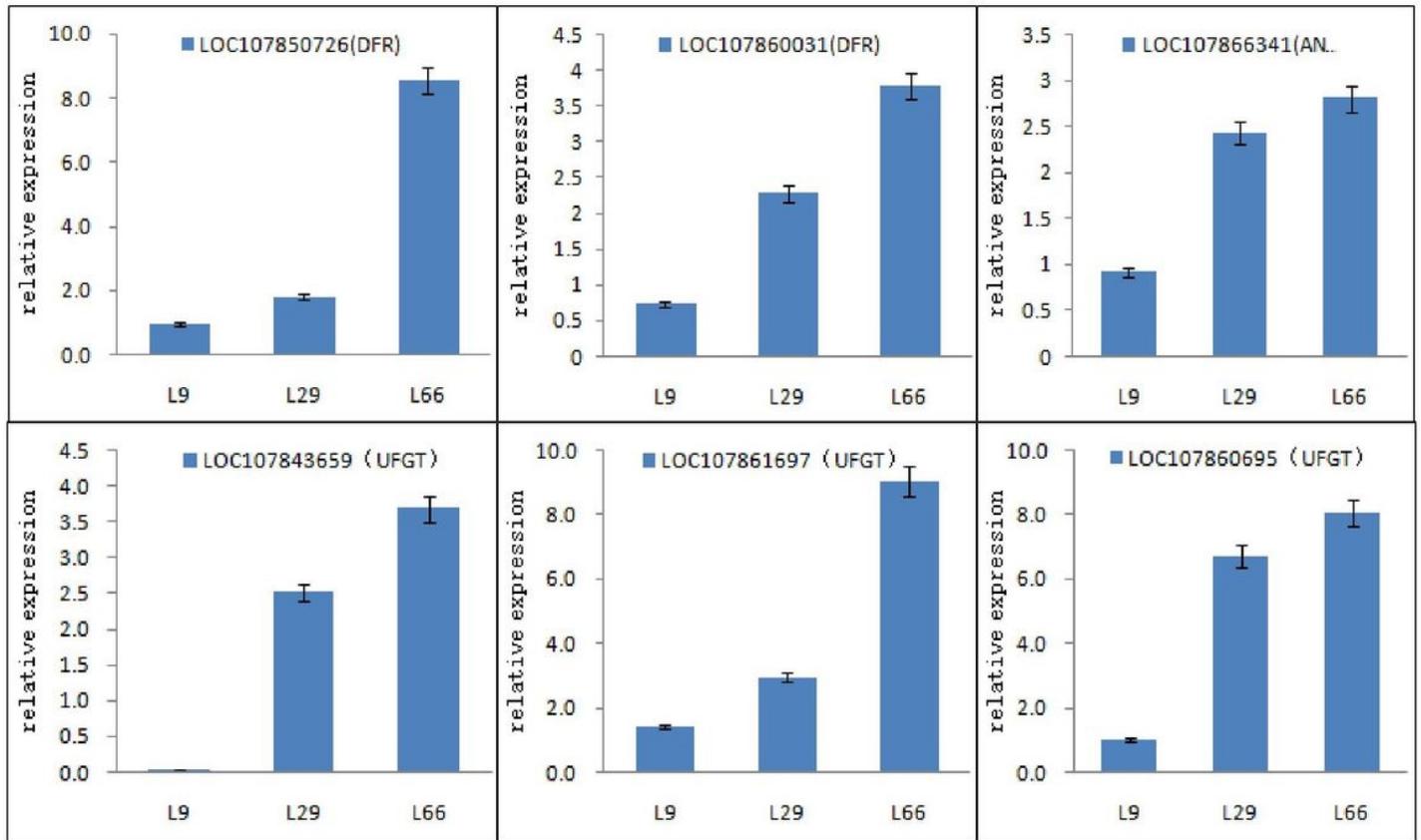


Figure 4

Expression of six genes in purple and green pepper fruits in the process of anthocyanin synthesis. L29 and L66 have purple pericarps. L9 has a green pericarp.

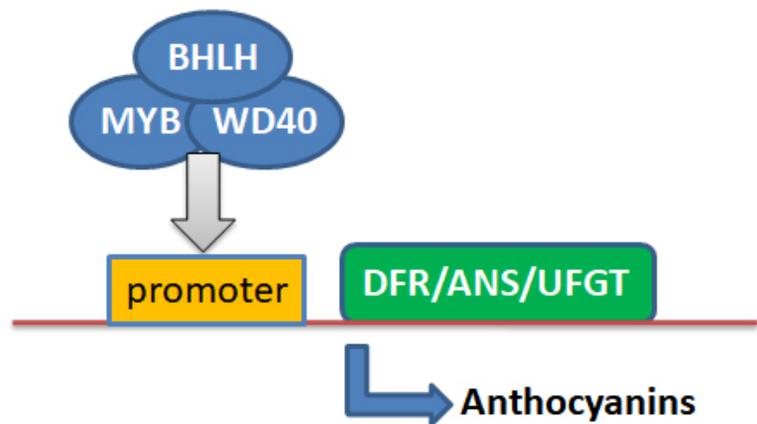
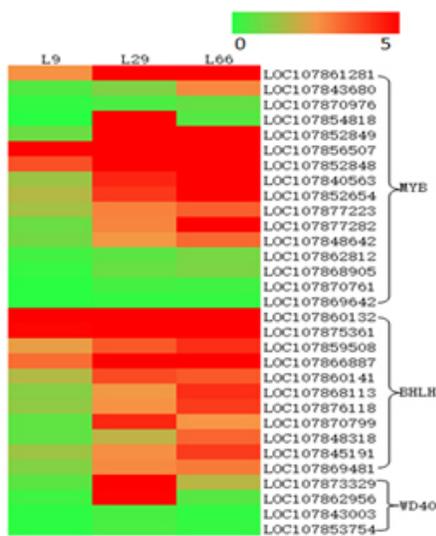


Figure 5

Heat map of the candidate genes of transcription factors MYB, BHLH, and WD40 involved in anthocyanidin biosynthesis in pepper pericarp and the diagram of the biosynthesis of anthocyanidins in peppers. (A) Heat map of the candidate genes of transcription factors MYB, BHLH, and WD40 involved in anthocyanidin biosynthesis in pepper pericarp. L9 has a green pericarp. L29 and L66 have purple pericarps. (B) Diagram of anthocyanidin biosynthesis in pepper pericarp.



Figure 6

The phenotype of purple and green pepper fruits.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [S1.cleanread.xlsx](#)
- [S2.totalmap.xlsx](#)

- [figS1.pdf](#)
- [Table1.Typeandcontentofanthocyaninsintwopurpleandonegreenpeppercultivars.xlsx](#)