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Research Article

Extensive transcriptome changes underlying the fruit skin colour intensity variation in purple eggplant

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Abstract

Fruit skin colour intensity is one of the most important economic traits of purple eggplant. A wide diversity for fruit skin colour intensity exists in purple eggplant and the accumulation of anthocyanins and chlorophylls of fruit skin mainly affected colour intensity. However, limited information is available contributing to the molecular mechanisms underlying fruit skin colour intensity variation in purple eggplant. In the present study, variation of two purple eggplant advanced lines EP26 and EP28, with different fruit skin colour intensity was investigated. Higher anthocyanin contents and lower chlorophyll contents were observed in EP26 with deeper fruit skin colour intensity at two developmental stages. Comparative transcriptome analysis of EP26 and EP28 identified a total of 2218 differential expressed genes (DEGs) at two developmental stages. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that these DEGs were mainly involved in flavonoid biosynthesis and photosynthesis. In addition, a total of 131 transcription factors including MYB, bHLH, WRKY, and NAC exhibited dynamic changes, which might be responsible for the variation of fruit pigments accumulation between EP26 and EP28. Taken together, these results expand our knowledge of molecular mechanisms underlying fruit skin colour intensity variation in eggplant, which allowing for improvement of fruit coloration in eggplant breeding.

Keywords: anthocyanin; colour intensity; eggplant; flavonoid biosynthesis; transcriptome

Introduction

Eggplant (*Solanum melongena* L.) belongs to the Solanaceae family, is an important vegetable crop and is of substantial economic importance in Asia, Africa, and the subtropics (Toppino *et al.*, 2020). The fruit skin colour of eggplant ranges from white to dark purple, and is one of the primary exterior quality and economic characteristics. Although there is a big diversity of eggplant varieties varying in shape and colour, the dark purple type receives more attention for high levels of anthocyanin accumulation (Li *et al.*, 2018). A broad spectrum of colour intensity exists within the group of dark purple-coloured cultivars, but intensive, uniform and lasting fruit colour types are most preferred by consumers than the paler types, especially in China (He *et al.*, 2019).

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The dark purple colour of eggplant fruit skin has been contributed to the accumulation of both anthocyanins and chlorophylls (Stommel *et al.*, 2015). Anthocyanins are an important class of flavonoids that represent a large group of plant secondary metabolites (Liu *et al.*, 2018). The anthocyanin biosynthesis pathway has been extensively studied (Zhang *et al.*, 2014). It is involved at least two types of genes: structural genes and regulatory genes which have been identified in many species (Boss *et al.*, 1996; Kim *et al.*, 2003; Wei *et al.*, 2011; Jiang *et al.*, 2016a). Structural genes encode the enzymes directly participate in the biosynthesis of anthocyanins, including phenylalanine ammonia lyase (*PAL*), 4-coumarate-CoA ligase (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxyl enzyme (*F3H*), dihydroflavonol reductase (*DFR*), anthocyanin synthase (*ANS*) and flavonoid 3-O-glucosyltransferase (*UFGT*).

Regulatory genes control the transcription of structural genes and it has been reported that DNA-binding MYB transcription factors, basic helix-loop-helix (bHLH) transcription factors, and WD40 repeat proteins form a ternary MYB-bHLH-WD40 (MBW) transcriptional complex, to activate the transcription of structural genes in the anthocyanin pathway (Liu *et al.*, 2018; Li *et al.*, 2021). Also, many factors have been reported to regulate the anthocyanin biosynthesis, such as light, temperature, sucrose and plant hormones (Qi *et al.*, 2011; Li *et al.*, 2012; Xie *et al.*, 2012; Hu *et al.*, 2016).

Chlorophylls play an important role in solar light harvesting and energy transport to the reaction centers during photosynthesis (Reinbothe *et al.*, 2010). Chlorophyll metabolic pathway can be divided into three phases: biosynthesis of chlorophyll *a* from glutamate, interconversion between chlorophyll *a* and *b*, and degradation of chlorophyll *a* into a non-fluorescent chlorophyll catabolite (Jin *et al.*, 2018). Almost all enzymes involved in the pathway have been identified (Beale, 2005). Several transcription factors have been reported as regulators of the chlorophyll biosynthesis pathway, such as LONG HYPOCOTYL5 (*HY5*), GOLDEN2-LIKE (*GLK*) (Lee et al., 2007; Waters et al., 2009; Ohmiya *et al.*, 2017). However, limited information is available about the regulation of chlorophyll metabolism in eggplant fruit skin pigmentation.

In recent years, more researches have focused on exploring the molecular regulatory mechanisms associated with the accumulation of anthocyanins of eggplant peel induced by light (Jiang *et al.*, 2016b; Li *et al.*, 2017; Li *et al.*, 2018; He *et al.*, 2019; Li *et al.*, 2021). In addition, low temperature was reported to induce anthocyanin accumulation in eggplant (Jiang *et al.*, 2016a) and high temperature down-regulated most of the genes in the anthocyanin biosynthetic pathway of eggplant (Zhang *et al.*, 2019). However, the mechanism underlying fruit skin colour intensity variation among dark purple type of eggplant remains poorly understood.

In the present study, comparative transcriptome analysis was conducted between two purple type materials of eggplant, EP26 and EP28, with different intensity of fruit skin coloration, resulting in a comprehensive data set for the identification of candidate genes responsible for the control of pigmentation intensity in purple eggplant. The results would provide better insights into the molecular basis underlying the intensity variation of fruit peel in purple eggplant.

Materials and Methods

Plant materials

Two dark purple eggplant advanced lines EP26 and EP28 were used in this study. Both of the two materials were obtained from Jiangsu Academy of Agricultural Science. EP26 and EP28 were both Chinese type with long fruit and purple calyx. The colour intensity of EP26 fruit is deeper and more stable than that of EP28. Well-developed plants of EP26 and EP28 were grown in a greenhouse at Jiangsu Academy of Agricultural Sciences. Because the fruit pigmentation of commercial stage is most valued, the samples were collected at two different developmental stages: young fruit, 15 days after anthesis (DAA15) and commercial maturity, 25 days after anthesis (DAA25). Three fruits were mixed as a sample and three samples were set as biological replicates (Li *et al.*, 2018). The fruit peel samples of each stage were divided into 3 groups, one for transcriptome sequencing, one for anthocyanin and chlorophyll content measurement, as well as HPLC

analysis, and the other for quantitative RT-PCR analysis. The fruit peels were collected and immediately frozen in liquid nitrogen and stored at – 80 °C until use. Three independent biological replications were performed for each stage mentioned above.

Fruit skin colour intensity measurement

The fruit peel colour intensity of EP26 and EP28 of each stage was analysed according to Gao *et al.* (2016). The L^* (lightness), a^* (redness and greenness), and b^* (yellowness and blueness) were measured using a hand-held spectrophotometer (CR-400, Minolta, Japan). The L^* is an indicator of fruit peel colour intensity, as lower lightness generally means deeper colour. The measurement was performed with three fruits of each stage of EP26 and EP28. Three independent biological replications were performed.

Total anthocyanin and chlorophyll analysis

The total anthocyanins of EP26 and EP28 at two different stages (DAA15 and DAA25) were measured using spectrophotometric differential pH method according to Zhang *et al.* (2014). The extraction of chlorophyll of EP26 and EP28 at two different stages was performed according to the method described by Li *et al.* (2018). The analysis mentioned above was performed in three independent biological replicates.

ESI-Q TRAP-MS/MS analysis of anthocyanins

The sample preparation, anthocyanin identification and quantification were performed at Wuhan MetWare Biotechnology Co., Ltd. (www. metware.cn) following the standard procedures. LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), API 6500 Q TRAP LC/MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in a positive ion mode and controlled by Analyst 1.6 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray, source temperature 500 °C, ion spray voltage (IS) 5500 V, ion source gas I (GSI), gas II(GSII), curtain gas (CUR) was set at 55, 60, and 25.0 psi, respectively. The collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi. DP and CE for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

RNA extraction, library preparation and transcriptome sequencing

The total RNA extracted from fruit peels of EP26 and EP28 was used to construct RNA-Seq libraries, and every sample was analysed in three biological replicates. Total RNA of each sample was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA quantity, integrity and purity were analysed using Nanodrop and Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA), respectively. The libraries were sequenced and analysed by Biomarker Corporation (Beijing, China) on an Illumina HiSeq 4000 platform.

Bioinformatics analysis

Filtered clean reads were mapped to an eggplant reference genome (http://eggplant.kazusa.or.jp). FPKM (fragments per kb per million fragments) was used to calculate unigene expression levels (Florea and Salzberg, 2013). DESeq2 was used for identifying differentially expressed genes (Love et al., 2014). The FDR (False Discovery Rate) control method was applied to determine the threshold of p value and Benjamini-Hochberg method was used to adjust the p value. An FDR < 0.01 and the absolute value of log2Ratio ≥ 1 were used as the threshold for the judgment of the significance of the gene expression differences. GO enrichment analysis of DEGs was performed using the topGO method based on Wallenius non-centreal hyper-genometric distribution with p≤ 0.05. For KEGG Ontology enrichment analysis of DEGs, KOBAS 2.0 (*http://www.biostars.org/p/200126*) was used and a threshold of FDR ≤ 0.05 was defined (Xie *et al.*, 2011).

Quantitative Real-Time PCR analysis

A total of thirteen differentially expressed unigenes were selected for quantitative real-time PCR (qRT-PCR) analysis. First-strand cDNA was generated from the total RNA isolated from fruit peels of EP26 and EP28 at DDA15 and DDA25, respectively, using the ReverTran Ace qPCR RT kit (Toyobo). Specific primers were designed using Primer 3 (*http://primer3.ut.ee/*). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene of eggplant was used as internal control. qRT-PCR was performed on the Roche lightcycle 480 system II using a SYBR Green-based PCR assay. Three independent biological replicates of each sample and three technical replicates of each biological replicate were used for qRT-PCR analysis. The reaction containing 10 μL of diluted cDNAs, and 0.4 μL of each primer to a final volume of 20 μL was performed. The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2013 and SPSS statistical software (IBM SPSS 19, Chicago, IL, USA). Significant differences were estimated using *t-test*, and $p < 0.05$ was considered to be significantly different.

Results

Phenotypic characterization of EP26 and EP28

In the present study, two dark purple eggplant advanced lines EP26 and EP28, with different fruit peel colour intensity were compared (Fig. 1a). Based on the lightness (L^*) of fruit peel, the colour intensity of EP26 was deeper than that of EP28 both at DDA15 and DDA25 stage (Figure 1b). The total contents of anthocyanin and chlorophyll from the two different stages (DDA15 and DDA25) of EP26 and EP28 were measured, respectively. The contents of anthocyanin of EP26 at DDA15 and DDA25 were higher than those of EP28 (Figure 1c). However, the level of chlorophyll contents of EP26 was lower than that of EP28 at both stages (Figure 1d). In addition, a total of eight anthocyanin compounds in fruit skin of EP26 and EP28 at DDA25 were detected, among which delphinidin 3-O-rutinoside was the most abundant both in EP26 and EP28 (Table 1).

Table 1. Anthocyanins detected in EP26 and EP28 at DDA25

Index	Compounds	EP26 Relative content	EP28 Relative content
pmb2961	Peonidin O-malonylhexoside	2.26E+04	3.58E+04
pme0443	Malvidin 3-O-galactoside	3.53E+05	4.58E+05
pme1398	Delphinidin 3-O-glucoside	3.99E+06	2.36E+06
pme1773	Cyanidin 3-O-rutinoside	8.23E+04	4.86E+04
pme3256	Delphinidin 3-O-rutinoside	2.23E+07	1.70E+07
pme3609	Cyanidin	9.00E+00	1.94E+04
pmf0116	Delphin chloride	1.51E+06	9.92E+05
pmf0615	Petunidin 3, 5-diglucoside	1.30E+04	6.82E+03

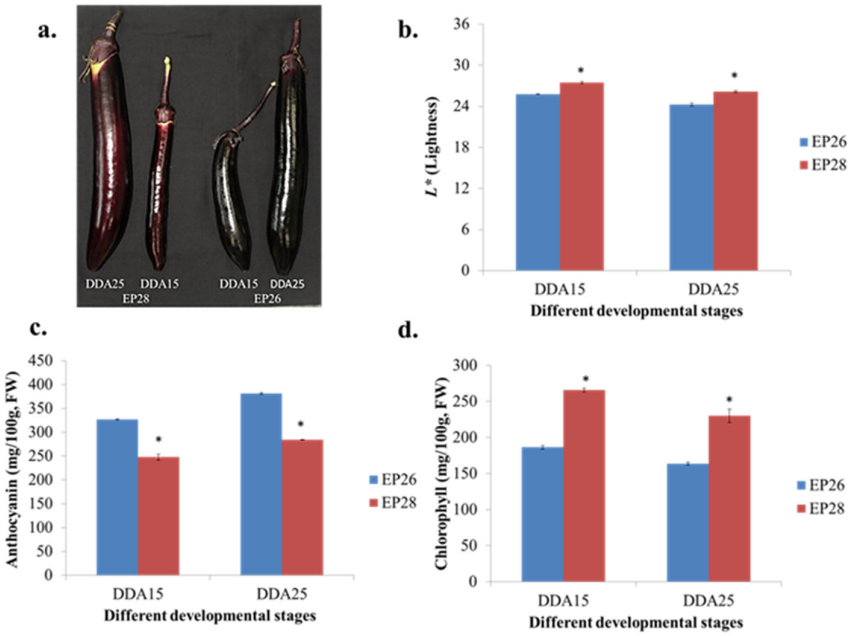

Figure 1. Characterization of EP26 and EP28 used in this study (A) Phenotypes of EP26 and EP28; (B) fruit peel colour intensity of EP26 and EP28 at DDA15 and DDA25; (C) anthocyanin contents of fruit peel in EP26 and EP28 at DDA15 and DDA25; (D) chlorophyll contents of fruit peel in EP26 and EP28 at DDA15 and DDA25

* Represents significant difference.

Identification of differentially expressed genes (DEGs) between EP26 and EP28

In order to systematically study the phenotypic differences between EP26 and EP28, the transcriptomes of four eggplant fruit peel samples with three repeats (A: EP26 at DAA15, B: EP26 at DAA25, C: EP28 at DAA15, D: EP28 at DAA25) were obtained using RNA-Seq. In total, twelve libraries (two materials × two developmental stages × three biological replicates) were constructed. Differences in gene expression in the fruit peel of EP26 and EP28 at two developmental stages were assessed (A vs C and B vs D). DEGs were identified with the expression fold (log2Ratio ≥ 1) and false discovery rate ≤ 0.01 as the thresholds.

A total of 1456 DEGs (913 up-regulated and 543 down-regulated) and 1163 DEGs (821 up-regulated and 342 down-regulated), were detected in EP26 vs EP28 at DDA15 and EP26 vs EP28 at DDA25, respectively (Figure 2a). Overall, a total of 2218 DEGs were found between EP26 and EP28 at the two stages. Of all these DEGs, 401 genes with 231 up-regulated and 111 down-regulated were shared at both stages (Figure 2b).

Gene ontology and KEGG pathway analyses of differentially expressed genes

The functions of the DEGs detected in the two developmental stages between EP26 and EP28 were classified according to the GO database, respectively. In the biological process category, the DEGs of the two stages were most enriched in the oxidation-reduction process (GO: 0055114). In the molecular functional category, the highly enriched terms among the two stages were ATP binding (GO: 0005524) and structural constituent of ribosome (GO: 0003735). In the cellular component category, the GO terms cell periphery (GO: 0071944) was the most enriched in EP26 vs EP28 comparison at DDA15, while the GO terms including photosystem I (GO: 0009522), photosystem I reaction center (GO: 0009538), chloroplast thylakoid membrane (GO: 0009535) and photosystem II (GO: 0009523) were highly enriched terms in EP26 vs EP28 comparison at DDA25.

KEGG pathway enrichment analyses were performed according to the DEGs in the two stages respectively, to further explore the biological functions. As shown in Figure 3, the pathways of phenylpropanoid biosynthesis and flavonoid biosynthesis involved in anthocyanin biosynthesis were highly enriched in the fruit skin of EP26 at DDA15. Particularly, the pathways of photosynthesis-antenna proteins, photosynthesis, and flavonoid biosynthesis were highly enriched in the EP26 vs EP28 comparison at DDA25.

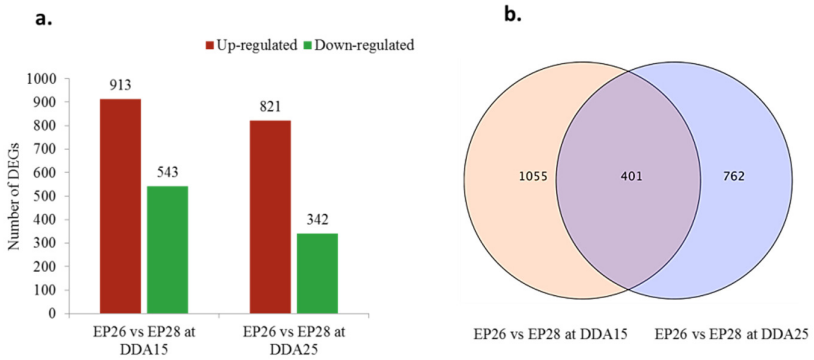

Figure 2. Differential gene expression profiles of EP26 and EP28 at two developmental stages
(A) The numbers of up- and down- regulated genes of EP26 vs EP28 comparison at DDA15 and DDA25, respectively;
(B) Venn diagram of differentially expressed genes of EP26 vs EP28 comparison at DDA15 and DDA25.

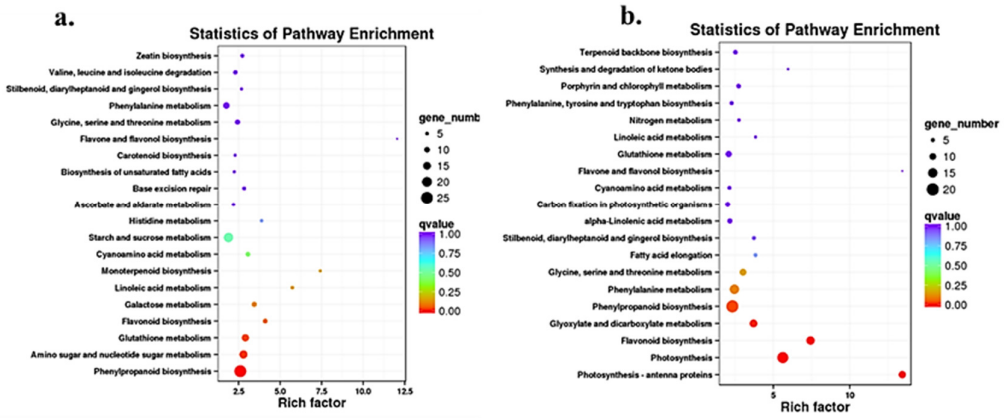

Figure 3. KEGG pathway enrichment analysis of DEGs (A) EP26 vs EP28 comparison at DDA15; (B) EP26 vs EP28 comparison at DDA15

Genes involved in anthocyanin biosynthesis

The anthocyanin biosynthetic pathway is an extension of the general flavonoid pathway (Liu et al., 2018). In the present study, structural genes participated in each step of the anthocyanin biosynthesis pathway were identified. Compared to EP28, the predicted encoding genes of *PAL*, *4CL*, *CHI*, *DFR*, *F3H*, and *UFGT* were up-regulated in EP26 at both two stages. At commercial maturity stage (DDA25), two unigenes Sme2.5_02154.1_g00001 and Sme2.5_01077.1_g00016 encoding *CHS* and one unigene *F3′5′H* were also up-regulated in EP26 compared to EP28, but did not change expression in the comparison of EP26 vs EP28 at DDA15. Notably, all the identified structural genes, including *CHS*, *CHI*, *F3H*, *F3′5′H*, *DFR*, *ANS*, and *UFGT*, in EP26 were higher than those in EP28 at DDA25 (Figure 4).

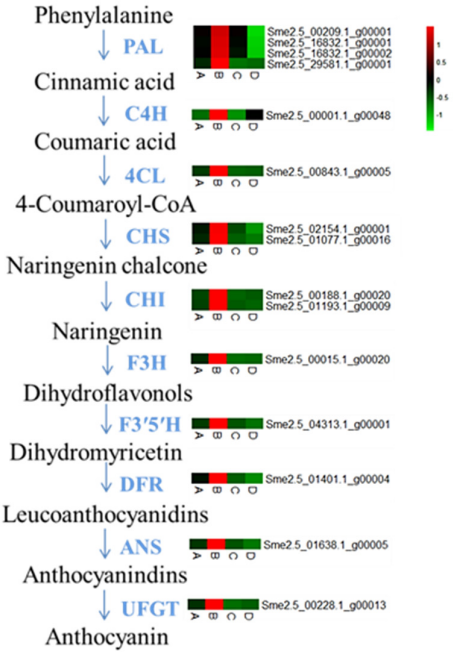

Figure 4. Expression profiles of DEGs involved in anthocyanin biosynthesis between EP26 and EP28 (A: EP26 at DDA15, B: EP26 at DDA25, C: EP28 at DDA15, D: EP28 at DDA25). PAL: phenylalanine ammonia lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumaroyl: CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3′5′H: flavonoid 3′5′-hydroxylase; DFR: dihydroflavonol-4-reductase; ANS: anthocyanin synthase; UFGT: flavonoid 3-O-glucosyltransferase.

Genes involved in chlorophyll synthesis

In current study, DEGs related to chlorophyll biosynthesis were identified based on KEGG pathway annotations. Compared to EP28, only four genes involved in chlorophyll biosynthesis including *HEMA* (encoding glutamyl-tRNA reductase), *CHLM* (encoding Mg-protoporphyrin IX methyltransferase), *CRD* (encoding Mg-protoporphyrin IX monomethylester cyclase), and *PORA* (encoding protochlorophyllide oxidoreductase A) were up-regulated in EP26 at DDA25 stage. However, none of DEGs involved in chlorophyll biosynthesis were identified between EP26 and EP28 at DDA15 stage (Table 2). Moreover, compared to EP28, the expression levels of DEGs related to photosynthesis were mostly up-regulated in EP26 at DDA25 based on KEGG enrichment. Particularly, a total of 11 unigenes encoding chlorophyll a-b binding protein, which transfer excitation energy to the reaction centers in photosystem I and photosystem II, showed increased expression levels in EP26 at DDA25 (Table 2).

Identification of differentially expressed transcription factors

Transcription factors play important roles in regulating gene expression in anthocyanin synthesis. In the present study, a total of 131 TFs were found to be differentially expressed in EP26 at least one pairwise combination (EP26 vs EP28 at DDA15 and EP26 vs EP28 at DDA25). These TFs could be classified into 29 families, among which bHLHs constituted the largest group (20 genes), followed by MYB (16 genes), HD-ZIP (9 genes), ERF (8 genes), NAC (8 genes), and WRKY (7 genes) (Figure 5a).

Among the 20 DEGs encoding bHLH TFs, only one DEG Sme2.5_03973.1_g0005 was shared by both stages, which was down-regulated in EP26 compared with EP28. The expression level of Sme2.5_00592.1_g00005, which is orthologous to *AtTT8* increased in the comparison of EP26 and EP28 at DDA25. Among the 16 unigenes encoding MYB TFs, two unigenes Sme2.5_05099.1_g00002 and Sme2.5_00492.1_g00008 were shared by both EP26 vs EP28 at DDA15 and EP26 vs EP28 at DDA25 comparison. Sme2.5_05099.1_g00002, annotated as *SmMYB1*, was up-regulated, which has been reported to

be involved in the control of anthocyanin biosynthesis. Sme2.5_00492.1_g00008 was orthologous to Arabdopsis *MYB124*, which was down-regulated in EP26 at both stages (Figure 5b).

Table 2. Differential expressed genes related to chlorophyll metabolism in EP26 vs EP28 at DDA25

Gene	Log2FC	FDR	Description
Sme2.5_01284.1_g00015	1.29	2.53E-04	HEMA
Sme2.5_00125.1_g00021	1.41	5.30E-04	CHLM
Sme2.5_00085.1_g00024	1.07	0.003817	CRD
Sme2.5_24391.1_g00001	2.06	2.14E-08	PORA
Sme2.5_00087.1_g00019	2.33	4.80E-13	chlorophyll a-b binding protein
Sme2.5_00136.1_g00024	1.02	0.005778	chlorophyll a-b binding protein
Sme2.5_00225.1_g00030	2.12	1.74E-05	chlorophyll a-b binding protein
Sme2.5_00225.1_g00032	1.94	5.43E-08	chlorophyll a-b binding protein
Sme2.5_01099.1_g00009	1.20	7.34E-04	chlorophyll a-b binding protein
Sme2.5_01235.1_g00007	1.66	9.07E-06	chlorophyll a-b binding protein
Sme2.5_03537.1_g00010	2.30	7.49E-06	chlorophyll a-b binding protein
Sme2.5_03628.1_g00001	1.35	1.48E-04	chlorophyll a-b binding protein
Sme2.5_04919.1_g00005	1.51	2.24E-04	chlorophyll a-b binding protein
Sme2.5_04919.1_g00006	1.39	0.002511	chlorophyll a-b binding protein
Sme2.5_06484.1_g00010	1.11	0.002765	chlorophyll a-b binding protein

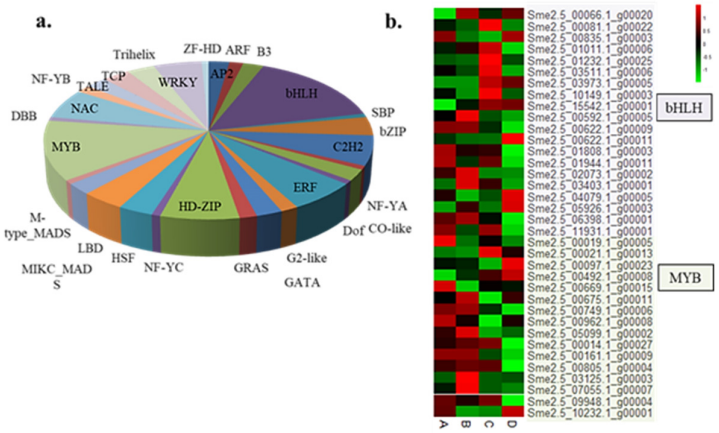

Figure 5. Differential expressed transcription factors (TFs) between EP26 and EP28
(A) Number of differentials expressed genes between EP26 and EP28 at two developmental stages; (B) Expression profiles of bHLH and MYB TFs between EP26 and EP28 at two developmental stages (A: EP26 at DDA15, B: EP26 at DDA25, C: EP28 at DDA15, D: EP28 at DDA25).

Validation of differential expressed genes by qRT-PCR

Based on the RNA-Seq results, gene expression of thirteen selected DEGs were further validated using qRT-PCR with gene-specific primers. These genes included anthocyanin biosynthetic genes and regulatory genes, as well as genes involved in photosynthesis. A linear regression analysis showed an overall correlation coefficient of R^2=0.7501, indicating the results of qRT-PCR were consistent with those of the transcriptome analysis (Figure 6).

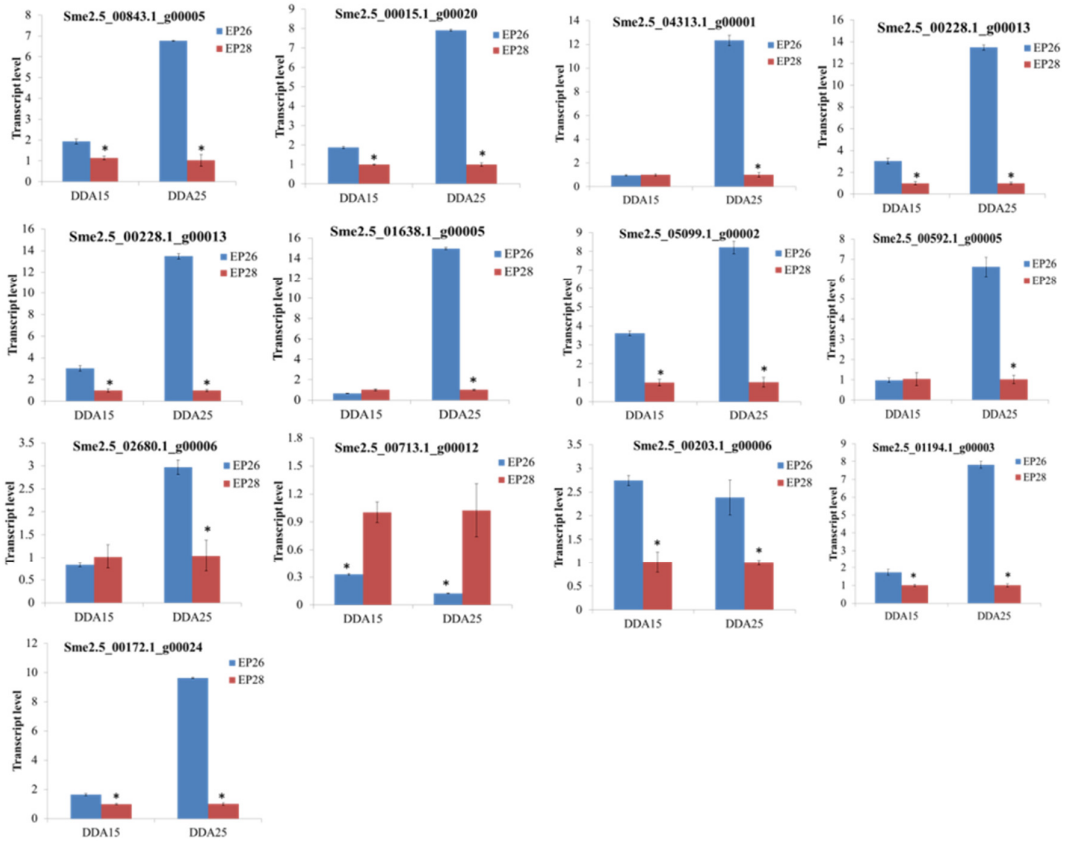

Figure 6. Quantitative real-time PCR analysis of the thirteen DEGs between EP26 and EP28 at two different stages

Discussion

Fruit peel colour intensity is a key trait for dark purple type of eggplant. Despite the importance of eggplant worldwide as a food crop and considerable interest in eggplant fruit secondary metabolites for their nutritive value, limited research has been conducted to understand the molecular mechanism underlying the fruit peel colour intensity variation. In the present study, EP26 exhibits good and stable performance on fruit peel colour intensity compared to EP28. Also, comparative transcriptome analysis identified a set of key genes for variation of fruit skin colour intensity between EP26 and EP28. The analysis of identified DEGs, together with phenotypic characterization of EP26 and EP28 will provide insights into the molecular basis underlying the intensity variation in fruit skin pigmentation of eggplant.

Fruit skin colour intensity variation between EP26 and EP28

It is reported that the highest level of anthocyanin was accompanied by the highest chlorophyll level of fruit peel in 11 eggplant cultivars tested (Nothmann *et al.*, 1976). However, in our study, although the anthocyanin contents of EP26 with deeper colour intensity were higher than those of EP28 at both stages, the chlorophyll contents of EP26 were lower than that of EP28 at two developmental stages. Moreover, in our preliminary study, the fruit peel colour intensity and anthocyanin contents, as well as chlorophyll contents of a set of purple advanced lines were also measured, and we found that there is a significant positive correlation between colour intensity and anthocyanin content in eggplant fruit peel (data not published). These results

suggest that the anthocyanin content variation rather than the chlorophyll content variation contributes to fruit peel colour intensity variation at the physiological level.

A total of eight kinds of anthocyanins were found both in the fruit peel of EP26 and EP28. The major anthocyanin identified was delphinidin 3-rutinoside, which is different with the findings of Zhang *et al.* (2014), Stommel *et al.* (2015), and Li *et al.* (2017). The anthocyanin detected in the materials used in their studies for light-induced analysis was delphinidin 3-(p-coumaroylrutinoside) -5- glucosid. It has been reported that delphinidin 3-(p-coumaroylrutinoside) -5- glucoside is the major anthocyanin in Japanese type eggplant and delphinidin 3-rutinoside is the major anthocyanin in non-Japanese type eggplant (Sadilova *et al.*, 2006; Azuma *et al.*, 2008).

Structural genes involved in anthocyanin biosynthesis

Most of the structural genes involved in anthocyanin biosynthesis have been isolated and cloned in eggplant. Zhang *et al.* (2014) found that the expression levels of all anthocyanin biosynthetic genes except PAL were up-regulated in purple eggplant fruit peel compared with those in white eggplant fruit peel. In our study, the expression levels of *SmCHI*, *SmF3H*, *SmF3'5'H*, *SmDFR*, and *SmANS*, except for *SmCHS* were all up-regulated in EP26 compared to EP28 at two developmental stages. Moreover, comparing our transcriptome data with previous studies of transcriptomic analysis for light-induced anthocyanin biosynthesis in eggplant (Li *et al.*, 2017; Li *et al.*, 2018), we found that a total of 322 DEGs were shared. Among these shared DEGs, almost all of the structural biosynthetic genes from our study, including *CHS*, *CHI*, *F3H*, *F3'5'H*, *DFR*, *ANS*, *UFGT* were differentially expressed under light treatment in previous studies. The up-regulation of these structural biosynthetic genes suggested that fundamental transcriptional regulation of anthocyanin biosynthetic pathways could be a major factor in EP26 for deeper fruit peel colour intensity.

In addition, previous studies indicated that *DFR*, *F3H*, and *UFGT* were considered as key enzymes in eggplant fruit anthocyanin biosynthesis (Cericola *et al.*, 2014; Toppino *et al.*, 2016). Stommel *et al.* (2015) suggested that higher expression of *CHS*, *DFR*, and *ANS* in eggplant fruit with violet vs. white fruit color result in high vs. very low anthocyanin concentration. In our study, the expression levels of *CHI* (Sme2.5_00188.1_g00020), *F3H* (Sme2.5_00015.1_g00020), and *UFGT* (Sme2.5_00228.1_g00013) increased in parallel with the accumulation of anthocyanins, indicating these three genes might be the key structural genes underlying fruit peel color intensity variation between EP26 and EP28.

Regulatory genes involved in anthocyanin biosynthesis

It has been revealed that R2R3-MYB and bHLH transcription factors, together with WDR proteins play an important role in regulating the transcription of structural genes of anthocyanin biosynthesis (Liu *et al.*, 2018). In eggplant, *SmMYB1* (Sme2.5_05099.1_g00002) was firstly found to be involved in regulation of anthocyanin accumulation (Zhang *et al.*, 2014). Besides *SmMYB1*, Li *et al.* (2017) reported another three MYB TFs including *SmMYB35* (Sme2.5_00216.1_g00001), *SmMYB44* (Sme2.5_00029.1_g00032), and *SmMYB86* (Sme2.5_09948.1_g00004) might be involved in light-induced anthocyanin biosynthesis pathway. In this study, a total of 16 MYB TFs with different expression patterns were identified, among which *SmMYB1* (Sme2.5_05099.1_g00002) was up-regulated at both stages and its transcript level increased in accordance with anthocyanin accumulation. *SmMYB35* and *SmMYB44* were not found differentially expressed in our study, while *SmMYB86* was only up-regulated in EP26 vs EP28 at DDA25. This indicated that the molecular regulatory mechanism of fruit peel colour intensity variation between purple eggplants may be different with that of light-induced anthocyanin accumulation.

Among the 20 DEGs of bHLH TFs identified in our study, only one down-regulated bHLH Sme2.5_03973.1_g0005 was shared by both stages. The expression level of Sme00592.1_g00005, an orthologue of *TT8*, was up-regulated in EP26 at DDA25, but did not expressed differentially in EP26 vs EP28 at DDA15. TT8 was first reported to positively regulate the expression of anthocyanin biosynthetic genes in *Arabidopisis*, and was potentially involved in the regulation of anthocyanin biosynthetic genes in pak choi

(Zhang *et al.*, 2017) and peanut (Huang *et al.*, 2019). Sme00592.1_g00005 was also reported to be involved in light-induced anthocyanin biosynthesis in eggplant (Li *et al.*, 2017; Li *et al.*, 2018). These findings suggested that the bHLH family is involved in the regulation of anthocyanins synthesis by different expression pattern.

Transcription factors of WRKY, NAC, bZIP families have also been reported to play important roles in regulating anthocyanin biosynthesis. In apple, overexpression of *MdWRKY11* promoted the expression of *F3H*, *FLS*, *DFR*, *ANS*, and *UFGT* and increased the accumulation of flavonoids and anthocyanin (Wang *et al.*, 2018). The family genes of NAC were found to be highly up-regulated in blood-fleshed peaches compared to non-red-fleshed peaches (Zhou *et al.*, 2015). *SmHY5* (Sme03211.1_g00004) was identified to function as anthocyanin regulation factor in response to light signal in eggplant (Jiang *et al.*, 2016b). In this study, *SmHY5* and 7 WRKYs, together with 8 NAC TFs were also found differentially expressed in EP26 vs EP28, these transcription factors might indirectly regulate anthocyanin biosynthesis via MBW complex in response to various environmental conditions. Some TFs belong to the same family showed different expression patterns, indicating a complex transcriptional regulatory network.

DEGs involved in chlorophyll metabolism

In the present study, only four genes including *HEMA*, *CHLM*, *CRD*, and *PORA* involved in chlorophyll (Chl) biosynthesis were found down-regulated in EP28 compared to EP26 at DDA25. The *HEMA* gene catalyses the initial enzymatic step of tetrapyrrole biosynthesis, which eventually leads to chlorophyll production (Kumar and Söll, 2000). However, the expression pattern of these genes was not associated with Chl content between EP26 and EP28. Notably, a total of 11 unigenes encoding chlorophyll a/b-binding protein were down-regulated in EP28 compared to EP26 at DDA25. Previous study showed that coordinated synthesis of chlorophyll and the chlorophyll a/b-binding proteins is critical to the development of functional light-harvesting complexes (McCormac and Terry, 2002). Considering anthocyanin biosynthesis often affected by light (Jiang *et al.*, 2016b; Li *et al.*, 2017; Li *et al.*, 2018;), the extensive alteration in the expression of genes involved in photosynthesis possibly reduced the photosynthetic capacity, which in turn affected the biosynthesis of anthocyanin in EP28.

Conclusions

In this study, we have improved our knowledge on the variation of fruit peel colour intensity of purple eggplant. The deeper pigmentation of EP26 fruit peel was a result of higher anthocyanin levels associated with higher expression of anthocyanin biosynthetic genes. In addition, some candidate transcription factors to be responsible for these differences were also identified. Our findings provide a better understanding of molecular mechanism underlying the intensity variation of fruit peel in purple eggplant, which may thus facilitate future genetic and breeding improvement.

Authors' Contributions

Conceptualization: YZ; Data curation and Writing-Original draft: XHZ; Investigation: SYL and YPL; Methodology: DW and JL; Writing-review and editing: YY. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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