High temperature reduces peel color in eggplant (*Solanum melongena*) as revealed by RNA-seq analysis

Journal:	<i>Genome</i>
Manuscript ID	gen-2019-0021.R2
Manuscript Type:	Note
Date Submitted by the Author:	06-May-2019
Complete List of Authors:	Lv, Ling Ling; Chinese Academy of Tropical Agricultural Sciences, South Subtropical Crops Research Institute Feng, Xue Feng; Shangqiu University Li, Wei; Chinese Academy of Tropical Agricultural Sciences, South Subtropical Crops Research Institute Li, Ke; Chinese Academy of Tropical Agricultural Sciences, South Subtropical Crops Research Institute
Keyword:	eggplant, RNA-seq, peel coloring, high temperature stress, anthocyanin
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

High temperature reduces peel color in eggplant (*Solanum melongena*) as revealed by RNA-seq analysis

Ling Ling Lv^{1*}, Xue Feng Feng², Wei Li¹, Ke Li¹

¹ South Subtropical Crops Research Institute, CATAS, Zhanjiang, Guangdong 524091, China
/
Hai-nan province key laboratory for postharvest physiology and technology of tropical horticultural products, Zhanjiang, Guangdong 524091, China

² Shangqiu University, Shangqiu, Henan 476000, China

* Correspondence: 15133253@qq.com

Draft

Abstract

To acquire the eggplant transcriptome under high temperature stress, eighteen cDNA libraries were constructed and sequenced. A total of 136.31 Gb clean data was obtained and 88.86~92.35% of the clean reads were mapped to the eggplant reference genome. Under high temperature, the number of down-regulated genes was more than that of up-regulated genes and there were more differentially expressed genes on the 10th day after flowering than on the 15th and 20th days after flowering. On the 10th day after flowering, the key genes *CHI*, *3GT*, *F3'5'H*, *DFR2*, *ANS*, *F3H* in anthocyanin synthetic pathway and most ERF, WRKY, bHLH and MYB transcription factors were all down-regulated. High temperature significantly decreased the total anthocyanin content in peels. The results showed that at the early stage of peel coloring, high temperature inhibited the expressions of key genes in anthocyanin biosynthetic pathways through the regulation of transcription factors, leading to a significant decrease in total anthocyanin content, which might reduce the peel color in eggplant.

Key words: eggplant, RNA-seq, peel coloring, anthocyanin, transcription factor, high temperature stress

Introduction

Eggplant (*Solanum melongena* L.) is a thermophile vegetable and is grown throughout China. However, consumers in different regions vary preference for the shape and color of eggplant. For example, green ovate eggplant is planted in Northeast China, purple eggplant in East China, green long eggplant in Central Plains, blackish purple eggplant in Southwest China and purplish red eggplant in South China. So the fruit color is one of the important factors in eggplant breeding. The optimum temperature for eggplant peel coloring is from 25 to 30 °C, and the peels color will turn

light even white when the temperature is over 35 °C. High temperature (HT) affects the appearance quality and reduces the commodity value of eggplant.

Flavonoids, including flavone, flavonol, isoflavone, flavanol, flavanone and anthocyanin, are a major class of secondary metabolites existed in all higher plants. They are very important pigments that contribute to a range of fruit colors from yellow to purple (Treutter 2005; Harborne and Williams 2000). Anthocyanins are one of the most abundant natural pigments and provide color to most flowers and fruits. Flavonoids, especially anthocyanins, are also thought to play important roles in protecting plant tissues from the damage of coldness and UV irradiation (Schulz et al. 2016; Takeda et al. 1994; Feild et al. 2001). The color of plant tissues is affected by intrinsic gene regulation and external environmental factors, such as light, temperature et al (Li et al. 2018; Ilk et al. 2015). Overexpression of *MYB10.1/ bHLH3* and *MYB10.3/bHLH3* activated anthocyanin production by up-regulating *NtCHS*, *NtDFR* and *NtUFGT*, leading to pigmented peach fruit (Rahim et al. 2014).

The effects of HT on the anthocyanin content or the expression of genes in anthocyanin biosynthesis have been reported in some crops. In apple, HT caused a dramatic reduction of both peel anthocyanin concentration and transcripts of the genes in anthocyanin biosynthetic pathway (Lin-Wang et al. 2015). HT (maximum 35 °C) reduced the total anthocyanin (TA) content to less than half of that in the control grape berries (25 °C) (Mori et al. 2007). In rose flowers, transcript levels of *CHS* and dihydroflavonol reductase (*DFR*) decreased by 50% after 39°C heat treatment, suggesting that the decreased anthocyanin concentration is in part due to reduced transcription (Dela et al. 2003). However, no related research was reported in eggplant. The objective of this study is to understand which genes were up-regulated or down-regulated and which genes played key roles when HT stress caused the light peel color in eggplant. To understand the mechanism will be important for breeding new eggplant varieties with purplish red peel.

Materials and methods

Plant materials

Eggplant homozygous line E30 (*Solanum melongena* L.) with purplish red peel (**Fig. 1A**) was used as material. E30 that supports the findings of this study has been deposited in the CNGBdb (<https://db.cngb.org/>) with the accession code CNSebb2005576. When the plants had flower buds and did not flower, the six plants had been treated at 35 °C / 25 °C (light 12 h / dark 12 h) and the other six plants used as control were treated at 25 °C / 20 °C (light 12 h / dark 12 h) in the growth chambers respectively until the fruits were mature. On the 10th day after flowering (10DAF), 15DAF and 20DAF, the peels were sampled and frozen in liquid nitrogen immediately, then stored at -80 °C until use. Each sampling point contained three independent biological replicates. When suffers HT stress, the peel color of E30 will turn light even white (**Fig. 1B**).

RNA isolation, library construction and RNA sequencing

Total RNA was isolated from peels using plant RNA extraction kit (TIANDZ, Inc Beijing, China, with DNase). The quantity and quality of the RNA samples were verified using Enzyme-linked immunosorbent analyzer (Spark 10 M, Switzerland) and agarose gel electrophoresis (1.0 %, w/v). Eighteen libraries were constructed and sequenced with PE150 using Illumina HiSeq 4000 platform by Genepioneer Biotech Corporation (Nanjing, China).

Data processing and mapping

Raw reads were cleaned by removing adaptor reads, ambiguous reads ('N' (represents A or T or C or G or U) > 3 %), and low-quality reads (i.e., more than 50% of bases in a read had a quality value (Q-score) ≤ 30) using perl script. High-quality reads were mapped to the eggplant reference genome (Hirakawa et al. 2014) on October 2017, using HISAT2 software (Kim et al. 2015).

Functional annotation and classification

All mapped genes and new genes found in this study were compared with public databases including the Non-redundant nucleotide and protein database (Altschul et al. 1997), Swiss-Prot database (Altschul and Gish 1996), the Clusters of Orthologous Groups of proteins database (Tatusov et al. 2003), Pfam database (Finn et al. 2016), using BLASTX, respectively, with an *E*-value of $1e^{-5}$. Then Web Gene Ontology Annotation Plot was used to obtain the GO functional classifications for all annotated genes (Ye et al. 2006). A Python script was used to retrieve Kyoto Encyclopedia of Genes and Genomes (KEGG) information from blast results and establish pathway associations between annotated genes and databases. Finally KEGG classification map was drawn.

Differentially expressed genes (DEGs) analysis

Fragments Per Kilobase of transcript per Million fragments mapped (FPKM) was applied to calculate and normalize the expression of the genes (Trapnell et al., 2010). The DESeq2 package (Love et al. 2014) was used to obtain the "base mean" value for identifying DEGs. Genes with False Discovery Rate (FDR) ≤ 0.05 and the absolute value of $\log_2$ (Fold Change) ≥ 1 were considered significant difference between the two libraries.

GO enrichment analyses of DEGs were performed using Singular Enrichment Analysis method with *P*-value < 0.01 and FDR < 0.01 by agriGO (Du et al. 2010). The hypergeometric Fisher exact test (*P*-value < 0.01) and Benjamini (FDR < 0.05) were performed to detect statistically significant enrichment of KEGG pathway. GO and KEGG enrichment analyses were performed using the whole eggplant genome as reference. Transcription factors (TFs) were identified using Plant TFDB 4.0 with default parameters (Jin et al. 2017). Heat maps of the gene sets were analyzed using R package 'pheatmap' (Kolde 2012). The gene expression levels were transformed to $\log_2$ (Fold Change) using three biological replicates.

Real-time quantitative PCR (qRT-PCR) analysis

The first-strand cDNA was synthesised from 2 μg of total RNA using Prime Script RT Reagent Kit (Takara, Dalian, China). The qRT-PCR reactions were performed in 96-well plates using the ABI

7500 fast Real-Time PCR system (Applied Biosystems, USA) with the SYBR Premix Ex Taq™ Kit (Takara, Dalian, China). The *18S rRNA* was used as an internal control for normalization of gene expression (Gantasala et al. 2013). The relative mRNA expression levels were calculated using the 2-ΔΔCt method (Livak and Schmittgen 2001). Three biological replicates and three technical repeats were performed for each gene and sample. Twenty microliters of the qRT-PCR reaction volume contained 10.0 μl SYBR Premix Ex Taq™ II, 0.4 μl ROX Reference Dye II (50×), 0.8 μl PCR forward primer and 0.8 μl PCR reverse primer (10 μM), 6.0 μl ddH₂O, and 2.0 μl cDNA (70 ng). The qRT-PCR parameters were: 95 °C for 5 min, then 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Melting curve and agarose gel electrophoresis analysis were performed to confirm the PCR specificity. The Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) was supplemented as **Table S1**.

The primers were designed using NCBI primer-BLAST (Ye et al. 2012). The information of genes and primer sequences were listed in supplemental **Table S2**.

Extraction and analysis of total anthocyanin

TAs were extracted from the peels according to the method described by Shao (2013) with minor modifications. In short, anthocyanins were extracted in 4 mL of acidic methanol (2 % HCl, w/v) by incubating 100 mg of finely grounded peels at 32 °C for 2 h in dark, then clarified by centrifugation at 8,000 × g for 10 min at 4 °C. The volume of the extract was made up to 10 mL with acidic methanol (2 % HCl, w/v). The content of TA was quantified based on the absorption of the extracts using the equation:

$$\text{The content of TA (mg / g)} = (A_{530} - 0.25 \times A_{700}) \times \text{FW}^{-1}$$

Where A_{530} and A_{700} are the absorptions at 530 nm and 700 nm respectively; FW is the fresh weight of plant materials (g). Anthocyanins were quantified as triplicates of three independent biological replicates

Statistical analysis

Statistical analysis was conducted with DPS version 7.05 software (Tang and Feng 2007). One-way ANOVA was used to analysis the TA content and differences between the sampling datas were estimated with the Duncan test ($p<0.01$).

Results

RNA-seq data output, quality assessment and mapping

In this study, eighteen cDNA libraries were constructed and sequenced on Illumina HiSeq 4000. The detailed information of the RNA-seq data was listed in **Table 1**. The rawdata was submitted to NCBI and the BioProject ID is PRJNA531285. A total of 136.31 Gb clean data was obtained. On average, 7.57 Gb clean data was obtained from each sample. The percentages of Phred-like quality score at the Q30 level (an error probability of 1%) ranged from 88.24% to 93.83 % and the average GC content was 42.54%. Among the 18 samples, 88.86% to 92.35% of the clean reads were mapped to the eggplant reference genome and about 69.13% of the mapped reads were located at the exon region. The RNA-seq data showed that the sequencing quality was high and sequencing depth was sufficient for transcriptome coverage.

Table 1 Summary of the sequencing data of 18 eggplant cDNA libraries

Samples	ID	Reads number (PE)	Base number	GC content	%≥Q30	Reads aligned	Exonic
s25-10-1	T01	28405481	8521644300	42.90	93.70	52051438(91.62%)	34,695,222(70.03%)
s25-10-2	T02	31048198	9314459400	42.71	93.11	56876777(91.59%)	37,571,420(69.33%)
s25-10-3	T03	28121326	8436397800	42.82	93.31	51404232(91.40%)	33,619,685(68.55%)
s25-15-1	T04	22032970	6609891000	42.60	91.66	40194817(91.22%)	26,700,374(69.71%)
s25-15-2	T05	24308360	7292508000	42.75	93.80	44574698(91.69%)	29,778,364(70.13%)
s25-15-3	T06	25843906	7753171800	42.59	88.24	46720964(90.39%)	30,647,828(68.76%)
s25-20-1	T07	23646322	7093896600	42.40	90.90	43109128(91.15%)	28,224,594(68.75%)
s25-20-2	T08	25182444	7554733200	42.60	91.54	44754546(88.86%)	29,603,628(69.55%)
s25-20-3	T09	24578380	7373514000	42.71	91.58	44830771(91.20%)	29,966,142(70.29%)
s35-10-1	T10	26158670	7847601000	42.32	90.61	47817035(91.40%)	30,726,052(67.31%)
s35-10-2	T11	25959333	7787799900	42.45	91.26	47944959(92.35%)	31,340,645(68.56%)
s35-10-3	T12	23620728	7086218400	41.96	91.01	43078441(91.19%)	26,612,496(64.71%)
s35-15-1	T13	25163461	7549038300	42.36	90.42	45896870(91.20%)	30,110,034(68.81%)
s35-15-2	T14	23181129	6954338700	42.38	90.74	42322650(91.29%)	28,131,775(69.67%)
s35-15-3	T15	24558310	7367493000	42.17	90.24	44712476(91.03%)	29,396,120(68.91%)
s35-20-1	T16	21676922	6503076600	42.57	91.04	39381141(90.84%)	26,283,534(70.10%)
s35-20-2	T17	25364209	7609262700	42.64	93.77	46484804(91.63%)	31,289,063(70.68%)
s35-20-3	T18	25504099	7651229700	42.75	93.83	46754110(91.66%)	31,385,277(70.54%)
average		25241902.67	7572570800	42.54	91.71	46050548(91.21%)	30337903(69.13%)
total		454354248					

Analysis of total anthocyanin

At different stages of fruit development and different temperatures, the changes of TA content were showed in **Fig. 1C**. At 25 °C (Control), the TA content in peels increased gradually with the fruit development. Compared to control, the TA content significantly decreased at all three time points under HT stress. The results showed that HT significantly decreased the TA content in peels.

Identification of DEGs

A total of 9,065, 5,845 and 6,629 genes were differentially expressed under 35 °C stress compared to 25 °C on 10DAF, 15DAF and 20DAF respectively. The number of up-regulated genes was less than that of down-regulated genes, which suggested HT might suppress the expression of many peel coloring related genes. There were more DEGs on 10DAF than 15DAF and 20DAF, which implied that HT had a severe effect at the early stage of peel coloring. As shown in **Fig. 2**, under HT stress, there were 497 co-up-regulated genes and 1,328 co-down-regulated genes at three time points.

Gene ontology (GO) classification and enrichment analysis of DEGs

Under HT stress, 5,086, 3,267 and 4,036 DEGs were significantly enriched on 10DAF, 15DAF and 20DAF respectively, which were classified into 76, 47, and 117 GO terms respectively (**Fig. S1A-C**). On 10DAF and 15DAF, the DEGs related to peel coloring were presented in the biological process, and in Anthocyanin-containing compound biosynthetic process (GO: 0009718, **Fig. S1A-B**), sixteen and fourteen DEGs were enriched respectively. Among the annotated DEGs, only one gene (Sme2.5_00096.1_g00007.1) was up-regulated, while the other DEGs were all down-regulated. The co-down-regulated DEGs were focused on anthocyanidin-3-O-glucosyltransferase-like (3GT,

207 Sme2.5_06210.1_g00004.1), flavonoid 3',5'-hydroxylase (*F3'5'H*, Sme2.5_04313.1_g00001.1,
 208 XLOC_046562, XLOC_046563), *DFR2* (Sme2.5_01401.1_g00004.1) and anthocyanidin synthase (*ANS*,
 209 Sme2.5_01638.1_g00003.1, Sme2.5_01638.1_g00005.1, XLOC_025201).

210 On 20DAF, most of the terms in biological process were closely related to defense, such as
 211 Response to blue light (GO: 0009637) and Defense response to bacterium (GO: 0042742).
 212 Anthocyanin-containing compound biosynthetic process (GO: 0009718) and Flavonoid biosynthetic
 213 process (GO: 0009813) were also enriched in GO classification but not showed in **Fig. S1C**.

214 KEGG Pathway Classification and Enrichment Analysis of DEGs

215 All DEGs were BLAST against the KEGG Ontology database. On 10DAF, 1,233 DEGs were
 216 classified into 13 KEGG categories. The top 8 categories were presented in **Fig. 3A** and there were 20
 217 DEGs in Flavonoids biosynthesis (ko00941). On 15DAF and 20DAF, there were 12 and 14 DEGs in
 218 Flavonoids biosynthesis respectively (**Fig. 3B-C**).

219 The Flavonoid biosynthesis category was found at all three time points (**Fig. 3**) and 22 DEGs
 220 were involved (**Fig. 4**). Caffeoyl-CoA O-methyltransferase (*CCoAOMT*) and Cytochrome P450
 221 (*CYP*) were all down-regulated by HT stress. *CYP 98A3-2*, *F3H* and *CHI* were down-regulated just
 222 on 10DAF. The key genes *CHS* (Sme2.5_01077.1_g00016.1, Sme2.5_13923.1_g00001.1), *DFR2*, *ANS2*,
 223 *ANS*, *F3'5'H* and *F3'H* were down-regulated on 10DAF and/or 15DAF, but up-regulated on 20DAF.

224 Validation of RNA-seq data by qRT-PCR analysis

225 To validate the expression data of RNA-seq, twelve DEGs were selected for qRT-PCR analysis
 226 (**Fig. 5**), including two TFs (*SmMYB12* and *SmWD40*), six genes involved in anthocyanin
 227 biosynthesis (*SmDFR2*, *SmANS2*, *SmANS*, *SmF3'5'H*, *SmCHS* and *SmCHS2*), one regulating
 228 anthocyanin metabolism (purple acid phosphatase 1, *SmPAP1*), one in lignin biosynthesis
 229 (*SmCCoAOMT-3*), one in isoflavone biosynthesis (*SmCYP-2*) and one flavonol synthase (*SmFLS*). To
 230 compare the expression data between RNA-seq and qRT-PCR, the relative expression level was

transformed to log₂ (Fold Change). As **Fig. 5** showed, the expressions of *SmF3'5'H1*, *SmCHS*, and *SmMYB12* were higher in RNA-seq than in qRT-PCR. In contrast, the expressions of *SmCCoAOMT-3*, *SmCYP-2* and *SmWD40* were lower in RNA-seq than in qRT-PCR. Though the expression trends of these genes were different, the qRT-PCR results showed a high consistency with RNA-seq data which suggested that RNA-Seq data was credible and could be used for subsequent experiments.

DEGs related to transcription factors

Under HT stress, comparing with control, a total of 205, 131 and 154 TFs were differentially expressed on 10DAF, 15DAF and 20DAF respectively (**Fig. 6**). Among the differentially expressed TFs, the top 5 of ERF, WRKY, bHLH, MYB and NAC families were enriched at all three time points.

Discussion

The peel color is an important economic trait for eggplant and is essentially determined by the components and content of anthocyanins which are modulated by the genes in flavonoid biosynthetic pathway or the TFs. Structural genes *SmF3'5'H*, *SmDFR*, *Sm3GT* and *Sm5GT* were highly expressed in peels of purple eggplant (Wang 2016). *SmCHS*, *SmCHI*, *SmDFR*, *SmF3'5'H* and *SmANS* had the highest expression in purple peels which showed correlation with anthocyanin content (Jiang et al. 2016). Stommel (2015) reported that anthocyanin content increased significantly in developing purple eggplant fruit, levels nearly 500-fold greater than those measured in white fruit. Coincident with anthocyanin content, the transcript levels of biosynthetic genes *CHS*, *DFR*, *ANS* were significantly higher in violet tissue than in white tissue (Stommel and Dumm 2015). Transcript levels of *CHS* and *DFR* decreased by 50% after heat treatment, suggesting that the decreased anthocyanin concentration in rose flower is in part due to reduced transcription (Gal et al. 2003).

In this study, on 10DAF and 15DAF, in Flavonoid biosynthetic category, only one annotated DEG (*CHS*, *Sme2.5_00283.1_g00002.1*) was up-regulated, and the other DEGs were all

down-regulated under HT stress (**Fig. 4**). Most of the down-regulated DEGs (other *CHS* homologs, *CHI*, *F3H*, *DFR2*, *ANS*, *F3'5'H* and *F3'H*) are the key genes in anthocyanin synthetic pathway (**Fig. 7**). *CYPs* and *CCoAOMTs* were the key genes in lignin biosynthetic pathway (**Fig. 7**) which played important roles in abiotic stresses (Lin et al. 2012). Winkel-Shirley (2001) reported that methylation mediated by *CCoAOMT* could improve the stability of anthocyanin. In this study, *CCoAOMTs* were down-regulated by HT stress, which was unfavourable for anthocyanin accumulation. Under HT stress, the TA content significantly decreased in eggplant peels at all three time points (**Fig. 1C**). Pavel (2018) reported that the color of grain was determined mainly by anthocyanin content and composition. The above results indicated that HT inhibited the expressions of anthocyanin synthesis related genes and decreased the TA content, which might lead to the light peel color in eggplant. The results are agreement with previous studies (Lin-Wang et al. 2015; Mori et al. 2007; Wang 2016; Jiang et al. 2016; Stommel and Dumm 2015; Gal et al. 2003; Rowen et al. 2009; Poudel et al. 2009). On 20DAF, the key genes in anthocyanin biosynthesis, such as *CHS*, *DFR2*, *ANS*, *ANS2* and *F3'H* were up-regulated (**Fig. 4**), resulting in anthocyanins accumulation, which might protect the eggplant fruits from the damage of HT. The similar phenomenon was reported in *Arabidopsis* that most flavonols and anthocyanins accumulated upon cold exposure (Schulz et al. 2016).

TFs have already been reported to play important roles not only in abiotic stresses but also in flavonoids or anthocyanin biosynthesis. The expressions of *SmMYB1*, *SmMYB2* and *SmAN11* in purple eggplant were higher than those in white eggplant; Overexpression of *SmMYB1* promoted anthocyanin accumulation in the regenerating shoots of eggplant (Zhang et al. 2014). Overexpression of *VvbHLH1* significantly increased the accumulation of flavonoids and enhanced salt and drought tolerance in transgenic *Arabidopsis thaliana* (Yang et al. 2016). *PbMYB10b* regulated the anthocyanin and proanthocyanidin pathways by inducing the expression of *PbDFR*; *PbMYB9* induced the synthesis of anthocyanins and flavonols in pear fruits (Zhai et al. 2016). *VvWRKY26* is involved in flavonoid biosynthesis (Amato et al. 2016). The heterodimer of a NAC TF and *PpNAC1*

can activate the transcription of *PpMYB10.1*, resulting in anthocyanin pigmentation in tobacco (Zhou et al. 2015).

In this study, under HT stress, ERF, WRKY, bHLH, MYB and NAC were differentially expressed compared with control (Fig. 6). All the differentially expressed ERFs, WRKYs, MYBs and bHLHs were showed in Figure S2-S5 respectively and the number of down-regulated TFs was more than that of up-regulated, especially on 10DAF. There were four TFs (WIN1-2, WRKY19-2, MYB12 and MYB113) up-regulated at three time points, which might negatively regulate the peel coloring. HT significantly down-regulated the expression of ERF1B-1, ERF4, WRKY45, WRKY31-2, MYB39, MYB108, bHLH14 and NAC1, which might postively regulate the peel coloring. In a word, differentially expressed ERF, WRKY, NAC, MYB and bHLH might play important roles in peel coloring, especially at the early stage of fruit development.

The results indicated that on the one hand HT stress inhibited the expressions of anthocyanin synthesis related genes (Fig. 4) through the regulation of TFs, on the other hand, HT significantly decreased the TA content in peels (Fig. 1C), which might lead to the light peel color in eggplant.

Acknowledgements

This work was funded by the Project of Natural Science Foundation of Guangdong Province (2015A030310452), the Fundamental Research Funds for South Subtropical Crops Research Institute , CATAS (1630062017030). The authors are grateful to Xiao Xi Ou, Li Ming Zhi and Gao Xiao Min for their technical assistance.

Conflict of Interest: The authors declare no conflict of interest.

References

Altschul, S. F., and Gish, W. 1996. Local alignment statistics. Method Enzymol. 266:460–480. doi: 10.1016/S0076-6879(96)66029-7

- 305 Altschul, S. F., Madden, T. L., Schaeffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. 1997. Gapped
306 BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:
307 3389–3402. doi: 10.1093/nar/25.17.3389
- 308 Amato, A., Cavallini, E., Zenoni, S., Finezzo, L., Begheldo, M., Ruperti, B., Tornielli, G.B. 2016. A grapevine
309 TTG2-like WRKY transcription factor is involved in regulating vacuolar transport and flavonoid
310 biosynthesis. *Front. Plant Sci.* **7**:1979. doi:10.3389/fpls.2016.01979
- 311 Dela, G., Or, E., Ovadia, R., Nissim-Levi, A., Weiss, D. & Oren-Shamir, M. 2003. Changes in anthocyanin
312 concentration and composition in ‘jaguar’ rose flowers due to transient high-temperature conditions. *Plant*
313 *Sci.* **164**(3):333-340.
- 314 Du, Z., Zhou, X., Ling, Y., Zhang, Z., Su, Z. 2010. agriGO: a GO analysis toolkit for the agricultural community.
315 *Nucleic Acids Res.* **38** (suppl_2):W64-W70. doi: 10.1093/nar/gkq310
- 316 Feild, T. S. , Lee, D. W., Holbrook, N. M. 2001. Why leaves turn red in autumn. the role of anthocyanins in
317 senescing leaves of Red-osier Dogwood. *Plant Physiology.* **127**(2):566-574. doi: [https://doi.org/10.1104/](https://doi.org/10.1104/pp.010063)
318 [pp.010063](https://doi.org/10.1104/pp.010063)
- 319 Finn, R. D. , Coghill, P. , Eberhardt, R. Y. , Eddy, S. R. , Mistry, J. , Mitchell, A. L. et al. 2016. The Pfam protein
320 families database: towards a more sustainable future. *Nucleic Acids Research.* **44** (D1):
321 D279–D285. <https://doi.org/10.1093/nar/gkv1344>
- 322 Gal, D., Etti, O., Rinat, O. 2003. Changes in anthocyanin concentration and composition in Jaguar rose flowers
323 due to transient high-temperature conditions. *Plant Sci.* **164**:333-340.
- 324 Gantasala, N.P., Papolu, P.K., Thakur, P.K., Kamaraju, D., Sreevathsa, R., Rao, U*. 2013. Selection and
325 validation of reference genes for quantitative gene expression studies by real-time PCR in eggplant
326 (*Solanum melongena* L). *BMC Res Notes.* **6**: 312. <https://doi.org/10.1186/1756-0500-6-312>
- 327 Harborne, J.B., Williams, C.A. 2000. Advances in flavonoid research since 1992. *Phytochemistry.* **55**:481-504.
328 [https://doi.org/10.1016/S0031-9422\(00\)00235-1](https://doi.org/10.1016/S0031-9422(00)00235-1)
- 329 Hirakawa, H., Shirasawa, K., Miyatake, K., Nunome, T., Negoro, S., Ohyama, A. et al. 2014. Draft genome
330 sequence of eggplant (*Solanum melongena* L.): the representative solanum species indigenous to the old
331 world. *DNA Res.* **21** (6):649-660. <https://doi.org/10.1093/dnares/dsu027>
- 332 Ilk, N., Ding, J., Ihnatowicz, A., Koornneef, M. & Reymond, M. 2015. Natural variation for anthocyanin
333 accumulation under high-light and low-temperature stress is attributable to the *enhancer of ag-4 2* (*HUA2*)

- locus in combination with *production of anthocyanin pigment1 (PAP1)* and *PAP2*. *New Phytol.* **206**(1): 422-435.
- Jiang, M., Liu, Y., Ren, L., Lian, H., Chen, H. 2016. Molecular cloning and characterization of anthocyanin biosynthesis genes in eggplant (*Solanum melongena* L.). *Acta Physiol. Plant.* **38**:163. <https://doi.org/10.1007/s11738-016-2172-0>
- Jin, J, Tian, F., Yang, D.C., Meng, Y.Q., Kong, L., Luo, J.C., Gao, G. 2017. Plant TFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res.* **45** (D1): D1040-D1045. <https://doi.org/10.1093/nar/gkw982>
- Kim, D., Langmead, B., Salzberg, S.L. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods.* **12** (4): 357-360. doi:10.1038/nmeth.3317
- Kolde, R. 2012. pheatmap: Pretty Heatmaps. R package version 061.
- Li, J., He, Y.J., Zhou, L.; Liu, Y., Jiang, M.M., Ren, L., Chen, H.Y. 2018. Transcriptome profiling of genes related to light-induced anthocyanin biosynthesis in eggplant (*Solanum melongena* L.) before purple color becomes evident. *BMC Genomics.* **19**:201. <https://doi.org/10.1186/s12864-018-4587-z>
- Lin J.S., Lin C.C., Lin H.H., Chen Y.C., Jeng S.T. 2012. MicroR828 regulates lignin and H₂O₂ accumulation in sweet potato on wounding. *New Phytologist.* **196**:427-440. doi:10.1111/j.1469-8137.2012.04277.x
- Lin-Wang, K., Micheletti, D., Palmer, R., Volz, J., Lozano, L., Espley, R. et al. 2011. High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. *Plant Cell & Environ.* **34**:1176-1190. doi:10.1111/j.1365-3040.2011.02316.x
- Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using Real-Time quantitative PCR and the 2^{(-Delta Delta C(T))} method. *Methods.* **25** (4): 402-408. doi: 10.1006/meth.2001.1262
- Love, M.I., Huber, W., Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**(12):550. <http://dx.doi.org/10.1186/s13059-014-0550-8>
- Mellway R. D., Tran L. T., Prouse M. B., Campbell M. M. and Constabel C. P. 2009. The wound-, pathogen-, and ultraviolet B-responsive MYB134 gene encodes an R2R3 MYB transcription factor that regulates proanthocyanidin synthesis in poplar. *Plant Physiology.* **150**: 924-941.
- Mori, K., Goto-Yamamoto, N., Kitayama, M., Hashizume, K. 2007. Loss of anthocyanins in red-wine grape under high temperature. *J. Exp. Bot.* **58**:1935-1945. doi:10.1093/jxb/erm055

- 362 Pavel, Z. , Elena, A. , Anna, L. , Natalia, T. , & Anatoly, V. 2018. Anthocyanin composition and content in rye
363 plants with different grain color. *Molecules*. **23**(4), 948. doi: 10.3390/molecules23040948
- 364 Poudel, P.R., Mochioka¹, R., Beppu, K. and Kataoka, I. 2009. Influence of temperature on berry composition of
365 interspecific hybrid wine grape 'Kadainou R-1' (*Vitis ficifolia* var. *ganebu* × *V. vinifera* 'Muscat of
366 Alexandria'). *J Japan Soc Hort Sci*. **78**:169-174.
- 367 Rahim, M.A., Busatto, N. & Trainotti, L. 2014. Regulation of anthocyanin biosynthesis in peach fruits. *Planta*.
368 **240**: 913. <https://doi.org/10.1007/s00425-014-2078-2>
- 369 Rowen, D.D., Cao, M.S., Lin-Wang, K., Cooney, J.M., Jensen, D.J., Austin, P.T. et al. 2009. Environmental
370 regulation of leaf colour in red 35S:PAP1 *Arabidopsis thaliana*. *New Phytol*. **82**:102-115.
- 371 Schulz, E., Tohge, T., Zuther, E., Fernie, A.R., Hinch,* D.K.2016. Flavonoids are determinants of freezing
372 tolerance and cold acclimation in *Arabidopsis thaliana*. *Sci. Rep*. 34027.
- 373 Shao, W.T. 2013. Cloning and function analysis of anthocyanin-related transcription factor gene *SmMYB* in
374 eggplant (*Solanum melongena*). Master thesis, Shanghai Jiao Tong University, Shanghai. pp:16.
- 375 Stommel, J.R. & Dumm, J.M. 2015. Coordinated regulation of biosynthetic and regulatory genes coincides with
376 anthocyanin accumulation in developing eggplant fruit. *J. Am. Soc. Hortic. Sci*. **140** (2):129-135.
- 377 Takeda, K., Tochika, Y., Fukazawa, R., & Mori, T. 1994. Flavonoids as uv-protectant. *Acta Hortic*. **83** (381):
378 348-354.
- 379 Tang, Q.Y., Feng, M.G. 2007. DPS data processing system: Experimental design, statistical analysis and data
380 mining. Beijing: Science Press.
- 381 Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V. et al. 2003. The COG
382 database: an updated version includes eukaryotes. *BMC Bioinformatics*. **4**:41. doi: 10.1186/1471-2105-4-41
- 383 Trapnell, C, Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J. et al. 2010. Transcript
384 assembly and quantification by RNA Seq reveals unannotated transcripts and isoform switching during
385 cell differentiation. *Nature Biotechnology*. **28**(5):511-515.
- 386 Treutter, D. 2005. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant*
387 *Biol*. **7**: 581-591. doi:10.1055/s-2005-873009
- 388 Wang, F., Zhu, H., Chen, D., Li, Z., Peng, R., Yao, Q. 2016. A grape bHLH transcription factor gene, *VvbHLH1*,
389 increases the accumulation of flavonoids and enhances salt and drought tolerance in transgenic
390 *arabidopsis thaliana*. *Plant Cell Tiss. Org*. **125** (2): 387-398. doi: <https://doi.org/10.1007/s11240-016-0953-1>

- 391 Wang, S.J. 2016. Screening and cloning of key MYB transcription factor of anthocyanin biosynthesis in eggplant
392 (*Solanum melongena* L.). Master thesis, Shandong Agricultural University, Taian.
- 393 Winkel-Shirley B.2001. Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and
394 biotechnology. *Plant Physiol.* **126**(2): 485-493.
- 395 Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z. et al. 2006. WEGO: a web tool for plotting GO
396 annotations. *Nucleic Acids Res.* **34** (2): W293-297. doi:10.1093/nar /gkl031
- 397 Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L. 2012. Primer-BLAST: A tool to
398 design target-specific primers for polymerase chain reaction [J]. *BMC bioinformatics.* **13**:134-144. PMID:
399 22708584
- 400 Zhai, R., Wang, Z., Zhang, S., Meng, G., Song, L., Wang, Z. et al. 2016. Two MYB transcription factors regulate
401 flavonoid biosynthesis in pear fruit (*pyrus bretschneideri rehd.*). *J. Exp. Bot.* **67**:1275-1284. doi: 10.1093/jxb/
402 erv524
- 403 Zhang, Y.J., Hu, Z.L., Chu, G.H., Huang, C., Tian, S.B., Zhao, Z.P. and Chen, G.P. 2014. Anthocyanin
404 accumulation and molecular analysis of anthocyanin biosynthesis-associated genes in eggplant (*Solanum*
405 *melongena* L.). *J. Agric. Food Chem.* **2**(13): 2906-2912. doi:10.1021/ jf404574c
- 406 Zhou, H., Lin-Wang, K., Wang, H., Gu, C., Dare, A.P., Espley, R.V. et al. 2015. Molecular genetics of
407 blood-fleshed peach reveals activation of anthocyanin biosynthesis by NAC transcription factors. *Plant J.*
408 *Cell & Mol. Bio.* **82**(1): 105. doi:10.1111/ tpj.12792
409
410
411
412
413
414
415
416
417

Captions

Fig. 1 (A): Eggplant E30 with normal purplish-red peel; **(B):** Eggplant E30 with poor peel color under high temperature; **(C):** The content of total anthocyanin in eggplant peels between 35 °C and 25 °C treatments. 10DAF: on the 10th day after flowering; 15DAF: on the 15th day after flowering; 20DAF: on the 20th day after flowering. HT: 35°C high temperature treatment; Control: 25°C treatment. Values are the means ± SD of three biological replicates. Asterisks indicate a significant difference determined by the Duncan test ($p < 0.01$)

Fig. 2 Differentially expressed genes of E30 (*Solanum melongena*) between 35 °C and 25 °C treatments. 10DAF_up, 15DAF_up, 20DAF_up: the up-regulated genes on the 10th, 15th and 20th days after flowering respectively. 10DAF_down, 15DAF_down, 20DAF_down: the down-regulated genes on the 10th, 15th and 20th days after flowering respectively.

Fig. 3 KEGG Pathway Classification of differentially expressed genes in *Solanum melongena* between 35 °C and 25 °C treatments. **(A):** on the 10th day after flowering; **(B):** on the 15th day after flowering; **(C):** on the 20th day after flowering.

Fig. 4 Heat map depicting log₂ (Fold Change) of differentially expressed genes in Flavonoid biosynthetic category of *Solanum melongena* between 35 °C and 25 °C treatments.

Fig. 5 qRT-PCR validation of RNA-Seq data using 12 peel-coloring related genes in *Solanum melongena*. 18S rRNA expression values were used as the internal reference. Values are the means ± SD of three biological replicates. 10DAF: on the 10th day after flowering; 15DAF: on the 15th day after flowering; 20DAF: on the 20th day after flowering.

Fig. 6 The KEGG pathway enrichment scatter plot of differentially expressed transcription factors in *Solanum melongena* between 35 °C and 25 °C treatments. **(A):** on the 10th day after flowering; **(B):** on the 15th day after flowering; **(C):** on the 20th day after flowering.

446 **Fig. 7** Biosynthetic pathways of anthocyanin, flavonol and lignin (Mellway et al. 2009)

447 ANS, Anthocyanidin synthase; CCoAOMT, Caffeoyl-CoA O-methyltransferase; CHI, Chalcone isomerase; CHS,

448 Chalcone synthase; CYP, Cytochrome P450; C3H, Coumaric acid-3-hydroxylase; C4H, Cinnamate -4-hydroxylase; DFR,

449 Dihydroflavonol reductase; F3'5'H, Flavonoid 3',5'-hydroxylase; F3'H, Flavonoid 3'-hydroxylase; F3H, Flavanone

450 3-hydroxylase; FLS, flavonol synthase; PAL, Phenylalanine ammonialyase

451

Draft

Draft

Draft