Plant Growth Regulation (2018) 84:437–450
https://doi.org/10.1007/s10725-017-0351-7

ORIGINAL PAPER

Transcriptome profiling of *Litchi chinensis* pericarp in response to exogenous cytokinins and abscisic acid

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Received: 22 August 2017 / Accepted: 2 December 2017 / Published online: 6 December 2017

Abstract

Anthocyanin biosynthesis in *Litchi chinensis* is promoted by exogenous abscisic acid (ABA) treatment and inhibited by exogenous *N*-(2-chloro-pyridin-4-yl)-*N*′-phenylurea (CPPU) application. However, the mechanisms by which ABA or CPPU regulates anthocyanin biosynthesis are still unclear. To understand the global molecular events of these physiological changes, transcriptome profiling was analyzed in *L. chinensis* cv. Feizixiao pericarps after 0, 10, and 20 days of exogenous ABA (25 mg/L ABA) and CPPU (4 mg/L) treatment using RNA-seq. Compared with the control, a total of 579 and 827 genes were differently expressed [$|\log_2$ fold change$| \geq 1$ and P value ≤ 0.005] in ABA- and CPPU-treated pericarp, respectively. Exogenous ABA up-regulated the expressions of genes involved in flavonoid and anthocyanin biosynthesis, including *PAL, C4H, CHS, CHI, DFR, LDOX*, and *GTs*. In contrast, exogenous CPPU induced genes related to carbon metabolism, amino acids biosynthesis, and photosynthesis, and down-regulated genes related to anthocyanin biosynthesis. Comparison of transcriptomes in responses to individual treatments with ABA or CPPU revealed that there were cooperative and antagonistic interplay between ABA and cytokinins in litchi fruit ripening. ABA treatment had no significant effect on the genes related to chlorophyll catabolism. On the other hand, CPPU treatment significantly increased the expression of chlorophyll synthesis genes and inhibited the expression of chlorophyll degradation gene (*SGR*). In addition, ABA and CPPU treatment also affected gene expression in other plant hormone signaling pathways, such as auxin, GA, and ethylene, forming a complex network to regulate anthocyanin biosynthesis. This study provides a valuable overview of global molecular events for studying the mechanisms by which ABA and cytokinins influence anthocyanin biosynthesis in litchi and other fruit trees enriched with anthocyanins.

Keywords ABA · Anthocyanin biosynthesis · CPPU · Chlorophyll degradation

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
BA	6-benzylaminopurine
bHLH	Basic helix-loop-helix
CTK	Cytokinin
CPPU	*N*-(2-chloro-pyridin-4-yl)-*N*′-phenylurea
DEGs	Differentially expressed genes
DFR	Dihydroflavonol-4-reductase
GAs	Gibberellins
IAA	Indole-3-acetic acid
MeJA	Methyl jasmonate
NAA	Naphthalene acetic acid
NDGA	Nordihydroguaiaretic acid
PGRs	Plant growth regulators
TF	Transcription factor

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10725-017-0351-7) contains supplementary material, which is available to authorized users.

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Introduction

Litchi (*Litchi chinensis* Sonn.) is an important fruit crop, originated in China and commercially grown in the tropical and subtropical regions of the world. China has the largest litchi industry in terms of cultivated area and production (Huang et al. 2005). Litchi fruit has a succulent edible flesh surrounded by a leathery pericarp and a dark brown seed. The color of red pericarp is an important quality attribute for the market acceptance of litchi fruit (Singh et al. 2014). The pink/red color of the pericarp is a consequence of anthocyanin biosynthesis and accumulation in the cells (Lee and Wicker 1991). Cyanidin-3-glucoside and cyanidin-3-rutinoside are the main anthocyanins in the red pericarp of litchi (Li et al. 2016a). The quantity and composition of anthocyanins in litchi varied widely among different varieties and also strongly depend on various environmental factors (Singh et al. 2014; Zhang et al. 2016). Therefore, a better understanding of the regulation of anthocyanin biosynthesis in litchi is of both scientific and economic importance.

Anthocyanin biosynthesis is mainly regulated at the transcriptional level. Three kinds of transcription factors (TFs) including MYB, basic helix-loop-helix (bHLH), and WD40 repeat (WDR) forming the MBW (MYB–bHLH–WD40) complex to regulate the expression of genes (*CHS, CHI, F3H, F3'H, DFR, UFGT*) that encode enzymes for the anthocyanin pathway (Xu et al. 2015; Lloyd et al. 2017). Increasing evidences indicate that the expression of MBW complex is modulated by environmental and biological factors such as light, sugars, and plant hormones (Teng et al. 2005; Loreti et al. 2008; Li et al. 2016b; Rodyoung et al. 2016). In our previous study, a R2R3–MYB TF *LcMYB1* was isolated from litchi pericarp and was proved as the major determinant regulatory gene in the anthocyanin biosynthesis (Lai et al. 2014). Cis-acting regulatory elements involved in plant hormones responsiveness were identified in the promoter of *LcMYB1*. Exogenous ABA and CPPU application could up- and down-regulate the expression of *LcMYB1*, respectively, resulting in enhanced or reduced anthocyanin accumulation (Lai et al. 2014). However, the influence of exogenous plant growth regulators (PGRs) on anthocyanin biosynthesis has not yet fully understood.

Numerous studies have indicated that the anthocyanin biosynthesis is strongly influenced by plant hormones (Loreti et al. 2008; Jeong et al. 2010; Das et al. 2012; Thiruvengadam et al. 2016). The impact of exogenous PGRs application on anthocyanin accumulation, however, is still controversial (Loreti et al. 2008). Deikman and Hammer (1995) observed that the synthetic cytokinin (CTK) BA induced anthocyanin accumulation in Arabidopsis. The promotion of anthocyanin production by BA in a dose-dependent manner was also observed in red-fleshed apple callus cultures (Ji et al. 2015). In contrast, several studies reported CTK as a negative regulator of anthocyanin accumulation (Kim et al. 2006; Loreti et al. 2008). Treatments with BA in non-chlorophyllous corn (*Zea mays* L.) inhibited anthocyanin accumulation (Kim et al. 2006). Likewise, the application of BA and CPPU markedly suppressed anthocyanin accumulation in litchi pericarp (Wei et al. 2011). In addition, the effects of exogenous synthetic GAs, ethylene, and auxin (2, 4-D, NAA) on anthocyanin accumulation are contradictory (Loreti et al. 2008; Jeong et al. 2010). The published evidences are mainly from exogenous synthetic PGRs treatments, and some discrepancies might be explained by considering the different plant species or organs (vegetative/reproductive) used.

Increasing evidences indicate that ABA accumulation appears to be critical during fruit ripening in both climacteric and non-climacteric fruits (as reviewed by Leng et al. 2014). There are notable physiological changes in fruit color during berry ripening. Color change in fruit ripening is achieved by chlorophyll degradation (degreening) and secondary color metabolites biosynthesis such as carotenoids and anthocyanins (McAtee et al. 2013; Kumar et al. 2014). There is evidence that the increase of ABA during fruit ripening is associated with anthocyanin accumulation (Jia et al. 2011). Many studies have reported that exogenous ABA application could increase anthocyanin contents in strawberry, grape, sweet cherry, and litchi (Jiang and Joyce 2003; Wang et al. 2007; Wheeler et al. 2009; Shen et al. 2014; Singh et al. 2014). Meanwhile, application of nordihydroguaiaretic acid (NDGA; an inhibitor of ABA biosynthesis) inhibits anthocyanin production (Medina-Puche et al. 2014; Shen et al. 2014). However, the molecular mechanisms from ABA perception and signal transduction to anthocyanin biosynthesis pathway remain to be elucidated.

In our previous study, exogenous ABA and CPPU application enhanced and reduced anthocyanin accumulation in litchi pericarp, respectively. To further understand the molecular basis of exogenous ABA and CPPU in regulating the anthocyanin biosynthesis of litchi fruit, we used RNA-seq to establish seven transcriptomes of harvested litchi fruits in response to individual treatments with ABA or CPPU, and we then carried out comparative analysis of these transcriptomes. This study will provide insight into the regulation of anthocyanin biosynthesis in litchi pericarp in response to exogenous ABA and CPPU.

Materials and methods

Plant materials and treatments

Fifteen-year-old *L. chinensis* cv. Feizixiao trees of similar vigor were used in the study. The experiment was carried out at Yongfa Fruit Farm, Haikou, China (110.21° N; 19.77° E) during 2013 season. Three trees under integrated orchard management practices were randomly selected for each treatment. Fifteen fruit clusters (10–15 fruits/cluster) from each direction were randomly tagged and received the same treatment. 25 mg/L (ABA, Sigma-Aldrich), 4 mg/L CPPU (Sigma-Aldrich) or tap water (used as control) were sprayed to the litchi fruits at color break stage (about 3 weeks before commercial harvest). Fruit clusters were sprayed with a handheld sprayer until runoff. Pericarp discs were sampled at 0, 10 and 20 days after treatments, respectively. All samples were frozen in liquid nitrogen immediately and kept at − 80 °C before use.

Determination of anthocyanin and chlorophyll content

Total anthocyanin content in pericarps was quantified as described by Wrolstad et al. (1982) with some modifications. Anthocyanin was extracted in 6 mL of methanol/water/HCl (3 mL, 85:12:3, v/v) for 5 h at 25 °C in darkness, and the extracts diluted with pH 1.0 and pH 4.5 buffers were measured at 520 nm with a UV2550 spectrophotometer (Shimadzu, Kyoto, Japan). Each sample was replicated three times.

For measurement of chlorophyll contents, pericarp disks (0.5 g) suspended in 10 mL 80% acetone for 24 h in darkness and the extracts were measured at 645 and 663 nm, respectively. Total chlorophyll content was calculated according to the method described by Arnon (1949). Each sample was replicated three times.

Endogenous ABA analysis

ABA content was determined using gas chromatography–mass spectroscopy (GC–MS) as described by Jia et al. (2011) with some modifications. Briefly, 1 g frozen samples were mixed with polyvinylpolypyrrolidone (PVPP), a small amount of 80% cold methanol, and 50 ng D3-ABA as an internal standard (Icon Services Inc., USA), and then grounded to homogeneity at 4 °C. The mixture was mixed with 80% methanol and soaked overnight at 4 °C. Then the mixture was filtered with a C18 column (Waters Corporation, Milford, MA, USA) and was concentrated to < 1 mL using speed vacuum at 35 °C. The residual was adjust to pH 2.5–3.0 by phosphate buffer, and extracted three times with equal volumes of ethyl acetate. The organic phase was dried by speed vacuum and dissolved with ethyl acetate, transferred to a capillary tube, and concentrated to determine the ABA content by GC–MS (Agilent Technologies, Santa Clara, CA, USA). This entire process was repeated three times.

RNA extraction, library construction and RNA-Sequencing

Seven samples were chosen for high-throughput RNA sequencing, Cont-0 (samples before sprayed), Cont-10 (samples of 10 days after water treatment), Cont-20 (samples of 20 days after water treatment), ABA-10 (samples of 10 days after ABA treatment), ABA-20 (samples of 20 days after ABA treatment), CPPU-10 (samples of 10 days after CPPU treatment), CPPU-20 (samples of 20 days after CPPU treatment). Total RNA was extracted using a Total RNA Purification Kit (TIANDZ, Beijing, China) from mixed samples of three biological replicates. The total RNA was treated with DNase I (TaKaRa Biotechnology, Dalian, China) to degrade DNA contamination and quantified using a NanoPothometer spectrophotometer (IMPLEN, CA, USA). Approximately 3 µg of total RNA was used to construct RNA-seq libraries. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform (San Diego, CA, USA) and 125/150 bp paired-end reads were generated.

RNA sequencing data analysis

Raw reads were processed to remove low-quality reads and reads with only the adaptors, resulting in clean reads. Clean reads were mapped to the litchi reference genome (unpublished) using TopHat v2.0.12. HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. And then number of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Differential expression analysis of two conditions was performed using the DEGSeq R package (1.20.0). The P values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.005 and |log2 fold change| of 1 was set as the threshold for significantly differential expression. TBtools v0.47 software was used to draw Venn diagrams and test the statistical enrichment of differential expression genes in KEGG pathways.

Gene functional annotation

Functional annotation of the differentially expressed genes (DEGs) was annotated based on the highest similarity in the following databases: Nr (NCBI non-redundant protein sequences, http://www.ncbi.nlm.nih.gov), Nt (NCBI non-redundant nucleotide sequences, http://www.ncbi.nlm.nih.gov), COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG), KO (KEGG Orthology database, http://www.genome.jp/kegg) and GO (Gene Ontology). Gene annotation was done using Blast2Go using BLASTX (1.0E-5 cut-off value).

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was used to validate the digital gene expression data obtained by RNA sequencing. Total RNA was isolated as described above and reverse transcribed with oligo $(dT)_{18}$ primers using M-MLV reverse transcriptase (Invitrogen, USA). The cDNA was used as templates in each qPCR reaction with the SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China). And the reactions were run in Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corporation, Beverly, MA, USA). Genes and primers were listed in Supplemental Table S1. All qPCR reactions were normalized using the Ct value corresponding to the *LcACTIN* gene (HQ615689). Relative expression level of candidate genes were calculated with the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Statistical analysis

Data were expressed as mean ± standard error (SE) using the SigmaPlot software version 11.0 (Systat Software Inc., San Jose, CA, USA). The data were analysed by ONE-WAY ANOVA with $P < 0.05$. Significant differences between treatments were determined with Duncan's multiple range tests using IBM SPSS Statistics software 19.0 (SPSS Inc., USA) for Windows.

Results

Changes in phenotype, pigment contents and endogenous ABA levels of 'Feizixiao' litchi pericarp in response to exogenous ABA and CPPU

Ten days after treatments, the ABA-treated fruits and control began to color (Fig. 1a), though there was no significant difference in anthocyanin content (Fig. 1b). However, the CPPU-treated fruits were only a little red at the fruit shoulder (Fig. 1a), and the anthocyanin content was much lower than those in ABA-treated fruits and control (Fig. 1b). Twenty days after treatments, the ABA-treated fruits became evenly red, and CPPU-treated fruits were still green with a little red (Fig. 1a). At the same time, the control fruit had an uneven red color (Fig. 1a), which is a typical feature of cv. Feizixiao. As shown in Fig. 1b, ABA treatment significantly promoted anthocyanin accumulation in litchi fruit pericarp after 20 days application, whereas CPPU treatment significantly inhibited it. In contrast to the anthocyanin content changes, the chlorophyll contents decreased during fruit ripening. Obviously, the ABA treatment accelerated chlorophyll degradation and CPPU treatment delayed this process (Fig. 1c).

There was no remarkable difference in endogenous ABA level between the ABA-treated fruits and control, whereas the CPPU-treated fruits had lower levels of ABA compared with those of ABA-treated fruits and control (Fig. 1d).

RNA sequencing and mapping of reads to the reference genome

Seven libraries (Cont-0, Cont-10, Cont-20, ABA-10, ABA-20, CPPU-10, CPPU-20) were constructed and subject to 150 bp paired-end reads using Illumina Hiseq platform. After eliminating adapters, ambiguous nucleotides and low-quality sequences, the total number of filtered clean reads in each library was 47,692,286, 50,199,598, 55,371,028, 47,120,500, 50,749,538, 44,194,082, and 47,962,430, respectively (Table 1). The average GC content was 45.36% (Table 1). The filtered clean reads were mapped to the litchi reference genome (unpublished) and there were at least 74.7% of total clean reads mapped (Table 1). Of these, about average 28 million were uniquely and average 7 million were multiple mapped (Table 1).

Global analysis of gene expression profiles of 'Feizixiao' litchi pericarps in response to exogenous ABA and CPPU

Many differentially expressed transcripts [$|\log_2$ fold change$| \geq 1$ and P value ≤ 0.005] at 0, 10, and 20 days after treatment among the three groups of fruit pericarps were found (Fig. 2). A total of 2263 DEGs were identified in control during litchi pericarp pigmentation (Fig. 2a). Besides there were 1986 and 2862 DEGs at the three stages after ABA (Fig. 2b) and CPPU (Fig. 2c) treatment, respectively.

To identify DEGs involved in ABA-/CPPU-regulated anthocyanin biosynthesis, DEGs of 10 and 20 days after ABA/CPPU treatment were selectively compared to those of the control, respectively (Supplemental Fig. S1). When compared to the control, 579 and 827 DEGs were significantly differentially transcribed in ABA- and CPPU-treated pericarp, respectively. Expression patterns analysis indicated that the numbers of up-regulated transcripts were larger than

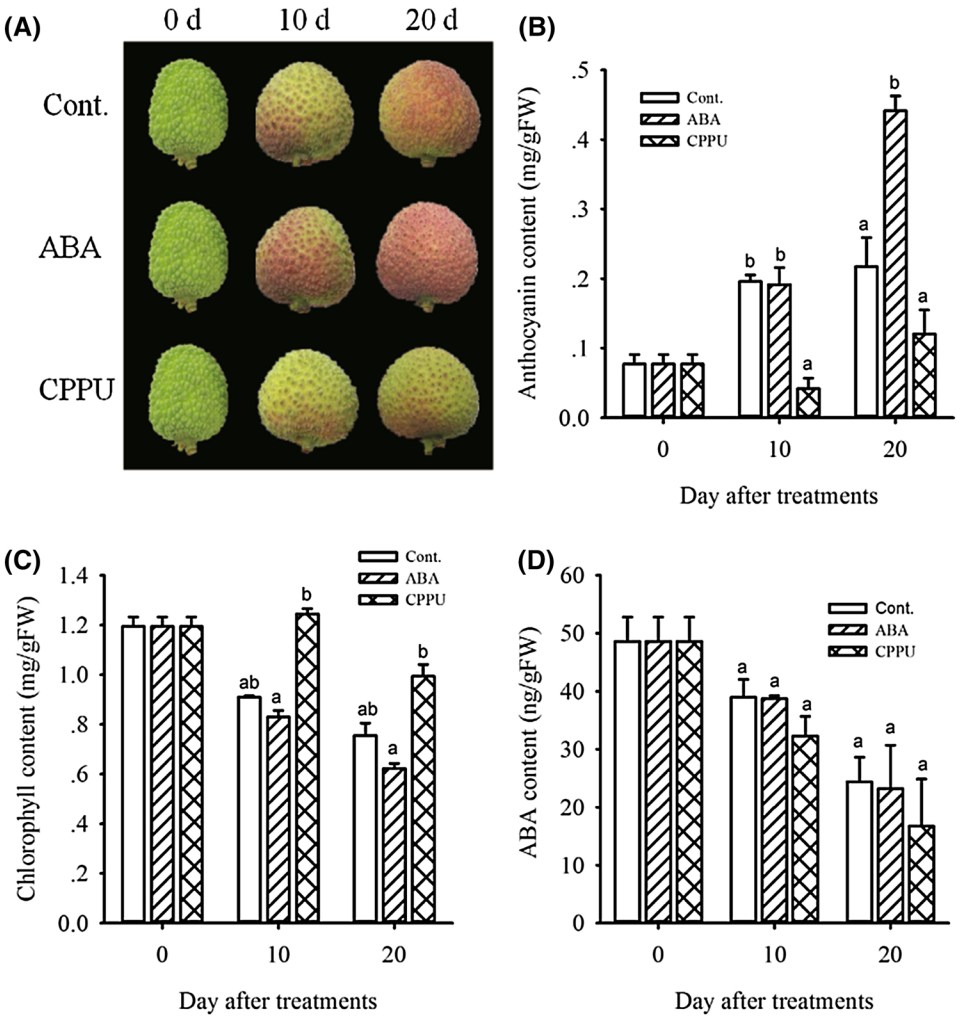

Fig. 1 The effects of exogenous ABA and CPPU on pericarp color (**a**), anthocyanin contents (**b**), chlorophyll contents (**c**), and endogenous ABA levels (**d**), in litchi cv. Feizixiao. PGR treatments include 25 mg/L ABA, 4 mg/L CPPU, and tap water used as control. Data were obtained after 0, 10, and 20 days of treatment. Error bars represent the SE of three replicates. Different letters indicate significant differences between treatments according to Duncan's multiple range test ($P < 0.05$, $n = 3$)

Table 1 RNA-seq reads in seven RNA-seq libraries

Summary	Cont_0d	Cont_10d	Cont_20d	ABA_10d	ABA_20d	CPPU_10d	CPPU_20d
Raw reads	51,633,460	53,875,410	60,107,870	50,986,200	54,646,196	46,673,842	50,293,142
Total number of clean reads (% of raw reads)	47,692,286 (92.37)	50,199,598 (93.18)	55,371,028 (92.12)	47,120,500 (92.42)	50,749,538 (92.87)	44,194,082 (94.69)	47,962,430 (95.37)
GC content (%)	45.00	45.76	45.80	45.71	45.79	44.84	44.63
Total number of mapped reads (% of clean reads)	35,772,746 (75.01)	37,503,329 (74.71)	41,877,713 (75.63)	34,813,835 (73.88)	38,455,578 (75.78)	33,014,533 (74.7)	36,558,560 (76.22)
Total number of uniquely mapped reads (% of clean reads)	28,041,034 (58.80)	29,040,839 (57.85)	32,473,659 (58.65)	27,222,561 (57.77)	29,588,006 (58.30)	26,093,184 (59.04)	28,830,892 (60.11)
Total number of multiple mapped reads (% of clean reads)	7,731,712 (16.21)	8,462,490 (16.86)	9,404,054 (16.98)	7,591,274 (16.11)	8,867,572 (17.47)	6,921,349 (15.66)	7,727,668 (16.11)

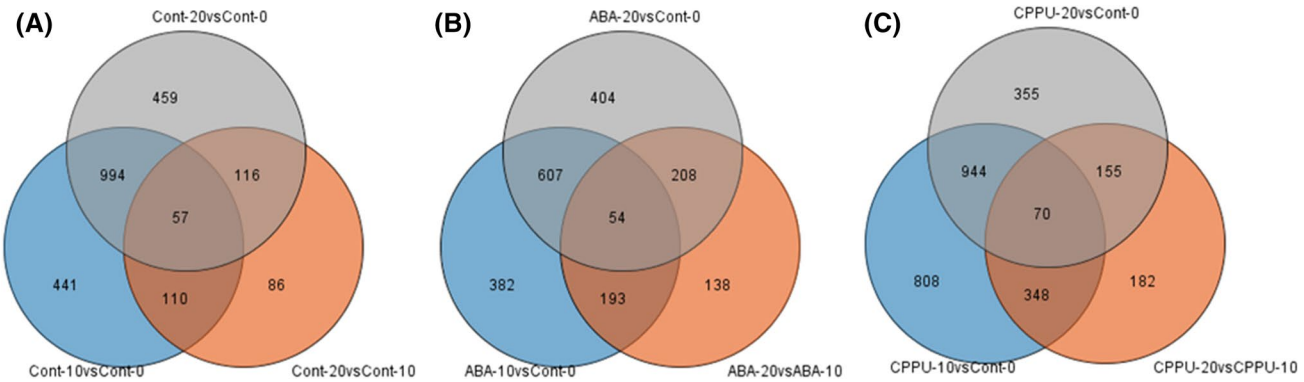

Fig. 2 Venn diagram showing the number of the differentially expressed genes among the control, ABA, and CPPU application

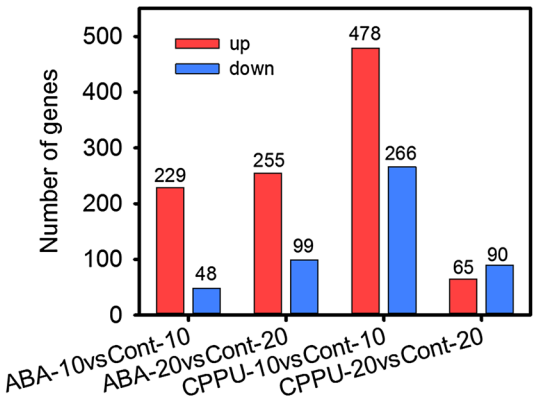

Fig. 3 The numbers of up- or down-regulated DEGs in ABA and CPPU treated fruit pericarps compared with control

those of the down-regulated ones at both 10 and 20 days after ABA application (Fig. 3). The number of up-regulated transcripts at 20 days after CPPU treatment was much lower when compared with that in response to ABA application (Fig. 3).

Expression analysis of 'Feizixiao' litchi pericarps in response to exogenous ABA

The genes with significantly different expression patterns were subjected to GO analysis (Supplemental Fig. S2). An enrichment for GO terms related to cellular process, metabolic process, cell, cell part, catalytic activity, and transporter activity was found in response to exogenous ABA treatment. The DEGs were also subjected to KEGG pathway enrichment analysis. 351 of 579 DEGs were assigned to 101 pathways, with the top five pathway groups were plant–pathogen interactions (49 DEGs), plant hormone signal transduction (32 DEGs), flavonoid biosynthesis (27 DEGs), protein processing in endoplasmic reticulum (27 DEGs), carbon metabolism (20 DEGs), starch and sucrose metabolism (20 DEGs) (Supplemental Table S2).

Among the DEGs in response to exogenous ABA, most of the DEGs related to anthocyanin biosynthesis were up-regulated, such as *PAL, C4H, CHS, CHI, DFR, LDOX*, and *GTs* (Table 2). Moreover, flavonol and proanthocyanidin synthesis genes, such as *FLS* and *LAR*, and lignin synthesis genes, such as *C3'H, COMT, F5H, CCR, CAD*, were also up-regulated by ABA treatment (Table 2). Another class of significantly differentially expressed unigenes upon ABA treatment was involved in plant hormone metabolism and signaling (Table 2). Two *NCEDs* unigenes (Litchi_GLEAN_10061007 and Litchi_GLEAN_10027836) were significantly up-regulated. *NCEDs* encode the key enzyme in ABA biosynthesis pathway (Jia et al. 2011). However, the genes involved in ABA signaling had no clear change rule after exogenous ABA treatment. Exogenous ABA treatment also affected the expression of genes related to auxin signaling pathways (Table 2). For example, two unigenes (Litchi_GLEAN_10043879 and Litchi_GLEAN_10023217) belonging to the Aux/IAAs family found up-regulated, especially at the late stage of litchi pericarp development after ABA treatment. In addition, the unigenes Litchi_GLEAN_10004071 and Litchi_GLEAN_10055239 encoding the DELLA proteins which are negative regulators of GA signaling (Sun and Gubler 2004) were up-regulated by ABA treatment.

Expression analysis of 'Feizixiao' litchi pericarp in response to exogenous CPPU

The DEGs between CPPU and control groups were subjected to GO analysis (Supplemental Fig. S3). There was no obviously difference compared with exogenous ABA treatment. The DEGs were also subjected to KEGG pathway enrichment analysis. 493 of 897 DEGs were assigned to 117 pathways, with the top six pathway groups were carbon metabolism (54 DEGs), plant–pathogen interactions (49 DEGs), photosynthesis (42 DEGs), biosynthesis of amino

Table 2 Differently expressed genes potentially related to flavonoid biosynthesis, anthocyanin biosynthesis, and plant hormone signaling transduction, in response to exogenous ABA (|log2 fold change| ≥ 1 and P value ≤ 0.005)

Gene ID	ABA-10 vs Cont-10		ABA-20 vs Cont-20		Putative functional identification
	log2 (fold change)	*P* value	log2 (fold change)	*P* value	
Litchi_GLEAN_10007133	–	–	1.781	0.000	Phenylalanine ammonia-lyase (PAL)
Litchi_GLEAN_10032621	–	–	1.620	0.000	Phenylalanine ammonia-lyase (PAL)
Litchi_GLEAN_10058183	–	–	1.033	0.000	Trans-cinnamate 4-monooxygenase (C4H)
Litchi_GLEAN_10014497	1.717	0.000	3.423	0.000	Chalcone synthase (CHS)
Litchi_GLEAN_10039644	–	–	1.829	0.000	Chalcone synthase (CHS)
Litchi_GLEAN_10039645	–	–	1.954	0.000	Chalcone synthase (CHS)
Litchi_GLEAN_10020220	–	–	2.093	0.000	Chalcone synthase (CHS)
Litchi_GLEAN_10014033	–	–	2.398	0.000	Chalcone isomerase (CHI)
Litchi_GLEAN_10052224	–	–	1.909	0.000	Chalcone isomerase (CHI)
Litchi_GLEAN_10042770	–	–	1.416	0.000	Chalcone isomerase (CHI)
Litchi_GLEAN_10064182	–	–	1.602	0.000	Chalcone isomerase (CHI)
Litchi_GLEAN_10058324	–	–	1.744	0.000	Flavanone 3-hydroxylase (F3H)
Litchi_GLEAN_10055014	–	–	1.353	0.000	Flavanone 3-hydroxylase (F3H)
Litchi_GLEAN_10003542	–	–	1.400	0.000	Flavonoid 3′-monooxygenase
Litchi_GLEAN_10004559	–	–	1.555	0.000	Flavonoid 3′-monooxygenase
Litchi_GLEAN_10032519	4.911	0.000	–	–	Flavonoid 3′-monooxygenase
Litchi_GLEAN_10024039	–	–	2.260	0.000	dihydroflavonol 4-reductase (DFR)
Litchi_GLEAN_10020768	–	–	1.684	0.000	dihydroflavonol 4-reductase (DFR)
Litchi_GLEAN_10003990	–	–	2.718	0.000	Leucoanthocyanidin dioxygenase (LDOX)
Litchi_GLEAN_10006612	–	–	3.031	0.000	Leucoanthocyanidin dioxygenase (LDOX)
Litchi_GLEAN_10009630	–	–	1.825	0.000	Flavonol 3-*O*-glucosyltransferase
Litchi_GLEAN_10002144	–	–	2.001	0.000	Anthocyanidin 3-*O*-glucosyltransferase
Litchi_GLEAN_10019268	1.747	0.000	1.390	0.000	Cyanidin-3-*O*-glucoside 2″-*O*-glucuronosyltransferase (UGAT)
Litchi_GLEAN_10047196	5.018	0.000	–	–	Cyanidin-3-*O*-glucoside 2″-*O*-glucuronosyltransferase (UGAT)
Litchi_GLEAN_10048303	1.327	0.001	2.613	0.000	Flavonol synthase (FLS)
Litchi_GLEAN_10062493	2.029	0.001	4.706	0.000	Flavonol synthase (FLS)
Litchi_GLEAN_10059439	1.553	0.000	–	–	Coumaroylquinate3′-monooxygenase
Litchi_GLEAN_10025449	–	–	1.064	0.000	Leucoanthocyanidin reductase (LAR)
Litchi_GLEAN_10018694	–	–	2.762	0.000	Leucoanthocyanidin reductase (LAR)
Litchi_GLEAN_10018690	–	–	1.285	0.000	Leucoanthocyanidin reductase (LAR)
Litchi_GLEAN_10061007	2.690	0.000	–	–	9-cis-epoxycarotenoid dioxygenase (NCED)
Litchi_GLEAN_10027836	2.424	0.000	1.908	0.003	9-cis-epoxycarotenoid dioxygenase (NCED)
Litchi_GLEAN_10015505	− 1.581	0.000	–	–	Abscisic acid receptor PYR/PYL family (PYL)
Litchi_GLEAN_10021571	− 1.462	0.000	–	–	Abscisic acid receptor PYR/PYL family (PYL)
Litchi_GLEAN_10062299	1.176	0.000	–	–	Protein phosphatase 2C (PP2C)
Litchi_GLEAN_10018088	1.386	0.000	–	–	Protein phosphatase 2C (PP2C)
Litchi_GLEAN_10043879	–	–	3.307	0.000	Auxin-responsive protein (IAA)
Litchi_GLEAN_10023217	1.204	0.000	1.301	0.000	Auxin-responsive protein (IAA)
Litchi_GLEAN_10009788	1.856	0.000	2.378	0.000	Auxin responsive GH3
Litchi_GLEAN_10004071	–	–	1.532	0.000	DELLA protein
Litchi_GLEAN_10055239	1.280	0.000	1.555	0.000	DELLA protein
Litchi_GLEAN_10003352	− 1.051	0.000	–	–	Serine/threonine-protein kinase (CTR1)
Litchi_GLEAN_10036596	− 1.168	0.000	–	–	EIN3-binding F-box protein (EBF1/2)
Litchi_GLEAN_10015119	1.986	0.001	2.898	0.000	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10056928	1.072	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10024159	–	–	2.910	0.000	Ethylene-responsive transcription factor 1 (ERF1)

Table 2 (continued)

Gene ID	ABA-10 vs Cont-10		ABA-20 vs Cont-20		Putative functional identification
	log2 (fold change)	*P* value	log2 (fold change)	*P* value	
Litchi_GLEAN_10012957	–	–	− 1.029	0.000	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10013413	1.910	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10023937	1.563	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10012956	− 1.242	0.000	− 1.039	0.000	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10029442	− 1.157	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10050388	− 1.174	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10022435	2.704	0.000	1.585	0.000	Ethylene-responsive transcription factor 2 (ERF2)
Litchi_GLEAN_10037034	2.591	0.000	1.834	0.000	Ethylene-responsive transcription factor 2 (ERF2)
Litchi_GLEAN_10034558	1.329	0.000	–	–	Ethylene-responsive transcription factor 2 (ERF2)

– indicate no significant differences between treatments and control

acids (38 DEGs), phenylpropanoid biosynthesis (33 DEGs), plant hormone signal transduction (32 DEGs) (Supplemental Table S3).

Different with ABA treatment, exogenous CPPU application up-regulated many genes involved in carbon metabolism, amino acids biosynthesis, and photosynthesis, especially at 10 days after CPPU treatment (Supplemental Table S4). For example, the unigene Litchi_GLEAN_10019646 was significantly up-regulated after 10 days of exogenous CPPU application by 6.1-fold, which encodes a light-harvesting complex II chlorophyll a/b binding protein 1 (Supplemental Table S4). Another class of significantly differentially expressed unigenes upon exogenous CPPU treatment was involved in chlorophyll biosynthesis metabolism (Table 3). After treated by CPPU, most of the DEGs involved in flavonoid and anthocyanin biosynthesis were down-regulated, such as *PAL, C4H, CHS* and *LDOX*. Moreover, flavonol and proanthocyanidin synthesis genes, such as *FLS* and *LAR* were also down-regulated (Table 3). There were 29 DEGs mapped to plant hormone signal transduction pathway (Table 3). Among them, two ABA receptors PYR/PYL in ABA signaling were both down-regulated by CPPU. In addition, most of the gene related to auxin, GA, and ethylene signaling were also down-regulated.

Expression analysis between ABA- and CPPU-treated 'Feizixiao' litchi pericarp

There were 199 DEGs response to both exogenous ABA and CPPU (data no shown). Most of these unigenes in response to ABA treatment displayed changes similar to those responding to the treatment with CPPU. It is noteworthy that ten unigenes showed an opposite pattern in the transcript levels (Supplemental Fig. S4). Among them, the unigene encoding (Litchi_GLEAN_10051861) GST4 protein was reported to be associated with vacuolar transfer of anthocyanins in litchi (Hu et al. 2016).

One of the significant differences between ABA treatment and CPPU treatment was about chlorophyll metabolism. Twenty-four candidate genes related to chlorophyll biosynthesis and degradation were identified and their expression patterns in response to exogenous ABA and CPPU were showed in Fig. 4. Overall, ABA treatment had no significant effect on the gene expression involved in chlorophyll biosynthesis and degradation. Compared with control, CPPU treatment significantly increased the most chlorophyll synthesis genes (*HEMA, GSA, HEMB, HEMC, HEME, HEMG, CHLD, CHLI, CHLM, CRD1, POR*, and *CHLG*), and down-regulated the TF SGR (Litchi_GLEAN_10003651).

Notably, the expression of some unigenes involved in flavonoid biosynthesis pathway was differently altered by the treatment of exogenous ABA and CPPU (Fig. 5). For the structural genes in litchi flavonoid biosynthesis, more than one gene was identified and different gene family members showed different expression patterns. According to our previous study reported by Lai et al. (2015), the members related to flavonoid biosynthesis was chosen for further analysis. As shown in Fig. 5, ABA treatment up-regulated the structural genes in litchi flavonoid biosynthesis, while CPPU inhibited their expression, especially *PAL, CHS* and *F3′H*.

qRT-PCR validation of RNA-seq-based gene expression

qRT-PCR was performed on 9 genes significantly differentially expressed as revealed above. They were HEMA (Litchi_GLEAN_10019172), CBR (Litchi_GLEAN_10058844), DFR (Litchi_GLEAN_10024039), CHI (Litchi_GLEAN_10064182), UFGT (Litchi_GLEAN_10002144), chloaophyllase (Litchi_GLEAN_10023168, Litchi_GLEAN_10031899), RCCR (Litchi_GLEAN_10033424), and SGR (Litchi_GLEAN_10003651). Overall, the RT-PCR results were consistent with the RNA-seq data (Fig. 6). Linear regression

Table 3 Differently expressed genes potentially related to chlorophyll metabolism, flavonoid biosynthesis, anthocyanin biosynthesis, and plant hormone signaling transduction, in response to exogenous CPPU (|log2 fold change| ≥ 1 and P value ≤ 0.005)

Gene ID	CPPU-10 vs Cont-10		CPPU-20 vs Cont-20		Putative functional identification
	log2 (fold change)	*P* value	log2 (fold change)	*P* value	
Litchi_GLEAN_10029712	−2.021	0.000	–	–	Cytochrome c oxidase assembly protein subunit 15 (COX15)
Litchi_GLEAN_10031899	1.417	0.000	–	–	Chlorophyllase
Litchi_GLEAN_10059162	1.662	0.000	–	–	Chlorophyll(ide) b reductase (NOL, NYC1)
Litchi_GLEAN_10032621	−1.136	0.000	–	–	Phenylalanine ammonia-lyase (PAL)
Litchi_GLEAN_10039644	−1.203	0.000	–	–	Chalcone synthase (CHS)
Litchi_GLEAN_10007251	2.073	0.000	−2.005	0.000	Leucoanthocyanidin dioxygenase (LDOX)
Litchi_GLEAN_10009089	−2.286	0.000	–	–	Leucoanthocyanidin dioxygenase (LDOX)
Litchi_GLEAN_10035828	−3.582	0.000	−1.873	0.000	Leucoanthocyanidin dioxygenase (LDOX)
Litchi_GLEAN_10051436	−3.260	0.000	–	–	Leucoanthocyanidin dioxygenase (LDOX)
Litchi_GLEAN_10012382	–	–	−3.348	0.000	Flavonol synthase (FLS)
Litchi_GLEAN_10017397	–	–	−2.943	0.000	Flavonol synthase (FLS)
Litchi_GLEAN_10034912	4.756	0.000	–	–	Flavonol synthase (FLS)
Litchi_GLEAN_10053074	1.401	0.000	–	–	Flavonol synthase (FLS)
Litchi_GLEAN_10001826	3.086	0.000	–	–	Flavonoid 3′-monooxygenase (TT7)
Litchi_GLEAN_10004559	−1.329	0.000	–	–	Flavonoid 3′-monooxygenase (TT7)
Litchi_GLEAN_10035742	−2.317	0.000	–	–	Shikimate *O*-hydroxycinnamoyltransferase (HCT)
Litchi_GLEAN_10048489	−1.998	0.000	–	–	Shikimate *O*-hydroxycinnamoyltransferase (HCT)
Litchi_GLEAN_10052163	−1.266	0.000	–	–	Shikimate *O*-hydroxycinnamoyltransferase (HCT)
Litchi_GLEAN_10048017	−1.298	0.000	–	–	Leucoanthocyanidin reductase (LAR)
Litchi_GLEAN_10043902	1.008	0.000	–	–	anthocyanidin 3-*O*-glucoside 5-*O*-glucosyltransferase (UGT75C1)
Litchi_GLEAN_10042641	1.104	0.000	–	–	anthocyanidin 5,3-*O*-glucosyltransferase (GT1)
Litchi_GLEAN_10047196	2.706	0.000	–	–	Anthocyanidin 3-*O*-glucoside 2‴-*O*-xylosyltransferase (UGT79B1)
Litchi_GLEAN_10012621	−1.608	0.000	–	–	Auxin responsive GH3 gene family
Litchi_GLEAN_10051017	3.466	0.000	–	–	Arabidopsis histidine kinase (cytokinin receptor) (AHK2/3/4)
Litchi_GLEAN_10029435	−1.081	0.000	–	–	Gibberellin receptor (GID1)
Litchi_GLEAN_10036019	−1.531	0.000	–	–	Gibberellin receptor (GID1)
Litchi_GLEAN_10062537	2.921	0.000	–	–	Gibberellin receptor (GID1)
Litchi_GLEAN_10064575	−2.073	0.000	–	–	Gibberellin receptor (GID1)
Litchi_GLEAN_10015505	−1.938	0.000	–	–	Abscisic acid receptor PYR/PYL family (PYR/PYL)
Litchi_GLEAN_10021571	−2.020	0.000	–	–	Abscisic acid receptor PYR/PYL family (PYR/PYL)
Litchi_GLEAN_10003352	−1.204	0.000	–	–	Serine/threonine-protein kinase (CTR1)
Litchi_GLEAN_10061797	1.114	0.000	–	–	Serine/threonine-protein kinase (CTR1)
Litchi_GLEAN_10036596	−1.995	0.000	–	–	EIN3-binding F-box protein
Litchi_GLEAN_10049734	−1.463	0.000	–	–	EIN3-binding F-box protein
Litchi_GLEAN_10008035	−1.874	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10012957	–	–	−1.725	0.000	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10029441	–	–	−1.541	0.000	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10043480	1.552	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10046547	−1.479	0.000	−1.376	0.001	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10050388	−1.749	0.000	–	–	Ethylene-responsive transcription factor 1 (ERF1)
Litchi_GLEAN_10011675	1.017	0.000	–	–	Ethylene-responsive transcription factor 2 (ERF2)
Litchi_GLEAN_10023938	−1.804	0.000	–	–	Ethylene-responsive transcription factor 2 (ERF2)
Litchi_GLEAN_10034458	−1.994	0.000	–	–	Ethylene-responsive transcription factor 2 (ERF2)
Litchi_GLEAN_10053348	−1.128	0.000	–	–	Protein brassinosteroid insensitive 1 (BRI1)

Table 3 (continued)

Gene ID	CPPU-10 vs Cont-10		CPPU-20 vs Cont-20		Putative functional identification
	log2 (fold change)	*P* value	log2 (fold change)	*P* value	
Litchi_GLEAN_10057780	− 1.023	0.000	–	–	Protein brassinosteroid insensitive 1 (BRI1)
Litchi_GLEAN_10030835	1.148	0.000	–	–	Protein brassinosteroid insensitive 1 (BRI1)
Litchi_GLEAN_10047205	1.595	0.000	–	–	Protein brassinosteroid insensitive 1 (BRI1)
Litchi_GLEAN_10017208	− 1.023	0.000	–	–	Jasmonate ZIM domain-containing protein (JAZ)
Litchi_GLEAN_10019091	− 1.619	0.000	–	–	Jasmonate ZIM domain-containing protein (JAZ)
Litchi_GLEAN_10060070	− 1.203	0.000	–	–	Jasmonate ZIM domain-containing protein (JAZ)
Litchi_GLEAN_10025051	− 1.855	0.000	–	–	Transcription factor MYC2

– indicate no significant differences between treatments and control

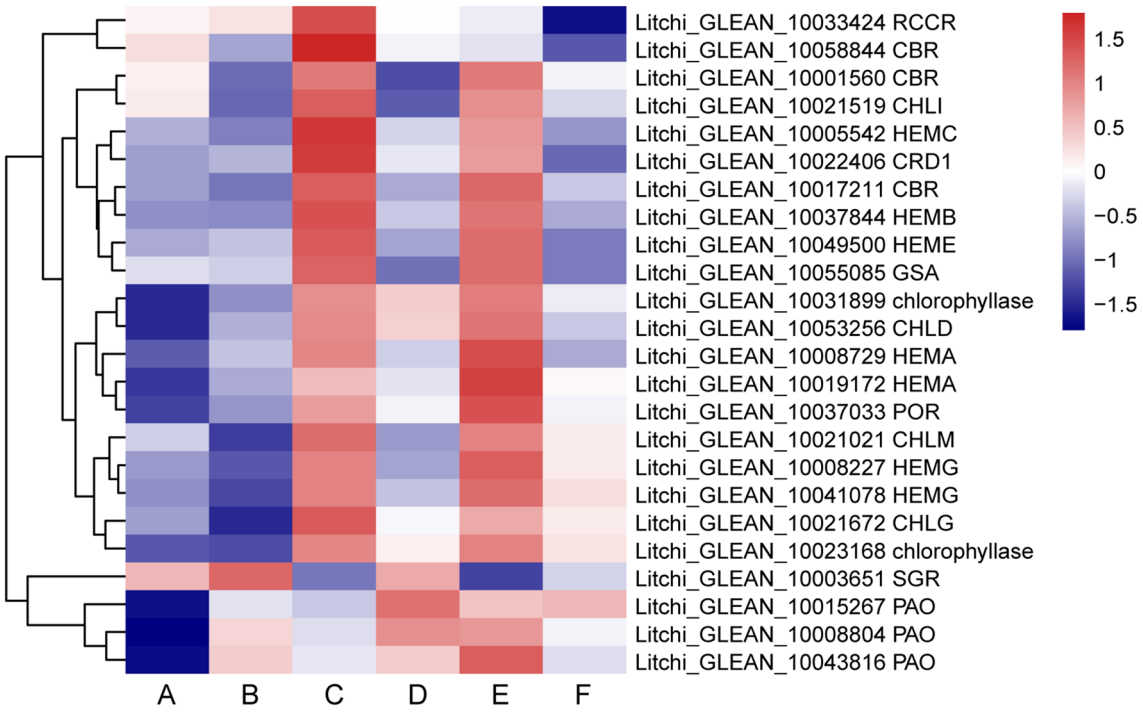

Fig. 4 Heat map diagram of relative gene expression levels of DEGs related to chlorophyll biosynthesis and degradation in litchi pericarp after exogenous ABA and CPPU treatment. ABA-10 and Cont-10 (*A*), ABA-20 and Cont-20 (*B*), CPPU-10 and Cont-10 (*C*), CPPU-20 and Cont-20 (*D*), CPPU-10 and ABA-10 (*E*), CPPU-20 and ABA-20 (*F*). Red color indicates a relative increase in expression, and blue color represents a relative decrease in expression. (Color figure online)

analysis indicated that there was a high correlation between the RT-PCR data and the RNA-seq data (Supplemental Fig. S5).

Discussion

Pericarp color of litchi is an important aspect of fruit quality, which might affect consumers' buying decision. The concentration and composition of anthocyanins determining color in litchi and other fruits is influenced by several internal and external factors, such as genotype, temperature, light, and plant hormones (Wei et al. 2011; Singh et al. 2014; Li et al. 2016a, b; Zhang et al. 2016). Natural plant hormones (auxins, CTK, GAs, ABA, ethylene) and synthetic PGRs with similar biological effects could affect anthocyanin biosynthesis both internally and externally. Our previous study showed that exogenous ABA application enhanced anthocyanin accumulation in litchi pericarp, while exogenous CPPU treatment inhibited this process. Our studies also revealed that exogenous ABA and CPPU application could up- and down-regulate the anthocyanin biosynthesis structural gene

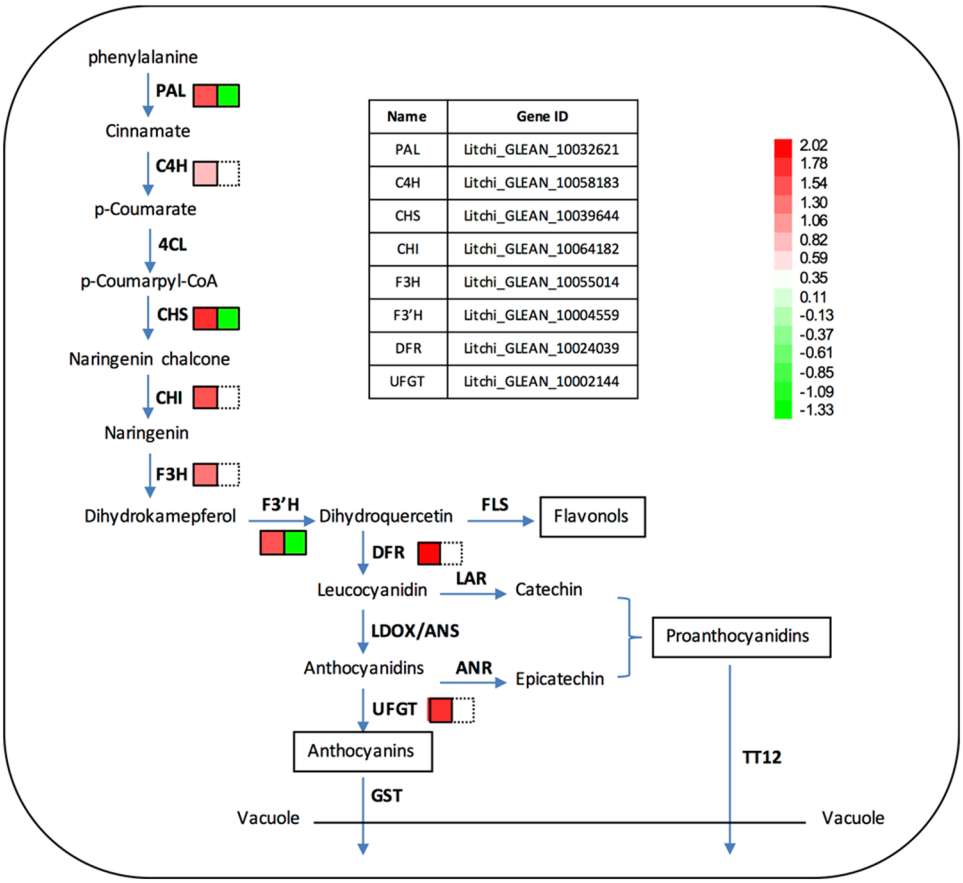

Fig. 5 Overview of the DEGs involved in flavonoid biosynthesis pathway in litchi pericarp after exogenous ABA and CPPU treatment. For each gene, the left square represents ABA treatment and the right square represents CPPU treatment. Red color indicates a relative increase in expression, green color represents a relative decrease in expression, and the dotted square represents no significant differences. *PAL* phenylalanine ammonia lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumaroyl-coA synthase, *CHS* chalcone synthase, *CHI* chalcone-flavanone isomerase, *F3H* flavanone-3-hydroxylase, *F3'H* flavonoid-3′-hydroxylase, *FLS* flavonol synthase, *DFR* dihydroflavonol-4-reductase, *LDOX* leucoanthocyanidin dioxygenase, *LAR* leucoanthocyanidin reductase, *ANR* anthocyanidin reductase, *UFGT* UDP-glucose:flavonoid-3-*O*-glucosyltransferase, *GST* glutathione-S-transferase, *TT12* transparent testa 12. (Color figure online)

(*LcUFGT*), transportation related gene (*LcGST4*), as well as regulatory gene (*LcMYB1*) (Wei et al. 2011; Lai et al. 2014; Hu et al. 2016). However, the earlier works just focused on the expression of single or few gene of litchi pericarp in response to exogenous ABA and CPPU. In the present study, transcriptome analysis was adopted to understand the global molecular events of litchi pericarp after exogenous ABA and CPPU treatment. This study would provide useful information for revealing the mechanism of anthocyanin biosynthesis regulated by plant hormone in litchi and other fruits.

Litchi is a non-climacteric fruit, whose ripening is thought to be ethylene-independent (Jiang et al. 1986). Ethrel, an ethylene releasing compound, has showed no effect on anthocyanin levels in pericarp, but it accelerated chlorophyll degradation (Wang et al. 2007; Singh et al. 2014). Compared with ethylene, ABA has a more dominant role during fruit ripening in non-climacteric fruits (McAtee et al. 2013; Leng et al. 2014; Kumar et al. 2014). However, supporting molecular evidences that link ABA signaling with anthocyanin biosynthesis have been lacking to date in litchi. In this study, the transcriptome profiling after ABA treatment indicated that ABA promoted the expression level of genes involved in flavonoid biosynthesis and sugar metabolism (Table 2, Supplemental Table S2). Indeed, our previous study indicated that the endogenous ABA concentrations in litchi increased in parallel with sugar accumulation (Wang et al. 2007). All these results indicate that the increase of endogenous ABA content during fruit maturation is the key signal triggering anthocyanin biosynthesis in litchi and exogenous application of ABA accelerates this process.

Several genes related to ABA metabolism and signaling were regulated by ABA treatment, including *NCEDs, PYR/PYLs*, and *PP2Cs* (Table 2). It was similar to the results in strawberry. Chen et al. (2016) reported that the exogenous ABA treatment moderately up-regulated genes involved in ABA biosynthesis and signaling in strawberry. In non-climacteric fruit, ABA interacts with other plant hormones to regulate anthocyanin biosynthesis and fruit ripening. For example, IAA has been reported to cross-talk with ABA during ripening in strawberry (Chen et al. 2016). In this study, exogenous ABA treatment significantly up-regulated *GH3* gene related to auxin conjugation and the genes (Aux/IAAs) encoding auxin signal repressors (Table 2), indicating the ABA has cross-talk with IAA in litchi fruit ripening. ABA also affected gene expression in ethylene signaling pathway (Table 2). However, the roles of both ABA and ethylene in the anthocyanin biosynthesis and fruit ripening of litchi are complex (Wang et al. 2007; Singh et al. 2014). Wang et al. (2007) pointed out that litchi fruit maturation may not be

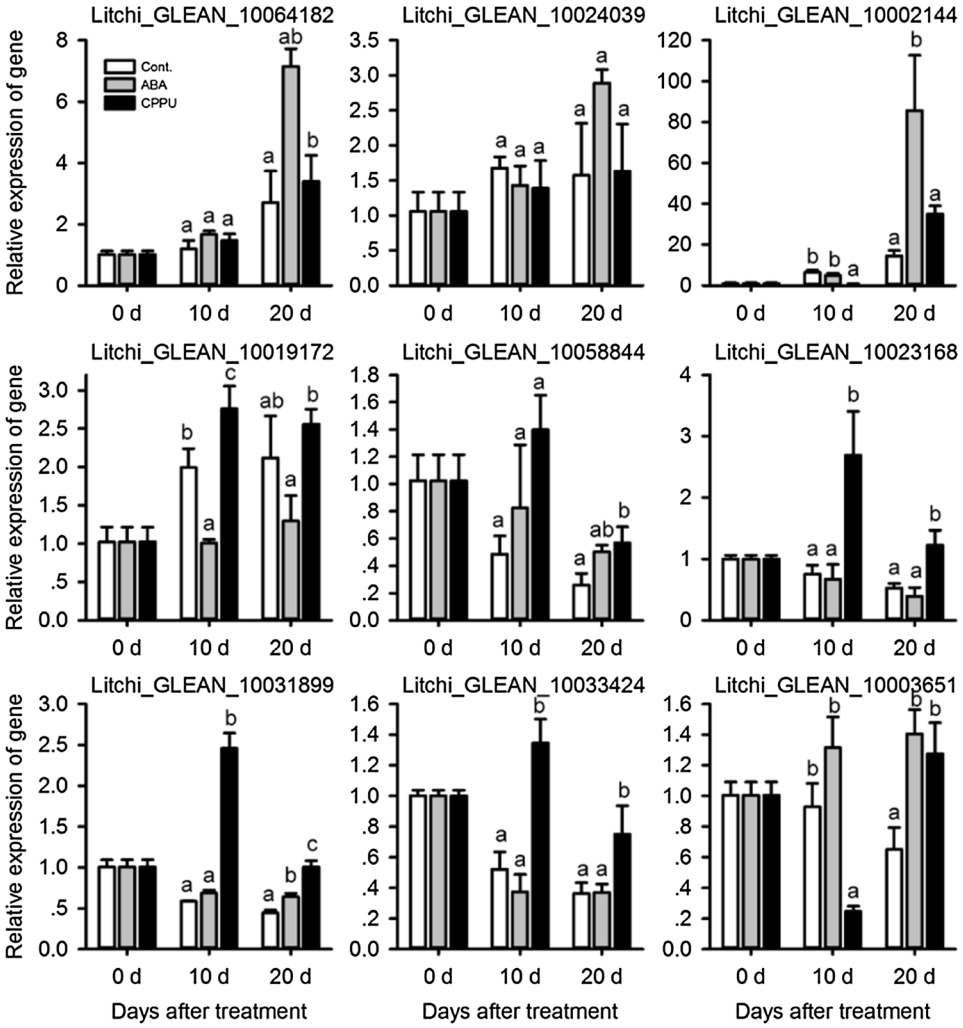

Fig. 6 qRT-PCR analysis of DGEs related to chlorophyll degradation and anthocyanin biosynthesis in litchi pericarps response to exogenous ABA and CPPU treatment. *Lcactin* was used as reference gene to normalize gene expression levels under identical conditions. The vertical bars represent the standard error of triplicate experiments

strictly independent of ethylene, and ethylene also participates in anthocyanin biosynthesis coupled with ABA.

In Arabidopsis, CTK has been reported to promote anthocyanin accumulation in light-grown seedlings with a dose-dependent manner (Deikman and Hammer 1995). Transgenic Arabidopsis overexpressing the *isopentenyltransferase* (*IPT*) gene, which is involved in CTK biosynthesis, showed a deeper red color in the shoot than wild-type plants (Guo et al. 2005), indicating that the endogenous increase in CTK induced more anthocyanins in shoots. However, the effects of CTK on the anthocyanin biosynthesis in fruits might be deferent. Multiple studies have shown CTK plays an important role in the stimulation of cell division during fruit development (as reviewed by McAtee et al. 2013). For fruit maturation, the role of CTK is less well documented, but there were studies found that the free CTK level was decreased before ripening in orange and grape (Minana et al. 1989; Bottcher et al. 2011). It is also noteworthy that the possible interaction between CTK and ABA during fruit ripening. CPPU appeared to stimulate ABA depletion via sustained catabolism in ripening avocado fruit (Cowan et al. 1999). In the present study, the CPPU-treated fruits had lower endogenous ABA level and anthocyanin biosynthesis was markedly suppressed (Fig. 1). CPPU treatment significantly increased the expression of chlorophyll synthesis genes and inhibited the expression of chlorophyll degradation gene *SGR*, which was suggested a key regulator in chlorophyll degradation in the litchi pericarp (Lai et al. 2015) (Table 3). Studies have been clearly demonstrated that exogenous application of CTK could delay leaf senescence by mediating chlorophyll degradation associated genes expression (Gan and Amasino 1995). During litchi fruits ripening, chlorophyll is degraded and then anthocyanin is synthesized. However, CPPU treatment represses chlorophyll degradation, leading to the inhibition of anthocyanin biosynthesis. The interplay of chlorophyll catabolism and anthocyanin biosynthesis during fruit ripening is worth further study.

Conclusion

Exogenous ABA application at color break stage improved the color of litchi cv. Feizixiao, whereas exogenous CPPU treatment inhibited the anthocyanin accumulation. Global molecular events were analyzed in litchi pericarps treated with exogenous ABA and CPPU by RNA-seq. Based on our results, the ABA treatments had a remarkable effect on the expression of genes involved in anthocyanins biosynthesis and sugar metabolism. On the other hand, exogenous CPPU treatment disturbed the chlorophyll catabolism, by increasing the expression of chlorophyll synthesis genes and inhibiting the expression of chlorophyll degradation gene (*SGR*). In addition, ABA and CPPU might interact with other hormone signaling pathways, such as auxin, GA, and ethylene, forming a complex network to regulate anthocyanin biosynthesis.

Acknowledgements The paper was supported by the Pearl River S&T Nova Program of Guangzhou (No. 201610010147), China Litchi and Longan Industry Technology Research System (Project No. CARS-32-05), and YangFan Innovative & Entrepreneurial Research Team Project (No. 2014YT02H013).

Availability of supporting data All of the raw reads are available in the NCBI Sequence Read Archive database (Accession Number PRJNA415698).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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