



Article

Transcriptomic and Metabolomic Analyses Reveal That Proanthocyanidin Treatment Delays Pericarp Browning of Litchi Fruit

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Abstract

Proanthocyanidins (PAs) display antioxidant properties and can partially delay pericarp browning in postharvested litchi (*Litchi chinensis* Sonn.) fruits. In this study, litchi fruit was treated with 3 g/L PA and then stored at 25 °C for 10 days. Differential metabolites and differentially expressed genes were comprehensively analyzed in litchi pericarp across four stages of browning progression. A total of 630 metabolites were identified through untargeted metabolomic analysis, with 237 of them being annotated in the Kyoto encyclopedia of genes and genomes (KEGG) database. The abundance of flavonoids (kaempferol), six types of anthocyanins, cyanidins, (–)-epicatechin, and (+)-catechin in the PA biosynthetic pathway was notably reduced in PA-treated pericarp compared to that of the control fruit. Transcriptomic analysis also revealed that five genes in the phenylpropanoid biosynthesis pathways and seven genes of phenoloxidase were significantly downregulated. In addition, correlation analysis revealed significant positive correlations between the browning index and genes like *LcFLS* (flavonol synthase), *LcANS* (anthocyanidin synthase), *LcUFGT* (UDP-glucose:anthocyanidin 3-O-glucosyltransferase), *LcPPO* (polyphenol oxidase), and *LcLAC7* (laccase), and a significant negative correlation with PAs and (–)-epicatechin. In conclusion, PA treatment can efficiently delay pericarp browning, mainly by reducing brown substrate accumulation and oxidation, and decreasing PA oxidation and polymerization. This study delves into the regulatory mechanisms through which PA delays litchi pericarp browning.

Keywords: litchi fruit; proanthocyanidin; transcriptome; metabolome; browning



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1. Introduction

Litchi (*Litchi chinensis* Sonn.), native to Yunnan, is highly favored among consumers for its appealing taste, attractive appearance, and presence of nutraceutical elements like proanthocyanidins [1–3]. Following harvest, litchi pericarp rapidly browns, undergoing a range of physiological and biochemical alterations. The formation of brown-colored compounds during this phase leads to a decline in quality and a reduction in international trade worth [4,5]. The synthesis of brown products is a result of multiple factors, such as anthocyanidin degradation, brown substrate oxidation, and improved respiration physiology [6,7]. Polyphenol oxidase (PPO), peroxidase (POD), and laccase (LAC) are the primary catalytic enzymes responsible for the oxidative browning of litchi pericarp [5].

Among them, PPO catalyzes the direct oxidation of (–)-epicatechin to form *o*-quinone, thereby promoting the oxidation of anthocyanins and leading to the formation of brownish compounds [8]. POD has the ability to catalyze the degradation of anthocyanins when H₂O₂ and guaiacol are present [9]. LAC facilitates the breakdown of anthocyanins and the formation of flavonoid polymers through (–)-epicatechin [10,11]. Therefore, inhibiting oxidative enzymes and decreasing the formation of browning substrates like anthocyanins and (–)-epicatechin can effectively postpone postharvest browning in litchi fruit, leading to an extension of their shelf life.

Polyphenols, plentiful secondary metabolites, are extensively employed as exogenous antioxidants to mitigate oxidative stress and prolong the senescence of fruits [12,13]. The most consumed polyphenols in the human diet are proanthocyanidins (PAs), which are derived from the condensation of widely distributed flavan-3-ols (e.g., epicatechin) in plants [14,15]. Exogenous PA treatment was found to initiate endogenous PA signaling, enhance total antioxidant capacity, inhibit phenol oxidase activities, reduce respiratory metabolism, and suppress ethylene biosynthesis. Consequently, this treatment effectively retards fruit browning and senescence [7,13,16]. Moreover, Duan et al. [17] documented that phenolics derived from litchi pericarp tissue inhibited the browning of litchi fruit. Huang et al. [18] reported that PA can form complexes with PPO, competitively inhibiting the latter's activity and effectively delaying the postharvest browning of loquat fruit. Wei et al. [11] revealed that during litchi pericarp browning, PAs undergo oxidative polymerization by LAC, resulting in a brown color. These results suggest that the biosynthesis and accumulation of PA play a crucial role in delaying fruit browning.

PA biosynthesis originates from the phenylpropanoid and flavonoid biosynthesis pathway, which has been extensively studied, culminating in the discovery of multiple enzymes and coding genes [19]. Leucoanthocyanidin reductase (LAR), dihydroflavonol-4-reductase (DFR), anthocyanidin reductase (ANR), and anthocyanidin synthase (ANS) have been identified as the catalysts for generating flavan-3-ol monomers, namely (–)-epicatechin and (+)-catechin, that are subsequently polymerized into PA [19]. The crucial genes *LcLAR1/2* and *LcANR1a/2a* were identified as strongly associated with the levels of (+)-catechin, (–)-epicatechin, and PAs in litchi pericarp [20]. Zhou et al. [21] also discovered that the genes phenylalanine ammonia lyase (*PAL1/2*), *DFR*, flavanone 3-hydroxylase (*F3H*), and *LAR*, identified through transcriptome analysis, respond to banana procyanidin treatment and are pivotal in controlling PAs accumulation during senescence. In addition, Qu et al. [22] demonstrated that litchi pericarp browning is associated with a higher rate of anthocyanidin degradation than biosynthesis, as evidenced by transcriptomic and LC-MS analyses. Therefore, it is necessary to explore the pivotal gene and its regulatory mechanism using omics analysis to understand how endogenous PA prevents litchi pericarp browning.

To explore the PA-induced differentially expressed genes (DEGs) and differential metabolites (DMs), metabolomic and transcriptomic analyses were performed in the present study. Then, the mechanism by which PA treatment is associated with endogenous PA accumulation and oxidative browning in litchi pericarp during room temperature storage was investigated. Our results offer an important theoretical foundation for comprehending the molecular mechanism underlying the PA-mediated regulation of litchi pericarp browning, and could potentially enhance litchi storage technology.

2. Results

2.1. Analysis of Fruit Browning and Proanthocyanidins Content

During room temperature storage, an increase in the browning index was observed in the CK and PA-treated groups, as depicted in Figure 1A. However, the increase rate for fruit subjected to PA was markedly lower than that of the CK ($p < 0.05$). From the fourth

day onwards, the browning index showed a highly significant contrast ($p < 0.001$), with a value of 1.18 in the PA-treated fruit on the fourth day and a higher level of 1.72 in the CK group. The inner proanthocyanidins content exhibited an inverse relationship with browning progression; however, PA treatment notably postponed ($p < 0.05$) the decline (Figure 1B).

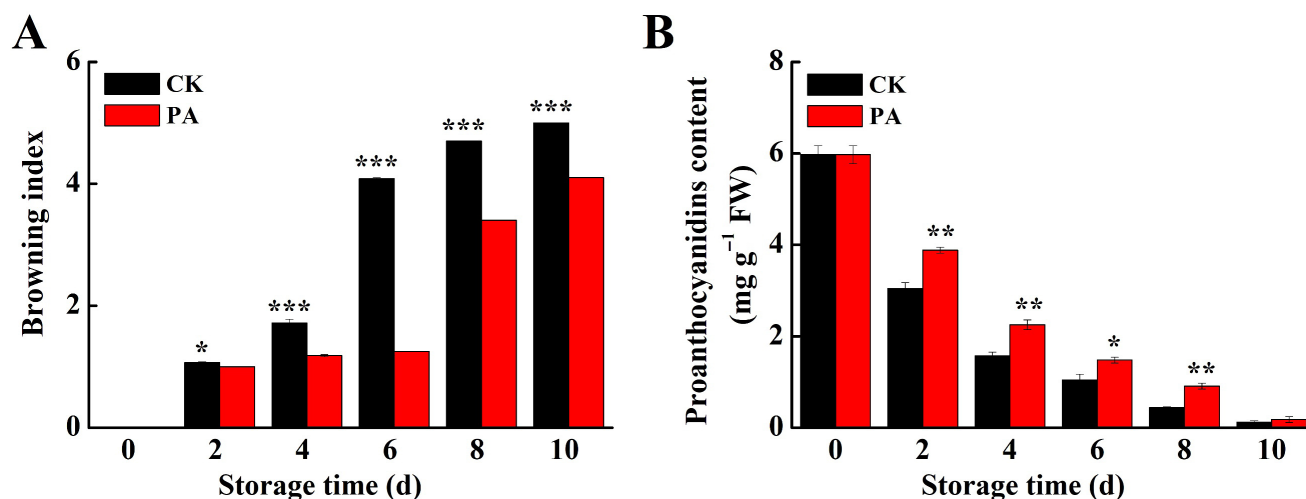


Figure 1. Browning index (A) and proanthocyanidin content (B) in ‘Feizixiao’ litchi fruit stored at 25 °C following PA treatment. The data are displayed as the mean $\pm$ standard error, with significance levels indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) for comparisons within one day.

2.2. DM Analysis of Litchi Fruit During Browning

Metabolites in the litchi pericarp under control and PA treatment were assessed using UPLC-ESI-MS/MS to elucidate the physiological transformations associated with browning. Principal component analysis (PCA) was conducted to facilitate the subsequent metabolite analysis step (Figure 2A). The outcomes validated the distinct separation of PA-treated samples from the CK groups along PC1 (17.52%) (Figure 2A). A total of 630 metabolites (positive-ion mode) were identified by aligning the MS2 spectra with the in-house database, with 237 being annotated using the KEGG compound database (Table S1). The metabolites were categorized into 18 superclasses, with lipids and lipid-like molecules accounting for 22.38%, benzenoids for 13.65%, organic acids and derivatives for 11.59%, phenylpropanoids and polyketides for 10.32%, organoheterocyclic compounds for 9.52%, and organic oxygen compounds for 4.60%, representing the largest group (Figure 2B,C).

In the PA2d_vs_CK2d groups, 161 differential metabolites (DMs) were identified, with 64 showing downregulation and 97 showing upregulation. For the PA4d_vs_CK4d groups, 120 DMs exhibited differences, with 46 downregulated and 74 upregulated. In the PA8d_vs_CK8d groups, 578 DMs displayed differences, with 322 downregulated and 256 upregulated. Similarly, in the PA10d_vs_CK10d groups, 518 DMs showed differences, with 255 downregulated and 263 upregulated (Table S2). This result also demonstrates the quantity of metabolite alterations during the browning process. To investigate the essential metabolic processes associated with delaying browning in PA-treated litchi pericarp, DMs for each comparison group were identified using volcano plots. As shown in Figure 2D, the largest number of down-regulated DMs was observed in the comparisons of PA8d_vs_CK8d and PA10d_vs_CK10d samples. Further, the comparison between PA and CK at all the sampling points revealed a higher number of down-regulated metabolites in the phenylpropanoids and polyketides pathways compared to up-regulated metabolites. This suggests the significant role of PA treatment in phenolic substrate biosynthesis and the inhibition of browning.

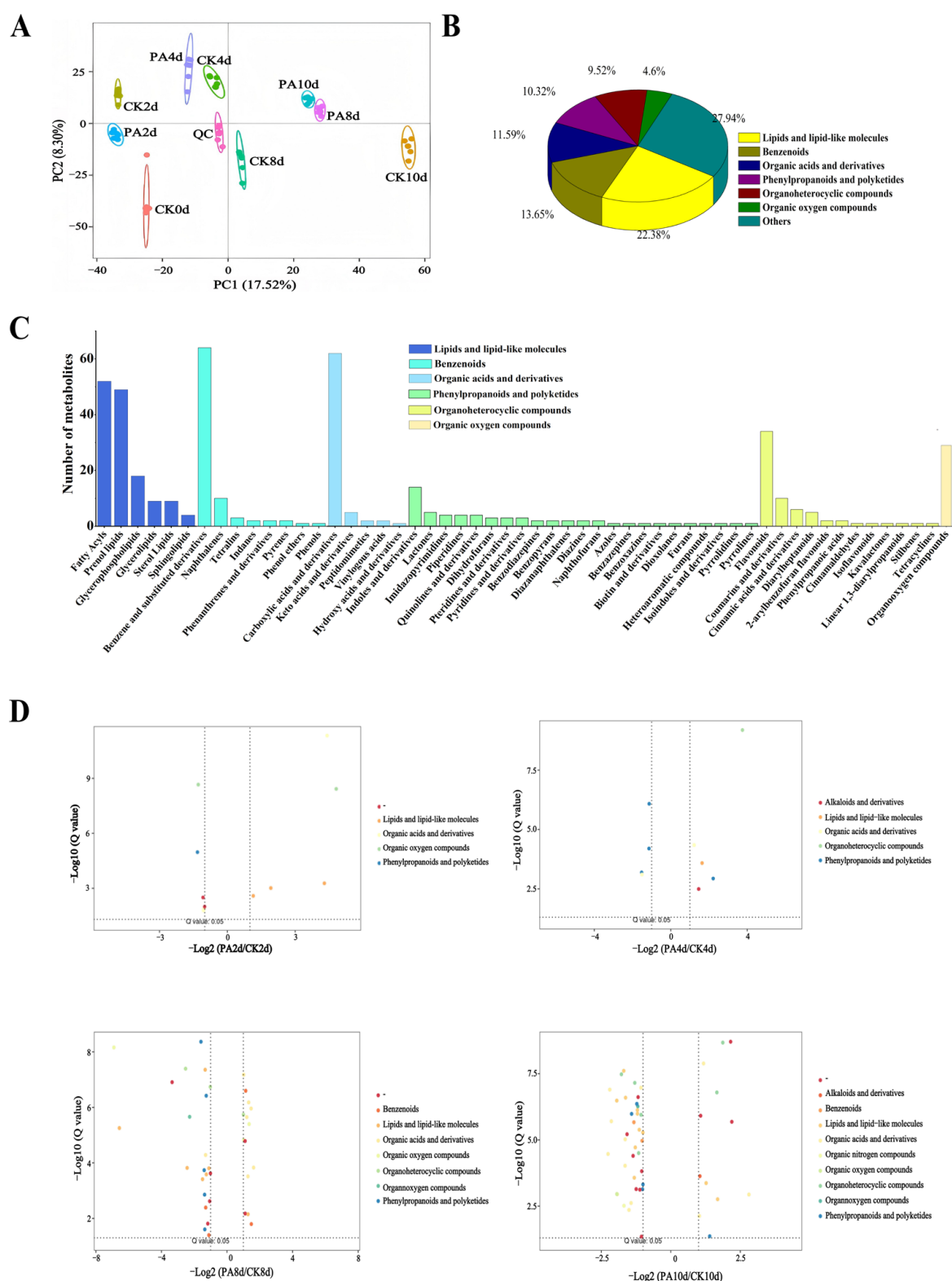


Figure 2. Untargeted metabolomic analysis of ‘Feizixiao’ litchi fruit under PA treatment. (A) PCA. (B) Pie chart of the metabolite proportions in the treatment group versus the control group. (C) Classification bar chart of main metabolites. (D) Volcano plot between the treatment groups (PA) and the control group (CK).

2.3. Transcriptome Analyses of Litchi Fruit During Browning

To identify candidate genes linked to pericarp browning in ‘Feizixiao’ litchi, RNA-seq analysis was carried out on 27 samples taken at 0, 2, 4, 8, and 10 days post CK and PA

treatment, each with three biological replicates. PCA was performed to determine the subsequent phase of transcriptome analyses (Figure 3A), and the results unequivocally distinguished the PA samples from CK based on PCA1 (91.34%). The results indicate a substantial disparity between the PA-treated and CK groups. In Figure 3B, the quantity of differentially expressed genes (DEGs) detected in the CK and PA groups is illustrated. The most notable change was observed in CK2d, with 1358 genes showing upregulation and 1005 genes displaying downregulation in comparison to PA2d (Figure 3B). Throughout the storage period, the quantity of upregulated genes was higher in the CK group than in the PA-treated group, except on day 8 (Table S3). Volcano maps were created to delineate the distinct gene distribution between the CK_vs_PA groups (Figure S1). The blue dots depict genes that are down-regulated ($\text{Log}_2\text{FC} < -1$), red dots indicate genes that are up-regulated ($\text{Log}_2\text{FC} > 1$), and gray dots represent genes with insignificant expression levels. A large number of DEGs was observed throughout the browning process, with the peak number of DEGs occurring at the onset of browning on day 2.

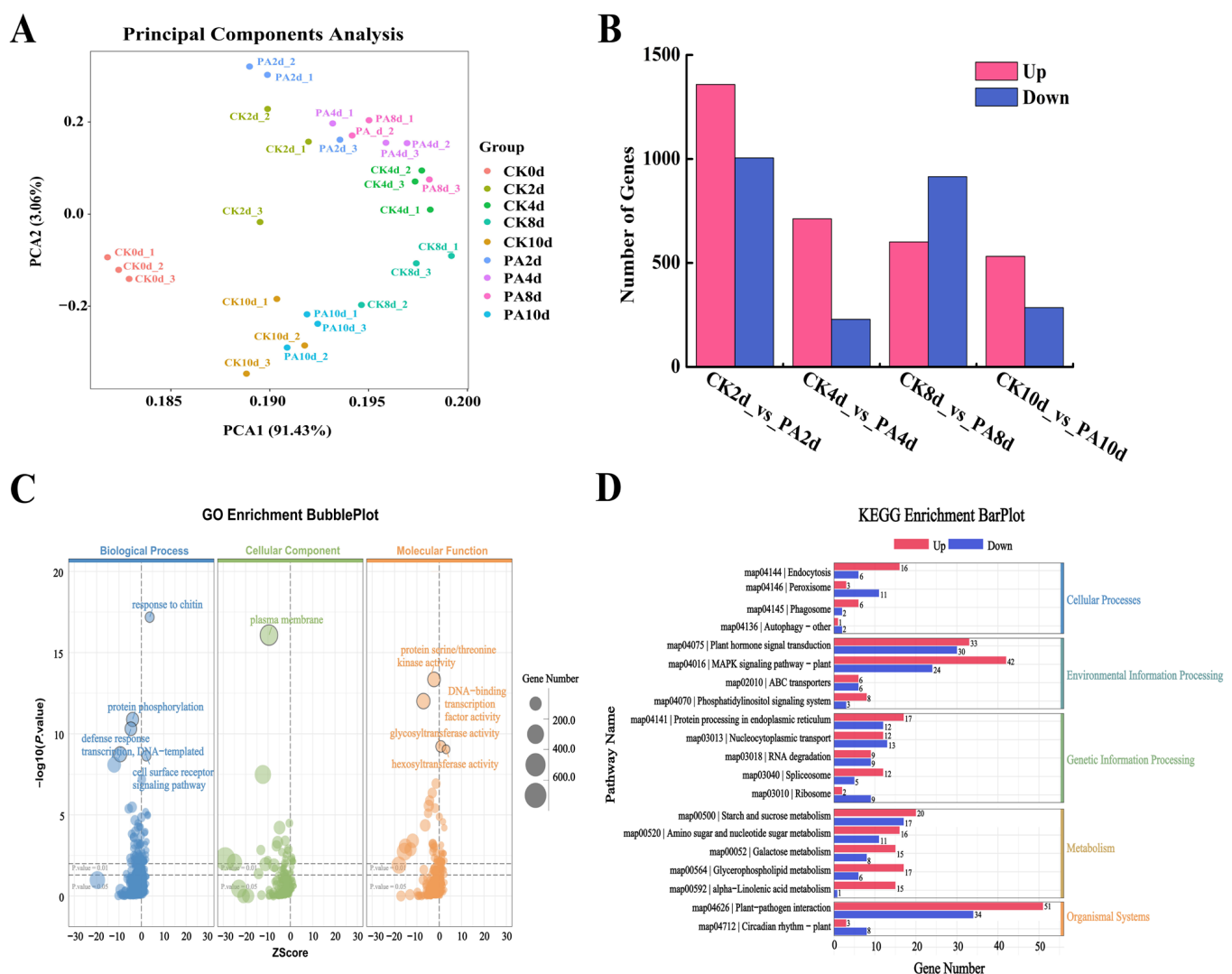


Figure 3. Preliminary analysis of transcriptomic data detailing the alterations in litchi fruit throughout storage at 25 °C. (A) Principal components analysis plot of the litchi pericarp after PA treatment. (B) Number of DEGs. (C) GO enrichment bubble plot in the CK2d_vs_PA2d group. (D) KEGG enrichment analysis of DEGs in the CK2d_vs_PA2d group.

DEGs were additionally examined through GO and KEGG analysis. DEGs from the CK2d_vs_PA2d comparison were categorized according to molecular function, cellular

component, and biological process, revealing 272 significant DEGs at $p < 0.05$, as illustrated in Figure 3C. Of these, 138 DEGs are activated by biological process, with the most prevalent being response to chitin, protein phosphorylation, defense response, transcription (DNA-templated), and cell surface receptor signaling pathways. The cellular component consisted of 38 DEGs, with the plasma membrane being the most enriched. In terms of molecular function, 96 DEGs were detected, with DNA-binding transcription factor activity, protein serine/threonine kinase activity, glycosyltransferase activity, and hexosyltransferase activity being the most prevalent. Additionally, distinct biological processes such as oxalate metabolism and the ethylene-activated signaling pathway in CK4d_vs_PA4d, obsolete oxidation–reduction process, cellular response to hypoxia, hydrogen peroxide catabolic process, and glutathione metabolic process in CK8d_vs_PA8d, as well as responses to abiotic stress in CK10d_vs_PA10d, were specifically identified, contrasting with CK2d_vs_PA2d (Figure S2A).

As shown in Figure 3D, samples in the CK2d_vs_PA2d group exhibited enrichment in 64 pathways based on KEGG enrichment analysis, including the MAPK signaling pathway, plant–pathogen interaction, and plant hormone signal transduction in environmental information processing, as well as starch and sucrose metabolism in the metabolic pathway. Notably, the phenylpropanoid biosynthesis pathway was enriched in the CK_vs_PA groups following 4 days of storage, with a substantial number of upregulated DEGs identified (Figure S2B). This observation suggests that the phenylpropanoid biosynthesis pathway was downregulated by the PA treatment.

2.4. Integrating Related Genes and Metabolites in the Phenylpropanoid Pathway

DMs in the phenylpropanoid pathway were evaluated to uncover the potential impacts of PA treatment on proanthocyanidin and browning substrate biosynthesis. The abundance of each metabolite was normalized using log₂, and their respective roles in the phenylpropanoid pathway are depicted in Figure 4A–J. Following PA treatment, both phenylalanine and 4-coumaric acid demonstrated a similar pattern, being suppressed during the first 4 days of storage and subsequently upregulated by PA (Figure 4A,B). The naringin content did not show a significant variance between the CK and PA treatments, although a downregulation in naringenin-7-*O*-glucoside content was observed following PA treatment (Figure 4C,D). Dihydrokaempferol content was reduced from day 2 to day 4 by PA, with no notable variation between the groups following 8 days of storage (Figure 4E). Similar trends were observed in the contents of kaempferol and cyanidin-3-(2*G*-glucosylrutinoside), showing significant retardation throughout the entire storage period due to PA treatment (Figure 4F,G). Notably, the behavior of cyanidins, (–)-epicatechin, and (+)-catechin differed from that of other metabolites upon exposure to PA treatment. Their levels increased on day 4 but decreased during the later storage period (8–10 days) (Figure 4H–J). Five additional anthocyanins were identified, namely cyanidin-3-arabinopyranoside, cyanidin-3-glucoside, delphinidin-3-glucopyranoside, petunidin-3-glucoside, and peonidin-3-glucoside. All of these anthocyanidins accumulated during the brown stage but exhibited significant decreases upon treatment with PA (Figure S3).

DEGs were identified through transcriptome analysis, focusing on genes associated with proanthocyanidin biosynthesis exhibiting maximum FPKM values > 5 and a maximum fold change (PA/CK) > 1.5 between groups. Genes meeting these criteria were marked with an asterisk and chosen for validation through real-time quantitative PCR (RT-qPCR). (Figure 4K). The expression of *LcFLS* increased as the storage period was extended (Figure 5A). PA treatment markedly suppressed *LcFLS* gene expression on both days 2 and 8. The trends of *LcANS* and *LcUGT* paralleled that of *LcFLS* throughout storage at room temperature (Figure 5B,C). PA treatment markedly decreased the expression of

the *LcANS* and *LcUGFT* genes on day 2, and continued to significantly inhibit *LcANS* expression from days 8 to 10 (Figure 5B). The expression of *LcLAR* significantly decreased after 8 days of storage following PA treatment (Figure 5D). Conversely, the expression of *LcANR* in PA-treated pericarp showed fluctuating changes compared to the control, with downregulation observed on days 2 and 8 (Figure 5E).

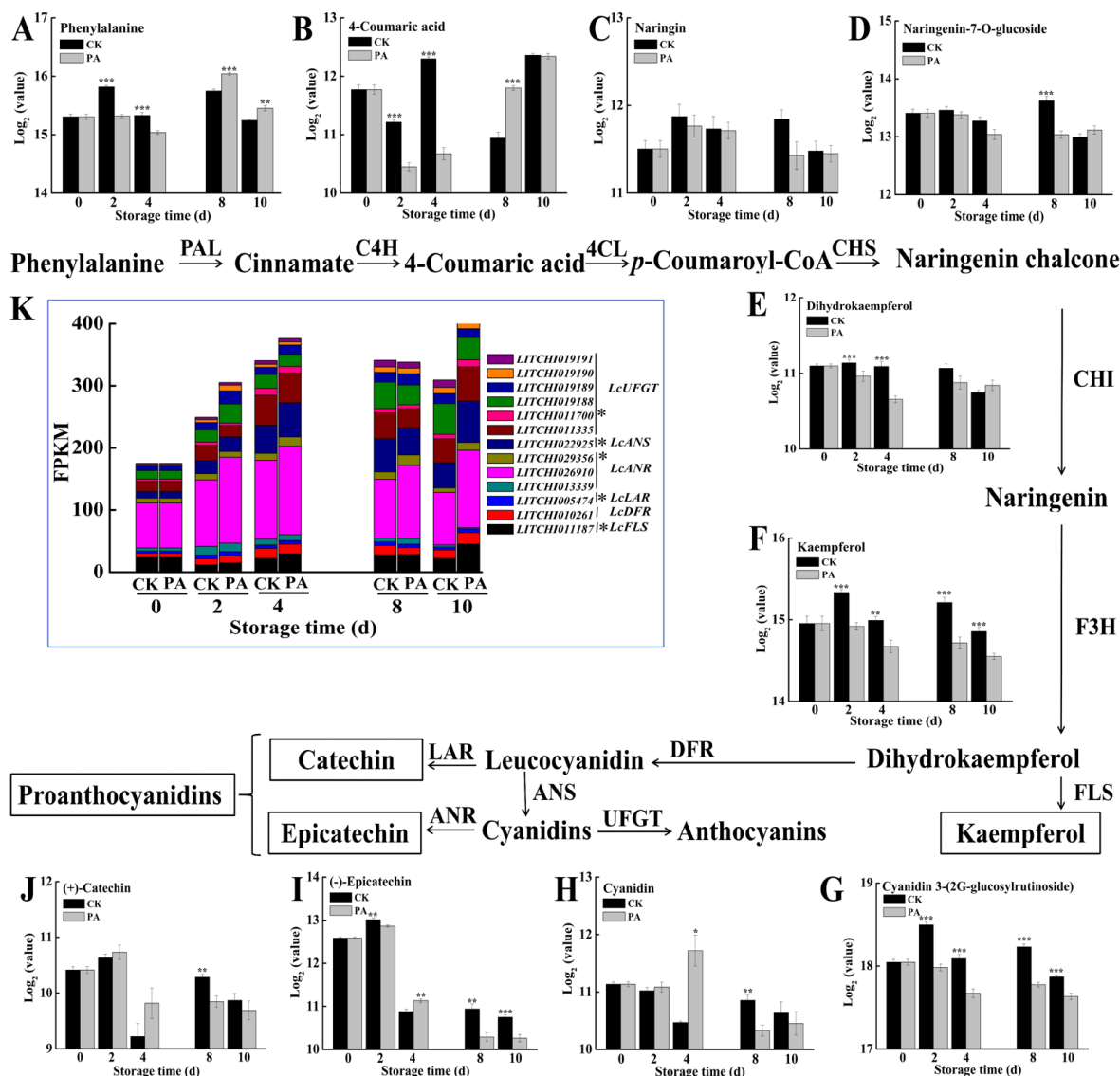


Figure 4. DEGs and DMs within the phenylpropanoid and flavonoid biosynthesis pathway. (A–J) Log₂ abundances values of the selected DMs within the proanthocyanidin biosynthesis pathway. (K) DEGs with maximum FPKM > 5 were evaluated through transcriptome analysis; genes marked with an asterisk denote those chosen for further investigations. The data are displayed as the mean ± standard error, with significance levels indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

2.5. Screening of Key Members in Phenoloxidase-Related Gene Family

In total, 31 phenoloxidase-related gene members were identified with maximum FPKM > 5. Subsequent investigations centered on polyphenol oxidase (*LcPPO*), laccase (*LcLAC14-2* and *LcLAC7*), and peroxidase (*LcPOD1-7*) due to their maximum $|\log_2\text{FC}| > 1$ and the significant difference between groups (Figure 6A). The expression of specific genes in both groups was additionally confirmed through RT-qPCR. As shown in Figure 6B, the expression of *LcPPO* increased with the storage period's extension, whereas PA treatment

notably inhibited *LcPPO* expression. Both *LcLAC14-2* and *LcLAC7* displayed similar patterns, peaking on the eighth day before declining thereafter. PA markedly reduced the expression of both of these genes. The expression levels of most *LcPODs* in PA-treated pericarp were notably lower compared to those in the CK, especially during the initiation of browning (2 d). The expression levels of *LcPOD1* and *LcPOD7* differed significantly from those of other *LcPODs* upon exposure to PA treatment, showing upregulation. This suggests that *LcPOD1* and *LcPOD7* are not associated with pericarp browning.

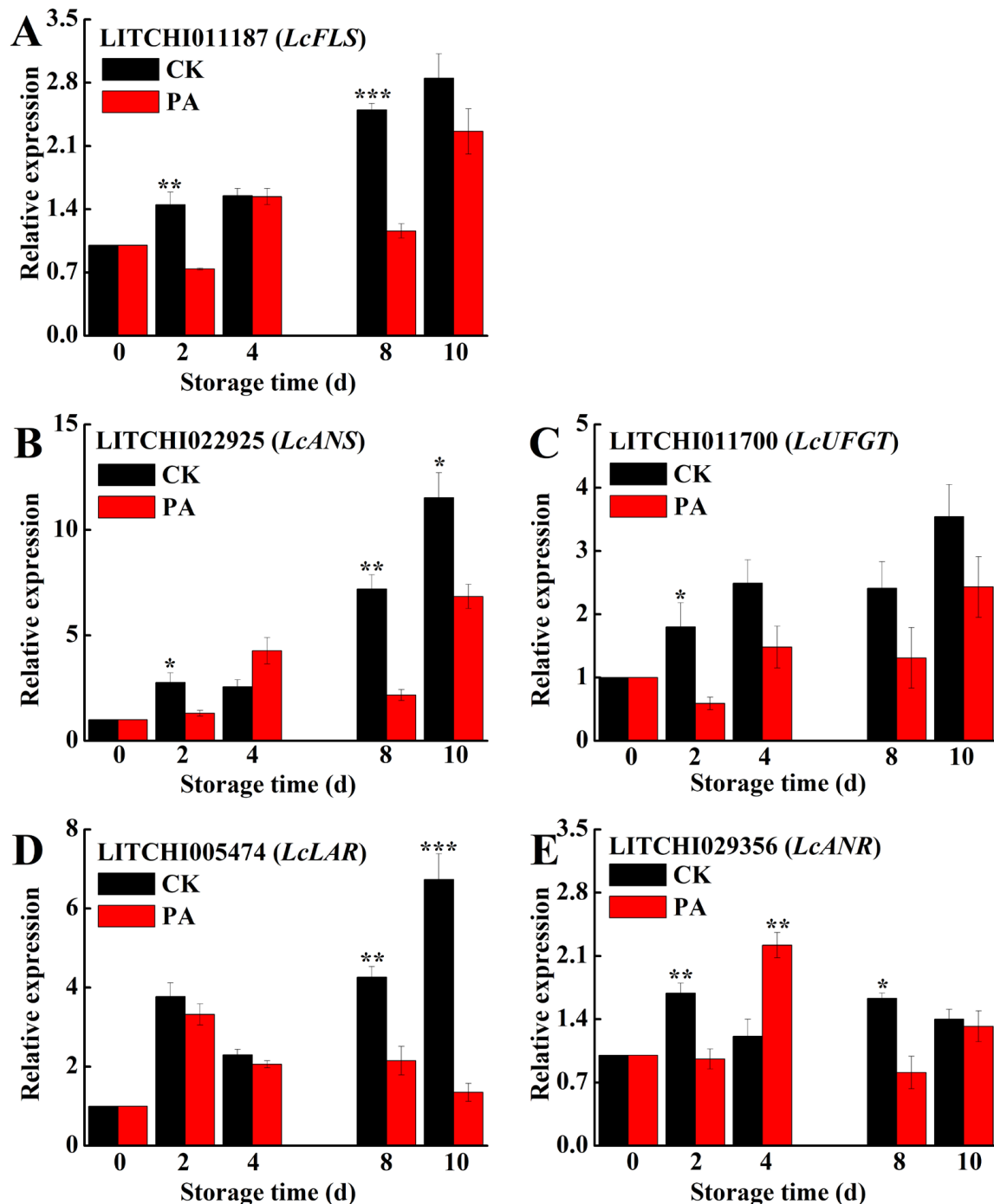


Figure 5. RT-qPCR validation of (A) *LcFLS*, (B) *LcANS*, (C) *LcUFGT*, (D) *LcLAR*, and (E) *LcANR* genes in the proanthocyanidin biosynthesis pathway: 0-day pericarp was used as the calibration sample; *LcActin* was designated as the internal reference gene for normalizing transcript profiles using the $2^{-\Delta\Delta C_t}$ method. The data are displayed as the mean $\pm$ standard error, with significance levels indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

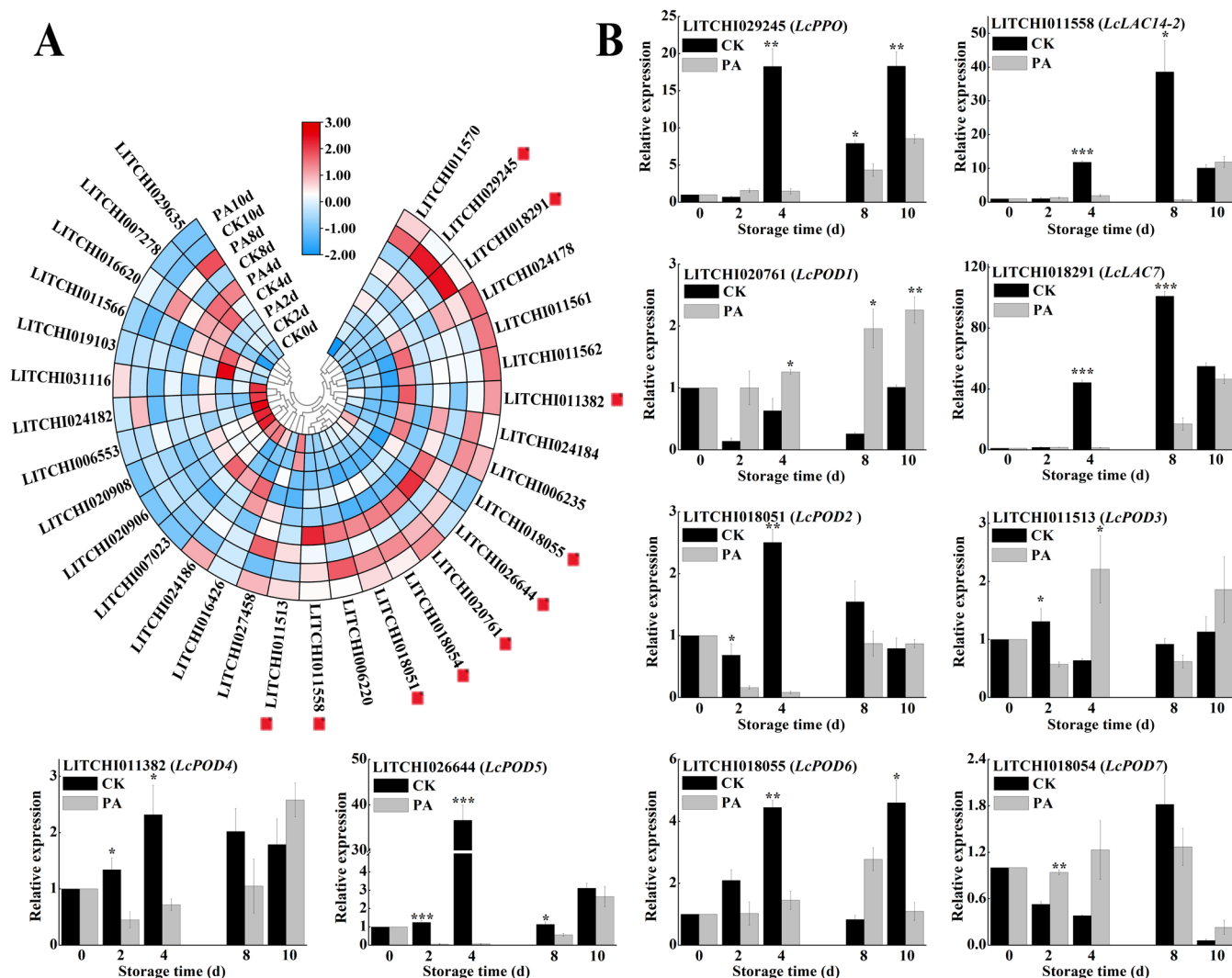


Figure 6. Screening of crucial members in phenoloxidase-related DEGs during litchi pericarp browning. (A) Heatmap displaying *LcPPO*, *LcLACs*, and *LcPODs* characterized by maximum FPKM > 5; genes denoted by red squares indicate the specific genes chosen based on a maximum $|\log_2FC| > 1$. (B) Gene expression of selected genes detected through RT-qPCR. The data are displayed as the mean $\pm$ standard error, with significance levels indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

2.6. Correlation Analysis of Browning Characteristics in PA-Treated Litchi Pericarp

Based on the RT-qPCR results and metabolite profile data, the coefficients of the correlations between DEGs and DMs during the litchi pericarp browning process were determined. As shown in Figure 7A, the results indicate a notable inverse correlation between the browning index and the abundance of proanthocyanidin and (–)-epicatechin, while showing a positive correlation with the gene expression of *LcFLS*, *LcANS*, *LcUFGT*, *LcPPO*, and *LcLAC7*. This suggests that pericarp browning is primarily linked to pericarp proanthocyanidin accumulation and the oxidation of the browning substrate, (–)-epicatechin. Moreover, the proanthocyanidin content exhibited significant positive correlations with cyanidin 3-(2G-glucosylrutinoside) and (–)-epicatechin, while showing negative correlations with the gene expressions of *LcANS*, *LcPPO*, and *LcLAC7*. This indicates that the biosynthesis and browning of proanthocyanidins were affected by the biosynthesis and oxidation of (–)-epicatechin.

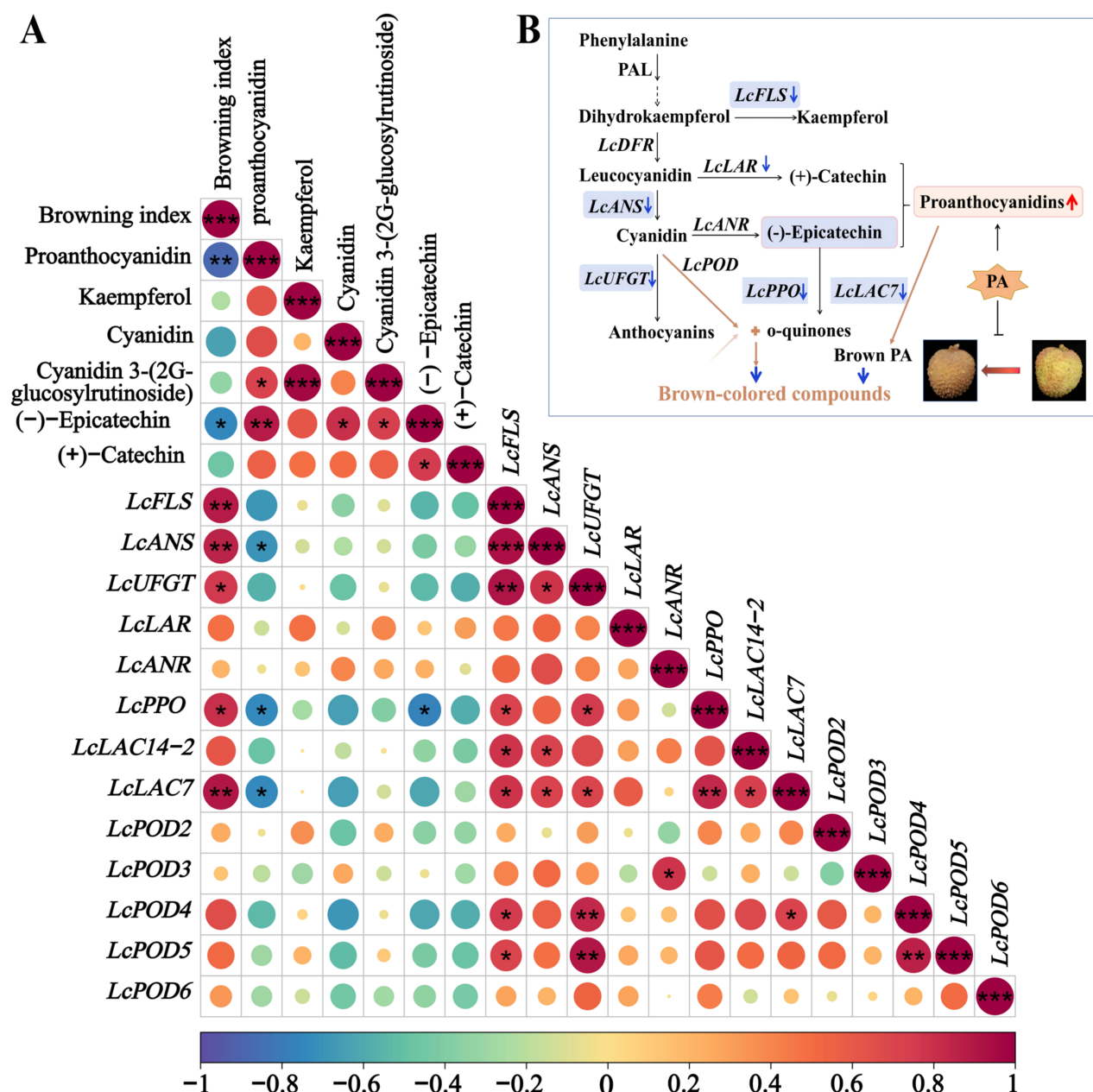


Figure 7. (A) Correlations between candidate genes and metabolites in litchi pericarp following PA treatment. Positive or negative correlation coefficients are depicted through a virtual color scheme. Statistical significance levels are denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Proposed model of the potential mechanism by which PA treatment delays the browning process in ‘Feizixiao’ litchi pericarp by downregulating the phenylpropanoid and flavonoid biosynthesis pathway and phenol oxidation. The blue arrow signifies inhibitory or delaying effects; the red arrow represents activating effects. The dashed line represents the results inferred from correlations. The blue and pink box denotes significant correlations with the browning index.

3. Discussion

3.1. PA Treatment Delays Litchi Pericarp Browning and the Decrease in PA Content

The browning of litchi pericarp is the primary physiological process leading to a decline in quality and a reduction in its international trade value [4]. This includes weight loss, brown pigments generation, a decline in nutritional content, a decrease in phenolic compounds, and increases in ethylene and the respiration rate [5,7,23]. Previous studies have revealed that exogenous PA treatment can effectively suppress color transition in

fruits such as banana [24], strawberry [25], and litchi [7,13]. In this study, comparable findings were observed, indicating a delayed browning phenomenon in postharvest litchi fruit following PA treatment. This effect was notably heightened after 6 days of storage (Figure 1). Furthermore, the pericarp PA content exhibited a significant negative correlation with the browning index. PA content decreased during browning but was higher in PA-treated fruits, consistent with prior studies that showed enhanced PA levels in litchi and banana peels, leading to delayed browning and senescence [7,16,22]. This implies the signaling pathways of endogenous PA and its potential role in anti-browning mechanisms.

3.2. PA Reduces Brown Substrate Accumulation

Metabolomics based on LC-MS has been established to identify DMs in litchi species during pericarp browning [22,26]. In this investigation, an untargeted metabolomic approach was employed to analyze the metabolic changes in stored litchi pericarp (CK and PA-treated) over 10 d. There were 630 metabolites across 14 superclasses identified in positive ion mode. The abundance of primary flavonoids and anthocyanins in the phenylpropanoid pathway was notably reduced in the PA group compared to the CK group, indicating the suppression of brown substrate biosynthesis due to PA treatment.

He et al. [26] identified, through metabolomic analysis, that epicatechin and anthocyanins were the primary brown substrates that decreased during the brown stage. Cyanidin-3-rutinoside constituted the primary anthocyanins present in litchi pericarp [10,26]. Following the metabolomic analysis, it was noted that the abundance of (–)-epicatechin and cyanidin in PA-treated pericarp rose on day 4 but declined thereafter, whereas the initial increase in most anthocyanins during the first 2 days was suppressed by PA. Other studies have also reported that the degradation of anthocyanin exceeds biosynthesis during the rapid browning period [22]. It is postulated that the rise in (–)-epicatechin and cyanidin, coupled with the decline in anthocyanins during the early storage period, is linked to PA accumulation. The subsequent decrease is associated with the brown substrate inhibition caused by PA treatment.

3.3. PA Suppresses the Expression of Phenylpropanoid Pathway Genes

We now turn to the biosynthesis of brown substrate and colorless PAs derived from the phenylpropanoid pathway [15,19]. Numerous evidence strongly supports a notable association between the presence of PAs in litchi fruit and key genes, including *LcFLS*, *LcDFR*, *LcANS1*, *LcLAR1/2*, *LcANR1a/2a*, and *LcUFGT* [20,26,27]. We observed that genes including *LcFLS*, *LcANS*, *LcUFGT*, *LcLAR*, and *LcANR* exhibited strong inhibition during the later brown stage, aligning with the decrease in primary brown substrates and PAs (Figures 4 and 5). Moreover, the browning index exhibited a positive correlation with *LcFLS*, *LcANS*, and *LcUFGT*, indicating that the biosynthesis of flavonoids and anthocyanins contributed to litchi pericarp browning. It is noteworthy that *LcANR*, the key gene for (–)-epicatechin biosynthesis, exhibited a notable upregulation following PA treatment on day 4, suggesting its potential role in promoting PA accumulation during the early browning stage (Figure 5). The research by Zhong et al. [20] revealed analogous findings, demonstrating a significant positive correlation between *LcANR1a* (LITCHI029356) and the levels of (–)-epicatechin and PAs. Thus, we concluded that genes implicated in the PA biosynthesis pathway may play a crucial role, as the inhibition of these metabolite-related genes by PA could attenuate the browning process.

3.4. PA Hinders the Expression of Phenoloxidase Genes

The browning of litchi fruit peel is commonly associated with the oxidation of anthocyanidins and (–)-epicatechin into brown-colored compounds, a process primarily catalyzed by phenol oxidase enzymes such as PPO, POD, and LAC [5]. The encoding genes,

including *LcPPO*, *LcLAC7*, and *LcPOD2-6*, exhibited significant downregulation in response to PA treatment, particularly evident from days 2 to 4. In addition, *LcPPO* and *LcLAC7* showed a positive correlation with the browning index but a negative relation with PA accumulation. Additionally, (–)-epicatechin displayed a negative correlation with *LcPPO* (Figure 7A). Previous studies have shown that gene-edited litchi calli with the *LcPPO* gene exhibited decreased PPO activity, resulting in reduced oxidation of (–)-epicatechin and a decelerated browning process [28]. *LcLAC7* exhibits catalytic activity for (–)-epicatechin and plays significant roles in converting colorless PAs into brown PA derivatives [29]. These findings suggest that PA can postpone (–)-epicatechin degradation by inhibiting *LcPPO* and decrease the polymerization of PAs by decreasing *LcLAC7* expression, consequently lowering browning occurrence. In addition, PAs, similar to hesperetin, can bind with PPO to competitively hinder its catalytic activity, consequently reducing fruit browning [18,30]. Therefore, we hypothesize that PA can combine with PPO and inhibit PPO activity.

4. Materials and Methods

4.1. Plant Material and Treatments

‘Feizixiao’ Litchi (*Litchi chinensis* Sonn.) fruits with similar color and size were collected from a commercial orchard in Haikou City, Hainan Province, China. The fruit was delivered to the laboratory within 1 h, and the fruit sample was subsequently split into two groups, each consisting of roughly 240 individual fruits. Groups were immersed individually in tap water (CK) or a 3 g/L proanthocyanidin (PA, food grade) solution for 10 min. The fruit was air-dried at room temperature, then transferred into polyethylene bags, and kept under storage conditions of 25 °C and 85–90% RH for 10 days. The pericarp browning index and proanthocyanidin content were assessed at two-day intervals, with 30 fruits acquired for measuring the browning index. Pericarp tissues were sampled and sent for transcriptomic and metabolomic sequencing.

4.2. Determination of Litchi Pericarp Browning and Proanthocyanidin Content

Litchi pericarp browning was assessed using established protocols outlined in a prior study by Shen et al. [7]. The browning index was assessed through a five-class method that evaluated the extent of browning on individual fruits. The pericarp proanthocyanidin content was quantified following the procedures outlined in Wang et al. [31]. First, 0.1 g of frozen pericarp powder was added to 10 mL of absolute ethanol, ensuring thorough mixing, and the mixture was subsequently allowed to stand in darkness overnight. After collection, 0.5 mL of the supernatant was mixed with 3 mL of a 4% (*w/v*) vanillin solution and 1.5 mL of concentrated hydrochloric acid. The absorbance at 500 nm was recorded after incubating the reaction mixture at room temperature for 15 min. A standard curve was constructed using standard proanthocyanidins, following the equation $y = 1.766x + 0.0069$.

4.3. Metabolome Analysis

Based on the browning and proanthocyanidin content, six biological replicates (each containing five fruits) were collected from fruit exposed to controlled storage (CK0d, CK2d, CK4d, CK8d, and CK10d) and pericarp treated with PA (PA2d, PA4d, PA8d, and PA10d). Metabolites were extracted with 120 µL of methanol buffer that had been pre-cooled. Additionally, a pooled quality control (QC) sample was also prepared by mixing 10 µL from each extraction mixture. Utilizing an LC-MS/MS system consisting of an ACQUITY UPLC HSS T3 column (1.8 µm, 2.1 mm × 100 mm, Waters, Milford, UK) coupled to a TripleTOF 5600 Plus high-resolution tandem mass spectrometer (Sciex, Warrington, UK), untargeted metabolomics analysis was conducted by the LC-Bio Technology company (Hangzhou, China). The mobile phase in this study comprised water (0.1% formic acid)

and acetonitrile (0.1% formic acid). Under gradient elution conditions at a flow rate of 0.4 mL/min, the following protocol was applied: 5% B for 0 to 0.5 min, 5–100% B from 0.5 to 7 min, 100% B from 7 to 8 min, 100–5% B from 8 to 8.1 min, and 5% B from 8.1 to 10 min. MS/MS was carried out in positive and negative ionization modes. The gas 1 and gas 2 pressure were maintained at 60 PSI, the curtain gas pressure was kept at 30 PSI, and the ion spray floating voltage was adjusted to 5 kV or −4.5 kV. The temperature of the interface heater was adjusted to 650 °C.

The MS data were processed through XCMS software (version 4.7.3); features detected in <50% of the QC samples or 80% of the test samples were removed, and the values for missing peaks were extrapolated with the k-nearest neighbor algorithm to further improve the data quality, while metabolite identification relied on an MS2 database according to Chen et al. [32]. PCA was employed for outlier and batch effect detection, after which supervised Partial Least Squares Discriminant Analysis with metaX was applied to pinpoint more precise distinctions among the groups [33]. Data normalization was performed on all samples using the probabilistic quotient normalization algorithm. Then, the *p*-value was determined using Student *t*-tests, which were then adjusted for multiple tests using a false discovery rate (FDR) (Benjamini–Hochberg) for the DM selection. DMs were significantly selected in each group based on a VIP cut-off criterion of 1.0. The DMs were then annotated to the KEGG database, and *p*-values were used for the selection of DMs.

4.4. Transcriptome Analysis

Three biological replicates collected from both control and PA-treated pericarp at the sampling points of 0 d, 2 d, 4 d, 8 d, and 10 d were used for RNA sequencing. Total RNA was extracted utilizing Trizol reagent (ThermoFisher, Waltham, MA, USA) as per the manufacturer's instructions. The RNA quality was evaluated using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA, USA). The mRNA was purified using Dynabeads Oligo (dT) from Thermo Fisher in California, Waltham, MA, USA. cDNA was generated using SuperScriptTM II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Following the addition of an A0base, the blunt ends of each strand were ligated to the indexed adapters. Finally, an Illumina NovaseqTM 6000 from LC-Bio Technology company (Hangzhou, China) was utilized to sequence the ligated cDNA libraries. Through the use of Cutadapt (version 1.9), clean reads were acquired through the filtration of low-quality bases (Table S4). Paired-end reads were aligned to the litchi genome database, accessible at <https://ngdc.cnbc.ac.cn/tcod/litchi?lang=en> (accessed on 6 July 2023), using HISAT2 (version 2.2.1) as described by Kim et al. [34].

FPKM values were determined for gene expression abundance utilizing StringTie (version 2.1.6). DEGs between the CK and PA-treated groups were analyzed using DESeq2 software (version 4.5) [35]. DEGs meeting the criteria of an FDR < 0.05 and an absolute fold change ≥ 2 were obtained. Finally, PCA was performed using R (<http://www.r-project.org/>, version 4.2), followed by GO and KEGG enrichment for the biological functional analysis of the DEGs [36,37].

4.5. RT-Quantitative PCR Validation of DEGs

The expression patterns of 10 DEGs involved in phenol oxidation and 5 DEGs related to the proanthocyanidin biosynthesis pathway were validated through real-time quantitative PCR (RT-qPCR). Utilizing the online Primer 3 platform, gene-specific primers were designed, and the primer details can be found in Table S5. Melting curves were used to validate the specificity of the primers. Total RNA extraction, cDNA synthesis, and the RT-qPCR program were carried out following the methodology detailed by Shen et al. [7].

The gene expression levels were normalized utilizing *LcActin*, with day 0 as the baseline set to an expression intensity of 1. Three biological replicates were conducted for each group.

4.6. Statistical Analysis

Significance analyses were performed using IBM SPSS Statistics 26.0. The CK and PA treatments were compared through an analysis of variance (ANOVA) using Duncan's multiple range test at a significance level of $p < 0.05$. The means $\pm$ standard errors of triplicates are presented, and the figures were produced utilizing Origin 8.0 (Microcal Software Inc., Northampton, MA, USA). The candidate genes and metabolites in litchi pericarp post-PA treatment were assessed for correlations through Pearson's correlation test.

5. Conclusions

In conclusion, this study demonstrates that PA treatment delays the degradation of endogenous PAs and suppresses browning in litchi pericarp. DEGs and DMs in the PA biosynthesis pathway exhibited decreased expression following PA treatment. The abundance of flavonoids (kaempferol) and six anthocyanins notably decreased throughout the treatment period, whereas cyanidins, (–)-epicatechin, and (+)-catechin exhibited a significant decrease after 8 days of PA treatment. Moreover, the expression of PA biosynthetic genes (such as *LcFLS*, *LcANS*, *LcUGT*, *LcLAR*, and *LcANR*) and phenoloxidase genes (including *LcPPO*, *LcLAC7*, and *LcPOD2-6*) is significantly downregulated in response to PA treatment. These findings suggest that PA treatment effectively delays pericarp browning by enhancing endogenous PA levels, decreasing the accumulation of brown substrates, and suppressing the expression of phenoloxidase genes. Therefore, the decrease in brown substrate accumulation and the reduction in brown substrate oxidation impeded the browning process (Figure 7B). In general, this study enhances our comprehension of the metabolic and molecular processes underlying PA-delayed pericarp browning, providing a basis for PAs and other polyphenols to prevent browning during litchi fruit storage.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052123/s1>.

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