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An in-depth study of anthocyanin synthesis in the exocarp of virescens and nigrescens oil palm: metabolomic and transcriptomic analysis

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Abstract

Background Oil palm (*Elaeis guineensis* Jacq.) is a very important tropical woody oil plant with high commercial and ornamental value. The exocarp of the oil palm fruit is rich in anthocyanosides and proanthocyanidins, which not only give it a bright colour, but also mark the maturity of the fruit. The study of the dynamic change pattern of anthocyanoside content and important anthocyanoside metabolism-related regulatory genes during oil palm ripening is conducive to the improvement of the ornamental value of oil palm and the determination of the optimal harvesting period of the fruits.

Methods We analyzed the virescens oil palm (AS) and nigrescens oil palm (AT) at 95 days (AS1, AT1), 125 days (AS2, AT2) and 185 days (AS3, AT3) after pollination were used as experimental materials for determining the changes in the total amount of anthocyanins as well as their metabolomics and transcriptomics studies by using the LC-MS/MS technique and RNA-Seq technique.

Result The results showed that the total anthocyanin content decreased significantly from AS1 (119 μg/g) to AS3 (23 μg/g), and from AT1 (1302 μg/g) to AT3 (170 μg/g), indicating a clear decreasing trend during fruit development. Among them, the higher flavonoids in AS and AT included anthocyanins such as peonidin-3-O-rutinoside (H35), pelargonidin-3-O-rutinoside (H21), and cyanidin-3-O-glucoside (H7), as well as condensed tannins such as procyanidin B2 (H47), procyanidin C1 (H49), and procyanidin B3 (H48). Notably, nine genes involved in the anthocyanin biosynthetic pathway exhibited up-regulated expression during the pre-development stage of oil palm fruits, particularly during the AS1 and AT1 periods. These genes include: Chalcone synthase (CHS; LOC105036364); Flavanone 3-hydroxylase (F3H; LOC105054663); Dihydroflavonol 4-reductase (DFR; LOC105040724, LOC105048473); Anthocyanidin synthase (ANS; LOC105035842), UDP-glucose: flavonoid 3-O-glucosyltransferase

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(UFGT; LOC105039612); Flavonoid 3',5'-hydroxylase (F3'5'H; LOC105036086, LOC105044124, LOC105045493). In contrast, five genes demonstrated up-regulated expression as the fruits developed, specifically during the AS3 and AT3 periods. These genes include: Chalcone synthase (CHS; LOC105036921, LOC105035716); Chalcone isomerase (CHI; LOC105045978); UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT; LOC105046326); Flavonoid 3'-hydroxylase (F3'H; LOC105036086).

Conclusion Most of differentially expressed genes exhibited up-regulation during the early stages of fruit development, which may contribute to the elevated anthocyanin content observed in oil palm fruits of both types during the pre-developmental period. Furthermore, the expression levels of most genes were found to be higher in the AT fruit type compared to the AS fruit type, suggesting that the differential expression of these genes may be a key factor underlying the differences in anthocyanoside production in the exocarp of oil palm fruits from these two fruit types. The findings of this study provide a theoretical foundation for the identification and characterization of genes involved in anthocyanin synthesis in oil palm fruits, as well as the development of novel variations using molecular biology approaches.

Keywords Oil palm, Virescens, Nigrescens, Anthocyanins, Metabolomics, Transcriptomics

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a tropical palm family tropical commercial crop known as the "World Oil King [1]. Oil palm fruits are classified according to their color and can be divided into two fruit types: virescens and nigrescens. The nigrescens oil palm is the most widely cultivated variety globally, followed by the virescens oil palm, which is currently cultivated in Thailand, Malaysia, Nigeria, and Costa Rica. Furthermore, cultivation of the virescens oil palm also extends to several provinces in China, including Hainan, Guangxi, Guangdong, Yunnan, and Fujian [2]. A notable distinction exists between the fruit colors of the virescens oil palm and the nigrescens oil palm. The virescens oil palm fruit undergoes a series of color transformations as it matures, progressing from green to yellow-green and eventually to orange-yellow. In contrast, the nigrescens oil palm fruit exhibits a range of deeper, richer hues, including purple-black, purple-red, and orange-red. The vibrant and varied coloration of the fruit not only serves as an indicator of its maturity but also confers a high ornamental value to the oil palm, making it a popular choice as an ornamental tree species in landscaping applications.

The peel color of fruits is a crucial indicator of their quality, and it is one of the most significant factors directly influencing the productivity of fruit-bearing plants. The accumulation of anthocyanins is responsible for imparting a range of colors to the fruit. Notably, anthocyanin also possess a multitude of bioactive properties, including anti-inflammatory, antioxidant, and cardiovascular disease prevention functions [3, 4]. The content of anthocyanin has a profound impact on the quality of fruits and vegetables, with moderate consumption of anthocyanin-rich foods such as blueberries, mulberries, and black wild cherries having been shown to have beneficial effects on cardiovascular disease prevention, obesity management, and immune system regulation [5–7]. Anthocyanin's are secondary plant metabolites belonging to the flavonoid group of the polyphenols, responsible for plants' blue, red and purple colors [8]. They are glycosides of anthocyanidins. The anthocyanidins (aglycons), the basic structures of the anthocyanins, consist of a heterocyclic ring (C) that contains oxygen, bonded with an aromatic ring (A), and connected from the other side by a carbon-carbon bond to a third aromatic ring (B) [9]. The sugar moiety of anthocyanins is usually conjugated to the skeleton of anthocyanidin via the C3 hydroxyl group in ring C. The conjugated bonds in the anthocyanin structures are responsible for fruits and vegetables' bright red and blue colors [10]. At least 635 anthocyanidins have been identified in nature [8], and despite the increasing number of anthocyanidin structures, they are derived from only about 30 different anthocyanins, the most common of which comprise six classes: cyanidin, delphinidin, pelargonidin, peonidin, petudinin, and Malvidin [11, 12]. In contrast, proanthocyanidins, also known as condensed tannins, are polymeric flavonoids composed of flavan-3-ol units such as catechin and epicatechin. These compounds do not contribute to pigmentation but are significant for their astringency, bitterness, and notable antioxidant properties [13]. Understanding these distinctions is crucial for the accurate interpretation of plant biochemistry and the health benefits associated with these compounds [14].

Oil palm fruits are rich in anthocyanins. Anthocyanin biosynthetic pathway involved in oil palm fruit color formation. The biosynthesis of anthocyanins is transcriptionally regulated by a complex of transcription factors (TFs), specifically the MBW (MYB-bHLH-WDR) complex. This complex comprises a combination of MYB, bHLH, and WDR proteins. Research has identified the *virescens* gene as an R2R3-MYB transcription factor that plays a crucial role in regulating the expression of enzyme genes involved in the anthocyanin biosynthetic pathway

of oil palm. Notably, normal expression of the virescens gene is associated with the nigrescens fruit type, whereas mutations in this gene result in the virescens fruit type Similarly, Suraninpong and Nuanlaong (2022) found that up-regulated expression of *C4H* (Cinnamate-4-hydroxylase), *CHS* (Chalcone Synthase), *F3H* (Flavanone 3-hydroxylase), *F3′H* (Flavonoid 3′-Hydroxylase), *F3′5′H* (Flavonoid 3′,5′-hydroxylase), *ANS* (Anthocyanidin synthase), *ANR* (Anthocyanin reductase) and other enzyme genes promotes anthocyanin synthesis and accumulation in the pericarp of oil palm. Thus, it is clear that the anthocyanin biosynthetic pathway plays a crucial role in oil palm fruit formation.

Anthocyanidin biosynthesis pathway is one of the flavonoid synthesis pathways and is formed through the phenylacetone pathway. The first substance in this metabolic pathway is naringenin chalcone, which is produced by catalyzing the enzyme chalcone synthase (*CHS*) from three malonyl CoA molecules and one para coumarin CoA molecule. Chalcone isomerase (*CHI*) catalyzes the following step, which entails changing naringenin chalcone into naringenin flavanone. The production of dihydroflavonol is then regulated by flavonoid 3 '- hydroxylase (*F3'H*), while the synthesis of colorless anthocyanins is regulated by dihydroflavonol 4-reductase (*DFR*). Leucoanthocyanin dioxygenase (*LDOX*) or anthocyanin synthase (*ANS*) oxidizes products to produce colored anthocyanins. Stable anthocyanins are formed through glycosidic connections between these products, catalyzed by the enzyme UDP-glycose flavonoid glycosyltransferase (*UFGT*) [15].

Despite the limited research on the anthocyanin synthesis pathway in oil palm exocarps, existing studies have primarily focused on identifying key enzyme genes involved in the anthocyanin pathway and transcription factors that regulate these genes. To address this knowledge gap, this study aimed to investigate the differences in total anthocyanin content between virescens and nigrescens fruit types of oil palm during exocarp development: To achieve this, we employed a comprehensive approach comprising three main components: (1) anthocyanin content analysis, (2) metabolomic and transcriptomic analysis of anthocyanins and proanthocyanidins, and (3) screening for key genes involved in the anthocyanidin synthesis pathway of oil palm exocarps. This study is expected to contribute to the improvement of the ornamental value of oil palm, the determination of the optimal harvesting period of the fruits, and provide a reference for screening oil palm resources with higher anthocyanoside content and the utilization of the important components of anthocyanosides.

Results

Changes of total anthocyanin in exocarp of oil palm fruits at different developmental periods

The color of plants is significantly influenced by the presence of naturally occurring pigments. The oil palm fruit's outer layer was rich in anthocyanins, giving its flesh a vivid color. The exploration illustrates how fruits from AS and AT undergo color changes (Fig. 1A). The fruit's outer peel in AS was initially green, gradually turning yellow, and then turning orange yellow when fully developed. The fruit's outer peel in AT was typically yellow at the base, deep purple to black at the tip, gradually turning red and ultimately orange red at the tip when mature. Fruit maturity is indicated by a change in peel color,

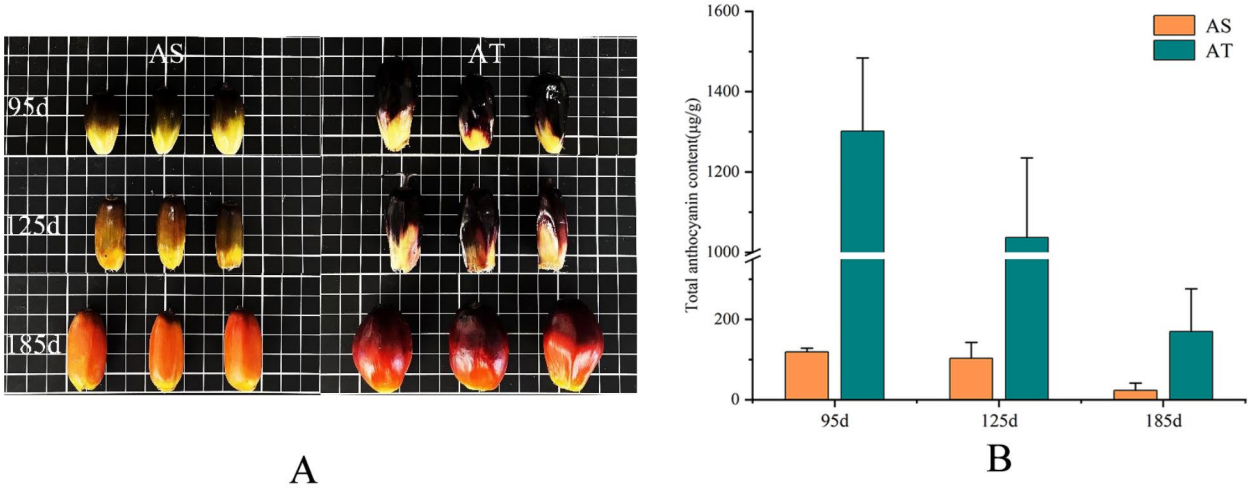

Fig. 1 Phenotypic characteristics and total anthocyanin content of exocarp of oil palm fruits at 95 days, 125 days and 185 days of development. (**A**), oil palm fruit, virescens oil palm fruit on the left, nigrescens oil palm fruit on the right; (**B**), total anthocyanin content, the length of the error bar represents the size of the standard deviation

which contributes to predict harvesting period and prevents decreases in productivity in oil palm plants from picking unripe or overripe fruits, which also diminishes fruit quality. The total anthocyanin content of AS1, AS2, AS3, AT1, AT2, AT3 period was extracted using total anthocyanin content extraction kit and the results were as follows: the contents of total anthocyanins in AS1-AS3 were119 μg/g, 103 μg/g, 23 μg/g, respectively; the total anthocyanins contents of AT1-AT3were found to be 1302 μg/g, 1037 μg/g, and 170 μg/g, respectively(Fig. 1B). The AS1-AS2 and AT1-AT2 stages had the highest total anthocyanin contents among them, whereas AS3 and AT3 had the lowest. Additionally, AS1 and AT1's exocarps had 5, 17 and 7, 66 times more total anthocyanosides than AS3 and AT3, respectively. Additionally, the total anthocyanin content fluctuation range in AT was 170 μg/g -1302 μg/g and in AS it was 23 μg/g-119 μg/g. Overall, there were substantially more total anthocyanins in AT than in AS, indicating a greater accumulation of anthocyanins during fruit growth.

Classification and analysis of anthocyanins and proanthocyanidins in exocarp of oil palm fruits at different developmental periods

A thorough investigation of the oil palm fruit exocarp during anthocyanidin formation revealed the presence of seven distinct classes of compounds, comprising: 10 kinds of cyanidin derivatives, 7 kinds of delphinidin and its derivatives, 2 kinds of malvidin derivatives, 10 kinds of pelargonidin derivatives, 8 kinds of peonidin and its derivatives, 6 kinds of petunidin derivatives and 6 kinds of procyanidins (Additional File 1). The total content of each anthocyanin class was calculated by summing the individual components, and the trends are illustrated in Fig. 2.Among the seven classes of substances, the content of procyanidins was the highest, followed by the cyanidin derivatives, and the lowest by the mallow malvidin

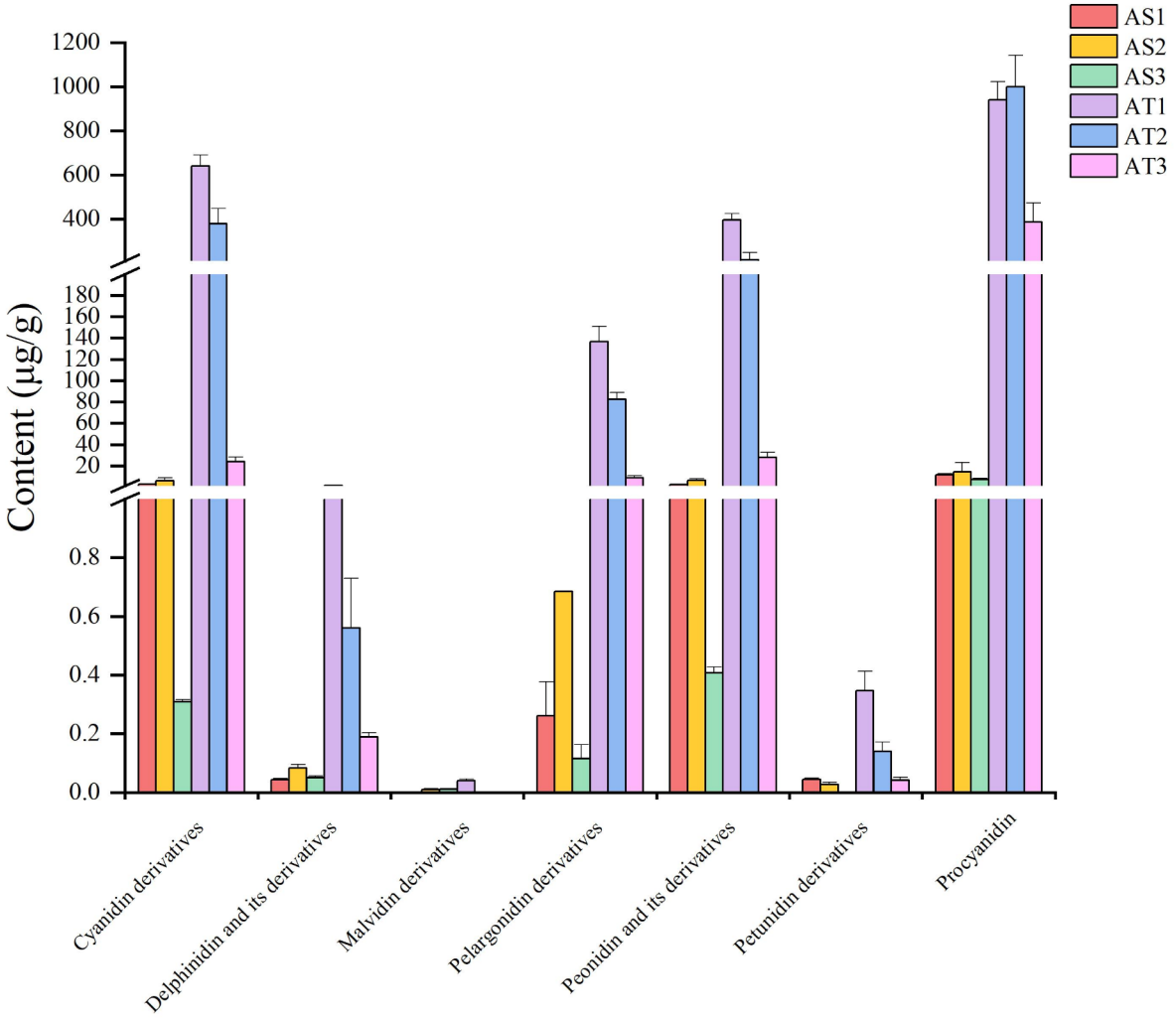

Fig. 2 Dynamics of seven anthocyanins in the exocarp of oil palm fruits at different developmental stages (AS1, AS2, AS3, AT1, AT2 and AT3)

derivatives. Among them, procyanidins were lower in the AS phase and higher in the AT phase, especially reaching 939.07 μg/g in the AT1 period and 999.22 μg/g in the AT2 period. Similarly cyanidin derivatives were higher in AT1 and AT2 periods and could reach 639.34 μg/g and 379.11 μg/g. Pelargonidin derivatives content was 136.73 μg/g and 82.51 μg/g in AT1 and AT2 periods, respectively. Peonidin and its derivatives content was 395.21 μg/g in AT1 and AT2 periods, respectively, and 214.79 μg/g. In summary, in the comparison of AS and AT, we found that the contents of six classes of anthocyanosides and proanthocyanidins were generally higher in AT than in AS, and the differences in the contents of all anthocyanosides and proanthocyanidins were large, except for the malvidin and petunidin derivatives. In addition, a comparison of different periods of the same fruit type revealed that the contents of exocarp anthocyanosides and proanthocyanidins in the first two periods were generally higher than those in the third period, both in AS and AT. These results were consistent with the trend of total exocarp anthocyanoside content in oil palm.

Significant difference metabolite analysis in exocarp of oil palm fruits at different developmental periods

The study found that 33 metabolites were significantly differentially expressed and common between the AS and AT oil palm exocarp developmental phases when compared using a combination of orthogonal partial least squares discriminant analysis (OPLS-DA) VIP≥1, difference multiplicity value (Fold_Change)≥2, and Fold_Change≤0.5 through comparison, specifically including procyanidin B1(H46), procyanidin C1(H49), procyanidinB3(H48),peonidin-3-O-rutinoside(H35),cyanidin-3-O-glucoside(H7), etc. (Fig. 3A). The detection of 49 significantly differentially expressed metabolites between AS1_vs_AT1 (44 up-regulated and 5 down-regulated), 46 significantly differentially expressed metabolites between AS2_vs_AT2 (39 up-regulated and 7 down-regulated), and 42 significantly differentially expressed metabolites between AS3_vs_AT3 (36 up-regulated and 7 down-regulated) (Fig. 3B). This shows that there are consistently more up-regulated differential metabolites than down-regulated differential metabolites in comparisons between different developmental periods of the fruit.

Difference analysis of anthocyanin and proanthocyanidin contents in exocarp of oil palm fruits at different developmental periods

According to the analysis of anthocyanins and proanthocyanidin components in the outer peel of oil palm fruits at different developmental stages, 43 anthocyanins and 6 proanthocyanidins were detected by AS and AT, among which 25 anthocyanins and 6 proanthocyanidins were detected in AS; 39 anthocyanins and 6 proanthocyanidins were detected in AT. In derivatives of cyanidin, the content of cyanidin-3-O-glucoside (H7) was the highest in both AS and AT, however it was higher in AS2 and AT1,in AT1, the content can reach up to 484.15 μg/g,

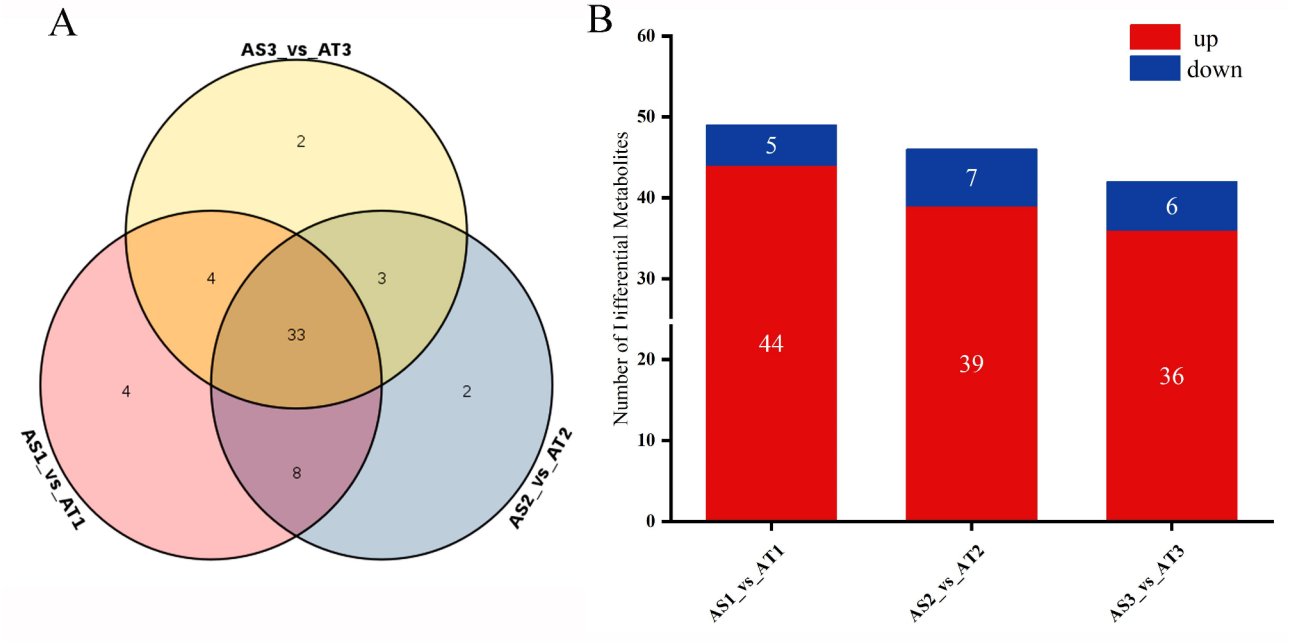

Fig. 3 Differential metabolite statistics of anthocyanins in the exocarp of oil palm fruits at different developmental periods (AS1_vs_AT1, AS2_vs_AT2, and AS3_vs_AT3). (**A**) differential metabolites Venn figure; (**B**) differential metabolites histogram

with variances between the AS and AT; Among the delphinidin and its derivatives, delphinidin-3-O-galactoside (H16) was the most abundant in AS and its concentration was higher than that of all periods in AT, while delphinidin-3-O-rutinoside (H14) was the most abundant in AT, with substantial variations between the two fruit kinds of oil palm; The content of AS and AT in malvidin derivatives was very low, and the difference was not statistically significant; The content of pelargonidin-3-O-galactoside (H21) in pelargonidin derivatives was the highest in both fruit types of oil palm, and the differences in the contents of this substance in AS and AT were significant, especially in the period of AT1, where its content was approximately 892 times higher than that in the period of AS1; peonidin-3-O-rutinoside (H35) and its derivatives showed significant differences in AS and AT developmental periods, followed by peonidin-3-O-(6-O-p-coumaroyl)-glucoside (H37) and peonidin-3-O-glucoside (H33) respectively. petunidin derivatives showed similar variation as malvidin derivatives, with low levels in both AS and AT, but no statistically significant differences. The content of procyanidin B2 (H47) in procyanidin was the highest in AS and AT, peaking at 7.08 μg/g and 622.38 μg/g in the period of AS2 and AT2, respectively, and then declining in the period of AS3 and AT3, indicating a general trend of increasing and then decreasing, and the content of procyanidin B2 (H47) was the highest of all the AT and AS anthocyanosides (Additional File 1).

The most frequent anthocyanosides and proanthocyanidins in AS were procyanidin B2 (H47), procyanidin C1 (H49), peonidin-3-O-rutinoside (H35), procyanidin B3 (H48), and cyanidin-3-O-glucoside (H7), while the highest amounts in AT were procyanidin B2 (H47), cyanidin-3-O-glucoside (H7), procyanidin C1 (H49), pelargonidin-3-O-rutinoside (H21), and procyanidin B3 (H48). The exocarp of oil palm fruit types showed significant differences in anthocyanosides and proanthocyanidins, except for petunidin derivatives and mallow pigment. Attributing to the AT's higher anthocyanoside and proanthocyanidin contents, the contents of each anthocyanoside and proanthocyanidin were generally higher in AT than AS.

The study categorized 43 anthocyanosides and 6 proanthocyanidins into two groups after normalization and clustering to examine their contents in the exocarp of each developmental phase of AS and AT (Fig. 4). The 42 anthocyanosides and proanthocyanidins in Group 1 showed reduced contents and less change during the development of AS. However, the concentrations of AT were generally larger during the AT1 phase, and gradually declined through the AT2 to AT3 stages. Only three compounds were found in greater concentrations on the AT3: delphinidin-3-O-rhamnoside (H11), peonidin-3-O-5-(6-O-coumaroyl)-diglucoside (H32), and petunidin-3-O-glucoside (H41). The concentration of seven anthocyanosides in Group 2 is higher in AS, indicating significant differences between AS and AT. During the AT1 and AT2 periods, anthocyanosides and proanthocyanidins with increased AT content were more abundant, whilepeonidin-3-O-5-O-(6-O-coumaroyl)-diglucoside(H32), delphinidin-3-O-rhamnoside (H11), and petunidin-3-O-glucoside (H41) fluctuated and peaked in the AT3 phase. Similar to AT, AS additionally revealed a rising anthocyanoside concentration trend. The content of proanthocyanidins and anthocyanosides in Group 1 was higher in AT and generally lower in AS, whereas the content of anthocyanosides in Group 2 was higher in AS and generally lower in AT when all stages of development of AS and AT were combined. The study revealed a significant difference in the concentration of anthocyanoside and proanthocyanidin in the exocarp of AS and AT.

Analysis of differentially expressed genes in exocarp of oil palm fruits at different developmental periods

A total of 9,757 significantly differentially expressed genes were found using the screening criteria of (False Discovery Rate, FDR)<0.05 and |log2Fold Change|≥1, with 1,592 significantly differentially expressed genes discovered across the three comparison groups of AS and AT oil palm exocarp (Fig. 5A). There were 5093 significantly differentially expressed genes between AS1_vs_AT1, with 2426 up-regulated and 2667 down-regulated; 5188 significantly differentially expressed genes between AS2_vs_AT2, with 2416 up-regulated and 2772 down-regulated; and 5610 significantly differentially expressed genes between AS3_vs_AT3, with 3180 up-regulated and 2430 down-regulated (Fig. 5B). The aforementioned results showed that AS3_vs_AT3 exhibited the highest number of up-regulated significant differentially expressed genes, which accounted for the largest percentage of the most significant differentially expressed genes.

Enrichment analysis of significantly differentially expressed genes in exocarp of oil palm fruits at different developmental periods

KEGG metabolic pathway analysis showed that the AS1_vs_AT1 comparative group was enriched to 133 pathways (Fig. 6A); The AS2_vs_AT2 comparative group enriched 132 pathways (Fig. 6B); The AS3_vs_AT3 comparative group enriched 130 pathways (Fig. 6C). The gene enrichment pathways annotated by the three comparison groups are mainly "metabolic pathways", "biosynthesis of secondary metabolites" and "plant-pathogen interaction". The anthocyanoside biosynthetic pathway is one of the flavonoid synthesis pathways. By analyzing the two pathways related to flavonoid biosynthesis and

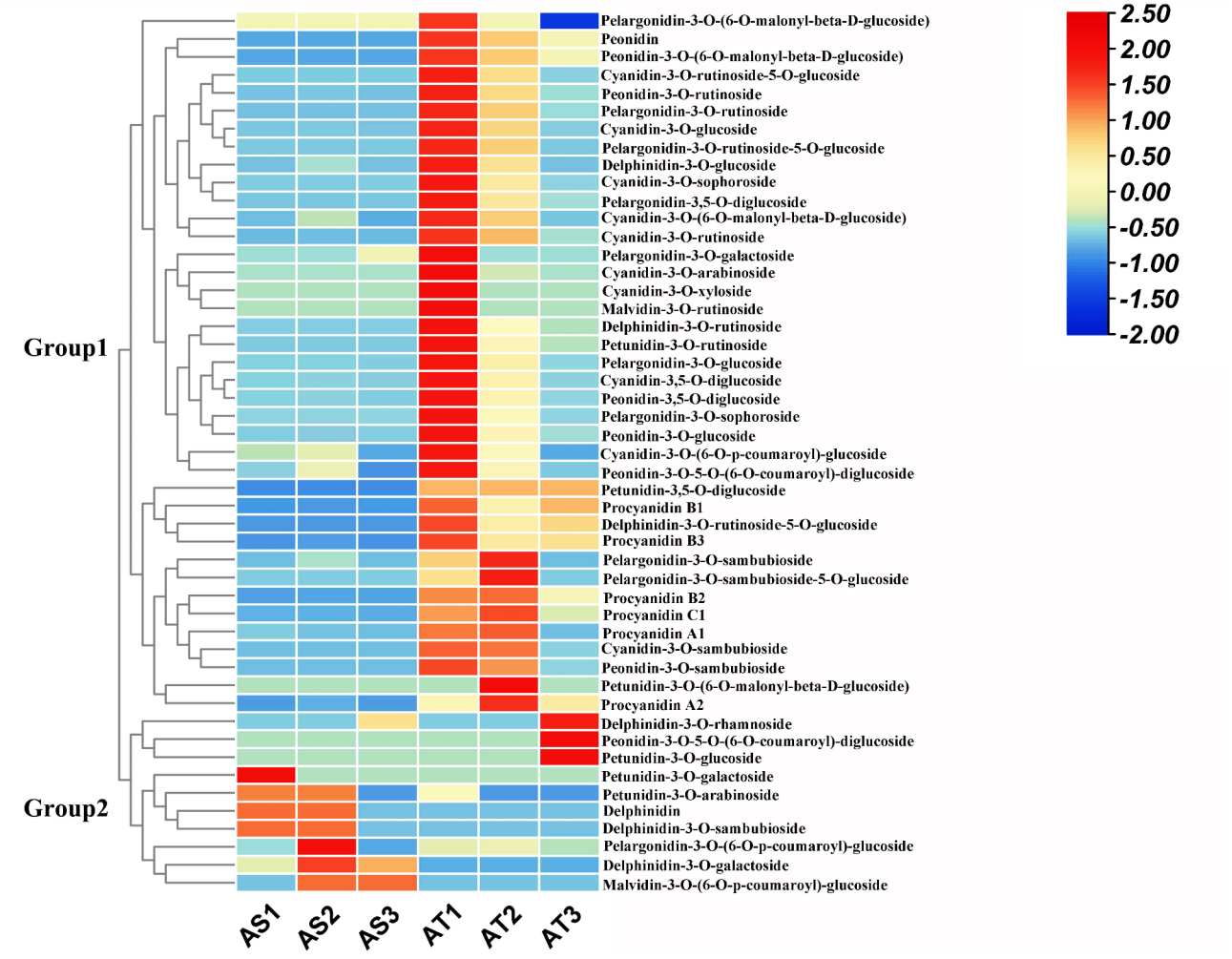

Fig. 4 Clustering heatmap of anthocyanin and procyanidin components in the exocarp of oil palm at different developmental stages (AS1, AS2, AS3, AT1, AT2, and AT3). The components are listed on the left side of the heatmap. The color of each square in the heatmap represents the relative abundance of the corresponding anthocyanin in the corresponding sample. The colors range from blue (low abundance) to red (high abundance). Two groups labeled on the left side, Group1 and Group2. Group1 appears to have higher levels of many anthocyanins compared to Group2

anthocyanin biosynthesis, it was obtained that, in Flavonoid biosynthesis, 35 significant differential genes were enriched in AS1_vs_AT1, 41 significant differential genes were enriched in AS2_vs_AT2, and AS3_vs_AT3 to 41 significantly different genes. In Anthocyanin biosynthesis, AS1_vs_AT1 was enriched for 1 significant difference gene with gene ID LOC105039612, AS2_vs_AT2 was enriched for 2 significant difference genes with gene ID LOC105039612 and LOC105046326, and AS3_vs_AT3 was enriched for 1 significant difference gene with gene ID LOC105039612 (Table 1). A gene LOC105039612 was found to co-exist in the comparison of three sets of data on the synthetic pathway of Anthocyanin biosynthesis, which was annotated as *UFGT* by NR (Non-Redundant Protein Database). It is hypothesized that this gene may play a key role in the synthesis of anthocyanins in the exocarp of oil palm.

Analysis of anthocyanin synthesis pathway in oil palm exocarp

In this study, we identified 14 enzyme genes involved in the anthocyanin production pathway of AS and AT fruits, including 3 *CHS* genes (LOC105036921, LOC105035716, LOC105036364), 1 *CHI* gene (LOC105045978), 1 *F3H* gene (LOC105054663), 2 *DFR* genes (LOC105040724, LOC105048473), 1 ANS gene (LOC105035842), 2 *UFGT* genes (LOC105039612, LOC105046326),3 *F3'5'H* genes (LOC105036086, LOC105044124, LOC105045493) and 1 *F3'H* gene (LOC105058071). The expression of these genes in the six periods of AS1, AS2, AS3, AT1, AT2 and AT3 are shown in Additional File 2. The highest expression levels in AS1 stage were found in the upstream *CHS* (LOC105036364) and *F3H* (LOC105054663), followed by the downstream *ANS* (LOC105035842) and individual *F3'H* (LOC105058071). In AT, the early and middle stages

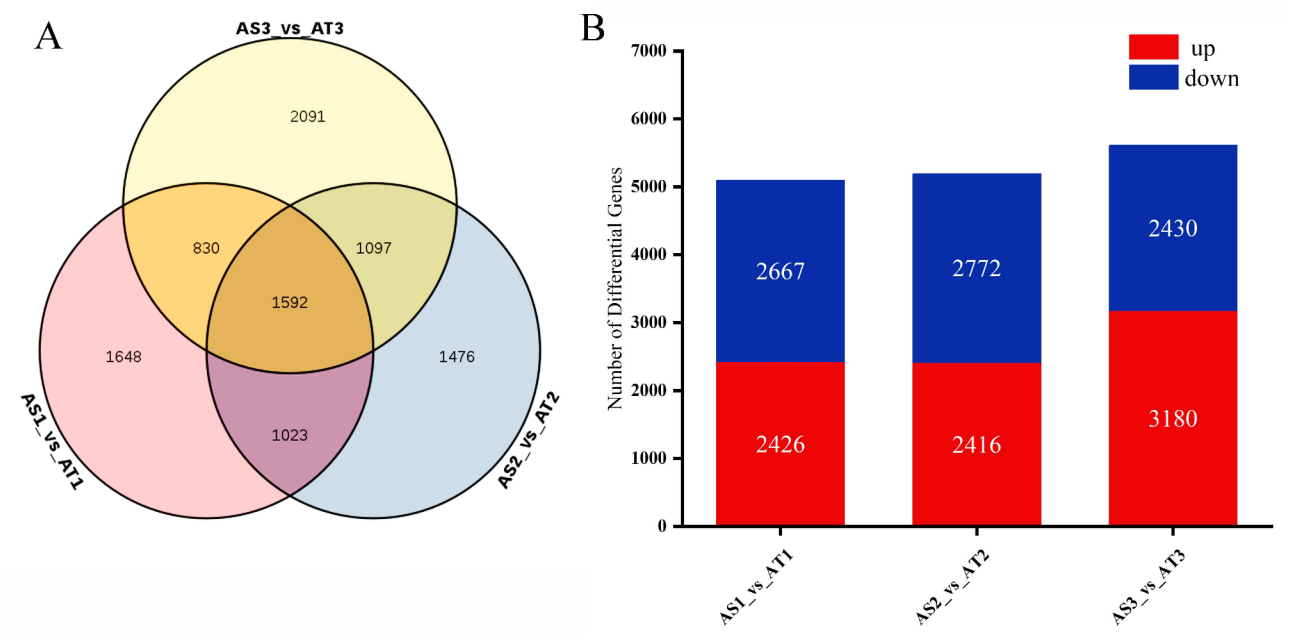

Fig. 5 Statistics of differential genes in the exocarp of oil palm fruits at different developmental periods (AS1_vs_AT1, AS2_vs_AT2, and AS3_vs_AT3). (**A**) differential gene Venn; (**B**) differential gene histogram

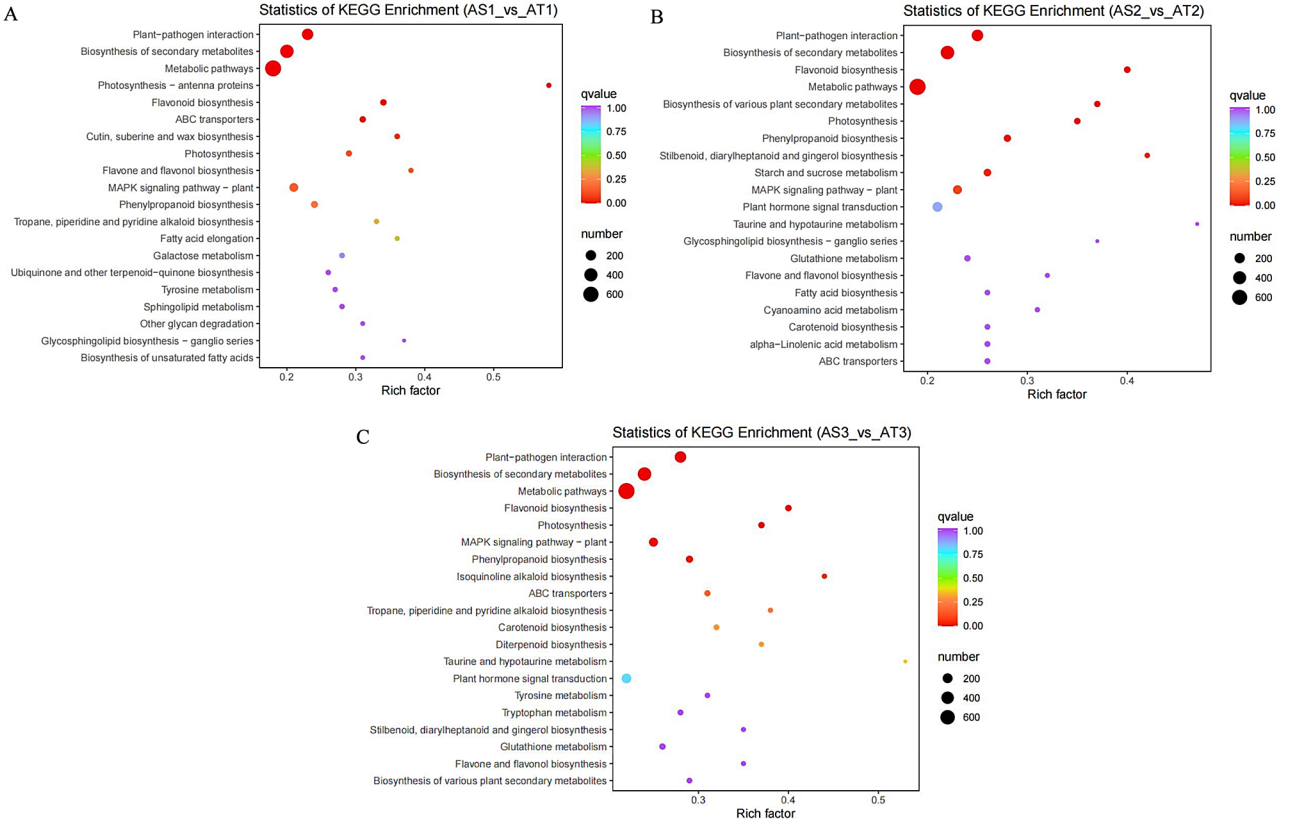

Fig. 6 Bubble map of KEGG enrichment in oil palm exocarp at different developmental periods. The size of the circle indicates the number of different genes, and the larger the circle, the more genes. q value is a p-value that has been verified by multiple factors. The value range is [0, 1]. It is represented by color. A red color indicates a smaller q value, indicating more obvious enrichment. (**A**) differential gene KEGG results for AS1_vs_AT1; (**B**) differential gene KEGG results for AS2_vs_AT2; (**C**) differential gene KEGG results for AS3_vs_AT3

Table 1 Differential genes enriched in flavonoid biosynthesis and anthocyanin biosynthesis in AS1_vs_AT1, AS2_vs_AT2 and AS3_vs_AT3

Comparable group	Biosynthetic pathway	Genes ID
AS1_vs_AT1	Flavonoid biosynthesis	LOC105045995;LOC105052309;LOC105050451;LOC105051171;LOC105051169;LOC105035716;LOC105036921;LOC105034617;LOC105050962;LOC105034618;LOC105060373;LOC105045834;LOC105054281;LOC105058232;LOC105044602;LOC105043935;LOC105055971;LOC105035639;LOC105037012;LOC105042335;LOC105060826;novel.515;novel.588;LOC105041053;LOC105048528;LOC105040723;LOC105040722;LOC105032764;LOC105053152;LOC105036086;LOC105044124;LOC105037552;LOC105045493;LOC105045491;LOC105036168
	Anthocyanin biosynthesis	LOC105039612
AS2_vs_AT2	Flavonoid biosynthesis	LOC105054663;LOC105059410;LOC105057212;LOC105045995;LOC105052309;LOC105050451;LOC105051171;LOC105051169;LOC105034618;LOC105060373;LOC105045978;LOC105041019;LOC105052295;LOC105054281;LOC105035842;LOC105058232;LOC105055971;LOC105035639;LOC105052441;LOC105033349;LOC105060153;LOC105060154;LOC105034344;LOC105037012;LOC105043757;LOC105039824;LOC105059098;LOC105036623;novel.588;LOC105041053;LOC105040723;LOC105040724;LOC105040722;LOC105048473;LOC105053152;LOC105053154;LOC105044124;LOC105037552;LOC105045493;LOC105045491;LOC105036168
	Anthocyanin biosynthesis	LOC105039612;LOC105046326
AS3_vs_AT3	Flavonoid biosynthesis	LOC105054663;LOC105059410;LOC105045995;LOC105052309;LOC105049276;LOC105051182;LOC105051169;LOC105036364;LOC105035716;LOC105036416;LOC105036921;LOC105034198;LOC105050962;LOC105034618;LOC105060373;LOC105045978;LOC105052295;LOC105035842;LOC105043935;LOC105055971;LOC105058071;LOC105035639;LOC105033349;LOC105060153;LOC105034344;LOC105037012;LOC105043757;LOC105059098;LOC105036623;LOC105042335;novel.515;LOC105041053;LOC105040724;LOC105040722;LOC105048473;LOC105053154;LOC105036086;LOC105044124;LOC105037552;LOC105045491;LOC105036168
	Anthocyanin biosynthesis	LOC105039612

of nigrescens oil palm growth (AT1, AT2), called the pigment accumulation stage, had high expression levels of the *CHS* (LOC105036364), *F3H*(LOC105054663), 2 *DFR* (LOC105040724,LOC105048473), *ANS*(LOC105035842), and *UFGT*(LOC105039612). Especially the expression of the *ANS* (LOC105035842) and *F3H* (LOC105054663). *CHS* (LOC105036921, LOC105035716), *CHI* (LOC105045978), and *F3'5'H* (LOC105036086) displayed greater expression levels in the late stage of fruit development (AT3). In conclusion, the majority of genes tended to express themselves highly in AT exocarps, which may be attributed to the abundance of anthocyanins in AT oil palm fruit. It is assumed that the genes of the same gene family play different roles in the accumulation of anthocyanins in the peel of the two different oil palm fruits based on the varied expression trends of the *CHS*(LOC105036921, LOC105035716, LOC105036364), *UFGT* (LOC105039612, LOC105046326), and *F3'5'H* (LOC105036086, LOC105044124, LOC105045493) in the two oil palm varieties. It is proposed that the genes from the same gene family perform various regulatory functions in the accumulation of oil palm fruit peel based on the variable expression levels of those genes in the same oil palm fruit. Combining the metabolome's composition and metabolite expression with the trend in gene expression, the aforementioned genes, which include the two downstream genes *ANS* (LOC105035842) and *F3'5'H* (LOC105036086) and three upstream genes *CHS* (LOC105036364), *CHI* (LOC105045978), and *F3H* (LOC105054663), significantly regulate the biosynthesis of anthocyanins (Additional File 3a-c, Fig. 7). The indicator of anthocyanin accumulation in fruit peel may be the anthocyanin expression level. The synthesis of anthocyanins in the oil palm peel is impacted by these genes with tremendous variability in expression.

QRT-PCR of the transcriptomic data

To validate the accuracy of the RNA-Seq expression profiles, six differentially expressed genes (LOC105045978, LOC105037862, LOC105050451, LOC105052309, LOC105052441, and LOC105055415) involved in related metabolic pathways were randomly selected and analyzed by quantitative real-time PCR (qRT-PCR) (Fig. 8A-F). The results demonstrated a strong correlation between the qRT-PCR and RNA-Seq data, with a coefficient of determination (R^2) greater than 0.8, indicating a high degree of consistency between the two methods.

Discussion

Difference analysis of total anthocyanin content in the exocarp of oil palm fruit

The concentration of anthocyanins is proportional to the depth of fruit peel color. The overall anthocyanin concentration in this study went from high to low:

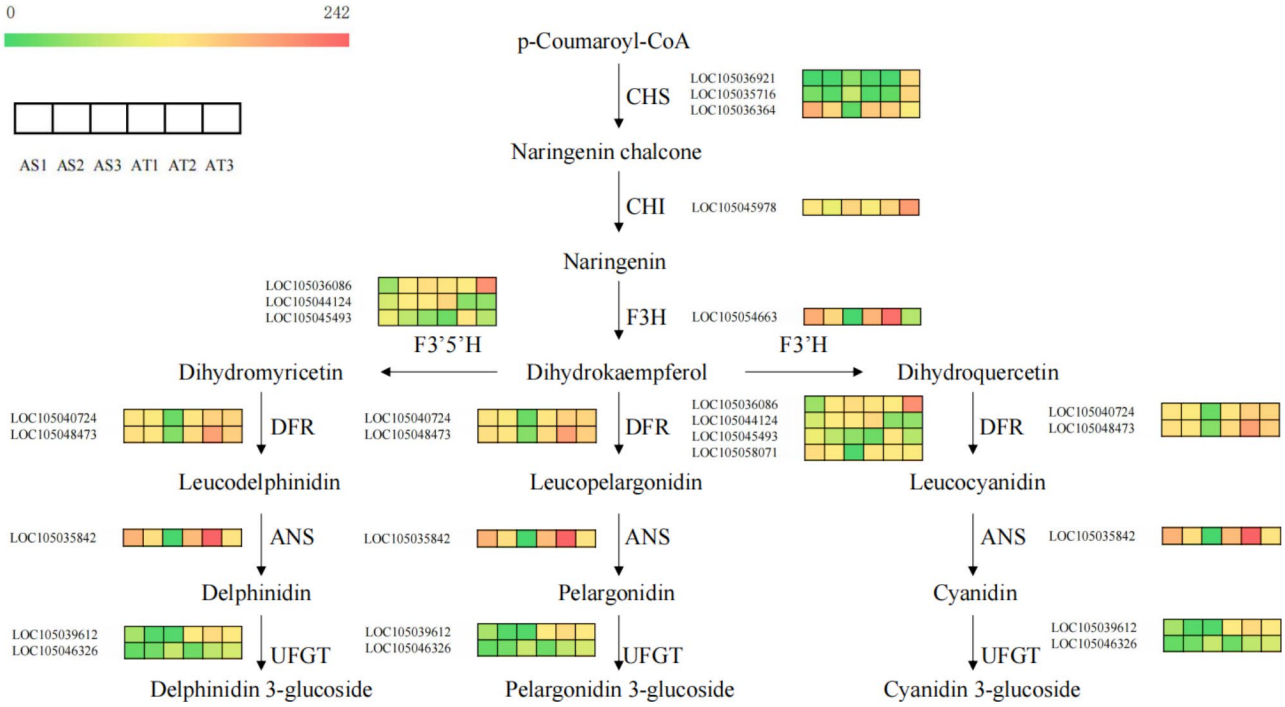

Fig. 7 Anthocyanin biosynthesis pathway in oil palm exocarp. Coloured squares represent clustered heatmaps of FPKM values for AS1, AS2, AS3, AT1, AT2 and AT3 periods. Green indicates gene down regulation, red indicates gene up regulation. Enzymes in this pathway are shown as follows: *CHS*, Chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone 3-hydroxylase; *F3′H*, flavonoid 3′-hydroxylase; *F3′5′H*, flavonoid 3′,5′-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase; *UFGT*, UDP-glucose: flavonoid 3-O-glucosyltransferase

purple black>purple red>orange red>green>yellow green>orange yellow, which corresponds to the shift in depth of fruit peel color. This effect is congruent with the conclusion reached after determining the total anthocyanin content in various carnation cultivars [16]. Furthermore, we discovered that as the oil palm fruit matured, the total anthocyanin content in its outer peel decreased, consistent with previous research suggesting that anthocyanin accumulation in pineapple peel decreases with fruit maturity [17]. For instance, previous studies, such as Hazir et al. (2012), have already investigated the changes in flavonoids and anthocyanins during oil palm fruit maturation, and the differentially expressed genes involved in anthocyanins biosynthesis in both virescens and nigrescens oil palms have been reported. The amount of anthocyanins in the AS peel grew from 17.37 μg/g in AS1 to 27.76 μg/g in AS2, and decrease to 8.19 μg/g in AS3, whereas it declined in AT from 2112.35 μg/g in AT1 to 1676.37 μg/g in AT2 to 447.90 μg/g in AT3. The AT peel contained 50–120 times more anthocyanins overall than the AS peel. The anthocyanin content in the outer peel of AS fruits with yellow peel color is relatively low, primarily due to the composition of flavonoids and carotenoids in yellow pigments [18], which is consistent with the research results on changes in anthocyanin composition and content during honeysuckle flowering [19]. Overall, it is speculated that the anthocyanin synthesis pathway is mainly involved in the formation of peel color in the AT, which is consistent with the research results of Suraninpong et al. [13].

Identification of anthocyanins in oil palm fruit peel

The oil palm exocarp is rich in anthocyanins, which are involved in the formation of fruit color. In recent years, metabolomics has been widely used in the study of plant color. Utilizing UPLC-MS/MS technology, a metabolomics investigation was carried out on the Hong caitai and Caixin [20]. According to the findings, the distribution of chemical content in the two forms of Caixin differs dramatically. A total of 170 distinct flavonoid metabolites, including 32 flavonoids, 38 anthocyanins, and 56 flavonol metabolites, were found. Gao et al. [21] laid the groundwork for further investigation into the metabolic regulatory mechanism of mango fruit coloration by using carotenoid metabolomics to analyze the commercial and physiological ripening stages of mango flesh and finding 68 types of secondary metabolites of carotenoids in the flesh. This study utilized UPLC-MS/MS technology to explore the metabolomics of anthocyanins and proanthocyanidins in the outer peel of AS and AT fruits, showing scant earlier research on oil palm peel color composition. The presence of anthocyanins and proanthocyanidins was found in the AS and AT from the early to late stages of development,

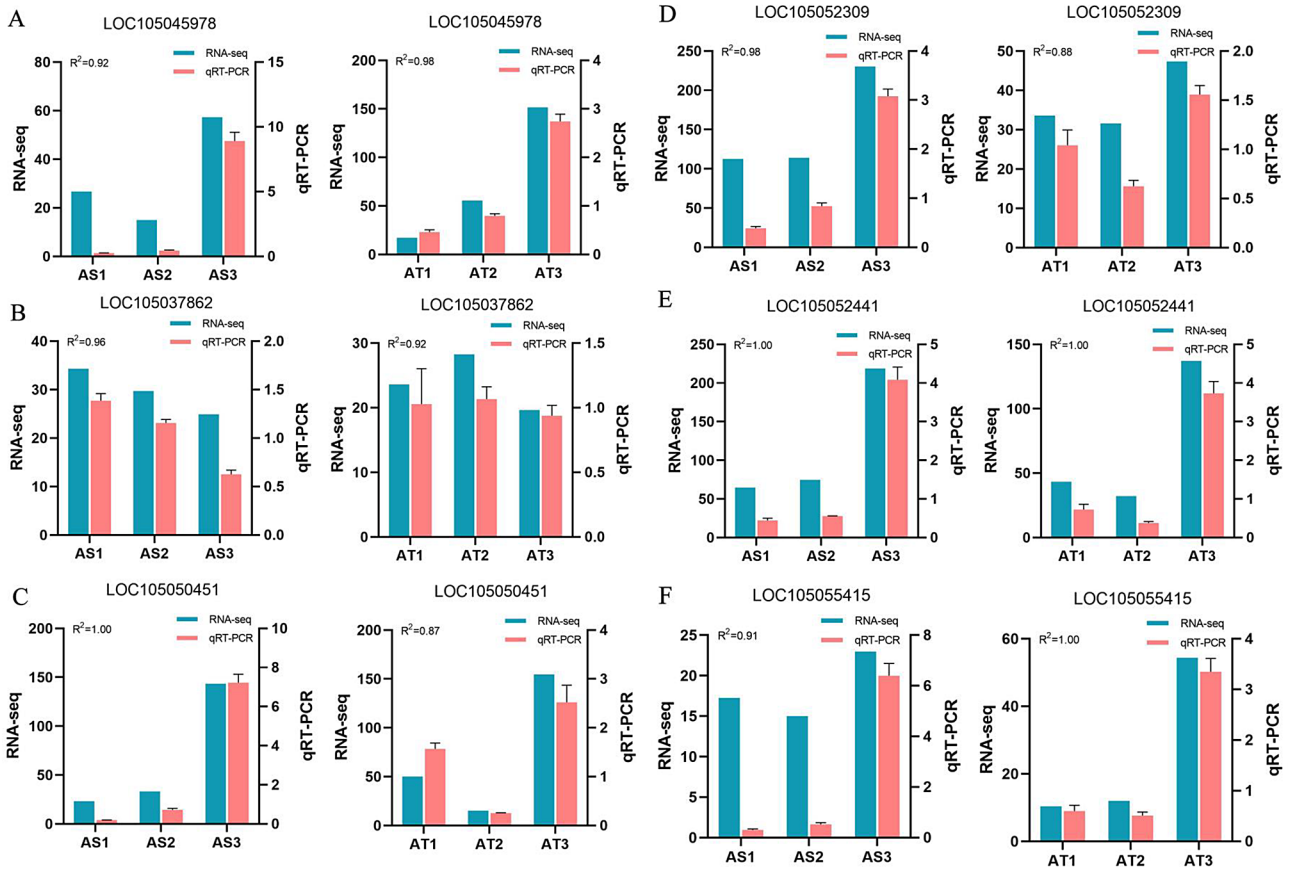

Fig. 8 Relative expression levels of LOC105045978 (**A**) LOC105037862 (**B**) LOC105050451 (**C**) LOC105052309 (**D**) LOC105052441 (**E**) and LOC105055415 (**F**) genes during the synthesis of oil palm exocarp at different developmental times (AS1, AS2, AS3, AT1, AT2, and AT3).R2 represents the correlation coefficient between FPKM value and RNA seq value, with $R^2 > 0.8$ indicating that the expression trends of the two tend to be consistent

although the content and types of anthocyanins and proanthocyanidins were constantly changing. A total of 43 anthocyanins, including cyanidin derivatives, delphinidin and its derivatives, mallow pigment derivatives, petunia derivatives, geranium derivatives, paeoniflorin and its derivatives, and six proanthocyanidins, are present in AS and AT. It was found that AT had 45 compounds compared to AS's 31. Proanthocyanidin concentration in AT is around 70 times greater than in AS. In addition, no cyanidin monomers were detected in this study, but 10 cyanidin derivatives were detected, including cyanidin-3,5-O-diglucoside(H1), cyanidin-3-(6-O-p-p-coumarin)-glucoside(H2),cyanidin-3-O-xyloside(H3),cyanidin-3-O-arabinoside(H4),cyanidin-3-O-(6-O-malonyl)-β-D-glucoside(H5),cyanidin-3-O-sophoroside(H6),cyanidin-3-O-glucoside(H7),cyanidin-3-O-rutoside-5-O-glucoside(H8),cyanidin-3-O-rutoside (H9), and cyanidin-3-O-sambucoside (H10) are due to the cleavage of glycosidic bonds caused by cyanidin derivatives in acidic mobile phases, resulting in the formation of cyanidin monomers [22]. In this study, the metabolomics detection approach used a mobile phase of 0.1% formic acid with low acidity, making it difficult for cyanidin derivatives to hydrolyze. As a result, numerous distinct cyanidin derivatives were discovered, but cyanidin monomers were not. This is congruent with the findings of Lu et al. [23] transcriptome and metabolome investigation on the mechanism of anthocyanin alterations in red maple leaves.

In addition, procyanidin content was generally higher in the exocarp of oil palm in both fruit types compared to other anthocyanins. Among them, procyanidin B2 (H47) and procyanidin C1 (H49) were higher at 622.38 μg/g and 315.52 μg/g during AT2, while procyanidin B3 (H48) was higher at 84.29 μg/g during AT1. The above results showed that the content of proanthocyanidins was significantly higher than that of other types of anthocyanin metabolites in both AS and AT fruit types of oil palm. This is in line with the results that the peel of apple is also rich in proanthocyanidins [24].

Genes involved in anthocyanin synthesis in oil palm peel

The discovery of potential genes for the anthocyanin biosynthetic pathway in this plant has been made possible by mining differentially expressed genes associated with anthocyanin synthesis based on transcriptome

sequencing. Transcriptome data analysis reveals distinct genes involved in anthocyanin production pathways in red pear, apple, and myrtle fruits [25–27]. In this study, the *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, *UFGT*, *F3'5'H*, and *F3'H* genes were identified through the transcriptome as possible regulators of exocarp color formation in AS and AT. It was found that *CHS*, *CHI*, *F3H*, *F3′H*, *F3′5′H*, *ANS* and *UFGT* regulate anthocyanin synthesis in kiwifruit, mulberry, aubergine, banana and litchiand other plant fruit anthocyanin synthesis, which are involved in the formation of fruit color [28–33].Similarly, it was found in this study that the *CHS* gene (LOC105036364) expression level was positively correlated with the content of anthocyanins in AS and AT, indicating its high expression during early fruit development, which is consistent with the positive correlation between *CHS* gene expression and anthocyanin accumulation in flesh peach fruits [34]. When compared to the expression of the *CHS* gene (LOC105036364 LOC105036921, LOC105035716) is significantly accumulated in the latter stages of development, indicating that different genes from the same gene family play diverse roles in producing oil palm peel color. Similarly, *CHS* synthesis of anthocyanins in eggplant peel shows two or more expression patterns [35]; A number of genes, including *CHS* (LOC105036364), *F3H* (LOC105054663), two *DFR* genes (LOC105040724, LOC105048473), *ANS* (LOC105035842), and *F3′H* (LOC105058071), may regulate the production of anthocyanins in the peel of two different types of oil palm fruits, causing the peel to turn orange red when AT fruits mature and orange yellow when AS fruits grow. This is consistent with studies that emphasize the formation of cherry red and yellow peels is mediated through different enzyme genes in anthocyanin biosynthesis pathways, including *CHS*, *CHI*, *F3H*, *DFR*, and *ANS* [36]; The expression levels of *CHS* (LOC105036364), *F3H* (LOC105054663), and *ANS* (LOC105035842) in the early stages of oil palm fruit development are 3–30 times higher than other enzyme genes, and their expression patterns are consistent. All were elevated in the initial stages of development and down regulated in the end. Therefore, the early phases of the development of the peel of oil palm fruit may be influenced by the formation of anthocyanin by *CHS* (LOC105036364), *F3H* (LOC105054663), and *ANS* (LOC105035842); The expression patterns of *CHI* (LOC105045978) and *F3'5'H* (LOC105036086, LOC105044124, LOC105045493) genes were comparable throughout two types of oil palm fruits, reaching peak levels in the latter stages, 3–15 times higher than other enzyme genes. The expression levels of 14 differential genes in AS are typically higher than in AT, indicating that the anthocyanin production pathway is primarily involved in fruit peel color development in the nigrescens oil palm, and these genes are speculated to be responsible for high anthocyanin accumulation in the later stages of oil palm fruit development.

In addition, the KEGG-enriched anthocyanin biosynthesis pathway revealed the co-existence of the gene *UFGT* (LOC105039612) in comparisons of AS1_vs_AT1, AS2_vs_AT2, and AS3_vs_AT3. The gene *UFGT* was lowly expressed in all three periods of AS, and conversely, it was highly expressed in AT, especially in the AT1 and AT2 period. This expression trend was consistent with the trend of Cyanidin-3-O-glucoside (H7), which was low in AS but high in AT1 (484.15 μg/g) and AT2 (273.87 μg/g). It has been reported that Cyanidin can form Cyanidin-3-O-glucoside (H7) catalyzed by *UFGT*(LOC105039612).Yi et al. [37] similarly found that the *UFGT* gene was significantly up-regulated and expressed during the formation of red exocarps in litchi, which plays a key role in the formation of Cyanidin-3-O-glucoside. Thus, it is speculated that the high expression in AT in this study may have a promoting effect on the formation of red exocarps in oil palm fruits.

Conclusion

In this study, we investigated the biosynthetic pathway of anthocyanins in the exocarp of virescens and nigrescens oil palm varieties. Our results identified key genes involved in anthocyanin biosynthesis—CHS (LOC105036364), F3H (LOC105054663), DFR (LOC105040724, LOC105048473), ANS (LOC105035842), UFGT (LOC105039612), and F3'5'H (LOC105036086, LOC105044124, LOC105045493)—which were up-regulated and expressed predominantly during the pre-middle stages of fruit development (AS1, AS2, AT1, and AT2). This gene expression pattern aligns with the observed decrease in total anthocyanin content as the oil palm fruit matures, with levels decreasing from 119 μg/g (AS1) to 23 μg/g (AS3) in green-fruited palms, and from 1302 μg/g (AS1) to 170 μg/g (AS3) in black-fruited palms.

Our findings suggest that these genes play a crucial role in regulating anthocyanin biosynthesis during the early stages of oil palm fruit development, a period characterized by rapid anthocyanin accumulation. These insights provide a foundation for future genetic transformation efforts aimed at improving oil quality by enhancing anthocyanin content.

Additionally, the study observed a significant decrease in total anthocyanin content during fruit maturation, with the highest levels detected in the early stages. The most prominent anthocyanins included peonidin-3-O-rutinoside (H35), pelargonidin-3-O-rutinoside (H21), and cyanidin-3-O-glucoside (H7), along with condensed tannins such as procyanidin B2 (H47), procyanidin C1 (H49), and procyanidin B3 (H48).

In conclusion, this study provides a comprehensive understanding of the anthocyanin biosynthetic pathway in oil palm exocarps and highlights the changes in anthocyanin content throughout fruit development. These findings offer valuable insights for the oil palm industry, informing strategies to enhance the ornamental value of oil palms, optimize fruit harvesting, and explore the potential of anthocyanins for various applications. The research provides a scientific basis for targeted improvements in the color of oil palm exocarps and the selection and breeding of oil palm varieties with high anthocyanin content.

Materials and methods

Plant materials

Fresh oil palm fruits were harvested at the National Tropical Palm Germplasm Resource Nursery, Wenchang, Hainan Province, China (110.8°latitude, 19.6°longitude). A total of 30 AS and AT oil palm trees were chosen randomly and split into three groups. In order to determine the total anthocyanin content, the outer peel of each group was chosen 95 days (early development stage of oil palm fruits, AS1 and AT1), 125 days (medium development stage of oil palm fruits, AS2 and AT2), and 185 days (late development stage of oil palm fruits, AS3 and AT3) following pollination. The fresh outer peel was also liquid nitrogen-frozen and kept in a -80 °C refrigerator for later metabolomics and transcriptomics research. Sample collection and determination of oil palm fruit exocarp at each period followed the principle of biological replicates and three parallel replicates.

Determination of total anthocyanoside content

Determination of total anthocyanins in the exocarp of AS and AT using a total anthocyanoside content assay kit (Suzhou Grace Biotechnology Co., Ltd.).The total anthocyanin content of oil palm exocarp was determined at six periods, AS1, AS2, AS3, AT1, AT2 and AT3.The frozen oil palm exocarp samples should be taken out of the refrigerator at -80 °C, weighed 0.1 g of the sample, and then 1 mL of the extraction solution added. The samples should then be shaken and extracted at 75 °C for 25 min, at room temperature, and 12,000 rpm for 10 min. After more than 30 min of preheating, distilled water was used to zero the UV-visible spectrophotometer. 200 μL of supernatant should be pipetted into the appropriate measurement and control tubes. After that, 600 μL of reagent I and 600 μL of reagent II were added to the measurement tube and control tube, respectively. Reagent I and Reagent II are reagents that come with the kit. The entire cleared liquid was placed into a 1 mL glass cuvette (optical diameter), shielded from light, and allowed to equilibrate at room temperature for 60 min. The total anthocyanoside content of the samples was then calculated using the absorbance at 530 and 700 nm.

Total anthocyanoside content (mg/g)= $(\Delta A \div (\varepsilon \times d) \times V2 \times 103 \times Mr) \div (W \times V1 \div V) \times D$(ε: extinction coefficient of Cyanidin-3-O-glucoside, 29600 L/mol/cm; Mr: molecular weight of Cyanidin-3-O-glucoside, 449.2; d: optical range; D: dilution; W: weight of the sample, g; V: sample extract, 1 mL; V1: volume of supernatant spiked with samples, 200μL$=2\times10^{-4}$ L; V2: total volume of the assay, 800 μL$=8\times10^{-4}$ L).

Metabolite extraction

Anthocyanins were extracted from the exocarp of oil palm at six periods, AS1, AS2, AS3, AT1, AT2 and AT3, and the experiment was repeated three times in parallel for each period. The sample was freeze-dried, ground into powder (30 Hz, 1.5 min), and stored at -80 °C until needed. 50 mg powder was weighted and extracted with 0.5 mL methanol/water/hydrochloric acid (500:500:1, V/V/V). Then the extract was vortexed for 5 min and ultrasound for 5 min and centrifuged at 12, 000 g under 4 °C for 3 min. The residue was re-extracted by repeating the above steps again under the same conditions. The supernatants were collected, and filtrated through a membrane filter (0.22 μm, Anpel) before LC-MS/MS analysis.

Metabolomics analysis and data processing

A MWDB (Metware Database) was constructed based on the standards to qualitatively analyze the data detected by mass spectrometry. The data were analyzed quantitatively using Multiple Reaction Monitoring (MRM) mode of triple quadrupole mass spectrometry. The data were acquired with Analyst 1.6.3 (Sciex) software, and the mass spectrometry data were processed with MultiQuant 3.0.3 (Sciex) software. The retention time and peak shape information of the standards were referenced, and the integral correction was applied to the chromatographic peaks of the analytes to be tested that were detected in different samples, in order to ensure the accuracy of the qualitative and quantitative analyses. After obtaining the mass spectrometry data of different samples, the chromatographic peaks of all the targets were integrated and quantified by standard curves. MRM model represents the quantitative results of metabolites (Additional File 4a-b).The anthocyanin contents detected at AS1, AS2, AS3, AT1, AT2 and AT3 periods are shown in Supplementary Table S1, with three parallel replicates of values for each period. To determine significant changes in metabolite abundance, we applied a fold change threshold of ≥ 2 or ≤ 0.5, along with a statistical significance threshold of $p \leq 0.05$.Analysis of variance (ANOVA) and significance of differences were tested by one-way ANOVA test for differential metabolites using SPSS 26.0.

Total RNA extraction and high-throughput sequencing

Total RNA was extracted from 18 samples from six periods, AS1, AS2, AS3, AT1, AT2 and AT3 using the instructions of RNA extraction kit(Tengen Biochemical Technology Co.). Transcriptome sequencing of AS and AT oil palm exocarp was performed using the Illumina HiSeq platform. Assembled of Clean Reads to the reference genome(Genomeassembly EG5 http://www.ncbi.nlm.nih.gov/assembly/GCA-000442705) was performed using HISAT2 to obtain positional information on the reference genome or gene as well as information on sequence features specific to the sequenced samples. Gene expression was calculated using FPKM (Fragments Per Kilobase of transcript per Million fragments mapped).The quality of RNA-seq is shown in Additional File 5.

Transcriptome data processing and differentially expressed genes analysis

Significantly differentially expressed genes between AS and AT oil palms at different developmental periods were analyzed using DESeq2/edgeR, with a screening threshold of FDR (False Discovery Rate)<0.05 and |log2Fold Change| ≥ 1. Enrichment analyses based on the hypergeometric test were performed by applying the hypergeometric test to Pathways in the KEGG database (https://www.genome.jp/kegg) to identify Pathways significantly enriched in differentially metabolised and differentially expressed genes compared to the whole genomic background. For the number of shared KEGG pathways exceeding 20, only the top 20 pathways in terms of (significance of enrichment) P-value are shown [36, 38]. Pearson correlation coefficient was used to calculate the quantitative correlation between the absolute content of free fatty acid metabolites and the expression of related genes. Correlation results with correlation coefficient>0.80 and pvalue<0.05 were selected.

Real-time quantitative PCR

Quantitative PCR was run using oil palm exocarp RNA from six periods, AS1, AS2, AS3, AT1, AT2 and AT3, as templates. Six genes with potential roles during anthocyanoside synthesis in oil palm exocarps were selected for real-time quantitative PCR reactions. qRT-PCR primers were designed using NCBI (Additional File 6) and Actin was used as an internal reference gene [39]. The QuantStudioTM7 Real-Time PCR Instrument was used to determine the relative expression of selected genes in 96 microtiter plates. The relative expression of the selected genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The qRT-PCR reaction system used in this study was 10 μL, in which SYBR® Select Master Mix (2X) 5 μL, cDNA template 0.5 μL, positive primer 0.5 μL, water (without RNAase) 3.5 μL, and negative primer 0.5μL. The reaction conditions were as follows: phase I UDG activation at 50 °C for 2 min; phase II UP activation at 95 °C for 2 min; and phase III UP activation at 95 °C for 2 min. 95 ℃ for 2 min; the third stage of denaturation 95 ℃ for 15 s for a total of 40 cycles. The fourth stage of annealing/extension was 60 °C for 1 min [40].

Data analysis

The statistical analysis comprised a three-pronged approach, encompassing metabolomics analysis via Multiple Reaction Monitoring and MultiQuant software, with significance testing performed using one-way ANOVA in SPSS 26.0; transcriptome data processing utilizing DESeq2/edgeR, with enrichment analyses conducted using the hypergeometric test, and real-time quantitative PCR calculation utilized the 2-ΔΔCt method. Heat maps were generated using the Complex Heat map package in R software Differential metabolites were annotated and presented using KEGG database. Venn diagram package (v1.6.20) in RStudio for Venn diagram generation and ggplot2 package (v3.3.2) and cowplot package (v1.0.0) in RStudio for figure generation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05607-2.

Supplementary Material 1

Author contributions

C.Y. S.Z. X.F. X.L. S.C. conducted the experiments and performed the data analysis. H.C. organized and supervised the overall project. X.L. guided the analysis of experimental data. C.Y. and S.Z. wrote the manuscript. J.M. edited the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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