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Combined transcriptome and metabolome analysis of sugar and fatty acid of aromatic coconut and non-aromatic coconut in China

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ABSTRACT

Sugar and fatty acid content are among the important factors that contribute to the intensity of flavor in aromatic coconut. Gaining a comprehensive understanding of the sugar and fatty acid metabolites in the flesh of aromatic coconuts, along with identifying the key synthetic genes, is of significant importance for improving the development of desirable character traits in these coconuts. However, the related conjoint analysis of metabolic targets and molecular synthesis mechanisms has not been carried out in aromatic coconut until now. UPLC-MS/MS combined with RNA-Seq were performed in aromatic coconut (AC) and non-aromatic coconut (NAC) meat at 7, 9 and 11 months. The results showed that D-fructose in AC coconut meat was 3.48, 2.56 and 3.45 fold higher than that in NAC coconut meat. Similarly, D-glucose in AC coconut meat was 2.48, 2.25 and 3.91 fold higher than that in NAC coconut meat. The NAC coconut meat showed a 1.22-fold rise in the content of lauric acid compared to the AC coconut meat when it reached 11 months of age. Myristic acid content in NAC coconut meat was 1.47, 1.44 and 1.13 fold higher than that in AC coconut meat. The palmitic acid content in NAC coconut meat was 1.62 and 1.34 fold higher than that in AC coconut meat. The genes *SPS*, *GAE*, *GALE*, *GLCAK*, *UGE*, *UGDH*, *FBP*, *GMLS*, *PFK*, *GPI*, *RHM*, *ACC*, *FabF*, *FatA*, *FabG*, *and FabI* exhibited a negative correlation with D-fructose ($r = -0.81$) and D-glucose ($r = -0.99$) contents, while showing a positive correlation ($r = 0.85–0.96$) with lauric acid and myristic acid. Furthermore, *GALE*, *GLCAK*, *FBP*, *GMLS*, and *ACC* displayed a positive correlation ($r = 0.83–0.94$) with palmitic acid content. The sugar/organic acid ratio exhibited a positive correlation with *SPS*, *GAE*, *UGE*, *FabF*, *FabZ* and *FabI*.

1. Introduction

The coconut (*Cocos nucifera* L. 2n = 32) is a monophyletic genus within the Arecaceae family (Nair et al., 2016). Coconut, widely cultivated in tropical and subtropical regions, is a renowned tropical fruit tree, woody oil crop, and a significant source of energy. Due to its versatility and numerous uses, coconut is frequently stated as the "tree of life" (Foale, 2003). The coconut fruit is composed of different layers, including the inner layer (endocarp), middle layer (mesocarp) and outer layer (exocarp), which encloses the kernel or endosperm (Chakraborty and Mitra, 2008). Inside the endosperm of the coconut, a distinction exists between the liquid endosperm, often referred to as coconut water and the solid endosperm, commonly known as coconut meat. In the early stages, the coconut meat typically possesses a semi-solid texture that progressively thickens as the fruit matures; partly decrease the volume of water (Reddy and Lakshmi, 2014).

The composition of both coconut meat and water includes high fibers, vitamins, phytohormones, phytochemicals, sugars, fatty acids and amino acids contents (Tuyekar et al., 2021). Incorporating coconut meat and water into one's regular diet can offer ample nutrition to the body and contribute to disease prevention. Studies have demonstrated that the presence of crude fiber and protein in coconut meat can effectively lower cholesterol absorption and facilitate its excretion from the body, thus aiding in the reduction of blood lipids. Predominantly composed of saturated fatty acids, the free fats found in coconut meat primarily consist of lauric acid and myristic acid. Coconut water is rich in essential mineral ions, with magnesium and potassium being the most abundant (Kumar et al., 2021). These minerals play a crucial role in reducing low-density lipoprotein, stabilizing platelets, and regulating blood coagulation (Mineo, 2020). Potassium, as the primary cation in intracellular

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fluid, helps to sustain acid-base balance, osmotic pressure, and water balance within the body (Yamada and Inaba, 2021). It also enhances muscle excitability, sustains regular heartbeats, and actively participates in protein, carbohydrate, and heat energy metabolism (DebMandal and Mandal, 2011).

Aromatic coconut, originally from Thailand, is a special variety of green dwarf coconut (Yang et al., 2020). Aromatic coconut exhibits exceptional traits of early maturation, high dwarf coconut yield, and possesses a delightful fragrance in both its water and meat. The meat of aromatic coconut is regarded as one of the finest natural green foods because of high sugars (Bauwens et al., 2023, Phonphoem et al., 2022). The introduction of aromatic coconut to China took place in 1998, followed by its plantation at the Coconut Research Institute (CRI), Chinese Academy of Tropical Agricultural Sciences (CATAS) in 2001. Subsequently, in 2006, these aromatic coconut trees successfully bloomed and bore fruit. Due to its distinctive aroma, aromatic coconut has gained significant popularity in the market. Simultaneously, aromatic coconut demonstrates both high productivity and early fruiting capabilities. In Hainan province, the market value of fresh aromatic coconuts significantly surpasses that of local coconut varieties. After pollination, the coconut fruit takes about 12 months to fully mature. At 6 months, solid endosperm begins to form on the inner wall of the embryo sac cavity. The endosperm first appears at the top of the fruit and gradually extends towards the peduncle. At 7 months, a thin layer of gelatinous material is formed, and the sugar content gradually increases. The sugar content begins to decrease at 9 months, and stabilizes at 11 months. Sugars and acids activate taste receptors, and flavor can be attained by increasing sugar and acid contents in many fruits. Flavor compounds in tender coconut water may be formed from the degradation of fatty acids, i.e. oxidative metabolism involving mechanisms such as p-oxidation and lipoxygenase (LOX) pathways (Jirapong et al., 2015). Additionally, fatty acids play a crucial role in influencing the sensory attributes of coconut. Besides, the maturity and quality of a specific variety of fruit relies on sugar to fatty acids ratio (Furuta et al., 2023). Genes responsible for encrypting the aluminum-activated malate transporter are also known to be associated with the regulation of fatty acid levels (Zhang et al., 2019). Exploring the roles of organic acids and sugars as chemical messengers in morphogenesis of plant poses a significant challenge, particularly in understanding their roles and relationships with other cellular communication pathways (Igamberdiev and Bykova, 2023, Jawad et al., 2020). Transcriptomic technologies have been utilized to elucidate the genes associated with the metabolism of fatty acids and sucrose (He et al., 2023, Liu et al., 2020). Thus, this study proposes a plausible mechanism for recognizing prominent candidate genes that are accountable for determining sugar and fatty acid content. The metabolite profiles of aromatic coconut and non-aromatic coconut fruits were assessed using UPLC-MS/MS, whereas the expression levels of specific genes that encode metabolic enzymes were assessed during three distinct stages of development.

2. Results

2.1. Identification of metabolites

Our metabolomic study identified a total of 204 metabolites including 20 alkaloids, 19 lipids, 30 amino acids and derivatives, 12 nucleotides and derivatives, 3 lignans and Coumarins, 5 flavonoids, 38 organic acids, 23 phenolic acids and 54 others. The detailed information on metabolites identified in this study is provided in supplementary Table 1.

2.2. Sugar and fatty acid profiles during fruit ripening of AC and NAC

From 7 to 11 months, there was a consistent upward trend observed in the levels of lauric acid, myristic acid, palmitic acid, D-glucose and D-fructose in both AC and NAC coconut fruit meat (Fig. 1). At all three evaluated developmental stages, the content of D-glucose and D-fructose in AC coconut meat exceeded that of NAC coconut meat (Fig. 1A and B). Conversely, NAC coconut meat exhibited greater amounts of palmitic acid, myristic acid, and lauric acid compared to AC coconut meat (Fig. 1C-E). In terms of specific performance, the content of D-fructose in AC coconut meats surpassed that in NAC coconut meat by 3.48-fold, 2.56-fold, and 3.45-fold at 7, 9, and 11 months, respectively. Similarly, D-glucose in AC coconut meats exhibited 2.48-fold, 2.25-fold, and 3.91-fold higher levels compared to NAC coconut meat at 7, 9, and 11 months, respectively. At 11 months, NAC coconut meat demonstrated a 1.22-fold rise in lauric acid content compared to AC coconut meat. Additionally, myristic acid content in NAC coconut meat was 1.47-fold,

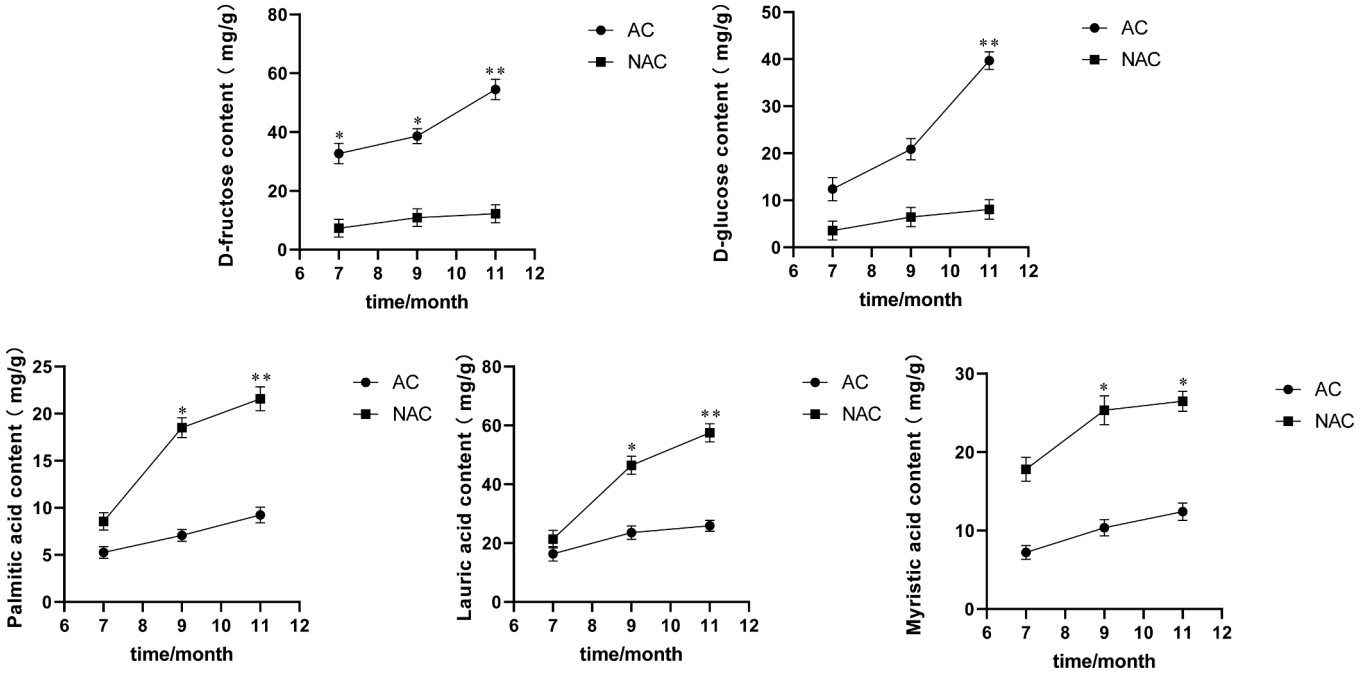

Fig. 1. Developmental profiles of D-fructose, D-glucose, Lauric acid, Myristic acid and Palmitic acid contents, in AC and NAC fruits at three developmental stages.

1.44-fold, and 1.13-fold greater than that in AC coconut meat at 7, 9, and 11 months, respectively. Moreover, palmitic acid content in NAC coconut meat exceeded that in AC coconut meat by 1.62-fold and 1.34-fold, at 9, 11 months respectively.

2.3. Transcriptome analysis of AC and NAC coconut meat

For transcriptome analysis, a total of 18 RNA samples were obtained from three biological replicates of AC and NAC coconut meat at 7, 9, and 11 months. These samples yielded a combined total of 774.59 million clean reads, accounting for approximately 116.21 gigabases of clean bases (Supplementary Table 2). The average clean bases per sample were approximately 6.46 gigabases, with a Q30 percentage exceeding 92.00 %. The clean reads ratio ranged from 93.11 % to 98.52 %.

2.4. Analysis of differentially expressed genes

The transcriptomic data obtained from three developmental stages of two genotypes of coconut fruit meat were categorized into seven groups for the identification of differentially expressed genes (DEGs) (NAC7-vs-AC7, NAC9-vs-AC9, NAC11-vs-AC11, AC7-vs-AC9, AC9-vs-AC11, NAC7-vs-NAC9, and NAC9-vs-NAC11) (Fig. 2). At 7, 9, and 11 months, significant changes in the number of Differentially Expressed Genes (DEGs) were observed in AC and NAC coconut meat, with 773, 155, and 2744 transcripts identified, respectively (Fig. 2A). AC7-vs-AC9 and NAC7-vs-NAC9 displayed 1332 and 1920 DEGs, respectively. Conversely, AC9-vs-AC11 and NAC9-vs-NAC11 exhibited a higher number of DEGs (3982 and 7195, respectively). Among these, the number of DEGs shared between AC and NAC at 7, 9, and 11 months was 41 (Fig. 2B), whereas DEGs specific to NAC7-vs-NAC9 and NAC9-vs-NAC11, AC7-vs-AC9 and AC9-vs-AC11 were 859 and 303, respectively (Fig. 2B). Overall, the up-regulated genes outnumbered the down-regulated genes.

2.5. Identification of key processes associated with sugar and fatty acid accumulation in AC and NAC coconut meat

Functional enrichment analysis of the differentially expressed genes (DEGs) was performed using Gene Ontology (GO) terms to identify the main functional categories. The top five significantly improved Gene Ontology (GO) terms were noted in the biological process, cellular component, and molecular function categories for AC and NAC at 7, 9, and 11 months (Fig. 3). In the biological process category, GO terms related to translation regulator activity, nucleic acid binding, ATP synthesis-coupled proton transport, organic cyclic compound binding, translation initiation factor activity, and translation regulator activity were prominent. Within the cellular component category, GO terms associated with protein-containing complex, cellular anatomical entity, photosystem II, intracellular, and eukaryotic 48S preinitiation complex were highly enriched. In the molecular function category, GO terms such as cellular nitrogen compound biosynthetic process, primary metabolic process, organic substance metabolic process, protein-containing complex assembly, and NADH dehydrogenase were significantly enriched. Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to examine molecular interactions among the DEGs. The top ten significantly enriched KEGG pathways with a p-value < 0.01 were identified (Fig. 4). Fatty acid metabolism, carbon metabolism, and TCA cycle metabolism were found to be shared in AC and NAC at 7, 9, and 11 months.

2.6. Transcription expression analysis of DEGs involved in sugar and fatty acid metabolisms

The transcriptional expression of sugar metabolism genes in AC and NAC coconut meat at 7, 9, and 11 months was assessed using a heat map (Fig. 5). In AC coconut meat, three sucrose synthase genes (*SUSs*: CCG000118.1, CCG003753.1, and CCG009611.1), two hexokinase genes (*HKs*: CCG012119.1 and CCG009388.1), and two UDP-glucose dehydrogenase genes (*GPIs*: CCG004035.1 and CCG022773.1) were significantly up-regulated compared to NAC coconut meat at all three time points. Conversely, two sucrose-phosphate synthase genes (*SPSs*: CCG009666.1 and CCG011040.1), two glucosidase genes (*GAEs*: CCG015836.1 and CCG015837.1), one acid beta-fructofuranosidase gene (*GALE*: CCG001199.1), two beta-mannosidase genes (*GLCAKs*: CCG004035.1 and CCG012050.1), two glycogen phosphorylase genes (*UGEs*: CCG020587.1 and CCG011135.1), two glucose phosphate isomerase genes (*UGDHs*: CCG019301.2 and CCG014763.1), two fructose-1,6-bisphosphatase deficiency genes (*FBPs*: CCG018678.1 and CCG027455.1), two *GMLS* genes (CCG024536.1 and CCG005508.1), one α-mannosidase gene (*MAN*: CCG008581.1), two 6-phosphofructokinase genes (*PFKs*: CCG003753.1 and CCG009411.1), two UDP-glucose dehydrogenase genes (*GPIs*: CCG004035.1 and CCG022773.1), two glucuronic acid kinase/glucuronokinase genes (*RHMs*: CCG004965.2 and CCG005083.1), and one GA2ox1ectopic gene (*TPSA*: CCG002791.1) were significantly down-regulated in AC coconut meat compared to NAC coconut meat across all time points. Regarding the expression levels of fatty acid metabolism genes, two acetyl coA carboxylase genes (*ACCs*: CCG002805.1 and CCG024567.1), three 3-oxoacyl-ACP reductase genes (*FabGs*: CCG022773.1, CCG003753.1, and CCG009411.1), two acyl-carrier-protein dehydratase genes (*FabZs*: CCG023098.1 and CCG009681.1), and two fattyacyl-ACP thioesterase B genes (*FatBs*: CCG01867.1 and CCG023098.1) were significantly up-regulated in AC coconut meat compared to NAC coconut meat at 7, 9, and 11 months (Fig. 6).

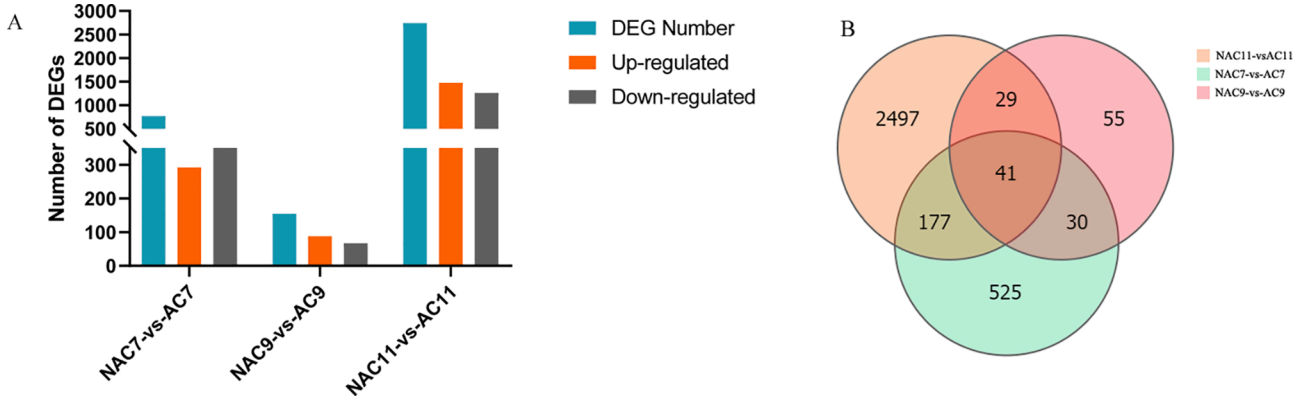

Fig. 2. Summary of the number of differentially expressed genes (DEGs) identified by RNA-seq analysis in coconut fruits of AC and NAC at three developmental stages. The number of total DEGs, upregulated DEGs, and downregulated DEGs are presented by histograms (A). The Venn diagrams represent the number of common DEGs shared by NAC vs. AC at three developmental stages (B).

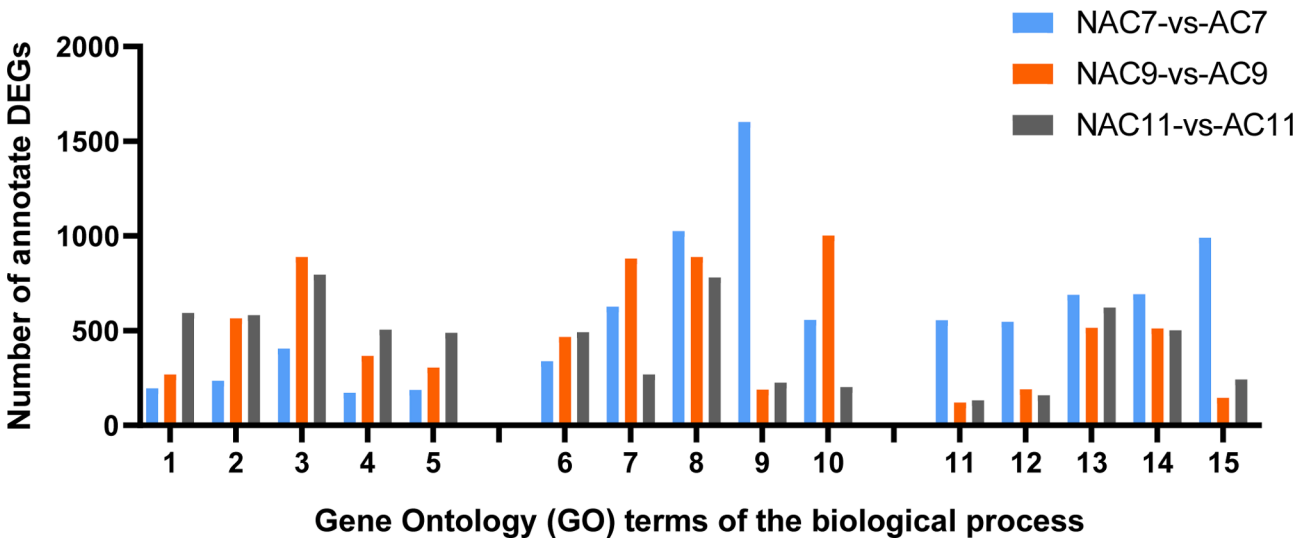

Fig. 3. Fisher's exact test for the significant (top five) enrichment analysis of Gene Ontology (GO) terms of the biological process, cellular components, and molecular function categories of the annotated DEGs in NAC and AC at three developmental stages. 1: GO:0090079 translation regulator activity, nucleic acid binding; 2: GO:0005488 ATP synthesis-coupled proton transport; 3: GO:0097159 organic cyclic compound binding; 4: GO:0003743 translation initiation factor activity; 5: GO:0045182 translation regulator activity; 6: GO:0032991 protein-containing complex; 7: GO:0110165 cellular anatomical entity; 8: GO:0071540 photosystemII; 9: GO:0005622 intracellular; 10: GO:0033290 eukaryotic 48S preinitiation complex; 11: GO:0044271 cellular nitrogen compound biosynthetic process; 12: GO:0044238 primary metabolic process; 13: GO:0071704 organic substance metabolic process; 14: GO:0065003 protein-containing complex assembly; 15: GO:0001732 NADH dehydrogenase.

2.7. Key candidate DEGs responsible for sugar and fatty acid accumulation in AC and NAC coconut meat

To examine the association among gene expression levels and sugar/fatty acid synthesis, correlation analysis was conducted amid transcriptional expression of genes and the content of sugar and fatty acids in AC and NAC coconut meat. The results presented in Supplementary Table 3 revealed that *SPS* (CCG011040.1), *GAE* (CCG015836.1), *GALE* (CCG001199.1), *GLCAK* (CCG004035.1 and CCG012050.1), *UGE* (CCG020587.1), *UGDH* (CCG019301.2), *FBP* (CCG018678.1), *GMLS* (CCG024536.1), *PFK* (CCG009411.1), *GPI* (CCG022773.1), *RHM* (CCG005083.1), *ACC* (CCG024567.1), *FabF* (CCG000421.1), *FatA* (CCG004035.1), *FabG* (CCG003753.1), and *FabI* (CCG01135.1) exhibited negative correlations with D-fructose (r = -0.81) and D-glucose (-0.99) contents, while showing positive correlations (r = 0.85 to 0.96) with lauric acid and myristic acid. Additionally, Palmitic acid content was positively correlated (r = 0.83 to 0.94) with *GALE* (CCG001199.1), *GLCAK* (CCG004035.1 and CCG012050.1), *FBP* (CCG018678.1), *GMLS* (CCG024536.1), and *ACC* (CCG002805.1). Furthermore, the sugar/organic acid ratio exhibited positive correlations with *SPS* (CCG009666.1), *GAE* (CCG015836.1), *UGE* (CCG020587.1), *FabF* (CCG002218.1), *FabZ* (CCG023098.1), and *FabI* (CCG19301.2).

In order to delve deeper into the examination of candidate genes within intricate regulatory networks, annotation information for these genes was obtained from the African oil palm reference genome database. Twelve genes, comprising eight genes related to sugar metabolism and four genes related to fatty acid metabolism (Figs. 5 and 6), were analyzed for their qRT-PCR expression. The relative expression levels at 7, 9, and 11 months were in line with the RNA sequencing results (Fig. 7).

3. Discussion

The characteristics of coconut fruit are significantly influenced by the pivotal role of sugar metabolism. Apart from serving as an energy source, sugars also act as signaling molecules involved in fruit ripening, aging, and stress responses (Kanwar and Jha, 2019, Ruan, 2014, Zimmer et al., 2021). The sweetness of fresh aromatic coconut is a vital parameter used to assess its quality. The main sugar components found in coconut meat are glucose and fructose. The sugar/acid ratio is a measure of the balance between sweetness and acidity, that can affect the taste, quality and shelf life of food. While the flavor characteristics of coconut meat are influenced by a combination of various metabolites, the sugar/fatty acid ratio plays a pivotal role in determining the overall sensory experience. This ratio significantly impacts consumer preferences, leading to repeat purchases and fostering customer loyalty (Li et al., 2021, Wang et al., 2015). Palmitic acid, myristic acid, and lauric acid are the predominant fatty acids found in coconut meat (Furuta et al., 2023). The current study was, therefore, meant to examine the sugar and fatty acid profiles of aromatic coconut and non-aromatic coconut fruit meat at three different developmental stages. Additionally, the study aimed to explore the genes responsible for sugar and fatty acid metabolism in these coconuts.

Aromatic coconut meat is renowned for its delightful sweetness, soft texture, and nutritional value, making it an exceptional natural green food choice. The flavor intensity of aromatic coconut meat is primarily influenced by the intricate interplay between sugar and fatty acid. Consequently, the sugar to fatty acid ratio emerges as an essential basis for the overall sensory experience (Beckles, 2012). The contemporary study focused on characterizing the sugar and fatty acid content in the meat of both aromatic and non-aromatic coconut varieties. At 7, 9, and 11 months, the levels of D-glucose and D-fructose were considerably elevated in aromatic coconut compared to non-aromatic coconut (Fig. 1). D-fructose in AC coconut meat was 3.48, 2.56 and 3.45 fold higher than that in NAC coconut meat at 7, 9, and 11 months, respectively. Similarly, D-glucose in AC coconut meat was 2.48, 2.25 and 3.91 fold higher than that in NAC coconut meat. Non-aromatic coconut exhibited higher levels of palmitic acid, myristic acid, and lauric acid compared to aromatic coconut. There was a 1.22-fold rise in the content of lauric acid compared to the AC coconut meat when it reached 11 months of age. Myristic acid content in NAC coconut meat was 1.47, 1.44 and 1.13 fold higher than that in AC coconut meat. The palmitic acid content in NAC coconut meat was 1.62 and 1.34 fold higher than that in AC coconut meat. An enhanced sweetness in aromatic coconut can be attributed to the sugar/fatty acid ratio.

SUS is a highly regulated cytosolic enzyme that catalyzes the

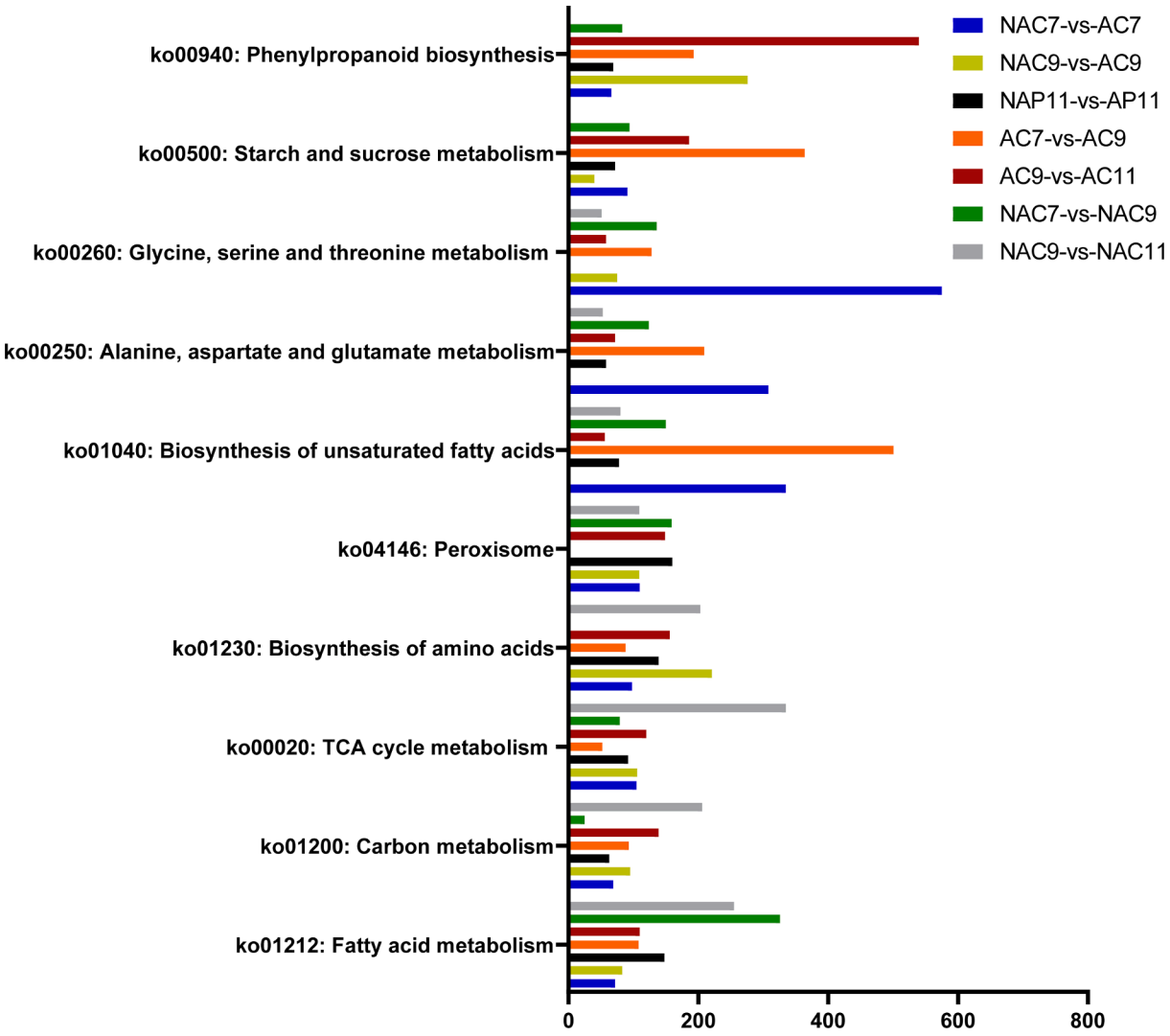

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the annotated DEGs in NAC and AC at three developmental stages.

reversible conversion of sucrose and UDP into UDP-glucose and fructose (Coleman et al., 2009). By means of its decomposition activity, SUS facilitates the hydrolysis of sucrose into UDPG (uridine diphosphate glucose) and fructose. Moreover, it actively participates in starch and sugar synthesis processes (Stein and Granot, 2019). The enhanced expression of sucrose synthase 3 in *Nicotiana tabacum* L. led to an expedited hydrolysis of sucrose and a rise in the fructose content (Daloso et al., 2016). In our study, fruits of aromatic coconut, three *SUS* genes (CCG000118.1, CCG003753.1, and CCG009611.1) exhibited significant upregulation compared to non-aromatic coconut at 7 months. Among these, *SUS* (CCG000118.1) displayed a positive correlation (r = 0.88) with the sugar/fatty acid ratio. The results indicate that *SUS* (CCG000118.1) has a notable involvement in the accumulation of fructose and glucose within the fruits.

The genes *FatI and FatB* are key regulators engaged in the synthesis and accretion of medium-chain fatty acids (C8 to C14) in coconut meat. Their role is crucial in this process (Shi et al., 2021). The expression of *CpuFatI* and *CvFatB1* genes led to the production of capric acid (10:0) in Camelina oil, whereas the expression of *CpuFatB4* gene ensued the production of myristic acid (14:0) and increased levels of palmitic acid (16:0) (Kim et al., 2015). Knocking out the *FatB* gene in Arabidopsis leads to a significant reduction of two-thirds in saturated fatty acids (Bonaventure et al., 2004). In this study, the genes *ACC* (CCG024567.1), *FabF* (CCG000421.1), *FatA* (CCG004035.1), *FabG* (CCG003753.1), and *FabI* (CCG01135.1) exhibited negative correlations with D-fructose (r = –0.81) and D-glucose (–0.99) contents, while positively correlating (r = 0.85 to 0.96) with lauric acid and myristic acid. Consequently, the genes *FabI* (CCG01135.1) and *FatB* (CCG023098.1) participate in the accumulation of lauric acid and myristic acid, potentially exerting a substantial influence on the sugar/fatty acid ratio.

The synthesis and degradation of sugar and fatty acid in plants involve the activity of numerous enzymes. In our study, through the down-regulation of *GALE*, *UGE*, *RHM*, *GlCAK*, *USP*, and *UDGH* expression, there is potential to enhance the accumulation of uridine diphosphate glucose, resulting in elevated levels of trehalose. The down-regulation of *TPSA* further influences this regulatory process. The outcomes imply that the synchronized regulation of the mentioned genes can result in an upsurge in trehalose expression.

The intricate metabolic traits of sugar/fatty acid content fluctuations are regulated by complex gene networks. By studying the attributes of genes, we can enhance our understanding of the molecular mechanisms that underlie the biosynthesis of sugar and fatty acids.

4. Conclusion

The results of the study revealed almost 3.5x and 4x rise in D-fructose and D-glucose of aromatic coconut (AC) meat as compared to the non-aromatic coconut (NAC) meat at 11 months age. On the contrary, the

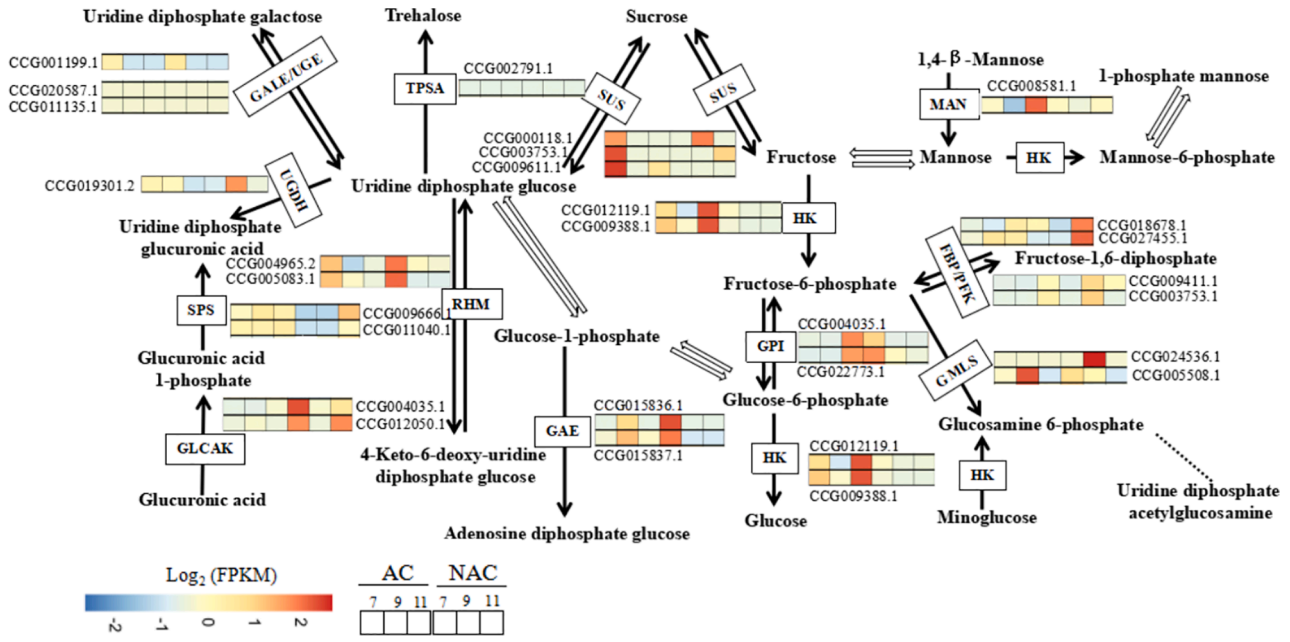

Fig. 5. Differential expression of genes involved in sucrose metabolism pathway in NAC and AC at three developmental stages. Heat maps depict the normalized gene expression values, which represent the means ± SD of three biological replicates. Expression values of 18 libraries are presented as FPKM normalized log2-transformed counts.

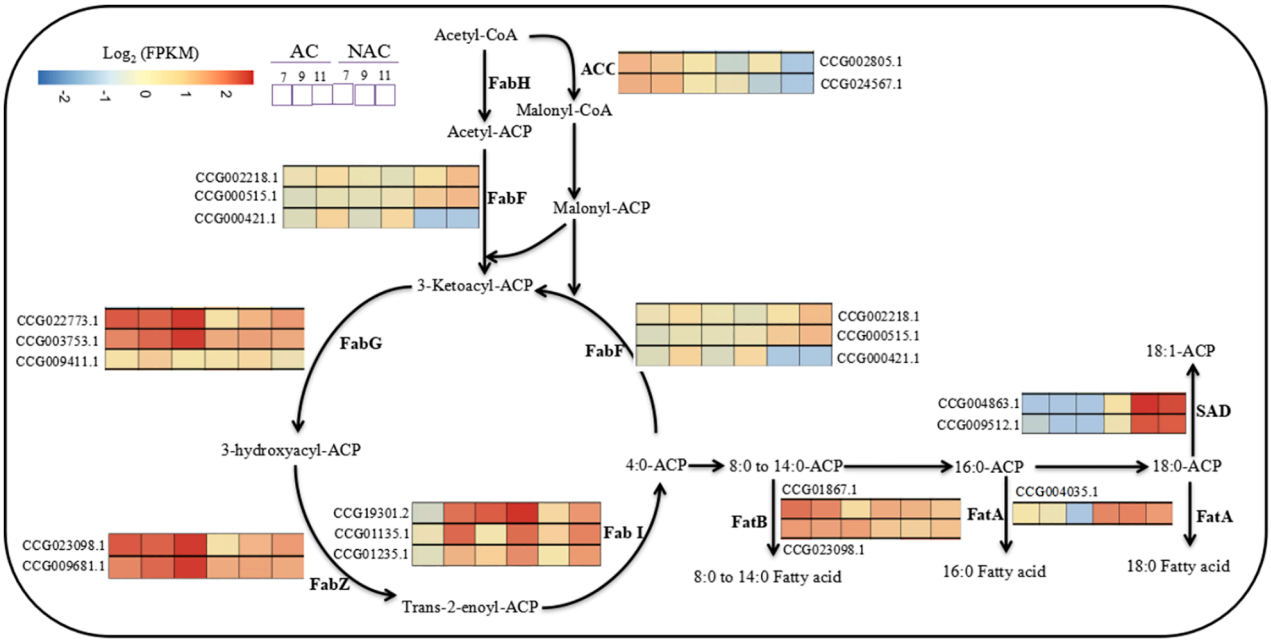

Fig. 6. Differential expression of genes involved in fatty acid metabolism pathway in NAC and AC at three developmental stages. Heat maps depict the normalized gene expression values, which represent the means ± SD of three biological replicates. Expression values of 18 libraries are presented as FPKM normalized log2-transformed counts.

observed rise in palmitic, myristic and lauric acids were 1.34x, 1.13x and 1.22x in NAC coconut meat in comparison to AC coconut meat at 11 months age. The molecular data exhibited that genes *SPS*, *GAE*, *GALE*, *GLCAK*, *UGE*, *UGDH*, *FBP*, *GMLS*, *PFK*, *GPI*, *RHM*, *ACC*, *FabF*, *FatA*, *FabG*, and *FabI* exhibited a negative correlation with D-fructose and D-glucose contents, which means that up-regulation of these genes slowed the production of sugars in the NAC meat. However, the expression of these genes in NAC enhanced the production of lauric acid and myristic acid in the meat. Moreover, the expression of *GALE*, *GLCAK*, *FBP*, *GMLS*, and *ACC* can be responsible for high palmitic acid contents in NAC meat as it displayed a positive correlation with it. This study concludes that a number of genes are responsible for sugar and fatty acid accumulation and metabolism in AC and NAC coconut meat. Also, the data obtained from this research provide a valuable groundwork for future investigations, which will focus on utilizing molecular biology techniques to unravel the structures and functions of the mentioned genes in the context of fruit quality in economically significant plant species.

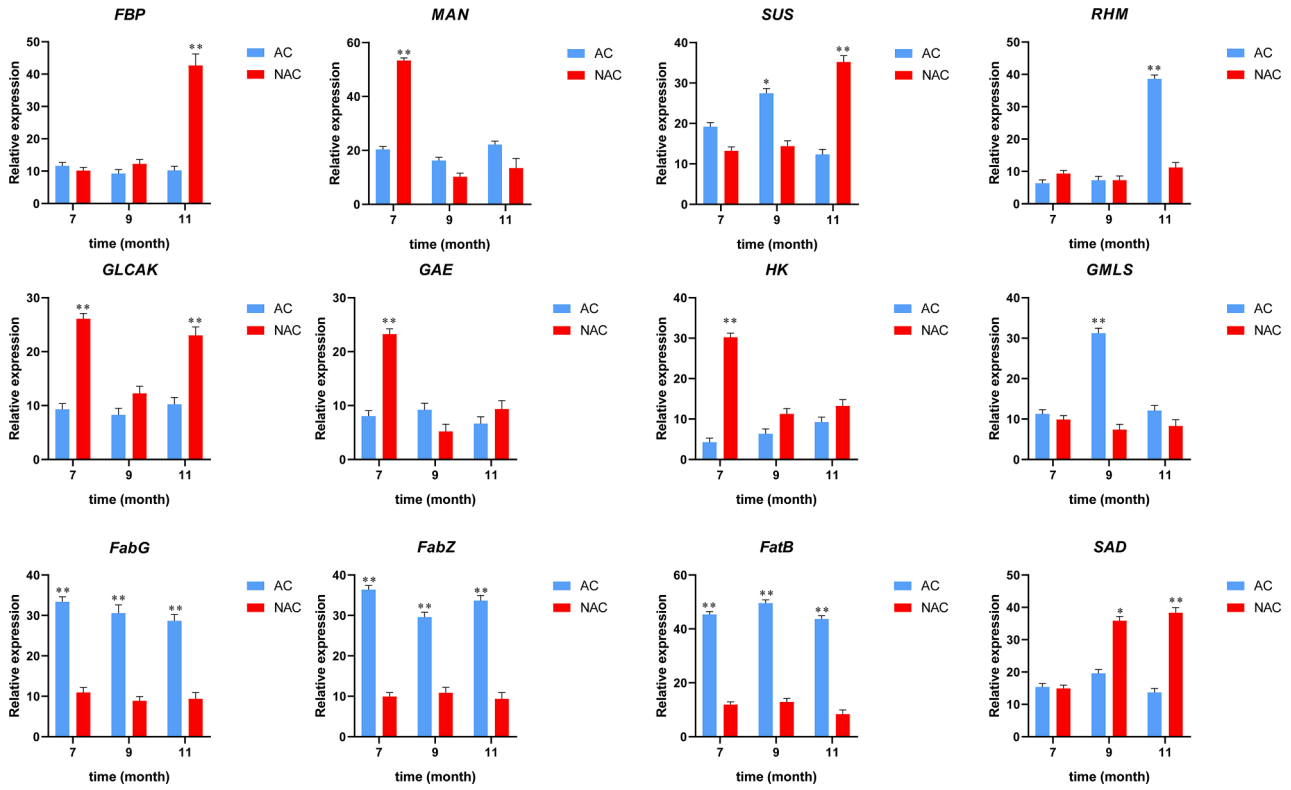

Fig. 7. Qrt-pcr assay, the mean was calculated from three biological replicates, each with three technical replicates (n = 9). These replicates were then normalized relative to the expression of Actin.

5. Materials and methods

5.1. Plant materials

The cultivation of aromatic coconut (AC) and non-aromatic coconut (NAC) took place in the nursery of the CRI, CATAS, Wenchang, Hainan, P.R. China. In brief, for each cultivar, a total of six fruits were randomly collected as a biological replicate at the ages of 7 months, 9 months, and 11 months, respectively. Each genotype was represented by three biological replicates. After collection, all the samples of coconut meat were quickly frozen using liquid nitrogen and subsequently stored at a temperature of −80 °C for consequent analysis.

5.2. Metabolite extraction and UPLC-MS analysis

Fifty mg of coconut meat was weighted and placed in a 1.5 mL eppendorf tube. 800 μL of extract (methanol: water = 7:3, v/v, −20 ℃ precooling), 20 μL of internal standards and two small steel balls were then added. The mixture was ground in a Tissue grinder (50 Hz, 5 min) followed by ultrasonic treatment with water bath at 4 ℃ for 30 min and kept in the refrigerator at −20 ℃ for 1 h. Subsequently, the samples were centrifuged at 4℃ for 15 min with shaking at 24,000*g*. Finally, 600 μL of supernatant was taken and filtered through a 0.22 μm membrane. The filtered sample was placed in a vial (1.5 mL) for LC-MS analysis. 20 μL of each sample was mixed into a QC sample to evaluate the repeatability and stability of LC-MS analysis. A LC-MS system consisting of Waters 2D UPLC (waters, USA) and Q exactive high resolution mass spectrometer (Thermo Fisher Scientific, USA) was used for metabolite separation and detection.

LC conditions: A Hypersil GOLD aQ column (100*2.1 mm, 1.9 μm, Thermo Fisher Scientific, USA) was used and the mobile phase consisted of 95 % of solvent A (water/formic acid, 99.9/0.1 (v/v)) and 5 % of solvent B (acetonitrile/formic acid, 99.9/0.1 (v/v)). Gradient elution conditions were set as follows: 0–2 min, 5 % phase B; 2–22 min, 5 % to 95 % B; 22–27 min, 95 % B; 27.1–30 min, 5 % B. The flow rate was 0.3 mL/min. The column oven was maintained at 40 ℃. The injection volume was 5 μL.

MS conditions: A Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) was used to obtain MS1 and MS2 data. The MS scan method was in the range of *m/z* 150–1,500, The MS1 resolution was 70,000, AGC was 1e6, and the maximum injection time was 100 ms. According to the precursor ion intensity, Top 3 ions were selected for MS2 analysis, MS2 resolution was 35,000, AGC was 2e5, maximum injection time was 50 ms, and collision energy (stepped an nce) were set as: 20, 40 and 60 eV. The parameters of ESI were: sheath gas of 40 L/min, aux gas of 10 L/min, spray voltage (|KV|) of 3.80 in positive ion mode and of 3.20 in negative ion mode, capillary temperature of 320 ℃ and aux gas heater temperature of 350 ℃.

5.3. RNA isolation and high-throughput sequencing

Total RNA was extracted from approximately 200 mg fruit samples using an RNAprep Pure Plant Plus Kit (TIANGEN). Extracted RNA samples were further assessed by agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, Waltham, United States) for quantity and quality check.

The six triplicate samples (AC and NAC fruits at three developmental stages) yielded 18 non-directional cDNA libraries using illumina HiSeq 2500 platform by signal end read libraries method of the Sequencing By Synthesis (SBS) technology, which was performed at Beijing Genomics Institute (BGI) in Shenzhen, China. The raw reads were cleaned, and the clean reads were aligned onto the reference genome of coconut (Accession: PRJNA374600) (Xiao et al., 2017).

The raw data of metabolome analysis and RNA sequencing was deposited in the China National GeneBank DataBase (https://db.cngb.org/cnsa, accession number CNP0004618) (accessed on 25 July 2023).

5.4. Metabolomics data analysis

Differential metabolites underwent clustering analysis, with the data subjected to $\log_2$ conversion and zero mean normalization treatments. Hierarchical Cluster, utilizing the Euclidean distance calculation, was employed as the clustering algorithm. Correlation study was carried out to assess the association among the differential ions, and the Pearson correlation coefficient was applied to calculate the degree of correlation. The criteria used to screen for differential metabolites involved a minimum fold change of $\geq$1.2 or $\leq$0.83, along with a p-value threshold below 0.05. Based on the KEGG database, a biochemical pathway functional enrichment analysis of the altered metabolites was conducted, identifying the metabolic pathways that exhibited significant enrichment of altered metabolites with a p-value of less than 0.05.

5.5. Differential gene expression analysis

An overall 18 non-directional cDNA libraries were constructed from the coconut meat samples (AC-7, AC-9, AC-11, NAC-7, NAC-9, and NAC-11), resulting in a combined clean data output of 116.21 Gb (Supplementary Table 2). The high-quality reads were mapped to the reference genome of coconut (Xiao et al., 2017). Differentially expressed genes (DEGs) were identified using a screening criterion of fold change $\geq$2 and a false discovery rate (FDR) of less than 0.01. Gene expression was normalized using the threshold of FPKM (Fragments Per Kilobase of transcript per Million mapped reads) $\geq$ 5.0. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were utilized to categorize coconut genes into GO categories and conduct pathway analyses using the KEGG database.

5.6. Combined analysis of transcriptome and metabolome

Co-joint analyses were employed to explore the differentially expressed genes and accumulated metabolites, aiming to assess the extent of pathway enrichment. For the construction of the transcript-metabolite network, gene-metabolite pairs exhibiting an absolute correlation coefficient greater than 0.9 and a p-value less than 0.05 were utilized.

5.7. RNA isolation and qRT-PCR analysis

The QRREM method, as outlined by Iqbal et al. (2019), was employed for the extraction of total RNA from the coconut meat. Following extraction, the RNA samples were subjected to agarose gel electrophoresis and assessed using a Nanodrop spectrophotometer to verify their quantity and quality. The synthesis of cDNA was carried out using 1 μg of RNA and the MightyScript first-strand cDNA synthesis kit, which includes gDNA digester, following the instructions provided by the manufacturer. Quantitative real-time PCR reactions were conducted using the 2 × SYBR Green qPCR ProMix (low ROX) protocol in 96-well optical plates, utilizing a Mastercycler ep realplex4 machine. The QuantPrime qPCR primer designing tool was utilized to design gene-specific primers for the experiment (Supplementary Table 4). The amplification reactions were carried out in a final reaction volume of 10 μL, following the conditions of 95 °C for 5 s, 55 °C for 15 s, and 68 °C for 20 s. The melting curve stage involved a gradual increase in temperature from 60 °C to 95 °C within 20 min. For each reaction, three biological replicates and three technical replicates were performed. The expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method, with the Actin gene serving as the internal control (Xia et al., 2014). Statistical significance was determined using one-way ANOVA with a significance level set at $p < 0.05$, and the analysis was conducted using SPSS software.

Author contributions

The experiments were conceived and designed by LZ, while XS conducted the experiments and YY supervised the project. Data analysis was performed by LZ, RY, YY and QW, while LZ, JL and YY and were responsible for the preparation and composition of the original manuscript draft. The entire manuscript was subsequently revised by RY and AI. The final version of the manuscript underwent comprehensive review and received approval from all authors.

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8. Publisher's note

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CRediT authorship contribution statement

Lixia Zhou: Data curation, Writing – original draft. **Xiwei Sun:** Data curation. **Rajesh Yarra:** Formal analysis, Writing – review & editing. **Amjad Iqbal:** . **Qiufei Wu:** Data curation. **Jing Li:** Data curation. **Yaodong Yang:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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