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Integration of Phenotypes, Phytohormones, and Transcriptomes to Elucidate the Mechanism Governing Early Physiological Abscission in Coconut Fruits (*Cocos nucifera* L.)

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Abstract: The abscission of fruits has a significant impact on yield, which in turn has a corresponding effect on economic benefits. In order to better understand the molecular mechanism of early coconut fruit abscission, the morphological and structural characteristics, cell wall hydrolysis and oxidase activities, phytohormones, and transcriptomes were analyzed in the abscission zone (AZ) from early-abscised coconut fruits (AFs) and non-abscised coconut fruits (CFs). These results indicated that the weight and water content of AFs are significantly lower than those of CFs, and the color of AFs is a grayish dark red, with an abnormal AZ structure. Cellulase (CEL), polygalacturonase (PG), pectinesterase (PE), and peroxidase (POD) activities were significantly lower than those of CFs. The levels of auxin (IAA), gibberellin (GA), cytokinins (CKs), and brassinosteroid (BR) in AFs were significantly lower than those in CFs. However, the content of abscisic acid (ABA), ethylene (ETH), jasmonic acid (JA), and salicylic acid (SA) in AFs was significantly higher than in CFs. The transcriptome analysis results showed that 3601 DEGs were functionally annotated, with 1813 DEGs upregulated and 1788 DEGs downregulated. Among these DEGs, many genes were enriched in pathways such as plant hormone signal transduction, carbon metabolism, peroxisome, pentose and gluconate interconversion, MAPK signaling pathway—plant, and starch and sucrose metabolism. Regarding cell wall remodeling-related genes (PG, CEL, PE, POD, xyloglucan endoglucosidase/hydrogenase (XTH), expansin (EXP), endoglucanase, chitinase, and beta-galactosidase) and phytohormone-related genes (IAA, GA, CKs, BR, ABA, JA, SA, and ETH) were significantly differentially expressed in the AZ of AFs. Additionally, BHLH, ERF/AP2, WRKY, bZIP, and NAC transcription factors (TFs) were significantly differently expressed, reflecting their crucial role in regulating the abscission process. This study's results revealed the molecular mechanism of early fruit abscission in coconuts. This provided a new reference point for further research on coconut organ development and abscission.

Keywords: coconut; fruit abscission; phytohormones; transcriptome; enzyme activities

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1. Introduction

Abscission is a fundamental process in plant biology, representing the evolutionary adaptation of plants, which allows for the abandonment of aging or physiologically damaged organs such as leaves, petals, and fruits [1,2]. Organ abscission is a crucial process in the complex gene regulation of the plant kingdom. Some regulatory factors, including external environmental disturbances and internal signals, may lead to abscission at different developmental stages [3]. Abscission is precisely regulated by structural, physiological, biochemical, and molecular changes, ultimately leading to the abscission of plant organs [4,5]. Abscission is a complex and highly coordinated physiological process. Organ abscission can be classified into three categories, including normal abscission (such as mature fruit

and seed abscission), metabolic abscission or physiological abscission (such as fruits of premature abscission and unpollinated flowers), and induced abscission (induced by high temperatures, low temperatures, light intensity, or pathogens) [6,7]. Abscission causes organ separation, which occurs in a special location called the abscission zone (AZ). The AZ undergoes a series of physiological events during the abscission process, including abscission signal transduction, AZ cell differentiation, the activation of organ separation, and the formation of protective layers for organs [8,9].

Plant hormones play a crucial role in plant growth and development throughout the entire plant growth cycle [10]. Plant hormones, as signaling substances during the shedding process, play an important role in regulating flower and fruit drop, as well as promoting flower and fruit preservation. Among these plant hormones, some have the effect of promoting flower and fruit drop, while others have the effect of inhibiting flower and fruit drop. Among these hormones, common ones related to flower and fruit drop include IAA, GA, CKs, ETH, ABA, etc. [2], and their mechanisms of action are also different. The response of AZ cells to internal and external shedding trigger signals is mediated by plant hormones [1]. ETH, JA, and ABA are abscission promoters, while IAA, GA, and BR inhibit abscission [1,11,12].

Fruit set is the process of transforming ovary tissue into fruit. As is well known, auxin and GAs play a crucial role in the induction stage of fruit set. Some studies have shown that auxin triggers cell division, and their interaction with GAs maintains cell expansion [13]. However, the negative controls for auxin/indole-3-acetic acid (AUX/IAA) and auxin response factor (ARF) proteins inhibited the transformation of tomato and Arabidopsis ovaries into fruits, which can be eliminated through pollination/fertilization or auxin treatment, leading to cell proliferation and fruiting. Moreover, the biosynthesis and action of ETH and ABA are significantly downregulated [14,15], while the biosynthesis and action of auxin and GAs are activated. This indicates that plant hormones play an important role in fruit development. Some reports suggest that the plant hormones auxin, ETH, and ABA seem to play a major role, while GAs, CKs, SA, and JA have also been reported to be involved [16–19].

The changes in minerals and carbohydrates also play an important role in fruit growth and abscission [20–22]. During the flower bud development stage of fruit trees, a large amount of nutrients is required for the development of ovules. During this period, if the quantity of nutrients required for bud development is insufficient, the formation of flower buds will be limited, and the fertility of flowers will decrease, thereby affecting pollination and fertilization, and causing the abscission of flowers and fruits [23]. There is also a possibility that nutrient deficiency induces changes in the expression of genes related to auxin signaling transduction in plants [24], leading to metabolic disorders in cells and programmed cell death, resulting in flower and fruit abscission. In addition, low sugar content in plants can induce the production of reactive oxygen species (ROS), which in turn can lead to the abscission of flowers and fruits.

The cell wall contains typical components such as CEL, hemi-CEL, PE, PG, proteins, and phenolic compounds. During the process of plant organ abscission, a large amount of cell wall hydrolytic enzymes are synthesized and enzyme activity is increased, which may be the reason for the loosening of the primary cell wall in the intermediate layer degradation and separation layer [25]. CEL and PG are two major cell wall hydrolases that have been extensively studied in different plants and play important roles in plant organ abscission [26]. In addition, EXP, XTH, and POD also play important roles in the process of plant organ abscission [27]. During ethylene induction or low-level auxin initiation, the degradation enzyme genes of plant cell walls are also upregulated, leading to plant organ abscission [28].

The abscission of plant organs involves multiple metabolic pathways and is related to various genes and compounds. Therefore, the use of high-throughput sequencing methods to study plant organs has been favored by researchers, and studying the abscission of plant organs at the transcriptome level is one of the commonly used methods. By using

transcriptome sequencing technology and setting up experimental and control groups, genes that may be related to plant organ abscission can be identified from a large number of genes. Then, the most likely genes can be selected from these possible genes and validated using experimental techniques. In the past decade, transcriptome and molecular biology studies have identified key molecular components involved in AZ development or abscission regulation in model plants (tomato and Arabidopsis) [29–32]. The ethylene response factor (ERF) family gene (SlERF52) has been identified as a connector between the abscission induction signal and the abscission process [29], while factors regulating AZ activity (transcription factors KNOX and bHLH, leucine-rich repeat (LRR) receptor-like kinase, and small-signal peptide IDA) have also been reported [30–33]. In citrus, transcriptome analysis found that genes regulated in the process of ETH promoting abscission, including ETH-responsive transcription factor activation, genes involved in defense, cell wall degradation, and secondary metabolism, and genes involved in starch/sugar biosynthesis and growth-promoting hormone synthesis were downregulated [34,35].

Sugar metabolism also plays an important role in the response process of fruit abscission. The content of sorbitol, glucose, fructose, and sucrose in abscised fruits is lower than that in continuous fruits [36]. Transcriptomic analysis showed that genes encoding trehalose-6-phosphate synthase, sorbitol transporter, UDP glycosyltransferase, and UDP-GLC-4-exoisomerase were upregulated in apples [37]. These enzyme genes are also controlled by sugar starvation and participate in resource mobilization in other species [38–40].

Coconuts are an important tropical fruit and oil crop known for their nutritional and industrial value. However, abnormal fruit abscission often greatly reduces coconut yield. Previous studies have shown that fruit abscission is not sufficient to achieve better economic benefits [41]. Fruit abscission is a highly regulated developmental process that is influenced by internal and environmental factors [3,42]. Its regulatory mechanism is relatively complex and involves multiple factors. Therefore, in order to comprehensively understand fruit abscission related to coconuts at the physiological and molecular levels, we sequenced the transcriptome of normally developing and early physiologically abscising fruits and identified differentially expressed genes (DEGs) between them to fully understand the differences in gene expression and explore the reasons for early fruit abscission in coconuts. In addition, this study also measured the phenotype of normally and early physiologically abscised fruits, as well as physiological indicators such as plant cell antioxidant enzymes and plant hormone content. To fully understand the physiological characteristics of coconut fruit drop and its association with related genes, this study helps deepen our understanding of fruit development and early fruit physiological shedding mechanisms, and also reveals the molecular mechanisms of coconut fruit abscission. The results of this study will be of great significance for improving coconut yield.

2. Results

2.1. Morphological Comparison of Fruits

According to the observation of fruit morphological characteristics, non-abscised fruit (CF) peels were smooth, while abscised fruit (AF) peels were dull and slightly wrinkled, the sepals turned gray/black, and the tissue in the abscission zone became necrotic (Figure 1a). In addition, the average weight of 10 AFs (28.352 g) was significantly lower than that of CFs (36.068 g). The water content of AFs (25.68%) was significantly lower than that of CFs (36.35%) (Figure 1b). Furthermore, the anatomical structure of the coconut fruit abscission zone shows that AZ cells in CFs were small and dense, with most cells appearing as short ellipses and distributed in a rotating shape (Figure 1a), while AZ cells in AFs were large and loose, appearing as long ellipses and distributed side by side (Figure 1a). These results indicated that the loss of substances and water from abscised fruits led to premature fruit abscission. At the same time, it also indicated that there may be a significant connection between coconut fruit abscission and AZ cell structure.

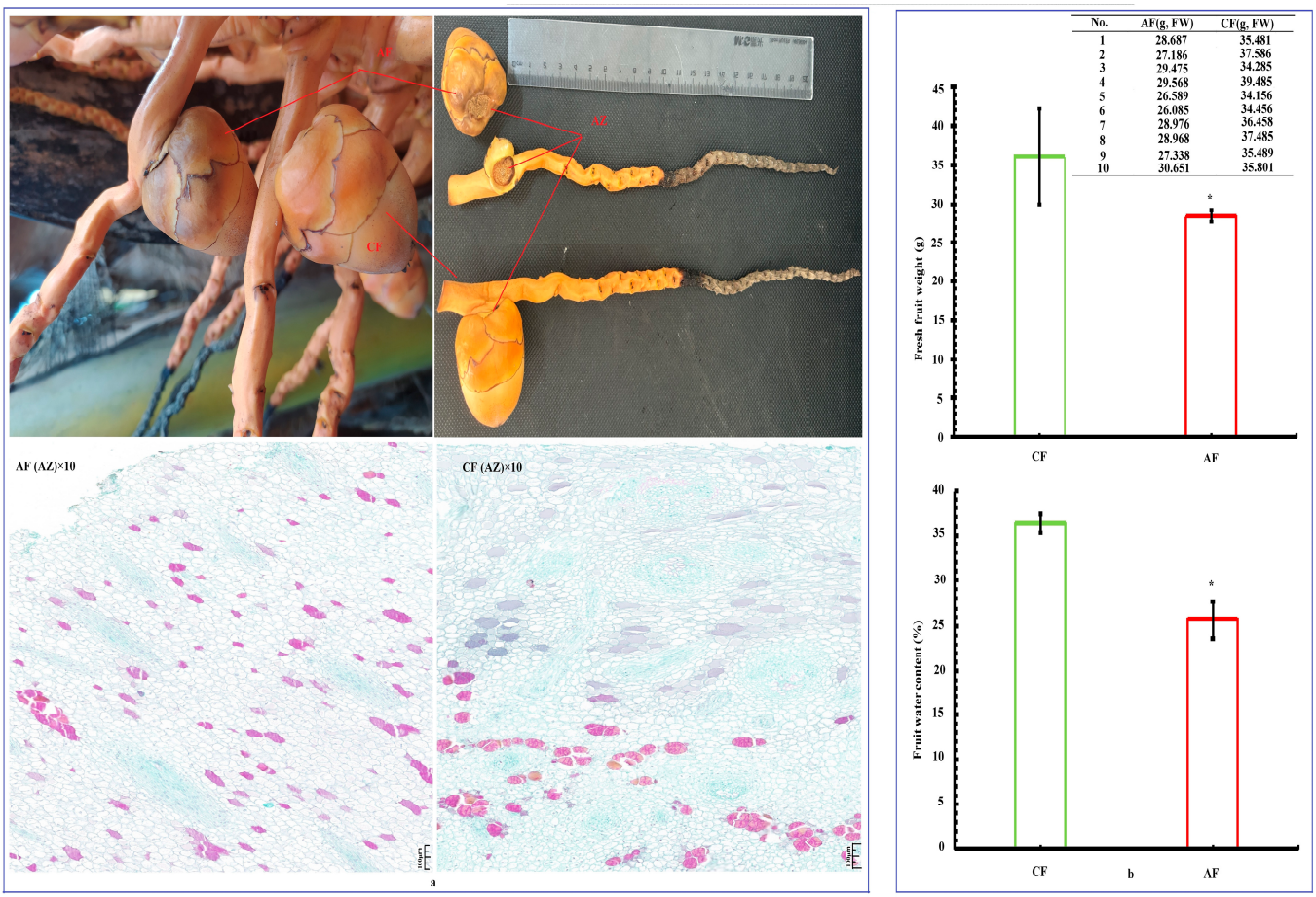

Figure 1. Development of early-abscised coconut fruits (AFs) and non-abscised coconut fruits (CFs). (**a**) The anatomical structure of fruit morphology and abscission zone (AZ). (**b**) Fresh weight and water content of coconut fruits. The data represent the mean ± standard deviation (SD) of ten samples, and the significance of fresh weight and water content of coconut fruits between CFs and AFs was determined using Student's *t*-tests. * significant at $p < 0.05$.

2.2. Enzyme Activity and Phytohormone Content in Fruits

In order to investigate whether the activities of cell wall hydrolases and antioxidant enzymes in the AZ were related to the abscission of coconut fruit, enzyme activities were measured in this study. The results showed that the activities of CEL and PG in the AZ of AFs were significantly higher than those in CFs, but the activities of PE and POD were significantly lower than those in CFs (Figure 2). This result suggested that the decrease in PE and POD in AFs was likely due to the rapid hydrolysis of cell walls in the AZ, leading to loss. But, at the same time, it also generated more CEL and PG. These results indicated that the abscission of coconut fruits was closely related to the physiological and biochemical metabolism and cellular structure of the AZ.

To further investigate the hormone regulation of coconut physiological fruit abscission, the levels of phytohormones such as IAA, ABA, GA, CKs, BR, JA, SA, and ETH in the AZ of AFs and CFs were analyzed. The contents of IAA, CKs, GA, and BR in the AZ of AFs were significantly lower than those in CFs. However, the contents of ABA, JA, SA, and EHT were significantly higher than those in CFs (Figure 2).

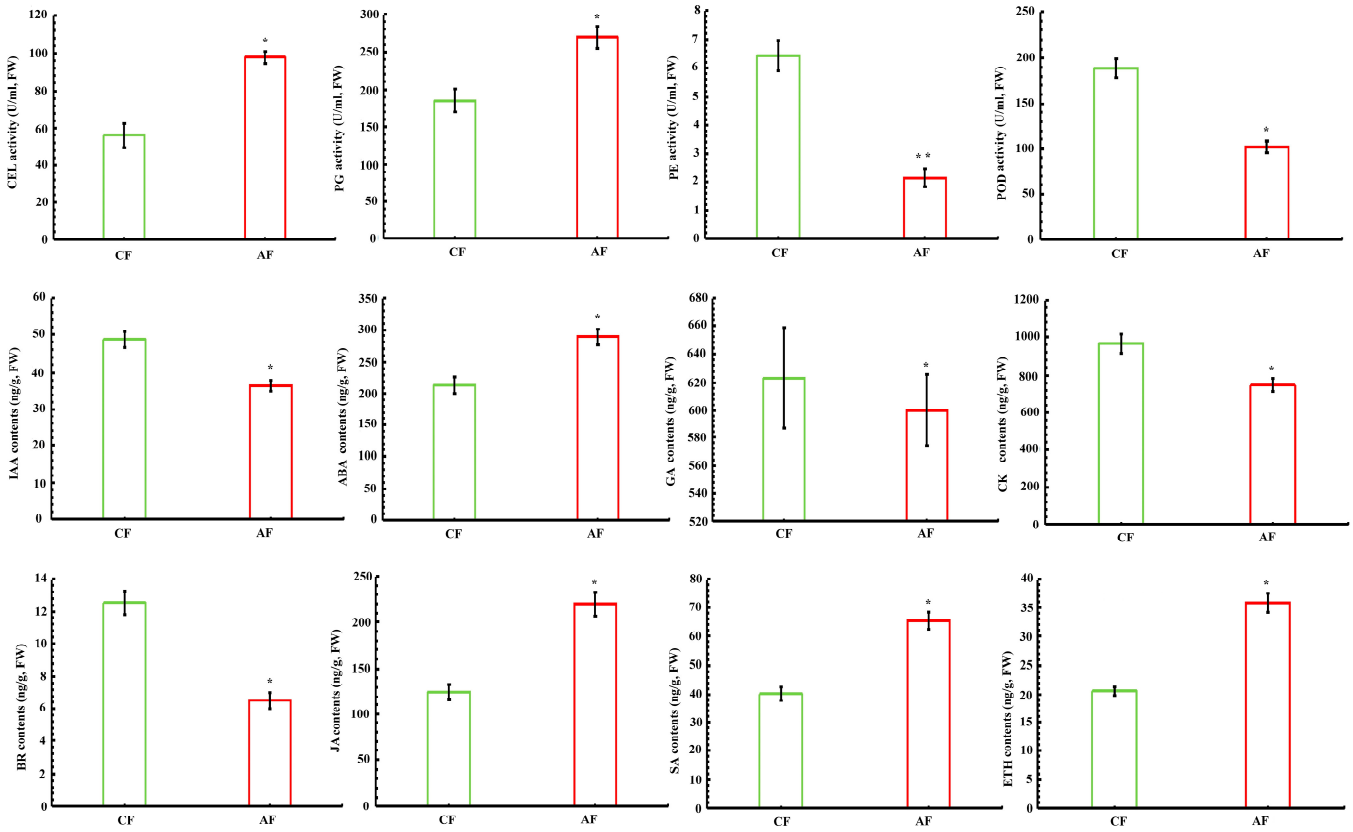

Figure 2. Enzyme activity and phytohormones in AZ of AFs and CFs. CEL, cellulase; PG, polygalacturonase; PE, pectinesterase; POD, peroxidase; IAA, auxin; GA, gibberellin; CK, cytokinin; BR, brassinosteroid; ABA, abscisic acid; ETH, ethylene; JA, jasmonic acid; SA, salicylic acid. The data represent the mean ± standard deviation (SD) of three biological replicates, and the significance of enzyme activity and plant hormone contents in AZ between CFs and AFs was determined using Student's *t*-tests. * significant at $p < 0.05$. ** significant at $p < 0.01$.

2.3. Transcriptome

2.3.1. Evaluation of Transcriptome Sequencing Data

Six samples (three biological replicates from two treatments (CFs and AFs)) were processed and subjected to sequencing quality control, resulting in a total of 39.74 Gb of clean data. The Q30 base percentage of each sample was not less than 93.83% (Table 1). According to the comparison results, the alignment efficiency between the reads of each sample and the reference genome ranges from 93.84% to 94.57% (Table 2). A total of 23,727 genes with appropriate FPKM values were detected by RNA-seq in CFs vs. AFs (Table S1). Using FPKM > 1 as the threshold for determining gene expression, the FPKM values in CF samples were higher than those in AF samples, and the FPKM values in AF samples were more dispersed (Figure S1a). Spearman's correlation coefficient was used as an evaluation metric for biological repeat correlation. The result further revealed the high correlation between the three biological repeats for each treatment (Figure S1b). The PCA results showed that CF and AF samples were clustered separately, indicating significant differences in gene expression between the sample groups (Figure S1c). The three biological replicates of CFs and AFs were strictly clustered together, indicating high biological reproducibility in each group of processed samples. Additionally, significant differences in gene expression were observed between CFs and AFs (Figure S1c). Figure S2 shows a volcano plot with significant upregulated and downregulated differences between the two groups.

Table 1. Overview of RNA-seq data statistics from CFs and AFs.

Samples	Clean Reads	Clean Bases	GC Content	% ≥ Q30
AF1	25,706,542	7,648,661,534	0.485	0.9497
AF2	21,558,918	6,435,636,332	0.4855	0.948
AF3	24,423,723	7,296,011,600	0.4946	0.9489
CF1	20,557,756	6,141,122,658	0.4904	0.9466
CF2	19,627,078	5,860,569,898	0.4931	0.9383
CF3	21,330,627	6,362,206,920	0.4853	0.9454

Table 2. Overview of compared statistics from sequence alignment results of seq-data and selected reference genomes between CF and AF samples.

Sample	Total Reads	Mapped Reads	Uniq Mapped Reads	Multiple Map Reads	Reads Map to '+'	Reads Map to '−'
AF1	51,413,084	48,391,912 (94.12%)	45,777,509 (89.04%)	2,614,403 (5.09%)	24,041,632 (46.76%)	24,079,623 (46.84%)
AF2	43,117,836	40,482,546 (93.89%)	38,332,913 (88.90%)	2,149,633 (4.99%)	20,126,344 (46.68%)	20,099,546 (46.62%)
AF3	48,847,446	45,864,997 (93.89%)	43,494,824 (89.04%)	2,370,173 (4.85%)	22,808,379 (46.69%)	22,800,063 (46.68%)
CF1	41,115,512	38,884,564 (94.57%)	36,707,903 (89.28%)	2,176,661 (5.29%)	19,238,804 (46.79%)	19,339,734 (47.04%)
CF2	39,254,156	36,835,121 (93.84%)	33,592,867 (85.58%)	3,242,254 (8.26%)	17,692,349 (45.07%)	17,969,384 (45.78%)
CF3	42,661,254	40,290,145 (94.44%)	37,962,049 (88.98%)	2,328,096 (5.46%)	19,867,801 (46.57%)	19,992,141 (46.86%)

2.3.2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analysis of DEGs

In the transcriptome obtained from the AF and CF treatment groups, differentially expressed gene (DEG) screening was based on $|\log_2 FC| \geq 1$, and FDR < 0.01 was the threshold (Figure S2, Tables S2 and S3). In CF vs. AF, a total of 3949 DEGs were detected, of which 1944 DEGs were upregulated and 2005 DEGs were downregulated (Figure S2b, Table S2). Functional classification and statistics of the DEGs showed that the most abundant (COG) categories were signal transduction mechanisms and carbohydrate transport and metabolism (Figure S2d, Table S2). Cluster analysis heatmaps showed that some DEGs were expressed differently between CFs and AFs (Figure S2c). In the CF vs. AF group, 3601 DEGs were functionally annotated, with 1813 DEGs upregulated and 1788 DEGs downregulated (Table S3). A total of 3059 DEGs were annotated to 55 GO items, including 21 in the biological process (BP) category, 18 in the cellular component (CC) category, and 16 in the molecular function (MF) category. In the BP category, the genes with the highest abundance belonged to "cellular process", followed by "metabolic process", "single-organism process ", and "biological regulation". In the CC category, "cell" and "cell part" have the highest number of genes, followed by "organelle", "membrane", and "membrane part". The main terms in the MF category were "binding" and "catalytic activity" (Figure S2). In order to classify the functions of DEGs, enrichment analysis of the DEGs' annotated GO pathway gene functions was performed (FDR < 0.05) (Figure 3).

In the BP category, gene expression was significantly upregulated in "translation", "regulation of jasmonic acid-mediated signaling pathway", "tricarboxylic acid cycle", and "regulation of defense response" (FDR < 0.05, Table S4). In the CC category, there were significantly upregulated genes in "chloroplast", "peroxisome", "cytosolic small ribosomal subunit", "ribosome", "chloroplast stroma", "cytosolic large ribosomal subunit", and "presequence translocase-associated import motor" (FDR < 0.05, Table S4). In the MF category, the significantly upregulated genes mainly included "structural constituent of ribosome", "pyridoxal phosphate binding", "protein serine/threonine phosphatase activity", "magnesium-dependent protein serine/threonine phosphatase activity", "trehalose-phosphatase activity", "L-lysine transmembrane transporter activity",

"[acyl-carrier-protein] S-malonyltransferase activity", "L-alanine transmembrane transporter activity", "arginine transmembrane transporter activity", and "hydro-lyase activity" (FDR < 0.05, Figures 3 and S3a, Table S4). The genes significantly downregulated in the BP category included "xyloglucan metabolic process", "plant organ morphogenesis", "microtubule-based movement", "microtubule-based process", "microtubule cytoskeleton organization", "auxin-activated signaling pathway", "anatomical structure morphogenesis", "leaf development", "pectin catabolic process", "steroid biosynthetic process", "root system development", "cell wall modification", "cell wall organization", "spindle assembly", "lignan metabolic process", "lignan biosynthetic process", "multicellular organism development", "SCF-dependent proteasomal ubiquitin-dependent protein catabolic process", "leaf morphogenesis", and "cell morphogenesis involved in differentiation" (FDR < 0.05, Table S4). In the CC category, "microtubule", "plasma membrane", "kinesin complex", "anchored component of plasma membrane", "cell wall", "plasma membrane part", "integral component of membrane", "cell periphery", "extracellular region", "cytoskeleton", "apical plasma membrane", "nuclear RNA-directed RNA polymerase complex", "apical part of cell", "intrinsic component of plasma membrane", "Golgi apparatus", "SCF ubiquitin ligase complex", "MCM complex", "membrane part", and "spindle" (FDR < 0.05, Table S4) were also significantly downregulated. In the MF category, significantly downregulated genes included "microtubule binding", "protein kinase activity", "ATP binding", "structural constituent of cytoskeleton", "microtubule motor activity", "pectinesterase inhibitor activity", "transmembrane receptor protein serine/threonine kinase activity", "pectinesterase activity", "aspartyl esterase activity", "RNA-directed 5′-3′ RNA polymerase activity", "xyloglucan:xyloglucosyl transferase activity", "hydrolase activity hydrolyzing O-glycosyl compounds", "transcriptional activator activity RNA polymerase II transcription regulatory region sequence-specific", "binding", and "ubiquitin-like protein transferase activity" (FDR < 0.05, Figures 3 and S3b, Table S4).

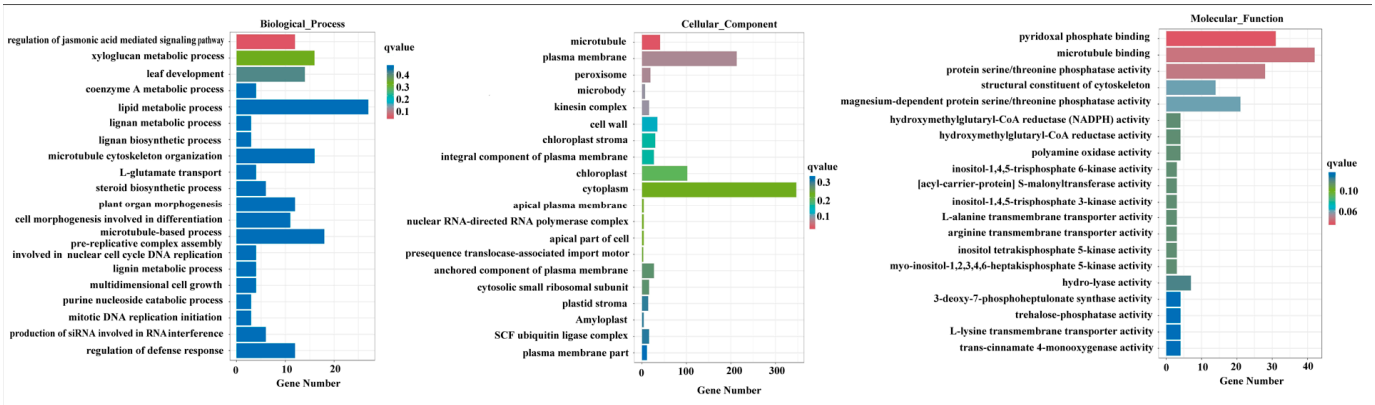

Figure 3. Differentially expressed genes (DEGs) enriched in the top 20 enriched GO terms in terms of biological processes, molecular functions, and cellular components in AFs and CFs.

Through KEGG pathway enrichment analysis, a total of 1372 DEGs were assigned to 128 KEGG pathways in the CFs vs. AFs comparison group, involved in metabolism, genetic information, tissue systems, environmental information processing, and cellular processes (Figure 4a). A total of 804 upregulated DEGs were allocated to 120 KEGG pathways, and 568 downregulated DEGs were allocated to 112 KEGG pathways (Table S5). Among these DEGs, many upregulated genes were enriched in pathways such as "ribosome (ko03010)", "cysteine and methionine (ko00270)", "carbon metabolism (ko01200)", "biosynthesis of amino acids (ko01230)", "citrate cycle (TCA cycle) (ko00020)", and "peroxisome (ko04146)" ($p < 0.05$, Figure 4c, Table S5). Conversely, the most common downregulated DEG-enriched KEGG pathways were "plant hormone signal transduction (ko04075)", "pentose and glucuronate interconversions (ko00040)", "plant–pathogen interaction (ko04626)", and "flavonoid biosynthesis (ko00941)" ($p < 0.05$, Figure 4d, Table S5). Furthermore, many downregulated and

upregulated DEGs were enriched in pathways such as "phenylpropanoid biosynthesis (ko00940)", "amino sugar and nucleotide sugar metabolism (ko00520)", "MAPK signaling pathway—plant (ko04016)", and "starch and sucrose metabolism (ko00500)" ($p < 0.05$, Figure 4b, Table S5). These results indicated that the above pathways were the main regulatory pathways for physiological fruit abscission and played a significant role in physiological fruit abscission.

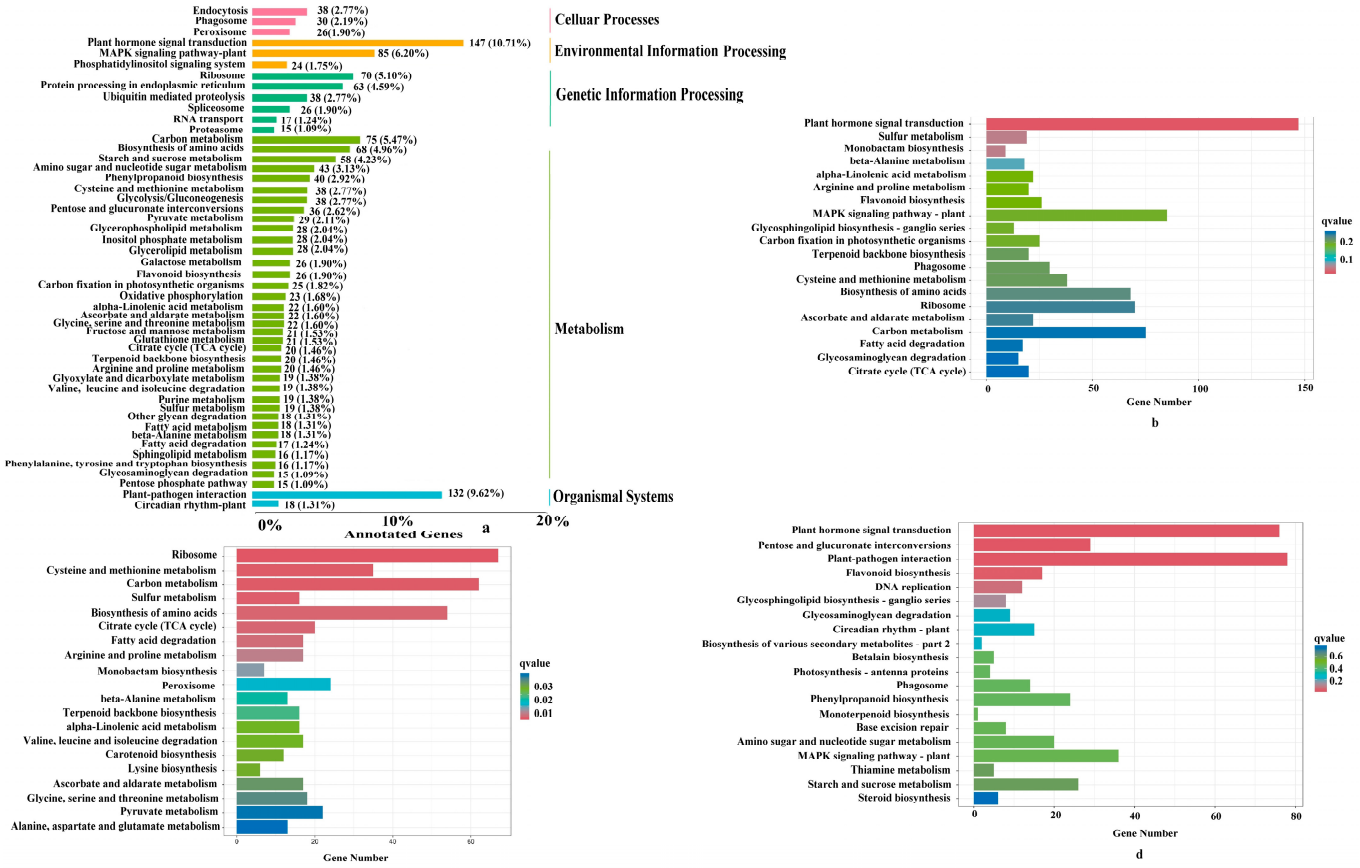

Figure 4. KEGG analysis from CF vs. AF group. (**a**) KEGG classification chart of DEGs. (**b**) KEGG enrichment bar chart of all DEGs. (**c**) KEGG enrichment bar chart of upregulated DEGs. (**d**) KEGG enrichment bar chart of downregulated DEGs.

2.3.3. DEGs Related to the Most Enrichment Pathways

Most upregulated DEGs were identified to be involved in pathways such as "ribosome", "cysteine and methionine", "carbon metabolism", "biosynthesis of amino acids", "citrate cycle (TCA cycle)", and "peroxisome" (Figure 4, Tables S5 and S6). Significantly, in the peroxisome pathway, 24 DEGs were upregulated, and 3-ketoacyl-CoA thiolase 2 (PED1), short-chain dehydrogenase reductase 3c (SDR3c), long-chain acyl-CoA synthetase 6 (LACS6), peroxisomal (S)-2-hydroxy-acid oxidase (GLO5), peroxisomal 2,4-dienoyl-CoA reductase (At3g12800), superoxide dismutase (SODA), F-box protein (At2g26850), alpha/beta hydrolase (LOC105038263), and serine-glyoxylate aminotransferase (AGT1) were significantly upregulated ($\log_2$FC > 2, Figure 5).

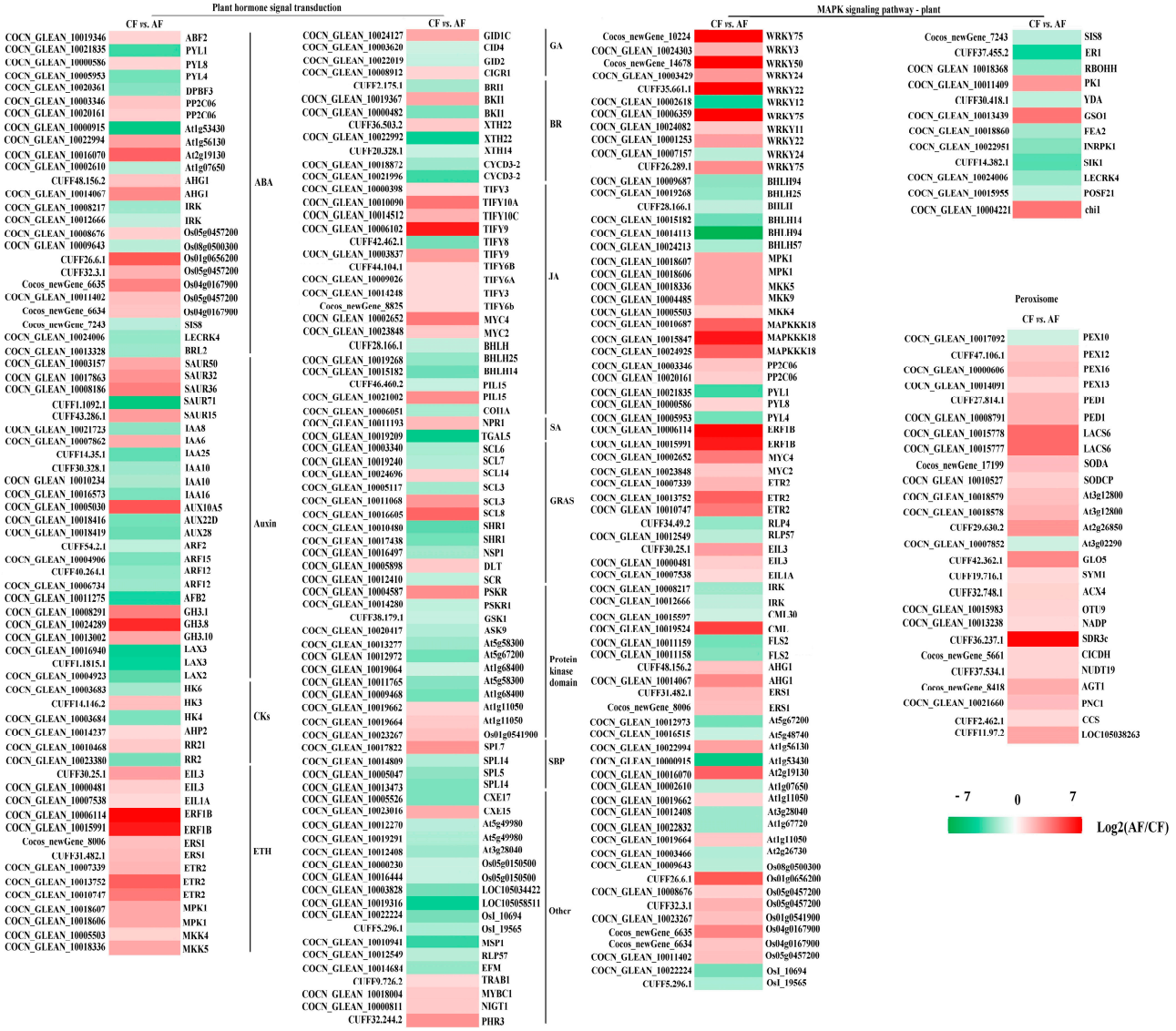

Figure 5. Expression of identified DEGs involved in the main KEGG enrichment pathways in CF vs. AF group.

Nonetheless, we also detected that the majority of downregulated DEGs were involved in "plant hormone signal transduction" (76 DEGs), "pentose and glucuronate interconversions" (29 DEGs), "plant–pathogen interaction" (78 DEGs), and "flavonoid biosynthesis" (17 DEGs) (Figure 4, Tables S5 and S6). In the plant hormone signal transduction pathway, 147 genes were differentially expressed. Among them, 76 DEGs were downregulated and 71 DEGs were upregulated. These DEGS were related to ABA (25), IAA (25), CKs (6), ETH (14), GA (4), BR (8), JA (18), SA (2), protein kinase (16), GRAS domain family (11), squamosa promoter-binding-like protein (4), etc. For ABA, 14 DEGs were upregulated, among which the expressions of probable protein phosphatase 2C (Os01g0656200, Os05g0457200, Os04g0167900, and AHG1), probable LRR receptor-like serine/threonine-protein kinase (At1g56130), and G-type lectin S-receptor-like serine/threonine-protein kinase (At2g19130) were significantly upregulated. However, 11 DEGs were downregulated, with significant downregulation in the expression of abscisic acid receptor (PYL1 and PYL4), abscisic acid-insensitive 5 (DPBF3), probable LRR receptor-like serine/threonine-protein kinase (At1g53430), G-type lectin S-receptor-like serine/threonine-protein kinase (LECRK4), and serine/threonine-protein kinase BRI1-like 2 (BRL2) ($\log_2$FC > 2, Figure 5). For auxin, nine DEGs were upregulated, among which DEG-encoded auxin-responsive protein (SAUR36,

SAUR32, SAUR50, SAUR15, and IAA6), auxin-induced protein (AUX10A5), and probable indole-3-acetic acid-amido synthetase (GH3.1, GH3.8, and GH3.10) were significantly upregulated. Nonetheless, 16 DEGs were downregulated, among which DEGs encoding auxin transporter-like protein (LAX2 and LAX3), auxin-responsive protein (IAA8, IAA25, IAA16, SAUR71), auxin-induced protein (AUX22D and AUX28), auxin response factor (ARF12 and ARF15), and protein auxin signaling F-BOX (AFB2) were significantly downregulated ($log_2FC > 2$, Figure 5). For CKs, three DEGs were upregulated and three DEGs were downregulated, with significantly downregulated expression of the coding probable histidine kinase (HK4) and two-component response regulator ORR2 (RR2). For ETH, all DEGs (12) were upregulated, among which the expression of ethylene-insensitive 3-like 3 protein (EIL3), ethylene-responsive transcription factor (ERF1B), ethylene receptor (ETR2), mitogen-activated protein kinase (MPK1), and mitogen-activated protein kinase (MKK5) were significantly downregulated. For GA, the expression of chitin-inducible gibberellin-responsive protein 1 (CIGR1) and gibberellin receptor (GID1C) encoded by two DEGs was significantly upregulated. However, the expression of two DEGs encoding polyadenylate-binding protein-interacting protein 4 (CID4) and F-box protein (GID2) was downregulated. For BR, six DEGs were downregulated, and one DEG encoding brassinosteroid LRR receptor kinase (BRI1), two DEGs encoding xyloglucan endotransglucosylase/hydrolase protein (XTH22 and XTH14), two DEGs encoding cyclin-D3-2 (CYCD3-2), and one DEG encoding BRI1 kinase inhibitor 1 (BKI1, Gi:COCN_GLEAN_10019367) were significantly downregulated. However, the expression of one DEG encoding BRI1 kinase inhibitor 1 (BKI1, Gi:COCN_GLEAN_10000482) was significantly upregulated. For JA, 12 DEGs were upregulated, among which DEGs encoding protein TIFY (TIFY10A and TIFY9) and transcription factor (MYC4, PIL15) were significantly upregulated in expression. Conversely, six DEGs associated with JA were downregulated, with significant downregulation of protein TIFY (TIFY8) and transcription factor (BHLH14 and bHLH25). The DEGs related to SA, encoding the BTB/POZ domain and ankyrin repeat-containing protein (NPR1), were upregulated. However, the expression of transcription factor (TGAL5) was significantly downregulated. For protein kinase, eight DEGs were upregulated, with significant upregulation of phytosulfokine receptor (PSKR), but eight DEGs were downregulated, including significant downregulation of probable inactive receptor kinase (At5g67200 and At5g58300) and probable leucine-rich repeat receptor-like protein kinase (At1g68400). For the GRAS domain family, five DEGs were upregulated, with two DEGs encoding scarecrow-like protein (SCL3 and SCL8) showing significant upregulation. Nonetheless, seven DEGs were downregulated, and the expression of two DEGs encoding the protein SHORT-ROOT (SHR1) was significantly downregulated. For squamosa promoter-binding-like protein, the expression of one DEG encoding the squamosa promoter-binding-like protein (SPL7) was significantly upregulated. Conversely, the expression of SPL5 and SPL14 encoded by two DEGs was significantly downregulated. In addition, some essential genes were also differentially expressed in the plant hormone signal transduction pathway. The expression of six DEGs encoding proteins (LOC105058511, OsI_10694 and LOC105034422), leucine-rich repeat receptor protein kinase (MSP1), probable carboxylesterase (CXE17), and Myb family transcription factor (EFM) was significantly downregulated. The expression of two DEGs encoding probable carboxylesterase (CXE15) and protein phosphate starvation response (PHR3) was significantly upregulated.

Furthermore, we also found that many downregulated and upregulated DEGs were enriched in pathways such as "phenylpropanoid biosynthesis" (24 DEGs), "amino sugar and nucleotide sugar metabolism" (20 DEGs), "MAPK signaling pathway—plant" (36 DEGs), and "starch and sucrose metabolism" (26 DEGs) (Figure 4, Tables S5 and S6). In the MAPK signaling pathway—plant pathway, 49 DEGs were upregulated; the DEGs encoding transcription factor (WRKY75, WRKY3, MYC4, WRKY24, ERF1B, and WRKY22), ethylene-insensitive 3-like 3 protein (EIL3), probable protein phosphatase 2C (Os01g0656200, Os05g0457200, Os04g0167900, and AHG1), probable LRR receptor-like serine/threonine-protein kinase (At1g56130), putative receptor protein kinase (PK1), G-type lectin S-receptor-like serine/threonine-protein kinase

(At2g19130), mitogen-activated protein kinase (MAPKKK18, MPK1, MKK9, MPK1, and MKK5), ethylene receptor 2 (ETR2), and endochitinase (chi1) were significantly upregulated. Conversely, the expression of 36 DEGs was downregulated; the DEGs encoding transcription factor (bHLH94, BHLH25, BHLH14, and WRKY12), receptor-like protein 4 (RLP4), probable inactive receptor kinase (At5g67200), LRR receptor-like serine/threonine-protein kinase (ER1, At1g53430, At1g67720, FLS2, and SIK1), G-type lectin S-receptor-like serine/threonine-protein kinase (LECRK4), putative respiratory burst oxidase homolog protein H (RBOHH), abscisic acid receptor (PYL1 and PYL4), protein G1-like7 (OsI_10694), and receptor-like protein kinase (INRPK1) were significantly downregulated ($log_2FC > 2$, Figure 5).

2.3.4. DEGs Related to Cell Wall Remodeling

Cell wall remodeling is one of the methods by which cells regulate abscission. We performed RNA-seq analysis on AZ tissues of CFs and AFs (Figure 6, Table S7). Among these DEGs associated with cell wall remodeling, five polygalacturonases (GSVIVT00026920001), ten glucan endo-1,3-beta-glucosidases (At4g34480(3), At1g11820(2), At3g13560(2), At5g58480, At5g56590, At1g32860), one mannan endo-1,4-beta-mannosidase (MAN1 (CUFF3.296.1)), two endoglucanases (GLU2 and Os09g0533900), one endochitinase A (ECHITA), three beta-galactosidases (Os03g0165400 (2) and Os01g0875500), eleven xyloglucan endotransglucosylase/hydrolase proteins (XTH28, XTHB, XTH22, XTH8, XTH7(3), XTH29, XTH14, XTH32, and XXT2), three probable xyloglucan glycosyltransferases (CSLC5), one xyloglucan galactosyltransferase (GT19), one protein-altered xyloglucan (AXY4), three expansins (EXPA4), thirteen pectinesterase/pectinesterase inhibitors (PME12(2), PPE8B(3), PME, PME9, PME3(2), PECS-1.1, PME51, PME53, PMEI3, PMEI9, and PME2.1), and ten peroxides (PER47, PER42 (2), PER17, PER72(2), PER15, PER63, PER35, and PER48) were downregulated. Conversely, the expressions of two polygalacturonases (PG and PGIP), three glucan endo-1,3-beta-glucosidases (GLC1, BANGLUC, and At2g27500), one mannan endo-1,4-beta-mannosidase (MAN1 (Cocos_nucifera_newGene_4975)), two acidic endochitinases (CHIT3), three chitinases (chi1(2) and chi2), five beta-galactosidases (Os03g0255100 (4) and Os01g0580200), two xyloglucan endotransglucosylase/hydrolase proteins (XTH3 and XTH22), one expansin (EXPA8), three L-ascorbate peroxidases (APX1, APX6, and APX8), and one cationic peroxidase (Sb03g046810) were upregulated.

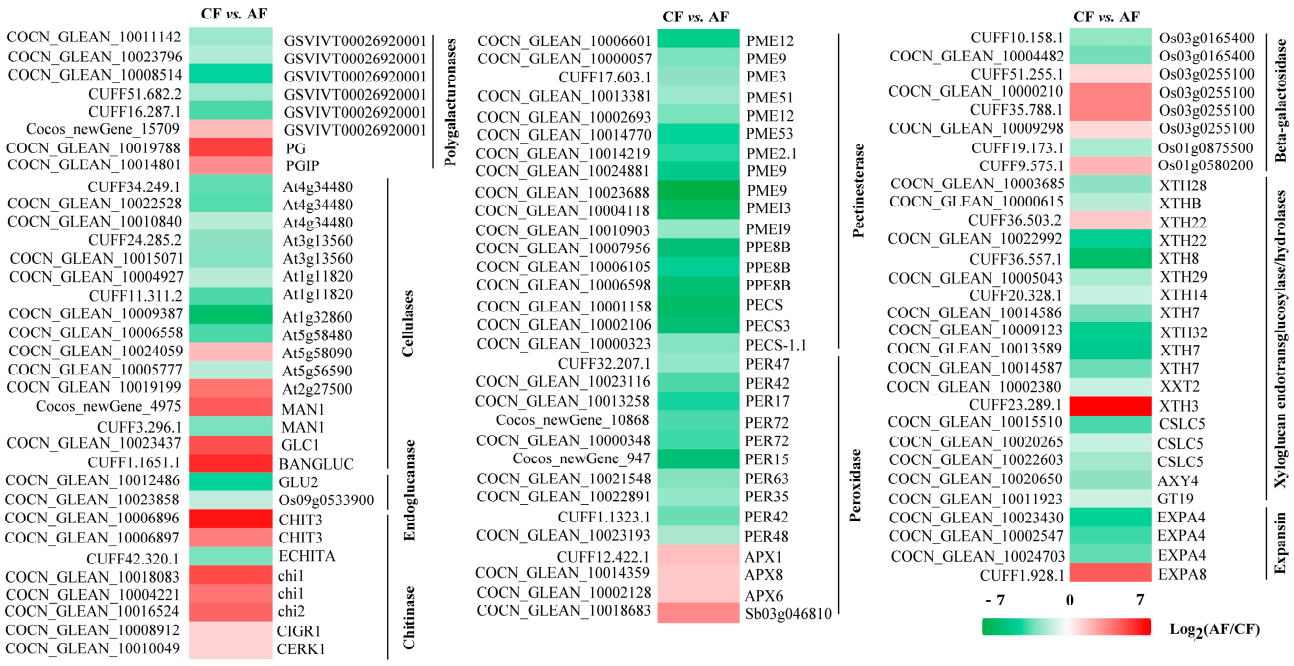

Figure 6. Expression of identified DEGs involved in cell wall modification in CF vs. AF group.

2.3.5. DEGs Related to Phytohormones

To determine the expression and regulation of various phytohormone-related genes in normal and physiological fruit abscission of coconuts, phytohormone-related genes such as auxin (43), ABA (112), ETH (30), CKs (12), GAs (11), BR (16), JA (14), and SA (5) were analyzed, as shown in Figure 7 and Table S8.

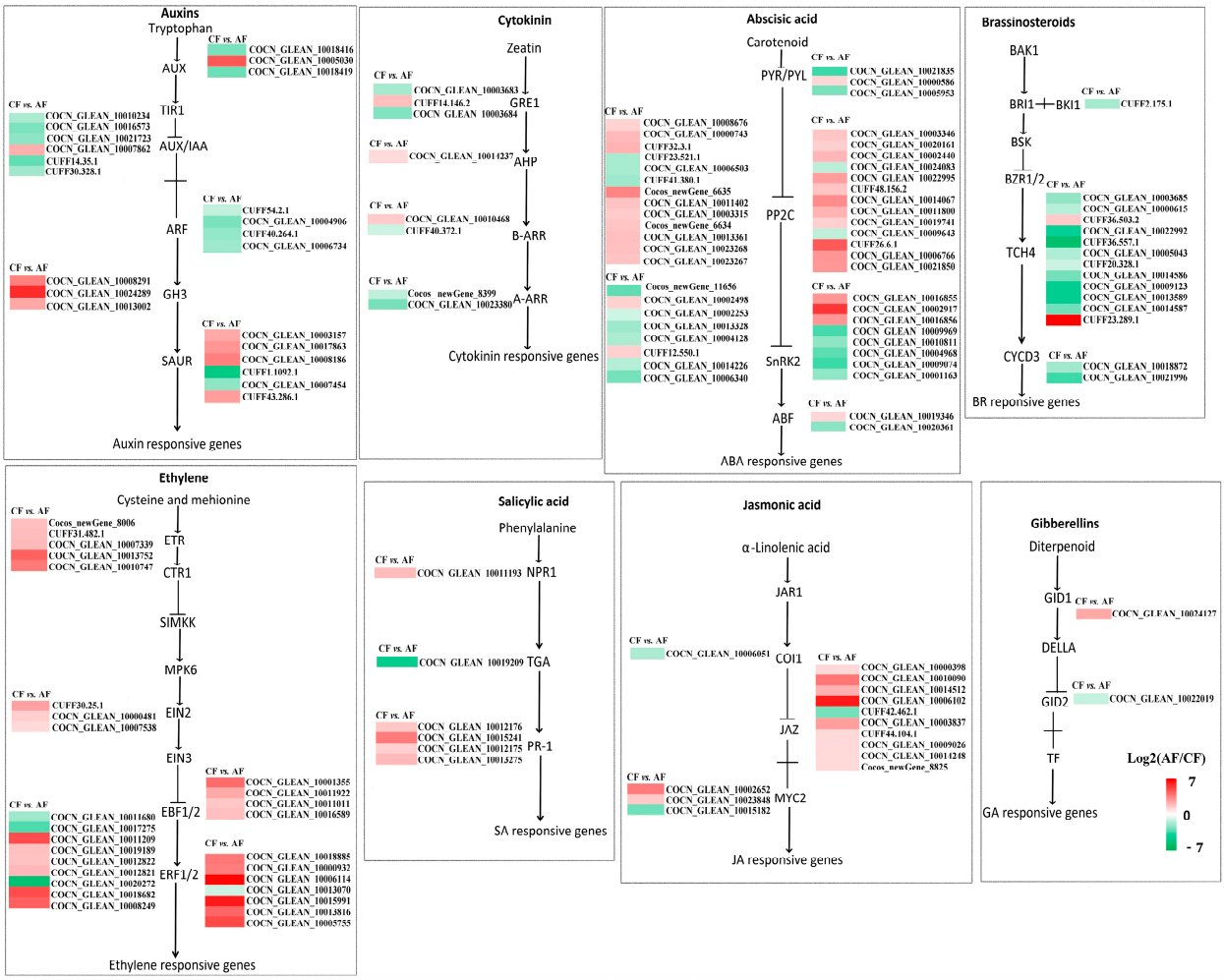

Figure 7. Heatmap of relative changes in expression patterns of 8 phytohormone-related genes in AZ of CF vs. AF group. The color scales on each heatmap display their expression values.

In the auxin pathway, 7 out of 12 DEGs related to auxin-responsive proteins were downregulated, and the DEGs encoding IAA16, IAA8, IAA25, SAUR71, and SAUR76 were significantly downregulated. However, 5 out of 12 DEGs related to auxin-responsive proteins were upregulated, and the DEGs encoding IAA6, SAUR50, SAUR32, SAUR36, and SAUR15 were significantly upregulated. In addition, four DEGs associated with auxin response factors were downregulated, and the three DEGs encoding ARF15 and ARF12 were significantly downregulated. In addition, two out of three DEGs related to auxin-induced proteins encoding AUX22D and AUX28 were significantly downregulated. However, the DEG encoding AUX10A5 was significantly upregulated. Additionally, three DEGs encoding GH3.1, GH3.8, and GH3.10 related to indole-3-acetic acid-amido synthetase were significantly upregulated. Moreover, the three DEGs related to the transport inhibitor response were downregulated. Furthermore, differential expression of 18 DEGs associated with other auxin proteins also occurred, with 7 out of 18 DEGs upregulated, and the DEGs encoding PILS1, PILS6, PILS7, and ARGOS were significantly upregulated. A total of 11 out of 18 DEGs were downregulated, and the DEGs encoding PIN3A, LAX3, LAX2, BG1, AFB2,

and ABP19A were significantly downregulated. The bidirectionality of gene expression in the auxin signaling pathway indicated the complexity of its regulatory mechanism on early physiological fruit abscission in coconuts.

In the ABA pathway, one DEG encoding CYP707A3 related to abscisic acid 8′-hydroxylase was significantly downregulated. However, CYP707A7 was significantly upregulated. Additionally, two DEGs encoding PYL1 and PYL4 related to abscisic acid receptors were significantly downregulated. However, PYL8 was upregulated. Moreover, 21 out of 26 DEGs associated with probable protein phosphatase 2C were upregulated; in particular, the DEGs encoding BIPP2C2, WIN2, AHG1, Os05g0134200, Os01g0656200, Os02g0690500, Os05g0457200, and Os04g0167900 were significantly upregulated. In addition, 53 out of 76 DEGs related to serine/threonine-protein kinase were downregulated; in particular, the DEGs encoding NEK2, UNC, AGC1-5, MHK, BRL2, CR4, PBL8, PBL26, GSO2, RGI3, FLS2, GHR1, ER1, FEI1, SIK1, IRK, ALE2, BAM3, BAM1, LECRK4, and CIPK21 were significantly downregulated. However, 23 out of 76 DEGs were upregulated, and the DEGs encoding CCR4, PBL19, WNK5, PIX7, GSO1, SD18, RBK1, and CIPK14 were significantly upregulated. In addition, two DEGs encoding NCED related to 9-cis-epoxycarotenoid dioxygenase were significantly upregulated; however, NCED1 was significantly downregulated. Furthermore, one DEG encoding abscisic acid-insensitive 5-like protein 5 (ABF2) was upregulated. In addition, one DEG encoding abscisic acid-insensitive 5-like protein 2 (DPBF3) was significantly downregulated. The bidirectional expression of ABA pathway genes indicated that the regulatory mechanism of early physiological fruit abscission in coconuts is complex.

In the ETH pathway, 16 out of 22 DEGs related to ethylene-responsive transcription factors were upregulated, and the DEGs encoding ERF1, ERF4, ERF110, ERF008, ERF1B, ERF071, ERF113, RAP2-1, and RAP2-3 were significantly upregulated. However, 6 out of 22 DEGs were downregulated, and the DEGs encoding ERF118, ERF023, and WRI1 were significantly downregulated. In addition, two DEGs encoding EIL3 and EIL1A that were related to ethylene-insensitive 3-like 3 protein were upregulated. Moreover, two DEGs encoding probable ethylene response sensor 1 (ERS1) were upregulated. Additionally, three DEGs encoding ethylene receptor 2 (ETR2) were significantly upregulated. From this, it can be seen that most ETH pathway genes are induced, indicating their positive regulatory effect on early physiological fruit abscission in coconuts.

In the CK pathway, 8 out of 13 DEGs were upregulated, and the DEGs encoding probable cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOGL1) and cytokinin dehydrogenase (CKX6 and CKX9) were significantly upregulated. However, 5 out of 13 DEGs were downregulated, and the DEGs encoding probable histidine kinase 4 (HK4) and two-component response regulator ORR2 (RR2) were significantly downregulated.

In the GA pathway, one DEG encoding gibberellin 20 oxidase 1-D (GA20ox1D), five DEGs encoding gibberellin 2-beta-dioxygenase (GA2OX6, GA2OX1, GA2OX5 (2), GA2OX8)), one DEG encoding gibberellin-regulated protein 13 (GASA13)), one DEG encoding gibberellin receptor (GID1C), and one DEG encoding chitin-inducible gibberellin-responsive protein 1 (CIGR1) were upregulated; in particular, GA20ox1D, A2OX6, GA2OX1, GA2OX5, GA2OX8, GASA13, and GID1C were significantly upregulated. However, one DEG encoding gibberellin-regulated protein 12 (GASA12) and one DEG encoding F-box protein (GID2) were downregulated, and GASA12 was significantly downregulated.

In the BR pathway, one DEG encoding gibberellin-regulated protein 12 (GASA12), one DEG encoding brassinosteroid-responsive RING protein 1 (BRH1), ten DEGs encoding xyloglucan endoglucosidase/hydrolase protein (XTH28, XTHB, XTH22, XTH8, XTH29, XTH14, XTH7 (3), and XTH32), and two DEGs encoding cyclin-D3-2 (CYCD3-2) were downregulated, and BRH1, XTH28, XTH22, XTH8, XTH7, XTH32, XTH7, and CYCD3-2 were significantly downregulated. However, one DEG encoding xyloglucan endotransglucosylase/hydrolase protein 3 (XTH3) was significantly upregulated.

In the JA pathway, eight DEGs encoding proteins TIFY (TIFY10A, TIFY10C, TIFF9 (2), TIFY6B (2), TIFY6A, and TIFY3) and two DEGs encoding transcription factors (MYC2 and MYC4) were upregulated, and TIFY10A, TIFY10C, TIFY9, and MYC4 were signifi-

cantly upregulated. However, DEGs encoding TIFF8, MYC3, and coronatine-insensitive protein homolog 1a (COI1A) were significantly downregulated, and TIFY8 and MYC3 were significantly downregulated.

In the SA pathway, all identified DEGs related to pathogenesis-related genes encoding transcriptional activator (PTI6 (3) and PTI5 (1)) and BTB/POZ domain and ankyrin repeat-containing protein (NPR1) were upregulated, and PTI5 was significantly upregulated.

2.3.6. TFs

Transcriptional regulation plays a crucial role in a series of complex events leading to the abscission of plant organs. Therefore, TFs also play an important role in this process. The differential expression of TFs was analyzed to determine the TFs involved in the early physiological abscission of coconut fruits (Figure 8, Table S9). We identified 136 TFs in differential expressions, and most of them showed significant differences in expressions. This included BHLH (35), MYB (24), ERF/AP2 (22), WRKY (17), GATA (7), bZIP (6), HSF (6), NAC (4), NFY (4), TCP (2), PCF (2), and other (7) TFs. For BHLH, 10 were upregulated and 25 were downregulated. For MYB, 15 were upregulated and 9 were downregulated. For ERF/AP2, 16 were upregulated and 6 were downregulated. For WRKY, 11 were upregulated and 6 were downregulated. For GATA, two were upregulated and five were downregulated. For bZIP, four were increased and two were decreased. For HSF, two were upregulated and four were downregulated. For NAC, there were four upregulations. For NFY, there were three upregulations and one downregulation. For TCP, there were two downregulations. For PCF, there was one upregulation and one downregulation. For other TFs, two were upregulated and five were downregulated. The above results indicated that these TF families may also play a key role as transcriptional regulatory genes in the early stages of coconut fruit development and abscission.

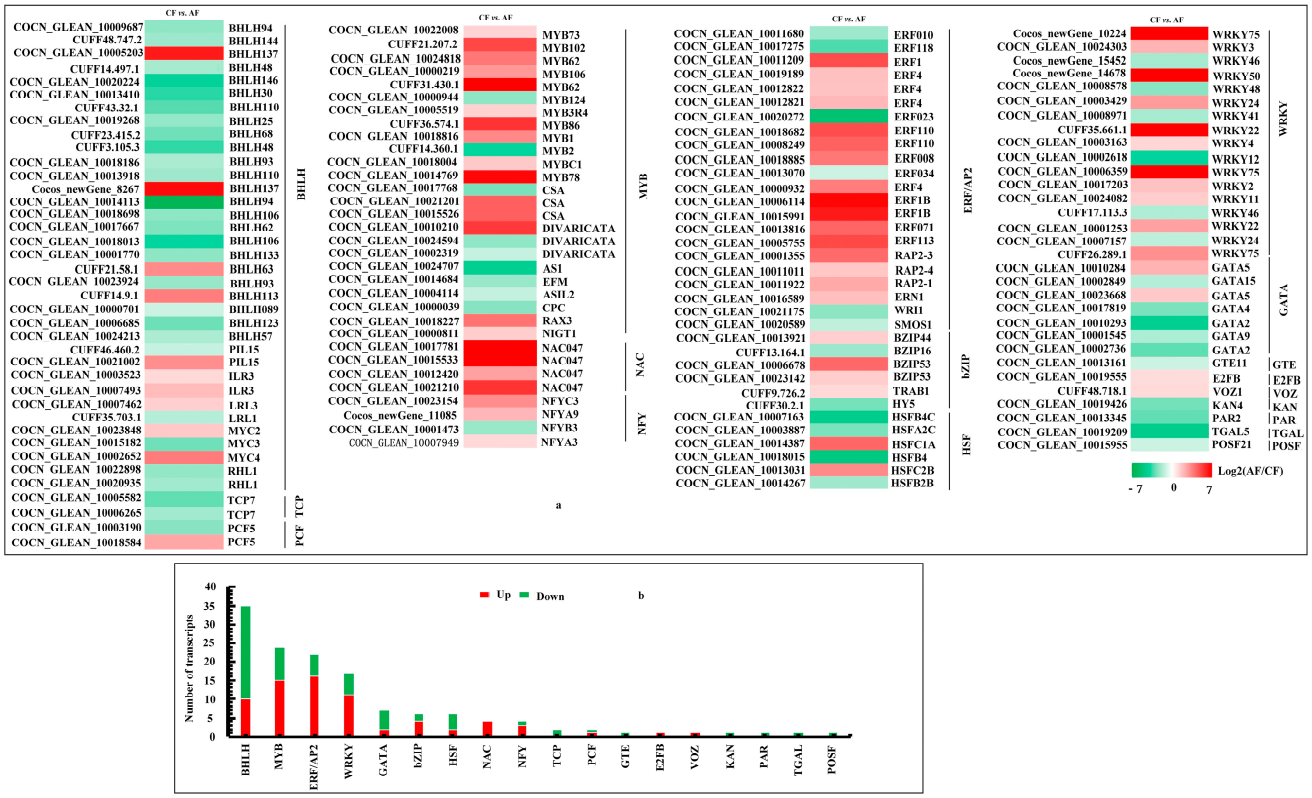

Figure 8. Transcription factor analysis in CF vs. AF group. (**a**) Expression of identified DEGs involved in transcription factors in CF vs. AF group. (**b**) Distribution of overexpression of the regulatory transcription factor family in CF vs. AF group.

2.4. DEG Validation by RT-qPCR

To confirm the gene expression results obtained from transcriptome data, we selected 11 DEGs related to plant organ abscission for qRT-PCR (Table S10). These DEGs were mainly involved in plant hormone signal transduction (IAA8, SAUR76, BIPP2C2, RR2, and BRH1), plant cell wall remodeling (XTH7, GSVIVT00026920001, GLU2, and EXPA4), and transcriptional regulation (ERF4 and MYC4). Compared to CF, BIPP2C2, ERF4, and MYC4 were upregulated in AZ tissues of AFs. However, IAA8, SAUR76, RR2, BRH1, XTH7, GSVIVT00026920001, GLU2, and EXPA4 were downregulated; these genes may regulate the abscission of early coconut fruits. According to the results of Seq-RNA and qRT-PCR, the expression trends in these 11 DEGs are similar, indicating the accuracy of transcriptome analysis (Figure 9).

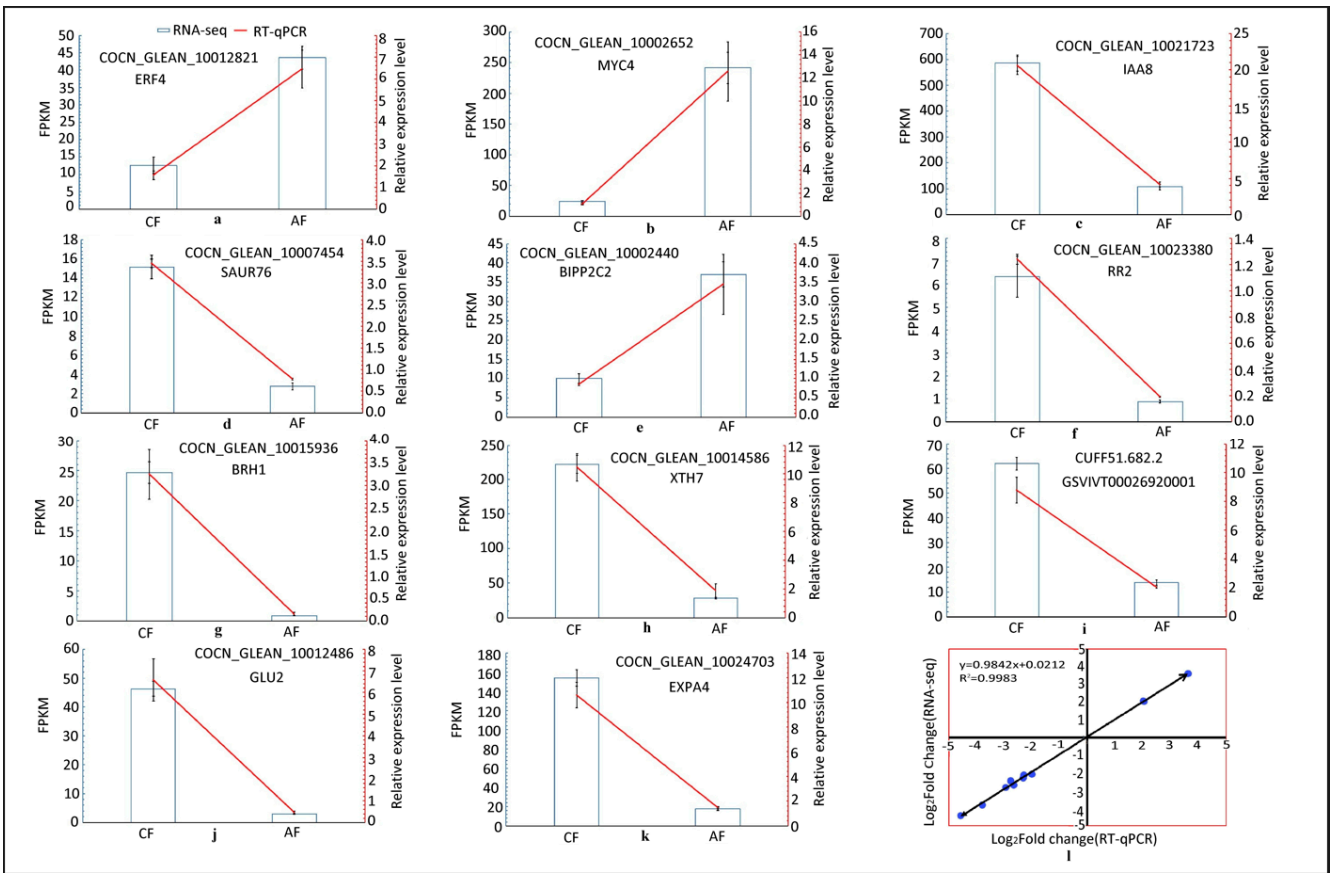

Figure 9. Verification of the expression of 11 coconut fruit abscission-related genes through qRT-PCR analysis. The bar chart represents the value of FPKM. The line graph represents qRT-PCR values. The error bar represents the standard deviation of three biological replicates (**a**–**k**). Correlation of expression changes observed through RNA-seq (*y*-axis) and qRT-PCR (*x*-axis) (**l**).

3. Discussion

There is a good correlation between fruit abscission and the polar transport of auxin, carbohydrates, and ABA. Abscission is a process that is influenced by both external and internal factors. This process involves complex mechanisms or modification processes, including cell wall modification, plant hormone biosynthesis, signal transduction pathways, and pathogen defense regulation [4,5]. This study combined morphological, transcriptomic, plant hormone, and enzyme activity analyses to reveal the molecular mechanism of early fruit abscission in coconuts. Our research suggested that the abscission of early coconut fruits may be related to the biosynthesis and signal transduction of plant hormones, cell wall remodeling, and transcription factors (Figure 10). Understanding the molecular

mechanisms of early coconut fruit physiological abscission was of great significance for regulating fruit physiological abscission.

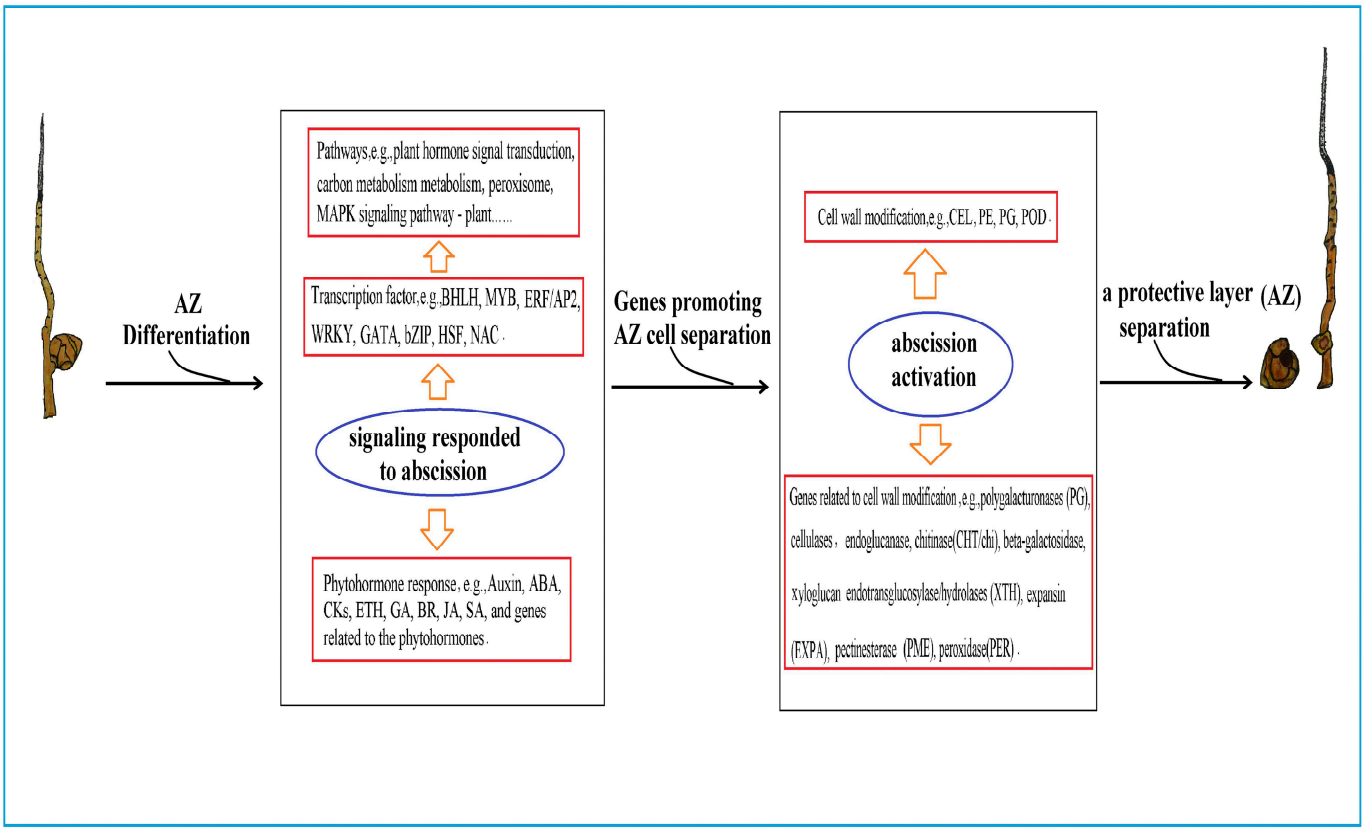

Figure 10. A hypothetical model for coconut fruit abscission.

3.1. Crucial Enrichment Pathways

The crucial KEGG enrichment analysis showed that many upregulated genes were enriched in pathways such as "ribosome", "cysteine and methionine", "carbon metabolism", "biosynthesis of amino acids", "citrate cycle (TCA cycle)" and "peroxisome". Nonetheless, the most common downregulated DEG-enriched KEGG pathways were "plant hormone signal transduction", "pentose and glucuronate interconversions", "plant–pathogen interaction", and "flavonoid biosynthesis". In addition, many downregulated and upregulated DEGs were enriched in pathways such as "phenylpropanoid biosynthesis", "amino sugar and nucleotide sugar metabolism", "MAPK signaling pathway—plant", and "starch and sucrose metabolism" ($p < 0.05$, Figure 4, Table S5). These results indicated that the above pathways were the main regulatory pathways for physiological fruit abscission and played a significant role in physiological fruit abscission. These pathways were mainly involved in physiological metabolism, plant hormone signal transduction, cell wall modification, transcription factor regulation, pathogen defense regulation, etc. [5].

3.2. Phytohormones

The regulatory role of plant hormones is crucial throughout the entire process of plant organ abscission, as they mediate the response of plant organs to stress. Plant hormones may play a role in promoting or inhibiting abscission signaling, depending on different tissues, concentrations, homeostasis, and their receptor affinity, transport, or interactions with each other, and the response is complex [3]. Some plant hormones, including ETH, ABA, JA, and methyl jasmonate (MeJA), act as abscission-acceleration signals [3,11], while IAA, GA, CKs, and polyamines are considered abscission inhibitors [3]. Due to the involvement of plant hormones throughout the entire plant development cycle, several genes controlling

abscission also form part of the biosynthesis of plant hormones and signal transduction pathways, or affect their metabolism [43].

Auxins are involved in the abscission of plant organs. Transcriptome analysis showed that auxin may regulate the expression of early auxin-responsive gene families, including upregulation of AUX/IAA, GH3, and small auxin RNA (SAUR) expression. Auxin regulates the expression of various ARFs during the early abscission of tomato, while ETH has the opposite effect on most of these genes in tomato [44]. *Slarf* has overlapping functions during the detachment process. The abscission of tomato flowers is related to the expression levels of AUX/IAA genes [30]. The GH3 gene is also involved in the abscission of fruits or flowers. LcAUX/IAA1 may play a more important role in lychee abscission than LcGH3.1, as LcAUX/IAA1 and LcSAUR1 are more expressed in the AZ [24]. Through transcriptome analysis, it was found that two SAUR genes are involved in induced abscission in apples [45]. SAUR36 has been reported to be involved in leaf senescence in Arabidopsis [46]. In this study, in the auxin pathway, IAA16, IAA8, IAA25, SAUR71, SAUR76, ARF15, ARF12, AUX22D, AUX28, PIN3A, LAX3, LAX2, BG1, AFB2, and ABP19A were significantly downregulated. However, IAA6, SAUR50, SAUR32, SAUR36, SAUR15, AUX10A5, GH3.1, GH3.8, GH3.10, PILS1, PILS6, PILS7, and ARGOS were significantly upregulated (Figure 7). The bidirectionality of gene expression in the auxin signaling pathway indicated the complexity of its regulatory mechanism on early physiological fruit abscission in coconuts.

The important role of ABA in the process of abscission has been widely studied in different species. Exogenous ABA treatment can induce apple fruit abscission, and ABA may be involved in the upstream induction of abscission. An increase in ABA levels was observed in apple fruits treated with ABA, along with upregulation of the ABA-responsive 9-cis-epoxycarotenoid dioxygenase 1 (MdNCED1) gene, indicating activation of the indirect biosynthesis pathway of ABA [47]. In this study, the genes related to ABA such as CYP707A3, PYL1, PYL4, NEK2, UNC, AGC1-5, MHK, BRL2, CR4, PBL8, PBL26, GSO2, RGI3, FLS2, GHR1, ER1, FEI1, SIK1, IRK, ALE2, BAM3, BAM1, LECRK4, CIPK21, NCED1, and DPBF3 were significantly downregulated. However, CYP707A7, PYL8, BIPP2C2, WIN2, AHG1, Os05g0134200, Os01g0656200, Os02g0690500, Os05g0457200, Os04g0167900, CCR4, PBL19, WNK5, PIX7, GSO1, SD18, RBK1, CIPK14, NCED, and ABF2 were significantly upregulated (Figure 7). The bidirectional expression of ABA pathway genes indicated that the regulatory mechanism of early physiological fruit abscission in coconuts is complex.

The ETH signaling pathway is significantly enhanced in peach fruit abscission, and high expression of some ETH signaling genes is shown. The mitogen-activated protein kinase (MAPK) gene is upregulated in abscised fruits [19] and is a key element in the ETH signaling pathway, possibly involved in ETH-ABA crosstalk [48]. ETH, as a signaling molecule, induces cell separation and promotes abscission [49]. Arabidopsis mutants (*ein2*, *ein3*, *etr1*, and *ers2*) lack ETH receptors or downstream pathway members and exhibit varying degrees of delay in flowering organ abscission [49]. ABA and CKs are key plant hormones that regulate plant abscission. However, current evidence suggests that the effects of ABA and CKs on plant organ abscission may be mediated by IAA or ETH rather than directly acting [1]. In this study, the genes related to ETH such as ERF1, ERF4, ERF110, ERF008, ERF1B, ERF071, ERF113, RAP2-1, RAP2-3, EIL3, EIL1A, ERS1, and ETR2 were significantly upregulated. However, ERF118, ERF023, and WRI1 were significantly downregulated (Figure 7). From this result, it can be seen that most ETH pathway genes were induced, indicating their positive regulatory effect on early physiological fruit abscission in coconuts.

The signaling pathways of CKs, JA, SA, and BR are also involved in the abscission of pecan fruits [19]. The correlation between response variables and expression data of genes such as CKs, JA, SA, and BR is detected in abscised apple fruits [37]. In this study, 8 out of 13 DEGs were upregulated in the CK pathway, and LOGL1, CKX6, and CKX9 were significantly upregulated. However, 5 out of 13 DEGs were downregulated, and HK4 and RR2 were significantly downregulated (Figure 7). CKs have been reported

as abscission-promoting factors, but high concentrations of CKs can inhibit abscission. In cotton, exogenous treatment of ETH promotes the degradation of endogenous CKs and enhances the production of endogenous ETH, leading to cell wall damage and cell separation. CKX catalyzes the breakdown metabolism of CKs into inactive products; therefore, activation of CKX leads to a decrease in endogenous CKs in plant cells [50].

GA and BRs have been shown to be inhibitors of fruit abscission [12–14]. In the GA pathway, GA20ox1D, GA2OX6, GA2OX1, GA2OX5, GA2OX8, GASA13, GID1C, and CIGR1 were upregulated; in particular, GA20ox1D, A2OX6, GA2OX1, GA2OX5, GA2OX8, GASA13, and GID1C were significantly upregulated. However, GASA2 and GID2 were downregulated, and GASA2 was significantly downregulated (Figure 7). Similarly, previous studies have found that some GA biosynthetic enzyme components, such as GA20 oxidase (GA20ox), are involved in the later stages of GA biosynthesis and are only enriched in mature fruits. Compared with fruit AZs, the highest GA level detected in mature olive fruits is consistent. On the contrary, GA2ox5 is a transcript involved in the inactivation of biologically active GAs, expressed only in the AZ, indicating that GA2ox plays a major role in the AZ [51]. Correspondingly, the changes in genes related to GA (such as GA20ox1D, A2OX6, GA2OX1, GA2OX5, and GA2OX8) may indicate their important role in early coconut fruit abscission (Figure 7). In the BR pathway, BRI1, BRH1, XTH28, XTHB, XTH22, XTH8, XTH29, XTH14, XTH7, XTH32, and CYCD3-2 were downregulated, and BRH1, XTH28, XTH22, XTH8, XTH7, XTH32, XTH7, and CYCD3-2 were significantly downregulated. However, XTH3 was significantly upregulated (Figure 7). Some studies have found that BRs inhibit ETH, inducing fruit abscission through transcriptional repression of LcACS1/4 and LcACO2/3 genes in lychee (litchi) mediated by LcBZR1/2 [52].

The response of fruit AZ cells to internal and external abscission trigger signals is mediated by plant hormones [1]. To a certain extent, ETH, JA, SA, and ABA are abscission promoters [11,12]. In this study, the JA and SA contents in the AZ of early-abscised coconut fruits (AFs) were significantly higher than those in normal fruits (CFs) (Figure 2). In the JA pathway, TIFY10A, TIFY10C, TIFF9, TIFY6B, TIFY6A, TIFY3, MYC2, and MYC4 were upregulated, and TIFY10A, TIFY10C, TIFY9, and MYC4 were significantly upregulated. However, TIFF8, MYC3, and COI1A were significantly downregulated, and TIFF8 and MYC3 were significantly downregulated (Figure 7). In the SA pathway, all identified DEGs related to pathogenesis-related genes such as PTI6, PTI5, and NPR1 were upregulated, and PTI5 was significantly upregulated (Figure 7). The above research results also indicated that JA and SA hormones may play important roles in the process of coconut fruit abscission.

3.3. Cell Wall Remodeling-Related Genes

Abscission is an active physiological process that dissolves cell walls at predetermined locations, namely abscission zones (AZs) [25]. The most direct cause of plant organ abscission is the degradation of cell walls due to changes in the activity of cell wall hydrolytic enzymes. There are several genes that regulate the function of plant cell walls. The changes in its expression are related to aging, organ growth and development, fruit ripening, and organ abscission [26]. However, it is worth noting that most enzymes involved in cell wall degradation show an upregulating trend, including CEL, PE, PG, and beta-galactosidase, which play a major role in cell wall degradation [25,26]. In addition, it has been confirmed that the EXP is related to the wall extension process during cell growth [27]. However, it is clear that EXP also makes important contributions to the fruit softening process, including wall rupture rather than expansion. Research has found that expansion proteins play an important role in ethylene-mediated abscission processes. The function of elastin may increase the disorder of cellulose crystals, making it easier for glucan chains to hydrolyze. XTH is one of the main hemicellulose components in the primary cell wall of dicotyledonous plants, accounting for 10%–20% of the cell wall composition [27]. XTHs belong to a multigene family and play important roles in several different processes of cell wall modification. These factors include the softening tension of the fruit, the formation of wood, and the shedding of petals. This suggests that the changes mediated by XTHs may make it

easier for the cell wall to approach other cell wall hydrolytic enzymes, thereby accelerating shedding [53,54].

Similar to previous results, this study also showed that the activities of CEL and PG in the AZ in AFs were significantly higher than those in CFs, but the activities of PE and POD were significantly lower than those in CF (Figure 2). Among these DEGs related to cell wall remodeling, five polygalacturonases, ten glucan endo-1,3-beta-glucosidases, one mannan endo-1,4-beta-mannosidase (MAN1 (CUFF3.296.1)), two endoglucanases (GLU2 and Os09g0533900), one endochitinase A (ECHITA), three beta-galactosidases (Os03g0165400 (2) and Os01g0875500), eleven xyloglucan endotransglucosylase/hydrolase proteins, three probable xyloglucan glycosyltransferases (CSLC5), one xyloglucan galactosyltransferase (GT19), one protein-altered xyloglucan (AXY4), three expansins (EXPA4), thirteen pectinnesterase/peptinesterase inhibitors, and ten peroxidases were downregulated. It is incredible that the expression of genes related to polygalacturonase, glucan endo-1,3-beta-glucosidase, mannan endo-1,4-beta-mannosidase, chitinase, beta-galactosidase, xyloglucan endotransglucosylase/hydrolase protein, expansin, and peroxidase was both upregulated and downregulated (Figure 6). The expression of these cell wall remodeling genes underwent bidirectional changes. This phenomenon may not only be related to the ongoing abscission process but also to the gradual development of organs that have not yet shed. The differential expression of genes in the above results may be a regulation of cell wall remodeling. These results indicated that genes related to cell wall remodeling played an important role in the process of fruit abscission. Regardless of the differential expression of these cell wall remodeling enzyme genes, the cell wall was reconstructed, leading to degradation of the cell wall or intermediate layer, resulting in cell separation and fruit abscission.

3.4. TFs Related to Fruit Abscission

Transcriptional regulation plays a crucial role in a series of complex events leading to the shedding of plant organs. Therefore, transcription factors also play an important role in this process. In the study, we found that BHLH, MYB, ERF/AP2, WRKY, GATA, bZIP, HSF, and NAC were the main transcription factors in AFs (Figure 8). BHLH plays a crucial role in flower and fruit abscission [30–33]. In the early-abscised coconut fruits, 35 BHLH gene expression differences were also found, with 10 BHLH genes upregulated and 25 BHLH genes downregulated. For other TF families, ERF1 inhibits BGLA by activating the transcription inhibitor ERF4, thereby suppressing pectin degradation and petal abscission [55]. In tomatoes, SlERF52 regulates the transcription of SlTIP1, thereby increasing the content and permeability of hydrogen peroxide in the cytoplasm and accelerating the process of pore shedding [56]. The lychee gene LcERF2 is a member of the ethylene-responsive AP2/ERF family, which regulates fruit abscission by directly targeting UDP-glucose-4-isomerase. Overexpression of LcERF2 promotes fruit abscission and reduces the levels of galactose and pectin in the cell walls of flower stems [57]. According to reports, ethylene-insensitive 2 (EIN2) acts as a positive regulator of the ethylene signaling pathway downstream of CTR1. EIN3 acts downstream of EIN2. EIN3 encodes downstream components of the ethylene signaling pathway, ultimately activating ETH-responsive genes. EILs are positive regulatory factors for various ethylene reactions throughout plant development [58]. A total of 22 ERF/AP2 genes were found, and compared with CF, 16 ERF/AP2 genes were upregulated in AF, but 6 genes were downregulated. These genes may be involved in the balance of plant hormones and the hydrolysis of cell walls.

Most MYB family members are upregulated in fruit AZs. The MYB protein is a key component of various hormone-regulated transcriptional cascades and cell wall biogenesis, regulating the shedding of tomato flowers, leaves, and fruits [58]. At present, in our comparison of the AZs in CFs and AFs, 15 MYB TFs are upregulated, while 9 are downregulated (Figure 8). This also indicates that MYB plays a role in regulating the early physiological fruit abscission of coconuts.

According to reports, GhWRKY51 can directly activate the expression of the key synthetic gene for salicylic acid (SA) SID2, thereby promoting the accumulation of SA and mediating the balance of cotton plant growth and immune response [59]. The expression of WRKY70 regulated by SA is positively correlated with the activation of pathogen-related genes [60]. We found that 11 WRKY genes were upregulated, but 6 WRKY genes were downregulated (Figure 8). This also suggests that WRKY may have a certain regulatory effect on the early physiological fruit abscission of coconuts.

In this study, four bZIP TFs were upregulated and two were downregulated (Figure 8). Similarly, the bZIP gene (BG631669) was downregulated in the early stages of tomato stem abscission [30]. This suggests that these TF genes may be positive regulators of the abscission signaling pathway [58]. Our research findings suggested that different bZIP TFs may mediate the transcriptional process of fruit abscission response. The homologous gene ANAC072 of GhNAC72 has been reported to promote chlorophyll degradation and participate in leaf senescence in Arabidopsis [61]. Additionally, GhNAC72 is also highly expressed in the yellow leaves of cotton, which may be related to leaf senescence [62]. In this study, we also found that four NACs were upregulated in the AZ of AFs compared to CFs (Figure 8). This also suggested that NAC may be related to early physiological fruit abscission of coconut. Moreover, this study also found differential expression of GATA and HSF TFs during the early fruit abscission of coconuts (Figure 8). This also indicated that these TFs may play a key role in the transcriptional regulation of genes.

4. Materials and Methods

4.1. Plant Materials

We used "Wenye No. 2" red dwarf coconuts growing in Wenchang, Hainan Province, China, as the raw material, and took early-abscised coconut fruits (AFs) and non-abscised coconut fruits or normal fruits (CFs) 15 days after coconut flower pollination. The length and width of the fruits were about 4.5 cm and 3.0 cm, respectively. A total of 10 plants with similar growth were selected for AF and CF treatments. And 1 AF and 1 CF were chosen from each coconut plant. In all, 10 AFs and 10 CFs from 10 plants were sampled to observe the shape of the AFs and CFs, determine the fresh fruit weight, and measure the water content of the fruits. Some fresh AF and CF samples were fixed in FAA. The volume ratio of formalin/glacial acetic acid/50% alcohol in FAA was 8:58:7. Some AF (3 fruits) and CF (3 fruits) samples from 10 plants were cut from the fruit abscission zone (AZ) in liquid nitrogen and then brought back to the laboratory. They were stored in a refrigerator at −80 °C for subsequent analysis preparation.

4.2. Measurement of Enzyme Activity

AZ samples from AFs and CFs were taken and CEL, PE, PG, and POD activities were measured using a reagent kit (Solario, Beijing, China). Each measurement indicator was tested three times. These indicators were tested by Nanjing Jiancheng Biotechnology Research Institute (http://www.njjcbio.com/ (accessed on 15 August 2022)). A total of 0.1000 g of AZ tissue was accurately weighed and mixed with pre-cooled PBS at a weight-to-volume ratio of 1:10. The sample was ground at high speed and centrifuged at 2500 rpm for 10 min. This was measured using 50 µL of supernatant and 0.2 mol/L pH 6.0 HAc-NaAc buffer solution. The reagent kit adopted a double-antibody one-step sandwich enzyme-linked immunosorbent assay (ELISA). The sample, standard, and HRP-labeled detection antibody were added in sequence to the pre-coated micropores with CEL, PE, PG, and POD antibodies, incubated, and washed. Using substrate TMB for color development, TMB was converted to blue under the catalysis of peroxidase and to the final yellow under the action of acid. The depth of color was positively correlated with the presence of polygalacturonase (PG) in the sample. The CEL, PE, PG, and POD absorbance (OD value) at 550, 540, 450, and 420 nm wavelength were measured using an enzyme-linked immunosorbent assay (ELISA) reader (DG5033A, Nanjing Huadong Electronics Group Medical Equipment, Nanjing, China) and the sample activity was calculated. The unit of

the CEL, PE, PG, and POD activity was U/mL. The time, liquid dosage, and sequence indicated in the instructions for incubation operation were strictly followed. All liquid components were shaken thoroughly before use. All measurements were taken within 10 min of adding the termination solution. The concentration/activity were calculated based on the absorbance value according to the manufacturer's formula.

4.3. Measurement of Phytohormones

The contents of phytohormones (IAA, ABA, ETH, GA, CKs, BR, JA, and SA) were determined using liquid chromatography–mass spectrometry (LC-MS) using equipment from the United States, following the methods of Balcke and Owen [63,64]. Between 0.5 and 1.0 g of the same sample was powdered in liquid nitrogen and added to 5 mL of pre cooled 80% methanol for transcriptome sequencing. Then, the sample was rinsed with 3 mL and 2 mL of methanol, respectively, transferred to a 50 mL centrifuge tube, placed on ice, and left in the dark for 12 h at 4 °C for leaching. Then, the sample was centrifuged at 10,000× 9 *g* for 10 min, the supernatant was transferred to a 50 mL centrifuge tube, and it was placed in the dark at 4 °C. Then, 5 mL of pre-cooled 80% methanol was added and leached in the dark for 12 h at 4 °C. The sample was then centrifuged at 10,000× 9 *g* for 10 min, the supernatant was collected, and the supernatant was mixed for the first time. The test tube containing the sample was placed in the refrigerator, and then it was shaken well in the dark at 100 rpm/min for 1 h. Then, the sample was centrifuged at 10,000× 9 *g* for 10 min, and the supernatant was poured into a C18 SPE column. The effluent solution was collected in a new 50 mL centrifuge tube. Then, the solution was covered in preservatives, and a precise hole was made in the middle with a toothpick. Then, it was rapidly freeze-dried in liquid nitrogen and transferred to a freeze-dryer for more than 36 h. Then, 1 mL of pre-cooled methanol was added to completely dissolve the freeze-dried powder sample. Finally, the sample solution was aspirated using a 2.5 mL syringe and passed through a 0.45 μ m organic ultrafiltration membrane to determine the levels of different hormones. Each sample underwent three repeated technical measurements.

4.4. Transcriptomics Analysis

RNA extraction from frozen samples was conducted utilizing an enhanced cetyltrimethylammonium bromide (CTAB) method. The quality and intactness of the RNA were assessed through agarose gel electrophoresis. Subsequently, the concentration of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), while the Agilent 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA, USA) was employed for quantifying RNA integrity. Library construction and RNA-seq analysis were carried out at Beijing Biomarker Biotechnology Company and Beijing Biomarker Cloud Technology Company, both located in Beijing, China. The NEBNext® Ultra™ II RNA Library Prep Kit was utilized to generate the RNA libraries, with index codes added to individual samples. Subsequently, sequencing was conducted on the Illumina® HiSeq2500 platform (San Diego, CA, USA). For each sample, three replicates of sequencing were conducted. Raw reads underwent filtration to eliminate low-quality reads and adapters. The resultant clean reads were aligned to the reference coconut genome [65] using the HISAT2 (hierarchical indexing for spliced alignment of transcripts) program [66]. Gene functional annotation was performed utilizing various databases, including Clusters of Orthologous Groups of proteins (COG/KOG), NCBI non-redundant protein sequences (Nr), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-PROT protein sequence database, Gene Ontology (GO), and Pfam (homologous protein families) [67,68]. The RESM software (3.8.6) was employed to compute the fragments per kilobase of transcript per million fragments mapped (FPKM) for each transcript region [69]. Differential gene expression analysis between samples was conducted utilizing DESeq software (1.6.3), and its significance was determined using the Benjamini–Hochberg method. DEGs were defined based on $|\log_2(FC)| \geq 1$ and a significance level of $p < 0.01$ [70]. GO term enrichment analysis of DEGs was conducted using the GOseq R software package (2.18.0) [71,72]. Additionally, KEGG pathway enrichment

analysis on DEGs was performed using the KEGG Orthology-Based Annotation System (KOBAS) software (3.0) [73].

4.5. Validation of DEGs in Coconut Seedlings via qRT-PCR

The DEGs in coconut seedlings were confirmed as detected by RNA sequencing through validation with quantitative real-time polymerase chain reaction (qRT-PCR). We also designed gene-specific qRT-PCR primers (Table S10). qPCR photocycling was performed on a 96-well plate using the 480II real-time system (Roche, Carlsbad, CA, USA) and Hieff qPCR SYBR green master mix (NotRox) from Yasen Biotechnology (Shanghai, China), following the manufacturer's protocol. The thermal cycling protocol comprises initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 30 s. All qRT-PCR analyses were conducted with 3 biological replicates and 3 technical replicates. The data were normalized using a reference gene (β-actin). Relative gene expression levels were calculated by using the $2^{-\Delta\Delta CT}$ method [74].

4.6. Statistical Analysis of Data

The information was presented as the average value ± standard deviation (SD) obtained from three separate repetitions. The statistics were performed using SPSS software (version 20.0; SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to evaluate variations among samples. Student's *t*-tests were applied to ascertain the significance of differences when $p < 0.05$. A significance level of $p < 0.05$ was considered statistically meaningful. Vector graphs and data tables were generated using Excel 2020.

5. Conclusions

This study aimed to elucidate the molecular mechanism of early coconut fruit abscission (AF) from the perspectives of morphology and structural characteristics, cell wall hydrolysis and oxidase activity, plant hormones, and the transcriptome of the abscission zone (AZ). The results indicated that early fruit abscission in coconuts was related to fruit morphology, biomass, and structural characteristics. The changes in cell wall remodeling enzymes and oxidases (PG, CEL, PE, and POD) and phytohormones (IAA, GA, CKs, BR, ABA, JA, SA, and EHT) have a significant impact on the early physiological abscission of coconut fruits (AFs). Transcriptome analysis showed that many upregulated DEGs were enriched in pathways such as "ribosome", "cysteine and methionine", "carbon metabolism", "biosynthesis of amino acids", "citrate cycle (TCA cycle) ", and "peroxisome". However, the most commonly downregulated DEG-enriched KEGG pathways were "plant hormone signal transduction", "pentose and glucuronate interconversions", "plant–pathogen interaction", and "flavonoid biosynthesis". Moreover, many downregulated and upregulated DEGs were enriched in pathways such as "phenylpropanoid biosynthesis ", "amino sugar and nucleotide sugar metabolism", "MAPK signaling pathway—plant", and "starch and sucrose metabolism". Furthermore, BHLH, ERF/AP2, WRKY, bZIP, and NAC transcription factors also showed significant differential expression, indicating their roles in early coconut fruit abscission. This study's results combined morphology, cytology, and transcriptional regulation to reveal the molecular mechanism of early fruit abscission in coconuts (Figure 10). This provides a theoretical basis for further research on the abscission of other organs in coconuts.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15081475/s1, Figure S1. Gene expression analysis in CFs and AFs. (a) The gene expression distribution in CFs and AFs. (b) Correlation analysis of the genes in CFs and AFs. (c) Principal component analysis (PCA) of expressed genes in CFs and AFs. Figure S2. Differential expression analysis of genes. (a) Differentially expressed gene (DEG) volcano map in CFs vs. AFs. (b) Bar chart of DEG statistics. (c) Cluster diagram of DEGs. (d) Statistical chart in COG annotation classification of DEGs. (e) Statistical chart in GO annotation classification of DEGs. Figure S3. Bar chart in GO enrichment of DEGs. (a) Bar chart in GO enrichment of upregulated DEGs. (b) Bar chart in GO enrichment of downregulated DEGs. Note: The horizontal axis represents GeneNum,

which is the number of genes of interest annotated in this entry, and the vertical axis represents each GO annotation entry. The color of the column represents the q-value of the hypergeometric test. Table S1. Genes with FPKM values in RNA-seq in CF vs. AF group. Table S2. Differentially expressed genes (DEGs) with FPKM values in RNA-seq in CF vs. AF group. Table S3. Annotatable DEGs with FPKM values in RNA-seq in CF vs. AF group. Table S4. Analyses of top 20 GO enrichment pathways in CF vs. AF group. Table S5. Analyses of KEGG enrichment pathways in CF vs. AF group. Table S6. Identified differentially expressed genes involved in the main KEGG enrichment pathways in CF vs. AF group. Table S7. Identified differentially expressed genes involved in cell wall modification in CF vs. AF group. Table S8. Identified differentially expressed genes involved in phytohormones in CF vs. AF group. Table S9. Identified differentially expressed genes involved in transcription factors in CF vs. AF group. Table S10. Primers used in qRT-PCR validation under CF vs. AF group.

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