*Article*

# Comparative Transcriptome Analysis Reveals Sex-Biased Expression of Hormone-Related Genes at an Early Stage of Sex Differentiation in Red Bayberry (*Morella rubra*)

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**Abstract:** The molecular mechanism of sex development and differentiation in the economically important dioecious fruit tree, red bayberry (*Morella rubra*), was revealed using next-generation transcriptome sequencing (NGS), and comparative analyses were used to identify differentially expressed genes (DEGs) in female and male flower buds. A total of 7029 of these DEGs were identified at two early development stages. KEGG pathway enrichment analysis revealed that plant hormone signal transduction was significantly overrepresented, and 91 genes related to hormones were identified. An analysis of 7029 DEGs revealed 161 hormone-related genes, with the 42 related to auxin and 26 related to ethylene being the most highly represented. A total of 62 genes were significantly up-regulated in females and 29 were in males, with 18 of them specifically expressed in females and 10 in males. A total of 415 transcription factors were identified, with 129 genes up-regulated in females and 53 in males. Moreover, 38 had female-specific expression and 18 had male-specific expression. Using weighted gene co-expression network analysis (WGCNA), two modules were found to be associated with sexual type. In the module coded light-green, there were five genes related to hormones, one to flower development and ten transcription factors with four genes specifically expressed in the males and four in females. The hub gene in the light-green module is *MR0TCONS_00017483.1* (*ACO*), which is involved in ethylene biosynthesis and had male-specific expression. Among the transcription factors, three of the four male-specific expressed genes involved in flavonoid biosynthesis, including the MYB gene *MR1TCONS_00020658.1* and two BHLH genes, *MR6G001563.1* and *MR8G020751.1*, played important roles in male floral differentiation. In the dark-cyan module, six hormone-related genes, five transcription factors and three flower development genes were identified with the hub gene *MR1G019545.1* (*ETR1*), which participates in the ethylene signaling pathway, and *MR4G023618.1*, which encodes the C3H zinc finger transcription factor. These results indicate that ethylene is the key hormone that interacts with other hormones and transcription factors to regulate sex differentiation in the red bayberry, which also provides new insights into the mechanism of sex determination and differentiation in the red bayberry.

**Keywords:** *Morella rubra*; sex determination and differentiation; hormones; ethylene; transcription factor

## 1. Introduction

The evolution of sex differentiation is an interesting topic because it is important to strategies for outbreeding, the prevention of inbreeding depression and a vital source

of genetic variation. About 10% of flowering plants have separate unisexual flowers in different individuals (dioecy) or in the same individual (monoecy) [1]. For the mechanisms of unisexual flower development, two categories of unisexual flowers have been defined [2,3]. In type I, bisexual flowers become unisexual through the termination of the development of the androecium or gynoecium. The sex differentiation occurs at the particular developmental stage when one of the reproductive organs is arrested. Flowers of type II are unisexual from inception, and sex differentiation occurs before the formation of female or male organ primordia [3].

Sex determination has evolved multiple times, independently, suggesting various genetic mechanisms of unisexual flower formation are at play in different species [3,4]. The genetic mechanism of sex determination in dioecy is often thought to be controlled by a putative sex chromosome. This includes a dominant XY system (male heterogamety), such as the one found in diploid persimmon [5] and papaya [6], and, in a few cases, a ZW system (female heterogamety), as found in the red bayberry [7] and wild species of the allo-octoploid strawberry [8]. Sex-determining genes are located in the non-recombining region of the Y or W chromosome. For example, in garden asparagus [9,10] and kiwifruit [11,12], the two sex-determining genes are in the sex determination region (SDR), consistent with the classical 'two-mutation model' for the evolution of dioecy from hermaphroditism via gynodioecy.

Hormones and transcription factors are coded with genetic factors, and can also regulate sex differentiation [13,14]. Several sex-differentiating genes have been found to be involved in hormone biosynthesis or signaling pathways. In kiwifruit, a cytokinin response regulator *Shy Girl* (*SyGI*) has been identified as a sex-determining gene that acts as the suppressor of feminization [11]. In the cucumber, four of five genes controlling flower sex type encode the key enzymes involved in ethylene biosynthesis, including *CsACS1G* (*F*) [15], *CsACS2* (*M*) [16], *CsACS11* (*A*) [17] and one *CsACO2* [18]. In addition, the zinc finger transcription factor *WIP1* suppresses the female flower to control unisexual flower development in the cucumber and melon [18].

*Morella rubra*, commonly known as the red bayberry, is the only cultivated edible fruit species in the Myricaceae family, and it is widely distributed in south China [19,20]. Most species in Myricaceae are dioecious, with very few monoecious individuals [21,22], and with male, female and monoecious flowers on different plants. It is difficult to identify sex before flowering, as there is no obvious difference between males and females. The sex type of the red bayberry is controlled by the sex chromosomes: ZW for females, ZZ for males. A 59 kb non-recombining region in the W chromosomes harboring seven candidate genes has been identified [7]. The red bayberry flower is a type II unisexual flower, in which only female or male organ primordia are initiated. The male flowers are compound catkins, which are surrounded by bracts without receptacles or pedicels, and each of them has two stamens (Figure 1a). The female flowers are catkins (Figure 1b), and the ovary is unilocular with a Y-shaped stigma [20]. A time course experiment indicated that the critical period for flower bud development is from July to September. The red bayberry is also a good species for sex determination mechanistic studies because of its small genome size (320 Mb), ZW chromosome and viable WW genotype (super female) individuals [7,23]. Although seven female-specific gene and molecular markers linked to the sex type have been identified [7], the molecular mechanism and the associated and regulated genes that govern sex determination are not well understood in the red bayberry.

With the continuous progress observed in high-throughput sequencing, transcriptome analyses have been reported in many species to unravel the molecular process regulating the determination of unisexual flowers, such as in papaya [24], spinach [25,26] and *Jatropha curcas* L. [27]. Although a comparative analysis of gene expression between female and the monoecious mutant red bayberry flowers has been reported [28], the flower samples used were taken during late stages (December) of flower development, when sex differentiation is completed and the key genes regulating sex determination might be no longer expressed. In the present study, transcriptomes analyses of red bayberry floral

buds from female and male trees were performed, before the initiation of reproductive organ primordia, aiming to capture useful information to understand the mechanism of sex differentiation and floral development in the red bayberry. The results of this study can also be used to guide cross-breeding between two cultivars by manually inducing male flowers in female plants.
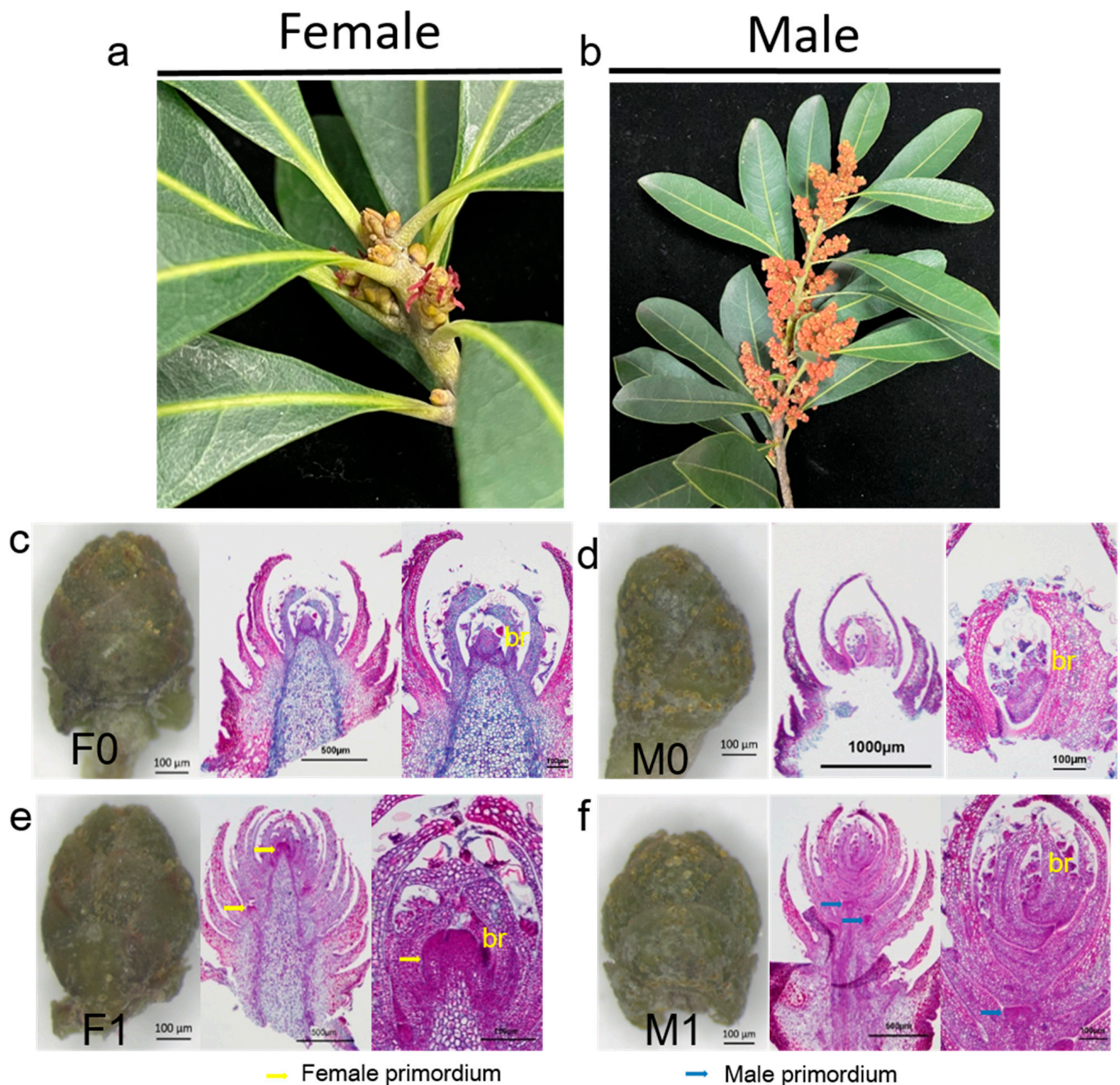


**Figure 1.** The male and female floral buds of the red bayberry. Female flower (**a**) and male flower (**b**) of the red bayberry. Male and female floral buds before sexual differentiation (**c,d**), and with female and male primordia (**e,f**). br, bracts. The figures (**e,f**) have been published in a previous study [7].

## 2. Materials and Methods

### 2.1. Flower Sample Collection and Morphological Observation

The male accession 'H2011-12' of a red bayberry, an approximately 30-year-old tree which was used for whole-genome sequencing [7], and the female individual 'H2011-11', grown at Zhejiang University Campus (Hangzhou, China), were used for all experiments.

Floral buds were collected on two dates in 2017: 13 July and 27 August. The male and female floral buds were sampled in three replicates. The samples were immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. The male and female flowers were screened under a stereoscopic microscope. Paraffin sections were used as in a previous study [29] to confirm the developmental stages of male and female buds. We used floral buds before sexual differentiation (F0 for female and M0 for male floral buds) and the early emergence of female and male primordia (F1 for female and M1 for male floral buds) for transcriptomic analysis to uncover the molecular regulatory mechanisms of unisexual flower development and sexual differentiation in the red bayberry.

### 2.2. RNA Extraction and Transcriptome Sequencing

The total RNA of male and female flowers was extracted by using a Quick RNA isolation Kit (Waryong, Beijing, China) according to the manufacturer's manual. The quality of total RNA was checked on 1% denatured agarose gel and then assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The pair-end cDNA sequencing libraries of four floral buds were prepared with three biological replicates by the Beijing Genome Institution (BGI, Beijing, China) and then sequenced at BGI using an Illumina HiSeq 2500 platform (Illumina) with PE125.

### 2.3. RNA-Seq Data Analysis

Adapter sequences, ambiguous nucleotides, and low-quality sequences in raw reads were removed with SOAPnuke (version1.4.0) [30] and Trimmomatic (v0.36) [31]. The paired-end clean reads from each library were mapped to the reference genome (https://www.ncbi.nlm.nih.gov/genome/?term=Morella+rubra, accessed on 15 February 2022) using HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts) to calculate the mapping ration [32]. Clean reads were aligned to complete the reference using Bowtie2 [33], and RSEM (RNA-Seq by Expectation Maximization) was used to estimate the abundance of the genes [34]. Read counts were normalized by calculating the FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) value of all the transcripts in the samples. Gene function was annotated based on NCBI nonredundant protein sequences (Nr), the protein family (Pfam), the Swiss-Prot protein database and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Gene ontology (GO) annotation was used for sequences with a match in the Nr database by using Blast2GO v.3.0 [35]. Differential expression between the two groups was analyzed with the DESeq R package version 1.30.0 [36]. Genes with a minimal two-fold difference in expression ($|\log2 \text{Ratio}| \geq 1$) and padj $\leq 0.01$ were considered to be differentially expressed genes (DEGs). Heatmaps (scaled by row) were prepared with TBtools and used for GO enrichment analysis and KEGG pathway enrichment analysis [37]. For the weighted gene co-expression network analysis (WGCNA), an R package (version 1.0.7) [38] was used to construct a potential regulatory network of sex differentiation in the red bayberry.

### 2.4. Validation of RNA-Seq Data

Quantitative real-time PCR (qRT-PCR) was used to validate the RNA-seq data. The Total RNA of F0, F1, M0 and M1 were extracted by using the Quick RNA isolation Kit (Waryong, Beijing, China) according to the manufacturer's manual, and the concentration was checked on a BioDrop spectrophotometer (Biochrom, Cambridge, UK). First-strand cDNA synthesis was performed with 2 μg RNA with EasyScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen Biotech, Beijing, China). The qRT-PCR primers were designed using an online tool, Primer 3 (https://primer3.ut.ee/, accessed on 15 February 2022). The PCR products of qRT–PCR were verified by sequencing. The qPCR was performed with the SYBR Premix Ex Taq™ (Takara, Ohtsu, Japan) and CFX Connect™ real-time PCR system (Bio-Rad, Hercules, CA, USA) with the reaction and program detailed as previously published by Ni et al. [39]. Actin

was used for normalization, and the expression data were calculated with the $2^{-\Delta\Delta Ct}$ formula [40].

## 3. Results

### 3.1. Morphological Observation and Dissecting Red Bayberry Flower Buds

Red bayberry floral meristems were initiated in the bract axils of both male and female plants. At the early stage of floral development, the inflorescence rachis became elongated (Figure 1c,d), and this was followed by the flower initiation stage, with the female and male flower primordia forming between the two bracts (Figure 1e,f). At this stage, there was no obvious morphological difference between male and female flowers.

### 3.2. Sequencing of Red Bayberry Flower Buds

A total of 562.3 million raw sequencing reads were obtained (Table S1), and the clean reads were aligned to the red bayberry reference genome and used to quantify the expression levels of genes using HISAT and Bowtie2. About 83.5% of reads could be mapped to the reference genome (Table S1). A principal component analysis (PCA) based on FPKM values separated the samples into four distinct groups, with each sample making a separate group with its replicates (Figure S1). The total variation portion (46.2%) consists of two principal components (PC1: 27.6%, PC2: 18.6%), with PC1 separating females from males. The distributions of the number of genes at different expression levels among the four groups were similar (Figure 2a). The number of genes not expressed (FPKM < 1) accounted for the highest proportion, followed by those genes expressed at a low level ($1 \leq$ FPKM < 10) and mid-level ($10 \leq$ FPKM < 100). The highly expressed genes with FPKM $\geq$ 100 accounted for the smallest proportion. About 85% of genes were expressed in both female and male floral buds, and 1979 genes were specifically expressed in female and 2015 in male buds (Figure 2b).

### 3.3. Gene Ontology (GO) and KEGG Enrichment Analysis of Highly Expressed Genes Revealed Rapid Growth in Female and Male Buds

A total of 633, 677, 521 and 558 highly expressed genes were detected in F0, F1, M0 and M1 floral buds, respectively, and 61.3% of them were shared between female and male floral buds (Figure 2c). The GO enrichment analysis and KEGG pathway enrichment analysis of the highly expressed genes in female and male floral buds were performed. The results from the GO enrichment analysis of the highly expressed genes showed that genes involved in cell proliferation, such as the 'structural constituent of ribosome' and 'structural molecule activity' belonging to molecular function categories, 'ribosomal subunit' and 'ribosome' belonging to the cellular component and the 'peptide biosynthetic process' and 'peptide metabolic process' belonging to the biological process, were more abundant (Table S2). This was in agreement with the results of the KEGG enrichment analysis, with ribosome overrepresented in the four floral samples (Table S3). In these analyses for mid-level expressed genes in four floral samples (Tables S4 and S5), the KEGG enrichment results indicated that ribosome was overrepresented in the four floral samples (Table S5). These results suggest that floral buds at these two development stages undergo rapid cell division and expansion.

### 3.4. Identification of Differentially Expressed Genes

We compared female vs. male floral buds in pair-wise analyses of M0 vs. F0 and M1 vs. F1. We also compared floral buds within a sexual type, F1 vs. F0 and M1 vs. M0. In all, 8889 genes were significantly expressed, with | Log2FC | $\geq$ 1 and *p*-value < 0.01 in four pairs (Figure S2), including 4970 DEGs in M0 vs. F0, 5421 DEGs in M1 vs. F1, 2659 DEGs in F1 vs. F0 and 2888 DEGs in M1 vs. M0 (Figure 2d). The number of DEGs between female and male was greater than those within one sex in an inner comparison, suggesting that a very complex physiological and biochemical process occurs during sexual differentiation and unisexual flower development.
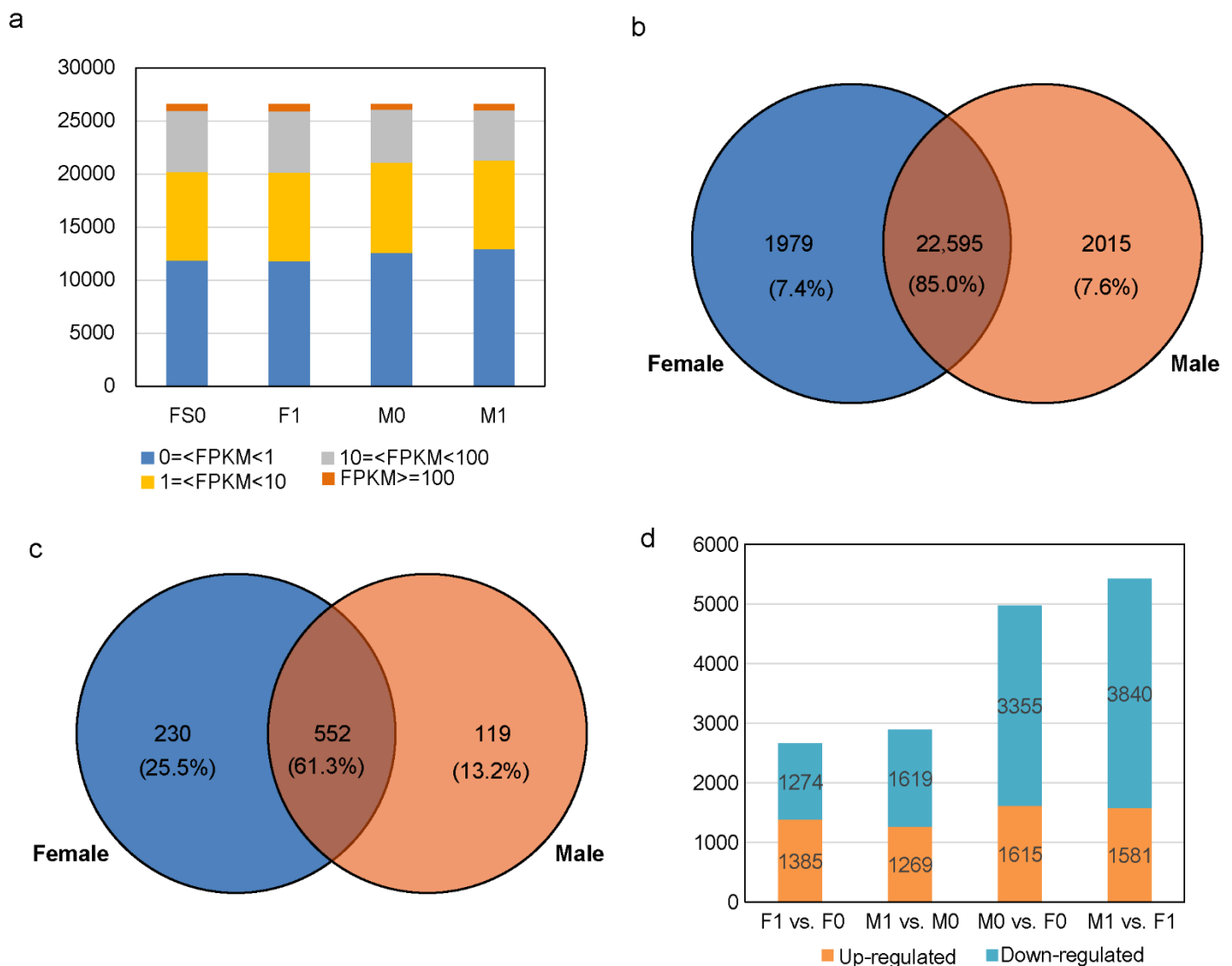
a



b



c



d



**Figure 2.** Overview of red bayberry M0, M1, F0 and F1 floral bud transcriptome. (**a**) Distribution of genes with high expression (FPKM ≥ 100), mid-level expression (100 > FPKM ≥ 10), low expression (10 > FPKM ≥ 1) and no expression (1 > FPKM ≥ 0). Venn diagram of genes expressed in female and male libraries (**b**) and genes with high expression in at least one library (**c**). (**d**) Distribution of up- and down-regulated DEGs in the pair-wise F1 vs. F0, M1 vs. M0, M0 vs. F0 and M1 vs. F1 analyses.

Because we aim to identify genes related to sexual differentiation in the red bayberry, we only focus on an analysis of DEGs between M0 vs. F0 and M1 vs. F1. We further used GO and KEGG enrichment to analyze DEGs between M0 vs. F0, M1 vs. F1, and all the 7029 DEGs of the two compared groups. GO enrichment results showed that catalytic activity was overrepresented in molecular functions (Table S6). KEGG pathway enrichment analysis showed that plant hormone signal transduction (ko04075) was significantly overrepresented in M0 vs. F0 and M1 vs. F1 and all 7029 DEGs (Figure S3), suggesting that plant hormones play a role in sex differentiation in the red bayberry. A total of 91 genes were identified in this category, 35 associated with auxin, 14 involved in CK, 10 with ABA and JA, and the remainder associated with gibberellin, BR, ethylene, and SA (Table S7).

*3.5. DEGs Are Involved in Plant Hormone Biosynthesis and Signalling*

To investigate hormone function during the process of sexual differentiation, hormone-related genes were identified in the red bayberry. A total of 161 hormone-related genes were

identified in 7029 DEGs (Table S8), and the distribution for different hormones is shown in Figure 3a: 42 genes related to auxin were most represented, followed by 26 related to ethylene, 20 to CK, 18 to SA, 17 to ABA, 15 to GA and JA, and 8 to brassinosteroid. Among the 161 hormone-related genes, 62 DEGs were significantly up-regulated in females and 29 were in males (Table S8). Moreover, a total of 18 genes displayed female-specific expression and 10 genes were expressed only in males (Table S9).
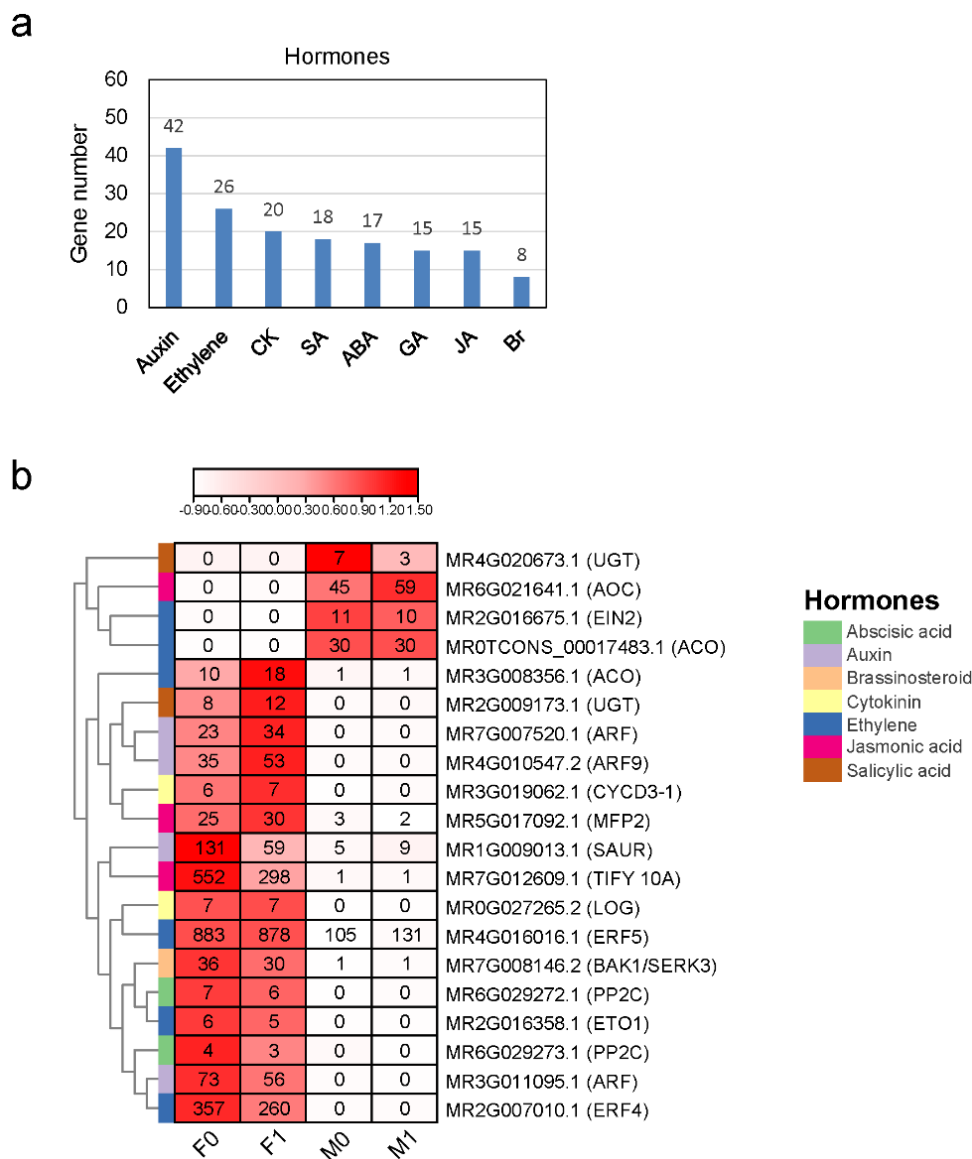


**Figure 3.** The distribution and expression of hormone-related genes. (**a**) The distribution of 184 hormone-related genes. (**b**) Heat map of sex-biased DEGs related to hormones, with the FPKM value of genes shown in the box.

The expression of 13 out of 42 auxin-related genes was significantly up-regulated in females. *MR1G009013.1* (*SAUR*) was expressed at a much higher level in females than in males (Figure 3b). Five of them were only expressed in females, including three *ARF* genes: *MR3G011095*.1, *MR4G010547.2* and *MR7G007520.1*; one IAA gene *MR3G001167.1* and one *YUC* gene *MR1G027448.1* (Table S9). The expression of six auxin-related genes was significantly up-regulated in males, including the *GH3.9* gene *MR8G002059.1*, which was only expressed in males. In the present study, 12 out of 26 ethylene-related genes were significantly up-regulated in females, including 4 female-specific expressed genes, *MR1G003948.1* (*ETO1*), *MR2G007010.1* (*ERF4*), *MR2G016358.1* (*ETO1*), and *MR3G008357.1*

(*ACO*) (Figure 3b and Table S9). The ethylene biosynthesis gene *ACO* (*MR3G008356.1*) and ethylene signal transduction gene *ERF5* (*MR4G016016.1*) were expressed at a much higher level in females than in males (Figure 3b). Four ethylene-related genes were significantly up-regulated in the male buds, including three male-specific expressed genes: *MR0TCONS_00017483.1* (*ACO*), *MR6G009324.1* (*ACO*) and *MR2G016675.1* (*EIN2*). The jasmonic acid biosynthesis gene *MR6G021641.1* (*AOC*) also had male-specific expression (Figure 3b). The cytokinin biosynthesis gene *MR0G027265.2* (*LOG*) and signal transduction gene *MR3G019062.1* (*CYCD3-1*) were only expressed in females, while two *UGT* genes involved in SA biosynthesis gene had the opposite expression pattern: *MR2G009173.1* was only expressed in females and *MR4G020673.1* was only expressed in males. The brassinosteroid signal transduction gene *BIM1* (*MR5G011875.1*) showed male-specific expression and BAK (*MR7G008146.2*) had much higher expression in females than in males (Figure 3b).

### 3.6. Transcription Factors Differentially Expressed in Male and Female Buds

Transcription factors (TFs) play important roles in flower development. A function annotation of the 7029 DEGs revealed that 415 transcription factors belong to 55 gene families (Table S10). The MYB family, AP2/EREBP family and bHLH family were over-represented (Figures 4a and S4). Among the transcription factors, the expression of 129 transcription factors genes were significantly up-regulated in females, and 53 were down-regulated. On further analysis of the expression of the sex-type-specific genes, 38 were found to be only expressed in females and 18 in males (Table S11).

Of the 58 MYB family genes, there was a significant female-biased expression of 12, and 6 had significant male-biased expression. The *MR4G023331.1* and *MR4G009025.1* gene expression was female-specific, while the *MR1TCONS_00020658.1* was only expressed in males; its homolog in *Arabidopsis AT3G13540.1* (*AtMYB5*) is involved in flavonoid biosynthesis. Among 36 genes in the bHLH gene family, 6 genes expressed were female-specific, including *MR1G020885.1, MR6G026964.1, MR7G020473.2, MR2G024394.1, MR4G009030.1* and *MR8TCONS_00067952.1*, and 3 genes were male-specific, including *MR5G011875.1*, *MR6G001563.1* and *MR8G020751.1* (Figure 4b). Four of AP2/EREBP genes displayed female-specific expression, including *MR0G006452.1, MR1G017646.1, MR2G007010.1* and *MR8G022041.1*. Moreover, *MR2G007010.1* was highly expressed in females with FPKM values of 357 and 260 for F0 and F1, respectively (Figure 4b).

MADS-box genes are involved in floral development and floral organ identity. In this study, we identified 10 MADS-box genes differentially expressed in the two groups. The *SOC1* gene *MR0G005926.1* was only expressed in males (Figure 4b); SOC1 activates the floral meristem identity gene *LEAFY* (*LFY*). Two C2C2-CO-like zinc finger genes, *MR6G018079.1* and *MR3G018958.1*, were specifically expressed in females, and *MR8G027384.1* was expressed only in males. We also found that two C2C2-Dof genes, *MR5G011870.1* and *MR3G009872.2*, and one C2C2-GATA gene, *MR6G010822.1*, were only expressed in females.

Among the 415 transcription factors, the expression of 24 of them was high or medium in females and were not expressed in males (Figure 4b). The hormone-related MYC, MYB, STRE, ABRE and TF transcription factor binding sites in the promoters were also enriched (Table S12).

### 3.7. Genes in the Non-Recombining Region of the Red Bayberry W Chromosome

The 59 kb FSR (female-specific region) had seven predicted genes, and the expression levels of seven female-specific genes are shown in Table 1. The expression of all the female-specific genes in F0 and F1 was very low, with the FPKM value < 2, except for *MrTFIID2*. The expression of these genes was consistent with those previously reported in red bayberry floral buds using qRT-PCR [7], and further confirmed that the gene expression profile of the RNA-seq data was reliable.
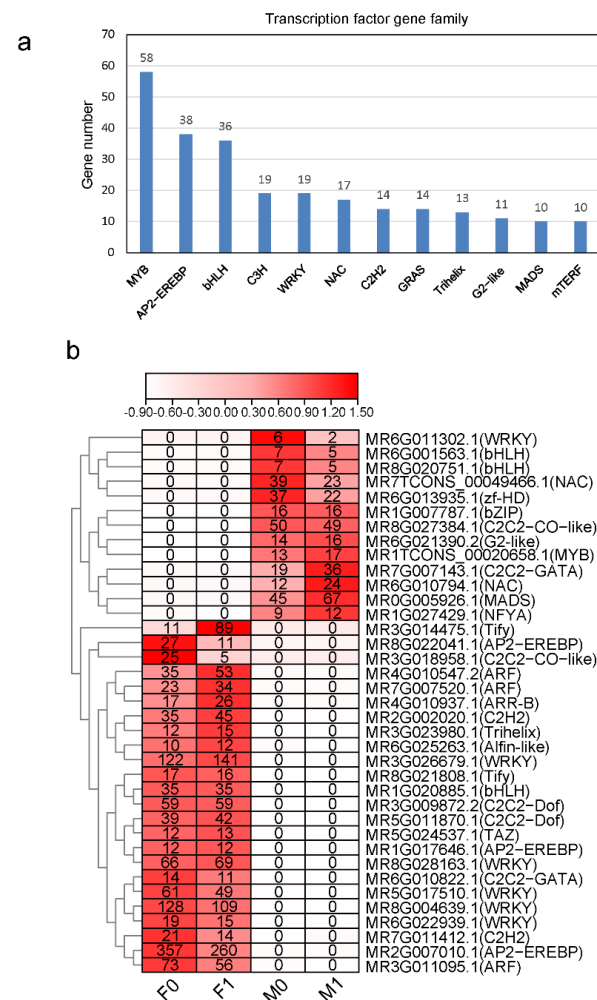
**Figure 4.** The distribution and expression of transcription factor genes in all 8889 DEGs. (**a**) The distribution of 547 transcription factor genes (top 12 families). (**b**) Heat map of sex-biased DEGs related to transcription. The FPKM value of genes is shown in the box.

**Table 1.** List of the expression level of seven female-specific genes in red bayberry floral buds.

| Gene Name | Gene_Id | M0_FPKM | M1_FPKM | F0_FPKM | F1_FPKM |
|-----------|---------|---------|---------|---------|---------|
| *MrCPS2* | *MR8G025874.1* | 0 | 0 | 0.00 | 0.04 |
| *MrCKA2* | *MR8G025875.1* | 0 | 0 | 0.71 | 0.60 |
| *MrTFIID2* | *MR8G025876.1* | 0 | 0 | 4.90 | 4.33 |
| *MrASP2* | *MR8G025877.1* | 0 | 0 | 0.53 | 1.14 |
| *MrSAUR2* | *MR8G025878.1* | 0 | 0 | 0.44 | 2.06 |
| *MrLsd90-2* | *MR8G025879.1* | 0 | 0 | 0.43 | 0.59 |
| *MrFT2* | *MR8G025880.1* | 0 | 0 | 0.23 | 0.25 |

### 3.8. Co-Expression Networks of Female and Male Buds

We used weighted gene co-expression network analysis (WGCNA) [38] to construct a potential regulatory network of sex differentiation in the red bayberry. A total of 42 modules were established using WGCNA in four male and female flower buds at early development stages (Figure 5a). The genes in the same module had strong connectivity (Figure 5b). The expression pattern of each module was analyzed in all samples. The module–trait relationships showed that the light-green ($r = 0.97$, $P = 6 \times 10^{-5}$) and dark-cyan ($r = -0.75$, $P = 0.03$) modules were highly associated with the sex phenotype (Figure 5c), so these two modules may play important roles in regulating sex differentiation and flower development.
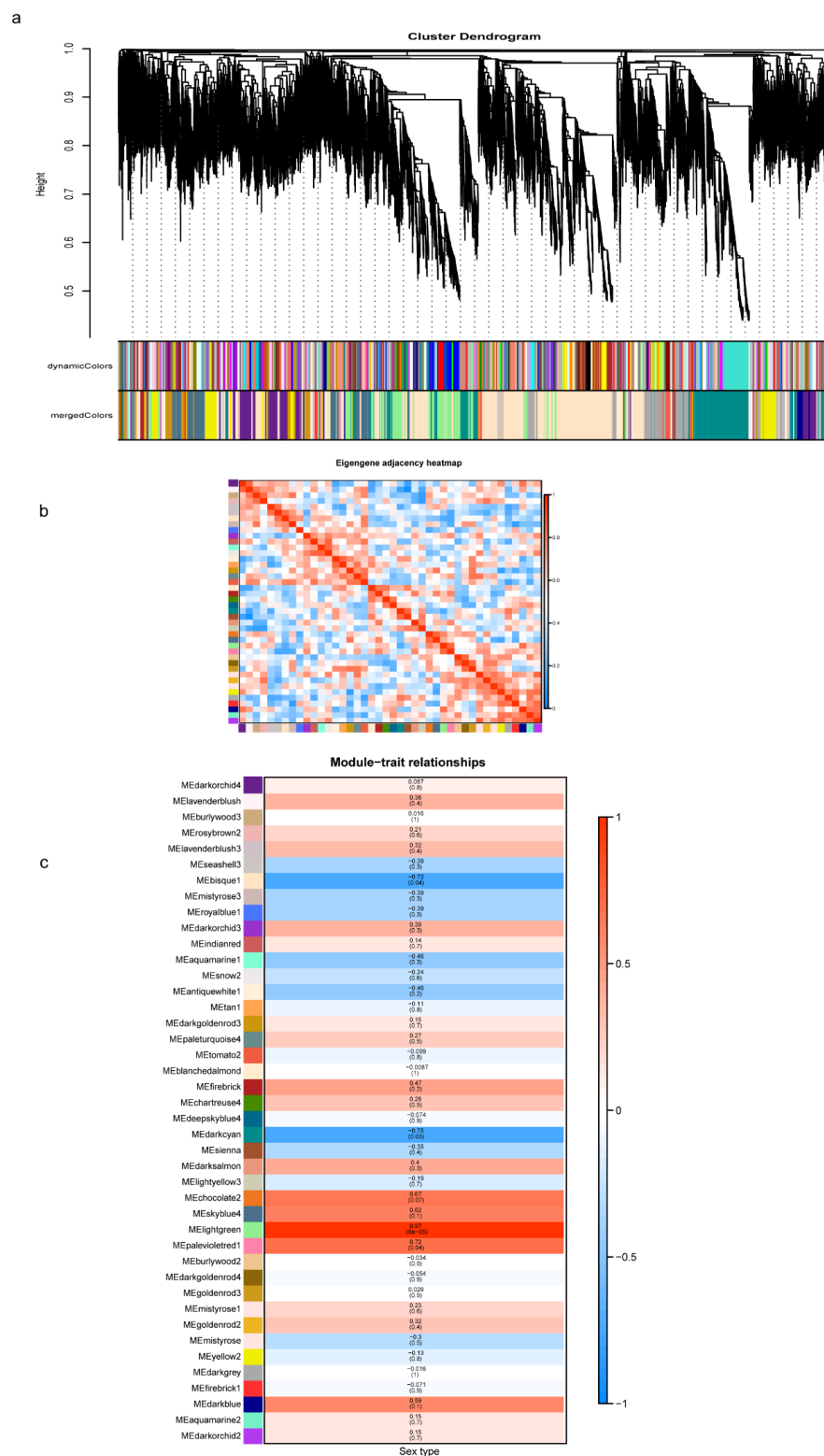
a



b

c

**Figure 5.** Weighted gene co-expression network analysis. (**a**) Hierarchical cluster tree showing co-expression modules identified using WGCNA; 'DynamicColors" represents modules divided according to clustering results, and "MergedColors" represents the merging of the module with a similar expression pattern according to the module similarity, with the analysis conducted according to the merged module. (**b**) Module gene correlation analysis; each row and column represent a gene; the darker the color of each point indicates a higher connectivity between the two genes. (**c**) Sample expression pattern analysis.

We screened the genes related to hormone and transcription factors in the two modules (Table S13). A total of 55 DEGs were selected from the light-green and 126 DEGs from the dark-cyan modules. As shown in Figure 6a and Table S14, the hub gene in the light-green module was *MR0TCONS_00017483.1,* which encodes 1-aminocyclopropane-1-carboxylate oxidase (ACO), which is a key gene in ethylene biosynthesis, and is only expressed in males (Figure 3b). In addition, the module included genes involved in ethylene biosynthesis (*MR2G016358.1* (*ETO1*), which is only expressed in females (Figure 3b)), ABA signaling (*MR8G022503.2* and *MR7G016748.1* (*PP2C*)) and CTK signaling (*MR7G024824.1* (*ARR5*), a male-specific expressed gene (Figure 6a)). Nine transcription factors were identified in the light-green module co-expression network, including three zinc finger genes (*MR5G011870.1* (Dof), *MR8G027384.1* (*COL4*) and *MR1G019332.1* (*C3H*)), one *MYB* gene (*MR1TCONS_00020658.1*), one *AP2/EREBP* (*MR2G007010.1*), one *PHD* gene (*MR5G024537.1*), one Tify (*MR8G021808.1*), one GRF (*MR4G021188.1*) and two *bHLH* genes (*MR6G001563.1* and *MR8G020751.1*). The floral meristem determinacy gene *MR6G008503.1* (*ULT1*) was also in the light-green module co-expression network (Figure 6a and Table 2).
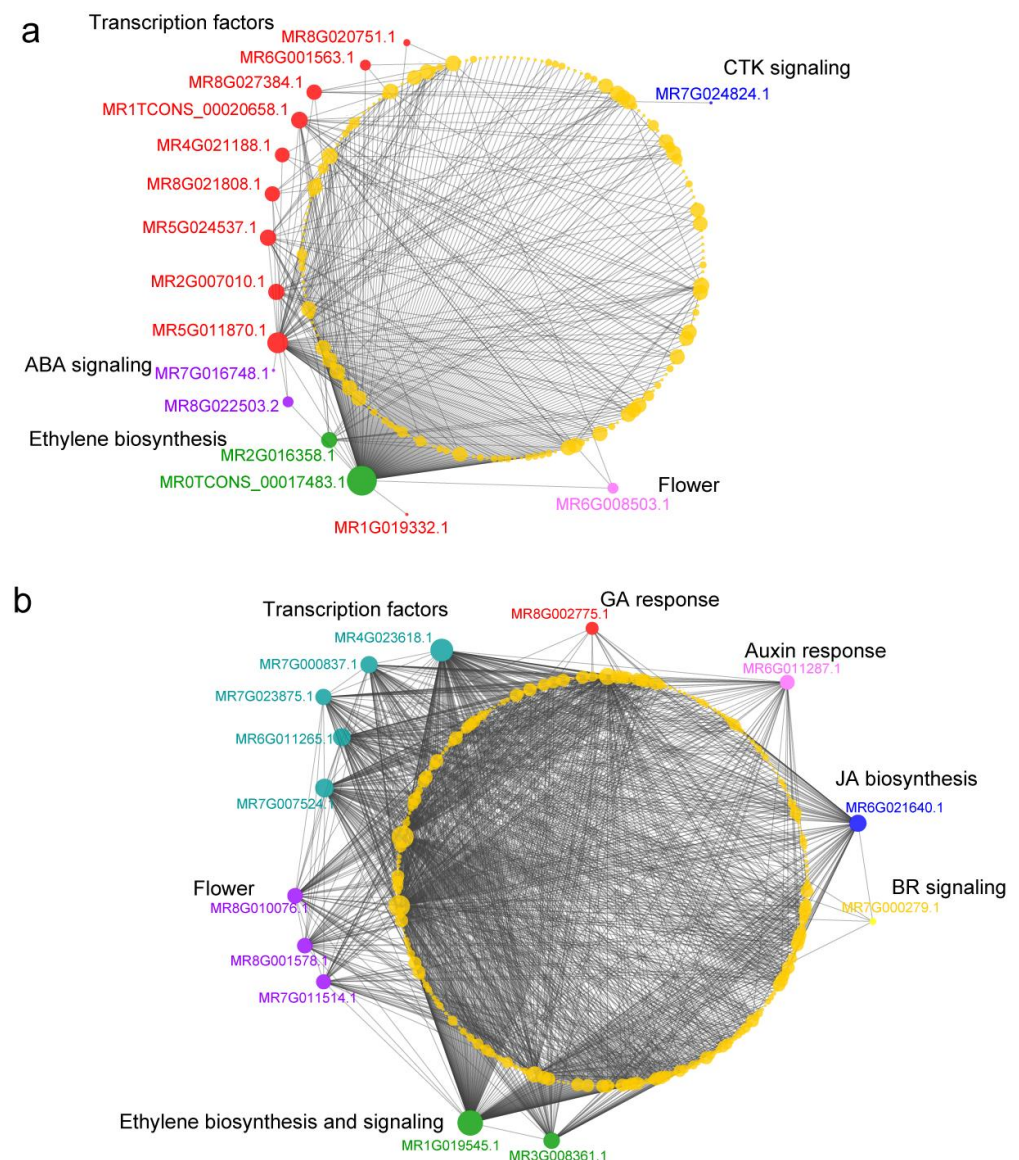


**Figure 6.** Visualization of co-expression network. (**a**) Co-expression network of genes in light-green modules. (**b**) Co-expression network of genes in dark-cyan modules.

**Table 2.** List of hormone-related genes, transcription factors and flowering-related genes in two modules.

| Module | Gene ID | Gene Name | Specifically Expressed in | Involved Process |
|---|---|---|---|---|
| Light green | *MR0TCONS_00017483.1* | ACO | male | ET biosynthesis |
| | *MR2G016358.1* | ETO | female | |
| | *MR8G022503.2* | PP2C | | ABA signaling |
| | *MR7G016748.1* | PP2C | | |
| | *MR7G024824.1* | ARR5 | male | CTK signalling |
| | *MR5G011870.1* | dof | female | Transcription factor |
| | *MR1TCONS_00020658.1* | MYB | male | |
| | *MR2G007010.1* | AP2/EREBP | female | |
| | *MR5G024537.1* | TAZ | female | |
| | *MR8G021808.1* | Tify | female | |
| | *MR8G027384.1* | C2C2-CO-like | male | |
| | *MR4G021188.1* | GRF | | |
| | *MR6G001563.1* | bHLH106 | male | |
| | *MR8G020751.1* | bHLH106 | male | |
| | *MR1G019332.1* | C3H | | |
| | *MR6G008503.1* | ULT | male | Flower development |
| Dark cyan | *MR1G019545.1* | ETR | - | ET biosynthesis and signaling |
| | *MR3G008361.1* | ACO | | |
| | *MR8G001578.1* | SPL | | Flower development |
| | *MR7G011514.1* | VRN1 | | |
| | *MR8G010076.1* | UGT | | |
| | *MR6G021640.1* | AOC | | Jasmonic acid biosynthesis |
| | *MR6G011287.1* | AUX22D | | Auxin response |
| | *MR8G002775.1* | GASA1 | | GA response |
| | *MR7G000279.1* | ASK7 | | Gibberellin |
| | *MR7G007524.1* | AP2-EREBP | | Transcription factor |
| | *MR4G023618.1* | C3H | | |
| | *MR7G000837.1* | ZF-HD | | |
| | *MR7G023875.1* | ZF-HD | | |
| | *MR6G011265.1* | bzip | | |

The hub genes in the dark-cyan co-expression network include *MR1G019545.1*, which encodes ethylene response 1, with its homolog *AtETR1* (AT1G66340), which is involved in ethylene signaling pathways, and *MR4G023618.1*, which encodes the C3H zinc finger transcription factor. The dark-cyan module also included five hormone-related genes, four transcription factors and three flower development genes (Figure 6a and Table S15). The details are shown in Table 2: *MR3G008361.1* (*ACO*) is involved in ethylene biosynthesis; *MR6G021640.1* (*AOC*) is involved in JA biosynthesis; *MR7G000279.1* (*ASK7*) is involved in the brassinosteroid signal transduction pathway; and *MR6G011287.1* (AUX22D) and *MR8G002775.1* (*GASA1*) are involved in the auxin and gibberellin response, respectively. With regard to the transcription factors, *MR7G007524.1* belongs to the AP2/EREBF gene family and *MR6G011265.1* encodes the *bZIP* transcription factor. *MR7G000837.1* and *MR7G023875.1* belongs to the zinc finger gene family. There were three genes related to flower development: *MR8G010076.1*, which encodes homologs of *AtUGT87A2* (*AT2G30140*), which regulate flowering time via *FLOWERING LOCUS C*; *MR7G011514.1* the homolog of *Arabidopsis AtVRN1*; and the *MR8G001578.1* homolog in *Arabidopsis* is *AtSPL14* (*AT1G20980*). These results showed that ethylene biosynthesis and signaling pathways may play vital roles in sex differentiation and flower development, with the transcription factor playing vital roles in regulating flower buds.

*3.9. Validation of Gene Expression Level in Male and Female Red Bayberry Floral Buds*

To confirm the gene expression pattern of the male and female floral buds identified in the RNA-Seq data, ten DEGs were randomly selected for validation by using qRT-PCR and the primers were shown in Table S16. The results of the qPCR were consistent with the gene expression patterns from RNA-seq (Figure S5), which confirmed that the gene expression profile of the RNA-seq data was reliable.

**4. Discussion**

In the present study, we constructed female and male *Morella rubra* floral bud libraries representing two early development stages, and used transcriptomes to analyze the genes differentially expressed in female and male floral buds to identify sex differentiation- and flower development-related genes.

The identification of sex-biased genes related to flower development is helpful to reveal the sex differentiation mechanisms. Here, we found 8889 DEGs in pair-wise analyses of F1 vs. F0, M1 vs. M0, M0 vs. F0 and M1 vs. F1 that may be related to sex differentiation and flower development. KEGG analysis indicated these DEGs were functionally enriched in plant hormone signal transduction (ko04075) (Figure S3). Phytohormones regulate various developmental processes, including floral development and sex differentiation, and the effects of plant hormones on sex differentiation vary among plant species [41]. Ethylene has been demonstrated to play a key role in the sex determination of cucumber and melon [15,42,43]. In maize, GA suppresses stamen development, while brassinosteroid and jasmonate coordinately suppress tassel development [44,45]. The exogenous application of ethrel, an auxin inhibitor, can induce male-to-female sex reversal in papaya [24]. In the red bayberry, the exogenous application of a GA inhibitor can induce female-to-male sex reversal in the 'Dongkui' and other cultivars (http://patents.google.com/patent/CN10758 1062A/en, accessed on 15 February 2022).

Hormones can crosstalk to regulate floral organ development. For example, ethylene can control floral transition via the DELLA-dependent regulation of floral meristem identity genes LEAFY (LFY) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) [46], and salicylic acid regulates flowering [47,48]. In this study we found 161 DEGs that were related to hormones, with 18 female-specific expressed genes and 10 male-specific expressed genes. Those genes, for instance, ethylene-related genes *MR2G016358.1* (*ETO1*) and *MR0TCONS_00017483.1* (*ACO*), are good candidates for studying the mechanism of red bayberry sex differentiation.

That transcription factors play vital roles in the regulation of sex differentiation and flower development has been reported in many plants. In the present study, we identified 415 transcription factors among the 7029 DEGs. Transcription factors interact with hormone-related genes to control sex identity. Moreover, the rest of three male-specific expressed genes, including one MYB gene (*MR1TCONS_00020658.1*) and two BHLH genes (*MR6G001563.1* and *MR8G020751.1*), have been reported to regulate flavonoid biosynthesis. Male floral differentiation has been found to be significantly related to flavonoid biosynthesis in *Jatropha curcas* L., and the inhibition of flavonoid biosynthesis causes male sterility in Petunia [27,49]. Our results suggest that transcription factors related to flavonoids may play roles in male floral differentiation.

For example, the sex *G* (*gynoecious*) gene *CmWIP1*, a C2H2-type zinc finger transcription factor, is a central integrator of the transcriptional networks, leading both to the inhibition of carpel development and the control of the expression of the stamen inhibitor *CmACS-7* in melon [50]. The female-specific expressed gene *MR2G002020.1* encodes the WIP2 protein identified in the present study. Based on the WGCNA results, a total of 15 transcription factors were identified, including four genes specifically expressed in females and four in males (Table 2). The homologue of the male-specific gene *MR8G027384.1* in *Arabidopsis* is *COL4* (*AT5G24930.1*), which can regulate flowering time [51]. The homolog of the zinc finger gene *MR3G018958.1*, also specifically expressed in the female (encoding a CONSTANS-like protein), in rice is *OsCOL16*, which represses flowering by up-regulating

*Ghd7* expression [52]. We also identified the male-specific expression of certain genes. The homologs of the *MADS-box* gene *MR0G005926.1* in *Arabidopsis* is *SOC1*, which is a core gene in the floral induction pathway [53,54]. Those sex-specific expressed transcription factor genes may play a role in the induction of female and male primordia in the red bayberry. Clearly, this sex-biased expression of transcription factors may interact with other genes to regulate red bayberry sex differentiation, and we will verify their function in future research.

To narrow down the key genes regulating sex differentiation in the red bayberry, we constructed the regulation network of sex differentiation in the red bayberry using WGCNA (Figures 5 and 6). Interestingly, we found modules coded light green and dark cyan to be associated with sex type. Both hub genes in the two modules were ethylene-related genes, and the homolog of *MR0TCONS_00017483.1* in the light-green module network was *AtACO*, which is a key gene in ethylene biosynthesis. Moreover, *MR0TCONS_00017483.1* was expressed specifically in the male bud. In the dark-cyan module network, *MR1G019545.1* is homologous to *AtETR1*, which is involved in ethylene signaling pathways (Figure 6 and Table 2).

Sex differentiation genes have been found to be involved in ethylene biosynthesis and signaling pathways in Cucurbitaceae species. In the cucumber, sex differentiation is mainly controlled by three *ACS* (1-aminocyclopropane-1-carboxylate (ACC) synthase) genes, including the *F* (*CsACS1G*), *M* (*CsACS2*) and *A* (CsACS11) genes. Additionally, *ACO* is also essential for the development of female flowers [18]. A recent study has shown that the ethylene receptors *CpETR1A* and *CpETR2B*, highly homologous to *AtETR1* and *AtETR2*, cooperate in the control of sex determination in *Cucurbita pepo* [55]. In present study, the male-specific expressed gene *MR0TCONS_00017483.1* (*ACO*) and female-specific expressed gene *MR2G016358.1* were identified in light-green module. Therefore, we can speculate that ethylene may be the key hormone regulating red bayberry male and female flower differentiation. We found GA-, auxin-, ABA-, CTK-, SA- and Br-related genes in both modules and transcription factors. We also identified three genes involved in floral identity and development. The homolog of *MR8G010076.1* is *AtUGT87A2*, which can regulate flower time via *FLC* (*FLOWERING LOCUS C*) [56], while the *MR7G011514.1* homolog in *Arabidopsis*, *AtVRN1*, acts as a key gene in the vernalization pathway, mediated by the floral pathway integrator *FT* (*FLOWERING LOCUS T*) and targeted to *FLC* to regulate flower development [57]. The homolog of *MR8G001578.1* (*SPL*) in *Arabidopsis* is a negative regulator of inflorescence identity [58]. Those genes may play roles in red bayberry floral development.

Sex-determining genes are often located in the non-recombining region of sex chromosomes. In the red bayberry, our previous study showed seven putative genes in the 59 Kb female-specific region of the W chromosome [7]. We found that all the female-specific genes in F0 (before sexual differentiation) and F1 (flower initiation) displayed low expression with the FPKM value < 5 (Table 1). Hermaphrodite *35S-MrFT2*-transformed *Arabidopsis* plants exhibited a slight reduction in the numbers of stamens (data not shown). Based on the above results, we speculate that ethylene-related gene *MR0TCONS_00017483.1* (*ACO*) may be downstream of the sex-determining gene, and it may interact with the transcription factor *MR8G027384.1* (*COL4*) to control sex differentiation in the red bayberry. This hypothesis needs to be verified in future work and can be tested with external ethylene application in an attempt to alter sex. This could provide effective technical support for cross-breeding between varieties.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae8020183/s1, Figure S1. Principal component analysis (PCA) of red bayberry floral buds; Figure S2. Venn diagram of the 8889 DEGs in the pair-wise analyses of F1 vs. F0, M1 vs. M0, M0 vs. F0, and M1 vs. F1; Figure S3. KEGG pathway enrichment analysis of the DEGs in M0 vs. F0 (a), M1 vs. F1 (b) and all the 7029 DEGs in the pair-wise M0 vs. F0, and M1 vs. F1 comparisons (c); Figure S4. Distribution of the 415 transcription factors genes in 7029 DEGs; Figure S5. Validation of the expression patterns of ten DEGs via qRT-PCR. Capital letters above the

black bars represent significant differences between the samples, and values are mean $\pm$ SE (n = 3); Table S1. Raw data of sequencing sample in the study; Table S2. Level 2 gene ontology annotation of highly expressed genes in four floral buds; Table S3. KEGG enrichment analysis of highly expressed genes in four floral buds; Table S4. Level 2 gene ontology annotation of middlingly expressed genes in four floral buds; Table S5. KEGG enrichment analysis of middlingly expressed genes in four floral buds; Table S6. Level 2 gene ontology annotation of the DEGs between M0 vs. F0, M1 vs. F1, and 7029 DEGs of the two compared groups in ontologies; Table S7. List of DEGs enriched in plant hormone synthesis and the signal transduction pathway; Table S8. List of hormone-related genes identified in the 7029 DEGs; Table S9. List of hormone-related genes specifically expressed in males and females; Table S10. List of transcription factors identified in the 7029 DEGs; Table S11. List of transcription factor genes specifically expressed in males and females; Table S12. The element enriched in promoter region of hormone-related genes; Table S13. List of genes in the light-green and dark-cyan networks; Table S14. Annotation of genes in light-green module; Table S15. Annotation of genes in dark-cyan module; Table S16. List of primers used for qRT-PCR.

**Author Contributions:** H.J. (Huimin Jia) and Z.G. designed this study and supervised the work. H.J. (Huimin Jia) performed the bioinformatic analyses and wrote the manuscript. L.Z., Y.W., H.W., H.Z. and H.J. (Huijuan Jia) performed the bioinformatic analyses. H.J. (Huijuan Jia), Y.Z., Y.J., G.W., C.Z. and C.H. collected the samples and generated the raw sequence data. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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