

Article

Deep Sequencing and Analysis of Transcriptomes of *Pinus koraiensis* Sieb. & Zucc.

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Abstract: The objective of this research was to study the differences in endogenous hormone levels and the genes related to reproductive development in Chinese pinenut (*Pinus koraiensis*) trees of different ages. The apical buds of *P. koraiensis* were collected from 2-, 5-, 10-, 15-, and 30-year-old plants and also from grafted plants. There were three replicates from each group used for transcriptome sequencing. After assembly and annotation, we identified the differentially expressed genes (DEGs) and performed enrichment analysis, pathway analysis, and expression analysis of the DEGs in each sample. The results showed that unigenes related to reproductive development, such as *c64070.graph_c0* and *c68641.graph_c0*, were expressed at relatively low levels at young ages, and that the relative expression gradually increased with increasing plant age. In addition the highest expression levels were reached around 10 and 15 years of age, after which they gradually decreased. Moreover, some unigenes, such as *c61855.graph_c0*, were annotated as abscisic acid hydroxylase genes, and the expression of *c61855.graph_c0* gradually declined with increasing age in *P. koraiensis*.

Keywords: Floral primordia; floral meristem; inflorescence development; ABC model; gene regulation; *Pinus koraiensis*

1. Introduction

During the process of reproductive development, plants need to attain a certain size, age, or level of maturity before they can flower, and this pre-flowering period is called the vegetative stage. The first step in flower development is the transition from the vegetative stage (producing buds and leaves) to the reproductive stage (starting to flower) [1]. In most cases, whether or not a plant can become reproductive after a certain stage of growth is determined by environmental factors such as light and temperature [2]. Many plants respond to changes in a well-defined range of relative day and night lengths (photoperiod) and temperature. Under the combined effect of these two factors, plants can enter the reproductive stage and flower buds differentiate [3]. Once this conversion is complete, the floral meristem identity genes promote the formation of individual flowers [4]. Many studies have examined the roles of flower-related genes such as *APETALA1* (*AP1*) [5], *APETALA2* (*AP2*) [6], *CAULIFLOWER* (*CAL*) [5], *LEAFY* (*LFY*) [7], *FRIGIDA* (*FRI*) [8], Arabidopsis *CONSTANS* (*AtCO*) [9], and *UNUSUAL FLORAL ORGANS* (*UFO*) [10]. At least two genes, *LFY* and *AP1*, are necessary not only for flower initiation, but are also sufficient to induce flowering in lateral shoots when overexpressed in transgenic plants [11]. Soon after floral meristem formation, floral organs began to form in a concentric

order around the periphery of the meristem. Each floral organ originates from a small number of progenitor cells. These cells then follow a specific developmental pattern to form a recognizable floral primordium through cell proliferation, and then develop from the floral primordium into each organ of the flower [12]. Once all of the primordia are activated, the relative position and the total number of floral organs, as well as the basic structure, leaf order, and symmetry of the flower, are determined, and the flower buds open under appropriate conditions [13]. Thus, in the process of floral organ formation, at the molecular level the transcriptional activity is increased, and the biological pathways are activated [14]. In addition, plant hormones are involved in the process of flower formation; for example, the spatial accuracy of organ initiation depends to a large extent on the antagonistic interaction of the two plant hormones auxin and cytokinin [15]. The auxin maximum is caused by the biosynthesis and polar transport of auxin, which specifies initiation of the floral organs [16]. Related research has shown that *YUCCA* (*YUC*) [17] and *PIN* (*PIN*) [18] play important roles in controlling auxin biosynthesis and polar transport. In addition, competition between apical and lateral root auxins (apical buds vs. axillary buds) leads to the inhibition of bud growth [19]. At the same time, cytokinin (CK) helps to maintain the auxin maxima by forming an inhibitory domain on the organ primordium [20]. Perturbation of the cytokinin signaling pathway will affect the arrangement of flower organs [20].

The ABC model [21] of flower organ formation describes the genes and mechanisms that regulate flower development. In the process of flower formation, the floral organs are generated from the outside to inside, and this process can be divided into four rounds; sepal (round 1), petal (round 2), stamen (round 3), and carpel (round 4), controlled by three homologous genes (A, B, C) in which A controls round 1, A and B control round 2, B and C control round 3, and C controls round 4 [22]. It also can be explained by saying that sepal formation is controlled by gene A, petal formation is controlled by genes A and B, stamen formation is controlled by genes B and C, and carpel formation is controlled only by gene C. This is the classic ABC model [22]. Studies have shown that the ABC genes in *Arabidopsis* correspond to *AP2*, *AP3/PI*, and *AG*, respectively. Among them, *APETALA1* (*AP1*) and *APETALA2* (*AP2*) are functional genes, and *APETALA1* (*AP1*) not only controls the characteristics of the sepals and petals, but is also related to the floral meristem [23]. In the process of transformation from vegetative growth to reproductive growth, *AP1* is equivalent to the organizer, and its ectopic expression will cause many plants to advance to flowering [24]. In addition, *AP1* is also a transcriptional activator that regulates *AP3* and *PI* [25,26]. Class B genes such as *APETALA3* (*AP3*) [26] and *PISTILLATA* (*PI*) [25] specify the development of petals and stamens. *AGAMOUS* (*AG*) is the only C function gene. Except for *AP2*, all of these genes are members of the MADS-box transcription factor family [27]. The MADS-box gene family plays an important role in the transition from vegetative growth to reproductive growth, fruit development, and especially in the formation of floral organs [5]. In addition, another group of MADS-box genes, *SEP1*, *SEP2*, and *SEP3* [28], were found to be essential for petal, stamen, and carpel development; in other words, the *SEP* gene products are required for B and C gene activity. [28].

Pinus is an important genus of conifers in the pine family (Pinaceae) with a total of 109 species recorded [29]. Pine species are important both ecologically and economically. *Pinus koraiensis* Siebold & Zucc. ($2n = 2x = 24$) [30] is widely distributed in Northeast China, Japan, the Korean Peninsula, and the Russian Far East [31]. *P. koraiensis* is also a nationally protected plant in China that has attracted attention from researchers because it produces economically valuable wood and edible seeds. However, *P. koraiensis* grows slowly, reaches sexual maturity late, and is difficult to select early in breeding. The tree properties that researchers are most interested in require years of growth to be reliably assessed. Compared to angiosperms, the molecular mechanisms underlying the reproductive development of gymnosperms are not well known. The MADS-box gene family has been characterized in Norwegian spruce and *P. radiata* [32]. Similar to many angiosperms, the “flowering” of *P. radiata* starts with the transformation of an indeterminate axillary apex into a determinate reproductive apex, which forms the male and female strobili (cones) [33]. Some studies have shown that the *P. radiata* gene belonging to the meristem-identity family of *FLO/LFY*-like genes is a true functional homolog of the

Arabidopsis LFY gene [34]. However, research on the internal molecular mechanism of *P. koraiensis* is still not comprehensive. In this study, we examined the changes in expression of flowering-type genes and hormones at different ages and in different parts of *P. koraiensis*. Our findings will be of great significance in understanding reproductive development in pines and will accelerate the breeding process and shorten selection time in *P. koraiensis*. The above-mentioned genes were summarized in Table 1.

Table 1. Genes associated with flower development.

Related Gene	Function
APETALA1 (AP1)	control the characteristics of sepals and petals, be related to the meristem of flowers, a transcriptional activator that regulates AP3 and PI
APETALA2 (AP2)	class A genes, control the characteristics of sepals and petals
APETALA3 (AP3)	class B genes, specify the development of petals and stamens
PISTILLATA (PI)	class B genes, specify the development of petals and stamens
AGAMOUS (AG)	class C genes, control the characteristics of stamens and carpel
SEP1, SEP2, and SEP3	were found to be essential for petal, stamen, and carpel development, that is the SEP gene product was required for B and C gene activity
CAULIFLOWER (CAL)	confer floral identity to emerging floral primordia, a paralogous genes of APETALA1 (AP1), act redundantly to control inflorescence architecture by affecting the domains of LFY expression as well as the relative levels of its activities
LEAFY (LFY)	is expressed throughout the flower, participates in the activation of homeotic genes, are expressed in specific regions of the flower, encodes a transcription factor that determines a meristem will generate flowers, is a direct upstream regulator of floral homeotic genes
FRIGIDA (FRI)	the flowering time gene, alleles were associated with accelerated flowering
<i>Arabidopsis</i> CONSTANS (AtCO)	regulate flowering time, accelerates flowering in response to long days, exerts its inhibitory effect on tuber formation by acting in the leaves
UNUSUAL FLORAL ORGANS (UFO)	controls meristem identity and organ primordia fate, related to floral-organ type
YUCCA (YUC)	spatially and temporally regulated auxin biosynthesis, is essential for the formation of floral organs and vascular tissues
PIN (PIN)	auxin efflux transport proteins, controlling auxin polar transport

2. Materials and Methods

P. koraiensis used in this study was from the Maoershan Forest Farm of Northeast Forestry University. A total of six samples of *P. koraiensis* apical buds were collected from 2-, 5-, 10-, 15-, and 30-year-old trees and also from grafted trees. Each sample consisted of three replicates. Among the samples, the 2-year-old and 5-year-old trees were at the early vegetative stage, the 10- and 15-year-old trees were at the transition from vegetative to reproductive growth, while the 30-year-old trees were at reproductive stage. The scions on the grafted trees were >50 years old.

Total RNA was extracted using the CTAB method [35], and the purity and concentration of the total RNA samples were determined by absorbance at 260 nm using a NanoDrop™ 2000 UV spectrophotometer. For transcriptome sequencing, an equal amount of total RNA was collected from each sample.

RNA sequencing libraries were generated using the NEBNext RNA Library Prep Kit for Illumina (New England Biolabs, USA). Briefly, mRNA was purified from 18 µg of total RNA using poly-T oligo-attached magnetic beads. The RNA samples were fragmented using divalent cations at elevated temperature in NEBNext Reaction Buffer (5X), and the short fragments were then converted to double-stranded cDNA using random hexamer primers and reverse transcriptase. The remaining overhangs were converted to blunt ends via exonuclease/DNA polymerase enzymes. After adenylation of the 3' ends of the DNA fragments, the NEBNext Adaptor with a hairpin loop structure was ligated to the fragments to prepare for hybridization. The library fragments (240 bp) were purified with the

AMPure XP system (Beckman Coulter, Beverly, USA) and amplified by PCR to construct the cDNA library. Finally, the PCR products were purified using the AMPure XP system and the library quality was assessed on the Agilent Bioanalyzer 2100 system.

The library preparations were sequenced on an Illumina HiSeq 2000 instrument to generate the raw sequencing reads. The raw reads were then filtered to remove low quality reads, reads consisting of adapters, and reads containing runs of poly-Ns (unknown bases) to give clean reads. At the same time, the Q20 and Q30 scores, the GC-contents, and the level of sequence duplication in the clean data were calculated for each sample. Trinity [36] was used for transcriptome assembly to generate libraries of non-redundant unigenes.

All non-redundant transcripts were subjected to Basic Local Alignment Search Tool (BLASTx) [37] searches (E-value < 10^{-5}) against public protein databases, including the NCBI non-redundant (nr) [38], the Swissprot protein (Swiss-Prot) [39], the Gene Ontology (GO) [40], the Clusters of Orthologous Groups (COGs) [41], KOG [42] (the database of Clusters of Protein homology), eggNOG4.5 (a database of orthologous groups of genes), [43] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [44]. The predicted amino acid sequences of the unigenes were then annotated using HMMER [45]. GO analysis was used to assign putative gene functions to the uncharacterized sequences. We used the information from the Nr annotation to categorize the unigenes into many functional groups using the topGO R package [46].

In order to fully understand the transcriptomic changes in the apical buds at the different developmental stages, we used DESeq2 [47] to identify DEGs between the apical buds in the different samples compared to 2-year-old trees. We compared the reads which obtained from sequencing to the unigene library, and based on the comparison results, combined with RSEM [48] to estimate the expression level. The expression abundance of the corresponding unigene was expressed by the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) [49]. The DEGs were also annotated using the GO (Gene Ontology) database, and the numbers of DEGs in each GO term were calculated. GO enrichment analysis of the DEGs (DEGs) was implemented by the topGO R package based on the Kolmogorov-Smirnov test [50]. KEGG pathway analysis of the DEGs was performed to identify the associated biochemical and signal transduction pathways. We used KOBAS [51] software to test the statistical enrichment of differentially expressed genes in the KEGG pathways. We used the STRING database [52] to form protein-protein interactions for the DEGs followed by visualization with Cytoscape v3.6 [53].

3. Results

3.1. RNA Sequencing and De Novo Transcriptome Assembly

A total of 18 cDNA libraries were sequenced on an Illumina HiSeq2000 instrument, and we obtained a total of 134.62 Gb of clean reads with an average number of 6,410,479,768 bases in each library. The average GC content was 45.57%. We obtained a total of 100,585 unigenes using Trinity [36] with a total length of 83,979,734 bp. The average and N50 lengths of the unigenes were 834.91 bp and 1561 bp, respectively. Of the resulting unigenes, 33,408 (33.21%) were between 200 and 300 bp in length; 24,178 (24.04%) were 300–500 bp; 18,224 (18.12%) were 500–1000 bp; 14,194 (14.11%) were 1000–2000 bp; and 10,581 (10.52%) were >2000 bp (Figure 1).

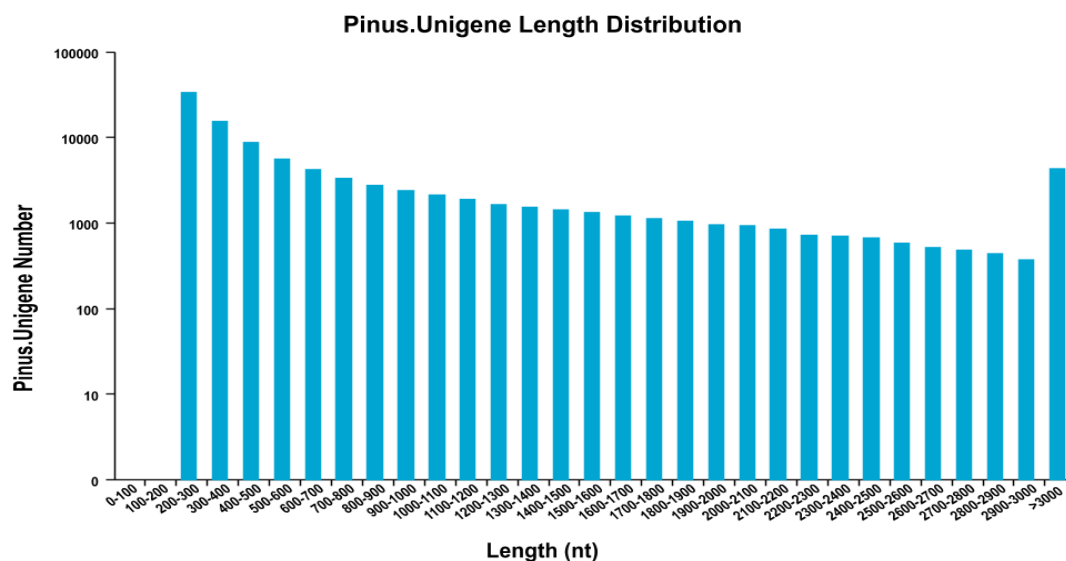


Figure 1. Length distribution of *P. koraiensis* unigenes. The x-axis shows the length intervals of the unigenes, and the y-axis shows the number of unigenes.

3.2. Functional Annotation of the *P. Koraiensis* Unigenes

For the unigene annotation, BLASTX [37] was used to search each database and HMMER was used to annotate the amino acid sequences. The results show that a total of 58,706 (58.36%) unigenes were annotated by at least one of the above seven databases.

In the GO annotation, most of the unigenes in the “biological process” category were sub-categorized into metabolic process, cellular process and single-organism process, followed by localization, biological regulation and response to stimulus. In the “cellular component” category, the GO terms “cell”, “cell part”, “membrane, and organelle predominated. While, catalytic activity, binding and transporter activity were the three most represented GO terms in the “molecular function” category. A total of 33,277 unigenes were annotated in the GO analysis.

In addition to GO annotation, a total of 18,577, 51,957, 19,726, 32,241, 30,528, 52,872, and 38,956 unigenes were annotated by the COG, eggNOG, KEGG, KOG, Swissprot, nr, and Pfam databases, respectively (Table 2). In terms of E-value distribution, 55.15% of the homologs ranged between $1e-11$ and $1e-50$, while a fraction of the sequences (44.85%) showed a threshold e-value $< 1e-50$ indicating inferior homology (Figure 2a). Among the unigenes annotated using the Nr database, 62.13% had more than 60% similarity with the corresponding gene sequence (Figure 2b). Overall, the unigene sequences exhibited most similar BLASTx matches to gene sequences from *Picea sitchensis* (20.39%), followed by those from *Amborella trichopoda* (3.84%), *Aureobasidium pullulans* (2.27%), *Aureobasidium melanogenum* (1.96%), *Nelumbo nucifera* (1.92%) and *Prunus persica* (1.77%) genes (Figure 2c).

Table 2. *P. koraiensis* unigenes annotated using seven protein databases.

Anno_Database	Annotated_Number
COG_Annotation	18,577
GO_Annotation	33,277
KEGG_Annotation	19,726
KOG_Annotation	32,241
Pfam_Annotation	38,956
Swissprot_Annotation	30,528
eggNOG_Annotation	51,957
nr_Annotation	52,872
All_Annotated	58,706

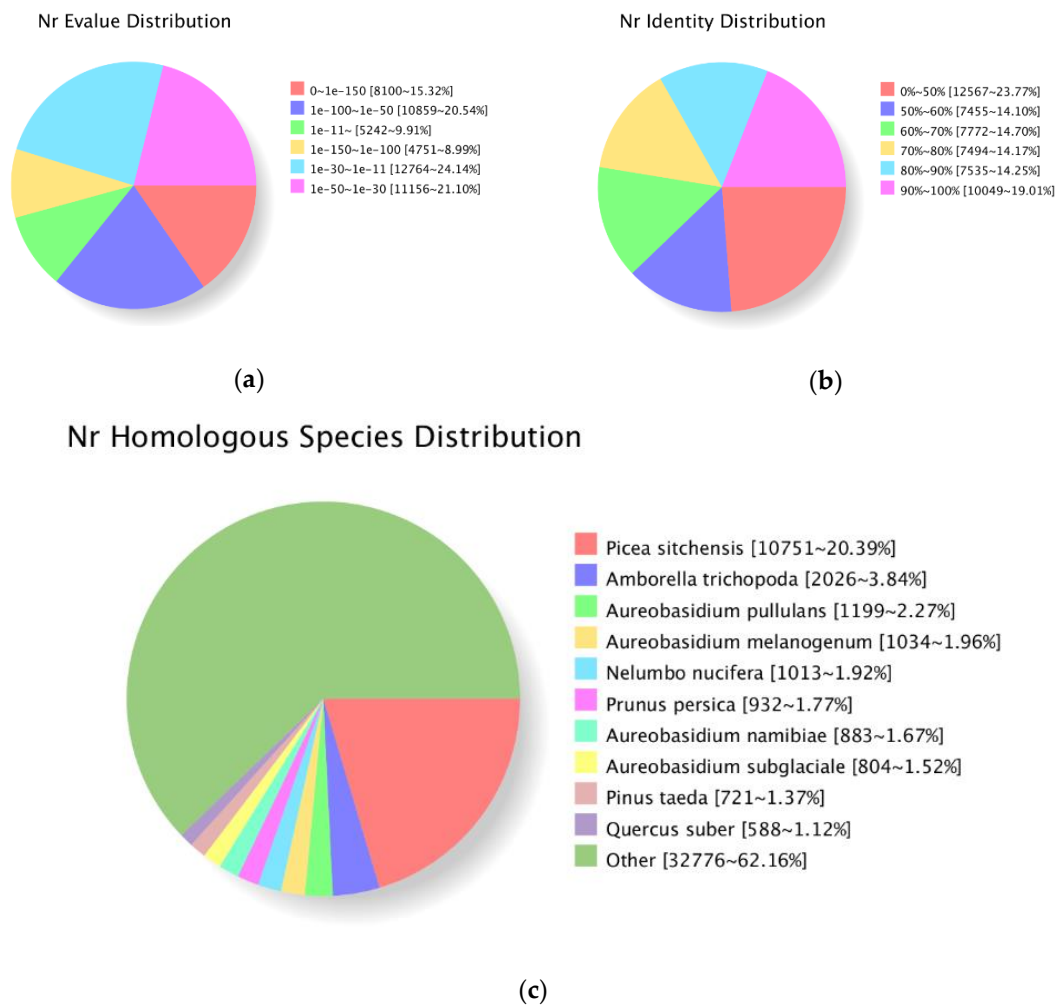


Figure 2. Feature of homology search of unigenes against Nr database. (a) E-value distribution of BLAST hits for each unique sequence with a cut-off E-value of 1×10^{-5} ; (b) Similarity distribution of top BLAST hits for each unigene; (c) Species distribution of the top BLAST hits for each unigene with a cut-off E-value of 1×10^{-5} .

3.3. Identification of Differentially Expressed Genes (DEGs)

The results of the DEG analysis in the five sample comparisons are shown in Table 3 and Figure 3. Using the 2-year trees as the control, we identified 267 (117 up-regulated, 150 down-regulated), 736 (480 up-regulated, 256 down-regulated), 1485 (725 up-regulated, 760 down-regulated), 1152 (578 up-regulated, 574 down-regulated) and 827 (598 up-regulated, 229 down-regulated) DEGs in the 5-, 10-, 15-, 30- and 50-year-old grafted trees, respectively. A total of 671 DEGs were identified by comparing RNA samples from the upper and lower apical buds sampled from the 30-year-old trees; of these DEGs, 378 were up-regulated and 293 were down-regulated.

Table 3. Statistical table of the number of DEGs.

DEG_Comparison	All_DEGs	Up-Regulated	Down-Regulated
Y2_vs_Y5	267	117	150
Y2_vs_Y10	736	480	256
Y2_vs_Y15	1485	725	760
Y2_vs_Y30	1152	578	574
Y2_vs_grafted	827	598	229

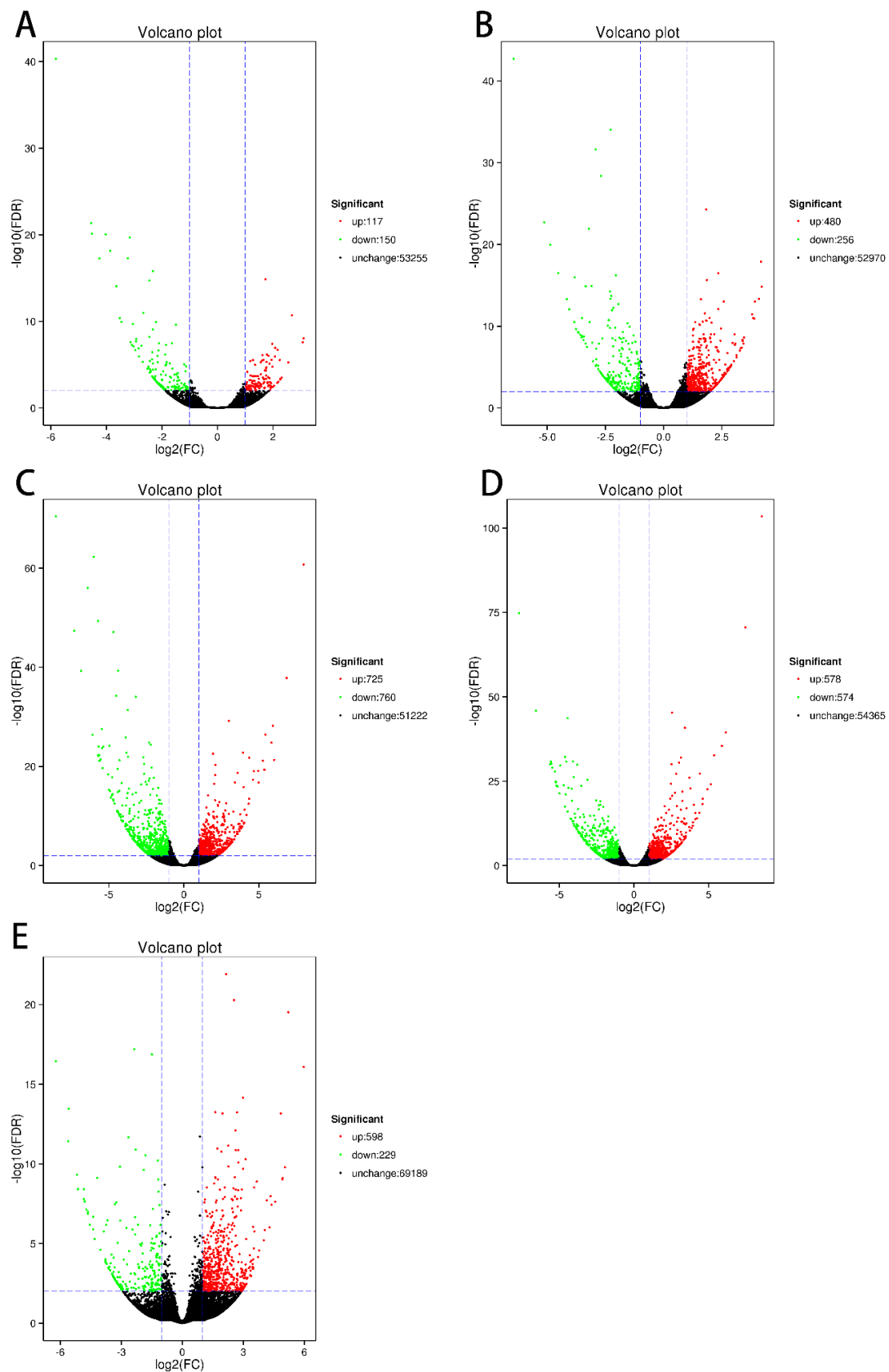


Figure 3. The volcano diagram of differential expression between samples. The green and red dots in the figure indicate significant DEGs, green indicates down-regulated, red indicates up-regulated, and black dots indicate no significant DEGs. (A) Y2_vs_Y5; (B) Y2_vs_Y10; (C) Y2_vs_Y15; (D) Y2_vs_Y30; (E) Y2_vs_grafted.

3.4. Gene Ontology (GO) Enrichment of the DEGs

GO enrichment analysis was performed for the DEGs identified in comparisons of RNA samples between the 2-year-old trees and the five other years using hypergeometric tests [54] (Table S1). The results indicated that GO terms in “biological process” such as “nucleocytoplasmic transport”, “ATP hydrolysis coupled proton transport”, “regulation of transcription from RNA polymerase II promoter”, “proteasomal ubiquitin-independent protein catabolic process”, and “misfolded or incompletely synthesized protein catabolic process” were significantly enriched in each sample. The P-values for these GO terms in the 5-year-old trees were the smallest, which means that the transcription, translation, and energy metabolism activities are strong in the vegetative stage in *P. koraiensis*. Also, the P-values of GO terms such as “transmembrane transport”, “protein folding”, “carbohydrate metabolic process”, and “intracellular protein transport” were lower in the 10- and 15-year-old trees, indicating that utilization of substances and protein post-processing are active in the transition from vegetative to reproductive growth. In addition, the term “negative regulation of short-day photoperiodism, flowering” was enriched in the “biological process” category in the 15-year-old samples, which indicates that the transition from vegetative to reproductive growth occurs at the age of 15 years in *P. koraiensis* (Table S1). According to the homology-based annotation, the unigene in this GO term, *c68641.graph_c0*, may be related to embryogenesis, gibberellin metabolism, negative regulation of floral organ shedding, auxin stimulation, and seed maturation (Table S2). In addition, the gene *c68641.graph_c0* was annotated in the Swissprot database as the agamous-like MADS-box protein AGL15. Interestingly, the expression level of this gene in 15-year-old trees is almost 4-fold higher than the average gene expression level (Table S3), indicating that the 15-year-old trees are undergoing differentiation of the reproductive structures. At the same time, the GO terms “maturation of LSU-rRNA from tri-cistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)” and “reproductive fruiting body development” were enriched in 30-year-old trees. GO enrichment of the DEGs in the grafted trees compared with 2-year trees also identified an important GO term, “regulation of flower development” (Table S4). The unigene *c64070.graph_c0* for this GO term showed the highest expression level in the grafted (50-year-old) trees, and expression in the 5-, 10-, 15-, and 30-year-old trees was relatively stable. The unigene *c64070.graph_c0* was annotated by the NR database as the hypothetical protein GOBAR_AA39818 from *Gossypium barbadense*. Furthermore, another GO term “response to abscisic acid” was also enriched, and a unigene (*c73235.graph_c0*) in this GO term had a higher expression level in the grafted trees (Table S4). The unigene *c73235.graph_c0* was annotated by the Swissprot database as the CBL-interacting protein kinase 5 from the *Oryza sativa*.

3.5. KEGG Pathway Enrichment Analysis of the DEGs

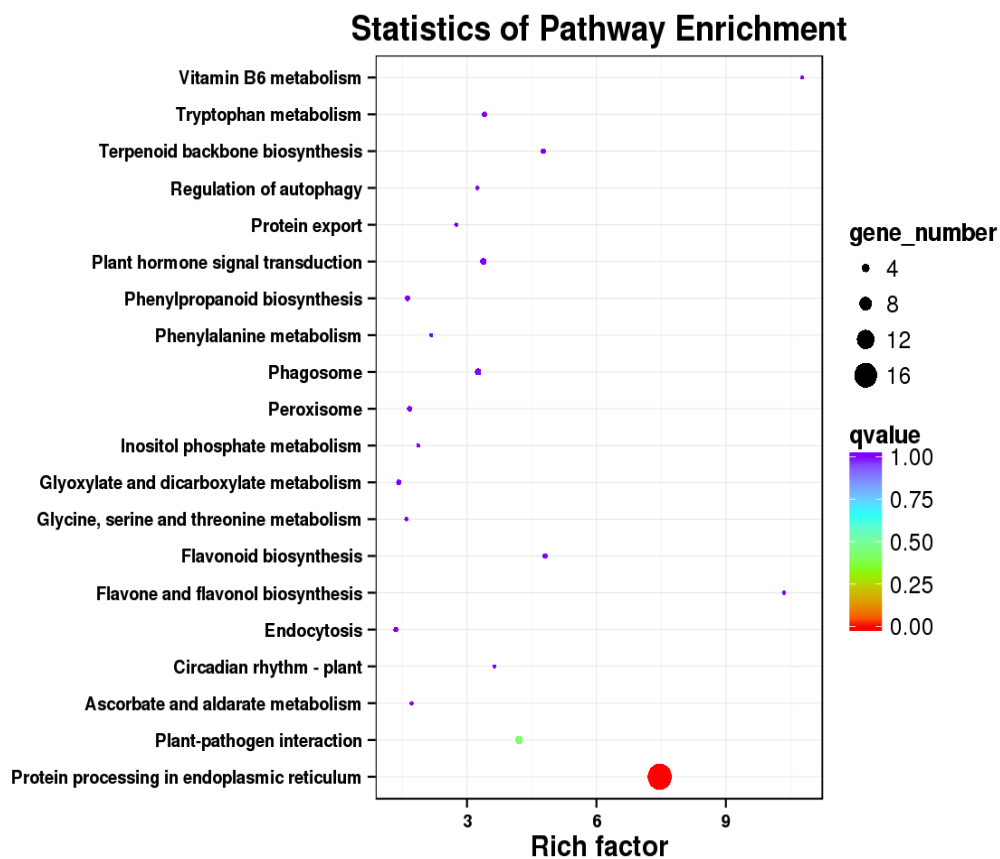
Pathway enrichment analysis was performed on the *P. koraiensis* DEGs using the KEGG database [44] to determine hormone-related pathways. The results of KEGG enrichment in trees of various ages is shown in Figure 4, which shows the top 20 pathways with the most significant enrichment levels. In the 5-year sample, 66 DEGs were associated with 34 KEGG pathways. The most significantly enriched pathways were “vitamin B6 metabolism”, “flavone and flavonol biosynthesis”, “protein processing in the endoplasmic reticulum”, “terpenoid backbone biosynthesis”, and “plant-pathogen interactions”. The pathway containing the most DEGs was found to be “protein processing in the endoplasmic reticulum” (17 DEGs).

The same analysis showed that, in the 10-year-old trees, 189 DEGs were associated with 58 KEGG pathways (Figure 4b). Among these, “protein processing in endoplasmic reticulum”, “plant-pathogen interaction”, “glycosphingolipid biosynthesis”, “carotenoid biosynthesis”, and “brassinosteroid biosynthesis” were the most prominent. The highest number of DEGs (27) were in the “endoplasmic reticulum protein processing” pathway. In the 15-year-old sample, 413 DEGs were associated with 86 KEGG pathways (Figure 4c), of which “betalain biosynthesis”, “diterpenoid biosynthesis”, “brassinosteroid biosynthesis”, “protein processing in endoplasmic reticulum”, “plant-pathogen interaction”, and “plant hormone signal transduction” were the most abundant. Similarly, the pathway

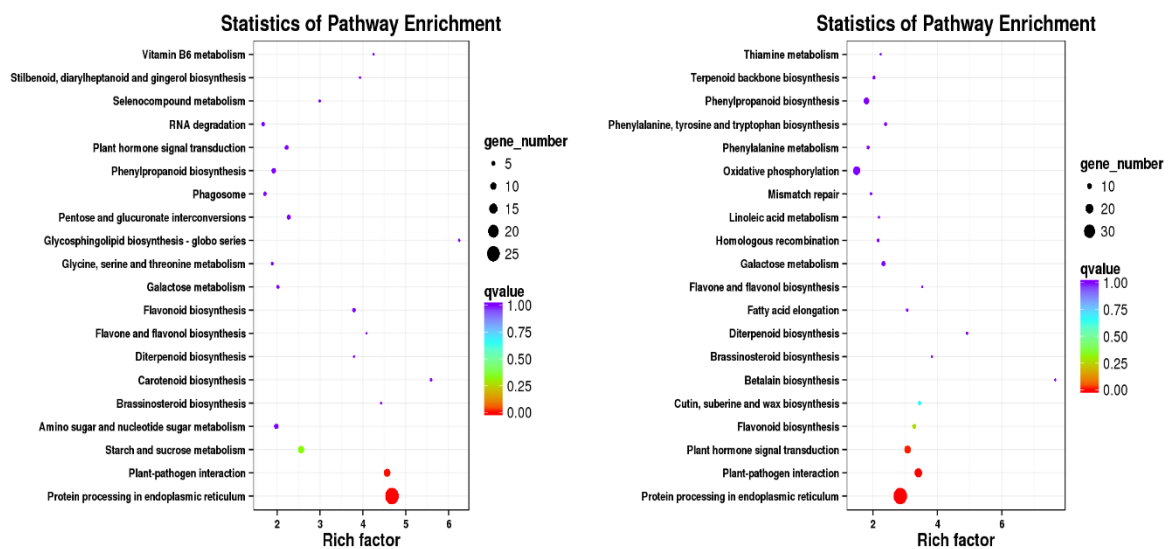
enriched for the most DEGs (38) was also “protein processing in the endoplasmic reticulum”. In the 30-year-old sample, 255 DEGs were associated with 63 KEGG pathways (Figure 4d). Among them, “carotenoid biosynthesis”, “flavone and flavonol biosynthesis”, “brassinosteroid biosynthesis”, “plant-pathogen interaction”, “diterpenoid biosynthesis”, and “ABC transporters” were the most enriched, with “plant-pathogen interaction” containing the most (16) DEGs. Obviously, pathways such as “plant-pathogen interaction”, “protein processing”, “organic acid synthesis and metabolism”, “starch and sugar metabolism”, and “plant hormone signal transduction” were enriched in each sample. Moreover, it can be seen from the results that the KEGG pathways are biased towards biosyntheses, such as the biosynthesis of flavonoids and terpenoid skeletons, in the 5-year-old sample. The pigment and hormone biosynthesis pathways in the 10-year-old samples were enriched for carotenoid synthesis and brassinosteroid synthesis, respectively. Moreover, both DEGs involved in carotenoid biosynthesis were up-regulated, indicating that the carotenoid content in the 10-year-old trees is predicted to be higher than that in the 2-year-old sample. Only one DEG was enriched in the brassinosteroid synthesis pathway, and it is the down-regulated gene *c55558.graph_c0*. and the gene *c55558.graph_c0* was a cytochrome P450 gene resembling CYP85A1 from *Musa acuminata*. The relative expression levels of this gene were 4.36, 1.14, 0.64, 0.41, 0.25 and 0.39 in the 2-, 5-, 10-, 15-, 30- and 50-year-old (grafted) samples, respectively. We observed that the expression level of this gene decreases with age. The 15-year and 30-year samples are roughly the same as the 10-year-old sample, except that the 15-year-old sample is more biased towards amino acid metabolism, while “ABC transporter” appears in the 30-year-old sample. In the grafted tree sample, 193 DEGs were associated with 60 KEGG pathways (Figure 4e), and the first five pathways with the most significant enrichment are “plant-pathogen interaction”, “diterpenoid biosynthesis”, “amino sugar and nucleotide sugar metabolism”, “taurine and hypotaurine metabolism”, and “vitamin B6 metabolism”. The grafted tree appears to be more inclined toward the metabolism of various substances, such as glucose and taurine. Also, the ABC transporter pathway appears only in the 30-year-old and grafted tree samples, but the significance of this observation is unclear and needs further research. Finally, the phytohormone signal transduction pathways in the six samples were found to be significantly enriched, and the most DEGs (16) were found in the 15-year-old sample. This indicates that hormone signaling pathways are involved in the regulation of development at different ages in *P. koraiensis*.

3.6. The Expression Level of Genes Related to Flowers and Hormones

We found 134 DEGs that are related to the development of reproductive structures or hormones in the annotation, and their expression levels in each of the tree RNA samples are shown in Table S5. Here, we selected 30 genes (Figure 5), of which some unigenes, including *c65543.graph_c1*, *c77350.graph_c2* and *c72714.graph_c0*, are annotated as encoding MADS-box transcription factors. The expression levels of these genes in the 2- and 5-year-old trees was low, and the relative expression levels gradually increased with increasing age. Interestingly, the expression levels peaked in the 10- and 15-year-old trees. We interpreted this to mean that the genes were more actively transcribed during bud differentiation. The expression levels of these genes in the grafted trees was also high, and even exceeded that in the 30-year-old trees. In addition, some unigenes, such as *c61855.graph_c0*, were annotated as abscisic acid hydroxylase genes. The enzyme encoded by *c61855.graph_c0* is related to the decomposition of abscisic acid, and we found that the expression of this gene gradually declined with increasing age of the trees. However, the expression level of *c61855.graph_c0* was very high in the grafted trees, almost equal to that in the 2-year-old RNA sample. This shows that the expression of the abscisic acid hydroxylase gene is higher in younger trees, which leads to a lower abscisic acid content. As the trees age, the expression level of the abscisic acid hydroxylase gene declines, and the content of abscisic acid therefore increases significantly. We also found that the abscisic acid content in the grafted tree is low due to a higher relative expression level of *c61855.graph_c0*.



(a)



(b)

(c)

Figure 4. Cont.

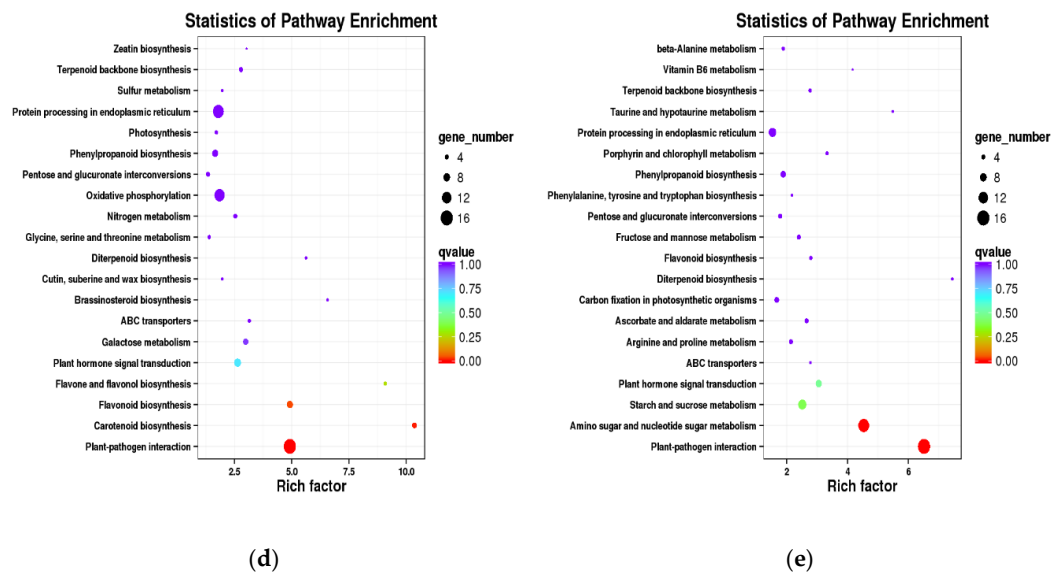


Figure 4. KEGG pathway enrichment analysis of the *P. koraiensis* DEGs showing the top 20 pathways. The abscissa shows the enrichment factors, the ordinate shows the different pathways, the circle sizes represents the number of genes, and the colors represent the q-values. (a) Y2_vs_Y5; (b) Y2_vs_Y10; (c) Y2_vs_Y15; (d) Y2_vs_Y30; (e) Y2_vs_grafted.

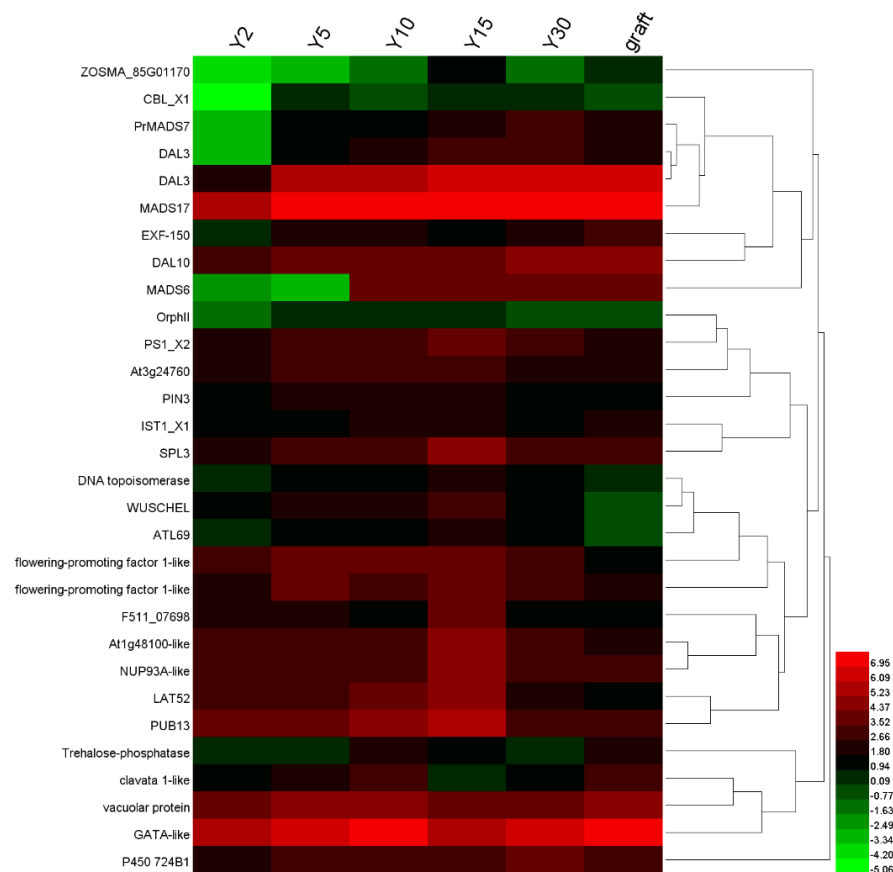


Figure 5. Relative expression levels of DEGs related to reproduction and plant hormones. The rows in the heat map represent the individual gene IDs, and the columns represent the samples taken from 2-year-old (Y2), 5-year-old (Y5), 10-year-old (Y10), 15-year-old (Y15), 30-year-old (Y30), and 50-year-old grafted (graft) trees. The changes in expression are indicated by colors ranging from green (down-regulated) to red (up-regulated).

4. Discussion

4.1. Analysis of DEGs that Show Changes in Expression during Reproductive Development

Genes related to flower development have been well studied in model angiosperms. Plants can regulate flower development through the ABC model, in which the A gene controls sepal formation, the A and B genes jointly control petal formation, the B and C genes together control stamen formation, and the C gene controls carpel formation [28]. In this study, DEGs were found at different ages (2-year-old trees were the control and were compared with 5-, 10-, 15-, and 30-year-old trees and also grafted trees). Among these, a total of 267, 736, 1485, 1152, and 827 DEGs were identified in the 5-, 10-, 15-, and 30-year-old trees and the grafts, respectively. It is worth noting that the number of DEGs gradually increased with increasing age, which indicates that growth and development continue for a long time in *P. koraiensis*. Among the 5-, 10-, and 15-year-old seedless (non-reproductive) samples, the number of up-regulated DEGs (480) in the 10-year-old sample was far greater than the number of the down-regulated DEGs (256), and the number of DEGs peaked in the 15-year-old sample, of which 725 and 760 were up- and down-regulated, respectively. A previous study reported that the age of reproductive maturity in *P. koraiensis* is about 12 years [55]. Based on this, we interpreted our results as showing that the expression level of the same gene is expected to gradually increased with increasing tree age. Relative gene expression levels are low during vegetative growth and are highest during differentiation of the reproductive structures.

4.2. Tree Age Affects Flowering Time

It is well known that age is one of the main factors in determining the flowering period of a plant. It can be seen from the GO and KEGG analyses in this experiment that as age increases in *P. koraiensis*, the tree gradually reaches the reproductive stage. Pathways are mainly shifted away from the metabolism of sugars and organic acids to the metabolism of structural and genetic materials. This is because the plant needs to accumulate a certain amount of nutrients before it can transition to the reproductive growth stage in response to the corresponding signal stimulation, and as the plant ages, the sugar and organic acid contents that are involved in its metabolism increase due to inhibition in some metabolic pathways. Therefore, the transformation in individual plants is reflected in the developmental structures that are related to growth and reproduction. At the gene level, the expression of unigene c64070.graph_c0 increases with increasing age and is related to the regulation of pollen germination and the development of reproductive structures.

4.3. Plant Hormones Related to Flowering

Previous studies have shown that ABA and cytokinin (CK) play important roles in the flowering and development of plants, and they are antagonistic to each other [56]. In this study, we found that the genes c82790.graph_c0 which was related to hormone-mediated signaling pathways resembling a histidine kinase 5 from *Herrania umbratica*. The gene c76037.graph_c0 was related to cytokinin metabolism, and similar to CKX3 from *Pinus tabulaeformis*. The gene c55708.graph_c0 was related to ABA-activated signaling pathways, which was resembling the abscisic acid receptor PYL8 from *Arabidopsis thaliana*. To the best of our knowledge, the effects of flowering are mainly focused on three hypotheses; the anthesis hypothesis [57], the nutrition accumulation hypothesis [58], and the multi-factor hypothesis [59]. At present, the multi-factor hypothesis is widely accepted, and shows that in the process of plant flower formation, the accumulation of nutrients is as important as are plant hormones. The GO enrichment analysis of each *P. koraiensis* RNA sample showed that processes related to energy metabolism and cell development were significantly enriched. This is because the development of reproductive structures requires not only the accumulation of nutrients, but also a direct supply of energy-rich substances such as ATP [60]. The DEGs enriched in organic acid and pyruvate metabolism pathways are related to this. Also, a large amount of ATP produced during metabolism is not only related to the energy supply, but is also one of the important raw materials

for the *de novo* biosynthesis of CK [61]. In summary, we can conclude that unigenes *c82790.graph_c0*, *c76037.graph_c0*, and *c55708.graph_c0* are related to the development of reproductive structures in *P. koraiensis*. The gene arrangement mentioned in the article was shown in Table 4.

Table 4. Gene ID and description.

Gene_ID	Description
c64070.graph_c0	Hypothetical protein GOBAR_AA39818, related to pollen germination and regulates flower development
c68641.graph_c0	The SRF-type transcription factor and agamous-like MADS-box protein AGL15, and related to somatic embryogenesis.
c65543.graph_c1	It has a K-box region and is a SRF-type and MADS-box transcription factor
c77350.graph_c2	It has a K-box region and is a MADS-box transcription factor DAL1 (<i>Picea abies</i>)
c72714.graph_c0	It is SRF-type and MADS-box transcription factor with transcription factor activity
c76037.graph_c0	CKX3 [<i>Pinus tabulaeformis</i>], which is involved in the metabolism of cytokinins and has oxidoreductase activity.
c55708.graph_c0	The abscisic acid receptor PYR/PYL family, involved in the abscisic acid-activated signaling pathway.
c61855.graph_c0	It participates in abscisic acid catabolic process and has the activity of abscisic acid 8' hydroxylase
c73235.graph_c0	It is related to the signaling mechanism and responds to abscisic acid
c82790.graph_c0	Related to hormone-mediated signaling pathway, histidine kinase 5 (<i>Herrania umberrima</i>)
c55558.graph_c0	Predicted as CYP85A1(<i>Musa acuminata</i>), which is a brassinosteroid-6-oxidase and involved in the synthesis of sterol hormones.

5. Conclusions

In this study, high-throughput RNA sequencing was used to generate a *P. koraiensis* transcriptome dataset containing 100,585 predicted transcripts, and analysis of differentially expressed genes in trees of different ages provided clues to the molecular mechanisms underlying the transition from vegetative to reproductive growth. We conclude from the results of GO and KEGG enrichment analyses that the vegetative period is more directed towards the accumulation of energy and materials, the “flowering” pathway is more active during differentiation of the reproductive buds, and the fruit ripening pathway is more obvious during the reproductive stage. In addition, the molecular mechanisms in the grafted tree are similar to those in the 30-year-old tree. Dynamic changes in gene expression levels were observed, and we identified genes encoding hormone signal elements and MADS-box transcription factors. The results presented here provide a valuable resource for studying the molecular biology of reproductive development and hormone levels in *Pinus* species.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/3/350/s1>, Table S1: GO enrichment analysis using the 2-year-old sample as the control. Each sample comparison was mapped to the DEG statistics table in each GO term. Table S2: In the GO enrichment, the GO annotation of each differentially expressed gene. Table S3: Expression levels of all DEGs identified in this study. Table S4: KEGG pathway enrichment and the DEGs contained in it. Table S5: Expression levels of genes involved in reproduction in *P. koraiensis* and annotations from each database.

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