

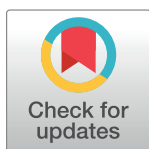
RESEARCH ARTICLE

Comparative transcriptome analysis of differentially expressed genes and pathways in male and female flowers of *Fraxinus mandshurica*

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Abstract

Fraxinus mandshurica Rupr. (*F. mandshurica*) is a dioecious tree species with important ecological and application values. To delve deeper into the regulatory pathways and genes responsible for male and female flowers in *F. mandshurica*, we conducted transcriptome sequencing on male and female flowers at four distinct stages. The analysis revealed that the female database generated 38,319,967 reads while the male database generated 43,320,907 reads, resulting in 2930 differentially expressed genes with 1441 were up-regulated and 1489 down-regulated in males compared to females. Following an analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), four distinct pathways (hormone signal transduction, energy metabolism, flavonoid biosynthesis, and photoperiod) linked to female and male flowers were identified. Subsequently, qRT-PCR verification revealed that *FmAUX/IAA*, *FmEIN3*, and *FmA-ARR* genes in hormone signal transduction pathway are related to female flower development. Meanwhile, *FmABF* genes in hormone signal transduction pathway, *FmGS* and *FmGDH* genes in energy metabolism pathway, *FmFLS* genes in flavonoid biosynthesis pathway, and *FmCaM*, *FmCRY*, and *FmPKA* genes in photoperiod pathway are related to male flower development. This study was the first to analyze the transcriptome of male and female flowers of *F. mandshurica*, providing a reference for the developmental pathways and gene expression levels of male and female plants.

1. Introduction

The *F. mandshurica* is a member of the *Fraxinus* genus within the Oleaceae family, one of northeastern China's three hardwood broadleaf tree families [1]. It is a crucial broad-leaved timber species and a key species in the climax forest community in Northeast China [2, 3]. Additionally, because of the beautiful pattern and hard material features, it has significant

economic value as an excellent precious wood in production practice [4]. These unique and valuable characteristics make it essential to study species value and ecological diversity.

The sexual development of higher plants is primarily evident in a specialized organ of flower [5]. *F. mandshurica* typically takes 15–20 years of growth before it blooms for the first time, and before that it is difficult to identify gender [6]. *F. mandshurica* is a dioecious plant, meaning male and female flowers are present on separate individuals. Dioeciousness is a means to avoid inbreeding and maintain genetic diversity, and it is possible that dioecious plants descended from hermaphroditic ancestors [7, 8]. Female plants are primarily responsible for population reproduction, while male plants are more commonly used for economic growth, reforestation, and timber production [9]. As a result, the study of molecular, genetic [10], and ecological behavior has been extensively conducted on both bisexual reproduction and dioeciousness [11, 12]. Therefore, we conducted a transcriptome analysis study on the male and female flowers of *F. mandshurica*.

Recently, RNA-seq has become a popular method for analyzing plant transcriptomes, allowing researchers to identify key pathways and genes [13, 14]. RNA-seq results can be used for various analyses such as de novo transcription assembly [15], transcription factor analysis [16], differential expression detection [17], and gene annotation [18]. While sex chromosomes have not been found in *F. mandshurica*, previous transcriptome studies have shown that female and male plants differ greatly in energy metabolism and flavonoid biosynthesis [19, 20]. It has been demonstrated that the plant hormone and photoperiod play a crucial role in flower development [21, 22], for instance, genes such as cytokinin [23], *FLCs* [24], and *PRR37* [25] respectively play important roles in the above process. These differences suggest that sex-specific adaptability to different environments might exist [26]. In addition, research has shown that flowering regulation in *Arabidopsis thaliana* involved at least six pathways, including the photoperiod, vernalization, ambient temperature, autonomous, gibberellin, and age pathways [27].

In previous research, we closely examined the morphology of male and female flowers in *F. mandshurica* during various stages of development [28]. To further refine the understanding of the genetic differences between male and female flowers in *F. mandshurica*, the genes of transcriptome of male and female flowers were annotated and analyzed. Using these data, four pathways related to flower development were further analyzed as hormone signal transduction pathway, energy metabolism pathway, flavonoid biosynthesis pathway, and photoperiod pathway. Additionally, we compared the expression of genes in the above pathways in adult male and female leaves as well as young leaves to better identify the difference genes in flower development.

2. Materials and methods

2.1 Plant material

The materials utilized in this study were sourced primarily from the Qingshan forest seed orchard of Weihe Forestry Bureau, located in Shangzhi City, Heilongjiang Province, China. In order to ensure consistency, 30 *F. mandshurica* with similar growth conditions in the same provenances were selected as experimental materials, including 15 male and 15 female trees. The male flower contains four cell stages, namely archesporial cell phase, pollen mother cell phase, meiosis cell, and cell of loose pollen phase. Similarly, the female flower has four cell stages: archesporial cell phase, embryo-sac mother cell phase, meiosis cell, binucleated cell [29]. The leaves were collected from adult male and female trees, as well as young trees (2 years old). Samples were manually picked, frozen immediately in liquid N₂ and stored at -80°C until further use. Three parallel experiments were conducted to ensure the accuracy of the results.

2.2 RNA extraction and library preparation

The TaKaRa MiniBEST universal RNA extraction kit (TaKaRa, Tokyo, Japan) was used to extract the total RNA from male and female flowers, and simultaneously remove DNA from RNA. The RNA concentration was measured using a Nanodrop 2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). To assess the quality of RNA, agarose gel electrophoresis was performed (S1 Fig). Two samples of female and male flowers were prepared by mixing RNA samples from 4 different periods of male and female flowers with equal quality, respectively.

Magnetic beads were used to enrich the mRNA of eukaryotes by using Oligo-dT [30]. Illumina HiSeq/MiSeq sequencing was then conducted on the cDNA library [31, 32]. The original sequence was sequenced, and sequences containing joints, an uncertain base ratio greater than 10%, and low quality were eliminated. All these procedures were performed by Novogene Co., Ltd. (Beijing, China). Additionally, all reads of transcriptome sequencing have been uploaded to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI, SRA accession number PRJNA1122990).

2.3 Analysis of illumina transcriptome sequencing results

A blast search of transcriptomes was performed on 7 databases: NR, NT, KO, Swiss-Prot, PFAM, GO, and KOG. Gene function was categorized using GO and KOG analysis. Subsequently, the sequence was annotated by pathway through KEGG analysis. This helped in analyzing both the metabolic pathway of gene products and compounds in cells and the function of these gene products. The gene expression levels were normalized into RPKM values [33]. p-values and q-values were calculated to identify differentially expressed genes (DEGs). To eliminate biological variation, a threshold was set: $|\log_2(\text{fold change})| > 1$ and $q\text{-value} < 0.005$. This helped in identifying significant differences between the two levels of assessment and the difference in gene screening.

2.4 Functional annotation and pathway prediction of DEGs

The male and female DEGs were analyzed using GO annotation and Pathway analysis to better understand the differences in gene function between the sexes in *F. mandshurica*. GO analysis allowed us to examine the distribution of differential genes in the Gene Ontology (<http://www.geneontology.org/>) and identify significantly enriched GO terms using the Goseq method with a p-value threshold of ≤ 0.05 . This provided insight into the major biological functions of DEGs. Moreover, The important biological pathways involved in DEGs through significant pathway enrichment analysis of KEGG (<http://www.genome.jp/kegg/>) were identified [34].

2.5 Primer design and qRT-PCR validation

According to the transcriptome analysis, the genes that need further verification were selected out. Use Primer 5.0 software to design fluorescent quantitative primers (S3 Table), the product length was between 100–300 bp, and the annealing temperature was limited to 55–65°C. The primer was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan) according to the kit instructions [35].

Real-time quantitative PCR was conducted on a 96-well plate and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit using the Applied Biosystems 7500 real-time PCR system. The 20 µL reaction mixture included 1 µL cDNA (< 100ng), 0.8 µL each of upstream and downstream primers, 10 µL SYBR® Premix Ex Taq™ II, and 0.4 µL Rox Reference Dye II. The PCR

procedure involved the following steps: 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 34 seconds, which was repeated for 40 cycles. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression and the internal reference gene was tubulin. All qRT-PCR reactions were performed with the three biological replicates.

2.6 Statistical analysis

Data analysis was conducted using SPSS 27 (SPSS, Inc., Chicago, IL, USA), t-test was used to determine the statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Furthermore, image generation were performed using Origin 2021 (OriginLab, Northampton, MA, USA) and Excel 2019 (Microsoft, Redmond, WA, USA).

3. Results

3.1 Transcriptome sequencing data results

Through transcriptome sequencing, the female and male flower databases generated 38,319,967 and 43,320,907 reads, respectively. Upon screening, it was found that female and male flowers contained 3.66 GB and 3.92 GB of nucleotides, respectively. The error rates of both male and female flower sequences were less than 0.06, with Q20(%) at around 95%, and the GC content at approximately 43% (S1 Table). Moreover, the proportion of clean reads in both samples accounted for more than 90% of raw reads, which proves the higher sequencing quality for both samples. Therefore, the number of clean reads obtained could meet the experiment's requirements (Fig 1).

To study the functions of the unigenes and gather information about their characteristics, the assembled Unigene was compared with seven public databases using Blast and Blastx methods. The results were then analyzed and counted. Out of the seven databases, 32,311 NR (35.69%), 14,051 NT (15.52%), 9,084 KO (10.03%), 22,583 SwissPro (24.94%), 22,589 PFAM (24.95%), 25,961 GO (28.67%), and 10,879 KOG (12.01%) had sequences that matched. All Unigene sequences obtained homologous alignment information (Table 1).

To evaluate the transcriptome annotations' completeness and validity, the annotated genes was categorized into KOG database. Out of 10,879 sequences, 2,047 genes (16.73%) fell into the General function prediction only (R) category, indicating that there are still many new

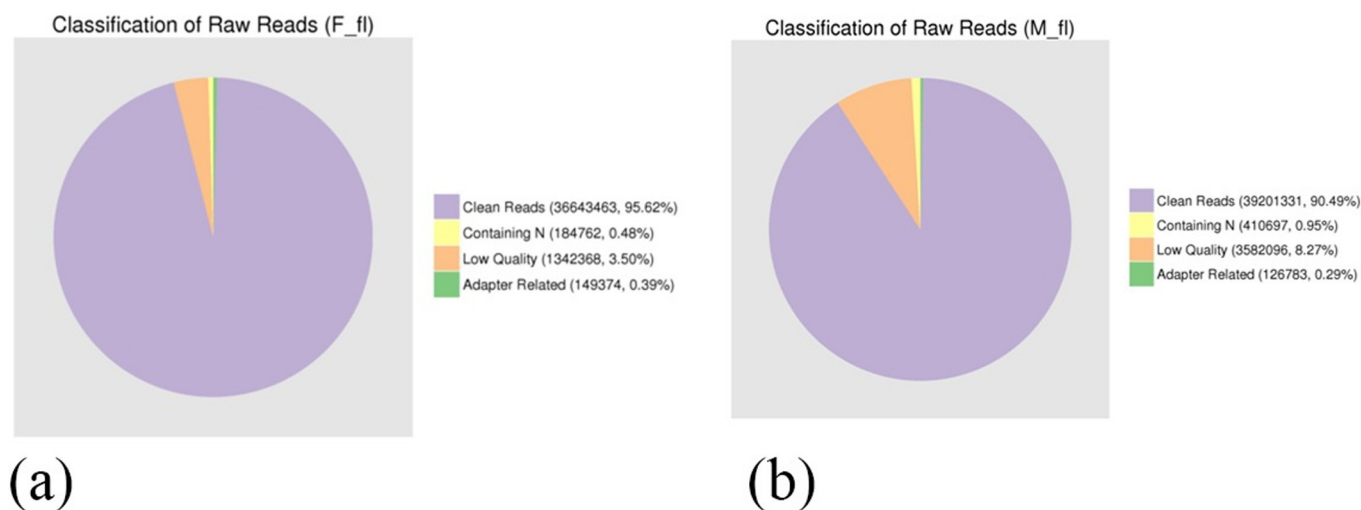


Fig 1. The composition of raw sequence data. (a) The composition of female flower data. (b) The composition of male flower data.

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the female and male databases, respectively. In *F. mandshurica* with RPKM >60, only 3.5% were observed, and the gene expression level followed a normal distribution. The volcano map revealed 2930 DEGs after the screening, with 1,441 genes (49.18%) up-regulated and 1,489 genes (50.82%) down-regulated in females compared to males (Fig 3A). Overall, the DEGs showed a distribution of $|\log_2(\text{Fold Change})|$ basic concentration values between 1 ~ 4, indicating a difference ratio of 2 ~ 16 times. A hierarchical clustering analysis was conducted based on RPKM values of the DEGs between males and females, revealing a large number of DEGs in the heat map (Fig 3B).

3.3 GO and KEGG analysis of DEGs

To facilitate a more intuitive understanding of gene expression differences, GO analysis was conducted on the up-regulated and down-regulated DEGs separately (Fig 4A and 4B). The differential genes were classified into three functional classes: biological processes (2,081 GO functional annotations, 59.27%, BP), cell components (406 GO functional annotations, 11.6%, CC), and molecular functions (1,024 GO functional annotations, 29.17%, MF). Among the up-regulated genes, significant enrichment was observed in oxidation-reduction processes (BP), lipid metabolic processes (BP), and oxidoreductase activity (MF). Similarly, among the down-regulated genes, there was significant enrichment in carbohydrate metabolic processes (BP) and single-organism carbohydrate metabolic processes (BP). The GO functional annotation analysis showed a large level of genetic divergence in biological processes.

1,612 DEGs were discovered by KEGG Pathway analysis, which was mapped to 151 pathways. The pathways for starch and sucrose metabolism, as well as glycolysis/gluconeogenesis, were significantly down-regulated. Conversely, metabolic pathways and biosynthesis of secondary metabolites were significantly up-regulated (Fig 4C and 4D). Based on biological functions, the DEGs can be classified into 4 categories-metabolism, genetic information processing, environmental information processing, and cellular processes and 22 sub-categories, including transport and metabolism, signal transduction, folding, sequencing, and degradation, as well as carbohydrate metabolism, among others. These indicates that the

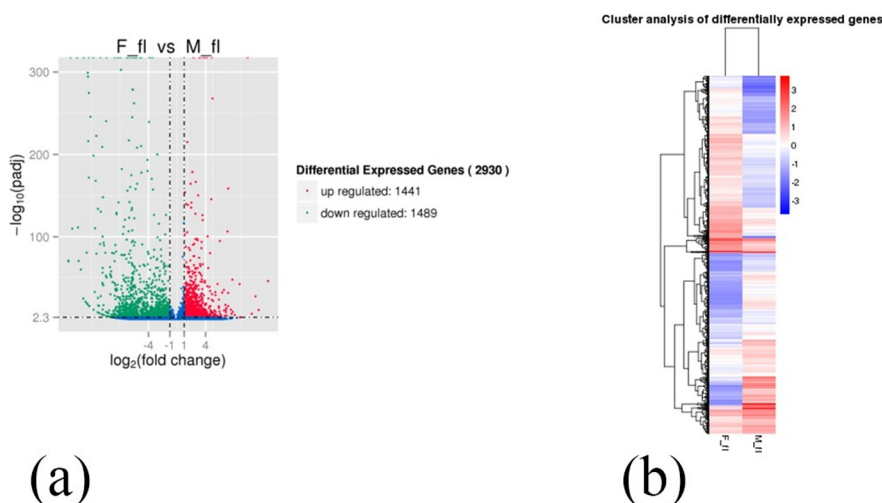


Fig 3. Analysis of DEGs. (a) Volcano map of DEGs. DEGs that may be false positives were shown in blue, up-expressed genes were shown in red, and down-expressed genes were shown in green. (b) Heat map of cluster analysis of DEGs. In the overall RPKM hierarchical clustering graph, red indicated up-regulated genes and blue indicated down-regulated genes.

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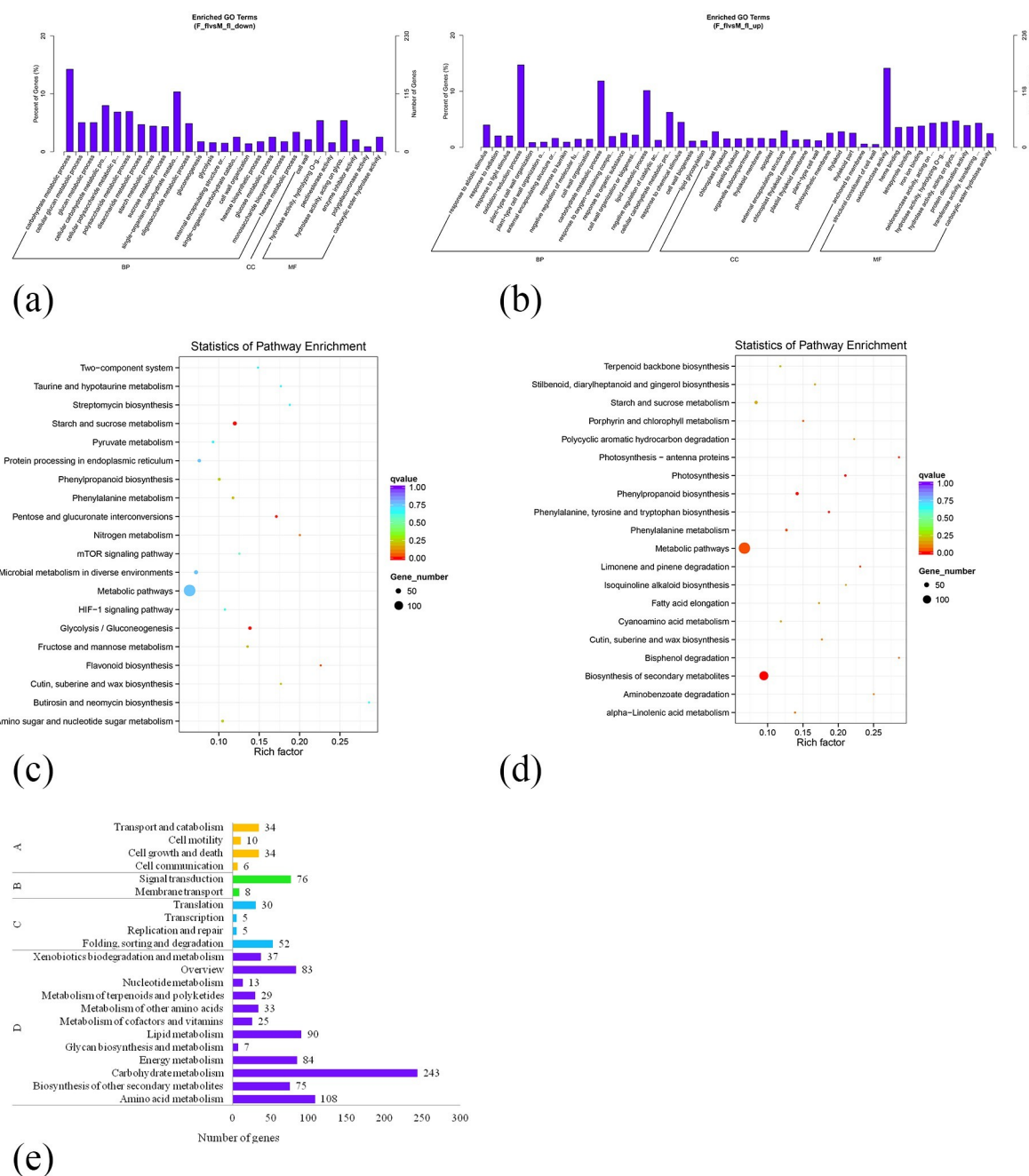


Fig 4. GO and KEGG analysis of DEGs. (a) GO analysis of down-regulated genes. (b) GO analysis of up-regulated genes. (c) KEGG analysis of down-regulated genes. (d) KEGG analysis of up-regulated genes. (e) KEGG classification of DEGs. A: Cellular Processes, B: Environmental Information Processing, C: Genetic Information Processing, D: Metabolism.

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development of male and female *F. mandshurica* involves complex physiological and biochemical processes (Fig 4E).

3.4 Analysis of DEGs in the pathways

Out of the 151 pathways of differential genes analyzed, it was found that 59 pathways had more genes expressed in down-expressed, while 10 pathways had equal amounts of genes

expressed by both expression. The remaining 82 pathways showed more genes expressed in up-expressed. In terms of female and male flowers development, the plant hormone signal transduction pathway had up to 20 up-expressed genes, while the other three pathways: the flavonoid biosynthesis pathway, the energy metabolism pathway (carbon metabolism and nitrogen metabolism), and the photoperiod pathway had more down-expressed genes (Table 2). Among the above four pathways related to flower development, genes with large multiples of difference were screened out (S2 Table). And 16 genes among these genes were selected for primer design and quantitative analysis (S3 Table).

3.5 The DEGs were verified by qRT-PCR

Fluorescence quantitative analysis was conducted on 16 genes in the flowers and leaves of male and female *F. mandshurica* plants over four stages. Based on the consistency and significance results of fluorescence quantitative analysis of flowers and leaves of *F. mandshurica*, 10 significantly expressed genes were obtained (Fig 5). There was hormone signal transduction (*FmAUX/IAA*, *FmA-ARR*, *FmEIN3*, and *FmABF*), energy metabolism (*FmGS* and *FmGDH*), flavonoid biosynthesis (*FmFLS*), and photoperiod (*FmCaM*, *FmPKA*, and *FmCRY*). The expression of these genes was significantly different between male and female plants, that *FmAUX/IAA*, *FmEIN3*, and *FmCRY* genes were significantly expressed in females, and *FmA-ARR*, *FmABF*, *FmGS*, *FmGDH*, *FmFLS*, *FmCaM*, and *FmPKA* genes were significantly expressed in males. These studies indicate that the transcriptome sequencing data of male and female flowers of *F. mandshurica* are appropriate and accurate, and the flower development process is complex, involving multiple pathways and genes.

4. Discussion

The use of transcriptome or expression profiles for screening DEGs has become widespread [36–38]. However, transcriptome analysis of flowers of the dioecious plant *F. mandshurica* has not been reported. Our study can provide a reference for further research on the developmental pathways and gene expression of female and male flowers of *F. mandshurica*. In one study, Maria et al. analyzed the gene expression of female and male gametophytes of *Fucus vesiculosus* brown alga and identified 28 female and 92 male over-expressed genes [39]. In another study, He et al. conducted transcriptome analysis on the leaves of *F. mandshurica* and hybrid seedlings, revealing 11,321 DEGs in the leaf transcriptome of 1601 and M8 with 6,008 genes up-regulated, and 5,313 down-regulated [40]. In this study, a total of 2,930 DEGs were identified from the transcriptome with 1,441 genes up-regulated, and 1,489 genes down-regulated. The transcriptome analysis provided sufficient DEGs for further analysis and predicted four pathways of gender-related: plant hormone signal transduction, energy metabolism, flavonoid biosynthesis, and photoperiod pathways.

Table 2. Analysis of differentially expressed genes in the pathways.

Pathway	Differential genes	down-expressed genes	up-expressed genes	Difference magnitude
Hormone signal transduction	26	20	6	0.231
Nitrogen metabolism	12	5	7	0.583
Carbon metabolism	32	15	17	0.531
Flavonoid biosynthesis	9	2	7	0.778
Photoperiod	4	1	3	0.750

Note: Difference magnitude = up-regulated genes/total differential genes in a pathway.

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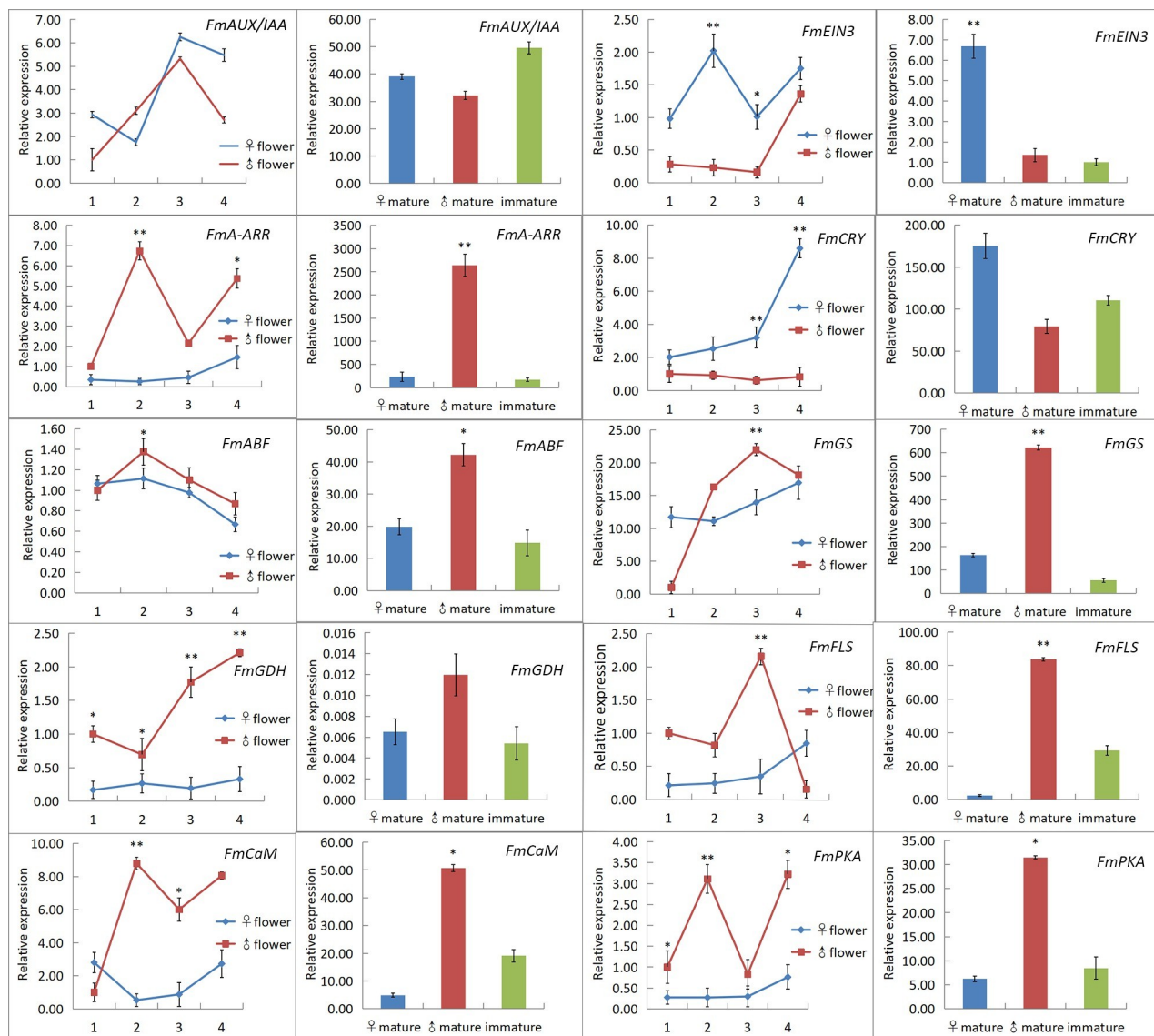


Fig 5. 10 genes that were consistently and significantly expressed in flowers and leaves of *F. mandshurica*. In the two graphs of the same gene, the left line graph shows expression in flowers, and the right bar graph shows expression in leaves. 1, 2, 3, and 4 represent the four stages of flower development, respectively, σ_1 : archesporial cell phase, σ_2 : pollen mother cell phase, σ_3 : meiosis cell, and σ_4 : cell of loose pollen phase. φ_1 : archesporial cell phase, φ_2 : embryo-sac mother cell phase, φ_3 : meiosis cell and φ_4 : binucleated cell. The left side of the same gene represents gene expression in the leaves of adult females, adult males, and young trees. Blue represents females, red represents males, and brown represents seedlings. A p-value ≤ 0.05 was considered significant (*), and a p-value ≤ 0.01 was considered highly significant (**).

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Plant hormones play a significant role in the regulation of sexual development and flowering in plants [41, 42]. Among these hormones, auxin and ethylene are considered to be the primary phytohormones associated with sex development [43–45]. Specifically, auxin is crucial to the development of female plants in hemp [46] and cactus [47], and its signal is up-regulated during the transformation of *Ricinus communis* pistil from leaf tip to flower bud [48]. Ethylene, has a strong effect on female development in cucumbers [49, 50] and melon [51]. Interestingly, female flowers were even found on the male plants of mulberry under a certain concentration of ethylene [52]. This effect is due to the transmission of the ethylene signal, which promotes the expression of the *EIN3* gene and the development of female flowers. In this study, it was

found that *FmAUX/IAA* and *FmEIN3* genes were preferentially expressed in females, indicating that the auxin and ethylene signal transduction pathways were stronger in females and promoted their development.

Studies have shown that cytokines (CTK) can have an impact on the gender expression of plants [53], with some evidence suggesting that it could promote female development in *Mercurialis annua* [54]. Furthermore, CTK treatment of male flowers in hemp, grape, and spinach plants has been shown to lead to the transformation of male flowers into female flowers [55]. Absciscic acid (ABA) is known to play a role in regulating various aspects of plant growth and development, including flower bud differentiation, seed formation, and responses to environmental stresses. Sladky's study found that a certain level of ABA is necessary for the differentiation of female flower buds, but excessive accumulation can halt the process [56]. This study found that the expression of *FmA-ARR* and *FmABF* genes was prominent in males, and the negative feedback mechanism of *A-ARR* regulating *B-ARR* inhibiting its expression was the reason for the significant expression of *A-ARR* genes. These findings suggest that cytokinin can stimulate the production and development of female flowers, while absciscic acid is important for the development and stress resistance of male flowers. More information about the regulatory networks of plant hormone signal transduction pathway is available at KEGG website (https://www.kegg.jp/kegg-bin/show_pathway?ko04075).

The development trends in energy metabolism and flavonoid biosynthesis differ between male and female plants. When nutrients are limited, female plants prioritize nutrient acquisition, while male plants allocate more resources to above-ground growth [57]. Flavonoids have been found to protect plant DNA from UV damage [58], and UV-stressed *Arabidopsis* plants have been observed to have higher flavonoid content [59]. Additionally, flavonoids have important effects on plant reproductive activities, promoting functional pollen tube formation and pollen germination [60]. In plants, FLS catalyzes dihydroquercetin to form flavonols such as kaempferol, quercetin and myricetin [61]. In cells, NH_4^+ is assimilated into glutamine under the action of glutamine synthetase (GS), which uses ATP to provide energy. Glutamate synthase (GOGAT) catalyzes the formation of glutamate from glutamine [62]. In this study, *FmGS* and *FmGDH* genes of energy metabolism pathway and *FmFLS* genes of flavonoid biosynthesis pathway were significantly expressed in male flowers of *F. mandshurica*. This suggests that male plants have a higher level of nitrogen metabolism and flavonoid biosynthesis, potentially contributing to their rapid growth and pollen formation.

The length of daylight, or photoperiod, has a notable impact on the flower development in plants [63]. In a study of *Arabidopsis thaliana*, researchers discovered that longer days encouraged flowering, while shorter days inhibited it [64]. The photoperiod pathway primarily detects and reacts to light signals through photoreceptors and circadian clocks to stimulate the transition to flowering [65]. In a gene chip screening of DEGs between male and female *Populus tomentosa*, researchers found that *PIL5* expression was notably increased in male flowers [66]. This study found that *FmCRY* gene was prominently expressed in females, which consequently hindered the function of the Clock-Bmal1 heterodimer and reduced the translation of Cry and period genes. Calcium and Ca^{2+} signaling plays a crucial role in flagellar and sperm motility, and given that *FmCaM* and *FmPKA* genes were highly expressed in males, it is believed that this dominant expression of photoperiod-related genes in male flowers may be linked to their more active biological activities.

5. Conclusions

In this study, we analyzed the transcriptome sequencing data of male and female flowers at four different developmental stages of *F. mandshurica*. 2930 DEGs were yielded, with 1441

genes in up-regulated and 1489 genes in down-regulated. Through GO and KEGG analysis, DEGs were enriched in four pathways related to flower development. Further validation through qRT-PCR demonstrated that 10 genes in these pathways showed significant differences, aligning with the transcriptome analysis. There were *FmAUX/IAA*, *FmA-ARR*, *FmEIN3*, *FmABF* genes in hormone signal transduction; *FmGS* and *FmGDH* genes in energy metabolism; *FmFLS* gene in flavonoid biosynthesis; and *FmCaM*, *FmPKA*, and *FmCRY* genes in photoperiod. Our findings offer valuable insights into the differential pathways and gene expression patterns of male and female flowers of *F. mandshurica* during development and serve as a reference for further studies on plant sex development.

Supporting information

S1 Fig. RNA quality testing for *F. mandshurica*.

(DOCX)

S1 Table. Summary of the *F. mandshurica* transcriptome.

(DOCX)

S2 Table. Flower development related pathways and genes.

(DOCX)

S3 Table. Genes and primer sequences of qRT-PCR.

(DOCX)

Author Contributions

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Formal analysis: Kaifang Zhang, Fanqiu Kong, Fenghui Qi.

Funding acquisition: Yaguang Zhan.

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Software: Kaifang Zhang, Yuan Cao, Xinyue Guo, Fanqiu Kong, Hongran Sun.

Supervision: Tianzhong Jing, Yaguang Zhan, Fenghui Qi.

Writing – original draft: Hongran Sun.

Writing – review & editing: Kaifang Zhang, Fenghui Qi.

References

1. Chen X, Wang H, Li X, Ma K, Zhan Y, Zeng F. Molecular cloning and functional analysis of 4-Coumarate: CoA ligase 4(4CL-like 1) from *Fraxinus mandshurica* and its role in abiotic stress tolerance and cell wall synthesis. *BMC Plant Biology*. 2019; 19(1). <https://doi.org/10.1186/s12870-019-1812-0> PMID: 31159735
2. Hu L-J, Uchiyama K, Shen H-L, Saito Y, Tsuda Y, Ide Y. Nuclear DNA Microsatellites Reveal Genetic Variation but a Lack of Phylogeographical Structure in an Endangered Species, *Fraxinus mandshurica*, Across North-east China. *Annals of Botany*. 2008; 102(2):195–205. <https://doi.org/10.1093/aob/mcn074> PMID: 18477559
3. Hao J, Chen N, Yan P, Xu K, Zhang L, Zhang H. Study on the variation in and selection of *Fraxinus mandshurica* provenances and families in northeast China. *Journal of Forestry Research*. 2022; 34(2):519–29. <https://doi.org/10.1007/s11676-022-01478-1>

4. Liu Y, Wei C, Wang H, Ma X, Shen H, Yang L. Indirect somatic embryogenesis and regeneration of *Fraxinus mandshurica* plants via callus tissue. *Journal of Forestry Research*. 2020; 32(4):1613–25. <https://doi.org/10.1007/s11676-020-01199-3>
5. Xiong E, Wu X, Shi J, Wang X, Wang W. Proteomic identification of differentially expressed proteins between male and female plants in *Pistacia chinensis*. *PLoS One*. 2013; 8(5):e64276. <https://doi.org/10.1371/journal.pone.0064276> PMID: 23691188.
6. Han-ying S, Dai-bin L. Influence of main site factors on *Fraxinus mandshurica* (Oleaceae) plantation. *Journal of Forestry Research*. 2003; 14(1):83–6. <https://doi.org/10.1007/BF02856770>
7. Guttman DS, Charlesworth D. An X-linked gene with a degenerate Y-linked homologue in a dioecious plant. *Nature*. 1998; 393(6682):263–6. <https://doi.org/10.1038/30492> PMID: 9607762
8. Cherif E, Zehdi S, Castillo K, Chabrilange N, Abdoukader S, Pintaud J-C, et al. Male-specific DNA markers provide genetic evidence of an XY chromosome system, a recombination arrest and allow the tracing of paternal lineages in date palm. *New Phytologist*. 2013; 197(2):409–15. <https://doi.org/10.1111/nph.12069> PMID: 23231423
9. Agren J. Sexual Differences in Biomass and Nutrient Allocation in the Dioecious *Rubus Chamaemorus*. *Ecology*. 1988; 69(4):962–73. <https://doi.org/10.2307/1941251>.
10. Vagera J, Paulíková D, Doležel J. The Development of Male and Female Regenerants by In Vitro Androgenesis in Dioecious Plant *Melandrium album*. *Annals of Botany*. 1994; 73(4):455–9. <https://doi.org/10.1006/anbo.1994.1056>
11. Edward DA, Chapman T. The evolution and significance of male mate choice. *Trends in Ecology & Evolution*. 2011; 26(12):647–54. <https://doi.org/10.1016/j.tree.2011.07.012> PMID: 21890230
12. Li Z, Wu N, Liu T, Tang M, Chen H. Gender-related responses of dioecious plant *Populus cathayana* to AMF, drought and planting pattern. *Scientific Reports*. 2020; 10(1). <https://doi.org/10.1038/s41598-020-68112-0> PMID: 32661316
13. Wang J, Zhang B, Guo H, Chen L, Han F, Yan C, et al. Transcriptome Analysis Reveals Key Genes and Pathways Associated with the Regulation of Flowering Time in Cabbage (*Brassica oleracea* L. var. capitata). *Plants*. 2023; 12(19). <https://doi.org/10.3390/plants12193413> PMID: 37836153
14. Zhang X, Wang G, Zhang S, Chen S, Wang Y, Wen P, et al. Genomes of the Banyan Tree and Pollinator Wasp Provide Insights into Fig-Wasp Coevolution. *Cell*. 2020; 183(4):875–89.e17. <https://doi.org/10.1016/j.cell.2020.09.043> PMID: 33035453
15. Robertson G, Schein J, Chiu R, Corbett R, Field M, Jackman SD, et al. De novo assembly and analysis of RNA-seq data. *Nature Methods*. 2010; 7(11):909–12. <https://doi.org/10.1038/nmeth.1517> PMID: 20935650
16. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011; 12(1):323. <https://doi.org/10.1186/1471-2105-12-323> PMID: 21816040
17. Leng N, Dawson JA, Thomson JA, Ruotti V, Rissman AI, Smits BMG, et al. EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics*. 2013; 29(8):1035–43. <https://doi.org/10.1093/bioinformatics/btt087> PMID: 23428641
18. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*. 2010; 28(5):511–5. <https://doi.org/10.1038/nbt.1621> PMID: 20436464
19. Jiao P, Chaoyang L, Wenhan Z, Jingyi D, Yunlin Z, Zhenggang X. Integrative Metabolome and Transcriptome Analysis of Flavonoid Biosynthesis Genes in *Broussonetia papyrifera* Leaves From the Perspective of Sex Differentiation. *Frontiers in plant science*. 2022; 13. <https://doi.org/10.3389/fpls.2022.900030> PMID: 35668799
20. Yang C, Xia L, Fu M, Chen Y, Kong X, Zhang S. DNA methylation-mediated phenylpropane and starch metabolism causes male poplars to be more tolerant to nitrogen deficiency than females. *Plant Physiology and Biochemistry*. 2023; 195:144–54. <https://doi.org/10.1016/j.plaphy.2023.01.004> PMID: 36638604
21. Guo Q, Liu L, Liu J, Korpelainen H, Li C. Plant sex affects plant-microbiome assemblies of dioecious *Populus cathayana* trees under different soil nitrogen conditions. *Microbiome*. 2022; 10(1). <https://doi.org/10.1186/s40168-022-01387-9> PMID: 36333709
22. Shin S, Lee H, Lee J-W, Kang H. Sex-related demographics in two remnant populations of a dioecious tree *Ilex cornuta* (Aquifoliaceae): implications for conservation. *Journal of Ecology and Environment*. 2019; 43(1). <https://doi.org/10.1186/s41610-019-0131-6>
23. Karami O, Rahimi A. The end of flowering: interactions between cytokinin and regulatory genes. *Trends in Plant Science*. 2022; 27(9):840–2. <https://doi.org/10.1016/j.tplants.2022.05.011> PMID: 35701292

24. Zhao N, Liu C, Meng Y, Hu Z, Zhang M, Yang J. Identification of Flowering Regulatory Genes in Allopolyploid *Brassica juncea*. *Horticultural Plant Journal*. 2019; 5(3):109–19. <https://doi.org/10.1016/j.hpj.2019.04.003>.
25. Reig-Valiente JL, Borredá C, Talón M, Domingo C. The G123 rice mutant, carrying a mutation in SE13, presents alterations in the expression patterns of photosynthetic and major flowering regulatory genes. *PLoS one*. 2020; 15(5):e0233120. <https://doi.org/10.1371/journal.pone.0233120> PMID: 32421736
26. Qi H-D, Lin Y, Ren Q-P, Wang Y-Y, Xiong F, Wang X-L. RNA Splicing of FLC Modulates the Transition to Flowering. *Frontiers in plant science*. 2019; 10. <https://doi.org/10.3389/fpls.2019.01625> PMID: 31921267
27. Fornara F, de Montaigu A, Coupland G. SnapShot: Control of Flowering in Arabidopsis. *Cell*. 2010; 141(3):550–e2. <https://doi.org/10.1016/j.cell.2010.04.024> PMID: 20434991
28. Zhu Z, Qi F, Yan C, Zhan Y. Sexually different morphological, physiological and molecular responses of *Fraxinus mandshurica* flowers to floral development and chilling stress. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*. 2016; 99:97–107. <https://doi.org/10.1016/j.plaphy.2015.12.006> PMID: 26744995
29. Han Y, Hu M, Ma X, Yan G, Wang C, Jiang S, et al. Exploring key developmental phases and phase-specific genes across the entirety of anther development in maize. *Journal of Integrative Plant Biology*. 2022; 64(7):1394–410. <https://doi.org/10.1111/jipb.13276> PMID: 35607822
30. Kashima M, Deguchi A, Tezuka A, Nagano AJ. Low-cost and Multiplexable Whole mRNA-Seq Library Preparation Method with Oligo-dT Magnetic Beads for Illumina Sequencing Platforms. *Bio-protocol*. 2020; 10(12):e3496. <https://doi.org/10.21769/BioProtoc.3496> PMID: 33659317
31. Tvedte ES, Michalski J, Cheng S, Patkus RS, Tallon LJ, Sadzewicz L, et al. Evaluation of a high-throughput, cost-effective Illumina library preparation kit. *Scientific Reports*. 2021; 11(1):15925. <https://doi.org/10.1038/s41598-021-94911-0> PMID: 34354114
32. Li Y-F, Zhao M, Wang M, Guo J, Wang L, Ji J, et al. An improved method of constructing degradome library suitable for sequencing using Illumina platform. *Plant Methods*. 2019; 15(1):134. <https://doi.org/10.1186/s13007-019-0524-7> PMID: 31832076
33. Takase S, Kera K, Hirao Y, Hosouchi T, Kotake Y, Nagashima Y, et al. Identification of triterpene biosynthetic genes from *Momordica charantia* using RNA-seq analysis. *Bioscience, Biotechnology, and Biochemistry*. 2019; 83(2):251–61. <https://doi.org/10.1080/09168451.2018.1530096> PMID: 30317922
34. Du J, Li M, Yuan Z, Guo M, Song J, Xie X, et al. A decision analysis model for KEGG pathway analysis. *BMC Bioinformatics*. 2016; 17(1):407. <https://doi.org/10.1186/s12859-016-1285-1> PMID: 27716040
35. Lim J, Shin SG, Lee S, Hwang S. Design and use of group-specific primers and probes for real-time quantitative PCR. *Frontiers of Environmental Science & Engineering in China*. 2011; 5(1):28–39. <https://doi.org/10.1007/s11783-011-0302-x>
36. Li K, Tian H, Tahir MM, Li S, Chen S, Fan L, et al. Transcriptome analysis reveals that cytokinins inhibit adventitious root formation through the MdRR12-MdCRF8 module in apple rootstock. *Plant Science*. 2022; 318. <https://doi.org/10.1016/j.plantsci.2022.111220> PMID: 35351311
37. Tian Y, Rao S, Li Q, Xu M, Wang A, Zhang H, et al. The coloring mechanism of a novel golden variety in *Populus deltoides* based on the RGB color mode. *Forestry Research*. 2021; 1(1):1–13. <https://doi.org/10.48130/fr-2021-0005>
38. Wang X, Wang Y, Wang R, Gong L, Wang L, Xu J. Genome-Wide Analysis of Alternative Splicing Events Responding to High Temperatures in *Populus tomentosa* Carr. *Forests*. 2023; 14(9). <https://doi.org/10.3390/f14091878>
39. Martins MJF, Mota CF, Pearson GA. Sex-biased gene expression in the brown alga *Fucus vesiculosus*. *BMC genomics*. 2013; 14(1):294. <https://doi.org/10.1186/1471-2164-14-294> PMID: 23634783
40. He L, Yan J, Lu H, Su W, Gao S, Wang Y, et al. Phenotypic Differences of Leaves and Transcriptome Analysis of *Fraxinus mandshurica* × *Fraxinus sogdiana* F1 Variety. *Forests*. 2023; 14(8). <https://doi.org/10.3390/f14081554>
41. Delph LF, Arntz AM, Scotti-Saintagne C, Scotti I. The Genomic Architecture of Sexual Dimorphism in the Dioecious Plant *Silene latifolia*. *Evolution*. 2010;no-no. <https://doi.org/10.1111/j.1558-5646.2010.01048.x> PMID: 20550575
42. Jaillais Y, Chory J. Unraveling the paradoxes of plant hormone signaling integration. *Nature Structural & Molecular Biology*. 2010; 17(6):642–5. <https://doi.org/10.1038/nsmb0610-642> PMID: 20520656
43. Calderón Villalobos LIA, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, et al. A combinatorial TIR1/AFB–Aux/IAA co-receptor system for differential sensing of auxin. *Nature Chemical Biology*. 2012; 8(5):477–85. <https://doi.org/10.1038/nchembio.926> PMID: 22466420
44. Dennis Thomas T. In Vitro Modification of Sex Expression in Mulberry (*Morus Alba*) by Ethrel and Silver Nitrate. *Plant Cell, Tissue and Organ Culture*. 2004; 77(3):277–81. <https://doi.org/10.1023/B:TICU.0000018390.65934.93>

45. Jaiswal VS, Kumar A, Lal M. Role of endogenous phytohormones and some macromolecules in regulation of sex differentiation in flowering plants. *Proceedings / Indian Academy of Sciences*. 1985; 95(6):453–9. <https://doi.org/10.1007/BF03053684>
46. Heslop-Harrison J. Auxin and Sexuality in *Cannabis sativa*. *Physiologia Plantarum*. 2006; 9:588–97. <https://doi.org/10.1111/j.1399-3054.1956.tb07821.x>
47. Orozco-Arroyo G, Vázquez-Santana S, Camacho A, Dubrovsky JG, Cruz-García F. Inception of male-ness: auxin contribution to flower masculinization in the dioecious cactus *Opuntia stenopetala*. *Planta*. 2012; 236(1):225–38. <https://doi.org/10.1007/s00425-012-1602-5> PMID: 22328126
48. Tan M, Xue J, Wang L, Huang J, Fu C, Yan X. Transcriptomic Analysis for Different Sex Types of *Ricinus communis* L. during Development from Apical Buds to Inflorescences by Digital Gene Expression Profiling. *Frontiers in Plant Science*. 2016; 6(1208). <https://doi.org/10.3389/fpls.2015.01208> PMID: 26904031
49. Rudich J, Halevy AH, Kedar N. Ethylene Evolution from Cucumber Plants as Related to Sex Expression 1. *Plant Physiology*. 1972; 49(6):998–9. <https://doi.org/10.1104/pp.49.6.998> PMID: 16658100
50. Yamasaki S, Fujii N, Matsuura S, Mizusawa H, Takahashi H. The M Locus and Ethylene-Controlled Sex Determination in Andromonoecious Cucumber Plants. *Plant and Cell Physiology*. 2001; 42(6):608–19. <https://doi.org/10.1093/pcp/pce076> PMID: 11427680
51. Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, et al. A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. *Science*. 2008; 321(5890):836–8. <https://doi.org/10.1126/science.1159023> PMID: 18687965.
52. Tanurdzic M. Sex-Determining Mechanisms in Land Plants. *The Plant Cell Online*. 2004; 16(suppl_1): S61–S71. <https://doi.org/10.1105/tpc.016667> PMID: 15084718
53. Li XG, Su YH, Zhao XY, Li W, Gao XQ, Zhang XS. Cytokinin overproduction-caused alteration of flower development is partially mediated by CUC2 and CUC3 in *Arabidopsis*. *Gene*. 2010; 450(1–2):109–20. <https://doi.org/10.1016/j.gene.2009.11.003> PMID: 19913077
54. Irish EE, Nelson T. Sex Determination in Monoecious and Dioecious Plants. *The Plant Cell*. 1989; 1(8):737–44. <https://doi.org/10.1105/tpc.1.8.737> PMID: 12359907
55. Dellaporta SL, Calderon-Urrea A. Sex determination in flowering plants. *The Plant Cell*. 1993; 5(10):1241–51. <https://doi.org/10.1105/tpc.5.10.1241> PMID: 8281039
56. Sladký Z. The role of endogenous growth regulators in the differentiation processes of walnut (*Juglans regia* L.). *Biologia Plantarum*. 1972; 14(4):273–8. <https://doi.org/10.1007/BF02935852>
57. Randriamanana TR, Nybakken L, Lavola A, Aphalo PJ, Nissinen K, Julkunen-Tiitto R. Sex-related differences in growth and carbon allocation to defence in *Populus tremula* as explained by current plant defence theories. *Tree Physiology*. 2014; 34(5):471–87. <https://doi.org/10.1093/treephys/tpu034> PMID: 24852570
58. Stapleton AE, Walbot V. Flavonoids Can Protect Maize DNA from the Induction of Ultraviolet Radiation Damage. *Plant Physiology*. 1994; 105(3):881–9. <https://doi.org/10.1104/pp.105.3.881> PMID: 8058838
59. Ormrod DP, Landry LG, Conklin PL. Short-term UV-B radiation and ozone exposure effects on aromatic secondary metabolite accumulation and shoot growth of flavonoid-deficient *Arabidopsis* mutants. *Physiologia Plantarum*. 1995; 93(4):602–10. <https://doi.org/10.1111/j.1399-3054.1995.tb05106.x>
60. Mo Y, Nagel C, Taylor LP. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proceedings of the National Academy of Sciences*. 1992; 89(15):7213–7. <https://doi.org/10.1073/pnas.89.15.7213> PMID: 11607312
61. Liu W, Feng Y, Yu S, Fan Z, Li X, Li J, et al. The Flavonoid Biosynthesis Network in Plants. *International Journal of Molecular Sciences*. 2021; 22(23):12824. <https://doi.org/10.3390/ijms222312824> PMID: 34884627
62. Fortunato S, Nigro D, Lasorella C, Marcotuli I, Gadaleta A, de Pinto MC. The Role of Glutamine Synthetase (GS) and Glutamate Synthase (GOGAT) in the Improvement of Nitrogen Use Efficiency in Cereals. *Biomolecules*. 2023; 13(12):1771. <https://doi.org/10.3390/biom13121771> PMID: 38136642
63. Xiang C, Qu L-j, Gao Y-m, Shi Y-y. Flower Development and Photoperiodic Control of Flowering in Rice. *Rice Science*. 2013;20(2):79–87. doi: 10.1016/s1672-6308(13)60112-2.
64. Luo X, Yin M, He Y. Molecular Genetic Understanding of Photoperiodic Regulation of Flowering Time in *Arabidopsis* and Soybean. *International Journal of Molecular Sciences*. 2021; 23(1). <https://doi.org/10.3390/ijms23010466> PMID: 35008892
65. Valverde F. CONSTANS and the evolutionary origin of photoperiodic timing of flowering. *Journal of Experimental Botany*. 2011; 62(8):2453–63. <https://doi.org/10.1093/jxb/erq449> PMID: 21239381
66. Song Y, Ma K, Ci D, Chen Q, Tian J, Zhang D. Sexual dimorphic floral development in dioecious plants revealed by transcriptome, phytohormone, and DNA methylation analysis in *Populus tomentosa*. *Plant Molecular Biology*. 2013; 83(6):559–76. <https://doi.org/10.1007/s11103-013-0108-2> PMID: 23860796