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Genetic and Molecular Mechanisms of Sex Differentiation in *Eucommia ulmoides*

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Abstract *Eucommia ulmoides*, a dioecious tree species endemic to China, holds significant medicinal and economic value. This study delves into the genetic and molecular mechanisms underlying sex differentiation in *E. ulmoides*. Utilizing advanced genomic technologies, a high-quality chromosome-level genome assembly for both male and female *E. ulmoides* was achieved, revealing key insights into sex differentiation. Comparative transcriptome analyses identified differentially expressed genes (DEGs) between male and female individuals, highlighting the role of MADS-box transcription factors and specific genes such as *EuAP3* and *EuAG* in sex determination. Additionally, molecular markers linked to sex determination were developed, facilitating early sex identification and improving breeding efficiency. The findings from this study provide a valuable genomic resource for further molecular biological studies and practical applications in the breeding and commercial production of *E. ulmoides*.

Keywords *Eucommia ulmoides*; Sex differentiation; MADS-box transcription factors; Genomic assembly; Molecular markers

1 Introduction

Eucommia ulmoides, also known as the hardy rubber tree, is a dioecious perennial woody plant native to China and widely distributed in subtropical and temperate regions. It is the only species in the Eucommiaceae family and is renowned for its medicinal and industrial value, particularly in rubber production and traditional Chinese medicine. However, the male and female flowers of *Eucommia ulmoides* grow on separate male and female trees, and its long growth cycle complicates early gender identification and breeding efforts (Du et al., 2023; Wang et al., 2023). Since it is difficult to distinguish the sex of the plant during the seedling stage based on external morphology, in-depth research into the mechanisms of sex differentiation in *Eucommia ulmoides* is not only important for improving breeding efficiency and utilizing the plant's dioecious characteristics but also provides a reliable scientific basis for large-scale commercial production.

Understanding the mechanisms of sex differentiation in *E. ulmoides* is crucial for several reasons. The dioecious nature of the plant poses challenges for breeding and economic cropping, as sex cannot be determined through morphological observation at early developmental stages. Elucidating the genetic and molecular pathways involved in sex differentiation can facilitate the development of molecular markers for early sex identification, thereby improving breeding efficiency and commercial production. Moreover, insights into sex differentiation mechanisms can contribute to the broader understanding of plant reproductive biology and may have applications in other dioecious species (Zhang et al., 2023).

Recent advancements in genomic technologies have provided significant insights into the genetic and molecular mechanisms underlying sex differentiation in plants. In *E. ulmoides*, high-quality chromosome-level genome assemblies for both male and female individuals have been generated, revealing key genes and pathways involved in sex differentiation (Du et al., 2023). For instance, transcriptome analysis has identified *EuAP3* and *EuAG* as potential regulators of sex differentiation in this species. Genome-wide identification and expression analysis of MADS-box transcription factors have highlighted their critical role in sex determination, with specific genes showing male- or female-biased expression patterns. Furthermore, double-digest restriction site-associated DNA sequencing (ddRAD-seq) has been employed to identify sex-linked molecular markers, such as the male-specific locus MSL4, which can reliably distinguish male from female seedlings (Lan et al., 2019; Wang et al., 2023). These findings collectively enhance our understanding of the genetic and molecular basis of sex differentiation in *E. ulmoides* and provide valuable tools for its breeding and cultivation.

This study aims to deeply analyze the mechanisms of sex differentiation in *Eucommia ulmoides*, identifying key sex-determining genes and molecular pathways. Through these investigations, the study seeks to provide new strategies for the breeding and commercial production of *Eucommia*, improving breeding efficiency and reducing production costs. Additionally, it aims to offer insights for the study of sex differentiation in other dioecious plants, further advancing the scientific understanding of plant sex determination mechanisms.

2 Botanical and Biological Characteristics of *Eucommia ulmoides*

2.1 General morphology and reproductive system

Eucommia ulmoides, commonly known as the hardy rubber tree, is a dioecious plant species endemic to China, meaning it has distinct male and female individuals. This species is highly valued for its medicinal and economic benefits, particularly due to its production of α-linolenic acid and gutta-percha, a rubber-like substance (Wang et al., 2020; Du et al., 2023). Ecologically, *E. ulmoides* plays a crucial role in its native habitats, contributing to biodiversity and ecosystem stability. The species is well-adapted to various environmental conditions, which makes it a resilient component of the local flora (Xu et al., 2004).

Morphologically, *E. ulmoides* is characterized by its unisexual flowers, which are produced on separate male and female trees from the earliest stages of stamen and pistil primordium formation. The male flowers typically contain stamens, while the female flowers contain pistils, and this differentiation occurs very early in the plant's development. This early differentiation is crucial for the plant's reproductive strategy and has significant implications for breeding and cultivation practices (He et al., 2014; Wang and Zhang, 2017).

2.2 The reproductive system of *Eucommia ulmoides*

The reproductive system of *E. ulmoides* is complex and involves various genetic and molecular mechanisms. The flower, as the unique reproductive organ of seed plants, plays a critical role in seed plant breeding research. A complete flower consists of five whorls: sepals, petals, stamens, carpels, and ovules. However, in *Eucommia ulmoides*, the flower contains only stamens or carpels. Therefore, understanding the genes involved in the development of stamens and carpels in *Eucommia* may help uncover the genetic and molecular mechanisms behind sex differentiation in this species. Flower organ development is an extremely complex process. Currently, the most widely applied model for flower development is the ABCDE model or AE model, which is based on studies of model plants such as *Arabidopsis*, snapdragon (*Antirrhinum majus*), and petunia (*Petunia hybrida* (Hook.) E. Vilm.). In this model, A-class genes primarily regulate the development of the sepals and petals. In Arabidopsis, the representative A-class gene is *APETALA1* (AP1). B-class genes regulate the development of petals and stamens and, in some species, also affect sepal development (e.g., in tulips). In Arabidopsis, the representative B-class genes are *APETALA3* (AP3) and *PISTILLATA* (PI). C-class genes control the development of stamens, carpels, and ovules, although in some plants, they regulate only the stamens and carpels. In Arabidopsis, the representative C-class gene is *AGAMOUS* (AG).

D-class genes control the development of ovules, and E-class genes inhibit the expression of A-class genes in petals and stamens. E-class genes are also involved in the formation of floral organs in each whorl and work together with A, B, and C genes to form the “quartet model” complex. In the AE model, these five classes of genes do not act independently to regulate flower organ development but instead function cooperatively across the five whorls of floral structures. Mutations in any of these gene classes can lead to abnormal floral morphology.

Recent studies have identified several sex-biased genes and transcription factors that play critical roles in sex determination. For instance, MADS-box transcription factors have been shown to be involved in the regulation of floral organ identity and sex differentiation. Specific genes such as *EuAP3* and *EuAG* have been implicated in the regulation of sex differentiation, with *EuAP3* being a male-biased gene associated with the development of male floral organs (Hussain et al., 2016; Wang et al., 2020).

The use of advanced genomic technologies has provided deeper insights into the genetic basis of sex differentiation in *E. ulmoides*. High-quality genome assemblies for both male and female individuals have been developed, revealing significant differences in gene expression between the sexes. For example, the female genome assembly is approximately 1.01 Gb with 31 665 protein-coding genes, while the male genome assembly is larger at 1.24 Gb with an increased number of predicted genes (Du et al., 2023). These genomic resources are invaluable for understanding the molecular mechanisms underlying sex differentiation and for facilitating the breeding of superior varieties.

2.3 Sexual differentiation of *Eucommia ulmoides*

Sex differentiation in *Eucommia ulmoides* is a complex process regulated by various genetic and molecular mechanisms. The species is strictly dioecious, meaning that individual trees are either male or female, and this sexual dimorphism is evident from the earliest stages of flower development. Recent genomic studies have provided significant insights into the sex differentiation mechanisms in *E. ulmoides*. For instance, the high-quality chromosome-level genome assemblies for both male and female trees have revealed key genes involved in sex differentiation, such as *EuAP3* and *EuAG* (Goldberg et al., 2017; Du et al., 2023). Comparative transcriptome analyses have identified differentially expressed genes (DEGs) between male and female individuals, including sex-biased genes like the MADS-box gene *APETALA3*, which is likely involved in sex determination (Nakazawa et al., 2009; Zhang et al., 2023).

3 Genetic Basis of Sex Differentiation in *Eucommia ulmoides*

3.1 Overview of sex chromosomes and sex-determining regions

Eucommia ulmoides is a dioecious plant, meaning it has distinct male and female individuals. The sex chromosomes and sex-determining regions in *E. ulmoides* have been a focal point of recent genomic studies. The high-quality chromosome-level genome assemblies for both male and female *E. ulmoides* have provided significant insights into the genetic basis of sex differentiation (Hu et al., 2023). The female genome assembly is 1.01 Gb with 17 pseudochromosomes, while the reassembled male genome is 1.24 Gb, indicating notable differences in genome size and structure between the sexes (Takeno et al., 2008; Du et al., 2023). Additionally, the identification of sex-specific markers, such as a 350 bp male-specific AFLP marker and a 569 bp pistillate-specific RAPD marker, has facilitated early sex identification, which is crucial for breeding programs (Wang et al., 2018).

3.2 Key genes involved in sex differentiation

Several key genes have been implicated in the sex differentiation of *E. ulmoides*. Transcriptome analyses have identified differentially expressed genes (DEGs) between male and female plants, including the MADS-box gene *APETALA3*, which is male-biased and likely involved in sex determination (Figure 1) (Zhang et al., 2023). Other studies have highlighted the role of MADS-box transcription factors, with specific genes such as *EuMADS39* and *EuMADS65* showing male-biased expression, suggesting their critical role in the regulation of sex differentiation (Miljković et al., 2019; Jin et al., 2020). Furthermore, genes like *EuAP3* and *EuAG* have been identified as potential regulators of sex differentiation based on their expression patterns.

3.3 Comparative genomics with other dioecious plants

Comparative genomics has revealed that *E. ulmoides* shares several genomic features with other dioecious plants. For instance, the presence of sex-biased gene expression and the involvement of MADS-box genes in sex determination are common themes across dioecious species (Jin et al., 2020). The study of chromatin landscapes in other dioecious systems, such as the UV sex determination system in brown algae, has provided insights into the epigenomic changes associated with sex differentiation, which may also be relevant to *E. ulmoides*. These comparative studies help in understanding the evolutionary conservation and divergence of sex-determining mechanisms in dioecious plants.

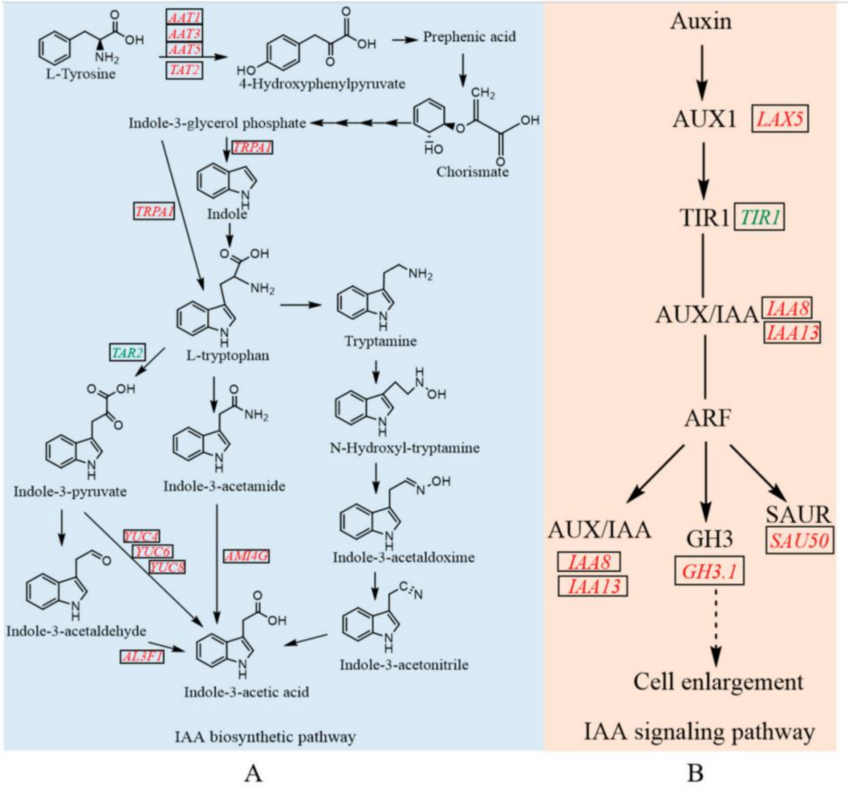

Figure 1 DEGs were involved in IAA biosynthesis pathway and signaling pathway in CK vs. T1 on day 4 (Adopted from Zhang et al., 2023)

Image caption: A: DEGs participated in IAA biosynthesis and metabolism pathway; B: DEGs participated in IAA signaling pathway; The different font color represents the genes that are regulated in CK vs. T1 (red indicated upregulation; green indicated downregulation) (Adopted from Zhang et al., 2023)

3.4 Recent advances in genomic studies of *Eucommia ulmoides*

Recent advances in genomic technologies have significantly enhanced our understanding of the genetic and molecular mechanisms underlying sex differentiation in *E. ulmoides*. The use of PacBio and Hi-C technologies has led to the development of high-quality genome assemblies, which have been instrumental in identifying sex-specific regions and genes. Additionally, the application of *de novo* transcriptome sequencing has provided a comprehensive view of the sex-biased gene expression landscape in *E. ulmoides* (Li et al., 2020). These genomic resources are invaluable for further studies on sex differentiation and for the genetic improvement of *E. ulmoides* through breeding and biotechnological approaches.

4 Molecular Pathways and Mechanisms

4.1 Hormonal regulation of sex differentiation

Hormonal regulation plays a crucial role in the sex differentiation of *Eucommia ulmoides*. The involvement of phytohormones in the differentiation process is evident from the presence of phytohormone-response cis-elements in the MADS-box transcription factors, which are key regulators of floral organ development. These transcription factors exhibit differential expression patterns between male and female flowers, suggesting that hormonal signals may influence the expression of these genes, thereby contributing to sex differentiation (Zhang et al., 2023). Additionally, the high expression of certain genes, such as the ω-3 fatty acid desaturase coding gene *EU0103017*, has been linked to specific metabolic pathways that may be hormonally regulated, further underscoring the importance of hormonal control in sex differentiation (Du et al., 2023).

Cucumber sex differentiation is primarily determined by the F, M, and A genes. Both the F (*CsACS1G*) and M (*CsACS2*) genes encode two ACC synthases (key enzymes in the ethylene biosynthesis pathway) that regulate the expression of female sex traits in cucumbers. The F gene promotes the development of female flowers, while the

M gene inhibits the development of stamens in flower buds. In contrast, the A gene suppresses female flower development and promotes the formation of male flowers. The interaction among the F, M, and A genes ultimately determines various sex phenotypes in cucumbers. However, gibberellins (GA) may promote masculinization in cucumbers via an ethylene-dependent pathway by altering the expression of the M (*CsACS2*) gene, ethylene receptor CsETR1, and ethylene response transcription factors. GA may also suppress female expression through an ethylene-independent pathway by regulating the expression of the class C floral homeotic gene *CAG2*. The fact that plant hormones like gibberellins (GAs) and ethylene promote the development of male and female flowers, respectively, highlights the significant role hormones play in regulating sex differentiation (Pelaz et al., 2000; Zhang et al., 2017).

4.2 Epigenetic modifications and their role in sex determination

The essence of epigenetic regulation is to regulate the spatiotemporal activity of genes through structural changes in chromatin. Typical epigenetic events include DNA methylation, histone covalent modification, non-coding RNA (ncRNAs) silencing, and chromatin remodeling (mammalian epigenetics) (Tachibana, 2015). Recent studies have shown that epigenetic regulation plays an indispensable role in the sexual expression of many species. For example, in non-mammalian vertebrates, the gene *cyp19a1*, which encodes an important steroidogenic enzyme that converts androgens into estrogens, plays a vital role in the temperature-induced masculinization process. Exposure to high temperatures during the critical period of sexual determination in European sea bass leads to increased DNA methylation levels in the promoter region of the *cyp19a1* gene, thereby inhibiting its expression and ultimately leading to masculinization. In the model plant melon of the Cucurbitaceae family, the gene *CmACS-7*, which encodes an ethylene biosynthetic enzyme, interacts with the transcription factor CmWIP1 to control the development of male and female flowers and hermaphrodites (Pawełkowicz et al., 2019).

The transposon Gyno-hAT was inserted into the CmWIP1 promoter region, triggering the spread of DNA methylation to the CmWIP1 promoter, resulting in the transformation of male melon plants into female plants. In the gonadal somatic cells of wild-type XY mice, the enrichment of H3K9 demethylases induced a significant increase in the H3K9 dimethylation level and a decrease in the H3K4 trimethylation level at the Sry gene locus, resulting in the inhibition of Sry gene expression, thereby hindering the formation of testes and causing the mice to reverse from male to female sex (Liu et al., 2019). Epigenetic modifications, including DNA methylation and histone modifications, may play an important role in the sex determination of *Eucommia ulmoides*. Although the specific epigenetic mechanisms were not described in detail in the current study, the complex protein-motif composition and exon-intron structure of MADS-box genes suggest that epigenetic regulation may be a key factor. These modifications can affect gene expression patterns without changing the DNA sequence, leading to the sexual dimorphism observed in *Eucommia ulmoides* (Zhang et al., 2023). Future studies targeting the epigenetic landscape could shed more light on how these modifications regulate sex-specific gene expression.

4.3 Transcriptomic and proteomic analyses of male and female differentiation

Transcriptomic analyses have revealed significant differences in gene expression between male and female *E. ulmoides*. Comparative transcriptome analyses identified 116 differentially expressed genes (DEGs) between males and females, with 73 male-biased and 43 female-biased genes. Notably, the male-biased DEG *APETALA3*, a B class floral organ identity gene, has been implicated in sex determination. Proteomic analyses, although not explicitly detailed, would complement these findings by identifying sex-specific proteins and their post-translational modifications, providing a more comprehensive understanding of the molecular mechanisms underlying sex differentiation (Wang and Zhang, 2017). The integration of transcriptomic and proteomic data is essential for elucidating the complex regulatory networks involved in this process.

4.4 Role of microRNAs and other non-coding RNAs in sex differentiation

MicroRNAs (miRNAs) and other non-coding RNAs (ncRNAs) are emerging as important regulators of gene expression in sex differentiation. These small RNA molecules can modulate the expression of target genes by binding to their mRNA transcripts, leading to degradation or inhibition of translation. Although specific miRNAs and ncRNAs involved in the sex differentiation of *E. ulmoides* have not been identified in the current studies, their potential role is supported by the differential expression of key regulatory genes such as MADS-box transcription factors. Future research focusing on the identification and functional characterization of miRNAs and ncRNAs will be crucial for understanding their contribution to the regulation of sex-specific gene expression in *E. ulmoides* (Devlin and Nagahama, 2002).

5 Environmental Influences on Sex Differentiation

5.1 Impact of environmental factors on sex expression

Environmental factors such as temperature, light, and soil nutrients play an important role in the sex expression of *Eucommia ulmoides*. Studies on a variety of species have shown that temperature can affect the expression levels of sex determination-related genes, leading to changes in the sex ratio within a population (Schenkel et al., 2022). For example, in fish and scallops, temperature has been found to affect the fate of somatic and germ cells in the primitive gonads, indicating that environmental conditions can alter genetic sex determination pathways (Devlin and Nagahama, 2002; Edelaar et al., 2017). Papaya is a trisexual plant with three sex forms: male, female, and hermaphrodite. Although the sex of papaya is mainly determined by genes, the expression of its phenotypic sex can also be affected by environmental factors. Low temperature induces changes in the methylation level of histone H3-K9 in male flowers of papaya, prompting the reprogramming of plant hormone signal transduction pathways in hermaphrodite flowers and changing the expression levels of class I KNOX gene family, floral organ identity genes, and polarity-related genes, resulting in the conversion of male papaya plants to hermaphrodite plants (low temperature-induced sex reversal) (Lin et al., 2016). Similarly, in *Eucommia ulmoides*, phenotypic traits such as leaf, fruit, and seed size are associated with geographical and climatic factors, indicating that environmental conditions such as annual average temperature and rainfall significantly affect phenotypic changes and may affect sexual differentiation (Wang et al., 2023).

5.2 Interaction between genetic and environmental factors in sex differentiation

The interaction between genetic and environmental factors is crucial in the sex differentiation process of *Eucommia ulmoides*. Mixed systems that incorporate both genetic and environmental components are more common than previously thought, and environmental effects on gene expression can lead to evolutionary divergence of sex determination mechanisms within species (Schenkel et al., 2022). In *Eucommia ulmoides*, genetic studies have identified key genes such as *EuAP3* and *EuAG* that are involved in regulating sex differentiation (Du et al., 2023). These genetic components may interact with environmental factors, such as temperature and light, to influence the expression of sex-biased genes, thereby affecting the overall sex differentiation process. This interaction is also observed in other species, where environmental factors like temperature and social interactions can activate or suppress various male and female pathways depending on the genetic background.

5.3 Case studies and experimental findings

Several case studies and experimental findings highlight the impact of environmental factors on sex differentiation in *Eucommia ulmoides* and other species. For example, a study on the housefly demonstrated that temperature-dependent expression levels of specific genes could predict the occurrence of different sex determination mechanisms along environmental gradients (Figure 2) (Schenkel et al., 2022). In *Eucommia ulmoides*, comparative transcriptome analyses have revealed differentially expressed genes between male and female individuals, indicating that both genetic and environmental factors contribute to sex differentiation. Additionally, genome-wide analyses of MADS-box transcription factors in *Eucommia ulmoides* have shown that certain genes exhibit sex-biased expression, which may be influenced by environmental conditions. These findings underscore the complex interplay between genetic and environmental factors in determining sex differentiation in *Eucommia ulmoides* and other species.

6 Biotechnological Approaches to Study and Manipulate Sex Differentiation

6.1 CRISPR/Cas9 and gene editing in sex determination studies

The CRISPR/Cas9 system has revolutionized the field of genome editing, providing a powerful tool for precise manipulation of specific genomic elements. This technology employs a Cas9 nuclease guided by a customizable

RNA sequence to introduce double-strand breaks at target sites, which are then repaired by cellular mechanisms, leading to desired genetic modifications (Sander and Joung 2014; Zhang et al., 2014). In the context of sex determination studies, CRISPR/Cas9 can be utilized to knock out or modify genes implicated in sex differentiation pathways. For instance, in *Eucommia ulmoides*, key genes such as *EuAP3* and *EuAG* have been identified as regulators of sex differentiation, and CRISPR/Cas9 could be employed to elucidate their specific roles by creating targeted mutations. Despite its potential, the application of CRISPR/Cas9 in sex determination studies must address challenges such as off-target effects and mosaicism, which can complicate the interpretation of results (Liang et al., 2016).

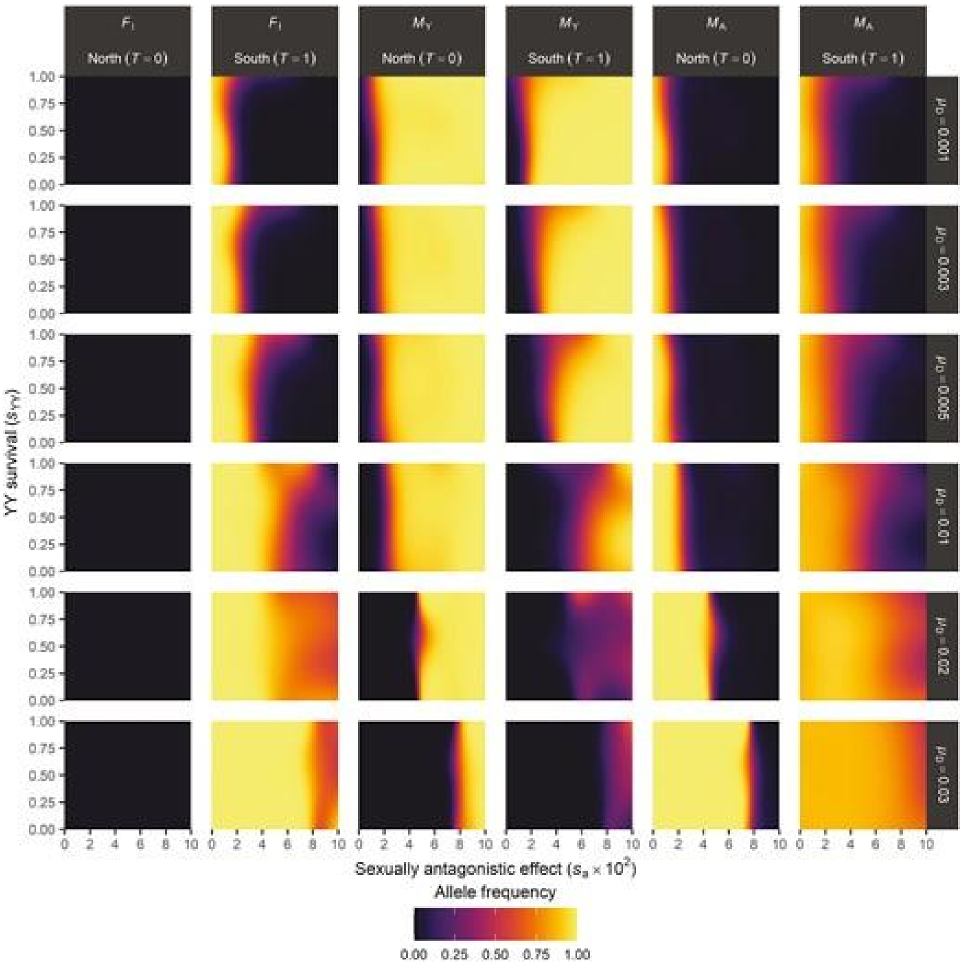

Figure 2 Y-chromosomal fitness effects alter the scope for SD transitions (Adopted from Schenkel et al., 2022)

Image caption: Shown are the predicted equilibrium frequencies of F_I in females (maternally inherited alleles), M_Y and M_A in males (paternally inherited alleles); we restrict our analysis to the maternal (F_I) c.q. paternal (M_Y, M_A) alleles to account for their (potential) sex-specific transmission. Horizontal labels indicate locus and temperature, vertical labels the M_A activation rate μD. Further parameter values used in simulations: $\theta F=1.2$; $\theta M=0.3$; $\beta=0.5$. Predicted allele frequencies were smoothed with binomial generalized additive models (Adopted from Schenkel et al., 2022)

6.2 Development of sex-specific markers and their application in breeding

The development of sex-specific markers is crucial for the efficient breeding of dioecious plants like *Eucommia ulmoides*. These markers can be used to identify the sex of plants at an early stage, thereby facilitating selective breeding and cultivation practices. Advances in genomic technologies, such as high-throughput sequencing and bioinformatics, have enabled the identification of sex-linked markers. For example, the chromosome-level genome assembly of *E. ulmoides* has provided valuable insights into the genetic basis of sex differentiation, allowing researchers to pinpoint specific markers associated with male and female traits (Belhaj et al., 2015). These markers can be applied in breeding programs to ensure the propagation of desired sex types, thereby optimizing yield and resource allocation.

6.3 Potential for manipulation of sex differentiation for cultivation and yield improvement

Manipulating sex differentiation in plants like *Eucommia ulmoides* holds significant potential for improving cultivation practices and yield. By understanding the genetic and molecular mechanisms underlying sex differentiation, it is possible to develop strategies to control the sex ratio of plant populations. For instance, targeted gene editing using CRISPR/Cas9 can be employed to induce sex reversal or to create monoecious plants that bear both male and female flowers, thereby enhancing reproductive efficiency and fruit production (Belhaj et al., 2015). Additionally, the use of sex-specific markers can aid in the selection of high-yielding female plants, which are often preferred for their higher fruit and seed output. Overall, the ability to manipulate sex differentiation through biotechnological approaches offers promising avenues for the cultivation and yield improvement of economically important dioecious plants like *Eucommia ulmoides* (Heisler et al., 2022).

7 Implications for Conservation and Breeding Programs

7.1 Significance of understanding sex differentiation for conservation strategies

Understanding the genetic and molecular mechanisms of sex differentiation in *Eucommia ulmoides* is crucial for effective conservation strategies. The identification of sex-specific markers, such as the male-specific locus MSL4 and the pistillate-specific SCAR marker SCARmr, allows for early sex identification, which is essential for maintaining genetic diversity and ensuring balanced sex ratios in conservation programs (Figure 3) (Du et al., 2023). Additionally, the high genetic diversity observed within populations, as indicated by microsatellite markers, suggests that conservation efforts should focus on preserving this genetic variability to enhance the resilience and adaptability of *E. ulmoides* populations (Zhang et al., 2017). The development of molecular markers and the understanding of sex-biased gene expression provide valuable tools for monitoring and managing the genetic health of both wild and cultivated populations (Wang et al., 2011).

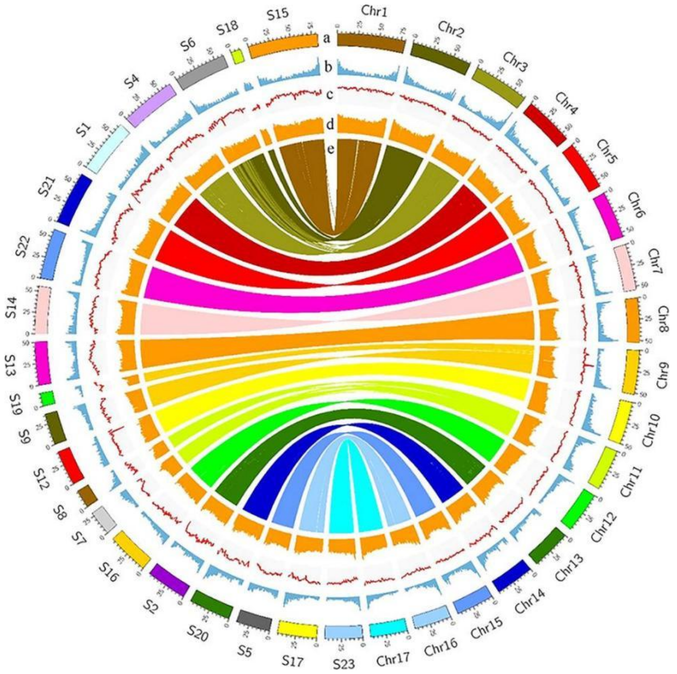

Figure 3 Landscape of Female V1 and Male V2 genome (Adopted from Du et al., 2023)

Image caption: The circle from outside to inside represents; A: Chromosomes of Female V1 and Superscaffolds of Male V2; B: gene density; C: GC content; D: repeat abundance; E: synteny information; All distributions were drawn in a window size of 1 Mb (Adopted from Du et al., 2023)

7.2 Application of genetic knowledge in breeding programs for *Eucommia ulmoides*

The application of genetic knowledge in breeding programs for *Eucommia ulmoides* can significantly enhance the efficiency and effectiveness of these programs. The identification of key genes involved in sex differentiation, such as *EuAP3* and *EuAG*, and the development of sex-linked molecular markers enable breeders to select desired traits at early developmental stages, thus accelerating the breeding process (Zhang et al., 2023). The construction of high-density genetic maps and the identification of quantitative trait loci (QTLs) associated with growth traits provide a solid foundation for marker-assisted selection, allowing for the precise breeding of superior varieties with desirable characteristics such as increased growth rates and higher α-linolenic acid content (Liu et al., 2022) Furthermore, the ability to identify and propagate female plants, which are economically more valuable, can optimize resource allocation and improve the economic viability of *E. ulmoides* cultivation (Xu et al., 2004).

7.3 Future directions for research and development

Future research on *Eucommia ulmoides* should focus on further elucidating the molecular mechanisms underlying sex differentiation and their interaction with environmental factors. Advanced genomic and transcriptomic analyses, such as genome-wide association studies (GWAS) and CRISPR-Cas9 gene editing, could provide deeper insights into the regulatory networks controlling sex determination and differentiation (Piferrer, 2013). Additionally, expanding the genetic studies to include more diverse populations and environmental conditions will help in understanding the adaptive significance of sex differentiation mechanisms. Integrating these findings into breeding programs through the development of more sophisticated molecular markers and breeding strategies will enhance the production of high-quality *E. ulmoides* varieties. Moreover, interdisciplinary approaches combining genetics, ecology, and conservation biology will be essential for developing comprehensive conservation strategies that ensure the long-term sustainability of *E. ulmoides* populations (Zhang et al., 2013).

8 Concluding Remarks

The study of sex differentiation in *Eucommia ulmoides* has yielded significant insights into the genetic and molecular mechanisms underlying this process. Comparative transcriptome analyses have identified 116 differentially expressed genes (DEGs) between male and female individuals, including genes related to floral organ identity and gutta content. High-quality chromosome-level genomes for both male and female *E. ulmoides* have been assembled, revealing key genes such as *EuAP3* and *EuAG* that are involved in sex differentiation. Additionally, genome-wide analyses of MADS-box transcription factors have highlighted their critical role in sex determination, with specific genes showing male- or female-biased expression. Molecular markers such as the male-specific locus MSL4 have been developed for early sex identification, facilitating breeding programs. Furthermore, the construction of a high-density genetic map has provided a solid foundation for future genetic studies and breeding efforts.

Despite these advancements, several challenges remain in the study of sex differentiation in *E. ulmoides*. One major challenge is the complexity of the genetic mechanisms involved, which requires comprehensive and integrative approaches combining genomics, transcriptomics, and proteomics. The long juvenile phase of *E. ulmoides* also poses a challenge for early sex identification and breeding programs. However, the development of molecular markers and high-density genetic maps offers new opportunities for overcoming these challenges. The availability of high-quality genome assemblies provides a valuable resource for further research into the molecular mechanisms of sex differentiation and the identification of additional sex-linked genes. Additionally, the integration of advanced technologies such as Hi-C and PacBio sequencing can enhance the resolution and accuracy of genetic studies.

The findings from these studies have significant implications for botany, genetics, and agriculture. Understanding the genetic and molecular mechanisms of sex differentiation in *E. ulmoides* not only advances our knowledge of plant reproductive biology but also has practical applications in agriculture. The ability to identify the sex of *E. ulmoides* at an early stage can greatly enhance breeding programs and improve the efficiency of commercial production. Moreover, the insights gained from these studies can be applied to other dioecious plant species, contributing to broader efforts in plant breeding and conservation. The integration of genomic, transcriptomic, and proteomic data provides a comprehensive framework for studying complex biological processes, paving the way for future research in plant genetics and molecular biology. Overall, these studies underscore the importance of interdisciplinary approaches in addressing key questions in plant science and highlight the potential for translating basic research into practical applications.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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