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# Molecular Regulatory Networks of Fruit Development and Oil Accumulation in Walnut (*Juglans regia*)

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**Abstract** This study summarizes the main growth characteristics of walnut fruits from the formation of the shell to the gradual enrichment of the kernel, introduces the key metabolic steps of carbon source supply, fatty acid synthesis and triacylglycerol (TAG) assembly in the process of lipid formation, and sorts out the core regulatory factors such as WRI1, LEC1/LEC2 and ABI3. It also summarizes the main roles of structural genes such as ACCase, KAS, and LACS in lipid accumulation, summarizes the participation modes of epigenetic regulation, hormone signaling, and miRNA networks in fruit development and lipid metabolism, discusses how environmental factors such as temperature, moisture, and light affect fatty acid composition and lipid content, and combines existing multi-omics achievements. It demonstrates the overall regulatory model of walnut fruit development and oil accumulation. This research aims to provide theoretical support for the molecular breeding and precise improvement of high-oil and high-quality walnut varieties.

**Keywords** Walnut (*Juglans regia*); Fruit development; Lipid biosynthesis; Gene regulatory networks; Multi-omics analysis

## 1 Introduction

Walnut (*Juglans regia* L.) is a common and important nut tree species worldwide and also one of the main woody oil crops. The oil content of walnut kernels is very high, reaching about 70%, mainly unsaturated fatty acids such as linoleic acid, oleic acid and alpha-linolenic acid, and has very good nutritional value (Cittadini et al., 2020). Walnut oil is not only widely used in food processing, but also extensively applied in medicine and functional foods because it contains active substances such as phenols, sterols, and vitamins. Due to its high oil content and good fatty acid composition, walnut has also become a key object in oil crop research and molecular breeding (Cittadini et al., 2020; Hao et al., 2024).

In recent years, with the development of omics technology, people have gained a better understanding of the molecular mechanisms of walnut fruit development and oil accumulation. Many studies on transcriptomics, metabolomics and multi-omics integration have identified a number of key genes related to lipid synthesis, such as ACCase, KAS, LACS, SAD, FAD2, PDAT, etc., and have also found multiple related transcription factors. Such as WRI1, LEC1, FUS3, ABI3, etc. (Shi et al., 2022; Hao et al., 2024). The role of miRNA in lipid accumulation has also been gradually revealed. They affect lipid synthesis by regulating target genes (Zhao et al., 2020). In addition, the external environment (such as light and temperature) can also affect oil synthesis, and its molecular response mechanism is receiving increasing attention.

However, there are still many unknowns in the regulatory network of walnut oil accumulation. For instance, the differences in the expression of key genes among different varieties or at different developmental stages, how miRNAs interact with target genes, and the specific mechanisms of oil body formation and lipid changes have not been fully clarified (Hao et al., 2024; Zhu et al., 2025). The functions of some key genes still need to be further verified through gene transformation or gene editing (Zhao et al., 2020).

This study summarizes the main molecular regulatory mechanisms related to walnut fruit development and oil accumulation, with a focus on sorting out the functional characteristics and interrelationships of key enzyme genes, transcription factors, miRNAs and their regulatory modules. Meanwhile, by integrating multi-omics data and network analysis, we identified the core regulatory nodes in oil synthesis and accumulation. This study aims to provide a theoretical basis and important molecular targets for molecular breeding to improve the high oil content and oil quality of walnuts.

## 2 Biological Basis of Walnut Fruit Development

### 2.1 Structural development of hull, shell, and kernel

The walnut fruit is mainly composed of three parts: the outer green skin (exocarp), the hard shell (endocarp), and the oil-rich kernel (endosperm) inside. During the early stage of fruit development, approximately 7 weeks after pollination, the germ begins to form and rapidly enlarges inside the cuticle. Meanwhile, the fruit shell begins to develop and gradually becomes woody. Capsid cells continuously differentiate, secondary walls thicken, and eventually form a hard protective structure. A mature walnut shell is usually divided into three layers: the outer layer is the stone cell layer, the middle layer is the thick-walled cell layer, and the inner layer is the thinner contractile cell layer. The thickness and hardness of the shell will directly affect the protective ability and commercial value of the kernel (Zhao et al., 2019). The microstructure of the fruit shell shows a "gradient" distribution: the outer layer is denser and firmer, the middle layer is more porous, and the inner layer is thinner. Therefore, it can take into account both protection and a certain degree of elasticity at the same time. This structure is called "functionally graded material" (Nicolás-Bermúdez et al., 2022).

### 2.2 Physiological dynamics during kernel filling

The development of walnut kernels generally goes through several stages, including cell division, nutrient accumulation and structural stability. About 60 days after pollination, the originally gel-like kernels start to solidify more and the oil begins to accumulate rapidly. When the fruit is ripe, the oil content in walnut kernels can reach 65% to 70%. Walnut oil mainly consists of unsaturated fatty acids, such as linoleic acid. Proteins and amino acids are synthesized in large quantities during the period of nutrient accumulation. They are important precursors for subsequent lipid synthesis and the formation of antioxidant substances. During the development of kernels, antioxidant activity first decreases and then increases, water content gradually decreases, while the proportion of dry matter and fat continuously increases (Christopoulos et al., 2021; Shi et al., 2022; Hao et al., 2024).

### 2.3 Genetic and environmental determinants of fruit development

The fruit traits of walnuts, such as thick shells, heavy kernels, and oil content, are simultaneously influenced by genetic and environmental factors. A variety of genes (such as MYB, NAC, UGP, FAD, etc.) are involved in the secondary wall and lignin formation of the fruit shell, as well as the lipid metabolism and stress resistance regulation of the kernel. Traits such as shell thickness and shell binding strength are more closely related to some specific genes, such as JrPXC1, MYB308, NAC043, etc. The external environment, such as light, temperature, soil conditions and management measures (such as ring peeling), can also affect the development rate, shell thickness and oil accumulation of walnut fruits. Due to different genetic backgrounds, different varieties will show significant differences in fruit size, shell thickness, kernel rate, etc. In hybrid breeding, the influence of the mother on fruit traits is usually quite significant (Bernard et al., 2021; Soveili and Khadivi, 2023; Kumar et al., 2024; Özcan et al., 2025).

## 3 Oil Biosynthesis Pathways in Walnut

### 3.1 Precursor supply and carbon metabolic flow

The formation of walnut oil is highly dependent on the supply of carbon sources and the regulation of metabolic flow. When fruits are developing, sugar metabolism provides the main raw materials and energy for lipid synthesis. Pyruvate produced by glycolysis (involving genes such as ENO and PK) is converted into acetyl-CoA (acetyl-CoA) through pyruvate dehydrogenase (PDH), which is A direct precursor for fatty acid synthesis. The high expression of genes related to the tricarboxylic acid cycle (TCA) can also provide sufficient carbon skeletons and energy for fatty acid synthesis. As the fruits gradually ripen, the contents of oil and protein continue to increase, while soluble sugar and starch gradually decrease, indicating that the carbon flow begins to be directed more towards lipid synthesis (Shi et al., 2022).

### 3.2 Fatty acid synthesis and triacylglycerol (TAG) assembly

The synthesis of fatty acids mainly occurs in plasmids, and enzymes such as ACC, KASII, and LACS are crucial at this stage. The synthesized fatty acids are transported to the endoplasmic reticulum, where they are further processed by enzymes such as GPAT, LPLAT, PAP, DGAT, and PDAT, and eventually assembled into TAG. During the process of walnut oil accumulation, the expression levels of desaturases such as SAD, FAD2, and FAD3 are relatively high, which can promote the production of polyunsaturated fatty acids. Research suggests that the PDAT pathway plays a particularly important role in the formation of walnut oil. In addition, oil-related proteins, such as oleosin, caleosin, and steroleosin, are involved in the formation of oil bodies and are also important for energy storage (Huang et al., 2020; Zhao et al., 2020; Hu et al., 2022; Hu et al., 2023).

### 3.3 Characteristics of walnut oil composition

The most distinctive feature of walnut oil is that it contains a very high proportion of unsaturated fatty acids. Among them, linoleic acid (C18:2) usually reaches about 60%, alpha-linolenic acid (C18:3) is between 7% and 15%, and the total proportion of unsaturated fatty acids often exceeds 90%. In addition, walnut oil also contains fatty acids such as oleic acid (C18:1), palmitic acid (C16:0), and stearic acid (C18:0). The main types of TAG include trilinolein (LLL), dilinoleoyl-linolenoyl glycerol (LLLn), olein-dilinolein (OLL), etc. Walnut oil is also rich in beneficial trace components such as γ -tocopherol (vitamin E) and β -sitosterol. Different varieties, environments and extraction methods will affect the specific proportion of oil, but "high polyunsaturated fatty acids (PUFA)" and "high nutritional value" have always been the core characteristics of walnut oil (Gao et al., 2019; Sun et al., 2020; Elouafy et al., 2022; Yang et al., 2022; Liu et al., 2024).

## 4 Regulatory Networks Controlling Oil Accumulation

### 4.1 Transcription factors governing lipid metabolism

A large number of studies have shown that transcription factors such as WRINKLED1 (WRI1), ABI3, LEC1/LEC2 and FUS3 are very crucial in the formation of walnut oil. These factors regulate many genes involved in fatty acid synthesis, such as ACCase, KASII, LACS, FAD2, FAD3, etc., thereby promoting the continuous accumulation of fats (Figure 1) (Li et al., 2022; Zhou et al., 2023). In addition, family members such as AP2/ERF and bHLH are often expressed together with lipid metabolism genes, indicating that they are also involved in the regulation of lipid synthesis (Li et al., 2022; Shi et al., 2022). miRNA also plays a role in this process. Some miRNAs target key metabolic genes, thereby affecting the rate of lipid accumulation and fatty acid composition (Zhao et al., 2020).

### 4.2 Gene expression patterns and co-expression modules

At different stages of walnut fruit development, oil-related genes show obvious temporal and tissue-specific expressions. WGCNA et al. Co-expression analysis showed that genes such as FAD2, FAD3, SAD, and PDAT were usually expressed at the highest level in the middle and late stages of fruit development, which exactly corresponds to the stage of rapid increase in oil (Jin et al., 2023; Zhu et al., 2025). In these co-expression modules, transcription factors and structural genes are often expressed in groups, forming a large regulatory network. For example, WRI1 is often expressed together with genes such as ACP, ENO, VAMP727, IDD14, etc., indicating that it is in a core position in the lipid metabolism network (Huang et al., 2020; Shi et al., 2022). Meanwhile, the expression levels of oil protein-related genes, such as OLE, CLO, and STE, are also very high during the lipid storage stage and are closely related to oil formation and lipid storage (Zhou et al., 2023; Hao et al., 2024).

### 4.3 Epigenetic and hormonal influences

Epigenetic regulation (such as DNA methylation and histone modification) as well as hormone signals (such as ABA, ethylene, GA, etc.) can also affect the accumulation of walnut oil. Hormone signals can regulate the expression of transcription factors and their downstream genes, thereby affecting fatty acid synthesis and oil body formation (Li et al., 2022). For example, the changes of ABA are closely related to the expression of ABI3 and FUS3, and these transcription factors in turn regulate the activity of lipid synthesis genes (Shi et al., 2022). In addition, external environments such as light can also affect hormone levels, thereby further influencing the accumulation of oils.

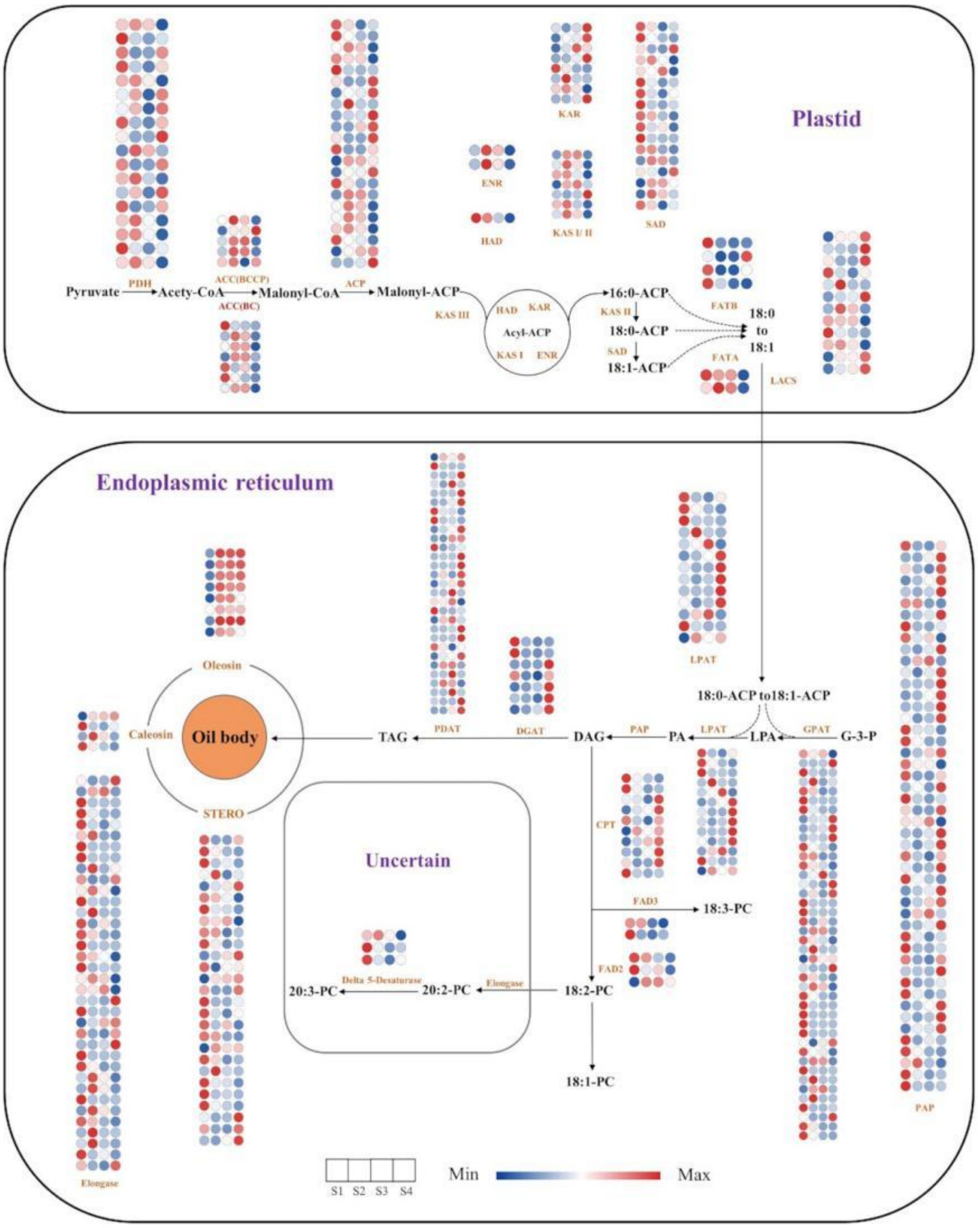

Figure 1 Comparative transcript analysis of genes involved in lipid biosynthesis of developing fruit (Adopted from Li et al., 2022)

## 5 Environmental and Stress-Responsive Regulation

### 5.1 Influence of temperature and water stress

Temperature changes, especially low temperatures and diurnal temperature differences, have a significant impact on the development of walnut seeds and the accumulation of oil. Low temperature can cause cell membrane damage, imbalance of osmotic regulation and protein degradation, thereby bringing a series of physiological and metabolic problems. The results of the transcriptome and miRNA show that at low temperatures, many metabolic pathways in walnut leaves are re-regulated, such as polysaccharide metabolism, starch and sucrose metabolism, and glycerophospholipid synthesis. The corresponding genes and miRNAs will also change their expression accordingly, and these changes help to enhance cold resistance and metabolic adaptation. In addition, in an environment with a large temperature difference between day and night, the expression level of heat shock proteins (HSPs) is higher. Such proteins may help protect seed development and lipid accumulation (Shi et al., 2022). The impact of water stress on the quality of walnuts is relatively small, but in cases of extreme drought or excessive irrigation, the size of the fruit, oil content and the proportion of fatty acids will all be affected. Studies have pointed out that the oil content of walnuts is more sensitive to temperature. Higher temperatures and mild drought can instead increase the oil concentration and improve the fatty acid composition (Calvo et al., 2023).

### 5.2 Light conditions and carbon allocation

Light intensity is also an important factor affecting the development of walnut fruits and the accumulation of oil. Shading treatment can significantly reduce the fat content in endosperm while increasing protein and soluble sugar. Shading can lead to a decrease in the expression of fatty acid desaturases (such as FAD2, SAD) and a reduction in the content of major fatty acids (such as oleic acid, linoleic acid and alpha-linolenic acid), indicating that light can affect lipid accumulation by regulating these lipid synthesis genes (Figure 2) (Liang et al., 2023). In the early stage of shading, the content of soluble sugar will increase, possibly because photosynthesis weakens and sugar cannot be smoothly converted into oil, showing a change in carbon distribution under low-light conditions.

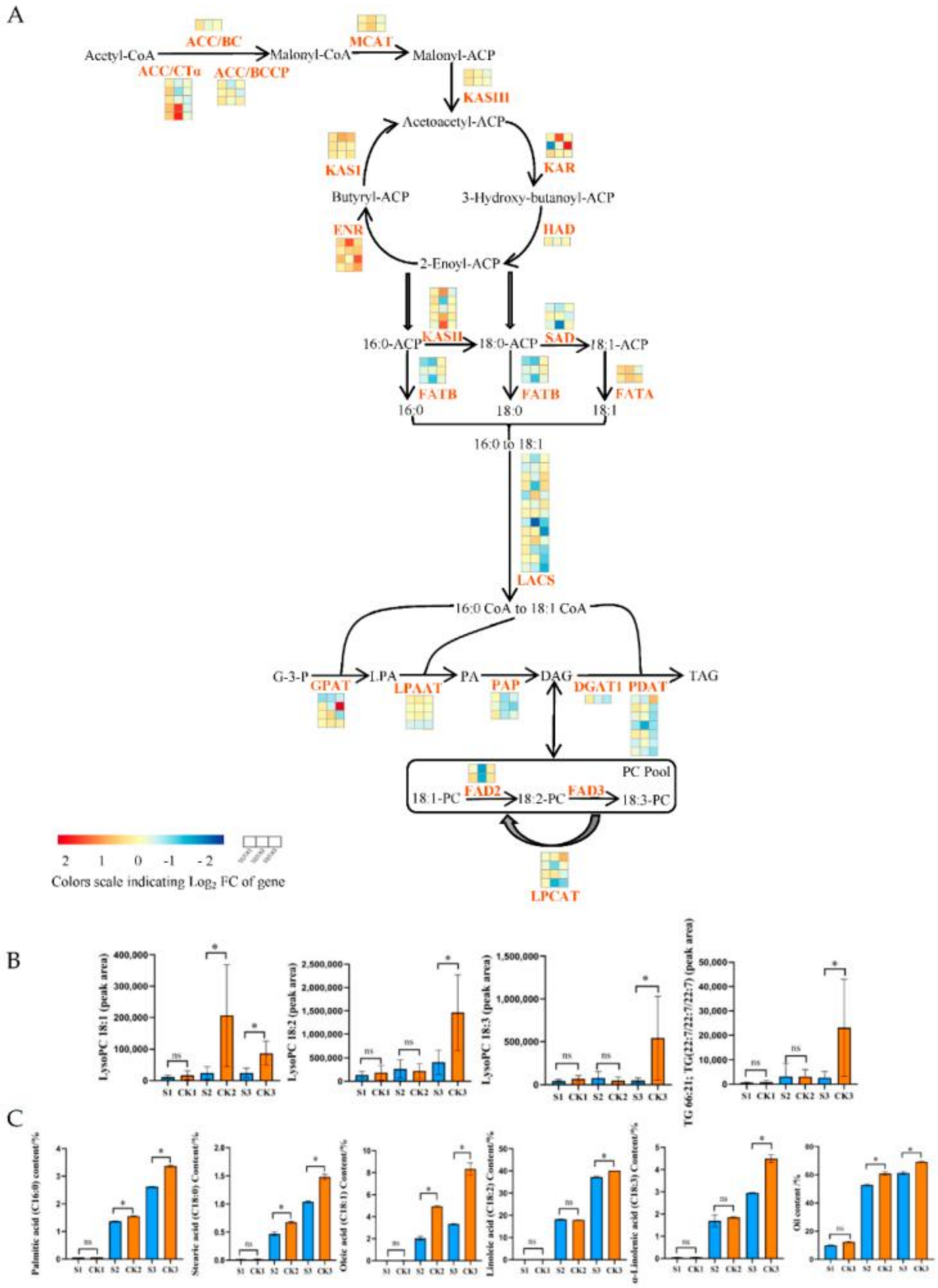

Figure 2 Effects of shade on lipid biosynthesis (Adopted from Liang et al., 2023)

### 5.3 Stress signaling and lipid metabolic adjustment

When walnuts are exposed to stress such as low temperature and shading, they activate multiple signaling pathways and transcription factors to regulate lipid metabolism genes. For example, under low-temperature conditions, the expression of fatty acid desaturase (FAD2, FAD3) is upregulated, which can promote the synthesis of polyunsaturated fatty acids and help maintain the fluidity of cell membranes, thereby enhancing cold resistance (Shi et al., 2022). Meanwhile, hormone signals such as ABA and ethylene, as well as transcription factors like AP2/ERF and bHLH, also participate in stress responses and regulate lipid synthesis. Under stress, walnuts also accumulate some antioxidant secondary metabolites, such as phenols and flavonoids, which can further enhance stress resistance (Terletskaya et al., 2024).

## 6 Multi-Omics Insights into Walnut Fruit and Oil Development

### 6.1 Genomic resources and identification of key gene families

High-quality walnut genomes and pan-genomes provide important tools for studying the genetic basis of fruit development and oil accumulation. Through GWAS and multi-omics integration, researchers have identified many gene families related to fruit shell development and lipid synthesis. Genes such as UGP, MYB308, MYB83, NAC043, NAC073, CCoAOMT1, CHS2, CESA7, LAC7, COBL4, IRX12, etc. are all related to the formation of secondary cell walls and lignin accumulation (Shi et al., 2024; Xi et al., 2025; Zhou et al., 2025). Some of these genes (such as JrUGP and JrMYB308) have been verified through heterologous system overexpression experiments, demonstrating that they can promote cell wall thickening and lignin synthesis, providing effective targets for molecular breeding.

### 6.2 Transcriptomic and metabolomic characterization

Combined transcriptomic and metabolomic analysis also demonstrated the dynamic changes of walnut fruits during development and oil accumulation. When the fruit ripens, the contents of oil and protein keep increasing. Eventually, the oil content can reach approximately 69%, mainly linoleic acid (18:2) in unsaturated fatty acids (Huang et al., 2020; Hao et al., 2024; Zhu et al., 2025). The transcriptome results showed that key enzyme genes such as ACCase, LACS, KASII, GPAT, SAD, FAD2, FAD3, and PDAT had the highest expression levels during the peak period of lipid synthesis, while transcription factors such as WRI1, ABI3, LEC1, and FUS3 played important regulatory roles in this process. More than 700 metabolites were detected in the metabolome, which are involved in pathways such as TAG synthesis, glycolysis and the TCA cycle. Many Tags and their precursors accumulate significantly in the later stage of the fruit.

### 6.3 Integrating omics for systems biology models

The integration of multi-omics enables researchers to understand the regulatory methods of walnut fruit development and oil accumulation from a systematic perspective. By comparing genomic variations, transcriptome expression, metabolite changes and epigenetic information, a regulatory network containing "gene - transcription factor - metabolite" can be constructed (Huang et al., 2020; Zhao et al., 2020; Zhou et al., 2023). For instance, co-expression analyses by WGCNA et al. revealed that transcription factors such as AP2/ERF and bHLH, in conjunction with key enzymes in lipid synthesis, regulate lipid accumulation. The regulatory relationship between miRNA and target genes has also been confirmed to be involved in fatty acid synthesis and lipid accumulation. These integrated analyses not only demonstrate the complexity of the regulatory network but also provide a theoretical basis for the quality improvement of walnuts and molecular design breeding.

## 7 Case Study: Molecular Mechanisms of a High-Oil Walnut Cultivar

### 7.1 Phenotypic and developmental features of the high-oil cultivar

The fruits of high-oil walnut varieties can accumulate a high amount of oil when they mature, with the oil content in the kernels generally reaching 69% to 70%. Among these oils and fats, polyunsaturated fatty acids (PUFAs) account for more than 90%, mainly linoleic acid, and also contain a relatively large amount of alpha-linolenic acid and oleic acid (Huang et al., 2020; Hakimi et al., 2024). During the fruit development process, the content of oil and protein will continuously increase, while the content of sugar and starch will gradually decrease. This indicates that glucose metabolism provides the main raw materials and energy for lipid synthesis (Shi et al., 2022). High-oil varieties usually also have excellent traits such as plump kernels, lighter kernel color, and easy kernel extraction (Sarikhani et al., 2021; Hakimi et al., 2024).

### 7.2 Enhanced transcriptional and metabolic pathways

Among these high-oil varieties, the expression of genes related to lipid synthesis is generally higher. Multiple transcriptome and metabolome studies have found that key genes for fatty acid neosynthesis (such as ACCase, KASII, LACS) and TAG assembly-related genes (such as DGAT2, PDAT2, LPAAT1) And the oil body protein genes (such as oleosin, caleosin, steroleosin) were all significantly upregulated in high-oil varieties. Meanwhile, the high expression of desaturases such as FAD2 and FAD3 will promote the increase of polyunsaturated fatty acids. Transcription factors such as WRI1, ABI3, FUS3, LEC1, AP2/ERF and bHLH also play a key role in regulating the expression of these structural genes (Huang et al., 2020; Shi et al., 2022). In addition, miRNA and its target gene relationship also play a regulatory role in fatty acid synthesis and lipid accumulation (Zhao et al., 2020).

### 7.3 Breeding implications derived from the case study

The research on the molecular mechanisms of these high-oil walnut varieties provides useful information for molecular breeding and genetic improvement. Screening key structural genes, transcription factors and miRNA regulatory modules can provide clear targets for molecular marker-assisted selection and genetic engineering improvement (Zhao et al., 2020; Huang et al., 2020; Shi et al., 2022). Combining phenotypic data and molecular data also helps to select superior germplasms more accurately, thereby accelerating the breeding of new varieties of high-yield and high-oil walnuts (Başak et al., 2021; Sarikhani et al., 2021; Hakimi et al., 2024). In the future, GWAS and multi-omics integration can be utilized to identify more important genes related to oil accumulation, further enhancing the oil quality and yield of walnuts.

## 8 Breeding and Biotechnological Applications

### 8.1 Marker-assisted and genomic selection approaches

High-density SNP chips and GWAS are now very commonly used tools in walnut breeding. The emergence of Axiom™ J.regia 700K SNP genotyping chip has significantly improved the efficiency of genetic diversity analysis, family structure inference and trait mapping, and has also laid the foundation for mark-assisted selection (MAS) and genomic selection (GS) (Bernard et al., 2021). Many studies have identified gene loci related to walnut fruit traits through GWAS, such as shell thickness, fruit weight, oil content, etc., and developed molecular markers that can be used for early screening (Marrano et al., 2019; Bernard et al., 2020). These molecular tools can significantly improve breeding efficiency and accelerate the selection and breeding of superior varieties.

### 8.2 CRISPR/Cas-based modification of regulatory pathways

CRISPR/Cas gene editing provides a more precise method for improving walnuts. It can target and regulate key genes such as lipid synthesis, fatty acid composition and stress resistance, thereby increasing the yield and quality of walnut oil. With the discovery of more and more functional genes for lipid biosynthesis, CRISPR/Cas is expected to be used to knock out unfavorable genes, enhance the expression of beneficial genes, and cultivate new walnut varieties with high oil content, high nutrition, and low allergy (Li et al., 2024). In addition, gene editing can also be applied to other pathways of fruit development, such as cell wall and lignin synthesis, providing more possibilities for improving traits such as fruit shell and fruit shape.

### 8.3 Strategies for precision improvement of walnut oil quality

The quality of walnut oil is mainly determined by its fatty acid composition, including linoleic acid, oleic acid, alpha-linolenic acid, etc. Multi-omics analysis has identified many key genes affecting lipid synthesis, such as FAD2, FAD3, SAD, PDAT, etc. At the same time, several important transcription factors have also been discovered, including WRI1, ABI3, FUS3, etc. Their expressions at different developmental stages are closely related to lipid accumulation (Zhao et al., 2020; Zhou et al., 2023; Hao et al., 2024). This information provides reliable molecular targets for the improvement of oil quality. Through MAS, GS and gene editing, precise improvement of target traits such as high oil content, high monounsaturated fatty acids (MUFA), or low polyunsaturated fatty acids (PUFA) can be achieved. In addition, by using the superior genotypes in natural germplasm resources and combining hybridization with molecular breeding, it is also helpful to cultivate new walnut varieties with strong adaptability and good quality (Poggetti et al., 2018).

## 9 Conclusion

In recent years, people have gained a clearer understanding of the molecular regulatory network of walnut fruit development and oil accumulation. Multi-omics analysis shows that lipid synthesis involves multiple steps, including the generation of fatty acids, elongation, desaturation, triacylglycerol (TAG), and storage in the oil body. The expression of core structural genes such as ACCase, LACS, and PDAT changes at different stages of fruit development. These changes affect the rate of lipid accumulation and also alter the composition of fatty acids. Transcription factors form a rather complex regulatory network by regulating the expression of these genes. Meanwhile, the participation of miRNA has also been confirmed. Some miRNA-mRNA combinations directly act on genes related to fatty acid metabolism and regulate lipid production. The expression of oil-related proteins is related to whether the oil can be stably stored in the kernel. Environmental factors such as light and temperature can also affect the expression of these key genes, thereby altering oil accumulation and fruit quality.

To increase the oil content and quality of walnuts in the future, several aspects can be considered. Gene editing technologies such as CRISPR/Cas can be utilized to precisely regulate key genes like ACCase, FAD2, FAD3, and PDAT or their upstream transcription factors, thereby improving oil content and the proportion of fatty acids. It is also possible to systematically explore miRNAs that regulate lipid synthesis and verify their target genes, providing more targets for molecular breeding. Combining multi-omics data such as transcriptomics, metabolomics, and epigenomics can also help us understand how the interaction between genes and the environment affects lipid accumulation, thereby guiding cultivation management and variety improvement. Further research on the functions and regulatory methods of oil body proteins can also help improve the stability of oil storage and the nutritional quality of fruits. Finally, the development of molecular markers related to oil content and fatty acid composition can help accelerate the breeding of high-oil and high-quality walnut varieties.

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### Conflict of Interest Disclosure

The author affirms 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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