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# The Development of Seeds in a Medicinal and Edible *Ficus* Species: Transcriptome Analysis Focusing on Hormone Dynamics and Related Signaling Genes in *Ficus hispida*

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## Abstract

Fig trees (*Ficus*) are widely used in landscaping, traditional medicine, and food. Their obligate mutualism with fig wasps makes them key models for coevolution studies. In dioecious figs, pollinators enter both female and male figs, but only female figs produce seeds—yet the mechanism remains unclear. In *Ficus hispida*, we found pollen tubes reached the ovaries and fertilization completed within 3-day. Integrated transcriptomic and metabolomic analysis indicated that the content of auxin (IAA) and abscisic acid (ABA) increased after pollination, while 1-aminocyclopropane-1-carboxylic acid (ACC) and gibberellin-7 (GA7) decreased. Trans-zeatin (TZ) content remained stable compared to unpollinated figs. In the early stage of pollination, the female flower may have activated the processes of ovary growth and seed formation by significantly enhancing the expression of the auxin early responder gene *SAUR71/GH3.9*, the gibberellin receptor gene *GID1*, and the ethylene response factor *ERF1*. Meanwhile, significantly enriched pathways-including amino sugar and nucleotide sugar metabolism, pentose and glucuronate interconversions, tyrosine metabolism, and nitrogen metabolism which supply energy and precursors for female flower development and seed formation. Our results show that pollination reprograms hormonal signaling and metabolism in female flowers, directing them toward seed development instead of gall formation. This advances understanding of how pollinators regulates plant development.

*Keywords:* Dioecious fig; Pollination; Seed formation; Phytohormone; Transcriptome.
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## 1. Introduction

Pollination is a key ecological process that sustains plant reproduction and the functioning of terrestrial ecosystems.[1,2] Approximately 35% of global food production directly depends on animal-mediated pollination services, encompassing edible crops (*e.g.* fruits, seeds, and vegetables) as well as plants that supply fiber, pharmaceuticals, and biofuels.[3] Pollination, fertilization and seed formation are critical life history stages that determine the survival, dispersal, and reproduction of plant species. These stages also form an essential foundation for maintaining biodiversity.[4] Presently, the deliberate introduction of pollinating insects has emerged as a pivotal strategy to augment plant production efficiency. For example, the successful introduction of the fig-pollinating wasp (*Blastophaga psenes*) into California in 1899, facilitated the commercial cultivation of figs in the region.[5] Concurrently, artificial pollination experiments offer quantifiable experimental methods for research of plant developmental biology, cytology, and ecology. However, the current state of knowledge regarding the pollination biology and seed formation mechanisms of numerous plant species remains quite limited.

The genus *Ficus* L. comprises over 800 species, and is one of the largest and ecologically most significant genera in the Moraceae family,[6,7] widely distributed across tropical and subtropical regions.[8] Species within this genus are of great importance as ornamental plants. Additionally, they act as critical food sources for a variety of frugivorous animals and provide habitats for parasitic and epiphytic plants, thus

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playing a key role in maintaining the ecological balance of tropical rainforest ecosystems.[9] The fruit of *Ficus* plants, commonly known as a fig, is botanically classified as a syconium. The flowers are situated on the inner surface of the fleshy receptacle, often referred to as the "fruit wall", which fully encloses the inflorescence. The interior of the syconium is largely isolated from the external environment, with only a small ostiole at the apex of the bracts permitting limited interaction with the outside. Figs are exclusively pollinated by fig wasps belonging to the family Agaonidae (Chalcidoidea).[10] Generally, each fig species is predominantly pollinated by a specific mutualistic fig wasp species, with only a few exceptions.[11] These pollinators can only reproduce by laying eggs, with their offspring developing within the ovule of female flowers inside the host syconium.[11,12] This highly specialized and obligate mutualism is widely regarded as one of the most intricate and representative examples of mutualistic symbiotic systems in nature.[13,14]

*Ficus hidpida* Linn.f., a dioecious small tree belonging to the genus *Ficus*, is widely distributed from Sri Lanka to India, southern China, Southeast Asia to Australia. It is commonly found in diverse habitats, such as secondary forests and disturbed areas, exhibiting remarkable ecological adaptability.[15,16] Concurrently, it functions as a pioneering species, bearing fruit year-round and providing habitat and food source for numerous animal species. The dense foliage also helps to regulate microclimate, soil conservation and urban greening. The figs has also been used in Chinese medicine, with its figs, leaves, and roots have the effects of clearing heat, promote diuresis, eliminate food stagnation, and resolve phlegm. Consequently, it is regarded as a prime candidate for use in nutritional health products and medicinal applications.[17] Furthermore, the seeds of mature female figs are frequently consumed directly by people.[18] The development of the figs from the formation of the inflorescence to maturity goes through five stages:[10] (1) Pre-female phase: This stage spans from the germination of the syconium to the entry of pollinating fig wasps into it. During this phase, the ostiole (bract opening) remains tightly closed, and neither male nor female florets have yet bloomed. (2) Female phase: Female flowers mature and open; the ostiolar bract channel begins to loosen and releases attractant compounds. Pollinating fig wasps locate the syconia via these volatile cues, enter female syconia to complete pollination, and infiltrate male fig for oviposition. (3) Interfloral phase: In female figs, pollinated female flowers initiate seed development; in male figs, oviposited female flowers develop into gall flowers, and male flowers also gradually mature. During this stage, figs exhibit the highest growth rate, with a notable thickening of the pericarp. (4) Male phase: the development and maturation of male fig wasps occurs in synchrony with the male flowers. The seeds within the female figs gradually harden, while male fig wasps emerge from the male figs. Subsequent to mating, the female wasps collect pollen and depart the syconium, subsequently taking to the skies in search of figs in their female flowering phase, thereby initiating a new cycle. (5) Post-floral phase: Seeds complete maturation, and mature syconia either abscise or are dispersed via frugivory. Consequently, in the male syconia of *F. hispida*, both female and male flowers undergo normal development. Following pollination and oviposition by pollinating fig wasps within the ovaries of female flowers in male syconia, offspring fig wasps are ultimately produced. Male flowers undergo normal development and produce viable pollen, yet no seeds are set. In female syconia, only female flowers undergo normal development. Following pollination by pollinating fig wasps entering the female fig, seed-bearing fruits are ultimately produced; fig wasps that access the fig cavity fail to reproduce.[19]

The female phase of syconia is the critical period for receive pollen to produce seeds. as well as for oviposition and offspring propagation by pollinating fig wasps. In most *Ficus* species, the female phase of syconia typically persists for 7 days. In the absence of pollinating fig wasp entry into the syconial cavity for oviposition or pollination, this phase can be extended to 15 days. However, shortly thereafter (by day 18), unpollinated female flowers will senesce to brown, followed by the yellowing and abscission of the entire syconium.[20,21] Due to the constraints of a limited number of female flowers and a restricted pollination period, *Ficus* syconia rely on precise spatiotemporal regulatory mechanisms to initiate female flower developmental programs and allocate reproductive functions, thereby maintaining population stability for both mutualistic partners.[19,22] Research has revealed that the floral pollination phase represents a critical phase for morphological and metabolic reprogramming, involving processes such as pollen germination, pollen tube elongation, hormonal signaling regulation, and high-energy metabolic demands.[23-25] For instance, during the flowering phase of Arabidopsis, upregulates the transport and metabolism of carbohydrates and amino acids to fulfill energy requirements and facilitate biomass synthesis, which is subsequently to support pollination and embryo development.[26] Following fertilization, auxin (IAA) is synthesized in the endosperm and subsequently activates downstream pathways, including gibberellin (GA) biosynthesis, thereby facilitating the transformation of the ovule integument into the seed coat.[27,28] In the research on the molecular mechanism of unisexual fruiting in melons, Both plant growth regulator GA4+7 and pollination induce melon fruit setting through the synergistic action of gibberellin and auxin. Early-stage GA4+7 treatment is dependent on gibberellin signaling, whereas auxin signaling is more

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pronounced during the late phase of pollination.[29] Many studies have also utilized metabolomics and transcriptomics technologies to examine the transcriptional profiles and metabolic dynamics underlying floral development transitions.[30,31] And has been analyzed the molecular mechanisms governing seed dormancy and germination in various crop species, including Arabidopsis, soybean, oil palm, and strawberry.[25,32-34] However, it remains poorly understood how female flowers coordinate a series of transcriptional and metabolic changes following pollination by pollinating fig wasps entering the syconium, so as to provide the necessary support for subsequent fertilization, ovule development, and seed formation.

The molecular mechanisms by which pollinating fig wasps enter female figs of dioecious Ficus species for pollination and oviposition, and how female figs can only produce seeds but fail to support the development of wasp offspring, remain unclear. This study aims to analyze the hormonal and metabolic regulatory mechanisms of pollination on seed development. The obtained results emphasize the significant role of hormone signal reprogramming triggered by pollination in the development of female flowers and seed formation. It reveals the intrinsic mechanism by which pollinators regulate the growth and development of plants, providing a theoretical basis for the study of plant - pollinator interactions.

## 2. Materials and methods

### 2.1 Experimental site and climatic condition

All methods were performed in accordance with institutional and national guidelines. The *F. hispida* trees and their obligate pollinator fig wasps (*Ceratosolen solmsi marchali* Mayr) were used as experimental material. All the experiments were performed using *F. hispida* plants grown in the Xishuangbanna Tropical Botanical Garden, located in Mengla County, Yunnan Province, Southwest China (21 °55′N, 101 °15′E, altitude 555 m). The Xishuangbanna region has a subtropical climate characterized by mild winters and hot, rainy summers. All experiments were conducted on clear, windless mornings during the local dry-hot season (March to May).

### 2.2 Material and sample preparation

#### 2.2.1 Collecting obligate pollinating fig wasps

The pollinating fig wasps used in the pollination experiment were collected as follows: On the day prior to the field experiment, three *F. hispida* figs (from one male tree) approaching the male phase were identified and individually enclosed in clean netting bags. Typically, after mating, the pollinating fig wasps carry pollen and emerge from the figs through the bracts between 6 and 8 a.m. the following morning. They should subsequently be transported to the field for the controlled pollination experiments.

#### 2.2.2 Detection of female flower receptivity

Three healthy, normally developed female *F. hispida* trees were selected for the field experiment. When the figs development to the pre-female floral phase, each fig was individually enclosed in netting bags to prevent interference from fig wasps and other insects. The ostiolar bract of each fig was observed daily, and upon noticing loosening, a single pollinating fig wasp was placed on the fig wall. If the wasp crawled toward the bract and exhibited entry behavior, It is considered that the figs has just developed to the female phase and is in a pollinatable state. If no entry behavior was observed within 5 minutes, the procedure was repeated the following day. In addition, three healthy female figs of similar size and shape were chosen for the experiment. The pollination experiments were carried out during the female phase and three days after the female phase, respectively. Ten fig wasps were examined in each fig. Each wasp was individually placed on the fig wall, and the total duration of the entire process, from the start of its search on the fig wall to its arrival at the ostiole, was recorded for each wasp. Once it was observed that the previous wasp had entered the bract opening and exhibited pollination behavior, the wasp was promptly removed, and the experimental process was repeated to evaluate the pollinability of the female flower.

#### 2.2.3 Controlled pollination experiments

Prior studies have demonstrated that three pollinating fig wasps can successfully pollinate the majority of female flowers.[35] On the first day of female phase, three pollinating fig wasps were sequentially introduced into the fig. To minimize potential interference between the wasps, the second and third wasp was placed on the fig wall only after the first wasp had fully entered the bracts. Subsequently, the figs were re-enclosed with netting bags to ensure their normal development. Based on the fruiting condition of the trees, at least 30 figs were retained on each tree to ensure an adequate supply of experimental materials for subsequent studies (Fig. 1).

### 2.3 Fig morphology and pollen tube aniline blue staining

The figs that are in the first day of female phase but have not been pollinated are referred to as day 0 figs. Figs are classified as day 1 figs 24 hours after being pollinated by fig wasps. Observations of fig growth conditions were conducted on days 0, 1, 3, 6, 9, 12, 15, and 18, during which the diameters and weights of the figs were also recorded, with 30 figs recorded each time. Additionally, three figs were collected at each time point, immediately transported to the laboratory for dissection, and female flowers were harvested. Subsequently, the flowers were fixed in a 50% FAA solution for 24 hours, rinsed with distilled water, treated in a 2 mol/L sodium hydroxide solution at 45 ℃ for 15 minutes, and then rinsed three times with distilled water. The flowers were subsequently stained in the dark for 10 hours using a 0.1% aniline blue solution (prepared by dissolving 1 g of aniline blue in 1000 ml of 0.1 mol/L K3PO4 buffer). Thereafter, the flowers were mounted on a slide, and the elongation of pollen tubes was observed and




**Fig. 1:** (A) Experimental tree of *F. hispida*. (B) Bagged figs for controlling experiment. (C) Pollinating fig wasp is attempting to enter the fig from bracts.

photographed under a fluorescence microscope (Zeiss Axio Imager. A2, ZEISS, America) with an excitation wavelength ranging from 330 to 385 nm.

### 2.4 RNA-Seq and transcriptomic analysis

On the third day after pollination (*i.e.* the third day of the female phase), the female flower inflorescences inside the figs were collected and used as the pollinated group (PO). Female flower inflorescences that had developed for the same period but were not pollinated were collected and used as the unpollinated group (CK). First, all the figs were collected within 2 hours. Figs were transported to the laboratory in ice packs, dissected immediately to isolate the female flower within the fig cavity, and ≥ 0.5g of female flowers were transferred into 2ml centrifuge tubes and rapidly submerged in liquid nitrogen for preservation. Subsequently, the samples were stored at -80 ℃ for subsequent transcriptome sequencing and plant hormone analysis. Each treatment included three biological replicates. The choice of the third day of female phase was informed by prior fluorescence microscopy studies, which demonstrated that pollen tubes reached the ovule by this time point, ensuring the completion of fertilization (Fig. 2). Furthermore, no significant difference in the receptivity of unpollinated female flowers between the female phase and three days after the female phase (Fig. S1).

Total RNA was extracted from the collected female flower samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Approximately 0.5 g of female flowers from each treatment was ground into a fine powder under liquid nitrogen for RNA extraction. The quality of the RNA samples was assessed by determining their purity, concentration, and integrity using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Only RNA samples with a total amount of ≥ 1 μg per sample were used for subsequent analysis. RNA libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs Inc, Ipswich, MA, USA). The cDNA libraries were then sequenced in paired-end mode (PE) on the Illumina HiSeq sequencing platform. Clean reads obtained after filtering were mapped to the *F. hispida* reference genome (available at https://ngdc.cncb.ac.cn/gwh/Genome/422/show).

### 2.5 Screening and analysis of DEGs

The gene expression levels of the samples were quantified using both the original reads count and fragments per kilobase of transcript per million mapped reads (FPKM).[36] Differential gene expression analysis between unpollinated female flowers and pollinated female flowers was conducted using the DESeq2 software, the calculated *P*-value was adjusted through false discovery rate (FDR) correction, and DEGs were identified based on an adjusted *P*-value < 0.05 and |log2 fold change| > 1.[37] Gene Ontology (GO) enrichment analysis of the DEGs was performed using the topGO software,[38] while KOBAS software was employed for Kyoto Encyclopedia of

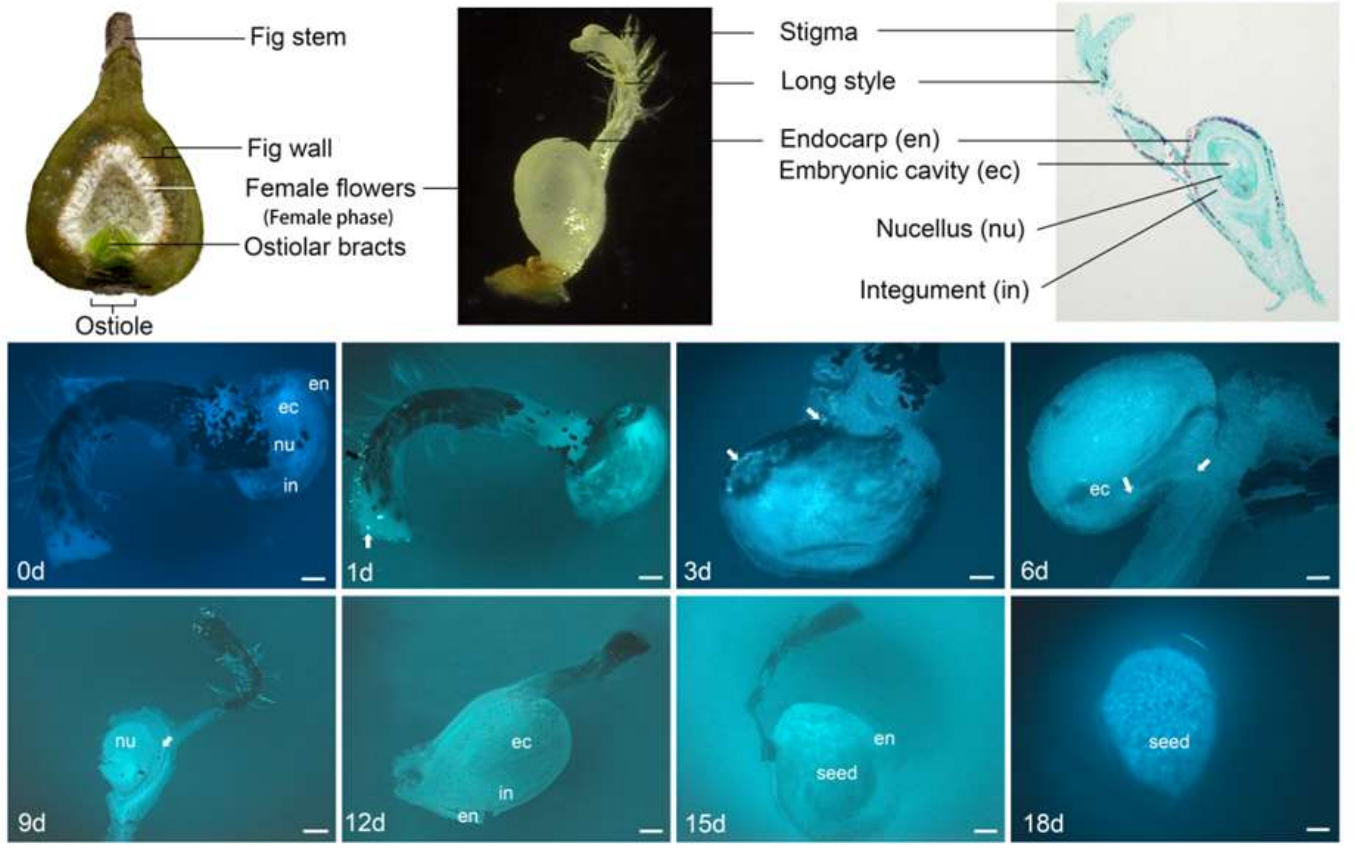



**Fig. 2:** The eight phases of pollen tube germination and seed morphogenesis. The scales are 50um (3d), 100um (0, 1, 6d) and 200um (9-18d); white arrow, pollen tube.

Genes and Genomes (KEGG) functional enrichment analysis.[39] Groups with *P*-value < 0.05 were considered significantly enriched.

### 2.6 Phytohormone extraction and analysis

Samples (0.2 g) of female flowers from unpollinated and pollinated figs were freeze-dried, ground in liquid nitrogen, and transferred to 2 mL centrifuge tubes. Subsequently, 1 mL of methanol was added to each tube, and the samples were vortexed at 2000 rpm for 10 min. The suspensions were then centrifuged at 12,000 rpm for 10 min at 4 ℃, and the supernatants were carefully collected. The supernatants were filtered through 0.22 μm filter membranes and transferred to injection vials for LC-MS/MS analysis.[40]

### 2.7 Ultra performance liquid chromatography (UPLC) conditions

The filtered mixture was analyzed using ultra-high performance liquid chromatography (UPLC, ExionLC UPLC system, AB Sciex, Framingham, MA, USA) coupled with tandem mass spectrometry (MS/MS, AB Sciex Triple Quadrupole 6500+, Framingham, MA, USA). The UPLC conditions were as follows: (1) column: Waters ACQUITY UPLC® CSH C18 (1.7 μm, 2.1 × 150 mm); (2) mobile phase: 2 mM ammonium formate in water containing 0.05% formic acid (phase A) and methanol containing 0.05% formic acid (phase B); (3) elution gradient: 0–2 min, 10% B; 2–4 min, 10–30% B; 4–19 min, 30–95% B; 19–19.10 min, 95–10% B; 19.10–22 min, 10% B; (4) flow rate: 0.25 mL/min; (5) column temperature: 40 °C; and (6) injection volume: 2 μL. The MS/MS conditions were as follows: electrospray ionization source temperature of 400 °C, MS voltage of +4500 V (positive mode) or −4500 V (negative mode), curtain gas pressure of 40 psi, nebulizer gas pressure of 40 psi, and auxiliary gas pressure of 25 psi. The quantification of the hormones was performed by analyzing the extracted ion chromatograms and comparing the peak positions with those of standard solutions. Each sample was analyzed in triplicate from independent experiments.

### 2.8 Statistical analysis of data

The data were pre-processed using Microsoft Excel 2010. Statistical analysis was carried out using SPSS software (version 26.0). General linear models (GLMs) were employed to analyze the influence of pollination (with the development time of pollinated female figs as the independent variable) on the development of female figs (taking the diameters and weights of the figs as the response variables). Graphs were created using Origin software (version 2019b). T-tests were conducted to investigate the differences in hormone levels between pollinated and unpollinated female flowers, and also the differences in the time needed for pollinating fig wasps to

search from the fig wall to the bract ostiole on figs at different female phases. The significance level was set at $P < 0.05$ for all analyses.

## 3. Results

### 3.1 Pollen tube germination and seed morphogenesis

Successful pollination of female flowers is a critical determinant for ensuring normal fig development and seed formation. Through controlled experiments, female flowers at specific developmental stages after pollination were collected, and the pollen germination process was observed using fluorescence microscopy. The results revealed that on the first day post-pollination (1d), pollen tubes began to germinate. By the third day post-pollination (3d), the pollen tubes were directed toward the ovules to facilitate fertilization. On the sixth day post-pollination (6d), the ovary was stimulated to gradually enlarge, supplying essential nutrients required for embryo development. From the ninth to the twelfth day post-pollination (9d-12d), the female flower entered the differentiation and growth phase, thereby promoting seed formation and expansion. Finally, from the fifteenth to the eighteenth day post-pollination (15d-18d), seed morphology became fully established and approached maturity (Fig. 2).

### 3.2 The impact of pollination on the development of female figs

After pollination by fig wasps, the diameter and weight of figs continued to increase. There was a significant correlation between the diameter of female figs and their development time (GLM, $\beta \pm SE = 0.20 \pm 0.07$, $t = 30.11$, $P < 0.01$). Similarly, the weight of figs also significantly increased after pollination (GLM, $\beta \pm SE = 0.24 \pm 0.12$, $Z = 20.12$, $P < 0.01$) (Fig. 3).

### 3.3 Analysis of the impact of pollination on the gene expression profile during seed formation

To identify key genes involved in regulating the seed formation of unpollinated and pollinated figs, we conducted RNA-seq analysis with three biological replicates for each treatment to ensure data reliability. Using the Illumina HiSeq platform, a total of 46.64 - 52.75 million raw reads were generated per sample. After filtering out low-quality reads, the

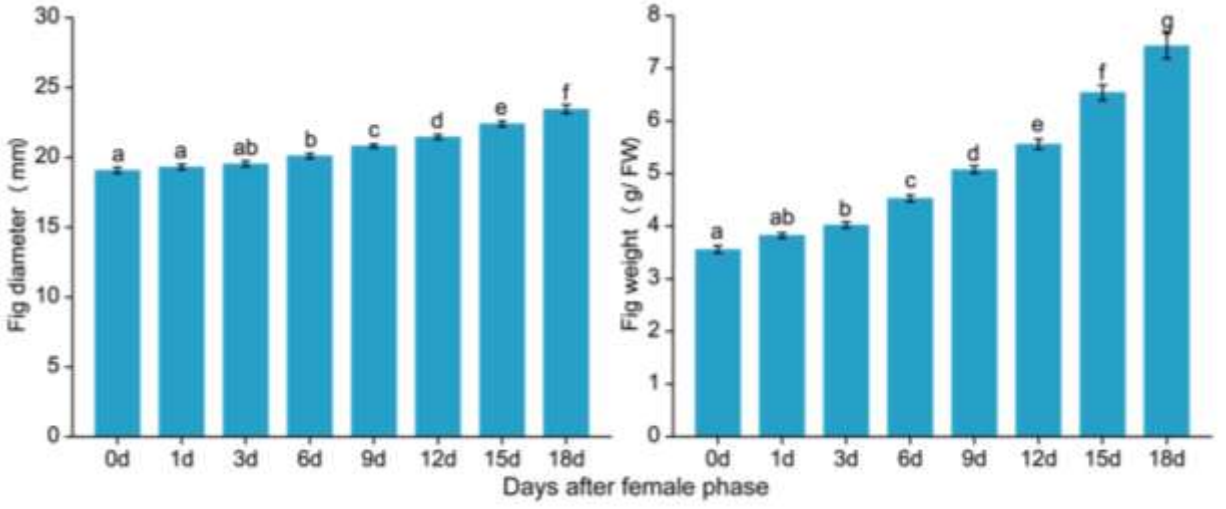



**Fig. 3:** The changes in diameter and weight of female figs after pollination (10 figs per female tree, n = 30).

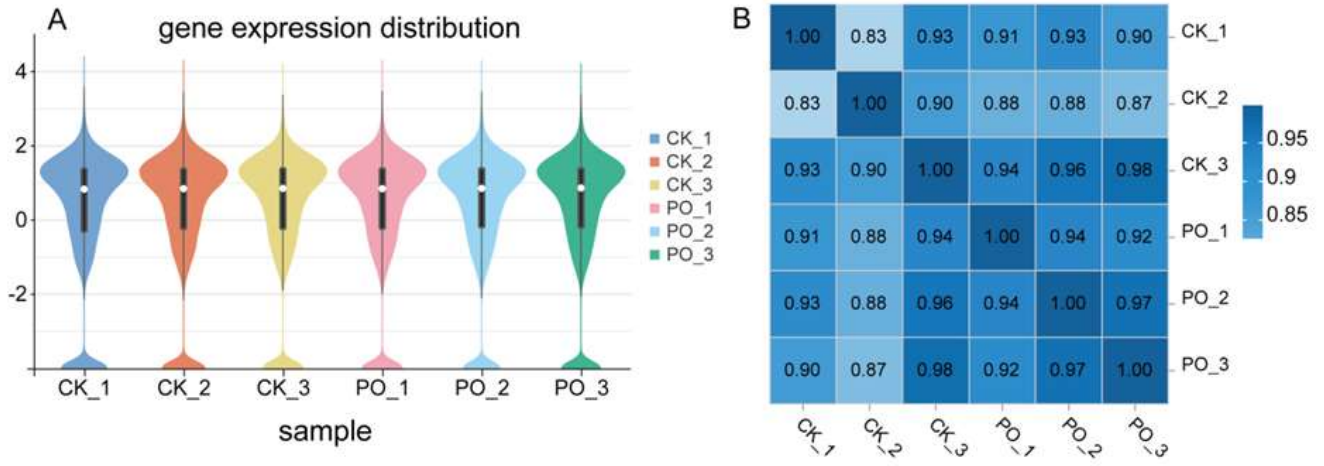



**Fig. 4:** Gene expression data from unpollinated and pollinated figs. (A) Gene expression distribution in each samples. (B) The correlation of gene expression pattern between all samples.

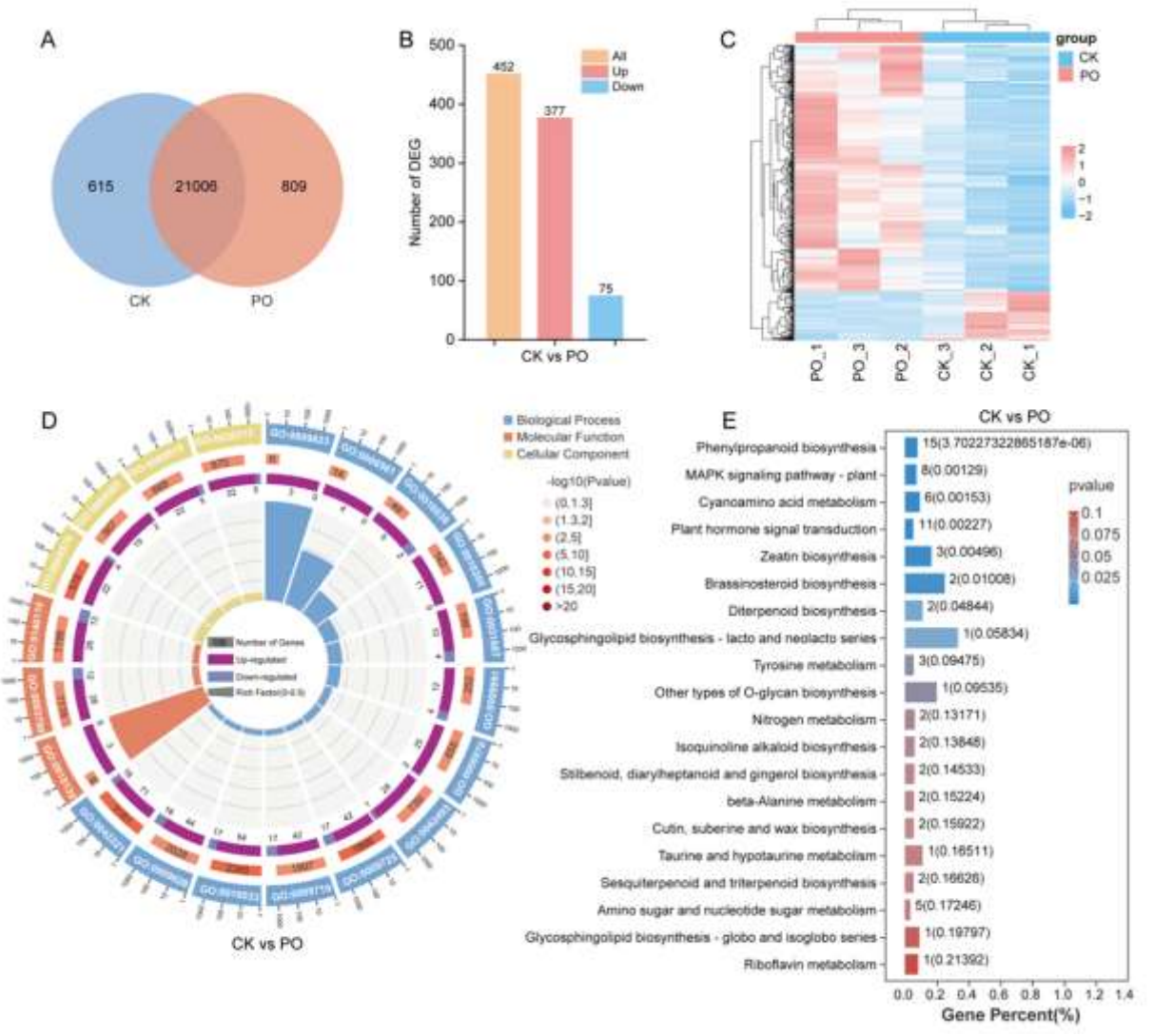



**Fig. 5:** Transcriptome analysis of unpollinated and pollinated groups. (A) Analysis of DEGs in Venn. (B) Statistics of gene number differences, with values represented as log2 (fold change). (C) Heatmap of different expression profile of DEGs. (D) GO enrichment circle diagram. The first circle highlights the top 20 pathways, with the number of genes indicated by the outer circle's scale. Different colors correspond to distinct categories; the second circle displays the quantity and *P*-value of pathways within the background gene set, where a greater number of genes is represented by a longer bar, and the *P*-value is indicated by color intensity, with more significant values shown in red; the third circle illustrates the gene ratio percentages, with dark purple denoting the gene ratio percentage and light purple indicating the gene ratio circle; the fourth circle represents the rich factor values for each pathway, with the background grid lines indicating increments of 0.1. (E) KEGG enrichment bar diagram. The Y axis represents the KEGG pathway. The X axis represents the DEGs numbers mapped to the reference pathway, and the color of bar represents the *P*-value, with more significant values shown in blue.

number of clean reads per library ranged from 43.43 - 43.88 million, with Q30 percentages ranging from 91.84% - 92.56%. The clean reads were successfully mapped to the reference genome at rates of 94.77% - 95.96% (Table S1). Based on the FPKM values, gene expression levels were found to beconsistent across all samples (Fig. 4A). Pearson correlation analysis further demonstrated high similarity ($R^2 > 0.8$) among biological replicates within the same group, confirming that the data were reliable and suitable for subsequent transcriptome analysis (Fig. 4B).

Our study revealed the presence of detectable expression signals in a total of 22,430 genes, as shown in Fig. 5A. A total of 615 and 809 genes were only found to be expressed in unpollinated flowers, and pollinated flowers, respectively, while overlap of 21,006 genes indicates significant interaction between pollinated and unpollinated flowers. DEGs were screened based on two aspects of log2 (fold change) $>$ 1 and *P*-value $<$ 0.05, 452 DEGs (377 up-regulated and 75 down-regulated) were identified by comparing the CK and PO groups (Fig. 5B, Table S2), and the expression of these DEGs in those two groups significantly differences (Fig. 5C).

To further investigate the biological processes associated with seed formation ,we examined the roles of 452 key DEGs using GO enrichment analysis. The DEGs were classified into

three major categories: biological process (BP), cellular component (CC), and molecular function (MF). Among the top 20 enriched GO terms, 13 belonged to biological processes, 4 to cellular components, and 3 to molecular functions (Fig. 5D). Specifically, the BP category was primarily related to responses to stimuli, chemicals, and phytohormones; the CC category was mainly associated with cell parts; and the MF category was predominantly linked to transcription regulator activity, cytokinin dehydrogenase activity, and DNA-binding transcription factor activity. This findings suggest that early pollination may activate genes involved in cell growth, division, metabolism, and hormone signaling, which are critical for regulating seed development in *F. hispida*. Additionally, Gene Ontology (GO) enrichment analysis was performed on the upregulated genes in unpollinated flowers, revealing significant enrichment of terms including gravitropism, response to auxin, and developmental process ($P < 0.05$). This suggests that unpollinated female flowers remain engaged in active morphological establishment and structural development, and have not yet initiated large-scale degeneration programs related to senescence and cell death (Fig. S2).

KEGG analysis provided further insights into the enriched biological pathways. As depicted in Fig. 5E, the significantly enriched pathways annotated by CK and PO treatments were primarily phenylpropanoid biosynthesis (ko00940), MAPK signaling pathway-plant (ko04016), cyanoamino acid metabolism (ko00460), and plant hormone signal transduction (ko04075) ($P < 0.05$).

### 3.4 The impact of pollination on the regulation of plant hormone signaling associated DEGs in seed formation

To achieve a more precise understanding of the major phytohormone signal transduction pathways regulating seed formation in pollinated female figs, we reconstructed the plant hormone signal transduction network. Gene function annotation and KEGG enrichment analysis indicated that 11 DEGs were significantly enriched in pathways related to plant hormone signal transduction. Specifically, there was a pronounced enrichment in IAA, cytokinin (CTK), GA, abscisic acid (ABA), and ETH signal transduction pathways (Fig. 6A, Table S3).

In the auxin signaling pathway, the expression of multiple genes in pollinated female flowers exhibited significant changes. Specifically, auxin response factors repressors Aux/IAA exhibited a decreased expression pattern in the pollinated flowers. Meanwhile, the expression of downstream genes of auxin early response (*SAUR71* and *GH3.9)* was upregulated. The expression level of the auxin-binding gene *GH3.1* was reduced, which increased accumulation of active auxin. Collectively, these changes in expression patterns enhanced the ability of pollinated female flowers to perceive, transduce, and respond early to auxin signals (Fig. 6B).

The key negative regulatory factors in the abscisic acid signaling pathway, protein phosphatase 2C (*PP2C7* and *PP2C24*), were significantly upregulated in the samples after pollination, this upregulation inhibit the activity of downstream genes. Additionally, the coding of the gibberellin receptor *GID1* in the gibberellin signaling pathway is upregulated in the female flowers after pollination, which may enhance the efficiency of gibberellin signal transduction. The downstream target gene of the ethylene signaling pathway, ethylene response factor C3 (*ERF1*), was also upregulated in the pistils after pollination; while the histidine kinase 4 (*AHK4*) gene of the cytokinin signaling pathway was downregulated after pollination (Fig. 6B).

### 3.5 The effect of pollination on endogenous hormones in female flowers

The results of RNA-Seq analysis revealed the regulatory effect of the pollination process on hormone signaling in figs. To investigate the changes in five hormones in both unpollinated

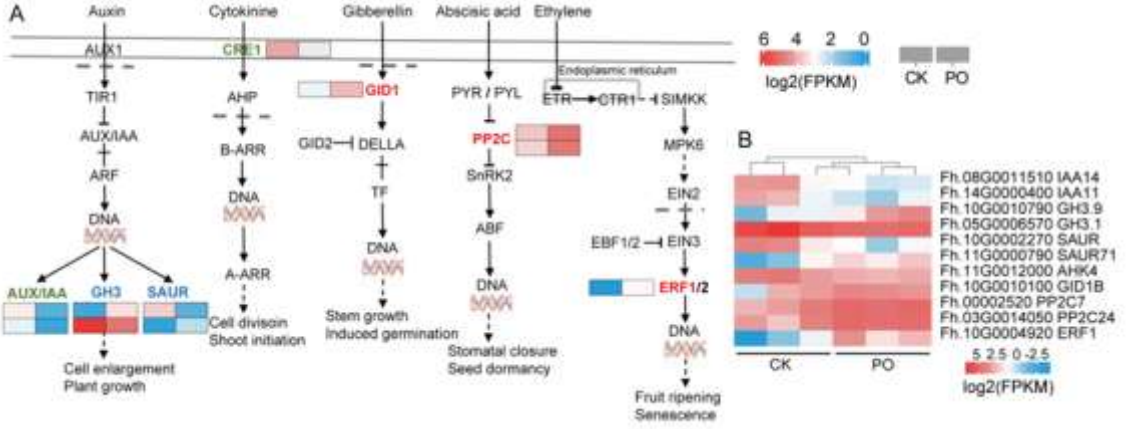



**Fig. 6:** Transcript profiling of genes in the plant hormone signaling pathway of pollinated and unpollinated flowers. (A) The DEGs expression enriched in IAA, CTK, GA, ABA and ETH signaling pathways. The genes of red font indicate up-regulated, the green font was down-regulated, the blue font has both up- and down-regulated genes in pollinated figs. (B) Heatmap representation of plant hormone-related gene expression patterns. Each row represents a single gene, the IDs and names of selected DEGs are indicated to the right of the histograms, and each column represents a sample.

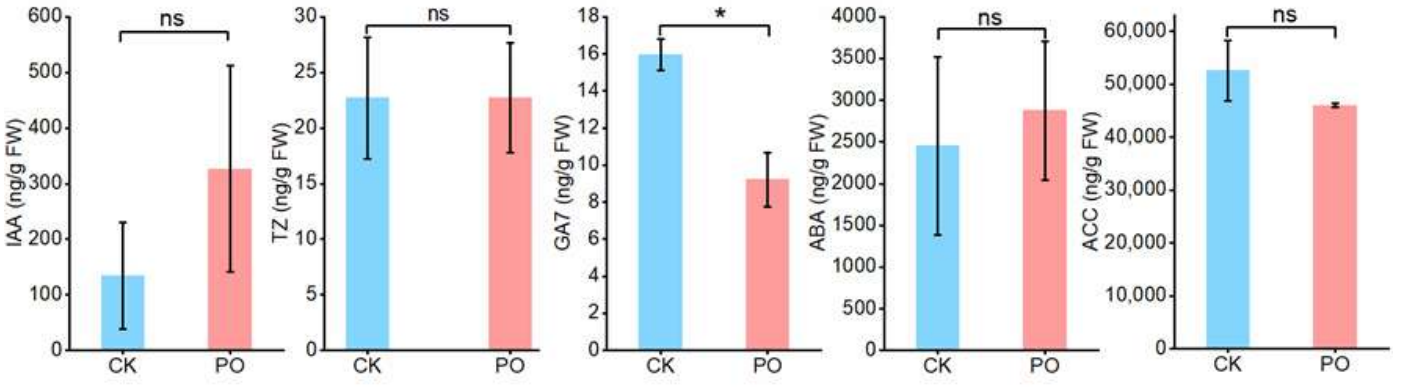



**Fig. 7:** The changes of IAA, TZ, GA7, ABA and ACC contents among unpollinated and pollinated flowers (10 figs per female tree, n = 30). Values are means ± SE (n=3). The star indicated the differences between unpollination and pollination flowers.

and pollinated flowers, we employed targeted metabolomics technology to quantitatively measure the endogenous hormone levels in female flowers. The results revealed that after pollination, the concentrations of IAA and ABA increased, while those of gibberellin-7 (GA7) and 1-aminocyclopropane-1-carboxylic acid (ACC) decreased. The content of trans-zeatin (TZ) remained relatively stable. Notably, only the concentration of GA7 showed a statistically significant difference between unpollinated and pollinated flowers ($P<0.05$) (Fig. 7).

### 3.6 The impact of pollination on the remodeling and modification of cell walls during seed formation

To future enhance the understanding of the transcriptional regulation of cell walls during the *F. hispida* seed formation. GO enrichment analysis revealed that a total of 35 DEGs (27 up-regulated and 8 down-regulated) were significantly enriched in biological processes, molecular functions and cellular components related to the cell wall (Fig. 8A, Table S4). As depicted in Fig. 8B, key enzyme-encoding genes involved in cell wall hydrolysis, modification, and degradation, including three peroxidase genes (*PER15, PER21, PER27*), Two α-L-arabinofuranosidases (*abfA*), one cysteine endopeptidase (*CYSEP*), one extracellular ribonuclease gene (*RNLE*), one mannan endo-1,4-β-mannosidase (*MAN6*) and multiple glycoside hydrolase-encoding genes were exhibited significantly higher expression levels in the pollinated female flowers, and they may play a core role in the decomposition and remodeling of plant cell walls.

### 3.7 The impact of pollination on nutrient activation and energy metabolism during seed formation

Pollination may play a role in the seed formation process by influencing the DEGs associated with the nutrient and energy metabolism pathway. KEGG enrichment analysis reveals that the three major functional categories, namely amino acid metabolism, carbohydrate metabolism, and energy metabolism, are all involved in the regulation of seed formation (Fig. 9A).

Among the 8 pathways related to amino acid metabolism, a total of 12 DEGs were identified (8 up-regulated and 4 down-regulated) (Table S5). Among them, the pathways of tyrosine metabolism, lysine degradation, and arginine and proline metabolism were the most significant. Notably, two genes encoding polyphenol oxidases (*PPO*), one encoding alcohol dehydrogenase-like3 (*ADHL3*), and one encoding methionine γ-lyase (*MGL*) were significantly upregulated in the pollinated female flowers, potenyially linked to plant antioxidant defenses (Fig. 9B).

Among the 8 pathways related to carbohydrate metabolism, a total of 15 DEGs were identified (13 up-regulated and 2 down-regulated) (Table S6). Among them, the pathways of pentose and glucuronate interconversions, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, and fructose and mannose metabolism were significantly enriched. Notably, one gene encoding chitin endochitinase (*CHI2*), two genes encoding pectin methylesterases (*PME*), one gene encoding mannan endo-1,4-β-mannosidase (*MAN6*), and one gene encoding alcohol dehydrogenase (*ADHL3*) were significantly upregulated in pollinated female flowers (Fig. 9C).

Among the three pathways related to energy metabolism, a total of five DEGs (4 up-regulated and 1 down-regulated) were identified (Table S7). Among them, the nitrogen metabolism, oxidative phosphorylation and photosynthesis pathways were significantly enriched. One α-carbonic anhydrase 7 (*cah*) and one plasma membrane-type ATPase 11 (*PMA11*) genes were both significantly up-regulated in pollinated female flowers (Fig. 9D).

## 4. Discussion

*Ficus* species are a crucial category of landscaping trees. They are also edible and medicinal plants, which hold considerable practical value. A single mature fig typically contains hundreds to thousands of female flowers. After being pollinated by fig wasps, the plant can yield thousands of seeds, thus laying a vital foundation for the natural regeneration of vegetation and the development of forest food resources. Nevertheless, there remains a lack of research on the internal

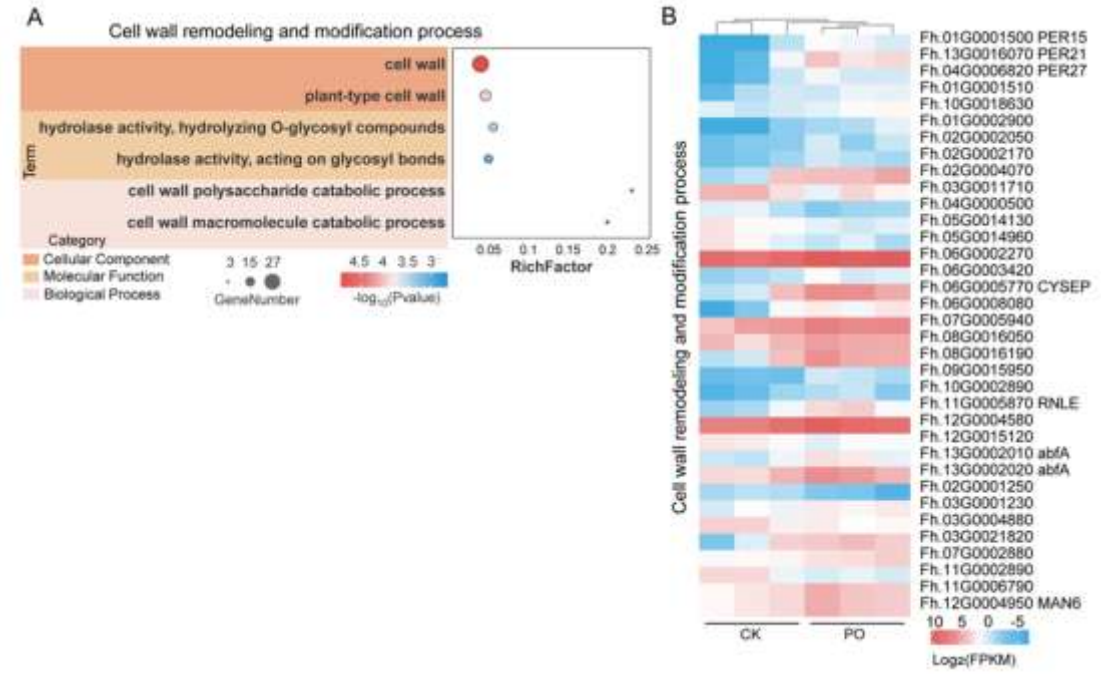



**Fig. 8:** Seed formation of female figs was accompanied by remodeling and modification of cell wall. (A) The GO terms involved in cell wall remodeling and modification process during seed formation, including cellular component, molecular function and biological process. The larger the rich factor is, the greater the enrichment. The smaller the *P*-value is, the more significant the enrichment. (B) Heatmap analysis of DEGs associated with cell wall remodeling and modification process in GO terms. Each column represents a sample, each row represents a single gene, the IDs of selected DEGs are indicated to the right of the histograms, the names are indicated those significantly up-regulated in pollinated figs.

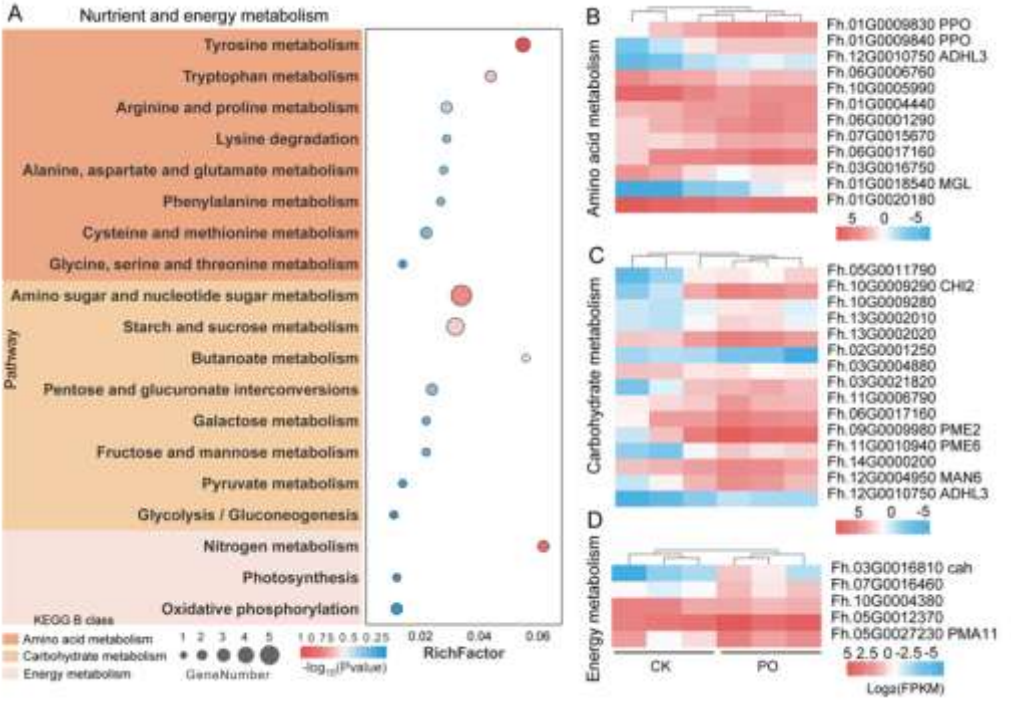



**Fig. 9:** Nutrition and energy metabolism were activated during seed formation of female figs. (A) KEGG analysis of pathways involved in nutrition and energy metabolism during seed formation, the enriched pathways were screened based on KEGG B calsss. The larger the rich factor is, the greater the enrichment. The smaller the *P*-value is, the more significant the enrichment. (B) Heatmap analysis of DEGs associated with amino acid metabolism. (C) Heatmap analysis of DEGs associated with carbohydrate metabolism. (D) Heatmap analysis of DEGs associated with energy metabolism. Each column represents a sample, each row represents a single gene, the IDs of selected DEGs are indicated to the right of the histograms, the names are indicated those significantly up-regulated in pollinated flowers.

mechanisms underlying a series of key developmental events in female flowers. These events encompass initiating ovule development, completing fertilization, regulating embryo and endosperm formation, and finally attaining functional seed maturation. In this study, the differential gene expression pattern between pollinated and unpollinated female flowers was analyzed, revealing that the pollination-triggered processes of female flower development and seed formation are associated with plant hormone signal transduction. Notably, in pollinated female flowers, genes involved in auxin and gibberellin signal transduction pathways were significantly upregulated, whereas endogenous hormone levels remained largely unchanged or even decreased. This efficient regulatory mechanism characterized by "low hormone level- high signal response" is crucial for seed formation in female figs. It indicates that in female figs, the influence of hormone signal reprogramming on seed formation is far greater than that of the hormone levels themselves. Concurrently, the upregulation of genes associated with cell wall degradation and modification, along with the substantial enrichment of carbohydrate and amino acid metabolic pathways, jointly support flower development, seed formation, and defense responses. These mechanisms offer structural and physiological protection for seeds during crucial developmental stages.

Previous studies have shown that the signal transduction pathways of various plant hormones are intricately interconnected, forming a complex regulatory network. The dynamic balance among these hormones is critical for embryo development.[33] Auxin functions as a polar transport molecule.[41] High concentrations of IAA can direct the polarized growth of pollen tubes, enabling them to successfully target the ovule and promoting its subsequent development into a seed.[42] In this study, it was detected that although IAA content exhibited an upward trend following pollination, this did not differ significantly from unpollinated female flowers. The auxin response factor repressors *AUX/IAA* are downregulated, and the early auxin-responsive genes *SAUR71* and *GH3.9* are upregulated. These genes enhance responsiveness to auxin signalling. Concurrently, the downregulation of the auxin-binding gene *GH3.1* will further maintain a consistently high levels of free auxin in the extracellular space, thereby contributing to the homeostasis of active IAA.[43] Based on the results of pollen tube polar growth on the third day after pollination, auxin plays a crucial role in pollination-induced ovary growth and inflorescence initiation. Concurrently, ABA plays a key role in various physiological processes throughout the plant life cycle, including seed dormancy, germination, lateral root formation, light signal integration, and the regulation of flowering time.[44-46] Excessive ABA can damage developmental processes such as senescence, reduce pollen fertility, and induce seed dormancy and increased susceptibility to pathogen infection.[47] In pollinated female flowers, ABA content are also elevated, and the key negative regulatory factor *PP2C7* and *PP2C24* in its signaling pathway is also upregulated. As an upstream component of the ABA signaling pathway, *PP2C* negatively regulates ABA signal transduction by dephosphorylating downstream signaling molecules (*e.g. SnRK2*), thereby contributing to the maintenance of homeostasis in plants.[48] It is therefore hypothesised that ABA upregulates the expression of *PP2C* in its signalling pathway, potentially activating a negative regulatory system. When the ABA content in pollinated female flowers reaches sufficiently high levels, it avoid excessive inhibition of the initiating seed developmental program. Therefore, the negative feedback regulation mediated by *PP2C* helps maintain the balance of ABA signaling.

Notably, the content of other growth-promoting phytohormones, such as GA7, exhibited a significant decreasing trend following pollination, which contrasts with previous studies demonstrating that pollination promotes gibberellin accumulation in ovaries.[49-51] We hypothesize that gibberellin signal transduction depends not only on hormone levels, but also on receptor perception and downstream signaling cascades. The gibberellin receptor gene *GID1* recognizes and binds to gibberellins, thereby activating downstream signaling pathways and sustaining cellular responsiveness to gibberellins.[52] Transcriptome profiling data revealed that the *GID1* was significantly upregulated in pollinated female flowers. In Arabidopsis, *GID1A* has been demonstrated to play a dominant role in fruit set and early fruit development, whlie *GID1B* and *GID1C* are respectively involved in the regulation of seed development and silique elongation.[53] Thus, despite the overall content of GA7 decrease after pollination and fertilization, the gibberellin signaling pathway may enhance perception of and response to trace gibberellin by upregulating *GID1*, thereby exerting specific functions in maintaining cellular homeostasis, coordinating metabolic demands, and driving early seed development. Similarly, the content of ACC, the key precursor of ethylene biosynthesis, also decreases after pollination, and this compound is mainly responsible for the early production of ethylene after pollination.[54] This contributes to suppressing the burst of ethylene biosynthesis and preventing the premature activation of ripening and senescence programs. Studies on orchids have demonstrated that the synergistic interaction between ethylene and auxin is essential for ovary development and ovule differentiation.[55] Higher auxin/gibberellin and auxin/ethylene ratios are critical for promoting embryonic cell differentiation.[56] In pollinated female flowers of *F. hispida*, moderate increasing the content of auxin and reducing the contents of gibberellin and ethylene may better promote seed development. In conclusion, relying solely on alterations in hormone content is inadequate to comprehensively explain the regulation of pollination-induced seed formation. Significantly boosting the expression levels of plant hormone signal transduction genes may act more directly and sensitively on the reprogramming process of female flower development. For example, the notable up - regulation

of signal genes like auxin and gibberellin, while sustaining the high activity of auxin - like hormones, is beneficial for the formation of seeds in female figs instead of galls.

During plant reproduction, extensive cell wall degradation events are critical for flower development, organ abscission, pollen formation, and anther dehiscence.[57] Transcriptome analysis of pollinated female flowers revealed significant upregulation of genes involved in cell wall polysaccharide catabolism (*e.g. PER*, *CYSEP*, *abfA*, *RNLE*) and genes encoding cell wall modification and degradation-related genes (*e.g. PME*, *MAN6*, *CHI2*). These genes participate in pollen tube tip growth,[58] as well as cell wall modification, reorganization, and degradation processes,[59] thereby providing the requisite structural foundation for cell expansion and differentiation during the early stages of seed development. The availability of carbohydrates and their intricate regulatory mechanisms are key parameters in the process of plant pollination and fertilization.[60] In this study, multiple signaling pathways cooperatively regulate the growth and development of pollinated female flowers, with pathways such as amino sugar and nucleotide sugar metabolism, pentose and glucuronate interconversions, and starch and sucrose metabolism being significantly enriched. These metabolic pathways and related genes not only supply abundant activated monosaccharide precursors for cell wall polysaccharide biosynthesis, but also provides the reserve of energy for pollen germination and pollen tube growth.[61] Concurrently, during the transition of female flowers from pollination to fertilization, the metabolic activities of tyrosine, lysine, arginine, and proline were enhanced, with the expression of related genes being significantly upregulated. In particular, the genes encoding polyphenol oxidase (*PPO*) and methionine γ-lyase (*MGL*) were significantly upregulated, indicating that pollination induces the accumulation of phenolic compounds and their oxidation products. This change may, on one hand, be associated with the production of reactive oxygen species (ROS) during secondary wall formation,[62] and on the other hand, may stem from the fact that the micropyle and pollen grains serve as pathways and vectors for potential pathogens to invade the ovule.[63,64] A similar phenomenon has also been documented in ovule and seed development of *Ginkgo biloba*.[65] The upregulated amino acid metabolism in pollinated female flowers may not only support de novo protein biosynthesis in seeds but also maintain intracellular homeostasis by coordinating carbon and nitrogen metabolism, thus facilitating active cell division and tissue differentiation. In summary, pollinated female flowers adopt a strategy of "simultaneous construction and defense" by orchestrating multiple processes, including cell wall remodeling, carbohydrate provision, and amino acid utilization, thereby providing effective biological protection in the early stage of development.

## 5. Conclusion

This study integrated transcriptomic and targeted metabolomic analyses to compare transcriptional profiles and metabolic dynamics between unpollinated and pollinated female flowers. The results revealed that the entry of pollinating fig wasps into figs during the early pollination stage did not induce widespread accumulation of growth-promoting hormones; instead, it triggered efficient reprogramming of hormone signaling responses, thereby initiating the seed developmental program. In pollinated female flowers, the developmental program is initiated primarily by maintaining the homeostasis of active auxin: this is achieved via moderate elevation of auxin content coupled with upregulation of auxin response genes *SAUR71* and *GH3.9*. Concurrently, seed formation is regulated through a "low hormone level-high signal responsiveness" mechanism, which involves reduced GA7 and ACC contents alongside upregulation of signaling genes *GID1* and *ERF1*. Concurrently, pathways such as cell wall modification, amino sugar and nucleotide sugar metabolism, pentose and glucuronate interconversions, and tyrosine metabolism were significantly enriched. The expression of related genes was significantly upregulated in pollinated female flowers, which underscores the crucial roles of hormone signaling and nutrient metabolism in the seed development of *F. hispida*. Due to the current limitations in pollination - specific effects, temporal resolution, and functional validation of candidate factors, future research should integrate spatiotemporal transcriptomics and multi - species validation. Meanwhile, it should incorporate additional developmental time points and control groups to clarify the regulatory mechanisms underlying seed formation in figs. This study offers insights into the obligate fig - fig wasp mutualistic system and serves as a valuable reference for exploring the regulatory mechanisms underlying seed development in entomophilous plants.

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## Conflict of Interest

There is no conflict of interest.

## Supporting Information

Applicable.

## CRediT Statement

**Yuan Zhang and Zongbo Li:** Designed the research. **Yunfang Guan and Yanqiong Peng:** Original draft preparation.

**Yunfang Guan, Yanqiong Peng, Yuan Zhang and Zongbo Li:** Review and editing. **Yuan Zhang, Yanqiong Peng and Zongbo Li:** Provided the plant materials. **Yunfang Guan, Ying Zhang and Changqing Chen:** Performed the experiments and Analyzed the data. **Baige Miao and Jinxia Gao:** Contributed analysis tools and prepared the tables. All authors read and approved the final manuscript.

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