

# Research on the Morphogenesis of *Notopterygium incisum* Rhizomes and its Mechanism: Multiomics Integration Analysis Reveals the Formation Mechanism of Silkworm-like Rings

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## Research Article

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# Abstract

**Background** *N. incisum* (Umbelliferae) is a rare medicinal plant. Its rhizome product ‘Can Qiang’ is valued for its silkworm-like ring patterns. However, no studies have reported how these rings form.

**Result** The ring structure forms through alternating segments: enlarged stem nodes with root traces and vascular bundles and shortened internodes without accessory structures. The key differentiation period is 126–139 days after germination (around the beginning of autumn). Under certain climatic conditions, cork cambium activity and programmed cell death in the cortex lead to uneven secondary growth. This causes vascular tissue expansion and cortex degeneration, which are facilitated by mechanical pressure from the secondary xylem. Metabolomic and transcriptomic analyses revealed many differentially accumulated metabolites and differentially expressed genes. Flavonoids, coumarins, simple phenylpropanoids, and terpenoids presented the greatest differential accumulation. During development, phenylpropanoid biosynthesis was enriched, structural defense pathways were upregulated, and energy storage pathways were activated. Among these organs, root stem tissues exhibit active proliferation and carbohydrate storage, whereas roots specialize in defense synthesis and nutrient assimilation.

**Conclusion** The silkworm-like ring likely results from a carbon flow allocation strategy triggered by early autumn environmental signals. These signals activate the phospholipid system and transcriptional network, redirecting phenylpropane pathway carbon flux. Lignin precursors accumulate, regulating cork cambium activation and secondary xylem proliferation. This forms multilayered rings and cortical cavities. Lignified rings improve mechanical strength; a hollow cortex buffers heat stress. Together, they maintain organ stability and allow the compartmentalized storage of medicinal components. This study provides a theoretical basis for the cultivation, breeding, and harvesting of *N. incisum*.

## 1 Introduction

*Notopterygium incisum* Ting ex H. T. Chang is a traditional Chinese medicinal herb belonging to the Umbelliferae family. One of its commercial specifications, ‘Can Qiang’, is classified as premium grade owing to its dense ring-like patterns on the surface and significantly higher content of essential oils and active coumarins (such as isoimperatorin and notopterol) compared with other commercial grades. Modern pharmacological studies have demonstrated that ‘Can Qiang’ also has prominent anti-inflammatory and analgesic activities<sup>[1][2]</sup>. The international market demand for *N. incisum* is showing steady growth, with exports mainly to Europe, Southeast Asia, and other regions, where it is used in dietary supplements and traditional medicine<sup>[3]</sup>. The “China *N. incisum* International ISO Standard” project is progressing smoothly and has entered the international expert voting stage<sup>[4]</sup>. Overall, *N. incisum* has gained international recognition in terms of traditional medicine applications, modern scientific research verification, growing market demand, and standardized certification. It is expected to continue to develop in the expansion of the natural medicine market in the future.

The silkworm-like ring, as a specific phenotype of the underground stem, is the core morphological identifier and quality indicator of high-quality 'Can Qiang'. Notably, the formation of the silkworm-like ring has significant specificity—this phenotype is only stably expressed under high-altitude environmental stress and exhibits obvious germplasm dependence, suggesting that its formation is jointly regulated by genetic factors and environmental factors<sup>[5]</sup>. Wild *N. incisum* resources are on the verge of depletion due to excessive harvesting, while artificial cultivation faces core issues such as large variations in rhizome morphology and low silkworm-like ring formation rates, severely limiting the sustainable production of high-quality 'Can Qiang'. Currently, research on *N. incisum* has focused mainly on chemical composition analysis, pharmacological activity evaluation, and cultivation technique exploration<sup>[6][7][8]</sup>. The anatomical structure and molecular regulatory mechanisms underlying the development of the silkworm-like ring, a key morphological feature, remain unclear.

The enlargement of plant rhizomes involves anatomical restructuring, such as secondary growth driven by vascular cambium activation<sup>[9][10]</sup>, and metabolic reprogramming, such as the transport of photosynthetic products underground and the accumulation of secondary metabolites<sup>[11]</sup>.

Rhizome morphology is often closely associated with secondary metabolism. For example, there is a strong correlation between phenotypic variation in *Atractylodes lancea* rhizomes after cultivation and genes involved in the biosynthesis of sesquiterpene compounds<sup>[12]</sup>. Studies on the development of underground organs of crops such as potatoes have shown that hormone signals such as auxin and gibberellin, sugar metabolism, and cell wall modification are crucial to the enlargement process<sup>[13][14][15]</sup>.

This study aimed to elucidate the molecular mechanism of silkworm-like ring formation in *N. incisum* and its association with secondary metabolite metabolism. By combining multiomics analysis (transcriptomics, metabolomics), we conducted an in-depth analysis of *N. incisum* rhizomes at different developmental stages and under key environmental changes. This study provides a theoretical basis for elucidating the genetic basis and environmental interactions involved in the morphogenesis of the silkworm-like ring, laying the foundation for the targeted cultivation of *N. incisum* medicinal materials with high silkworm-like ring formation rates and high quality.

This study aimed to elucidate the anatomical structure and molecular mechanisms underlying the formation of the *N. incisum* ring, as well as its association with secondary metabolite metabolism. Through multiomics analysis, we conducted an in-depth analysis of the rhizomes of *Ligusticum chuanxiong* at different developmental stages. This study provides a theoretical basis for elucidating the genetic basis of morphogenesis of the *N. incisum* ring, laying the foundation for the targeted cultivation of high-quality *N. incisum* medicinal materials with high *N. incisum* ring formation rates.

## 2 Materials and methods

### 2.1 Plant materials

The *N. incisum* material used in the experiment was cultivated at the project team's controlled experimental base located in Tanchang County, Gansu Province, China. The species was identified and confirmed by Associate Professor Wenlong Zhao. The experiment was conducted outdoors under conditions simulating the natural environment and strictly excluded external interventions such as fertilization and irrigation. Only competitive weeds were removed periodically to ensure that the root stem development process was not affected by differences in nutrient supply, thereby accurately reflecting their spontaneous characteristics. A two-year repeated experimental design was adopted: in the first year, systematic monitoring during the reproductive period revealed that the morphogenesis of the characteristic root stem structure, the silkworm-like ring, was concentrated in late summer and early autumn; in the second year, high-frequency sampling was carried out during the critical morphogenesis window period to accurately capture its dynamic formation process. Plant sample collection involves two key periods during rhizome development that exhibit significant phenotypic differences: (1) the seedling stage (T1A), in which the underground tissues of seedlings are in the early growth stage following seed germination; (2) the maturity stage (T2), in which the underground parts of plants are collected at the end of summer and further subdivided into rhizomes with ring structures (T2RH), in which rhizome tissues form characteristic silkworm-like rings (note that this structure is unique to rhizomes); and the root system (T2RO), in which the root tissues and complete underground organs (T2A) include whole samples containing both rhizomes and root systems. All fresh samples were immediately frozen in liquid nitrogen after collection and stored at -80°C in an ultralow-temperature environment for subsequent transcriptomic sequencing and metabolomic analysis.

## 2.2 Morphology and Anatomy of *N. incisum*

To analyze the spatiotemporal characteristics of rhizome morphogenesis, high-frequency monitoring was conducted during the critical developmental window (late summer to early autumn). Rhizome samples (n = 20) were systematically collected every 7 days from consistent growth sites, and the maximum transverse diameter of the rhizomes was measured via a digital caliper (accuracy  $\pm 0.01$  mm). Continuous time series data were used to construct dynamic diameter change curves to quantify the radial expansion patterns during the formation of the silkworm-like ring.

The root and root samples from typical developmental nodes were selected. The samples were fixed at room temperature for 48 hours in FAA fixative, dehydrated with graded ethanol, clarified with xylene, and embedded in paraffin. A Leica RM2265 rotary microtome was used to prepare 5  $\mu$ m serial transverse sections. After the sections were spread and dried, tissue-specific staining was performed with 0.1% toluidine blue (pH 4.0) to reveal the structural differentiation characteristics of the cortex and vascular tissue. The stained sections were mounted with neutral balsam and imaged under a Leica DMLB compound optical microscope (40 $\times$  objective, 10 $\times$  eyepiece) in brightfield mode. Five random fields of view were selected from each sample, and the following quantitative measurements were performed via the SlideViewer 3.0 image analysis system: cortex area (from the inner cortex to the epidermis), vascular column area (including phloem, xylem, and cambium), and calculation of the cortex/vascular area ratio

(C/V value) to characterize the tissue allocation pattern during organ development. All measurement data were averaged from three replicates.

### 2.3 Metabolomic analysis of *N. incisum*

The plant samples were rapidly frozen in liquid nitrogen, freeze-dried under vacuum conditions at -50°C for 48 hours, and then homogenized via a ball mill (30 Hz, 90 s). To extract metabolites, 50 mg of freeze-dried powder was weighed and extracted with 1.2 mL of prechilled extraction solution (70% methanol aqueous solution containing 0.1% formic acid and 0.1 mg/L reserpine as an internal standard). The extraction process was carried out through six cycles of shaking (30 seconds of vortexing followed by 30 minutes of ice bath cooling each time). After centrifugation (12,000 ×g, 10 minutes, 4°C), the supernatant was collected, filtered through a 0.22 µm microporous membrane, and stored at -80°C for chromatographic–mass spectrometric analysis. Six replicates of each biological sample were performed.

A complementary analytical strategy was employed to address the metabolic characteristics of different developmental stages and organs: nontargeted metabolomics analysis was applied to samples from the seedling stage (T1A) and mature plants (T2A) via an ultrahigh-performance liquid chromatography–quadrupole time–of–flight mass spectrometry system (UHPLC-Q-TOF MS; Thermo Vanquish-Bruker Impact II HD). Chromatographic separation was performed via a Phenomenex Kinetex C18 column (2.1 × 100 mm, 1.7 µm) at a flow rate of 0.3 mL/min with gradient elution: the initial 5% B phase (0.1% acetic acid in acetonitrile) was maintained for 1 min, linearly increased to 95% B over 14 min, held for 2 min, and then returned to the initial ratio over 2 min. Mass spectrometry data were acquired in dual mode ( $m/z$  50–1500) with optimized ion source parameters: capillary voltage  $\pm 3.5$  kV, desolvation oven temperature 350°C, and cone gas flow rate 50 L/h. Broad targeted quantitative analysis focused on specific metabolites in the rhizome (T2RH) and root system (T2RO) via the UHPLC-QqQ MS platform (SCIEX ExionLC-6500+). Multiple reaction monitoring (MRM) was employed, with retention times validated via a plant metabolite standard library and collision energy optimized (10–50 eV). The gradient program was compressed to 15 min to increase throughput: 5% B phase within 0.5 min, linear increase to 100% B over 11.5 min, and maintenance for 1.5 min, followed by rapid equilibration. The ionization conditions were set as follows: ion spray voltage  $\pm 5.5$  kV/-4.5 kV, source temperature 500°C, and curtain gas 35 psi.

The raw data were processed via XCMS software for peak extraction and alignment (quality deviation  $\pm 10$  ppm, retention time window 0.3 min). Differentially abundant metabolite screening was performed via the MetaboAnalyst 5.0 platform. First, an orthogonal partial least squares discriminant analysis (OPLS-DA) model was constructed, with a significance threshold of a VIP value  $> 1.0$  and  $p < 0.05$ . Subsequently, pathway enrichment analysis was performed via the KEGG Plant Metabolism Database (hypergeometric test, FDR-corrected  $p < 0.01$ ). The quality control samples were interspersed throughout the analysis to monitor system stability, ensuring that the relative standard deviation of the peak areas was  $< 15\%$ .

## 2.4 Transcriptomic analysis of *N. incisum*

Total RNA extraction was performed via frozen root stem tissue (ground in liquid nitrogen), which was separated via a plant RNA purification kit (Qiagen RNeasy Plant Mini Kit), and RNA integrity was assessed via an Agilent 2100 Bioanalyzer. Library construction was performed via the Illumina TruSeq Stranded mRNA LT Kit, which involves poly-A-enriched mRNA fragmentation, reverse transcription, and double-end adapter ligation. Sequencing was conducted on the Illumina NovaSeq 6000 platform in 150 bp double-end read length (PE150) mode, yielding an average of 6 Gb of raw data per sample. The raw sequences were quality controlled via Trimmomatic (v0.39): adapter sequences were removed (ILLUMINACLIP:2:30:10), low-quality bases were filtered via a sliding window (SLIDINGWINDOW:4:20), and reads shorter than 50 bp were excluded. High-quality sequences were assembled de novo via Trinity (v2.13.2) with the following optimized parameters: k-mer length, 25; minimum overlap length, 50 bp; and generation of nonredundant transcripts as reference sequences.

Differentially expressed genes (DEGs) were screened using a significance threshold of  $|\log_2FC| > 1$  and a P value  $< 0.05$ . Functional annotation was performed through database analysis. Homologous protein identification: BLASTX alignment based on the Nr database (E value  $< 1e-5$ ); functional domain analysis: Annotation of orthologous groups (COG/KOG) and metabolic pathways via eggNOG (v5.0); protein functional localization: mapping to the Swiss-Prot database to obtain manually verified functional descriptions; pathway enrichment: classification of metabolic and signaling pathways via the KEGG Orthology (KO) system; and ontology annotation: GO term enrichment analysis (Fisher's exact test, FDR  $< 0.05$ ) to reveal the biological processes, molecular functions, and cellular component characteristics of DEGs.

To investigate the dynamic patterns of differentially expressed genes, we first used online tools to perform cluster analysis on the basis of their expression profile trends, dividing them into different clusters with similar expression patterns. For each significantly enriched gene cluster, we subsequently performed functional enrichment analysis via the KOBAS online platform to identify significantly enriched biological functions and signaling pathways in databases such as KEGG pathways (significance criteria:  $P < 0.05$  and FDR corrected).

Systematic family classification statistics of differentially expressed genes were performed via databases such as PlantTFDB and Pfam to clarify their functional group distribution characteristics. The coexpression analysis module of the Micro-Bio Alliance Cloud Platform ([www.microbioinfo.com](http://www.microbioinfo.com)) was subsequently used to construct a gene interaction network on the basis of Pearson correlation coefficients (threshold  $> 0.8$ ). Finally, the network topology structure was visualized via Cytoscape software (v3.9.1), and the built-in tools were used to analyze the core hub genes (Hub genes).

## 2.5 qRT–PCR analysis

Total RNA was extracted from plant leaves via a kit, followed by quality verification via electrophoresis and spectrophotometry. Reverse transcription was performed with a Quansijin Bio Kit to synthesize

cDNA. qPCR was conducted via SYBR Green premix with gene-specific primers under standard cycling conditions. Relative expression levels were calculated via the  $2^{-\Delta\Delta CT}$  method on the basis of recorded CT values.

## 2.6 Multiomics analysis

To analyze the gene–metabolism regulatory network during silkworm-like ring development, this study adopted a multiomics integration strategy to analyze key pathways systematically. Spearman rank correlation analysis was used to quantify the associations between DEGs and DAMs, generating a correlation heatmap. The gene expression and metabolite concentration matrices were normalized via min–max normalization, and Ward's D2 hierarchical clustering with Euclidean distance measurement was employed for clustering. On the basis of the KEGG database, pathway enrichment mapping was performed for differentially expressed genes and differentially accumulated metabolites. Significantly enriched pathways were screened via the hypergeometric test (phyper function) ( $p < 0.01$ , FDR-corrected). For highly enriched metabolic pathways, a gene–metabolite joint heatmap was constructed via z score standardization to display the synergistic change patterns of DEGs and DAMs across developmental stages (T1A/T2A/T2RH/T2RO).

# 3 Results

## 3.1 Dynamic characteristics of the rhizome morphology of *N. incisum*

This study, through two years of dynamic observation (2023–2024), revealed the developmental process of the locally formed morphological feature of the silkworm-like ring in the rhizomes of *N. incisum*. This feature is a prominent ring-shaped structure that appears in underground rhizomes at a specific stage of development (Fig. 1). The silkworm-like ring is formed by extremely shortened internodes and significantly enlarged stem nodes arranged alternately and closely in a horizontal direction, resulting in a ring-like appearance. The protruding parts correspond to enlarged stem nodes, where root traces, buds, and leaf scars can be observed; the concave areas correspond to shortened internodes, which have the typical primary structure of stems but lack accessory structures such as root traces. The regular compression and accumulation of this “swollen node-shortened internode” unit formed the unique root stem morphology known as the silkworm-like ring. According to observations of the development process, 0–75 days after germination (early development), the diameter of the underground rhizome slowly increases to 1.65 mm, with only primary structures present. During the rapid expansion period, vascular cambium activation drives secondary xylem proliferation, with the diameter significantly increasing to 2.8 mm ( $p < 0.05$ ); 126–139 days (beginning of autumn) is the critical period for the differentiation and maturation of the silkworm-like ring morphology, with the root stem diameter significantly thickening to 4.6 mm (Fig. 1B, Table S1). The unique climatic conditions of late summer and early autumn trigger the activity of the cork cambium, which produces 5–8 layers of highly corkified cells (stained with toluidine blue); the mechanical pressure generated by the rapid proliferation of secondary xylem and the programmed death of cortical cells together leads to the formation of cavities in the

cortical region. Microscopic quantitative analysis revealed that the proportion of vascular tissue in the silkworm-like ring region (5.0%) was significantly greater than that in the roots of the same plant (1.5%), whereas the proportion of the cortex decreased significantly to 0.95%. This finding indicates that the imbalance between vascular tissue and the cortex in secondary growth is the structural basis for the formation of ring patterns on the rhizome.

Dynamic observations of silkworm-like ring morphology (A) and root stem diameter statistics (B), root stem-root organ anatomical section strategy and microstructure (C), silkworm-like ring enlarged area/depressed area and root cross-sectional area (D), and cortex area/cross-sectional area ratio statistics (E).

### 3.2 Spatiotemporal heterogeneity of secondary metabolism in *N. incisum*

UPLC–MS/MS nontargeted metabolomics analysis revealed significant metabolic reprogramming in the underground organs of *N. incisum* during the critical period of secondary growth from July (T1A group) to August (T2A group) (Fig. S1–S2, Fig. 2), with a total of 1,219 DAMs identified (Table S2). This process is characterized by the synergistic regulation of secondary metabolites and amino acid pathways: Coniferaldehyde (id 25,  $\text{LOG}_2\text{FC}=0.43$ ) and coniferin (id 193,  $\text{LOG}_2\text{FC}=0.22$ ) are upregulated in the accumulation of lignin precursors, which is directly associated with the extremely significant enrichment of the phenylpropanoid biosynthesis pathway ( $p=1.22\times10^{-8}$ ); simultaneously, the increased accumulation of l-tryptophan (id 694,  $\text{LOG}_2\text{FC}=2.56$ ) and l-proline (id 18,  $\text{LOG}_2\text{FC}=5.99$ ) is coupled with the systematic activation of amino acid biosynthesis pathways (e.g., valine/leucine/isoleucine pathway enrichment factor 0.39,  $p<10^{-7}$ ) and synergistically enhances aminoacyl-tRNA biosynthesis ( $p=3.34\times10^{-8}$ ) and D-amino acid metabolism ( $p=1.29\times10^{-8}$ ), collectively driving protein synthesis demand. Energy metabolism reprogramming is characterized by the downregulation of succinic acid (id 2,  $\text{LOG}_2\text{FC} = -1.84$ ) and raffinose (id 281,  $\text{LOG}_2\text{FC} = -1.89$ ) accumulation, which, together with 2-oxoglutarate metabolism ( $p = 1.17 \times 10^{-5}$ ), promotes the transfer of carbon sources to secondary growth, whereas the upregulation of transferulic acid (id 49,  $\text{LOG}_2\text{FC}=1.20$ ) accumulation synergizes with the enrichment of the ABC transporter ( $p=1.45\times10^{-7}$ ) and flavonoid biosynthesis ( $p=7.27\times10^{-5}$ ) pathways, highlighting the integrated strategy of nitrogen metabolism and secondary metabolism in summer stress responses.

To analyze the mechanism of organ functional differentiation, a broad-target metabolomics analysis of August rhizomes (T2RH group) and roots (T2RO group) (Fig. S1–S2, Fig. 2) identified 380 DAMs, of which 50.3% were flavonoids, coumarins, simple phenylpropanoids, and terpenoids (Table S3). Metabolite accumulation and pathway enrichment jointly reveal organ functional specialization: The upregulation of the accumulation of coniferaldehyde (A3D0,  $\text{Log}_2\text{FC} = +1.26$ ) and transferulic acid (A3D1,  $\text{Log}_2\text{FC} = +0.30$ ) in roots is directly associated with their specific enrichment in the phenylpropanoid biosynthesis pathway ( $p = 0.002$ ), whereas the accumulation of delphinidin (A0D9,  $\text{Log}_2\text{FC} = +1.62$ ), okanin (A0F0,  $\text{Log}_2\text{FC} = +1.24$ ), and other phytoprotective compounds are synergistically associated with secondary metabolite biosynthesis pathways (involving 28 compounds,  $p = 0.043$ ) and taurine metabolism ( $p = 0.021$ ), collectively driving root stress resistance functions; the significant accumulation

of pinoresinol diglucoside (B8S8,  $\text{Log}_2\text{FC} = -2.23$ ) and quercetin (A3R2,  $\text{Log}_2\text{FC} = -0.85$ ) in the rhizomes is coupled with the specific enrichment of the flavonoid/flavonol biosynthesis pathway (enrichment factor 0.098,  $p = 0.0001$ ), collectively driving their role in storing bioactive compounds. Hormone network differentiation further reinforces organ specialization: the accumulation of abscisic acid (B1Z8,  $\text{Log}_2\text{FC} = +0.63$ ) and 5'-deoxy-5'-methylthioadenosine (A3K9,  $\text{Log}_2\text{FC} = +1.81$ ) in roots is upregulated in synergy with the activation of the zeatin synthesis pathway ( $p = 0.006$ ), which jointly regulates vascular maturation. Moreover, the root stem maintains meristematic activity through zeatin-riboside (A3T8,  $\text{Log}_2\text{FC} = -0.51$ ), which maintains meristematic activity, whereas organ-specific enrichment of the ABC transporter pathway ( $p = 0.009$ ) supports the root's advantage in nutrient transport.

Differentially abundant metabolite volcano plot for T1A vs. T2A (A) and T2RH vs. T2RO (B); DMA classification characteristics between T1A vs. T2A (C) and T2RH vs. T2RO (D); heatmap of metabolite clusters showing differences between T1A vs. T2A (E) and T2RH vs. T2RO (F); KEGG pathway enrichment of T1A vs. T2A (G) and T2RH vs. T2RO (H).

The metabolic shifts from July to August are characterized by the activation of the phenylpropanoid pathway and the accumulation of defense compounds. The metabolic profiling of rhizomes and roots in August revealed the physiological basis for secondary growth (enhanced lignification) and organ functional specialization (rhizome enrichment of defense compounds and root maintenance of hormonal regulation). These spatiotemporal differences in metabolic trajectories provide molecular-level evidence for understanding the morphogenesis of *N. incisum* rhizomes.

### 3.3 Transcriptomic analysis of the gene regulatory network of *N. incisum*

RNA was extracted from samples used for metabolomics analysis and analyzed via RNA-seq. The raw read counts in the samples ranged from 40,273,901 to 48,120,703. After low-quality reads were removed, the Q30 score for all samples was greater than 94%, indicating that high-quality gene sequencing results were obtained for downstream analysis (Table S4). A total of 125,158 assembled single genes with an average length of 886.81 bp were compared with multiple databases, including NR, GO, KEGG, eggNOG, Swiss-Prot, and Pfam, to annotate the functions of the single genes, among which 67,481 single genes were located in at least one database (Table S5, Fig. S3).

In the PCA, the biological replicate samples clustered together, indicating low variability in the unigene spectrum (Fig. S4). Using thresholds of absolute  $\log_2$ -fold change ( $\text{FC}$ )  $\geq 1$  and adjusted  $p$  value  $< 0.05$ , 24,461 differentially expressed genes (DEGs) were identified in the comparison between T1A and T2A, and 12,289 DEGs were identified in the comparison between T2RH and T2RO (Fig. 3A).

On the basis of a systematic analysis of the high-dimensional correlation coefficient matrix between samples (Fig. 3B), the transcriptomics data revealed clear spatiotemporal patterns in the development of *N. incisum* underground organs: the technical replicate samples presented extremely strong intragroup correlations, confirming detection stability. In the temporal dimension, the July samples (T1A) and August samples (T2A) presented significant negative correlations (T1\_2 vs T2\_5:  $r=0.1797$ ), indicating

intense metabolic reprogramming between July and August. In the organ dimension, the August rhizomes (containing the silkworm-like ring, T2RH) and the same plant roots (T2RO and T2A roots) presented structural separation in their metabolic profiles (T2RH\_1 vs T2RO\_1:  $r=0.1270$ ), whereas the August rhizome samples (T2RH and T2A) presented high intergroup clustering (T2RH\_1 vs T2\_2:  $r=0.4325$ ), including the root samples (T2RO and T2A roots), which formed their own cluster (T2RO\_5 vs T2A\_5:  $r=0.0489$ ). Additionally, the degree of organ differentiation in August (T2RH vs T2RO average  $r=0.13$ ) significantly exceeded that in July (T1A group-internal organ-to-organ  $r>0.991$ ). This spatiotemporal coupling of metabolic trajectory differences—namely, global reprogramming in July–August and rapid differentiation between rhizomes and roots in August—statistically confirms the synchrony between morphological development and metabolic restructuring during the formation of the Silkworm-Like Ring.

To systematically explore the biological functions of the DEGs potentially involved in the underground parts of *N. incisum* at different growth stages, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the DEGs generated from paired comparisons between different groups (Fig. 3C, D). The analysis revealed significant metabolic reprogramming related to developmental stage transition and organ functional differentiation. During the transition from July to August, the overall upregulated genes in the underground parts of August plants were significantly enriched in pathways regulating developmental processes (plant hormone signal transduction), enhancing structural defense (phenylpropanoid biosynthesis), and storing energy (starch and sucrose metabolism, diterpene biosynthesis), whereas the downregulated genes indicated a reduction in basal metabolic activities (such as protein synthesis represented by ribosomes and membrane lipid metabolism represented by unsaturated fatty acid biosynthesis). In particular, the comparison of rhizomes and roots within the underground organization of August revealed the rhizome-specific functions underlying the formation of the silkworm-like ring: rhizome tissue (as the substrate for silkworm-like ring formation) exhibited significant upregulation of DNA replication, recombination, and repair pathways, providing the core molecular basis for active cellular proliferation in the silkworm-like ring region; simultaneously, the upregulation of carbohydrate storage-related pathways (starch and sucrose metabolism) in the root stem further indicated that it serves as the essential energy source for the expansion and accumulation of the silkworm-like ring. In sharp contrast, pathways related to support structure formation and defense (benzene propane biosynthesis, flavonoid biosynthesis), nutrient absorption and assimilation (nitrogen metabolism, alanine/aspartate/glutamate metabolism), and the stress response ( $\alpha$ -linolenic acid metabolism) were significantly upregulated in root tissues during the same period.

This high degree of spatiotemporal coordination between gene expression and metabolite accumulation, such as the universal activation of the phenylpropanoid pathway, stage-specific reprogramming of energy metabolism, and organ differentiation of DNA replication and starch metabolism, has established a multiomics evidence-based regulatory network for the secondary development of underground organs in *N. incisum*.

### 3.4 Expression trends of DEGs in different growth stages and organs of *N. incisum*

To assess gene expression patterns during development, differentially expressed genes were grouped into clusters on the basis of their expression patterns, yielding gene clustering results (Fig. 4, Table S6). During the critical developmental stage of silkworm-like ring formation, cluster 8 genes presented the lowest expression levels in undifferentiated underground organs (T1A) in July but were significantly upregulated by August during the silkworm-like ring formation stage in fully developed underground organs (T2A); these genes reached their expression peak in root stem tissues with silkworm-like ring structures (T2RH), whereas their expression levels significantly decreased in root tissues without silkworm-like ring structures (T2RO). These findings indicate that the expression intensity of these genes is correlated with the developmental maturity of the silkworm-like ring structure.

Among the 8 genes exhibiting the expected expression pattern, functional enrichment analysis revealed their significant involvement in core biological processes related to energy metabolism regulation and signal transduction: the genes were highly enriched in the “metabolic pathways” (ath01100, corrected  $P = 0.018$ ), indicating that this gene cluster is widely involved in the regulation of fundamental material and energy metabolism networks; the synergistic enrichment of “phosphatidylinositol signaling system” (ath04070) and “inositol phosphate metabolism” (ath00562) (corrected  $P = 0.028$ ) suggested that these genes regulate cellular stress responses by modulating phospholipid-derived second messengers (such as IP3, PIP2, etc.); the core genes FAB1D (phosphatidylinositol kinase) and PTEN1 (lipid phosphatase) simultaneously drive the aforementioned pathways, suggesting their critical regulatory role in maintaining the dynamic balance of the membrane signaling microenvironment; “purine metabolism” (ath00230, corrected  $P = 0.032$ ) enriched the genes APT5 (adenosine phosphate transferase) and APY2 (pyrophosphatase), indicating their involvement in ATP synthesis and maintenance of nucleotide pool homeostasis; and the enrichment of the ubiquinone (ath00130) and porphyrin (ath00860) pathways further supported that this gene cluster supports energy conversion

### 3.5 Regulatory network of DEG families involved in the formation of silkworm-like rings

By integrating developmental dynamics (T1A: presilkworm-like ring stage; T2A: silkworm-like ring stage) and tissue specificity (T2RH: rhizome; T2RO: root) coexpression networks (Fig. 5, Table S7), the core regulatory mechanism underlying silkworm-like ring formation was revealed: During the T2A stage, the Whirly family hub gene TAR1 initiates ring formation by activating the cell wall synthesis gene UXS4 ( $r = 0.858$ ,  $p = 1.08E-08$ ) and inhibiting the RNA-binding protein CHIC ( $r = -0.872$ ,  $p = 3.03E-09$ ); the MYB family member BABL shows a strong positive correlation with the stress response factor ERF71 ( $r = 0.897$ ,  $p = 2.44E-10$ ), integrating environmental signals with developmental processes; the ERF family member PIP14 promotes the expression of the cell proliferation gene RAB1C ( $r = 0.866$ ,  $p = 5.53E-09$ ) by activating the MYB-related gene CAP9 ( $r = 0.699$ ,  $p = 4.99E-05$ ).

In the root stem tissue (T2RH) that forms the silkworm-like ring, the CHIT gene from the NAC family is strongly positively correlated with the LAC9 gene from the ERF family ( $r = 0.763$ ,  $p = 3.71E-06$ ). These two genes synergistically drive lignin deposition, directly participating in the secondary lignification process of vascular tissue and providing mechanical support for the ring-shaped structure of the

silkworm-like ring. Additionally, the NF-YB family gene 7DLGT further enhances this pathway by positively regulating CHIT ( $r = 0.804$ ,  $p = 4.37E-07$ ), ensuring the spatial specificity of the lignification process. Most importantly, a unique NAC-CHIT→ERF-LAC9→G2-like-ARAK cascade pathway (average correlation coefficient  $r=0.87$ ) is present in the root stem and is completely absent in the root tissue (T2RO). Genes related to lignin synthesis (GO:0009809) were significantly enriched in this network (FDR=3.4E-06). High-intensity regulatory relationships ( $|r| > 0.8$ ) are enriched 3.2-fold in the rhizome (Fisher's test  $p = 0.002$ ), directly supporting the functional specialization of rhizome tissue in achieving ring-like structure formation through the reinforcement of lignification. In root tissue (T2RO), ERF family members are more inclined to participate in the regulation of pathways related to defense substance synthesis and nutrient absorption: the phenylpropanoid/flavonoid synthesis and nitrogen assimilation pathways enriched in roots are coupled with the expression patterns of ERF family stress response factors, which integrate environmental signals to activate the synthesis of phytoalexins, thereby enhancing the root stress defense functions; simultaneously, the activation of the abscisic acid and brassinosteroid synthesis pathways in roots is also associated with the regulation of hormone signaling by ERF family genes, ensuring the functional specialization of root nutrient absorption and vascular maturation. This NAC-ERF transcription hub exhibits differential expression and regulatory network separation between rhizomes and roots, directly driving the functional polarization of rhizomes toward "lignification ring formation and energy storage" and that of roots toward "defense substance synthesis and nutrient assimilation," serving as the core molecular basis for organ functional differentiation. In rhizomes, NAC-CHIT specifically upregulates ERF-LAC9, thereby regulating the G2-like-ARAK cascade pathway (average Pearson correlation coefficient  $r = 0.87$ ,  $p < 0.01$ ). This pathway is completely absent in root tissues. The T2RHvT2RO network was significantly enriched with lignin synthesis-related genes (GO:0009809, FDR=3.4E-06), and high-strength edges with  $|r|>0.8$  were enriched 3.2-fold in T2RH (Fisher's exact test  $p=0.002$ ), confirming that ring formation depends on root-stem-specific transcriptional programming.

### 3.6 Validation of RNA-Seq Data by qRT-PCR

To validate the accuracy of the RNA-Seq data, we selected four DEGs (AB11B, DTX40, JAL1, and U76F1) for qRT-PCR analysis. Our results indicated that the levels of expression of the chosen genes were largely congruent with the expression levels in the transcriptome, indicating that the results of the transcriptome sequencing analysis were reliable (Fig. 6).

### 3.7 Key pathways involved in the development of *N. incisum*

Multomics analysis revealed the synergistic reconstruction of basic metabolic and secondary metabolic networks during the development of the silkworm-like ring. At the basic metabolic level, the histidine metabolic pathway and the ABC transporter system form a spatiotemporally coupled regulatory network. A time series dynamic analysis revealed that HSN7, a key enzyme in His synthesis, is highly expressed in undifferentiated underground tissues (T1A) in July, where it drives the initial synthesis of His. However, after entering the formation period (T2A) in August, the expression of this gene is completely silenced, indicating the closure of the synthesis pathway. Moreover, the expression level of the ABC transporter

ABCB9 doubled (48.3 → 96.6 TPM), and ABCB11 was significantly upregulated (178.1 TPM, a 107% increase compared with T1A). The concentration of the metabolite C00135 (histidine) increased from 0.0001 nmol/mg in T1A to 0.0016 nmol/mg in T2A. The organ distribution characteristics further indicate that the histidine concentration in the root stem region (T2RH) reached a peak of 0.0071 nmol/mg, which was strongly positively correlated ( $r = 0.98$ ,  $p < 0.001$ ) with the highly specific expression of ABCB11 in this region (582.8 TPM), confirming the root stem-directed enrichment mechanism mediated by ABCB11 (Fig. 7, Table S8). Notably, with increased transport, the histidine-degrading enzyme BALDH was activated in the T2A phase (0→26.04 TPM), promoting the accumulation of the downstream metabolite C00137 (carnosine) in T2RH (0.00110 nmol/mg, a 64.2% increase compared with that in T1A). This cascade reaction of “synthetic shutdown-transport enhancement-degradation activation” achieves the redistribution of nitrogen sources to functional organs while enhancing biological stress defense capabilities through the accumulation of carnosine.

At the secondary metabolism level (Fig. 8, Table S9), the phenylpropanoid and flavonoid synthesis pathways exhibit developmentally dependent activation and organ functional differentiation characteristics. The PAL1 gene encoding the rate-limiting enzyme in the phenylpropanoid pathway presented a 35.1-fold increase in expression during the T2A phase, and the downstream 4CL2 gene expression was simultaneously upregulated by 12.5-fold (reaching 3983.4 TPM), driving the accumulation of the lignin monomer precursor coniferone (C01494) in the root stem (T2RH: 0.0134 nmol/mg) and roots (T2RO: 0.0165 nmol/mg), representing an increase of more than 100-fold compared with the T1A basal level (0.0001 nmol/mg). In sharp contrast, although the key enzyme in flavonoid synthesis, CHS1, was upregulated by 39.6-fold in the T2A phase, the concentrations of the flavonoid aglycones apigenin (C10028) and quercetin (C00389) were significantly reduced in the rhizome region (T2RH), whereas they remained stable in the root region (T2RO). Multiomics correlation analysis revealed that this spatial heterogeneity originated from the specific expression of the glycosyltransferase At4g26220 in the roots (4.78 TPM) and the suppression of hydroxycinnamoyltransferase HCT expression in the root stems (T2RH: 13.4 TPM → T2RO: 92.4 TPM). This hierarchical regulation enables the precise localization of defense substances—the rhizome accumulates coniferone aldehyde to strengthen cell wall lignification (mechanical support function), whereas the root retains a pool of flavonoid aglycones to respond to oxidative stress (chemical defense function).

In summary, silkworm-like ring development achieves a dual adaptation strategy through multimodule metabolic reprogramming: basal metabolic reorganization (histidine transport–degradation axis) optimizes nitrogen source allocation efficiency, and secondary metabolic partitioning (phenylalanine–flavonoid diversion axis) establishes an organ-specific defense system, providing a synergistic molecular basis for underground organ morphology construction and ecological adaptation.

## 4 Discussion

The silkworm-like ring structure of *N. incisum* rhizomes is characterized by extremely shortened nodes and internodes, which form silkworm-like ring patterns. This phenomenon is the ultimate manifestation of the coupling between morphological development and secondary metabolism in terms of time and space. This study integrates morphological dynamics, metabolic reprogramming, and transcriptional regulation to reveal that the core formation mechanism is resource allocation and organ function polarization triggered by environmental signals. Unlike the homogeneous enlargement pattern of root tubers such as *Ipomoea batatas*<sup>[16]</sup>, the formation of the silkworm-like ring is essentially the result of the precise differentiation of vascular tissue and the cortex during development. This differentiation process involves strict phenological coupling, such as the correlation between hypocotyl growth in *Arabidopsis* and the light–temperature cycle<sup>[17]</sup>. This study revealed that the beginning of autumn triggers periodic activation of the cork cambium, producing a multilayered corkified "ring" structure. This process coincides with the critical period of underground organ material accumulation, and the phenomenon of secondary xylem cell proliferation squeezing the cortex to form a hollow space confirms the resource reallocation strategy of "shortening internodes and expanding radially" in plant evolution—allocating limited resources to vascular tissue (mechanical support function) rather than cortical storage tissue<sup>[18]</sup>  
[19].

Metabolic reprogramming provides a molecular-level explanation for morphological differentiation. During the transition period from July to August, the spatiotemporal dynamics of 1,219 DAMs showed significant regularity: in the temporal dimension, the phenylpropane pathway became the main channel for carbon flow allocation, and the explosive accumulation of coniferone and coniferone glycoside provided building materials for secondary wall deposition, which is consistent with the classic regulatory pattern of the lignin biosynthesis pathway<sup>[20][21]</sup>; the simultaneous decline in basic energy metabolites such as succinic acid echoes the core principle of resource competition and redistribution in the "source–sink" theory<sup>[22]</sup>. In terms of organ dimensions, the functional polarization of roots and rhizomes is particularly prominent, similar to the compartmentalization of ginsenoside synthesis in the roots of *Panax ginseng*<sup>[23]</sup>. This study revealed that rhizomes specifically accumulate cell wall strengthening factors (such as pinoresinol diglucoside), whereas roots shift to phytochemical synthesis (such as anthocyanin accumulation) and nitrogen metabolism pathway activation. This differentiation is precisely regulated by hormone gradients—the mechanism by which cornusol nucleoside maintains meristem activity in rhizomes is highly similar to the mode of action of cytokinins in potato tuber development<sup>[24]</sup>.

Transcriptome analysis revealed that the development of underground organs in *N. incisum* has strict spatiotemporal reprogramming characteristics. RNA-seq data revealed that global transcriptional reprogramming occurs during the transition period from July (T1A) to August (T2A). During this phase, genes enriched in hormone signaling, phenylpropanoid synthesis, and starch metabolism are upregulated, driving structural reinforcement and energy storage; moreover, basic metabolic pathways such as ribosome synthesis are downregulated, indicating a shift in developmental focus toward secondary growth. At the organ differentiation level, the rhizome (T2RH) and root (T2RO) undergo structural separation. The rhizome significantly activates DNA replication and repair (supporting cell

proliferation) and starch metabolism (providing energy for enlargement), whereas the root is enriched in the phenylpropane/flavonoid synthesis and nitrogen assimilation pathways. Relationship between NACs and ERFs. The analysis of gene regulatory networks further revealed the command system for phenotype formation. The root-specific expression of the NAC-ERF-bHLH transcription hub constitutes a key molecular switch, and the function of NAC family genes in promoting lignification has been verified in studies on wood formation in poplar trees<sup>[25]</sup>. The regulation of lignification inhibitory factors by bHLH family genes has expanded the understanding of the role of this family in secondary growth. Notably, the enrichment intensity and tissue-specific expression pattern of this network in root stems confirmed, from a genetic perspective, that silkworm-like ring formation is a root stem-specific programming process. In terms of developmental timing, the modular relay regulation between the early material storage stage (starch-sucrose metabolism and activation of ABC transporters) and the late vascular expansion stage (progressive upregulation of the phosphatidylinositol signaling pathway) reproduces the common developmental patterns of storage organs in tuber crops<sup>[26]</sup>. The ERF-PIP14→MYB-CAP9→RAB1C cell proliferation axis revealed in this study shares similar regulatory patterns with the cascade amplification mechanism of the temperature response pathway in potato tuber formation<sup>[27]</sup>; however, the homology between the two has not yet been verified by molecular evolutionary analysis or functional complementation experiments. This time-sensitive constraint on signal transmission (premature activation leads to resource misallocation, whereas delayed activation makes it difficult to withstand cold damage) has driven *N. incisum* to develop a unique adaptive structure: multilayered ring-shaped cork tissue not only enhances mechanical resistance but also the insulating cavities it forms buffer freeze–thaw damage. The essence of this structure–metabolism dual-function design lies in the synergy between vascular tissue proliferation and metabolic product storage<sup>[28]</sup>.

The formation of the silkworm-like ring is essentially an evolutionary adaptation strategy of *N. incisum* to alpine habitats. Its 'extremely shortened internodes and thickened ring patterns' morphological structure achieves dual ecological benefits: on the one hand, the shortened internodes significantly reduce intercellular fluid mobility at low temperatures, enhancing cold tolerance by minimizing freeze–thaw damage; on the other hand, the periodic accumulation of lignified ring patterns (activated by the cork cambium in autumn) enhances the root stem shear strength, effectively resisting physical stress from strong high-altitude winds and gravel compression. Compared with *Stephania kwangsiensis*<sup>[29]</sup>, which achieves uniform expansion by inhibiting lignification, *N. incisum*'s strategy reinforces the lignification of the vascular system to construct a mechanical skeleton while sacrificing the formation of the cortex to create lightweight cavities that are more resistant to wind and frost heave stress in cold environments. Of particular note is the dual metabolic channelization phenomenon in the phenylpropanoid pathway: this pathway simultaneously points to the synthesis of structural components (lignin) and defense molecules (coumarins), providing a new paradigm for understanding the “morphological–metabolic coevolution” of high-altitude medicinal plants. Its mechanism is similar to metabolic flux partitioning regulation in the synthesis of flavonoid compounds<sup>[30]</sup>.

The remaining issues focus on two dimensions: environmental signal decoding and structural function quantification. The correlation patterns of environmental factors at the omics level need to be analyzed in combination with simulated experiments in artificial climate chambers. Moreover, the spatial expression pattern of phenylalanine synthase and the influence of the lignin monomer ratio on mechanical properties can be studied by referring to the biomechanical model of secondary cell walls in trees<sup>[31]</sup>. Future research should combine single-cell sequencing to construct a vascular microzone metabolic map<sup>[32]</sup> for single-cell analysis of *Arabidopsis* roots or use synthetic biology to reconstruct the NAC-ERF module<sup>[33]</sup> to elucidate the evolutionary driving forces behind the silkworm-like ring.

## 5 Conclusion

On the basis of these multiomics correlations, we propose that during the development of *N. incisum* rhizomes, carbon source allocation is directed toward the phenylpropanoid biosynthesis pathway from the basic energy metabolism pathway, which drives vascular system lignification rather than cortex expansion. This directed allocation is spatially specialized through the NAC–ERF–bHLH transcriptional hub, ultimately forming a heterogeneous structure in which the secondary xylem compresses the cortex. The increased lignification downstream of the phenylpropanoid pathway and the periodic activation of the cork cambium are synergistically triggered during the window of the beginning of autumn, directly promoting the formation of ring patterns on silkworm cocoons. On the basis of assumptions related to phenological periods, subsequent environmental control experiments are needed for verification. These findings provide a theoretical basis for the ecological cultivation (e.g., temperature and water-fertilizer regulation during temperature fluctuations) and quality improvement (targeted enhancement of coumarin accumulation in the ring pattern zone) of *N. incisum* herbal materials. Further validation is needed to elucidate the specific roles of environmentally signaled transcription factor cascades in the initiation of secondary growth.

## Declarations

### Acknowledgments

We thank all the authors for their contributions to this study.

### Author contributions

W.L.Z. and J.G performed the bioinformatics data analysis and wrote the manuscript. H.G.C. and J.B.Z. prepared the plant materials. W.W.L. assisted with bioinformatics data analysis. W.L.Z. and L.J. conceived the study and supervised the entire process. All the authors read and approved the final manuscript.

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### Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The raw RNA-seq data were deposited in the NCBI SRA database (accession number: PRJNA1300633).

The morphological image records and descriptive data of the *Notopterygium incisum* rhizomes that support the species identification have been deposited in the Figshare repository under the identifier 10.6084/m9.figshare.30811628.

### Ethics approval and consent to participate

All *Notopterygium incisum* materials used in this study were cultivated at the controlled experimental base of the project team in Tanchang County, Gansu Province, China, and were not collected from the wild. No specific permissions were required for plant collection.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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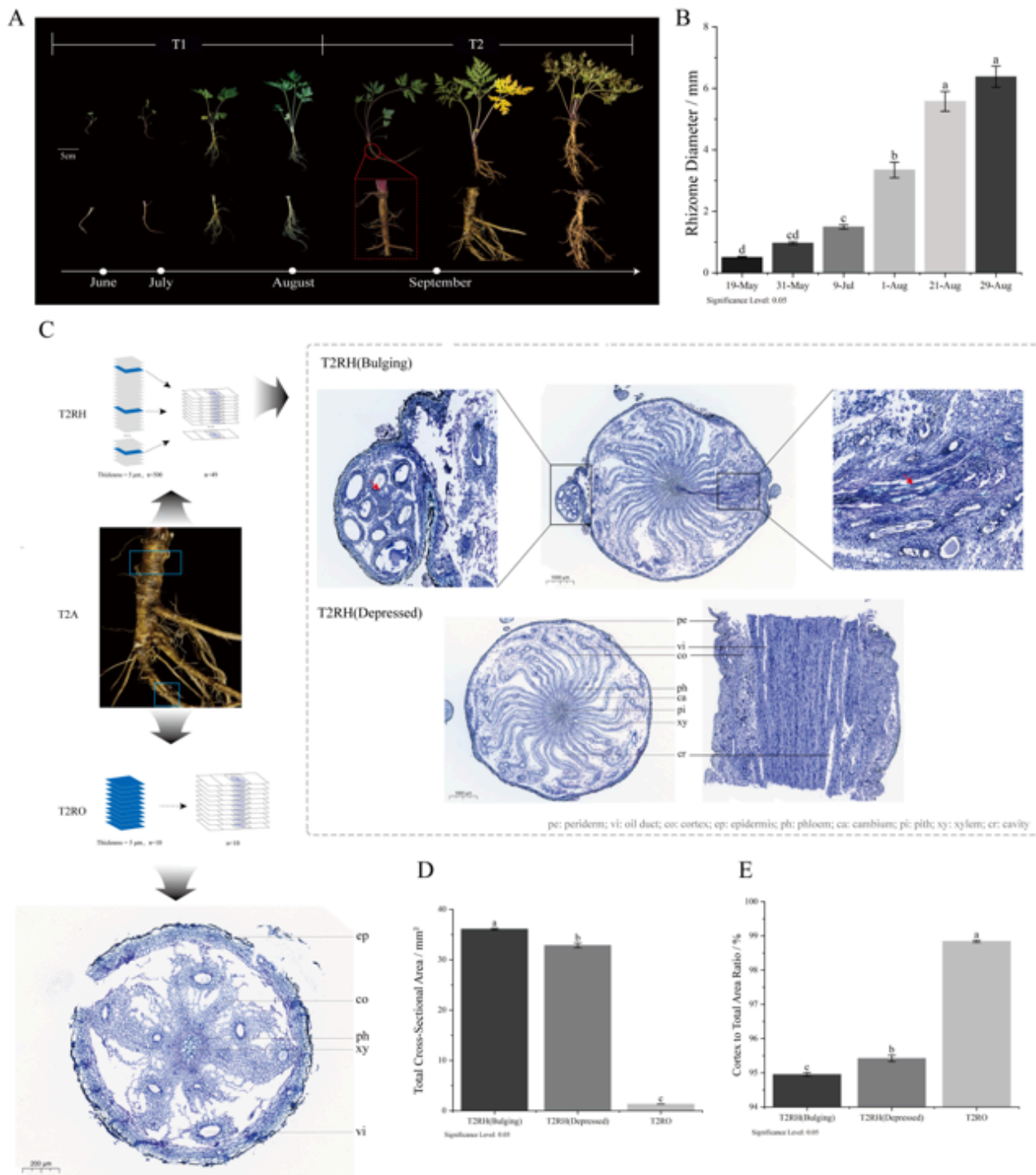
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## Figures



**Figure 1**

Morphogenesis and anatomical characteristics of the silkworm-like ring structure of the rhizome of *N. incisum*

Dynamic observations of silkworm-like ring morphology (A) and root stem diameter statistics (B), root stem-root organ anatomical section strategy and microstructure (C), silkworm-like ring enlarged

area/depressed area and root cross-sectional area (D), and cortex area/cross-sectional area ratio statistics (E).

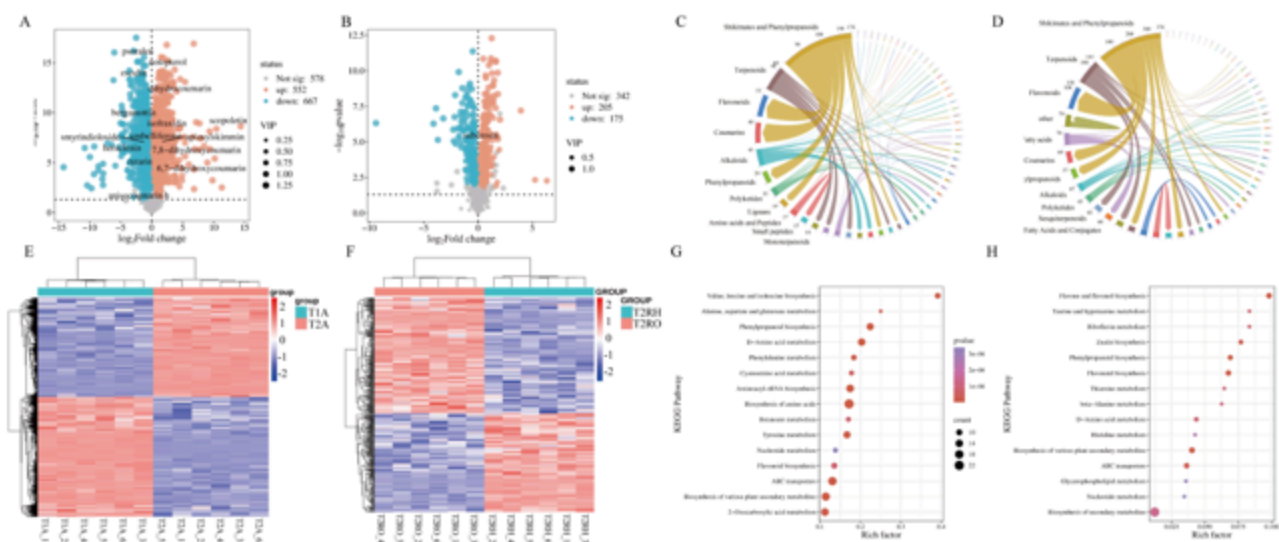
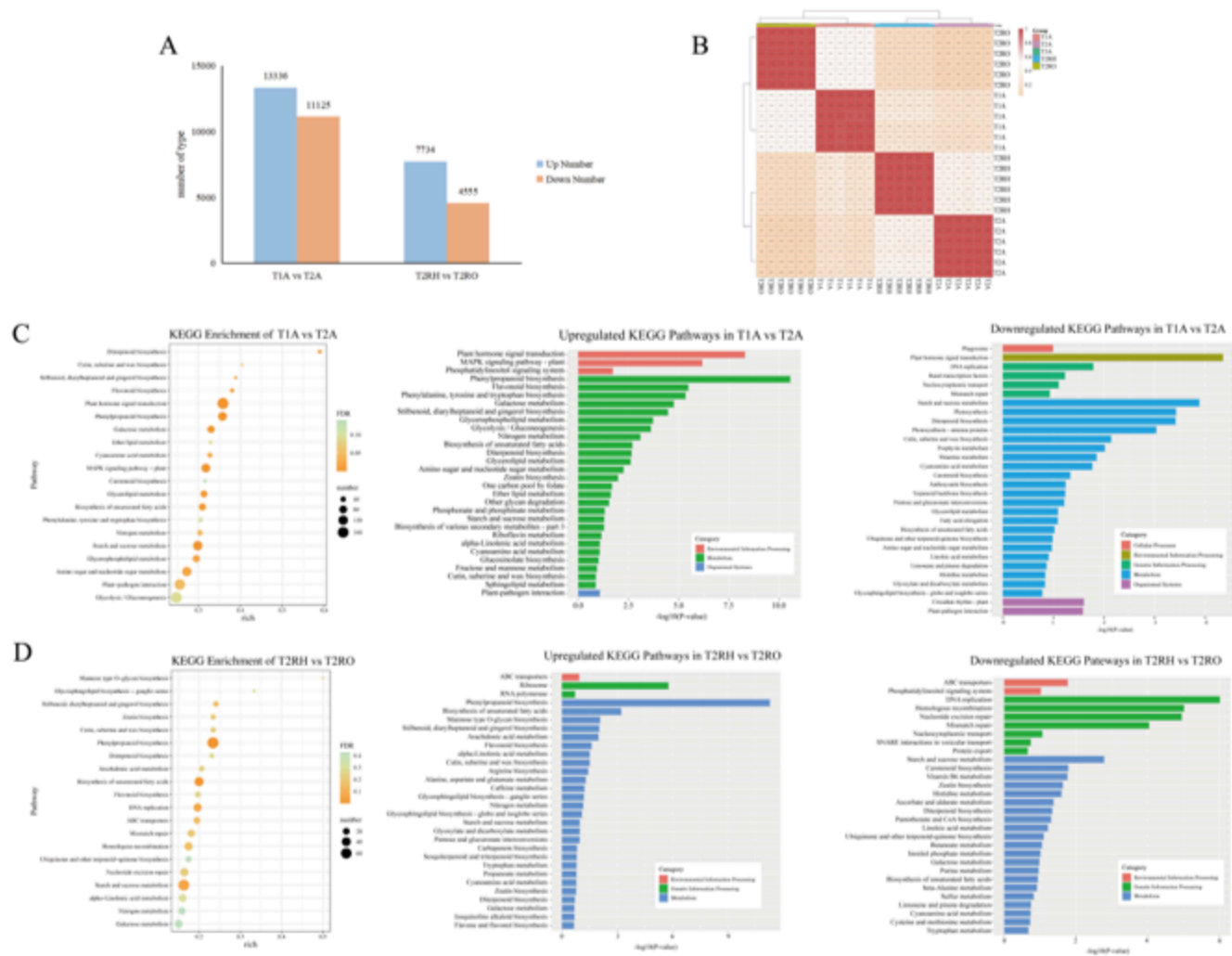


Figure 2

Metabolomic analysis of *N. incisum*

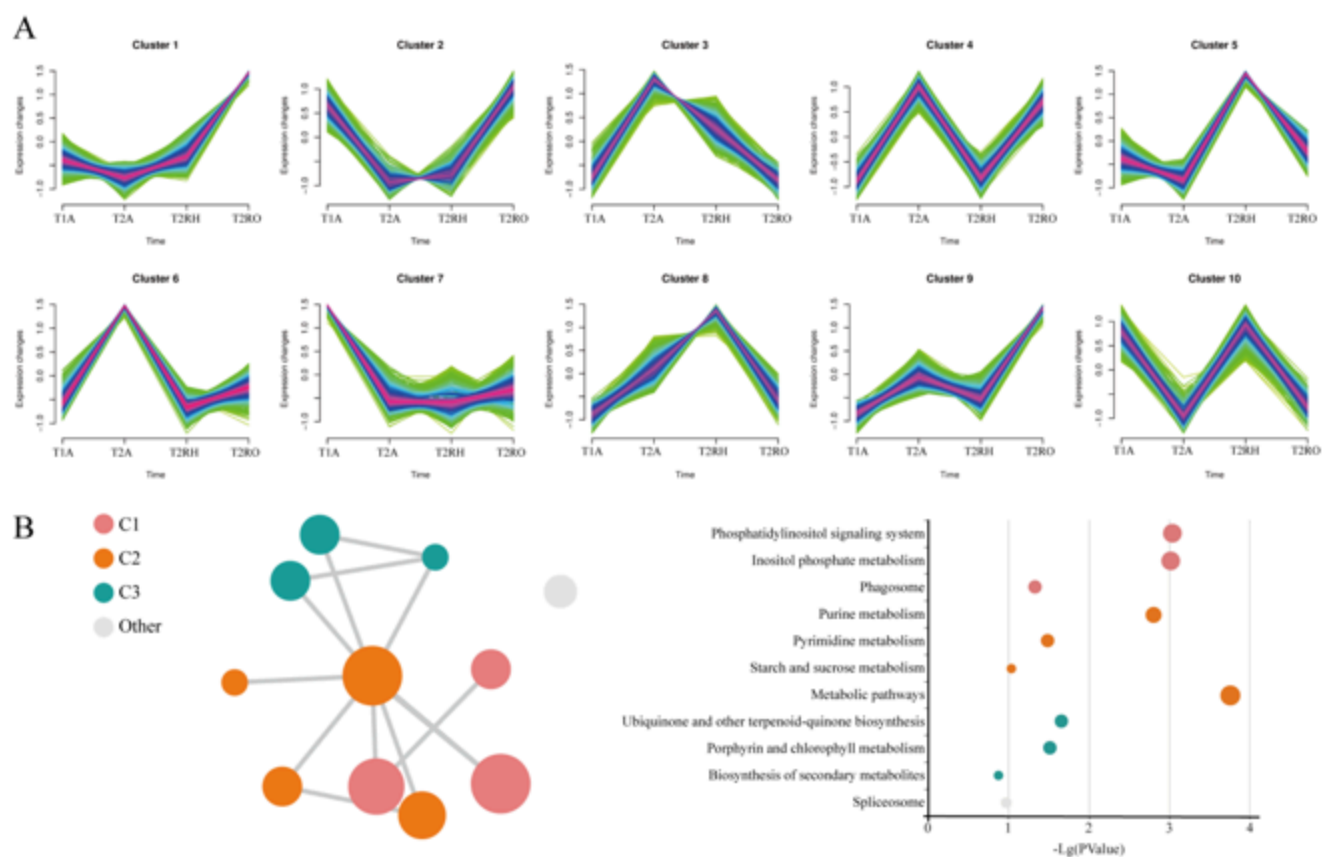
Differentially abundant metabolite volcano plot for T1A vs. T2A (A) and T2RH vs. T2RO (B); DMA classification characteristics between T1A vs. T2A (C) and T2RH vs. T2RO (D); heatmap of metabolite clusters showing differences between T1A vs. T2A (E) and T2RH vs. T2RO (F); KEGG pathway enrichment of T1A vs. T2A (G) and T2RH vs. T2RO (H).



**Figure 3**

# Transcriptomic analysis of *Notopterygium incisum*

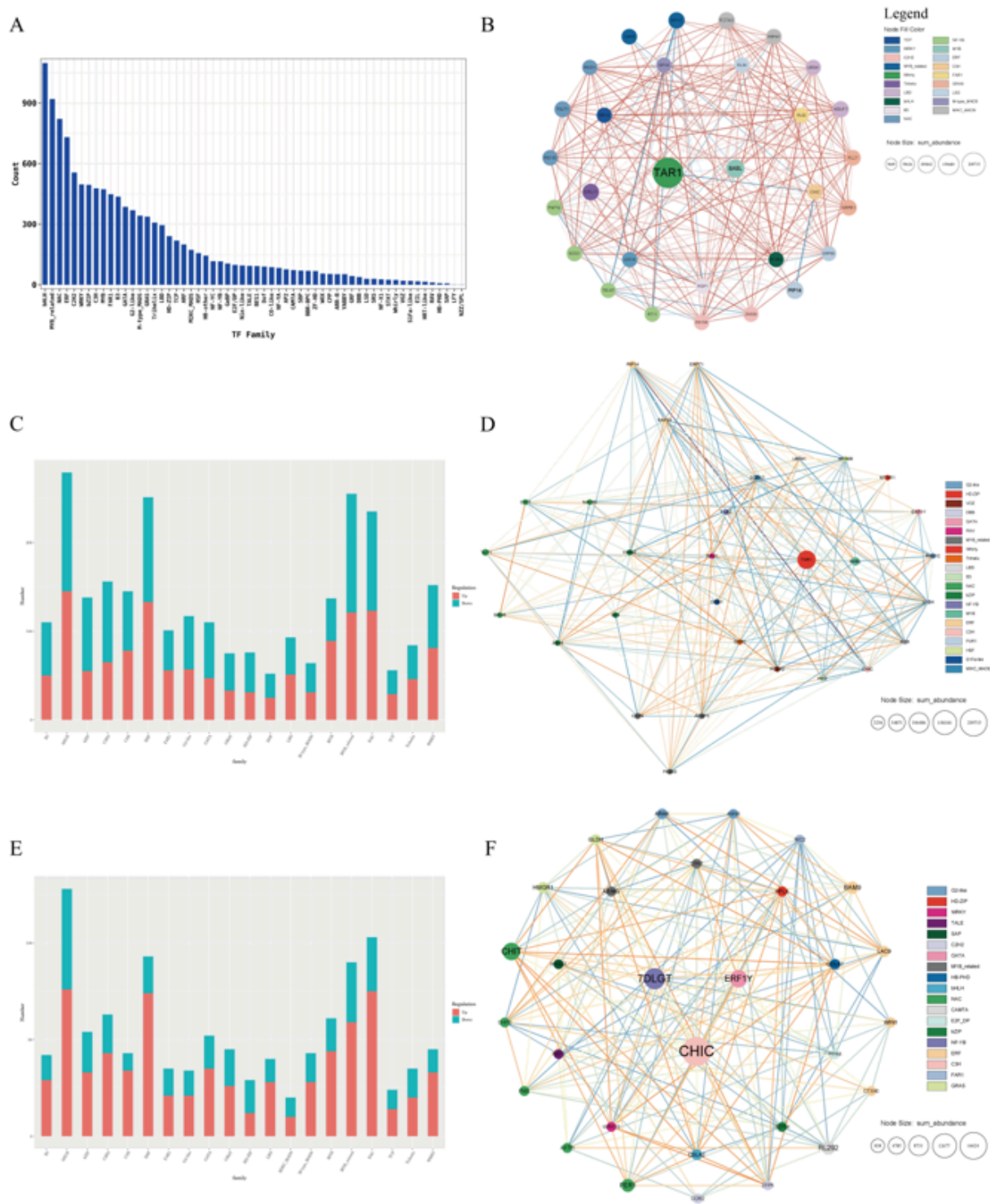
Statistics on the number of DEGs between transcriptomic comparison groups (A); correlation analysis of transcriptomic expression profiles between samples (B); DEG pathway enrichment and upregulated and downregulated gene function distributions between T1A vs. T2A (C) and T2RH vs. T2RO (D).



**Figure 4**

Analysis of DEG expression trends

Gene cluster trends (A); correlations and pathways between trends (B).



**Figure 5**

# Analysis of DEG families

Statistical analysis of all DEG families (A) and coexpression analysis (B); statistical analysis of differentially expressed gene families (C) and coexpression analysis (D) between the T1A and T2A

groups; statistical analysis of differentially expressed gene families (E) and coexpression analysis (F) between the T2RH and T2RO groups.

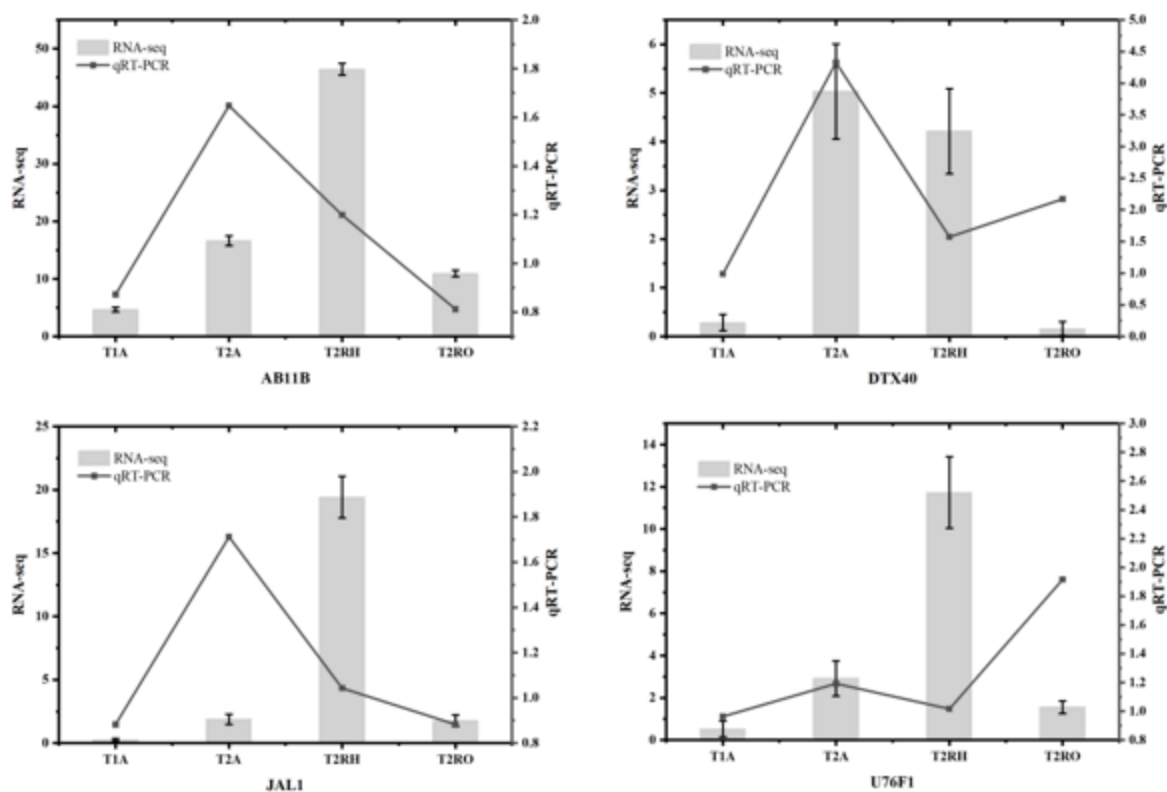


Figure 6

qRT-PCR validation of the 4 DEGs

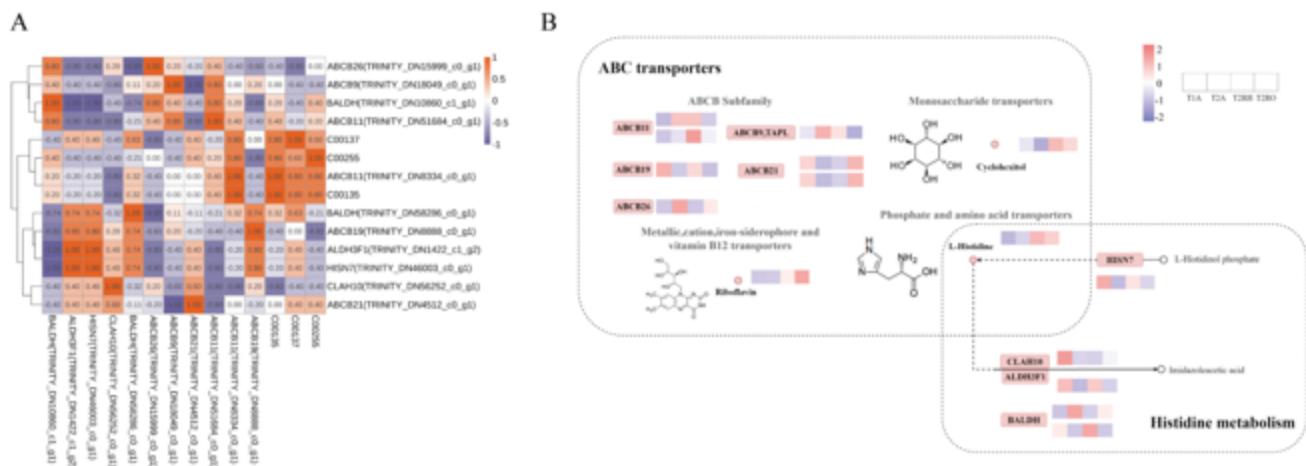


Figure 7

Histidine metabolism and the systemic function of ABC transporters

Heatmap showing the correlation between DEGs and DEMs (A); synergistic change patterns of DEGs and DAMs (B).

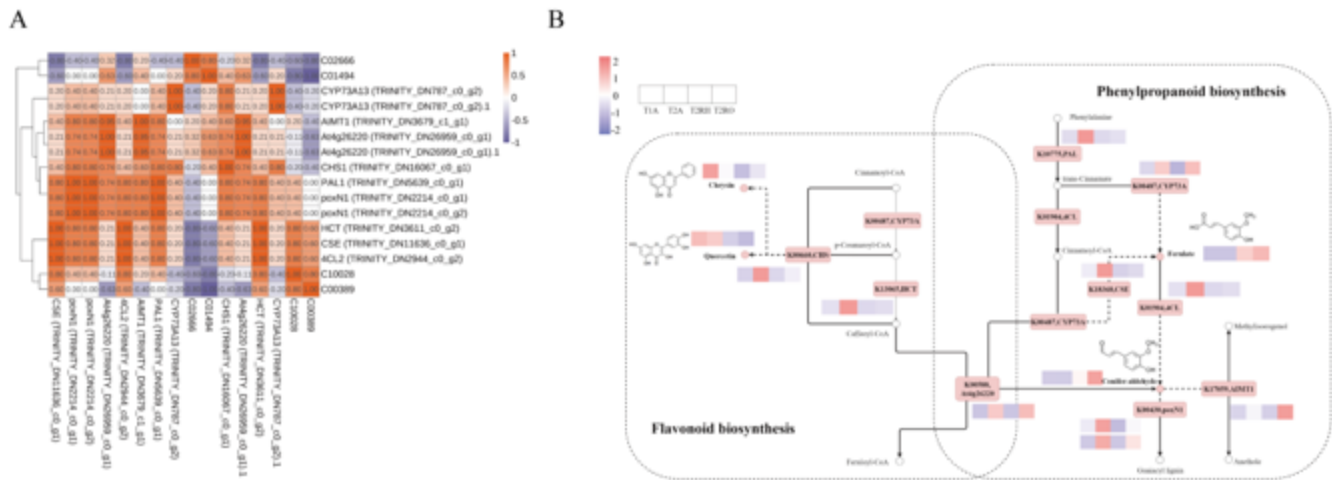


Figure 8

Ecological defense network of phenylpropanoid and flavonoid synthesis

Heatmap showing the correlations between DEGs and DEMs (A); synergistic change patterns of DEGs and DAMs (B).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1TableS9.xlsx](#)
- [Fig.S1Fig.S4.pptx](#)