https://doi.org/10.17221/131/2025-HORTSCI

Combined analysis of transcriptomics and metabolomics reveals the regulatory mechanism of branch bending on leaf bud development in *Carya illinoinensis* (Wangenh.) K. Koch

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Citation: Wu W., Hu K., Gong M., Yao X.H., Yang S., Song Q., Li X.Y., Yang Q., Wang Y. (2026): Combined analysis of transcriptomics and metabolomics reveals the regulatory mechanism of branch bending on leaf bud development in *Carya illinoinensis* (Wangenh.) K. Koch. Hort. Sci. (Prague), 53: 290–305.

Abstract: Branch bending is an important cultivation practice that manipulates plant growth by promoting lateral bud growth. Although this practice has been applied to various plant species, no research has been conducted to identify its effect on the molecular regulatory mechanism in *Carya illinoinensis* (Wangenh.) K. Koch leaf bud development. This study analysed dynamic changes in hormone concentrations, differentially expressed genes (DEGs), and differentially accumulated metabolites (DAMs) by integrating transcriptome, widely targeted metabolome, and targeted hormone metabolome after branch bending in leaf buds. Key regulatory pathways were also investigated using multi-omics analysis. Transcriptome analysis identified 132 DEGs (117 up-regulated, 15 down-regulated) between treated and control groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis indicated that these DEGs were mainly enriched in sesquiterpenoid and triterpenoid biosynthesis and pentose and glucuronate interconversion pathways. Widely targeted metabolome screened 62 DAMs, which were mainly enriched in flavonoid-related biosynthetic pathways, including biosynthesis of quercetin aglycones I, biosynthesis of chalcone II, and flavone and flavonol biosynthesis pathways. Targeted hormone metabolome showed branch bending increased auxin (IAA), cytokinins acid (CTK), and jasmonate (JA) concentrations, but reduced abscisic acid (ABA), gibberellins (GA), and salicylic acid (SA) concentrations. Multi-omics integration identified 7 core co-enriched pathways, namely carotenoid biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, α-linolenic acid metabolism, plant hormone signal transduction, biosynthesis of secondary metabolites, and metabolic pathways. These main pathways work together to facilitate the influence of branch bending on enhanced leaf bud development by regulating hormone homeostasis, the production of secondary metabolites, and signal transduction. This study discusses the molecular and metabolic mechanisms involved in branch bending and its role in regulating the development of *C. illinoinensis* leaf buds. It also offers key basic data, theoretical support for optimising its high-quality cultivation techniques, as well as significant practical guidance for the optimisation of branch bending technology through the regulation of leaf bud development.

Keywords: pecan; shoot orientation; plant hormones; transcriptome; metabolome; bud morphogenesis

Supported by the Agriculture Research System of Anhui Province (AHNYCYTX-10) and the Achievement Transformation Project of Anhui Academy of Agricultural Sciences "Transformation and Application of Sightseeing Agriculture and Planning Technology for Beautiful and Harmonious Villages" (Project No. 2025ZH034).

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https://doi.org/10.17221/131/2025-HORTSCI

The pecan [*Carya illinoinensis* (Wangenh.) K. Koch] tree is one of the most highly versatile species belonging to the *Carya* genus. This tree is commercially valuable and is cultivated for its nuts, oils, and wood products. Its growth and yield are crucial for its economic value (Gao et al. 2024). The effective growth and development of leaf buds is pivotal to the quality of the fruit and wood produced. Leaf buds grow to form new branches and leaves, which are parts of the plant necessary for photosynthesis and nutrient synthesis. To promote the effective growth and development of buds, internal hormonal signals and external environmental factors coordinate in a complex, tightly regulated process. Plant growth prioritises vertical growth over lateral growth through a process called apical dominance, with bud growth patterns being influenced by apical dominance to maintain this growth pattern. Auxin (IAA) is the main hormone produced by apical buds to regulate the apical dominance process by inhibiting the outgrowth of nearby lateral buds. Together with cytokinin (CTK), abscisic acid (ABA), and strigolactone (SL), these hormones form a complex system that integrates the plant's internal regulatory system and its external environmental signals to effectively control the growth and development of the plant (Domagalska, Leyser 2011; Cao et al. 2023).

Similar to most trees, *C. illinoinensis* is shown to prioritise vertical growth and polar growth characteristics. The plant synthesises high amounts of IAA, which promotes apical dominance and inhibits lateral growth. This phenomenon leads to "bare" branches, which can result in substantially reduced fruit yield (Cai et al. 2021). In large-scale cultivation practice, branch bending is a highly efficient method for shaping and pruning, effectively breaking apical dominance. Growth direction and spatial distribution of the branches can be changed to enhance tree vigour, increase light penetration and ventilation conditions of the canopy, optimise the distribution of nutrients and induce flowering and fruit production (Zhou et al. 2025; Yang, Yang 2019). Practice has shown that reasonable branch bending can significantly increase the germination rate of lateral buds and the number of medium-short branches (Su et al. 2021). Its physiological mechanism may be related to the reduction in the efficient polar transportation of IAA, and the regulation of both hormone balance in buds and the expression of functional genes; however, the specific molecular regulatory network remains largely unexplored (Li et al. 2015). Currently, there is limited systematic analysis of the gene expression patterns and metabolome profile changes in leaf buds following branch bending. This gap significantly limits the understanding of synergistic mechanisms present in signalling pathways, thereby restricting the advancement of effective techniques that optimise the regulation of tree growth.

Omics technologies have made remarkable progress, providing powerful tools for dissecting the mechanisms of plant growth and development (Xiong et al. 2025). While the analysis of a single transcriptome is essential for elucidating the spatiotemporal dynamics of gene expression, it fails to establish a link between gene expression and phenotypic development (He et al. 2018). In contrast, metabolomics can directly characterise physiological changes by identifying metabolites and their compositions to link genotypes with phenotypes (Liu et al. 2024a). Therefore, integrating transcriptome and metabolome analysis can provide comprehensive and accurate information on plant molecular mechanisms driving plant biological activities, and aid in interpreting the relation between gene expression and metabolite accumulation. This combined strategy is expected to be used to study the mechanism of walnut bud germination and clarify the synergistic regulatory relationship between genes and hormones (Zhao et al. 2025). Notably, hormone metabolomics and widely targeted metabolomics can accurately measure key hormones and various metabolites, respectively, which provides the basis for the study of metabolic and hormone regulation (Ning et al. 2024; Shang et al. 2024). Despite advancements made towards understanding hormone signalling and transcription factor regulatory modules in various plants (such as strawberry axillary bud development) (Han et al. 2024), research that combines transcriptome and metabolome analyses to assess the impact of branch bending in *C. illinoinensis* remains insufficient. Based on this, our research used the excellent variety 'Hebo 1' of *C. illinoinensis* as the experimental material. We screened differentially expressed genes (DEGs) in leaf buds via transcriptomic sequencing, identified differentially accumulated metabolites (DAMs) using metabolomics, and conducted correlation analysis between DEGs and DAMs. Our objective is to

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examine the core mechanisms involved during the transition from apical dominance to branch-bending-induced lateral development of leaf buds using both molecular expression and metabolic regulation. Additionally, we aim to identify important key regulatory genes, characteristic metabolites, and core pathways, to provide methodological references for the study of branch management and bud development in woody plants, as well as theoretical support for optimising tree regulation methods.

MATERIAL AND METHODS

Plant materials. This experiment was conducted at the Qinglongdian Pecan (*C. illinoinensis*) Planting Base in Bailong Town, Feidong County, Hefei City, Anhui Province, China (32°19'N, 117°38'E). The base is situated at an altitude of 65.5 m, with an average temperature of 15.9 °C and an annual average precipitation of 999.4 mm. The soil type was acidic claypan yellow-brown soil.

In early March 2024, six 4-year-old *C. illinoinensis* 'Hebo No.1' pecan trees with uniform growth free from pests or diseases were selected and randomly divided into two groups: the branch bending group (T) and the control group (CK), with 3 trees in each group. *C. illinoinensis* 'Hebo No.1' is an excellent pecan cultivar that has been independently bred by Anhui Shuiqing Muhua Ecological Technology Co., Ltd. It was selected for this experiment due to its outstanding agronomic advantages, which include: (1) early staminate flowering: its staminate flowers fully bloom in late April, which can provide pollination for most pecan cultivars; (2) early fruit maturation: the earliest maturation time occurs in mid-to-late September, approximately 15 days earlier than that of other conventional cultivars; (3) high kernel oil content: the kernel oil content in this cultivar can reach up to 73%, and also possesses a sweet taste that is ideal for consumption. On March 10, 3 branchless lateral branches with similar thickness (approximately 1 cm in diameter) and length were selected from each tree in the branch bending group for branch bending treatment. The initial growth angle of the branches relative to the main trunk was 30 °, and the branches were bent by an additional 30 ° according to the principles of consistent orientation and height (Figure 1). The branches assigned to the control group did not undergo branch bending. On April 10, well-developed leaf buds were collected from the upper-middle segments of each lateral branch. Buds from the 3 lateral branches of the same tree were mixed to form one biological replicate sample, resulting in a total of 6 samples (3 from the branch bending group and 3 from the control group). After sampling, the outermost scales of the buds were removed immediately. The buds were then quickly frozen in liquid nitrogen and transferred to an ultra-low temperature refrigerator at –80 °C for storage. All samples were sent to Metway Biotechnology Co., Ltd. (Wuhan, China) for transcriptome and metabolome analyses. This company provides professional omics analysis services for academic research. The experimental design and the generated data in this study comply with the general technical specifications for transcriptomics and metabolomics research, and the data quality is sufficient to support subsequent scientific conclusions.

Transcriptome sequencing and analysis. Total RNA was extracted from leaf bud samples. Using a Qubit 4.0 fluorometer (Thermo Fisher Scientific, MA, USA), we measured the RNA concentration



Figure 1. Schematic diagram of branch bending treatment

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and verified the absence of RNA degradation using a Qseq400 bioanalyzer (BiOptic, Taiwan, China). Then, high-quality RNA was chosen for mRNA enrichment. Following this, it was fragmented into short sections by adding a fragmentation buffer, after which first-strand cDNA was synthesised using random hexamers (Thermo Fisher Scientific, MA, USA) as primers. Subsequently, second-strand cDNA was synthesised by adding buffer, dNTPs, and DNA polymerase I, followed by purification of the double-stranded cDNA using Hieff NGS® DNA Selection Beads (No. 12601ES75; YEASEN Biotech Co., Ltd., Shanghai, China). After purification, terminal repair and 3'-end adenylation were performed, and Illumina sequencing adapters were ligated to the cDNA fragments. Fragment size selection was conducted using magnetic beads, and the final cDNA library was obtained through PCR enrichment. After library construction, initial quantification was carried out using the Qubit dye method, and the insert size was measured using a fragment analyser. The effective concentration (> 2 nM) of the library was accurately measured using qPCR. After the library was qualified, paired-end sequencing (PE150) was performed on the Illumina NovaSeq 6000 platform.

Quality filtering of the sequencing data was performed to remove adapter sequences, reads with an N content > 10%, and low-quality reads (where bases with Q20 < 20 accounted for > 50%), thereby obtaining Clean Reads. HISAT2 (version 2.2.1) was used to align the Clean Reads to the *C. illinoinensis* reference genome (*C. illinoinensis* Pawnee_588_v1.0) (Kim et al. 2015). FeatureCounts (version 2.0.3) was utilised to calculate the fragments per kilobase of transcript per million mapped reads (FPKM) values of genes, which were used to normalise gene expression levels (Zhao et al. 2025). Pearson correlation coefficient (PCC, r) and principal component analysis (PCA) were used to evaluate the correlation between biological replicates. DESeq2 (version 1.22.1) was used for inter-group differential analysis to screen for genes with $|\log_2$ fold change (FC)$| \geq 1$ and false discovery rate (FDR) < 0.05, resulting in DEGs. ClusterProfiler (version 4.6.0) was used to perform gene ontology (GO) enrichment analysis [covering biological processes (BP), molecular functions (MF), and cellular components (CC)] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis ($P < 0.05$) (Wu et al. 2021). The top 20 significantly enriched terms/pathways were visualised.

Targeted metabolomic analysis of hormone contents. The samples stored at −80 °C were ground into powder using a grinder (30 Hz, 1 min) in liquid nitrogen. A 50 mg aliquot of the powder was weighed in liquid nitrogen and transferred to a 2 mL centrifuge tube. Subsequently, 1 mL of a methanol/water/formic acid mixture (15 : 4 : 1, V/V/V) was added, followed by the addition of 10 µL of internal standard mixture (100 ng/mL). The mixture was vortexed for 10 min and then centrifuged at 12 000 r/min at 4 °C for 5 minutes. The supernatant was transferred to a new centrifuge tube and concentrated to near dryness. The residue was reconstituted with 100 µL of 80% methanol (V/V), and the reconstituted solution was filtered through a microfiltration membrane (0.22 µm). The filtrate was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using an ExionLC™ AD UPLC system and a QTRAP® 6500+ mass spectrometer (both from Sciex, https://sciex.com.cn/).

The LC conditions were as follows: chromatographic column, Waters ACQUITY UPLC HSS T3 C18 (1.8 µm, 100 × 2.1 mm); column temperature of 40 °C; mobile phase system, water containing 0.04% acetic acid (A) and acetonitrile containing 0.04% acetic acid (B); gradient program, 95 : 5 (V/V) A/B for 0 min, 95 : 5 (V/V) A/B for 1.0 min, 5 : 95 (V/V) A/B for 8.0 min, 5 : 95 (V/V) A/B for 9.0 min, 95 : 5 (V/V) A/B for 9.1 min, and 95 : 5 (V/V) A/B for 12.0 min; flow rate of 0.35 mL/min; and an injection volume of 2 µL. The MS conditions were as follows: ion source, electrospray ionisation (ESI); ESI temperature of 550 °C; ion spray voltage (IS), 5 500 V (positive ion mode)/−4 500 V (negative ion mode); and curtain gas (CUR) 35 psi. Quantitation was performed using the multiple reaction monitoring (MRM) mode of a triple quadrupole MS. Each MRM ion pair was scanned and detected based on the optimised declustering potential (DP) and collision energy (CE).

Qualitative analysis of the MS data was performed by matching against the Metware Database (MWDB) constructed using standard reference materials. Chromatographic peaks were integrated using MultiQuant software (version 3.0.3), and hormone concentrations were calculated using standard curves. Orthogonal partial least squares discriminant analysis (OPLS-DA) was first used for

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preliminary screening, and differential hormones between the two groups were further determined based on fold change (FC ≥ 2 or FC ≤ 0.5).

Widely targeted metabolomics analysis. The samples were freeze-dried for 63 h using a vacuum freeze dryer (Scientz-100F, Scientz Biotechnology, Ningbo, China), and then ground into a powder with a grinder (MM 400, Retsch, Germany) at 30 Hz for 1.5 minutes. A precise 50 mg of the powder was measured and subsequently dissolved in 1.2 mL pre-cooled (–20 °C) 70% methanol solution (V/V). The mixture was vortexed once every 30 min, with each vortexing lasting 30 s, and this process was repeated 6 times. Subsequently, the samples were centrifuged at 12 000 r/min for 3 min at 4 °C. The supernatant was aspirated, filtered through a microfiltration membrane (0.22 μm), and stored in a sample vial for UPLC-MS/MS analysis.

The LC conditions were as follows: chromatographic column, Agilent SB-C18 (1.8 μm, 100 × 2.1 mm); column temperature of 40 °C; mobile phase system, pure water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B); gradient program, initial conditions of 95% A and 5% B, an applied linear gradient at 95% B for 9 min, concentration held at 5% A and 95% B for 1 min, concentration adjusted to 95% A and 5.0% B for 1.1 min, with the conditions maintained for 2.9 min; a flow rate of 0.35 mL/min; and an injection volume of 2 μL. The MS conditions were as follows: ESI temperature of 550 °C; IS 5 500 V (positive ion mode)/–4 500 V (negative ion mode); gas source I (GSI) 50 psi, gas source II (GSII) 60 psi, and CUR 25 psi; and high collision energy dissociation parameters.

Metabolite identification was conducted based on a self-established MWDB using secondary MS spectral information. For metabolite quantification, MRM analysis with a triple quadrupole mass spectrometer was employed. Raw data were imported into the metabolomics data processing software Analyst (version 1.6.3) for further data processing, which included baseline filtering, peak detection, peak integration, retention time correction, peak alignment, and normalisation. DAMs were screened using the OPLS-DA model, with the screening criteria of variable importance in projection (VIP) > 1 and FC ≥ 2 or FC ≤ 0.5. Lastly, DAMs enriched in KEGG pathway analysis were identified using the hypergeometric distribution test to determine significantly enriched metabolic pathways ($P < 0.05$) (Lv et al. 2024).

Combined analysis of transcriptomics and metabolomics. KEGG pathway annotations were performed separately for DEGs and DAMs, and the pathways significantly enriched by both were screened out. The PCC between DEGs and DAMs was calculated using the core function in R software (version 4.2.0). The screening threshold was set as $|PCC| > 0.8$ and $P < 0.05$ to further analyse the associations between DEGs and DAMs.

RESULTS

Phenotypic traits of lateral shoot formation in response to branch bending. To establish a phenotypic basis for interpreting the multi-omics data, we systematically investigated the effects of branch bending on lateral shoot formation. In three independent biological replicates, the number of sprouted shoots formed on the branches under the branch bending treatment ranged from 15 to 18, whereas in the control groups, it was only 6 to 8 (Figure 2). These phenotypic results show that branch bending is an effective method for facilitating lateral branch growth. The observed morphological differences in lateral shoot formation between the treatment and control groups bridge the gap between the morphological performance and molecular regulatory mechanisms of lateral shoot formation and provide direct phenotypic evidence for subsequent correlation analysis with omics findings.

Transcriptome analysis. In this study, sequencing analysis was conducted on 6 leaf bud samples from the branch-bending group (T) and the control group (CK). After quality filtering, a total of 50.7 GB of Clean Reads were obtained, with each sample having a data volume of no less than 6 GB. For all samples, the proportion of Q20 bases (error rate ≤ 1%) exceeded 98%, and the proportion of Q30 bases (error rate ≤ 0.1%) was over 96% (Table S1 in Electronic Supplementary Material (ESM)). These results indicate that the sequencing data exhibited high accuracy and a low error rate, thereby fulfilling the criteria for subsequent analysis. The Clean Reads were aligned with the reference genome of *C. illinoinensis* (*C. illinoinensis* Pawnee_588_v1.0), and the alignment efficiency of samples in both groups ranged from 95% to 97%, with a unique alignment rate no less

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Figure 2. Observed sprouting shoots in the treatment group (left) versus the control group (right) in three replicates

than 93% (Table S1 in ESM). This demonstrates a high matching degree between the sequencing sequences and the reference genome, and an ideal genomic positioning effect, which can be used for subsequent gene expression quantification and functional annotation analyses. The results of sample correlation analysis showed that the PCC of the three biological replicates within each group and between groups were ≥ 0.99, indicating a strong consistency in the gene expression patterns of the replicate samples, significant differences between groups, reliable biological replication of the experiment, and no abnormal samples interfering with the analysis. The two-dimensional score plot of PCA showed that samples from the branch bending group (T) and the control group (CK) clustered separately in different quadrants, and the contribution rate of the first principal component (PC1) was 34.96% (Figure S1 in ESM), which could clearly distinguish the two groups of samples. This result further confirmed that the branch bending treatment had a significant regulatory effect on the gene expression of leaf buds, providing a reasonable basis for the subsequent screening of DEGs.

With the screening criteria of $|\log_2 FC| \geq 1$ and FDR < 0.05, a total of 132 DEGs were identified. Following the branch bending treatment, 117 DEGs were up-regulated, and 15 DEGs were down-regulated; the number of upregulated DEGs was roughly 7.8 times greater than that of the downregulated DEGs (Figure 3). This result indicates that the branch bending treatment can significantly alter the gene expression pattern of leaf buds, mainly by activating gene expression. Detailed information on all DEGs has been compiled into Table S2 in ESM. After performing GO enrichment analysis on the DEGs, a total of 94 GO annotation entries were obtained. These entries were classified into three major categories: BP, CC, and MF. The top 15 GO terms with the most significant enrichment in each category were selected for visualisation (Figure 4A). The results showed that in the BP category, DEGs were mainly concentrated in basic life activity-related entries such as cellular process, metabolic process, and response to stimulus, suggesting that branch

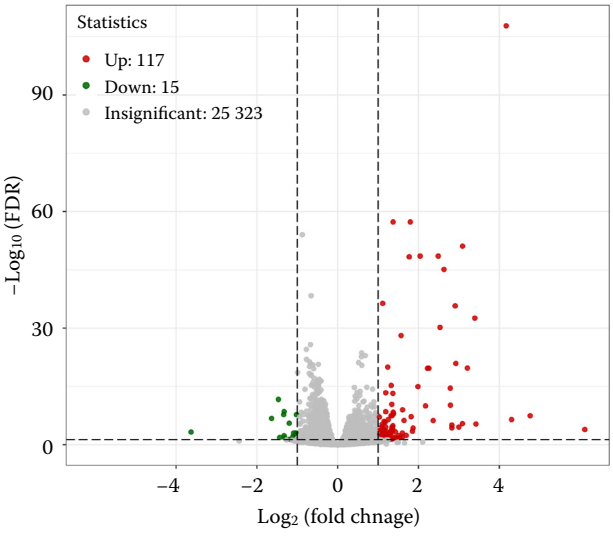

Figure 3. Volcano map of gene expression in the control group and the branch bending group

The red, green, and grey dots represent upregulated, downregulated, and insignificantly expressed genes, respectively; FDR – false discovery rate

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bending may have a broad regulatory effect on core biological processes such as leaf bud growth and development. In the CC category, only a few DEGs were enriched in the protein-containing complex entry, suggesting that branch bending may affect the structure or function of protein complexes within leaf bud cells. In terms of MF, the DEGs were significantly enriched in terms like binding, transcription regulator activity, catalytic activity, and transporter activity. This demonstrated that branch bending could affect the physiological metabolism and various processes of leaf buds by regulating the expression of genes associated with proteins that participate in transportation and act as enzymes in leaf buds. KEGG enrichment analysis of DEGs identified 49 pathways related to leaf bud development. The 20 most significantly enriched pathways included sesquiterpenoid and triterpenoid biosynthesis, glucosinolate biosynthesis, cyanoamino acid metabolism, ABC transporters, D-amino acid metabolism, tyrosine metabolism, 2-oxocarboxylic acid metabolism, betalain biosynthesis, metabolic pathways, pentose and glucuronate interconversions, alpha-Linolenic acid metabolism, biosynthesis of secondary metabolites, biosynthesis of unsaturated fatty acids, isoquinoline alkaloid biosynthesis, cysteine and methionine metabolism, flavonoid biosynthesis, glycerophospholipid metabolism, fatty acid elongation, nitrogen metabolism, and cutin, and suberine and wax biosynthesis (Figure 4B). Among these pathways, the sesquiterpenoid and triterpenoid biosynthesis pathway exhibited high significance, while the pentose and glucuronate interconversions pathway showed a high enrichment level. This showed that these pathways participated significantly in the physiological metabolism of leaf buds from the branch-bending group. Branch bending may regulate the expression of genes involved in these pathways, thereby altering the physiological processes of leaf buds, such as substance synthesis and energy metabolism.

Metabolomics analysis. Targeted hormone detection was carried out on leaf bud samples from the branch-bending group and the control group using LC-MS/MS technology, identifying a total of 46 hormones and their derivatives. Using FC ≥ 2 or FC ≤ 0.5 as the screening criteria, 15 significantly different hormones were ultimately obtained (Table S3 in ESM). These differential hormones belong to six major categories: ABA, IAA, CTK, gibberellin (GA), jasmonate (JA), and salicylic acid (SA). According to the detection results, after branch bending treatment, the accumulation of IAA, CTK, and JA in leaf buds increased significantly, while the synthesis of ABA, GA, and SA was markedly inhibited. It is inferred that branch bending may promote leaf bud development by enhancing the effect of growth-promoting hormones and weakening the influence of dormancy-regulating hormones.

To reveal the effects of the branch bending group and the control group on metabolic changes during leaf bud germination, a widely targeted plant

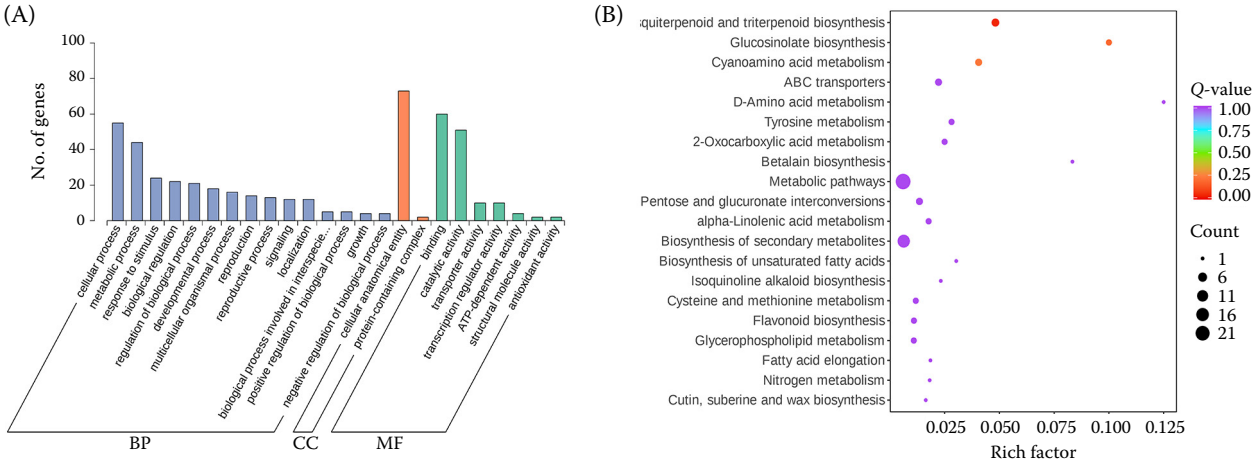

Figure 4. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of differentially expressed genes (DEGs) in the control group and the branch bending group: GO enrichment analysis, the top 10 GO terms with the highest richness in each GO category (A), KEGG enrichment analysis, the top 20 KEGG pathways with the smallest *P*-values (B)

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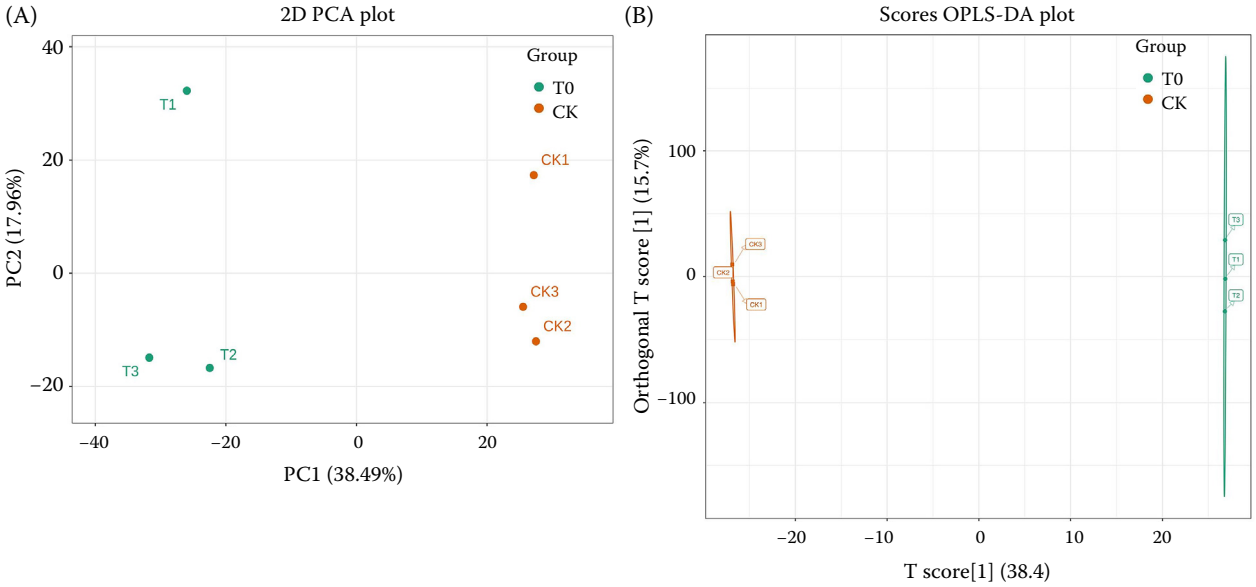

Figure 5. Principal component analysis (PCA) score plot (A) and orthogonal partial least squares discriminant analysis (OPLS-DA) score plot (B)

metabolomics analysis was performed to determine metabolite differences. PCA results showed that the samples of the branch bending group and the control group were significantly separated in the score plot, with good aggregation of samples within each group (Figure 5A). OPLS-DA further revealed significant differences in the scores between the two sample groups. Furthermore, the samples within each group exhibited a stable distribution and clear separation according to the principal components (Figure 5B). These results confirmed that branch-bending treatment significantly changed metabolite composition during leaf bud development. DAMs were screened based on the VIP values of the OPLS-DA model, and a total of 2 251 metabolites were identified. Using VIP > 1 and FC ≥ 2 or FC ≤ 0.5 as the screening criteria, 62 DAMs were finally obtained (Table S4 in ESM), among which 37 were upregulated, and 25 were downregulated. These DAMs were classified into 11 categories: flavonoids (25, 40.32%), terpenoids (7, 11.29%), lignans and coumarins (6, 9.68%), alkaloids (4, 6.45%), quinones (4, 6.45%), tannins (4, 6.45%), amino acids and derivatives (3, 4.84%), lipids (3, 4.84%), phenolic acids (2, 3.23%), organic acids (1, 1.61%), and others (3, 4.84%). Among them, flavonoids were the most dominant category of DAMs, while most of them were downregulated. Terpenoids, lignans, and coumarins are important secondary metabolites in plants (Anjali et al. 2023), and their expressions were observed to be mostly upregulated. The differential expression patterns of these key secondary metabolite categories suggest that they may play a core regulatory role in the process of branch bending, thus regulating leaf bud development. Mapping of the 62 DAMs to the KEGG database revealed that the significantly enriched pathways were mostly associated with substance metabolism and synthesis (Figure 6). Flavonoid-related biosynthetic pathways, such as biosynthesis of quercetin aglycones I, biosynthesis of chalcone II, and flavone and flavonol biosynthesis, had relatively small *P*-values and high rich factors. This demonstrated that branch bending treatment exerts a significant impact on the flavonoid biosynthetic pathway. Based on related studies, it is speculated that changes in the expression of key enzyme genes may drive the substantial accumulation of metabolites such as flavones and flavonols. These metabolites work synergistically with the genes to jointly regulate the relevant processes of this pathway (Liu et al. 2025).

Correlation analysis between DEGs and DAMs. By comparing the pathway enrichment results of DEGs and DAMs, it was found that DEGs were enriched in a total of 49 pathways, while DAMs were enriched in 11 pathways. After performing an intersection analysis of the two types of an-

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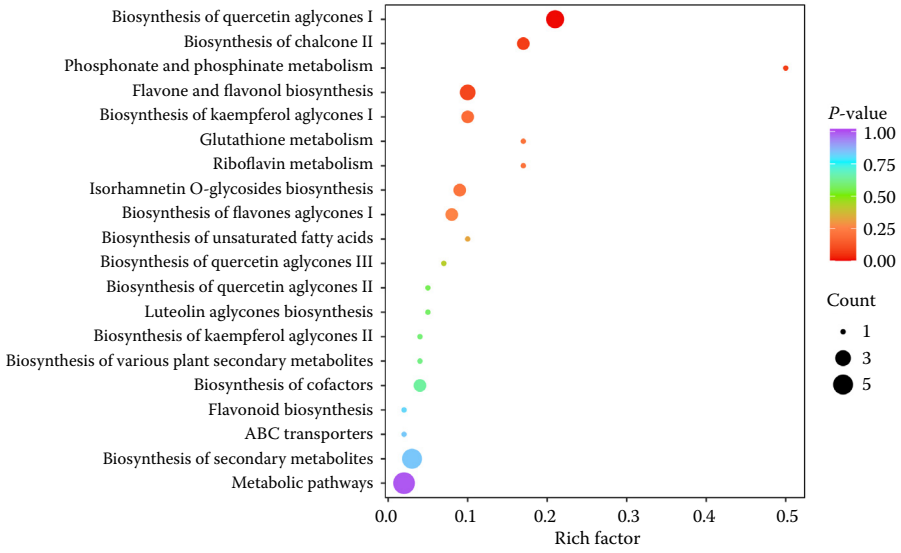

Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially accumulated metabolites (DAMs) in the control group and the branch bending group

notation results, 7 core common pathways were identified (Figure 7), namely carotenoid biosynthesis (ko00906), phenylpropanoid biosynthesis (ko00940), phenylalanine metabolism (ko00360), alpha-linolenic acid metabolism (ko00592), plant hormone signal transduction (ko04075), biosynthesis of secondary metabolites (ko01110), and metabolic pathways (ko01100). The metabolites in these pathways play regulatory roles during leaf bud development.

DISCUSSION

Leaf bud development is a critical stage in the growth of *C. illinoinensis*, marking not only the transition from vegetative growth to reproductive growth but also directly affecting the overall yield and economic value of the trees. Branch bending

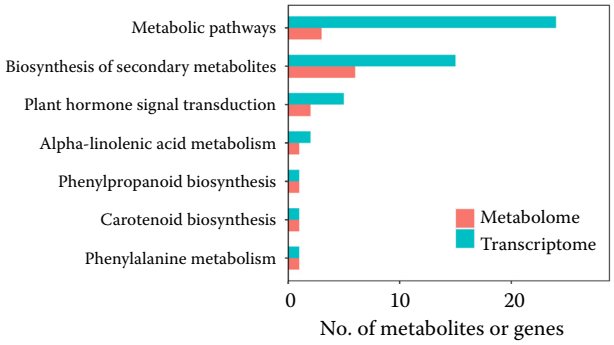

Figure 7. Core common pathways enriched with differentially expressed genes (DEGs) and differentially accumulated metabolites (DAMs)

can regulate the internal physiological metabolic processes and gene expression of trees by adjusting the growth angle of the branches, which in turn has a significant impact on the development of leaf buds. As shown in Figure 2, this may be phenotypically detected by the increased number of shoots sprouting from the branches that were treated with branch bending. This study innovatively employed a combined analysis of transcriptomics and metabolomics to systematically explore the underlying mechanisms by which branch bending regulates leaf bud development in *C. illinoinensis*. The results of this study can provide theoretical support for optimizing high-quality and efficient cultivation techniques for *C. illinoinensis*, while also offering a new research approach to understanding the molecular regulatory networks of bud development in woody plants.

Response mechanisms of different hormones in leaf buds under branch bending. The content and balance of endogenous hormones are key to regulating bud dormancy induction (Wojtania et al. 2022). The regulatory impact of branch bending on leaf bud hormone balance was the main focus of this work, which employed targeted hormone metabolomics technology to systematically measure endogenous hormone levels in leaf buds of the treatment group and the control group. The results showed significant differences in the levels of IAA, CTK, JA, ABA, GA, and SA contents between the two groups of leaf buds. After branch bending, IAA levels in leaf buds increased significantly, which may be directly related to the mechanical stimulation caused by branch bend-

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ing (Maimaiti et al. 2013). Previous studies have shown that mechanical signals can interfere with the polar transport of IAA by regulating the localisation and activity of PIN proteins, thereby promoting the local accumulation of this hormone in target tissues such as leaf buds (Julien et al. 2019). Notably, branch bending treatment not only increased CTK concentration but also enhanced the synergistic effect of IAA and CTK in inhibiting leaf bud dormancy. CTK can directly break bud dormancy (Xiong et al. 2025) and interact with IAA to work together to regulate lateral bud germination and elongation (Wu et al. 2024). This provides important hormonal evidence for the promotion of leaf bud activity by branch bending. The significant increase in JA concentration is a typical stress response of leaf buds to the mechanical stimulation of branch bending. As a core hormone regulating plant defence and development, JA can rapidly activate responses to mechanical damage (Liu et al. 2024b; Li et al. 2025b) and influence bud dormancy through interactions with other hormones such as brassinosteroids (BR) (Wang et al. 2024). These findings reflect the integration of stress response and developmental regulation signals.

Branch bending treatment significantly decreased the accumulation of ABA, GA, and SA, while increasing the concentration of IAA, CTK, and JA. This phenomenon indicates that the hormone network within plants can maintain a stable state favourable for growth through adaptive regulation. The decrease in ABA content is a key change, as it can break the plant's dormancy, thereby promoting growth and development (Tang et al. 2023; Ding et al. 2024). This effect exhibits a synergistic enhancement with the growth-promoting signals of IAA/CTK, forming a key hormonal regulatory node for branch bending to break dormancy. Active GAs (such as GA3 and GA4) have been shown in several studies to have physiological roles that disrupt dormancy and promote bud germination (Li et al. 2021; Zhang et al. 2021; Yue et al. 2025). However, in our study, the overall GA concentration decreased after branch bending, and different subtypes of GA showed differential changes. For example, GA15 (a precursor for active GA synthesis) (Zhang et al. 2020) and GA34 (a metabolite of active GA) (Tian et al. 2024) were significantly downregulated, while GA29 (an inactive form in the 13-hydroxylation pathway of the GA metabolism pathway) (Hartmann et al. 2011) was significantly upregulated. This result reveals that branch bending may reduce the effective accumulation of active GA in plants through a dual regulatory pathway of "inhibiting the supply of active GA synthesis precursors + accelerating the inactivation of active GAs". Additionally, studies have pointed out that at specific developmental stages, a moderate reduction in the concentration of active GAs can relieve their antagonistic effect on growth-promoting hormones such as CTK (Liu et al. 2023), thereby creating favourable conditions for leaf bud germination. Various studies have shown that SA (a hormone that functions as a defence and developmental regulator) participates in controlling the breaking of bud dormancy and the subsequent germination processes (Orrantia-Araujo et al. 2021; Yang et al. 2025). The findings from our study verified that when components of the SA hormone were significantly downregulated following branch bending, leaf germination was promoted. This occurred through the inhibition of the defence-related hormone signals.

Consequently, the mechanical stimulations associated with branch bending induce various hormonal regulation mechanisms. This process creates a boost of growth-promoting signals by facilitating local accumulation of IAA and upregulation of CTK. Additionally, the integrated defence and developmental regulation through the JA stress response promotes reducing dormancy inhibition through the cooperative downregulation of ABA, active GA forms, and SA. Finally, it enables conditions conducive to germination by promoting the hormonal balance in the leaf buds, thus establishing a stable signal environment for further growth and development.

Gene expression regulatory characteristics of leaf bud development under branch bending. In this study, transcriptome sequencing analysis was employed to determine the DEG expression characteristics in leaf buds following branch bending treatment. Of the significantly up-regulated DEGs, 117 showed a coordinated expression pattern. This result suggests that these genes act as key regulators by enabling leaf buds to respond to branch-bending treatment. Furthermore, these genes also play a vital role in leaf bud development.

Additional GO functional enrichment analysis of DEGs showed that they were primarily enriched in BPs such as cellular process, metabolic process,

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and response to stimulus. It can be observed that branch bending acts as a mechanical stimulus, inducing gene expression changes in the leaf buds. This leads to their adaptation to branch bending by controlling key biological processes like growth and development, as well as substance metabolism (Darwish et al. 2022). In the CC category, these genes were primarily enriched in the protein-containing complex. This finding indicates that branch bending may participate in the modulation of hormone signalling pathways related to plant development by regulating the assembly or activity of protein complexes (Shimada et al. 2015). Furthermore, the genes were significantly enriched in catalytic activity and transporter activity in the MF category. Bud dormancy can be efficiently disrupted by the upregulation of genes encoding catalytic GA synthases (such as *GA20ox* and *GA3ox*) (Zheng et al. 2018). Moreover, changing the expression of genes associated with transporter activity (like ABC transporter genes) can regulate the transmembrane transport efficiency of signalling substances such as hormone molecules and secondary metabolites (Behrens et al. 2019; Devi et al. 2025; Li et al. 2025a). This process ensures the maintenance of hormone homeostasis in the leaf buds, thereby facilitating the growth and development of the leaf buds.

Based on the findings from KEGG pathway enrichment analysis, DEGs were also found to be significantly enriched in pathways such as pentose and glucuronate interconversions and sesquiterpenoid and triterpenoid biosynthesis. Terpenoids are essential secondary metabolites in plants with a variety of biosynthetic pathways that give plants more metabolic adaptability (Xue et al. 2025). It was previously determined that some terpenoids show unique properties that control plant growth and development due to their involvement in the synthesis of precursor substances for hormones like GAs, ABA, and BRs (Bajguz et al. 2020; Liu et al. 2022). Pentose and glucuronate interconversions are important branches of the network of carbohydrate metabolism (Wang et al. 2025), which play a fundamental regulatory role in breaking bud dormancy (Zhuang et al. 2015). Soluble sugars, which act as both an energy source and a signalling molecule, are known to facilitate the breaking of bud dormancy (Liang et al. 2019; Wojtania et al. 2022). The notable enrichment of this pathway in our study indicates that branch bending could influence the expression of essential enzyme genes within the pathway, thereby modifying the pattern of soluble sugar accumulation and distribution in leaf buds, and ultimately controlling leaf bud growth and development (De Rosa et al. 2022; Zhu et al. 2024).

Dynamic changes of DAMs in leaf buds under branch bending. A widely targeted metabolomics analysis identified 62 DAMs in both the branch-bending and the control groups. The results showed a higher number of upregulated metabolites relative to downregulated ones. Furthermore, the regulatory patterns of various secondary metabolite classes were strongly linked to this outcome. Flavonoids were the most common differential category among these DAMs, followed by terpenoids, lignans, and coumarins. In particular, most flavonoids were downregulated, whereas most terpenoids, lignans, and coumarins were upregulated. This pattern directly contributed to a higher number of the total up-regulated metabolites. From our results, it can be determined that branch bending has a notable effect in regulating the secondary metabolism of leaf buds, with substantially different impacts in the regulatory directions for different classes of secondary metabolites. Based on KEGG pathway enrichment analysis, flavonoid-related biosynthesis pathways were identified to be significantly enriched. Although most flavonoids were down-regulated, branch bending was observed to still impact the balance of flavonoid synthesis and accumulation by controlling these crucial pathways. In addition to participating in physiological functions like antioxidant and stress resistance, flavonoids are important secondary metabolites in plants that may significantly influence plant growth and development (Mierziak et al. 2014; Patil et al. 2024). From these findings, it can be surmised that branch bending enhances leaf bud growth of *C. illinoinensis* by differentially regulating the production of secondary metabolites, like flavonoids and terpenoids. This may be based on the findings that the majority of terpenoids, lignans, and coumarins were upregulated. However, more investigations are required to elucidate the unique regulatory mechanisms and synergistic modes of the different metabolites.

Potential mechanism of branch bending on leaf bud development. We compared the enriched pathways of DAMs and DEGs in leaf buds between

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the branch bending group and the control group using the combined transcriptomics and metabolomics analysis, and identified 7 key common pathways. The identified pathways included carotenoid biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, and α-linolenic acid metabolism. Their significant enrichment reveals the participation of branch bending in the regulation of leaf bud development by multidimensionally controlling substance synthesis and physiological activity. Carotenoids are crucial for photosynthetic pigment and a fundamental precursor of plant signalling molecules; thus, changes in the carotenoid synthesis pathway following branch bending show the influence branch bending has on the light capture efficiency of leaf buds. Additionally, it controls the supply of hormone precursors like ABA and SLs, offering an effective control of hormone levels (Ruiz-Sola, Rodríguez-Concepción 2012; Moreno et al. 2020; Ezquerro et al. 2023;). Secondary metabolite synthesis is closely associated with both phenylpropanoid biosynthesis and phenylalanine metabolism pathways. By regulating the expression patterns of key genes in these two pathways, branch bending is thought to modify the accumulation levels of secondary metabolites like flavonoids and lignin, improving the stress resistance of leaf buds and modulating their growth status (Shi et al. 2022). The enrichment of the α-linolenic acid metabolism pathway suggests that branch bending may play a role in controlling the growth and development process by regulating the synthesis and transduction of signalling molecules, such as JA and the biosynthesis of membrane lipid components in leaf buds (Gabay et al. 2019; Griffiths 2020). Its role in leaf bud development was further verified by the enrichment of the plant hormone signal transduction pathway. Through hormone signal cascades, this pathway can directly control the expression of functional genes linked to leaf bud development and directly mediate changes in the levels of key hormones such as IAA and CTK induced by the process of branch bending. Moreover, the involvement of two other pathways (biosynthesis of secondary metabolites and metabolic pathways) shows that branch bending may activate multiple ones to regulate leaf bud development. Several organized activations of the main metabolic network and the secondary metabolic system are involved in facilitating leaf bud growth and development. DAMs and DEGs together form a regulatory network that synergistically mediates the development process of *C. illinoinensis* leaf buds in the core pathways identified in this study.

Limitations. Using a combined transcriptomics and metabolomics analysis, this study has identified the possible mechanisms that show the impact of branch bending on the leaf bud development of *C. illinoinensis*; however, several limitations should be acknowledged. First, the sample size used in this study was relatively small, which inevitably reduced the statistical power of the analyses and compromised the generalizability of the findings. Expanding the size with more biological replicates in future studies will be critical to enhance the robustness of the results and strengthen the reliability of the conclusions. Second, this study was conducted exclusively on the 'Hebo No.1' pecan cultivar. Given the genetic diversity across different pecan genotypes, the current findings may not be directly extrapolated to other cultivars with distinct genetic backgrounds. Thus, future research should extend the experimental scope to include a wider variety of pecan genotypes to determine the genotype-specific reactions to the branch bending treatment. Third, comparisons with alternative bending angles were not included in this study. Future research should thoroughly assess the impacts of various bending angles on branch development and fruit production to provide a more improved strategy for orchard management. Fourth, although this study identified potential "gene-metabolite" regulatory relationships based on multi-omics correlation analysis, these associations merely reflect correlative links rather than definitive causal relationships between the identified DEGs and DAMs. As a result, direct functional verification of these interactions is lacking. In subsequent studies, functional verification assays such as gene overexpression, gene silencing, or metabolite supplementation experiments targeting the core genes and key metabolites in identified pathways should be performed. This will clarify their specific molecular functions in branch-bending-regulated bud development, thereby further verifying and refining the regulatory mechanism proposed in this study. Lastly, samples in this study were collected only at a single time point (one month post-bending). The lack of various time-series sampling limits our ability to track and analyse the progressive changes in hormone levels, gene expression patterns, and

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metabolite profiles occurring during the complete process of bud development induced by branch bending. Future studies could incorporate multi-time point sampling covering the pre-germination, germination, and post-germination stages. This will facilitate the construction of a more comprehensive and temporal regulatory network of branch-bending-mediated bud development.

CONCLUSION

This study identified the hormone regulatory pattern of branch bending on leaf bud development by targeted determination of hormone contents in leaf buds of *C. illinoinensis* between the branch bending group and the control group. Branch bending treatment effectively promoted leaf bud development by significantly increasing the contents of IAA, CTK, and JA, while also decreasing the contents of ABA, GA, and SA. Combined analysis of metabolomic and transcriptomic data from leaf buds showed that the pathways of carotenoid biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, α-linolenic acid metabolism, plant hormone signal transduction, biosynthesis of secondary metabolites, and metabolic pathways played key regulatory roles in the process of branch bending, thus regulating leaf bud development. These pathways collectively mediated the promoting effect of branch bending on leaf bud development by coordinately regulating hormone homeostasis, secondary metabolite synthesis, and signal transduction. The results of this study not only provide key basic data and theoretical support for elucidating the molecular regulatory network of *C. illinoinensis* leaf bud development, but also offer important practical guidance for the optimization of branch bending technology based on regulating leaf bud development and the construction of a precise cultivation technology system in *C. illinoinensis* cultivation.

Acknowledgments: The authors thank 51edit.com for the English editing service for this manuscript.

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Received: November 28, 2025
Accepted: February 18, 2026
Published online: October 2, 2026