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Dual Melatonin Enhances Coordination Between Carbon and Nitrogen Assimilation in Soybean

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Abstract: Soybean production is currently insufficient to meet global demand, highlighting the need for strategies to enhance growth. Melatonin (MT) has emerged as a promising solution due to its growth-promoting properties. This study investigated the effects of a dual MT treatment—combining seed soaking and foliar spraying—on soybean carbon and nitrogen metabolism using metabolomics analysis. The results demonstrated that MT treatment significantly upregulated the TCA cycle, providing energy and precursors for amino acid and carbohydrate synthesis. Key amino acid pathways, including histidine and phenylalanine metabolism, were enhanced, with histidine metabolism stimulating purine synthesis to improve biological nitrogen fixation and phenylalanine metabolism promoting secondary metabolite production to support growth. Additionally, carbohydrate pathways such as starch and sucrose metabolism and glycolysis/gluconeogenesis were positively regulated, ensuring energy supply and carbon homeostasis. Overall, dual MT treatment enhanced soybean metabolic capacity by promoting amino acid and carbohydrate metabolism, stimulating purine and secondary metabolite production, and maintaining carbon and nitrogen balance. These findings underscore melatonin's regulatory role in soybean growth and provide insights for improving crop productivity.

Keywords: soybeans; melatonin; metabolomics; amino acid metabolism; carbohydrate metabolism

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Academic Editor: Ju-Kyung Yu

Received: 21 February 2025
Revised: 16 March 2025
Accepted: 20 March 2025
Published: 23 March 2025

Citation: Wang, Y.; Jin, X.; Zhang, Y. Dual Melatonin Enhances Coordination Between Carbon and Nitrogen Assimilation in Soybean. *Agriculture* **2025**, *15*, 681. https://doi.org/10.3390/agriculture15070681

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1. Introduction

Soybean [*Glycine max* (L.) Merr.] is a crucial oil crop and a primary protein source for both human consumption and animal feed [1]. Recently, soybean cultivation has significantly expanded, resulting in a global yield surpassing 300 million tons by 2019. However, with the increasing global population, soybean production still falls short of meeting the current demand [2]. Therefore, it is essential to enhance soybean growth and increase its productivity.

Melatonin (MT), an indolic compound with hormone-like properties, is structurally similar to other biologically significant molecules such as tryptophan, serotonin, and indole acetic acid (IAA) [3]. Initially discovered in plants in 1995, MT has since been recognized as a versatile metabolite extensively distributed across various plant organs, including leaves, stems, roots, fruits, and seeds [4,5]. Extensive research has demonstrated the protective role of MT in various physiological processes, including germination, photosynthesis, water regulation, primary and secondary metabolisms, as well as the growth and development of vegetation [6]. For instance, He et al. [7] demonstrated that silencing COMT1, a key gene involved in MT biosynthesis in tomato plants, led to impaired endogenous MT synthesis,

resulting in delayed fruit development, reduced fruit weight, and lower seed-setting rates. Similarly, Samani et al. [8] reported that exogenous MT application significantly enhanced biomass productivity in microalgae compared to untreated controls. These findings highlight the multifaceted roles that MT across different biological systems and its potential as a growth regulator in plants. In addition to its physiological roles, MT has been shown to modulate key metabolism pathways in plants, including amino acids, carbon fixation, sugar metabolism, phenylpropane, and REDOX. For example, MT has been reported to enhance the activity of the ascorbate–glutathione (ASA-GSH) cycle under stress conditions, promoting the biosynthesis of ascorbic acid (AsA) and glutathione (GSH) and maintaining cellular redox homeostasis [9]. Furthermore, MT has been shown to regulate respiratory metabolism and energy status in Chinese cabbage [10]. Additionally, MT influences the levels of metabolites associated with the tricarboxylic acid (TCA) cycle, amino acid biosynthesis, and the production of flavonoids and phenylpropanoids [11,12]. These regulatory effects highlight the central role of MT in coordinating plant metabolism and stress responses, further emphasizing its potential as a key metabolic modulator in plants.

Maintaining a balance between carbon and nitrogen metabolism is crucial for regulating plant growth and development [13]. Nitrogen is an essential element for plants, serving as a fundamental component of amino acids, proteins, nucleic acids, chlorophyll, and plant hormones [14]. These processes of nitrogen assimilation and amino acid biosynthesis rely heavily on reducing agents, cellular energy, and a carbon framework, all of which are derived from photosynthesis and mitochondrial respiration [15]. Erdal [16] explored the influence of MT on nitrogen assimilation, mitochondrial respiration, and photosynthesis in maize seedlings, emphasizing the role of MT in maintaining the necessary balance between carbon and nitrogen metabolisms for plant growth. Similarly, Ren et al. [17] illustrated that MT alleviated growth inhibition and enhanced drought tolerance in maize by modulating carbon and nitrogen metabolism. Additionally, Xu et al. [18] explored the effects of exogenous MT on amino acid metabolism and nitrogen utilization in cucumber seedlings under stress environments, underscoring the function of MT in enhancing nitrogen and carbon metabolism for the enhancement of plant growth and stress tolerance. Together, these studies underscore the pivotal role of MT in optimizing carbon and nitrogen metabolism to enhance plant growth and stress resistance.

In recent years, MT has been shown to enhance soybean's resistance to abiotic stress, improve carbon and nitrogen metabolism, and promote growth and development [19–21]. However, its effects on soybean growth under normal (non-stress) conditions remain poorly understood. Metabolites, as downstream end products of genes and protein activity, serve as critical indicators of physiological and biochemical processes, as their composition and abundance directly influence phenotypic outcomes [22]. To investigate the impact of MT on soybean metabolism and growth, this study employed a non-targeted metabolomics approach. The soybean variety "Suinong 26" was selected as the test variety, and a dual strategy was implemented. This strategy involved MT treatment, where seeds were soaked in MT at a concentration of 500 μmol/L, and foliar spraying with MT at a concentration of 100 μmol/L at the V2 stage of soybean growth.

2. Material and Experimental Design

2.1. Plant Materials

This study was conducted at the experimental base of Heilongjiang Bayi Agricultural University, Heilongjiang Province, Northeast China (124°19′–125°12′ E, 45°46′–46°55′ N). The site is in a temperate continental monsoon climate zone, with an average annual accumulated temperature of 2689 °C, a daily average temperature of 23.6 °C in summer,

and an average temperature difference of 8 °C between day and night. All potted plants were placed under a canopy in a carefully controlled environment.

The soybean variety "Suinong 26" was selected for this study, which was provided by the Soybean Research Institute of Daqing Branch of Heilongjiang Academy of Agricultural Sciences. The soil used in the experiment was chernozem soil, taken from Chunlei Farm in Daqing City, Heilongjiang Province, and the previous crop was corn. The basic fertility of the soil was as follows: alkaline nitrogen 88.7 mg/kg, available potassium 18.9 mg/kg, available phosphorus 113.8 mg/kg, organic matter 4.1 mg/kg, and pH 7.2.

2.2. Experimental Design

For the experiment, uniform and fully developed soybean seeds were selected. These seeds were first soaked in 75% alcohol for 2 min, followed by a 5 min soak in 5% NaClO solution. Afterward, the seeds were thoroughly rinsed with distilled water several times. The cleaned seeds were then placed into beakers.

To prepare the pots for the experiment, they were initially sprayed with 75% alcohol. Subsequently, they were placed in a natural environment to air dry before the start of the experiment. The pots had a diameter of 30.0 cm and a height of 33.0 cm. We prepared a total of 24 pots; six seeds were sown evenly in each pot, and three homogeneous seedlings were retained at the cotyledon stage. The specific treatment setup included 12 pots with normal water treatment (CK) and 12 pots with dual MT treatment (CKM). For each treatment group, the second or third leaves from the top were harvested every 5 days, resulting in a total of 5 harvests.

2.3. Melatonin (MT) Treatment

Melatonin (MT) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Under dark conditions, a 10 mmol/L MT solution was prepared, placed in a brown bottle, and stored in a refrigerator at 4 °C as a stock solution. The dual MT treatment involved both seed soaking and foliar spraying. For the soybean seed soaking treatment, sterilized soybean seeds were immersed in a 500 μmol/L MT solution for 6 h, with the volume of the MT solution being approximately 3 times that of the soybean seeds. After the soaking process, the seeds were placed on a super-clean work table to air dry naturally. Regarding the foliar spraying treatment, a 100 μmol/L MT solution was uniformly sprayed on the front and back sides of stage V1 soybean leaves at 21:00 for 3 consecutive days under dark conditions, ensuring that the droplets reached the surface of the leaves.

2.4. Sample Preparation for LC-MS

The soybean leaves (0.5 g) samples for the third time (15 d after treatment) after being treated with H_2O (CK) and dual MT were grounded and mixed and then sent to BmK company for nontargeted LC-mass spectrometry (MS) detection. Each treatment was set up for six replicates. Data analysis was performed using BMK Cloud (www.biocloud.net accessed on 17 March 2025).

2.5. The Content of Components in the TCA Cycle

The soybean leaves (0.1 g) sampled for the third time after being treated with CK and double MT were ground and mixed to determine the content of components in the TCA cycle, including citric acid, malic acid, oxoglutaric acid, and fumaric acid. These components were quantified using assay kits obtained from Hefei Laier Biotechnology Co., Ltd., Hefei, China, and Beijing Solarbio Science & Technology Co., Ltd., Beijing, China, and Beyotime Biotech Inc., Shanghai, China, respectively, following the manufacturer's instructions.

2.6. Statistical Analysis

Data were expressed as mean ± standard error (SE) after a one-way analysis of variance (ANOVA) using the SPSS 17.0 program (SPSS Inc. Chicago, IL, USA). The data were statistically analyzed using the multiple range test of Duncan ($p < 0.05$). The PCA and OPLS-DA were performed using SIMCA v13.0.3 software (Umetrics, Umea, Sweden). Heatmaps were constructed using TBtools-II software (v2.056) [23].

3. Results

3.1. Metabolic Profiling and Quality Control

To investigate the impact of dual melatonin (MT) application on soybean leaf metabolites through seed soaking and leaf spraying, non-targeted metabolomics analysis under two different treatments was conducted: (1) water (CK); and (2) dual MT treatment (CKM). The results of the orthogonal partial least squares-discriminant analysis (OPLS-DA) score plots for the CK/CKM comparisons showed high, Q2Y (0.983) and R2Y (0.998) scores, indicating the robustness and dependability of the models for identifying differential metabolites (Figure 1A).

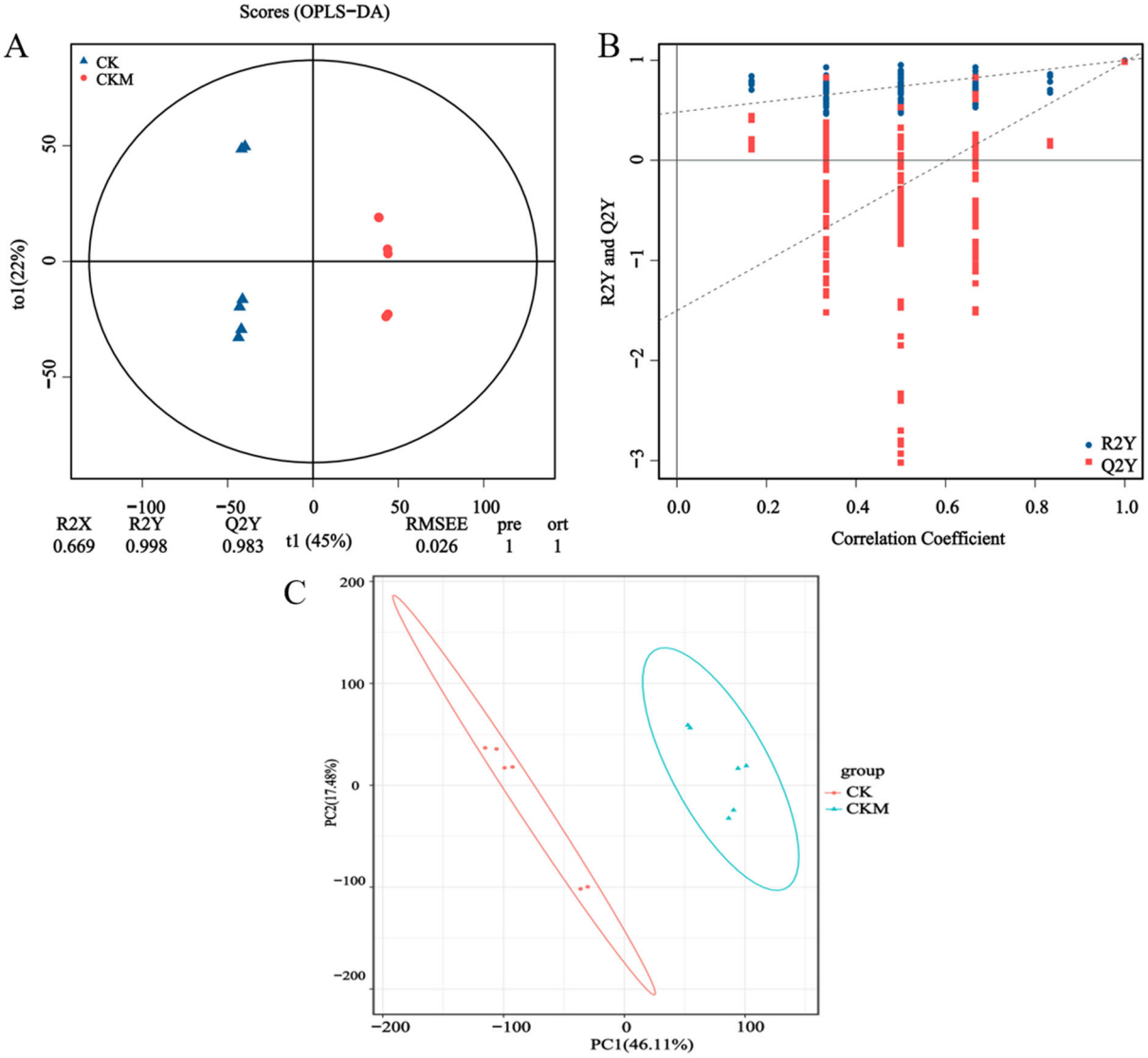


Figure 1. (**A**) The Scatter plots of OPLS-DA of the samples, (**B**) model permutation verification diagram, and (**C**) principal component analysis (PCA) of the CK/CKM group.

The validation of the OPLS-DA models was performed using permutation tests, as illustrated in Figure 1B. In the figure, blue dots and red dots represent the R^2Y and Q^2Y values of the model after permutation, respectively. The two dotted lines correspond to the regression lines fitted for R^2Y and Q^2Y. The positive slope of the Q^2Y regression line indicates that the model is statistically meaningful. Furthermore, the blue dots are predominantly positioned above the red dots, suggesting that the modeling training set and test set are independent, which further supports the robustness of the model.

Additionally, the Principal component analysis (PCA) in Figure 1C illustrates significant differences between the CK and CKM groups, indicating that the metabolic profile of soybean leaves was indeed affected by the dual MT treatment. The consistent repeatability of each treatment further strengthens the findings, suggesting that under normal conditions, the application of dual MT disrupted the metabolic balance in soybean leaves. These results provide valuable insights into the impact of dual MT treatment on soybean leaf metabolism and highlight its potential implications for plant physiology and stress responses.

3.2. Analysis of the Differential Metabolites (DMs)

In the CK/CKM comparisons, a total of 2123 differential metabolites (DMs) were identified, meeting the criteria of $|\text{Fold change (FC)}| \geq 1$ and $p < 0.05$ for both treatment groups. Among these DMs, 904 were significantly upregulated, while 1219 were downregulated (Figure S1). Additionally, the study identified 10 DMs with the most pronounced differences, based on the smallest *q*-value. These metabolites are: 2-oxo-2,3-dihydrofuran-5-acetate, 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate, D-1-aminopropan-2-ol o-phosphate, 3-hydroxy-3-methylglutarate, nagilactone B, D-lactic acid, dTDP-3-N, N-dimethylamino-2,3,6-trideoxy-4-keto-D-glucose, aldosterone, 5-N-Acetyl-7-N-(D-alanyl)-legionaminic acid, and coptisine, respectively (Figure 2). These metabolites exhibited the most significant variations between the CK and CKM groups, and 7 metabolites were notably enriched in metabolic pathways (Table S1), offering essential insights into the specific metabolic changes induced by the dual MT treatment in soybean leaves. Additionally, compared to CK, 5 DAMs were significantly upregulated under CKM treatment. Among these, 3-hydroxy-3-methylglutarate is involved in fatty acid metabolism and energy production, while D-lactic acid plays an important role in lactic acid fermentation and glucose metabolism. These results suggest that CKM treatment helps optimize nutrient accumulation and energy balance during soybean growth.

3.3. Metabolic Pathways Enrichment Analysis

The KEGG enrichment bubble map has provided valuable insights into the pathways significantly enriched with the annotated DMs in the CK/CKM comparisons. Specifically, DMs were found to significantly enrich the pathways including biotin metabolism, nicotinate and nicotinamide metabolism, arginine biosynthesis, monobactam biosynthesis, and pentose and glucuronate interconversions (Figure 3). These pathways promote the biosynthesis and metabolism of soybeans. Additionally, among the top 20 significantly enriched pathways, we found 5 pathways were linked to amino acid metabolism, such as arginine biosynthesis, tyrosine metabolism, lysine biosynthesis, histidine metabolism, and glutathione metabolism (Figure 3, marked in red). Moreover, 8 pathways related to carbohydrate metabolism were identified, including the pentose phosphate pathway, pyruvate metabolism, C5-branched dibasic acid metabolism, citrate cycle (TCA cycle), starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, ascorbate, and aldarate metabolism, and butanoate metabolism (Figure 3, marked in blue). This highlights the significant impact of dual MT treatment on the carbon metabolism of soybean seedlings.

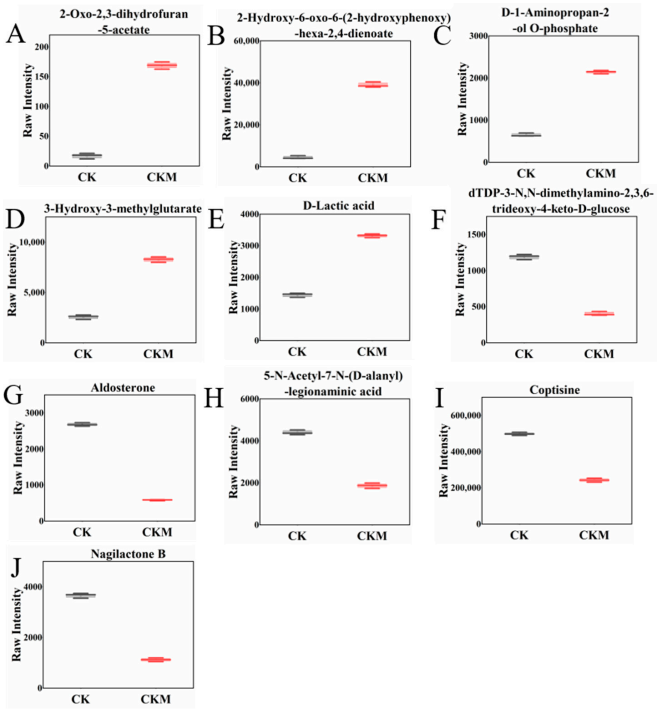


Figure 2. Up-regulated and down-regulated the top 10 DMs (n = 6). (**A**–**J**) The raw intensity of 2-oxo-2,3-dihydrofuran-5-acetate, 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate, D-1-aminopropan-2-ol o-phosphate, 3-hydroxy-3-methylglutarate, nagilactone B, D-lactic acid, dTDP-3-N,N-dimethylamino-2,3,6-trideoxy-4-keto-D-glucose, aldosterone, 5-N-acetyl-7-N-(D-alanyl)-legionaminic acid, and coptisine, respectively.

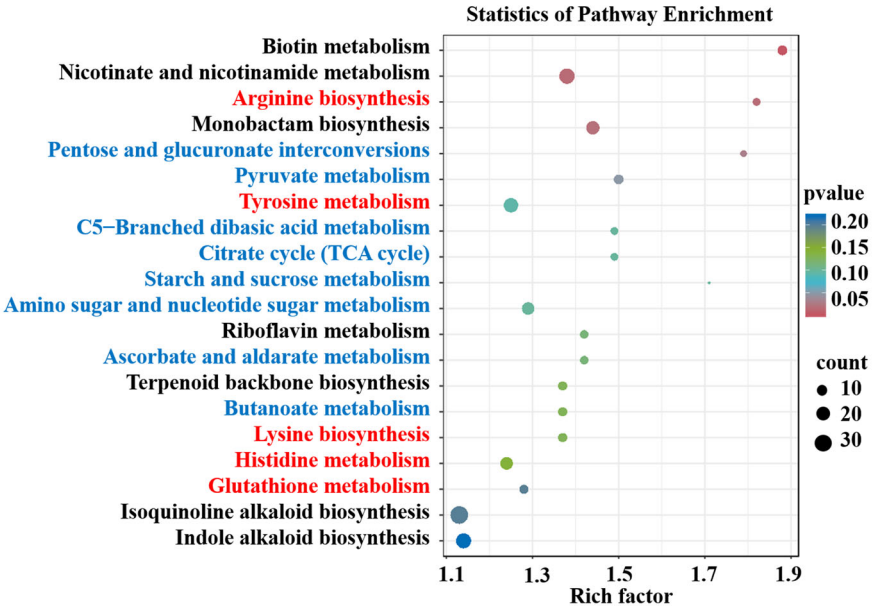


Figure 3. The top 20 KEGG enrichment map of differential metabolites. The blue rectangle represents the pathway associated with amino acids, and the red rectangle represents the pathway associated with carbohydrates.

Amino acid and carbohydrate metabolism play crucial roles in regulating nitrogen and carbon source assimilation, as well as the transport of these sources and pools in soybeans. These metabolic pathways provide essential precursors and energy for plant growth, development, and nutrient formation. Consequently, dual melatonin treatment enhances the growth and development of soybean seedlings and promotes nutrient formation.

3.4. Amino Acid Metabolic Pathways Enrichment Analysis

In this study, we identified a total of 140 amino acids and amino acid derivatives, of which 73 exhibited significant upregulation and 67 showed significant down-regulation (Table S2). These DMs were involved in 20 distinct amino acid pathways, primarily including arginine biosynthesis, lysine biosynthesis, glutathione metabolism, tyrosine metabolism, and histidine metabolism (Figure S2). Notably, arginine and tyrosine are positioned upstream of the tricarboxylic acid (TCA) cycle, whereas lysine is located downstream. The TCA cycle serves as a central hub in plant metabolic processes. As illustrated in Figure 4A,B,D, 10, 12, and 23 DMs within arginine biosynthesis, lysine biosynthesis, and tyrosine metabolism, respectively, were significantly induced. These findings suggest that dual MT treatment positively modulates nitrogen assimilation and energy production in soybeans, thereby directly contributing to enhanced growth rates and stress tolerance. Furthermore, Glutathione, a critical antioxidant, plays an essential role in the glutathione-ascorbic acid cycle, where it collaborates with ascorbic acid to maintain cellular redox stability. As demonstrated in Figures 4C and S3, dual MT treatment significantly upregulated gamma-L-glutamyl-L-cysteine (the biosynthetic precursor of glutathione) and dehydroascorbic acid. These results indicate that dual MT treatment enhances redox homeostasis, a key mechanism for preserving cellular integrity under oxidative stress conditions. Additionally, amino acid metabolism is integral to the regulation of nitrogen assimilation, as well as the transport and allocation of nitrogen sources in soybeans. Pathways such as histidine metabolism, valine metabolism, cysteine and methionine metabolism, phosphonate and phosphinate metabolism, phenylalanine metabolism, and valine, leucine, and isoleucine biosynthesis were positively influenced by dual MT treatment (Figures 4E and S4A–D). Collectively, these findings underscore the beneficial effects of dual MT treatment in optimizing amino acid metabolism, thereby improving nitrogen assimilation, antioxidant capacity, and overall growth performance in soybeans.

3.5. Carbohydrate Metabolites Analysis

In this study, we identified a total of 15 carbohydrate-related metabolic pathways, which play critical roles in carbohydrate utilization and energy production within the cell. These pathways include starch pentose and glucuronate interconversions, starch and sucrose metabolism, pyruvate metabolism, citrate cycle (TCA cycle), C5-branched dibasic acid metabolism, ascorbate and aldarate metabolism, butanoate metabolism, amino sugar, and nucleotide sugar metabolism, fructose and mannose metabolism, propanoate metabolism, galactose metabolism, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, pentose phosphate pathway, and inositol phosphate metabolism (Figure S5). These pathways are crucial for carbohydrate utilization and energy production within the cell. Notably, 12 out of the 15 pathways were positively regulated by dual MT treatment, except for C5-branched dibasic acid metabolism, galactose metabolism, butanoate metabolism (Figures 5A–E and S6A,B). This widespread upregulation suggests that dual MT treatment significantly enhances carbohydrate metabolism, which is essential for supporting energy production and biomass accumulation in soybean plants.

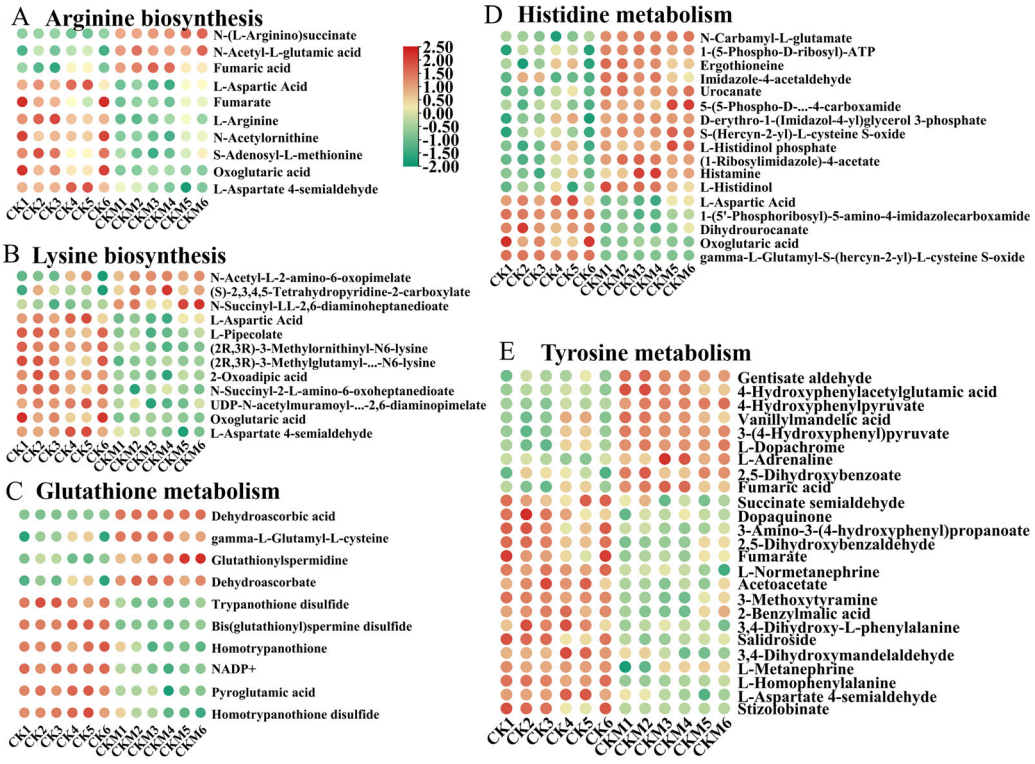


Figure 4. (**A**–**E**) Heat map of DMs of arginine biosynthesis, lysine biosynthesis, glutathione metabolism, histidine metabolism, and tyrosine metabolism, respectively (n = 6).

Among the 15 pathways, we identified 77 DMs, with 49 significantly upregulated and 28 significantly down-regulated (Figure S7). With $|\log2FC| \geq 2$ and q-value < 0.05, we identified 17 distinct carbohydrates were obtained, of which 13 were significantly upregulated, These included thiamin diphosphate, 3-carboxy-1-hydroxypropyl-ThPP, L-malic acid, d-xylonate, manninotriose, dehydroascorbic acid, L-arabinono-1, 4-lactone, N-glycoloylneuraminate, N-acetyl-D-mannosamine, 2-deoxy-5-keto-d-gluconic acid, mesaconate, citraconic acid, and 2-methylmaleate. Notably, 3-carboxy-1-hydroxypropyl-ThPP and L-malic acid are key intermediates in the TCA cycle (Figure S8), further confirming that dual MT treatment positively regulates this central metabolic pathway. This finding underscores the broader impact of dual MT treatment on enhancing energy metabolism and overall metabolic efficiency in soybean plants.

To validate the reliability of the LC-MS data, we quantified the levels of key TCA cycle intermediates, including citric acid, malic acid, fumaric acid, and oxoglutaric acid, to examine their content changes after dual MT treatment. As shown in Figure S9, the results were consistent with the LC-MS data: malic acid and fumaric acid levels increased significantly, while oxoglutaric acid levels decreased and citric acid levels remained unchanged. These findings provide further evidence that dual MT treatment enhances TCA cycle activity, thereby improving energy production and metabolic flux in soybean plants.

Overall, this study demonstrates that dual MT treatment positively regulates carbohydrate metabolism, leading to enhanced carbohydrate accumulation and improved energy availability. These metabolic changes are likely to contribute to increased biomass production and overall growth performance in soybean plants. By optimizing carbohydrate utilization and energy metabolism, dual MT treatment offers a promising strategy for enhancing soybean productivity and nutritional quality.

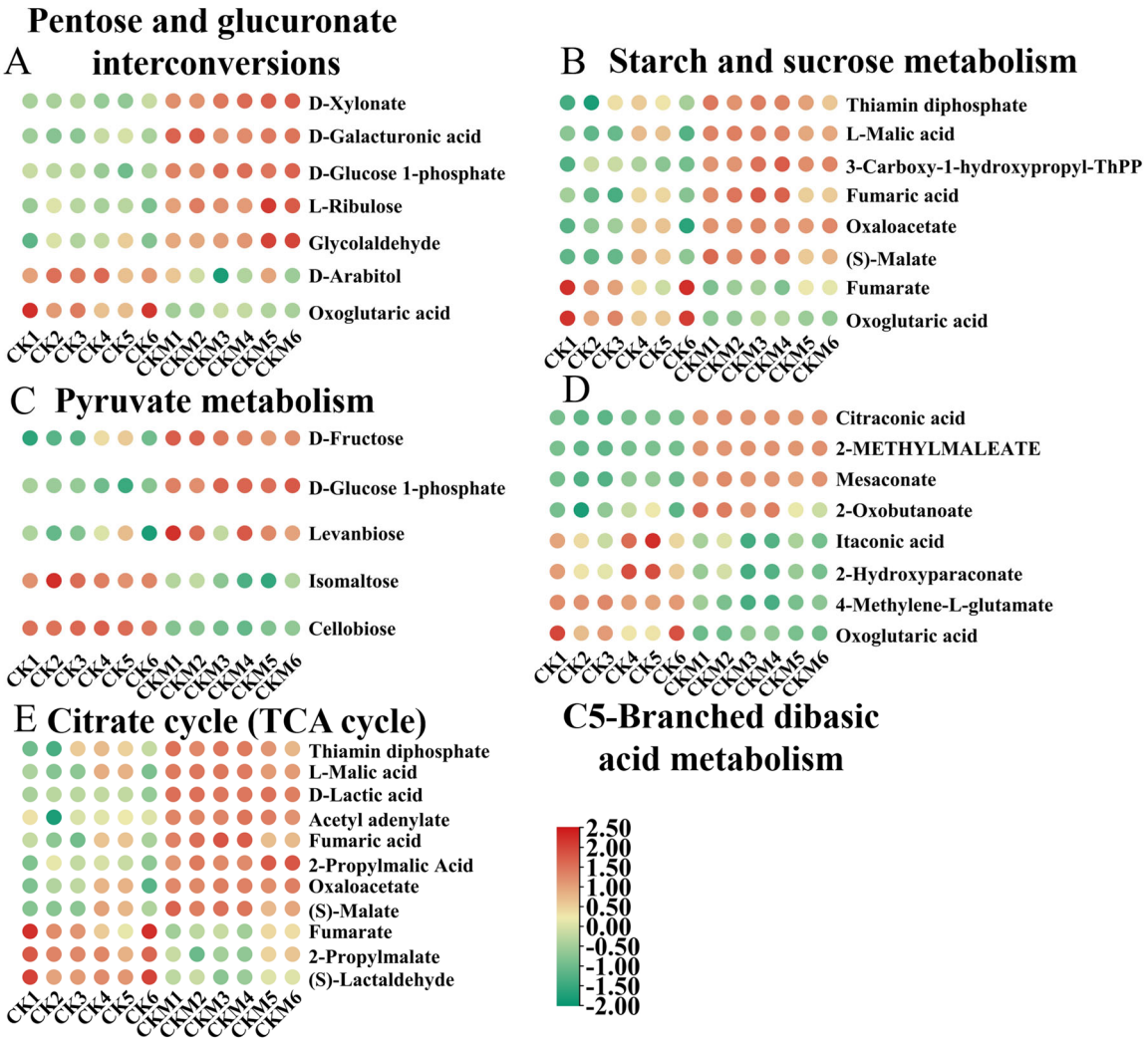


Figure 5. (**A**–**E**) Heat map analysis of DMs enriched in the pathway of pentose and glucuronate interconversions, starch and sucrose metabolism, pyruvate metabolism, citrate cycle (TCA cycle), c5-branched dibasic acid metabolism, and ascorbate and aldarate metabolism, respectively (n = 6).

4. Discussion

Soybean (*Glycine max* (L.) Merr.) is a vital oil crop and a primary source of protein for both humans and animals. Therefore, enhancing soybean growth and yield is a significant challenge in agricultural production. Melatonin (MT), a biostimulant, has garnered increasing attention in recent years and is recognized as an innovative plant growth regulator. It significantly enhances substance accumulation by improving carbon and nitrogen metabolism, maintaining redox balance, and promoting secondary metabolism [3]. To address the challenges in soybean cultivation, a dual MT treatment (soaking seeds and foliar spraying) was employed, and LC-MS was used to analyze the metabolites in soybean leaves treated with H_2O (control, CK) and dual MT treatment. This study showed that dual MT treatment significantly regulated 20 amino acid and 15 carbohydrate metabolic pathways, and significantly upregulated the TCA cycle, the central hub of cell metabolism.

The TCA cycle plays a crucial role in delivering energy to various organelles and maintaining diverse physiological functions [24]. Key intermediates such as malic acid, fumaric acid, oxaloacetate, etc. are integral to the TCA cycle, serving as reductants, osmotic regulators, and pH balancers in plant metabolism [25]. In our study, dual MT treatment significantly elevated the level of thiamin diphosphate, L-malic acid, 3-carboxy-1-hydroxypropyl-ThPP, fumaric acid, oxaloacetate, (S)-malate. This suggests that dual

melatonin treatment can increase the efficiency of the TCA cycle, thereby providing energy for soybean growth and development, and promoting various physiological activities, which is consistent with previous findings [11,12]. In addition to energy supply, the TCA cycle is also a hub for carbohydrate and amino acid metabolism to connect and convert to each other. For example, oxaloacetic acid serves as a precursor for gluconeogenesis, which is used for glucose synthesis, as well as for the synthesis of aspartate and asparagine [26–28]. Therefore, the enhancement of the TCA cycle by dual melatonin treatment is key to improving both carbohydrate and amino acid metabolism. Zhao et al. [29] also demonstrated that enhanced TCA cycle activity may lead to stronger nitrogen assimilation.

Amino acids are critical products of nitrogen assimilation, serving as fundamental building blocks for protein synthesis and precursors for various metabolites. They play essential roles in multiple key metabolic processes during plant growth and development [19,30]. Cao et al. [31] demonstrated that melatonin can enhance amino acid synthesis to increase soybean biomass under drought stress. In this study, we found that dual MT treatment significantly regulated multiple amino acid metabolic pathways, including histidine metabolism, phenylalanine metabolism, valine metabolism, and glutathione metabolism, etc. These results indicate that dual melatonin treatment also has a positive regulatory effect on soybean biomass under normal growth conditions. Specifically, in histidine metabolism, dual MT treatment notably enhanced the level of phosphoribosyl-ATP and phosphoribosyl-formimino-AICAR-P, thereby reinforcing the purine metabolism (Figure S10). Purines play a crucial role in biological nitrogen fixation and root nodules development in soybeans [32,33]. Therefore, dual MT treatment may enhance purine metabolism, promoting biological nitrogen fixation, root nodule growth, and overall plant growth regulation. Furthermore, phenylpropane is a key precursor of a variety of secondary metabolites, such as flavonoids, anthocyanins, and isoflavones [34–36], and its metabolism was significantly upregulated by dual MT treatment. This upregulation likely facilitates the biosynthesis of these secondary metabolites, which enhance soybean adaptability to environmental stress and support normal growth and development [37,38]. In glutathione metabolism, the gamma-L-Glutamyl-L-cysteine (The precursor of glutathione) and dehydroascorbic acid were significantly upregulated by dual MT treatment. Simultaneously, dual MT treatment positively regulated ascorbate and aldarate metabolism (Figure S11). The glutathione-ascorbate cycle is at the heart of a network of cellular REDOX systems that support energy transfer in different cellular regions and integrate plant signaling pathways [39]. Glutathione and ascorbate as a multifunctional metabolite that regulates plant growth and development [40]. Therefore, dual MT treatment may modulate soybean physiological processes by activating glutathione and ascorbic acid signaling pathways, maintaining cellular REDOX homeostasis, and promoting growth and development.

The TCA cycle plays a central role in carbohydrate metabolism [41]. Carbohydrates are broken down into pyruvate via glycolysis, and pyruvate is further converted into acetyl-CoA, which enters the TCA cycle. Ultimately, it is fully oxidized to CO_2 and H_2O, generating large amounts of ATP and reducing power (NADH and $FADH_2$). This process not only provides energy for plant growth but also supplies carbon skeletons for pathways such as amino acid synthesis [42,43]. Our research found that double melatonin (MT) treatment significantly upregulated key metabolites in the TCA cycle (such as L-malate and fumarate), indicating that MT enhances the activity of the TCA cycle, thereby promoting the efficient utilization of carbohydrates and energy supply. In this study, dual MT treatment positively influences carbohydrate catabolism, affecting processes such as starch and sucrose metabolism, pyruvate metabolism, fructose and mannose metabolism, propanoate metabolism, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, and pentose phosphate pathway. This suggests that double MT treatment promotes carbo-

hydrate synthesis, which is consistent with Cao et al. [31]. Carbohydrates are essential substances for plant life activities, and as the main products of photosynthesis, they not only provide the necessary energy for plant growth but also affect plant biomass formation by maintaining cell osmotic potential [44–47]. In soybeans, starch and sucrose metabolism are key carbohydrate processes occurring through distinct pathways [48]. Our study observed an increase in fructose levels, with fructose-6-phosphate, produced by the Calvin cycle, being converted to glucose-6-phosphate and subsequently used to synthesize starch (Figure S12). Carbohydrates like starch and sucrose are oxidized and decomposed through glycolysis/gluconeogenesis and the TCA cycle to provide energy for plants [49]. Glycolysis and gluconeogenesis boost ATP production, enhancing plants' adaptability and growth. Wei et al. [50] proposed that MT may enhance soybean growth by upregulating genes involved in photosynthesis, carbohydrate metabolism, and ascorbic acid metabolism. Our study confirms that dual MT treatment upregulates metabolites related to carbohydrate metabolism, indicating its potential to improve soybean growth and yield, though further research is needed to fully understand the process.

5. Conclusions

In this study, the effects of dual MT treatment—that is seeding with MT and foliation at the V2 stage—on soybean growth were studied, and metabolomics was used to analyze the regulation of dual MT on soybean metabolic pathways and metabolites. Our results indicate that dual MT treatment activated the TCA cycle by increasing the content of L-malic acid, 3-carboxy-1-hydroxypropyl-ThPP, fumaric acid, oxaloacetic acid, and (S)-malic acid. This provides energy and precursor substances for the synthesis of amino acids and carbohydrates, enhancing soybean carbon and nitrogen metabolism. This also promoted the nitrogen-fixing ability of soybean nodules and provided precursors for the synthesis of secondary metabolites. Therefore, dual melatonin treatment has the potential to promote soybean growth and development and increase yield. The study provides valuable insights into the impact of MT on soybean metabolism, growth, and development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15070681/s1, Figure S1. Volcanic map of DAMs in CK /CKM group. Red circles indicate up-regulation of metabolites, and blue circles indicate down-regulation; Figure S2. The KEGG enrichment map related to amino acid metabolism; Figure S3. The pathways of the glutathione metabolism. The colored circles in the figure represent metabolites with significant differences in the CK/CKM comparison group, with red indicating up-regulation and green indicating down-regulation; Figure S4. (A–D) heat map of DMs of cysteine and methionine metabolism, phosphonate and phosphinate metabolism, phenylalanine metabolism and valine, leucine, and isoleucine biosynthesis, respectively. Data are the means of six replicates. Red circles indicate up-regulation of metabolites, and green circles indicate down-regulation; Figure S5. The KEGG enrichment map related to carbohydrate Metabolites; Figure S6. (A,B) heat map of DMs of galactose metabolism, butanoate metabolism. Data are the means of six replicates. Red rectangles indicate up-regulation of metabolites, and green rectangles indicate down-regulation; Figure S7. Heat map of carbohydrate. Data are the means of six replicates. Data are the means of six replicates. Red rectangles indicate up-regulation of metabolites, and green rectangles indicate down-regulation; Figure S8. The pathways of the TCA cycle. The colored circles in the figure represent metabolites with significant differences in the CK/CKM comparison group, with red indicating up-regulation and green indicating down-regulation; Figure S9. (A–D). The content of citric acid, malic acid, fumaric acid, and oxoglutaric acid. Data are the means of three replicates with SE, and asterisk (*) indicate a significant difference ($p < 0.05$); Figure S10. The pathways of histidine metabolism and purine metabolism. The colored circles in the figure represent metabolites with significant differences in CK/CKM comparison group, with red indicating up-regulation and green indicating

down-regulation; Figure S11. The pathways of ascorbate and aldarate metabolism. The colored circles in the figure represent metabolites with significant differences in CK/CKM comparison group, with red indicating up-regulation and green indicating down-regulation; Figure S12. The pathways of starch metabolism and sucrose metabolism. The colored circles in the figure represent metabolites with significant differences in CK/CKM comparison group, with red indicating up-regulation and green indicating down-regulation; Table S1: Enrichment analysis of 10 DMs with the most significant differences; Table S2: Information on amino acids and their derivatives.

Author Contributions: Validation, formal analysis, investigation, resources, visualization, data curation, writing—original draft, Y.W.; Conceptualization, software, methodology, and supervision, Y.W. and X.J.; Funding acquisition: Y.W. and Y.Z.; Project administration and writing—review and editing, Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the China Agriculture Research System of MOF and MARA (CARS-04-PS18), Heilongjiang Province's "Revealing the List and Commanding the Leaders" scientific and technological research project (2021ZXJ05B02), Open Project of Heilongjiang Provincial Key Laboratory of Modern Agricultural Cultivation and Crop Germplasm Improvement (109201705), and Intramural Cultivation Project of Heilongjiang Bayi Agricultural University (XZR2016-02).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author/s.

Conflicts of Interest: The authors declare no conflicts of interest.

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