The Effect of Nitrogen Fertilizer on Rhizome Quality and Starch Physicochemical Properties in *Nelumbo nucifera*

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**Abstract:** Optimal nitrogen (N) supply significantly increases the starch content, components, and yield of *Nelumbo nucifera*. However, the underlying transcriptional mechanism and starch accumulation under dose-dependent nitrogen fertilizer are poorly understood. In this study, we found that the optimal nitrogen fertilizer (N2, 30 kg/667 m²) was more beneficial to improve the stomatal conductance (Gs), leaf intercellular CO₂ concentration (Ci), transpiration rate (Tr), net photosynthetic rates (Pn), chlorophyll content, starch content, and plot yield. What is more, N2-fertilizer treatment induced a higher number of starch granule, AP2 content, and RVA curve peaks. Then, the transcriptomic analyses performed in control (CK) and N2-fertilizer treatment (N2) showed that the expressions of many differentially expressed genes (DEGs) were significantly induced by N2. KEGG and GO enrichment analysis showed that these DEGs were significantly enriched in biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, carbon metabolism, carbon fixation in photosynthetic organisms, plant hormone signal transduction, and starch and sucrose metabolisms, suggesting that nitrogen fertilizer induced alterations of photosynthesis- and starch accumulation-related gene expression profiles. Finally, six photosynthesis-related genes and fourteen starch synthesis-related genes were confirmed to be required for starch accumulation in *Nelumbo nucifera* rhizome under N-fertilizer treatment. qPCR analysis of six starch accumulation-related genes demonstrated the accuracy of the transcriptome.

**Keywords:** N-fertilizer; starch; transcriptomic analyses; *Nelumbo nucifera*

1. Introduction

*Nelumbo nucifera* Gaertn, commonly known as lotus, is a kind of perennial aquatic vegetable, belonging to the genus *Nelumbo* in the family *Nelumbonaceae* [1–4]. According to the different phenotypes that are the resulted from different directions of artificial cultivation and breeding, the *Nelumbo nucifera* could be divided into three types: rhizome, flowers, and seeds lotus [5]. The *Nelumbo nucifera* rhizome recognized as nourishing foods which riches in starch, protein, vitamins, flavonoid, and mineral substances, was famous for its nutritional and medicinal values, containing leaf, seed, and rhizome [6,7]. According to “Compendium of Materia Medica” written by Shizhen Li, “The lotus decocted with water could detoxify fungus poison and prevent blood collapse and blood drowning.” According to China characteristic vegetable industry technology system statistics, the planting area of lotus has reached 600,000 hectares in 2021 in China, and the productions of lotus are exported to Japan, South Korea, the United States, Great Britain, and other countries or states.
The development stage of lotus root could be classified into three stages, early swelling (S1), middle swelling (S2), and later swelling stage (S3). Lotus root at each stage needs a lot of fertilizer to promote its growth and development. Fertilizer is an important for farming regulation and has become the focus of research on crop plant growth and basic metabolic processes [8,9]. Especially, nitrogen (N), as one of the three macronutrient elements (nitrogen, phosphorus, and potassium) of plants, and the main component of essential compounds such as chlorophyll, nucleotides amino acids, and some plant hormones, plays an important role in the whole life cycle of plant growth and development [10,11]. Urea is the widely used nitrogen-containing fertilizer, effectively increasing crop yields [12]. However, the disastrous effects of excessive application of N-fertilizer gradually stand out because of residual nitrogen left in the soil and water [13]. Water eutrophication, soil acidification, contamination of groundwater, environmental degradation, and crop reduction often happened with excess nitrogen use [14–16]. Therefore, the optimum N rates and the mechanisms of nitrogen absorption and utilization became the research focus to resolve the above problems [9,17].

Nitrogen is the main component of photosynthetic machinery and enzymes, and is closely related to leaf photosynthesis that determines crop yield [18]. The photosynthetic rate of plants has a significant positive correlation with the nitrogen content of leaves [19]. Plant starch, one of the final product of photosynthesis, accounted for about 70% of the total dry matter of rhizome, whose content, composition, and structure were closely related to the quality of rhizome products [20]. The synthesis of plant starch carried out in chloroplasts and amyloplasts is regulated by environmental conditions, gene transcription levels, and metabolism levels in the plant [21,22]. In addition, sucrose, the important production of photosynthesis, became an indispensable resource involved in the synthesis of starch in amyloplasts. Hence, the photosynthesis (chloroplast)-sucrose/starch-starch (amyloplast) is an important model in plants. However, the molecular mechanism of nitrogen regulating photosynthesis and starch synthesis was still poorly understood. Given starch content is the main quality indicator of *Nelumbo nucifera*, the research of nitrogen regulating photosynthesis and starch synthesis is extremely important.

In this study, different concentrations of nitrogen were used to treat lotus at the different growth stages. The photosynthetic characteristics of lotus leaves and starch content of rhizome development were tested. The optimal nitrogen fertilizer was confirmed based on photosynthetic characteristics, starch content, and plot yield. We further characterized the regulatory genes by transcriptome and identified the important regulatory genes of lotus starch. We considered that these genes might be subjected to be further investigated to increase photosynthesis efficiency and starch accumulation.

### 2. Materials and Methods

#### 2.1. Plant Materials and Nitrogen Treatment

The main cultivar of lotus “MRH” was selected as the material and planted in the experimental station of aquatic vegetables, Yangzhou University, Yangzhou.

The 15 lotus plants were planted in a 2.5 m × 2.0 m regular plot and treated with nitrogen fertilizer. The test set urea (N > 46%) 0 kg/667 m² (CK), 20 kg/667 m² (N1), 30 kg/667 m² (N2), 40 kg/667 m² (N3), and 50 kg/667 m² (N4) as five concentrations of nitrogen treatment, and each treatment was repeated 3 times. To decrease the waste of nitrogen and pollution for water, we conducted the mixture (wet soil:nitrogen = 2:1) and then spread evenly over the plot. In this way, the nitrogen could sink directly to the bottom of the plot water. Rhizomes were sampled across three stages: early swelling (S1) (Figure 1A), middle swelling (S2) (Figure 1B), and later swelling (S3) (Figure 1C) stages throughout the growth season.
2.2. Determination of Photosynthetic Characteristics

The lotus leaf photosynthetic characteristics, leaf net photosynthetic rate, transpiration rate, intercellular CO$_2$ concentrations, and stomatal conductance, were tested by LI-6400 xt portable photosynthesis system (LI-COR companies, Lincoln, NE, USA) after nitrogen treatment 2, 4, 6, and 8 days (the determination time at 9 to 11 a.m. per day).

2.3. Determination of Starch Content

Make the standard curve before measuring rhizome starch. (1) 0.1 g starch was added into 2 mL water, (2) 3.2 mL 60% perchloric acid was added into above mixture while stirring until all mixture dissolved, (3) add water to 250 mL, (4) suck the solution with different gradients (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL) into the centrifuge tube, (5) add 3 mL distilled water and 2 mL iodine reagent into above mixture, (6) add water to 10 mL. Using distilled water as control, the OD values of each standard liquid were measured at 660 nm. The standard curve was calculated by the formula, $y = 0.013x - 0.0028$, $R^2 = 0.9996$. Then the total starch content was determined using iodine colorimetry based on the previous method with a few modification [5,23,24]. Starch content (%) = $R/$(sample weight $\times 0.01 \times 0.05 \times 106) \times 100$, R: Concentration measured on the standard curve (mg/kg).

Amylose content was determined using dual-wavelength spectrophotometry based on the previous method with slight modification [5,25]. First, 50 mg of the sample was weighed into a beaker, and 0.5 mL of 45% HClO$_4$ solution was added to the beaker, then shaken slightly, and left for 4 min. After the starch sample was completely dissolved, 8 mL of distilled water was added, and then mixed evenly. At the same way, in another beaker, 0.5 mL of 45% HClO$_4$ and 8 mL of distilled water was added to mix well and make a blank control. Next, 24 μL each of the mixed samples and the control was taken and put into 2 cuvettes, and then 300 μL iodine solution (2 g KI + 1 g I$_2$ into 300 mL distilled water) was added with 2376 μL distilled water in the cuvette. The solution was mixed well and OD values at 550 nm and 618 nm were measured. Amylose content (%), accounting for total starch) = $(3.5 - 5.1R)/(10.4R - 19.9)$, $R = 618$ nm OD value/550 nm OD value. Additionally, the hypothetical test of the data, as shown above, was analyzed by software SPSS. Means not sharing a common letter are significantly different between groups at $p = 0.05$ as determined by LSD after a one-way ANOVA test.

2.4. Transcriptome Sequencing

Based on the starch content results, 12 libraries, CK (0 kg/667 m$^2$ urea) and NT (30 kg/667 m$^2$ urea) groups at early swelling (S1) and middle swelling (S2), were sent to Gene Denovo (Guangzhou, China) to prepare for RNA-seq with three biological replicates per group. The raw reads produced by the sequencing instrument were filtered to remove...
adaptors, low-quality sequences with unknown nucleotides N, and read with more than 20% low quality bases (base quality < 10). What is more, the raw data can be obtained on NCBI SRA (accession number is PRJNA807076). The high quality clean reads were assembled into unigenes using the short reads assembling program Trinity [26]. The gene functions and classification were analyzed based on searches against the following databases: the National Center for Biotechnology Information (NCBI) non-redundant nucleotide [27], the Swiss-Prot database, EuKaryotic Orthologous Groups (KOG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Next, we used the Blast 2 GO program to obtain a GO annotation of the transcripts [28], and the GO function was classified by the WEGO software [29]. The coding sequences (CDSs) were predicted by using the best matched fragments of the annotated unigenes that were obtained using BLASTX with a threshold E-value of $10^{-5}$ against protein databases in Nr, Swiss-Prot, KEGG, and KOG. Proteins showing the highest scores in the BLASTX results were used to determine the CDSs of the unigenes. The CDSs of the remaining un-annotated unigenes were predicted using ESTScan [30].

2.5. Differentially Expressed Genes (DEGs)

The expression level of each gene was calculated and normalized by fragments per kilobase of transcript per million mapped reads (RPKM), and differential expression analyses among the NCKS1-NTS2, NCKS1-NTS1, NCKS1-NCKS2, and NCKS2-NTS2 were identified by comparing their FPKM values when the p-value was <0.05 and the absolute value of the log2(fold change) was $>1$ [31,32]. To analyze the expression profiles of NCKS1-NTS2, NCKS1-NTS1, NCKS1-NCKS2, and NCKS2-NTS2 based on the RPKM values, Short Time-series Expression Miner (STEM) software (http://www.cs.cmu.edu/~jernst/ stem/ accessed on 25 March 2022) was used to display the trends in these two stages [33]. All genes were divided into several modules according to the expression pattern, and the p-value of each module was calculated using Permutation Test method. 97% DEGs were mapped to terms in the “Kyoto Encyclopedia of Genes and Genomes” (KEGG) database (https://www.kegg.jp/, accessed on 25 March 2022), and we looked for significantly enriched pathways in DEGs using the hypergeometric test. The pathways with p $\leq$ 0.05 were defined as the significantly changed KEGG pathways.

2.6. Real-Time Quantitative PCR (qRT-PCR)

Total RNA was isolated from treated rhizome using TaKaRa MiniBEST Plant RNA Extraction Kit (Takara, Dalian, China). Reverse transcription of RNA into cDNA was performed using the Prime Script RT reagent Kit (TAKARA, Beijing, China) with corresponding primers. Primers for qRT-PCR were designed using Primer 5.0 software (Premier Biosoft, San Francisco, CA, USA), and are listed in Table S1. The housekeeping gene was TURA, which encode the Nelumbo nucifera tubulin alpha-3 chain. Plus, the mastermix used for qRT-PCR was SYBR qPCR Master Mix kit (Vazyme, Nanjing, China). Three independent PCR reactions on the 7500 Fast Real-Time PCR System. The qRT-PCR assays were carried out with three biological and technical replicates. According to $2^{-\Delta\DeltaCT}$ method, gene expression levels were calculated [34].

3. Results

3.1. Dynamic Changes of Photosynthesis Characteristics under Nitrogen (N)-Fertilizer Treatment

Photosynthesis, the fundamental needs for animals and plants, directly affects the crop production [18]. Detecting dynamic changes of photosynthesis characteristics can apparently reflect the influence of N-fertilizer treatment for Nelumbo nucifera rhizome production and quality. By combining five traits, stomatal conductance (Gs), leaf intercellular CO2 concentration (Ci), transpiration rate (Tr), net photosynthetic rates (Pn), and chlorophyll content, it is possible to predict the photosynthetic capacity. Compared with CK, the four levels of N-fertilizer treatment (N1, N2, N3, and N4) increased the values of Gs, Ci, Tr, Pn, and chlorophyll content, and N2 displayed the most remarkable impact (Figure 2). For
example, Gs was increased and reached the maximum when the application rate of urea was 30 kg/667 m² (N2) on the sixth day (Figure 2A), and Ci, Tr, and Pn exhibited the same trend (Figure 2B–D). Meanwhile, N fertilizer kept chlorophyll content at a higher level compared with CK, and chlorophyll content displayed two peaks on the fourth day and eighth day (Figure 2E). These results demonstrated that N-fertilizer promoted photosynthesis related traits improvement, and the positive effects of N2 (30 kg/667 m² urea) was relatively obvious.

Figure 2. The photosynthesis-related physiological index under N-fertilizer treatment. (A) stomatal conductance (Gs), (B) leaf intercellular CO₂ concentration (Ci), (C) transpiration rate (Tr), (D) net photosynthetic rates (Pn), and (E) chlorophyll content. CK: Control, N1–N4: four levels of N-fertilizer treatment. Different letters indicate a significant difference at the 5% level.

3.2. Effects of N-Fertilizer Treatment for Rhizomes Starch Accumulation and Yield

N-fertilizer treatment could increase the crop yield and change the content and component of starch [35]. Therefore, the starch content in rhizomes grown under different concentrations of N-fertilizer was detected. Compared with CK, N-fertilizer treatment was more beneficial to the accumulate of total starch, amylose, amyllopectin, and plot yield (Figure 3A–C). Interestingly, N2 was the most outstanding in both photosynthetic traits and rhizomes starch content and yield among four levels N-fertilizer treatment, which suggested that N2 could be used as the optimal concentration of nitrogen fertilizer treatment for *Nelumbo nucifera* production. To elucidate these changes, the CK and N2 were selected to be further investigated at the transcriptional level.
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**Figure 3.** Starch content and plot yield under N-fertilizer treatment. The content of (A) total starch, (B) amylose, (C) amylopectin, and (D) plot yield. Different letters indicate a significant difference at the 5% level.

3.3. Analysis of Grain Morphology and Starch Structure

To further investigate the effect of N-fertilizer treatment on starch changes, isolated starch was used for SEM observation. The starch granules contained two types, including long oval and ground (Figure 4A,B). The starch granule number was then calculated, and the number of two type starch from CK was both lower compared with the N2-fertilizer treatment (Figure 4C).

To determine the effect of CK and N2 on starch fine structure, the starch samples were debranched and measured by GPC. The CK showed a higher AP1 but lower AP2 than N2 (Figure 4D,E), suggested that N2-fertilizer treatment changed the starch structure and further influence the rhizome cooking and starch pasting characteristic.

To evaluate rhizome quality under CK and N2, the pasting property which can reflect the rhizome texture was tested. The starch of N2 exhibited higher RVA curve peaks (PKV, HPV, BDV and FV) than that in CK (Table 1). However, the changes of SBV, Ptime, and PT in N2 were lower than that in CK, which suggests that N2 changed the starch component.

**Table 1.** Pasting properties of endosperm flour and starch and the thermal properties of starch from different NILs.

| Treatments | PKV (cP) | HPV (cP) | BDV (cP) | FV (cP) | SBV (cP) | Ptime (min) | PT (°C)  |
|------------|---------|---------|---------|---------|---------|------------|---------|
| CK         | 1400 ± 13.6 b | 1012 ± 15.6 b | 388 ± 10.5 b | 1331 ± 16.4 b | −69 ± 3.5 a | 4.8 ± 0.1 | 81.1 ± 0.1 a |
| N2         | 2873 ± 20.0 a | 1875 ± 16.1 a | 998 ± 13.9 a | 2507 ± 19.8 a | −366 ± 5.7 b | 4.4 ± 0.1 | 77.85 ± 0.1 b |

PKV, peak viscosity; HPV, hot paste viscosity; FV, final viscosity; BDV, breakdown viscosity; SBV, setback viscosity; Ptime, peak time; PT, pasting temperature. Different letters indicate a significant difference at the 5% level.
Venn diagram analysis showed that N2-fertilizer treatment induced 1266 differentially expressed genes (1378 up-regulated and 877 down-regulated) at stage S1, while 2255 differentially expressed genes (560 up-regulated and 706 down-regulated) at stage S2 (Figure 5B), which meaning many genes induced by N2-fertilizer treatment occurred at a key stage S2.

3.4. Transcriptome Sequencing and Analysis of DEGs

To evaluate the gene expression profile, mRNAs from two rhizomes developmental stages (NCKS1 and NCKS2) and two N2-fertilizer treatments (NTS1 and NTS2) were sequenced. In all, 4563 differential genes (the threshold change fold > 1 and p value < 0.05) containing up- and down-regulated from 12 cDNA libraries were identified (Figure 5A). Venn diagram analysis showed that N2-fertilizer treatment induced 1266 differentially expressed genes (560 up-regulated and 706 down-regulated) at stage S1, while 2255 differentially expressed genes (1378 up-regulated and 877 down-regulated) at stage S2 (Figure 5B), which meaning many genes induced by N2-fertilizer treatment occurred at a key stage S2.

Figure 4. SEM of starch and starch granular number. (A,B) SEM micrographs of purified starch, (C) the number of long oval and ground starch granule, and (D,E) relative molecular weight distributions. AP1 and AP2 indicate amylopectin fractions with short and long chain lengths, respectively. Different letters indicate a significant difference at the 5% level.

Figure 5. Summary of the differentially expressed genes among different stages under CK and N-fertilizer treatment. (A) The Venn diagram of DEGs of NCKS1-NTS2, NCKS1-NCKS2, NCKS1-NTS1, and NCKS2-NTS2, and (B) the number of up-regulated and down-regulated DEGs of four comparative groups.
3.5. Functional Analysis of DEGs under N2-Fertilizer Treatment

To further investigate the biological functions of the DEGs, we performed GO enrichment analyses (Figure 6), which showed that the DEGs were enriched in several key GO terms. Both CK and N2-fertilizer treatment, the top five biological processes were metabolic, cellular, single-organism, response to stimulus, and biological regulation. The catalytic activity and binding related DEGs were the overwhelming preponderant in ontology of molecular function. The cellular component mainly contained cell, cell part, organelle, and membrane which were the foundation component of plant life. Interestingly, compared with CK, N2-fertilizer treatment exhibited more advantages in inducing more genes expression in ontology of biological processes, molecular function, and cellular component, especially at stage S2 (Figure 6).

![Figure 6. The GO enrichment analyses of DEGs among four levels of N-fertilizer treatment. The blue bar represents the number of down regulated genes and the yellow bar represents the number of up regulated genes.](image)

In addition, to better understand the biological function of DEGs involving in rhizome development, the Kyoto Encyclopedia of Genes and Genes (KEGG) analysis was performed. Compared with NCKS1, the most DEGs of NTS1, NCKS2, and NTS2 were enriched in biosynthesis of secondary metabolites, metabolic pathways, phenylpropanoid biosynthesis, plant-pathogen interaction, and starch and sucrose metabolism (Figure 7C).

Similar to GO enrichment analysis results, N2-fertilizer treatment was also an important factor inducing more development- and stress-related genes expression, especially at stage S2. As shown in Figure 7C,D, after N2-fertilizer treatment, the pathways enriched by differentially expressed genes were changed, which mainly involved in carbon metabolism, carbon fixation in photosynthetic organisms, and plant hormone signal transduction, suggesting that nitrogen fertilizer induced alterations of photosynthesis- and starch accumulation-related gene expression profiles in *Nelumbo nucifera*. Other important pathways, including the MAPK signaling pathway, glycolysis/gluconeogenesis, and brassinosteroid biosynthesis, were also enriched.
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Figure 7. KEGG pathway enrichment analysis of the DEGs. (A) DEGs between NCKS1 and NTS1, (B) DEGs between NCKS1 and NCKS2, (C) DEGs between NCKS1 and NTS2, and (D) DEGs between NCKS2 and NTS2.

3.6. Identification of Starch Accumulation-Related Genes Induced by Nitrogen Treatment

To uncover the potential biological regulatory networks modulating the expression of the photosynthesis- and starch accumulation-related genes by nitrogen treatment, the two corresponding groups of DEGs were identified. In photosynthesis, *PSBP* has specific and important roles in stabilizing the active form of the PSII-light-harvesting complex II (LHCII) increasing the photosynthesis rates [36,37]. N-fertilizer may promote the photosynthesis by increasing the expression levels of photosynthesis-related genes including *LHCA4*, *PSAF*, *LHCBS5*, *CAB6A*, and *PETH* (Table 2).
Table 2. The photosynthesis- and starch-related DEGs of NT-NCK.

| ID            | Symbol  | log₂(NT/NCK) | p Value     |
|---------------|---------|--------------|-------------|
| evm.model.LG07.1627 | PSBP    | 2.588565     | 0.007947    |
| evm.model.LG04.953  | LHCA4   | 1.137687     | 0.008232    |
| evm.model.LG07.1688 | PSAF    | 1.269197     | 0.009753    |
| evm.model.LG05.284  | LHCB5   | 1.440339     | 0.021165    |
| evm.model.LG03.3983 | CAB6A   | 2.235426     | 0.030319    |
| evm.model.LG01.4474 | PETH    | 1.658211     | 0.036832    |
| evm.model.LG01.1391 | TPP2    | 9.789534     | 0.00106     |
| evm.model.LG01.1419 | At1g64390 GH9 | 6.118941 | 7.03 × 10⁻⁶⁶ |
| evm.model.LG01.2619 | At1g11820 GHF17 | 1.193103 | 8.63 × 10⁻¹⁰ |
| evm.model.LG01.3730 | AGPS1   | 1.271413     | 0.018608    |
| evm.model.LG01.475  | BAM3    | 1.474745     | 1.62 × 10⁻⁶  |
| evm.model.LG01.759  | INV     | 1.997073     | 4.06 × 10⁻⁹  |
| evm.model.LG02.1694 | gluA    | 1.005513     | 1.94 × 10⁻⁵  |
| evm.model.LG03.2635 | TPS9    | 1.129111     | 4.56 × 10⁻⁸  |
| evm.model.LG03.952  | SS      | 1.035091     | 1.18 × 10⁻¹⁶ |
| evm.model.LG04.1643 | BGLU44  | 1.085905     | 0.014078    |
| evm.model.LG05.1660 | DPEP    | 1.03929      | 1.60 × 10⁻²⁴ |
| evm.model.LG05.2134 | PGI     | 1.298246     | 6.99 × 10⁻²⁶ |
| evm.model.LG06.1346 | TPPG    | 1.250168     | 0.000414    |
| evm.model.LG08.1469 | TPS9    | 1.337351     | 2.64 × 10⁻⁵  |

Analysis of the lotus starch and sucrose metabolism pathway, we identified 14 differentially expressed structural genes, including AGPS1, SS, INV, GPI, AMY, BAM3, and DPEP (Table 2 and Figure S1). Though the key genes of starch synthesis, SBE and WAXY, not exhibited significant difference, they maintained high expression levels under both CK and N-fertilizer treatment (Table S2).

We further selected 8 genes involved in the starch and sucrose synthesis for the qRT-PCR analysis (Figure 8). The photosynthesis- and starch accumulation-related genes, INV, SUS, UGP, BAM, SS3, and GPI, were upregulated by N-fertilizer, which is consistent with the RNA-seq results. These results may give the possible directions and candidate genes for further investigating the lotus development and metabolism under N-fertilizer treatment. We hypothesized that N-fertilizer increased the starch content by promoting the photosynthesis rates, thus leading to an increase in sucrose which was the main resource of starch synthesis.
Nitrogen is an essential macronutrient for crop growth and development [11,13]. Though the application of nitrogen fertilizer is often used as an important method to improve crop yield and agronomic production [38–40], excessive application of nitrogen fertilizer occurs resulting in crop yield and quality reduction, resources waste, and economic benefits decline, and more seriously, and leading to environmental problems, such as soil and water pollution [41–43]. Researchers found that both low and overuse of nitrogen fertilizers resulted in lower crop yield than optimization nitrogen fertilization [39]. Therefore, it is necessary and urgent to determine the optimization nitrogen for vegetables and crops. In this research, we conducted five concentrations of N-fertilizer, labeled as CK, N1, N2, N3, and N4, respectively. The results showed that N-fertilizer increased the photosynthesis characteristics (Gs, Tr, Ci, Pn, and chlorophyll content) (Figure 2). Stomatal conductance plays an important role in controlling both CO2 uptake and H2O transpiration, and thus affects photosynthesis [44]. Similarly, increased leaf intercellular CO2 concentration, transpiration rate, and net photosynthetic rates also promote the photosynthesis [45,46]. Hence, N2-fertilizer (30 kg/667 m²) was identified as the optimization nitrogen fertilization for lotus growth and development. In addition, compared with CK, the total starch, amylose, amyllopectin, and plot yield under N-fertilizer significantly increased (Figure 3), which was consistent with previous research that the application of nitrogen fertilizer could improve crop yield [47–49].

Nitrogen-promoted crop metabolism is a complex process, in which many genes and proteins are involved [13,50]. Overexpressing the genes involved in nitrogen metabolism could influence plant growth and development, crop yield, and starch accumulation [51]. In barley, 1000 genes were induced under nitrate application treatment [52]. In potato, the DEGs participating the processes of transport and N metabolism were identified under sufficient N fertilizer and deficient N fertilizer treatment [53,54]. Two GS1 (Glutamine Synthetase 1) genes were functioned which played an important role at the balance between...
carbon and nitrogen metabolic which regulated rice growth and yield [51]. The expression level of NRT1.1 was induced by nitrate application treatment [55,56]. To identify the DEGs involving nitrogen fertilizer-photosynthesis-starch model, the RNA-seq of lotus cultivar “MRH” was conducted. Additionally, 4563 DEGs were identified between CK and N-fertilizer treatment (Figure 5) and functionally analyzed (Figures 6 and 7). Compared with CK, N2-fertilizer treatment exhibited more advantages in inducing more genes expression in ontology of biological processes, molecular function, and cellular component (Figure 6). After N2-fertilizer treatment, the pathways enriched by DEGs were changed, which mainly involved in carbon metabolism, carbon fixation in photosynthetic organisms, and plant hormone signal transduction (Figure 6). These results suggested that nitrogen fertilizer induced alterations of photosynthesis- and starch accumulation-related gene expression profiles in *Nelumbo nucifera*. Similarly, the previous research showed that the N-fertilizer was closely linked with photosynthesis and starch accumulation [57]. However, the relationship between photosynthesis and starch accumulation still needs to be further studied.

In this study, the identified genes, *PSBP*, *LHCA4*, *PSAF*, *LHCB5*, *CAB6A*, and *PETH*, are closely related to photosynthesis [36,37,58–63]. N-fertilizer treatment increased their expression levels (Table 1) and enhanced photosynthesis (Figure 2) suggesting that N-fertilizer may enhanced photosynthesis by affecting photosynthesis-related genes. Furthermore, the correlation between photosynthesis and starch was also indicated by the DEGs (Table 2). *INV* (*INV1*, *INV3* and *INV2*), playing pivotal roles in sucrose signaling, controlling grain size and starch content [64,65]. *AGPL1* and *AGPS1* were responsive to N-fertilizer treatment and their encoded enzymes were located at chloroplast and catalyzed the rate-limiting step of starch biosynthesis [66]. *Arabidopsis TPS9* was involved in the glucose and sucrose metabolism [67]. *Ss*s as the vital genes in starch accumulation affected the balance of amylose and amylopectin and the crop yield [68,69]. The above genes were verified by qRT-PCR significantly increased under N-fertilizer treatment (Figure 8 and Table 2). Though *SBEs* and *GBSSs* did not show a significant difference between CK and N-fertilizer treatment, their expression levels remain high from S1 to S2 (Table S2). Based on the above research, we established a hypothesis that N-fertilizer increased the starch content by promoting the photosynthesis rates leading to an increase in sucrose which was the main resource of starch synthesis.

This research provides an updated understanding of gene expression profiles of starch accumulation under CK and N-fertilizer treatment. The data led to many genes identified for further functionally investigation in *Nelumbo nucifera*. Our work and mRNA-seq datasets will better promote the development of *Nelumbo nucifera* research.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/agronomy12040794/s1](https://www.mdpi.com/article/10.3390/agronomy12040794/s1), Table S1: Primer sequences for qRT-PCRused in this study; Table S2: The gene expression profiles between CK and N-fertilizer treatment; Figure S1: The KEGG of starch and sucrose metabolism. Genes highlighted in red are the up-regulated genes.

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