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The Impact of Different Nitrogen Levels on the Tuber Yield and Anthocyanin Synthesis of Purple Potatoes

Zhaojuan Zhang [1], Binbin Cai [1], Yiling Guo [1], Tiancang Na [2,*] and Yuchun Guo [1,*]

[1] Key Laboratory of Crop Genetics, Breeding and Comprehensive Utilization, Ministry of Education, College of Agriculture, Fujian Agriculture and Forestry University, Fuzhou 350002, China; zhaojuan@fafu.edu.cn (Z.Z.); yiling_guo@fafu.edu.cn (Y.G.)
[2] Academy of Agriculture and Forestry Sciences, Qinghai University, Xining 810016, China
* Correspondence: 1996990029@qhu.edu.cn (T.N.); ycguo168@fafu.edu.cn (Y.G.)

Abstract: The biosynthesis of anthocyanins is influenced by external environmental conditions such as light, temperature, and nitrogen level, with nitrogen level being a key factor in anthocyanin synthesis and accumulation. Nitrogen level regulates the transcription factors involved in the anthocyanin synthesis pathway, with low nitrogen levels promoting anthocyanin accumulation, while high nitrogen levels have the opposite effect. Purple potatoes are a type of cultivated crop that is rich in anthocyanins and has unique economic value. Nitrogen fertilizer is crucial to improve the agronomic traits, yield, quality, and anthocyanin content of purple potatoes. In this study, the impact of four different nitrogen concentrations—0 kg/hm^2 (N0), 90 kg/hm^2 (N1), 225 kg/hm^2 (N2) and 360 kg/hm^2 (N3)—on the agronomic traits, yield, quality, and anthocyanin content of purple potatoes, 'Huasong 66', at different stages were investigated by using physiological index measurement and RNA-seq technology. It was found that the purple potato 'Huasong 66' was more sensitive to low nitrogen (N1). Under N1 level of nitrogen fertilization, 'Huasong 66' possessed the finest agronomic traits, yield, and quality, and the total anthocyanins in the tubers were significantly increased. Furthermore, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that nitrogen levels in purple potato tubers primarily affect genes related to nutrient transport and metabolism by regulating carbon and nitrogen metabolism, enzyme catalysis and binding, and signal transduction. In addition, nine candidate genes related to the anthocyanin synthesis pathway had been preliminarily screened. These results provide a basis to understand the impact of different nitrogen levels on the tuber yield and anthocyanin synthesis of purple potatoes.

Keywords: purple potato; nitrogen fertilizer; anthocyanin; RNA-seq

Citation: Zhang, Z.; Cai, B.; Guo, Y.; Na, T.; Guo, Y. The Impact of Different Nitrogen Levels on the Tuber Yield and Anthocyanin Synthesis of Purple Potatoes. *Agriculture* **2024**, *14*, 125. https://doi.org/10.3390/agriculture14010125

Academic Editors: Agnieszka Faligowska and Katarzyna Panasiewicz

Received: 22 November 2023
Revised: 5 January 2024
Accepted: 10 January 2024
Published: 15 January 2024

1. Introduction

Potatoes (*Solanum tuberosum* L.) belong to the Solanaceae family and are among the top ten most nutritious foods worldwide [1]. They are rich in polyphenols, carotenoids, flavonoids, and Vitamin C, among other antioxidant compounds [2]. Potatoes with purple, red, black, yellow, and other colored peels and flesh are collectively known as colored potatoes. Colored potatoes are cultivated varieties that contain high levels of anthocyanins and have unique economic value in the potato family [3]. Studies show that colored potatoes contain anthocyanin, which is distributed in all tissues and organs of colored potatoes [4]. It is mainly concentrated in tuber tissue and has tissue specificity in its distribution [5]. In cells, anthocyanins are mainly concentrated in the vacuole [6]. Anthocyanins show antimutagenic, antioxidant, antibacterial, antitumor, and antiproliferative activities and perform functions such as preventing cardiovascular disease and protecting the liver [2,7–9].

Anthocyanidins are a class of water-soluble pigments widely found in plants and in nature, and they belong to the flavonoid polyphenolic compound [10]. Anthocyanidin combines with various monosaccharides through glycosidic bonds to form anthocyanins [11].

Anthocyanins primarily include six types of anthocyanidins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin [12]. Colored potatoes are produced largely due to differences in cell fluid metabolite composition within tissues [13]. For example, purple potatoes contain malvidin, whereas red potatoes have pelargonidin. Anthocyanidins in colored potatoes vary in their types and contents. Even tubers of the same color may contain different anthocyanidins [4]. It has been shown that anthocyanins are one of the most effective compounds available to date as free radical scavengers [14,15], and thus, they are used to reduce aging, prevent arteriosclerosis, and regulate immune function. Previous studies have also indicated that anthocyanins directly or indirectly prevent nearly 100 types of diseases and are regarded as potential anticancer compounds [16–18]. In addition, anthocyanins extracted from purple potatoes have widely been applied as natural pigment additives replacing chemical pigments. Due to their excellent thermostability, anthocyanins in purple potatoes offer significant processing advantages over ordinary potatoes and other pigment crops, as well as significant potential for development and application.

Previous studies have indicated that different environmental factors (such as temperature, light, ultraviolet rays, fertilization status, hormone levels) could regulate plant anthocyanidin metabolism through activating or inhibiting different types of regulatory factors and thus affecting the biosynthesis of anthocyanidins. Flavonoids, especially red and purple anthocyanins, can be accumulated due to deficiencies of various nutrients in plants [19]. Moreover, the accumulation of anthocyanins plays a key role in plants' tolerance to low-nitrogen stress [20]. A low nitrogen level promotes anthocyanidin synthesis, while a high nitrogen level inhibits its biosynthesis process. Other studies have revealed that nitrogen levels regulate the expression levels of transcription factors involved in the anthocyanin synthesis process, such as MYB, bHLH, and WD40 [21]. Anthocyanidin biosynthesis in Arabidopsis thaliana was shown to be regulated by the TTG1-GL3/TT8-PAP1 complex in [22]. Under high-nitrogen conditions, the expression of R2R3-MYB transcription factors *PAP1* and *TT3* genes was down-regulated, which resulted in reduced anthocyanidin synthesis. On the contrary, the expression of *PAP1* and *TT3* genes was up-regulated under low-nitrogen conditions, leading to anthocyanidin accumulation. In addition, the GA-DELLA pathway positively regulated anthocyanidin accumulation induced by nitrogen deficiency [23]. The DELLA protein enhanced the transcriptional level of anthocyanidin biosynthesis genes by interacting directly with *PAP1*, thereby positively regulating anthocyanidin accumulation caused by nitrogen deficiency.

At present, transcriptome studies on colored potato anthocyanidin synthesis at different nitrogen levels have rarely been reported. The molecular regulation of purple potatoes and the synthesis of their anthocyanins based on nitrogen level is still unclear. Hence, further research is needed to explore the impact of varying nitrogen levels on anthocyanin accumulation in purple potatoes, as well as to investigate whether the biosynthesis of anthocyanins in purple potatoes is also regulated by the transcription factors MYB, bHLH, and WD40. In this study, the impacts of applying four different nitrogen levels in the cultivation period on the agronomic traits, growth, and anthocyanin contents of purple potato plants of the 'Huasong 66' variety were studied during six critical periods: at 45 d, 60 d, 75 d, 90 d, 105 d, and 120 d. Furthermore, RNA-Seq technology was used to analyze the differential expression of key genes within the anthocyanidin metabolic pathways. Subsequently, a comprehensive analysis of the key candidate genes within these pathways, combined with physiological and biochemical indexes, was conducted to understand the underlying molecular mechanism of anthocyanidin biosynthesis by nitrogen levels. This study aims to provide a fertilization strategy that is effective for the future cultivation of purple potatoes that contain anthocyanins.

2. Materials and Methods

2.1. Plant Materials

The experimental materials were 'Huasong 66' potatoes, a virus-free purple potato variety provided by Huasong Seed Industry Co., Ltd. (Beijing, China). The crops were

planted at the experimental teaching site of Fujian Agriculture and Forestry University from 2018 to 2020 (119.24 E, 26.09 N). The experimental field was composed of clay loam soil which was derived from former rice crops and had uniform soil fertility, soil tillage layer (0–20 cm, pH 4.73), organic matter content (3.98%), total nitrogen content (0.125%), alkali-hydrolyzable nitrogen (230.7 mg/kg), available phosphorous (82.9 mg/kg), and available potassium (174.0 mg/kg).

2.2. Experiment Design

The random block arrangement design was employed with four nitrogen fertilizer treatments set up in three replicates, leading to a total of 12 plots, with each plot being 17.85 m long and 1.2 m wide, occupying 21.42 m^2, one replicate serving as the root digging test area, and the remaining two serving as the production experiment areas. The formula ratio of N, P_2O_5, and K_2O was set up as 1:0.5:1.6 according to the local medium fertility soil formula. Based on relevant articles and our preliminary experimental results regarding the nitrogen fertilizer requirements of 'Huasong 66' potatoes, which were derived from experiments conducted in our research laboratory [24,25], the phosphorus and potassium fertilizers were kept the same as they were in those experiments. Phosphate (superphosphate 12.5%, 112.5 kg/hm^2), potassium (potassium sulfate 50%, 360 kg/hm^2), and nitrogen fertilizers (urea 46.4%, 0 kg/hm^2, 90 kg/hm^2, 225 kg/hm^2, 360 kg/hm^2) were applied (indicated in this paper by N0 (CK), N1, N2, and N3). Fertilizer was applied between two rows of potatoes, and all fertilizer was applied at the same time as a base fertilizer. Method of determining soil physical and chemical properties [26]: The pH value of the soil was calculated using a water/soil ratio of 2.5:1. The determination of organic matter was based on the potassium dichromate volumetric method. Alkaline hydrolytic nitrogen adopts an alkali solution diffusion method and standard acid titration. We used a flame photometer to determine the amount of available potassium. The molybdenum antimony anticolorimetric method was used to determine available phosphorus.

2.3. Determination of Agronomic Traits, Yield, and Quality of Purple Potatoes at Different Nitrogen Levels

At each growth stage, under different nitrogen levels, three plants with similar growth were selected in each area and used to measure plant height, stem and leaf fresh weight, and tuber fresh weight. Each area was subjected to three biological replicates, and the average value was taken. The purple 'Huasong 66' potatoes showed yellow leaves after entering the physiological maturity stage. Weight and yield were measured and calculated after harvesting the purple potatoes when 50% of the leaves turned yellow (removal of diseased, rotten, and moth-eaten potatoes). For each group, we selected tubers of the same size and determined their quality by conventional methods. In brief, the starch content was measured by using the iodine colorimetric method [27], the crude protein content was determined by using the Coomassie brilliant blue method [28], the vitamin C content was measured by using the xylene extraction colorimetric method [29], and the reduced sugar content was determined by using the 3,5-dinitrosalicylic acid (DNS) method [30].

2.4. Determination of the Nitrogen Contents of the Plants

After a whole plant was dug out, we removed the soil from it, cleaned it, and placed it in a drying box (Bluepard instruments Co., Ltd., Shanghai, China) after weighing. To record the plants' dry matter weight values, the following drying conditions were employed: First, 105 °C for 10 min. Then, drying at 80 °C for 4 h to a constant weight after weighing. Finally, the samples were crushed and digested by using the H_2SO_4-H_2O_2 digestion method. Each plant's nitrogen content was determined by using the Full-automatic Kjeldahl method (Foss Kjeltec Auto N Analyzer, Beijing, China) [31].

2.5. Assay of Anthocyanin, Chlorophyll, and Flavonoids in Purple Potatoes under Various Nitrogen Levels

The total anthocyanin contents of the purple potatoes were determined by using hydrochloric acid. Hydrochloric acid solution (1%) and 1 g tubers were mixed in a ratio of 1:40 and extracted at room temperature for 2 h in a dark environment. Subsequently, 10 mL of the extract solution was taken and centrifuged at 4000 rpm for 10 min. The obtained supernatant was subjected to a colorimetric assay at 525 nm, and 1% hydrochloric acid was used as the control. Chlorophyll content and flavonoid content in purple potatoes were measured using a plant chlorophyll content detection kit and a plant flavonoid content detection kit, respectively (Solarbio Science and Technology Co., Ltd.; Beijing, China), following the manufacturer's instructions. For chlorophyll content detection, the pulverized potato tubers (1 g) were placed in a mortar and homogenized in liquid nitrogen with 2 mL distilled water and 40 mg of reagent 1 under dark conditions. The homogenate was transferred to a 50 mL centrifuge tube. The mixture was extracted in the dark for 3 h, with the centrifuge tube being subjected to two gentle shakes during this process. After the residual tissue at the bottom of the tube turned nearly white, centrifugation was performed at 4000 rpm for 5 min. The supernatant was collected as a source of chlorophyll. Absorbance was measured at 645 and 663 nm (AllSheng Company, Hangzhou, China) [32]. For flavonoid content detection, the potato tubers (1 g) were placed in a mortar and ground thoroughly in liquid nitrogen. The sample was then transferred to a 50 mL centrifuge tube, and 10 mL of extraction solvent was added. Ultrasound-assisted extraction was carried out with a power of 300 W and a temperature of 60 °C for 30 min. After extraction, the sample was centrifuged at 12,000 rpm for 10 min at 25 °C, and the supernatant was collected for absorbance measurement at 470 nm [33].

2.6. RNA Isolation, Transcriptome Sequencing, and Data Analysis

We selected the potato tubers 60 days after sowing under different nitrogen level treatments. Total RNA was extracted by using the modified Trizol method and stored at −80 °C [34]. The concentration and purity of total RNA were measured and analyzed by using a Nano-400A Ultramicro nucleic acid analyzer (AllSheng Company, Hangzhou, China) and 1.0% agarose gel electrophoresis, respectively. The integrity of total RNA was evaluated using an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Inc., Beijing, China). The cDNA library was constructed by using Qubit 2.0 for preliminary quantification and Agilent 2100 for the detection of library insert size. The effective concentration of the library was accurately quantified by using the qRT-PCR method. Sequencing was conducted on the Illumina Hiseq platform (Illumina, San Diego, CA, USA). The resulting Clean Reads were used to perform sequence alignment with the potato reference genome available at http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml (accessed on 6 June 2021) using TopHat2 [35]. Mapped reads aligned with the reference genome were then compared with a public database using BLASTX (Q-value $\leq 10^{-5}$). The protein annotation information with the highest matching degree for each Unigene was obtained and used to perform functional annotation and classification. The databases used for comparison included GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), Pfam (a database of conserved Protein families or domains), Swissprot (a non-redundant protein database), and NR (NCBI non-redundant proteins). The *p*-value of the original hypothesis test was used to correct and generate the FDR (False Discovery Rate) by using the Benjamini–Hochberg method. Lastly, differential genes were screened according to the criteria of FDR < 0.01 and Fold Change $\geq$ 1.5.

2.7. Quantitative Real-Time PCR (qRT-PCR) Validation

Total RNA was extracted using the modified Trizol method as described above. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Shanghai, China) according to the manufacturer's instructions. Ten differentially expressed genes were selected to verify the RNA-Seq results using the primers

designed by Primer Premier 5.0 software (Table 1). The primer sequences were synthesized by BioSune (BioSune Co., Ltd.; Shanghai, China). qRT–PCR assays were performed using a LightCycler 96 (LightCycler® 96, Roche Company, Basel, Switzerland), and the SYBR Green Master Mix Kit (Thermo Fisher Scientific, Shanghai, China) was used for the qRT-PCR assay. The qRT-PCR conditions were as follows: predenaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, 40 cycles, dissolution curve at 95 °C for 30 s, 60 °C for 15 s. The experiment had three biological replicates, and each biological replicate had three technical replicates. *EF1α* was used as a reference gene, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [36]. The data were analyzed by variance using Microsoft Excel 2016 software.

Table 1. Real-time fluorescent quantitative primers.

Primer Name	Forward Primer Sequence (5′→3′)	Reverse Primer Sequence (5′→3′)
EF1α	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA
PGSC0003DMG400004109	GACCCTGTTGATGGAGAAGGA	GCATTGCACTCTGGTGGTAG
PGSC0003DMG400037860	CCTCCTCAACAATGGACCCT	CGCCCATCTCGAAGTTTACC
PGSC0003DMG401005729	CCTCTCTGCCAGCTGTTGAT	CACTGGAAAGCCAACTCTGC
PGSC0003DMG401020664	CAACACCGGCTGATGTTTCT	TGCTGGTGCCATCATTCCTA
PGSC0003DMG400008000	CGTGGCAGCAGTTATGGAAT	AGCAATAAGCCCAGCAAACC
PGSC0003DMG400026032	GGTACATTGAGACGGAGGCT	TGAACCTCCACTTCCCACTC
PGSC0003DMG400021691	TGTGTTGTTGGATTTGTTGTGC	TGCATTCTCATCCGATGCTG
PGSC0003DMG400029130	TGTAAGTCCTTGTGGTTGGC	CGACACCTTTAAGAGTTTCCGT
PGSC0003DMG400027438	CCTCGTCCAGGTTCAAAGGA	ACCTTCTCTGCAACTTCACC
PGSC0003DMG400024644	GCTTATTGGTGAGCCTGGTG	AGCCAAGTTACTCGGGACAT

2.8. Data Processing and Statistical Analysis

Microsoft Excel software and DPS V9. 05 software were used to statistically analyze and graph the data for each measurement item. The Least Significant Difference (LSD) method was used for multiple comparisons.

3. Results

3.1. Effects of Different Nitrogen Levels on the Agronomic Traits, Yield, and Quality of Purple Potatoes

3.1.1. Different Nitrogen Levels Affect Plant Height of Purple Potato

As shown in Figure 1, the height of the plants in the N1 and N2 treatment groups was significantly higher than those in the N0 and N3 treatment groups, and the plant height in the N3 treatment group was significantly higher than that in the N0 treatment group throughout the growth period. At 60 d and 75 d, the plant height in the N2 treatment group was significantly higher than that in the N1 treatment group. However, at 90 d, 105 d, and 120 d, the height of the plants in the N1 treatment group appeared to be significantly higher than in the N2 treatment group. Compared with the control group N0 (CK), the plant height of the N1 treatment group increased by 34.21%, the plant height of the N2 treatment group increased by 20.00%, and the plant height of the N3 treatment group increased by 8.42%. In addition, purple potatoes in each treatment group entered the slow growth stage at different times. As shown in Figure 1, purple potatoes in the N0 (CK) treatment group entered the slow stage after 60 days, whereas the plant height of the N1 treatment group tended to plateau 90 days later. Ultimately, maximum plant height in the N1 treatment group could be achieved at 120 days. The results indicated that nitrogen application played an important impact on the growth of purple potato plants.

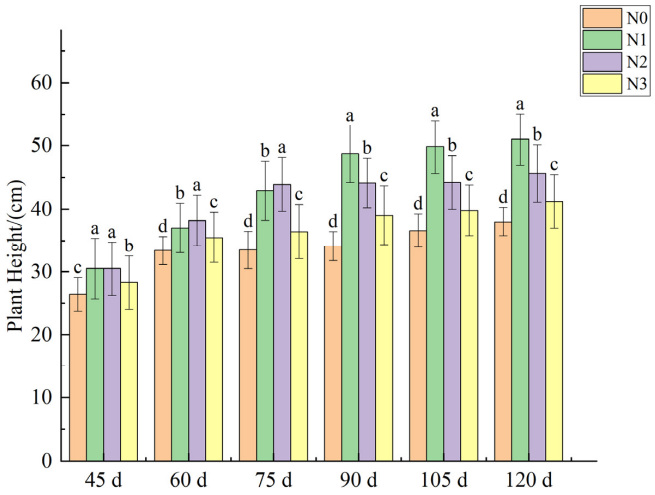


Figure 1. The effects of different nitrogen levels on the height of purple potato plants. Note: Different lowercase letters in the figure indicate significant differences between the treatments ($p < 0.05$, LSD).

3.1.2. Different Nitrogen Levels Affect the Fresh Weight of Purple Potato Stems and Leaves

The results showed that different nitrogen levels have a significant impact on the fresh weight of the stems and leaves of purple potatoes (Figure 2). The fresh weight values of the stems and leaves in each treatment group generally showed a trend in which they first increased and then decreased. However, the weight values of the stems and leaves in each treatment group reached their maximum at different times. The maximum weights of the stems and leaves could be observed at 75 days for N1, 90 days for N2, and 105 days for N3, while the weights of the stems and leaves in the control group N0 reached their maximum level at 75 days. Compared with the control group N0, the fresh weights of the stems and leaves in the N1 treatment group increased by 21.91%; in the N2 treatment group, they increased by 30.77%, and in the N3 treatment group, they increased by 18.59%. In addition, the fresh weights of the stems and leaves in the N0 and N1 treatment groups decreased rapidly at 90 days. On the contrary, a significant decrease in the fresh weight values for the N2 and N3 groups was found at about 120 days. It is worth noting that the fresh weight values of the stems and leaves in the N3 treatment group did not show any obvious changes at 75–105 days.

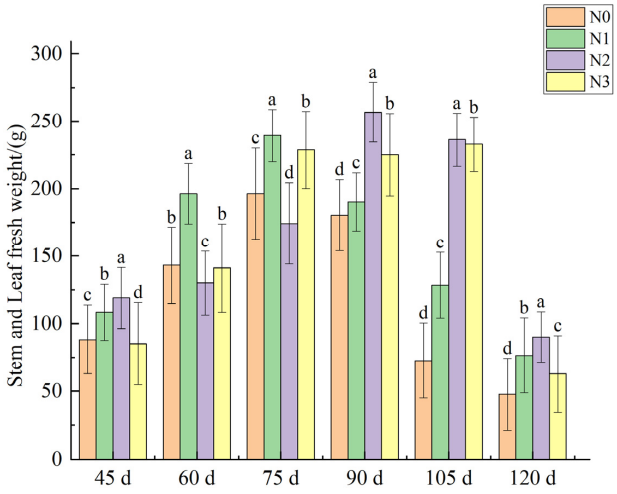


Figure 2. The effects of different nitrogen levels on fresh weights of the stems and leaves of purple potato plants. Note: Different lowercase letters in the figure indicate significant differences between the treatments ($p < 0.05$, LSD).

3.1.3. Different Nitrogen Levels Affect Tuber Fresh Weight

Under four different nitrogen levels, the fresh weight values of purple potato tubers showed a similar change trend, initially increasing and then decreasing (Figure 3). A maximum fresh weight of tubers of 217.6 g could be observed for the control group N0 at 90 days. Regarding the other nitrogen treatment groups, the fresh weights of the purple potato tubers exhibited rapid increases in the N1 and N2 treatment groups between 90 and 105 days, and maximum tuber weight values of 343.6 g and 502.8 g were obtained at 105 days for N1 and N2, respectively, indicating that nitrogen application can improve tuber weight. However, the N3 treatment group reached its maximum fresh weight value at 120 d. Compared with the control group N0, tuber fresh weight in the N1 treatment group increased by 57.90%, while that of the N2 treatment group increased by 131.07%, and that of the N3 treatment group increased by 51.15%. This indicates that increased nitrogen application can promote purple potato tuber accumulation.

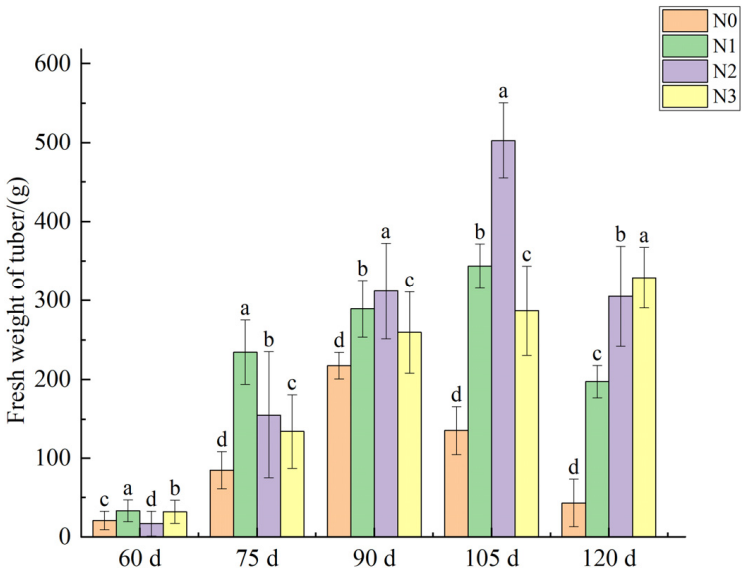


Figure 3. The effects of different nitrogen levels on the fresh weight of tubers. Note: Different lowercase letters in the figure indicate significant differences between the treatments ($p < 0.05$, LSD).

3.1.4. Different Nitrogen Levels Affect the Yield of Purple Potatoes

Values pertaining to the yield and commercial rate of purple 'Huasong 66' potato plants were determined, and these values are shown in Table 2. The results show that the average yields of N1, N2, and N3 were significantly higher than those of N0 (CK). A maximum yield of 19,808.55 kg/hm^2 for N2 was obtained, and an increase of 132.67% when compared with the control group N0. High commodity rates of 92.79% and 92.80% were achieved by N1 and N2, respectively, superior to those of N3 (87.31%) and N0 (69.34%). These results indicate that reasonable nitrogen application can improve the yield of purple potatoes.

Table 2. Assay results regarding tuber yield at different nitrogen levels.

Treatment Group	Yield (kg/hm^2)	Average Yield (kg)	Commodity Rate (%)
N0 (CK)	8513.70	9.09 b	69.34
N1	17,164.35	18.33 a	92.79
N2	19,808.55	21.15 a	92.80
N3	16,984.50	18.14 a	87.31

Note: Different lowercase letters in the table indicate significant differences between the treatments ($p < 0.05$, LSD).

3.1.5. Different Nitrogen Levels Affect the Quality of Purple Potatoes

The crude protein, reduced sugar, starch, and vitamin C contents in the purple potato plants were determined to evaluate the effects applying different nitrogen levels had on their quality. The results showed that the crude protein and reduced sugar contents in the purple potatoes increased with an increase in the nitrogen application levels (Table 3). While the starch content and VC content first increased and then decreased with the increase in nitrogen application (Table 3). Compared with the control group N0, the reduced sugar content, crude protein content, starch content, and vitamin C content of the N1 treatment group increased by 158.33%, 1.66%, 20.52%, and 17.40%, respectively. The contents of reduced sugar, starch, and vitamin C increased significantly. In the N2 and N3 treatment groups, although the reduced sugar content and crude protein content were significantly higher than those in the N0 treatment group, the starch content and vitamin C content were significantly lower than those in the N0 treatment group.

Table 3. Assay results regarding purple potato quality at different nitrogen levels.

Treatment Group	Reduced Sugar Content (g/100 g)	Crude Protein Content (mg/g)	Starch Content (mg/g)	Vitamin C Content (mg/100 g)
N0	0.12 ± 0.01 d	10.26 ± 0.09 c	11.45 ± 0.10 b	14.08 ± 0.11 b
N1	0.31 ± 0.00 c	10.43 ± 0.12 c	13.80 ± 0.09 a	16.53 ± 0.02 a
N2	0.40 ± 0.01 b	11.45 ± 0.06 b	10.43 ± 0.09 c	12.00 ± 0.10 c
N3	0.55 ± 0.02 a	13.80 ± 0.20 a	10.26 ± 0.09 c	9.84 ± 0.02 d

Note: Different lowercase letters in the table indicate significant differences between the treatments ($p < 0.05$, LSD).

3.2. Nitrogen Uptake and Utilization by Purple Potato Plants at Different Nitrogen Levels

3.2.1. Nitrogen Contents of Purple Potato Plants at Different Growth Stages

During the whole growth period, each treatment had the same nitrogen content (Figure 4). The nitrogen content of the whole plant was highest at the seedling stage (45 d), and then decreased with the growth and development of the plant. The nitrogen content of the whole plant decreased the fastest in N0 at 45–60 d and 90–105 d, which was significantly different from the other three treatments. This indicates that the nitrogen uptake rate of the N0 treatment group was lower than that of the other nitrogen treatment groups. In comparison with the control group N0, the nitrogen content of the whole plant in the N1, N2, and N3 treatment groups increased by 10.43%, 42.82%, and 47.71%, respectively, during the harvest period.

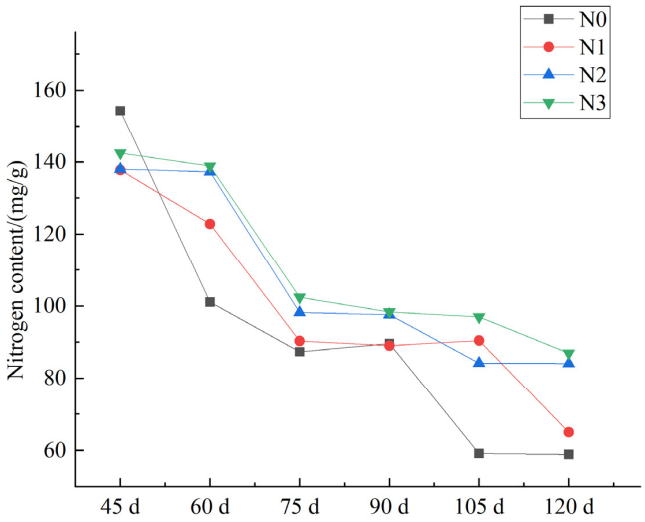


Figure 4. Nitrogen content of the whole plant varied with different nitrogen levels during different growth periods.

3.2.2. Nitrogen Utilization Efficiency of Purple Potato Plants at Different Nitrogen Levels

In purple potatoes, nitrogen application levels significantly affected nitrogen utilization efficiency (Table 4). With the increase in nitrogen application levels, partial factor productivity from applied N (PFPN) and nitrogen agronomic efficiency (NAE) decreased gradually; of the different treatment groups, the N1 treatment group performed the best in this respect, with values of 188.72 kg/kg and 94.12 kg/kg, respectively. The nitrogen physiological efficiency (NPE) values for the N2 and N3 treatments were 89.55% and 77.89% lower than that of the N1 treatment, respectively. In addition, the nitrogen harvest index (NHI) of N1 was the highest, 30.88% higher than N0 (CK). The N2 treatment was 5.88% higher than N0 (CK); the N3 treatment was 11.77% lower than N0 (CK).

Table 4. Nitrogen utilization efficiency of plants at different nitrogen levels.

Treatment Group	PFP_N (kg/kg)	NPE	NAE	NHI
N0 (CK)	-	-	-	0.68 ± 0.00 c
N1	188.72 ± 1.44 a	5524.57 ± 4.33 a	94.12 ± 0.42 a	0.89 ± 0.01 b
N2	76.29 ± 0.31 b	577.27 ± 1.22 c	38.45 ± 0.27 b	0.72 ± 0.00 b
N3	55.02 ± 2.36 c	1221.44 ± 3.29 b	31.37 ± 0.33 c	0.60 ± 0.02 d

Note: Different lowercase letters indicate significant differences ($p < 0.05$). PFP_N: Partial factor productivity from applied N; NPE: Nitrogen physiological efficiency; NAE: Nitrogen agronomic efficiency; NHI: Nitrogen harvest index; "-" indicates that no detection was performed.

3.3. Different Nitrogen Levels Affect Tuber Anthocyanidin Accumulation

The period from budding to harvest (60–120 days), in the context of purple potatoes. is critical for tuber development and quality formation, as well as anthocyanidin accumulation. As shown in Figure 5, anthocyanidin accumulation at four different nitrogen levels exhibited a trend in which it first increased at 60–70 days and then decreased at 90–120 days. The accumulation of anthocyanidin began when the underground stolons in the budding stage developed into tubers. The period of anthocyanidin accumulation in purple potatoes was between 60 days and 75 days. Maximum anthocyanidin contents in the purple potatoes were achieved at about 75 days for four groups. Moreover, the plants in the N1 treatment group showed significantly higher anthocyanidin contents than those in the other treatment groups. At the harvest period (120 d), the anthocyanidin content of the N1 treatment group was 31.91 mg/100 g, and the N2 and N3 treatment group showed similar anthocyanin contents of 29.88 mg/100 g and 28.68 mg/100 g, respectively. While the lowest anthocyanidin content of 26.37 mg/100 g was achieved by the control N0 group.

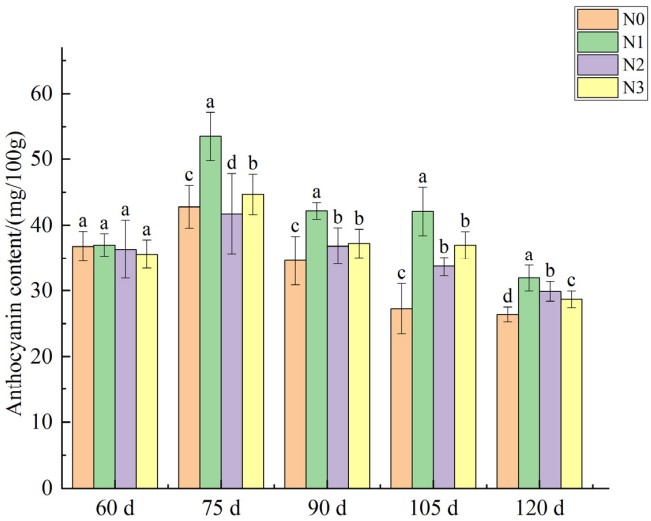


Figure 5. Time-course of total anthocyanin accumulation at different nitrogen levels. Note: Different lowercase letters in the figure indicate significant differences between the treatments ($p < 0.05$, LSD).

3.4. Chlorophyll and Flavonoid Contents in Purple Potatoes at Different Nitrogen Levels

The chlorophyll contents of purple potatoes generally showed a declining trend during the entire growth period (45–120 days), and the nitrogen fertilization treatments exhibited higher chlorophyll contents in comparison to the control N0 group (Figure 6a). At 60–105 d, the relative chlorophyll content of the N2 treatment group was higher than that of the other treatment groups, while the relative chlorophyll contents of the N1 and N3 treatment groups were not significantly different at this stage. The minimum contents of chlorophyll for each treatment group were observed at 120 d.

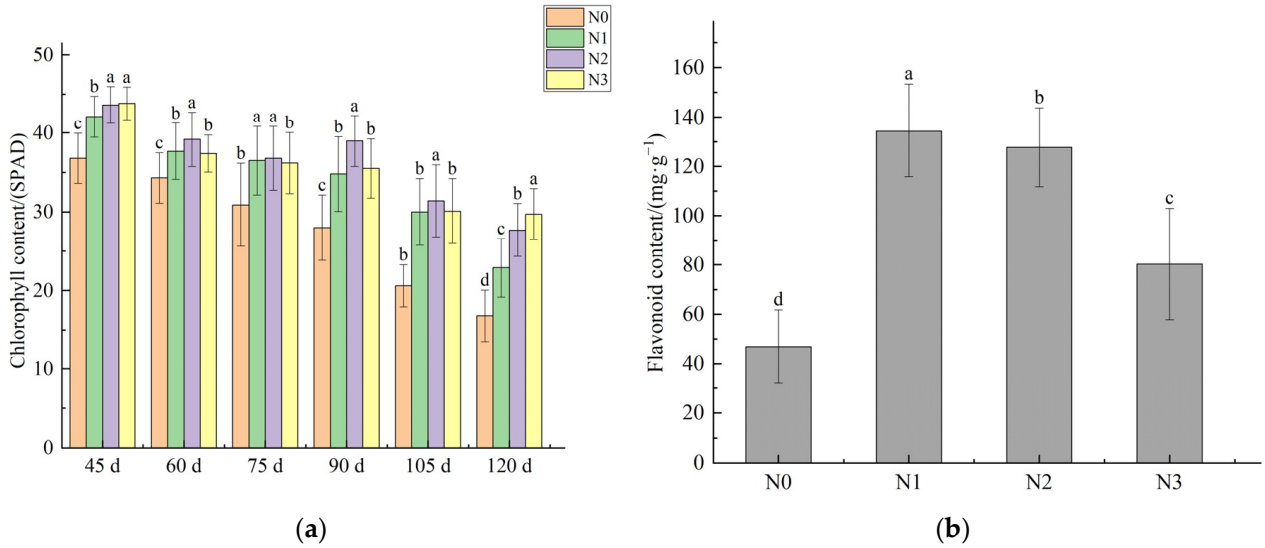


Figure 6. Effects of different nitrogen levels on chlorophyll content (**a**) and flavonoid content (**b**). Note: Different lowercase letters in the figure indicate significant differences between the treatments ($p < 0.05$, LSD).

The flavonoid contents of the purple potatoes in each treatment group under different nitrogen concentrations showed significant differences with the following order: N1 > N2 > N3 > N0 (Figure 6b). The highest flavonoid content of 134.63 mg/g was determined for the N1 treatment group, followed by the N2 group, which had a flavonoid content of 127.76 mg/g, and the lowest flavonoid content of 46.82 mg/g was achieved by the control N0 group. The results indicate that flavonoid accumulation in purple potatoes can be improved by nitrogen application, especially the application of low nitrogen levels.

3.5. Transcriptome Analysis

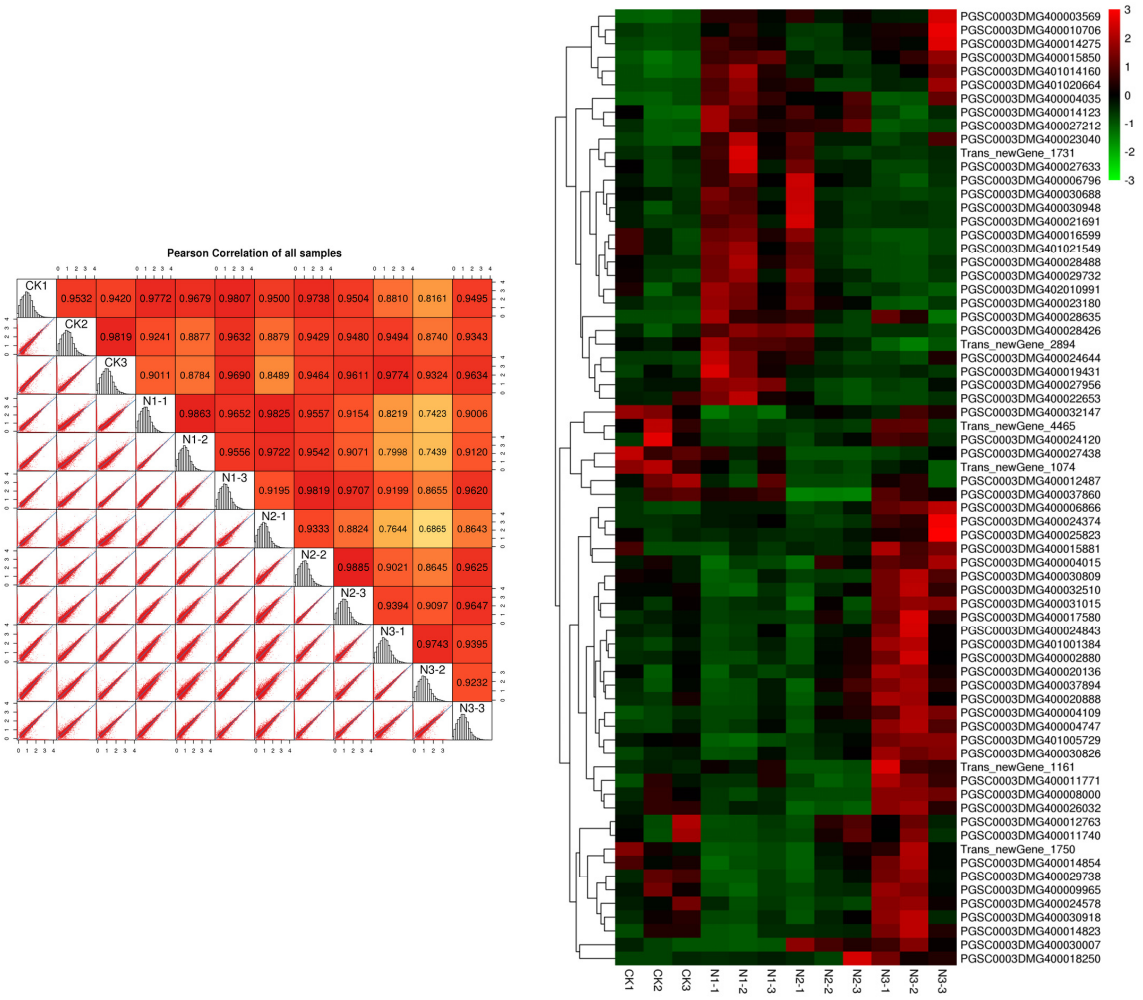
3.5.1. Evaluation of the Transcriptome Sequencing Results

RNA-seq sequencing technology was used to sequence four purple potato tubers with different nitrogen levels during the tuber formation stage, and the Clean Reads were 22.18~30.98 Mb. The total number of bases reached 6.65~9.29 Gb; Q20 was above 98%, and Q30 was above 94% (Supplementary Table S1). After using TopHat2 to align the Clean Reads with the reference genome, the results showed that the average alignment rates of N0, N1, N2, and N3 were 69.05%, 66.48%, 67.87%, and 70.02%, respectively, demonstrating that the data between samples are comparable (Supplementary Table S2). In addition, the unique alignment rates of the reads obtained with the reference genome were all above 99.69%, and the alignment rates among samples were even, showing that the transcriptome sequencing data have a high utilization rate.

3.5.2. Differentially Expressed Genes (DEGs) Analysis

A total of 2836 DEGs were identified from the transcriptome sequencing results regarding the purple potato samples at the tuber formation stage under four different nitrogen

levels, of which 202 new genes were identified and annotated. In order to handle expression differences due to biological variability, Pearson's r (Pearson correlation coefficient) was used to evaluate the repeated correlations between treatments. The repeated correlations between the samples were all above 0.85, which indicated that there was good biological repeatability between the treatments and thus guaranteed subsequent differential gene screening (Figure 7a).



(**a**) (**b**)

Figure 7. DEGs analysis. (**a**) Heatmap of the expression correlations for the pairwise samples. (**b**) Heatmap analysis of different genes under different nitrogen treatments.

Four different nitrogen level treatment groups were analyzed for pairwise comparisons between the groups (N0 vs. N1, N0 vs. N2, N0 vs. N3, N1 vs. N2, N1 vs. N3, and N2 vs. N3) (Supplementary Figures S1–S6). In the N0 vs. N1 comparison group, 454 genes were up-regulated and 361 genes were down-regulated. In the N0 vs. N2 comparison group, 78 genes were up-regulated and 118 genes were down-regulated. In the N0 vs. N3 comparison group, 112 genes were up-regulated and 66 genes were down-regulated. In the N1 vs. N2 comparison group, 22 genes were up-regulated and 78 genes were down-regulated. The comparison group with the most differentially expressed genes was the N1 vs. N3 comparison group, with 1259 up-regulated genes and 1023 down-regulated genes. In the N2 vs. N3 comparison group, 135 genes were up-regulated and 66 genes were down-regulated. In addition, the overall expression pattern of the genes in the N0 treatment group was significantly different from that in the other treatment groups (Figure 7b). The

expression levels of most genes in the N0 group were down-regulated, and only 14 genes were up-regulated, of which 3 genes (PGSC0003DMG400032147, PGSC0003DMG400027438, Trans_newGene_1074) exhibited a significantly increased expression. On the contrary, the expression levels of these genes were down-regulated in other three treatment groups. In the N1 group, the expression levels of 29 genes were up-regulated. The expression levels of most genes in N2 were down-regulated, except for eight genes whose expression was up-regulated. The expression of 39 genes was up-regulated in the N3 treatment group, of which 26 genes were significantly up-regulated, and their expression was down-regulated in other treatment groups. Meanwhile, it can also be seen that the expression of each gene in the N1 and N3 treatment groups was basically opposite, which indicates that significant differences existed between the two treatments.

3.5.3. GO and KEGG Analysis of DEGs

In order to comprehensively analyze the DEGs, pairwise comparisons were performed among the four different nitrogen treatment groups (N0 vs. N1, N0 vs. N2, N0 vs. N3, N1 vs. N2, N1 vs. N3, and N2 vs. N3). GO classification analysis showed that a total of 23 biological processes, 15 cellular components, and 15 molecular functions involved the different nitrogen levels (Supplementary Figure S7a–f). In terms of biological processes, the DEGs are mainly involved in metabolic processes, biological regulation, cellular processes, stress processes, and the organization or biogenesis of cellular components. Among the cellular components, the DEGs are mainly involved in cell and cell membrane formation. Regarding molecular functions, the DEGs are mainly involved in binding and catalysis.

KEGG pathway analysis was performed to analyze the DEGs, and the results showed that the DEGs are involved in metabolic activities such as carbohydrate metabolism, energy metabolism, the biosynthesis of secondary metabolites, amino acid metabolism, and sugar metabolism in purple potatoes (Supplementary Figure S8a–f). Moreover, some defense-related pathways, such as the phenylpropanoid biosynthesis pathway, the glutathione metabolism pathway, and the plant hormone signal transduction pathway, involved the DEGs. The DEGs involved in genetic information processing, such as translation and transcription, were also significantly enriched.

3.5.4. Screening of the DEGs Involved in Anthocyanidin Biosynthesis and Metabolism in Purple Potato Tubers

In nature, most higher plants perform biological functions through the collective effect of multiple genes or metabolic pathways. Therefore, to reveal the potential regulatory mechanism of anthocyanin accumulation during the tuber formation process, GO and KEGG pathway analysis were performed on the DEGs of purple potato tubers with different nitrogen levels. A total of nine differentially expressed Unigenes that may be involved in the anthocyanidin biosynthesis pathway were identified (Supplementary Table S3). These included *LAR* (1) of the bHLH family; the structural genes of *DFR* (1), *AT* (1), *CHS* (1), *UFGT* (2), and *F3H* (3); encoding dihydroflavonol reductase; anthocyanin acyltransferase; chalcone synthase; glucosyltransferase; and flavanone 3-hydroxylase.

3.6. Verification of Selected DEGs via qRT-PCR

To further verify the reliability and accuracy of the transcriptome sequencing results, the expression patterns of these DEGs at different nitrogen levels were randomly selected and determined (Figure 8). Ten DEGs or transcription factors were selected for real-time fluorescence quantitative PCR verification experiments (Figure 8a–h). The verification results indicated that the expression pattern between the qRT-PCR results and RNA-seq data was basically consistent, although their fold differences were different. Therefore, the use of RNA-seq technology to analyze the differential expression of purple potato tuber genes with different nitrogen levels can be relied upon.

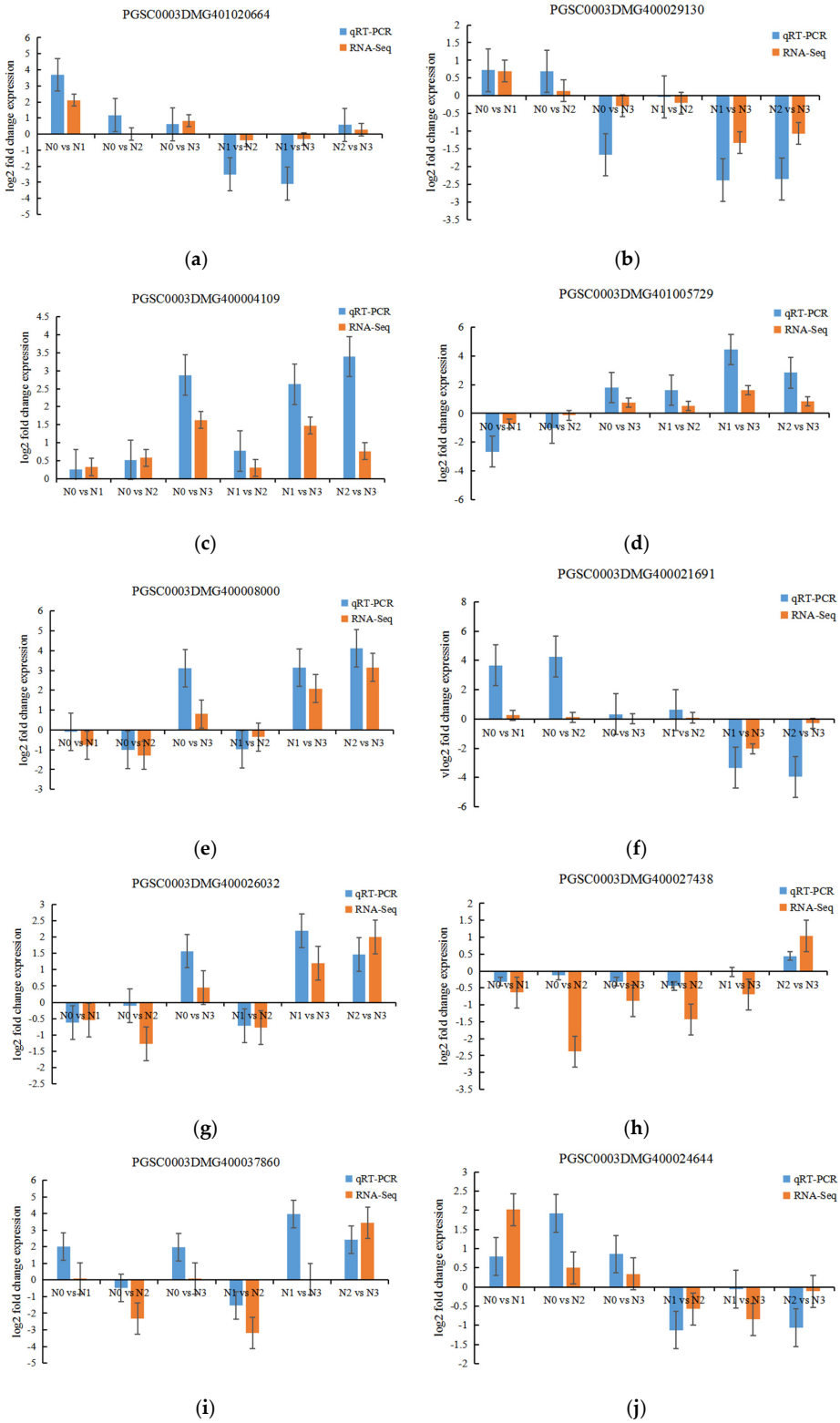


Figure 8. Results of qRT-PCR verification at different nitrogen levels. (**a**) PGSC0003DMG401020664 (binding protein), (**b**) PGSC0003DMG400029130 (glucosyltransferase), (**c**) PGSC0003DMG400004109 (xyloglucan endotransglucosylase), (**d**) PGSC0003DMG401005729 (wall-associated kinase), (**e**) PGSC0003DMG400008000 (L-asparaginase), (**f**) PGSC0003DMG400021691 (lipid-binding protein), (**g**) PGSC0003DMG400026032 (carotenoid cleavage dioxygenase 7), (**h**) PGSC0003DMG400027438 (transcription factor), (**i**) PGSC0003DMG400037860 (eukaryotic initiation factor 4A-15), (**j**) PGSC0003DMG400024644 (101 kDa heat shock proteins).

4. Discussion

4.1. The Agronomic Traits, Yield, and Quality of Purple Potatoes under Different Nitrogen Levels

Previous studies have shown that potatoes are nitrogen-sensitive crops and that nitrogen application has a significant impact on potato yield and quality [37]. In the absence of nitrogen fertilizer, plants may suffer premature senescence in the middle and later stages of growth due to insufficient nitrogen supply, thus resulting in yellow leaves and limited material accumulation in underground tubers [38]. According to the results of the current study, different nitrogen application levels can affect the plant height, fresh weight of stems and leaves, tuber fresh weight, yield, and quality of purple 'Huasong 66' potato plants. Among the different nitrogen treatment groups, the N1 and N2 treatment groups performed better in terms of the agronomic traits, yield, and quality of the purple potatoes. In terms of agronomic traits, the plant height values of the N1 and N2 treatment groups increased by 34.21% and 20.00%, the fresh weight of the stems and leaves increased by 21.91% and 30.77%, and the fresh weight of tubers increased by 57.90% and 131.07%. In terms of yield and quality, the yields of the N1 and N2 treatment groups increased by 101.61% and 132.67%, respectively, and the commodity rate was similar. However, the N1 treatment group was not only higher than the N0 treatment group in crude protein content, starch content, and vitamin C content, but also the starch and vitamin C content were significantly higher than the N2 treatment groups. In addition, purple potatoes can obtain nitrogen from soil organic matter and show a nitrogen harvest index of 0.68%. However, with plant growth and development, nitrogen uptake decreases. Nitrogen fertilizer application can increase the uptake of nitrogen by plants. When 90 kg/hm^2 (N1) and 225 kg/hm^2 (N2) of nitrogen fertilizer were applied, the nitrogen harvest index increased by 30.88% and 5.88%, respectively, compared with N0. The nitrogen utilization indexes of the N1 treatment group were significantly higher than those of other treatment groups. It has been reported that nitrogen fertilizer application can improve potato height and promote chlorophyll synthesis in plant leaves [39]. The photosynthetic efficiency of potato leaves can increase significantly by applying nitrogen [38]. Furthermore, a significant increase in potato leaf area index, SPAD value, and biological yield was observed after applying certain levels of nitrogen in [37,40,41]. In this study, the relative chlorophyll content (SPAD) of the purple potato plants in the nitrogen treatment groups was higher than that in N0 during the whole growth period. This was consistent with the fact that the plant height of the purple potato plants in the nitrogen treatment groups was higher than that in the N0 treatment group. This shows that even if nitrogen was present in the experimental field, the absence of nitrogen fertilizer was also not conducive to plant growth and underground tuber accumulation. Based on the above, we believe that the level of nitrogen applied to the plants in the N1 treatment group is the most suitable for the cultivation of 'Huasong 66' purple potatoes, as this treatment group performed well in terms of agronomic traits, yield, and quality.

4.2. Tuber Anthocyanidin Content and Related Differentially Expressed Genes in Anabolic Pathways

Nitrogen plays a crucial role in plant life. It promotes plant growth and development through nutrient absorption, transport, assimilation, and utilization [42]. In addition, nitrogen is also one of the key elements regulating anthocyanin synthesis. In general, high-nitrogen conditions will inhibit anthocyanin biosynthesis, while low-nitrogen conditions will promote it [43]. Researchers have shown that apples, radishes, and grapes can accumulate anthocyanin under low-nitrogen stress [44–46]. In this study, the anthocyanin contents in the purple potatoes were in the order of N1 > N0 (CK) > N2 > N3 at the tuber formation stage (60 d). This shows that purple potatoes can induce anthocyanin formation under nitrogen deficiency or low-nitrogen conditions. Each treatment group reached peak anthocyanin content at 75 days, indicating that the period of the rapid accumulation of anthocyanin in purple potatoes occurred between the tuber formation period and the tuber expansion period (60 to 75 d). However, with the development of

tubers, the anthocyanin content gradually decreased. At the harvest stage (120 d), the total anthocyanin content of the tubers in the N1, N2, and N3 treatment groups was significantly higher than that in the N0 treatment group. Meanwhile, the anthocyanin content of the N1 treatment group was higher during the entire growth period than that of the other treatment groups. This indicates that low-nitrogen conditions can promote anthocyanin synthesis in purple potatoes.

The results of our GO and KEGG enrichment analysis showed that the different nitrogen levels regulated carbon and nitrogen metabolism, enzyme catalysis and binding, and signal transduction in the purple potato tubers, and thus affected nutrient transport and metabolism-related genes in the purple potato tubers. The anthocyanin synthesis pathway in plants has been clearly described. Anthocyanin synthesis is regulated by the transcription factors R2R3 MYB, bHLH, and WD40 to form the MBW complex (MYB-bHLH-WD40) [21,47]. In potatoes, anthocyanin biosynthesis may be related to three R2R3 MYB encoding genes (*StAN1*, *StMYBA1*, and *StMYB113*), two bHLH encoding genes (*StJAF13* and *StbHLH1*), and one WD40 encoding gene (*StWD40*) [48]. In addition, recent studies have shown that the potato WRKY encoding gene (*StWRKY13*) can promote anthocyanin biosynthesis by activating the transcription of *StCHS*, *StF3H*, *StDFR*, and *StANS* in potato tubers [49]. Although genes related to anthocyanin synthesis in potatoes are constantly being explored and verified, the molecular regulation mechanism of the effect of nitrogen levels on anthocyanin content in potato has rarely been reported. The DELLA protein in Arabidopsis can directly interact with *PAP1* to enhance its transcriptional activity on the expression of anthocyanin biosynthesis genes *F3′H* and *DFR*, thereby positively regulating anthocyanin accumulation caused by nitrogen deficiency [22]. In *Malus spectabilis*, miR858 is a key microRNA that regulates anthocyanin biosynthesis under low nitrogen conditions, and MiR858 overexpression increases anthocyanin content in *Malus spectabilis* [43]. A number of genes have been found to be important in anthocyanin synthesis in colored potato tubers, including chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), glutathione S-transferase (*GST*), flavonoid 3′,5′-hydroxylase (*F3′5′H*), and anthocyanidin synthase (*ANS*) [50]. Therefore, in the current study, nine candidate genes related to the anthocyanin synthesis pathway were screened through KEGG pathway analysis. These genes mainly affected the second, third, and fourth stages of the anthocyanin synthesis pathway. At different nitrogen levels, these candidate genes have a similar pattern regarding total anthocyanidin levels, showing that anthocyanins accumulate at low nitrogen levels and that anthocyanin reduction takes place at high nitrogen levels. However, these candidate genes need further validation.

5. Conclusions

Nitrogen is essential for the growth, development, and anthocyanin synthesis of 'Huasong 66' purple potatoes. In this study, four different nitrogen levels affected the agronomy traits, yield, quality, and anthocyanin contents of the purple potatoes. Compared with the N0 treatment group (without nitrogen fertilizer), the results for the low-nitrogen treatment group (N1) showed that applying low levels of nitrogen can significantly promote the growth of purple potato plants, increase their yields, improve their tuber quality, and enhance their anthocyanin accumulation. Moreover, nine genes related to the anthocyanin biosynthesis pathway were screened via a transcriptome analysis, and low nitrogen (N1) could promote anthocyanin accumulation in 'Huasong 66' purple potatoes via regulating the transcription and expression levels of the regulatory factors and related genes involved in the anthocyanin biosynthesis pathway.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14010125/s1. Table S1: Quality statistics of filtered reads; Table S2: Statistical data of read mapping; Table S3: Different genes related to anthocyanidin synthesis pathways in purple potato tubers with different nitrogen levels; Figure S1: Volcano plots (a) and MA plot (b) of the DEGs up-regulated and down-regulated between the N0 VS N1 treatments; Figure S2: Volcano plots (a) and MA plot (b) of the DEGs up-regulated and down-regulated between

the N0 VS N2 treatments; Figure S3: Volcano plots (a) and MA plot (b) of the DEGs up-regulated and down-regulated between the N0 VS N3 treatments; Figure S4: Volcano plots (a) and MA plot (b) of the DEGs up-regulated and down-regulated between the N1 VS N2 treatments; Figure S5: Volcano plots (a) and MA plot (b) of the DEGs up-regulated and down-regulated between the N1 VS N3 treatments; Figure S6: Volcano plots (a) and MA plot (b) of the DEGs up-regulated and down-regulated between the N2 VS N3 treatments; Figure S7: GO annotation of the DEGs. (a) GO annotation of the DEGs of N0 and N1. (b) GO annotation of the DEGs of N0 and N2. (c) GO annotation of the DEGs of N0 and N3. (d) GO annotation of the DEGs of N1 and N2. (e) GO annotation of the DEGs of N1 and N3. (f) GO annotation of the DEGs of N2 and N3; Figure S8: KEGG enrichment analysis of the DEGs. (a) KEGG enrichment analysis of the DEGs of N0 and N1. (b) KEGG enrichment analysis of the DEGs of N0 and N2. (c) KEGG enrichment analysis of the DEGs of N0 and N3. (d) KEGG enrichment analysis of the DEGs of N1 and N2. (e) KEGG enrichment analysis of the DEGs of N1 and N3. (f) KEGG enrichment analysis of the DEGs of N2 and N3.

Author Contributions: Y.G. (Yuchun Guo) and T.N. conceived this study and designed the experiments; Z.Z. performed the assays of the agronomic traits, yield, quality, and anthocyanin content of purple potatoes ('Huasong 66') at different stages; Z.Z. and B.C. performed the analysis of transcriptional data; Y.G. (Yiling Guo) performed qRT-PCR experiments; Y.G. (Yuchun Guo) and T.N. wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Sub-Topic of Major Grain Special Project of Fujian Provincial Department of Science and Technology (2017NZ0002-2) and Fujian Provincial Seed Innovation and Industrialization Engineering Project-Breeding and Industrial Development of High-Quality Special-Purpose Potatoes (KLd21H09A).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The detailed data that generated the results presented in this study are available from the corresponding author upon request.

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

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