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ARTICLE

Silencing of the Nonspecific Phospholipase C6 (*NPC6*) Gene Induces Ricinoleic Acid Accumulation in Castor Seeds

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ABSTRACT

Castor, scientifically known as *Ricinus communis* L., is among the top ten oil crops globally. It is considered a renewable resource and is commonly referred to as 'green oil'. Castor seeds contain castor oil as their main component, which is predominantly composed of ricinoleic acid. This study utilized RNAi technology to silence the *NPC6* gene in NO.2129 castor, resulting in the creation of mutant plants L1 and L2. The weight of 100 dry seed kernels from L1 and L2 exceeds that from NO.2129. The crude fat and ricinoleic acid levels of L1 and L2 were higher than those of NO.2129 at various developmental stages. In the proteomics analysis of 60-day-old castor seeds, a total of 21 differentially expressed proteins were identified, out of which 19 were successfully recognized. Eleven of the differentially expressed proteins identified were legumins, which play a crucial role in nutrient storage within the seed. Silencing the *NPC6* gene results in the accumulation of ricinoleic acid in castor seeds. The findings of this study not only enhance our knowledge of *NPC6*'s role in regulating castor seed oil synthesis but also offer fresh perspectives for investigating oil synthesis and accumulation in other plant species.

KEYWORDS

Ricinus communis L; ricinoleic acid; *NPC6*; RNAi

1 Introduction

Castor (*Ricinus communis* L.) belongs to the *Euphorbiaceae* family and is primarily cultivated in China, India, and Brazil. Castor seeds have high economic value because they contain a high-value oil. The ricinoleic acid content in castor oil can reach 89%–92% [1]. Ricinoleic acid is an unusual unsaturated fatty acid found in castor oil, with a hydroxyl group on the C12 atom. Ricinoleic acid is a crucial raw material in the biodiesel, cosmetics, and other industries. Previous studies have shown that ricinoleic acid possesses bactericidal, anti-inflammatory, and anti-herpes properties attributed to the presence of functional groups such as -COOH, -OH, and -C=C [1]. In addition, it can be used to generate the important industrial raw material sebacic acid through a series of chemical reactions [2,3].

Phospholipase C (PLC) is a crucial enzyme that plays a significant role in regulating various plant cell functions. It is responsible for controlling signal transduction, lipid metabolism, growth, and development in plants. The process of PLC hydrolysis plays a significant role in various physiological functions within cells. It involves the hydrolysis of phospholipid molecules, resulting in the formation of sn-1,2-diacylglycerol (DAG) [4,5]. PLC can be divided into two subtypes, namely, phosphoinositide-specific PLC (PI-PLC) and nonspecific PLC (NPC). PI-PLC is evolutionarily conserved and specifically targets the hydrolysis of phosphatidylinositol phosphate (PIP). On the other hand, the hydrolysis of phospholipids by NPC is not specific [6,7]. PI-PLC contains a PH domain, catalytic X-Y domain, and C2 domain, while NPC only contains one phosphatase domain [8]. These differences indicate that NPC may function using different structures. Previous studies have shown that NPC is a calcium-independent phospholipase. Six and five NPC genes were found in *Arabidopsis* and rice, respectively. In *Arabidopsis*, NPC plays a crucial role in multiple biological processes [9], such as root development [10], flower development [11], and seed oil production [12]. NPC1 is responsible for the plant response to heat [13], NPC2 plays a role in gametophyte development and glycerol metabolism [14], and NPC3 is involved in root development and brassinolide signal transduction [15]. The study found that *Arabidopsis* NPC4 is highly effective in breaking down PC and PG, indicating that its acts preferentially on substrates that have these specific acyl chain components [16,17]. Additionally, research has revealed that *NPC4* and *NPC5* mutants exhibit a decrease in diacylglycerol (DAG) species [17,18]. The *NPC6* gene plays a crucial role in various biological processes, such as gametophyte development, glycerolipid metabolism, root development, seed yield, and oil production [19–21]. Overexpression of *NPC6* in oilseed Brassicaceae plants results in an increase in seed oil content, seed weight, and yield. Conversely, knockout of *NPC6* leads to a decrease in seed oil content and seed size [21].

This study found that *NCP6* plays a vital role in regulating the metabolic pathways of oil in seeds. Despite progress made in researching the oil synthesis pathway, the molecular mechanism regulating ricinoleic acid synthesis in castor seeds remains largely unknown. This study investigated the impact of silencing the *NPC6* gene on the accumulation of ricinoleic acid in castor seeds. In this study, we used RNAi technology to silence the *NPC6* gene in castor plants. The silenced plants were then subjected to biochemical testing to assess the accumulation of fatty acids. To evaluate proteomic differences between mutant and wild-type mature seeds, two-dimensional gel electrophoresis was utilized. The findings of this research will enhance our understanding of the role of NPC6 in regulating castor seed oil synthesis.

2 Materials and Methods

Castor NO.2129 was provided by Tongliao Academy of Agricultural Sciences, Inner Mongolia, China. It is widely planted in the Inner Mongolia and Jilin regions of China.

2.1 Construction of the RNAi Vector for the NPC6 Gene

The C1 fragment of the sense orientation of *NPC6* (ID: 8269216) was PCR-amplified from castor variety NO.2129 using the primer pair C1-S and C1-X (Supplementary Table 1). The primer C1-S was used to

introduce a SpeI restriction site at one end of the fragment. The C2 fragment of NPC6 in antisense orientation was PCR-amplified from castor variety NO.2129 using the primer pair C2-S and C2-X (Supplementary Table 1). To introduce a SmaI restriction site at one end of the fragment, the primer C2-S was utilized. The intron fragment was acquired through double digestion of pHANNIBAL using the HindIII and KpnI restriction enzymes (Supplementary Fig. 1). Similarly, the pBI-121 vector was linearized through the use of the XbaI and SmaI restriction enzymes. The hairpin RNAi vector pBI-C1-IN-C2 was obtained by cloning C1, the intron, and C2 into the pBI-121 vector using T4 DNA ligase.

2.2 Transformation of Castor

Agrobacterium GV3101 containing the plasmid pBI-C1-IN-C2 was utilized for the transformation of the cotyledon nodes of castor plant NO.2129. NO.2129 castor seeds were inoculated in germination medium (30 g/L sucrose, 5.6 g/L agar, pH 5.6–5.8). After the seeds had germinated, the cotyledon nodes were cut off and inoculated into culture medium (1/8 MS, 10 g/L agar, 20 g/L saccharose, 8.0 mg/L ZT, 1.0 mg/L NAA, 100 μm/L AS, pH 5.6–5.8) and cultivated for 2 days. The castor bean cotyledon nodes were soaked in Agrobacterium GV3101 for 20 min and then inoculated in screening medium (1/8 MS, 10 g/L agar, 20 g/L saccharose, 8.0 mg/L ZT, 1.0 mg/L NAA, 25 mg/L Kan, pH 5.6–5.8) for 30 days. The surviving castor plants in Kan medium were transplanted into rooting medium (1/16 MS, 10 g/L agar, 20 g/L saccharose, 0.4 mg/L NAA, pH 5.6–5.8). Once the roots grew 2–3 cm, they were into soil. Castor plants were grown in greenhouses under natural sunlight at 28°C–35°C.

2.3 PCR Analysis of Transgenic Castor Plants

The molecular characterization of putative transgenic plants involved PCR analyses. PCR amplification was performed using genomic DNA and the template primers CT-S and CT-X (Supplementary Table 1). The expected product was a 1595 bp fragment from the pBI-C1-IN-C2 vector. In this study, DNA was isolated from the leaves of putative transgenic plants using a genomic DNA extraction kit (Beijing Zoman Biotechnology Co., Ltd., Beijing, China). The PCR (50 μL) mixture contained 200 ng of template DNA, 10 pmol of each primer, and 25 μL of TaKaRa Taq Version 2.0 plus dye (TaKaRa, Beijing, China). The PCR was set up as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 60 s, and a final extension step at 72°C for 10 min.

2.4 qRT–PCR

A total plant RNA extraction kit (Beijing Zoman Biotechnology Co., Ltd., Beijing, China) was used to separate RNA from transgenic and wild-type seeds, and the mRNA was purified with an mRNA kit (Qiagen). Determination of the concentration and quality of the mRNA was conducted using a microplate reader (Bio-Rad Microplate Reader 550). A cDNA reverse transcription kit (Takara) was used to reverse transcribe 1000 ng of mRNA to cDNA. The qRT–PCR (50 μL) contained 100 ng of template cDNA, 10 μL of SYBR Premix Ex Taq (Takara), 10 pmol of each primer, and 0.4 μL of ROX Reference Dye II. P-S and P-X were the primers used for the NPC6 gene, and 18s-s and 18s-x were the primers for the ribosomal 18S reference gene (Supplementary Table 1). The $2^{-\Delta\Delta CT}$ method was used for relative quantification. All qRT–PCR experiments were performed with four biological replicates.

2.5 Average 100-Kernel Weight

To further measure the weight of castor seeds produced by the plants, 100 seeds were randomly selected in each developmental period to measure the average weight. The seed coat was removed, and the weight of 100 fresh kernels (g) was measured. This process was repeated three times to determine the average value. Fresh kernel percentage (%) = weight of 100 fresh kernels (g)/weight of 100 fresh seeds (g) × 100%. Then, the fresh kernels were dried in a 65°C constant temperature oven, the weight of 100 dried kernels was

measured, and this process was repeated three times to obtain the average value. Dried kernel rate (%) = dried kernel weight (g)/fresh seed weight (g) × 100%.

2.6 Determination of Crude Fat and Fatty Acid Content in Castor Seeds

The determination of crude fat in castor seeds was performed according to the Chinese standard GB/T2906-82. The Soxhlet extraction method was used to determine the crude fat content of seeds [22]. Dried seed kernels (5 g) were ground to powder, put into a dry filter paper cylinder, and extracted with petroleum ether for 8 h in a Soxhlet extractor. The extracted crude fat was dried in an oven and weighed.

The determination of fatty acids in castor seeds was performed according to the Chinese standard GB5009.168-2016. The method for determining fatty acids involved saponification and methyl-esterification of castor kernel crude fat under alkaline conditions, resulting in the production of fatty acid methyl ester. The fatty acid methyl ester content was quantitatively determined by capillary column gas chromatography. The chromatographic column was an HP-INNOWAX (15.0 m × 250 μm × 0.25 μm) column. The gas chromatography conditions were as follows: injection temperature of 250°C, column flow rate of 1.0 mL/min, column box temperature: 180°C retention for 5 min, 180°C~230°C 3°C/min.

2.7 Proteomics Analysis of Castor Seeds

The total protein from 60-day-old castor seeds was separated by the phenol extraction method [23]. Proteins (1 g) were dissolved in the lysate (7 M urea, 2 M thiourea, 4% CHAPS (W/V), 40 mM DTT, 2% pharmalyte 3–10, 4% protease inhibitor). The dissolved protein was subjected to one-dimensional isoelectric focusing gel electrophoresis (IEFGE) at the following voltages: 30 V 8 h, 50 V 4 h, 100 V 1 h, 300 V 1 h, 600 V 1 h, 1000 V 2 h, 8000 V 12 h, and 1000 V 10 h. After one-dimensional electrophoresis, a 12% polyacrylamide gel was used for two-dimensional electrophoresis [24]. The gel was stained with Coomassie brilliant blue R250 after electrophoresis. The stained gel was photographed with a white light scanner. Image Master 2D Platinum 7.0 software (GE Healthcare) was used to analyze the gel (fold change ≥ 2-fold and $p < 0.05$), and differential protein spots were identified for mass spectrometry analysis.

3 Results

3.1 L1 and L2 Castor Generated by Silencing NPC6

The RNAi vector pBI-C1-IN-C2 was transformed into castor cotyledon nodes using *Agrobacterium tumefaciens* (Fig. 1). PCR amplification from genomic DNA of T2 generation plants subjected to *NPC6* gene silencing yielded 1595 bp bands for only the L1 and L2 plants (Fig. 2B). The expression level of the *NPC6* gene in L1 and L2 was found to be significantly lower than that in wild-type NO.2129 through qRT–PCR analysis (Fig. 2C). In summary, L1 and L2 are *NPC6* gene mutant plants.

Meng-Yuan et al. found that seed weight was significantly positively correlated with seed oil content [25]. In this study, the 100-seed weight of castor seeds at different developmental stages was determined (Table 1). Table 1 shows that the fresh weight of seeds reaches its maximum on the 40th day of development, and gradually decreases from 50 to 60 days, with significant differences between the three varieties ($p < 0.05$). The weight of fresh kernel increases with the development of seeds but not significantly. The weight of dry kernel also increased with the development of seeds, and the difference was significant ($p < 0.05$). Interestingly, the fresh and dry kernel weights of L1 and L2 were both greater than those of NO.2129, and there is a significant difference between L1 and L2 compared to NO.2129 ($p < 0.05$) The above results indicate that silencing the *NPC6* gene promotes dry matter accumulation in castor seeds.

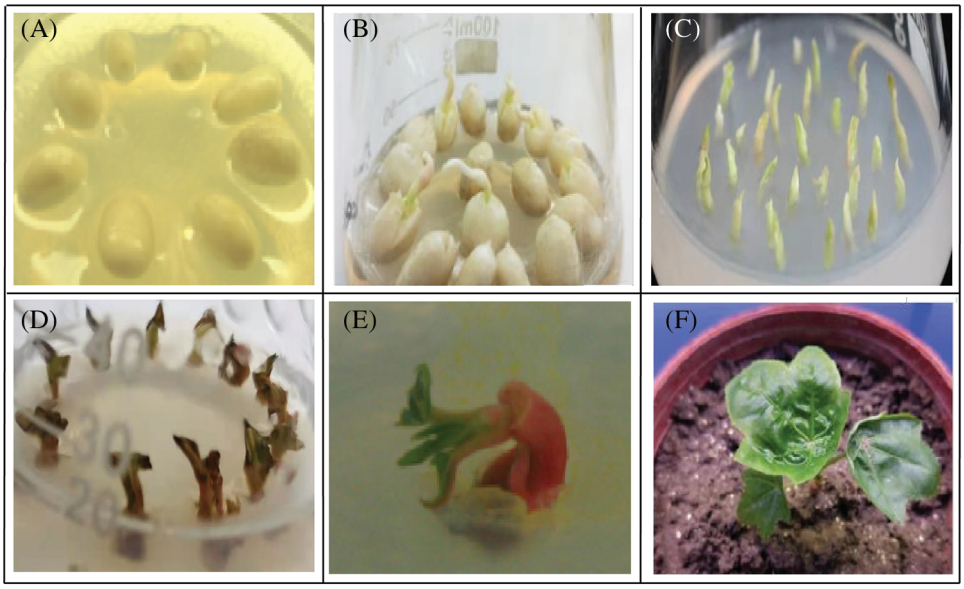

Figure 1: Genetic transformation of castor cotyledon nodes with the pBI-C1-IN-C2 vector. (A) NO.2129 seeds were inoculated into germination medium after removing the seed coat. (B) Seed germination. (C) The cotyledon nodes of castor seeds were cut and inoculated in medium. (D) Castor cotyledon nodes were inoculated into a screening medium containing Kan after being impregnated with *Agrobacterium tumefaciens*. (E) Kan-resistant seedlings. (F) Putative transgenic plant culture

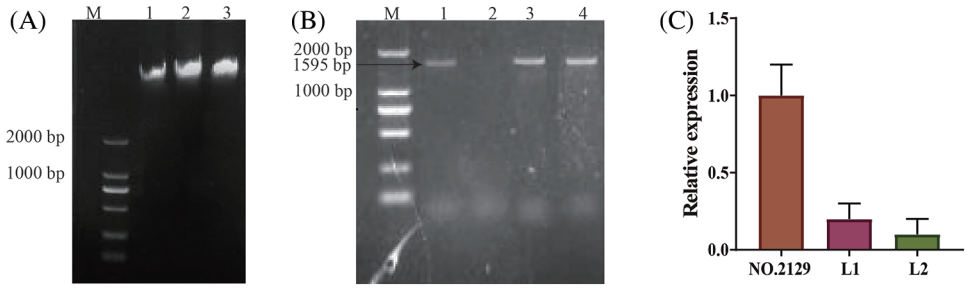

Figure 2: PCR-based identification of transgenic plants. (A) Genomic DNA electrophoresis results for castor seeds; M: DL2000 DNA marker, 1: L1 genomic DNA, 2: L2 genomic DNA, 3: NO.2129 genomic DNA. (B) PCR amplification of partial fragments of the pBI-C1-IN-C2 vector using genomic DNA as a template; M: DL2000 Marker, 1: positive control, 2: negative control, 3: L1 seed DNA PCR, 4: L2 seed DNA PCR. (C) qRT–PCR analysis of the relative expression levels of the *NCP6* gene in L1, L2, and NO.2129 seeds

3.2 Crude Fat and Fatty Acids in Seeds

In this study, the crude fat content of castor seeds at different developmental stages was determined (Table 2). Crude fat accumulated with the growth of castor seeds. Seed crude fat accumulated rapidly during the 30 to 40 days of development. The determination of the crude fat content of L1, L2, and NO.2129 showed that the crude fat content of L1 and L2 seeds from 20 to 60 days of development was higher than that of NO.2129, but the difference between L1 and L2 was not significant. According to the above results, *NPC6* silencing promotes the accumulation of crude fat in castor seeds.

Table 1: Average weight of one hundred castor seeds

Development time (d)	Material	Fresh seed weight (g)	Fresh kernel weight (g)	Fresh kernel weight rate (%)	Drying seeds kernel weight (g)	Drying seeds kernel rate (%)
20	L_1	46.2510 ± 0.0372b	27.5911 ± 0.0232b	59.6551 ± 0.0225b	3.7719 ± 0.0012a	8.1553 ± 0.0039a
	L_2	46.2817 ± 0.0353b	27.6149 ± 0.0247b	59.6671 ± 0.0400b	3.7842 ± 0.0028b	8.1764 ± 0.0055c
	2129	44.3916 ± 0.0301a	26.3107 ± 0.02482a	59.2696 ± 0.0304a	4.0954 ± 0.0011c	9.2257 ± 0.0067c
30	L_1	47.0828 ± 0.0009b	29.5964 ± 0.0035b	62.8602 ± 0.0415b	15.1348 ± 0.0001b	32.1450 ± 0.0150b
	L_2	47.0934 ± 0.0005b	29.6073 ± 0.0055b	62.8692 ± 0.0425b	15.1465 ± 0.0071c	32.1627 ± 0.0148c
	2129	46.4696 ± 0.0279a	27.9502 ± 0.0503a	61.7922 ± 0.1322a	13.5822 ± 0.0168a	30.0274 ± 0.0321a
40	L_1	49.0183 ± 0.0239b	35.4075 ± 0.0246b	72.2276 ± 0.0177b	28.2130 ± 0.0022b	57.5563 ± 0.0312b
	L_2	49.0282 ± 0.0296b	35.4119 ± 0.0220b	72.2333 ± 0.0368b	28.2244 ± 0.0066c	57.5677 ± 0.0364c
	2129	47.2710 ± 0.0598a	33.0625 ± 0.0255a	69.9426 ± 0.0391a	26.7292 ± 0.0016a	56.5447 ± 0.0688a
50	L_1	38.5770 ± 0.0007b	30.8562 ± 0.0071b	79.8031 ± 0.0079b	28.2743 ± 0.0099b	73.0783 ± 0.0250b
	L_2	38.6904 ± 0.0029c	30.8762 ± 0.0016b	79.9859 ± 0.0967c	28.4605 ± 0.0095c	73.7761 ± 0.1514c
	2129	37.7165 ± 0.0332a	30.0259 ± 0.0023a	79.6094 ± 0.0675a	27.3322 ± 0.0189a	72.4674 ± 0.0377a
60	L_1	38.3312 ± 0.0068b	32.0872 ± 0.0111b	83.7359 ± 0.0117b	30.2858 ± 0.0026b	79.0108 ± 0.0209b
	L_2	38.6120 ± 0.0078c	32.0972 ± 0.0121b	85.3907 ± 0.1769c	30.4976 ± 0.0038b	79.6932 ± 0.2410c
	2129	37.2764 ± 0.0020a	30.8096 ± 0.0588a	82.6517 ± 0.0417a	28.7067 ± 0.0889a	77.0103 ± 0.0342a

Note: a, b, and c represent significant differences $p < 0.05$. Comparison of lowercase letters among three varieties (L1, L2, and 2129) with the same developmental time and variable.

Table 2: Castor seed crude fat content at different developmental stages

Material	20 d (g)	30 d (g)	40 d (g)	50 d (g)	60 d (g)
L1	0.1795 ± 0.005b	9.0218 ± 0.0021b	18.3356 ± 0.0014b	18.3783 ± 0.0065b	19.3205 ± 0.0024b
L2	0.1801 ± 0.001c	9.0288 ± 0.0042c	18.3430 ± 0.0043c	18.4993 ± 0.0062c	19.3531 ± 0.0578c
NO.2129	0.1589 ± 0.004a	8.5731 ± 0.0106a	17.1200 ± 0.0010a	17.6757 ± 0.0122a	18.7223 ± 0.0048a

Note: a, b, and c represent significant difference $p < 0.05$. Comparing lowercase letters between different varieties at the same developmental time.

For further investigation, we examined the levels of different types of fatty acids in crude fat (Table 3), including palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid, arachidonic acid, behenic acid, and ricinoleic acid. The most abundant fatty acid was ricinoleic acid. Table 3 shows that fatty acids accumulate rapidly in seeds from 30 to 40 days of development, while slowly accumulating from 40 to 60 days. This is consistent with the crude fat determination results. In addition, the levels of ricinoleic acid, arachidonic acid, oleic acid, and palmitic acid in L1 and L2 seeds were higher than those in NO.2129, but the difference in ricinoleic acid was the largest. These results confirmed that NPC6 gene silencing promoted ricinoleic acid accumulation in castor seed crude fat.

3.3 Castor Seed Proteomic Analysis

We performed proteomic analysis on L1, L2, and NO.2129 seeds developed for 60 days (Fig. 3). To assess variations in protein amounts, we performed pairwise alignment based on fold change ≥ 2 and $p < 0.05$. In all, 21 differentially expressed proteins were detected: nine in L1-*vs*.-NO.2129, four in L2-*vs*.-NO.2129, and eight in L1-*vs*.-L2. Mass spectrometry analysis of 21 differentially expressed proteins successfully identified 19 proteins (Table 4 and Supplementary Fig. 2). A heatmap was drawn based on the expression levels of these 19 proteins (Fig. 4). Of the 12 proteins, 7 were found in L1-*vs*.-NO.2129, 2 were found in L2-*vs*.-NO.2129, and the remaining 3 were found in L1-*vs*.-L2 (Table 5). The expression levels of legumins in L1 and L2 were found to be higher than those in NO.2129. Furthermore, upregulation of nucleolar proteins was also observed in L1 and L2.

Table 3: Fatty acid content of castor seeds at different developmental stages

Development Time	Material	Palmitic acid (mg)	Stearic acid (mg)	Oleic acid (mg)	Linoleic acid (mg)	Linolenic acid (mg)	Arachidic acid (mg)	Arachidonic acid (mg)	Behenic acid (mg)	Ricinoleic acid (mg)
20 d	L1	34.2 ± 0.0b	3.6 ± 0.0b	27.8 ± 0.0b	70.9 ± 0.0b	27.6 ± 0.0b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	15.2 ± 0.0b
	L2	34.4 ± 0.0c	3.6 ± 0.0c	27.9 ± 0.0c	71.1 ± 0.0c	27.7 ± 0.0c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	15.3 ± 0.1c
	NO.2129	28.4 ± 0.0a	2.8 ± 0.0a	21.5 ± 0.0a	59.7 ± 0.0a	24.6 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	15.0 ± 0.0a
30 d	L1	117.2 ± 0.0b	90.2 ± 0.0b	369.9 ± 0.0b	523.2 ± 0.0b	63.1 ± 0.0b	9.0 ± 0.0b	54.1 ± 0.0b	9.0 ± 0.0b	7749.8 ± 0.0b
	L2	117.3 ± 0.0b	90.2 ± 0.0b	370.1 ± 0.1b	523.6 ± 0.2b	63.2 ± 0.0b	9.0 ± 0.0b	56.0 ± 0.0c	9.0 ± 0.0b	7800.9 ± 0.3c
	NO.2129	94.3 ± 0.1a	85.7 ± 0.0a	317.2 ± 0.3a	454.3 ± 0.0a	42.8 ± 0.0a	8.5 ± 0.0a	42.8 ± 0.0a	8.5 ± 0.0a	7527.1 ± 0.9a
40 d	L1	183.3 ± 0.0b	201.7 ± 0.0b	770.1 ± 0.0b	858.4 ± 0.0b	91.7 ± 0.0b	18.4 ± 0.0b	91.6 ± 0.0b	18.4 ± 0.0b	16080.3 ± 11.2b
	L2	183.4 ± 0.0c	201.7 ± 0.0c	770.4 ± 0.1c	858.8 ± 0.3b	91.7 ± 0.0b	18.5 ± 0.0c	91.7 ± 0.0c	18.5 ± 0.0c	16086.8 ± 11.9c
	NO.2129	171.2 ± 0.0a	171.2 ± 0.0a	684.8 ± 0.4a	838.8 ± 0.8a	85.6 ± 0.0a	18.3 ± 0.0a	85.6 ± 0.0a	18.3 ± 0.0a	15082.7 ± 10.5a
50 d	L1	183.7 ± 0.0b	202.1 ± 0.0b	716.7 ± 0.2b	882.1 ± 0.2b	75.1 ± 0.0b	18.1 ± 0.0b	91.8 ± 0.0b	18.1 ± 0.0b	16209.6 ± 5.6b
	L2	184.9 ± 0.0c	203.5 ± 0.0c	721.4 ± 0.2c	887.9 ± 0.3c	75.9 ± 0.0c	18.3 ± 0.0c	92.4 ± 0.0c	18.3 ± 0.0c	16316.4 ± 5.4c
	NO.2129	173.9 ± 0.2a	186.7 ± 0.0a	707.0 ± 0.4a	830.7 ± 0.5a	73.5 ± 0.0a	17.0 ± 0.0a	70.7 ± 0.0a	17.0 ± 0.0a	15589.9 ± 10.7a
60 d	L1	192.4 ± 0.0b	211.6 ± 0.0b	711.8 ± 0.0b	923.5 ± 0.2b	86.9 ± 0.0b	19.2 ± 0.0b	96.9 ± 0.0b	19.2 ± 0.0b	17027.8 ± 2.1b
	L2	193.5 ± 0.5c	212.8 ± 0.0b	716.0 ± 0.0c	928.9 ± 0.1b	87.4 ± 0.0c	19.3 ± 0.0c	97.4 ± 0.0c	19.3 ± 0.0c	17127.5 ± 2.4c
	NO.2129	187.2 ± 0.0a	187.2 ± 0.0a	705.5 ± 0.2a	879.9 ± 0.7a	84.8 ± 0.0a	18.7 ± 0.0a	94.8 ± 0.0a	18.7 ± 0.0a	16624.2 ± 4.2a

Note: a, b, and c represent significant difference $p < 0.05$. Comparison of lowercase letters among three varieties (L1, L2, and 2129) with the same developmental time and variable.

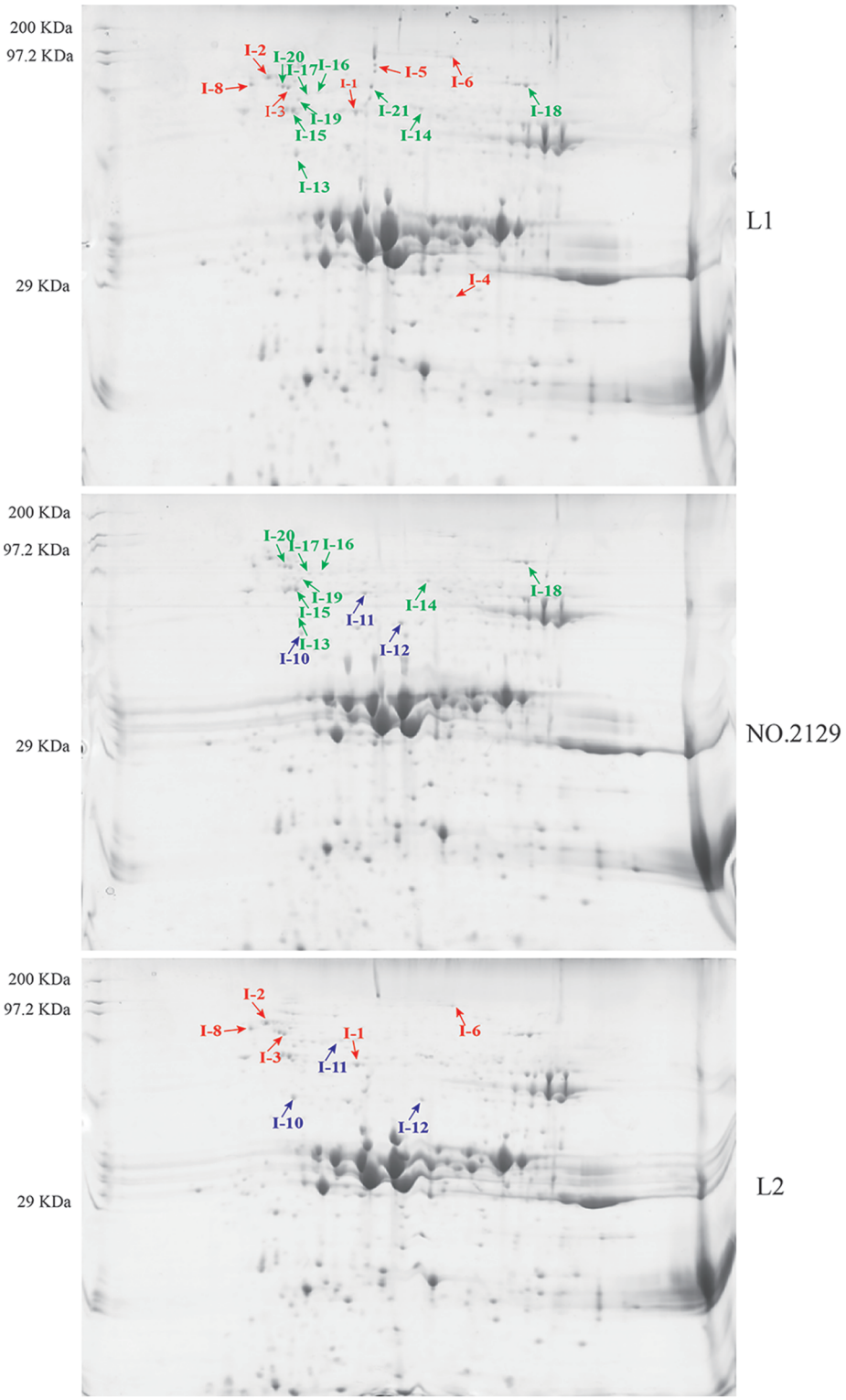

Figure 3: Two-dimensional electrophoresis analysis of castor seeds grown for 60 days. "→ : L1-*vs*.-L2 differentially expressed proteins." "→ : L_1 *vs*. NO.2129 differentially expressed proteins." "→ : L2-*vs*.-NO.2129 differentially expressed proteins."

Table 4: Identification of differentially expressed proteins in castor seeds by two-dimensional gel electrophoresis

No.	Category and name	NCBI accession no.	Theoretical KDa/PI	Score/threshold	SC[a] (%)
I-1	Legumin A	XP_002524604.1	53.91/6.88	377/58	9
I-2	Legumin B	XP_015583443.1	55.57/7.71	541/57	19
I-3	Legumin B	XP_015583443.1	55.57/7.71	298/57	10
I-4	Superoxide dismutase	EEF41564.1	26.72/5.72	454/57	27
I-5	Heat shock protein	EEF44084.1	71.30/6.10	71/40	4
I-6	Nucleolar protein nop56	XP_002524752.1	64.47/6.56	375/58	6
I-8	Protein disulfide isomerase	EEF48327.1	55.76/4.91	605/57	19
I-10	Legumin B precursor	EEF28917.1	45.65/7.86	311/57	15
I-11	Legumin A	XP_002524605.1	57.87/8.03	251/58	8
I-12	Vicilin-like seed storage protein	XP_015578419.1	44.23/5.04	238/41	8
I-13	Legumin B	EEF28918.1	56.34/7.68	171/58	6
I-14	Legumin B	NP_001310671.1	54.03/8.65	43/58	11
I-15	Legumin A	XP_002524604.1	53.91/6.88	312/58	7
I-16	Legumin A	XP_002524605.1	57.87/8.03	504/59	14
I-17	Legumin B	XP_015583443.1	55.57/7.71	517/58	17
I-18	Nucleolar protein nop56	XP_002524752.1	64.47/6.56	655/58	18
I-19	Legumin B	XP_015583444.1	55.57/7.71	596/58	19
I-20	Nucleolar protein nop56	XP_002524752.1	64.47/6.56	203/58	5
I-21	Legumin B	NP_001310671.1	54.03/8.65	491/59	16

Note: a: protein sequence coverage.

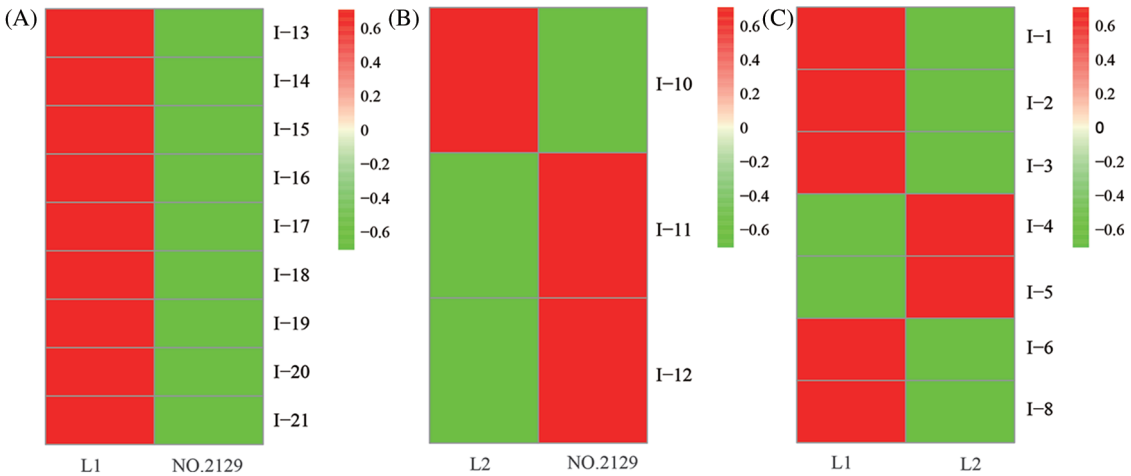

Figure 4: Heatmap of 19 differentially expressed proteins. (A) The differentially expressed proteins between L1 and NO.2129; (B) The differentially expressed proteins between L2 and NO.2129; (C) The differentially expressed proteins between L1 and L2

Table 5: Differentially expressed proteins expressed in L1, L2, and NO.2129

	Spot no.	Name	Function	Expression changes
L1-*vs*.-NO.2129	I-13	Legumin B	Nutrient storage	+
	I-14	Legumin B	Nutrient storage	↑
	I-15	Legumin A	Seed storage protein	↑
	I-16	Legumin A	Seed storage protein	↑
	I-17	Legumin B	Nutrient storage	↑
	I-18	Nucleolar protein	Storage nutrients	↑
	I-19	Legumin B	Nutrient storage	↑
	I-20	Nucleolar protein	Storage nutrients	↑
	I-21	Legumin B	Nutrient storage	+
L2-*vs*.-NO.2129	I-10	Legumin B precursor	Fat body storage protein	↑
	I-11	Legumin A	Seed storage protein	↓
	I-12	Vicilin-like seed storage protein	Storage nutrients	↓
L1-*vs*.-L2	I-1	Legumin A	Seed storage protein	↑
	I-2	Legumin B	Storage nutrients	↑
	I-3	Legumin B	Storage nutrients	↑
	I-4	Superoxide dismutase	Free radical scavenging	–
	I-5	Heat shock protein	2-alkenal reductase	–
	I-6	Nucleolar protein	Storage nutrients	↑
	I-8	Protein disulfide isomerase	Disulfide bond rearrangement	↑

Note: "+" means detected in L1 but not in NO.2129; "↑" means increased expression, "↓" means decreased expression; "–" means detected in L1 but not in L2.

4 Discussion

NPC is a type of nonspecific PLC that plays an important role in many biological processes, including in plant photosynthesis, root growth, response to heat stress, phosphorus deficiency, and salt stress [26–30]. This paper reports a new function of castor *NPC6*, which regulates the biosynthesis of ricinoleic acid. This work contributes to a better understanding of the function of the *NPC6* gene and provides new mechanistic insights into castor oil biosynthesis.

RNAi was utilized to silence the castor *NPC6* gene, resulting in two mutant plants, L1 and L2, which exhibited higher crude fat content than NO.2129. The analysis of the castor seeds fatty acid determination results revealed that L1 and L2 had higher ricinoleic acid levels than NO.2129. Studies on oilseed Brassicaceae plants showed that knockout of *NPC6* reduced the production of fatty acids from phospholipids and galactolipids to triglycerides, but the result of overexpression of the *NPC6* gene was the opposite [21]. This is consistent with our research results. It is well known that 2-oleoyl PC is the substrate for ricinoleic acid synthesis, but *NPC6* can promote the conversion of 2-oleoyl-PC to

triacylglycerol [31]. Therefore, *NPC6* gene silencing may increase the content of ricinoleic acid in seeds by preventing nonhydroxy fatty acids from entering triacylglycerol synthesis (Fig. 5).

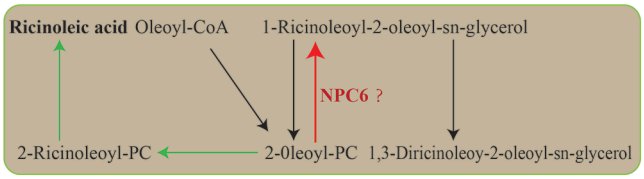

Figure 5: Ricinoleic acid biosynthesis pathway. The green arrow represents the synthesis pathway of ricinoleic acid. The red arrow represents the metabolic processes that NCP6 may regulate

This study analyzed the proteome of castor seeds from the L1, L2, and NO.2129 varieties. Twelve legumins were found to have differential expression, with higher expression in L1 and L2 compared to NO.2129. The synthesis and accumulation of storage proteins require a large amount of energy and substances, and lipid metabolism can provide energy and substances for their synthesis [32]. NPC not only produces energy in the process of hydrolyzing membrane phospholipids but also stimulates the release of Ca^{2+} stored in the endoplasmic reticulum (ER), which increases the concentration of cytoplasmic Ca^{2+} [33,34]. Therefore, *NCP6* gene silencing may increase the concentration of Ca^{2+} in the ER, thereby promoting the expression of storage proteins and processing enzymes in seeds, but the specific regulatory mechanism still needs to be further studied.

The proteomics research found that legumin expression was upregulated in L1 and L2. Nucleolar proteins also exhibited the same trend. Nucleolar proteins are a class of proteins that exist in the nucleus and play a crucial role in the formation and functional regulation of nucleoli. In addition, nucleolar proteins play an important role in ribosome biosynthesis, including in transcription, processing, and assembly of rRNA (ribosome RNA) [35,36]. Therefore, it is speculated that nucleolar proteins regulate the expression of legumin by participating in ribosome biosynthesis. However, further in-depth research is needed on how NPC6 regulates nucleolar proteins and legumin.

5 Conclusion

In this study, RNAi was used to silence the *NPC6* gene and increase the content of ricinolic acid in castor seeds. *NPC6* gene silencing may not only increase the content of ricinolic acid in seeds but also promote the accumulation of nutrients by preventing nonhydroxy fatty acids from entering triacylglycerol synthesis.

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Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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