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Overexpression of the Peanut *AhDGAT3* Gene Increases the Oil Content in Soybean

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Abstract: Soybean (*Glycine max*) is the main oilseed crop that provides vegetable oil for human nutrition. The main objective of its breeding research is to increase the total oil content. In the Kennedy pathway, Diacylglycerol acyltransferase (DGAT) is a rate-limiting enzyme that converts diacylglycerol (DAG) to triacylglycerol (TAG). Here, the *AhDGAT3* gene was cloned from peanut and overexpressed in the wild-type (WT) *Arabidopsis*. The total fatty acid content in T_3 *AhDGAT3* transgenic *Arabidopsis* seeds was 1.1 times higher on average than that of the WT. Therefore, *AhDGAT3* was transferred into the WT (JACK), and four T_3 transgenic soybean lines were obtained, which proved to be positive using molecular biological detection. Specific T-DNA insertion region location information was also obtained via genome re-sequencing. The results of high-performance gas chromatography showed that the contents of oleic acid (18:1) composition and total fatty acids in transgenic soybean plants were significantly higher than that of the WT. However, linoleic acid (18:2) was much lower compared to the WT. The agronomic trait survey showed that the quantitative and yield traits of *AhDGAT3* transgenic soybean were better than those of the WT. These results suggest that fatty acids in transgenic soybeans, especially oleic acid and total fatty acid, are enhanced by the over-expression of *AhDGAT3*.

Keywords: *AhDGAT3* gene; gene expression; oil content; peanut; transgenic soybean; triacylglycerol (TAG)

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1. Introduction

Soybean (*Glycine max*) and peanut (*Arachis hypogaea* L.) are the main sources of vegetable protein and oil, which are the most important bioenergy resources for humans to achieve sustainable development in the world. Peanut is recognized as one of the world's major oil crops and is widely planted and cultivated in China. The oil content of peanut seeds accounts for more than 50% of the seeds' dry weight. Among several common edible oil crops, the total oil content of peanut is second only to sesame (*Sesamum indicum* L.) but is higher than many other common oil crops, such as rapeseed (*Brassica campestris* L.), soybean, and cottonseed (*Gossypium* spp.) [1]. More than 80% of unsaturated fatty acids with high quality are stored in peanut seeds, which is approximately 2.5 times more than the two major oil crops, soybean and corn [2]. Therefore, it will be an innovative discovery if the functional genes that regulate the oil content in peanuts can be transformed into other common oil crops.

Soybean is a diploid plant that developed from an ancient tetraploid and contains a set of pathways to synthesize complex lipids. The oil synthesis pathways of soybean involve the process from synthesis to desaturation and the final formation of TAG. There are two

methods for the synthesis of soybean fatty acids: one method involves their function as glycerin and phospholipid to form cell membranes, and the other is their storage in seeds, mainly to form TAG [3]. TAG is the main form of oil and fat stored in all kinds of living creatures and is very important for the formation of oil in plant seeds [4]. In addition, many enzymes are involved in the lipid synthesis pathway, including the expression and regulation of different genes. Therefore, it is much more complex to reveal the molecular regulation mechanism of changes in the content of total fatty acids and various fatty acid compositions [5].

DGAT is a rate-limiting enzyme in the Kennedy pathway and plays a key role in the synthesis and accumulation of TAG, which is mainly responsible for the conversion of DAG to TAG. There are four types of *DGATs* in the *DGAT* family, including *DGAT1*, *DGAT2*, *DGAT3*, and *WS/DGAT* [6]. *DGAT1* is a member of the Acyl-CoA cholesterol acyltransferase family, which was first cloned in mice and then in *Arabidopsis* [7,8]. The *DGAT1* gene was subsequently cloned in many other plants, including nasturtium, castor, maize, tobacco, and rape [9–13]. The *DGAT2* gene was cloned in *Mortierella ramanniana* and *Arabidopsis* [14,15], castor [16], and tung tree [17]. The *DGAT3* gene has only been identified in peanut [18] and *Arabidopsis* [19]. However, the DGAT3 protein sequence showed low homology with those of the *DGAT1* and *DGAT2* subfamilies. *WS/DGAT* was cloned in fungal microorganisms and has rarely been studied so far.

There are also four types of *DGAT* genes in peanuts. *AhDGAT1* is involved in the lipid synthesis of yeast [20]. *AhDGAT2* was found to significantly increase the content of fatty acids in Escherichia coli [21]. The *AhDGAT3* gene is a soluble enzyme located in the cytoplasm with an unclear function [18]. However, the in-depth functional research on *AhWSD/DGAT* is still very limited. Peanut oil has a high nutritional value, and it is easily digested and absorbed by the human body from the composition and proportion of fatty acids in peanut seeds. Peanut oil is mainly composed of 12 fatty acid compositions, in which the sum of palmitic acid (16:0), oleic acid (18:1), and linoleic acid (18:2) accounts for about 90% of the total fatty acids. Kamisaka et al. found that DGAT was purified from the liposome in a lipid-producing fungus [21]. Lardizabal observed that overexpression of UrDGAT2A significantly increased the oil content in transgenic soybean seeds [22]. Chen et al. found that *GmDGAT2D* was overexpressed in hairy roots, increasing the 18:1 and 18:2 TAG content, whereas overexpression of *GmDGAT1A* increased the 18:3 TAG content. The overexpression of *GmDGAT2D* increased 18:1 TAG production and decreased 18:3 TAG in mutant seeds [23]. However, no study has reported that the *AhDGAT3* gene is heterologously overexpressed in soybean or verified its further functions and regulation mechanisms so far.

In our study, the *AhDGAT3* gene was cloned from peanut seeds, and the role of *AhDGAT3* in regulating the total fatty acid content, fatty acid compositions, growth, and development was characterized in transgenic soybean by heterologously overexpressing *AhDGAT3* to provide a theoretical basis for further verifying its function in soybean.

2. Materials and Methods

2.1. Plant Materials

Total RNAs were extracted from 30-day-old immature peanut seeds after flowering and used for cloning *AhDGAT3* by RT-PCR. Mature seeds of WT soybean (JACK) were used as a negative control for subsequent experiments. JACK was a common soybean variety and receptor for soybean cotyledon node genetic transformation because of its high genetic transformation efficiency. T_3 *AhDGAT3* transgenic soybean lines were selected and randomly sampled for specificity analyses of gene expression levels and fatty acid compositions. All soybean and peanut seeds were planted in the crop genetics and breeding station in May. Tissues including roots, stems, leaves, flowers, different period of pods after flowering, and mature seeds were sampled randomly for our experiments at particular time. *Arabidopsis* (Col-0) was used to transform the *AhDGAT3* gene and measure the content of fatty acid compositions. The specific disinfection, vernalization, and culture methods

of *Arabidopsis* referred to the relevant literature [24]. The mature seeds of the WT and 5 transgenic *Arabidopsis* lines after harvesting were selected to extract their DNA, RNA, and protein and measure the total fatty acid content and 5 key fatty acid compositions. All samples were selected and collected randomly from 3 different lines and stored in −80 °C freezer after freezing in liquid nitrogen.

2.2. Isolation and Sequence Analysis of AhDGAT Gene in Peanut

The complete coding sequence of the *AhDGAT3* gene was inquired, obtained, and accessed on 4 May 2020 using the NCBI Nucleotide Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and peanut genome database (http://www.peanutbase.org). The conserved sequences and regions of DGAT3 in different plants were aligned, analyzed and accessed on 5 May 2020 using the online software MEME 5.5.4 (https://meme-suite.org/meme/tools/meme). Total RNAs were sampled and extracted from 30-day-old immature peanut seeds after flowering. Then, the extracted RNAs were reverse-transcribed into cDNAs, which were used as a template for cloning the *AhDGAT3* using RT-PCR method. The specific PCR primers were designed according to the cDNA sequence using Primer Premier 5.0 software. All the RT-PCR primer pairs are provided in Table 1. The RT-PCR reaction contained the forward and reverse primers, cDNA, dNTP mixture, PCR reaction buffer, and double distilled water. After being sequenced in the Biosciences Company (Comate, Changchun, China) for accuracy, the PCR product containing target gene would be constructed to the cloning vector pMD18-T for further studies.

Table 1. Gene specific primer pairs and information used for PCR and molecular detection.

Purpose	Gene Name (Amplification Length)	Accession Number	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
RT-PCR	*AhDGAT3* (1438 bp)	XM_016339125	AATAGAAATAGAAATGTGATAATGG	ACAAATCAGGCTCTGGAAGTT
qRT-PCR	*AhDGAT3* (140 bp)	XM_016339125	AGAATGGAACCGCTATGT	CTCTGCCCTTACTTGCTC
	β-Tubulin (185 bp)	GMU12286	GGAAGGCTTTCTTGCATTGGTA	AGTGGCATCCTGGTACTGC
	Actin (155 bp)	J01298	GTCCTTTCAGGAGGTACAACC	CCTTGAAGTATCCTATTGAGC
Detection	*Bar* (220 bp)		GTCTGCACCATCGTCAACCACTACA	AGACGTACACGGTCGACTCGGCCGT
	AhDGAT3 (304 bp)	XM_016339125	AGAATGGAACCGCTATGT	CTCTGCCCTTACTTGCTC

2.3. Arabidopsis and Soybean Transformation of AhDGAT3

The open reading frame of the *AhDGAT3* gene was constructed into an overexpression vector pTF101-35s using heat-shock method, and the DH5α competent cells were treated by $CaCl_2$, which needed to be prepared in advance. Then, the resultant plasmid pTF101-AhDGAT3 was transformed into *Agrobacterium* tumefaciens EHA105 using heat-shock method for the genetic transformation of *Arabidopsis* and soybean. Then, the resultant plasmid was transformed into *Arabidopsis* Col-0 using *Agrobacterium* transformation and floral-dip method [25] and transformed into soybean JACK (WT) using *A*. tumefaciens to infect the soybean cotyledon nodes. The transformed *Arabidopsis* and soybean were screened on the MS medium, which contained 5 mg L^{-1} glufosinate-ammonium. Then, T_0 transgenic *Arabidopsis* and soybeans were detected using PCR and *bar* strip, which is a simple and accurate method to identify the positive plants. All the positive *AhDGAT3* transgenic *Arabidopsis* and soybean lines were bred to T_3 generation for subsequent experiments.

2.4. Molecular Detection of AhDGAT3 Transgenic Soybean

The regenerated plants were transplanted and cultivated in the plant tissue incubator. Leaves of T_0 transgenic plants were sampled and identified using PCR and *bar* strip for

positive detection. Transgenic plants were tested via RT-PCR, from which the DNA of *AhDGAT3* transgenic soybean leaves was extracted and used as the PCR template. All the specific primer pairs were provided in Table 1 and used to amplify the *AhDGAT3* gene (1438 bp) and *bar* gene (220 bp). Southern blot was performed to verify that the *AhDGAT3* gene has been transformed into soybean successfully at the DNA level. The *bar* and *AhDGAT3* genes were chosen as DNA probes, which referred to the DIG High Prime DNA Labeling and Detection Starter Kit II. Western blot was performed to verify the expression level of AhDGAT3 protein in transgenic soybeans on a qualitative level. Protein of JACK leaves was extracted as the negative control. The primary monoclonal antibody, which was required for the experiment, was Mouse Monoclonal with 1:5000 titers. The HRP Goat Anti-Mouse lgG was chosen to be the secondary antibody with 1:6000 titers. ELISA was performed to determine whether the PAT protein was expressed in transgenic soybeans on a quantitative level. The specific experiment methods all referred to the relevant literature [24].

2.5. Genome Re-Sequencing

Four T_3 *AhDGAT3* transgenic soybean strains were sent to Biotechnology company (Biomarker, Beijing, China) for genome re-sequencing analysis. After the Genomic DNA samples were qualified, the DNA was fragmentated using ultrasonic mechanical interrupt method, and then the end of the DNA fragment was purified and repaired. Then, 3' and A ends of the DNA were sequenced, connected with joints, and then the DNA fragment was selected and sequenced. Finally, the genomic library was constructed, qualified, and sequenced with Illumina instrument. After the quality of the sequenced original reads (double-ended sequences) was evaluated, the clean reads were selected to be compared with the reference genome sequences, including the mutation detection and annotation of SNP. InDel and mutations were carried out on the basis of sequence alignment. The functional genes were finally discovered and annotated at DNA level.

The *AhDGAT3* transgenic soybeans (R01–R04), respectively, were aligned to the reference genome and exogenous T-DNA sequence data, according to the comparison results. The short end of two kinds of matching sequences was found, and the sample data were aligned to the reference genome and the exogenous insertion sequence, respectively. According to the result of alignment, two types of paired ends were found. The insertion sequence was aligned to the reference genome by blast for homology assessment. The assembled contig sequences were aligned to the reference genome by blast, and then the contig sequences were selected to be compared to the regions of chromosomes according to the alignment results. These regions were verified by IGV screenshots, and then the region location information where *AhDGAT3* was inserted was obtained.

2.6. Quantitative RT-PCR Analysis

Total RNAs were extracted from the WT soybean, peanut, and *AhDGAT3* transgenic soybean tissues, including vegetative growth (root, stem, and leaf) and reproductive growth (different period of pods after flowering and mature seeds). Then, RNAs were reverse-transcribed into cDNAs using RNAiso Plus and M-MLV Kit. All the qRT-PCR primer pairs of the *AhDGAT3* and internal reference genes including *β-Tubulin* and *Actin* genes, which were used to standardize the data [26–28], are provided in Table 1. The qRT-PCR conditions referred to the instructions of TAKARA Biotechnology Company. All the qRT-PCR samples followed the principles of three biological and technical replicates.

2.7. Measurement of Fatty Acid Content in Arabidopsis and Soybean

The total fatty acids of *AhDGAT3* transgenic *Arabidopsis* were extracted using the methylester method. The fatty acid samples were tested and analyzed using a flame ionization detector (Agilent 7890A GC system, Santa Clara, CA, USA) and referring to the methods described in the literature [29]. Five key fatty acid compositions (16:0, 18:0, 18:1, 18:2, and 18:3) were standardized as the control. The total fatty acids of mature seeds in

AhDGAT3 transgenic soybeans were extracted using the hydrolysis method (GB5009.168-2016). Two standard sample solutions of the two methods were formulated and used to measure different peak values at different time. And then, we plotted the standard curve in different concentration gradients. All the values were plugged into the standard curve for calculation. The specific experiment methods both referred to the relevant literature [24].

2.8. The Agronomic Trait Analysis of WT and Transgenic Soybeans

After JACK and T_3 *AhDGAT3* transgenic soybean plants matured, the following agronomic traits were analyzed for each plant, including plant height, main stem node number, effective branch number, 100-seed weight, pod number per plant, seed number per plant, seed weight per plant, and podding height. We randomly selected 30 WT (JACK) and 40 T_3 transgenic soybeans (10 of each line). Each result was the average of one soybean line. The 10-day to 50-day pods after flowering of JACK and *AhDGAT3* transgenic soybeans were randomly selected and assessed for pod length and width. The pods were measured 3 times. And then, the average length and width of a single soybean were calculated. The results of the agronomic traits were determined by tape measure, vernier calipers, grain counting machines, rulers, and scales.

2.9. Statistical Analysis

All statistical data were calculated and analyzed using SPSS 20.0 (IBM Corp, Armonk, NY, USA) software. Significant differences among the means of samples were compared at $p < 0.05$ (significant difference) or $p < 0.01$ (extremely significant difference), based on an independent-sample *t*-test.

3. Results

3.1. Isolation and Characterization of AhDGAT3 Gene

In order to verify the functions of *AhDGAT3*, all the *AhDGAT* family genes were searched, obtained, and accessed on 4 May 2020 from the plant comparative genome database, Phytozome (http://phytozome-next.jgi.doe.gov/), and the Peanut genome database, (http://www.peanutbase.org/), for homologous search. Four candidate genes were identified, named *AhDGAT1* (XM_016346849), *AhDGAT*2 (XM_016095296), *AhDGAT3* (XM_016339125), and *AhWSD/DGAT* (XM_016109460). The *AhDGAT1*, *AhDGAT*2, and *AhDGAT3* genes were closely related but were far related to the *AhWSD/DGAT*. In the DGAT3 conserved domain, Motif1 contains the characteristic fatty-acid-binding protein sequence KSGSIALLQEFERVVGAEG, and Motif2 contains the CKCMGKCKSAPNVRIQN-STAD conserved sequences. Motif3 contains the speculated structural motif NPLCIGV-GLEDVDAIVA, and Motif4 contains the DDLQGNLTWDAAEVLMKQLEQVRAEEKELKK-KQKQEKKAKL conserved sequences. The subfamily also contains the KKRVLFDDL active sites of the acyltransferase family [15]. In addition, this subfamily also contains the thioesterase characteristic sequence TNPDCESSSSSSSSESESES (Figure 1).

3.2. Oil Content in Seeds of Different Genotype Arabidopsis Plants

To reveal and verify the functions of *AhDGAT3* in the accumulation of oil in seeds, the *AhDGAT3* gene was transformed into the WT *Arabidopsis* (Col-0) plants. Mature seeds from T_3 *AhDGAT3* transgenic lines were sampled to measure the oil content. The results showed that the content of 18:1 composition in *AhDGAT3* transgenic *Arabidopsis* seeds was significantly higher than that of the WT, reaching 1.3 times on average, respectively (Figure 2A). However, the contents of 16:0, 18:2, and 18:3 compositions in the transgenic lines were slightly lower than those in the WT. The total fatty acid content in four *AhDGAT3* transgenic soybean lines was also enhanced a lot (Figure 2B).

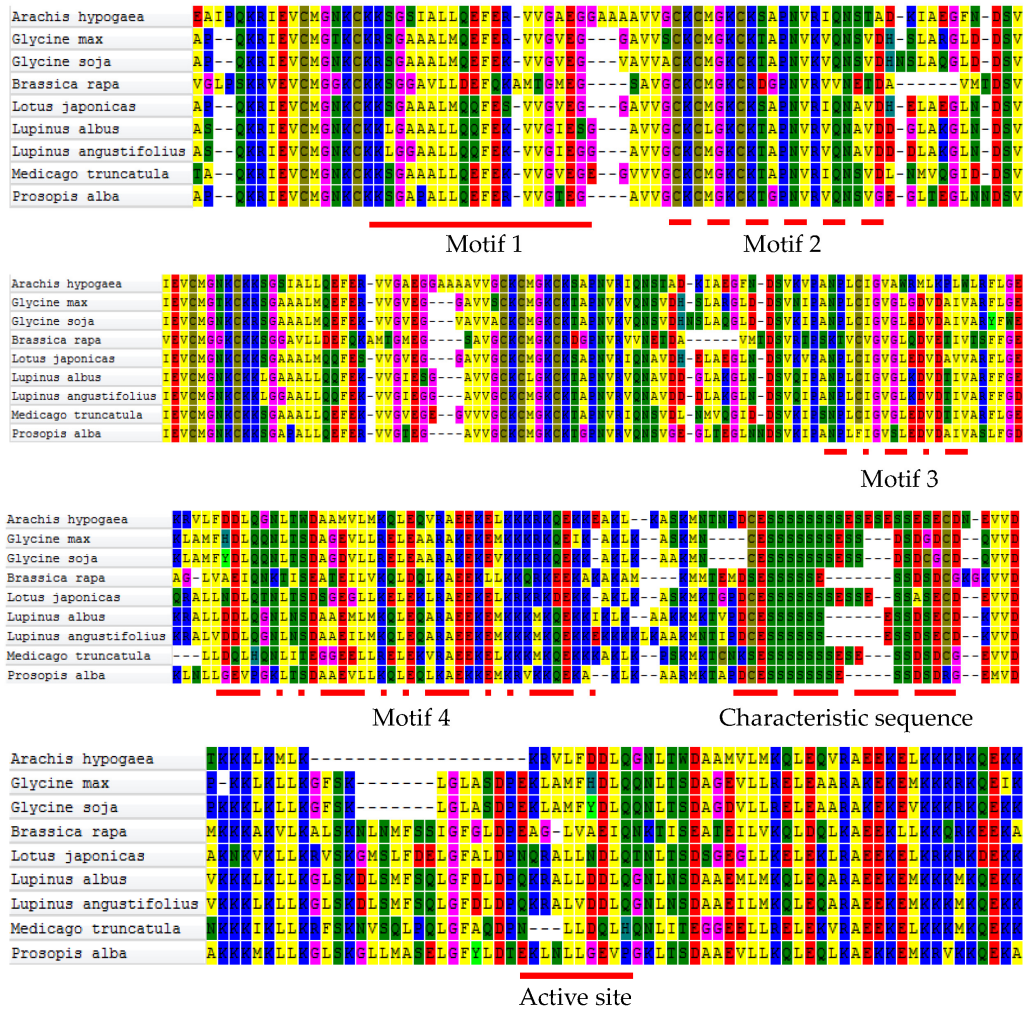

Figure 1. Partial alignment of DGAT3 deduced amino acids in different plants. Sequences were aligned using MEGA 7.0 software. Different shapes of lines represent four different special functional domains and two special sites. Accession numbers of 9 genes in GenBank are as follows: *Arachis hypogaea*, AAX62735.1; *Glycine max*, XP_003542403.1; *Glycine soja*, XP_028209585.1; *Brassica rapa*, RID49571.1; *Lotus japonicas*, AFK37850.1; *Lupinus angustifolius*, XP_019419028.1; *Medicago truncatula*, XP_003609890.1; *Prosopis alba*, XP_028760027.1.

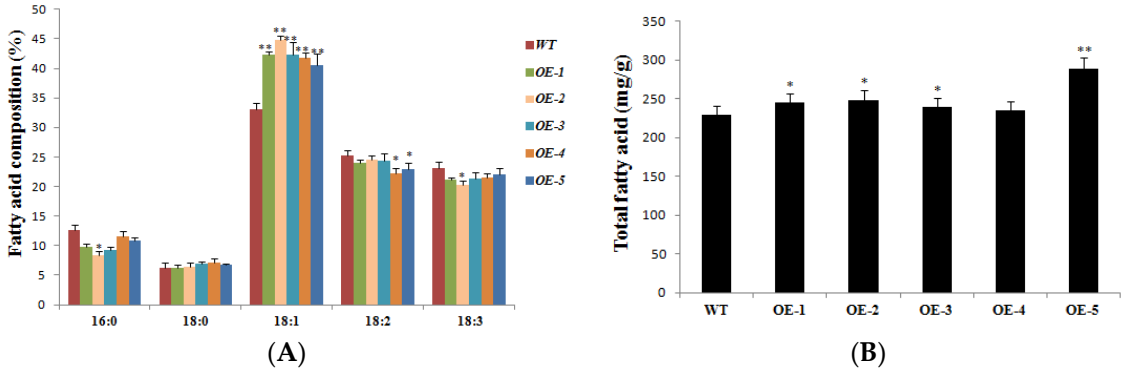

Figure 2. (**A**) Content of 5 fatty acid compositions in the WT and *AhDGAT3* transgenic *Arabidopsis* lines. (**B**) Total fatty acid content in the WT and *AhDGAT3* transgenic *Arabidopsis* seeds. The data represent the average of 3 independent repetitions of experiments. Error bars indicate the standard error. * $p < 0.05$ and ** $p < 0.01$ represent the significant difference. The peaks and peak time of each group were palmetto acid (16:0), 11.672 min; stearic acid (18:0), 16.011 min; oleic acid (18:1), 16.471 min; linoleic acid (18:2), 17.779 min; and linolenic acid (18:3), 20.345 min.

3.3. Overexpression AhDGAT3 Gene in Soybean

To verify the further functions of *AhDGAT3* in soybean, four T_3 *AhDGAT3* transgenic soybean lines were obtained and detected via a series of molecular biology methods, including the *bar* strip, Southern blot, and Western blot. The results showed that *AhDGAT3* was transformed into a JACK cultivar background (Figure 3). ELISA analysis revealed that the expression level in transgenic soybeans was higher than that of the WT (Table 2).

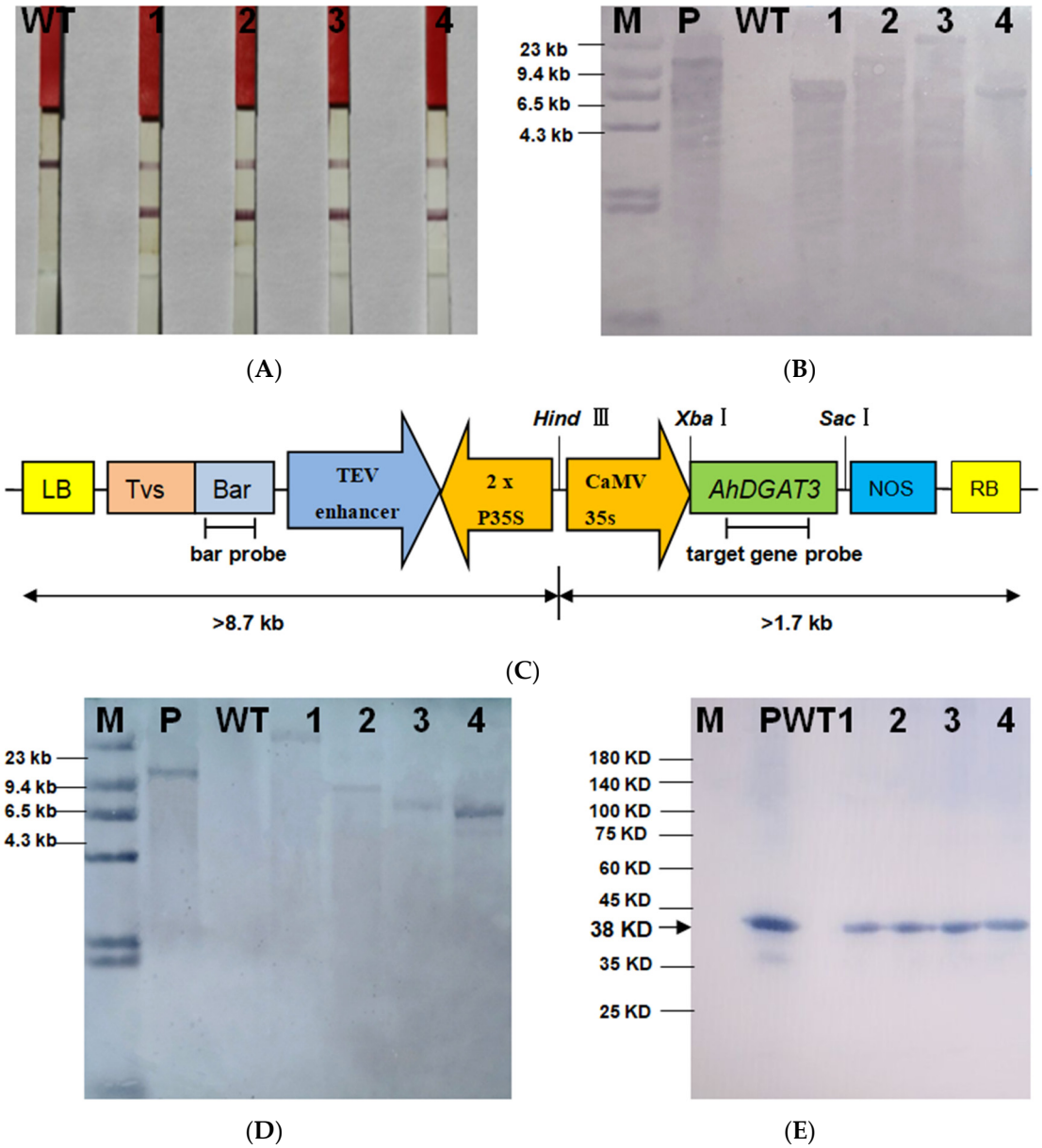

Figure 3. Positive detection of *AhDGAT3* transgenic soybean lines. (**A**) *Bar* strip analysis of 4 *AhDGAT3* transgenic soybeans. (**B**) Southern blot of T_3 transgenic soybeans using the *bar* gene fragment as a probe. M represents DNA marker; P represents recombinant plasmid (positive control); WT represents JACK (negative control). (**C**) Map of pTF101-*AhDGAT3* recombinant plasmid in *AhDGAT3* transgenic soybeans. (**D**) Southern blot of T_3 transgenic soybeans using the target gene fragment as a probe. M represents DNA marker; P represents recombinant plasmid (positive control); WT represents JACK (negative control). (**E**) Western blot of T_3 transgenic soybeans. M represents pre-stained protein marker; P represents AhDGAT3 protein (positive control); WT represents JACK (negative control).

Table 2. AhDGAT3 protein levels of different tissues in JACK and 4 transgenic lines (OE-1 to OE-4). Data represent the average values of three biological replicates. NA means that the value is not detected and calculated through the standard curve, and there were no detection results.

Material	JACK	OE-1 (ng/g)	OE-2 (ng/g)	OE-3 (ng/g)	OE-4 (ng/g)
Nodules	NA	196.2 ± 10.2	220.6 ± 14.3	200.5 ± 13.8	233.9 ± 14.9
Root	NA	236.1 ± 19.2	389.5 ± 8.7	248.2 ± 1.5	415.5 ± 20.4
Stem	NA	203.7 ± 14.9	208.7 ± 6.9	200.6 ± 7.9	317.1 ± 11.7
Leave	NA	483.7 ± 16.7	523.7 ± 14.4	511.3 ± 10.2	655.1 ± 11.4
Flower	NA	288.3 ± 27.2	385.1 ± 17.7	348.6 ± 11.5	417.6 ± 10.5
10-day Pod	NA	803.8 ± 14.8	841.7 ± 9.2	816.9 ± 3.8	869.5 ± 6.9
20-day Pod	NA	676.1 ± 22.9	706.6 ± 10.5	796.1 ± 4.6	787.1 ± 27.1
30-day Pod	NA	700.6 ± 15.9	804.1 ± 5.4	753.5 ± 32.3	883.7 ± 8.8
40-day Pod	NA	913.2 ± 5	985.4 ± 3.8	951.4 ± 20.3	1014.4 ± 11.2
Seed	NA	949.3 ± 17.9	1096.9 ± 11.1	1004.6 ± 16.9	1166.1 ± 22.1

The 1038 bp *AhDGAT3* gene was inserted into the pTF101-35s vector with the *Xba* I/*Sac* I restriction site (Figure 3C). The distances between the *bar* gene probe and the left/right boundaries of T-DNA were approximately 9 kb and 2 kb, respectively. The distances between the target gene and the left/right boundaries of T-DNA were approximately 9.6 kb and 1 kb, respectively (Figure 3C). The results of the Southern blot showed that only one hybridization band appeared in four *AhDGAT3* transgenic lines (OE-1 to OE-4), and no hybridization band was detected in the WT. This suggested that exogenous T-DNA was integrated in the form of single-copy DNA in four *AhDGAT3* transgenic lines (Figure 3B,D). Specific insertion sites were analyzed and studied via genome re-sequencing in transgenic soybean. The *AhDGAT3* gene was translated into the protein and synthesized monoclonal antibodies to identify the transgenic soybeans. The Western blot results showed that there was an apparent hybridization signal at 38 KD, indicating that the *AhDGAT3* gene was over-expressed at the protein level in transgenic plants (Figure 3E).

The ELISA results showed that the AhDGAT3 protein was not expressed in the WT (negative control), and no value was calculated using a standard curve. The AhDGAT3 protein of four transgenic lines was detected and calculated using a standard curve. AhDGAT3 expression levels in the vegetative growth stage were lower than those of the reproductive growth stage. AhDGAT3 level accumulation was highest in mature seeds as the most important organ for oil storage, which was approximately two to five times higher than that of the nutritious organs. This result can further indicate that AhDGAT3 proteins are expressed in four transgenic soybean lines (Table 2).

3.4. Genome Re-Sequencing of AhDGAT3 Transgenic Soybean

In this analysis, the genomic DNA from four T_3 transgenic soybean leaves was re-sequenced. Clean data with a total data volume of 92.59 Gbp were obtained, and Q30 reached 90.51%. The average contrast ratio of the sample to the W82 reference genome was 98.88%. The depth of the average coverage was 21× and the depth of the genome coverage was 98.28%, which made sure at least one base was covered.

The re-sequencing data of four transgenic soybeans (R01–R04) were compared with reference genome and exogenous T-DNA sequences, and all short sequences that could be compared with exogenous T-DNA sequences were selected for the analysis. The assembled contig sequences were blasted to the reference genome. And then, the contig sequences were selected to be compared to the regions of chromosomes according to the alignment results. These regions were verified by IGV screen shots to obtain the location information of exogenous insertion fragments. All the results of gene insertion information are shown in Table 3.

Table 3. The insertion region location information of four *AhDGAT3* transgenic strains (R01–R04).

Sample	Chromosome	Integration Sites	Integrated Way
R01	3	36,280,392	Single copy
R02	10	19,795,197	Single copy
R03	11	32,500,049	Single copy
R04	14	8,451,776	Single copy

3.5. Expression Analysis of AhDGAT3 in WT and Transgenic Soybean

Tissues in vegetative and reproductive growth stages from JACK and transgenic soybeans were collected, and the total RNAs were extracted to verify gene over-expression in the transgenic soybean using qRT-PCR. The results showed that *AhDGAT3* expression in transgenic soybeans was significantly ($p < 0.01$) higher than that of the WT in the leaves and 10- and 40-day-old pods after flowering. The *AhDGAT3* expression level in the flower and 30-day-old pods of transgenic soybeans was significantly higher ($p < 0.05$) than that in the WT (Figure 4).

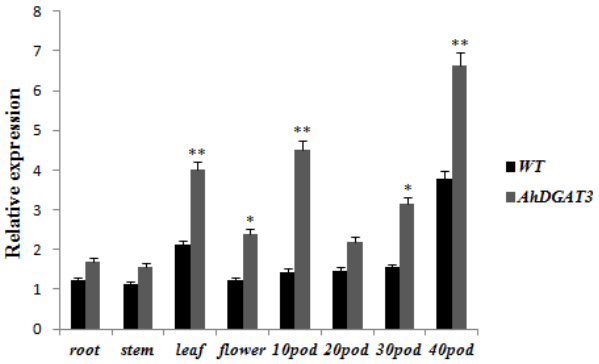

Figure 4. Expression levels of *AhDGAT3* in different tissues of soybean. The data represent the average of 3 independent experiments ± SD. Error bars indicate the standard error. * $p < 0.05$ and ** $p < 0.01$ represent the significant difference.

3.6. AhDGAT3 Over-Expression Enhances Fatty Acid Content in Transgenic Soybean

Mature soybean seeds (0.01 g) were collected and sampled with three biological replicates, including the WT (JACK) and *AhDGAT3* transgenic lines, which were used for oil determination and analysis. The results of high-performance gas chromatography (HPGC) showed no significant difference in the content of 16:0, 18:0, and 18:3 fatty acid compositions between *AhDGAT3* transgenic soybeans and JACK. However, the difference of the 18:1 content was significant, with the transgenic line being 27.3% higher than JACK, respectively. The content of the 18:2 fatty acid compositions was significantly lower than JACK. The total fatty acid content of the T_3 *AhDGAT3* transgenic soybean was up to 4% higher than that of JACK (Figure 5).

3.7. AhDGAT3 Overexpression Improved the Agronomic Traits of Transgenic Soybean

The analysis of the agronomic traits in the WT and *AhDGAT3* transgenic soybean seeds showed that plant height, effective branch number, pod number per plant, and seed weight per plant in the *AhDGAT3* transgenic soybean were significantly superior to the WT (Figure 6A and Table 4). The size of 10-day to 50-day pods after flowering of the *AhDGAT3* transgenic soybean was significantly bigger than the WT at different stages of reproductive growth (Figure 6B–D). The results showed that overexpression of the *AhDGAT3* gene may also affect the growth and development of soybean.

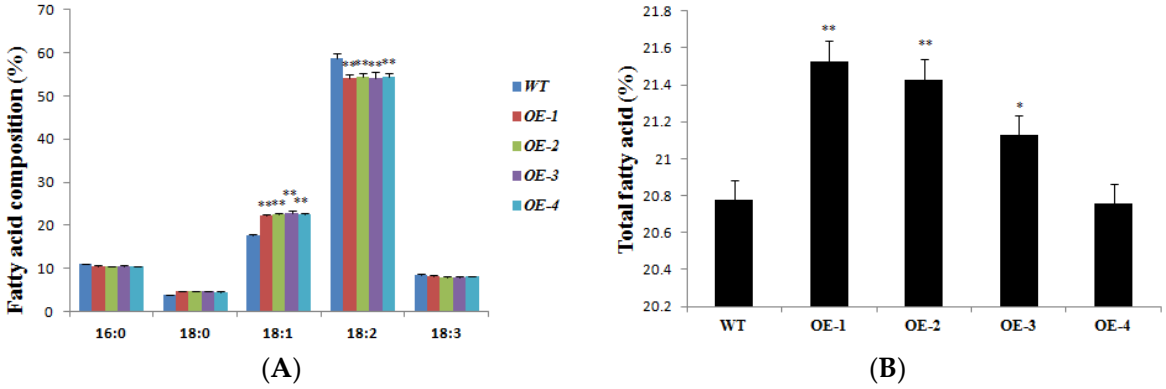

Figure 5. Content of different fatty acid compositions and total fatty acids in soybean seeds of different genotypes. (**A**) The content of different fatty acid compositions in JACK and 4 different *AhDGAT3* transgenic lines (OE-1 to 4) of soybean, measured by HPGC. (**B**) Total fatty acid of 4 *AhDGAT3* overexpressing soybean seeds. Error bars indicate the standard error. * $p < 0.05$ and ** $p < 0.01$ represent the significant difference.

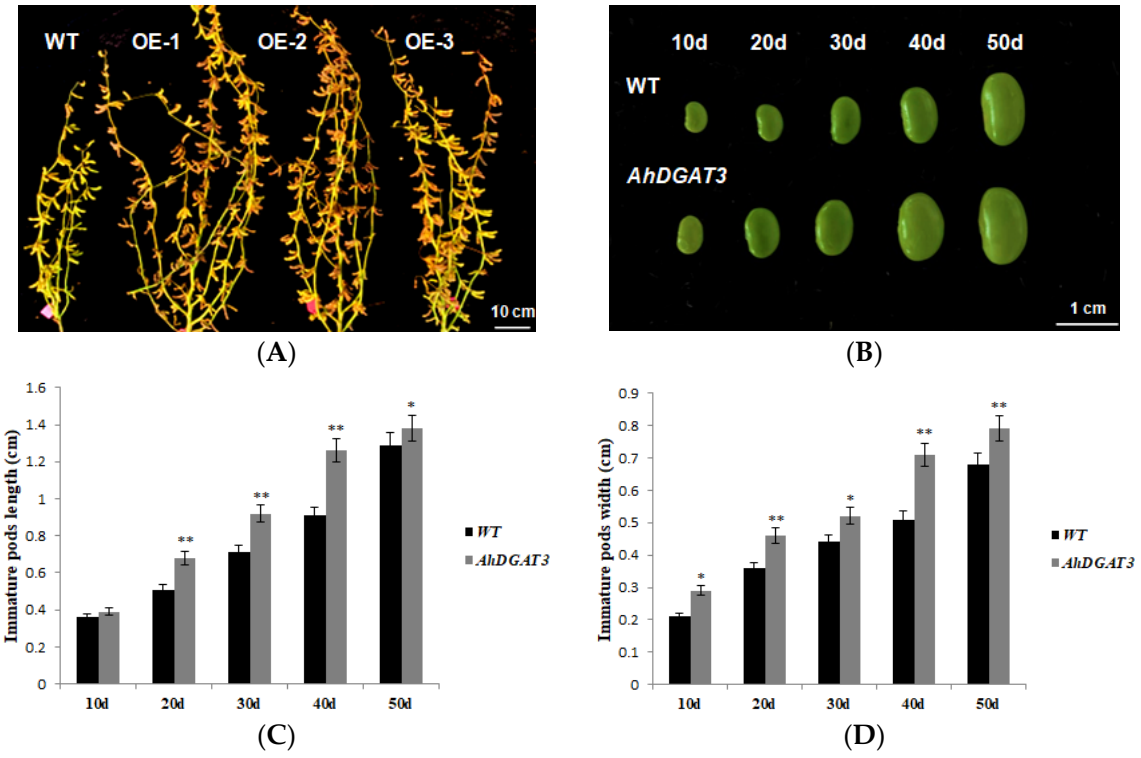

Figure 6. Phenotypic observation of *AhDGAT3* transgenic soybean and the WT. (**A**) Three *AhDGAT3* transgenic lines (OE-1 to OE-3) and the WT in mature period. (**B**) The size of 10-day to 50-day immature pods after flowering in the WT and *AhDGAT3* transgenic soybean. (**C**) The difference of 10-day to 50-day immature pods' length between the WT and *AhDGAT3* transgenic lines. (**D**) The difference of 10-day to 50-day immature pods' width between the WT and *AhDGAT3* transgenic lines. Error bars indicate the standard error. * $p < 0.05$ and ** $p < 0.01$ represent the significant difference.

Table 4. Agronomic traits in the WT and *AhDGAT3* transgenic soybean (OE-1 to 3). The data for sample mean ± standard error ($p < 0.01$). Significantly different results are indicated by different letters (a, b): a represents no significant difference; b represents significant difference.

Material	WT	OE-1	OE-2	OE-3
Plant height (cm)	101.4 ± 0.14 a	114.1 ± 0.17 b	109.2 ± 0.21 b	105.1 ± 0.27 a
Effective branch number	4 ± 0.21 a	8 ± 0.16 b	7 ± 0.28 b	6 ± 0.12 b
Pod number per plant	78 ± 0.14 a	188 ± 0.11 b	168 ± 0.18 b	161 ± 0.27 b
Seed number per plant	155 ± 0.16 a	316 ± 0.29 b	293 ± 0.19 b	289 ± 0.31 b
100-seed weight (g)	13.5 ± 0.21 a	16.3 ± 0.22 b	15.6 ± 0.24 a	16.9 ± 0.17 b
Seed weight per plant (g)	25.7 ± 0.17 a	57.2 ± 0.24 b	49.7 ± 0.26 b	45.7 ± 0.26 b
Main stem node number	18 ± 0.25 a	22 ± 0.26 b	21 ± 0.21 b	21 ± 0.24 b
Podding height (g)	8.4 ± 0.29 a	10.1 ± 0.31 a	9.9 ± 0.31 a	10.1 ± 0.27 a

4. Discussion

Soybean is an important food and oil crop in the world, accounting for a significant proportion [30]. How to effectively improve vegetable oil content and the composition ratio of unsaturated fatty acids is an important breeding goal for us. Heterologous gene expression is an important means to improve soybean oil content.

Recently, studies on how to improve the oil content of plant seeds have mainly focused on the lipid metabolism pathway. All kinds of functional enzyme genes and transcription factors, including *PDAT*, *FAD*, *SAD*, *LEC1*, *LEC2*, Dof, and ABI3, were cloned and studied, which proved to play an important role [31]. It is a major technical method to verify the coordinated expression of multiple genes in metabolic pathways via genetic transformation technology [32]. However, as an enzyme gene that plays a key catalytic role in the conversion process of DAG to TAG. *DGAT* plays an important role in regulating the accumulation of fatty acids and lipid synthesis in oil crops [33]. The oil content of seeds depends on quantitative heredity and is regulated by all kinds of factors, including the synthesis and accumulation of fatty acids and the development of seeds [34,35].

Compared to animal fatty acids, various unsaturated fatty acids in plants are very beneficial to our health. The demand for high-oleic-acid vegetable oil is also increasing with the improvement of our living standards. The results showed that *AhDGAT3* was over-expressed in *Arabidopsis* and soybean. The contents of 18:1 unsaturated fatty acid compositions in transgenic plant seeds were significantly increased. This result is consistent with recent research reports [23]. *AtDGAT3* was involved in a soluble cytosolic process in the circulation of linoleic and linolenic acid to TAG during the degradation and breakdown of seed oil [19]. However, no in-depth studies showed the functions of the *AhDGAT3* gene using heterologous transformation and genetic engineering technology between peanut and soybean. Most recent studies on *DGAT* genes mainly focus on functional analysis in *Arabidopsis*. The *UrDGAT2* gene of Umbelopsis ramanniana was successfully transferred into soybean and over-expressed in seeds. The *UrDGAT2* transgenic soybean showed a higher oil content [22]. TAG, as the main storage in oil crop seeds, provided enough C sources for seed germination and development. The up-regulation of the *DGAT1* gene was involved in the fatty acid mobilization and the catalyzation in the reverse reaction of TAG synthesis during seed germination and development [36,37]. The results of the agronomic traits showed that the phenotype of the *AhDGAT3* transgenic soybean was better than that of the WT soybean in terms of the size of different period pods, the number of effective branches, the number of seeds per plant, pod number per plant, main stem node number, and the weight of seeds per plant at harvest time. We speculated that *AhDGAT3* was also closely related to the growth and development of soybeans. Moreover, the next step is to study the internal regulation mechanism of *AhDGAT3*. We aimed to develop high-quality transgenic soybean lines with high oil content and yield, creating excellent soybean germplasm resources.

5. Conclusions

We characterized the major functions of *AhDGAT3* in *Arabidopsis* and soybean. The content of total fatty acids and 18:1 compositions in the *AhDGAT3*-overexpressed transgenic soybean was significantly higher than those of the WT (JACK). The re-sequencing of *AhDGAT3* transgenic soybean revealed that T-DNA was integrated into the soybean genome as a single copy. Overexpression of the *AhDGAT3* gene can affect the size of soybean immature pods and a series of agronomic traits. Therefore, we also speculate that this gene may also play a key role in soybean growth and development, which needs further study. Our results indicated that molecular-assisted breeding is a very efficient and fast way of increasing the oil content of soybean seeds, which can also be used in the modulation of other agronomic traits of soybean in the future.

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