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Overexpressing *Sesamum indicum* L.'s *DGAT1* increases the seed oil content of transgenic soybean

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Abstract Soybean (*Glycine max*) is an important oilseed crop that provides ~30% of the vegetable oil used for food, feed and industrial applications. Genetic engineering can produce precise changes in plants in a short period of time and complements conventional plant breeding methods. However, soybean has the lower transformation efficiency and more genotypic limitations than other species, which makes it more difficult to transform. In this study, we introduced diacylglycerolacyl transferase (DGAT) isolated from sesame (*Sesamum indicum* L.) into the soybean cultivar Dongnong 47, using *Agrobacterium*-mediated transformation to produce high-oil transgenic lines that are more suitable for breeding. After a 2-year single-location field trial, the transgenic soybean overexpressing *SiDGAT1* had increase seed oil content, by an average of over 1.0 percentage point, compared with wild-type plants. Additionally, the transgenic plants expressing *SiDGAT1* had significantly reduced protein and soluble sugar contents in mature seeds. *SiDGAT1*-overexpressing soybean exhibited an altered fatty acid composition, with increases in palmitic (C16:0) and linoleic (C18:2) acid contents and decreases in stearic (C18:0) and oleic (18:1) acid contents, but no major yield change. Thus, engineering the SiDGAT1 enzyme is an effective strategy to improve the oil content and value of soybean.

Keywords *SiDGAT1* · Overexpression · Soybean · Seed oil content · Yield

Introduction

Soybean (*Glycine max* (L.) Merrill) is an important oilseed crop worldwide that provides 27% of vegetable oil production (Chen et al. 2009). However, the oil content in soybean seeds (20%) is relatively low compared with most other oilseed crops, such as sesame (*Sesamum indicum* L.; 60%), even though they have a similar lipid biosynthetic pathway (Singh and Hymowitz 1999; Gupta 2008). This suggests that improving the soybean seed oil content through genetic engineering to regulate critical targets and control points in the lipid biosynthetic pathway could be effective (Savadi et al. 2017). Genetic engineering not only can complement conventional plant breeding methods to break yield barriers but also can precisely regulate trait expression in short periods of time without any lineage drag in crop plants (Savadi et al. 2017).

Seed oil is mainly composed of triacylglycerols (TAGs). There are two ways to regulate TAG biosynthesis: through substrate availability, allosteric effectors, and/or enzyme modifications or through the control of lipid-related enzyme synthesis and turnover rates. Diacylglycerolacyl transferase (DGAT) is the only

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enzyme solely committed to TAG formation, catalyzing diacylglycerol (DAG) from phospholipid synthesis into TAG (Yen et al. 2008; Courchesne et al. 2009). DGAT presents a key rate-limiting enzyme in the control of the flux toward TAG synthesis in the Kennedy pathway (Settlage et al. 1998; Cahoon et al. 2007; Courchesne et al. 2009; Abdullah et al. 2016). Furthermore, the key regulatory role of DGAT in TAG biosynthesis has been established (Guo et al. 2017; Abdullah et al. 2018). The overexpression of the *AtDGAT1* gene, driven by the Napin promoter, resulted in an increase of 11–29% in the oil contents of transgenic seeds of *Arabidopsis thaliana* (Jako et al. 2001). The overexpression of *AtDGAT1* and *Brassica napus DGAT1* resulted in seed oil content increases of up to 14% in transgenic *Arabidopsis* compared with the control plants (Weselake et al. 2008). Additionally, field trials of canola, rapeseed (Weselake et al. 2008; Taylor et al. 2009), maize (Zheng et al. 2008; Oakes et al. 2011), and soybean (Lardizabal et al. 2008; Roesler et al. 2016), significant seed oil improvements were achieved by the overexpression of *DGAT*.

In 2014, we isolated and identified *SiDGAT1*. The overexpression of *SiDGAT1* improved seed oil contents by 1.75 and 1.35 percentage points in T2 and T3 transgenic soybean lines, respectively, in a greenhouse (Wang et al. 2014). However, the receptor soybean cultivar used in that study was Dongnong 50, which has high genetic transformation efficiency, but the seeds are too small for soybean breeding applications. In this study, to breed high seed oil content soybean lines, *SiDGAT1* was overexpressed in soybean cultivar Dongnong 47, which is a high oil content cultivar, using the *Agrobacterium*-mediated cotyledonary node transformation method. Significant increases in seed oil content were achieved by overexpressing *SiDGAT1*, and a protein content decrease was also observed in two field trials. Moreover, other quality and agronomic traits of transgenic soybean were compared with wild-type soybean.

Materials and methods

Transformation and molecular characterization

To generate *SiDGAT1*-overexpressing transgenic soybean, the open reading frame of *SiDGAT1* was amplified using primer pair 5′-CGTAGATCTTTTAATGGCGATTTTGGAC-3′ and 5′-GTTGGTGACCGGGGTGTAGTATTCATTCCTC-3′ that contained *Bst*EII and *Bgl*II restriction enzyme sites, respectively. The amplified product was inserted downstream of the cauliflower mosaic virus (CaMV) 35S promoter of the pCAMBIA3301 vector, replacing the GUS-encoding gene *gusA*. The recombinant plasmid pCAMBIA3301-*SiDGAT1* was introduced into soybean "Dongnong 47" using an *Agrobacterium*-mediated transformation method described previously (Li et al. 2012a, b; Wang et al. 2014).

T3 generation plants were identified using PCR by selecting bar genes with the primer pair 5′-CCGGCAACAATTAATAGACT-3′ and 5′-TCCATAGTTGCCTGACTCCC-3′ (403 bp). T4 generation plants were identified using PCR tests by selecting bar genes and the 35S promoter with the primer pair 5′-TGTGATAACATGGTGGAGCAC-3′ and 5′-AAATCTCGGTGACGGGCA-3′ (1000 bp).

The T4 generation transgenic plants were further confirmed by Southern blot analysis. Genomic DNA (10 μg) from young leaves of homozygous T4 lines was digested with restriction enzymes *Eco*RI and *Hin*dIII, respectively at 37 °C overnight. Digested DNA was separated on 0.8% (*w*/*v*) agarose gels and blotted onto Hybond N^+ nylon membrane (Bio-Rad, Hercules, CA, USA) for hybridization with the DIG-labeled bar probe according to the protocol of the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Mannheim, Germany). SiDGAT1 protein expression was tested by western blot analysis in T4 generation plants. The proteins from young leaves were fractionated by dodecyl sulphate-polyarylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a Mighty Small II electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA, USA). The proteins were resolved on a slab gel (10 × 8 × 0.75 cm), consisting a 13.5% (*w*/*v*) separation gel and a 4% (*w*/*v*) stacking gel, and electroblotted onto pure nitrocellulose membrane (Midwest-Scientific, Valley Park, MO, USA). Immunoblot analysis was performed using SiDGAT1 protein antibodies made by Abmart Company (Shanghai).

Field trial methods

The T3 and T4 generation transgenic lines "4-1," "4-3," "4-4," and "74-3" that overexpress *SiDGAT1* and the "Dongnong 47" soybean (WT) were planted under natural conditions at the Transgenic Experimental Field of Northeast Agricultural University (45° 45′ 06″ N, 126° 43′ 21″ E), Harbin, China in 2017 and 2018. The experiment had a

completely randomized block design with three replicates, and plants were planted in rows 3-m long and 0.65-m apart, with 6 cm between plants. Sufficient nutrition and water were supplied throughout the field to avoid potential nutrient and drought stresses. In October, whole seeds were harvested from the plants and air-dried.

In each independent experiment, 10 WT plants and 10 of each transgenic line were randomly sampled at the mature stage. Plant height, number of primary branches, number of primary internode, pod number, seed number per plant, seed weight per plant, and 100-seed weight were measured. Three independent experiments were performed.

The analyses of seed oil and protein contents

Seeds from four transgenic lines and WT were harvested in October. The seed oil content of soybean was analyzed using the Soxhlet extraction method (Tan et al. 2011; Wang et al. 2014). The seed protein content was analyzed using the Kjeldahl method (Leng et al. 2007). The experiments were performed in triplicate, and mean values were used to calculate the oil and protein contents.

Fatty acid composition

Soybean seed powder (0.1 g) was used to analyze the fatty acid composition by gas chromatography (GC-14C, Shimadzu Company, Japan). The fatty acid extraction and analysis were performed according to the method of Zhang et al. (2008). The test columns all had nominal dimensions of 30 m × 0.125 m with a 0.13-μm film thickness. Operating conditions were as follows: carrier, hydric (400 ml/min) split injection, injection temperature 250 °C, detector temperature 250 °C, and column temperature 210 °C (Panthee et al. 2006; Xie et al. 2012). The experiments were performed in triplicate.

Soluble sugar contents

The soluble sugar was measured using anthrone colorimetry (Li et al. 2012a, b). In total, 50 mg soybean powder was used to analyze the soluble sugar contents. The experiments were performed in triplicate.

Statistical analysis

The statistical data analyses were performed using the IBM SPSS Statistics 19 (IBM, Armonk, New York, USA). The significant differences were determined using paired Student's *t* tests, and data were presented as means ± standard deviations. Values of $p < 0.05$ were considered to be statistically significant.

Results

Generation of the transgenic soybean overexpressing SiDGAT1

Transgenic soybean plants overexpressing *SiDGAT1*, driven by the CaMV 35S promoter, were generated by an *Agrobacterium*-mediated soybean cotyledonary node transformation system. Genomic DNA was isolated from WT and different transgenic plants. The PCR analysis was performed using bar gene-specific primers and Bar+35S promoter-specific primers (Fig. 1a, b). After the PCR analyses of T3 and T4 generation transgenic plants, four independent transgenic lines were obtained. These transgenic lines were further confirmed by Southern blot analysis. The Southern blot data indicated that each of the four transgenic lines contained a single insertion, and a similar single-band pattern was observed in all lines (Fig. 2a). To verify the expression of *SiDGAT1* in these transgenic lines, total leaf proteins from the four transgenic lines and WT were separated by SDS-PAGE, and the western blot analysis was performed using antibodies raised against SiDGAT1 (Fig. 2b). The antibody recognized a protein of ~55 kDa from the transgenic soybean leaf, which is similar to the molecular weight of SiDGAT1, while WT produced no reaction. Thus, the data indicated that *SiDGAT1* had been introduced into the WT genome, and the SiDGAT1 protein was expressed in transgenic plants. Transgenic lines 4-1, 4-3, 4-4, and 74-3 were chosen for the phenotype analysis.

Overexpression of SiDGAT1 significantly increases seed oil content

To determine if the overexpression of *SiDGAT1* led to a greater accumulation of seed oil in transgenic soybean, the seed oil contents of four independent transgenic lines were analyzed using the Soxhlet extraction method in 2017 and 2018. An increase in the seed oil content was consistently observed across two generations in a field environment (Fig. 3). In particular, in transgenic lines 4-1 and 4-4, the seed oil content increased by at least 1.0 percentage point

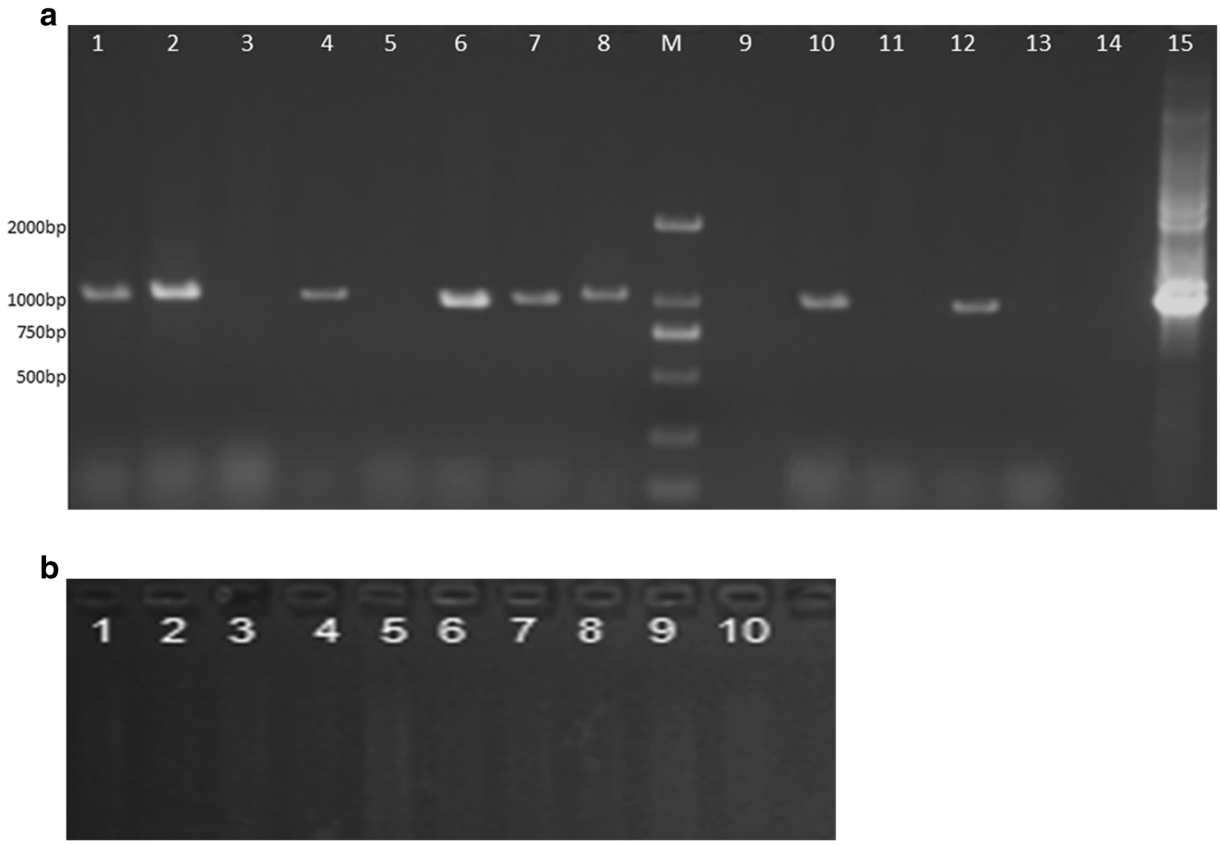

Fig. 1 Identification of transgenic soybean overexpressing *SiDGAT1* by PCR analysis. **a** PCR analysis of T3 generation transgenic lines for the *bar* gene and 35S promoter. M: DL2000 ladder, 15: positive control (plasmid as template), 14: negative control (H_2O as template), 13: negative control ("Dongnong 47" DNA as template), 1–12: transgenic plants. **b** PCR analysis of T4 generation transgenic lines for the bar gene. M: DL2000 ladder, 11: positive control (plasmid as template), 13: negative control (H_2O as template), 12: negative control ("Dongnong 47" DNA as template), 1–10: transgenic plants

compared with WT in the 2017 and 2018 seasons, indicating that this elevated level of seed oil content was retained in the progeny of these transgenic soybean plants. To determine whether *SiDGAT1* overexpression would affect fatty acid composition, we compared the fatty acid profiles of the seeds from four transgenic lines with those from WT. As shown in Fig. 4, there were significant increases in palmitic (C16:0) and linoleic (C18:2) acid contents and decreases in stearic (C18:0) and oleic (18:1) acid contents compared with WT. Thus, the overexpression of *SiDGAT1* changed the fatty acid composition of transgenic soybean seeds.

Overexpression of SiDGAT1 significantly decreases the seed protein content

With the increase in the seed oil content, a major impact on the protein level was observed in transgenic seeds. *SiDGAT1*'s overexpression decreased the protein content in transgenic soybean seeds compared with WT (Fig. 5). The decrease in the seed protein content in transgenic lines 4-1, 4-3, and 4-4 were statistically significant ($p < 0.01$). Thus, the increase in the oil content associated with this transgene produced a correlated decrease in the protein content, in contrast to results obtained from traditional breeding (Burton 1984; Wilcox 1998).

Overexpression of SiDGAT1 significantly changes seed total soluble sugar content

Mature seeds harvested from the field were subjected to an analysis of the soluble sugar contents. As shown in Fig. 6, the total soluble sugar content decreased significantly ($p < 0.01$) in the four transgenic lines compared with WT. The greatest reduction occurred in line 4-1, which decreased 6.63 percentage points (43.14%). Therefore, we

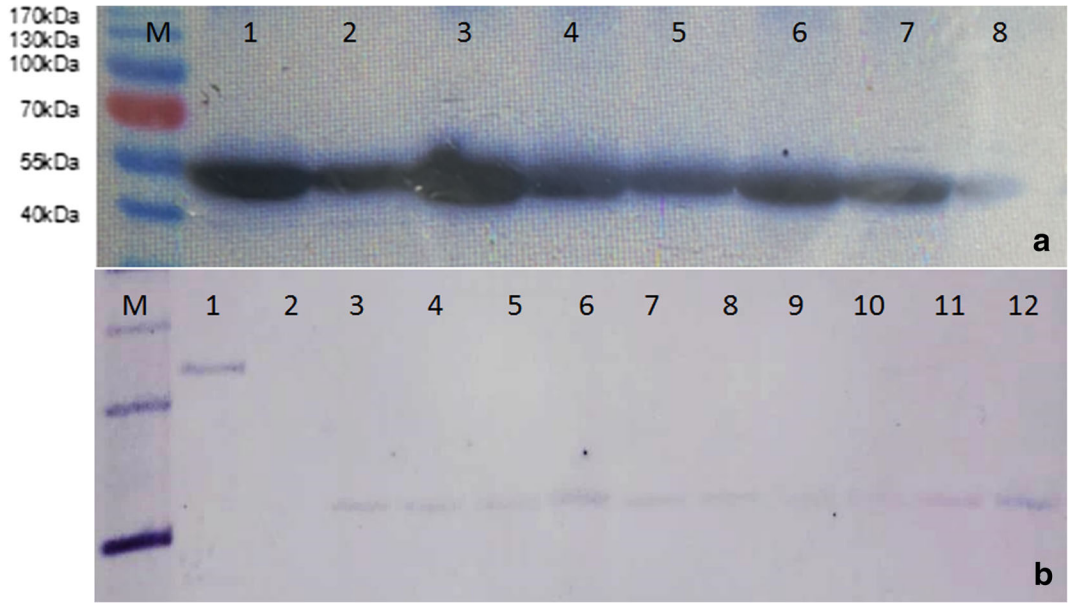

Fig. 2 Southern and western blot analyses of transgenic soybeans overexpressing *SiDGAT1*. **a** Genomic DNA isolated from leaves of wild type and T4 transgenic lines 4-1, 4-2, 4-4, and 74-3 that had been digested with *Eco*RI or *Hin*dIII and separated by agarose gel electrophoresis. M: marker, 1: positive control (plasmid DNA), 2: negative control ("Dongnong 47" DNA), 3–6: DNA from lines 4-1, 4-3, 4-4, and 74-3 digested by *Eco*RI. 7–12: genomic DNA from lines 4-1, 4-3, 4-4, and 74-3 digested by *Hin*dIII. **b** Total leaf proteins from wild type and T4 transgenic lines (4-1, 4-2, 4-4, and 74-3) fractionated by 13.5% sodium dodecylsulphate-polyarylamide gel electrophoresis and transferred to nitrocellulose membranes. M: markers. 8: negative control. 1–7: transgenic plants

speculated that the significant improvement in the seed oil content in overexpressing *SiDGAT1* soybean was accounted for by the concomitant reductions in the soluble sugar and protein contents.

Field trials

To determine whether the overexpression of *SiDGAT1* resulted in growth changes in the transgenic plants, the four *SiDGAT1* overexpression lines were grown at the Transgenic Experimental Field of Northeast Agricultural University for 2 years, as was the WT. The data analyses (Table 1) showed that *SiDGAT1* overexpression changed the agronomic traits of the transgenic plants. For example, overexpression line 4-1 had a statistically shorter plant height ($p < 0.05$), but a greater number of primary branches ($p < 0.01$) and seed number per plant ($p < 0.05$), as well as a greater seed weight per plant ($p < 0.05$), compared with

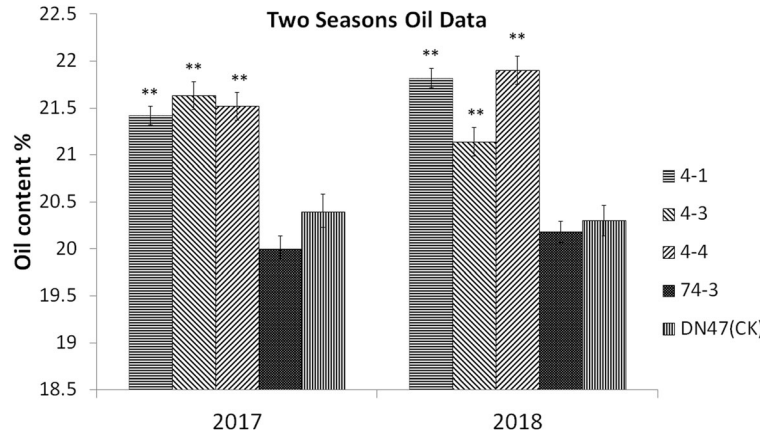

Fig. 3 Seed oil contents of four *SiDGAT1*-overexpressing transgenic and wild type plants over two seasons. The data represent the Means ± standard deviations of three replicate experiments, and the values are in dry weight (DW) for seeds. ** indicates significant differences compared with the wild type as determined by Student's *t* test ($p < 0.01$)

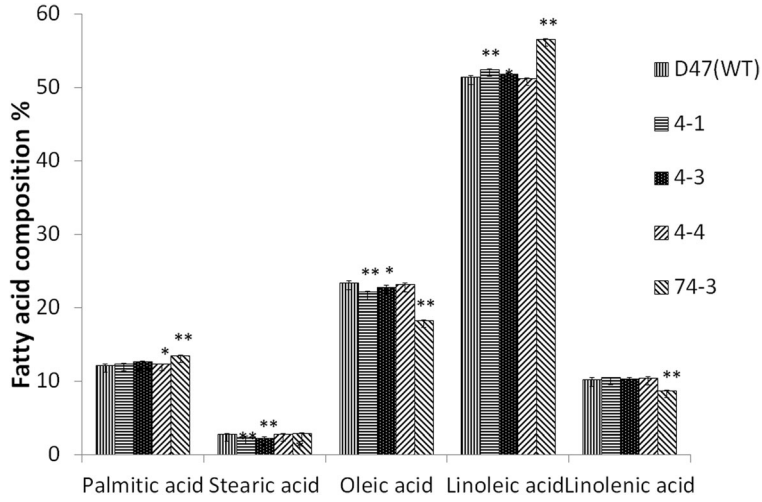

Fig. 4 Seed fatty acid composition of *SiDGAT1*-overexpressing transgenic and wild-type plants. The data represent the means ± standard deviations of three replicate experiments, and the values are in dry weight (DW) for seeds. Asterisk (*) indicates significant differences compared with the wild type as determined by Student's *t* test ($p < 0.05$)

WT. The number of primary branches per plant was significantly increased in all transgenic lines compared with WT plants. Seed weight per plant in the transgenic lines had also increased, but it was not statistically significant. Other yield traits did not show statistical changes in comparison with those of WT.

Discussion

DGAT is a very important rate-timing enzyme in the Kennedy pathway. Enhancing DGAT's activity could improve the carbon flux toward the synthesis of seed-storage lipids (Jako et al. 2001; Taylor et al. 2009). Additionally, the genetic manipulation of *DGAT* is a promising option for breaking the yield barriers of oilseed crops (Savadi et al. 2017; Wang et al. 2014; Xu et al. 2008; Lardizabal et al. 2008).

Our previous studies have demonstrated that the expression of *SiDGAT1* cDNA driven by the CAMV 35S promoter enhanced the seed oil content in transgenic *Arabidopsis* and soybean grown in a greenhouse (Wang et al. 2014). In this study, we replaced the receptor soybean cultivar Dongnong 50 with Dongnong 47 because the 100-seed weight of the former is just 10.0–11.0 g, which is too small for soybean breeding. The overexpression *SiDGAT1* in the Dongnong 47 cultivar also resulted in a significant increase in the transgenic seed oil content. However, the increased oil content in Dongnong 47 is less than that in Dongnong 50. This might be because Dongnong 50 is a low-oil soybean cultivar that has a seed oil content of just 17.54% when

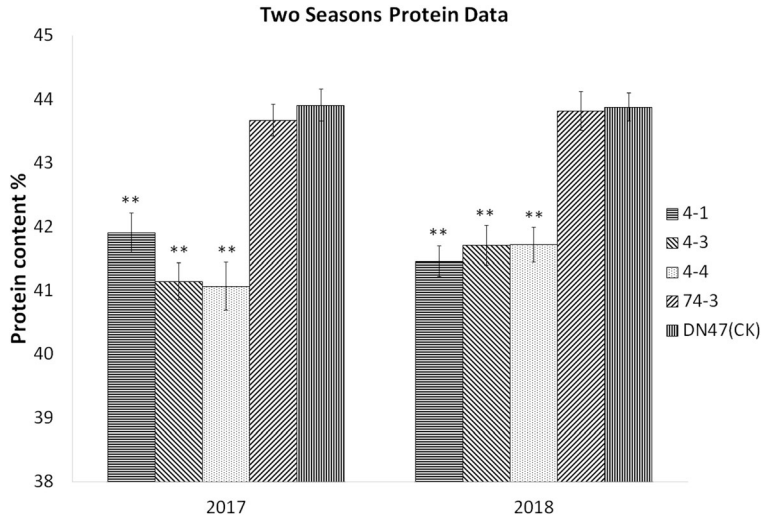

Fig. 5 Seed protein contents of four *SiDGAT1*-overexpressing transgenic and wild-type plants over two seasons. The data represent the means ± standard deviations of three replicate experiments, and the values are in dry weight (DW) for seeds. Double asterisks (**) indicates significant differences compared with the wild type as determined by Student's *t* test ($p < 0.01$)

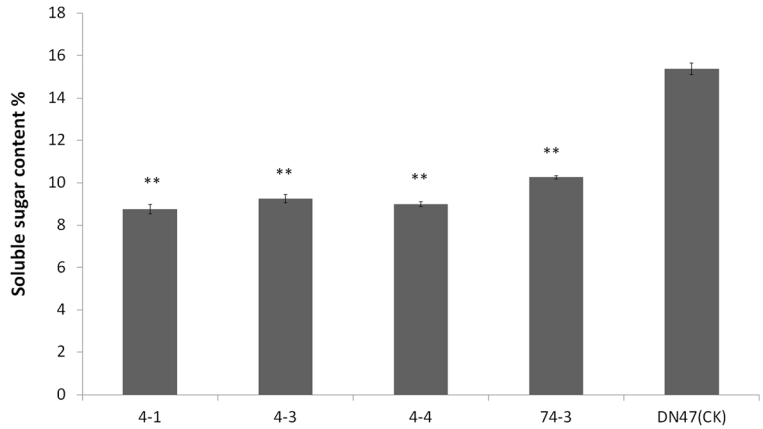

Fig. 6 Seed soluble sugar contents of four *SiDGAT1*-overexpressing transgenic and wild-type plants. The data represent the means ± standard deviations of three replicate experiments, and the values are in dry weight (DW) for seeds. Double asterisks (**) indicates significant differences compared with the wild type as determined by Student's *t* test ($p < 0.01$)

grown in the Transgenic Experimental Field of Northeast Agricultural University in 2018. We speculated that the very large increase in the seed oil content of high-oil soybean cultivars might be prevented owing to a "bottleneck," such as substrate concentration, that likely limits the increase. The overexpression of *DGAT1* changes not only the enzyme activity, but also the entire substrate–enzyme–product relationship (Taylor et al. 2009). Additionally, this experiment was conducted in a field, while the previous study was conducted in a greenhouse. The oil content is highly influenced by the environmental conditions (Canvin 1965; Hocking et al. 1997; Fayyaz-ul-Hassan et al. 2005). These two factors may explain why the increase rate in the oil content in Dongnong 47 is less than that in Dongnong 50.

The overexpression of *SiDGAT1* in Dongnong 47 increased the palmitic acid (C16:0) and linoleic acid (C18:2) contents and decreased stearic acid (C18:0) and oleic acid (18:1) contents in this study, in marked contrast to that of Roesler et al. (2016). The discrepancies could be due to the *DGAT1* from different species. DGAT1 from different species may have the different substrate specificities, and the selectivity of *DGAT1* genes varies (Weselake et al. 1991), such as the *DGAT1* from *B. napus*, which prefers 16:0-ACP and very long chain fatty acids, while the *DGAT1* of *Obroma cacao* prefers 18:0-ACP (Giffith and Harwood 1991; Li et al. 2012a, b). DGAT in *Arabidopsis* has preference for 20:1-CoA, instead of 18:2-CoA and 18:1-CoA (Katavic et al. 1995; Jako et al. 2001). SiDGAT1 comes from sesame, and modified GmDGAT1b comes from soybean. DGAT1 from different species has different enzymatic preferences for substrates during seed development. Hence, DGAT has effects on both oil quantity and quality (Savadi et al. 2016).

With the overexpression of *SiDGAT1* leading to a greater than 1.0 percentage point increase in seed oil content, the seed protein and soluble sugar contents suffered significant negative impacts, similar to previous results (Zhang et al. 2005; Savadi et al. 2016; Roesler et al. 2016). Zhang et al. (2005) found that the RNAi-mediated silencing of *AtDGAT1* in tobacco led to a reduction in the

Table 1 Agronomic performance of overexpressing *SiDGAT1* lines and wild-type plants in field

Genotype	Plant height cm)	Number of primary internode	Number of primary branches	Pod number per plant	Seed number per plant	Seed weight per plant (g)	Seed weight per 100 seeds (g)
4-1	75.56 ± 1.64*	16.76 ± 0.97	2.44 ± 1.45**	33.04 ± 11.78	93.92 ± 30.61*	19.99 ± 5.99*	21.67 ± 0.73
4-3	79.33 ± 5.30	17.00 ± 0.71	2.11 ± 1.03**	33.35 ± 3.73	86.21 ± 17.70	18.49 ± 3.71	21.75 ± 0.69
4-4	73.53 ± 1.79*	16.53 ± 0.99	1.60 ± 0.87	28.47 ± 6.89	59.80 ± 23.49	13.64 ± 3.83	23.67 ± 2.42
74-3	84.13 ± 1.47	19.60 ± 0.34	1.13 ± 1.02	46.07 ± 5.22*	70.13 ± 3.19	15.38 ± 0.22	22.14 ± 0.93
DN47(wild type)	81.20 ± 5.51	18.00 ± 2.00	0.60 ± 0.69	33.80 ± 8.61	64.20 ± 20.66	14.37 ± 3.53	22.98 ± 2.24

Values are means ± SD of three replicates. Ten plants were measured in each independent measurement. Asterisks (*) indicates significantly different from the wild type as determined by Student's *t* test ($p < 0.05$)

seed oil content, but increases in protein and sugar contents. Here, the decreases in the protein and soluble sugar contents could have contributed to the increase in the oil contents in transgenic soybean seeds. During seed maturation, seed storage compounds, like starch, oil, and protein, are formed from photosynthates derived from the silique wall and leaves (Meyer and Kinney 2010). Thus, the flow of carbon for TAG synthesis in seed is influence by other metabolic pathways. The overexpression of *SiDGAT1* gene in transgenic soybeans increased the assembly of glycerides, and at the same time formed effective competition with other metabolic pathways, such as protein biosynthesis and glucose metabolism, resulting in reduced protein and soluble sugar content in the seeds of transgenic soybeans. And, the reduction in soluble sugars and protein further suggests that seed oil accumulation in *SiDGAT1-ox* plants is limited by source strength. Additionally, there is a negative correlation between protein and oil contents in soybean (Fillo et al. 2001).

DGAT1's overexpression did not increase the transgenic soybean yield or 100-seed weight, contrary to previous studies in which the overexpression of *DGAT*s improved seed weights (Zou et al. 1997; Jako et al. 2001; Vigeolas et al. 2007; Wang et al. 2014). In this study, the 100-seed weight of *SiDGAT1*-overexpression lines did change compared with WT. There was also a small increase in seed weight per transgenic plant, but it was not always statistically significant. Savadi et al. (2016) found no correlation between seed weight and oil content. Thus, breeding for increased yield would not necessarily lead to the overexpression of *DGAT*, because the high energy content of oil means that a strong positive correlation between yield and oil content is unlikely (Roesler et al. 2016). Additionally, the results from greenhouse experiments are always different compared with those conducted in the natural environment.

The most important finding of this study is that the *SiDGAT1*-overexpression lines exhibited consistent increases in seed oil contents compared with the WT. Furthermore, the enhanced-seed oil content trend remained consistent. The overexpression of *SiDGAT1* can be used to enhance the seed oil content in the Dongnong 47 cultivar, and transgenic soybean plants generated in the study may be used for farming in the future.

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