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Overexpression of Soybean Transcription Factors *GmDof4* and *GmDof11* Significantly Increase the Oleic Acid Content in Seed of *Brassica napus* L.

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Abstract: Rapeseed (*Brassica napus* L.) with substantial lipid and oleic acid content is of great interest to rapeseed breeders. Overexpression of *Glycine max* transcription factors *Dof4* and *Dof11* increased lipid accumulation in *Arabidopsis* and microalgae, in addition to modifying the quantity of certain fatty acid components. Here, we report the involvement of *GmDof4* and *GmDof11* in regulating fatty acid composition in rapeseeds. Overexpression of *GmDof4* and *GmDof11* in rapeseed increased oleic acid content and reduced linoleic acid and linolenic acid. Both qPCR and the yeast one-hybrid assay indicated that GmDof4 activated the expression of *FAB2* by directly binding to the *cis*-DNA element on its promoters, while *GmDof11* directly inhibited the expression of *FAD2*. Thus, *GmDof4* and *GmDof11* might modify the oleic acid content in rapeseed by directly regulating the genes that are associated with fatty acid biosynthesis.

Keywords: *Brassica napus*; *GmDof4*; *GmDof11*; oleic acid; fatty acid composition

1. Introduction

Rapeseed (*Brassica napus* L.) is among the most important oil crops worldwide, providing high-quality edible oils and industrial raw materials [1–3]. The production and yield of rapeseed has rapidly increased in China in recent years [4]. Rapeseed oil is principally a mixture of seven main fatty acids [5], namely palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), eicosenoic acid ($C_{20:1}$), and erucic acid ($C_{22:1}$), of which oleic acid is the most abundant and has the highest nutritional value [6]. Therefore, creating new rapeseed varieties with a high seed oil content that is rich in oleic acid content is a primary goal for rapeseed breeders [7]. Remarkable progress in increasing the content of seed oil and proportion of oleic acid has been reported by traditional breeding and putative candidate genes have been dissected using quantitative trait loci mapping and molecular markers [8–11].

Genetic engineering is a potentially efficient method of modifying the expression of single or multiple genes that are involved in lipid metabolism [7,12]. In *B. napus*, the overexpression of genes encoding glycerol-3-phosphate dehydrogenase [13], acyl-CoA: lysophosphatidic acid acyltransferase [14], mitochondrial pyruvate dehydrogenase kinase [15], and diacylglycerol acyltransferases [16–19] significantly increased seed oil content. Liu et al. [20] overexpressed triacylglyceride (TAG) synthesis pathway genes in *B. napus*, including *BnGPDH*, *BnGPAT*, *BnDGAT*, and *ScLPAAT*, and found that the overexpression of a single gene could increase the content of seed oil, but the simultaneous overexpression of multiple genes may result in more substantial changes in oil composition.

Besides lipid synthase, a number of genes encoding seed-specific transcription factors (TFs) have been shown to play important roles in the regulation of lipid biosynthesis [7,21]. Previous reports have suggested that altering the expression levels of the plant-specific B3 domain family members LEAFY COTYLEDON 2, FUSCA3 and ABSCISIC ACID INSENSITIVE 3 [22–24]; NF-YB type TF LEAFY COTYLEDON 1 [7,25]; AP2/EREB domain TF WRI1 [26,27], *Arabidopsis* 6b-interacting protein 1-like 1 [28]; BnGRF2 (GRF2-like gene from *B. napus*) [21]; and, SHOOTMERISTEMLESS [29] resulted in a change in the proportions of seed storage materials. These genes could be used to genetically improve the oil content and composition of rapeseed.

Fatty acid dehydrogenase (FAD) and fatty acid elongase (FAE) are the key enzymes that determine fatty acid composition in seed oil. FAD catalyzes the biosynthesis of polyunsaturated fatty acids, such as linoleic and linolenic acid [30,31], while FAE catalyzes the chain elongation reaction, resulting in the formation of long-chain fatty acids, including eicosenoic and erucic acid [30,32]. Previous studies have suggested that inhibition of *FAE1* expression increases oleic acid and reduces erucic acid content in rapeseed seed oil [33,34], as did inhibition the expression of the *BnFAD2* gene in transgenic seeds [34]. Jung et al. [35] found that expression of the *B. rapa BrFAD2* gene in an antisense orientation increased the synthesis of oleic acid in *B. napus*. FAD3 desaturase is responsible for the synthesis of linolenic acid [36], in *BnFAD3* mutants of *B. napus*, the concentration of linolenic acid was significantly reduced [37].

Dof (DNA binding with one finger) is an important family of TFs in plants, with its members being widely involved in seed development, plant growth, morphogenesis, nutrient metabolism, and other processes [36,38–40]. As far as we know, comprehensive analysis of Dof family factors in *B. napus* has not been previously performed, with few reports of the function of *Dof* genes in *B. napus* [41], even though genome-wide analysis has been performed in other *Brassica* plants [42]. In soybean, 28 Dof members have been identified [43], and eight of them, including *GmDof4* and *GmDof11*, are strongly expressed in the flowers and pods of soybean. Wang et al. [44] found that fatty acid and seed oil content, and seed weight were significantly increased in *GmDof4* and *GmDof11* overexpressing lines of *A. thaliana*. Further studies showed that *GmDof4* and *GmDof11* directly downregulated the expression of the seed storage protein gene *CRA1*. Moreover, *GmDof4* and *GmDof11* have been shown to induce the expression of the β-subunit of the ACCase encoding gene *acetyl CoA carboxylase* (*accD*) and *long-chain-CoA synthetase gene 5* (*LACS5*), respectively [44]. These results indicate that *GmDof4* and *GmDof11* can simultaneously increase seed oil content by upregulating genes that are involved in fatty acid synthesis and downregulating genes associated with the accumulation of seed protein in Arabidopsis. In addition, increased lipid accumulation was demonstrated after heterologous expression of *GmDof4* in *Chlorella ellipsoidea* [45], indicating that *GmDof4* regulates seed oil content and composition both in higher and lower plants.

In the current study, using the rapeseed cultivar 'Yangyou 6' as receptor, we created *GmDof4* and *GmDof11* overexpression *B. napus* lines via *Agrobacterium*-mediated transformation. 'Yangyou 6' is a double low variety, which is widely planted in the Jiangsu province of China. Our results demonstrated that, when compared with non-transgenic lines, the content of oleic acid in the transgenic lines increased significantly, whereas the content of linoleic acid and linolenic acid were reduced. We found that *GmDof4* and *GmDof11* could activate or inhibit genes that are involved in fatty acid synthesis by directly binding to promoter regions. These findings indicate that *GmDof4* and *GmDof11* have the potential to improve the quality of rapeseed oil.

2. Materials and Methods

2.1. Plant Growth and Transformation

B. napus cv. 'Yangyou 6' plants were grown at 24 °C using a 16-h photoperiod in a growth chamber. *GmDof4* (Accession No: DQ857254) and *GmDof11* (Accession No: DQ857261) DNA sequences were cloned using the primer pairs: GmDof4-F: GACGCACTCACTGACATCAACACTAG, GmDof4-R:

GGTGAGATAAGATTTAGAAGAGGCGTG, and GmDof11-F: GAGACTTCGCAATTTGCATGACTC, GmDof11-R: CTAGCTACTGCTAGAGTGAAGTCATTG, respectively, as designed by Wang, et al. (2007) [44]. The Soybean cultivar 8904 was used for cloning *GmDof*. The overexpression vectors pBIN438-*GmDof4* and pBIN438-*GmDof11* were kindly gifted by Professor Shouyi Chen (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The vectors contained a *neomycin phosphate transferase II* (*NPT II*) gene as a selection marker. The *GmDof4* and *GmDof11* genes were driven using a CaMV 35S promoter. The vectors were introduced into the *Agrobacterium tumefaciens* strain GV3101 for genetic transformation into *B. napus*.

GmDof4 and *GmDof11* overexpressing lines of *B. napus* were generated as described by De Block et al. [46] with some modifications. Certified, uniform, and healthy seeds were surface-sterilized with sodium hypochlorite solution and then rinsed in sterile distilled water. The seeds were germinated in the dark on 1/2 MS basal medium containing 2% (*w*/*v*) sucrose. Seven-day-old hypocotyl explants (~15 mm) were prepared and cultured on co-cultivation medium [MS medium supplemented with 2% (*w*/*v*) sucrose, 1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), and 1 mg/L benzyladenine (BA); pH 5.8] for three days. Explants were then transferred to selection medium [MS medium supplemented with 2% (*w*/*v*) sucrose, 1 mg/L 2,4-D, 1 mg/L BA, 300 mg/L cephalosporin (Cef) and 30 mg/L G418; pH 5.8] and incubated at 25 °C. The explants with shoot initials were transferred to shoot outgrowth medium [MS medium supplemented with 2% (*w*/*v*) sucrose, 0.3 mg/L NAA, and 300 mg/L Cef; pH 5.8]. Finally, green shoots were transferred to root initiation medium [MS medium supplemented with 2% (*w*/*v*) sucrose, 0.3 mg/L NAA, and 300 mg/L Cef; pH 5.8]. All of the regenerated plantlets were transferred into pot containing nutritious soil after becoming fully developed.

2.2. PCR, Semi-Quantitative, and Quantitative Real-Time PCR Analyses

Total DNA was extracted from the young leaves of each transgenic plant using the CTAB method, as described by Porebski et al. [47]. PCR was performed to identify positive transformants using specific primers.

Total RNA was extracted from non-transgenic and *GmDof*-overexpression seedlings, and the young seeds of *B. napus* using an RNA isolator (Vazyme, Nanjing, China) in accordance with the manufacturer's instructions. First-strand cDNA synthesis was performed using the first-strand cDNA synthesis kit HiScript Q RT SuperMix with oligo$(dT)_{23}$ (50 μM) and Random hexamers (50 ng/μL) as primers for semi-quantitative PCR analysis (Vazyme, Nanjing, China). T_2 generation seeds were collected at 30 days after flowering (DAF), from which 30 seeds were randomly selected and used for RNA extraction.

Semi-quantitative PCR was performed, as follows: 95 °C for 3 min then 32 cycles of 95 °C for 30 s, annealing (56 or 58 °C; detailed information shown in Supplemental Table S1) for 30 s, polymerization at 72 °C for 30 s, followed by 72 °C for 5 min. Real-time PCR was performed in an Mx3500p (Agilent, Santa Clara, CA, USA) using FastStart Universal SYBR Green Master (Rox) (Roche Applied Science, Penzberg, Germany). *BnActin* transcripts were used as the internal reference [18,22]. Relative gene expression was calculated using the$2^{-\Delta\Delta Ct}$ method. qPCR was performed with three biological replicates and three technical replicates for every sample.

To identify whether the expression of genes related to fatty acid metabolism in transgenic seeds, namely, *FAB2* (*Fatty acid biosynthesis 2*), *FAD2*, *FAD3*, *FAD6*, *FAD7*, *FAD8*, *FAE1*, and *FAE7*, were regulated, the expression pattern of genes involved in lipid and fatty acid synthesis were analyzed in seeds at 30 DAF. Amplification primers of these genes were designed to amplify all homologous of specific gene. The primers used for qPCR and the gene ID are listed in Supplementary Table S1.

2.3. Determination of Seed Oil and Fatty Acid Composition

Total seed oil content of the transgenic and non-transgenic plants was determined using near-infrared reflectance (NIR) spectroscopy [48]. Fatty acid concentration was measured using the method that was described by Taylor et al. [49]. The seeds of transgenic (T_2 generation) and

non-transgenic plants were ground to a fine powder in a mortar. Five mL of *iso*-propanol were mixed with 0.5 g seed powder and incubated at 100 °C for 5 min. The solution was immediately cooled on ice and 2.5 mL of dichloromethane added. The samples were shaken at 200 rpm for 30 min at room temperature after which, 4 mL of dichloromethane and 4 mL of 1 mol/L KCl in 0.2 mol/L H_3PO_4 were sequentially added into each tube to separate the organic and aqueous phases. The samples were vortexed and centrifuged at 2000 rpm for 20 min. The supernatant was washed twice with 4 mL of dichloromethane, and the original organic phase combined with the washes and dried under nitrogen to yield triacylglycerol. The triacylglycerol was hydrolyzed and the fatty acid esterified, as described by Fatima et al. [50].

Fatty acid composition was analyzed using a gas chromatography-mass spectrometer (Trace GC DSQII, Thermo, Waltham, MA, USA) with a DB-WAX capillary column (30 m × 0.25 mm ID × 0.25 μm df) [49]. The peaks were identified by reference to the identified retention times of internal standard FAMEs (Sigma, Lot No.: 18919-1AMP, St. Louis, MO, USA). GC was performed using a gas carrier (helium) flow rate of 30 mL·min^{-1} and a column and injector temperature of 250 °C. Running temperatures were as follows: 50 °C for 2 min, increasing to 220 °C at a rate of 4 °C/min, and held at 220 °C for 7 min. Each experimental material was biologically replicated three times.

2.4. Detection of DNA Binding Specificity of GmDof4 and GmDof11 by Yeast One-Hybrid Assay

The yeast strain, Y1HGold (*MATα*, *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4*Δ, *gal80*Δ, *met-*, and *MEL1*) containing the AbA^r reporter gene was used as the assay system. *GmDof4* and *GmDof11* were amplified and fused to the GAL4 DNA binding domain on the pGADT7 plasmid. Two or three copies of the *cis*-DNA elements of interest at the promoter of potential targeted genes were synthesized, annealed, and cloned into the "prey" plasmid pAbAi. Then, the recombinant "prey" plasmid was then digested using *Bst*BI for 1 h and transfected into yeast Y1HGold cells according to the manufacturer's protocol (Clontech, Mountain View, CA, USA). The PCR-identified recombinant Y1HGold strains were then used to introduce pGADT7-*GmDof4* or pGADT7-*GmDof11* plasmids. The transfected yeast cells were then cultured in SD/-Leu/-Ura plates. Finally, cultures were placed on SD/-Leu/-Ura + AbA (0.2 mg/L) plates. Strains growing in colonies indicated positive *GmDof* binding on the corresponding *cis*-DNA element.

2.5. Statistical Analysis

All experimental data, including seed oil and fatty acid content analysis, were compared statistically using one-way analysis of variance (ANOVA) followed by Student's *t* test to determine significant differences among the means of different groups using Statistical Product and Service Solutions (SPSS) v16.0 software.

3. Results

3.1. Dof Family Numbers in B. napus

Based on the *Arabidopsis* annotated *Dof* genes, 134 homologous genes of *AtDof* were identified using BlastP (E-value $\leq 1 \times 10^{-5}$, identity $\geq$50% and coverage $\geq$50%) in the *B. napus* reference genome of Darmor-bzh [51] (Supplementary Table S3). BlastP results showed no homology of either *GmDof4* or *GmDof11* in the *B. napus* reference genome using the DNA sequences of *GmDof4* and *GmDof11* as query terms (results not shown).

3.2. Generation and Identification of B. napus Transgenic Plants

To investigate whether *GmDof4* and *GmDof11* could regulate lipid biosynthesis in rapeseed, they were transfected into rapeseed plants, under the control of the CaMV 35S promoter, while using the *Agrobacterium*-mediated method. *B. napus* L. cultivar "Yangyou 6" was used as the receptor and hypocotyl explants were prepared from seven-day-old seedlings. Each experiment was performed

using approximately 650 explants. Twenty and 12 rooted plantlets were obtained for *GmDof4* and *GmDof11* transformation, respectively. The existents of *GmDof4* and *GmDof11* in plantlets were identified using PCR (Figures S1 and S2), with the putative transformants then transferred into nutritious soil and placed in a green house. The RNA of the plantlets was extracted and first-strand cDNA synthesized, with the expression of *GmDof4* and *GmDof11* genes in individual transgenic plants detected by semi-quantitative PCR (Figure 1). Finally, seven and five overexpression lines of *GmDof4* and *GmDof11* were obtained, respectively. Two *GmDof4* transformants (*DOF4-2* and *DOF4-20*) demonstrated no *GmDof4* expression, and the expression of *GmDof11* in the *DOF11-12* transformant was very low. Based on the expression levels of *GmDof4* and *GmDof11*, the transgenic plants *DOF4-9*, *DOF4-13*, *DOF11-1*, and *DOF11-6* were further analyzed. When compared with the non-transgenic plants, no significant difference was observed in their growth and development. The presence of the *GmDof* transgene in T_1 generation transgenic plants was confirmed by PCR.

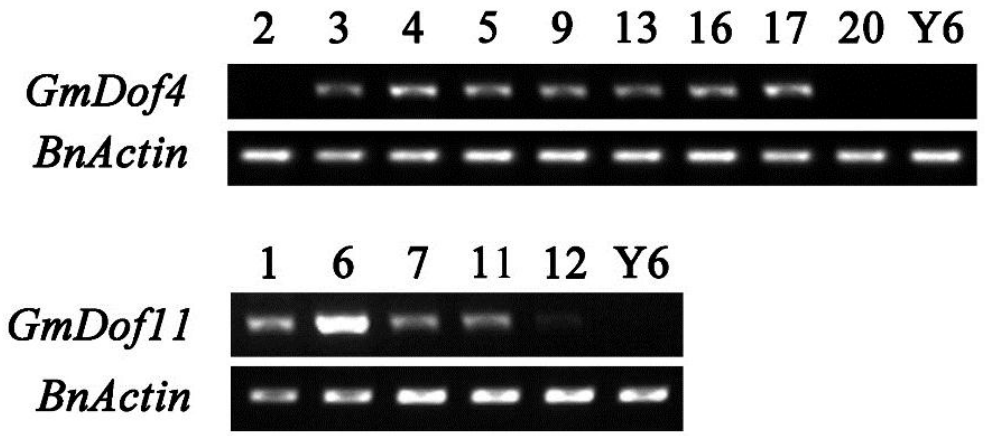

Figure 1. Semi-quantitative RT-PCR analysis of *GmDof4* and *GmDof11* transgenic plants. *BnActin* expression is displayed as the internal control. The lengths of the amplification products of *BnActin*, *GmDof4* and *GmDof11* were 144 bp, 135 bp and 186 bp respectively.

3.3. Changes in the Fatty Acid Composition of GmDof4 and GmDof11 Overexpressing Lines of B. napus

The relative content of the principal fatty acids in the seeds of *GmDof* transgenic and non-transgenic lines was analyzed using gas chromatography (GC). The T_1 and T_2 progenies of *DOF4-9*, *DOF4-13*, *DOF11-1*, and *DOF11-6* lines were produced by self-pollination. The seeds of homozygous T_1 lines that had no gene segregation were used for the fatty acid content determination. The results showed that the quantities of major unsaturated fatty acids, such as oleic, linoleic, and linolenic acid, underwent significant alteration in four transgenic lines compared with the non-transgenic plants. However, the content of the two principal saturated fatty acids, namely, palmitic acid and stearic acid, were consistent with those of the non-transgenic lines (Figure 2, Supplementary Table S2). Among the fatty acids, the content of oleic acid in four overexpression lines was significantly increased, from 62.8% in the non-transgenic rapeseed to 67.11–71.32%. Conversely, the content of linoleic and linolenic acid in the four overexpression lines was significantly lower than in the non-transgenic lines. In addition, total lipid content was measured in the seeds of the overexpression and non-transgenic plants by NIR. Seed oil content of the *GmDof4* and *GmDof11* overexpression lines was ~39%, being not significantly different than the non-transgenic lines (Figure 3). These results indicate that the expression of *GmDof4* and *GmDof11* stimulated the accumulation of oleic acid and regulated the fatty acid composition of rapeseed, but, neither gene could increase total seed oil content.

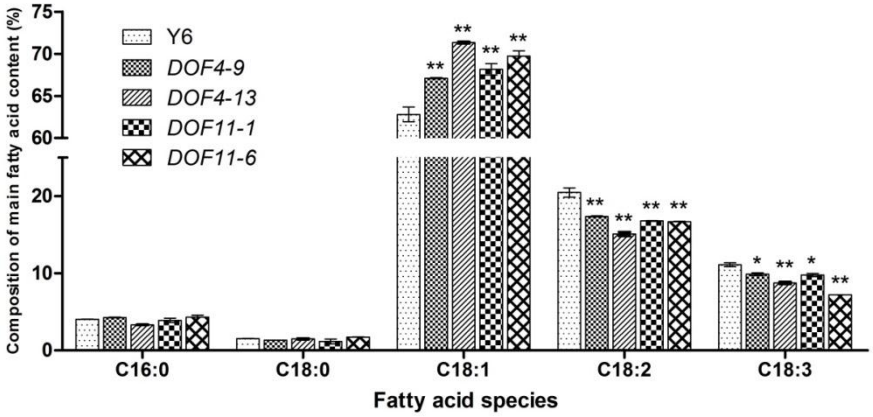

Figure 2. Composition of the major fatty acids in *GmDof4* and *GmDof11* transgenic seeds. The data represent the means ± SD of three replicate experiments and were analyzed by Student's *t*-test ($n = 3$). * $p < 0.05$; ** $p < 0.01$.

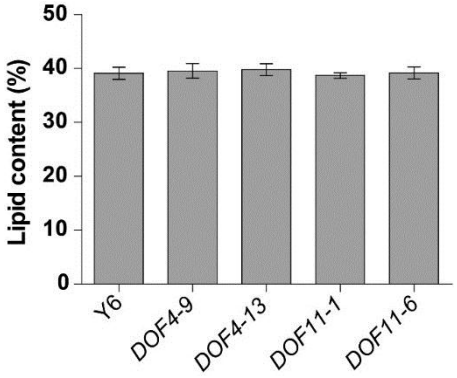

Figure 3. Total lipid contents in the seeds of *GmDof4* and *GmDof11* transgenic plants. The data represent the means ± SD of three replicate experiments and were analyzed by Student's *t*-test ($n = 3$).

3.4. Changes in the Expression of Fatty Acid Metabolism-Related Genes in GmDof4 and GmDof11 Transgenic Plants

Referring to previous reports, the expression of target genes that were directly controlled by *GmDof4* and *GmDof11* were additionally analyzed, including the 12S storage protein subunit encoding gene *CRA1*, the ACCase β subunit encoding gene *accD*, and *LCAS*5. The expression of *accD* was significantly upregulated in two *GmDof4* transgenic seeds compared with its expression in the non-transgenic plants. *FAB2*, which is responsible for oleic acid synthesis, *FAD3* and *FAD8*, which is responsible for the synthesis of linolenic acid from linoleic acid, were significantly upregulated by more than threefold in both lines (Figure 4). However, no significant difference was found in the expression of the other genes, except for *LACS5*, which was slightly upregulated. The expression of *accD* was also upregulated by approximately threefold in two *GmDof11* transgenic seeds. However, the expression of *FAD2* and *FAD6*, which are the coding genes responsible for the synthesis of linoleic acid, was inhibited (Figure 4). These results indicate that GmDof4 and GmDof11 do upregulate the expression of *accD*, and both genes jointly and specifically upregulate or downregulate the genes that are involved in the synthesis of fatty acids.

In addition, qPCR results demonstrated that the gene expression levels of *FAE1* and *FAE7*, which are responsible for the synthesis of eicosanoic acid, were lower than the detection limit of the qPCR technique (Ct > 40). Moreover, there was no expression of the *CRA1* gene in both the *GmDof4* transgenic and non-transgenic seeds, but it was detected in the two *GmDof11* transgenic seeds with slight expression (expression level relative to *BnActin* $\approx 10^{-4}$), indicating that *CRA1* might be upregulated slightly in the *GmDof11* transgenic plants.

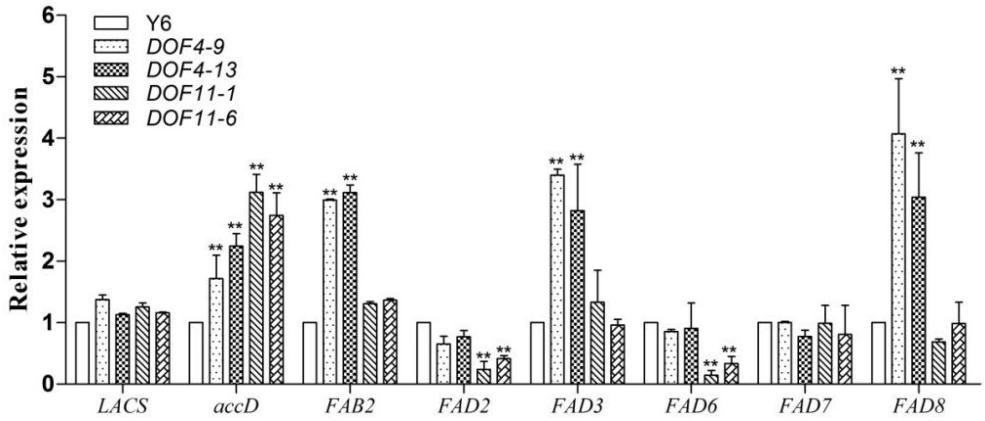

Figure 4. Gene expression detected by qRT-PCR in transgenic and non-transgenic seeds. RT-qPCR was used to determine the relative expression of genes related to lipid and fatty acid metabolism. Bars indicate SD (n = 3), Significant differences between transgenic and non-transgenic seeds are labelled with asterisks: ** $p < 0.01$ (Student's t test).

3.5. Yeast One-Hybrid Assay to Detect Target Genes of GmDof4 and GmDof11

Based on the transcriptome data in various tissues and organs of *B. napus* obtained earlier by our group, more than one copy of the *CRA1*, *FAB2*, and *FAD2* genes was found in *B. napus* (Table 1). Analysis of the promoters demonstrates numerous Dof-binding *cis*-DNA elements in the promoter regions of these genes (Table 1). To test whether GmDof4 and GmDof11 regulated the expression of the aforementioned genes by directly binding to their promoter regions, we investigated part of the putative Dof binding elements in the 1.5 kb promoter region of the *CRA1*, *FAB2*, and *FAD2* genes according to the binding features of GmDof4 and GmDof11. The results showed that GmDof4 protein could bind strongly to the *FAB2-1* and *FAB2-2 cis*-DNA elements (Figure 5a). These results suggest that GmDof4 protein may regulate *FAB2* by binding directly to their promoters. Analysis of GmDof11 protein binding activity demonstrated that GmDof11 binds strongly to *FAD2-1* but weakly to *CRA1-1* and *FAD2-2* (Figure 5a). These results indicate that the GmDof11 protein can directly regulate *CRA1* and *FAD2*.

Table 1. Number of transcripts of *GmDof* regulated genes in seeds at 34 days after flowering (DAF) and the *cis*-DNA elements of these genes.

		***Cis*-DNA Element**				**Sum**
		AAAAG	**TAAAG**	**CTTTT**	**CTTTA**	
FAB2	BnaA03g20420D	1	4	3	3	11
	BnaA05g03490D	6	5	3	5	19
	BnaC03g24420D	5	3	2	2	12
	BnaC04g03030D	3	0	3	2	8
FAD2	BnaA05g26900D	7	4	3	5	19
	BnaAnng09250D	4	3	5	2	14
	BnaC05g40970D	9	6	11	4	30

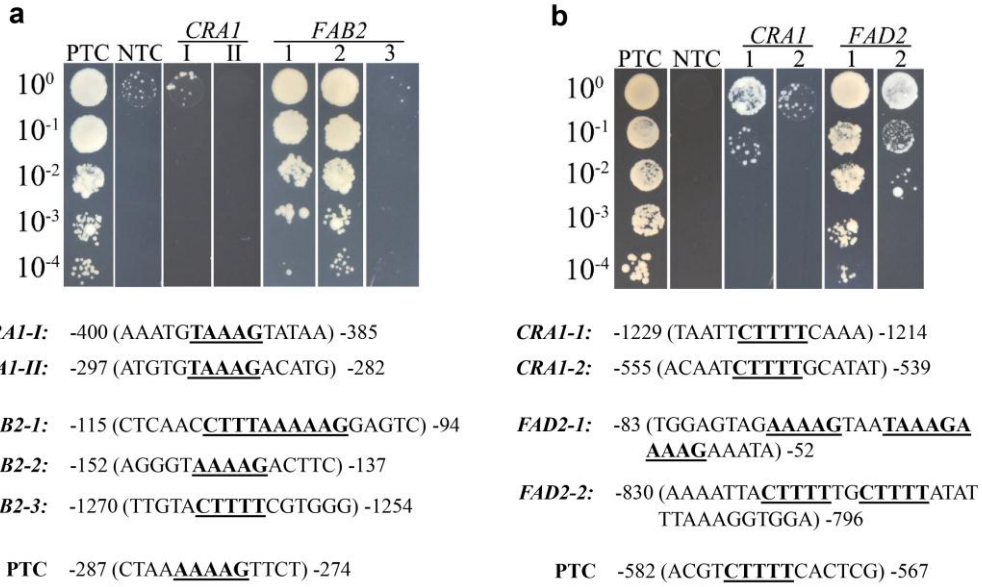

Figure 5. GmDof4 and GmDof11 interact with the *cis*-DNA elements in the promoter regions of downstream genes in the transgenic plants (**a**) Interaction between GmDof4 and the *cis*-DNA elements in the promoter regions of *CRA1* and *FAB2*. The bolded and underlined sequences indicate the core sequence of the Dof-binding elements. The putative Dof-binding elements were cloned into pAbAi, and these plasmids were transfected into yeast Y1HGold cells with pGADT7-*GmDof4*. Growth of the transfected yeast cells on the SD/-Leu/-Ura + AbA (0.2 mg/L) plates indicates that GmDof4 protein can bind to its corresponding *cis*-DNA element. PTC is a strain that contains pGADT7-*GmDof4* and a pAbAi plasmid with an element in the promotor region of *AtaccD*, which was confirmed to interact with the GmDof4 protein. NTC is a strain that contains pGADT7-*GmDof4* and an empty pAbAi plasmid. (**b**) Interaction between GmDof11 and the *cis*-DNA elements in the promoter regions of *CRA1* and *FAD2*. The bolded and underlined sequences indicate the core sequence of the Dof-binding elements. The putative Dof-binding elements were cloned into pAbAi, and these plasmids were transfected into yeast Y1HGold cells with pGADT7-*GmDof11*. Growth of the transfected yeast cells on SD/-Leu/-Ura + AbA (0.2 mg/L) plates indicates that the GmDof11 protein can bind to its corresponding *cis*-DNA element. PTC is a strain that contains pGADT7-*GmDof11* and a pAbAi plasmid with an element in the promotor region of *AtCRA1*, which was confirmed to be interacting with GmDof11 protein. NTC is a strain that contains pGADT7-*GmDof11* and an empty pAbAi plasmid.

4. Discussion

4.1. Overexpression of GmDof4 and GmDof11 Augmented the Oleic Acid in B. napus Seed Oil

GmDof4 and GmDof11 are TFs involved in the regulation of seed oil synthesis in soybean. Overexpression of *GmDof4* or *GmDof11* augments oil synthesis in transgenic *Arabidopsis* and the single-cell microalga *C. ellipsoidea* [44,45]. *GmDof4* and *GmDof11* overexpressed rapeseed plants were produced using *Agrobacterium*-mediated genetic transformation. However, no significant change was found in the seed oil content in the four transgenic lines. Comparison of the fatty acids in *GmDof4* and *GmDof11* transgenic and non-transgenic plants showed a significant change in fatty acid composition. The relative level of the monounsaturated fatty acid oleic acid increased, while the relative levels of the polyunsaturated fatty acids linoleic and linolenic acid decreased significantly in the *GmDof* transgenic plants compared with the non-transgenic plants. These results suggest that GmDof4 and GmDof11 may play a role in the late stage of fatty acid synthesis in *B. napus*, by regulating the synthesis of a few specific fatty acids rather than the carbon metabolic flux that would alter the relative levels of the major fatty acids. This phenomenon is different from those in *Arabidopsis* and *C. ellipsoidea*. The total lipid content increased significantly in transgenic *Arabidopsis* seeds and *C. ellipsoidea* cells, but the relative levels of each fatty acid did not change, except for linoleic acid in *Arabidopsis* overexpressing

GmDof4 [44,52]. The difference may be due to the large genome of *B. napus* and the complex network regulation of the synthesis and accumulation of oil during seed development. Therefore, increasing the oil content of rapeseed may require precise and more targeted genetic engineering.

4.2. GmDof4 and GmDof11 Regulated the Genes of Fatty Acid Synthesis by Binding to the Cis-DNA Elements in the Promoter Region of These Genes

In oil crops, lipid and fatty acid synthesis involves a number of enzymes [5,7,12]. *FAB2*, which encodes a stearoyl-ACP desaturase, catalyzes the synthesis of oleic acid. The expression level of *FAB2* affects the content of oleic acid [53,54]. Kachroo et al. found that the stearic acid content in the *FAB2* gene mutant (*ssi2*) was approximately 18 times higher than that in WT plants, and the content of oleic, linoleic, and linolenic acid was significantly reduced. Meanwhile, *FAB2* is involved in the activation of NPR1-dependent and -independent defense responses [55–57]. FAD2 and FAD3 are two important enzymes in the synthesis of unsaturated fatty acids in seed oils. They are integrated into the endoplasmic reticulum and they are responsible for the catalysis of the conversion of oleic acid to linoleic acid and then linolenic acid. Furthermore, in plastids FAD6 is an isoenzyme of FAD2, while FAD7 and FAD8 are isoenzymes of FAD3 [31,58]. FAD6, FAD7, and FAD8 are closely related to the synthesis of unsaturated fatty acids on chloroplast membranes [59]. The expression of these genes affect leaf lipids in *Arabidopsis* [31,56]. Therefore, we examined the expression of these desaturases in transgenic lines.

accD, which encodes the β-subunit of ACCase, was upregulated in seeds of the *GmDof4* transgenic plants at 30 DAF. This result is consistent with studies in *Arabidopsis*. In addition, *FAB2, FAD3,* and *FAD8* genes were significantly upregulated. This finding suggests that *GmDof4* likely increased oleic acid synthesis by increasing the expression of genes that are related to oleic acid synthesis, in spite of the upregulated *FAD3* not increasing the content of $C_{18:3}$, which is possibly due to the complexity of the regulation of fatty acid accumulation in seeds. The expression of *FAD2* and *FAD6* were downregulated in *GmDof11* transgenic seeds. The downregulation of *FAD2* may have caused a decrease in the content of linoleic acid and linolenic acid, thereby increasing the proportion of oleic acid. It is not clear whether the change in the expression of *FAD6* and *FAD8* in the overexpression plants changed the response to stress. Expression of *FAE1* and *FAE7* genes, which are responsible for erucic acid ($C_{22:1}$) synthesis, were not detected in all plants. This result may be due to the fact that the rapeseed variety that is used here has low erucic acid characteristics, and erucic acid synthesis genes are severely inhibited. In addition, although the expression of *accD* was upregulated in the seeds of the four transgenic lines, seeds oil content of did not increase. This result indicates that other regulation mechanisms in the seeds of *B. napus* related to the accumulation of oil.

In *Arabidopsis*, GmDof4 binds directly to the Dof-binding *cis*-DNA element in the promoter regions of the *accD* and *CRA1* genes, and GmDof11 directly regulates the expression of *LCAS* and *CRI1* genes [44]. In this study, we found that GmDof4 bound to the *cis*-DNA element in the promoter region of *FAB2*, whereas GmDof11 bound to the *cis*-DNA element in the promoter region of *CRA1* and *FAD2*. These results indicate that *GmDof4* and *GmDof11* regulated components of fatty acid synthesis in seed oil by regulating the expression of specific genes. Whether the slight upregulation of the *CRA1* gene in *GmDof11* transgenic seeds was caused by the direct interaction of GmDof11 and the *cis*-DNA element of *CRA1* should be further examined using a dual luciferase reporter system. Evaluation of the number of Dof binding elements (A/T)TTTG or CAAA(A/T) at the promoter regions of the potential target genes revealed that they contained a large number of Dof binding elements (Table 1). While considering that there are 134 putative *Dof* genes in *B. napus*, the existence of those elements indicates that the specific spatial and temporal expression of these genes may be regulated by various *Dof* TFs. This makes it possible to regulate the expression of the genes that are involved in lipid and fatty acid synthesis in *B. napus* by GmDof4 and GmDof11. Interestingly, except for *BnaC.accD.c* (BnaC09g27690D), which showed incomplete genome sequencing at the promoter region, the 1.5 kb promoter region of three *accD* duplicates in *B. napus* were identical (Figure S3). The homology of the

accD gene to *Arabidopsis* was 96.74%. In addition, it has been suggested that the 5′-untranslated region (UTR) of plant *FAD2* genes is evolutionarily conserved [35,60]. These results strongly suggest that the regulation of expression of the genes involved in fatty acid synthesis might also be highly conserved. GmDof4 and GmDof11 proteins increased oleic acid content in seed oil by activating or inhibiting genes that are associated with fatty acid synthesis in *B. napus*. Both proteins may be used as a genetic resource to improve the quality of rapeseed oil.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/10/222/s1, Figure S1: PCR analysis of putative transformants of *GmDof4*, Figure S2: PCR analysis of putative transformants of *GmDof11*, Figure S3: Homology of the *accD* gene promoters in *B. napus* and *A. thaliana*, Table S1: Amplification primers used for Semi-quantitative and quantitative RT-PCR, Table S2: Composition of the major fatty acids in *GmDof4* and *GmDof11* transgenic seeds, Table S3: the *Dof* genes in *B. napus*.

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