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CRISPR/Cas9-Mediated Mutagenesis of *GmFAD2-1A* and/or *GmFAD2-1B* to Create High-Oleic-Acid Soybean

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Abstract: Soybean (*Glycine max* (L.) Merr.) oil is an important source of vegetable oil for supporting the human diet. However, the high level of polyunsaturated fatty acids in natural soybean oil renders the oil unstable and thus susceptible to the development of unpalatable flavors and trans fatty acids. Therefore, reducing the content of polyunsaturated fatty acids and increasing the content of monounsaturated fatty acids is a longstanding and important target for soybean breeding. However, soybean varieties with a high oleic acid content are rare in soybean germplasm resources, which introduces substantial difficulties in the cultivation of high-oleic-acid soybeans. In this study, CRISPR/Cas9-mediated gene-editing technology was used to create targeted knockout of the soybean fatty acid desaturase encoding genes *GmFAD2-1A* and *GmFAD2-1B* that contribute to the formation of polyunsaturated fatty acids. We obtained *fad2-1a*, *fad2-1b*, and *fad2-1a/fad2-1b* homozygous mutants using two sgRNAs. We found that the oleic acid content increased from 11% to 40-50% in the *fad2-1a* and *fad2-1b* mutants and to 85% in the *fad2-1a/fad2-1b* mutants. We also generated transgene-free double mutants that conferred higher oleic acid, and the *fad2-1a/fad2-1b* mutant had no adverse phenotyping compared with the wild type. Our study provided new materials for the selection and breeding of high-oleic-acid soybean varieties.

Keywords: soybean; oleic acid; *GmFAD2-1*; genome editing; mutants

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important oil crops in the world. Unsaturated fatty acids account for about 85% of the total fatty acids in soybean seeds. High unsaturated fatty acid content is associated with strong antioxidant capacity and high stability, which is beneficial for human health [1]. Oleic acid is highly stable and its content is an important indicator for evaluating soybean oil quality, whereas polyunsaturated fatty acids are unstable and can cause undesired flavors for consumers. Thus, soybean oil with a high oleic acid content is far superior to traditional soybean oil in terms of oxidation stability. Reducing the synthesis of polyunsaturated fatty acids in soybean seeds can improve the oxidation stability of soybean oil without causing the production of trans fatty acids [2]. However, oleic acid accounts for only 20% of the total unsaturated fatty acids in soybean, which restricts the application of soybean oil in food and industry [3,4]. With the increasing demand for industrial uses of soybean oil and consumer-driven concerns about health issues related to edible oils, the development of soybean varieties with an ideal fatty acid composition has become one of the important goals of soybean genetic improvement [5].

Fatty acid desaturase 2-1 (*FAD2-1*) localizes to the endoplasmic reticulum and plays an important role in the initial desaturation of fatty acids to produce polyunsaturated fatty acids, particularly for converting oleic acid to linoleic acid [6–8]. As such, *GmFAD2-1*

activity determines the level of monounsaturated fatty acids in soybean oil. Six *GmFAD2* desaturase genes have been identified in the soybean genome, namely *GmFAD2-1A* (*Glyma.10G278000*), *GmFAD2-1B* (*Glyma.20G111000*), *GmFAD2-2A* (*Glyma.19G147300*), *GmFAD2-2B* (*Glyma.19G147400*), *GmFAD2-2C* (*Glyma.03G144500*), and *GmFAD2-2D* (*Glyma.09G111900*) [9,10].

Studies have shown that *GmFAD2-1* is seed-specific and is strongly expressed in developing seeds, while *GmFAD2-2* is expressed during seed development and in vegetative tissues. Both *GmFAD2-1A* and *GmFAD2-1B* are highly expressed during oleic acid synthesis and are the main genetic determinants of oleic acid and linoleic acid contents in soybean seeds [11]. Oleic acid content can be increased by downregulating the expression of *GmFAD2-1A* and *GmFAD2-1B*, which resulted in 80% oleic acid content upon *GmFAD2-1A* and *GmFAD2-1B* silencing [12–14]. Different methods have been used to develop soybean lines with an increased oleic acid content, including RNA interference (RNAi) and gene-editing technologies. RNAi can effectively inhibit *FAD2-1* expression levels to increase oleic acid [15–17]. Gene-editing technology is mediated by specific sequence nucleases such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) to generate mutants [17–20]. Such genetic strategies are promising routes to optimize the oleic acid content in soybean.

The objective of this work was to create high-oleic-acid soybean lines using gene-editing technology. In this study, we obtained three homozygous mutants that included *fad2-1a*, *fad2-1b*, and *fad2-1a/fad2-1b* using two sgRNAs for *FAD2-1A* and *FAD2-1B*. The oleic acid content was approximately doubled in the single mutants and was increased by nearly threefold in the double mutants. In addition, we also generated transgene-free mutants that retained the elevated high oleic acid with no adverse phenotyping. Thus, this study laid a foundation for accelerating the selection and breeding of high-oleic-acid soybean varieties.

2. Materials and Methods

2.1. sgRNA Design and Plasmid Construction

The sgRNA was constructed in the VK005 vector. The Cas9 sequence was driven by the CaMV 35S promoter and assembled with the respective sgRNA driven by the *Arabidopsis thaliana* U6 promoter. The selectable marker *bar* gene was also driven by the CaMV 35S promoter. The CRISPR/Cas9-based vectors were constructed according to our previous study [21]. The genetic information for the soybean genes *GmFAD2-1A* and *GmFAD2-1B* were downloaded from the Phytozome website. The sgRNA for the target site were designed using the web-based tool CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2) [22]. We designed two sgRNAs: one that targeted *GmFAD2-1A* alone and one that simultaneously targeted *GmFAD2-1A* and *GmFAD2-1B*.

2.2. Soybean Transformation

The two CRISPR/Cas9 vectors were transformed into the EHA105 *Agrobacterium tumefaciens* strain. The *Agrobacterium tumefaciens*-mediated transformation procedure of the soybean cultivar Jack was modified from a previously published protocol [23].

2.3. DNA Extraction and Mutant Identification

Genomic DNA was extracted from the leaves of transgenic soybean plants using the Genomic DNA Kit (Cwbiotech, Beijing, China) and was subsequently used for PCR detection of mutant alleles with gene-specific primers. The primers used for *FAD2-1A* were 5′-ACACATTCAGCAAAACAACTGAAAC-3′ (forward) and 5′-ACCTGTGTTGGAGTGATGGC-3′ (reverse). The primers used for *FAD2-1B* were 5′-AAGCCACTAGGCATGGTATGAT-3′ (forward) and 5′-ACCCACACGCCAGTAAGAAT-3′ (reverse). The PCR products spanning the target sites were sequenced and analyzed via sequence peaks.

To seek transgene-free plants, we detected two regions (part of the Cas9 coding sequence and the marker gene *bar*) using a PCR strategy (Tsingke Biotechnology Company)

and a bar test strip. The primers used for Cas9 were 5′-TTGGGGCTCACACCAAACTT-3′ (forward) and 5′-CGATCGCCTTCTTTTGCTCG-3′ (reverse).

2.4. Fatty Acid Analysis

The contents of five fatty acids—palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18: 3)—were determined in the soybean seed extracts. The heated methyl ester extraction method was used for fatty acid extraction. First, 20 soybean seeds were selected for each soybean line and ground into a fine powder with a grinding machine (RetschZM100, Φ = 1.0mm, Rheinische, Germany). Then, 0.03 g of soybean powder was placed in a 2 mL sterile centrifuge tube, and 1 mL of n-hexane was added into the centrifuge tube at 60 °C for 20 min with shaking every 5 min. Then, 1 mL of sodium methanol solution (0.5 mol/L) was added into each centrifuge tube and oscillated for 10 min to complete the methyl esterification. Following centrifugation at 13,000 rpm/min for 2 min, 200 μL of supernatant was absorbed into a special sample bottle for chromatographic analysis. The soybean fatty acid contents were determined using a gas chromatograph (GC-2010) from the Shimadzu company (Japan). The gas chromatographic analysis and detection conditions were: an RTX-Wax chromatographic column (30 m × 0.25 m × 0.25 m), injection port temperature of 250 °C, nitrogen at 54 mL/min, hydrogen at 40 mL/min, and air at 400 mL/min using a programmed temperature rise mode. The detector temperature was 300 °C, and the area normalization method was used to calculate the fatty acid content [24]. The samples of the genetically modified soybean lines consisted of three biological replicates; each replicate was tested three times. The average value of the three repeated tests was determined as the content of the fatty acid component, and the fatty acid content of each transgenic soybean line was based on the average value of the three repeated tests.

2.5. Phenotype Identification

The germination, flowering time, and maturity time were recorded in the wild type and *fad2-1a*/*fad2-1b* mutants. The flowering time was recorded when the first flower appeared at any node on the main stem. The maturity time was recorded when the first mature pod appeared at any node on the main stem. The statistical analyses were performed using Microsoft Excel.

3. Results

3.1. Generation of fad2-1a, fad2-1b, and fad2-1a/fad2-1b Mutants with the CRISPR/Cas9 System

The *GmFAD2-1A* and *GmFAD2-1B* genes were located on soybean chromosomes 10 and 20, respectively. Two target sites (named SPA and SPD) in the second exon were selected. SPA was used to target *GmFAD2-1A* (Figure 1a) alone, and SPD was used to target both *GmFAD2-1A* and *GmFAD2-1B* simultaneously (Figure 2a).

At the SPA target site, we obtained 14 T0-positive transgenic events. A total of 102 T1 plants from the different T0 generation plants were detected; 37 T1 plants were heterozygous and 65 plants were identified as the wild type. Thus, homozygous mutants were identified in the T2 generation. Among them, we detected 28 T2-generation homozygous *fad2-1a* mutants and obtained four types of mutations: a 1 bp deletion, a 2 bp deletion, an 11 bp deletion, and a 1 bp insertion (Figure 1b).

At the SPD target sites, we obtained 15 T0-positive transgenic events and identified mutations in both *GmFAD2-1A* and *GmFAD2-1B*. A total of 154 T1 plants were derived from 15 T0 transgenic events; 11 and 12 plants were heterozygous for single mutations to *GmFAD2-1A* and *GmFAD2-1B*, respectively, and 42 plants were heterozygous for both *GmFAD2-1A* and *GmFAD2-1B* mutant alleles. The seeds from heterozygous plants were collected and planted for further genetic segregation, and 37 homozygous mutants were identified in the T3 generation: 14 were homozygous *fad2-1a* mutants, 11 were homozygous *fad2-1b* mutants, and 12 were homozygous *fad2-1a*/*fad2-1b* mutants. The *fad2-1a* mutants consisted of three types of mutations: a 4 bp deletion, a 5 bp deletion, and a 10 bp dele-

tion/1 bp insertion (Figure 2b). The *fad2-1b* mutants showed five types of mutations: a 1 bp insertion, a 4 bp deletion, a 5 bp deletion, a 7 bp deletion, and a 17 bp deletion (Figure 2c). The *fad2-1a*/*fad2-1b* mutants showed a total of nine types of mutations that were combinations of two types of *fad2-1a* mutations (5 bp deletion and 10 bp deletion with 1 bp insertion) and six types of *fad2-1b* mutants (3 bp deletion, 4 bp deletion, 5 bp deletion, 7 bp deletion, 8 bp deletion, and 1 bp insertion) (Figure 2d). Using the CRISPR/Cas9 system, we obtained different materials that included *fad2-1a*, *fad2-1b*, and *fad2-1a*/*fad2-1b* mutants.

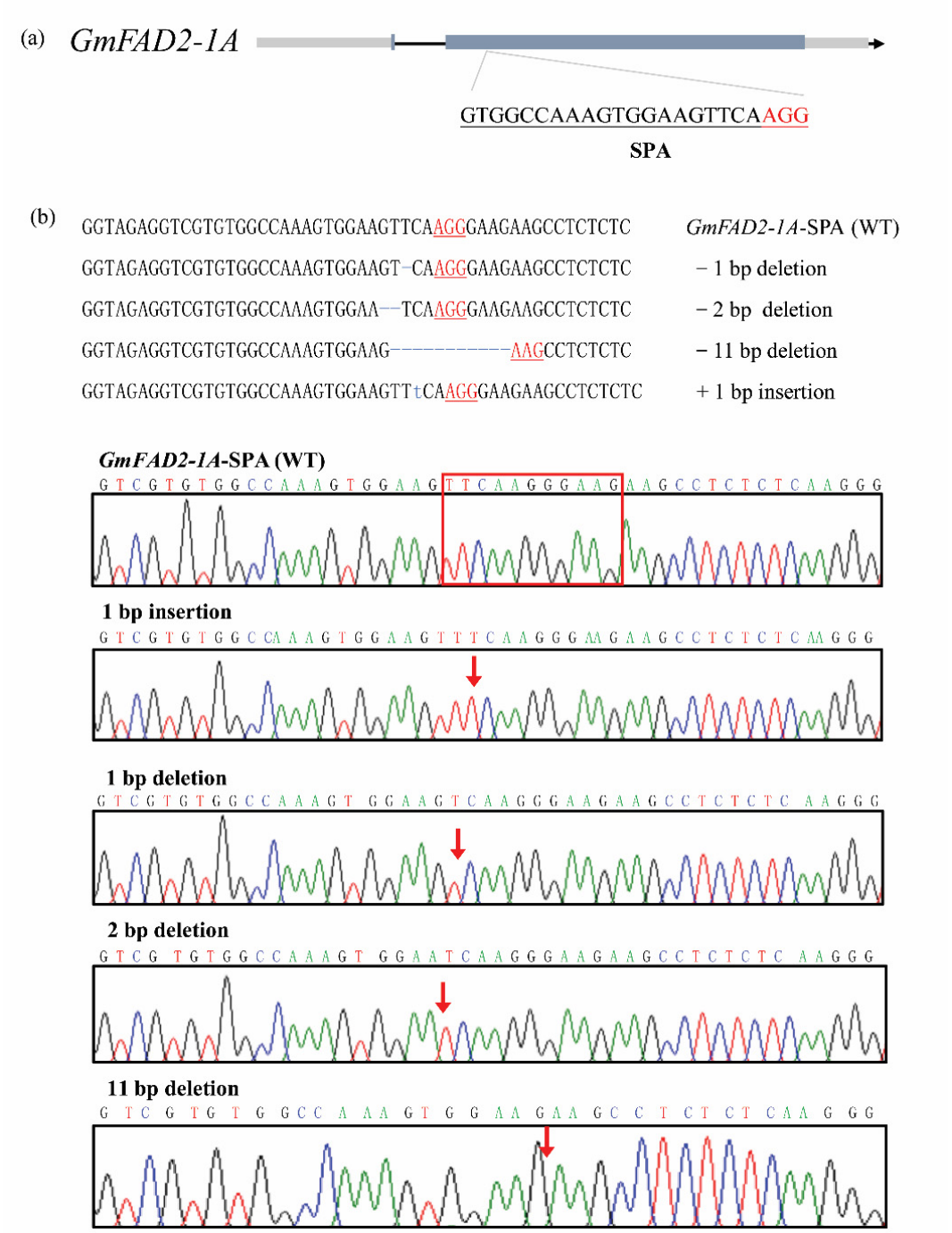

Figure 1. Homozygous targeted mutagenesis of *GmFAD2-1A* induced by CRISPR/Cas9. (**a**) Gene structures of *GmFAD2-1A* with target sites of CRISPR/Cas9. Black solid line: intron; blue stripe: CDS; gray stripe: UTR (untranslated region). (**b**) Sequencing of the mutants. Deletions and insertions are indicated as dashes and blue lowercase letters, respectively. The PAM is in red uppercase letters. The types of indels (insertions–deletions) are indicated in the right column.

Figure 2. Three simultaneously obtained homozygous mutants of *fad2-1a*, *fad2-1b*, and *fad2-1a*/*fad2-1b* using one target site. (**a**) Gene structures of *GmFAD2-1A* and *GmFAD2-1B* with the target sites of CRISPR/Cas9 indicated and schematic illustrating the target site sequence and corresponding PAM (red uppercase letters). (**b**) Sequencing results of the *fad2-1a* mutants. The types of indels are indicated in the right column. (**c**) Sequencing results of the *fad2-1b* mutants. (**d**) Sequencing results of the *fad2-1a*/*fad2-1b* mutants. Deletions and insertions are indicated as dashes and red lowercase letters, respectively.

3.2. Fatty Acid Profiles of Mutant Seeds

To detect the effects of inactivation of the *GmFAD2-1A* and *GmFAD2-1B* genes on the fatty acid content of soybean seeds, three *fad2-1a* mutants (SEA-73, SEA-81, and SEA-122), four *fad2-1b* mutants (PB-41, PB-86, PB-116, and PB-154) and three *fad2-1a/fad2-1b* mutants (JM-72, JM-90, and JM-264) in the T3 generation were selected for the determination of fatty acid content via gas chromatography. The editing genotypes of each line are shown in Table 1. The wild-type soybean Jack was included as a control.

Table 1. Mutants types used for determination.

Mutant Lines	Editing Types
SEA-73	fad2-1a (−2 bp)
SEA-81	fad2-1a (+1 bp)
SEA-122	fad2-1a (−1 bp)
PB-41	fad2-1b (−7 bp)
PB-86	fad2-1b (+1 bp)
PB-116	fad2-1b (−17 bp)
PB-154	fad2-1b (−4 bp)
JM-72	fad2-1a (−5 bp)/fad2-1b (−4 bp)
JM-90	fad2-1a (−5 bp)/fad2-1b (−8 bp)
JM-264	fad2-1a (−5 bp)/fad2-1b (+1 bp)

Our results showed that the oleic acid content was significantly higher in the mutant lines compared to that of the wild type. The oleic acid composition in the seeds of the *fad2-1a* mutant lines increased from ~21% in the wild type to ~35–50%, and the linoleic acid content decreased from about 57% in the wild type to 31-41% in the different mutant lines. The oleic acid content in the *fad2-1b* mutant seeds increased from ~21% in the wild type to 39–50%, and the linoleic acid content decreased from about 57% to 27–38% in the different mutant lines. The oleic acid content in the seeds of the *fad2-1a/fad2-1b* mutants greatly increased to ~85%, whereas the linoleic acid content decreased from about 57% to 2% in the double mutant relative to the wild type (Table 2).

Table 2. Fatty acid profiles in different homozygous mutants.

Mutant	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Linolenic (%)
WT	11.14 ± 0.22	4.29 ± 0.32	21.69 ± 0.84	57.22 ± 1.14	5.66 ± 0.37
SEA-73	10.32 ± 0.03 *	3.1 ± 0.02 **	35.64 ± 0.15 **	41.97 ± 0.08 **	8.97 ± 0.06 **
SEA-81	9.68 ± 0.06 **	3.48 ± 0.07 **	50.11 ± 0.28 **	31.12 ± 0.22 **	5.61 ± 0.08
SEA-122	11.12 ± 0.15	4.25 ± 0.07	38.28 ± 0.43 **	40.9 ± 0.23 **	5.44 ± 0.07
PB-41	8.63 ± 0.03 **	4.52 ± 0.02	54.08 ± 0.33 **	27.52 ± 0.27 **	5.25 ± 0.02
PB-86	8.51 ± 0.06 **	4.60 ± 0.03	53.30 ± 0.46 **	28.27 ± 0.38 **	5.32 ± 0.02
PB-116	10.44 ± 0.07 *	3.79 ± 0.06 *	39.95 ± 0.13 **	38.98 ± 0.18 **	6.84 ± 0.09 *
PB-154	8.88 ± 0.05 **	4.39 ± 0.11	50.67 ± 0.41 **	30.92 ± 0.49 **	5.14 ± 0.05
JM-72	6.11 ± 0.02 **	4.23 ± 0.01	84.55 ± 0.05 **	2.35 ± 0.01 **	2.76 ± 0.05 **
JM-90	6.13 ± 0.03 **	3.50 ± 0.04 **	85.42 ± 0.06 **	2.36 ± 0.06 **	2.59 ± 0.03 **
JM-264	6.70 ± 0.01 **	2.78 ± 0.03 **	84.69 ± 0.12 **	2.78 ± 0.05 **	3.04 ± 0.09 **

* Indicates significant difference compared with WT at 0.05 level; ** indicates significant difference compared with WT at 0.01 level; ± indicates standard error.

3.3. Identification of Transgene-Free fad2-1a/fad2-1b Mutant Plants

As the *fad2-1a/fad2-1b* mutant was obtained using the CRISPR/Cas9 system, the *Cas9* gene and *bar* selectable marker genes were integrated into the soybean genome during the transformation process, thereby producing transgenic plants. However, these genes can be separated while retaining the genetic modification at the genes of interest in the progeny of self-crossed plants. In order to obtain *fad2-1a/fad2-1b* mutants that did not contain *Cas9* and its associated selectable marker gene, the sequence encoding the *Cas9* gene was detected

via PCR (Figure 3a), and the bar strip test was used to detect the presence of the selectable marker gene (Figure 3b). In the T3 generation, two of the six tested lines were T-DNA-free, and 15 progeny plants in the T4 generation were transgene-free. Four of six T3 generation lines were T-DNA-positive, but some transgene-free mutants were identified in the T4 generation following self-crossing (Table 3). We obtained *fad2-1a/fad2-1b* transgene-free mutants through the selection.

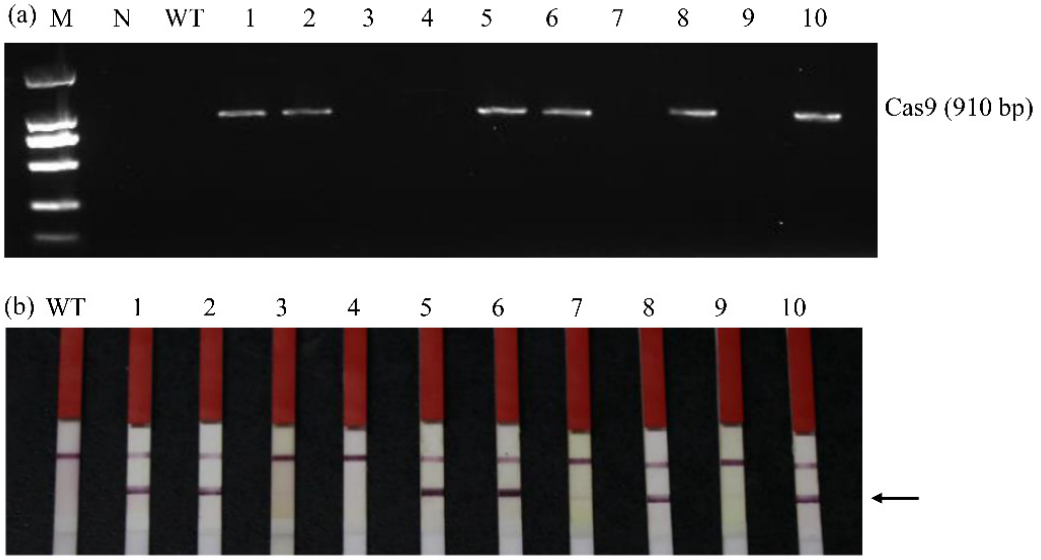

Figure 3. Identifying "transgene-free" mutant soybean lines of *fad2-1afad2-1b*. (**a**) PCR method to detect a partial sequence that encoded the Cas9 protein with an expected PCR product of 910 bp. M represents the marker, N represents the negative control, WT represents the wild type, and Lanes 1-10 are the respective tested transgenic lines. (**b**) The *bar* strip test to detect transgenic elements. The arrow indicates that the *bar* was positive for the presence of the transgene.

Table 3. The *fad2-1a/fad2-1b* mutants without transgenic elements in the T3 and T4 generations.

***fad2-1a/fad2-1b* Mutant Lines**	**T-DNA in the T3 Mutants**	**No. of Progeny Plants Identified**	**No. of T4 "Transgene-Free" Mutants**
fad2-1-SPD-JM-72	T-DNA-free	7	7
fad2-1-SPD-JM-264	T-DNA-free	8	8
fad2-1-SPD-JM-90	T-DNA-positive	10	3
fad2-1-SPD-JM-95	T-DNA-positive	9	0
fad2-1-SPD-JM-113	T-DNA-positive	8	1
fad2-1-SPD-JM-196	T-DNA-positive	12	4

3.4. No Adverse Phenotyping of fad2-1a/fad2-1b Mutant Plants

Germination testing is often the most reliable way of assessing viability. The seeds were germinated on filter paper for two days (Figure 4a). We counted the seeds with the hypocotyl. The germination rate was 95.56% and 93.33% in the wild type and *fad2-1a/fad2-1b* mutant, respectively (Figure 4b). There was no significant difference in the germination rate between the wild type and *fad2-1a/fad2-1b* mutant.

Under the natural conditions, the *fad2-1a/fad2-1b* mutants flowered at 29.4 days after emergence (DAE), while the WT plants flowered at 28.8 DAE (Figure 4c,d). The *fad2-1a/fad2-1b* mutants matured at 75.4 DAE, while the WT plants matured at 74.8 DAE (Figure 4e). The results of these experiments demonstrated that *fad2-1a/fad2-1b* mutant had no adverse phenotyping compared with the wild type.

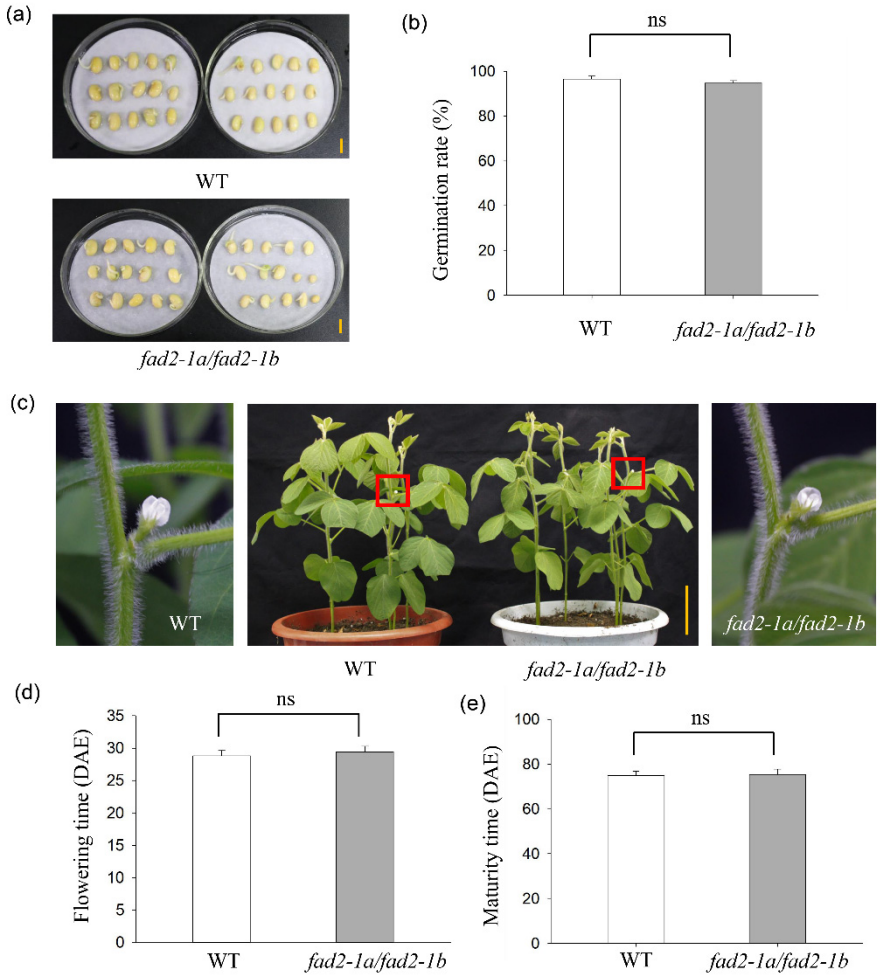

Figure 4. The phenotyping of the *fad2-1a/fad2-1b* mutant. (**a**) The seedlings of the wild type and *fad2-1a/fad2-1b* were germinated for 2 days. Scale bars = 1 cm. (**b**) The germination rate in the wild type and *fad2-1a/fad2-1b*. (**c**) The flowering of the wild type and *fad2-1a/fad2-1b*. Scale bars = 10 cm. (**d**) The flowering time in the wild type and *fad2-1a/fad2-1b*. (**e**) The maturity time in the wild type and *fad2-1a/fad2-1b*. The ns represents no significant differences.

4. Discussion

There are few soybean germplasm resources and mutagenic materials with a high oleic acid content and no soybean materials with oleic acid content higher than 80%. The content of oleic acid in the natural mutant is ~46% of the total fatty acids, which introduces great difficulties in the cultivation of high-oleic-acid varieties. Traditional breeding methods entail the crossing of the existing *Gmfad2-1a* and *Gmfad2-1b* mutants to create a double mutant [13,14]. However, crossing, genotyping, and phenotyping is a long and expensive process to create soybean lines with high oleic acid content through hybridization.

Genome-editing technology has revolutionized biological research and has been used to improve many agronomic traits [25,26]. Here, we used the CRISPR-Cas9 system to edit the *GmFAD2-1* genes in soybean. A single-guide-RNA construct was designed to target different positions of the *GmFAD2-1* genes in soybean. We chose two targets: one targeting *GmFAD2-1A* and the other targeting both *GmFAD2-1A* and *GmFAD2-1B*; transgenic plants were obtained through a soybean-transformation system. Through a double-knockout vector using only one sgRNA, we successfully obtained three mutants (*fad2-1a*, *fad2-1b*, and *fad2-1a/fad2-1b*). This shows that the vector could effectively edit two genes at the same time in soybean. The CRISPR/Cas9 system provides a fast and efficient method

to simultaneously edit homologous soybean genes, which can promote the breeding of important crops with complex genetic architecture.

Previous studies have been reported in which the CRISPR-edited homozygous mutants of both *GmFAD2-1A* and *GmFAD2-1B* were created by Do [20]. Do et al. designed two gRNAs to guide Cas9 to simultaneously cleave two sites within the second exons of *GmFAD2-1A* and *GmFAD2-1B*. The analysis of the fatty acid profile showed that the oleic acid content was dramatically increased to over 80% in the T1 seeds of both *GmFAD2* mutants. In our study, we achieved the simultaneous knockdown of the *GmFAD2-1A* gene and the *GmFAD2-1B* gene by designing only one target site to obtain the target plant with high oleic acid content. In addition, we also isolated and obtained the single mutants from the offspring of the heterozygous *fad2-1a/fad2-1b*.

Significant progress has been made to increase the oleic acid content of soybean seeds by downregulating the expression of *GmFAD2-1A* and *GmFAD2-1B*. Using RNAi technology, the oleic acid content in the obtained genetically modified soybean seeds was increased from 20% to 51.7% and 80% [15,17]. Haun used TALEN technology to design target sequences for recognizing and cleaving *GmFAD2-1A* and *GmFAD2-1B*, and the oleic acid content in the resulting soybean double-mutant seeds increased to 80% [18]. In this study, we used CRISPR/Cas9-mediated genome-editing technology to knock out *GmFAD2-1A* and/or *GmFAD2-1B* in the quality parent (Jack). The average relative contents of oleic acid and linoleic acid were significantly different from those of the unmodified Jack variety. The oleic acid content increased from ~21% to 40–50% in the *fad2-1a* and *fad2-1b* single mutants, whereas the oleic acid content in the *fad2-1a/fad2-1b* double-mutant seeds reached about 85%. The content of linoleic acid was reduced from ~57% to 2% in the double mutant as well. This study thus provided mutant materials with enhanced oleic acid contents, which is of great value in breeding high-quality soybean varieties with a high oleic acid content.

We also generated transgene-free double mutants that retained their higher oleic acid contents through self-crossing of the mutant lines. Cas9 and the associated selectable marker were removed in later generations via genetic segregation. Taken together, we obtained transgene-free soybean plants with a higher oleic acid content using homozygous targeted mutagenesis of endogenous *GmFAD2* homologs via CRISPR/Cas9 in this study. We also estimated the phenotyping of the *fad2-1a/fad2-1b* double mutant. The results showed no significant changes in the seeds' germination, flowering time, and maturity time. There was no adverse result in the pot experiment. In addition, we have obtained permission to conduct field trials for the *fad2-1a/fad2-1b* double mutant, so the intermediate experiments will be performed and evaluated in the field. Thus, this technology can provide excellent mutant materials for improving soybean quality with reduced safety concerns due to the absence of transgenes.

Author Contributions: W.H. designed and revised the manuscript; M.F. and L.C. carried out most of the experiments in this research and wrote the manuscript; Y.C. (Yupeng Cai) constructed the CRISPR/Cas9 vectors; Y.C. (Yingying Chen) and Q.S. participated in the phenotype investigations and experiments. All authors have read and agreed to the published version of the manuscript.

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

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