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By apmball for BRS Document Control Officer at 12:15 pm, Apr 23, 2020

□ April 2, 2020

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98 Riverdale,
MD 20737

Dear Ms. Juarez,

ToolGen Inc. is a biotechnology company located in Seoul, Korea, which has developed a soybean cultivar with a high oleic acid content in the soybean seed using the CRISPR/Cas9 technology. ToolGen would like to request confirmation from USDA-APHIS's Biotechnology Regulatory Service (BRS) that this soybean does not meet the definition of regulated articles under 7 CFR Part 340, since the selected cultivar does not include any foreign DNA.

ToolGen provides the information summarized in this letter to assist BRS in making its determination. Key information in support of our request includes:

1. In order to perform genome editing, we used disarmed *Agrobacterium tumefaciens* to deliver a plasmid vector that contains the gene cassette encoding CRISPR-Cas9, FAD2 targeting guide RNA, and selectable Bar gene.
2. The experimental methodology and process used to develop the high oleic acid content soybean seed did not introduce any DNA sequence from the plasmid vector genome into the plant.
3. No soybean species is currently listed as a Federal Noxious Weed or listed in any U.S. State as a noxious weed (USDA Natural resources conservation service), and the gene-edited soybean is not a plant pest and does not exhibit increased weed potential.

Sincerely yours,

Chee Hark Harn, Ph. D.
Director, Seed R&BD Headquarters
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Developer name and contact information, including email address

- Developer: OJ Koo, SJ Kim
- Responsible Party: ToolGen Inc.
- Contact info: Chee Hark Harn, ch.harn@toolgen.com

Taxonomic description of organism

Soybean: *Glycine max L.* (cultivar name: Kwangan). The Kwangan cultivar is a Korean soybean cultivar that belongs to the *Glycine max L* species. The cultivation of this soybean is popular in Korea, and its young sprouts are prepared as vegetable food.

The Intended Phenotype

In soybeans, the oleic acid content is determined by genes that are involved in the unsaturated fatty acid controlling system, in which fatty acid desaturase 2 (FAD2) is a key enzyme[1,2]. Since FAD2 catalyzes the desaturation of oleic acid (C18:1) to linoleic acid (C18:2), it is an important regulator of the oleic acid content of the soybean[3]. Thus, the FAD2 gene is a promising target to increase the levels of oleic acid in soybean, once this gene is knocked out by CRISPR/Cas9.

The intended phenotype after the genome editing experiment is a soybean cultivar with a high oleic acid content. We have obtained soybeans with oleic acid levels of approximately 80-85%. Furthermore, the palmitic, stearic, and linolenic fatty acid composition was not changed, while the levels of linoleic acid were significantly lowered. The phenotypic traits of the genome edited soybean (HO-0) cultivated in the field, however, were identical to the control cultivar, Kwangan.

The Intended Activity

ToolGen intends to work for a soybean breeding program with the HO-0 cultivar and commercialize a soybean variety, which has a high oleic acid content.

The Intended Genetic Change in the Final Product

The intended genetic changes are CRISPR/Cas9-mediated small deletions in the specific region of the FAD2 gene, which is complementary to the guide RNA.

Development of The FAD2 Knock-Out Soybean

To edit the FAD2 gene, we used a commercial soybean cultivar (*Glycine max L.* Kwangan), which is a well-known edible herb with high protein content. The components of the CRISPR/Cas9 system, the expression cassette of Cas9 endonuclease, and FAD2-targeted sgRNA were delivered into the plant cells by disarmed *Agrobacterium*-mediated transformation system with a binary vector and the co-culturing method. During the genome editing process via CRISPR/Cas9, the Cas9 protein recognizes the target site and generates blunt double strand breaks (DSBs) based on 20 nt of gRNA sequences (known as protospacer) and PAM sites (5'-NGG-3' for SpCas9)[4,5]. Subsequently, the DSB is repaired by the plant's natural DNA repair system, leaving small indels, but this frequently results in a frameshift that causes a knock-out of the intended target gene[6]. For this reason, the gRNAs targeting the FAD2 gene, known as a regulator of the unsaturated fatty acid content in soybean, were designed and used in the FAD2 knock-out via the *Agrobacterium*-mediated genetic transformation method. The edited soybeans were selected by next-generation sequencing (NGS)[7], and the transgene-free soybeans were obtained by self-crossing. Finally, from these

soybeans, the ones with the high content oleic acid were sorted out by HPLC[8].

Agrobacterium-mediated transformation is the most commonly used method for genome editing in plants to deliver components of the CRISPR/Cas9 system. However, occasionally, such transformation is accompanied by the integration of a segment of the plasmid vector DNA into the host plant genome, which frequently induces unwanted genetic alterations[9-11]. Since unwanted genome insertion into the host plant is frequently reported in *Agrobacterium*-mediated transformation, we analyzed the edited soybean genome to determine if the edited genome contained any segment of DNA from the plasmid vector via whole genome sequencing (WGS) of the edited soybean.

The detailed methods and the experimental results for the development of the FAD2 knock-out soybean cultivar are described below.

1. Transformant Vector and Production of T_0 Transformants.

To knock out the FAD2 gene, as shown in Table 1, guide RNA targeting the FAD2 gene was designed and cloned with a Cas9 protein in a pPZP vector, as pPZP-FAD2-7 (Figure 1). The detailed information about the vector used in this development, is presented in Table 2. The pPZP-FAD2-7 vector was transformed into *Agrobacterium tumefaciens* EHA105 cell line, and the *Agrobacterium* was inoculated in soybean seed explants (Figure 2). As shown in Figure 2, through the natural plant regeneration process, a total of 9 transformants (T_0) were obtained (Figure 3), and each transformant originated from an independent explant.

2. Determining Gene Transfer into T_0 Transformants

To determine whether a gene cassette between left border (LB) and right border (RB) was introduced into T_0 transformants, genomic DNA was isolated from FAD2-7 T_0. Then, PCR was performed targeting the specific sequences of gRNA of the FAD2-7, Bar, and Cas9 genes (Figure 4A). Specific primer sequences for each gene used for the PCR analysis are shown in Table 3. As shown in Figure 4B, the introduction of all genes was confirmed by the PCR experiment.

3. Analysis of Oleic Acid Content and Mutation of FAD2 Gene Sequence in the T_1 Generation.

T_0 transformants were self-crossed, and T_1 seeds were harvested from the FAD2-7-7 and FAD2-7-30 groups. To analyze the oleic acid content in T_1 seeds, fatty acids were extracted from T_1 seeds using the Extraction solution [chloroform:hexane:methanol, 8:5:2][8,12]. The extracted fatty acid samples were pre-treated with a Methylation solution [0.25 M methylsulfonylmethane:petroleum ether:ethyl ether, 1:5:2], thereby measuring the contents of oleic acid in T_1 seeds using gas chromatography (Agilent GC 7890A)[8,12]. The detailed running conditions of gas chromatography are described in Table 4. The oleic acid contents (18:1) in the FAD2-7-7 and FAD2-7-30 T_1 seeds were significantly higher than those in control seeds such as *Glycine max L.* Pungsan and *Glycine max L.* Kwangan (Table 5).

T_1 seeds were planted, and T_1 transformants were grown. The insertion and deletion (indel) frequencies and sequences were analyzed to confirm whether the genome editing induced any mutation in the FAD2 genes during the genetic transformation steps in Figure 2. Finally, T_1 transformants were analyzed through targeted deep sequencing to confirm whether mutations were induced in the FAD2 gene. One mutation was confirmed at the target site in the FAD2 gene in chromosome #10 (chr10) and chromosome #20 (chr20) in all T_1 transformants, except FAD2-7-7 #1-1 and FAD2-7-30 #2-4, #3-1, and #9-1 (Table 6 and 7).

4. Removal of the Introduced Gene in the Transgenic Soybean.

We examined if the inserted gene cassette between LB and RB of the plasmid vector, pPZP-FAD2-7, was removed after genome editing. Genomic DNA was isolated from the FAD2-7-7 and FAD2-7-30 T_1 transformants, and PCR was performed for detecting the introduced genes using the specific primers described in Table 3. As shown in Figure 5, the introduced genes were removed in 14 T_1 transformants (FAD2-7-7 #1-1, FAD2-7-30 #2-4, 3-1, 3-2, 8-1, 8-2, 8-3, 8-4, 8-5, 21-2, 21-3, and 21-4) and retained in 4 transformants (FAD2-7-30 #9-1, 19-1, 21-1, and 21-5).

5. Analysis of Possible Contamination by Plasmid DNA in the Soybean Genome.

During *Agrobacterium*-mediated transformation, a part of the plasmid vector DNA could also be introduced into the host genome[11]. In order to analyze if any segment of the plasmid vector DNA remained in the host soybean genome, WGS was performed on the genome of the [ ] and on the plasmid vector DNA using TruSeq Nano library (Illumina). The results of WGS were analyzed based on the methods described in Figure 6. CBI-deleted

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6. Analysis of the Oleic Acid Content and Mutation of FAD2 Gene Sequence in [ ] Seeds. CBI-deleted

T_1 soybean plants that lacked the inserted gene were self-crossed, and the seeds were secured through self-crossbreeding. This process was carried out up to the [CBI-deleted
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Figure 1. A schematic diagram of CRISPR/Cas9 vector for editing the soybean FAD2 gene; pPZP-FAD2-7.

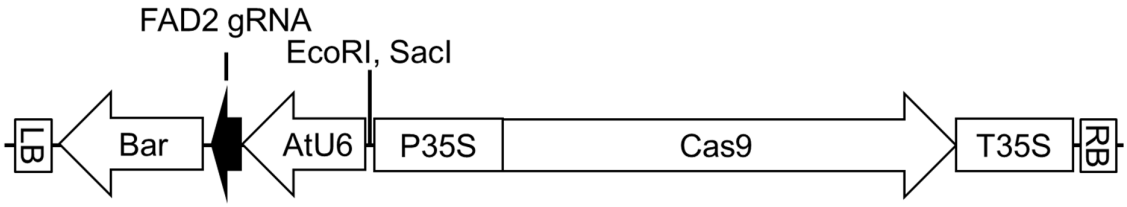


Figure 2. The developmental processes of soybean transformed plants prepared by the knockout of a FAD2 gene using pPZP-FAD2-7[14]. (a) Seed explants on co-cultivation medium (CCM), a-1: one day after inoculation with *Agrobacterium*, a-2: 5 days after inoculation, (b) Shoot induction medium (SIM) without DL-phosphinothricin (PPT), (c) SIM containing PPT 10 mg/L for Bar selection, (d) Shoot elongation medium containing PPT 5 mg/L, (e) Rooting in Rooting induction medium (RIM), (f) Acclimatized transformed plant in a small pot, (g) transformed plant in a big pot, and (h) PPT leaf painting, h-1: non-transformed plant (sensitive), h-2: transformed plant (resistant).

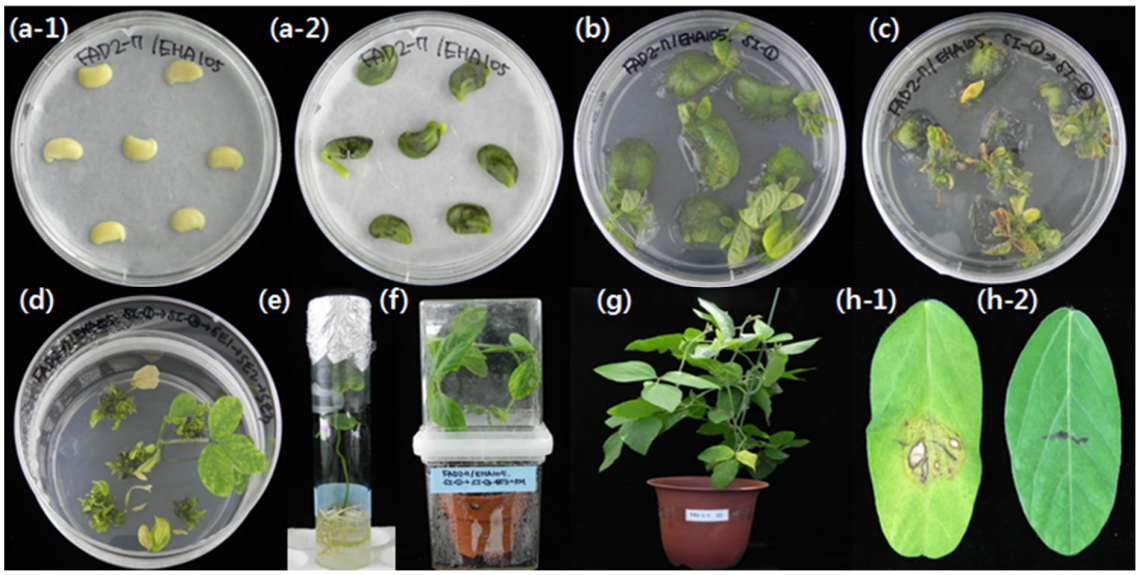


Figure 3. T_0 transformants obtained from the regeneration process. A total of nine soybean transformants were secured by PPT resistance screening.

Figure 4. PCR results for confirming insert genes in T_0 transformants. (A) Indication of designed PCR products: Bar (sequence from 12,968 bp to 13,375 bp), Cas9-① (sequence from 1,464 bp to 2,587 bp), Cas9-② (sequence from 2,304 bp to 4,380 bp), Cas9-③ (sequence from 4,185 bp to 5,633 bp), and gRNA (sequence from 85 bp to 356 bp). (B) The PCR results of each T_0 transformant; NT is *Glycine max L.* Kwangan (control).

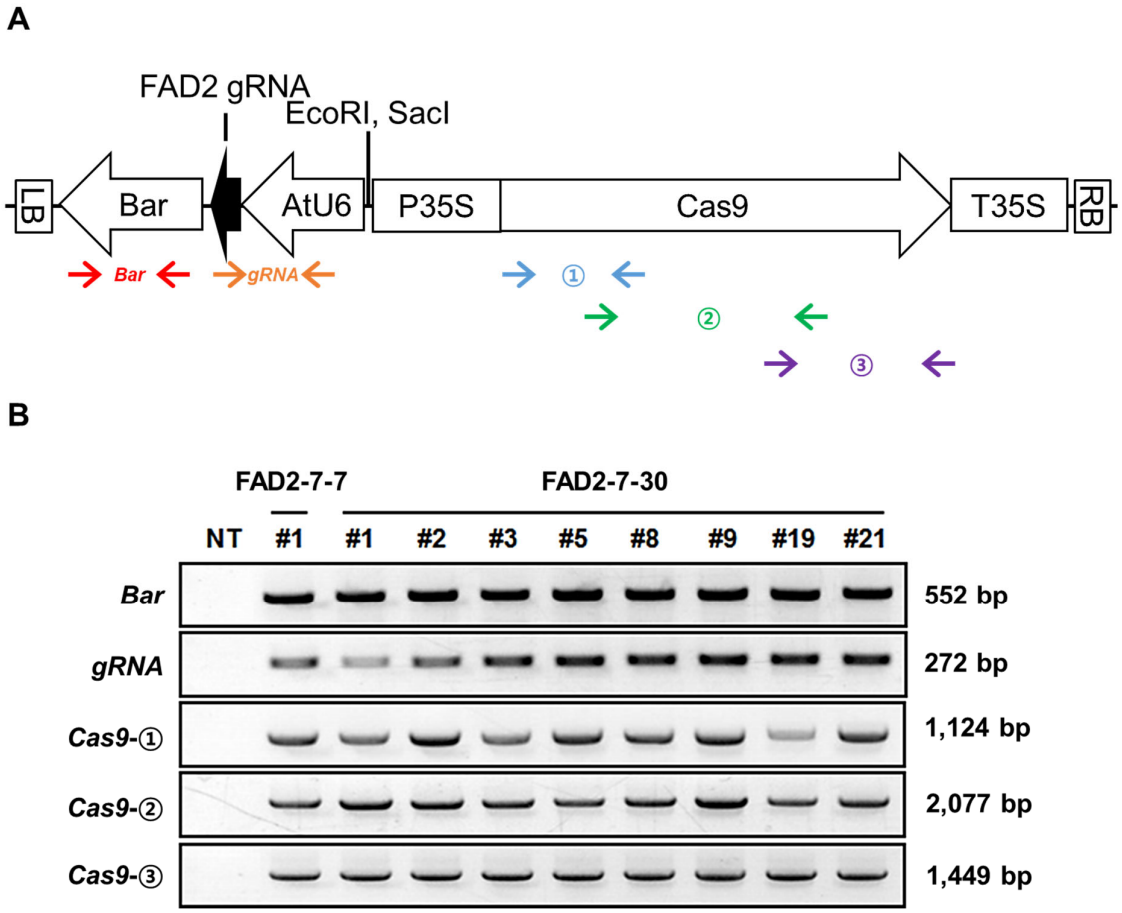


Figure 5. PCR analysis to confirm the removal of genes in pPZP-FAD2-7 from T_1 transformants. PCR products indicate the presence of genes in the pPZP-FAD2-7 vector. NT is *Glycine max L.* Kwangan; PC is positive control (pPZP-FAD2-7 vector).

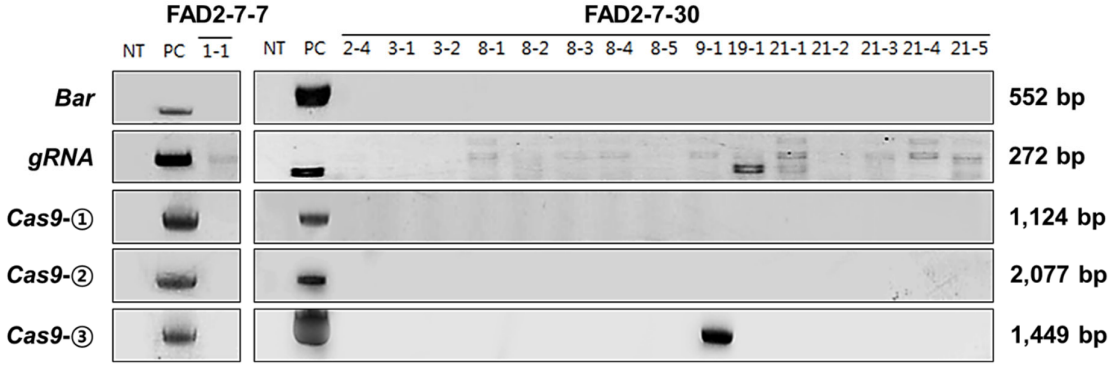
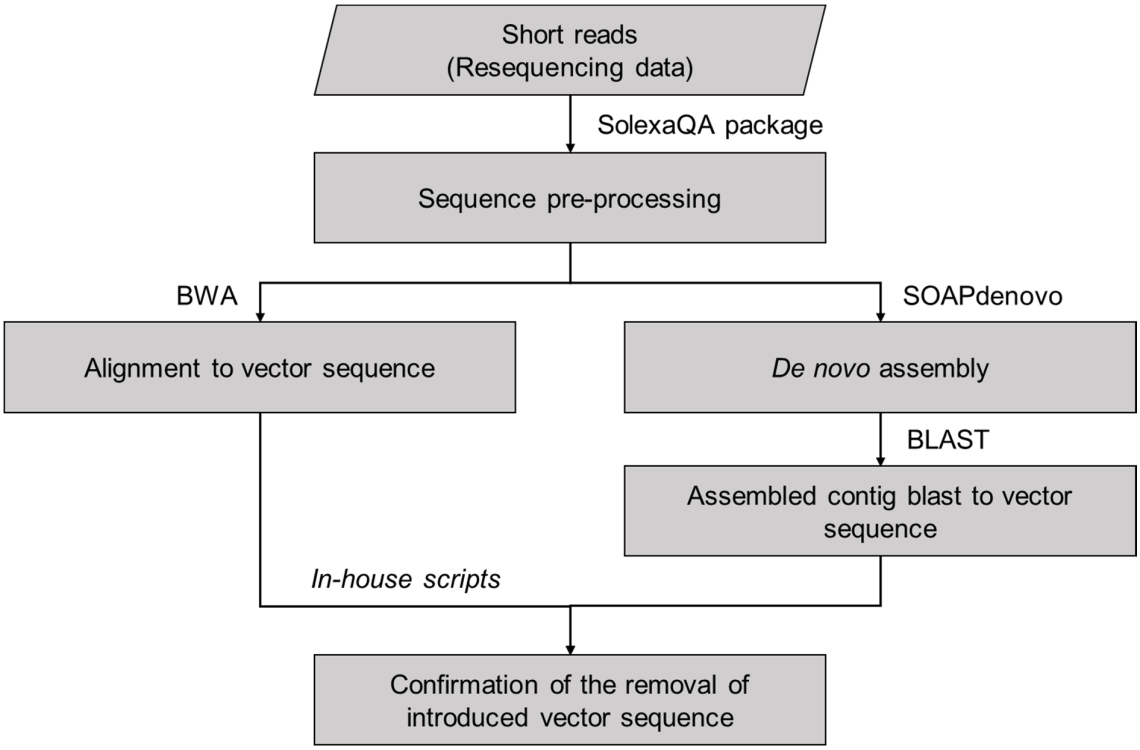


Figure 6. A Schematic diagram of pre-processing and WGS analysis. The DynamicTrim and LengthSort programs from the SolexaQA (v.1.13) package were used to remove PCR duplicate reads and later for quality trimming[15]. Sequencing data was refined using DynamicTrim to cut the bad-quality bases at both ends of the sequences with a phred score below 20. After that, in order to obtain high-quality clean reads, short-read sequences from the DynamicTrim process of length shorter than 25 bp were removed using LengthSort. After processing the sequencing data, it was analyzed in two ways: 1) Alignment of pre-processed sequences for each sample with pPZP vector sequence using BWA (0.6.1-r104)[16]; 2) BLAST the vector sequence by assembling the short contig of each sample using SOAPdenovo2 (version 2.04)[17]. The data obtained from the two analytical methods were compared and analyzed to confirm whether or not the introduced vector sequence was removed. In this way, we confirmed that plasmid DNA vector sequence does not exist in the [ ] soybean genome. CBI-deleted



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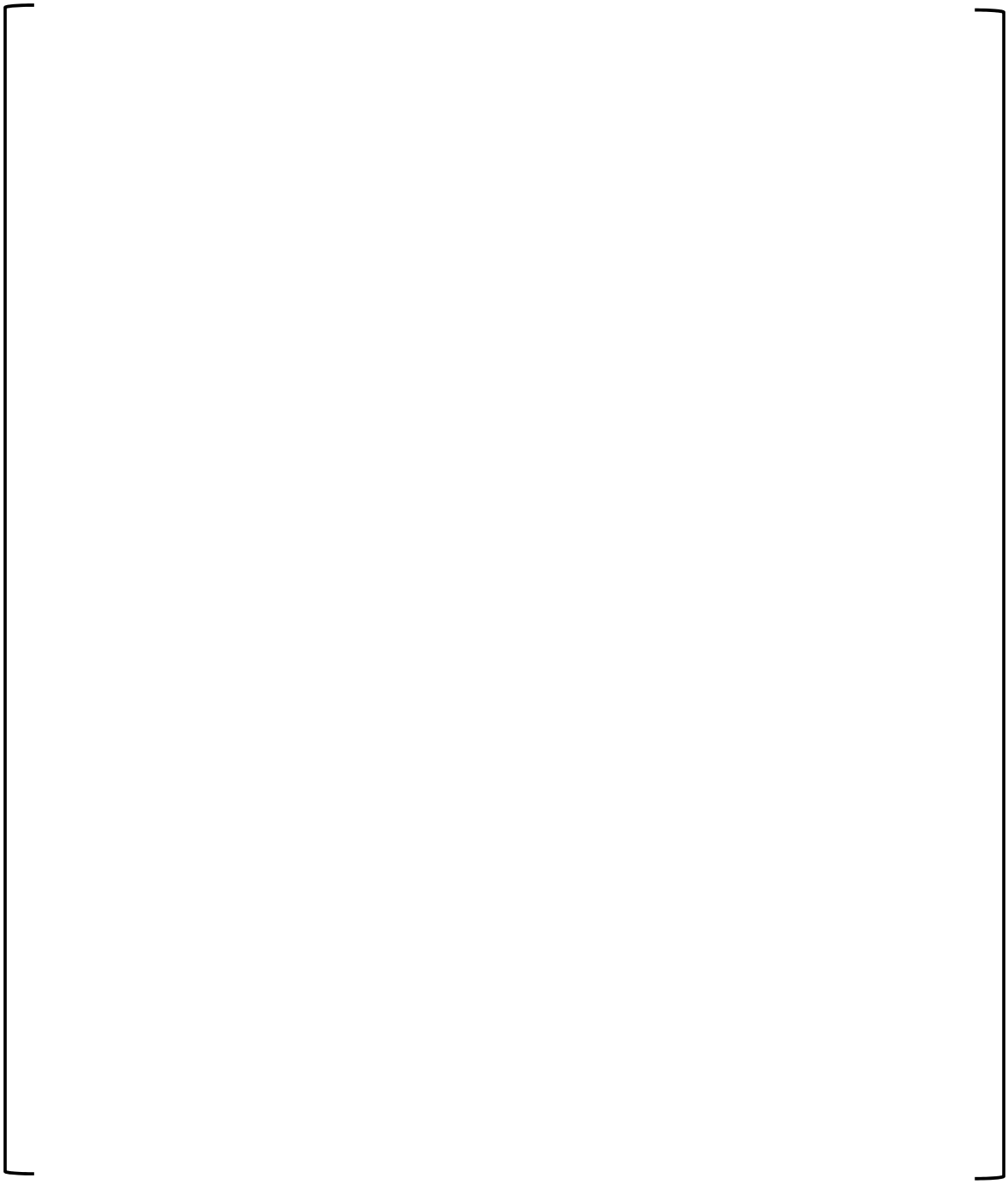

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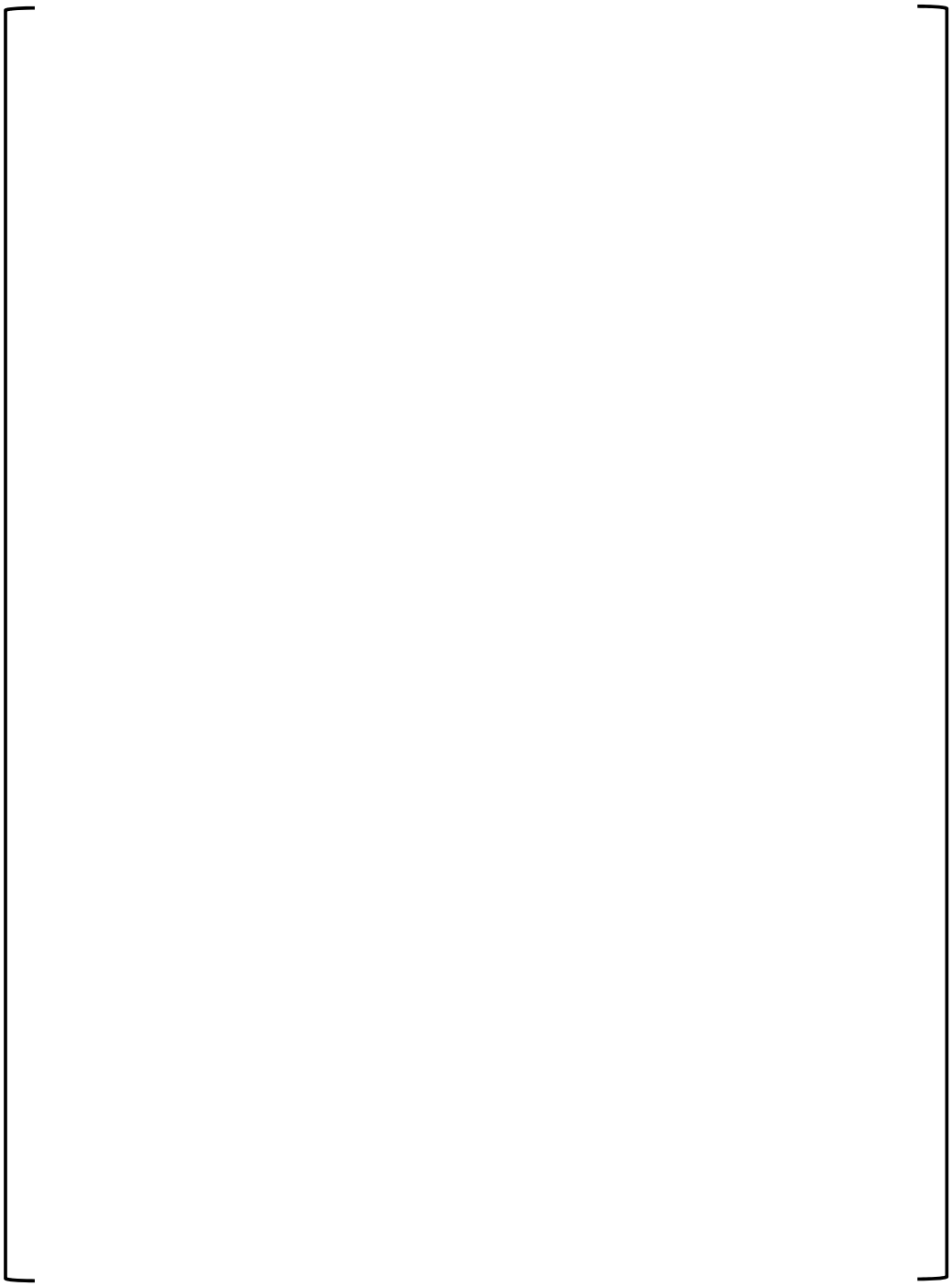

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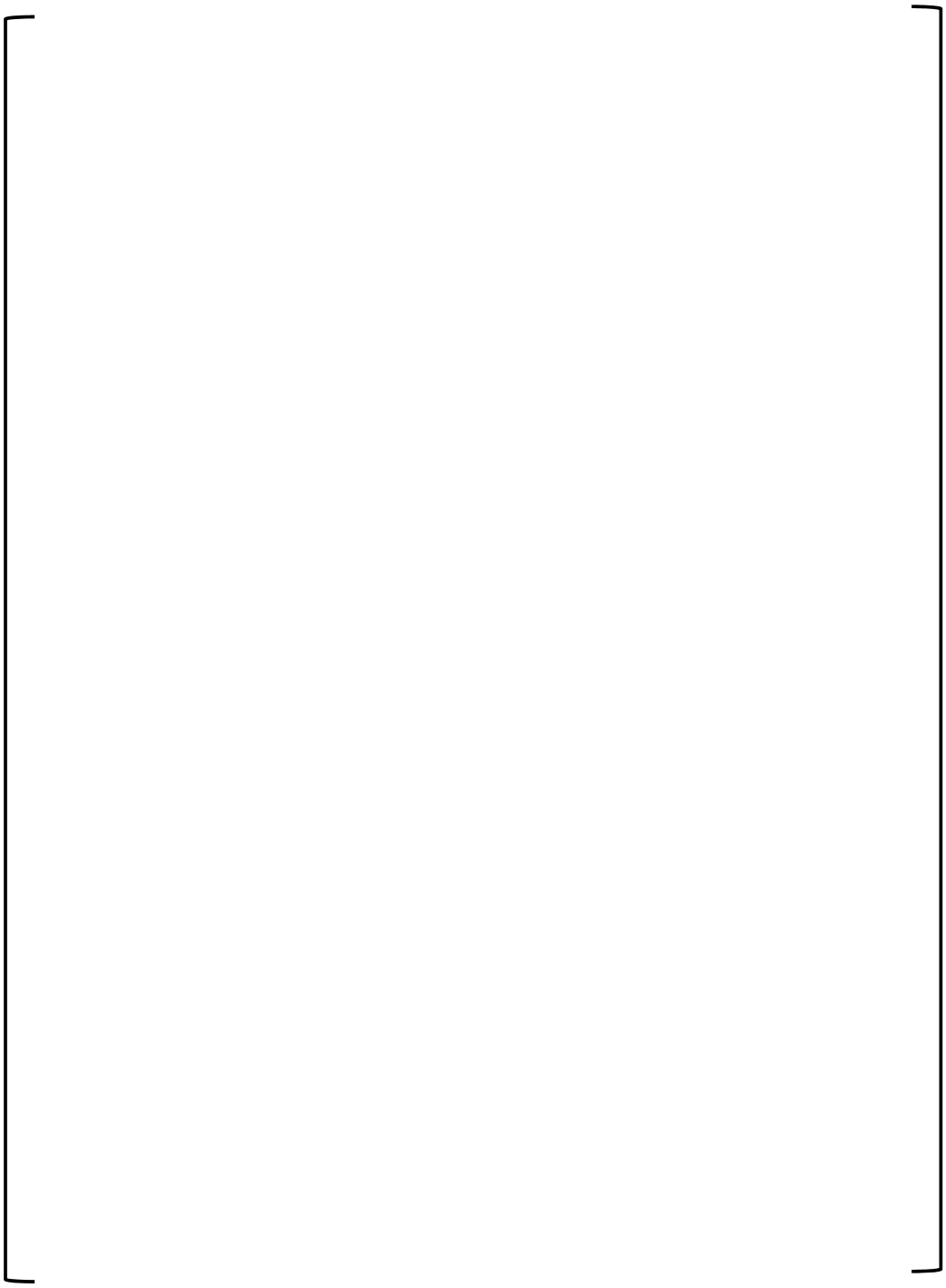

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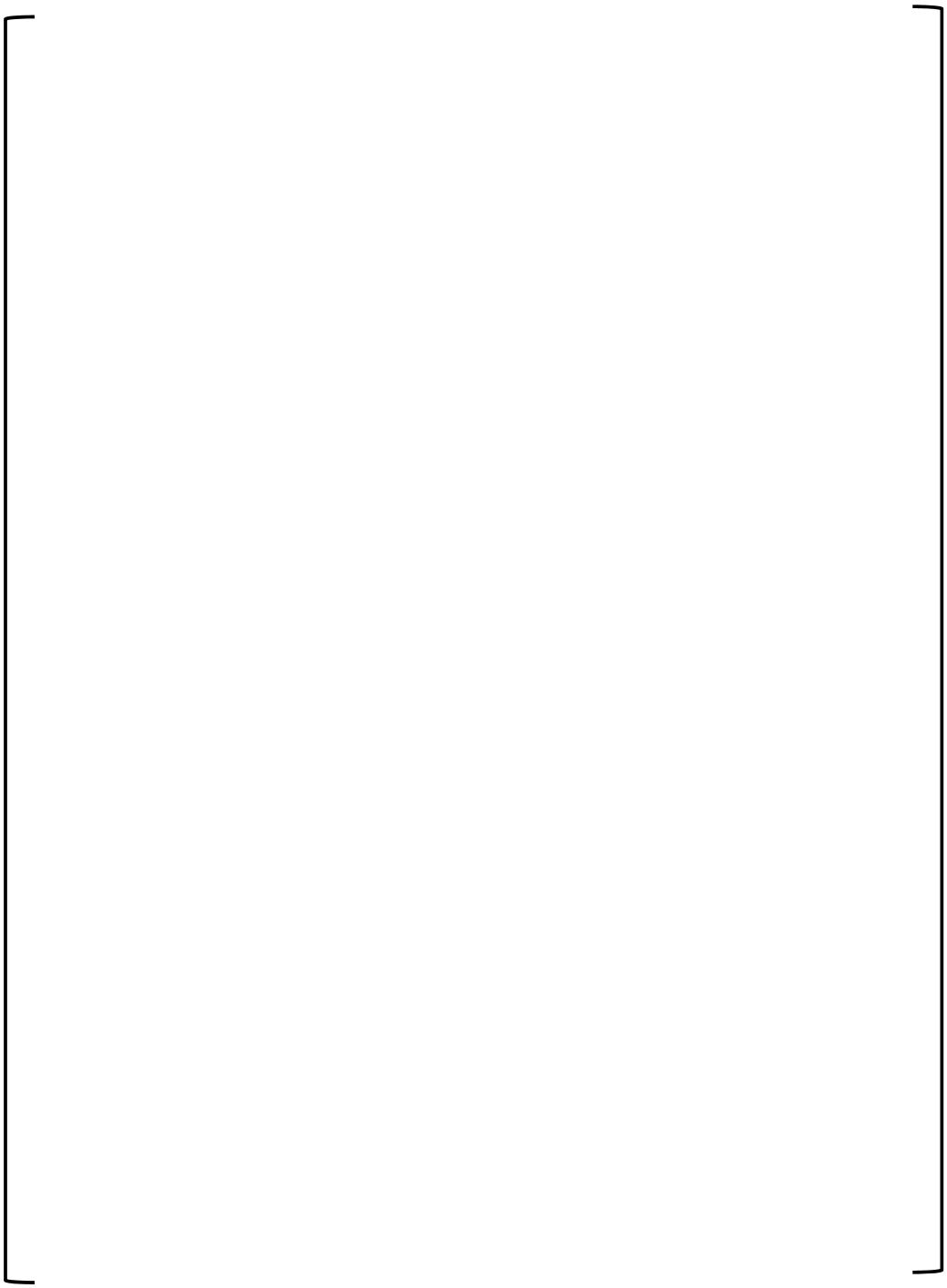

Table 1. FAD2 gene sequence targeted for genome editing.

gRNA name	Target sequence (including PAM)
FAD2 RG7	GTGTTTGGAACCCTTGAGAGAGG (SEQ ID NO: 7)

Table 2. Description of the genetic elements of the vector construct.

Vector	Genetic Element	Source	Function
pPZP-FAD2 vector	LB (Left border repeat of T-DNA)	*Agrobacterium tumefaciens*	Upon recognized by Ti plasmid in *Agrobacterium*, the region between the T-DNA border repeats is transferred to plant cells
	Bar	*Streptomyces hygroscopicus*	Allows plant to be resistant to phosphinothricin
	FAD2 RG7 (sgRNA)	*Streptococcus pyogenes*	FAD2 targeted guide RNA compatible with Cas9 derived from *Streptococcus* pyogenes
	AtU6 promoter	*Arabidopsis thaliana*	RNA Polymerase III promoter of the U6-26 small nuclear RNA gene, drives high-level expression of the gRNA
	P35S promoter	*Cauliflower mosaic virus*	Strong constitutive promoter, drives high-level gene expression in dicot plants
	Cas9	*Streptococcus pyogenes*	Cas9 derived from *Streptococcus* pyogenes, generates double-strand DNA breaks
	T35S terminator	*Cauliflower mosaic virus*	Transcription terminator signal, derived from Cauliflower mosaic virus and terminates the transcription of Cas9 gene
	RB (Right border repeat of T-DNA)	*Agrobacterium tumefaciens*	Upon recognized by Ti plasmid in *Agrobacterium*, the region between the T-DNA border repeats is transferred to plant cells

Table 3. Primers used in PCR analysis.

Use	Name	Sequence
Gene transfer analysis	gRNA forward primer (AtU6 promoter)[a]	5'-GAA TGA TTA GGC ATC GAA CC-3'
	gRNA reverse primer (FAD2 RG7)[a]	5'-AAA CTC CTC AAG GGT TCC AAA CAC-3'
	Bar forward primer	5'-TCC GTA CCG AGC CGC AGG AA-3'
	Bar reverse primer	5'-CCG GCA GGC TGA AGT CCA GC-3'
	Cas9-① forward primer	5'-ATG GAC AAG AAG TAC AGC ATC GGC-3'
	Cas9-① reverse primer	5'-AAC TTG TAG AAC TCC TCC TGG CTG-3'
	Cas9-② forward primer	5'-TTC AGG AAG TCC AGG ATG GTC TTG-3'
	Cas9-② reverse primer	5'-AGA ACT GGA AGT CCT TGC GGA AGT-3'
	Cas9-③ forward primer	5'-CTG AGC GAG CTG GAC AAG GCC GG-3'
	Cas9-③ reverse primer	5'-TTA GGC GTA GTC GGG CAC GTC GTA-3'

[a]gRNA forward primer includes a part of AtU6p and gRNA reverse primer includes a part of FAD2 gRNA.

Table 4. GC (Gas Chromatography) conditions for analyzing fatty acids in soybeans.

Item	Condition
Instrument	Agilent 7890A
Column	0.25 μm i.d. × 30 m DB-FFAP capillary column
Detector	Flame ionization detector
Oven temperature	230 ℃
Injection temperature	210 ℃
Detector temperature	250 ℃
Carrier gas	N_2 (1.5 mL/min)
Injection volume	1 μL

Table 5. Fatty acid contents in soybean T_1 seeds. A dramatic shift occurred in the contents from Linoleic acid to Oleic acid while the oleic acid levels increased.

Transformants number	Contents of fatty acids in T_1 seeds (%)				
	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	α-Linolenic (18:3)
FAD2-7-7 #1	7.8 8.3 8.4	2.6 2.6 2.6	83.0 79.8 78.6	2.1 3.5 3.8	4.6 5.8 6.5
FAD2-7-30 #1	8.6 8.1	2.2 2.0	78.7 80.1	4.1 2.7	6.4 7.1
FAD2-7-30 #2	8.2 8.2 8.2	2.9 2.9 3.1	79.4 82.6 78.4	7.0 1.8 4.3	5.5 4.5 5.9
FAD2-7-30 #3	8.1 8.7	3.0 3.3	80.9 76.9	2.5 4.3	5.5 6.8
FAD2-7-30 #5	8.4 10.1 8.8	3.2 3.0 2.9	79.1 73.4 74.4	3.3 5.9 6.0	6.0 7.7 7.9
FAD2-7-30 #8	8.2 10.1 8.8	3.3 3.1 3.1	82.3 83.3 83.1	1.7 1.6 1.5	4.5 4.3 5.1
FAD2-7-30 #9	8.0 8.3 7.8	3.1 3.1 3.8	80.1 78.7 82.4	3.9 3.5 1.6	4.9 6.4 4.5
FAD2-7-30 #19	8.5 8.5	3.4 2.9	75.2 75.5	6.3 6.1	6.7 7.1
FAD2-7-30 #21	7.8 9.2 8.1	2.9 3.0 2.9	82.4 70.9 79.6	2.5 7.5 3.3	4.3 9.4 6.0
Pungsan	10.6	3.6	23.5	52.3	10.0
Kwangan	12.5	3.4	38.2	36.2	9.5

Table 6. The indel frequency of FAD2 genes in T_1 soybean plants edited using CRISPR/Cas9. As a result, genome editing mutations induced deletion and insertion into a target site in the FAD2 gene in chromosome #10 (chr10) and chromosome #20 (chr20) of T_1 transformants. The colored lines are transformants that contain over 99% deletions in both chromosomes.

	T_1 transformants	Total reads	Insertions	Deletions	Indel frequency (%)
Chr 10	FAD2-7-7 #1-1	11,252	0	95	95 (0.8%)
	FAD2-7-30 #2-4	13,523	0	13,410	13,410 (99.2%)
	FAD2-7-30 #3-1	18,132	0	70	70 (0.4%)
	FAD2-7-30 #3-2	14,784	0	14,771	14,771 (99.9%)
	FAD2-7-30 #8-1	29,358	0	29,327	29,327 (99.9%)
	FAD2-7-30 #8-2	28,970	0	27,798	27,798 (96%)
	FAD2-7-30 #9-1	18,012	11	9,438	9,449 (52.5%)
	FAD2-7-30 #19-1	21,332	0	21,318	21,318 (99.9%)
	FAD2-7-30 #21-2	22,833	0	22,818	22,818 (99.9%)
	FAD2-7-30 #21-5	26,990	0	26,981	26,981 (100.0%)
	FAD2-7-30 #22-5	19,492	0	19,483	19,483 (100.0%)
	FAD2-7-30 #22-6	26,485	0	26,454	26,454 (99.9%)
Chr 20	FAD2-7-7 #1-1	22,943	0	22,931	22,931 (99.9%)
	FAD2-7-30 #2-4	23,466	0	11,463	11,463 (48.8%)
	FAD2-7-30 #3-1	24,145	0	73	73 (0.3%)
	FAD2-7-30 #3-2	25,790	18	25,742	25,760 (99.9%)
	FAD2-7-30 #8-1	19,141	22	19,111	19,133 (100.0%)
	FAD2-7-30 #8-2	26,126	54	26,063	26,117 (100.0%)
	FAD2-7-30 #9-1	23,439	23,294	129	23,423 (99.9%)
	FAD2-7-30 #19-1	15,524	7	15,511	15,518 (100.0%)
	FAD2-7-30 #21-2	18,724	5	18,719	18,724 (100.0%)
	FAD2-7-30 #21-5	23,165	2	23,163	23,165 (100.0%)
	FAD2-7-30 #22-5	4,582	2	4,580	4,582 (100.0%)
	FAD2-7-30 #22-6	3,360	0	3,360	3,360 (100.0%)

Table 7. Targeted deep sequencing results of FAD2 genes. The target sequences including PAM are marked in green, and the insertion nucleotides are marked in red. The colored FAD2-7-30 #21-2 lines were chosen for further study.

	T_1 transformants	Indel	Deletion and insertion in local sequence	
Chr 10	WT	-	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTCTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-7 #1-1	-	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTCTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #2-4	-8	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC--------TCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #3-1	-	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTCTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #3-2	-6	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC------GTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #8-1	-7	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC-------TTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #8-2	-2	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC--AAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #9-1	-7	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC-------TTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #19-1	-8	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC--------TCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #21-2	[	]	CBI-deleted
		[	]	CBI-deleted
	FAD2-7-30 #21-5	-2	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC--AAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #22-5	-2	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC--AAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
	FAD2-7-30 #22-6	-2	AAAGTGGAAGTTCAAGGGAAGAAGCCTCTC--AAGGGTTCCAAACACAAAGCCACCATTCACTGTGGCCAA	
Chr 20	WT	-	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-7 #1-1	-4	AAAGTTGAAATTCAGCAGAAGAAGCCTCTC----GGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #2-4	-	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #3-1	-	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #3-2	-2	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCT–AGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #8-1	-2	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCT–AGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #8-2	-2	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCT–AGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #9-1	+1	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCTTCAAGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #19-1	-3	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCT---GGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #21-2	[	]	CBI-deleted
	FAD2-7-30 #21-5	-11	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCT----------AACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #22-5	-10	AAAGTTGAAATTCAGCAGAAGAAGCCTC----------TCCAAACACAAAGCCACCATTCACTGTTGGCCA	
	FAD2-7-30 #22-6	-11	AAAGTTGAAATTCAGCAGAAGAAGCCTCTCT----------AACACAAAGCCACCATTCACTGTTGGCCA	

Table 8. [ ]

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Table 9. [ ]

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Table 10. [ ]

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Table 13. [

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Table 14. [ ]

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Table 15. [

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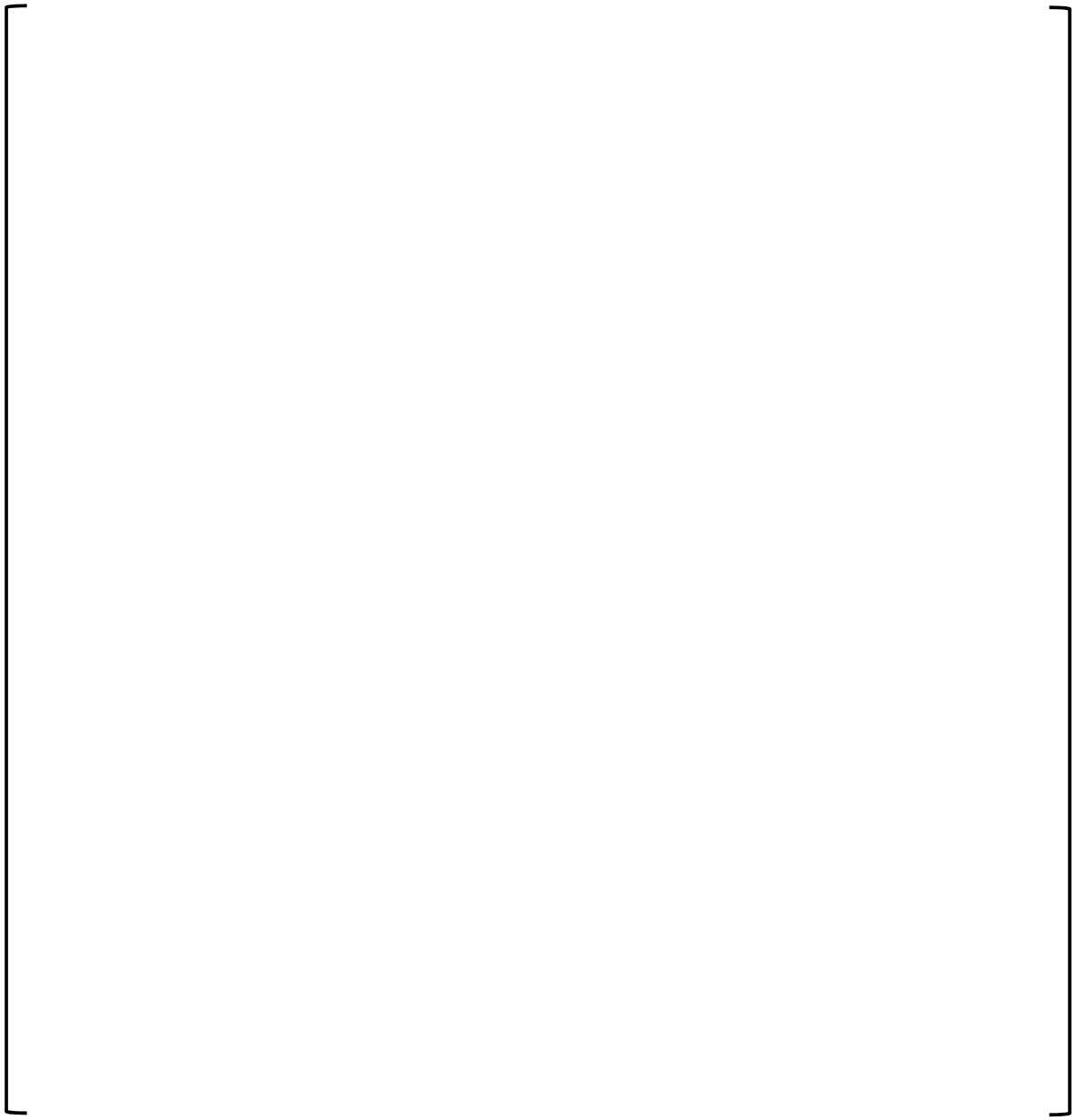

Soybean is Not a Regulated Article

Soybean is not a federal noxious weed pursued by 7 CFR 360.

No Plant Pest Risk or Increased Weed Potential

Since no foreign DNA was introduced during genome editing and genetic transformation, the high oleic soybean have no increased potential for plant pest risk or weed growth.

Conclusion

The CRISPR/Cas9 cassette was used to target the FAD2 gene involved in soybean fatty acid control. The gene expressing cassette for genome editing, including Cas9, FAD2-targeting guide RNA (FAD2-7), and selectable Bar gene was delivered to soybean plants through *Agrobacterium tumefaciens*. Subsequently, small deletions were successfully induced, and the FAD2 genes were knocked out by the introduced CRISPR/Cas9 system, and T_1 transgenic soybean seeds with a high content of oleic acid were selected. After that, through conventional breeding up to the [ ], we confirmed that 1) the introduced CRISPR/Cas9 expression cassette was removed from the genome-edited soybean, and 2) the [ ] soybean does not contain any foreign DNA sequences. CBI-deleted CBI-deleted CBI-deleted

In order to facilitate further studying and business process, ToolGen respectfully requests confirmation from BRS that the fatty acid content-modified soybean line described does not meet the definition of a regulated article under 7 CFR, Part 340.

References

1. Okuley J, Lightner J, Feldmann K, Yadav N, Lark E. Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell.* 1994;6(1):147-158.
2. Beló A, Zheng P, Luck S, et al. Whole genome scan detects an allelic variant of fad2 associated with increased oleic acid levels in maize. *Molecular Genetics and Genomics.* 2008;279(1):1-10.
3. Pham A-T, Lee J-D, Shannon JG, Bilyeu KD. Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with the high oleic acid seed oil trait. *BMC plant biology.* 2010;10(1):195.
4. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature.* 2014;513(7519):569-573.
5. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *science.* 2012;337(6096):816-821.
6. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology.* 2014;32(4):347.
7. Millas R, Espina M, Ahmed CS, et al. Detection of novel allelic variations in soybean mutant population using Tilling by Sequencing. *bioRxiv.* 2019:711440.
8. Kim H-J, Ha B-K, Ha K-S, et al. Comparison of a high oleic acid soybean line to cultivated cultivars for seed yield, protein and oil concentrations. *Euphytica.* 2015;201(2):285-292.
9. Nacry P, Camilleri C, Courtial B, Caboche M, Bouchez D. Major chromosomal rearrangements induced by T-DNA transformation in Arabidopsis. *Genetics.* 1998;149(2):641-650.
10. Clark KA, Krysan PJ. Chromosomal translocations are a common phenomenon in Arabidopsis thaliana T-DNA insertion lines. *The Plant Journal.* 2010;64(6):990-1001.
11. Jupe F, Rivkin AC, Michael TP, et al. The complex architecture and epigenomic impact of plant T-DNA insertions. *PLoS genetics.* 2019;15(1).
12. Seo J-D, Jo H, Kim M, Song JT, Lee J-D. Agronomic Traits and Forage Production in a Mixed-Planting with Corn for Forage Soybean Cultivars, Chookdu 1 and Chookdu 2. *Plant Breeding and Biotechnology.* 2019;7(2):123-131.

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14. Kim M-J, Kim JK, Kim HJ, et al. Genetic modification of the soybean to enhance the β-carotene content through seed-specific expression. *PLoS One.* 2012;7(10).
15. Cox MP, Peterson DA, Biggs PJ. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC bioinformatics.* 2010;11(1):485.
16. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics Oxf Engl 25: 1754–1760. 2009.
17. Luo R, Liu B, Xie Y, et al. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience.* 2012;1(1):2047-2217X-2041-2018.

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