agronomy

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Combining a Mutant Allele of *FAD2-1A* with *HD* Improves the ω-6/ω-3 Ratio in Soybeans

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Abstract: The intake of foods with unbalanced ω-6/ω-3 ratios causes various health problems. Commodity soybeans generally have a ω-6/ω-3 ratio of 6–7:1. The recommended ratio in terms of health benefits is <4:1. This study aimed to identify the appropriate combination of mutant alleles that can reduce the ω-6/ω-3 ratio using three segregating soybean populations. F_2 individuals from each population were genotyped for three different alleles of microsomal delta-12 fatty acid desaturase 2 enzyme (*FAD2-1A*) and an allele of homeodomain-like transcriptional regulator (*HD*) genes, and their five major fatty acids were assessed. F_2 seeds carrying both *fad2-1a* and *hd* had slightly different ω-6/ω-3 ratios according to the different *fad2-1a* alleles. The *fad2-1a*$_{DEL}$, *fad2-1a*$_{S117N}$, and *fad2-1a*$_{W293STOP}$ alleles combined with a *hd* allele resulted in ω-6/ω-3 ratios with a range of 1.9–2.7:1, 2.7–3.9:1, and 2.6–3.6:1 in soybean seeds, respectively. This study revealed that the induction of mutations in *FAD2-1A*$_{DEL}$ and *HD* was the most efficient strategy to improve the ω-6/ω-3 ratio and elevate the ω-3 fatty acid concentrations in soybean seeds. These results provide useful information in soybean breeding programs to release a new soybean cultivar with a lower ω-6/ω-3 ratio and elevated ω-3 fatty acids, which can be a beneficial ingredient for soybean-based foods.

Keywords: α-linoleic acid; *FAD2-1*; homeodomain transcription factor; soybean food; ω-6/ω-3 ratio

Citation: Kim, H.; Jo, H.; Lee, J.-D. Combining a Mutant Allele of *FAD2-1A* with *HD* Improves the ω-6/ω-3 Ratio in Soybeans. *Agronomy* **2023**, *13*, 913. https://doi.org/10.3390/agronomy13030913

Academic Editor: Hengyou Zhang

Received: 25 February 2023
Revised: 17 March 2023
Accepted: 17 March 2023
Published: 19 March 2023

1. Introduction

Soybeans [*Glycine max* (L.) Merr.] are cultivated in many countries because of their seed compositions with ~40% protein and ~20% oil [1]. Soybeans are used not only as animal feed and a source of oil but also as an ingredient in healthy soybean-based foods, such as soybean paste, soybean sprout, soy sauce, tofu, and soy milk. Soybeans contain 11% palmitic acid, 4% stearic acid, 23% oleic acid, 54% linoleic acid (ω-6), and 8% α-linoleic acid (ω-3). Among these fatty acids (FAs), ω-6 and ω-3 are important FAs that humans must consume from foods, as mammals do not express enzymes for FA biosynthesis which insert double bonds at positions 3 and 6 from the methyl end of the carbon chain. Thus, humans and animals cannot synthesize ω-3 and ω-6 FAs by themselves [2–4]. The ω-3 FAs in plants or crops mostly exists as α-linolenic acid which is a precursor of EPA and DHA. Many studies have demonstrated the health benefits of ω-3 FAs, such as in the context of coronary heart disease and brain development [5–11].

Previous studies have revealed that an unbalanced consumption of a ω-6/ω-3 ratio can cause cardiovascular, cerebrovascular, lung diseases, and cancer [12–14]. Therefore, a balanced intake of ω-6/ω-3 FAs is important for human health. In fact, the intake ratio of ω-6 and ω-3 FAs was ~1:1 for *Homo sapiens* in the past [15]. Currently, the World Health Organization announced that the recommended ω-6/ω-3 ratio for human diets is <4:1 [16]. Because of westernized eating habits, individuals consume a severely disproportionate ω-6/ω-3 ratio, i.e., ~10–20:1 [15]. Commodity soybean oil contains an ω–6/ω-3 ratio of

approximately 6–7:1. Therefore, it is necessary to adjust this ratio to <4:1 in soybean seeds for health benefits and their consumption as food.

Previous studies have shown that the microsomal delta-12 FA desaturase 2 (*FAD2*) and microsomal ω-3 FA desaturase (*FAD3*) genes are specifically involved in FA biosynthesis, especially that of unsaturated FAs, in plants. Mutations in either *FAD2-1A* (*Glyma.10g278000* in W82.a2.v1) or *FAD2-1B* (*Glyma.20g111000*) increase the oleic acid concentration by ~30–40% while decreasing that of ω-6 FAs [17–22]. The PE529 EMS mutant line, which carries a nonsense mutation in *FAD2-1A* (*fad2-1a*$_{W293STOP}$), produces about 45% oleic acid [18], whereas the M23 line with a deletion of *FAD2-1A* (*fad2-1a*$_{DEL}$) produces about 46% oleic acid [22] and the 17D line with a missense mutation (*fad2-1*$_{S117N}$) produces about 35% oleic acid [17]. In addition, PI283327, with a missense mutation in *FAD2-1B*, produces about 30% oleic acid [19–21]. Regarding ω-3 FAs, mutations occurring in the homeodomain-like transcriptional regulator (*HD*, *Glyma.05g221500*) gene, a transcription factor-encoding gene that may affect the expression level of *FAD3* in soybeans, increase the levels of ω-3 FAs by up to 14% [23].

The ω-6/ω-3 ratio in cultivated soybeans is ~6–7:1 [24,25]. However, the ω-3 FA level in wild soybeans (*Glycine soja* Sieb. and Zucc.) is almost two-fold higher than that in cultivated soybeans, resulting in a ω-6/ω-3 ratio of ~4:1 [24–28]. Several studies have reported quantitative trait loci (QTLs) that control ω-3 FA production in wild soybeans; in wild soybean lines such as Hidaka4, PI483463, and ZYD00463, multiple QTLs were found to be associated with ω-3 FAs, as assessed through genetic analyses [27,29,30]. These studies suggest that the high levels of ω-3 FAs in wild soybeans are controlled by polygenes. A previous study proposed that the ω-6/ω-3 ratio in soybean seeds was improved via the utilization of the *FAD2-1A*$_{DEL}$ allele together with ω-3 FA QTLs from wild soybeans [28]. In addition, Jo et al. [23] reported that a new system for lower ω-6/ω-3 ratios could be developed using two alleles from either *fad2-1a*$_{S117N}$ or *fad2-1b* with *hd* from EMS-mutated *G. max*. These systems can produce a lower ω-6/ω-3 ratio accompanied by elevated ω-3 FAs in soybean seeds.

The concentration of oleic acid in soybean genotypes containing mutated *FAD2-1* was increased at the expense of ω-6 FAs, resulting in a lower ratio of ω-6 to ω-3 FAs in soybean seeds [21,31]. In addition, elevating ω-6 FAs in soybean seeds is another strategy that can be used to decrease the ω-6/ω-3 ratio. To develop a new soybean cultivar with a lower ω-6/ω-3 ratio and elevated ω-3 FAs, the interaction of different allele combinations must be determined and optimized. Therefore, this study aimed to evaluate the ω-6/ω-3 ratio in soybean seeds with different combinations of three *fad2-1a* mutant alleles combined with the *hd* allele to determine the optimal allele combinations to produce an improved ω-6/ω-3 ratio with an elevated ω-3 FA concentration. We developed three different populations that segregated different alleles of *FAD2-1A* and an *hd* allele.

2. Materials and Methods

2.1. Plant Materials and Experimental Environment

To assess ω-6/ω-3 ratios, three different segregating soybean populations were developed, and the parental lines were crossed at the affiliated field of Kyungpook National University (KNU), Gunwi, Republic of Korea (36°06′45″ N, 128°38′34″ E) during the summer of 2021. The original source of the *fad2-1a*$_{W293STOP}$ allele was the PE529 line [18]. The S08-14719 line is a high-oleic-acid genotype that produces ~80% oleic acid and carries the *fad2-1a*$_{DEL}$ allele from M23 and *fad2-1b* allele from PI283327 [19]. Hosim is a high-oleic-acid cultivar that combines the *fad2-1a*$_{S117N}$ allele from 17D with the *fad2-1b* allele [32]. The JD17-20-2-39 breeding line has an elevated ω-3 FA level and is the source of a mutant allele of the homeodomain-like transcriptional regulator (*HD*) gene from the PE2166 line [23]. We developed populations 1, 2, and 3 from PE529 and JD17-20-2-39, S08-14719 and JD17-20-2-39, and Hosim and JD17-20-2-39, respectively (Table 1). Each population yielded an F_1 seed during the autumn of 2021. F_2; seeds were produced in the greenhouse of the KNU, Daegu, Republic of Korea (35°53′42″ N, 128°36′45″ E) during the winter of 2021–2022. In this

experiment, 96, 305, and 380 F_2 seeds of PE529 × JD17-20-2-39, S08-14719 × JD17-20-2-39, and Hosim × JD17-20-2-39 were used for genotype and phenotype determinations.

Table 1. Nomenclature, *FAD2-1A*, *FAD2-1B* and *HD* allele status, and soybean population information.

Name	Genotype			Fatty Acid Profile	Note	Reference
	***FAD2-1A* (*Glyma.10g278000*)**	***FAD2-1B* (*Glyma.20g111000*)**	***HD* (*Glyma.05g221500*)**			
PE529	*fad2-1a*$_{W293STOP}$	*FAD2-1B*	*HD*	Elevated oleic acid	Parent 1 (P1)	Jo et al. [18]
S08-14719	*fad2-1a*$_{DEL}$	*fad2-1b*	*HD*	High oleic acid	Parent 2 (P2)	Pham et al. [19]
Hosim	*fad2-1a*$_{S117N}$	*fad2-1b*	*HD*	High oleic acid	Parent 3 (P3)	Lee et al. [32]
JD17-20-2-39	*FAD2-1A*	*FAD2-1B*	*Hd*	Elevated ω-3	Parent 4 (P4)	Jo et al. [23]
Population 1	*FAD2-1A*/*fad2-1a*$_{W293WTOP}$		*HD*/*hd*		P1 × P4	
Population 2	*FAD2-1A*/*fad2-1a*$_{DEL}$		*HD*/*hd*		P2 × P4	
Population 3	*FAD2-1A*/*fad2-1a*$_{S117N}$		*HD*/*hd*		P3 × P4	

FAD2-1A indicates functional microsomal delta-12 fatty acid desaturase 2 gene which is similar to Williams 82 reference sequence for *Glyma.10g278000*. *fad2-1a*$_{W293STOP}$, *fad2-1a*$_{DEL}$, and *fad2-1a*$_{S117N}$ indicate the mutant alleles of *FAD2-1A*. *FAD2-1B* indicates functional microsomal delta-12 fatty acid desaturase 2 gene which is similar to the reference sequence for *Glyma.20g111000*. *fad2-1b* indicates the mutant alleles of *FAD2-1B*. *HD* indicates functional homeodomain-like transcriptional regulator which is similar to the reference sequence for *Glyma.05g1221500*. *hd* indicates the mutant alleles of *HD*.

2.2. FA Analysis and Genomic DNA Extraction

FA profiles of each F_2 seed were determined using gas chromatography (GC) with the Agilent Series 7890A capillary gas chromatographer (Agilent Technologies Inc., Wilmington, DE, USA) equipped with an ionization detector. Soybean oil was extracted from crushed seeds and then, those seeds were placed in 700 µL of an extracting solution (chloroform/hexane/methanol [8:5:2, *v/v*]) for 12 h, and 75 µL of a methylation reagent consisting of methanol methoxide/petrolether/ethyl ether [1:5:2, *v/v*] was added to ~200 µL of extracted solution from previous step. Subsequently, a total of 1 mL of solution was obtained by adding ~725 µL of hexane. The peaks of each FA including, palmitic, stearic, oleic, ω-6, and ω-3 FAs were separated using a DB-FFAP capillary Agilent column (30 m × 0.25 mm, 0.25 µm; Agilent Technologies Inc., Wilmington, DE, USA). The peak areas of each FA were determined according to standard curves of a FA solution (Fame #16, Restek). We isolated genomic DNA from the F_2 seeds of each population according to the manufacturer's protocol of the HiGene Genomic DNA Prep Kit (BioFACT Co., Daejeon, Republic of Korea).

2.3. Development of the HD Genotyping Assay

The SimpleProbe was designed using the LightCycler Probe Design Software 2.0 (version 1, Roche Applied Sciences, Penzberg, Germany) based on the position of mutation. The SNP position of *HD* was determined in our previous study [23]. The *HD* genotyping assay was performed using a 2:5 asymmetric mixture of 0.2 µM forward primer (5′–TGTTTGTGAAGCTCAAATGTTCT–3′) and 0.5 µM reverse primer (5′–CTTCACAATTTCCGGAGACG–3′). Moreover, we used 20 µM 5′–Fluorescein-SPC-AGAAACCTATCCATTTGCAACCTTGG-phosphate–3′ SimpleProbe. The total reaction volume was 20 µL, including genomic DNA, forward and reverse primers, SimpleProbe, titanium taq polymerase buffer, and titanium taq polymerase (BD Biosciences, Palo Alto, CA, USA). The polymerase chain reaction (PCR) was performed on a Roche LightCycler 480 II using a melting curve analysis at 95 °C for 3 min, 60 °C for 20 s, 72 °C for 20 s, and melting temperatures of 45–75 °C. The homozygous allele of *HD* gene showed a peak at 64°C. The homozygous *hd* allele showed a peak at 57 °C. The heterozygous alleles of a *HD* gene produced peaks at both temperatures of 57 °C and 64 °C.

2.4. *FAD2-1A*$_{W293STOP}$ Genotyping Assay

The *FAD2-1A*$_{W293STOP}$ genotype analysis [18] was performed using a 5:2 asymmetric mixture of 0.5 µM forward (5′–CCGTGTTGCAACCCTGAAAG–3′) and 0.2 µM reverse (5′–ACCATGATCGCAACAAGCTG–3′) primers. We also used the 5′–Fluorescein-SPC-

CCAAAGCTCCCTTCAGCCAG-phosphate–3′ SimpleProbe. The PCR was performed on a Roche LightCycler 480 II using a melting curve analysis at 95 °C for 3 min, 60 °C for 20 s, 72 °C for 20 s, and melting temperature from 50 °C to 72 °C to measure fluorescence at every 0.1 °C. The single nucleotide matches or inconsistencies with the SimpleProbe were identified by incorporating fluorescence extinction at different melting temperatures. The homozygous allele of *FAD2-1A* gene showed a peak at 64 °C. The homozygous *fad2-1a*$_{W293STOP}$ alleles produced a peak at 57 °C. The heterozygous alleles of a *FAD2-1A*$_{W293STOP}$ gene showed peaks at both temperatures of 57 °C and 64 °C.

2.5. *FAD2-1A*$_{DEL}$ Genotyping Assay

The *FAD2-1A*$_{DEL}$ genotype analysis [19] was performed using a 1:1:1:1 primer mixture as follows: 5′–ATCTTTAGATTTTTCACTACCTGGTTTAAAATTGAGGGATTG–3′, 5′–CTTTGCTAGACCCTGTGTCAAAGTATAAAC–3′, 5′–AACGTGAATGGGAATAGTTGC–3′, and 5′–GTATTCGATCTGCGAATGCC–3′. We used 2.5 µM Eva Green. The analysis was performed on a Roche LightCycler 480 II using a melting curve analysis at 95 °C for 5 min, 64 °C for 20 s, 72 °C for 20 s, and melting temperatures from 70 °C to 85 °C to measure fluorescence at every 0.1 °C. The homozygous allele of *FAD2-1A*$_{DEL}$ showed a peak at 77 °C. The homozygous *fad2-1a*$_{DEL}$ alleles produced two peaks at 79 °C and 82 °C. All peaks at three temperatures were showed for heterozygous alleles of *FAD2-1A*$_{DEL}$.

2.6. *FAD2-1A*$_{S117N}$ Genotyping Assay

The *FAD2-1A*$_{S117N}$ genotype analysis [20] was performed using a 5:1 asymmetric mixture of 0.5 µM forward primer (5′–CCAAGGTTGCCTTCTCACTGGT–3′) and 0.2 µM reverse primer (5′–TAGGCCACCCTATTGTGAGTGTGAC–3′). Moreover, we used 20 µM 5′–Fluorescein-SPC-GTACTTGCTGAAGGCATGGTGA-phosphate–3′ SimpleProbe. The PCR was performed on a Roche LightCycler 480 II using a melting curve analysis at 95 °C for 3 min, 65 °C for 20 s, 72 °C for 30 s, and melting temperatures from 50 °C to 68 °C. The homozygous functional allele of *FAD2-1A*$_{S117N}$ showed a peak at 62 °C. The homozygous *fad2-1a*$_{S117N}$ alleles produced a peak at 54 °C, and heterozygous alleles showed peaks at both temperatures of 54 °C and 62 °C.

2.7. *FAD2-1B* Genotyping Assay

The *FAD2-1B* genotype analysis [19–21] was performed using a 5:2 asymmetric mixture of 0.5 µM forward primer (5′–GGTTCTCCAAGGTTGCATTCTTACT–3′) and 0.2 µM reverse primer (5′–AGGGTTGTTCAGGTACTTGGTGT–3′). Moreover, we used 20 µM 5′–Fluorescein-SPC-AGTCCCTTATTTCTCATGGAAAATAAGC-phosphate–3′ SimpleProbe. The PCR was performed on a Roche LightCycler 480 II using a melting curve analysis at 95 °C for 3 min, 60 °C for 20 s, 72 °C for 20 s, and a melting curve from 50 °C to 68 °C to measure fluorescence at every 0.1 °C. The homozygous allele of *FAD2-1B* was detected at 62.5 °C, marked with a peak. Homozygous *fad2-1b* alleles produced a peak at 56.5 °C, and heterozygous alleles showed peaks at both temperatures.

3. Results

To improve the ω-6/ω-3 ratio of soybean seeds, three different *fad2-1a* alleles were combined with a single *hd* mutant allele to produce three different segregating populations. For population 1, 96 F_2 seeds were produced to segregate the *FAD2-1A*$_{W293STOP}$ and *HD* alleles from a cross between the PE529 and JD17-20-2-39 lines. Plants from the JD17-20-2-39 breeding line were found to carry a mutant *HD* allele, and the soybean seeds from these plants contained 16.6 ± 1.2% ω-3 FA. The phenotypic distribution of each FA is presented in Figure S1. The F_2 seeds contained 31.1–60.7% ω-6 FA and 7.9–17.8% ω-3 FA. The average FA levels in the 96 F_2 seeds in population 1 were as follows: 9.8% palmitic acid, 3.8% stearic acid, 25.0% oleic acid, 50.2% ω-6 FA, and 11.1% ω-3 FA. In the F_2 individuals, eight individual plants were wild-type homozygous for the *FAD2-1A*$_{W293STOP}$ and *HD* alleles (*AAHH*), seven were homozygous for the *FAD2-1A*$_{W293STOP}$ and *hd* alleles (*AAhh*), five were

homozygous for the *fad2-1a*$_{W293STOP}$ and *HD* alleles (*aaHH*), and four were homozygous for the *fad2-1a*$_{W293STOP}$ and *hd* alleles (*aahh*; Table 2). In the F_2 seeds of population 1, the ω-6 FA concentrations in *AAHH*, *AAhh*, *aaHH*, and *aahh* plants were 55.0 ± 1.8%, 59.3 ± 0.9%, 37.3 ± 4.5%, and 42.1 ± 5.6%, respectively. In addition, the ω-3 FA concentrations in *AAHH*, *AAhh*, *aaHH*, and *aahh* plants were 9.3 ± 0.8%, 13.8 ± 0.6%, 9.3 ± 0.5%, and 13.6 ± 0.2%, respectively. Interestingly, an F_2 seed carrying a heterozygous *FAD2-1A*$_{W293STOP}$ allele and a homozygous mutant allele of *HD* (*Aahh*) exhibited the highest ω-3 FA concentration in population 1 (Table 2). The ω-6/ω-3 ratios in the *AAHH*, *AAhh*, *aaHH*, and *aahh* plants were 5.9 ± 0.5:1, 4.3 ± 0.3:1, 4.0 ± 0.5:1, and 3.1 ± 0.4:1, respectively. The lowest ω-6/ω-3 ratio was 3.1 ± 0.4:1 in the *aahh* genotype group in population 1.

Table 2. Variation of fatty acids concentrations and ω-6/ω-3 ratio by genotypic groups in population 1 from a cross of PE529 and JD17-20-2-39.

ID	Genotypic Groups	Fatty Acid Concentration (%)					ω-6/ω-3
		Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid (ω-6)	α-Linolenic Acid (ω-3)	
Pungsannamul	*AAHH* (n = 13)	11.8 ± 0.4 f	2.9 ± 0.3 a	19.7 ± 2.3 cd	55.6 ± 1.9 efg	10.0 ± 0.8 abc	5.6 ± 0.5 ef
PE529	*aaHH* (n = 13)	9.0 ± 0.8 a	4.1 ± 0.6 bc	42.2 ± 4.9 h	33.7 ± 4.2 a	11.0 ± 0.6 c	3.0 ± 0.3 a
JD17-20-2-39	*AAhh* (n = 13)	11.2 ± 0.4 e	2.4 ± 0.2 a	11.6 ± 1.0 a	58.2 ± 0.8 gh	16.6 ± 1.2 f	3.5 ± 0.3 b
F_2 seed	*AAHH* (n = 8)	10.6 ± 1.2 d	5.0 ± 0.8 d	20.0 ± 2.9 cd	55.0 ± 1.8 ef	9.3 ± 0.8 a	5.9 ± 0.5 f
	AAHh (n = 7)	10.5 ± 0.4 cd	4.0 ± 0.8 bc	19.1 ± 1.2 c	55.9 ± 1.4 fg	10.4 ± 1.0 bc	5.4 ± 0.6 de
	Aahh (n = 7)	9.9 ± 0.4 bc	2.8 ± 0.2 a	14.2 ± 0.6 ab	59.3 ± 0.9 h	13.8 ± 0.6 d	4.3 ± 0.3 c
	AaHH (n = 9)	10.5 ± 0.9 cd	4.5 ± 0.5 cd	23.2 ± 3.2 e	52.0 ± 2.3 d	9.7 ± 0.7 ab	5.4 ± 0.3 de
	AaHh (n = 27)	9.9 ± 0.6 bc	4.0 ± 0.6 bc	22.4 ± 1.8 de	53.2 ± 1.7 de	10.5 ± 0.9 bc	5.1 ± 0.4 d
	Aahh (n = 11)	9.5 ± 0.4 ab	2.8 ± 0.4 a	15.3 ± 2.6 b	57.6 ± 1.5 fgh	14.8 ± 1.8 e	3.9 ± 0.5 bc
	aaHH (n = 5)	9.4 ± 0.4 ab	4.3 ± 0.3 c	39.6 ± 4.6 gh	37.3 ± 4.5 b	9.3 ± 0.5 a	4.0 ± 0.5 c
	aaHh (n = 18)	9.1 ± 0.4 a	3.7 ± 0.4 b	38.5 ± 3.9 g	38.0 ± 3.6 b	10.6 ± 0.8 bc	3.6 ± 0.4 b
	aahh (n = 4)	9.1 ± 0.3 a	2.8 ± 0.3 a	32.5 ± 5.8 f	42.1 ± 5.6 c	13.6 ± 0.2 d	3.1 ± 0.4 a

AA: wild-type *FAD2-1A* allele, *aa*: nonsense mutation of *FAD2-1A* allele from PE529, *HH*: wild-type *HD* allele, *hh*: mutant *HD* allele. The concentration of each fatty acid at the 5% level determined by Duncan's multi-range test is not significantly different if indicated by the same letter. PE529 is a line that includes *fad2-1a*$_{STOP}$ [18]. JD17-20-2-39 (*hh*) was derived from Pungsannamul × PE2166/Daepung [23]. PE2166 is a line containing 14% linolenic acid as a mutation caused by EMS treatment of Pungsannamul, and Daepung is a Korean soybean cultivar containing a normal linolenic acid concentration [23].

In population 2, F_2 seeds were genotyped for the *FAD2-1A*$_{DEL}$, *FAD2-1B*, and *HD* alleles (Figure S2 and Table 3). The F_2 seeds of population 2 were classified into seven genotypic categories: those with all wild-type alleles (A^1A^1BBHH); those with a functional *FAD2-1A*$_{DEL}$ allele, functional *FAD2-1B* allele, and mutant *HD* allele (A^1A^1BBhh); those with a functional *FAD2-1A*$_{DEL}$ allele, *fad2-1b* allele, and functional *HD* allele (A^1A^1bbHH); those with a functional *FAD2-1A*$_{DEL}$ allele, *fad2-1b* allele, and *hd* allele (A^1A^1bbhh); those with a *fad2-1a*$_{DEL}$ allele, functional *FAD2-1B*, and *hd* allele (a^1a^1BBhh); those with a *fad2-1a*$_{DEL}$ allele, a *fad2-1b* allele, and *HD* (a^1a^1bbHH); and those with all mutant alleles (a^1a^1bbhh; Table 3). The FA profile of the F_2 seeds in the genotypic group A^1A^1bbhh was as follows: 12.1%–19.9% oleic acid and 13.6–18.4% ω-3 FAs, whereas that of the a^1a^1BBhh group was 22.8%–32.1% oleic acid and 16.4–21.7% ω-3 FAs. Two mutant types (a^1a^1BBhh and A^1A^1bbhh) exhibited an FA profile with an ω-6/ω-3 ratio of <4.0 (Table 3). The comparison of the a^1a^1BBhh and A^1A^1bbhh genotypic groups revealed that the F_2 seeds with the a^1a^1BBhh genotype had a lower ω-6/ω-3 ratio than the seeds with the A^1A^1bbhh genotype. The ω-6/ω-3 ratio in the a^1a^1bbhh genotypic group was approximately 0.3 ± 0.1:1, which was the lowest ω-6/ω-3 ratio among all seven different genotypic categories, followed by F_2 seeds with a^1a^1bbHH (ω-6/ω-3 ratio of 0.4 ± 0.1:1) and a^1a^1BBhh (ω-6/ω-3 ratio of 2.2 ± 0.3:1) genotypes.

Table 3. Variation of fatty acids concentrations and ω-6/ω-3 ratio by genotypic groups in population 2 from a cross of S08-14719 and JD17-20-2-39.

ID	Genotypic Groups	Fatty Acid Concentration (%)					ω-6/ω-3
		Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid (ω-6)	α-Linolenic Acid (ω-3)	
Pungsannamul	A^1A^1BBHH (n = 13)	11.8 ± 0.4 f	2.9 ± 0.3 a	19.7 ± 2.3 cd	55.6 ± 1.9 efg	10.0 ± 0.8 abc	5.6 ± 0.5 ef
S08-14719	a^1a^1bbHH (n = 13)	7.9 ± 0.3 a	3.1 ± 0.3 cd	80.4 ± 1.2 e	2.4 ± 0.9 a	6.3 ± 0.9 a	0.4 ± 0.1 a
PI283327	A^1A^1bbHH (n = 13)	11.0 ± 0.3 b	4.5 ± 0.2 e	27.1 ± 2.6 d	47.0 ± 2.0 c	10.4 ± 0.6 c	4.5 ± 0.2 e
JD17-20-2-39	A^1A^1BBhh (n = 13)	11.2 ± 0.4 e	2.4 ± 0.2 a	11.6 ± 1.0 a	58.2 ± 0.8 gh	16.6 ± 1.2 f	3.5 ± 0.3 b
F_2 seed	A^1A^1BBHH (n = 4)	12.7 ± 0.8 d	3.0 ± 1.1 cd	20.2 ± 3.5 c	52.7 ± 4.1 d	11.3 ± 0.7 c	4.7 ± 0.6 e
	A^1A^1BBhh (n = 2)	13.4 ± 2.3 d	2.0 ± 0.8 a	12.2 ± 2.3 a	57.5 ± 2.4 ef	15.0 ± 1.6 d	3.9 ± 0.6 d
	A^1A^1bbHH (n = 7)	11.9 ± 0.5 c	3.5 ± 0.4 d	26.8 ± 3.9 d	47.0 ± 3.3 c	10.7 ± 1.1 c	4.4 ± 0.6 e
	A^1A^1bbhh (n = 11)	11.7 ± 1.0 bc	2.3 ± 0.5 ab	16.5 ± 2.5 b	53.0 ± 2.5 d	16.3 ± 1.5 e	3.3 ± 0.4 c
	a^1a^1BBhh (n = 6)	11.0 ± 1.2 b	2.9 ± 0.9 bc	26.1 ± 3.7 d	40.8 ± 3.3 b	18.6 ± 2.3 f	2.2 ± 0.3 b
	a^1a^1bbHH (n = 8)	8.2 ± 0.5 a	3.3 ± 0.5 cd	80.6 ± 1.4 e	2.2 ± 0.4 a	5.6 ± 0.3 a	0.4 ± 0.1 a
	a^1a^1bbhh (n = 5)	8.2 ± 0.3 a	1.8 ± 0.7 a	78.6 ± 1.5 e	2.4 ± 0.4 a	8.4 ± 0.9 b	0.3 ± 0.1 a

A^1A^1: wild-type *FAD2-1A* allele, a^1a^1: deletion of *FAD2-1A* allele from M23, *BB*: wild-type *FAD2-1B* allele, *bb*: missense *FAD2-1B* allele from PI283327, *HH*: wild-type *HD* allele, *hh*: mutant *HD* allele. The concentration of each fatty acid at the 5% level determined by Duncan's multi-range test was not significantly different if indicated by the same letter. S08-14719 is a line that includes $fad2\text{-}1a_{DEL}$ from M23 and $fad2\text{-}1b_{P137R}$ from PI283327 [19]. JD17-20-2-39 (*hh*) was derived from Pungsannamul × PE2166/Daepung. PE2166 is a line containing 14% linolenic acid as a mutation caused by EMS treatment of Pungsannamul, and Daepung is a Korean soybean cultivar containing a normal linolenic acid concentration [23].

In population 3, 380 F_2 seeds were genotyped for $FAD2\text{-}1A_{S117N}$, *FAD2-1B*, and *HD* alleles; moreover, a FA analysis was performed (Figure S3 and Table 4). The phenotypic distribution of the FA profile is shown in Figure S3. The average FA levels in the 380 F_2 seeds in population 3 were as follows: 9.9% palmitic acid, 3.1% stearic acid, 34.4% oleic acid, 42.0% ω-6 FAs, and 10.6% ω-3 FAs. After excluding F_2 seeds that were heterozygous for the evaluated alleles, a total of 45 F_2 seeds were homozygous for the $FAD2\text{-}1A_{S117N}$, *FAD2-1B*, and *HD* alleles (Table 4). The F_2 seed genotype A^2A^2bbhh had an average of 21.4% oleic acid, 53.0% ω-6 FAs, and 13.3% ω-3 FAs, whereas the average FA concentration in the a^2a^2BBhh genotypic group was 35.5% oleic acid, 39.8% ω-6 FAs, and 16.4% ω-3 FAs (Table 4). The comparison of the genotypes revealed that the A^2A^2bbhh, a^2a^2BBhh, and a^2a^2BBhh genotypes had lower ω-6/ω-3 ratios of 3.1 ± 0.7:1. The F_2 seeds in the A^2A^2BBhh genotypic group exhibited the highest ω-3 FA levels, at an average of 15.2%.

Table 4. Variation of fatty acids concentrations and ω-6/ω-3 ratio by genotypic groups in population 3 from a cross of Hosim and JD17-20-2-39.

ID	Genotypic Groups	Fatty Acid Concentration (%)					ω-6/ω-3
		Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid (ω-6)	α-Linolenic Acid (ω-3)	
Pungsannamul	A^2A^2BBHH (n = 13)	11.8 ± 0.4 f	2.9 ± 0.3 a	19.7 ± 2.3 cd	55.6 ± 1.9 efg	10.0 ± 0.8 abc	5.6 ± 0.5 ef
Hosim	a^2a^2bbHH (n = 13)	7.7 ± 0.1 ab	2.8 ± 0.1 bc	79.5 ± 0.7 h	3.6 ± 0.4 a	6.4 ± 0.4 a	0.6 ± 0.1 a
PI283327	A^2A^2bbHH (n = 13)	11.0 ± 0.3 ef	4.5 ± 0.2 f	27.1 ± 2.6 d	47.0 ± 2.0 c	10.4 ± 0.6 c	4.5 ± 0.2 e
JD17-20-2-39	A^2A^2BBhh (n = 13)	11.2 ± 0.4 e	2.4 ± 0.2 a	11.6 ± 1.0 a	58.2 ± 0.8 gh	16.6 ± 1.2 f	3.5 ± 0.3 b
F_2 seed	A^2A^2BBHH (n = 1)	10.6 ± 0.0	4.1 ± 0.0	25.0 ± 0.0	50.8 ± 0.0	9.4 ± 0.0	5.4 ± 0.0
	A^2A^2BBhh (n = 8)	10.2 ± 1.3 de	2.7 ± 0.4 bc	16.4 ± 3.7 b	55.4 ± 1.4 de	15.2 ± 2.4 e	3.7 ± 0.5 cd
	A^2A^2bbHH (n = 4)	10.2 ± 1.9 de	4.0 ± 0.6 e	32.8 ± 8.9 e	45.0 ± 7.4 c	8.0 ± 0.8 b	5.6 ± 0.6 f
	A^2A^2bbhh (n = 8)	9.6 ± 0.6 cd	2.8 ± 0.2 bc	21.4 ± 2.3 c	53.0 ± 2.2 d	13.3 ± 1.0 d	4.0 ± 0.3 d
	a^2a^2BBHH (n = 6)	10.1 ± 2.4 de	3.6 ± 0.9 de	40.3 ± 12.7 f	36.6 ± 10.3 b	9.2 ± 1.2 bc	4.0 ± 1.0 cd
	a^2a^2BBhh (n = 3)	8.7 ± 0.7 bc	2.8 ± 0.6 bc	35.5 ± 2.2 e	39.8 ± 0.3 b	13.3 ± 2.5 d	3.1 ± 0.7 b
	a^2a^2bbHH (n = 6)	8.4 ± 1.9 ab	3.5 ± 0.7 d	77.3 ± 3.6 gh	4.3 ± 1.6 a	6.5 ± 0.9 a	0.7 ± 0.2 a
	a^2a^2bbhh (n = 9)	7.6 ± 0.8 a	1.8 ± 0.4 a	74.8 ± 2.4 g	6.4 ± 2.2 a	9.3 ± 1.2 bc	0.7 ± 0.2 a

A^2A^2: wild-type *FAD2-1A* allele, a^2a^2: missense *FAD2-1A* allele with $fad2\text{-}1a_{S117N}$ from 17D, *BB*: wild-type *FAD2-1B* allele, *bb*: missense *FAD2-1B* allele from PI283327, *HH*: wild-type *HD* allele, *hh*: mutant *HD* allele. The concentration of each fatty acid at the 5% level determined by Duncan's multi-range test was not significantly

different if indicated by the same letter. Hosim is a line that includes *fad2-1a*$_{S117N}$ from 17D and *fad2-1b*$_{P137R}$ from PI283327 [17,19]. JD17-20-2-39 (*hh*) was derived from Pungsannamul × PE2166/Daepung. PE2166 is a line containing 14% linolenic acid due to a mutation caused by EMS treatment of Pungsannamul, and Daepung is a Korean soybean cultivar containing a normal linolenic acid concentration [23].

4. Discussion

Because soybeans are an important oil crop, most soybean researchers have been interested in decreasing ω-3 FA concentrations for the improvement of oxidative stability in soybean oil. However, soybeans are consumed directly in both fermented and non-fermented forms in many Asian countries and it can be a source of plant-based ω-3 FAs. Studies have demonstrated that intake of ω-6 and ω-3 FAs prevents inflammation, cardiovascular disease, and Alzheimer's disease [33,34]. Individuals consume these FAs at a severely disproportionate ω-6/ω-3 ratio (~10:1 to ~20:1) [15]. The WHO recommends an ω-6/ω-3 ratio of <4:1. Normal soybean seeds contain an ω-6/ω-3 ratio of 6:1–7:1. Therefore, it is necessary to adjust the ω-6/ω-3 ratio to <4:1 and to elevate ω-3 FA in soybean-based foods.

Soybeans with genotypes containing mutated *FAD2-1* have increased oleic acid and decreased ω-6 FA, resulting in a lower ω-6/ω-3 ratio in these soybean seeds [21,31]. In addition, increasing the ω-3 FA level in soybean seeds is another strategy for improving the ω-6/ω-3 ratio. Previous studies have established a seed-development system using wild soybeans as a genetic source for elevating ω-3 FA concentrations. However, nine estimated QTLs were found to be involved in the increase of ω-3 FA concentrations in the PI483463 wild soybean [27]. Using PI483463 plants as donor of genomic loci to increase ω-3 FAs and lower the ω-6/ω-3 ratio in soybean seeds was proposed using either of the *fad2-1a*$_{DEL}$ from M23 or *fad2-1b* from PI283327 combined with elevated ω-3 loci from wild soybeans [28]. Kulkarni et al. [28] demonstrated ω-6/ω-3 ratios of 2.5:1–3.9:1 via the use of the wild soybean, PI483463. The present study revealed that plants with the a^1a^1BBhh genotype carrying *fad2-1a*$_{DEL}$ and *hd* alleles in population 2 produced an ω-6/ω-3 ratio of 2.2:1 and an ω-3 FA concentration of 18.6%, which was significantly higher than that produced by the A^1A^1BBhh genotypic group (15.0%) in population 2 (Table 3).

The higher ω-3 concentrations detected in wild soybeans may be attributed to the different genes that control ω-3 biosynthesis [27,35]. Recently, a new system was established with PE2166, containing about 14% ω-3 FAs with mutations caused by EMS treatment of Pungsannamul [23]. The mutation in *HD* regulates the expression of *FAD3A*, resulting in an increase in ω-3 FA concentrations. Jo et al. [23] demonstrated the improvement of the ω-6/ω-3 ratio via the use of two genes, i.e., the *fad2-1a*$_{S117N}$ and *hd* mutant alleles, resulting in the production of an approximately 3.3:1 ω-6/ω-3 ratio in seeds. A similar result was obtained for the a^2a^2BBhh genotypic group in population 3, which produced an ω-6/ω-3 ratio of 3.1:1 and an increased concentration of ω-3 FA (13.3%) in soybean seeds (Table 4).

Our previous study discovered a new allele of *FAD2-1A* in the PE529 EMS-mutant line that yielded >40% oleic acid (*fad2-1a*$_{W293STOP}$) [18]. However, we reported that F_2 seeds with a homozygous *fad2-1a*$_{W293STOP}$, functional *FAD2-1B* and *HD* alleles in the three segregating populations produced 40.3%, 46.6%, and 30.4% oleic acid, respectively [18]. In addition, the PE529 line produced oleic acid concentrations of 49.1% and 43.4% in two growing years in a row, respectively. In the present study, the *aaHH* genotype yielded an oleic acid concentration of 37.3% (Table 2). Many studies have investigated the effects of environmental factors and temperature on FA profiles across various soybean genotypes because of the unstable production of FAs [36–41]. The instability of oleic acid may affect the ω-6/ω-3 ratio in soybean seeds. Thus, it is important to understand the stability of the FA profiles in genotypes containing the *fad2-1a*$_{W293STOP}$ allele together with the *hd* allele in different growing environments for further research.

To date, 12 different *fad2-1a* mutant alleles have been identified in natural germplasms and mutagenized populations, producing oleic acid at concentrations ranging from 27 to 37% [18,42–45]. Several studies demonstrated that combinations of different *fad2-1a* mutant alleles combined with a *fad2-1b* mutant allele produced >70% oleic acid concentration,

but produced slightly different oleic acid concentrations [19,20,31,42]. To determine the most effective strategy for a lower ω-6/ω-3 ratio, the *aahh*, a^1a^1hh, and a^2a^2hh genotypes from three different populations were compared in terms of the ω-6/ω-3 ratio and ω-3 FA concentrations in soybean seeds. This study is the first time that the *aahh*, and a^2a^2hh genotypes combination has been explored for ω-6/ω-3 ratios and seed ω-3 FA concentrations in soybean seeds. Among these genotypic groups, the highest ω-3 concentration in soybean seeds was observed in the a^1a^1hh group, which carried a deletion of the *FAD2-1A* allele from M23. In addition, the ω-6/ω-3 ratio of the a^1a^1hh genotypic group in population 2 was 2.2, which was lower than that observed for the *aahh* genotypic group (3.1) in population 1 and the a^2a^2hh genotypic group (2.1) in population 3. Regarding the ω-3 FA concentrations, the a^1a^1hh genotypic group (18.6%) produced an approximately 5% higher level of this FA than that produced by the *aahh* (13.6%) and a^2a^2hh (13.3%) genotypic groups. This study reported that the induction of mutations in $FAD2\text{-}1A_{DEL}$ and *HD* in soybean seeds is the most efficient strategy for improving the ω-6/ω-3 ratio and increasing the ω-3 FA concentrations. These results provide information that can be useful for developing novel soybean cultivars with a lower ω-6/ω-3 ratio and elevated ω-3 FA concentration, which can be a beneficial ingredient for soybean-based foods to enhance human health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030913/s1, Figure S1: Distribution of fatty acids for F_2 seeds of population 1 (PE529 × JD17-20-2-39). C1 means Pungsannamul, P1 means PE529, and P2 means JD17-20-2-39, and the average value of each check line is displayed; Figure S2: Distribution of fatty acids for F_2 seeds of population 2 (S08-14719 × JD17-20-2-39). C1 means Pungsannamul, C2 means PI283327, P1 means S08-14719, and P2 means JD17-20-2-39, and the average value of each check line is displayed; Figure S3: Distribution of fatty acids for F_2 seeds of population 3 (Hosim × JD17-20-2-39). C1 means Pungsannamul, C2 means PI283327, P1 means Hosim, and P2 means JD17-20-2-39, and the average value of each check line is displayed.

Author Contributions: H.K. and H.J. contributed equally to the study. Conceptualization, J.-D.L.; formal analysis, H.K. and H.J.; investigation and methodology, H.K.; writing—original draft preparation, H.K. and H.J.; writing—review and editing, H.J. and J.-D.L.; supervision, J.-D.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by a project to train professional personnel in biological materials by the Ministry of Environment and was supported by the Korea Basic Science Institute (National Research Facilities and Equipment Center) grant funded by the Ministry of Education (2021R1A6C101A416).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated in this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors acknowledge the personnel from the Plant Genetics and Breeding Laboratory at Kyungpook National University.

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

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