Chapter

Breeding Strategy for Improvement of Omega-3 Fatty Acid through Conventional Breeding, Genetic Mapping, and Genomics in Soybean

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Abstract

Plant-derived omega (ω)-3 polyunsaturated fatty acid is an essential fatty acid in human and animal diets and is a precursor of eicosapentaenoic acid and docosahexaenoic acid, which exists as α-linolenic acid (ALA, ω-3) in plant oil. Several epidemiological studies have revealed the health benefits of regular consumption of ω-3 fatty acid-containing diets. Soybean [*Glycine max* (L.) Merr.] is one of the major oil crops in the world and has around 8% ALA (ω-3) in seed oil. Soybean-derived ω-3 can be potential alternative sources of ω-3 fatty acids for populations living in countries with high risks of inadequate ω-3 intake. Therefore, increasing ω-3 concentration became an important goal in soybean breeding. Conversely, higher content of ω-3 fatty acids makes seed oil rancid, necessitating chemical hydrogenation, which generates *trans* fats. Since *trans* fats have been associated with the heart and other diseases, demand for soybeans with reduced ALA content is growing. In this book chapter, we described the importance of ω-3 fatty acid and consumption of diets with balanced ω-6/ω-3 ratio and discussed breeding and biotechnological means (and integrated approaches) for altering the ω-3 fatty acid content to avoid the need for chemical hydrogenation as well as to improve the ω-6/ω-3 ratio.

Keywords: soybean, fatty acid, omega-3, omega-6, breeding

1. Introduction

Soybean [*Glycine max* (L.) Merr.], an important oil crop, accounted for 28% of the total vegetable oil consumption in the world in 2019 [1]. The last 7 years have seen 22.5% increases in soybean oil consumption worldwide (**Figure 1**) and soybean oil production is expected to rise in the future. Commodity soybean seed contains 20% oil, 40% protein, and 12% soluble carbohydrates on a dry weight basis [2]. Fatty acid compositions of food and oil have received considerable attention in the past few decades for human nutrition and health concerns. Hence, improving the FA composition of the soybean oil is crucial to reduce the risks of associated coronary heart and other diseases.

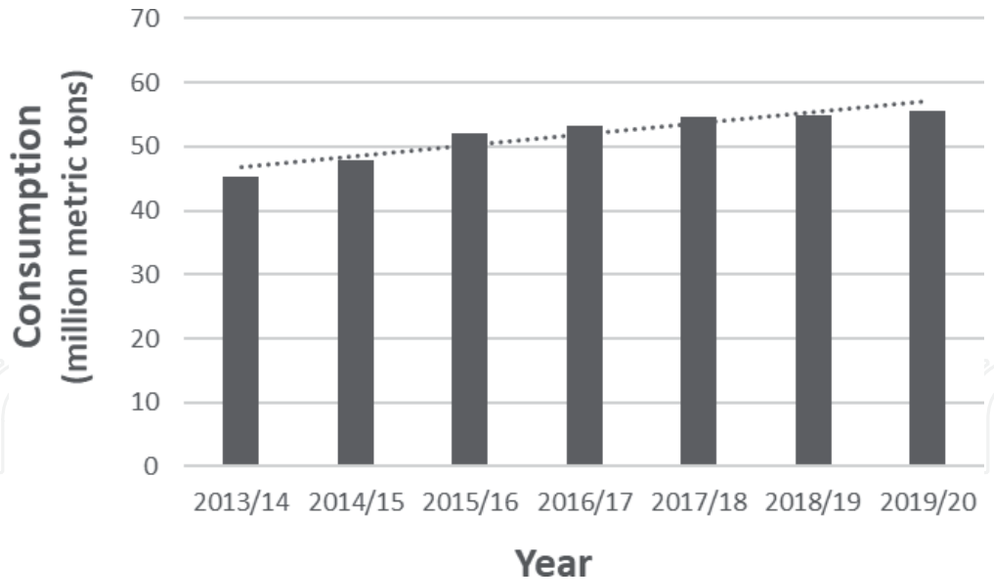

Figure 1.
Soybean oil consumption worldwide from 2013/14 to 2019/20 (in million metric tons). Retrieved from https://www.statista.com/statistics.

1.1 Fatty acid composition of important oil crops

Oil and fatty acids are essential elements for the development and growth of all the creatures. These elements are the structural components of the cellular membrane, as well as play a pivotal role in storing energy and involved in the cellular signaling processes. Known natural resources of oil and fatty acids are plants, animals, and oleaginous microorganisms. Oil and fatty acids played a crucial role in human life in several ways as food and fuel resource, mostly as a nutritional element of the diet. Edible oilseed crops (palm, olive, rapeseed, canola, sunflower, and soybean) primarily contain five fatty acids: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (ω-6, 18:2), and α-linolenic acid (ALA, ω-3, 18:3) in their seed oil. The fatty acid composition varies with the oilseed plant species as given in **Table 1**. Fatty acids accumulated in plants as a form of triacylglyceride, which consists of three fatty acids linked to glycerol as a backbone through ester bond [4, 5]. The triacylglyceride is a key source of renewable energy in the form of reduced carbon used as food, feedstock, and fuel.

In the plant fatty acids, basic biosynthetic pathways are well established but FA/lipid operating between the plastid and the endoplasmic reticulum remains to be determined [6, 7]. The fatty acid biosynthesis takes place in the chloroplast stroma of leaves and proplastids of seeds by *de novo* and further incorporation of triacylglyceride occurs in the endoplasmic reticulum [8]. Besides, polyunsaturated fatty acids (PUFA) synthesis in higher plants takes place by both prokaryotic and eukaryotic enzymatic pathways [7, 9]. These pathways are regulated through diverse sets of genes.

1.2 Polyunsaturated fatty acids in soybean

Soybean seed primarily contains two saturated fatty acids, which are palmitic acid, and stearic acid, and three unsaturated fatty acids, which are oleic acid, linoleic acid, and ALA. The relative ratio of these fatty acids commonly found in cultivated soybean are, 11% of palmitic acid, 4% of stearic acid, 23% of oleic acid, 55% of linoleic acid, and 8% of ALA [10]. However, wide variation for fatty acids content has been reported in several studies [11–13]. Commonly, the proportion

Crops	Saturated fatty acids (Palmitic and stearic acid) (%)	Unsaturated fatty acids (%)		
		Oleic acid	Linoleic acid	α-linolenic acid
Canola	7.4	61.8	18.6	9.1
Coconut	82.5	6.0	—	—
Corn	12.9	27.3	58.0	1.0
Cottonseed	25.9	19.0	54.0	1.0
Flaxseed	8.0	19.0	17.0	53
Olive	13.8	71.3	9.8	0.7
Palm	49.3	40.0	9.1	0.2
Peanut	20.3	46.5	31.4	—
Safflower	7.5	75.2	12.8	—
Sesame	—	36.2	44.2	0.3
Sunflower	—	25.4	59.9	0.1
Soybean	15.6	22.6	51.0	7.0

Data retrieved from Kim [3].

Table 1.
Fatty acid composition of major oilseed crops.

ratio of fatty acids in soybean is influenced by the genotypes as well as the environmental factors which are very crucial to determine the entire quality of the oil.

Linoleic acid and ALA are the PUFA, also termed essential fatty acids (EFA), present in the soybeans. Due to the presence of two or more double bonds between the carbons of fatty acid chains, PUFA are distinguished from saturated and monounsaturated fatty acids. The EFA is primarily classified into two forms, ω-6 and ω-3, which are metabolically interlinked but functionally diverse. ω-6 and ω-3 comprise long chains of carbon atoms with a carboxyl group at one end of the chain and a methyl group at the other end.

ω-3 fatty acids have a carbon–carbon double bond located between the third and fourth carbon atoms from the methyl end of the chain. ω-3 fatty acids exhibit *cis-trans* isomerism with its extension to *E-Z* configuration [14]. ALA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are the three important ω-3 fatty acids. The carbon backbone of these contains 18 carbon atoms, 20, and 22 for ALA, EPA, and DHA, respectively. Their structures are shown in **Figure 2**. ALA is the precursor of DHA and EPA, which are essential for the growth and development of the brain and retina in humans [15].

1.3 Sources of ω-3 fatty acids

Seafood products such as fish are the main sources of ω-3 fatty acids (ALA, EPA, and DHA). However, they are not a routine part of the traditional diet in many countries. The ω-3 fatty acid is abundantly available in nature and found in most of the oilseed crops. The ALA is also synthesized in the plants found in green leafy vegetables, and in the seeds of flax, as rapeseeds (*Brassica campestris*), chia (*Salvia hispanica*), perilla (*Perilla frutescens*), walnut (*Juglans sinensis*), and soybean. The ω-3 is dietary EFA for humans; however, because of the absence of delta-12 and delta-15 desaturase enzymes, humans and other animals are unable to synthesize ω-6 and ω-3 fatty acids. Therefore, these EFAs need to acquire through diet or dietary supplements [16].

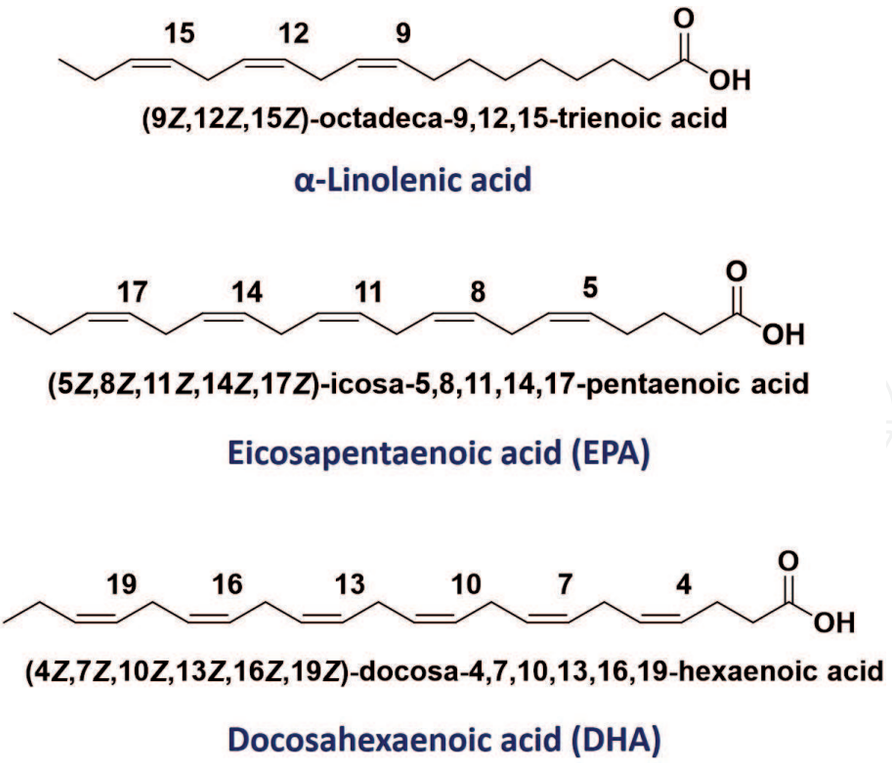

Figure 2.
Major three ω-3 polyunsaturated fatty acid structure: Three unsaturated fatty acids are shown with the cis configuration of the double bonds with a methyl end and a carboxyl (acidic) end represented with commonly followed nomenclature numerical scheme.

1.4 Importance of ω-3 fatty acid and ω-6/ω-3 ratio

Several studies reported the nutritional and health benefits of ω-3 in humans [17]. Besides, ω-3 fatty acids are known for therapeutic uses and to offer protection against numerous diseases [15]. Thus, the nutritional value of ω-3 fatty acids is now widely accepted. Earlier diets comprised meat, plants, eggs, fish, nuts, and berries, which contained substantial amounts of ω-3 fatty acid [17, 18]. With the changes in dietary habits, consumption of ω-6 fatty acid was enhanced, which consequently reduced the level of ω-3 fatty acids in human. Thus, the contemporary diets are now comprised of a high intake of saturated and ω-6 fatty acids, decreased ω-3 fatty acid intake, and an overuse of salt and refined sugar [19]. These dietary changes led to diets with an undesirable ω-6/ω-3 ratio up to 20:1 [20]. Ultimately, an altered ratio of ω-6/ω-3 is considered unhealthy and reported to be the prevalent cause of prothrombotic and proinflammatory diseases, such as atherosclerosis, obesity, and diabetes [16, 21–23]. Several studies have reported a positive correlation between lower ω-6/ω-3 ratio and reduced risks of cardiovascular disease (CVD), cancer, including breast, colon, prostate, liver, and pancreatic cancers, inflammation, favor apoptosis and exert antiproliferative effects cancers [24].

The balanced ω-6/ω-3 ratio is an important determinant in decreasing the risk for CVD [17]. Increased intake of linoleic acid is known to interfere with the incorporation of EPA and DHA (which have the most potent inflammatory effects) in cell membrane lipids, and causes platelet aggregation and oxidation of low-density lipoprotein. Intake of ω-3 fatty acids may help in preventing the development of CVD as well as other associated diseases. Therefore, it is highly essential to increase the intake of ω-3 fatty acids and to reduce the consumption of ω-6 fatty acids. It has been estimated that the present Western diets have a ω-6/ω-3 ratio of 15-20:1, which is highly imbalanced. Several studies in animals, such as *Caenorhabdtis*

elegans, rats, mice, and pigs have shown the importance of balanced ω-6/ω-3 ratio [25–27]. Experimental studies suggested the changes in mucosal inflammation and reduced the patient suffering from an increased ratio of ω-6/ω-3. The combination of rheumatoid arthritis drug treatment and an adequate ω-6/ω-3 ratio has been suggested to cause significant changes in inflammatory markers [24]. Such studies have provided evidence for the need to have a balanced ratio of ω-6/ω-3 (1:1 to 2:1) [20, 24]. Besides, ω-6 and ω-3 fatty acids are involved differentially in the cellular process. It was reported that ω-6 fatty acids increase triacylglyceride content in the cell through altering membrane permeability; whereas, ω-3 fatty acids lower fat deposition in adipose tissues by suppressing lipogenic enzymes and increasing β-oxidation [28].

2. Breeding goals for improvements in the ω-3 fatty acid content of soybean seeds

ALA (ω-3) in soybean oil is unstable and has undesirable flavors. Due to the presence of a double bond at the 12th carbon in the fatty acid hydrocarbon chain, the ALA is oxidized easily, which causes unwanted odor and off-flavors [29–31]. Ultimately, this reduces the functional quality of fry food or soy food items [31, 32]. Hence, ALA content is negatively associated with the stability and shelf life of soybean oil. To improve the shelf life, stability, desirable flavor, and palatability, soybean oil is chemically hydrogenated, which leads to the formation of *trans* fats. The *trans* fatty acids are linked to coronary heart diseases, and hence it is desirable to reduce the consumption of foods containing *trans* fats. Nearly 13 million Americans are reported to suffer from coronary heart disease, and over 500,000 die annually from causes related to coronary heart disease. For these reasons, the addition of *trans* fat labels to food nutrition labels directly under the line for saturated fat was started in 2006. This resulted in a change in the narrative of food industries to promote and explore substitutions for hydrogenated soybean oils. In the past few decades, soybean geneticists and breeders have shown that oil with reduced *trans*-fat content can be obtained by decreasing the content of ω-3, and ω-6 fatty acids through conventional and modern breeding approaches. Soybean oils derived from cultivars with reduced ω-3 fatty acid content are shown to increase stability, lower hydrogenation, and comprise reduced *trans*-fat levels [29, 33, 34]. This shifted the breeder's focus to develop soybeans with reduced ω-3 fatty acid content [35, 36]. In this section, the genetic basis of ALA (ω-3) content and breeding approaches fro altering ALA content are discussed.

2.1 Genetic basis of PUFA in soybean

The biosynthesis of PUFAs in soybean involves a variety of pathways, which are catalyzed by a complex series of desaturation and elongation steps [37]. Fatty acid desaturases introduce double bonds into the hydrocarbon chains of fatty acids to produce unsaturated fatty acids [38]. The delta-12 fatty acid desaturase-2 enzyme (*FAD2*) catalyzes the conversion of oleic acid to linoleic (ω-6) in the developing soybean seeds [39]. The microsomal ω-3 fatty acid desaturases (*FAD3*) catalyze the transformation of linoleic into ALA [36]. Thus, the genes coding for these fatty acid desaturases may act together to control the ALA content in soybean.

Two identical copies of FAD2 enzymes (FAD2-1, and FAD2-2) have been identified in the soybean. Five *FAD2* gene family members (two *FAD2-1* members: *FAD2-1A* and *FAD2-1B*, and three *FAD2-2* members: *FAD2-2A*, *FAD2-2B*, and *FAD2-2C*) are present in the soybean genome [39, 40]. Through syntenic, phylogenetic, and in silico analysis, Lakhssassi et al. [41] revealed two additional members

of the *FAD2* gene family: *FAD2-2D* and *FAD2-2E*, positioned on chromosomes 9 and 15, respectively. Of these *FAD-2* genes, *FAD2-1A* is highly expressed in developing soybean seeds [41]. The chromosomal locations and gene model names of these genes are given in **Table 2**. Mutations in one or more of these genes have been utilized to alter the fatty acid content of the soybean seeds.

The genetic basis ω-3 fatty acid trait in soybean has been identified based on the experimental study of gene information from the model plant *Arabidopsis thaliana* (L.) Heynh. and screening of lower ω-3 fatty acid mutant soybean lines. Arabidopsis contains a single gene encoding a microsomal ω-3 fatty acid desaturase and two chloroplast targeted enzymes (FAD7 and FAD8). At least three independent loci (*fan*, *fan2*, and *fan3*), influencing the ALA content have been identified [42]. Genes underlying all the three loci have been identified as homologous genes of *FAD3* [43]. In soybeans, the level of ALA is controlled by three *FAD3* genes: *FAD3A* (*Glyma.14g194300* for Wm82.a2.v1 assembly), *FAD3B* (*Glyma.02g227200*), and *FAD3C* (*Glyma.18g062000)*. Of these, *FAD3A* has been reported to show consistent high expression in developing seed, and hence have the greatest effect in controlling the accumulation of ALA contents [42, 44]. These three loci have a greater effect on ALA concentration, as combining mutant alleles of these genes resulted in soybean oil having ~1% ALA [45–48].

The full-length genomic DNA sequences for *FAD3A, FAD3B, FAD3C*, and *FAD3D* genes were found to share 78 to 95% similarity, and have similar structure, and contain eight exons [49]. These eight exons of *FAD3* genes are highly conserved in soybean and correspond to the sizes of exons in Arabidopsis. However, significant variation is found among the introns of *FAD3* genes. Based on the structure and similarity, the four *FAD3* genes could be separated into two groups: *FAD3A*/*FAD3B*, and *FAD3C*/*FAD3D.*

2.2 Reducing ω-3 fatty acid content to avoid the need for chemical hydrogenation

Initial breeding efforts were made by the USDA-ARS in 1952 to identify soybean germplasm with lower ω-3 content to replace chemical hydrogenation of soybean oil [50]. During that period, the cultivars with lower ω-3 levels were identified but

Gene family	Gene/ paralogs	Gene model (Wm82.a1.v1)	Gene model (Wm82.a2.v1)	Chromosome (Linkage group)
FAD2	*FAD2-1A*	Glyma10g42470	Glyma.10g278000	10 (O)
	FAD2-1B	Glyma20g24530	Glyma.20g111000	20 (I)
	FAD2-2A	Glyma19g32930	Glyma.19g147300	19 (L)
	FAD2-2B	Glyma19g32940	Glyma.19g147400	19 (L)
	FAD2-2C	Glyma03g30070	Glyma.03g144500	3 (N)
	FAD2-2D	Glyma09g17170	Glyma.09g111900	9 (K)
	FAD2-2E	Glyma15g23200	Glyma.15g195200	15 (E)
FAD3	*FAD3A*	Glyma14g37350	Glyma.14g194300	14 (B2)
	FAD3B	Glyma02g39230	Glyma.02g227200	2 (D1b)
	FAD3C	Glyma18g06950	Glyma.18g062000	18 (G)
	FAD3D	Glyma11g27190	Glyma.11g174100	11 (B1)

Table 2.
Chromosomal location and gene model information of FAD2 and FAD3 genes in the soybean genome.

no cultivar having <4% ω-3 were found. In 1981, USDA-ARS and North Carolina State University collaborated and developed the line N79-2245 having a reduced ω-3 content of 4.2% by recurrent selection approach [32]. The major cultivars/lines with low ω-3 content developed using conventional breeding, germplasm screening, mutation breeding, and recurrent selection [44, 51–53] are given in **Table 3**.

The natural accessions reported with the low ω-3 level in the USDA germplasm is known as PI 123440 and PI 361088B with allelic variant at *fan* locus. This mutation was reported as allelic or identical to the initial single recessive allele derived from the C1640 genotype [57, 61–63]. Burton et al. [71] used the PI 123440 as a parent source to develop a low ω- trait known as Soyola and Satelite.

Through the EMS and X-ray mutagenesis approach, several mutants were previously reported for the lower ω-3 fatty acid content ranging from <2.5% to 5.6% that are linked with the *Fan* loci such as C1640 (*fan*), A5 (*fan*), A23 (*fan2*), KL-8 (*fanx*), M-5 (*fan*), M-24 (*fanxa*) and RG10 (*fan-b*) [54, 58, 60, 64, 66, 67, 72]. Besides, mutants A16, A17, A29, MOLL, and LOLL with reduced ω-3 acid content showed allelic variation at *fan* loci [58, 64, 66, 67]. The RG10 line was developed from the mutagenesis of 4% ω-3 line C1640 [55, 60]. Several studies

Lines/ Cultivars	Selection Type	α-linolenic acid (%)	Reference
M5	Mutant	<4.6	[54]
N79-2245	Recurrent selection	4.2	[32]
C1640	Mutant	3.4	[55, 56]
A5	Mutant	4	[57]
A23	Mutant	5.6	[58]
IL-8	Mutant	4.5	[54]
M-24	Mutant	4.5	[59]
RG10	Mutant	< 2.5	[60]
PI123440	Germplasm	< 4.0	[61–63]
PI361088B	Germplasm	4	[61–63]
A16	Mutant	< 2.5	[58]
A17	Mutant	< 2.5	[58]
MOLL	Recurrent selection	< 3.0	[64, 65]
LOLL	Recurrent selection	< 3.0	[65, 66]
A29	Mutant	3.0	[67, 68]
CX1512–44	Mutant	3.0	[46]
J18	Mutant	3.0	[43]
PE1690	Mutant	3.7	[51, 58]
	Mutant	4.0	[69]
RCAT 0716L	Mutant	3.0	[45]
MS382	Mutant	<4.0	[65]
19,457	Mutant	3.9	[70]
18,777	Mutant	4.0	[70]
21,249	Mutant	4.5	[70]

Table 3.
List of soybean mutants and germplasm lines with low levels of α-linolenic acid in the seed oil.

used the RG10 line to develop the novel lines (*GmFAD3aabbcc*) with low ω-3 fatty acid content and also used for mapping and validating the quantitative trait loci (QTLs) and *FAD3* genes [45, 49, 73]. The EMS mutant line PE1690 with the reduced ω-3 fatty acid was reported to have a single base mutation in the *FAD3A* gene, resulting in the desaturase enzyme being nonfunctional [51]. Recently, Held et al. [69] identified a novel mutant allele of the *FAD3C* gene in a screen of a N-nitroso-N-methylurea (NMU)-mutagenized population. This allele resulted in 2 to 3% reduction in ω-3 FA levels.

2.3 Increasing ω-3 fatty acid content for improving ω-6/ω-3 ratio in soybean

Soybean production focuses on providing high protein meals for livestock and the manufacture of vegetable oils in both Western and Asian countries, while soybean has traditionally been used as a staple food in many Asian countries [2, 74]. The consumption of soy foods has been increasing in North America, following the recognition of the health benefits of soy foods.

Since the shortage of resources in cultivated soybean with elevated ALA content [75], researchers tried to find suitable genetic resources to develop new cultivars with high ALA concentrations in soybean breeding programs. Wild soybean can be a possible resource to achieve the goal to increase ALA concentration because those soybeans have an average of 15% ALA concentration, which is almost twice the ALA concentration present in the cultivated soybean [76]. Cultivated soybeans have an ω-6/ ω-3 ratio of 6–7:1, whereas wild soybeans have an ω-6/ ω-3 ratio of 3–4:1, which has better health benefits [76–78]. Thus, wild soybean can be exploited as a genetic resource to develop soybean lines with high ALA concentrations, although exploiting wild soybeans in breeding programs is challenging due to their poor agronomic traits. Several studies reported soybean lines with elevated ALA from wild soybean using conventional breeding methods. Asekova et al. [77] reported that three recombinant inbred lines with elevated ALA concentrations from an interspecific cross between *G. max* and *G. soja* were stable for the accumulation of ALA across the environments. Also, since *G. soja* as donor plant was backcrossed with three different cultivars, new genotypes with elevated ALA concentration and agronomically similar to the cultivated soybeans have been developed [78]. These soybean lines developed by classical breeding could be exploited as genetic resources for the development of novel soybean cultivars with high levels of ALA concentration, which could be sources of ω-3 fatty acids.

To date, there have been few genetic mapping studies with high ALA concentration in soybean. Shibata et al. [79] identified four QTLs controlling ALA concentration in the wild soybean accession Hidaka 4. Also, Ha et al. [80] identified nine putative QTLs controlling ALA concentration in a wild soybean accession PI 483463. According to these studies, high ALA concentrations in wild soybean were controlled by multiple QTLs. Besides, Pantalone et al. [81] suggested that high ALA concentration in wild soybean was controlled by a different set of desaturase alleles from cultivated soybean. Recently, the application of gamma-ray irradiation has generated new mutant soybeans with a high level of ALA concentration [82]. They concluded that the phenotype of high ALA concentration in these mutant lines was related to *FAD3* gene expression levels, although they observed no direct relationship between elevated gene expression level and gene sequence variations. Taken together, we assume that increased expression levels of *FAD3* genes during seed development may be associated with the gene expression regulators.

Since *FAD2* genes play an important role in regulating ω-6/ω-3 ratio in soybean, *FAD2* mutant alleles were found to increase in oleic acid and decrease in linoleic acid contents [40, 83, 84]. Populations segregating for *FAD2–1A* and *FAD2–1B*

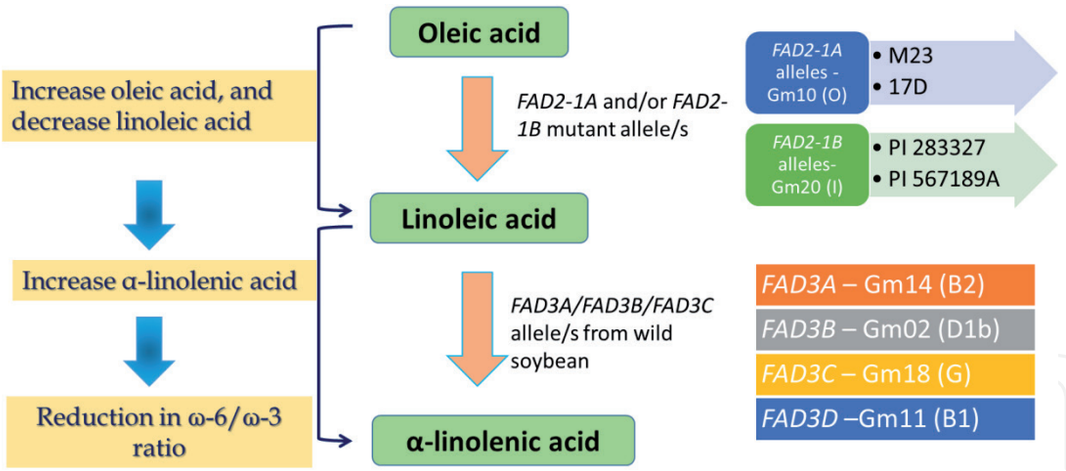

Figure 3.
Schematic representation of the genetic improvements in the steps that result in significant reductions in ω-6/ω-3 ratio in soybean. The mutant alleles of FAD2-1A or FAD2-1B increase the oleic and reduce linoleic acid content, whereas, various alleles of FAD3 cause increases in the α-linolenic acid contents. These improvements significantly alter the ω-6/ω-3 ratio in soybean seed oil.

mutant alleles have been investigated for increases in oleic acid content [40]. By combining either of a mutant allele in M23 (the deletion of the *FAD2–1A* gene) or 17D (*FAD2–1A* S117N) with either of a missense mutant allele in *FAD2-1B* from PI 283327 (*FAD2-1B* P137R) or PI 567189A (*FAD2-1B* I143T), soybean genotypes with 77.3–82.2% oleic acid content were developed [84]. The ω-6/ω-3 ratio in these lines ranged from 0.6 to 1.3. Similarly, progenies containing *FAD2-1A* allele from the 17D lines, and *FAD2-1B* allele from S08-14788 were found to show an ω-6/ ω-3 ratio in the range of 0.62-0.97 [85]. These soybean genotypes had high oleic acid content (~80%) and lower ω-6/ω-3 ratio, but the overall ω-3 acid content (~5%) was also very low [86]. Kulkarni et al. [87] suggested the genetic improvement of the system to increase ALA concentration with a balanced ω-6/ω-3 ratio. Soybean containing either of the *FAD2-1* mutant alleles with ALA-related alleles from wild soybean reduced the seed ω-6/ω-3 ratio as well as increased ω-3 fatty acid concentration. Among *FAD2* genes, soybean genotypes with a mutant allele of the *FAD2-1A* gene had higher oleic acid and ALA content in soybean oil than one with *FAD2-1B* mutant allele. Further genetic improvements in the FA biosynthetic pathways were made by combining mutant alleles of either of *FAD2-1A* or *FAD2-1B* genes with alleles governing ALA in wild soybeans to develop soybean genotype with lower ω-6 and higher ω-3, resulting in low ω-6/ ω-3 ratio (**Figure 3**; [87]). Similar genetic improvements involving new sources of ALA-controlling alleles from the wild soybeans can guide development of soybeans with a balanced ω-6/ω-3 ratio in their seed oils.

3. Biotechnological approaches for improving the fatty acid composition

3.1 Transgenic soybeans with improved fatty acid profile

Soybean is widely recognized as a dual-use crop because of its high protein and oil content [29], and several loci controlling both the traits have been identified. The negative correlation between these two traits [88] pose a challenge in genetic improvement programs. Introducing a transgene that can specifically modulate one pathway without disrupting the other can be useful to overcome the linkage between oil and protein. Several transgenic approaches have been tried to improve

seed oil content in oilseed crops, In Arabidopsis, transcription factor gene, *WRI1,* and metabolic enzyme, acetyl-CoA carboxylase have been targeted [89, 90]. In soybean, Lardizabal et al. [91] first reported the development of a transgenic soy crop with increased oil that shows no major impact on protein content or yield. They achieved an increase in oil by 1.5% (by weight) in the mature seed by expressing a codon-optimized version of a diacylglycerol acyltransferase (DGAT)-2A from the soil fungus *Umbelopsis ramanniana* in soybean seed during development. Later, increased oil content of soybean seeds by an average of 3% was also reported with the use of an improved variant of soybean type 1 DGAT [92].

In recent years, RNA interference (RNAi) has gained significant attention due to its success for efficient metabolic engineering across the plant species. RNAi uses small interfering RNAs (siRNAs) to mediate the degradation of mRNA to regulate the expression of a desired plant gene. Using this approach, Flores et al. [93] showed that silencing of *GmFAD3* by siRNA caused a reduction in the ALA contents in *fad3*-mutant. A similar approach was used by Wagner et al. [94] for simultaneous suppression of soybean *FAD2* and *fatty acyl-ACP thioesterase (FATB)* genes to produce soybean seed with low-saturated, low-polyunsaturated oil phenotype.

Many studies in the recent past have demonstrated the role of *GmFAD2* family members in metabolically engineered oilseed plants. Using antisense RNA mediated posttranscriptional gene silencing approach, Zhang et al. [95] were successful in inhibiting the expression of *Gmfad2-1b* to develop transgenic soybean lines with increased oleic acid contents up to 51.7%. To simultaneously elevate stearic acid and reduce PUFA content in soybean, Park et al. [96] introduced the mangosteen (*Garcinia mangostana*) stearoyl-ACP thioesterase into soybean and subsequently stacked it with a soybean event that is down-regulated in both palmitoyl-ACP thioesterase activity and Δ12 fatty acid desaturase activity in a seed-specific fashion. This approach generated soybeans with a seed lipid phenotype of approximately 11–19% stearic acid and approximately 70% oleic acid. Recently, the introduction of the *PfFAD3-1* gene from Lesquerella (*Physaria fendleri*) into soybean resulted in an increase in the ALA content up to 42% in the seeds of T_2 homozygous plants [97].

It is important to note that the transgenes expressing RNAi constructs are subject to variation in transgene expression, and hence a large number of events need to be screened to select the candidate providing stable expression. They also need to go through the regulation process, which is not only expensive but also time-consuming. Nevertheless, these approaches are expected to guide further improvement in the fatty acid composition without largely affecting the other traits, mainly the protein content and yield.

3.2 Targeted mutagenesis to improve ω-3 fatty acid contents

Targeted genome engineering (also known as genome editing) using designed nucleases has emerged as an alternative to conventional plant breeding and transgenic means to improve crop plants [98]. The discovery of sequence-specific nucleases (SSNs) such as TAL effector nucleases (TALENs) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas 9, made it possible to introduce targeted knockout mutations within gene/s of interest [99, 100]. These SSNs make DNA double-stranded breaks at defined genomic loci, which are subsequently repaired by two main DNA repair pathways, which result in frameshift mutations that often create genetic knockouts. Such knockout lines have been generated across the plant species, making genome editing an emerging tool for trait improvement.

Using genome editing approach, Haun et al. [101] engineered TALENs to recognize and cleave conserved DNA sequences in *FAD2–1A* and *FAD2–1B* genes. In the

plants that carried homozygous mutations in both *FAD2–1A* and *FAD2–1B* genes, oleic acid was increased from 20% to 80% and linoleic acid was reduced from 50% to 4%. Further reduction in the linoleic acid (up to 2.5%) was achieved by stacking mutations within *FAD2–1A* and *FAD2–1B* with mutations in *FAD3A* [44]. Such an approach of TALENs-mediated targeted mutagenesis of *FAD2* was found to be effective in the development of the high oleic peanut (*Arachis hypogaea* L.) varieties [102]. The low to average mutagenic frequency by TALENs has been observed in the genome editing studies done so far in peanut and soybeans. The efficiency of genome editing can further be enhanced by using the CRISPR/Cas system.

In recent years, the CRISPR/Cas9 system has revolutionized functional genomics due to its simplicity, efficiency, cost-effectiveness, and versatility [103]. The CRISPR system has two components: a nuclear-localized CRISPR-associated (Cas) 9 protein and a guide RNA (gRNA). Cas9 is a large protein containing two nuclease domains, whereas the gRNA is a synthetic 100 nucleotide RNA molecule, of which the first ~20 nucleotides are the targeting site, and the 3′ end forms a hairpin structure that interacts with the Cas9 protein [104]. The Cas9 and the gRNA interact to identify DNA sequences complementary to the gRNA and generate a DNA double-strand break, which, after a repair result in genomic insertion or deletion (indel) mutations.

In plants, the CRISPR-Cas9 system has been effectively used in many species such as *Arabidopsis thaliana*, *Nicotiana benthamiana*, rice, tobacco, sorghum, wheat, and maize [105]. In soybean, CRISPR/Cas9-mediated genome editing has been successful in targeting DNA mutations in genes for soybean hairy roots and flowering [106–109], plant architecture and yield [110], plant height [111], and seed storage protein genes [112]. However, researchers have started using this system to improve fatty acid composition. Do et al. [113], designed two gRNAs to guide Cas9 to simultaneously cleave two sites, spaced 1Kb apart, within the second exons of *GmFAD2–1A* and *GmFAD2–1B* to yield a high-oleic, low-linoleic, and low-ALA phenotype in soybean. In this study, dramatic increases in oleic acid content to >80%, and decreases of 1.3–1.7% in linoleic acid were observed in the T1 seeds derived from CRISPR-edited plants homozygous for both *GmFAD2* genes. In a similar study, increases in oleic acid from 17.10% to 73.50%, and decreases in the linoleic acid content from 62.91% to 12.23% have been reported by inserting mutations in *GmFAD2–1A* and *GmFAD2–2A* soybean fatty acid desaturase mutants based on CRISPR/Cas9 Technology [114]. Overall, these studies demonstrated the CRISPR/Cas9 system as a rapid and highly efficient method to simultaneously edit homeologous soybean genes to facilitate gene discovery and breeding programs.

4. Conclusions and perspectives

Altering the ω-6 and ω-3 fatty acid profile of the soybean seed/oil has been an important goal for soybean breeders. While low-ALA oils are better-suited for vegetable oil, genotypes with high ALA can be suited in food products that use whole soybeans in various fermented/non-fermented recipes. Therefore, breeding strategies according to the specific requirements are required. For these reasons, three major breeding strategies need be considered to achieve improvement in ω-3 fatty acid content in soybean. 1. To reduce ω-3 fatty acid for soybean oil, which is being achieved with the use of available several mutant lines with reduced ALA concentration in breeding programs. 2. To increase ω-3 fatty acid for soybean foods, which can be achieved by finding new alleles in wild soybeans, and introgressing such alleles in desired cultivars. However, there are many difficulties in this breeding process. Generating mutants with increased ω-3 fatty acid could be very

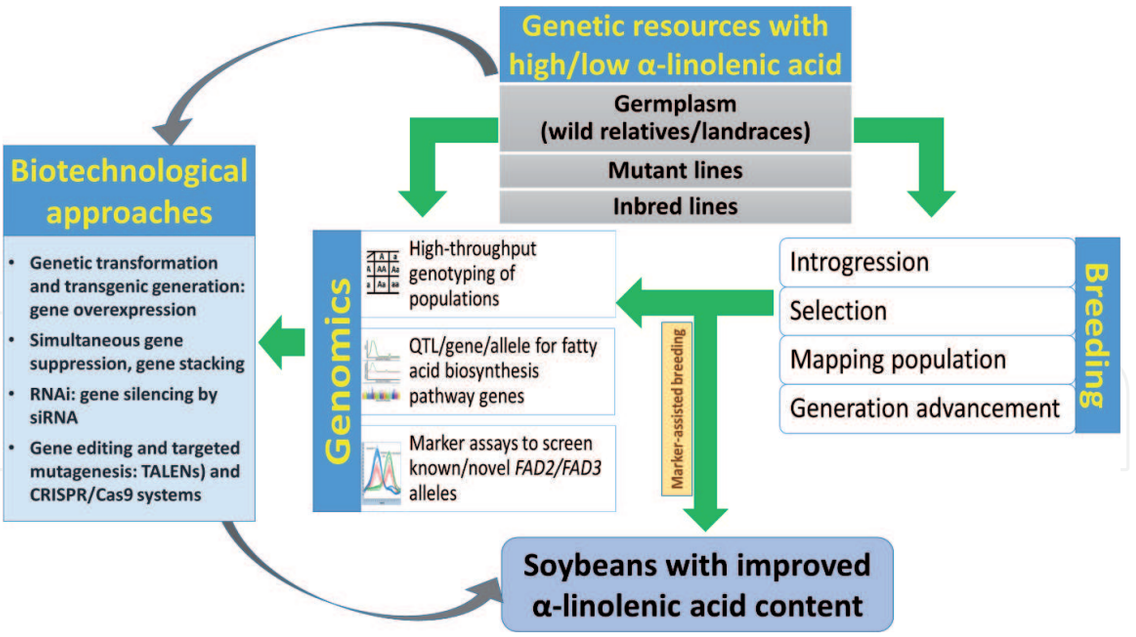

Figure 4.
Schematic representation of the integrated approach involving genomic, biotechnological, and breeding approaches for the improving α-linolenic acid in soybean cultivars.

crucial in achieving this goal. Wild soybeans [76] and some mutants [82] have relatively higher ω-3 fatty acid; however, there is still a lack of clarity and research information on the genes that regulate *FAD3* genes. Therefore, studies investigating the regulators controlling ω-3 fatty acid in soybean need to be carried out. 3. To increase ω-3 fatty acid along with decreasing ω-6/ω-3 ratio, which can be achieved by combining mutant alleles of either of *FAD2-1A* or *FAD2-1B* genes with alleles (genes) governing elevated ALA in wild and cultivated soybeans. The success of these three strategies rely on the availability of genetic and genomic information governing ALA content, which at the moment is limited. Hence, an integrated approach (**Figure 4**) comprising genetic dissection, breeding, and biotechnological approaches is necessary to develop soybeans with desired fatty acid profile.

In last two decades, advances in the genomic and DNA sequencing technologies facilitated the genetic discovery of fatty acid biosynthesis in soybean and other oilseed crops [115]. It is now feasible to screen a large germplasm and mutant collections in quick time using high-density genotyping platforms (such as Axiom SoyaSNP array; [116]), and use the data for genetic and association mapping. Several wild and cultivated soybean genotypes with varied seed fatty acid contents are already known and have been used to develop improved cultivars. Also, many artificial mutant lines have been used in developing segregating mapping populations to identify novel alleles, for which genotyping assays have been developed and used for introgression of desired fatty acid trait in a soybean cultivar. Besides, the recent success of gene-editing technologies in targeting selected sites in the genes regulating fatty acid composition traits has shown the potential to selectively insert mutations in target genes. TALENs, and CRISPR/Cas9 has shown a great potential in soybean for many agronomic traits, and need to be exploited for improving the seed fatty acid composition.

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