

Induced Mutations for Elevated Oleic Acid and Reduced Linolenic Acid Content in Soybean Seeds

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ABSTRACT

The high linolenic acid content (7-9%) is associated with poor flavor of soybean oil. Soybean oil with less than 2% linolenic acid would be desirable for improving the quality. Oil in which the linolenic acid (18:3) content has been reduced from 8 to 1% makes it possible to reduce or eliminate the need for chemical hydrogenation and henceforth can achieve the stability and increased shelf life. Oil with elevated levels of oleic acid is less susceptible to oxidative changes during refining, storage and frying. Oil in which the oleic acid (18:1) has been increased from 25 to 80% also have increased stability and longer shelf life. Natural genetic variability for fatty acid composition in commercial soybean seed oil is limited, therefore, additional variability for increasing economical usage is to be created. In this augmentation, induced mutagenesis has been seen to be an excellent tool for creating variability for fatty acid contents in soybean. Induced mutagenesis is regarded as a supplement to the conventional breeding method used to alter the concern genes for reduced linolenic acids and elevated oleic acid content in the cultivars currently grown for commercial production. A significant positive correlation of 100-seed weight with oleic acids and negative correlation with linolenic acids is also useful to select elevated oleic acid and low linolenic acid lines indirectly. The purpose of this paper is to review the methods that have been used to develop the fatty acid modifications, the inheritance of the modifications, the impact of the trait on agronomic and seed characteristics, the methods of phenotypic and genotypic selection, and the commercial status of the modified oils.

Keywords: linolenic acid, mutation, oleic acid, soybean

Abbreviations: EMS, ethyl methane sulphonate; FAD, fatty acid desaturase; HO, high oleic; HS, high stearic; MAS, marker-assisted selection; PTGS, post-transcriptional gene silencing; TILLING, Targeting Induced Local Lesions in Genomes

CONTENTS

INTRODUCTION.....	46
STRATEGIES USED FOR REDUCED LINOLENIC ACID CONTENT.....	47
STRATEGIES USED FOR HIGH OLEIC ACID WITH REDUCED LINOLENIC ACID CONTENT.....	48
STRATEGIES USED FOR ELEVATED OLEIC ACID CONTENT.....	49
AGRONOMIC TRAITS USEFUL FOR SELECTION OF DESIRABLE LINOLENIC AND OLEIC ACID MUTANTS.....	50
REFERENCES.....	50

INTRODUCTION

Soybean oil is the predominant vegetable oil in the world. Due to its neutral flavor and a competitive price, soybean oil is used extensively in food industry as cooking oil, baking and frying fat and has been the most consumed vegetable oil in the world (Soystats 2010). It is ranked number one in world oil production (48%) in the international trade markets among the major like cottonseed, groundnut, sunflower, rapeseed, coconut and palm kernel. Oilseed crops, grown all over the world, are an important source of edible and non-edible oils and fatty acids (Robbelen *et al.* 1989).

Oils and fats are important to human nutrition as they have a high-energy content. Soybean oil contains both saturated and unsaturated fatty acids. A common soybean cultivar contains 11% palmitic (16:0), 3% stearic (18:0), 22% oleic (18:1), 56% linoleic (18:2), and 8% linolenic acid (18:3) (Wilcox 1985). The soybean oil with high linolenic acid content (7-9%) is associated with poor flavor (fishy, painty-grassy-melony) stability (Dutton *et al.* 1951). Oil with elevated levels of oleic acid is less susceptible to oxidative changes during refining, storage and frying (Miller *et*

al. 1987; Laga *et al.* 2004). An increase in the oleic acid content and reduction in the linolenic acid contents would increase the stability of the oil and reduce or eliminate the need for chemical hydrogenation, which is responsible for production of undesirable *trans*-fatty acids. Oil with reduced linolenic acid was sold commercially from the crop grown in 1994 and high oleic acid oil from the crop of 2001. Research has been concentrated on rich contents soybean oil with high oleic acid, low linolenic acid. This can be done by changing the degree of unsaturation and by increasing or decreasing the fatty acid chain length (Topfer *et al.* 1995). Gene technology and plant breeding are combining to provide powerful means for modifying the composition of oilseeds to improve their nutritional value and provide the functional properties required for various food oil applications. Major alterations in the proportions of individual fatty acids have been achieved in a range of oilseeds using conventional selection and induced mutation. The survey indicated that mutagenesis has been an excellent tool for creating variability for fatty acid contents in soybean (Singh and Hymowitz 1999; Velasco *et al.* 1999). Subsequent research on induction of mutations for fatty acid composition was discussed by Robbelen (1990). Induced

mutations have been extensively used for genetic enhancement of the annual oilseed crops, and specially to modify the fatty acid composition of *Brassica* species, sunflower, linseed and soybean (Nichterlein *et al.* 1996). The first induced mutants altering fatty acid composition were reported by Rakow (1973). Several mutant lines with modified fatty acids have been registered as germplasm.

Major alterations in the proportions of individual fatty acids have been achieved in a range of oilseeds using post-transcriptional gene silencing (PTGS). PTGS can be invoked to modify seed oil fatty acid composition by seed-specifically expressing a DNA sequence that is complementary to the whole or part of the appropriate target fatty acid biosynthesis gene. Liu *et al.* (2002) applied hpRNA-mediated PTGS in cotton to down-regulate key fatty acid desaturase genes and develop nutritionally-improved high-oleic (HO) and high-stearic (HS) cottonseed oils (CSOs). Sense-mediated PTGS (co-suppression) targeted against the 12-desaturase that converts oleic acid to linoleic acid has resulted in the development of soybean, rapeseed (*Brassica napus*) and mustard (*B. juncea*) oils with very high levels of oleic acid (Stoutjesdijk *et al.* 2000). Hoshino *et al.* (2010) isolated novel fatty acid mutant using Targeting Induced Local Lesions In Genomes (TILLING) and a reverse genetic method.

Induced mutants altering the fatty acid composition have been extensively used in the breeding programmes to develop new cultivars. However, it was not always possible to ascertain the source of the altered fatty acid character in the new cultivars or hybrids, especially those developed by the seed industry, and trace it back to the original mutant.

STRATEGIES USED FOR REDUCED LINOLENIC ACID CONTENT

Soybean lines with low linolenic acid levels were obtained from the naturally occurring variability (Wilson *et al.* 1981; Rennie *et al.* 1988; Rennie and Tanner 1989b; Fehr *et al.* 1992). The soybean germplasm collection does not contain any line with low enough (<4%) linolenic acid (Hammond and Fehr 1975), less than 2% to overcome the flavor and stability problems of oil (Kleiman and Cavins 1982; Rahman *et al.* 1994). The high linoleic acid content of soybean oil is potentially nutritionally negative, because diets high in linoleic acid content may reduce the nutritionally positive effects of the health-beneficial ω -3 fatty acids such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA-3), and docosahexaenoic acid (DHA) in tissue (Friesen and Innis 2010; Blasbalg *et al.* 2011).

Mounts *et al.* (1988) identified one line, PI 361088B, with low linolenic (4.2%) content from northern and southern soybean germplasm. Beside these, mutagenesis has been applied as an important tool for obtaining low linolenic acid mutants (Hammond and Fehr 1983; Takagi *et al.* 1990). Linolenic acid concentration has been significantly altered by both X-rays and chemical mutagens (Hammond and Fehr 1975). Wilcox *et al.* (1984) identified a genetically stable low linolenic acid (3.4%) mutant from ca. 15,000 M₂ plants of cv. 'Century' treated with ethyl methanesulphonate (EMS) and from germplasm by X-rays or chemical mutagen treatments and further hybridization of low linolenic mutants (Takagi *et al.* 1990; Rahman and Takagi 1997; Rahman *et al.* 1998). A mutant line 'M-5' with reduced linolenic acid content was isolated from X-ray irradiation of the seeds of cv. 'Bay' (Takagi *et al.* 1990; Rahman *et al.* 1996). The low linolenic acid mutant lines such as: line 'RG10' with 4.3% (Stojsin *et al.* 1998b), lines 'C1640' and 'C1725' with 3-4% (Wilcox and Cavins 1985; Erickson *et al.* 1988), and line 'A5' with 4.1% (Hammond and Fehr 1983a) were obtained from the EMS treated population. The low linolenic acid mutant 'KL-8' (Rahman *et al.* 1996), 'A26' (Ross *et al.* 2000), and 'M-24', 'M5' (M923) (Rahman *et al.* 1998) were obtained by X-ray irradiation. Two mutants containing low levels of linolenate were identified by Hudson (2012) by using an *N*-nitroso-*N*-methylurea

(NMU)-treated soybean population.

Backcross breeding programs can also be used to introduce the low linolenic acid content trait into soybean seeds (Abelmon *et al.* 2003). Development of two lines (A16 and A17) with 2.5% linolenic acid from the cross between low linolenic acid mutant 'A5' and high palmitic acid mutant 'A23' has been reported by Hammond and Fehr (1983) and Fehr *et al.* (1992). A mutant 'A5' (M923) with low linolenic acid 4.1% (Hammond and Fehr 1983), mutants 'A23' and GLLA with low linolenic acid were developed from parent 'A5' (Rennie *et al.* 1988; Fehr *et al.* 1992). BARC-12 with low linolenic acid (3%) line was obtained by crossing and selecting the two full-siblines 'N85-2176' and 'N85-2124' (Leffel 1994). Low linolenic acid mutants N83-375 (5.5%), N89-2009 (2.9%) and N85-2176 (1.9%) were developed by Mounts *et al.* (1994). Mutant IL-8 with 4.5% (Rahman *et al.* 1996), and a mutant line isolated from 'M5' x 'M24' had 2.4% linolenic acid (Rahman *et al.* 1998). Mutant LOLL and MS382 with 2.7% linolenic acid, exhibited reduced yield and thus mutant MOLL was obtained from (K18 x M5) with 3.7% linolenic acid (Wasala *et al.* 1997). Lines with decreased levels of palmitic and linolenic acids have been developed by combining different mutant alleles through hybridization (Valerio *et al.* 2002). Wilcox and Cavins (1987) observed that linolenic acid content was controlled by the genotype of the embryo rather than by the genotype of maternal parent. Rahman *et al.* (1994) observed no maternal and cytoplasmic effects for linolenic acid content. Expression of linolenic acid genotype is complicated by maternal and environmental effects and cytoplasmic factors (Pleines and Friedt 1989). The low linolenic trait was found to behave like a quantitative character with a partial maternal effect (Graef *et al.* 1988). Low linolenic acid content is a quantitative trait reported by Fehr *et al.* (1992). Several mutations associated with the quantitative trait for low linolenic acid have been described and oil with lower linolenic acid had improved oxidative stability and flavor (Liu and White 1992). It is evident that linolenic acid in soybean seed oil is the end product in a successive desaturation of oleic acid (Dutton and Mounts 1966). The relative complexity of the biochemical effects and the genetics of this mutant suggest that the mutation may not directly affect a structural gene for a desaturase but may affect the regulation of expression of desaturase activity. The predominant gene responsible for the synthesis of linolenic acid in seed triacylglycerols is fatty acid desaturase (*fad3*), the structural gene for a microsomal 18:2 desaturase (Lemieux *et al.* 1990). Analysis of the overall fatty acid composition of seeds, leaves and roots of plant from *fad2* mutant indicated that the mutation is expressed in all tissues (Lemieux *et al.* 1990). The level of low linolenic acid found in different lines was controlled by single gene (Rennie and Tanner 1989b) two genes (Fehr *et al.* 1992) or by more than three genes (Takagi *et al.* 1999; Ross *et al.* 2000). Wilcox and Cavins (1987) assigned gene symbols *Fan Fan* for high levels linolenic acids contents ($X = 7.2 \pm 0.11\%$), *Fan fan* for intermediate levels ($X = 5.2 \pm 0.07\%$), *fan fan* for low levels ($X = 3.2 \pm 0.13\%$). It has also been reported that linolenic acid was controlled by the genotype of embryo rather than genotype of maternal parents. At least three independent genetic loci are associated with seed linolenic acid levels, with mutant alleles identified at *fan*, *fan2*, *fan3*, and *fanx* (Rahman and Takagi 1997; Ross *et al.* 2000) and the *fan* locus has been mapped (Brummer *et al.* 1995; Rahman *et al.* 1996; Stojsin *et al.* 1998). In soybean, a deletion in the *fad3* gene was associated with a reduced linolenic acid concentration and this deletion accounted for 67% of the variation for linolenic acid (Byrum *et al.* 1997). Three major alleles for reduced linolenate content have been developed through chemical mutagenesis: *fan1*, *fan1* (A5), and *fan2* (Walker *et al.* 1998). The *fan1* allele was developed by treating 'Century' with EMS (Wilcox and Cavins 1986). The *fan1* (A5) was obtained by treating 'FA9525' with EMS and *fan2* by treating 'FA47437' with EMS (Hammond and Fehr 1983; Fehr and Hammond 1996). Bilyeu *et al.*

(2005) identified mutations in two of the three soybean microsomal ω -3 *fad* genes that are associated with a greater than two third reduction in seed linolenic acid levels. Mutations resulting in the low linolenic oil phenotype in soybean have been shown to lie within the *FAD3* gene (Chappell and Bilyeu 2006; Reinprecht *et al.* 2009), which encodes for a delta 15 fatty acid desaturase that incorporates a third double bond into linoleic acid to produce linolenic acid.

Polyunsaturated fatty acids (PUFAs) play an important role in maintaining cellular membranes through the regulation of cholesterol synthesis and its transport (Simopoulos 1991). An adequate dietary intake of the n-3 PUFAs α -linolenic acid has many clinical benefits for our health (Crawford *et al.* 2000). There are two distinct pathways for the biosynthesis of PUFAs, one located in the microsomes and the other in the plastid membranes (Browse and Somerville 1991). The genes encoding both the microsomal and plastid forms of this desaturase have been cloned in soybean and other plants (Yadav *et al.* 1993). The plastid fatty acid composition is involved in many important physiological reactions, e.g. temperature tolerance (Kodama *et al.* 1995) and wounding response (Nishiuchi *et al.* 1997). Both chloroplast-targeted and microsomal ω -3 *fads* have been identified in plants, but the enzymes have been shown to be the major contributors to seed linolenic acid levels (Yadav *et al.* 1993). Microsomal ω -3 *fad* is an essential enzyme in the production of the n-3 PUFAs α -linolenic acid during the seed developing stage (Anai *et al.* 2003). Toyoaki *et al.* (2005) developed three low- α -linolenic acid soybean mutants, 'J18', 'M5', and 'M24' and characterized four cDNAs, *GmFAD3-la*, *GmFAD3-1b*, *GmFAD3-2a*, and *GmFAD3-2b*, encoding microsomal ω -3 *fad* from soybean developing seeds. A *fan3* allele for reduced linolenate was developed by treating seeds of the line 'A89-144003' with EMS (Fehr and Hammond 1998). Low linolenic acid soybean line 'C1640', allelic with 'A5', containing a mutation of the ω -3 *fad* gene (*GmFAD3A*) has been characterized. The genetic lesion in 'C1640' using *GmFAD3A* as a candidate gene was identified and distinguished between the wild type and mutant alleles using PCR followed by endonuclease digestion (Chappell and Bilyeu 2006). The molecular genetic basis for soybean line 'A29' containing 1% (10 g kg⁻¹) linolenic acid (three *GmFAD3* genes) in the seed oil along with SNP-based molecular markers identified alleles, utilizing the soybean homologues of *Arabidopsis FAD3* as candidate genes, the mutations were associated with the linolenic acid phenotype in segregating populations (Bilyeu *et al.* 2006). Marker-assisted selection (MAS) procedures to assess the accuracy of phenotypic selections in soybean for linolenic specific for mutations in two *fad* genes, *GmFAD3A* and *GmFAD3C* have been identified (Beuselinck *et al.* 2006). The use of MAS can accelerate and simplify breeding soybean with improved flavour. Sauer *et al.* (2008) developed *Fad3A* gene-based markers for linolenic acid content. The molecular genetic basis for low linolenic acid soybeans has been demonstrated to be due to mutations in one or more of the three ω -3 *fad* genes designated *GmFAD3A*, *GmFAD3B*, and *GmFAD3C* (Anai *et al.* 2005; Bilyeu *et al.* 2005, 2006; Chappell and Bilyeu 2006, 2007; Reinprecht *et al.* 2009). The *FAD3B* mutation is due to a mutation that disrupts a splice site, and the *FAD3C* mutation is due to a missense mutation in a conserved amino acid residue (Bilyeu *et al.* 2005; Reinprecht *et al.* 2009). Using a targeted gene silencing approach to deliberately down-regulate the *GmFAD3* family in a seed-specific manner, Flores *et al.* (2008) were able to generate an ultra-low-linolenic phenotype in soybean by effectively suppressing the expression of all three *GmFAD3* genes with a single RNA interference construct. The *GmFAD3B* molecular marker assay was designed to distinguish 'RG-10' derived mutant *GmFAD3B* alleles from wild-type *GmFAD3B* alleles (Reinprecht *et al.* 2009) based on resolution of allele-specific amplification products after the inclusion of GC-rich tails as previously described (Wang *et al.* 2005). *GmFAD3A* corresponds to the *Fan1* locus and is represented as Glyma14

g37350 in the soybean genome sequence; *GmFAD3B* is the *Fan3* locus and is represented as Glyma02 g39230; and *GmFAD3C* corresponds to the *Fan2* locus and is represented as Glyma18 g06950 (Schmutz *et al.* 2010). Many GenBank records of ω -3 *fad* genes exist. Recent release of a complete draft of soybean genome sequence opens new insights into genome organization of this ancient paleopolyploid (Schmutz *et al.* 2010).

Total elimination of linolenic acid from the seed oil is probably impossible, since it plays an essential role in photosynthesis (Tanhuanpaa and Schulman 2002) and furthermore, it is critical for pollen development (McConn and Browse 1996). The chloroplast membranes of all higher plants contain very high proportions of trienoic fatty acids (Routaboul *et al.* 2000). Trienoic fatty acids are important to ensure the correct biogenesis and maintenance of chloroplasts during growth of plants at low temperatures and in maintaining photosynthetic activity at low temperatures. Thies (1970) reported that accumulation of linolenic acid occurs only in the seed of oil-producing plants which possess green and photosynthetically active chloroplasts during their development, such as those of the families Fabaceae (soybean), Linaceae (flax, *Linum usitatissimum* L.) and Brassicaceae (rapeseed, mustard, *Brassica napus* L.). Thies (1970) also found a positive correlation between the chlorophyll content and the linolenic acid content present in rapeseed mutants. Since linolenic acid is the main fatty acid to produce the chlorophyll thylakoid membranes, he concluded that genotype with complete absence of linolenic acid was not possible to develop in these chloroplast-bearing species.

STRATEGIES USED FOR HIGH OLEIC ACID WITH REDUCED LINOLENIC ACID CONTENT

Linolenic acid has been identified as an unstable component of soybean oil that is responsible for the undesirable odors and flavors commonly associated with poor oil quality (Dutton *et al.* 1951; Smouse 1979). On the other hand oleic acid being less susceptible to oxidation imparts oxidative stability to soybean oil. Therefore, globally, soybean cultivars with low linolenic acid and high oleic acid content are being searched and developed (Ross *et al.* 2000; Rahman *et al.* 2001; Fehr and Curtiss 2004). Rahman *et al.* (2001) crossed 'M23' that has \approx 50% oleate to 'M5' that has 4.9% linolenate and recovered a line with 54.4% oleate and 4.2% linolenate. They crossed that line to one with 33.9% oleate and 2.9% linolenate and recovered a line with 56.3% oleate and 2.8% linolenate. Later, they crossed a line 'LPKCC-3' with the *fap1* and *sop1* alleles for reduced palmitate to a line DHL that has the *ol* allele for elevated oleate and the *fan1* and *fanxa* alleles for reduced linolenate (Rahman *et al.* 2004). They recovered a line LPDHL with 4.0% palmitate, 51.0% oleate, and 2.9% linolenate. A line low in linoleic and linolenic, and high in oleic (Hildebrand and Pfeiffer 1991) was developed from parent '9525' through EMS induced mutation.

Soybean with 1% linolenic acid content has been produced by combination of *Fan1*, *Fan2* and *Fan3* alleles (Roiss *et al.* 2000). However, soybean lines with low linolenic (1-3%) and high oleic acid (55-60%) in India are not available. Genetic variants have been developed with 80% oleic acid and 1% linolenic acid (Fehr and Curtiss 2004). The genetic relationship of the loci for high oleic and low linolenic acids in soybean is still unknown, although studies on relationships of low linolenic acid with low and high palmitic acid in soybean were conducted and cultivars were successfully developed (Nickell *et al.* 1991), but the levels of palmitic and linolenic acid appear to be inherited independently (Nickell *et al.* 1991). High oleic and low linolenic fatty acid composition was ascertained by expressed sequence tags (ESTs) in soybean (Bilyeu *et al.* 2003).

STRATEGIES USED FOR ELEVATED OLEIC ACID CONTENT

Vegetable oils with a high content of oleic acid are of interest for nutritional, industrial purposes and storage longevity (Schierholt *et al.* 2001; Warner and Gupta 2005). Oleic acid is more stable than the other unsaturated fatty acids because it is monounsaturated, and with only one double bond it is less susceptible to oxidation than linolenic acid (3 double bonds) during refining, storage and frying (Laga *et al.* 2004). The enhanced oxidative stability of soybean oil with higher oleic acid content will also open up a variety of food uses and industrial applications like spraying oils or machine lubricants (Butzen and Schnebly 2007). A diet containing a high content of oleic acid can reduce the content of the undesirable low-density lipoprotein cholesterol in blood plasma (Grundny 1986) and monounsaturated fatty acids more effectively prevent arteriosclerosis and coronary heart disease than PUFAs (Chang and Huang 1998). Scientists have therefore modified the seed trait of soybeans to increase the concentration of oleic acid.

Brossman and Wilcox (1984) noticed the lines with oleic acid concentration as high as 25.9% in soybean. No soybean lines exist in the USDA National Plant Germplasm System collection with the high oleic acid trait (oleic acid content above 70% of the oil fraction), although multiple lines contain elevated oleic acid levels (Lee *et al.* 2009). Rahman *et al.* (1996) obtained the mutants in soybean with high oleic acid content (M11 and M23 with 380 g kg⁻¹ and 46.1%, respectively) from X-ray irradiation. A distinct inverse relationship was observed between the oleic and linolenic acid content in the 'M-23' mutant. The same mutant showed high oleic acid content under different environmental conditions in M₃ generation (Rahman *et al.* 1996). The environmental effect on fatty acids of seed oil for seventeen soybean genotypes with normal and modified fatty acid profiles were accessed by Oliva *et al.* (2006). Under optimal environmental conditions, the 'M23' genotype and the other mid-oleic acid genotypes require pyramiding of multiple genetic alleles for full penetration of the mid-oleic acid phenotype (Bachlava *et al.* 2008b; Monteros *et al.* 2008).

Rucker and Robbelen (1995) selected several mutants with contents of 77 to 80% oleic acid in the seed oil in an EMS mutagenised population of winter oilseed rape. Hudson (2012) obtained 16 mutants containing high levels of oleic acid from an NMU-treated population. Kinney (1996) obtained a high oleic acid content (84%) soybean through particle-bombarded-mediated transformation. A soybean oil with >80% oleate was developed by DuPont Co. through genetic engineering (Kinney and Knowlton 1998). Oleate contents >90% have been reported by Buhr *et al.* (2002). The highest content of oleate that has been achieved by conventional breeding is >70% (Alt *et al.* 2005b). The high oleic acid transgenic lines have a greater oxidative and thermal stability than their wild-type counterparts have excellent agronomic properties (Mazur *et al.* 1999). The high oleic acid character is expressed exclusively in the tissues of the seeds (Garces *et al.* 1989). Oleic acid in soybean oil was found to be quantitatively inherited or there is involvement of more than one gene in biosynthesis of oleic acid (Carver *et al.* 1987). Takagi and Rahman (1996) first observed that the oleic acid content in mutant was controlled by a single recessive allele, designated as *ol*. Another allele *ol a* at the same *Ol* locus was found in mutant 'M11'.

In the past few years, it has been able to clone the genes encoding each of the soybean *FAD2* (flavin adenine dinucleotide 2) desaturases causing seed oleic acid concentrations to increase from 25% in wild-type to 85% in the transgenic lines (Mazur *et al.* 1999). In developing soybean seeds the *FAD2-1A* and *FAD2-1B* are considered to play an important role in controlling the oleic acid level, these are most closely related to one another, with a shared genomic organization containing a single intron and 99% identity in encoded amino acid sequence, and are present on homologous chromosome regions mapped to linkage group O

(chromosome 10) and I (chromosome 20), respectively (Schlueter *et al.* 2007). In Arabidopsis and maize only one single copy of a *FAD2* gene was identified (Belo *et al.* 2008), soybean appears to possess multiple copies of the gene due to the consequence of repeated rounds of genome duplication followed by limited sequence loss (Schlueter *et al.* 2007). In an effort to identify novel alleles of the soybean *FAD2-1A* and *FAD2-1B* genes, genomic DNA was characterized for the sequence of both genes from plant introduction (PI) lines selected from the National Genetic Resources Program containing elevated oleic acid levels from 27 to approximately 50% percent of the oil, while commodity soybeans typically produce 19-23% oleic acid (Lee *et al.* 2007). However, the previously characterized sources of elevated oleic acid soybean involved mutation of the *FAD2-1A* gene alone, which failed to achieve oleic acid levels stable above 60% of the oil (Anai *et al.* 2008; Scherder and Fehr 2008; Dierking *et al.* 2009) or utilized approaches that have proven to have limited usage in soybean breeding due to the complexity of the trait (Bachlava 2009). The level of oleic acid in the oil of soybean lines was particularly susceptible to environmental effects when compared to the X-ray *FAD2-1A* deletion line (Lee *et al.* 2009).

Several elevated oleic acid soybean lines have been characterized at the molecular level. Transgenic high oleic acid soybeans were produced using ribozyme-terminated antisense to down-regulate *FAD2-1* gene transcript levels (Buhr *et al.* 2002). A reverse genetics approach was utilized to identify a soybean line containing a missense mutation in *FAD2-1A* that associated with an elevated oleic acid content of the oil (Dierking *et al.* 2009). Two independent studies report that the combination of mutant *FAD2-1A* and mutant *FAD2-1B* alleles resulted in soybean lines with more than 80% oleic acid (Hoshino *et al.* 2010; Pham *et al.* 2010).

The microsomal ω -6 desaturase enzymes, which catalyze the desaturation of oleic acid to linoleic acid during fatty acid biosynthesis, are encoded by the *FAD2-1* and *FAD2-2* genes in soybean (Bachlava *et al.* 2008). The genotyping assays developed for the *FAD2-1A*, *FAD2-1B*, and *FAD2-2B* isoforms, as well as their linked simple sequence repeat markers, can be used in soybean breeding programs for the elevation of oleic acid seed content through marker-assisted selection.

Alt *et al.* (2005) used PCR-based markers for phenotypic and molecular analysis of oleate content in the mutant soybean line 'M23'. The mutation of the *ol* gene in 'M23' was the result of a deletion at the *Fad2-1a* locus (Sandhu *et al.* 2007). Monteros *et al.* (2008) used SSR markers to map and confirm the areas of the soybean genome associated with oleic acid content from the soybean line 'N00-3350' (~583 g kg⁻¹ oleic acid). Six QTLs have been mapped and confirmed for high 18:1 in line 'N00-3350' a derivative of 'N98-4445A' on LGs A1 (R² = 4%) at Satt211, D2 (R² = 6%) at Satt389, G (R² = 13%) at Satt394, G (R² = 7%) at Satt191, L (R² = 9%) at Satt418, and L (R² = 25%) at Satt561 (Monteros *et al.* 2008). Zhang *et al.* (2008) reported a newly identified soybean gene, designated *SACPD-C* that encodes a unique isoform of Δ^9 -stearoyl-ACP-desaturase, the enzyme responsible for converting stearic acid to oleic acid (18:1). The participation of *SACPD-C* in storage triacylglycerol synthesis is further supported by the observation of mutations in this gene in two independent sources of elevated 18:0 soybean germplasm, 'A6' (30% 18:0) and 'FAM94-41' (9% 18:0).

Pham *et al.* (2010) identifying and combining mutations in two delta-twelve fatty acid desaturase genes, *FAD2-1A* and *FAD2-1B* to produce the high oleic acid trait in soybeans. It has been shown that the more severe the mutations in either *FAD2-1A* or *FAD2-1B*, the higher and more stable is the oleic acid content in the soybean oil, due to residual enzymatic activity of *FAD2*. For instance, high oleic acid soybeans with the 17D-derived mutant *FAD2-1A* alleles containing a missense mutation showed a larger reduction of oleic acid content (more flux through the desaturase pathway) when grown in cooler environments, compared to

those that had the 'M23' derived *FAD2-1A* null alleles, which are completely deleted (Anai *et al.* 2008; Pham *et al.* 2010). A candidate gene approach was used to identify a mutant *FAD2-1B* gene in PI '283327' (a missense mutation causing a change of P137R in the amino acid sequence) and combine this gene with existing mutant *FAD2-1A* genes from two soybean lines, 'M23' (genomic deletion of 160 kb including *FAD2-1A*) and 17D (an S117N missense mutation) to produce a stable, non-transgenic high oleic acid (80%) content in soybean seed oil (Dierking and Bilyeu 2009; Pham *et al.* 2010; Bolon *et al.* 2011; Pham *et al.* 2011).

Hoshino *et al.* (2010) isolated novel mutant alleles of *GmFAD2-1b* from EMS treated soybean mutant populations through TILLING, a reverse genetic method and, successfully generated soybean lines with elevated >80% oleic acid content by combining *GmFAD2-1a* and *GmFAD2-1b* mutant alleles. A novel *FAD2-1A* allele was identified in a soybean plant introduction by Pham *et al.* (2011). Gamma rays-induced changes in oil yield and oleic acid content have been evaluated in other crops like *Guizotia abyssinica* by Shabnam *et al.* (2011).

AGRONOMIC TRAITS USEFUL FOR SELECTION OF DESIRABLE LINOLENIC AND OLEIC ACID MUTANTS

Carver *et al.* (1986) reported that increased oleic acid content in soybean did not affect yield. According to Kinney and Knowlton (1998) and Graef *et al.* (2009), the high oleate trait did not have any negative effects on yield or other agronomic traits. The yield drag associated with the mid-oleic acid soybean genotypes is not due to the fatty acid profile of the seed per se, given that targeted down-regulation of *FAD2-1* in a seed-specific manner results in seeds with greater than 80% oleic acid that do not have compromised agronomic performance (Graef *et al.* 2009). These studies suggest that selection of high oleic acid containing plants/mutants without altering yield. Significant positive correlation of 100 seed weight with oleic acids and negative correlation with linoleic and linolenic acids have been reported by Brossman and Wilcox (1984). According to him within the EMS population it may be possible to select low linolenic acid lines indirectly based on seed weight. He also found the lack of association of linolenic acid with agronomic characteristics suggesting that selection for favorable low levels of linolenic acid would not adversely alter yield, maturity or lodging. Scherder and Fehr (2008) evaluated the influence of elevated oleate content on agronomic and seed traits; the mean seed yield of mid oleate lines was significantly less by 12% than lines with conventional oleate content and high yielding lines in each population were of conventional oleate type. However the mean protein content of mid oleate were significantly greater and mean oil content significantly less than conventional oleate. Bachlava *et al.* (2008) observed a significant negative correlation between yield and oleate content, and positive correlations of yield with linoleate, linolenate and palmitate contents. Brace *et al.* (2011) identified a small but statistically significant reduction in both oil content and yield (less than 5% compared to those of the control soybean line with normal fatty acid composition) in high oleic acid soybeans. Recently, high yielding soybean plants with low linolenic acid content have been reported by Wu *et al.* (2012).

A highly significant negative correlation of oleic (MUFA) with linoleic and linolenic acid (PUFA) contents, is also important to increase MUFA and lower PUFA content or for selection of mutants with high oleic and low linolenic acid content to improve oxidative stability index (OSI) of soybean oil (Alt *et al.* 2005a; Wang 2006; Patil *et al.* 2007; Bachlava *et al.* 2008) in soybean. The oxidative stability index, broadly defined as the length of time before the onset of oxidation of an oil, is 8.9, 5.4, 22.7, and 16.7 h for ultra-low-linolenic, standard soybean oil, partially hyd-

rogenated soybean oil, and high oleic acid (80%) sunflower (*Helianthus annuus*) oil, respectively (Warner 2009).

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