J Am Oil Chem Soc (2019)
DOI 10.1002/aocs.12298

ORIGINAL ARTICLE

Genomic Regions Governing the Biosynthesis of Unsaturated Fatty Acids in Recombinant Inbred Lines of Soybean Raised across Multiple Growing Years

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Received: 15 May 2019 / Revised: 1 September 2019 / Accepted: 23 September 2019

Abstract The ratio of oleic acid to the combined value of linoleic and α-linolenic acids determines the oxidative stability, and the ratio of linoleic acid to α-linolenic acid is the key to the nutritional value of soybean oil. The present study was conducted to identify genomic regions associated with oleic, linoleic, and α-linolenic acids in recombinant inbred lines (RIL), developed from LSb1 × NRC7, across 5 cropping years. These RIL were genotyped using 105 polymorphic SSR markers across soybean genome and analyzed for fatty acid composition. SSR markers, namely, Satt245 (LGp M), Satt556 (LGpB2), Sat_042 (LGp C1), Staga002 (LGp D1b), Satt684 (LGp A1), and AI856415 (LGpD1b) showed significant ($P < 0.05$) association with oleic acid for all the 5 years, though this association was weak in the years when the growing temperature during active seed formation stage was high. Quantitative trait loci (QTL) linked to Satt684 (LGp A1), Satt556 (LGp B2), Sat_042 (LGp C1), and AI856415 (LGp D1b) showed pleiotropic influence on the levels of unsaturated fatty acids. Complementation of favorable QTL from LSb1 and NRC7 generated 60% oleic acid and less than 4% α-linolenic acid RIL, stable across 5 cropping years. New SSR markers, namely, Satt245, AI856415, and Staga002 identified to be associated with different unsaturated fatty acids may be useful in improving the efficiency of marker-assisted breeding for enhancing the monounsaturated to polyunsaturated fatty acids ratio of soybean oil.

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Keywords Soybean · Unsaturated fatty acids · Genomic regions · Temperature

J Am Oil Chem Soc (2019).

Introduction

Soybean is the mainstay of global oilseeds economy, contributing 60.25% and 27.7% to the worldwide oilseed (600.97 million metric ton) and edible oil (203.95 million metric ton) production in 2018–2019, respectively (United State Department of Agriculture, 2019). One of the major criteria for assessing the quality of soybean oil is the fatty acid composition as part of triacylglycerol moiety, with palmitic (11%), stearic (2%), oleic (23%), linoleic (52%), and α-linolenic acids (7%) as the major fatty acids. Palmitic and stearic acids are saturated fatty acids, while oleic, linoleic, and α-linolenic acids are unsaturated fatty acids. Oleic acid (18:1) is monounsaturated fatty acid (MUFA) with single unsaturation, while linoleic (18:2/omega-6) and α-linolenic acids (18:3/omega-3), the polyunsaturated fatty acids (PUFA), bear two and three unsaturation, respectively, across the fatty acid hydrocarbon chain. This variation in unsaturation causes 10.0 and 21.2 times faster oxidation in linoleic and α-linolenic acids than oleic acid. Furthermore, the ideal ratio of these important unsaturated fatty acids in vegetable oil is 5.0–10.0 (Word Health Organization, 2003), which is in the proximity of 7.0, the ratio of omega-6 to omega-3 fatty acid present in human cell membrane. Soybean oil is one of the very few vegetable oils that possess the ideal omega-6 to omega-3 ratio of 7.0; paradoxically, it suffers from poor oxidative stability, shelf life, and flavor due to the rapid oxidation of the PUFA.

Partial hydrogenation employed to improve the shelf life of soybean oil by lowering PUFA incurs extra cost and generates trans fats, which are diabetogenic, atherogenic, and carcinogenic (De Souza et al., 2015). Food safety regulatory bodies in several countries, including India, have set the thresholds for *trans* fats in commercial edible oils and the processed food products containing edible oil as the major ingredient and have made it mandatory to declare the level of trans fats on the nutrition facts label (Food and Drug Administration, 2003; Food Safety and Standards Authority of India, 2018; Ratnayake et al., 2014). Therefore, specialty soybean with high oleic acid and low α-linolenic acid, one of the most unstable unsaturated fatty acids, is becoming much sought-after commodity in oil extraction industry across the world. The oil extracted from such soybean genotypes is characterized by improved oxidative stability, flavor, and storability, and therefore, it obviates the need of cost-incurring and health hazardous process of partial hydrogenation. Several breeding strategies such as recurrent selection and transgenic approach have been followed to develop soybean genotypes with high oleic acid and low α-linolenic acid contents.

During soybean seed development, omega-6 fatty acid desaturase catalyzes the conversion of oleic acid (18:1) into linoleic acid (18:2) by inserting a double bond at 12th carbon from the carboxyl end of fatty acid hydrocarbon chain. Linoleic acid is further acted upon by omega-3 fatty acid desaturase, resulting in α-linolenic acid. Accumulation of oleic acid is determined by fatty acid desaturase activity, which is governed by two candidate genes, namely, *FAD2-1A* (Glyma10g42470) and *FAD2-1B* (Glyma20g24530) (Schlueter et al., 2007). Pham et al. (2011) demonstrated that soybean genotypes carrying mutated alleles of both *FAD2-1A* and *FAD2-1B* possess 82–86% oleic acid. Alternatively, transgenic plants homozygous for the cleaved conserved sequences in *FAD2-1A* and *FAD2-1B* with elevated levels of oleic acid have also been developed (Haun et al., 2014). On the contrary, development of low α-linolenic acid soybean has been accomplished by modulating the desaturase which inserts a double bond at 15th carbon from carboxy end, thereby converting linoleic to α-linolenic acid. The activity of this desaturase is governed by at least three loci, namely, *FAD3A/fan1* (Glyma.14 g194300), *FAD3B/fan2* (Glyma.02g227200), and *FAD3C/fan3* (Glyma.18g062000) present on LGp B2/chr14, LGp G/chr18, and LGp D1b/Chr2, respectively (www.soybase.org). Deletions, insertions, and nonsense mutation in *FAD3A* (Bilyeu et al., 2005; Chappell and Bilyeu, 2006, 2007), *FAD3B* (Reinprecht et al., 2009), and *FAD3C* (Bilyeu et al., 2005) have been reported to lower α-linolenic acid content. All the three *FAD3* mutant loci (Bilyeu et al., 2011) or *FAD3A* gene mutated by a transcription activator-like effector nuclease genome editing approach in the *FAD2-1A* and *FAD2-1B* genetic background (Demorest et al., 2016) has been reported to deliver soybean genotypes with less than 2% α-linolenic acid content. Thapa et al. (2018) reported three novel point mutations in *FAD3A* gene responsible for low α-linolenic acid content. Combination of mutations in *FAD2* and *FAD3* genes has been shown to produce soybean genotypes with high oleic and low α-linolenic acid soybean (Bilyeu et al., 2018). Therefore, high oleic acid and low α-linolenic acid soybean may result from the mutations in the above-mentioned known loci controlling the activities of fatty acid desaturases, catalyzing the biosynthesis of unsaturated fatty acids. Furthermore, temperature during the seed filling stage in soybean has been well demonstrated to be associated with the differences in the fatty acid composition of the mature seeds (Lee et al., 2009; McNaughton et al., 2015), indicating thereby the temperature-dependent expression of the loci, key to the biosynthesis of unsaturated fatty acids. Furthermore, Pham et al. (2011) hypothesized that the elevated oleic acid in several exotic soybean lines with no alteration in the *FAD2-1A* and *FAD2-1B* genes may be attributed to the factors regulating at the transcription or posttranscriptional and posttranslational levels of these target genes. Recently, Zhao et al. (2019) reported novel genes other than *FAD2-1A* and *FAD2–1B* underlying the synthesis of unsaturated fatty acid in a panel of 174 soybean accessions using genome-wide associations grown under two environments. Furthermore, Xia et al. (2017) reported an additive effect of four quantitative trait loci (QTL) on oleic acid and three QTL on α-linolenic acid content of soybean in three different growing years in 134 RIL derived from Suinong10 and L-9. Furthermore, several studies (Priolli et al., 2015; Smallwood et al., 2017) reported the pleotropic effect of QTL on oleic, linoleic, and α-linolenic acid contents in soybean. In brief, there is a need to investigate for identifying new loci or validate the reported genomic regions contributing the synthesis of oleic acid and α-linolenic acid. In the present study, recombinant inbred lines (RIL) developed using biparental crossing were genotyped, and subsequently phenotyped for the unsaturated fatty acids raised across 5 cropping years, leading to the identification of novel loci contributing to high oleic acid and low α-linolenic acid in some of the RIL across multiple years.

Material and Methods

Development of RIL

LSb1 was crossed with NRC7 to obtain F_1 plants, which gave F_2 seeds. F_2 population was raised in the field and the identity of F_2 individuals was maintained as the generations were advanced to F_9 through single seed descent to obtain RIL. Selection of LSb1 and NRC7 as parents was based upon their fatty acid composition reported in our earlier study (Kumar et al., 2004). Averaged across 5 years, LSb1 exhibited oleic, linoleic, and α-linolenic acids to the

magnitude of 38.74%, 39.56%, and 5.8%, respectively, while the corresponding values for these unsaturated fatty acids in NRC7 were 23.9%, 53.04%, and 6.66%, respectively. Both the parents and the RIL were planted in 3 m plot, maintaining row-to-row and plant-to-plant distance of 45 and 5 cm, respectively, in three replicates in complete random block design in the fields of ICAR-Indian Institute of Soybean Research, Indore, Madhya Pradesh, India, consecutively for 5 years in the last week of June. Average min. temperature during vegetative stage V4 to active seed formation stage was 22.38, 21.42, 21.47, 21.76, and 22.05 °C, while average max. temperature was 27.93, 27.82, 28.63, and 29.1 °C during 2012, 2013, 2014, 2015, and 2016, respectively. Average minimum humidity was 76.68%, 83.36%, 83.36%, 82.22%, 80.64%, and 83.05%, while maximum humidity was 91.14%, 85.56%, 82.98%, 82.21%, and 86.38%, for 2012, 2013, 2014, 2015, and 2016, respectively. Standard recommended agronomic practices for raising soybean in Central India were followed from sowing till harvesting of the crop.

Estimation of Unsaturated Fatty Acids by Gas Chromatography

Oil was extracted from the randomly selected mature seeds (20) of single plant of the RIL using petroleum ether (boiling point 50 °C). Fatty acid methyl esters prepared using 1N sodium methoxide were estimated through Gas Chromatography using a Shimadzu GC17A instrument (Kyoto, Japan), fitted with a capillary column (SGEBPX70, 30 m × 0.32 mm × 0.25 μm). For resolution of fatty acid methyl esters, oven temperature was programmed at 140 °C for 3.6 min, then increased to 170 °C at a rate of 13.5 °C min^{-1} and maintained for 3.8 min, and finally increased to 182 °C at a rate of 5 °C min^{-1}. The flame ionization detector and injector were maintained at 240 °C. Nitrogen was used as the carrier gas. Peaks obtained for fatty acid methyl esters were identified by comparing the retention times with those of standard fatty acid methyl esters (Sigma-Aldrich, Bangalore, India) (Kumar et al., 2004). The quantification of fatty acid methyl ester was carried out through Class GC 10 software. The data presented in Table 2 are the average content of three plants from each of the parents and RIL.

DNA Extraction and PCR Conditions

Genomic DNA was extracted from young leaves of RIL, following cetyl trimethyl ammonium bromide procedure (Doyle and Doyle, 1990). DNA was purified and its concentration was quantified using spectrophotometer (Model Lamda 35, Perkin Elmer, MA, United States) and final concentration was adjusted to ~25 ng μL^{-1}. Polymerase chain reaction (PCR) was carried out for amplification of the genomic DNA using SSR markers in 10 μL reaction mixture containing 2 μL DNA (25 ng μL^{-1}), 1 μL PCR 10× buffer, 1.1 μL $MgCl_2$ (25 mM), 0.1 μL dNTP (25 mM), 0.4 μL each forward and reverse SSR primers (30 ng μL^{-1}), 0.068 μL Taq DNA polymerase (3 U μL^{-1}), and 4.932 μL distilled water. DNA was denatured in the thermocycler (*Lifepro* Bioer) at 94 °C for 2 min, followed by 30 cycles each consisting of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 2 min, primer elongation at 72 °C for 3 min, and final elongation at 72 °C for 10 min. PCR products amplified through SSR markers were resolved on 3% metaphor agarose. The images were analyzed in Gel Documentation Unit (Syngene, Cambridge, United Kingdom). Six hundred SSR markers across the genome, selecting minimum 25 SSR markers from each of the 20 chromosomes, were employed for parental polymorphism survey.

Statistical Analysis

All the statistical analyses were carried out using SAS 9.3 software. Association of genomic regions with unsaturated fatty acids was assessed through single marker analysis using JMP genomics component of this software.

Results and Discussion

Variability for Unsaturated Fatty Acids and Transgressive Seggregants

Freshly harvested seeds of parents, namely, LSb1 and NRC7, and RIL derived from LSb1 × NRC7 across 5 years were analyzed for fatty acid composition. Data presented in Table 2 showed that oleic acid content was significantly ($P < 0.05$) higher than NRC7 across 5 growing years. Transgressive seggregants were observed for all the three unsaturated fatty acids in all the 5 years, which is in consonance with the earlier report (Bueno et al., 2018). Averaged across 5 years, eight RIL registered oleic acid content in the range of 45–50%, while two RIL were found to contain about 60% oleic acid. Ten RIL showed less than 35% linoleic acid, while three RIL showed less than 4% α-linolenic acid. Average fortnightly maximum and minimum temperatures commencing from 15th July (at vegetative stage V4) to 30th September (active seed formation stage) of each of the 5 cropping years (2012, 2013, 2014, 2015, and 2016) at the planting location is presented in Table 1. Across RIL and the parents, average oleic acid content in 2013, 2015, and 2016, when the temperature during active seed formation stage was high, was significantly ($P < 0.05$) higher

Table 1 Average maximum and minimum fortnightly temperature from 15th July (vegetative stage V4) to 15th September (active seed formation stage)

Period	Temperature (°C)									
	2012		2013		2014		2015		2016	
	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.
15–31 July	28.80	22.89	26.85	21.78	27.48	21.90	27.32	21.51	28.50	22.65
1–15 August	26.07	22.05	27.05	21.45	28.53	21.38	27.82	22.11	27.06	22.26
16–31 August	27.89	21.86	25.93	21.27	30.59	21.52	29.78	21.96	28.02	21.59
1–15 September	28.97	22.75	31.48	21.21	27.94	21.09	31.48	21.46	30.57	21.72

than 2014 and 2012. On the contrary, average linoleic and α-linolenic acid content was significantly ($P < 0.05$) low in 2013, 2015, and 2016 than 2014 and 2012. Across all the 5 growing years, both skewness and kurtosis for oleic acid and linoleic acid were less than 1.0, while these statistical parameters were, in general, less than 0.5 for α-linolenic acid in RIL (Table 2).

Genomic Regions Significantly Associated with Unsaturated Fatty Acids across Different Years

LSb1 and NRC7 surveyed for parental polymorphism using 600 SSR markers across the genome. This revealed 105 polymorphic SSR markers (4, 7, 5, 2, 6, 3, 6, 8, 2, 6, 6, 3, 11, 9, 2, 7, 6, 6, 3, and 3 SSR markers on *chr* 1, *chr* 2, *chr* 3, *chr* 4, *chr* 5, *chr* 6, *chr* 7, *chr* 8, *chr* 9, *chr* 10, *chr* 11, *chr* 12, *chr*13, *chr* 14, *chr* 15, *chr* 16, *chr* 17, *chr* 18, *chr* 19, and *chr* 20, respectively), which were employed for genotyping 108 RIL derived from crossing these 2 genotypes. SSR markers which have shown significant association with oleic, linoleic, and α-linolenic acids in all the five cropping seasons are given in Tables 3–5, respectively, though some SSR markers that were not found to be significantly associated with different unsaturated fatty acids in all the 5 years are not given in these tables. SSR markers, namely, Satt245 (53.54cM, LGp M), Satt556 (73.21cM, LGp B2), and Sat_042 (82.51cM, LGp C1) showed significant ($P < 0.05$) association with oleic acid in 2012, 2014, and 2016, though this association was weak in 2013 and 2015 (Table 3). SSR

Table 2 Unsaturated fatty acid content (%) of parent and RIL, including range, average, kurtosis, and skewness across different growing years

Year	Parent		Range in RIL	Average content of RIL	Kurtosis	Skewness
	LSb1	NRC7				
Oleic acid (%)						
2012	35.2a	23.2b	17.80–60.20	33.81a	−0.98	0.19
2013	40.1b	25.0c	17.48–61.70	35.80b	−0.97	0.46
2014	36.3a	21.4a	15.93–67.22	34.17a	−0.49	0.75
2015	40.8b	24.4b	17.22–63.99	36.15b	−0.83	0.66
2016	41.3b	25.5c	19.48–62.07	35.91b	−0.87	0.31
Linoleic acid (%)						
2012	45.2b	56.0b	22.70–59.20	46.44b	0.78	−0.77
2013	36.6a	52.0a	25.93–59.20	43.42a	−0.95	−0.63
2014	44.7b	55.3b	13.67–59.20	45.14b	0.52	−0.81
2015	35.3a	51.1a	22.85–59.27	43.14a	−0.32	−0.70
2016	36.0a	50.8a	25.10–58.89	43.91a	−0.66	−0.49
α-Linolenic acid (%)						
2012	6.5b	7.3b	3.10–9.50	6.21b	0.39	0.46
2013	5.6a	6.5a	3.55–9.98	5.51a	−0.26	0.26
2014	6.1b	7.0b	3.61–9.09	5.94a	−0.28	0.46
2015	5.3a	6.2a	2.21–8.79	5.45a	0.02	0.09
2016	5.5a	6.3a	3.45–8.34	5.36a	−0.41	0.50

Values (parents, RILS) for different unsaturated fatty acids with different alphabets within the same column are significantly different from each at $P < 0.05$.

marker Satt245 (LGp M) showed LOD score of 5.03 (R^2 = 17.92%), 5.5 (R^2 = 19.22), and 4.04 (R^2 = 15.4%) in 2012, 2014, and 2016, respectively, and exhibited weak association in 2013 and 2015 with LOD score of 1.48 and 1.46, respectively. Satt556 (LGp B2) showed LOD score of 4.21 (R^2 = 15.06), 3.46 (R^2 = 11.99), and 3.0 (R^2 = 10.5) in 2012, 2014, and 2016, but significantly lesser LOD score in 2013 (1.53) and 2015 (1.68). Similarly, Sat_042 (LGp C1) exhibited LOD score of 3.03 (R^2 = 10.24), 3.4 (R^2 = 11.63), and 2.9 (R^2 = 8.9) in 2012, 2014, and 2016, respectively, but lower LOD score of 1.50 and 1.56 in 2013 and 2015, respectively. Furthermore, average maximum temperature in 2012, 2013, and 2016 in the first fortnight of September, which coincided with the active seed formation stage of the crop growth, was 28.97, 27.94, and 30.57 °C, respectively, but exceeded 31 °C in both 2013 and 2015 cropping years (Table 1). A lower LOD score of the above-mentioned three SSR markers, namely, Satt245 (LGp M), Satt556 (LGp B2), and Sat_042 (LGp C1) for oleic acid content in 2013 and 2015 (compared with the years 2012, 2014, and 2016) indicates the thermo-sensitivity of these QTL at temperature more than 31 °C during active seed formation stage as average maximum temperature reached 31.48°C in both these years. Three SSR markers, namely, Staga002 (126.45cM, LG D1b), Satt684 (3.54cM, LGp A1), AI856415 (50.11cM, LG D1b) also showed LOD score of 3.0 or above for association with oleic acid content for the years 2012 and 2014 but showed relatively weak association in 2013, 2015, and 2016. This indicated the thermo-sensitivity of these three QTL associated with oleic acid content when the maximum temperature during active seed formation stage reached 30.57°C or above in the years 2013, 2015, and 2016.

Table 3 SSR markers significantly associated with oleic acid and their allelic effect among different RIL across 5 cropping years

SSR marker	Year	LOD	Average content (%) of RIL with allele 0	Average content (%) of RIL with allele 2	Difference[a]
Satt684 LG A1 3.54cM	2012	4.89	26.16	36.50	10.34
	2013	2.54	28.09	35.84	7.75
	2014	4.15	28.55	36.15	7.60
	2015	2.56	28.42	35.62	7.20
	2016	1.78	26.77	38.76	7.99
Satt556 LG B2 73.21cM	2012	4.21	25.3	36.20	10.90
	2013	1.53	28.68	35.48	6.80
	2014	3.46	28.78	35.91	7.13
	2015	1.68	29.44	35.07	5.63
	2016	3.00	27.35	38.10	10.75
Sat_042 LG C1 82.51cM	2012	3.03	41.23	28.96	12.27
	2013	1.50	39.89	30.51	9.38
	2014	3.40	36.82	32.09	4.73
	2015	1.56	39.17	30.13	9.04
	2016	2.90	40.58	31.86	8.72
AI856415 LG D1b 50.11cM	2012	3.50	37.45	30.86	6.59
	2013	1.42	34.88	33.86	1.02
	2014	3.00	36.58	32.40	4.18
	2015	1.48	37.14	30.50	6.64
	2016	1.42	39.13	32.55	6.58
Staga002 LG D1b 126.45cM	2012	4.66	31.41	40.04	8.63
	2013	2.10	31.67	39.12	7.45
	2014	4.30	33.60	36.34	2.74
	2015	1.43	31.59	40.66	9.07
	2016	1.49	32.55	43.54	10.99
Satt245 LG M 53.54cM	2012	5.03	36.88	22.91	13.97
	2013	1.48	35.54	27.65	7.89
	2014	5.50	36.34	25.50	10.84
	2015	1.46	35.88	26.68	9.20
	2016	4.04	39.07	24.78	14.29

[a]Difference in average content of oleic acid is attributed to allele 0 (LSb1) or 2 (NRC7) inherited from LSb1 or NRC7, respectively.

Table 4 SSR markers significantly associated with linoleic acid and their allelic effect among RIL across 5 cropping years

SSR marker	Year	LOD value	Average content (%) of RIL with Allele 0	Average content (%) of RIL with Allele2	Difference[a]
Satt684 LG A1 3.54cM	2012	3.55	51.32	44.98	6.34
	2013	1.62	48.86	43.96	4.90
	2014	1.50	45.33	42.48	2.85
	2015	1.60	46.18	43.49	2.69
	2016	1.42	48.67	44.47	4.2
Satt556 LG B2 73.21cM	2012	3.43	51.95	45.54	6.41
	2013	1.29	46.04	43.95	2.09
	2014	2.06	46.11	42.65	3.46
	2015	1.28	45.50	43.69	1.81
	2016	2.18	51.07	44.44	6.63
Sat_042 LG C1 82.51cM	2012	3.38	43.82	48.63	4.81
	2013	1.28	43.21	45.19	1.98
	2014	2.60	41.47	44.39	2.92
	2015	1.27	42.59	44.89	2.30
	2016	2.16	42.88	47.35	4.47
AI856415 LG D1b 50.11cM	2012	2.96	44.03	48.40	4.67
	2013	1.54	43.12	44.85	1.73
	2014	1.45	41.21	44.88	3.67
	2015	1.36	43.79	45.04	1.25
	2016	1.39	44.30	46.76	2.46
Staga002 LG D1b 126.45cM	2012	4.96	48.76	42.57	6.19
	2013	1.54	45.55	42.34	3.21
	2014	1.45	43.28	42.54	0.74
	2015	1.43	45.00	42.18	2.82
	2016	1.39	46.52	43.08	3.44
Satt245 LG M 53.54cM	2012	3.22	45.07	51.84	6.77
	2013	1.35	43.97	46.07	2.10
	2014	3.00	42.13	48.15	6.02
	2015	1.38	43.35	46.60	3.25
	2016	3.21	44.17	51.71	7.54

[a]Difference in average content of linoleic acid is attributed to allele 0 (LSb1) or 2 (NRC7) inherited from LSb1 or NRC7, respectively.

Association of Satt245 (LGp M), Satt556 (LGp B2), and Sat_042 (LGp C1) showed higher LOD value in 2012, 2014, and 2016 than 2013 and 2015 for linoleic acid also. Three SSR markers, namely, Staga002 (126.45cM, LGp D1b), Satt684 (3.54cM, LGp A1), and AI856415 (50.11cM, LGp D1b), which registered higher significance with oleic acid in 2012 and 2014 than 2013, 2015, and 2016, were found to have higher significance for linoleic acid for the year 2012 than the remaining 4 years as evident from the corresponding LOD score (Table 4). Satt245 (LGp M) and Staga002 (LGD1b), which were found to be associated with oleic acid and linoleic acid, did not show any significant association with α-linolenic acid (Table 5). The genomic regions may be synthesizing some regulatory proteins, which may significantly influence omega 6 desaturase but not omega3 desaturase activity. Satt556 (LGp B2) and Satt684 (LGp A1) showed significance ($P < 0.5$) with α-linolenic acid in all the 5 cropping years, and the LOD score of these QTL for each cropping year was higher than observed for the oleic and linoleic acids. AI856415 (LGp D1b) also showed significant association with α-linolenic acid in all the 5 years with LOD score of 2.0 or more. Sat_042 (LGp C1) showed slightly lesser LOD score for this fatty acid than noted for oleic acid. Table 5 also shows that in 2012, 2014, and 2016, LOD score of Satt556 (LGp B2), Satt684 (LGp A1), and Sat_042 (LGp C1) for association with α-linolenic acid was higher than 2013 and 2015 when maximum temperature exceeded 31 °C.

Allelic Contribution

As evident from Table 3, average oleic acid content in RIL with allele 0 (from LSb1) of Satt245 (LGp M) was significantly higher (11.2%) than in RIL with allele 2 (NRC7)

across 5 years, though the allelic effect varied significantly (13.97%, 7.89%, 10.84%, 9.20%, and 14.29%) across the years. On the contrary, RIL with allele 2 (from NRC7) of Satt556 (LGp B2), Satt684 (LGp A1), and Staga002 (LGp D1b) showed 8.24%, 8.16%, and 7.8% higher average oleic acid content than the RIL with allele 0 (LSb1) across 5 years, respectively. Similarly, RIL with allele 0 (from LSb1) of Sat_042 (LGp C1) and AI856415 (LGp D1b) showed 8.82% and 5.0% higher oleic acid than the RIL with allele 2 of these SSR markers, respectively. Table 4 summarizes the contribution of different QTL found to be significantly associated with linoleic acid. RIL with allele 0 (from LSb1) of Satt245 (LGp M), Sat_042 (LGp C1), and AI856415 (LGp D1b) registered 5.13%, 3.29%, and 2.75% average lower value for this trait, respectively, than RIL with allele 2 of these SSR markers. Conversely, allele 2 (from NRC7) of Satt556 (LGp B2), Satt684 (LGp A1), and Staga002 (LGp D1b) contributed marginally lower linoleic acid value than allele 0 (from LSb1). With regard to α-linolenic acid, RIL with allele 2 of Satt684 (LGp A1) and Satt556 (LGp B2) showed less phenotypic value than the RIL with allele 0 of the same loci (Table 5). On the contrary, RIL with allele 0 (from LSb1) of Sat_042 (LGp C1) and AI856415 (LGp D1b) showed slightly less average α-linolenic acid content than RIL with allele 2 of these SSR markers.

Synthesis of unsaturated fatty acids in developing soybean seeds is regulated by omega 6 fatty acid desaturase controlled by *FAD2-1A* and *FAD2-1B*, which convert oleic acid (MUFA) to linoleic acid and omega 3 fatty acid desaturase controlled by *FAD3* genes (*FAD3A*, *FAD3B*, and *FAD3C*) which convert linoleic to α-linolenic acid. About 180 QTL have been reported to be associated with the synthesis of unsaturated fatty acids in soybean till 2018 (www.soybase.org). However, very few of them have been tested under multiple growing environments/cropping years, especially under varying growing temperature. The objective of the present study was to investigate the identification of QTL associated with unsaturated fatty acids synthesis and assess their stability across the varying growing temperature in multiple years. With regard to oleic acid, some of the SSR markers reported earlier in the literature have been found to be associated with this trait in our study also. Kim et al. (2010) reported association of Satt556 (LGp B2, 73.21cM) with oleic acid in 117 RIL population derived from Keunolkong × Iksan10, but these authors did not test the association of this SSR marker in multiple years/multi-environments. In the present study, this genomic region was found to be significantly ($P < 0.05$) associated with oleic acid in 5 growing years (2012–2016), though with weak association in 2013 and 2015 when the average max. temperature reached 31.48 °C. Moreover,

Table 5 SSR markers significantly associated with α-linolenic acid and their allelic effect among RIL across 5 cropping years

SSR marker	Year	LOD value	Average content (%) of RIL with allele 0	Average content (%) of RIL with allele 2	Difference[a]
Satt684 LG A1 3.54cM	2012	10.68	6.84	5.12	1.72
	2013	2.30	6.89	5.98	0.91
	2014	4.89	6.92	5.54	1.38
	2015	3.34	6.45	5.20	1.25
	2016	3.55	6.16	5.46	0.7
Satt556 LG B2 73.21cM	2012	12.10	7.45	5.19	2.26
	2013	3.00	7.06	6.01	1.05
	2014	7.82	7.68	5.55	2.13
	2015	3.00	6.62	5.24	1.38
	2016	4.45	6.80	5.42	1.38
Sat_042 LG C1 82.51cM	2012	2.56	5.12	5.81	0.69
	2013	1.30	5.78	6.43	0.65
	2014	2.40	5.33	6.16	0.83
	2015	1.45	5.009	5.71	0.70
	2016	2.30	5.35	5.80	0.45
AI856415 LG D1b 50.11cM	2012	2.10	5.18	5.85	0.67
	2013	2.10	5.92	6.45	0.53
	2014	2.00	5.46	6.20	0.74
	2015	2.17	5.13	5.87	0.74
	2016	3.92	5.15	6.11	0.96

[a]Difference in average content of α-linolenic acid is attributed to allele 0 (LSb1) or 2 (NRC7) inherited from LSb1 or NRC7, respectively.

Satt556 (LGp B2, chr 14, start 39,579,320-end 39,579,361 bp) is positioned very close to a peak SNP, namely, rs39135305 on LGp B2 position at 39135305 bp reported significantly associated with oleic acid recently (Zhao et al., 2019). Monteros et al. (2008) reported significant association of Satt594 (52.9cM) and Satt303 (53.4cM), both on LGp G, in $F_{2:3}$ population of G99-G725 × N00-3350. In the proximity of these genomic regions, SSR marker Satt564 (57.3cM, LGp G) was found to be significantly associated with oleic acid for 2 years 2012 and 2013 in the present study (not given in Table 3). Similarly, Satt513 (LGp L) reported to be significantly associated with oleic acid by these authors was also found to be significantly associated with this trait in our study (not given in Table 3) but for 2 years only (2012 and 2014), not in those years (2013, 2015, and 2016) when the temperature during active seed formation reached 30.57°C. Sattt597 (LGp B1, chr. 11 start 27,036,582-end 27,036,737) found to be significantly associated with oleic acid for 3 years in our study is positioned near to the peak SNP rs24645193 controlling oleic acid synthesis as reported recently (Zhao et al., 2019). Satt245 (LGp M), which was found to be significantly associated with the oleic acid biosynthesis for all the 5 years in our study, has not been earlier reported.

With regard to linoleic acid, Satt564 (LGp G) observed to be significantly associated with this trait for 3 years in the present study was near to the SNP rs39638216 reported significantly associated with this trait (Zhao et al., 2019). For α-linolenic acid also, some of the SSR identified significantly associated with this trait are in the close proximity of the genomic regions reported in the earlier studies. Priolli et al. (2015) reported association of Satt294 (78.94cM, LGp C1) in 94 soybean accessions from Asian and American gene pool. In the proximity of this genomic region, Sat_042 (82.51cM, LGp C1) was found to be associated with this fatty acid in the present study. Xia et al. (2017) reported significant association of Satt459 (118.62cM, LGp D1b) in 134 RIL derived from Suinong × L-9. Sat_183 (112.63cM, LGp D1b), which is in the

Table 6 Complementation of different genomic regions in high oleic acid (>45%), low linoleic acid (<35%), and low α-linolenic acid (<4%) averaged across 5 cropping years

			Allele of SSR markers					
	RIL	Avg	Satt684 LG A1 3.54cM	Satt556 LG B2 73.21cM	Satt245 LG M 53.54cM	Sat_042 LG C1 82.51cM	AI856415 LG D1b 50.11cM	Satga002 LG D1b 126.45cM
High oleic acid (>45%)	P2-4	45.96	2	2	0	2	0	2
	P4-13	45.33	2	2	0	0	2	0
	P5-16	45.10	2	2	0	0	0	0
	P1-33	45.17	2	2	0	2	0	0
	P6-13	46.06	2	2	0	1	2	0
	P4-5	47.20	2	2	0	0	2	0
	P4-27	47.09	2	2	0	0	0	0
	P5-5	47.24	2	2	0	2	0	2
	P3-21	60.17	2	2	0	2	2	0
	P4-19	61.19	2	2	0	2	0	0
Low linoleic acid (<35%)	P3-31	28.82	2	2	0	0	0	0
	P6-13	30.16	2	2	0	1	2	0
	P4-4	31.09	2	2	0	0	2	0
	P4-20	31.74	2	2	0	0	1	2
	P2-4	32.60	2	2	0	2	0	2
	P1-39	33.88	2	2	0	2	0	2
	P4-19	30.27	2	2	0	2	0	0
	P2-1	34.04	2	2	0	0	0	0
	P3-40	34.34	2	2	0	0	2	2
	P1-33	34.50	2	2	0	2	0	0
Low α-linolenic acid (<4%)	P4-2	3.72	2	2	NS	0	0	NS
	P4-5	3.84	2	2	NS	0	2	NS
	P7-6	3.94	2	2	NS	2	0	NS

Allele 0 and 2 correspond to allele from LSb1 and NRC7, respectively. NS denotes nonsignificant.

proximity of Satt459, was found to be associated with α-linolenic acid for 4 years in our study. Satt684 (LGp A1) found to be associated with α-linolenic acid for all the 5 years is in consonance with the earlier report (Bachlava et al., 2009). Furthermore, Sat_042 (LGp C1 chr 4, start 44,370,764-end 44,371,032) observed to be significantly associated with α-linolenic acid for all the 5 years in our study was at a distance of about 4.4 Mbps from SNP rs48837377 identified significantly associated with this trait by Zhao et al. (2019). AI856415 (LGp D1b) and Staga002 (LGp D1b) found to be significantly ($P < 0.05$) associated with all the three unsaturated fatty acids, i.e., oleic, linoleic, and α-linolenic acids for all the 5 years in the present study have not been earlier reported.

Pleiotropy and QTL Complementation

We observed specific genomic regions significantly associated with all the three unsaturated fatty acids in all the 5 growing years, suggesting their pleiotropic role in the biosynthesis of these fatty acids. Satt684 (LGp A1), Satt556 (LGp B2), Sat_042 (LGp C1), and AI856415 (LGp D1b) showed significant ($P < 0.05$) association with oleic acid, linoleic acid, and α-linolenic acid for all the 5 years, indicating pleiotropic influence of these genomic regions on the synthesis of all the three unsaturated fatty acids. This may be attributed to the fact that during the seed development, omega-6 fatty acid desaturase mediates conversion of oleic acid (18:1) to linoleic acid (18:2) by inserting of two unsaturation, which further get converted into α-linolenic acid (18:3) by omega 3 fatty acid desaturase with the insertion of three unsaturation. Satt684 (LGp A1) and Sat_042 (LGp C1) are not located on the chromosomes of candidate genes, i.e., *FAD2-1A* (LGp O) or *FAD2-1B* (LGp I) or *FAD3A* (LGp B2), *FAD3B* (LGp D1B), *FAD3C* (LGp G), while Satt556 (LGp B2) and AI856415 (LGp D1b) are far distant from *FAD3A* and *FAD3B* on LGp B2 and LGp D1b, respectively. This suggested that these SSR markers may be synthesizing some transcription factors/modifiers modulating the activities of both omega-6 desaturase and omega-3 desaturase. Furthermore, Satt245 (LGp M) and Staga002 (LGp D1b) were found to be associated with oleic and linoleic acid but not α-linolenic acid, suggesting the influence of these QTL only on *FAD2-1A*/*FAD2-1B*. Staga002 (LGp D1b, base pair position: start 49,358,807-end 49,358,842) was found to be at a distance of about 4.8 Mbp from *FAD3B* (base pair position: start 44,497,249-end 44,501,613) on LGp D1b. These observations suggesting the pleiotropic effect of some of the genomic regions on the biosynthesis of both oleic and α-linolenic acids are in consonance with the recent studies (Priolli et al., 2015; Smallwood et al., 2017; Zhao et al., 2019), which reported the pleiotropic effect of some of the sequences in the genome-wide sequencing of panel of 194 soybean accessions.

Table 6 presents the allelic contribution of significantly associated SSR markers among RIL with high oleic acid (>45%), low linoleic acid (<35%), and low α-linolenic acid (<4%). Among all the high oleic acid RIL, allele 2 (from NRC7) of Satt684 (LGp A1) and Satt556 (LGp B2) complemented with allele 0 (from LSb1) of Satt245 (LGp M). However, alleles of Sat_042 (LGp C1) and AI856415 (LGp D1b) from either of the parents were present in the high oleic acid RIL. Barring two high oleic acid RIL, all the high oleic RIL inherited allele (0) of Staga002 (LGp D1b) from LSb1. All the RIL with low linoleic acid (< 35%) showed complementation of allele 2 of Satt684 (LGp A1) and Satt556 (LGp B2) with allele 0 of Satt245 (LGp M). However, alleles of Sat_042 (LGp C1), AI856415 (LGpD1b), and Staga002 (LGp D1b) from either of the parents were present in low linoleic RIL. In the three low α-linolenic acid RIL (<4%), allele 2 of Satt684 and Satt556 complemented with alleles of Sat_042 and AI856415 from either of the parents.

In conclusion, phenotyping of 108 RIL along with parents, raised across 5 cropping years, for unsaturated fatty acids in conjunction with their genotyping using 105 polymorphic SSR markers spanning across the genome led to the identification of QTL other than the candidate genes for oleic acid, namely, *FAD2-1A*, *FAD2-1B*, and α-linolenic acid, namely, *FAD3A*, *FAD3B*, and *FAD3C* biosynthesis. Allelic contribution of these genomic regions to different unsaturated fatty acids varied significantly ($P < 0.5$). However, the effect of these QTL was dependent on the temperature during the active seed formation stage of crop growth. Three novel genomic regions, namely, Satt245 (LGp M), AI856415 (LGp D1b), and Staga002 (LGp D1b) identified in the present study can be useful in improving the efficiency of marker assisting breeding for high oleic and low α-linolenic acid. Furthermore, favorable alleles from different genomic regions significantly associated with oleic acid and α-linolenic acid complemented to give 60% oleic acid and <4% α-linolenic acid RIL. These results suggested that it is possible to develop soybean genotypes with high oleic and low α-linolenic acid, without altering known candidate genes, namely, *FAD2-1A*, *FAD2-1B*, *FAD3A*, *FAD3B*, and *FAD3C* controlling their biosynthesis.

Conflict of Interest The authors declare that they have no conflict of interest.

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