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ORIGINAL ARTICLE

Effect of *ahfad2* genes on oil profile of advanced breeding lines in groundnut (*Arachis hypogaea* L.)

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Abstract

The oil quality of groundnut is largely determined by the balance between oleic and linoleic acids; higher oleic acid levels enhance nutritional value and shelf life. This study assessed the effects of two key genes, *ahFAD2A* and *ahFAD2B*, on fatty acid composition using 100 recombinant inbred lines derived from crosses between three popular cultivars (TAG 24, Dheeraj, Visishta) and a high-oleic donor (ICGV 181024). Oil quality was analysed using Near-Infrared Reflectance Spectroscopy and gene variants were tracked through marker-assisted selection. Results showed that *ahFAD2B* had a stronger influence than *ahFAD2A* on elevating oleic acid content. Selecting for *ahFAD2B* alone proved nearly as effective as pyramiding both genes, suggesting a simpler breeding strategy. Twenty-four high-oleic lines (>70% oleic acid) were identified and four promising RILs (16, 18, 71, and 86) exhibiting an oleic/linoleic ratio above 9. These lines offer valuable resources for developing groundnut varieties with enhanced oil quality, improved nutritional benefits and extended shelf life. This article aligns with SDG-2 (Zero Hunger) of the UN Agenda for Sustainable Development.

Keywords Groundnut · Oil quality · High Oleic Acid · *ahFAD2A* and *ahFAD2B* · O/L Ratio · SDG-2 (Zero Hunger)

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Significance Statement: This study highlights the key role of ***ahFAD2B*** in improving groundnut oil quality by increasing oleic acid content. Identified high-oleic lines offer valuable resources for breeding healthier, longer shelf-life groundnut varieties, providing a simpler, efficient genetic pathway for future oil quality improvement.

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Introduction

Groundnut (*Arachis hypogaea* L.), a widely cultivated oilseed crop, plays a vital role in the diets and livelihoods of millions of people, particularly in tropical and subtropical regions. It is an allotetraploid species ($2n = 4x = 40$, AABB) with a genome size of approximately 2.54 Gb [32, 33]. Brazil is considered the centre of origin, but more than 100 countries grow groundnut today due to its adaptability to

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warm climates [16, 28]. Often referred to as the "poor man's almond," groundnut offers a remarkable nutritional profile. A 100-gram serving contains around 49.2 g of fats, 25.8 g of protein, and 16.1 g of carbohydrates, along with dietary fibre, vitamins (B, C, E, and K), and essential minerals such as iron, zinc, calcium, and magnesium[1, 2, 9, 27]. These components make groundnut a valuable source of energy, promote metabolic health, and contribute to the prevention of cardiovascular and age-related diseases.

One of the most important qualities of groundnut oil is its fatty acid composition, particularly the balance between oleic acid (a monounsaturated fat) and linoleic acid (a polyunsaturated fat). A higher oleic to linoleic acid ratio improves the oil's nutritional value and extends its shelf life by reducing rancidity. This has driven global breeding efforts aimed at increasing oleic acid content in cultivated groundnut varieties. The conversion of oleic acid to linoleic acid is catalysed by the enzyme fatty acid desaturase (*FAD2*), which is encoded by two homologous genes: *ahFAD2A* on the A-genome and *ahFAD2B* on the B-genome [6]. These genes share over 99% sequence similarity [18, 22]. Naturally occurring mutations in these genes, specifically a G:C to A:T substitution in *ahFAD2A* and an A:T insertion in *ahFAD2B,* have been shown to disrupt desaturase activity, resulting in elevated oleic acid levels in the oil [5, 17, 26].

The development of the high oleic mutant 'F435', which contains nearly 80% oleic acid and only 2% linoleic acid, marked a breakthrough in groundnut breeding [25]. Since then, over 80 high oleic varieties have been released globally using traditional breeding, marker-assisted selection (MAS), backcrossing, and mutagenesis approaches [4, 7, 15, 19, 29].

Although these efforts have been successful, there remains a limited understanding of the relative impact of *ahFAD2A* and *ahFAD2B* on fatty acid composition across diverse genetic backgrounds. This knowledge is essential to optimize selection strategies and accelerate the development of improved varieties. Therefore, the present study aimed with the objectives to evaluate the individual and combined effects of mutations in *ahFAD2A* and *ahFAD2B* genes on oleic and linoleic acid content in groundnut advanced breeding lines and identify stable high oleic lines for future cultivation and varietal development.

Materials and methods

Experimental location

The field experiment was conducted during the kharif 2022 season at Field No. 38, Dryland Farm, Regional Agricultural Research Station (RARS), Tirupati, under Acharya N.G. Ranga Agricultural University (ANGRAU), Andhra Pradesh, India. The experimental site is situated at an elevation of 182.9 meters above mean sea level, with geographic coordinates of 13°N latitude and 79°E longitude.

Planting material

The experimental material comprised 100 promising F_6 recombinant inbred lines (RILs), selected from a larger population of 350 RILs developed under the high oleic acid (HOA) breeding program at the Groundnut Breeding Section, RARS, Tirupati. Selection was based on uniform pod maturity and the presence of desirable donor traits. These RILs were derived from three interspecific crosses viz., TAG 24 × ICGV 181024 (51 RILs), Dheeraj × ICGV 181024 (31 RILs), and Visishta × ICGV 181024 (18 RILs), and were advanced through the single seed descent (SSD) method. The female parents (TAG 24, Dheeraj, and Visishta) are high-yielding Spanish bunch-type cultivars widely grown in Andhra Pradesh. The male parent, ICGV 181024, is a high oleic acid donor line that was developed by ICRISAT in Patancheru. For phenotypic evaluation, three additional high oleic lines viz., Girnar 4, ICGV 201181, and ICGV 201291, along with ICGV 181024 were included as positive checks. Girnar 4 served as an internal control for molecular analysis.

Experimental design

The experiment was laid out in an augmented block design comprising ten blocks. Each RIL was sown in two rows of 3 m length, with a row-to-row spacing of 30 cm and plant-to-plant spacing of 10 cm. High oleic checks and the female parents were included once in each block after every ten RILs to maintain uniformity and enable reliable internal comparison.

DNA isolation and quantification

Genomic DNA was extracted from 0.5 g of young leaf tissue (15–20-day-old seedlings) using a modified cetyltrimethylammonium bromide (CTAB) method [10]. Leaf samples were ground in liquid nitrogen prior to extraction. DNA purity was assessed using a NanoDrop spectrophotometer, with a 260/280 ratio close to 1.8, indicating high-quality DNA. Concentrations were calculated using the formula: dsDNA (μg/mL) = 50 × OD260 × dilution factor.

PCR amplification of *ahFAD2* alleles

Allele-specific PCR was used to detect wild-type and mutant alleles of the *ahFAD2A* (mutant: *ahfad2A*) and *ahFAD2B* (mutant: *ahfad2B*) genes, following the protocol described by Yu et.al. [31]. Four separate PCR reactions were performed: Reaction I: *FAD2A* wild-type allele (Ol1); Reaction II: *FAD2A* mutant allele (ol1); Reaction III: *FAD2B* wild-type allele (Ol2) and Reaction IV: *FAD2B* mutant allele (ol2). PCR conditions included an initial denaturation at 94 °C for 1 minute, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 53 °C for 30 seconds, and extension at 72 °C for 90 seconds. A final extension was carried out at 72 °C for 5 minutes. Primer sequences are provided in Supplementary Table 1.

Phenotypic data collection

Phenotypic evaluation of oil quality traits, including kernel oil content (%), oleic acid (%), linoleic acid (%) in the parental lines and all RILs was performed using Near-Infrared Reflectance Spectroscopy (NIRS; DS2500, FOSS, Denmark). The oleic/linoleic (O/L) acid ratio was calculated from the respective fatty acid values. Palmitic and stearic acid contents were evaluated only in the high oleic acid lines (>70% oleic acid) and the parental lines.

Statistical analysis

Phenotypic data were analysed using descriptive statistics and analysis of variance (ANOVA) in IBM SPSS Statistics 20. Correlation among traits and distribution analyses were carried out using the “garpesAgril” package in R [13].

Results

Genotyping of parents and checks for *ahfad2a* and *ahfad2b* genes using allele-specific markers

Allele-specific markers were used to detect the presence of wild-type and mutant alleles of the *ahFAD2A* and *ahFAD2B* genes. The wild-type alleles were designated as Ol1 (for *ahFAD2A*) and Ol2 (for *ahFAD2B*), while the corresponding mutant alleles were denoted as ol1 and ol2, respectively. The high oleic acid (HOA) donor line ICGV 181024 was used as the male parent, while the popular low-oleic cultivars TAG 24, Dheeraj, and TCGS 1694 served as female parents. The released HOA variety Girnar 4 (ICGV 15083) was included as a reference check.

PCR-based screening confirmed that ICGV 181024 carried homozygous mutant alleles at both loci (ol1ol1 ol2ol2), consistent with its high oleic phenotype. In contrast, the female parents exhibited only the wild-type alleles (Ol1Ol1 Ol2Ol2), correlating with their low oleic acid content. Gel electrophoresis revealed amplification products at 557 bp (reaction II) and 539 bp (reaction IV) in ICGV 181024 and Girnar 4, indicating the presence of mutant alleles. Conversely, TAG 24, Dheeraj, and TCGS 1694 showed amplification in reactions I and III, corresponding to the wild-type alleles (Fig. 1). A summary of the parental genotypes is presented in Table 1.

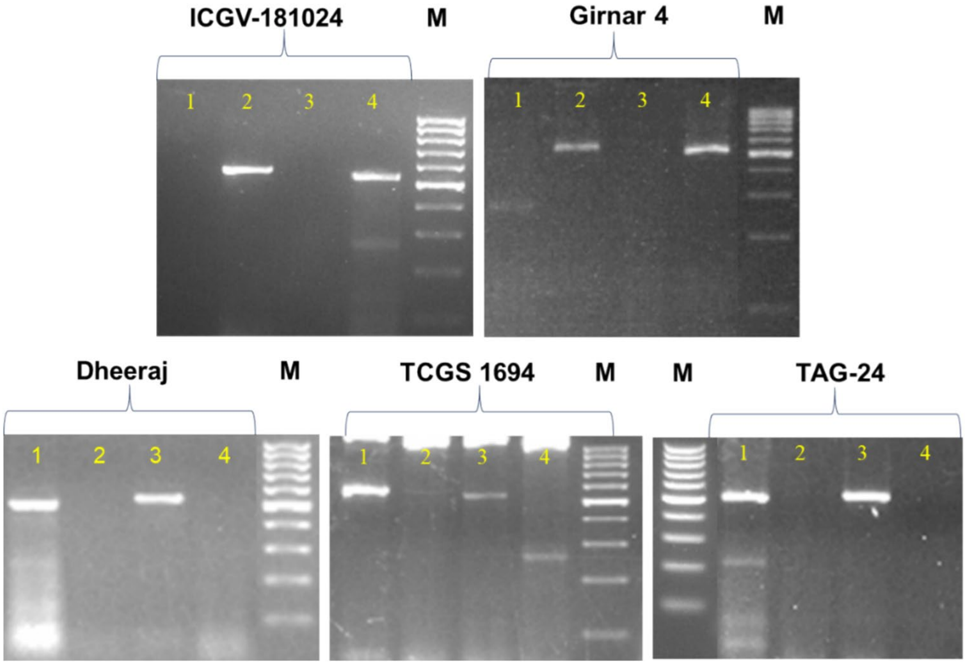

Fig. 1 Gel images showing the allelic pattern observed in *FAD2* genes (*FAD2A* and *FAD2B*) in male parent (ICGV 181024), female parents (Dheeraj, TCGS 1694, TAG 24) and high oleic check variety (Girnar 4). *Note* 1—Wild allele for *FAD2A* (Ol1) at 557 bp, 2—Mutant allele for *FAD2A* (ol1) at 557 bp, 3—Wild allele for *FAD2B* (Ol2) at 539 bp, 4—Mutant allele for *FAD2B* (ol2) at 539 bp, M: 100bp ladder

Table 1 Allele scoring of *FAD2* genes (*FAD2A* and *FAD2B*) and genetic nature of parents and check variety, Girnar 4

Parent	Alleles Scored				Genetic Nature	
	Ol1 (Wild) 557bp	ol1 (Mutant) 557bp	Ol2 (Wild) 539bp	ol2 (Mutant) 539bp	*FAD2A*	*FAD2B*
ICGV 181024	–	ol1	–	ol2	Homo (Mutant)	Homo (Mutant)
Dheeraj	Ol1	–	Ol2	–	Homo (Wild)	Homo (Wild)
TCGS 1694	Ol1	–	Ol2	–	Homo (Wild)	Homo (Wild)
TAG 24	Ol1	–	Ol2	–	Homo (Wild)	Homo (Wild)
Girnar 4	–	ol1	–	ol2	Homo (Mutant)	Homo (Mutant)

Genotyping of RILs for *ahFAD2A* and *ahFAD2B* genes

Allelic profiling of 100 recombinant inbred lines (RILs), derived from three crosses, revealed seven distinct genotypic combinations based on allele-specific marker analysis (Fig. 2; Tables 2 and 4). Fourteen RILs, namely RIL 8, 9, 11, 16, 18, 62, 71, 78, 85, 86, 101, 117, 193, and 254, carried homozygous mutant alleles for both *ahFAD2A* and *ahFAD2B* (ol1ol1 ol2ol2), indicating complete loss of fatty acid desaturase function in both sub-genomes (Table 4, Supplementary Table 2). These included 11 lines from TAG 24 × ICGV 181024, one from Dheeraj × ICGV 181024 and two from TCGS 1694 × ICGV 181024. Two other genotypic combinations were found in 16 RILs: ol1ol1 Ol2ol2 (homozygous mutant for *ahFAD2A*, heterozygous for *ahFAD2B*), and Ol1ol1 Ol2ol2 (heterozygous at both loci), suggesting partial disruption of desaturase function. Seventeen RILs showed the ol1ol1 Ol2Ol2 genotype, representing a complete mutation in *ahFAD2A* and wild-type alleles in *ahFAD2B*. Nine RILs carried the Ol1ol1 Ol2Ol2 genotype (heterozygous for *ahFAD2A*, wild-type for *ahFAD2B*), while 15 RILs had the Ol1Ol1 Ol2ol2 genotype (wild-type for *ahFAD2A*, heterozygous for *ahFAD2B*). The remaining 13 RILs exhibited homozygous wild-type alleles for both genes (Ol1Ol1 Ol2Ol2), indicating no disruption of *FAD2* function.

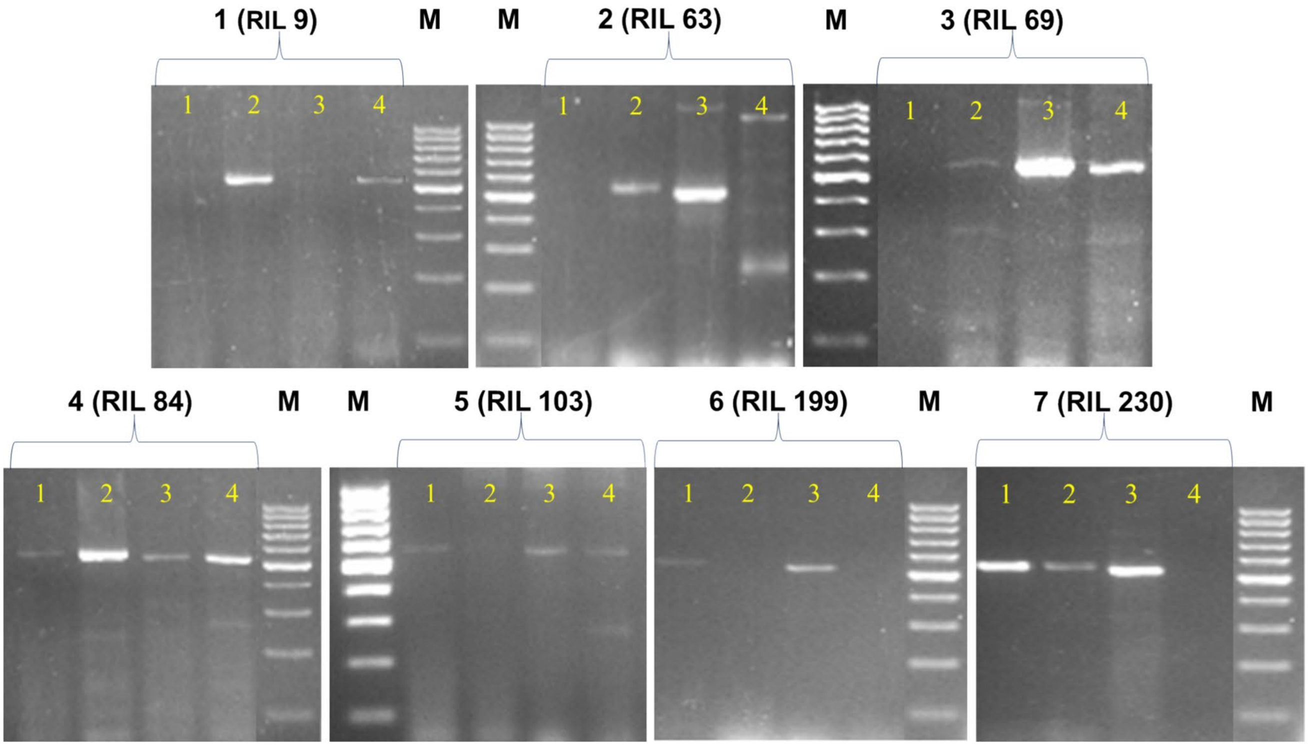

Fig. 2 Representative gel images showing seven kinds of allele patterns for *FAD2* genes (*FAD2A* and *FAD2B*) observed among 100 RILs. ^{1}Wild allele for *FAD2A* (Ol1) at 557 bp, ^{2}Mutant allele for *FAD2A* (ol1) at 557 bp, ^{3}Wild allele for *FAD2B* (Ol2) at 539 bp, ^{4}Mutant allele for *FAD2B* (ol2) at 539 bp. M: 100 bp Ladder

Table 2 Tabular representation of seven different allele patterns observed in 100 RILs

S. No	Representative Line in Fig 2.	Alleles Scored				Genetic nature		Alleles Scored
		Ol1 (Wild) 557bp	ol1 (Mutant) 557bp	Ol2 (Wild) 539bp	ol2 (Mutant) 539bp	*FAD2A*	*FAD2B*	
1	RIL 9	–	ol1	–	ol2	Homo (M)	Homo (M)	ol1ol1 ol2ol2
2	RIL 63	–	ol1	Ol2	–	Homo (M)	Homo (W)	ol1ol1 Ol2Ol2
3	RIL 69	–	ol1	Ol2	ol2	Homo (M)	Hetero	ol1ol1 Ol2ol2
4	RIL 84	Ol1	ol1	Ol2	ol2	Hetero	Hetero	Ol1ol1 Ol2ol2
5	RIL 103	Ol1	–	Ol2	ol2	Homo (W)	Hetero	Ol1Ol1 Ol2ol2
6	RIL 199	Ol1	–	Ol2	–	Homo (W)	Homo (W)	Ol1Ol1 Ol2Ol2
7	RIL 230	Ol1	ol1	Ol2	–	Hetero	Homo (W)	Ol1ol1 Ol2Ol2

These allele distributions highlight the genetic diversity present within the RIL population and underscore their potential impact on fatty acid composition and oil quality traits.

Phenotyping for kernel oil quality traits

Phenotypic evaluation of parents

Among the parental lines, ICGV 181024, carrying the double mutant alleles (ol1ol1 ol2ol2), exhibited the highest oleic acid content (78.18%) and an elevated oleic to linoleic acid (O/L) ratio of 9.42 (Fig. 3). This enhancement in oleic acid content is attributed to mutations in the *ahFAD2A* and *ahFAD2B* genes, which disrupt the fatty acid desaturase (FAD) enzyme, thereby limiting the conversion of oleic acid to linoleic acid. In contrast, the female parents viz., TAG 24, Dheeraj, and TCGS 1694, possessing wild-type alleles (Ol1Ol1 Ol2Ol2), showed lower oleic acid levels, ranging from 40.73% to 48.95%, and corresponding O/L ratios between 1.11 and 1.27 (Fig 3). Additionally, ICGV 181024 recorded a higher overall oil content (49.03%) compared to the female parents, which ranged from 45.28% to 48.37%.

Phenotypic evaluation of checks and RILs

The high oleic lines Girnar 4, ICGV 181024, ICGV 201181, and ICGV 201291 were used as positive controls. Among them, Girnar 4 exhibited superior kernel oil quality traits, recording the highest oil content (53.25%), oleic acid content

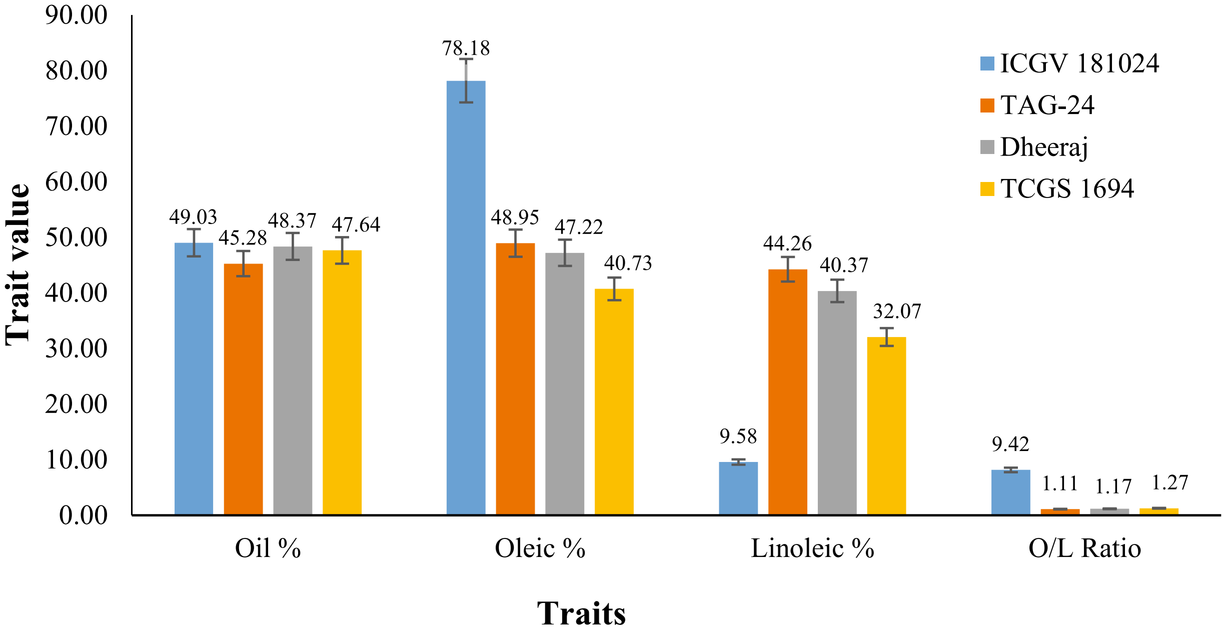

Fig. 3 Graphical representation of kernel oil quality traits of male parent, ICGV 181024 and female parents, TAG 24, Dheeraj and TCGS 1694)

(79.94%), and O/L ratio (33.61), along with the lowest linoleic acid content (2.38%) (Table 3). The remaining check lines showed oleic acid levels ranging from 76.66% to 78.18% and O/L ratios between 7.54 and 9.42, confirming their high oleic status.

Among the RILs, RIL 9 (TAG 24 × ICGV 181024) exhibited the highest oleic acid content (77.73%), while RIL 220 (Dheeraj × ICGV 181024) recorded the lowest (31.10%) (Table 4). Although Spanish bunch types generally exhibit oleic acid levels in the range of 30–50% [23], 47 RILs in the present study exceeded this baseline, with oleic acid content ranging from 60.00% to 77.73%. RIL 249 (TCGS 1694 × ICGV 181024) showed the highest linoleic acid content (56.43%), whereas RIL 86 (TAG 24 × ICGV 181024) had the lowest (5.39%). Notably, RIL 86 also recorded the highest oleic to linoleic acid (O/L) ratio (13.27), while RIL 249 had the lowest (0.60).

Among the 24 RILs identified with oleic acid levels exceeding 70%, the majority (21 lines; 87.5%) were derived from the TAG 24 × ICGV 181024 cross, while two originated from Dheeraj × ICGV 181024 and one from TCGS 1694 × ICGV 181024 (Supplementary Table 3). The improvement in oleic acid content among the RILs derived from the TAG 24 × ICGV 181024 cross was comparatively higher, ranging from 52.03% (RIL 52) to 77.73% (RIL 9). In contrast, the Dheeraj × ICGV 181024 cross showed a range of 31.10% (RIL 220) to 71.72% (RIL 147), while the TCGS 1694 × ICGV 181024 cross ranged from 32.52% (RIL 234) to 73.14% (RIL 254). (Table 4). This distribution indicates superior genetic compatibility and effective stabilization of the high oleic acid trait in the TAG 24 genetic background.

Across the entire RIL population, the oleic to linoleic acid (O/L) ratio ranged from 0.60 (RIL 249) to 13.27 (RIL 86). Notably, four RILs viz., RIL 16, RIL 18, RIL 71, and RIL 86, all derived from the TAG 24 × ICGV 181024 cross, exhibited O/L ratios above 9, indicative of enhanced oxidative stability and extended shelf life [8]. These lines showed O/L ratios ranging from 9.00 to 13.27, representing a 1.80 to 3.85 fold increase over the male parent, ICGV 181024. Additionally, 47 RILs with oleic acid content between 60.16% and 77.73% demonstrated a 25.04% to 60.99% (0.25 - 0.61 fold) increase over the low-oleic parents (47.22% - 48.95%), accompanied by a linoleic acid reduction ranging from 28.91% to 77.43% (0.29 - 0.77 fold). However, in the high oleic RILs (Supplementary Table 3), palmitic (6.26–8.52%) and stearic acid (2.65–3.49%) contents were less affected compared to oleic and linoleic acids in the parental lines following the incorporation of the *FAD2A* and *FAD2B* genes. Thus, the study emphasizes the oleic/linoleic (O/L) ratio in groundnut lines incorporating *FAD2* genes.

Table 3 Kernel oil quality traits recorded in four positive check genotypes

Check	Oil %	Oleic acid %	Linoleic acid %	O/L
ICGV 15083 (Girnar 4)	53.25	79.94	2.38	33.61
ICGV 181024*	49.03	78.18	9.58	9.42
ICGV 201181	50.94	76.66	9.68	7.92
ICGV 201291	47.95	77.09	10.25	7.54

* ICGV 181024 is the male parent and check in the current study

Analysis of Variance (ANOVA)

The effectiveness of any breeding programme largely depends on the extent of genetic variation present in the population. Greater genetic variability provides a broader base for the selection of superior genotypes. ANOVA was conducted for an augmented design comprising 100 RILs, four parental lines, and four check varieties, evaluated for four traits: oil content, oleic acid content, linoleic acid content and O/L ratio. Revealed statistically significant differences ($p \leq 0.01$) among blocks, treatments, checks and check vs. treatment comparisons (Table 5). These results confirm the presence of substantial genetic variability within the population for all studied traits.

Trait distribution

Trait distribution analysis offered valuable insights into the variability and selection potential within the RIL population. Most traits exhibited skewed distributions, reflecting opportunities for directional selection.

(i) Oil Content (%): The check variety Girnar 4 exhibited the highest oil content (53.25%) among all genotypes. The distribution curve showed positive skewness (0.94), indicating a concentration of genotypes with moderate to high oil content (Table 6, Fig. 4).
(ii) Oleic Acid (%): Girnar 4 also recorded the highest oleic acid content (79.94%). The distribution was negatively skewed (–0.56), suggesting that a substantial number of RILs had oleic acid levels exceeding 50%, which is favourable for improving oil quality.
(iii) Linoleic Acid (%): ICGV 201291 exhibited the highest linoleic acid content (10.25%) among both checks and RILs. The trait showed a positively skewed distribution (0.80), with the majority of genotypes exhibiting reduced linoleic acid levels consistent with the breeding goal of enhancing oleic acid content.
(iv) O/L Ratio: The highest oleic to linoleic acid ratio (33.61) was observed in Girnar 4, resulting in a strongly positively skewed distribution (3.72). The

Table 4 Allele Scoring of *FAD2* genes (*FAD2A* and *FAD2B*) and kernel oil quality traits of 100 RILs

S. No	RIL. No	Alleles Scored	Oil %	Oleic acid %	Linoleic acid %	O/L Ratio
TAG 24 × ICGV 181024						
1	2	ol1ol1 Ol2ol2	46.56	63.33	20.49	3.09
2	4	Ol1ol1 Ol2ol2	47.30	71.11	19.50	3.65
3	8	**ol1ol1 ol2ol2**	47.55	73.57	13.13	5.60
4	9	**ol1ol1 ol2ol2**	49.50	**77.73**	13.63	5.70
5	10	ol1ol1 Ol2ol2	49.19	74.49	17.04	4.37
6	11	**ol1ol1 ol2ol2**	50.16	76.15	11.87	6.42
7	13	ol1ol1 Ol2Ol2	47.28	53.93	26.13	2.06
8	14	Ol1ol1 Ol2ol2	46.27	54.13	21.40	2.53
9	15	ol1ol1 Ol2ol2	48.23	72.19	12.74	5.67
10	16	**ol1ol1 ol2ol2**	47.31	72.26	5.70	12.68
11	18	**ol1ol1 ol2ol2**	46.84	74.15	8.06	9.20
12	19	ol1ol1 Ol2ol2	48.43	72.14	15.56	4.64
13	33	Ol1ol1 Ol2Ol2	46.47	52.99	27.99	1.89
14	34	ol1ol1 Ol2ol2	48.42	63.79	22.30	2.86
15	36	ol1ol1 Ol2ol2	46.71	70.38	10.67	6.60
16	38	ol1ol1 Ol2ol2	44.73	68.51	10.26	6.68
17	44	ol1ol1 Ol2Ol2	48.00	66.77	19.36	3.45
18	50	ol1ol1 Ol2Ol2	47.14	65.26	17.03	3.83
19	51	ol1ol1 Ol2ol2	45.78	69.59	13.76	5.06
20	52	Ol1ol1 Ol2ol2	46.45	52.03	10.08	5.16
21	55	ol1ol1 Ol2ol2	44.02	62.30	14.98	4.16
22	56	Ol1ol1 Ol2ol2	46.51	72.51	15.82	4.58
23	57	Ol1ol1 Ol2ol2	47.72	69.54	13.88	5.01
24	58	Ol1Ol1 Ol2ol2	44.31	52.71	25.03	2.11
25	59	ol1ol1 Ol2ol2	43.70	73.35	23.37	3.14
26	62	**ol1ol1 ol2ol2**	47.84	74.66	13.14	5.68
27	63	ol1ol1 Ol2ol2	45.30	69.02	13.30	5.19
28	64	ol1ol1 Ol2ol2	45.47	70.51	10.57	6.67
29	65	Ol1ol1 Ol2ol2	44.54	60.37	22.87	2.64
30	67	Ol1Ol1 Ol2ol2	44.40	54.11	22.70	2.38
31	69	ol1ol1 Ol2ol2	45.22	62.47	12.61	4.95
32	71	**ol1ol1 ol2ol2**	48.15	75.30	7.92	9.51
33	78	**ol1ol1 ol2ol2**	46.12	71.01	8.61	8.25
34	79	ol1ol1 Ol2Ol2	43.92	66.84	28.92	2.31
35	84	Ol1ol1 Ol2ol2	44.65	70.53	13.86	5.09
36	85	**ol1ol1 ol2ol2**	48.22	71.81	11.37	6.32
37	86	**ol1ol1 ol2ol2**	46.64	71.51	5.39	13.27
38	88	ol1ol1 Ol2Ol2	44.96	57.33	20.08	2.86
39	93	ol1ol1 Ol2ol2	45.12	66.56	10.79	6.17
40	95	ol1ol1 Ol2Ol2	44.03	56.38	18.88	2.99
41	96	ol1ol1 Ol2Ol2	47.47	62.95	26.58	2.37
42	100	ol1ol1 Ol2Ol2	45.16	66.83	33.71	1.98
43	101	**ol1ol1 ol2ol2**	46.73	72.50	9.89	7.33
44	103	Ol1Ol1 Ol2ol2	45.48	56.91	15.98	3.56
45	269	Ol1ol1 Ol2ol2	46.64	65.60	14.77	4.44
46	274	ol1ol1 Ol2ol2	47.02	73.21	14.07	5.20
47	276	ol1ol1 Ol2Ol2	47.95	55.40	24.46	2.26
48	281	Ol1Ol1 Ol2ol2	46.82	56.55	31.41	1.80
49	284	ol1ol1 Ol2Ol2	46.95	53.47	19.57	2.73
50	286	Ol1ol1 Ol2ol2	45.08	59.75	13.16	4.54

Table 4 (continued)

S. No	RIL. No	Alleles Scored	Oil %	Oleic acid %	Linoleic acid %	O/L Ratio
51	289	ol1ol1 Ol2Ol2	47.56	53.96	20.42	2.64
Dheeraj × ICGV 181024						
52	20	Ol1ol1 Ol2ol2	46.36	62.16	14.23	4.37
53	22	Ol1ol1 Ol2Ol2	45.84	31.83	48.90	0.65
54	23	Ol1Ol1 Ol2Ol2	46.45	33.60	54.54	0.62
55	26	Ol1Ol1 Ol2Ol2	45.13	31.80	48.60	0.65
56	27	Ol1ol1 Ol2Ol2	46.14	33.22	45.99	0.72
57	105	Ol1ol1 Ol2Ol2	44.10	34.83	39.69	0.88
58	107	Ol1Ol1 Ol2Ol2	46.21	34.02	43.08	0.79
59	128	Ol1ol1 Ol2ol2	45.91	55.04	8.05	6.84
60	129	ol1ol1 Ol2Ol2	46.23	59.00	12.36	4.77
61	130	ol1ol1 Ol2Ol2	45.46	58.54	21.90	2.67
62	133	Ol1Ol1 Ol2ol2	45.82	53.49	27.34	1.96
63	140	ol1ol1 Ol2Ol2	47.64	62.90	32.46	1.94
64	144	ol1ol1 Ol2Ol2	45.41	64.49	27.99	2.30
65	147	Ol1ol1 Ol2ol2	47.30	71.72	18.96	3.78
66	171	Ol1Ol1 Ol2Ol2	45.87	34.83	39.69	0.88
67	173	Ol1Ol1 Ol2Ol2	45.24	33.54	46.19	0.73
68	174	Ol1Ol1 Ol2ol2	45.91	52.76	41.39	1.27
69	189	Ol1Ol1 Ol2ol2	50.89	57.28	34.20	1.67
70	193	**ol1ol1 ol2ol2**	45.09	70.61	11.65	6.06
71	198	Ol1Ol1 Ol2Ol2	46.19	32.44	48.13	0.67
72	199	Ol1Ol1 Ol2Ol2	45.77	33.87	45.44	0.75
73	201	Ol1Ol1 Ol2Ol2	45.57	31.56	45.92	0.69
74	202	Ol1Ol1 Ol2Ol2	47.51	34.16	43.02	0.79
75	204	Ol1ol1 Ol2Ol2	46.96	34.92	48.47	0.72
76	208	Ol1Ol1 Ol2ol2	45.47	52.40	20.77	2.52
77	210	Ol1Ol1 Ol2Ol2	46.62	31.47	51.31	0.61
78	212	Ol1ol1 Ol2ol2	43.57	69.01	16.54	4.17
79	217	Ol1Ol1 Ol2ol2	45.37	51.22	27.15	1.89
80	220	Ol1Ol1 Ol2Ol2	44.15	31.10	44.77	0.69
81	222	ol1ol1 Ol2ol2	47.51	64.69	8.68	7.45
82	224	Ol1ol1 Ol2ol2	47.15	63.50	15.60	4.07
TCGS 1694 × ICGV 181024						
83	116	Ol1Ol1 Ol2ol2	46.20	52.63	29.96	1.76
84	117	**ol1ol1 ol2ol2**	48.39	69.94	15.43	4.53
85	230	Ol1ol1 Ol2Ol2	46.30	34.91	40.14	0.87
86	234	Ol1ol1 Ol2Ol2	44.90	32.52	46.12	0.71
87	241	Ol1Ol1 Ol2Ol2	45.88	34.73	35.73	0.97
88	245	Ol1ol1 Ol2Ol2	49.44	52.19	22.27	2.34
89	249	Ol1ol1 Ol2Ol2	46.13	33.83	56.43	0.60
90	254	**ol1ol1 ol2ol2**	45.45	73.14	10.84	6.75
91	257	Ol1Ol1 Ol2Ol2	49.55	34.06	42.71	0.80
92	258	Ol1Ol1 Ol2ol2	49.01	52.92	29.35	1.80
93	292	Ol1ol1 Ol2ol2	43.76	51.83	16.44	3.15
94	294	ol1ol1 Ol2Ol2	43.56	57.43	21.94	2.62
95	295	Ol1Ol1 Ol2ol2	46.26	51.52	20.77	2.48
96	296	Ol1Ol1 Ol2ol2	42.81	52.29	20.11	2.60
97	297	Ol1Ol1 Ol2ol2	46.87	51.76	23.33	2.22
98	303	Ol1Ol1 Ol2ol2	46.64	52.70	25.91	2.03
99	304	Ol1ol1 Ol2ol2	45.24	56.89	10.68	5.33

Table 4 (continued)

S. No	RIL. No	Alleles Scored	Oil %	Oleic acid %	Linoleic acid %	O/L Ratio
100	305	ol1ol1 Ol2Ol2	44.36	52.69	22.96	2.29
Min.			**42.81**	**31.10**	**5.39**	**0.60**
Max.			**50.89**	**77.73**	**56.43**	**13.27**
Mean			**46.32**	**57.14**	**23.79**	**3.56**
SD			**1.64**	**14.22**	**13.42**	**2.70**
C. V%			**3.55**	**24.88**	**56.42**	**75.84**

Bold, homozygous mutants for *ahFAD2A* and *ahFAD2B*

Table 5 Analysis of variance for oil, oleic acid, linoleic acid and O/L ratio

Source	Df	Mean sum of squares			
		Oil	Oleic Acid	Linoleic Acid	O/L Ratio
Block (ignoring Treatments)	3	5.67**	5073.93**	3466.14**	124.89**
Treatment (eliminating Blocks)	107	4.85**	107.05**	108.27**	49.76**
Treatment: Check	3	21.66**	9.78**	52.71**	643.80**
Treatment: Test and Test vs. Check	104	4.36**	109.86**	109.88**	32.62**
Residuals	9	0	1.13	0.74	2.50

Table 6 Descriptive statistics of kernel quality traits

	Oil (%)	Oleic acid (%)	Linoleic acid (%)	O/L Ratio
Mean	46.89	59.85	21.86	5.27
Median	46.59	62.69	18.92	3.51
Mode	49.03	74.66	9.75	9.23
Standard Error	0.19	1.37	1.24	0.63
Standard Deviation	2.12	14.98	13.59	6.93
Sample Variance	4.50	224.26	184.79	48.08
Kurtosis	1.17	– 0.78	-0.38	15.12
Skewness	0.94	– 0.56	0.80	3.72
Range	10.44	49.19	55.07	42.78
Minimum	42.81	31.10	1.36	0.60
Maximum	53.25	80.29	56.43	43.38
Sum	5626.61	7181.42	2623.09	632.95
Count	120.00	120.00	120.00	120.00

presence of such outliers highlights the genetic potential for improving oxidative stability in groundnut oil.

Character associations

Correlation analysis is a vital tool for understanding interrelationships among traits, thereby guiding effective breeding strategies. In the present study, significant positive correlations were observed between oil content and oleic acid content (r = 0.440), oil content and O/L ratio (r = 0.447), and oleic acid content and O/L ratio (r = 0.450). These associations suggest that simultaneous improvement of these traits is feasible through selection, supporting the development of high-oil, high-oleic groundnut genotypes with enhanced oxidative stability (Table 7).

Conversely, significant negative correlations were found between oil and linoleic acid (r = – 0.336), oleic acid and linoleic acid (r = – 0.881), and linoleic acid and O/L ratio (r = – 0.550). These inverse relationships highlight the compensatory nature of oleic and linoleic acid biosynthesis and reinforce the value of selecting for increased oleic acid to enhance oil quality. Similar findings have been reported by Gangadhara *et al.* [11], supporting the robustness of the observed associations.

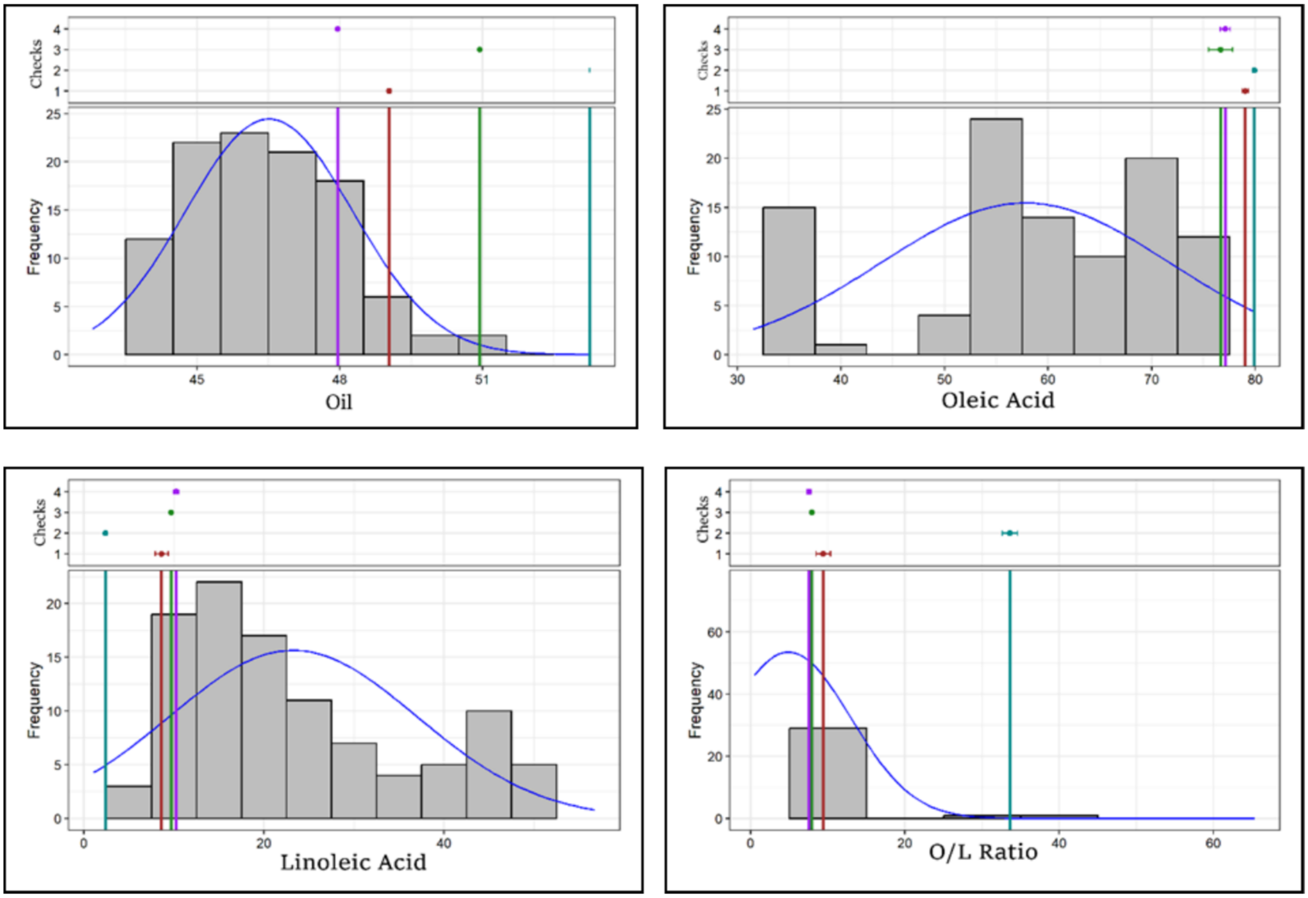

Fig. 4 Character distribution of oil, oleic acid, linoleic acid, O/L ratio. *Note* purple colour line/dot: ICGV 201291, red colour line/dot: ICGV 201181, green colour line/dot: ICGV 181024, blue colour line/dot: Girnar 4

Discussion

Wild-type *FAD2* alleles encode enzymes that convert oleic acid to linoleic acid, thereby lowering oleic acid levels in the seed. In contrast, mutations in *FAD2* genes disrupt this enzymatic activity, leading to oleic acid accumulation. In this study, the recombinant inbred lines (RILs) were specifically developed for high oleic acid content, which was reflected in the overall reduction of linoleic acid levels and an increase in the O/L ratio compared to their parental lines. This inverse relationship between oleic and linoleic acid was statistically supported by significant negative correlations, consistent with previous reports in groundnut [14], soybean [3] and sunflower [24].

Table 7 Character association of kernel quality traits (Oil, oleic acid, linoleic acid and O/L ratio)

Trait	Oil %	Oleic acid %	Linoleic acid %	O/L Ratio
Oil %	1	0.440**	– 0.336**	0.447**
Oleic acid %		1	– 0.881**	0.450**
Linoleic acid %			1	– 0.550**
O/L Ratio				1

**Correlation significant at P= 0.01 level.

The primary objective of this study was to evaluate the effects of single and double mutations in the *ahFAD2A* and *ahFAD2B* genes through an integrated approach, combining genotyping with gene/allele-specific markers and phenotyping of oil quality traits using NIRS. Among the 100 RILs evaluated, seven distinct genotypic combinations were observed: ol1ol1 ol2ol2 (14 RILs), ol1ol1 Ol2Ol2 (17 RILs), ol1ol1 Ol2ol2 (16 RILs), Ol1ol1 Ol2ol2 (16 RILs), Ol1Ol1 Ol2ol2 (15 RILs), Ol1Ol1 Ol2Ol2 (13 RILs), and Ol1ol1 Ol2Ol2 (9 RILs) (Fig. 5).

Of these, 24 RILs with genotypes ol1ol1 ol2ol2, ol1ol1 Ol2ol2, and Ol1ol1 Ol2ol2 exhibited oleic acid levels above 70% (RILs - 4, 8, 9, 10, 11, 15, 16, 18, 19, 36, 56, 59, 62, 64, 71, 78, 84, 85, 86, 101, 147, 193, 254, 274) classifying them as high oleic lines (Supplementary Table 3). The introgression of mutant alleles of *ahFAD2A* and *ahFAD2B*

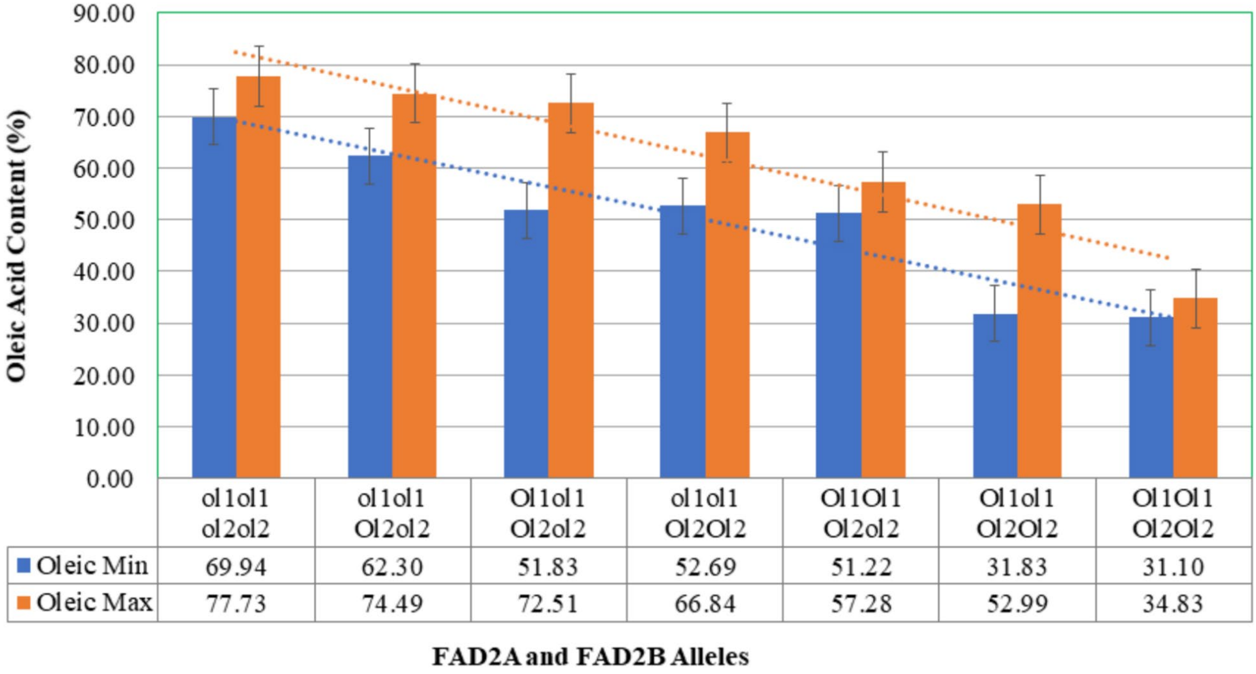

Fig. 5 Graphical representation of mean values of oleic acid, linoleic acid and O/L ratio in different genotypes

into TAG 24 × ICGV 181024 RILs successfully elevated oleic acid levels, with 24 lines recording values above the 70% threshold considered characteristic of the high-oleic phenotype (Supplementary Table 3). However, within this cross, oleic acid content varied from 70.38% to 77.73%, indicating a measurable degree of intra-cross variability. Such variation, despite the fixation of the two major loci, suggests that the oleic phenotype in groundnut is influenced by additional minor genetic and regulatory factors. Previous studies by Bertioli et al. [5] and Zhao et al. [12] have reported that the *Arachis* genome harbors multiple *FAD2* paralogs, in addition to *ahFAD2A* and *ahFAD2B*, some of which may retain residual or tissue-specific activity in oleic-to-linoleic acid conversion. The differential expression or partial activity of these paralogs could account for the modest but consistent variability observed across RILs. Importantly, the outcomes of the present investigation not only address the immediate objectives of trait characterization and genetic validation, but also establish a framework for future research aimed at fine-mapping minor modifier loci, functional characterization of candidate genes and elucidation of regulatory networks governing oil quality traits. These findings thus provide a strong foundation for advanced genomics-assisted breeding strategies in groundnut.

Furthermore, modifier loci and background genome effects inherited from the parental lines may interact with the high-oleic alleles to produce quantitative differences in fatty acid composition. Epistatic interactions among lipid metabolism genes and possible genotype × environment (G×E) effects may further contribute to the observed spread in oleic acid values. In addition, the identified genetic combinations and associated phenotypic variation offer valuable resources for subsequent studies focusing on gene expression profiling, epistatic interactions, and genotype × environment effects, thereby facilitating the development of stable, high-oleic groundnut cultivars with enhanced nutritional and industrial value.

Importantly, the variability is confined within a high-oleic range (>70%), affirming the effectiveness of *ahFAD2A* and *ahFAD2B* introgression while simultaneously highlighting the complexity of fatty acid regulation in groundnut. This intra-cross variation offers scope for selection of RILs with superior oleic acid content (>75%), which are desirable for breeding programs targeting improved oil stability, shelf life, and nutritional quality.

Notably, RILs carrying single *ahFAD2B* mutant allele (Ol1Ol1 Ol2ol2) showed oleic acid content ranging from 51.22% to 57.28% (Mean: 53.42%) (Fig. 5), while RILs with the single *ahFAD2A* mutant allele (Ol1ol1 Ol2Ol2) had a lower range of 31.83% to 52.99% (Mean: 37.92%). These results were supported by trait correlations: oleic acid content was negatively correlated with linoleic acid and positively correlated with the O/L ratio.

Further, it is observed that among the single mutants, *ahFAD2B* had a more pronounced impact on oil quality traits. Lines with the *ahFAD2B* mutation had a low mean linoleic acid content of 26.36% and a high mean O/L ratio of 2.14, whereas those with the *ahFAD2A* mutation showed higher linoleic acid (41.78%) and a lower O/L ratio (1.04). These findings align with previous studies [18, 20, 21, 28, 30], confirming that mutations in *ahFAD2B* have a stronger effect on increasing oleic acid and reducing linoleic acid than those in *ahFAD2A*.

Overall, our results indicate that a functional mutation in *ahFAD2B* alone can lead to a 15.50% higher oleic acid content compared to a mutation in *ahFAD2A*. This corresponds to a 40.88% greater improvement in oleic acid accumulation, highlighting *ahFAD2B* as a key target gene for enhancing oleic acid content in groundnut breeding programmes.

Conclusion

Our findings highlight that selecting for the *ahFAD2B* gene alone through marker-assisted breeding is nearly as effective as selecting for both *ahFAD2A* and *ahFAD2B* mutations. This approach can simplify the breeding process, saving time and effort without compromising the oil quality outcome. Out of the 24 high oleic RILs identified (oleic acid content between 70.38% and 77.73%), several lines carrying both mutant alleles stand out as candidates for advancement to yield trials. In particular, RILs 16, 18, 71 and 86, all from the TAG 24 × ICGV 181024 cross showed O/L ratios above 9, suggesting enhanced oil stability and longer shelf life. These lines meet the dual goals of improving nutritional quality and offering added value to farmers and processors through better storage potential and market appeal.

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Declarations

Conflict of interest A preprint version of this manuscript is available on [Preprint Server – Research Square, DOI/Link https://doi.org/10.21203/rs.3.rs-5717440/v1]. The authors declare that there is no conflict of interest related to this preprint.

Ethical approval Research does not involve human participants and/or animals.

Human and animal rights Research is not involving human participants and/or animals

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