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Tilling by sequencing (TbS) and association analysis in EMS induced M_2 population of groundnut TMV (Gn) 13 for higher oleic acid content

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Abstract

The present investigation was carried out in ground nut TMV (Gn) 13 to increase its oleic acid content. Its seeds were treated with EMS (ethyl methane sulfonate) at different concentration of 10 mM, 20 mM, 30 mM, 40 mM and 50 mM. Probit analysis conducted in the M_1 generation revealed an LD50 value of 39 mM. Consequently, the lower (30 mM) and upper (50 mM) limits of the LD50 were forwarded to the M_2 generation. Allele-specific primers were used for screening and analysis through TILLING (Target Induced Local Lesions in Genomes), utilizing Sanger sequencing. The analysis revealed mutations in the ahFAD2A and ahFAD2B genes, which are responsible for the conversion of oleic acid to linoleic acid, resulting in improved oleic acid levels in 7 putative mutants. Additional analyses, such as correlation and path analysis, were conducted using 16 yield-contributing traits. The number of pods, pod width, number of primary branches and number of secondary branches showed a positive correlation with pod yield per plant. It was observed that oleic acid had a negative correlation with linoleic acid content. Choosing traits that have a stronger correlation with pod yield per plant would speed up the improvement program for groundnuts. Therefore, potential mutants in M_2 with improved pod yield traits, characterized by high oleic and low linolenic levels, would be selected for advancement to the M_3 generation.

Keywords

association; groundnut; putative mutants; sanger sequencing; TILLING

Introduction

Groundnut, an incredible legume belonging to the Fabaceae family, is primarily cultivated for its oil but can also be consumed raw, boiled, or fried. China is the top producer and consumer of groundnuts, followed by India. This tropical plant grows well in hot climates with temperatures ranging from 30-35 °C on average. The focus for plant breeders is on enhancing the oil's quality by improving its flavour, shelf life and stability. Groundnut oil predominantly contains monounsaturated fatty acids, with saturated fatty acids comprising approximately 20%. In groundnut, the original homoeologous genes (ahFAD2A and ahFAD2B) are responsible for producing the delta-12-desaturase (oleoyl-PC desaturase) enzyme (1), which converts

oleic acid to linoleic acid by adding another double bond to its hydrocarbon chain (2). Within the group of unsaturated fatty acids (UFA), monounsaturated fatty acids and polyunsaturated fatty acids have nearly the same prevalence, with oleic acid and linoleic acid being the most abundant, respectively (3). PUFA are good for health, however, the more double bonds in the fatty acids, the more oxidation occurs. As a result, elevating the level of mono-unsaturated fatty acids would easily reduce oxidation activity. Mutations in ahFAD2A and ahFAD2B lead to the disruption of oleoyl-PC desaturase activity, resulting in reduced levels of oleic acid (4).

Reverse genetics is a valuable method for discovering new mutations in a target gene. TILLING, a reverse genetics technique, is applies to all plant species, regardless of their ploidy level and genomic structure (5). Tilling is focused on identifying nucleotide alterations caused by chemical mutagenesis within specific genes (6), allowing for potential modifications to protein functionality. EMS is commonly used as a mutagen in this approach because it causes a variety of mutations by chemically altering specific nucleotides, resulting in single nucleotide changes (7). The mutations in gene coding regions can be silent, nonsense, missense and splicing (8). Enhancing the oleic acid content in groundnut oil through traditional hybrid breeding or mutation breeding has significantly boosted the quality of the oil. Enzymes regulated by FAD genes play a role in transforming oleic acid into linoleic acid in oilseeds. A mutant FAD gene in groundnuts was discovered to contain approximately 80% oleic acid, while the wild type only has less than 45% (9). Two genetic mutation in the A and B genomes control the function of the ahFAD gene, blocking the transformation of oleic acid into linoleic acid. Enhancing a particular type should not sacrifice its grain production, which is a vital characteristic in all plant breeding efforts and highly significant. Because multiple genes control this trait, it is greatly affected by factors like environment, soil type, plant interactions and gene interactions. The objective of this research was to discover mutants using Tilling by sequencing for detecting mutations in the genes ahFAD2A and ahFAD2B and to examine association studies in the M_2 group of peanuts.

Materials and Methods

The present study was carried out at V.O.C Agricultural College and Research Institute Killikulam, Tamil Nadu Agricultural University. Low oleic acid content TMV (Gn) 13 a red seeded, bunch type, pureline selection from Pollachi local of Tamil Nadu with oil content of 51.40, Oleic acid (35 -40%) and Linoleic acid (30-37%) was used in this study. For Tilling analysis mutagenized population was developed by using EMS (Ethyl Methyl Sulphonate). The 100 seeds were pre-soaked in distilled water for atleast 3 h at room temperature and then dried off using tissue paper. After pre-treatment, the seeds were soaked in different doses of EMS viz., 10, 20, 30, 40 and 50 mM for 6 h at room temperature (10) to develop M_1 generation during Kharif of 2022 in field condition. This M_1 generation was subjected to probit analysis to identify its LD50 value (11). The lower (30 mM) and upper (50 mM) treatment values from LD50 were forwarded to next M_2 generation during Rabi of 2024. Then this generation were genotyped with allele-specific primers. The confirmed putative mutants varied from control (TMV (Gn) 13) were Sanger sequenced to identify and confirm its mismatch pairs. Positive putative mutants with mismatch pair in genes (ahFAD2A and ahFAD2B) and other desirable mutants were forwarded to develop the M3 population.

Molecular analysis

Samples of leaves from seedlings around 2 weeks old were gathered from the M_2 population and DNA extraction was carried out following the protocol (12). A 0.8% agarose gel and a nanodrop (Eppendorf bio spectrometer) were utilized to measure DNA quantity and the DNA concentration was normalized to 40 ng/µL using sterile distilled water or TE buffer (Tris Ethylenediamine tetraacetic acid). In order to identify the mutation in the A genome, specific primers (Table 1) such as aF19-F and 1056-R were used, while to detect the mutation in the B genome, specific primers like bF19-F and R1/FAD-R were utilized (13).The PCR cocktail included 2 µL of diluted NA, 1 µL of forward primer, 1 µL of reverse primer, 3 µL of master mix and 3 µL of PCR-grade water. The PCR procedure began with a 5 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 30 °C for 48 sec and extension at 72 °C for 1 min each and ended with a final extension at 72 °C for 7 min (14).

The products were separated in 3% agarose gel and documented. After AS-PCR all the PCR products and gel documentation the identified varied putative mutants with respective band size of FAD genes compared to control was further sequenced using Sanger sequencing (15) for confirming the mismatch pair in the genes (ahFAD2A and ahFAD2B). Mismatch pair in the putative mutants is analysed using Blast analysis and bio edit software to identify number of SNPs (16). Further oleic acid content of the obtained putative mutants was estimated by NIR Spectroscopy utilizing 5 g of grain sample (17).

Biometrical observations and statistical analysis

The observations was collected on each plant regarding its height, time taken to reach 50% flowering, secondary branch count, pod number, pod length, pod width, weight of a hundred kernels, length and width of kernels, percentage of shelling, content of oil, oleic acid, linoleic acid, score of late leaf spot and yield of pods per plant. Probit Analysis (18) method was used to determine LD 50 of various treatments. An online tool called GRAPES, based on R language, was utilized to compute correlation coefficients (19-21), while PB Perfect, another online tool, was used for path analysis by splitting the correlation coefficients into direct and indirect effects (22).

Results and Discussion

Probit analysis

Probit analysis is used to calculate LD50 which is the amount of a material, given all at once, which causes the death of 50% (one half) of a plant material. Anent to probit analysis the LD 50 was adjudged at 38.89 (Table 1), CMP (Corrected Mortality percentage) was given more importance and as such the 30 and 50 mM of EMS were forwarded to M_2 generation during rabi 2023.

Table 1. Probit analysis for calculating LD_{50} value for EMS treated TMV (Gn) 13 groundnut variety

Mutagens		EMS (*In vitro*)				
Treatments	**Control**	**10 mM**	**20 mM**	**30 mM**	**40 mM**	**50 mM**
L	-	1.00	1.30	1.48	1.65	1.76
OMP	10.9	18.75	29.76	42.56	50.16	60.56
CMP	-	8.89	21.13	35.76	44.13	55.89
EPU	-	4.56	4.76	4.97	5.35	5.78
LD_{50} value			38.89 mM			

L=Log $_{10}$ of doses, OMP=Observed mortality per cent, CMP - Corrected Mortality percentage, EPU Empirical Probit unit

Tilling by sequencing

TILLING is a broad reverse genetic method that involves combining chemical mutagenesis with PCR-based screening to detect point mutations in a specific gene. M_2 population is formed by EMS, which is an alkylating agent. It creates transition mutations (G/C: A/T) by transferring alkyl groups to DNA bases at 6-oxygen and 7-nitrogen, specifically targeting G residues. A total of 834 (403 from 30 mM and 431 from 50 mM) plants from M_2 generation was screened and analysed for TILLING. Seven putative mutants, 4 for A genome and 3 for B genome showed positive variation for the target allele in M_2 (Fig.1, 2) generation was obtained by screening the plants with allele-specific primers (Table 2). Similar studies to improve the oleic acid content in groundnut using the same primers were reported by (14).

Table 2. Allele specific markers for TILLING

S.No	Genes	Primer	Sequence (5' to 3')	Expected band size
1	ahFAD2A	aF19-Forward	ATCCAAGGCTGCATTCTCAC	826 bp
2		1056R-Reverse	TGGGACAAACACTTCGTT	
3	ahFAD2B	bF19-Forward	ATCCAAGGCTGCATTCTCAC	1214 bp
4		R1/FAD-Reverse	AACACTTCGTCGCGGTCT	

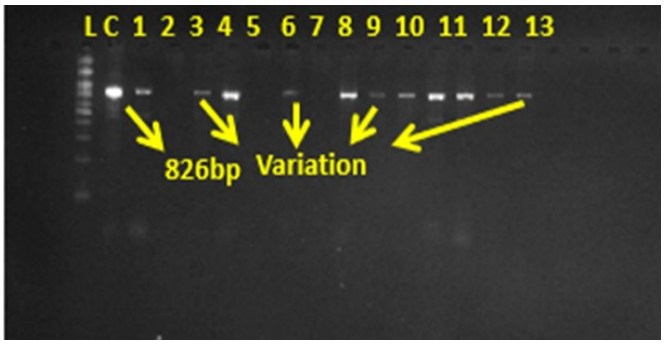

Fig. 1. Genotyping of M_2 for ahFAD2A allele: **L-** 100 bp ladder**, C-** TMV (Gn) 13, 1-14 putative mutants, PM 3, 6, 9,13 shows variation.

Fig. 2. Genotyping of M_2 for ahFAD2B allele: **L-** 100 bp ladder**, C-** TMV (Gn) 13, 1-11 putative mutants, PM 4, 1, 10 shows variation.

The obtained 7 putative mutants along with the control TMV (Gn) 13 was sequenced using Sanger sequencing (Fig. 3) to validate and confirm the mismatch pairs of nucleotide in the specific genes ahFAD2A and ahFAD2B. Similarly, in a study the mutants was confirmed using Sanger Sequencing (15). Putative mutants 3, 6, 9 and 13 shows mutation in ahFAD2A gene whereas putative mutants 4, 1 and 10 shows mutation in ahFAD2B gene. Further PM3, PM6, PM 9 and PM13 had 10,9,12 and 8 SNPs respectively for ahFAD2A gene whereas PM4, PM1, PM10 had 8, 6 and 9 SNPs respectively for ahFAD2B gene. Thus, these confirmed mutants showed higher oleic acid of up-to 15% increase in comparison with the control genotype (Table 3). As such, TILLING was successfully applied to different crop species such as sunflower (23), soybean (24) and in wheat (25).

Correlation: Inter-relationship between biometrical traits could shower the ways and means to manipulate individual traits towards yield or quality improvement. Correlation coefficient reported to range between -1 to +1 signifying the strength of linear relationship between the component traits. Positive correlation coefficient (+1) predicts the

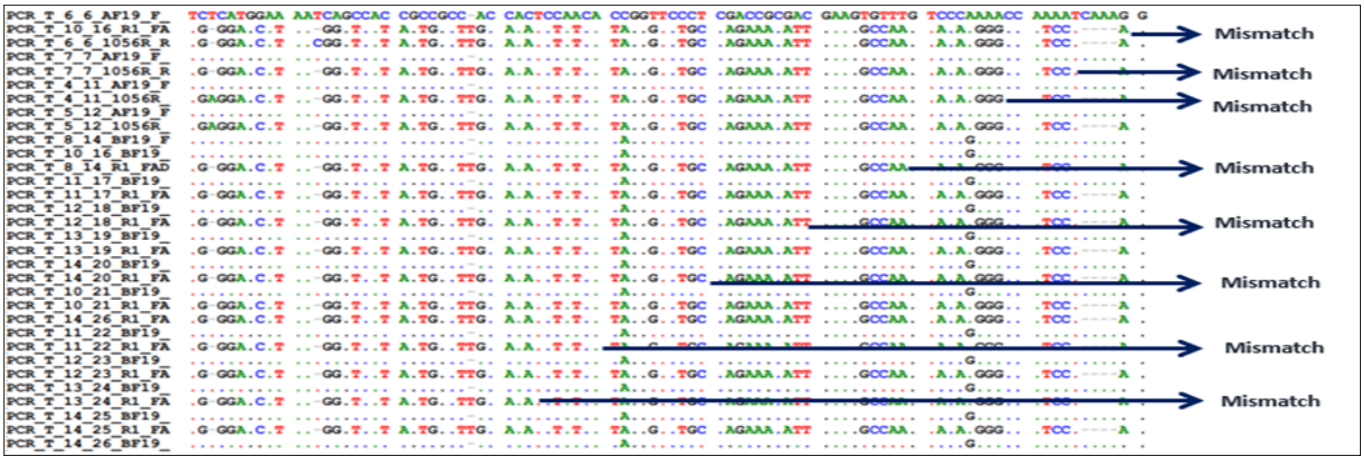

Fig. 3. Sanger sequencing of putative mutants for ahFAD2A and ahFAD2B allele: First- TMV (Gn) 13, Others-putative mutants.

Table 3. Oleic acid content in control and putative mutants and number of SNPs identified from Sanger sequencing

Genotype	Description	SNPs	Position of SNPs	Oleic acid (%)
Control	No mutation	Nil	-	39.6
PM3	ahFAD2A mutant	10	558,583,1637,1641,1650,1670,1695,1699,1702,1708	49
PM6	ahFAD2A mutant	9	3314,3352,3376,3437,3452,3460, 3474,3485,3495	47.8
PM9	ahFAD2A mutant	12	558,586,1606,1622,1640,1658,1673, 1679,1688,1692,1698,1702	49.3
PM13	ahFAD2A mutant	8	557,1646,1649,1681,1684,1690,1702,1705	47.9
PM4	ahFAD2B mutant	8	1652,752,719,688,675,606,479,495	48.9
PM1	ahFAD2B mutant	6	751,743,688,580,582,570	49.8
PM10	ahFAD2B mutant	9	1490,1596,1615,1620,1626,1643,1651,1662,1666	48.9
			Standard Deviation (SD)	**2.29**

improvement of 1 trait through working on the related traits. As well, a negative correlation coefficient (-1) construe that the improvement of 1 trait could have adverse effects on other traits. Positive correlations were found in the study between pod yield per plant and traits such as 100 pod weight (0.39), number of primary branches (0.28), number of secondary branches (0.28) and pod length (0.21). Plant height correlated positively with the number of secondary branches (0.37) and pod length (0.11) (Table 4). Additionally, significant positive correlations were observed between the number of primary branches and traits like the number of secondary branches (0.87) and pod length (0.37). The number of secondary branches, pod length and hundred pod weight demonstrated a positively significant correlation with pod yield as per (26). It was construed from the present study that 100 kernal weight had negatively correlated with number of primary branches and number of secondary branches while shelling percent had shown the negative correlation with number of primary branches, pod length and oil content (Fig. 4). Further the linoleic acid content had exhibited negative correlation coefficient for kernel weight, kernel length, oleic acid and pod length (27).

***Path analysis*:** Path analysis is a statistical method that separates correlation coefficients into direct and indirect effects to explain the cause and effect relationships. In plant breeding, it is utilized to assess how much independent traits, both direct and indirect, contribute to a dependent trait, the yield (28). When an independent trait has an impact on a dependent trait without any intermediaries, it is called a direct effect. If the impact is through an intermediary, it is known as an indirect effect. Table 5 provides the direct and indirect impacts of the 10 independent characteristics on the pod output per plant. The number of pods (0.48) and oil content (0.44) had the

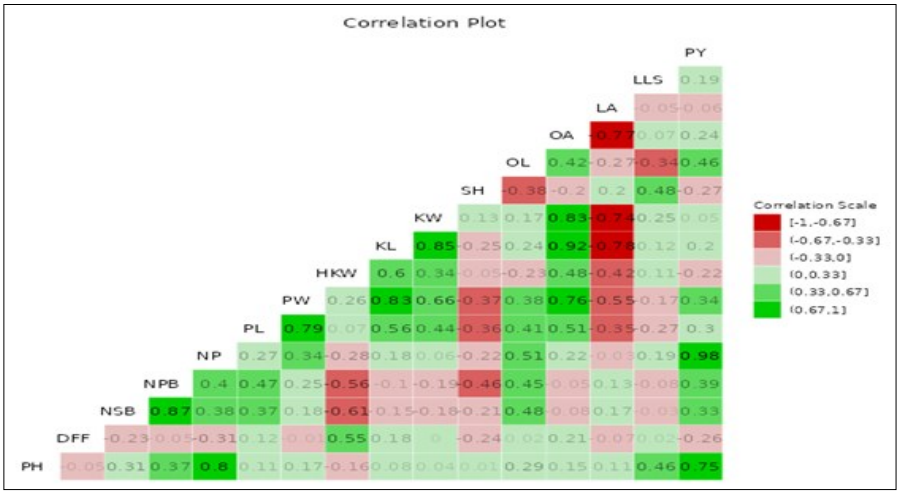

Fig. 4. Correlogram depicting the correlation coefficients of 16 traits.

Table 4. Correlation among yield and oil quality traits

	PH	DFF	NSB	NPB	NP	PL	PW	HKW	KL	KW	SH	OL	OA	LA	LLS	PY
PH	1.00															
DFF	-0.05	1.00														
NSB	0.31	-0.23	1.00													
NPB	0.37*	-0.05	0.87**	1.00												
NP	0.80**	-0.31	0.38*	0.40*	1.00											
PL	0.11	0.12	0.37*	0.47**	0.26	1.00										
PW	0.17	-0.01	0.18	0.25	0.34*	0.79**	1.00									
HKW	-0.16	0.56**	-0.61**	-0.56**	-0.28	0.07	0.26	1.00								
KL	0.08	0.18	-0.14	-0.10	0.18	0.56**	0.83**	0.60**	1.00							
KW	0.04	0.00	-0.18	-0.20	0.06	0.44**	0.66**	0.34*	0.85**	1.00						
SH	0.01	-0.24	-0.21	-0.46**	-0.22	-0.36*	-0.37*	-0.05	-0.25	0.13	1.00					
OL	0.28	0.03	0.48**	0.45**	0.51**	0.41*	0.38*	-0.23	0.24	0.17	-0.38*	1.00				
OA	0.15	0.21	-0.08	-0.05	0.22	0.51**	0.76**	0.48**	0.92**	0.82**	-0.20	0.42*	1.00			
LA	0.11	-0.07	0.17	0.13	-0.03	-0.35*	-0.55**	-0.42*	-0.78**	-0.74**	0.20	-0.27	-0.77**	1.00		
LLS	0.46**	0.02	-0.03	-0.08	0.19	-0.28	-0.17	0.11	0.12	0.24	0.48**	-0.34*	0.07	-0.05	1.00	
PY	0.44**	-0.26	0.33	0.39*	0.48**	0.30	0.34*	0.22	0.20	0.25	-0.27	0.46**	0.24	-0.06	0.19	1.00

Significance levels: p < .01 '**', p < .05 '*'

PH: Plant Height, DFF: Days to Fifty percent flowering, NSB: Number of secondary branches, NP: Number of pods, PL: Pod length, PW: Pod width, HKW: Hundred kernel weight, KL: Kernal Length, KW: Kernal Width, SH: Shelling Percentage, OL: Oil Content, OA: Oleic Acid content, LO: linoleic Acid content, LLS: Late leaf spot score and PY: Pod Yield

Table 5. Path analysis - effects among yield and oil quality traits

	PH	DFF	NSB	NPB	NP	PL	PW	HKW	KL	KW	SH	OL	OA	LA	LLS	Correlations PY
PH	-0.20	0.02	-0.03	-0.02	0.87	0.02	0.01	-0.02	-0.04	-0.03	-0.02	-0.09	0.04	-0.03	0.11	0.44**
DFF	0.01	-0.04	0.02	0.03	-0.34	0.03	-0.01	0.05	-0.09	0.00	0.04	-0.01	0.06	0.02	0.01	-0.26
NSB	-0.06	0.01	-0.09	-0.04	0.41	0.08	0.01	-0.05	0.07	0.02	0.03	-0.02	-0.02	-0.04	-0.01	0.33
NPB	-0.08	0.02	-0.08	-0.05	0.44	0.10	0.02	-0.05	0.05	0.02	0.07	-0.01	-0.01	-0.03	-0.02	0.39*
NP	-0.16	0.03	-0.04	-0.02	1.09	0.06	0.02	-0.03	-0.09	-0.01	0.03	-0.02	0.06	0.01	0.05	0.48**
PL	-0.02	-0.01	-0.03	-0.02	0.29	0.22	0.05	0.01	-0.27	-0.04	0.05	-0.01	0.14	0.08	-0.07	0.30
PW	-0.04	0.00	-0.02	-0.01	0.37	0.18	0.07	0.02	-0.40	-0.05	0.06	-0.01	0.21	0.01	-0.04	0.34*
HKW	0.03	-0.03	0.06	0.03	-0.30	0.01	0.02	0.09	-0.29	-0.03	0.01	0.07	0.13	0.01	0.03	0.22
KL	-0.02	-0.01	0.01	0.01	0.20	0.12	0.06	0.05	-0.48	-0.07	0.04	-0.07	0.25	0.02	0.03	0.20
KW	-0.01	0.01	0.02	0.01	0.07	0.10	0.05	0.03	-0.41	-0.09	-0.02	-0.05	0.23	0.02	0.06	0.25
SH	-0.03	0.01	0.02	0.02	-0.24	-0.08	-0.03	-0.04	0.12	-0.01	-0.15	0.01	-0.06	-0.01	0.12	-0.27
OL	-0.06	-0.01	-0.05	-0.02	0.56	0.09	0.03	-0.02	-0.12	-0.01	0.06	-0.03	0.12	0.01	-0.09	0.46**
OA	-0.03	-0.01	0.07	0.01	0.24	0.11	0.05	0.04	-0.45	-0.07	0.03	-0.01	0.28	0.02	0.02	0.23
LA	-0.02	0.01	-0.02	-0.01	-0.04	-0.08	-0.04	-0.04	0.38	0.06	-0.03	0.01	-0.21	-0.02	-0.01	-0.06
LLS	-0.09	-0.01	0.02	0.01	0.21	-0.06	-0.02	0.01	-0.09	-0.02	-0.07	0.01	0.02	0.01	0.24	0.19

Residuals: 0.226 , The last column is the correlations with the dependent variable.

PH: Plant Height, DFF: Days to Fifty percent flowering, NSB: Number of secondary branches, NP: Number of pods, PL: Poad length, PW: Pod width, HKW: Hundred kernel weight, KL: Kernal Length, KW: Kernal Width, SH: Shelling Percentage, OL: Oil Content, OA: Oleic Acid content, LO: linoleic Acid content, LLS: Late leaf spot score and PY: Pod Yield

greatest and moderate direct impact on pod yield as influenced by the number of primary branches. Minimal impacts were seen in pod length and linoleic acid content, as reported in reference (29). The residual influence was determined to be 0.23 and the combined impact of dependent and independent traits in the M_2 population accounted for 77% of total variability.

Conclusion

A rigorous selection process of potential mutants in a segregating mutant population is a critical step. Based on this research, selecting plants with desirable traits such as height, number of primary branches, 100 pod weight and high oleic acid content would be a yield compromising traits to increase the pod yield in ground nut. Enhancing the oil quality of ground nut is achieved by increasing its oleic acid content through tilling and sequencing. Further oil quality of ground nut is improved by increasing its oleic acid content which is achieved by TILLING followed by sequencing. Thus, the identified 7 mutants with altered genes (ahFAD2A and ahFAD2B) had improved its oleic acid content. These confirmed mutants can be used in further hybridization programme and can be forwarded to M_3 generation along with the other screened putative mutants.

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Authors' contributions

JRJ conducted the research experiments and wrote the manuscript. SJ, KG and SM helped in conducting the experiments. SS and MAP designed the study and supervised it. AK and JS helped in the statistical analysis and interpretation. SS helped in genotype collection and corrected and revised the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly in order to improve the language and readability. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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