Euphytica (2018)214:162
https://doi.org/10.1007/s10681-018-2241-0

Improving oil quality by altering levels of fatty acids through marker-assisted selection of *ahfad2* alleles in peanut (*Arachis hypogaea* L.)

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Received: 4 May 2018 / Accepted: 11 August 2018

Abstract Peanut plays a key role to the livelihood of millions in the world especially in Arid and Semi-Arid regions. Peanut with high oleic acid content aids to increase shelf-life of peanut oil as well as food products and extends major health benefits to the consumers. In peanut, *ahFAD2* gene controls quantity of two major fatty acids viz, oleic and linoleic acids. These two fatty acids together with palmitic acid constitute 90% fat composition in peanut and regulate the quality of peanut oil. Here, two *ahfad2* alleles from SunOleic 95R were introgressed into ICGV 05141 using marker-assisted selection. Marker-assisted breeding effectively increased oleic acid and oleic to linoleic acid ratio in recombinant lines up to 44% and 30%, respectively as compared to ICGV 05141. In addition to improved oil quality, the recombinant lines also had superiority in pod yield together with desired pod/seed attributes. Realizing the health benefits and ever increasing demand in domestic and international market, the high oleic peanut recombinant lines will certainly boost the economical benefits to the Indian farmers in addition to ensuring availability of high oleic peanuts to the traders and industry.

Keywords Peanut · Oleic acid · Oil quality · Marker-assisted selection (MAS) · *ahFAD2* gene

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10681-018-2241-0) contains supplementary material, which is available to authorized users.

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Published online: 20 August 2018

Introduction

Peanut (*Arachis hypogaea* L.) is cultivated in an area of ~ 25.44 m ha with a production of ~ 45.22 m tons (FAO 2014). Based on average production, the China, India, Nigeria, United States of America and Sudan are top five peanut producing countries in the world which together contribute ~ 70% to the global peanut production (FAO 2014). In USA and other European countries, major share of peanut produce (~ 75%) goes towards confectionary and other food purpose, while half of the produce (~ 49%) goes for oil extraction in two major groundnut producing countries viz, China and India. Fatty acid composition defines the peanut oil quality which contains 80% unsaturated fatty acids (UFA) and 20% saturated fatty acids (SFA). Oleic and linoleic acids constitute UFA, while palmitic, stearic, arachidic, gadoleic, behenic and lignoceric acids constitute SFA. Palmitic acid alone constitutes about 10% of SFA, while remaining five fatty acids together constitute remaining 10% of SFA (Kavera et al. 2014). Together oleic, linoleic and palmitic acids comprise ~ 90% of the total fatty acid composition and regulate the quality of peanut oil (Moore and Knauft 1989).

Peanut oil is preferred for cooking purpose due to its higher UFAs to SFAs ratio (Johnson and Saikia 2008). In general SFAs increase serum low-density lipoproteins (LDL) cholesterol level in the blood. Excess consumption of palmitic acid increases the risk of cardiovascular diseases (CVD) (WHO 2003). On the other hand higher linoleic acid in oil helps in oxidative rancidity and oil becomes thermodynamically unstable upon heating (Kratz et al. 2002). Furthermore, variation in linoleic acid content promotes formation of trans-fatty acid which also causes CVD. In contrast, high oleic acid content in cooking oil decreases the risk of CVD by reducing the level of serum LDL cholesterol and maintains level of high-density lipoproteins (HDL) (Rizzo et al. 1986; Wang 2009; Vassiliou et al. 2009). Besides, consumption of peanut products rich in oleic acid decreases tumorigenesis, and ameliorate inflammatory diseases (O'Byrne et al. 1997; Yamaki et al. 2005). Oleic acid has 10-fold higher auto-oxidative stability than linoleic acid (O'Keefe et al. 1993). Thus, peanut and its byproducts with high oleic acid as well as high oleic to linoleic acid ratio (O/L ratio) have longer shelf life than normal peanut (Mozingo et al. 2004). Therefore, breeding peanut varieties with high oleic acid and reduced level of linoleic and palmitic acids are essential to make peanut nutritionally more desirable to the consumers (Janila et al. 2016). Availability of F435, a peanut mutant with 80% oleic acid and 2% linoleic acid contents, helped in breeding high oleic peanuts, SunOleic 95R followed by SunOleic 97R in USA (Norden et al. 1987). Previous studies have reported that two alleles each in the A-genome (*ahFAD2A*) and in the B-genome (*ahFAD2B*) control fatty acids contents in peanut (Wang et al. 2013, 2015a). Subsequently, Chu et al. (2009) and Chen et al. (2010) developed linked cleaved amplified polymorphic sequence (CAPS) and allele-specific markers, respectively for both *ahFAD2A* and *ahFAD2B* alleles. Associated CAPS markers to high oleic acid in peanut helped to breed 'Tifguard High O/L' variety in USA (Chu et al. 2007). Recently, Janila et al. (2016) developed high oleic peanuts using both AS-PCR and CAPS markers. Here we used both AS-PCR and CAPS markers for breeding high oleic recombinants of ICGV 05141. The female parent, ICGV 05141 used here is different from Janila et al. (2016) and has high pod and oil yield together with desirable pod and seed features. High oleic peanut recombinant lines bred here are of different genetic background and under large-scale yield trials which would be released soon for cultivation to the Indian peanut farmers.

Materials and methods

Plant material

The ICGV 05141, a Virginia bunch genotype (*A. hypogaea* ssp. *hypogaea* var. *hypogaea*), is derived from the cross {{[(Robut 33-1 × NC Ac 316) × (Robut 33-1 × CS 9)] × ICGV 93023} × ICGV 99160}. ICGV 05141, a high oil (55.1%) and normal oleic acid content (55.8%) peanut breeding line was used as female parent. ICGV 05141 is one of the 52 high oil containing peanut breeding line developed by ICRISAT and tested for yield at ICAR–Directorate of Groundnut Research (ICAR-DGR), Junagadh, Gujarat over four seasons. Pod yield of ICGV 05141 was 2672 kg/ha and 2416 kg/ha during 2011 rainy and 2012 rainy seasons, respectively. While, in 2011 post rainy and 2012 post rainy seasons pod yield was

1486 kg/ha and 1875 kg/ha, respectively (Project report, 'Development and promotion of….Groundnut farmers in India' ICRISAT, 2012; unpublished). The SunOleic 95R was used as male parent which contains *ahfad2a* and *ahfad2b* alleles and characterized with low oil (45%) and high oleic acid ($\sim$ 80%) containing line. The SunOleic 95R was bred using F435 mutant at Florida Experimental Agriculture Station, USA and was characterized as a Virginia runner peanut (Gorbet and Knauft 1997). However, in our experiment we found Virginia bunch growth habit in SunOleic 95R.

Molecular markers

Breeding population in early generations (F_1 and F_2) was screened with two types of markers linked to *ahFAD2* gene. Plants with wild or mutant alleles were identified using the allele specific-polymerase chain reaction (AS-PCR) markers (Chen et al. 2010). While, plants with homozygous or heterozygous alleles were identified using the cleaved amplified polymorphic sequences (CAPS) markers (Chu et al. 2009) (Supplementary Table).

DNA extraction and marker genotyping

Tender leaf samples from 10 to 15 days old seedlings were collected. The DNA was extracted from the leaf samples of ICGV 05141, SunOleic 95R and segregating progenies using modified cetyltrimethyl ammonium bromide (CTAB) extraction method (Mace et al. 2003). The quality of DNA was tested on 0.8% agarose gel (Lonza, USA). The concentration of DNA was checked in ND100 Spectrophotometer (Nano Drop Technology, USA) and later concentration was normalized to $\sim$ 100 ng/µl for downstream application.

Genotyping with allele specific-polymerase chain reaction markers

Amplification of *ahfad2a* and *ahfad2b* alleles were checked using two different primer pairs, while a separate primer pair was used to amplify *ahFAD2A* or a*hFAD2B* alleles (Chen et al. 2010). In case of mutant allele (substitution from G:C→A:T) in the A-genome the primer combination, F435-F and F435SUB-R amplified a 203 bp fragment, while in case of mutant allele (A:T insertion) in the B-genome the primer combination, F435-F and F435INS-R amplified a 195 bp fragment. For wild type allele, the primer combination, F435-F and F435WT-R amplified a 193 bp fragment. F435-F and F435IC-R, amplified a 250 bp fragment and used as internal control to confirm successful amplification of alleles. The polymerase chain reaction (PCR) for AS-PCR markers was carried out in C1000 Thermal cycler (BIO-RAD, USA). The PCR reaction was setup in 25 µl volume using the protocol of KAPA3G Plant PCR Kit (KK7251, Kapa Biosystems, USA). Amplification of PCR assay was done using protocol as mentioned in Janila et al. (2016). The amplified DNA fragments along with 100 bp DNA marker (Thermo Scientific, USA) were size separated on a 2.0% horizontal agarose gel (Lonza, USA). Gel electrophoresis was carried out in 1X TBE buffer at 100 V current for one to two hours. The Ethidium bromide was used for staining the DNA fragments and gel was scanned using laser scanner (Fujifilm FLA 5100, Japan) for scoring.

Genotyping with CAPS markers

The PCR amplification was carried out in BIO-RAD C1000 Thermal cycler. For amplification of *ahFAD2A/ahfad2a* alleles the primer pairs aF19F and 1056R (IDT, USA) were used. Amplified product was further digested with Hpy99I (New England Bio Labs, UK) restriction enzyme having single recognition site to detect 448 G→A mutation. Similarly, the primers bF19F and R1/FADR (IDT, USA) were used to detect insertion mutation (insertion of single base 'A') at 441_442 bp in the *ahFAD2B/ahfad2b* alleles. The PCR reaction was setup in 25 µl volume using the protocol of KAPA3G Plant PCR Kit (KK7251, Kapa Biosystems, USA). Amplification of PCR assay was done using protocol as mentioned in Janila et al. (2016) and Chu et al. (2009). The PCR product was resolved on 2% agarose gel for confirming the amplification and digested with restriction enzyme after purification. Restriction digestion of the 10 µl of A-genome amplicon was done using 0.5 U of restriction enzyme Hpy99I (New England Biolabs, UK) by incubating at 37 °C for about 4 h. In case of *ahFAD2A* allele, the 826 bp fragment was digested to 598 bp and 228 bp, while *ahfad2a* allele had the 826 bp fragment intact. On the other hand, the restriction digestion of 10 µl of B-genome amplicon was done using 2.0 U of

restriction enzyme Hpy188I (New England Biolabs, UK) by incubating about 16 h at 37 °C. The 1214 bp *ahFAD2B* allele having five restriction sites cleaved into five fragments i.e., 736, 263, 171, 32 and 12 bp. While in case of *ahfad2b* allele, the 736 bp fragment had one additional restriction site which further cleaved into 550 and 213 bp. Thus, *ahfad2b* allele produced six fragments instead of five in case of *ahFAD2B* allele.

Back ground testing of recombinant lines was done with nine simple sequence repeat markers (SSRs). Nine SSRs (Supplementary Data) were picked up within the 20 cM of *ahFAD2* loci (Gautami et al. 2012). SSRs analysis was done in female parent and 11 recombinant lines (HOP-IL$_{_MAS}$-108, HOP-IL$_{_MAS}$-111, HOP-IL$_{_MAS}$119, HOP-IL$_{_MAS}$-120, HOP-IL$_{_MAS}$-130, HOP-IL$_{_MAS}$-154, HOP-IL$_{_MAS}$-181, HOP-IL$_{_MAS}$-201, HOP-IL$_{_MAS}$-123, HOP-IL$_{_MAS}$-144, and HOP-IL$_{_MAS}$-171). Recombinant lines used in background testing were selected based on existing DNA samples in the lab. Amplification of PCR assay was done using protocol as mentioned in Bera et al. (2016). The PCR product was resolved on 2% agarose gel and was scanned using laser scanner (Fujifilm FLA 5100, Japan).

Hybridization and development of MAS lines

Hybridization was done at ICRISAT, Patancharu, Telangana, India during 2011 rainy season. The F_1s were planted in pots kept inside net-house at ICAR-DGR, Junagadh, India during 2011 post rainy season. Individual F_1 plants were tagged and genotyped with allele specific markers. The F_1 plants having double mutant alleles were selfed and harvested single plant basis. F_2 onwards plants were planted in open field of ICAR-DGR.

A total of 204 F_2 plants were planted in 2012 rainy season and were genotyped with allele specific markers to identify double mutant lines. Further double mutant lines were genotyped using CAPS markers to select homozygous double mutant lines. Homozygous double mutant lines were further advanced to next generations during 2012 post rainy season. A total of 21 homozygous double mutant single plant progenies were advanced to F_4 generation in 2013 rainy season and subsequently phenotyped for oil content, protein content and fatty acid profile. Phenotyping for oil, protein and fatty acid profile was repeated for entire 21 lines in F_5 generation in 2013 post rainy season.

Field evaluation

Selected high oleic recombinant lines were compared with female parent and elite cultivar for yield and its related traits in 2014 rainy (F_6) and 2015 rainy (F_7) seasons. Recombinant lines, 21 in numbers, together with female parent (ICGV 05141) and an elite cultivar (GG 20) were planted in the farm of ICAR-DGR, Junagadh. Sowing of experiment was done in randomized block design (RBD) with three replications in second week of June 2014 and harvested in last week of October 2014. Each genotype was planted in four lines on four-metre beds. Line to line and plant to plant spacing were 45 and 10 cm, respectively. Recommended local crop management practices were followed for raising a healthy crop. Pod yield per plot (plot size was 7.2 m^2) was recorded at harvest. The experiment was repeated in 2015 rainy season following experimental design and crop management practices of previous season.

Characterization of genotype

Recombinant lines and ICGV 05141 were characterized based on 16 qualitative, 17 quantitative and two special features following peanut-descriptor (International Board for Plant Genetic Resources (IBPGR) and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) 1992) from five plant samples collected from field at vegetative, reproductive and harvesting stages.

Biochemical analysis for oil content and fatty acids

Sound matured kernels (10–15 g) harvested from $F_{3–4}$ and $F_{4–5}$ progenies were used for both oil content and fatty acid analysis. The fatty acid composition and oil content were estimated using Gas chromatography (GC 700, Thermo Fisher, USA) with flame ionization detector (FID). For estimation of the esters of fatty acids the fatty acid methyl esters were passed through capillary column (TR-wax) (Misra and Mathur 1998). The FID detector was set to 240 °C and oven at 190 °C. Carrier gas (nitrogen) and fuel gas (hydrogen) were maintained at 30 ml per min. Each sample was run for 12 min and the peaks were identified by

comparison to a FAME standard mix RM-3 (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

Mean differences among genotypes were done using Cropstat version 7.2 (IRRI 2007).

Results

Development of MAS progenies

Cross-seed, 11 in numbers, from a cross between ICGV 05141 and SunOleic 95R were collected from ICRISAT, Patancheru and were planted at ICAR-DGR, Junagadh. Upon genotyping of F_1s, eight plants with *ahfad2a* and *ahfad2b* alleles were identified. Here after, plants carrying both *ahfad2a* and *ahfad2b* alleles shall be referred as double mutant plant(s). All eight double mutant plants were selfed and advanced to F_2 generation.

A total of 204 F_2 plants were grown and genotyped with allele-specific and CAPS markers (Figs. 1, 2). Of these, 21 plants were homozygous double mutant plants and were selfed from F_3 generation to F_5 generation. Upon phenotyping for oil and oleic acid content consecutively for two years 21 recombinant lines with high oil and oleic acid content were selected. Selected recombinant lines and female parent were further screened with nine SSRs for selecting ideal recombinants. Out of which PM-170, AC3C07, TC5D06, Seq4G02, Seq7G02, GM2120, GM1893 markers amplified both in female parent and majority of the recombinant lines revealing transfer of the genomic region adjacent to *ahFAD2* loci from ICGV 05141 to the recombinant lines (Fig. 3).

Oil protein and fatty acid analysis of recombinant lines and their parents

A significant variation in oil content was observed among the recombinant lines and their parents which ranged from 49.7 to 57.9% with an average of 53.3% during 2013 rainy season (Table 1). Oil content in majority of the recombinant lines was at par with the female parent except HOP-IL$_{_MAS}$-116, HOP-IL$_{_MAS}$-120 and HOP-IL$_{_MAS}$-172 which had 49.7, 50.5 and 50.9% of oil, respectively. During 2013 post rainy season, oil content ranged from 45.0 to 57.6% with an average of 53.0% (Table 1). Unlike 2013 rainy season, majority of recombinant lines were at par with the female parent except HOP-IL$_{_MAS}$-116, HOP-IL$_{_MAS}$-119, HOP-IL$_{_MAS}$-172 and HOP-IL$_{_MAS}$-201 which had 49.3, 46.2, 45.0 and 46.3% of oil, respectively. All the recombinant lines had oil content at par with female parent except HOP-IL$_{_MAS}$-116 and HOP-IL$_{_MAS}$-172 which had 49.5% and 48.0% of oil, respectively. Pooled protein content in ICGV 05141 and SunOleic 95R were 25% and 26%, respectively. Nevertheless, a significant variation was observed for protein content among recombinant lines which varied from 21.25 to 25.01% (Table 1). ICGV 05141 had 55.7% oleic acid, 23% linoleic acid and 10.8% palmitic acid, while SunOleic 95R had 78.8% oleic acid, 3.7% linoleic acid and 7.6% palmitic acid. Oleic acid content in recombinant lines ranged from 57.8 to 80.5% during 2013 rainy season. While, in 2013 post rainy season, it varied from 55.6 to 82.0% (Figs. 4, 5). Stable quantity of oleic acid content in

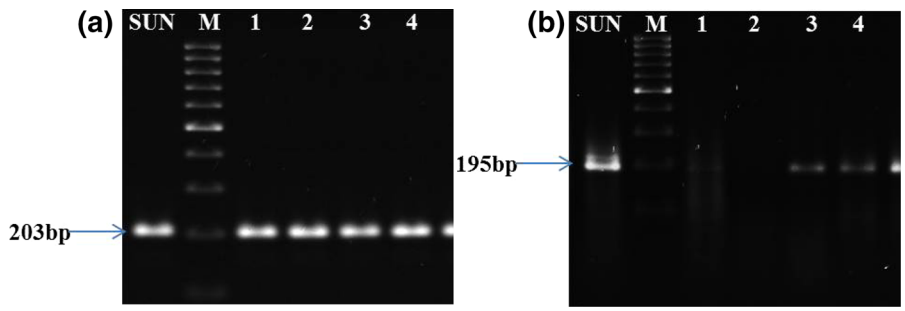

Fig. 1 AS-PCR assay; **a** Showing amplification of *ahfad2a* allele specific 203 bp fragmentin 1 to 4 F_1 plants **b** Amplification of *ahfad2b* allele specific 195 bp fragment in 3 and 4, while absent in 1 and 2 F_1 plants; Where SUN: SunOleic 95R, M:100 bp DNA ladder

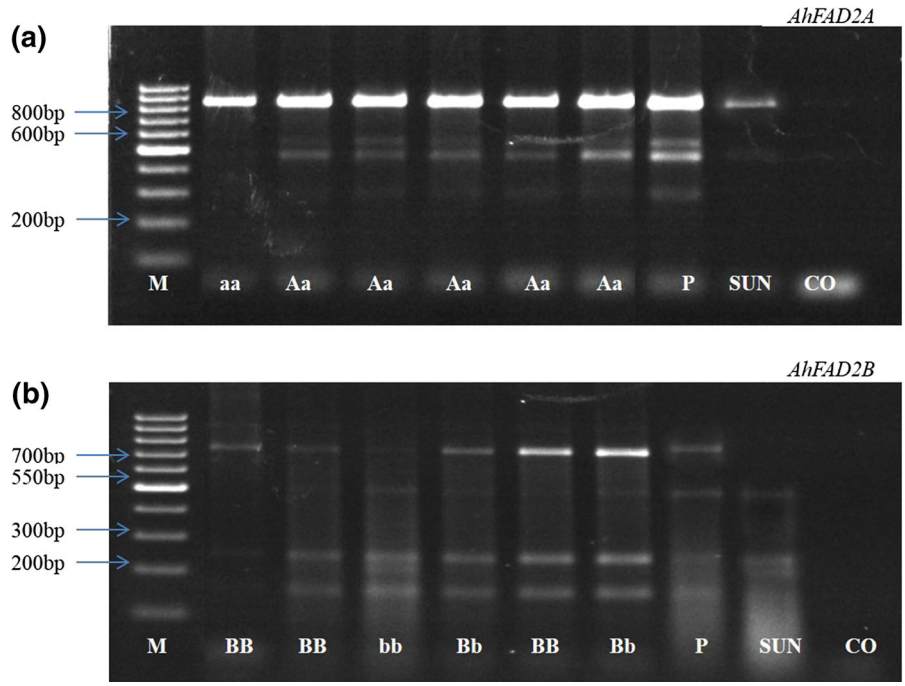

Fig. 2 CAPS assay; **a** Showing heterozygous and homozygous plants for *ahfad2a* allele. **b** Showing heterozygous and homozygous plants for *ahfad2b* mutant allele. Where SUN: SunOleic 95R, M: 100 bp DNA ladder, CO: Control, 'AA, BB': homozygous wild alleles, 'Aa, Bb': heterozygous alleles and 'aa, bb': indicates homozygous mutant alleles

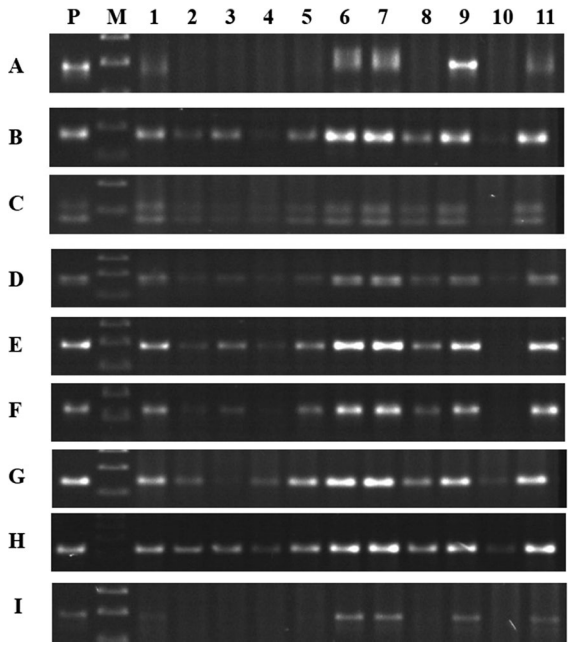

Fig. 3 SSR assisted background selection, Where A—gi-1107, B—PM-170, C—AC3C07, D—TC5D06, E—Seq 4G02, F—Seq 7G02, G—GM2120, H—GM1893, I—Seq 17C09. P—ICGV 05141, M—100 bp ladder, 1 to 11—Recombinant lines

both the seasons was noticed in majority of the recombinant lines, while it varied marginally in a few of them including SunOleic 95R over seasons. Based on oleic acid content pooled over two seasons, majority of the recombinant lines had > 70% oleic acid except HOP-IL$_{_MAS}$-123, HOP-IL$_{_MAS}$-144, HOP-IL$_{_MAS}$-164, HOP-Il$_{_MAS}$-166 and HOP-IL$_{_MAS}$-171 which had 61.0, 58.3, 56.7, 63.8 and 69.1% of oleic acid, respectively (Fig. 6). Wide range of variation for linoleic acid content was observed among recombinant lines during both 2013 rainy season and 2013 post rainy season. It varied from 2.3 to 21.6% in rainy season while from 2.2 to 24% in post rainy season. Indeed, majority of recombinant lines had < 10% linoleic acid (average of two seasons) except HOP-IL$_{_MAS}$-123, HOP-IL$_{_MAS}$-144, HOP-IL$_{_MAS}$-164, HOP-IL$_{_MAS}$-166 and HOP-IL$_{_MAS}$-171 which had 19.4%, 21.45%, 22.8% 16.9% and 12.25% of linoleic acid, respectively. Similar to oleic acid, linoleic acid content also differed between seasons in a few recombinant lines. While, linoleic acid content in ICGV 05141 and GG 20 remained almost stable between seasons. Besides, lower and comparatively stable linoleic acid content was observed in 10 recombinant lines irrespective of seasons (Table 2). In case of palmitic acid, marginal variation was observed in recombinant lines, irrespective of seasons. Over all, palmitic acid, pooled over two seasons, varied from 6.6 to 10.5% among recombinant lines (Fig. 6). We observed up to 44.2% increase in oleic

Table 1 Oil and protein content estimated at 5% of moisture in recombinant lines and parents

Genotype	Oil (%)			Protein (%)		
	2013 rainy	2013 post rainy	Pooled	2013 rainy	2013 post rainy	Pooled
HOP-IL$_{_MAS}$-108	53.3	56.8	55.1	24.3	23.1	23.7
HOP-IL$_{_MAS}$-109	57.2	55	56.1	24.1	23.8	24.0
HOP-IL$_{_MAS}$-111	52.4	56	54.2	23.8	22.4	23.1
HOP-IL$_{_MAS}$-116	49.7	49.3	49.5	22.4	23.1	22.8
HOP-IL$_{_MAS}$-119	56.3	46.2	51.2	23.6	21.0	22.3
HOP-IL$_{_MAS}$-120	50.5	55.6	53.1	21.8	21.7	21.8
HOP-IL$_{_MAS}$-123	52.4	51.3	51.9	25.6	24.4	25.0
HOP-IL$_{_MAS}$-125	53.7	55.6	54.7	23.4	23.4	23.4
HOP-IL$_{_MAS}$-130	54.8	54.6	54.7	21.7	22.1	21.9
HOP-IL$_{_MAS}$-138	52.5	55.5	54	23.9	23.3	23.6
HOP-IL$_{_MAS}$-144	53.7	54.1	53.9	23.3	21.2	22.2
HOP-IL$_{_MAS}$-145	51.8	57.2	54.5	21.5	21.2	21.4
HOP-IL$_{_MAS}$-154	52.6	54.5	53.5	22.9	23.1	23.0
HOP-IL$_{_MAS}$-163	52.7	52.5	52.6	23.6	22.5	23.1
HOP-IL$_{_MAS}$-164	51.5	55.7	53.6	24.0	24.6	24.3
HOP-IL$_{_MAS}$-166	57.9	57.6	57.7	22.8	23.0	22.9
HOP-IL$_{_MAS}$-171	53.3	52.5	52.9	23.0	23.6	23.3
HOP-IL$_{_MAS}$-172	50.9	45	48	22.1	22.6	22.3
HOP-IL$_{_MAS}$-181	51.6	50.4	51	22.6	22.7	22.6
HOP-IL$_{_MAS}$-191	53.2	53.1	53.2	21.3	21.2	21.2
HOP-IL$_{_MAS}$-201	57.2	46.3	51.8	22.4	20.8	21.6
ICGV 05141	55.1	54.3	54.7	24.6	25.5	25.0
SunOleic 95R	50.9	50.4	50.6	25.1	27.2	26.2
Mean	53.3	53.0	53.2	23.2	22.9	23.1
SE mean	1.42	1.56	1.19	1.27	1.18	1.14
CD (0.05%)	4.04	4.45	3.38	3.62	3.38	3.25
CV%	4.61	5.1	3.87	9.51	8.94	8.56

acid, while up to 89% and 39.06% decrease in linoleic and palmitic acid, respectively, in recombinant lines, as compared to ICGV 05141 (Table 2). Furthermore, these three fatty acids constituted $\sim$ 90% of total fat composition, and palmitic acid content remained almost constant (in between 6% and 10%) in recombinant lines and parents. Indeed peanut genotypes with both high oleic acid and high oleic to linoleic acid (O/L) ratio are more desirable. We observed O/L ratio of 15.8 in SunOleic 95R and 2.4 in ICGV 05141, while it varied from 2.5 to 30.9 in recombinant lines. Out of which nine lines (HOP-IL$_{_MAS}$-111, HOP-IL$_{_MAS}$-116, HOP-IL$_{_MAS}$-119, HOP-IL$_{_MAS}$-130, HOP-IL$_{_MAS}$-138, HOP-IL$_{_MAS}$-145, HOP-IL$_{_MAS}$-172, HOP-IL$_{_MAS}$-191, HOP-IL$_{_MAS}$-201) had O/L ratio more than 15.8 along with $\sim$ 80% oleic acid content (Fig. 7).

Pod yield

Pooled pod yield/plot was 1453 kg and 1323 kg in ICGV 05141 and GG 20, respectively, while it varied from 722 to 2151 kg among recombinant lines. Wide variation in pod yield was observed between genotypes as well as seasons. Significant yield superiority over female parent was observed in five recombinant lines (HOP-IL$_{_MAS}$-130, HOP-IL$_{_MAS}$-145, HOP-IL$_{_MAS}$-163, HOP-IL$_{_MAS}$-181 and HOP-IL$_{_MAS}$-

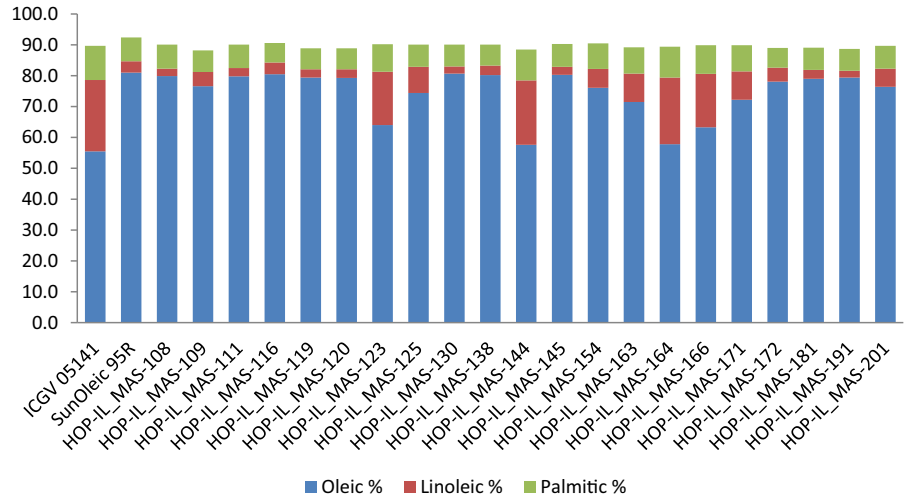

Fig. 4 Oleic, linoleic and palmitic acid content in recombinant lines and parents during 2013 rainy season

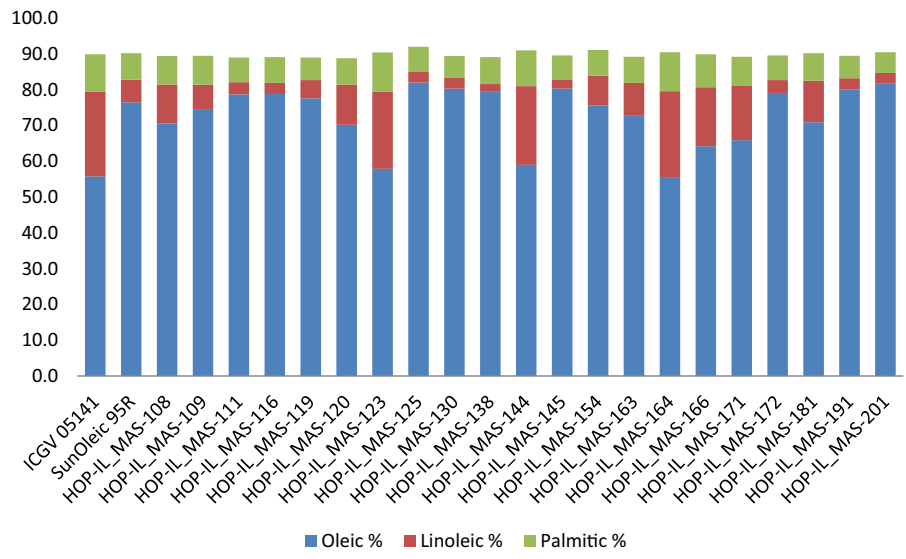

Fig. 5 Oleic, linoleic and palmitic acid content in recombinant lines and parents during 2013 post rainy season

191) (Table 3). Besides, additional five recombinant lines (HOP-IL$_{_MAS}$-116, HOP-IL$_{_MAS}$-144, HOP-IL$_{_MAS}$-171, HOP-IL$_{_MAS}$-172 and HOP-IL$_{_MAS}$-201) yielded at par with the female parent. Indeed, these 10 recombinant lines had yield superiority over GG 20. There was no significant difference in shelling percent between ICGV 05141 (68%) and GG 20 (66%), while it varied from 65 to 74% among recombinant lines.

Recombinant lines having superior or at par pod yield with ICGV 05141 also had superior or at par shelling percent with female parent except HOP-IL$_{_MAS}$-181 which had 65% shelling percent. Similarly, no significant variation in hundred kernel weight was observed between ICGV 05141 (38 g) and GG 20 (39 g), while it varied from 26 to 39 g among recombinant lines (Table 3).

Passport data of high oleate genotype

ICGV 05141 is a Virginia bunch genotype with decumbent-3 growth habit; alternate branching; dark green colour ovate shape leaf and simple inflorescence. The genotype produces 50% flowering at 26 days after germination and matures in 120 days. Average plant height is 36.0 cm; produces average six primary branches/plant and two to three flowers/inflorescence. Average leaflet length and width are

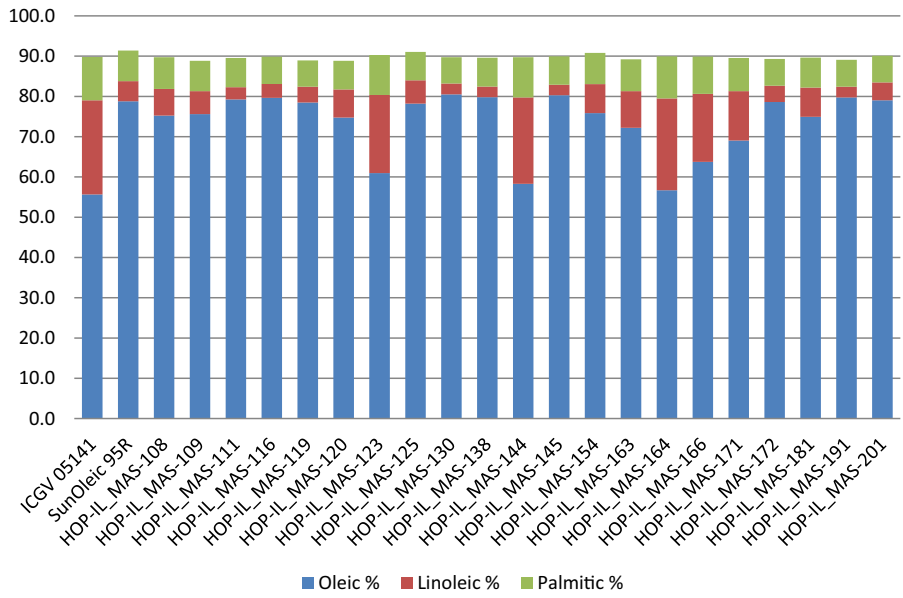

Fig. 6 Oleic, linoleic and palmitic acid content in recombinant lines and parents pooled over 2013 rainy and 2013 post rainy seasons

36.6 mm and 14.6 mm, respectively. Pods are mostly two seeded and average length and width of pods are 36.6 mm and 26.0 mm, respectively. Kernels are rose in colour, and average length and width of kernels are 28.0 mm and 16.0 mm, respectively (Fig. 8). It yields 202.0 g of pods per square meter area with 32% harvest index, 70% shelling, hundred kernels weight of 38 g, ~ 55% oil content and ~ 55.7% oleic acid content. All the recombinant lines developed under this study were Virginia bunch in growth habit. For qualitative traits, not much variation was observed between recombinant line and female parent. However, moderate to wide variation was observed between recombinant line and female parent and between recombinant lines in terms of quantitative traits and special features. Recombinant lines mostly differed in days to maturity, plant height, leaflet size, pod size and kernel size; besides pod yield, shelling percent and 100-kernel weight. In case of two special features, majority of recombinant lines were in combination of high oil content similar to ICGV 05141 and high oleic acid content similar to SunOleic 95R which revealed the success of marker assisted selection (Supplementary Data).

Discussion

In general, high oleic peanuts are preferred by the stakeholders due to its enhanced shelf life and multiple health benefits. Hence, improvement of oleic acid content in peanut is one of the important breeding objective worldwide (Janila et al. 2016). The fatty acid desaturase enzyme catalyzes the conversion of oleic acid to linoleic acid, and is encrypted by *ahFAD2A* and *ahFAD2B* homeologous alleles (Jung et al. 2000a, b; Yu et al. 2008). Both the *ahFAD2* alleles have 99% sequence homology (Jung et al. 2000a; Lopez et al. 2000) and inactivation of both the alleles stops conversion of oleic acid to linoleic acid, causing increased accumulation of oleic acid in peanut (Jung et al. 2000a, b). Development of molecular markers linked to *ahFAD2* genes has expedited the breeding high oleic acid content peanut more precisely in much shorter time and resources. Moreover, MABC breeding ensures transfer of desired gene/QTL keeping the other features of the female parent intact (Pandey et al. 2012; Varshney et al. 2013). Previously, nematode resistance (Simpson et al. 2003a), high oleic acid content (Chu et al. 2011; Janila et al. 2016) and rust resistance (Varshney et al. 2014) were transferred to elite peanut cultivars through molecular breeding. Transfer of high oleate trait into popular peanut genotypes has been achieved using both conventional and molecular breeding. The F435, a mutant with 80%

Table 2 Change in oleic acid, linoleic acid and palmitic acid content in recombinant lines with respect to ICGV 05141

Recombinant line	Oleic acid %	% increase in oleic acid	Linoleic acid %	% decrease in linoleic acid	Palmitic acid %	% decrease in palmitic acid
HOP-IL$_{_MAS}$-108	75.3	35.1	6.6	72	7.9	0.4
HOP-IL$_{_MAS}$-109	75.6	35.7	5.7	76	7.6	0.4
HOP-IL$_{_MAS}$-111	79.3	42.3	3.1	87	7.3	0.5
HOP-IL$_{_MAS}$-116	79.7	43.0	3.5	85	6.8	0.5
HOP-IL$_{_MAS}$-119	78.5	40.9	3.9	83	6.6	0.6
HOP-IL$_{_MAS}$-120	74.8	34.2	7.0	70	7.1	0.5
HOP-IL$_{_MAS}$-123	61.0	9.4	19.4	17	10.0	0.1
HOP-IL$_{_MAS}$-125	78.2	40.4	5.8	75	7.1	0.5
HOP-IL$_{_MAS}$-130	80.5	44.5	2.7	88	6.6	0.6
HOP-IL$_{_MAS}$-138	79.8	43.3	2.7	89	7.2	0.5
HOP-IL$_{_MAS}$-144	58.3	4.7	21.5	8	10.0	0.1
HOP-IL$_{_MAS}$-145	80.3	44.2	2.6	89	7.1	0.5
HOP-IL$_{_MAS}$-154	75.9	36.2	7.2	69	7.8	0.4
HOP-IL$_{_MAS}$-163	72.2	29.6	9.1	61	7.9	0.4
HOP-IL$_{_MAS}$-164	56.7	1.8	22.8	3	10.5	0
HOP-IL$_{_MAS}$-166	63.8	14.5	16.9	28	9.3	0.2
HOP-IL$_{_MAS}$-171	69.1	24.0	12.3	48	8.3	0.3
HOP-IL$_{_MAS}$-172	78.6	41.1	4.1	83	6.7	0.5
HOP-IL$_{_MAS}$-181	75.0	34.6	7.3	69	7.5	0.4
HOP-IL$_{_MAS}$-191	79.8	43.2	2.7	89	6.7	0.5
HOP-IL$_{_MAS}$-201	79.1	41.9	4.5	81	6.6	0.6
ICGV 05141	55.7	0	23.4	0	10.8	0

oleic acid content was the primary source for high oleate trait (Norden et al. 1987) and subsequently high oleate peanut lines such as, SunOleic 95R (Gorbet and Knauft 1997) and Tamrun OL01 (Simpson et al. 2003b) were developed using F435 through conventional breeding methods. Chu et al. (2007, 2009) first developed the molecular markers for *ahFAD2* genes and used them for increasing the high oleate trait in peanut using molecular breeding (Chu et al. 2011; Janila et al. 2016). Both CAPS and SNP markers, linked to high oleic acid content, were used to transfer the mutant alleles. Further selected lines were confirmed by HybProbeSNP assay (Bernard et al. 1998). In a separate study, Mienie and Pretorius (2013) selected heterozygous and homozygous lines for both the mutant alleles using multiplex real-time PCR assay, developed by Barkley et al. (2010). Thus, above studies showed that linked marker could be conveniently used to identify plants, carrying mutant alleles, in early generations of breeding program aimed at increasing oleic acid content. Furthermore, the time and the volume of breeding material in segregating generations reduced considerably. In our study ICGV 05141, a high oil containing peanut genotype, was targeted for improving its oil quality using marker assisted selection. Selection of breeding lines with desirable oil content and high oleic acid content was carried out by genotyping in early generations and phenotyping in advanced generations. Genotyping-based selection was done in early generations, to confirm plants with desirable genomic region. Subsequently, desirable lines were phenotyped in F_4 and F_5 generations for confirmation. High oil content and high oleic acid content peanuts are most suitable for oil industry to produce higher oil production along with improved oil quality. Furthermore, increased shelf-life and health benefits of food products made from high oleic peanuts are boon to all the stake holders including consumers. In this study we developed high oleic acid and oil content recombinants

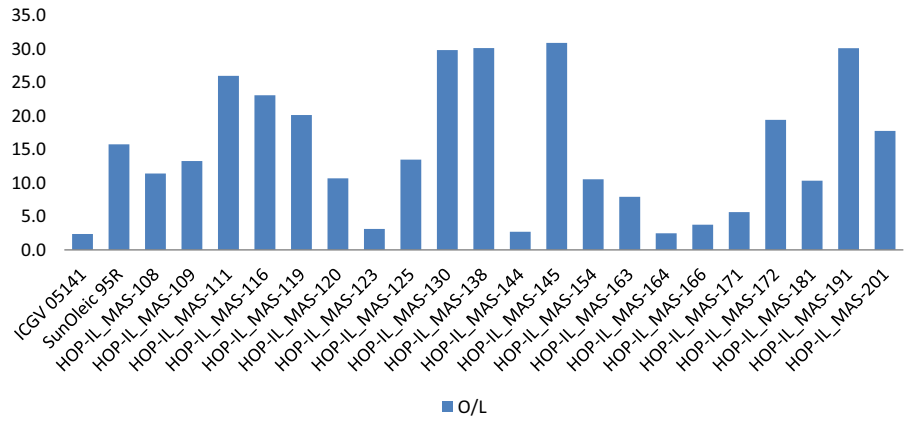

Fig. 7 Oleic to linoleic acid ratio in recombinant lines and parents pooled over 2013 rainy and 2013 post rainy seasons

using marker-assisted breeding. Realizing the importance of oil content for Indian consumers, the high oil content feature of the female parent has been successfully retained in majority of the recombinant lines. Furthermore, back ground selection with selected SSRs revealed the development of ideal recombinants carrying genomic region of female parent adjacent to *ahFAD2* loci along with high oleic acid as well as high oil content. Thus, we successfully transferred the high oleic acid content from SunOleic 95R in the genetic background of ICGV 05141, which resulted in development of recombinants of ICGV 05141 with high oleic acid. Up to 44% increase in oleic acid content was observed in the recombinant lines as compared to ICGV 05141. The increase in oleic acid content in recombinant lines simultaneously reduced the levels of linoleic acid. This was due to mutation in both the *ahFAD2* loci resulting in less/no production of fatty acid desaturase enzyme which stopped conversion of oleic acid to linoleic acid. Reduced linoleic and palmitic acid contents have additional health benefits to the consumers. However, so far available studies or data do not allow determination of the level of dietary linoleic acid needed for optimum health (Jandacek 2017). Linoleic acid in recombinant lines decreased up to 89% as compared to female parent. Similarly, palmitic acid in recombinant lines decreased up to 0.6% as compared to female parent. Thus, low linoleic acid and low palmitic acid traits have also been introgressed from SunOleic 95R. Studies concerning high oleic acid have mostly focused on the levels of oleic acid and linoleic acid in the recombinant lines. Very often it was observed that the change in one metabolite brought about by a change in the corresponding enzyme in a biosynthetic pathway, affected the levels of all other metabolites in the pathway. Earlier studies by Pandey et al. (2012) and Wang et al. (2015b) illustrated the effect of *ahfad2* alleles on palmitic acid levels. Here, we also observed significant reduction in palmitic acid simultaneously with the increase in oleic acid content in recombinant lines due to introgression of *ahfad2* alleles. Moreover, oil, oleic acid, linoleic acid and palmitic acid contents traits are quantitative in nature and would vary with the change in environments (Sarvamangala et al. 2011). The variation observed here in oil content among recombinant lines could be due to genotype × environmental interactions. Besides, Knauft et al. (1993) and Moore and Knauft (1989) reported that inheritance of high-oleate trait in F-435 mutant is controlled by two recessive genes. Later, Isleib et al. (1996) reported that oleic acid content in Virginia peanut was controlled by two loci but with modifiers and additional epistatic interactions. This could be one of the possible reason for variation in oleic acid (56 to 80%) content in recombinant lines.

Yield superiority and yield stability of a genotype over environments are two key parameters of a successful variety (Allard 1960). The genotypic selection combined with phenotypic selection was found effective in selecting recombinant lines with target traits, desired plant features and agronomic value. The recombinant lines were initially selected by genotyping and later tested for phenotypic traits, biochemical parameters, and yield. Selected 10 recombinant lines yielded either significantly higher or at par with female parent. Moreover, shelling percent and hundred-kernel weight of these recombinant lines were also either higher or at

Table 3 Yield and related traits of recombinant lines, ICGV 05141 and GG 20

Recombinant line	2014 rainy season			2015 rainy seasons			Pooled		
	Pod yield/plot[a] (g)	Shelling (%)	100-kernel weight (g)	Pod yield/plot[a] (g)	Shelling (%)	100-kernel weight (g)	Pod yield/plot[a] (g)	Shelling (%)	100-kernel weight (g)
HOP-IL$_{_MAS}$-108	948	70	27	800	72	27	874	71	27
HOP-IL$_{_MAS}$-109	750	69	26	688	73	24	719	71	25
HOP-IL$_{_MAS}$-111	1032	75	26	1200	73	28	1116	74	27
HOP-IL$_{_MAS}$-116	1400	70	30	1750	74	26	1575	72	28
HOP-IL$_{_MAS}$-119	644	68	27	800	66	27	722	67	27
HOP-IL$_{_MAS}$-120	1352	66	28	1100	68	28	1226	67	28
HOP-IL$_{_MAS}$-123	980	75	26	1248	73	30	1114	74	28
HOP-IL$_{_MAS}$-125	1218	72	32	1400	72	32	1309	72	32
HOP-IL$_{_MAS}$-130	1782	70	30	1500	72	36	1641	71	33
HOP-IL$_{_MAS}$-138	1132	70	35	1200	70	31	1166	70	33
HOP-IL$_{_MAS}$-144	1500	69	39	1344	73	39	1422	71	39
HOP-IL$_{_MAS}$-145	2064	68	29	2100	66	27	2082	67	28
HOP-IL$_{_MAS}$-154	1028	70	31	900	70	35	964	70	33
HOP-IL$_{_MAS}$-163	2100	72	28	2002	68	28	2051	70	28
HOP-IL$_{_MAS}$-164	1094	65	28	1400	65	22	1247	65	25
HOP-IL$_{_MAS}$-166	900	75	32	762	71	32	831	73	32
HOP-IL$_{_MAS}$-171	1426	74	35	1600	74	33	1513	74	34
HOP-IL$_{_MAS}$-172	1500	71	35	1378	71	41	1439	71	38
HOP-IL$_{_MAS}$-181	2004	66	26	2200	64	26	2102	65	26
HOP-IL$_{_MAS}$-191	2002	74	25	2300	68	31	2151	71	28
HOP-IL$_{_MAS}$-201	1400	68	25	1700	66	25	1550	67	25
ICGV 05141	1606	68	35	1300	68	41	1453	68	38
GG 20	1200	68	43	1446	64	35	1323	66	39
CD at 5%	239.77	2.44	2.61	294.25	2.34	2.53	125.07	2.45	2.45
CV%	10.75	2.23	4.63	12.81	2.16	4.12	5.67	2.12	4.94

[a]Plot size = 7.20 m^2

Fig. 8 Pods and kernels of selected high oleic content recombinant lines and ICGV 05141 showing variation in shape and size

par with the female parent. Besides, passport data reiterates that qualitative and quantitative traits of recombinant lines and ICGV 05141 did not vary much except oleic acid content. Thus, recombinant lines, bred here using marker assisted selection by cutting huge resources and time, would be high yielding peanut cultivar(s) with high oil and high oleic acid content to the peanut farmers of India.

Conclusion

Peanut with desirable fatty acid composition is key for sustaining the demand of consumers and traders in coming years realizing the tough competition from other oil crops. India being the second largest peanut producer holds great market potential of high oil and oleic peanut and therefore, we developed recombinants of ICGV 05141 with high oleic acid besides high oil, pod yield and yield related traits devoid of much time and resources. These recombinant lines will certainly extend additional economic benefits to the Indian farmers in addition to ensuring availability of high oleic peanuts to the traders and industry vis-à-vis peanut oil and other food products with extended shelf life and additional health benefits to the consumers.

Acknowledgements Authors acknowledge the financial support received vide no. 11-2/2010-Pul (TMOP), Govt. of India, Ministry of Agriculture, Department of Agriculture and Cooperation.

Compliance with ethical standards

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

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