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ORIGINAL ARTICLE

Development of high oleic peanut lines through marker-assisted introgression of mutant *ahFAD2* alleles and its fatty acid profiles under open-field and controlled conditions

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Abstract

Peanut is one of the most important oilseed crops grown worldwide. In this study, the mutant *ahFAD2* alleles conferring high oleic (HO) content are introgressed into an elite Indian cultivar GPBD4 which is also resistant to the foliar fungal diseases like rust and late leaf spot (LLS). The allele-specific PCR (AS-PCR) and cleaved amplified polymorphic sequences (CAPS) assays were used for the marker-assisted backcross (MABC) approach and 64 HO introgression lines (ILs) were generated. These ILs were tested for the FA compositions under the glasshouse and field conditions. The oleic acid and linoleic acid contents in the ILs were recorded to be between 68.94–82.33% and 1.74–10.87%, respectively, under glasshouse and 67.04–81.71% and 2.00–15.66%, respectively, under field conditions. The increase in the oleic acid content of the ILs over its recurrent parent (RP) was recorded to the tune of 28.78–53.80% and 33.70–62.96% under glasshouse and field conditions, respectively, indicating the stable expression of *ahFAD2B* gene in two different environments. On the contrary, linoleic acid showed 56.47–93.03% and 40.02–92.34% reduction in the ILs over its RP under glasshouse and field conditions, respectively. These ILs with a healthy FA profile can meet not only the nutritional requirements of a health-conscious society but also the industrial demands for better shelf life of oil and its products.

Keywords Allele-specific PCR · Fatty acid desaturase · Groundnut · High oleate · Introgression lines · MABC

Introduction

Peanut (*Arachis hypogaea* L.) is one of the major annual legume and oilseed crops, which is grown in nearly 100 countries (Mishra et al. 2015). Worldwide, it grows on 27.66 million ha, yielding 43.98 million metric tons of production, with average productivity of 1.91 metric tons per ha (FAOSTAT 2016). Apart from oil, it is also widely used for the production of snack products, peanut butter, desserts, and soups (Patil et al. 2018). The nutritional quality, shelf life, and flavor of peanut oil and its products are reliant on the presence of different proportions of saturated fatty acids (SFAs), monounsaturated FA (MUFA), and poly UFA (PUFA) (Derbyshire 2014; Nawade et al. 2018). Peanut oil contains nearly 11 different FAs, of which oleic acid (C18:1, Δ9), a MUFA and linoleic acid (C18:2, Δ9, Δ12), a PUFA contribute approximately 80% of the total oil composition (Nawade et al. 2018).

A high proportion of linoleic acid results in off flavors, rancidity, and short shelf life of manufactured food products due to its low oxidative and frying stability (Mondal et al. 2011). On contrary, oleic acid has tenfold higher auto-oxidative stability than linoleic acid due to which high oleic (HO) oils are in great demand by various industries including food (fried products and bakery), cosmetic (emulsions, soaps, and detergents) and oleochemical industries (lubricants, paints, and adhesives) (Abiodun

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2017). HO peanut encompasses a relatively longer shelf life along with neutral flavor and odor (O'Keefe et al. 1993), which also makes it an excellent solution for food industries, looking for healthy alternatives to saturated or hydrogenated oils (Cao et al. 2013). A diet with the HO acid can reduce the risk of heart diseases like the reduction of systolic blood pressure and slowing down of atherosclerosis (Vassiliou et al. 2009).

The first milestone in this aspect was achieved by Norden et al. (1987) who identified the first natural high oleate mutant lines, F435 with about 80% oleic acid and 2% linoleic acid, much higher compared to the traditional peanut genotypes having 36–70% oleic and 15–43% linoleic acid (Knauft et al. 1993). In peanut, two homeologous genes, *ahFAD2A* and *ahFAD2B* having 99% sequence similarity, are reported to regulate the desaturase activity (Jung et al. 2000b; Lopez et al. 2000). The coding sequences (CDS) of these genes consist of 1140 bp, encoding 379 amino acids with no introns in the coding region. A single base pair (bp) substitution (448G > A) mutation at 448 bp position in *ahFAD2A* gene, results in a missense amino acid from aspartic acid to asparagine (D150 N). While 1-bp insertion (441_442insA) mutation in *ahFAD2B* gene, at 442 bp position resulted in frame-shift mutation, which generates a premature stop codon (Jung et al. 2000b; Lopez et al. 2000). Recently, Wang et al. (2015b) identified two natural mutant lines (PI342664 and PI342666) with 80% oleic acid, having substitutions of G448A in *FAD2A* (same as previously identified) and C301G in *FAD2B* (new mutation) resulting in a missense amino acid substitution of D150 N, and H101D, respectively. The mutations in *ahFAD2* gene were found to affect the histidine motifs of metal ion complex, which is required for oxygen reduction and resulting in reduced enzymatic activity and HO content in the mutant genotypes (Jung et al. 2000a; Yu et al. 2008; Chu et al. 2009).

To enhance the efficiency of HO peanut breeding program, different molecular assays including CAPS for *ahFAD2A* (Chu et al. 2007) and *ahFAD2B* alleles (Chu et al. 2009), real-time PCR (Barkley et al. 2010; 2011), AS-PCR (Chen et al. 2010; Yu et al. 2013) and kompetitive allele-specific PCR (KASP) (Zhao et al. 2017) have been developed and successfully utilized for both screening of peanut genotypes (Chu et al. 2007; Wang et al. 2011, 2013; Mukri et al. 2012; Nawade et al. 2016) as well as marker-assisted selection (MAS) studies (Chu et al. 2011; Janila et al. 2016; Bera et al. 2018).

The first HO cultivar, SunOleic95R was released in the USA using the conventional breeding method (Gorbet and Knauft 1997). Since then, over 90 HO peanut cultivars have been developed, of which the majority were bred through traditional methods and some using the chemically induced mutagenesis approach (Nawade et al. 2018). Further, only a few cultivars were developed through the MAS approach namely, Tifguard High O/L (Chu et al. 2011), SA Juweel and ARC Oleic2 (Mienie and Pretorius 2013).

Recently, Nawade et al. (2016) characterized a total of 174 Indian peanut genotypes for *ahFAD2* allele polymorphism and its FA compositions, of which 80 were found to have the *ahFAD2A* mutant (448G > A) allele, while none recorded natural mutation in *ahFAD2B* (441_442insA) allele. The oleic acid content and O/L (oleic acid to linoleic acid) ratio of these genotypes ranged between 37.81–66.57% and 0.93–3.76%, respectively, which is much lower than the industrially acceptable O/L ratio of > 9 and/or 70% oleic acid content (Davis et al. 2013; Janila et al. 2016). Thus, looking at the increasingly global and domestic market demand for HO peanut, and unavailability of any such variety in India, the present investigation was undertaken to enhance the oleic acid content of an Indian peanut cultivar using the robust molecular breeding approach.

Materials and methods

Plant material

GPBD 4, a leading peanut variety of India with good agronomic features such as early to mid-maturity, and high yield (Sujay et al. 2012), but with a low O/L ratio was used as a recurrent parent (RP). In addition, it is highly resistant to various foliar fungal diseases and thus was used as a disease resistance check in the field trials of All India Coordinated Research Project on Groundnut (AICRP-G) in India (Gowda et al. 2002). Incidentally, in our previous work (Nawade et al. 2016), we found GPBD4 harboring a mutant *ahFAD2A* (448G > A) allele, while another *ahFAD2B* allele was normal, which results in the production of low oleic acid (55%) and more linoleic acid (26%) contents (Nawade et al. 2016). The first HO but poor yielder cultivar, SunOleic95R from the USA, having both *ahFAD2A* (448G > A) and *ahFAD2B* (441_442insA) mutant alleles in homozygous condition was used as a donor parent (Gorbet and Knauft 1997). The seeds of SunOleic95R were obtained from ICRISAT, Patancheru (India).

Hybridization and backcross breeding

The hybridizations were conducted in a controlled glasshouse, having 60–65% humidity and 30 ± 2 °C temperature at ICAR-Directorate of Groundnut Research, Junagadh, India (21°48′34″N; 70°44′07″E; 67 m amsl). Three seeds were sown per pot (30 × 35 cm dimension), containing a mixture of dry soil, fine sand and well-decomposed farmyard manure (3:1:1 proportion) and only one healthy plant was retained at a later stage for crossing. A total of 15 plants were used as the female parent (GPBD4), while 10 plants

were used as the male parent (SunOleic95R) and hybridization was performed in the Kharif season (June–October) in the year 2013. The emasculation was carried out between 16:30 h and 17:30 h, while pollination was conducted on the next day between 06:00 h and 08:00 h (Nigam et al. 1990). For backcrossing, the true hybrids of F_1, BC_1F_1, and BC_2F_1 generations were used as pollen parents, while RP (GPBD4) was used as the female parent. The hybrids and segregating populations (F_2, BC_1F_2, BC_2F_2, and BC_3F_2) generated after each crossing were grown for further molecular studies (Fig. 1).

Mass multiplication of hybrids using the stem cutting technique

The 10–15-cm-long stem cuttings having 2–3 internodes were prepared from the F_1 plants at the time of pod harvesting, as the plants were still green. A slanting cut was made at the base of each cutting, which were then immersed in 0.5% Bavistin (10–15 min), rinsed with distilled water and dipped (4–5 cm) in 1X Hoagland's solution supplemented with 1% naphthaleneacetic acid (NAA) for rooting, in 50 mL test tubes (Borosil, India) wrapped in aluminum foil to prevent algal contamination. Further, after every 6–7 days, the cuttings were transferred into a new set of tubes filled with fresh growth medium. The cuttings with adventitious roots and 2–3 newly generated shoots were then transplanted into the earthen pots filled with a sterilized mixture of soil and fine sand (1:2, v:v). Hoagland solution was used for watering (6–7 days) the rooted cuttings at the time of hardening, as it simultaneously provides the desired nourishment during establishment (Radhakrishnan et al. 1999). The hardened plants are allowed to complete its life cycle and set the seeds under controlled growth conditions (Fig. 2).

DNA extraction, polymerase chain reaction and genotyping

DNA was extracted from fresh leaves of the parental genotypes, F_1s and segregation populations using the cetyltrimethylammonium bromide (CTAB) extraction method (Cuc et al. 2008). The quality of DNA was checked on the agarose gel (0.8%, w/v), quantification was performed using NanoDropND-1000 (NanoDrop products, DE, USA) and working concentration was adjusted to 20 ng μL^{-1}. For the identification of mutant *ahFAD2A* and *ahFAD2B*alleles, all the hybrid plants were first screened using AS-PCR markers (Chen et al. 2010). Further, CAPS (Chu et al. 2007, 2009) and AS-PCR (Yu et al. 2013) assays (Table 1) were performed to identify the zygosity of ILs for the mutant *ahFAD2* alleles.

For AS-PCR analysis, PCR mixture (10.0 µL) containing template DNA (1.0 µL, 20.0 ng), 5 × PCR buffer (2.0 µL, Promega, USA), 25.0 mM $MgCl_2$ (0.8 µL, Promega, USA), 2.0 mM dNTP (0.7 µL, Thermo Fisher Scientific, USA), primers (0.5 µL, 25.0p moles), 5U Taq polymerase (0.2 µL, Promega, USA) and sterile ddH_2O (4.3 µL) were used. Amplification was performed in a thermal cycler

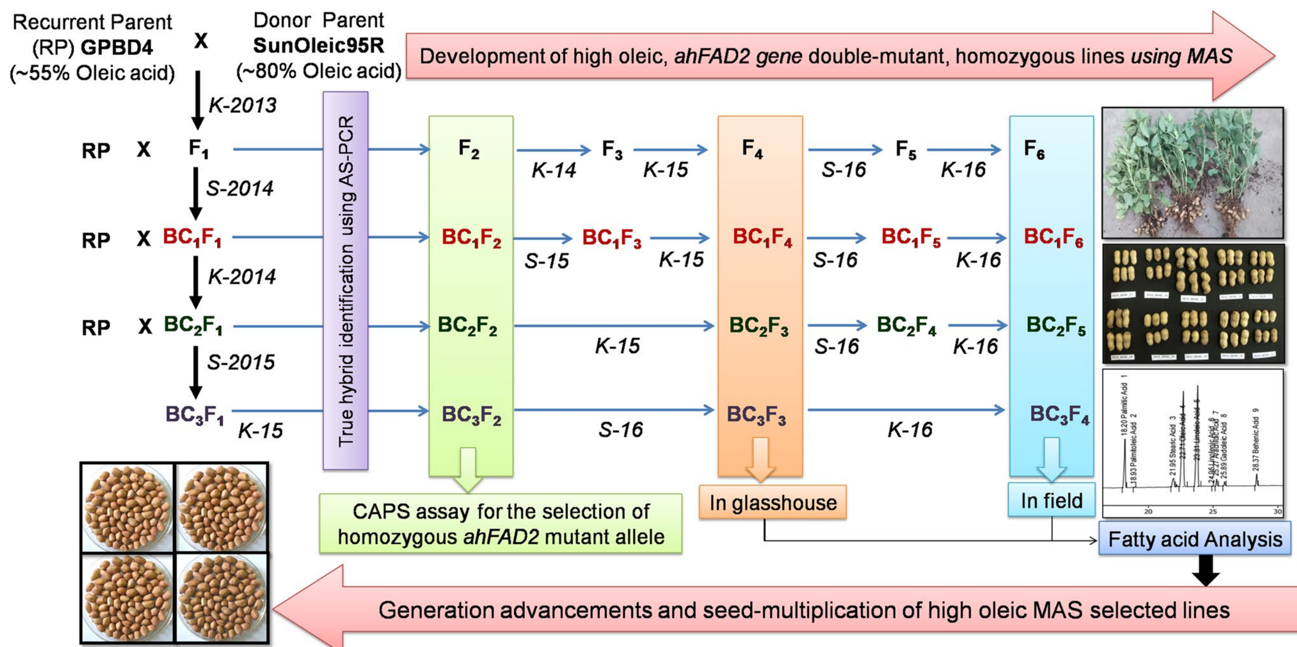

Fig. 1 Schematic presentation of the crossing and selection scheme used for the development of *ahFAD2* gene double-mutant HO peanut lines in different generations

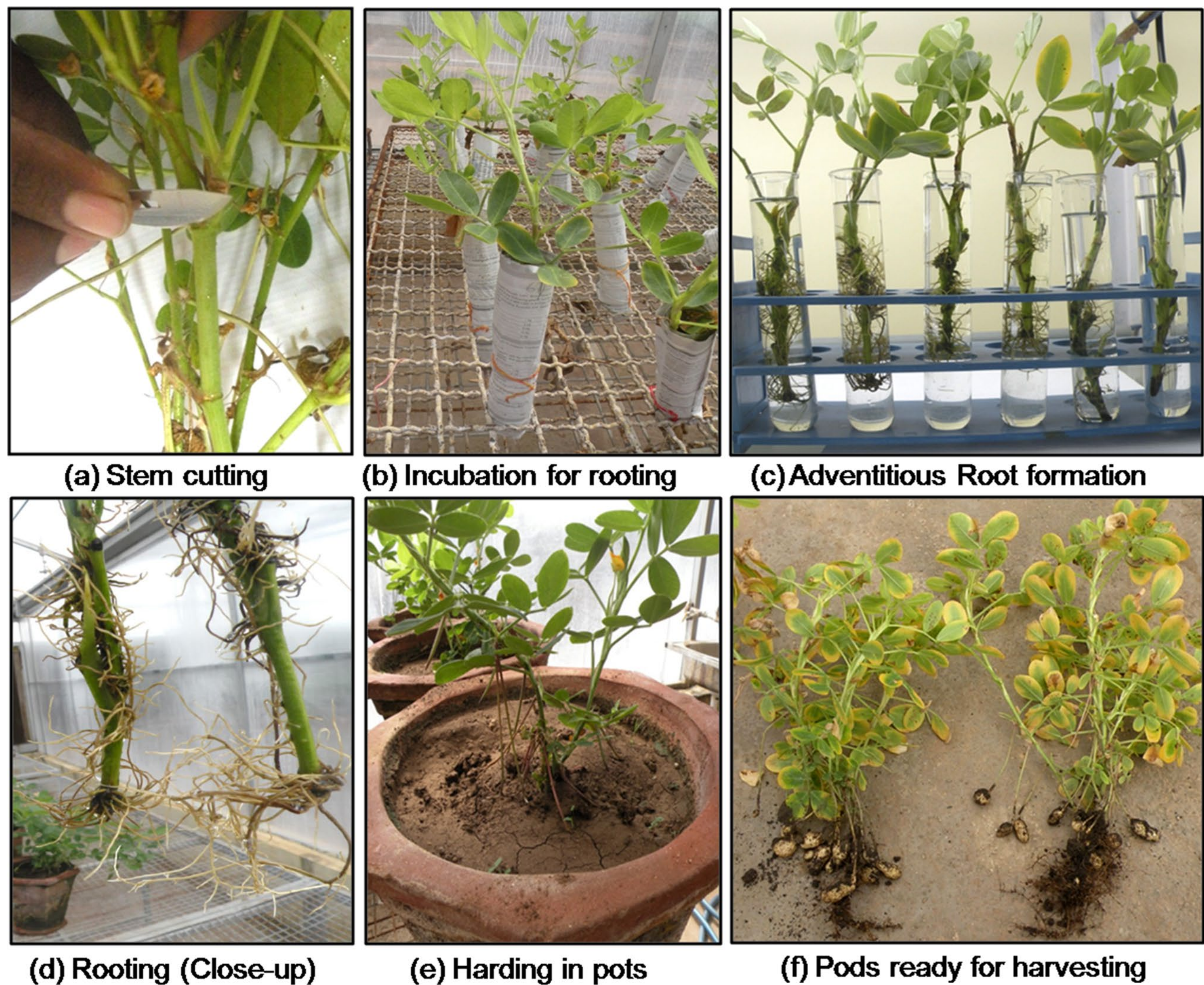

Fig. 2 Clonal multiplication of F_1 plants using stem cuttings for increasing the number of F_2 progenies

Table 1 Details of true hybrids, and homozygous *ahFAD2B* mutant lines obtained in each generation

Target	Generation	Seeds obtained	Plants germinated and screened	Plants found positive/homozygous	Season
Hybrid identification	F_1	8	6 (75.0%)	04 (66.7%)	Summer-2014
	BC_1F_1	6	5 (83.3%)	03 (60.0%)	Kharif-2014
	BC_2F_1	6	5 (83.3%)	03 (60.0%)	Summer-2015
	BC_3F_1	10	8 (80.0%)	05 (62.5%)	Kharif-2015
Total	–	30	24 (80.0%)	15 (62.5%)	–
Selection of *ahFAD2B* homozygous mutant	F_2	56	52 (92.9%)	09 (17.3%)	Kharif-2014
	F_2 (cutting)	148	112 (75.7%)	18 (16.1%)	Summer-2015
	BC_1F_2	62	58 (93.5%)	08 (13.8%)	Summer-2015
	BC_1F_2 (cutting)	70	69 (98.6%)	10 (14.5%)	Kharif-2015
	BC_2F_2	40	38 (95.0%)	04 (10.5%)	Kharif-2015
	BC_3F_2	86	83 (96.5%)	15 (18.1%)	Summer-2016
Total	–	462	412 (89.2%)	64 (15.5%)	–

(Eppendorf, USA) using thin-walled 96-well PCR plates (Sorenson™ Bioscience, USA). The touchdown PCR was done with an initial denaturation at 94.0 °C/3.0 min and then 5 cycles of 94.0 ºC/30 s (− 1.0 ºC reduction per cycle), 65–60 ºC/30 s and 72.0 °C/1 min. This was followed by another 35 cycles of 94.0 °C/30 s, 60 °C/30 s and 72.0 ºC/1.0 min of denaturation, annealing, and extension, respectively. The final extension was done at 72.0 ºC/10 min. Amplification was performed twice and amplified products were analyzed using 2% agarose gel in 1 × TBE buffer at 225 volts for 2.5–3.0 h and stained with ethidium bromide. The gels were documented in an automated gel documentation system (Fujifilm FLA-5000) and scored.

For CAPS analysis of *ahFAD2A* and *ahFAD2B* mutations, the PCR was performed with 4.0 µl 5 × PCR buffer (Promega, USA), 1.6 µL $MgCl_2$ (25 mM, Promega-USA), 1.4 µL dNTPs (2 mM, Thermo Fisher Scientific-USA), 1.0 µL each of forward and reverse primers (10 pmol), 0.2 µL Taq DNA polymerase (5U per µL, Promega-USA) polymerase and 6.6 µL sterile ddH_2O in a total PCR mixture volume of 20.0 µL.

Amplification conditions were initial denaturation at 94 °C/5.0 min followed by 35 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/1.0 min denaturation, annealing and extension, respectively. The final extension step was done at 72 °C/10 min. After confirmation of 1200 bp PCR amplification on a 2.0% agarose gel, 10.0 µL PCR products were digested with 0.4 µL of *Hpy188I* (4 U) restriction enzyme (New England, Biolabs, Ipswich, MA), in a solution containing digestion buffer (2.0 µL), 0.1% BSA (2.0 µL; Takara, Japan) and distilled water (5.6 µL); restriction digestion was done at 37 °C overnight. The digested products were separated on a 2% (w/v) agarose gel and wild-type allele resulted in five fragments of 736, 263, 171, 32, and 12 bp, while in the mutant allele the 736-bp fragment got further digested into 505 and 213 bp, producing six fragments of 505, 263, 213, 171, 32, and 12 bp. However, the larger three fragments could be resolved on a 2% (w/v) agarose gel (Fig. 3).

Fatty acid (FA) profiling

The FAs were analyzed using the gas chromatography system (Thermo Fisher, Trace GC 1100) equipped with flame ionization detector (FID), by passing the FA methyl esters through a capillary column (TR wax) (Misra and Mathur 1998). The temperature of the inlet and FID detector was set at 240 ºC, with oven at 190 ºC, whereas, the flow of carrier gas (nitrogen) and fuel gas (hydrogen) was maintained at 30 mL per min. Total run time for each sample was 12 min, and the peaks were identified by comparing to a FAME standard mix RM-3 (Sigma-Aldrich, St. Louis, MO). FA profiles of ILs and parents were estimated from the seeds of the plants which are grown under, (1) controlled conditions, in F_4, BC_1F_4, BC_2F_3, and BC_3F_3 generations; and (2) field conditions, in F_6, BC_1F_6, BC_2F_5, and BC_3F_4 generations.

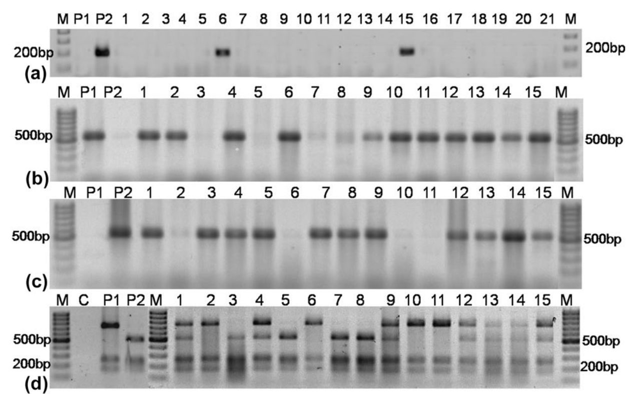

Fig. 3 Identification of homozygous *ahFAD2B* mutant allele **a** AS-PCR (Yu et al. 2013) for non-mutant *ahFAD2B* allele (557 bp) identification; **b** AS-PCR (Yu et al. 2013) for mutant *ahFAD2B* (441_442insA; 539 bp) allele identification; **c** CAPS assays for selection of heterozygous and homozygous (441_442insA) alleles; Lanes: M (100 bp DNA ladder); C (Control with water); P1 (GPBD4); P2 (SunOleic95R); 3, 5, 7, 8 (lines homozygous for the*ahFAD2B* mutant allele); 1, 4, 9, 12–15 (lines heterozygous for the *ahFAD2B* mutant allele); 2, 6, 10,11 (lines homozygous for the *ahFAD2B* wild allele)

Statistical analysis

The data were analyzed using the one-way analysis of variance (ANOVA) by XLSTAT software (Addinsoft XLSTAT 2017) to test the statistical differences ($p < 0.05$), and Tukey's HSD multiple comparison test was done to evaluate the significant differences in the means.

Results

Identification of true hybrids and implementation of MABC

Crosses were attempted between SunOleic95R and GPBD4 during *Kharif*-2013, F_{1s} were raised during the summer of 2014, and were tested for *ahFAD2B* insertion mutation using AS-PCR assay (Fig. 3). Further, various hybrids from different generations (BC_1F_1, BC_2F_1, and BC_3F_1), and the segregating populations (F_2, BC_1F_2, BC_2F_2 and BC_3F_2) were screened using AS-PCR and CAPS assays at 8–10 days after germination (DAG) for the presence of *ahFAD2B* mutant alleles and its zygosity was confirmed (Fig. 3). True hybrids and the plants homozygous for *ahFAD2* alleles from the segregating generations were transferred to the earthen pots (30 × 35 cm) for the completion of its life cycle, while other plants were discarded.

The true hybrid plants having a mutant *ahFAD2B* allele were then used as a pollen parent and GPBD4 used as RP at each backcross generation, thus the successive backcrossing-generated BC_1F_1, BC_2F_1, and BC_3F_1 generations were also shelfed to get segregation populations. Details about the number of seeds generated, plants analyzed for identification of true hybrids using AS-PCR marker in various F_1 generations (F_1, BC_1F_1, BC_2F_1, and BC_3F_1) and homozygosity test in different F_2 (F_2, BC_1F_2, BC_2F_2 and BC_3F_2) are given in Table 1. Only the confirmed homozygous *ahFAD2B* mutant allele lines identified from each segregation population through molecular maker assays were selected and multiplied as an individual line. Finally, a total of 64 lines were found homozygous for the mutant *ahFAD2A* and *ahFAD2B* alleles from all segregating generations Table 1. Pod and kernel features of some of the introgression lines are shown in supplementary figure S1.

Production of a large number of F_2 seeds in different generations, using the stem cutting method

Unlike *Arabidopsis* or rice, where a single F_1 plant can produce hundreds of seeds, in peanut, only 20–25 seeds can be obtained per plant under normal growth conditions. Therefore, to get more number of seeds of a segregating generation, we have resorted to our own stem cutting method for mass multiplication of any generation. We used plants of F_1 and BC_1F_1 generations, immediately after harvesting its seeds for the stem cutting experiment. A total of 52 (F_1) and 38 (BC_1F_1) cuttings were attempted which generated 40 and 25 cuttings, respectively, with well-elongated roots within 15–20 days after incubation in the rooting media (Table 2). The cuttings having adventitious roots and 2–3 new leaflets were selected for transplanting in the earthen pots (Fig. 2). On average, 12–15 stem cuttings were made from each hybrid plant. During hardening, 70% F_1 and 63% BC_1F_1 plants could survive and perform like a normal peanut plant for flowering, pegging, and pod development, but overall plant growth habit was quite poor. It was interesting to note that the cuttings from the primary and secondary branches get elongated, while main stem cuttings produced both primary and secondary branches. After 90–100 days, the plants get matured and yielded 148 F_2 and 70 BC_1F_2 seeds from 28 F_1 and 15 BC_1F_1 plants (Table 2). The use of stem cutting in peanut is reported only for the perennial *Arachis* species, soon after flowering, where seed setting is a major problem (Nigam 2014). However, we have optimized and successfully used the method of stem cutting using mature cultivated peanut plants for increasing the number of F_2 seeds. Although this approach needs an extra season, it is a good alternative to increase the number of F_2 seeds, especially when the number of plants in any F_1 generation is less.

Fatty acid analysis

The GC analysis of parents and 64 ILs detected various FAs, among which oleic, linoleic, and palmitic acids constituted more than 80% of total FAs (Table 3). The oleic acid content in ILs ranged from 68.94 to 82.33% with the mean of 78.54% under controlled conditions (Table 3), while under field conditions, it ranged from 67.04 to 81.71% with a mean of 77.63% (Table 4). The linoleic acid content ranged from 1.74 to 10.87% and 2.00 to 15.66% under controlled and field conditions, respectively. With respect to the RP, an increment of 28.78–53.80% has been recorded among the ILs for oleic acid content with an average increase of 46.74% under controlled condition. On the contrary, in field conditions, this increment was 33.70–62.96% with a mean of 54.82% over RP. While the linoleic acid showed 56.47–93.03% and 40.02–92.34% reduction over RP under controlled and field conditions, respectively, with the mean reduction of 86.66 and 82.81% in both the situations. The palmitic acid content varied from 5.85 to 9.92% with an average of 7.06%, which is a reduction of 31.49% compared to the RP in glasshouse conditions (Table 3), while it ranged from 5.99 to 9.75% with a mean of 6.94% under field conditions (Table 4).

Correlation among various FAs under different growth conditions

Pearson's correlation coefficient among all the ILs was carried out to find the effect of growth conditions, viz., controlled and field conditions on the variations recorded for different FAs. A highly significant negative correlation was observed between oleic and linoleic acid contents under both field ($r = -0.975$) and glasshouse ($r = -0.940$) conditions. Further, significant negative correlations have been recorded for oleic acid content with arachidic and behenic

Table 2 Details of the stem cutting used for the multiplication of F1 and BC1F1 plants

Generation	Number of SC made	Days to rooting	Number of cutting with AR	Plants survived during hardening	Total number of seeds
F_1	52	12–15	40 (76.9%)	28 (70%)	148
BC_1F_1	38	18–20	25 (65.8%)	15 (63)	70

where *SC* Stem cuttings; *AR* adventitious roots. F_1 and BC_1F_1 were raised during Rainy-2014 and Summer-2015, respectively

Table 3 Fatty acid composition (%) and O/L ratio of 64 introgression lines (ILs) and their parents grown under glasshouse conditions

ILS	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20:0)	Gadoleic acid (C20:1)	Behenic acid (C22:0)	Erucic acid (C22:1)	Lignoceric acid (C24:0)	O/L ratio
GPBD4	10.30	0.07	3.14	53.53	24.97	0.04	1.68	1.31	3.45	0.07	1.45	2.14
SunOleic95R	7.30	0.06	1.74	80.21	3.52	0.05	1.05	1.95	2.59	0.12	1.44	22.81
MAS_HOL_1	6.69	0.04	2.48	80.91	2.59	0.03	1.37	1.68	2.93	0.09	1.19	31.29
MAS_HOL_2	6.75	0.07	3.46	76.82	3.99	0.05	1.55	1.98	3.50	0.18	1.66	19.23
MAS_HOL_3	7.02	0.10	3.39	79.91	1.94	0.03	1.51	1.67	2.83	0.12	1.49	41.28
MAS_HOL_4	5.96	0.09	3.47	80.15	1.93	0.03	1.53	1.86	3.11	0.13	1.76	41.62
MAS_HOL_5	6.61	0.02	3.28	78.47	3.89	0.05	1.50	1.58	3.14	0.09	1.37	20.19
MAS_HOL_6	6.50	0.02	4.10	77.63	2.75	0.04	1.90	1.59	3.65	0.09	1.75	28.26
MAS_HOL_7	7.20	0.14	2.70	77.11	3.69	0.07	1.36	2.02	3.75	0.16	1.80	20.88
MAS_HOL_8	7.10	0.06	2.10	81.66	2.39	0.06	1.09	1.82	2.34	0.13	1.27	34.14
MAS_HOL_9	7.08	0.06	2.68	81.42	3.00	0.04	1.05	1.74	2.21	0.12	1.22	27.13
MAS_HOL_10	7.33	0.07	2.87	79.53	3.09	0.07	1.50	1.39	2.87	0.08	1.20	25.73
MAS_HOL_11	6.42	0.08	3.76	75.34	4.14	0.03	1.74	2.08	4.25	0.19	1.97	18.20
MAS_HOL_12	6.10	0.05	3.36	79.77	2.21	0.04	1.48	1.87	3.26	0.17	1.69	36.03
MAS_HOL_13	6.15	0.10	3.58	78.00	2.86	0.03	1.76	1.72	3.71	0.12	1.99	27.30
MAS_HOL_14	6.15	0.07	4.06	79.47	2.17	0.04	1.62	1.57	3.25	0.11	1.48	36.64
MAS_HOL_15	6.81	0.06	2.72	76.25	4.59	0.07	1.41	2.17	3.73	0.19	2.00	16.63
MAS_HOL_16	6.97	0.09	3.38	76.50	3.54	0.05	1.69	1.82	3.97	0.16	1.83	21.59
MAS_HOL_17	7.36	0.07	3.64	75.23	4.10	0.06	1.77	1.77	4.15	0.15	1.70	18.34
MAS_HOL_18	6.42	0.08	3.72	76.63	3.02	0.04	1.74	2.06	3.99	0.16	2.12	25.37
MAS_HOL_19	6.29	0.08	3.49	78.29	2.85	0.04	1.69	1.79	3.60	0.12	1.77	27.51
MAS_HOL_20	7.36	0.09	3.03	79.95	2.48	0.05	1.37	1.64	2.61	0.12	1.29	32.21
MAS_HOL_21	6.58	0.03	3.60	77.56	3.29	0.05	1.78	1.75	3.60	0.11	1.67	23.55
MAS_HOL_22	6.60	0.07	3.68	76.01	3.32	0.07	1.94	1.79	4.46	0.14	1.94	22.87
MAS_HOL_23	6.21	0.08	3.90	75.63	3.94	0.05	1.84	2.19	3.99	0.21	1.97	19.22
MAS_HOL_24	6.55	0.07	4.15	73.83	4.94	0.09	1.87	2.11	4.15	0.23	2.02	14.96
MAS_HOL_25	6.49	0.06	4.02	77.50	3.50	0.04	1.67	1.77	3.36	0.13	1.48	22.15
MAS_HOL_26	6.74	0.07	3.66	79.46	2.27	0.02	1.68	1.52	3.01	0.09	1.48	34.97
MAS_HOL_27	5.85	0.09	4.14	74.13	4.37	0.07	1.95	2.58	4.00	0.27	2.55	16.97
MAS_HOL_28	6.28	0.08	3.39	76.63	4.18	0.09	1.57	2.02	3.66	0.20	1.89	18.33
MAS_HOL_29	6.48	0.06	3.54	78.47	2.96	0.06	1.56	1.75	3.49	0.12	1.51	26.51
MAS_HOL_30	7.04	0.03	2.59	76.47	5.13	0.06	1.41	2.12	3.53	0.16	1.46	14.90
MAS_HOL_31	7.42	0.06	2.06	80.08	2.94	0.04	1.18	1.86	2.77	0.13	1.47	27.23
MAS_HOL_32	7.20	0.07	1.95	79.74	3.62	0.06	1.10	1.94	2.53	0.16	1.64	22.00

Table 3 (continued)

ILS	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20:0)	Gadoleic acid (C20:1)	Behenic acid (C22:0)	Erucic acid (C22:1)	Lignoceric acid (C24:0)	O/L ratio
MAS_HOL_33	6.89	0.07	1.97	80.12	2.83	0.05	1.14	2.15	2.81	0.19	1.80	28.36
MAS_HOL_34	6.41	0.04	2.35	80.72	2.22	0.03	1.27	2.16	2.93	0.17	1.70	36.33
MAS_HOL_35	7.66	0.12	2.13	81.04	2.47	0.06	1.06	1.71	2.26	0.11	1.38	32.76
MAS_HOL_36	7.52	0.09	2.32	79.68	2.67	0.04	1.25	1.88	2.96	0.14	1.46	29.90
MAS_HOL_37	6.23	0.03	1.90	81.96	2.72	0.05	1.04	1.98	2.55	0.15	1.40	30.13
MAS_HOL_38	7.73	0.12	2.02	80.63	2.27	0.08	1.17	1.94	2.38	0.13	1.56	35.49
MAS_HOL_39	6.75	0.05	1.94	81.20	2.68	0.04	1.17	1.92	2.71	0.13	1.40	30.36
MAS_HOL_40	6.82	0.04	2.23	81.70	1.80	0.03	1.33	1.78	2.90	0.11	1.26	45.29
MAS_HOL_41	7.79	0.13	2.81	80.41	1.77	0.05	1.51	1.51	2.69	0.11	1.22	45.41
MAS_HOL_42	6.93	0.13	2.75	79.30	3.33	0.04	1.62	1.64	3.31	0.08	1.31	23.82
MAS_HOL_43	7.95	0.07	2.78	71.29	10.62	0.04	1.45	1.52	2.96	0.06	1.28	6.71
MAS_HOL_44	7.01	0.07	2.69	80.26	2.82	0.05	1.44	1.53	2.66	0.08	1.39	28.48
MAS_HOL_45	6.34	0.07	3.28	75.11	4.49	0.07	1.64	2.60	3.88	0.27	2.26	16.73
MAS_HOL_46	6.67	0.06	2.87	78.40	3.83	0.05	1.38	1.94	3.21	0.15	1.60	20.45
MAS_HOL_47	7.14	0.08	2.73	80.33	2.04	0.05	1.34	1.79	2.74	0.11	1.66	39.35
MAS_HOL_48	8.13	0.09	2.66	79.62	1.76	0.05	1.47	1.48	3.14	0.08	1.47	45.13
MAS_HOL_49	6.84	0.07	2.69	80.93	2.26	0.04	1.43	1.54	2.68	0.08	1.44	35.83
MAS_HOL_50	9.92	0.10	3.06	68.94	10.87	0.05	1.47	1.30	2.85	0.12	1.33	6.34
MAS_HOL_51	7.91	0.12	2.77	71.32	9.96	0.06	1.45	1.61	3.10	0.10	1.60	7.16
MAS_HOL_52	8.04	0.08	2.46	79.48	2.07	0.02	1.36	1.73	3.03	0.13	1.59	38.40
MAS_HOL_53	7.19	0.07	2.47	79.85	2.72	0.05	1.28	1.81	2.77	0.16	1.50	29.36
MAS_HOL_54	6.58	0.07	2.72	81.55	2.11	0.04	1.40	1.58	2.62	0.08	1.25	38.67
MAS_HOL_55	8.30	0.14	2.35	80.11	2.01	0.03	1.29	1.54	2.72	0.08	1.43	39.80
MAS_HOL_56	7.99	0.11	2.34	75.39	5.85	0.05	1.40	1.80	3.13	0.12	1.84	12.90
MAS_HOL_57	8.00	0.11	2.47	80.36	1.93	0.06	1.18	1.73	2.70	0.10	1.38	41.62
MAS_HOL_58	9.33	0.25	2.37	78.18	2.85	0.07	1.29	1.37	2.80	0.07	1.44	27.41
MAS_HOL_59	7.27	0.06	2.61	79.57	2.69	0.04	1.33	1.83	2.88	0.13	1.58	29.56
MAS_HOL_60	6.26	0.03	1.92	82.33	1.74	0.04	1.28	1.93	2.98	0.11	1.41	47.43
MAS_HOL_61	7.85	0.09	2.29	80.18	2.51	0.07	1.28	1.66	2.67	0.10	1.33	31.96
MAS_HOL_62	6.56	0.04	1.91	80.92	3.02	0.04	1.11	2.09	2.63	0.16	1.50	26.78
MAS_HOL_63	7.00	0.10	1.63	81.87	2.29	0.03	1.13	1.85	2.50	0.12	1.47	35.72
MAS_HOL_64	8.85	0.20	2.04	79.52	2.38	0.04	1.15	1.74	2.50	0.12	1.47	33.45
Mean[a]	7.06	0.08	2.87	78.54	3.33	0.05	1.44	1.81	3.13	0.13	1.58	27.97
Minimum	5.85	0.02	1.63	68.94	1.74	0.02	1.04	1.30	2.21	0.06	1.19	6.34

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Table 3 (continued)

ILS	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20:0)	Gadoleic acid (C20:1)	Behenic acid (C22:0)	Erucic acid (C22:1)	Lignoceric acid (C24:0)	O/L ratio
Maximum	9.92	0.25	4.15	82.33	10.87	0.09	1.95	2.60	4.46	0.27	2.55	47.43
% increase/decrease[b]	−31.49	10.56	−8.57	46.74	−86.66	13.90	−14.21	38.04	−9.31	97.59	9.38	1205.19

[a]Values of both the parents are not considered

[b]Percent increase or decrease in mean fatty acid content of ILs with respect to recurrent parent

acids under both controlled and field conditions (Table S2). Palmitic acid content under controlled conditions showed a significantly negative correlation with SFAs such as stearic ($r = -0.390$), arachidic ($r = -0.310$) and lignoceric acids ($r = -0.486$). Further, behenic acid showed a significant positive correlation with stearic acid ($r = 0.725$) and arachidic acid ($r = 0.856$) under controlled conditions, while no such strong correlation was observed under field conditions. Moreover, under controlled and field conditions, erucic acid showed a significant positive correlation with gadoleic acid ($r = 0.779$ and 0.877, respectively) and lignoceric acid ($r = 0.725$ and 0.703, respectively). The linolenic acid also recorded a significantly positive correlation with gadoleic ($r = 0.279$), behenic ($r = 0.294$), erucic ($r = 0.465$), and lignoceric acid ($r = 0.366$), under controlled conditions. Similarly, stearic acid showed a significantly positive correlation with behenic ($r = 0.725$), erucic ($r = 0.312$), and lignoceric acid ($r = 0.537$), under controlled conditions, but under field conditions it reflected a significant negative correlation with gadoleic ($r = -0.468$), erucic ($r = -0.423$), and lignoceric acids ($r = -0.266$) (Table S2). Wang et al. (2015a) also reported a significant negative correlation of stearic acid with gadoleic and also with lignoceric acid under filed conditions, indicating more use of stearic and linolenic acid for long-chain FA production. But it still needs a further in-depth analysis to pinpoint the exact factors responsible for such expression.

The *ahFAD2* gene double mutant plant uses its resources for the production of more linoleic acid by mobilizing the palmitic acid towards the oleic acid formation (Wang et al. 2015a). Thus, increase in oleic acid also acts as a signal to trigger a negative feedback loop to deal with an excess of SFAs, which might lead to the negative correlation between oleic acid and SFAs (Lim et al. 2013; Harvey et al. 2010).

Discussion

The alteration of FA profiles aimed for higher oxidative stability and better dietary properties is an important and evolving theme to meet the nutritional needs and industrial criteria of the modern market. Henceforth, a concentrated effort has been made in our peanut breeding program, and an array of HO lines has been generated by introgressing mutant *ahFAD2B* gene through MAS.

Peanut being an allotetraploid crop contains two sets of alleles of any gene in its A and B genomes, and mutation in both *ahFAD2A* and *ahFAD2B* allele is required for the expression of HO trait in peanut line (Nawade et al. 2018). The understanding of the genetics and molecular basis of *ahFAD2* gene has led to the development of several molecular marker systems such as CAPS and allele-specific PCR (AS-PCR) assays for the accurate genotyping of *ahFAD2*

Table 4 Fatty acid composition (%) and O/L ratio of 64 introgression lines (ILs) and their parents grown under open-field conditions

ILS	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20:0)	Gadoleic acid (C20:1)	Behenic acid (C22:0)	Erucic acid (C22:1)	Lignoceric acid (C24:0)	O/L ratio
GPBD4	11.56	0.06	3.37	50.14	26.11	0.04	1.63	1.16	4.28	0.08	1.16	1.92
SunOleic95R	6.49	0.03	2.16	78.68	5.20	0.05	1.11	2.06	2.74	0.16	1.32	15.12
MAS_HOL_1	6.01	0.04	2.61	79.44	3.93	0.04	1.34	1.95	3.06	0.14	1.44	20.21
MAS_HOL_2	6.54	0.03	2.59	77.10	5.33	0.05	1.35	2.01	3.45	0.13	1.42	14.46
MAS_HOL_3	6.66	0.04	2.79	78.78	3.36	0.04	1.45	1.95	3.41	0.11	1.42	23.48
MAS_HOL_4	6.45	0.03	2.24	77.13	4.98	0.07	1.25	2.45	3.58	0.21	1.63	15.49
MAS_HOL_5	6.54	0.04	3.26	76.40	4.42	0.06	1.62	1.86	3.90	0.13	1.77	17.29
MAS_HOL_6	6.69	0.05	3.05	77.48	3.90	0.06	1.64	1.91	3.52	0.13	1.58	19.87
MAS_HOL_7	6.86	0.04	2.90	76.82	4.54	0.06	1.50	1.90	3.63	0.15	1.62	16.92
MAS_HOL_8	6.03	0.06	3.10	79.41	3.06	0.04	1.61	1.87	3.33	0.11	1.38	25.97
MAS_HOL_9	7.02	0.05	2.24	78.56	4.79	0.05	1.06	2.11	2.82	0.18	1.44	16.41
MAS_HOL_10	6.56	0.05	4.05	77.54	3.88	0.04	1.66	1.52	3.52	0.07	1.12	19.99
MAS_HOL_11	7.34	0.04	2.94	75.37	5.64	0.07	1.42	2.17	3.37	0.16	1.49	13.36
MAS_HOL_12	6.87	0.04	2.27	78.48	4.65	0.04	1.34	1.81	3.07	0.12	1.33	16.89
MAS_HOL_13	6.47	0.06	3.37	78.53	2.92	0.06	1.61	1.87	3.47	0.11	1.53	26.90
MAS_HOL_14	6.86	0.05	2.97	77.41	3.83	0.07	1.50	1.94	3.71	0.13	1.54	20.21
MAS_HOL_15	7.35	0.04	2.84	75.89	5.02	0.07	1.47	1.96	3.78	0.13	1.45	15.10
MAS_HOL_16	7.29	0.04	2.71	78.00	4.27	0.05	1.31	1.77	3.13	0.12	1.31	18.28
MAS_HOL_17	7.21	0.05	2.81	76.46	4.35	0.08	1.61	1.91	3.88	0.13	1.52	17.57
MAS_HOL_18	6.70	0.03	2.39	77.26	4.91	0.06	1.39	1.98	3.63	0.11	1.57	15.74
MAS_HOL_19	6.78	0.04	3.16	76.92	4.45	0.06	1.55	1.91	3.56	0.10	1.48	17.27
MAS_HOL_20	6.45	0.04	2.98	78.53	3.55	0.05	1.50	1.92	3.31	0.12	1.56	22.10
MAS_HOL_21	7.21	0.06	3.57	76.50	4.02	0.06	1.75	1.67	3.63	0.12	1.42	19.04
MAS_HOL_22	6.84	0.05	3.40	77.45	3.49	0.06	1.70	1.78	3.57	0.11	1.55	22.18
MAS_HOL_23	7.07	0.02	2.30	77.16	5.03	0.07	1.23	2.19	3.51	0.16	1.27	15.34
MAS_HOL_24	7.64	0.04	2.86	75.84	5.26	0.05	1.35	2.07	3.40	0.14	1.36	14.41
MAS_HOL_25	7.23	0.03	3.10	77.91	4.00	0.05	1.58	1.72	3.16	0.07	1.16	19.50
MAS_HOL_26	6.81	0.03	2.40	76.53	5.68	0.07	1.26	2.30	3.25	0.20	1.50	13.48
MAS_HOL_27	6.87	0.05	2.41	76.23	4.61	0.07	1.33	2.57	3.71	0.25	1.92	16.55
MAS_HOL_28	6.34	0.02	2.29	77.64	5.35	0.06	1.09	2.18	3.49	0.18	1.36	14.50
MAS_HOL_29	6.86	0.04	3.07	77.09	4.42	0.06	1.52	1.89	3.46	0.11	1.49	17.45
MAS_HOL_30	5.99	0.06	4.87	75.78	3.94	0.07	1.87	1.95	3.44	0.18	1.87	19.22
MAS_HOL_31	7.02	0.05	2.24	78.56	4.79	0.05	1.06	2.11	2.82	0.18	1.44	16.41
MAS_HOL_32	6.35	0.04	2.54	77.91	4.72	0.06	1.27	2.19	3.10	0.19	1.64	16.52

Table 4 (continued)

ILS	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20:0)	Gadoleic acid (C20:1)	Behenic acid (C22:0)	Erucic acid (C22:1)	Lignoceric acid (C24:0)	O/L ratio
MAS_HOL_33	6.04	0.02	2.78	79.60	3.78	0.05	1.33	2.01	2.87	0.15	1.37	21.04
MAS_HOL_34	6.47	0.05	2.77	78.00	3.83	0.05	1.65	1.92	3.54	0.13	1.59	20.36
MAS_HOL_35	6.36	0.03	3.37	78.58	3.65	0.05	1.59	1.76	3.12	0.12	1.36	21.55
MAS_HOL_36	6.62	0.05	3.26	77.63	3.91	0.06	1.56	1.92	3.52	0.13	1.35	19.85
MAS_HOL_37	6.55	0.04	2.83	78.79	3.75	0.05	1.41	1.99	3.03	0.14	1.43	21.03
MAS_HOL_38	6.63	0.04	2.58	77.13	5.04	0.07	1.32	2.20	3.15	0.16	1.67	15.31
MAS_HOL_39	6.69	0.05	2.97	78.64	3.74	0.05	1.48	1.89	2.99	0.13	1.38	21.05
MAS_HOL_40	6.60	0.05	2.75	79.93	3.47	0.04	1.41	1.58	2.96	0.08	1.14	23.05
MAS_HOL_41	6.79	0.06	2.91	81.27	2.10	0.05	1.48	1.53	2.62	0.07	1.13	38.79
MAS_HOL_42	6.38	0.10	2.64	79.96	2.80	0.05	1.63	1.70	3.34	0.09	1.32	28.53
MAS_HOL_43	8.29	0.07	2.76	68.91	13.02	0.04	1.45	1.39	2.88	0.03	1.18	5.29
MAS_HOL_44	6.99	0.08	2.62	81.29	2.25	0.05	1.45	1.41	2.46	0.07	1.34	36.18
MAS_HOL_45	9.75	0.18	2.93	74.27	5.62	0.06	1.54	1.29	3.05	0.12	1.28	13.22
MAS_HOL_46	6.76	0.06	2.23	78.86	4.16	0.06	1.17	2.07	2.81	0.17	1.64	18.94
MAS_HOL_47	6.70	0.05	2.67	78.61	4.33	0.06	1.36	2.13	3.28	0.18	1.63	18.17
MAS_HOL_48	6.75	0.10	3.79	79.57	2.00	0.04	1.72	1.52	2.97	0.14	1.46	39.75
MAS_HOL_49	6.94	0.06	3.48	78.56	3.46	0.05	1.58	1.44	3.20	0.07	1.15	22.71
MAS_HOL_50	8.03	0.07	2.83	67.04	15.66	0.03	1.40	1.17	2.54	0.05	1.19	4.28
MAS_HOL_51	7.67	0.02	2.31	71.22	10.42	0.07	1.18	2.16	3.30	0.16	1.48	6.83
MAS_HOL_52	6.56	0.06	2.53	81.71	2.01	0.04	1.28	1.55	2.73	0.10	1.42	40.71
MAS_HOL_53	7.00	0.05	2.28	78.55	4.10	0.06	1.24	2.03	2.98	0.13	1.55	19.14
MAS_HOL_54	6.71	0.07	2.75	80.45	2.72	0.04	1.41	1.65	2.78	0.10	1.32	29.60
MAS_HOL_55	6.91	0.08	2.61	80.68	2.51	0.05	1.40	1.64	2.73	0.10	1.29	32.09
MAS_HOL_56	7.50	0.07	2.56	72.34	9.84	0.05	1.40	1.64	2.91	0.11	1.58	7.35
MAS_HOL_57	7.52	0.09	2.58	80.36	2.29	0.06	1.25	1.67	2.27	0.11	1.39	35.09
MAS_HOL_58	7.02	0.08	2.70	80.21	2.76	0.07	1.33	1.62	2.68	0.13	1.40	29.08
MAS_HOL_59	7.05	0.04	2.46	77.24	5.58	0.03	1.39	1.77	2.91	0.10	1.43	13.84
MAS_HOL_60	8.30	0.14	2.35	80.11	2.01	0.03	1.29	1.54	2.72	0.08	1.43	39.80
MAS_HOL_61	8.64	0.11	2.30	78.15	3.00	0.04	1.25	1.83	2.85	0.15	1.66	26.01
MAS_HOL_62	6.61	0.06	2.55	79.92	3.27	0.06	1.17	1.97	2.57	0.15	1.69	24.42
MAS_HOL_63	7.02	0.09	1.97	80.68	2.85	0.04	1.22	1.85	2.69	0.12	1.48	28.30
MAS_HOL_64	6.97	0.08	2.85	78.17	3.82	0.04	1.44	1.75	3.03	0.11	1.48	20.46
Minimum	5.99	0.02	1.97	67.04	2.00	0.03	1.06	1.17	2.46	0.03	1.12	4.28
Maximum	9.75	0.18	4.87	81.71	15.66	0.08	1.87	2.57	3.90	0.25	1.92	40.71

Table 4 (continued)

ILS	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20:0)	Gadoleic acid (C20:1)	Behenic acid (C22:0)	Erucic acid (C22:1)	Lignoceric acid (C24:0)	O/L ratio
Mean[a]	6.94	0.06	2.81	77.63	4.49	0.05	1.42	1.86	3.18	0.13	1.45	20.31
% increase/decrease[b]	−39.98	−3.49	−16.73	54.82	−82.81	33.73	−12.46	61.28	−25.73	70.34	25.52	957.27

[a]Values of both the parents are not considered

[b]Percent increase or decrease in mean fatty acid content of ILs with respect to recurrent parent

genes. Based on our previous result (Nawade et al. 2016), we have selected GPBD4, multiple foliar fungal disease-resistant and high-yielding variety, having a natural mutation in the *ahFAD2A* gene. Furthermore, GPBD4 is extremely popular among the south Indian farmers because of its durable resistance to various foliar fungal diseases including rust and late leaf spot (LLS). The rust and LLS are prevalent in groundnut growing regions across the world causing yield loss up to 70% (Sujay et al. 2012). Further, the extent of economic losses to the tune of 467 m USD by rust and 599 m USD by LLS was estimated (Khera et al. 2016). Besides, adversely affecting productivity, they also affect the quality of the seeds and fodder, making it unsuitable for consumption. Henceforth, several popular groundnut varieties have been phased out of farmer's fields in the recent past due to heavy yield losses caused by foliar fungal diseases. We have focused on the selection and transfer of only one mutant allele, *ahFAD2B* from SunOleic95R into GPBD4 using the MABC approach (Fig. 1). Moreover, we have confirmed the presence and homozygosity of *ahFAD2A* allele using AS-PCR and CAPS markers in introgression lines.

Hybridization is the most critical step for the success of any peanut breeding program, as the peanut is a self-pollinated crop with cleistogamous pollination system (Othman 1979; Lim et al. 1980). Consequently, significant efforts are required during hybridization programs to produce sufficient true hybrid seeds for the development of mapping populations or to improve the desired trait of selection (Chu et al. 2016; Norden 1980). We got a hybridization success rate in the range of 3–6% (data not shown) which is in tune with the previous reports (Norden and Rodriguez 1971; Banks 1976). However, a high pollination success rate (25–70%) was also reported (Norden 1980; Kale and Mouli 1984; Nigam et al. 1990). This large variation could be due to the fact that the pollination in peanut is greatly influenced by a number of factors like humidity, temperature, crossing schedule, the integrity of emasculated flowers, the skill of the operator and the parental combinations (Chu et al. 2016).

The AS-PCR assay proficiently identified the true hybrids from putative F_1s, while, CAPS helped in timely identification of homozygous plants for both *ahFAD2* alleles, in the segregating generations within a week of peanut germination and enabled the availability of pollen parent for subsequent backcrossing. The AS-PCR assay (Yu et al. 2013) targeting the mutant and non-mutant *ahFAD2B* alleles in separate reactions was also validated in these populations (Fig. 3) and was found more convenient to perform over CAPS assay. The CAPS assay was first utilized by Chu et al. (2011) to generate 'Tifguard High O/L' genotype through three rounds of accelerated backcrossing. While Janila et al. (2016) introgressed the *ahFAD2* mutant alleles from SunOleic95R in the background of elite genotypes (ICGV06110, ICGV06142, and ICGV06420) using AS-PCR (Chen et al. 2010) and

CAPS assay (Chu et al. 2007, 2009) and generated a total of 469 ILs. Further, real-time PCR (Barkley et al. 2010; 2011) was also exploited to transfer the HO trait in South African peanut cultivars, 'SA Juweel' and 'ARC Oleic2' (Mienie and Pretorius 2013), whereas, Koilkonda et al. (2013) identified 9 *ahFAD2* homozygous lines from 205 BC_2F_2 plants. In this experiment, we have successfully introgressed high oleic trait through MABC and developed a total of 64 HO ILs.

Further, among the 64 ILs generated, only 3 lines; MAS_HOIL_43, 50 and 51 (from glasshouse) and 4 lines; MAS_HOIL_43, 50, 51 and 56 (from field conditions) recorded O/L ratio of the below industrially acceptable value of 9.0 (Tables 3, 4). Similarly, Janila et al. (2016) also reported considerable variations in the oleic and linoleic acid contents in the *ahFAD2* gene double mutant ILs which ranged from 62 to 82% and 2 to 20%, respectively. A 0.5–1.1-fold increase in the oleic acid content with concomitant reduction of linoleic acid by 0.4–1.0-fold among 82 MABC and 387 MAS-derived ILs compared to recurrent parents was also recorded by Janila et al. (2016). Moreover, many reports on *ahFAD2* gene double mutant recombinant inbred lines (RILs) also revealed substantial phenotypic variations for oleic and linoleic acid contents (Wang et al. 2015a; Pandey et al. 2014; Sarvamangala et al. 2011). Pandey et al. (2014) reported phenotypic variance for *ahFAD2B* gene (26.54%, 25.59% and 41.02%) and *ahFAD2A* gene (8.08%, 6.86% and 3.78%) for oleic acid (C18:1), linoleic acid (C18:2), and O/L ratio, respectively.

Furthermore, the double mutant line SunOleic95R when grown under US conditions was reported to have 79–81% oleic acid and 2.5–4.7% linoleic acid (Gorbet and Knauft, 1997; Andersen et al. 1998; Barkley et al. 2010, 2011). However, when same genotype is grown under Junagadh, India (21°47′73″N, 70°44′80″E; Nawade et al. 2016) and ICRISAT-Patancheru, India (17°50′28″N, 78°27′79″E; Janila et al. 2016) conditions, recorded 78.68%–80.21% and 78.30% oleic acid and 3.20%–7.34% and 5.00–6.00% linoleic acid, respectively. Furthermore, a few seeds having normal O/L were also identified in the seed lots of HO peanut cultivar 'Brantley' (Chamberlin et al. 2011). All these convincingly prove that multiple factors are involved in the regulation of oleic to linoleic acid flux, but a major role was played by the *ahFAD2* gene.

The ILs recorded higher mean SFA and MUFA contents under controlled conditions than under field conditions, which recorded a higher PUFA content (Fig. 4). The mean linoleic acid content of ILs showed an increment of 1.24% under field conditions, whereas palmitoleic, oleic, and lignoceric acid contents which along with O/L ratio showed a significant reduction under field conditions. Furthermore, other FAs did not show any significant change under both conditions. Thus, these HO lines under two different growth conditions showed the HO content indicating successful transfer and expression of the targeted trait in the RP. Moreover, the variations recorded are within the range of HO classification, which could be attributed to the growth conditions and other minor factors (Hinds 1995; Golombek et al. 1995; Singkham et al. 2010; Sun et al. 2014). The genetic factors and its interaction with the environmental factors are known to play a significant role in the formation of different FAs including oleic acid and linoleic acid (Singkham et al. 2010; Isleib et al. 2008; Andersen and Gorbet 2002).

The significant increment in the linoleic acid content under open conditions could be the effect of lower mean temperature during pod-filling stage during August, September and October months (27.4, 27.9 and 27.1 °C, respectively) of the year 2016 (Table S3 and Fig. 4). The lower temperature range (22–29 °C) is known to boost the oleate desaturase activity, thus promoting linoleic acid synthesis in peanut (Sogut et al. 2016; Andersen and Gorbet 2002). Moreover, the temperature in the glasshouse was set between 30 and 32 °C during the experiment period, which seems responsible for the lower linoleic acid content, due to the poor desaturase activity (Chaiyadee et al. 2013; Dwivedi et al. 1996; Golombek et al. 1995). This appears one of the very relevant reasons for peanuts generally showing higher O/L ratio when grown in warmer climatic conditions. Accordingly, Sun et al. (2014) have also reported a significant decrease in O/L ratio of HO cultivars under lower temperature, but in normal oleate cultivars, the corresponding decrease was not significant. It means there are certainly some other factors regulating the O/L ratio, along with the temperature. Thus, considering the influence of various abiotic factors on O/L flux underlines the importance of the selection of well-characterized parental lines and cultural practices according to the area so that it will minimize the environment-dependent negative modifications in the oil composition including O/L ratio (Nawade et al. 2018).

The variations in the oleic (67.04–82.33%) and linoleic acid (1.74–15.66%) contents in different ILs even in the presence of homozygous *ahFAD2* mutant alleles could also be due to the presence of some modifying genes and/or *ahFAD2* gene families (Nawade et al. 2016; Janila et al. 2016; Wang et al. 2015c). Recently, Wang et al. (2015c) reported six novel members of *the ahFAD2* gene family in peanut with varying expression in different plant parts and *ahFAD2-1* showed the highest expression. Besides, they also predicted the presence of more candidate genes controlling the oleate levels in developing seeds and/or presence of complex gene networks controlling the fluxes between the endoplasmic reticulum and the chloroplast within the peanut cells. The peanut whole genome sequence data also revealed the presence *FAD2* gene family consisting of two genes from *A. duranensis* and four from *A. ipaensis* (https://peanutbase.org/). The availability of peanut genome sequence and identification of different *ahFAD2* gene families is expediting the

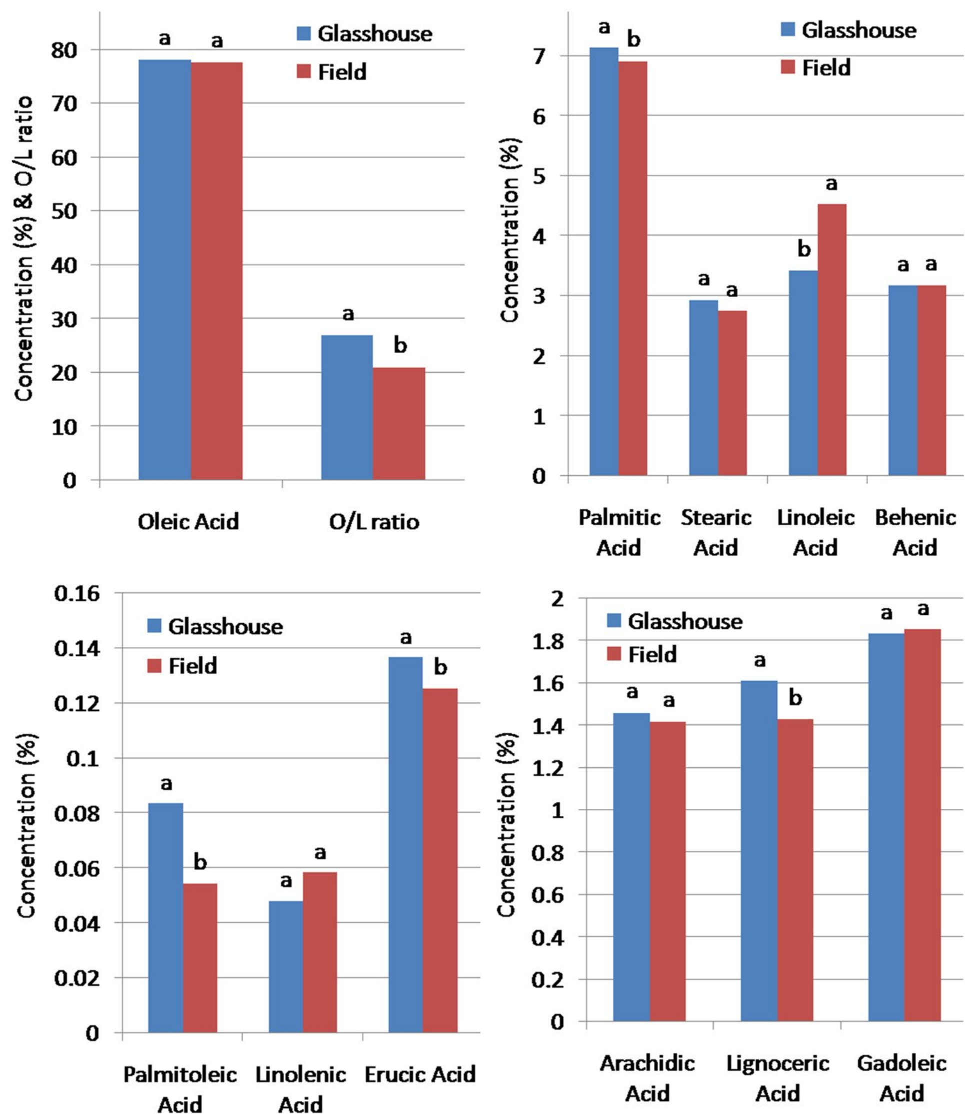

Fig. 4 Effect of growth conditions on FA composition and O/L ratio of peanut IL

research for detailed understanding of the O/L flux of HO peanut genotypes (Nawade et al. 2018).

Conclusions

In this age of capitalism and globalization, the role of edible oils and fats in health and related issues continues to evolve as further knowledge is gained about the significant interplay between health and dietary fats, FAs and chronic diseases (Huth et al. 2015). HO oils are increasingly demanded as a value-added product with wide applicability across industries. We have successfully developed high oleic ILs in the background of foliar fungal disease-resistant cultivar GPBD-4. The ILs lines identified through MAS performed excellently well in two different growth conditions for HO content. These improved lines could be the potential breeding material for further HO breeding programs and can also be released as a variety. These ILs having customized FA profile with lower SFA and higher MUFA contents are health friendly and simultaneously it will expand the applicability of peanut oil in different industries.

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