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Improvement of three popular Indian groundnut varieties for foliar disease resistance and high oleic acid using SSR markers and SNP array in marker-assisted backcrossing☆

Yaduru Shasidhar[a,b,1], *Murali T. Variath*[a,1], *Manish K. Vishwakarma*[a], *Surendra S. Manohar*[a], *Sunil S. Gangurde*[a,b], *Manda Sriswathi*[a], *Hari Kishan Sudini*[a], *Keshavji L. Dobariya*[c], *Sandip K. Bera*[d], *Thankappan Radhakrishnan*[d], *Manish K. Pandey*[a,*], *Pasupuleti Janila*[a,*], *Rajeev K. Varshney*[a,*]

[a]*International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India*
[b]*Department of Genetics, Osmania University, Hyderabad, India*
[c]*Junagadh Agricultural University, Junagadh, India*
[d]*ICAR-Directorate of Groundnut Research, Junagadh, India*

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ABSTRACT

Foliar fungal diseases (rust and late leaf spot) incur large yield losses, in addition to the deterioration of fodder quality in groundnut worldwide. High oleic acid has emerged as a key market trait in groundnut, as it increases the shelf life of the produce/products in addition to providing health benefits to consumers. Marker-assisted backcrossing (MABC) is the most successful approach to introgressing or pyramiding one or more traits using trait-linked markers. We used MABC to improve three popular Indian cultivars (GJG 9, GG 20, and GJGHPS 1) for foliar disease resistance (FDR) and high oleic acid content. A total of 22 BC_3F_4 and 30 BC_2F_4 introgression lines (ILs) for FDR and 46 BC_3F_4 and 41 BC_2F_4 ILs for high oleic acid were developed. Recurrent parent genome analysis using the 58 K Axiom_*Arachis* array identified several lines showing upto 94% of genome recovery among second and third backcross progenies. Phenotyping of these ILs revealed FDR scores comparable to the resistant parent, GPBD 4, and ILs with high (~80%) oleic acid in addition to high genome recovery. These ILs provide further opportunities for pyramiding FDR and high oleic acid in all three genetic backgrounds as well as for conducting multi-location yield trials for further evaluation and release for cultivation in target regions of India.

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* *Corresponding authors.*
E-mail addresses: m.pandey@cgiar.org, (M.K. Pandey), p.janila@cgiar.org, (P. Janila), r.k.varshney@cgiar.org. (R.K. Varshney).

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[1] Yaduru Shasidhar and Murali T. Variath contributed equally to this work.

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1. Introduction

Groundnut (*Arachis hypogaea*), also popularly known as peanut, is a major oilseed and food crop grown on ~27.9 Mha across 100 countries for a global production of 47 Mt. during 2017 [1]. The crop is consumed mainly as confectionery and in various food products in Western countries and is used for cooking oil and confectionery in the Indian subcontinent. The groundnut kernel contains fat (40%–55%), protein (20%–30%) and carbohydrates (10%–20%) along with several nutritional components (vitamin E, niacin, zinc, iron, calcium, magnesium, phosphorus, riboflavin, thiamine, and potassium) [2]. Malnutrition is the greatest challenge in most African and Asian countries, and this nutrition-rich crop has the potential to play a key role in combating malnutrition. The high-quality produce with oleic acid rich kernels and good quality fodder provides sustainable income and livelihood to the resource-poor farmers as well as ensure supply of quality groundnuts to the consumers and industry.

Groundnut is exposed to biotic and abiotic stresses that reduce its yield and quality [3,4]. Crop health, productivity, and quality are likely to be impaired in coming years owing to fluctuating climatic conditions such as uncertain rain and high temperature, especially in semi-arid regions of the world including India [5]. The co-occurrence of two foliar fungal diseases namely, rust (caused by *Puccinia arachidis*) and late leaf spot (LLS, caused by *Cercosporidium personatum*) causes chlorotic lesions leading to defoliation, which lowers crop yield and fodder quality. These two diseases infect plants especially during the seed setting stage and result in yield losses ranging from 15% to 59% for LLS and 10% to 52% for rust [6]. Although these diseases can be controlled by timely application of fungicides, however, such control measures are labor-intensive, increase the financial burden on resource-poor farmers, and are not environmentally benign.

High oleic acid has emerged in recent years as a key market trait, as it improves not only product shelf life but also enhances the oil quality and offers health benefits to consumers. Groundnut oil with high linoleic acid is prone to oxidation, leading to an unpleasant smell and taste and short shelf life of the oil and other groundnut products. Linoleic acid, which accounts for ~40% of total kernel oil content, is subject to oxidative rancidity when heated at high temperatures, resulting in changes in taste and odor of the oil and formation of trans-fatty acids, which cause cardiovascular disease [7]. For this reason, oleic-rich groundnuts are in high demand by consumers, traders and industry worldwide. This high oleic feature of new varieties is expected to provide more healthy cooking oil to the Indian consumers.

To achieve higher yield gains in farmers' fields and increased income to farmers, faster replacement of improved varities are required which can outyield under prevailing conditions [5]. Although conventional breeding approaches have played significant role in developing improved varieties, however, the current pace is not enough to match up with the required speedy and timely replacement of improved varieties in farmers field. On the other hand, genomics-assisted breeding (GAB) especially MABC for introgression of traits for disease resistance [8,9], high oleic acid [10–12] and nematode resistance [10] in groundnut in combination with rapid generation cycle turnover and other modern tools provide an opportunity to reduce the time required to develop new varieties. Some improved molecular breeding lines have recorded inscreased pod and haulm yield and have been released as cultivars, while others are in the pipeline for evaluation and release [13].

The linked and validated markers are available for resistance to rust, LLS [14–16] and high oleic acid [10]. The recent availability of reference genome sequences of diploid progenitors [17,18] and resequencing of diverse lines have facilitated discovery of millions of single nucleotide polymorphisms (SNPs) and development of high density SNP chip (Axiom_*Arachis* [19]) containing 58 K highly informative genome-wide SNP markers for use in trait mapping and molecular breeding in groundnut.

Lack of high genomic diversity, a large tetraploid genome, and a self-pollinating habit have hindered trait mapping progress in groundnut. Majority of the QTL mapping studies have produced sparse genetic maps with large QTL regions [14,15,20–22]. The first study on FDR mapping [14] identified a dominant SSR marker, IPAHM103, with phenotypic variation explained (PVE) of 55% from segregating population TAG 24 × GPBD 4. Later improved density of genetic maps with SSRs on segregating populations (TAG 24 × GPBD 4 and TG 26 × GPBD 4) identified SSRs (GM1536, GM2301, and GM2079) linked to FDR with 82% PVE. With the above linked and validated SSRs, MABC was initiated for transferring these FDR QTL to the popular cultivars. Very recently, sequencing-based approaches including QTL-seq [16,23] and low coverage/skim sequencing of a complete RIL population [24] helped in the identification of user-friendly markers for foliar fungal diseases and tomato spotted wilt virus resistance. These approaches led to rapid discovery of candidate resistance genes and diagnostic markers for use in breeding.

Here we report the improvement of three popular groundnut cultivars of Gujarat state of India: GJG 9, GG 20, and GJGHPS 1, for resistance to rust and LLS and increased oleic acid levels using MABC. This study also demonstrates the utility of high-density genotyping for performing background genome recovery and selecting promising MABC lines for further evaluation. The improved MABC lines developed through this study were multiplied for conducting further multi-location yield trials.

2. Materials and methods

2.1. Plant material

Three popular cultivars: GJG 9, GG 20, and GJGHPS 1, from Gujarat, the leading groundnut-producing state of India, were targeted for improving FDR and high oleic acid (Fig. 1-A). The Spanish bunch groundnut cultivar, GJG 9, is high-yielding and resistant to stem rot disease. This variety was developed from the cross GG-5 × ICGV 90116, has a pod yield of 1632 kg ha^{-1} and 48.7% seed oil content, and was released in 2012 [25]. The Virginia bunch groundnut variety, GG 20, is released in 1992. It was developed from the cross GAUG-10 × R-33-1 and has a

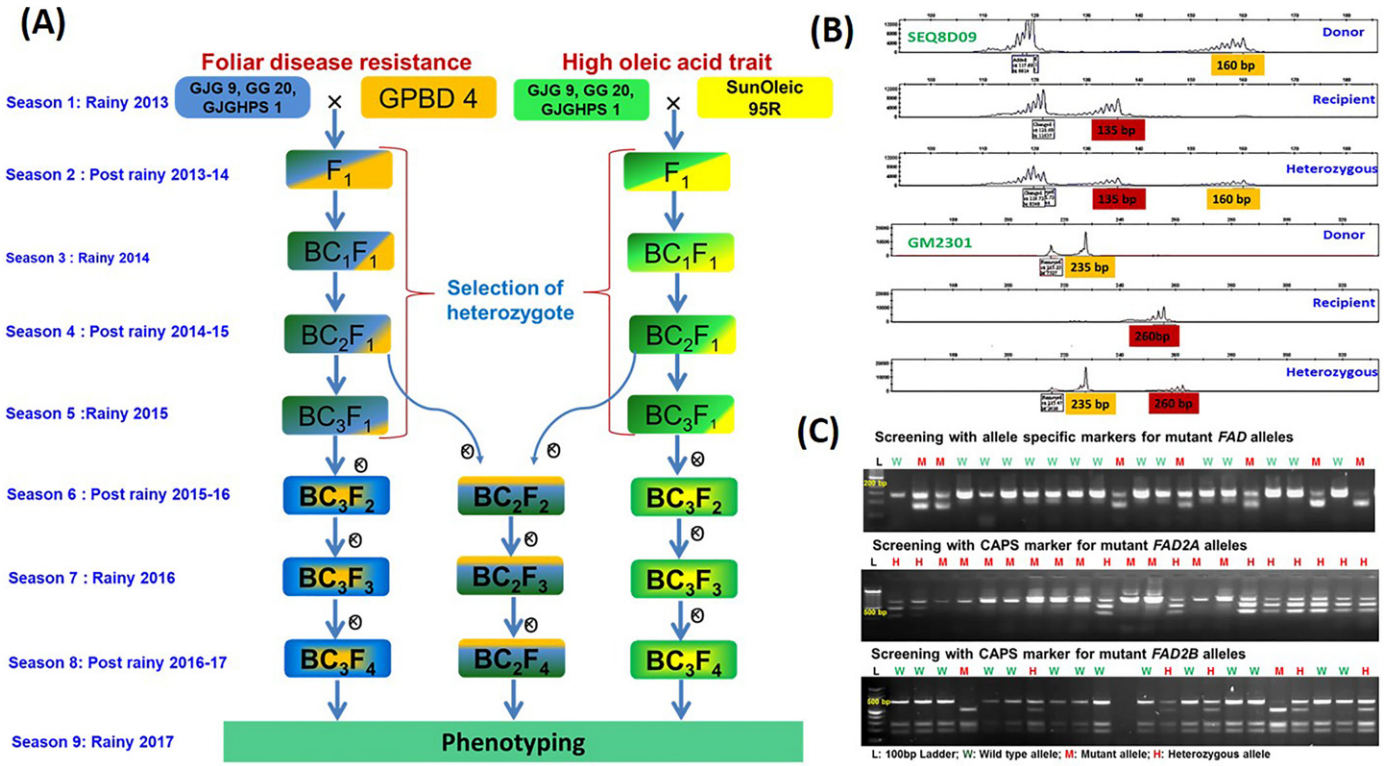

Fig. 1 – Marker-assisted breeding for increasing foliar disease resistance and oleic acid content in three elite and popular cultivars. (A) Flowchart of MABC for foliar disease (rust and LLS) resistance and high oleic acid. (B) Foreground genotyping of the backcross populations for FDR QTLs using SSRs (SEQ8D09, GM2301) (C) Foreground genotyping of backcrossed population for *FAD* mutants using allele-specific and CAPS marker.

pod yield of 1960 kg ha^{-1} and 50.7% seed oil content [25]. The Virginia runner groundnut variety, GJGHPS 1, is a bold-kernel cultivar released in 2010. It was developed from the cross JSP-21 × VG-5 and has a pod yield of 2125 kg ha^{-1} and 47.9% seed oil content [25]. Pure seed of these three cultivars was obtained from Junagadh Agricultural University (JAU), Junagadh, India.

The genotype GPBD 4, a foliar disease resistant variety, released in India for Karnataka state [26] was used as a donor parent in MABC program for introgression of quantitative trait locus (QTL) for FDR. It is derived from the cross KRG 1 × CS 16 (ICGV 86855). Along with FDR, it also has desirable traits like high yield, pod growth rate, oil content, and mid-early maturity [26]. Another genotype, SunOleic 95R, a high-oleic runner agronomic type [27] released in USA, was used as a donor for increasing oleic acid content. It is derived from the cross F435-2-3-B-2-l-b4-B-3-b3-l-B × F519-9 and carries the mutant alleles of the fatty acid desaturase (FAD) genes in both the sub-genomes of tetraploid groundnut.

2.2. Linked markers for FDR and high oleic acid

For FDR, linked markers for rust (IPAHM103, GM1536, GM2301, and GM2079) and LLS (SEQ8D09 and GM1009) [15] were initially used to track both QTLs. In later generations, newly developed allele-specific markers for rust (GMRQ517, GMRQ786, and GMRQ843) and LLS (GMLQ975) [16] were also included in the screening panel for performing foreground selection (Table S1). For high oleic acid, allele-specific markers for mutant *FAD* alleles for both the A and B sub-genomes [28] were used for screening both F_1s and backcrossed F_1s for identifying heterozygous plants. Because these markers could not differentiate homozygous and heterozygous mutant alleles in segregating backcross lines, cleaved amplified polymorphic sequences (CAPS) markers were used for genotyping segregating and advanced breeding lines (Table S1) [29].

2.3. DNA extraction and genotyping

DNA was extracted from 15-days-old plant leaves at the F_1 and each backcross generation along with the parents, using the modified cetyltrimethylammonium bromide (CTAB) extraction method [30]. DNA quality and quantity were checked on 0.8% agarose gel by electrophoresis at 100 V for 60 min. The final concentration of the diluted DNA for genotyping was around 5 ng μL^{-1}.

2.3.1. Genotyping for selecting resistance alleles controlling foliar disease resistance

The genotyping was performed with linked markers (Table S1) for FDR using touchdown PCR. The PCR mix consisted of 2–5 ng of DNA, 2 pmol L^{-1} of M13-labeled forward primer (F), 5 pmol L^{-1} of reverse primer (R), 2 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} dNTPs, 0.1 U of Taq DNA polymerase (KAPA Biosystems, Fisher Scientific, USA) and 1× PCR buffer. A standardized touchdown PCR program was used with 5 min initial denaturation, followed by 5 cycles of 94 °C for 20 sec, 65 °C for 20 sec, and 72 °C for 30 sec with 1 °C decrement for every cycle, followed by 40 cycles of 94 °C for 20 sec, constant annealing temperature at 59 °C and 72 °C for 30 sec, ending with extension for 20 min at 72 °C. PCR products were resolved on 1.5% agarose gel for confirming amplification. Amplified products were denatured and separated by capillary electrophoresis on an ABI 3700 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). These SSR primers were labeled with FAM, VIC and NED dyes, which were detected as blue, green, and black color peaks, respectively when capillary separated on sequencer. The GeneMapper software (Applied Biosystems, Foster City, CA, USA) was used to analyze the peaks, whereas, the allele-specific primers were separated on 2% agarose gel for identification of the ILs by presence vs. absence (Fig. 1-B).

2.3.2. Genotyping for selecting mutant alleles controlling high oleic acid

Genotyping of F_1s and MABC lines was performed for selecting *FAD* mutant alleles located on A09 and B09 chromosomes using two different marker types: allele-specific and CAPS markers for foreground selection. The primer pair F435 and F435SUB amplifies a 203-base pair (bp) mutant allele fragment in the A-genome (G:C to A:T) whereas another primer pair F435 and F435INS, amplifies the 195-bp mutant allele fragment in the B genome (A:T insertion). An internal control primer pair, F435 – F and F435IC-R, was used to avoid the false positives. The touchdown PCR program described above was used and 2% agarose gel was used to resolve the PCR products. For CAPS markers, the primer pair aF19F and 1056R was used to amplify the *FAD2A* allele and then the amplified PCR product was restriction-digested with *Hpy99I* at 37 °C for 6 h. The mutant *FAD2A* allele 826 bp remained uncleaved, whereas the wild-type allele was cleaved into two fragments of 598 and 228 bp (Fig. 1-C). For amplifying the *FAD2B* mutant allele of 1214 bp, the primers R1*FAD* and bF19F were used, followed by restriction digestion of the PCR product with *Hpy188I* at 37 °C for 16 h. The mutant allele formed 6 fragments: 550, 213, 263, 171, 32, and 12 bp, whereas the wild-type allele formed five fragments: 736, 263, 171, 32, and 12 bp [29].

2.3.3. High-throughput genotyping with Axiom_Arachis SNP array

The Axiom_*Arachis* SNP array was used to genotype second- and third-backcross homozygous lines using the Affymetrix GeneTitan system and polymorphic SNPs were identified using Axiom Analysis Suite 2.0 (Affymetrix, Thermo Fisher Scientific, USA). The 58 K SNPs array contains an average of ~2900 SNPs per chromosome. It contains an average of one SNP per 42 kb, similar to recently developed SNP arrays for major crops including maize, rice, and barley [31–33]. A total of 20 ng μL^{-1} DNA from each sample was used for genotyping with the Affymetrix SNP array using the Affymetrix GeneTitan system following Pandey et al. [19]. In brief, the cell intensity files (CEL) produced by the GeneTitan instrument were altered to genotype calls using the Axiom Genotyping Algorithm version 1 (Axiom GT1) available in the Affymetrix Power Tools or Genotyping Console v4.1 software package. Following the Axiom Best Practices Genotyping Workflow, SNPs were sorted into different classes [19]. The "Poly High Resolution" (PHR) SNPs which also passed all quality control (QC) were further filtered based on the proportion of calls between parents and

among introgression lines in respective recurrent parent background. These SNP calls were then used to assess the percentage of recurrent parent genome recovery among ILs in both the backcross generations.

2.4. Hybridization and backcross generation development

During season 1 (Rainy 2013), GPBD 4 and SunOleic 95R, donors for FDR and high oleic acid, respectively, were used as male parents, while the three targeted cultivars GJG 9, GG 20, and GJGHPS 1 were used as female parents (Fig. 1-A). There were total six cross combinations i.e. three crosses for FDR (GJG 9 × GPBD 4, GG 20 × GPBD 4, and GJGHPS 1 × GPBD 4) and three crosses for high oleic acid (GJG 9 × SunOleic 95R, GG 20 × SunOleic 95R, and GJGHPS 1 × SunOleic 95R). After ~40 days, the fully developed floral buds in recurrent parental genotypes were emasculated and were pollinated on the following day by squeezing pollen from the donor (GPBD 4 for FDR or SunOleic 95R for high oleic acid) onto the stigma of the emasculated flower. These F_1 pods were harvested and grown in the second season (Post-rainy 2013–2014).

During the second season, the F_1 seeds were planted and screened with the linked markers for the identification of the true F_1 plants. The identified true F_1s were then used as a male parent and the recurrent parents as the female parent to develop the first backcross F_1s (Fig. 1-A). At the end of the second season, the first backcross (BC_1F_1) pods were harvested. During the third cropping season (Rainy 2014), the harvested BC_1F_1 seeds from all six crosses were planted in the field and genotyped with the linked markers for the respective traits to confirm the true BC_1F_1 plants. The positive BC_1F_1 were used as a male parent to perform the second backcrossing using respective recurrent parents as female. The successful BC_2F_1 pods were harvested at the end of season 3. During the fourth season (Post rainy 2014–2015), the harvested BC_2F_1 plants were planted and genotyped with linked markers for both traits in the respective crosses. After identifying true BC_2F_1 plants, few BC_2F_1 plants were also selfed to produce the homozygous BC_2F_2 seeds while remaining plants were used for making third backcross (BC_3F_1) with the recurrent parents. These BC_3F_1 pods were harvested and used for growing in the next season.

During the fifth season (Rainy 2015), the BC_3F_1 seeds were planted in the field, followed by DNA isolation and foreground selection. The positive BC_3F_1 lines were selfed to produce homozygous BC_3F_2 seeds. During the sixth season (Post rainy 2015–2016), the homozygous BC_3F_2 and BC_2F_2 plants were again confirmed for homozygosity and generation advanced to BC_3F_4 and BC_2F_4, respectively. Based on the phenotyping for traits including plant morphology, pod size, pod shape, and pod number, homozygous ILs were selected for multiplication to generate uniform breeding lines. Finally, the homozygous lines for the six backcross lines were phenotyped for the respective target traits.

In summary, the foreground selection was performed in each generation i.e. hybrids (F_1), first (BC_1F_1), second (BC_2F_1), and third (BC_3F_1) backcross generations successfully. Finally, the homozygous BC_2F_2 and BC_3F_2 lines were generated for the three crosses for FDR and high oleic acid.

2.5. Phenotyping for foliar disease resistance and high oleic acid

MABC lines were screened for FDR in disease nursery plots using the spreader row technique [34] at Patancheru, India. TMV 2, a highly susceptible control for both rust and LLS diseases, was used as a spreader row. These spreader rows were planted as every tenth row in the nursery plot and on the borders to ensure high inoculum load. Forty-five days after sowing, plants were inoculated by spraying a spore suspension of rust and LLS spores and the infected plants from the greenhouse were transplanted between the spreader rows [14,15]. Disease scoring was performed at 75, 90, and 105 days after sowing, using a modified 1–9 points scale [35].

Phenotyping of the MABC lines for high oleic acid was estimated using near infrared reflectance spectroscopy (NIRS) (model using a XDS RCA instrument, FOSS Analytical AB, Hilleroed, Denmark) [36]. NIRS is non-destructive method, by which seed samples of 70–100 g were scanned for the major fatty acids oleic (C18:1), linoleic (C18:2), and palmitic (C16:0). A calibration equation having regression coefficient (R^2) values of 0.89 for palmitic acid and 0.96 each for oleic and linoleic acids was calibrated to predict palmitic, oleic and linoleic acid contents in the backcrossed populations. The efficiency of cross-validation of the selected equation, measured as coefficient of determination of cross-validation (1 – VR, where VR is variance ratio) was 0.94 for oleic and linoleic acids and 0.80 for palmitic acid.

3. Results

3.1. Development of MABC lines

Three crosses each for FDR and high oleic acid were made in parallel followed by backcrossing and foreground selections. The details of the plants selected at each stage and the numbers of confirmed plants identified at F_1, BC_1F_1, BC_2F_1, BC_2F_2, BC_3F_1, and BC_3F_2 are presented in Table 1.

During post-rainy 2013–2014, a total of 135 F_1 and 120 F_1 plants were planted for FDR and high oleic acid, respectively. As a result of screening with linked markers, 68 plants for FDR (20 plants from GJG 9 × GPBD 4, 23 plants from GG 20 × GPBD 4, and 25 plants from GJGHPS 1 × GPBD 4) and 46 for high oleic acid (24 plants from GJG 9 × SunOleic 95R, 11 plants from GG 20 × SunOleic 95R, and 11 plants from GJGHPS 1 × SunOleic 95R) were confirmed as true F_1 plants in post-rainy 2013–2014 (Table 1). These true F_1 plants were used as pollen parents to make the first backcross with the respective recurrent parents. The BC_1F_1 seeds from the pods were harvested.

During the rainy 2014, a total of 76 plants for FDR crosses and 67 plants for high oleic crosses were used for the foreground selection by using linked markers for the respective traits. As a result, 43 BC_1F_1 from FDR crosses (9 from GJG 9 × GPBD 4, 15 from GG 20 × GPBD 4, and 19 from GJGHPS 1 × GPBD 4) and 23 BC_1F_1 from high oleic acid crosses (6 from GJG 9 × SunOleic 95R, 11 from GG 20 × SunOleic 95R, and 6 from GJGHPS 1 × SunOleic 95R) were found heterozygous for the linked loci (Table 1). All the heterozygous plants were used

Table 1 – Summary of MABC generation advancement for foliar disease resistance and high oleic acid.

Generation	GJG 9		GG 20		GJGHPS 1	
	Number of plants screened	Number of plants selected after marker analysis	Number of plants screened	Number of plants selected after marker analysis	Number of plants screened	Number of plants selected after marker analysis
MABC for foliar disease resistance						
F_1	47	20	44	23	44	25
BC_1F_1	11	9	30	15	35	19
BC_2F_1	68	14	64	5	113	19
BC_3F_1	114	9	72	4	94	25
BC_2F_2	85	45	27	11	62	27
BC_3F_2	95	7	29	5	135	5
MABC for high oleic acid						
F_1	48	24	36	11	36	11
BC_1F_1	17	6	29	11	21	6
BC_2F_1	55	7	46	17	66	20
BC_3F_1	155	2	69	8	90	30
BC_2F_2	25	4	16	10	22	12
BC_3F_2	250	5	73	11	174	29

for making the second backcross (BC_2) with the respective parents, and pods were harvested from these backcrosses.

During post-rainy 2014–2015, a total of 245 BC_2F_1 plants for FDR crosses and 167 BC_2F_1 plants for high oleic crosses were used for conducting foreground selection by screening with linked markers. A total of 38 BC_2F_1 plants (14 from GJG 9 × GPBD 4, 5 from GG 20 × GPBD 4, and 19 from GJGHPS 1 × GPBD 4) were found heterozygous for FDR, while 44 BC_2F_1 plants (7 from GJG 9 × SunOleic 95R, 17 from GG 20 × SunOleic 95R, and 20 from GJGHPS 1 × SunOleic 95R) were heterozygous for high oleic acid (Table 1). These heterozygous plants from six respective crosses were selected to make the third backcross (BC3) and BC_3F_1 seeds were harvested. In parallel, BC_2F_1 plants were also selfed to produce homozygous BC_2F_2 pods.

During the rainy 2015, totals of 280 BC_3F_1 plants for FDR crosses and 314 BC_3F_1 plants for high oleic crosses were used for foreground selection. For FDR, 9 BC_3F_1 plants were found to be heterozygous from GJG 9 × GPBD 4, 4 BC_3F_1 plants from GG 20 × GPBD 4, and 25 BC_3F_1 plants from GJGHPS 1 × GPBD 4. For high oleic acid, 2 BC_3F_1 plants from GJG 9 × SunOleic 95R, 8 BC_3F_1 plants from GG 20 × SunOleic 95R, and 30 BC_3F_1 plants from GJGHPS 1 × SunOleic 95R were heterozygous (Table 1). These plants carrying targeted heterozygous loci were selfed for achieving homozygosity among the MABC derived lines.

During the post-rainy 2015–2016, 259 BC_3F_2 FDR homozygous lines were subjected to foreground selection and 17 of these lines (7 from GJG 9 × GPBD 4, 5 from GG 20 × GPBD 4, and 5 from GJGHPS 1 × GPBD 4) were found homozygous. Similarly, in case of high oleic acid, 497 BC_3F_2 homozygous lines were screened and 45 BC_3F_2 lines (5 from GJG 9 × SunOleic 95R, 11 from GG 20 × SunOleic 95R, and 29 from GJGHPS 1 × SunOleic 95R) were found to be homozygous (Table 1). In parallel, a total of 174 BC_2F_2 FDR plants were screened and 83 BC_2F_2 plants (45 from GJG 9 × GPBD 4, 11 from GG 20 × GPBD 4, and 27 from GJGHPS 1 × GPBD 4) were identified as homozygous for the target loci. Similarly, a total of 63 BC_2F_2 high oleic lines were screened and 28 BC_2F_2 plants (4 from GJG 9 × SunOleic 95R, 10 from GG 20 × SunOleic 95R, and 12 from GJGHPS 1 × SunOleic 95R) were identified as homozygous for the target loci (Table 1).

During rainy 2016, both the positive BC_3F_3 and BC_2F_3 homozygous ILs from these six crosses were multiplied to generate enough seeds for further phenotyping and yield trials. Based on morphological traits including plant type, pod shape, pod size, seed shape, and seed size, a total of 44 ILs (29 BC_2F_4 and 15 BC_3F_4 plants) in the genetic background of GJG 9, 42 ILs (11 BC_2F_4 and 31 BC_3F_4 plants) in the background of GG 20 and 22 ILs (16 BC_2F_4 and 6 BC_3F_4 plants) in the background of GJGHPS 1 were selected for FDR screening. Similarly, a total of 33 ILs (13 BC_2F_4 and 20 BC_3F_4) in the background of GJG 9, 51 ILs (44 BC_2F_4 and 7 BC_3F_4) in the background of GG 20, and 65 ILs (29 BC_2F_4 and 36 BC_3F_4) in the background of GJGHPS 1 were screened for high oleic acid.

3.2. Validation of introgressed traits under field conditions

3.2.1. Foliar disease resistance

Phenotyping for the FDR ILs was performed during rainy 2017 at ICRISAT, Patancheru. Disease scoring was performed manually around 75, 90, and 105 days after sowing (DAS). A total of 10 BC_3F_4 best ILs in the background of GJG 9 were identified as resistant to both foliar fungal diseases: LLS and rust. These ILs scored a minimum of 4–5 for LLS and 2 for rust at 105 DAS, while the recurrent parent scored 8 and 4 for LLS and rust, respectively at 105 DAS. In the case of GG 20, a total of 6 BC_3F_4 best ILs were found resistant to rust and LLS. One of these ILs, BC_3F_4 88, scored 4 for LLS and 2 for rust, similar to the donor parent GPBD 4, while GG 20 scored 8 for LLS and 5 for rust at 105 DAS. A total of 6 BC_3F_4 ILs in the background of GJGHPS 1 were found resistant to rust and LLS. Four of these ILs scored 4 for LLS and 2 for rust similar to the donor parent GPBD 4 while the GJGHPS 1 scored 7 for LLS and 5 for rust at 105 DAS (Table 2, Fig. 2, Table S2). In the case of BC_2F_4, most of the ILs of three crosses showed resistance to rust with minimum scores ranging from 2 to 3, but a few scored susceptible for LLS with the maximum disease score of 6 (Table S2).

Table 2 – Best performing five introgression lines with rust and LLS resistance and high background genome recovery of GJG 9, GG 20, and GJGHPS 1.

Cross details	% of RPG	75 DAS		90 DAS		105 DAS	
		Rust	LLS	Rust	LLS	Rust	LLS
GJG 9		1	4	5	6	5	8
GPBD 4		2	2	2	4	2	4
BC_3F_4_65	86.3	1	4	2	4	2	5
BC_3F_4_70	85.7	1	3	3	4	3	5
BC_3F_4_73	90.6	1	4	2	4	2	4
BC_3F_4_74	90.6	2	4	2	4	2	4
BC_3F_4_76	90.6	1	4	2	4	2	4
GG 20		4	3	4	7	5	8
GPBD 4		2	2	2	4	2	4
BC_3F_4_88	80.5	2	3	2	3	2	4
BC_3F_4_94	80.8	1	1	2	4	2	5
BC_3F_4_95	80.8	2	2	2	5	2	5
BC_3F_4_96	80.8	2	2	2	5	2	5
BC_3F_4_100	75.0	1	5	3	5	3	6
GJGHPS 1		2	3	4	5	5	7
GPBD 4		2	2	2	4	2	4
BC_3F_4_115	89.0	1	2	2	4	2	4
BC_3F_4_116	90.4	1	3	2	4	2	5
BC_3F_4_118	87.5	1	2	2	3	2	4
BC_3F_4_119	86.0	1	2	1	4	2	4
BC_3F_4_120	86.0	1	3	2	3	2	4

RPG, recurrent-parent genome at BC_3F_2; DAS, days after sowing; LLS, late leaf spot. 1–9 scale of disease scoring (1 represents highly resistant and 9 represents the highly susceptible).

3.2.2. *Fatty acid (palmitic, oleic, and linoleic) content*

Twelve BC_3F_4 ILs were identified in the background of GJG 9 with high oleic acid. The fatty acid composition in these ILs ranged from 63% to 84% for oleic, 1.4% to 19.5% for linoleic, and 6.9% to 12.3%for palmitic acid. A total of five best BC_3F_4 ILs with high oleic acid with maximum genome recovery were identified with better fatty acid chemistry than the recurrent parent, GG 20. These five ILs showed fatty acid composition ranging from 62.0%–80.4% of oleic, 2.9%–19.1% of linoleic, and 6.4%–8.7% palmitic acid. A total of 30 BC_3F_4 best ILs for high oleic acid which also showed better fatty acid chemistry than the recurrent parent, GJGHPS 1, were developed. Among these, five best ILs in the background of GJGHPS 1 with maximum recurrent genome recovery with improved fatty acid composition ranging from 75%–82% oleic, 3.2%–6.9% linoleic, and 6.0%–9.5% palmitic acid (Table 3, Fig. 3). The BC_2F_4 lines also showed high oleic acid levels under three recurrent background parents (Table S3).

RPG, recurrent-parent genome at BC_3F_2.

3.3. *High-density background genome recovery among MABC lines*

We deployed recently developed 58 K SNP array for tracking the background genomic recovery of the recurrent parent among ILs. We selected 7–8 best and promising lines from second (BC_2F_2) and third (BC_3F_2) backcrossed homozygous lines for foliar disease resistance (GJG 9 × GPBD 4, GG 20 × GPBD 4, and GJGHPS 1 × GPBD 4) and high oleic acid (GJG 9 × SunOleic 95R, GG 20 × SunOleic 95R, and GJGHPS 1 × SunOleic 95R) for genotyping with 58 K SNP array. For the six crosses, the number of polymorphic markers ranged from 2762 to 3925, while recurrent parent genome (RPG) recovery ranged from 71% to 94% among second backcross lines and 75%–92% across third backcross lines.

Among crosses for FDR, the highest polymorphic SNP markers were identified for GJGHPS 1 × GPBD 4 (3833 SNPs) followed by GG 20 × GPBD 4 (3714 SNPs) and GJG 9 × GPBD 4 (2762 SNPs) (Table 4). In the second backcross, RPG recovery ranged from 81 to 94% in the genetic background of GG 20 followed by GJG 9 (76.0%–87.5%) and GJGHPS 1 (71%–89%). In the third backcross, RPG recovery ranged from 83% to 90% in the genetic background of GJG 9, followed by GJGHPS 1 (72%–92%) and GG 20 (71.0%–85.5%) (Fig. 4, Table 5, Fig. S1). Among high-oleic crosses, the highest number of polymorphic loci were identified for GJG 9 × SunOleic 95R (3925 SNPs), followed by GJGHPS 1 × SunOleic 95R (3018 SNPs) and GG 20 × SunOleic 95R (2888 SNPs). In the second backcross, RPG recovery among ILs ranged from 72% to 85% for GJG 9 followed by GJGHPS 1 (87%–89%). Whereas in case of the third backcross, RPG recovery was highest for GJG 9 (90%–92%) followed by GG 20 (79%–86%) and GJGHPS 1 (77%–91%) (Fig. 4, Table 5, Fig. S2).

For the target chromosomes A02 and A03 for FDR, the polymorphic loci identified for GJGHPS 1 × GPBD 4 (413 and 223 SNPs) with average recoveries of 92% and 70%, respectively, followed by GG 20 × GPBD 4 (420 and 217 SNPs) with RPG average recoveries of 93% and 85%, respectively and GJG 9 × GPBD 4 (369 and 232 SNPs) with RPG average recoveries of 94% and 91%, respectively were observed across the ILs (Fig. 5, Fig. S3). For target chromosomes A09 and B09 for high oleic acid, the number of polymorphic loci identified for GJG 9 × SunOleic 95R (244 and 205 SNPs) with average recoveries of 92% and 88%, respectively, followed by GG 20 × SunOleic 95R (96 and 102 SNPs) with average recoveries of 82% and 85%, respectively and GJGHPS 1 × SunOleic 95R (90 and 114 SNPs) with average recovery of 78% from each chromosome were observed across the ILs (Fig. 5, Fig. S4).

4. Discussion

The biotic and abiotic stresses combined with erratic rains, poor crop management practices, and weak seed supply chain are the major constraints reducing groundnut yields in farmers' field. Foliar diseases, especially rust and LLS are the two major devastating diseases, causing significant yield loss and deteriorating quality of the produce throughout India. On the other hand, groundnut oil quality with essential fatty acids has created great demand for industries and household purposes. MABC is one rapid approach to strengthening existing cultivars for target traits in several crops [37]. Using MABC approach, we have successfully transferred target QTL/genes while retaining the maximum amount of the recurrent genome, allowing control of linkage drag. For this reason, we used MABC to improve FDR and oleic acid in three popular cultivars of Gujarat.

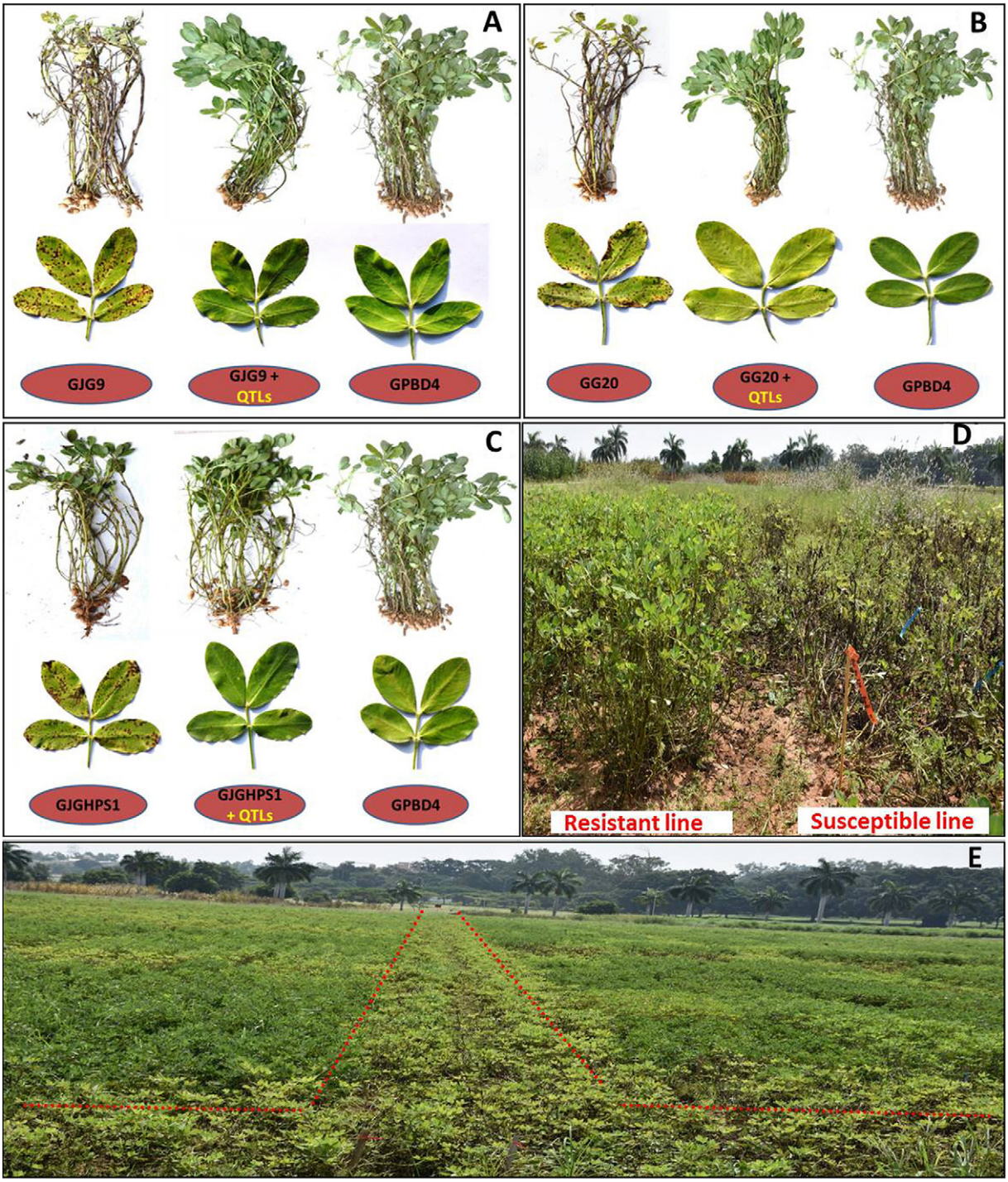

Fig. 2 – Disease-resistance screening of ILs with QTLs for rust and LLS resistance. (A, B, and C) Represent the ILs with the backgrounds of GJG 9, GG 20, and GJGHPS 1, respectively. (D) Represents the resistant and susceptible parent reactions. (E) Field view of disease nursery plot for rust and LLS screening. Red dotted lines indicate the infector row.

The first successful use of MABC for FDR was reported for improving three elite groundnut cultivars viz.: JL 24, TAG 24, and ICGV 91114, for rust resistance [8]. These MABC ILs also had 39%–79% higher mean pod yield and 25%–89% higher mean haulm yield than the recurrent parents [13]. Another report [9] described the improvement of genetic resistance for foliar diseases using MABC approach in one of the most popular and old groundnut variety, TMV 2, using the foliar disease-resistant donor GPBD 4. Selected MABC homozygous backcross lines such as TMG-29 and TMG-46 showed enhanced resistance to foliar fungal disease in addition to yield increase up to 71% over the original recurrent parent, TMV 2.

Table 3 – Best-performing five introgression lines with high oleic acid and high background genome recovery from GJG 9, GG 20 and GJGHPS 1.

Cross details	% of RPG recovered	Oleic acid (%)	Linoleic acid (%)	Palmitic acid (%)	Oil content (%)	Protein (%)
SunOleic 95R (Donor)		82.1	3.5	6.5	49.0	27.0
GJG 9 × SunOleic 95R						
GJG 9 (Recurrent)		37.1	42	12.3	49.0	26.0
BC_3F_4-206/1	90.6	83.2	3.0	8.8	53.8	31.2
BC_3F_4-206/3	90.6	81.7	5.1	9.3	46.5	22.9
BC_3F_4-206/5	90.6	77.6	8.2	9.7	52.6	24.8
BC_3F_4-206/4	90.6	77.2	9.2	5.8	46.1	27.4
BC_3F_4-209	92.3	72.5	11.2	7.02	44.6	25.7
GG 20 × SunOleic 95R						
GG 20 (Recurrent)		46.3	33.0	10.7	51	26.0
BC_3F_4-221	81.2	80.4	2.9	6.5	51.1	29.4
BC_3F_4-223	86.1	73.9	9.8	6.4	55.0	30.1
BC_3F_4-220	83.5	72.9	8.0	8.7	45.1	30.0
BC_3F_4-219	–	66.7	14.5	8.4	50.9	22.1
BC_3F_4-222	–	62.8	19.1	8.5	50.9	26.4
GJGHPS 1 × SunOleic 95R						
GJGHPS 1 (recurrent)		42.0	47.7	13.2	49.0	26.0
BC_3F_4-233	89.5	82.7	3.2	8.7	46.2	24.7
BC_3F_4-197/1	91.5	81.0	3.8	6.1	44.4	27.8
BC_3F_4-197/2	91.5	78.7	6.1	9.2	49.8	26.9
BC_3F_4-199/1	88.5	78.4	7.8	9.8	48.8	27.1
BC_3F_4-232	79.2	75.8	6.9	9.5	44.2	29.3

Several of these lines are in different stages of yield testing and some of the promising and best-performing lines may be released as new varieties for cultivation in India.

Improved oil quality with high oleic acid is an important trait attracting both industry and consumers. The high-oleic acid (~80%) mutant line F435 has been used for improving high oleic acid in peanuts [38]. These mutant lines lack a functional *FAD* gene, thereby preventing desaturation of oleic to linoleic acid and increasing oleic acid content in the oil. GAB approaches including MAS and MABC were used successfully in the development of high oleic cultivars [10–12]. Initially linked markers for mutant *FAD2* alleles were deployed for improving the nematode-resistant variety Tifguard by transferring mutant alleles using MABC, leading

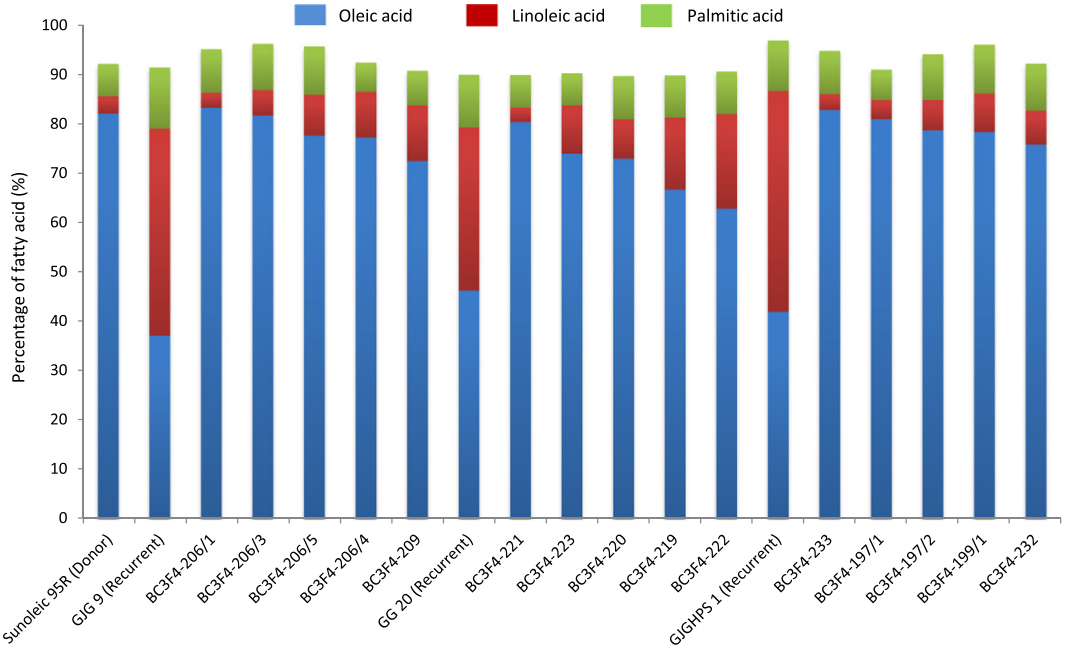

Fig. 3 – High oleic acid introgression lines developed in the background of GJG 9, GG 20, and GJGHPS 1.

Table 4 – Summary of the polymorphic SNPs identified in a genome recovery study for six crosses.

Chromosome	GJG 9 × GPBD 4	GG 20 × GPBD 4	GJGHPS 1 × GPBD 4	GJG 9 × SunOleic 95R	GG 20 × SunOleic 95R	GJGHPS 1 × SunOleic 95R
A01	63	89	110	117	112	128
A02	369	420	413	216	179	180
A03	232	217	223	196	168	182
A04	142	221	204	302	122	178
A05	121	135	163	197	145	125
A06	95	180	173	296	270	307
A07	121	188	189	184	124	125
A08	108	164	168	187	108	102
A09	119	204	204	244	96	90
A10	126	166	174	142	124	122
B01	86	101	110	116	127	137
B02	199	232	259	186	149	129
B03	163	160	175	170	192	179
B04	135	205	206	195	114	142
B05	92	112	133	189	132	130
B06	93	171	171	314	253	267
B07	97	187	175	175	139	122
B08	84	133	152	148	103	115
B09	214	266	274	205	102	114
B10	103	163	157	146	129	144
Total	2762	3714	3833	3925	2888	3018

to the development of the improved breeding line Tifguard High O/L [10]. Later [11] these linked markers were used in MABC and MAS approaches for converting three elite varieties, ICGV 06110, ICGV 06142, and ICGV 06420, into high-oleic lines. These high-oleic lines contained up to 80% oleic and reduced palmitic and linoleic acid, a perfect combination for industry and cooking oil use. Similarly, another recent report [12] described the development of a high-oleic version of the popular variety ICGV 05141, using MAS. Most importantly, these high oleic lines are also demonstrating high yield potential in addition to high oleic acid and many of these lines are in multi-location yield trials in the All India Co-ordinated Research Project on Groundnut (AICRP-G) of Indian Council of Agricultural Research (ICAR), India for testing and release. Two of these molecular breeding lines [11] namely ICGV 15083 (Girnar 4) and ICGV 15090 (Girnar 5) were identified for varietal release and cultivation in India. The Indian market and consumers would like to see more and more high oleic lines for all the major oilseed crops in coming years.

In view of the need to improve foliar fungal disease resistance and high oleic acid in India, the present study improved both traits using MABC approach in three popular Indian varieties: GJG 9, GG 20, and GJGHPS 1. We used first-generation SSR markers along with GMRQ517, GMRQ786, and GMRQ843 for rust and GMLQ975 for LLS. These were developed using the QTL-seq approach [16] and were subjected to genotyping with the 58 K Axiom_*Arachis* SNP array for estimating background genome recovery. Thus, this study represents the precise use of available genomic resources in groundnut breeding to develop lines with FDR and high oleic acid.

Phenotyping of backcross-derived lines identified ILs with rust resistance scores <3 and LLS resistance scores < 4, comparable with those of the resistant donor, GPBD 4. The co-occurrence of rust and LLS resulted in severe defoliation and decrease in chlorophyll area in susceptible plants. We also observed that the second backcross lines showed severe susceptibility especially for LLS despite carrying resistance QTL. This finding may be due to complex genetic control of LLS in addition to the background genome effect or to an allelic effect [39]. As expected, the third backcross lines more similarity with the respective recurrent parents and similar morphological characters but lines with prioritized yield traits especially pod shape, size, etc. were advanced. The selected MABC lines developed through this research work for FDR and high oleic acid are planned for further generation advance, followed by multi-location testing for identification of promising lines for potential release in India.

In high-oleic lines, given that oleic-acid content is a qualitative trait governed by mutant *FAD* alleles, the second and third backcross lines showed high oleic acid levels. ILs having homologous mutations on both subgenomes showed a greater percentage of oleic acid (>75%) due to the dosage of mutant alleles. As the fatty acid pathways are inter-linked, a few ILs with decreased (50%) palmitic acid levels were noticed, as also reported earlier [11,12]. In addition to high oleic acid, ILs showed 1%–3% increase in oil content compared to the parents (Table 3). These ILs with greater oil content will be promising especially for processors' benefits. Earlier [40] it was reported that a 1% increase in seed oil content increased groundnut processing benefit by 7%. These BC_3F_4 and BC_2F_4 ILs with high oleic acid combined with high oil content are currently under multiplication, and promising lines proper yield trials may replace the existing lines.

This study is the first to use the 58 K SNP array for assessing background genome recovery across the chromosomes, whereas earlier studies in groundnut were focused on targeted linkage groups. This whole genome based recovery enhances the selection accuracy of the ILs, avoiding linkage drag. The background genome recovery study helped in identifying several MABC lines with >85% recovery even in

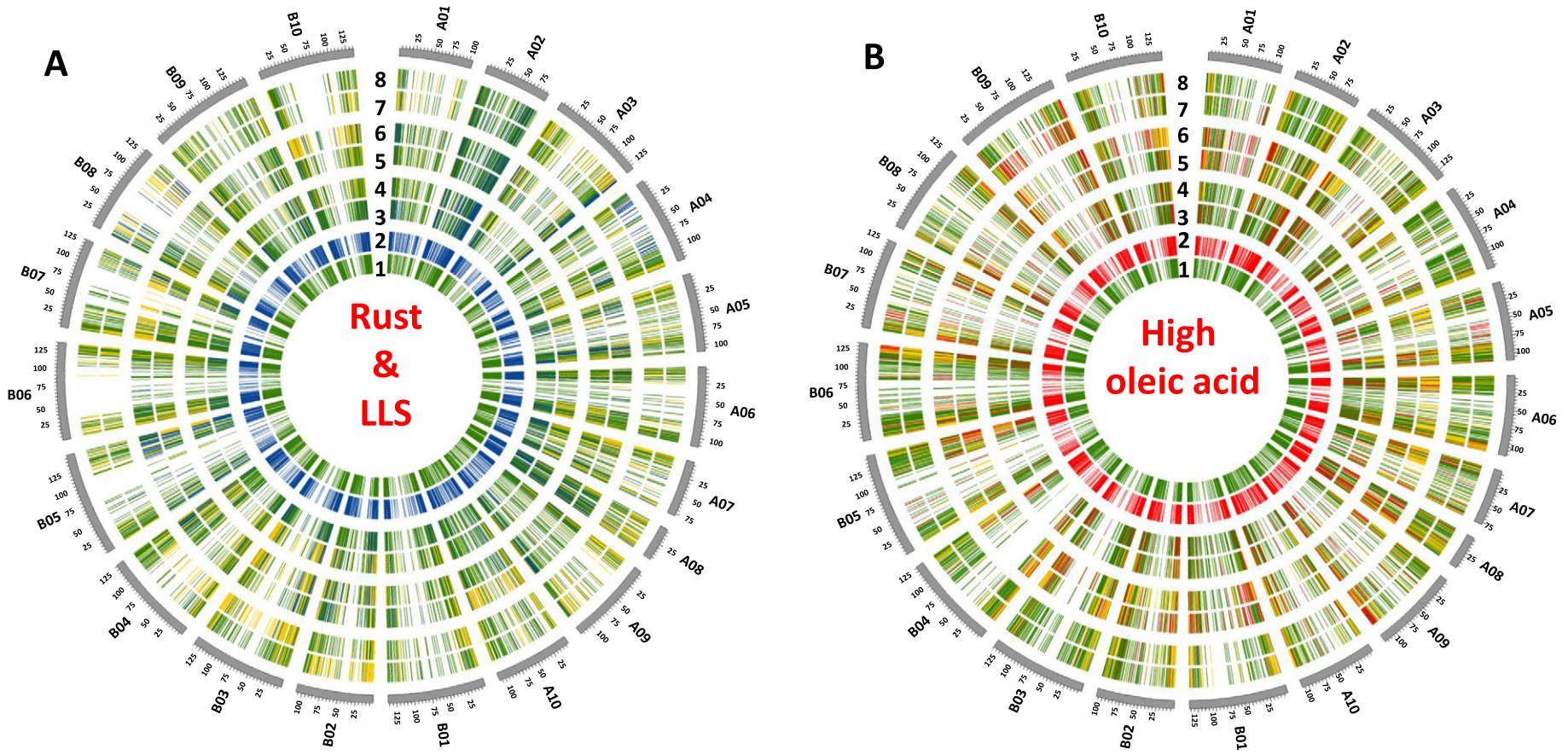

Fig. 4 – Circos plots depicting recurrent-parent genome recovery among selected ILs for resistance to rust and LLS and high oleic acid. In circular vizualization plot (A), the tracks from inside out depict (1) SNPs from recurrent parents (GJG 9, GG 20, and GJGHPS 1) in green, (2) SNPs from donor (GPBD 4) in blue, heterozygous SNPs in yellow, (3, 4) SNPs in ILs from GJG 9, (5, 6) SNPs in ILs from GG 20, and (7, 8) SNPs in ILs from GJGHPS 1. In circular vizualization plot (B), the tracks from inside out depict (1) SNPs from recurrent parents (GJG 9, GG 20 and GJGHPS 1) in green, (2) SNPs from donor (SunOleic 95R) in red, heterozygous SNPs in yellow, (3, 4) SNPs in ILs from GJG 9, (5, 6) SNPs in ILs from GG 20, and (7, 8) SNPs in ILs from GJGHPS 1.

Table 5 – Foliar disease-resistant and high-oleic introgression lines with high recurrent genome recovery based on the 58 K SNP array.

Line ID	% of recurrent parent genome recovered	Line ID	% of recurrent parent genome recovered	Line ID	% of recurrent parent genome recovered
GJG 9 × GPBD 4		GG 20 × GPBD 4		GJGHPS 1 × GPBD 4	
GJG 9_BC_2F_2_10	80.7	GG 20_BC_2F_2_106	81.1	GGJHPS 1_BC_2F_2_141	89.1
GJG 9_BC_2F_2_12	86.0	GG 20_BC_2F_2_90	81.6	GJGHPS 1_BC_2F_2_122	71.1
GJG 9_BC_2F_2_15	87.5	GG 20_BC_2F_2_91	88.5	GJGHPS 1_BC_2F_2_124	77.7
GJG 9_BC_2F_2_5	85.6	GG 20_BC_2F_2_92	87.2	GJGHPS 1_BC_2F_2_129	76.2
GJG 9_BC2F2_6	85.6	GG 20_BC_2F_2_93	87.9	GJGHPS 1_BC_2F_2_130	72.4
GJG 9_BC_2F_2_7	76.2	GG 20_BC_2F_2_94	94.2	GJGHPS 1_BC_2F_2_131	79.0
GJG 9_BC_2F_2_8	77.7	GG 20_BC_2F_2_95	81.0	GJGHPS 1_BC_2F_2_132	79.8
GJG 9_BC_2F_2_9	78.2			GJGHPS 1_BC_2F_2_133	72.1
GJG 9_BC_3F_2_26	86.3	GG 20_BC_3F_2_102	80.1	GGJHPS 1_BC_3F_2_198	90.4
GJG 9_BC_3F_2_39	89.6	GG 20_BC_3F_2_107	70.9	GGJHPS 1_BC_3F_2_204	87.5
GJG 9_BC_3F_2_40	82.9	GG 20_BC_3F_2_112	85.6	GGJHPS 1_BC_3F_2_227	86.5
GJG 9_BC_3F_2_71	84.2	GG 20_BC_3F_2_113	80.5	GGJHPS 1_BC_3F_2_231	86.6
GJG 9_BC_3F_2_75	85.7	GG 20_BC_3F_2_114	79.3	GGJHPS 1_BC_3F_2_232	92.4
GJG 9_BC_3F_2_82	83.3	GG 20_BC_3F_2_118	75.2	GGJHPS 1_BC_3F_2_238	78.5
GJG 9_BC_3F_2_89	86.3	GG 20_BC_3F_2_125	80.8	GGJHPS 1_BC_3F_2_253	85.3
GJG 9_BC_3F_2_99	90.6	GG 20_BC_3F_2_126	75.2	GJGHPS 1_BC_2F_2_134	72.7
GJG 9 × SunOleic 95R		GG 20 × SunOleic 95R		GJGHPS 1 × SunOleic 95R	
GJG 9_BC_2F_2_191	85.2	GG 20_BC_3F_2_536	83.6	GJGHPS 1_BC_2F_2_240	89.6
GJG 9_BC_2F_2_200	79.7	GG 20_BC_3F_2_544	79.9	GJGHPS 1_BC_2F_2_245	86.8
GJG 9_BC_2F_2_201	72.6	GG 20_BC_3F_2_549	80.3	GJGHPS 1_BC_2F_2_246	87.8
GJG 9_BC_3F_2_315	91.4	GG 20_BC_3F_2_580	81.8	GGJHPS 1_BC_3F_2_641	85.9
GJG 9_BC_3F_2_346	90.6	GG 20_BC_3F_2_581	82.7	GGJHPS 1_BC_3F_2_653	88.7
GJG 9_BC_3F_2_356	92.3	GG 20_BC_3F_2_590	81.3	GGJHPS 1_BC_3F_2_657	88.5
GJG 9_BC_3F_2_363	90.4	GG 20_BC_3F_2_591	83.5	GGJHPS 1_BC_3F_2_666	89.9
		GG 20_BC_3F_2_604	86.0	GGJHPS 1_BC_3F_2_670	88.5
				GGJHPS 1_BC_3F_2_675	79.2
				GGJHPS 1_BC_3F_2_676	77.4
				GGJHPS 1_BC_3F_2_687	91.5

second backcross lines, a finding that may help in shortening the future MABC programs by earlier selection and screening. We also observed that polymorphic loci were identified in telomere, in contrast to centromere, regions of all the chromosomes, indicating a high frequency of recombination events in telomeric regions. This high-density SNP array-based background genome recovery screening will be useful for qualitative traits such as high oleic acid. In case of MABC

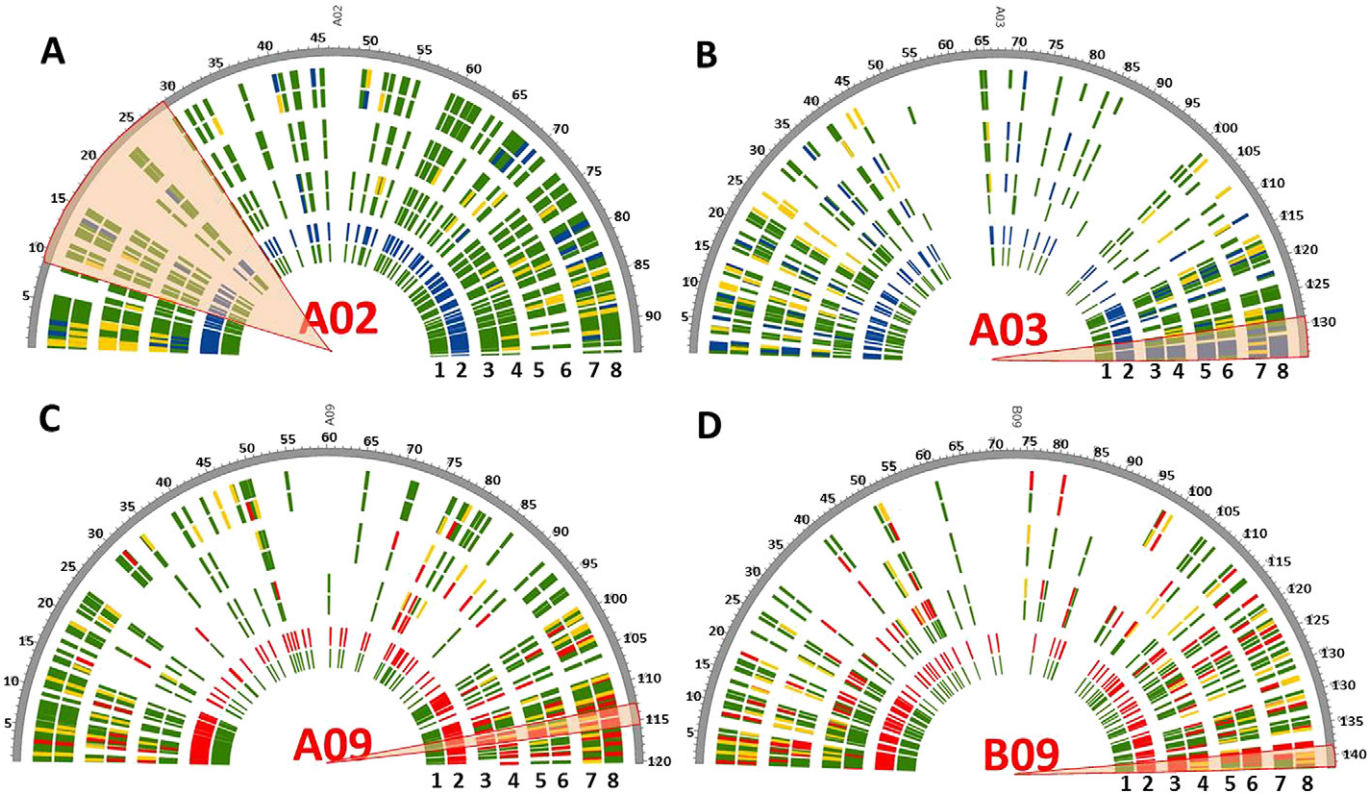

Fig. 5 – Semi-circos plots depicting the recovery of recurrent-parent genomic region on chromosome A02 and A03 for rust and LLS and on chromosome A09 and B09 for high oleic acid. The semi-circular vizualization plots (A) and (B) represents the SNPs from the genomic regions of chromosomes A02 and A03 and the tracks depict (1) SNPs from recurrent parents (GJG 9, GG 20 and GJGHPS 1) in green, (2) SNPs from donor (GPBD 4) in blue, heterozygous SNPs in yellow, (3, 4) SNPs in ILs from GJG 9; (5, 6) SNPs in ILs from GG 20 and (7, 8) SNPs in ILs from GJGHPS 1. Similarly, semi- circular vizualization plots (C) and (D) represent the SNPs from genomic regions of chromosomes A09 and B09 and the tracks depict (1) SNPs from recurrent parents (GJG 9, GG 20 and GJGHPS 1) in green, (2) SNPs from donor (SunOleic 95R) in red, heterozygous SNPs in yellow, (3, 4) SNPs in ILs from GJG 9; (5, 6) SNPs in ILs from GG 20 and (7, 8) SNPs in ILs from GJGHPS 1. QTL/gene regions are highlighted in red and physical positions are in megabases (Mb) on the circumference.

for high oleic acid, we identified a few lines with >85% background genome recovery and with >70% oleic acid in the second backcross.

Linkage drag is a general problem in plant breeding especially for gene or QTL introgression. In the present study, genotyping with 58 K SNP array enabled us to identify the parental origin of SNPs on each chromosome. We also put forward the idea of using foreground markers coupled with high density SNP arrays in current breeding programs to avoid linkage drag and also to recover the maximum recurrent-parent genome. These high-density SNP array genotyping will also help in the identification of recombinant individuals at target genes or QTL in early generations, which is the aim of backcross breeding programs. To our knowledge, this study is the first to use a large number of polymorphic markers including 3925 loci in background genomic screening of ILs not only in legumes but also in major crops. In future, the decrease in cost of genotyping will increase the frequency of the array application with varied SNP density in most crops for early selection or application of array at each stage in selection of line with maximum recovery. These SNP arrays can also enhance the accuracy of molecular breeding approaches such as MABC, gene pyramiding, MAGIC, and NAM. We recommend the new dimension of array- based selection of ILs with the aim of reducing breeding cycles, lowering the cost especially for qualitative traits in legumes and other major crops. Most importantly, high-quality reference genomes for both subspecies of cultivated tetraploid groundnut [41–43] have also become available in 2019 and will add further precision and accuracy to such applications in groundnut genetics and breeding studies.

5. Conclusions

Resistance to foliar fungal diseases and high oleic acid have been identified as key market traits for Indian groundnut varieties. This study improved three popular cultivars: GJG 9, GG 20, and GJGHPS 1 for resistance to foliar diseases and for high oleic acid using MABC. Improved ILs (BC_3F_4 lines) with FDR resistance and high oleic acid in addition to high recurrent-parent genome recovery were identified for further

evaluation and yield trials. Furthermore, inter-crossing between FDR and high-oleic ILs has generated pyramided lines in these backgrounds. These will now be evaluated in target locations to identify promising lines for further testing and release.

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2019.07.001.

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