Characterization of high oleic gene pool and validation of the identified genomic regions controlling oleic acid content in Sunflower (*Helianthus annus* L.)

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Abstract: The increase of oleic acid content has become one of the major goals of plant breeders to improve sunflower oil quality, as high content of oleic acid increases the oil's stability to oxidative degradation at high temperatures and as well has been suggested to reduce cholesterol in blood plasma thereby reduces the risk of coronary heart disease. In this study 120 inbred lines of high oleic gene pool were characterized for yield, its attributing traits, oleic content and then validated with two known microsatellite molecular markers linked to oleic acid content. High phenotypic and genotypic coefficients of variation as well high heritability and high genetic advance as percent of mean was recorded for oleic acid content. This indicated the presence of the additive type of gene action controlling the trait. Further, the two molecular markers under the study exhibited differentiating bands between all the high and low oleic inbred lines. Hence the validated markers from this study, linked to the high oleic acid trait could be further used in marker-assisted selection and would greatly contribute to develop stable high oleic acid breeding lines.

Key words: Oleic acid content, *Ol* gene, Inbred lines, microsatellite molecular markers and Sunflower

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1. Introduction

Oleic acid, a monounsaturated omega-9 fatty acid (18:1 cis-9) is found in many foods, but mainly in olive oil. Even if other mono-unsaturated fatty acids are present in olive and seed oil, oleic acid is receiving great attention worldwide for its beneficial health properties [1]. The FDA has determined the existence of realistic evidence to support a health claim associated to the oleic acid consumption and to the reduction of coronary heart disease risk [2]. For this reason, in the recent decades, plants with higher oleic acid content (up to 70% and more) have been selected, which opened up a new frontier to the possible uses of these crops taking advance of possible beneficial health effects and triggering at the same time the market interest for its wider use [3]. Similarly, in case of sunflower oil with a high oleic acid content in the range between 70 to 90 *per cent* is called as "high oleic content" sunflower oil and presents a fatty acid composition similar to that of olive oil. There were many attempts to carry out the genetic research with this trait in sunflower crop. The most of them were dedicated to a high oleic mutation of variety 'Pervenets', obtained by Soldatov, [4] and this was exclusively used as a donor of the high oleic trait in sunflower breeding programmes worldwide. Several genetic approaches have been developed to study the high oleic mutation and different conclusions are reported on the number of genes that control the trait and on their dominance. Research on genetic control of a high oleic mutation led to the hypotheses of one major gene *Ol* and gene-modifier *ml* [5]. Nevertheless, the genetic control of high oleic acid content is still not well understood, as high oleic acid content was initially identified as monogenic trait produced by dominant alleles *Ol*, but afterwards several modifying genes were identified that affect the *Ol* gene and produce reversal of the expected

dominance. This has complicated the practical management of the trait in breeding programmes. [6]**.** The oleic acid content, to a certain extent, be considered a semi-qualitative trait since OAC is dependent not only on the environment, but also on the genetic background of the receiver line. [7-9]. A partial duplication of the Fatty Acid Desaturase 2-1 (FAD2-1) allele caused by chemical mutation leads to an increase in OAC by silencing the FAD2-1 gene encoding FAD 2 [10]. This enzyme catalyzes the synthesis of linoleic acid from oleic acid and by silencing its activity oleic acid is accumulated. Different markers were employed in mapping and detecting the *Ol* mutation in sunflower, however Premnath et al. [11] identified two QTL's, HO_Fsp_b and ORS762 explaining about 60% of the phenotypic variation in OAC.

With this background, the objective of this study was to characterize the 120 inbred lines of high oleic gene pool for yield, its attributing traits, oleic acid content and as well to validate the reliability of two gene-based microsatellite molecular markers for utilization in marker assisted breeding programme to improve oil quality of sunflower.

2. Material and methods

Phenotypic characterization of high oleic gene pool:

The plant material of this study constituted of 120 inbred lines of R x R (Restorer x Restorer) high oleic gene pool developed at AICRP Sunflower, ZARS, GKVK, Bangalore. The characterization of these 120 inbred lines was carried out for yield, its attributing traits and oleic acid content in alpha lattice design in 12 blocks with 10 inbred lines each in a block with two replications during summer 2021.

Validation of microsatellite markers linked to oleic acid content:

From the study conducted by Premnath et al. [11] the *Ol* gene was mapped to linkage group (LG) 14 and tightly linked to the markers HO_Fsp_b and ORS 762. These two reported linked markers from this study were used to ascertain oleic acid content and demonstrate the utility of gene-based microsatellite molecular markers for accurate identification of high oleic lines. Hence the reliability of these identified markers was carried with 17 selected high oleic inbred lines (>70 %) and 13 low oleic inbred lines (<40 %) to find out whether the identified markers could discriminate the low and high oleic acid content of the inbred lines under study. The details of the inbred lines used for validation, along with respective oleic acid content is presented in Table 1 while the details of the microsatellite molecular markers are presented in Table 2.

Estimation of fatty acid composition

The validation was carried out using 30 samples with known oleic acid content as estimated using standard gas chromatography (GC) available at Indian Institute of Oilseed Research, Hyderabad.

Genomic DNA isolation

DNA was extracted from 15–20-day-old fresh fully expanded leaves of the 30 inbred lines using the modified cetyl trimethyl ammonium bromide (CTAB) extraction method as described in Doyle [12]. The DNA quality and quantity were checked on 0.8 % agarose gel and DNA concentration was normalized to 10 ng/L.

3. Results and discussion

The knowledge of genetic variability helps the breeder to improve the suitable breeding strategy, therefore it is necessary to know genetic variability, heritability and genetic advance as *per cent* in the available genetic material. Genetic variability together with heritability estimates would give a better idea on the amount of genetic gain expected out of selection [13-14]. Through this study an attempt was made to assess the mean performance and extent of variability in the 120 inbred lines of high oleic gene pool, for nine quantitative traits. The estimates of range, mean, phenotypic coefficients of variation (PCV), genotypic coefficient of variation (GCV), heritability (h^2 broad sense) and genetic advance as *per cent* of mean (GAM) are presented in Table 3.

The analysis of variance revealed significant differences among the entries for all the characters studied indicating the existence of a high degree of variability in the material. Genetic parameter studies showed that the magnitude of difference between PCV and GCV was relatively

low for all the traits and the magnitude of PCV was little bit higher than GCV for all the traits, which revealed less influence of environment on the expression of these traits.

The oleic acid content recorded a wide range of variation from 36 *per cent* (L-12-1) to 86.23 *per cent* (K-10) with a mean value of 56.20 *per cent*. The estimates of PCV (21.89) and GCV (21.76) were high, indicating wider variability. High heritability (98.85) accompanied by high genetic advance as *per cent* of mean (44.58) was observed for this trait indicating the role of additive gene action and also that the inheritance of this trait was less influenced by environmental effects and therefore selection of the inbred lines under the study, for oleic acid content would be effective. These findings were in line with the results obtained several other researchers [15-18].

Wider variability was observed in case of plant height, head diameter, seed yield plant^{-1} which indicated its amenability towards directional selection. stem girth and 100 seed weight recorded moderate variability whereas days to 50 *per cent* flowering, volume weight and oil content showed low variability. Plant height, head diameter, stem girth, seed yield plant^{-1}, 100 seed weight and oleic acid content showed high heritability coupled with high genetic advance of mean, suggesting the role of additive gene action in the inheritance of these traits and hence, improvement for these characters could be achieved by mass selection and progeny selection methods. The high heritability observed for most of the traits, which might not be realistic as the experiment was conducted in one location with two replications. More realistic estimates could be obtained by testing genotypes in a multi-environment trial. However, variability together with heritability and genetic advance gave some indication of the nature of gene action governing the traits.

Further from the same gene pool, 17 high oleic inbred lines (>70 %) and 13 low oleic inbred lines (<40 %) were selected and screened with previously reported microsatellite molecular markers to find out whether the identified markers could discriminate the low and high oleic acid content among these inbred lines. The markers under the study were basically derived from the study conducted by Premnath et al. [11]. They had developed an F_2 mapping population of sunflower and phenotyped for oleic acid content. The *Ol* gene was mapped to linkage group (LG) 14 and tightly linked to the marker HO_Fsp_b. In addition, two more quantitative trait loci (QTLs) for oleic acid content were identified in LG8 and LG9. Further the study was conducted with 13 genotypes differing in oil quality as well as quantity over three seasons to assess the reliability of the identified QTLs over seasons. It resulted in the identification of two potential QTLs for oleic acid content with the markers ORS 762 and HO_Fsp_b. These markers explained more than 57.6–66.6 *per cent* of phenotypic variation in their study. With this background to further validate the genomic regions controlling the oleic acid content the current study was envisaged utilizing the high oleic restorer gene pool.

The results from the current study revealed that both the markers exhibited differentiating bands between high and low oleic lines. The high oleic containing individual lines showed a specific band at about 890 bp length which was absent in low oleic lines (Fig. 1) for the primer HO_Fsp_b. Similarly, the high oleic containing individual lines showed a specific band at about 750 bp length which was absent in low oleic lines (Fig. 2) for the primer ORS 762. It was evident from the result that both the markers exhibited the expected amplicon size as per the study conducted by Premnath et al. [11] and successfully differentiated all the high oleic and low oleic lines.

4. Conclusion

The oleic acid content is highly influenced by environmental factors such as the temperature and the amount of moisture in the soil. In addition, high oleic genes show unstable expression for oleic acid content in different genetic backgrounds and therefore phenotypic selection for the high oleic acid trait could be difficult across different environments and seasons. The standard method to determine oleic acid content is Gas Chromatography (GC) which produces accurate result but is expensive, time consuming and involves hazardous chemicals. The DNA markers are not influenced by the environment and therefore selection based on molecular markers linked to the high oleic acid trait will allow further advance in breeding for this character. Hence the validated molecular markers from this study, linked to the high oleic acid trait could be further used in marker-assisted selection and would greatly contribute to develop stable high oleic acid breeding lines.

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Table 1. List of selected high and low oleic lines for validation of molecular markers for oleic acid

Sl. No	Code	Inbred Lines	Oleic acid content (%)
1	HO-1	K-10	87.50
2	HO-2	F-20	84.40
3	HO-3	L-3-1	83.20
4	HO-4	K-11	81.60
5	HO-5	G-5	81.05
6	HO-6	G-17-1	80.26
7	HO-7	L-17	78.85
8	HO-8	B-29-2	78.28
9	HO-9	G-12	76.75
10	HO-10	D-33-2	76.40
11	HO-11	N-16	75.85
12	HO-12	D-11	72.36
13	HO-13	C-30	74.10
14	HO-14	L-1-1	70.78
15	HO-15	A-16	70.75
16	HO-16	M-25	70.25
17	HO-17	M-19-1	70.50
18	LO-1	A-6	40.00
19	LO-2	B-22	39.50
20	LO-3	C-31	39.00
21	LO-4	F-6-2	37.50
22	LO-5	G-13-2	38.00
23	LO-6	K-6	38.50
24	LO-7	G-36-1	37.50
25	LO-8	I-18	39.50
26	LO-9	L-23	39.00
27	LO-10	M-21-1	40.00
28	LO-11	N-17	39.00
29	LO-12	RHA-95-C-1	36.00
30	LO-13	RHA-6D-1	38.00

*LO- Low Oleic Lines HO- High Oleic Lines

Table 2. The list of microsatellite molecular markers, primer sequence and expected PCR amplicon

Primer Name	Primer Sequence	Gene	Expected Amplicon Size
ORS 762	Forward- 5'-TGCACATGAGGGTATTCTTGTC-3' Reverse- 5'- TCGAGGAGAGTGTGACGTTG-3'	*Ol*	750 bp
Ho_Fsp_b	Forward- 5'- GCACCATGAGGGCTGTTATTGT-3' Reverse- 5'- TGCATGGAAGTGGAGTCTAT-3'	*Ol*	890 bp

Table 3. Mean performance and genetic variability of 120 sunflower inbred lines for yield, its attributing traits and oleic acid content

Character	Mean	Standard Error of Mean	Range		Coefficient of variation (%)		Heritability (%)	Genetic Advance as Mean (%)
			Low	High	Genotypic Coefficient of Variation	Phenotypic Coefficient of Variation		
Days to 50 % flowering	57.40	0.251	52.00	68.00	5.39	5.42	98.70	11.03
Plant height (cm)	117.88	0.812	92.50	144.50	11.38	11.43	99.27	23.37
Head diameter (cm)	10.95	0.577	5.00	15.60	21.66	22.02	96.76	43.90
Stem girth (cm)	1.78	0.048	1.30	2.37	11.01	11.66	89.07	21.41
Seed yield plant^{-1} (g)	11.89	0.308	6.00	19.10	20.45	22.04	86.07	39.10
Volume weight (g/100ml)	37.78	0.517	31.00	44.30	7.68	7.92	94.02	15.34
100 seed weight (g)	3.83	0.155	2.19	5.20	16.54	17.51	89.27	32.20
Oil content (%)	34.19	0.451	28.31	40.48	6.47	6.73	92.39	12.82
Oleic acid content (%)	58.33	0.968	36.00	86.23	21.76	21.89	98.85	44.58

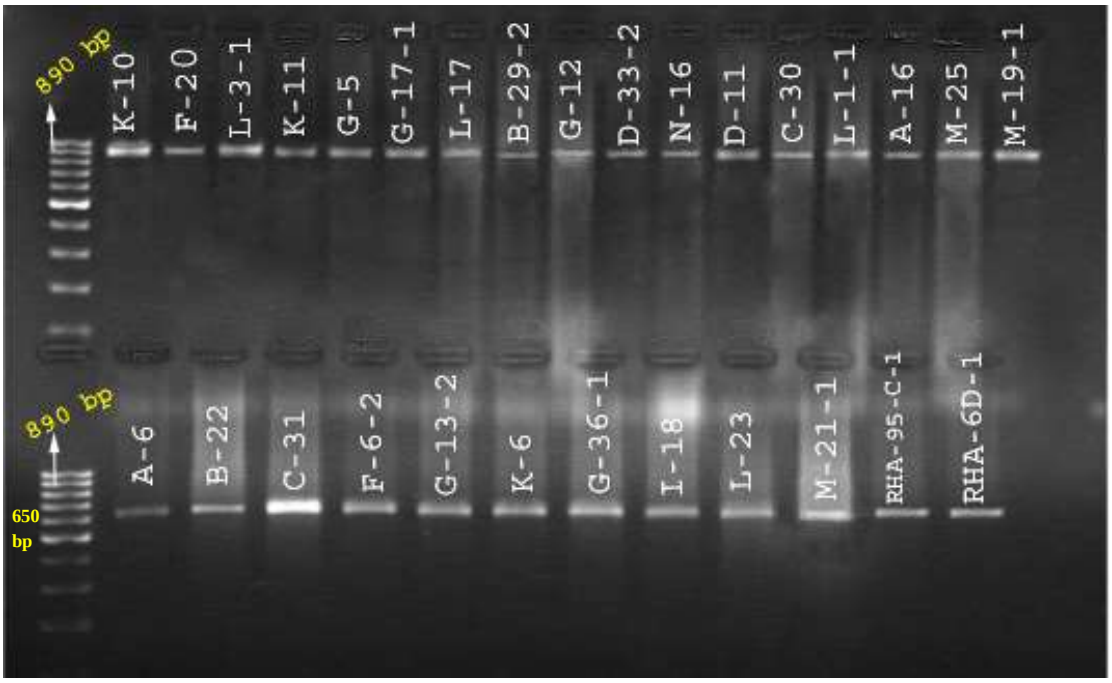

Fig 1. PCR amplification of High oleic and Low oleic lines for HO_Fsp_b marker

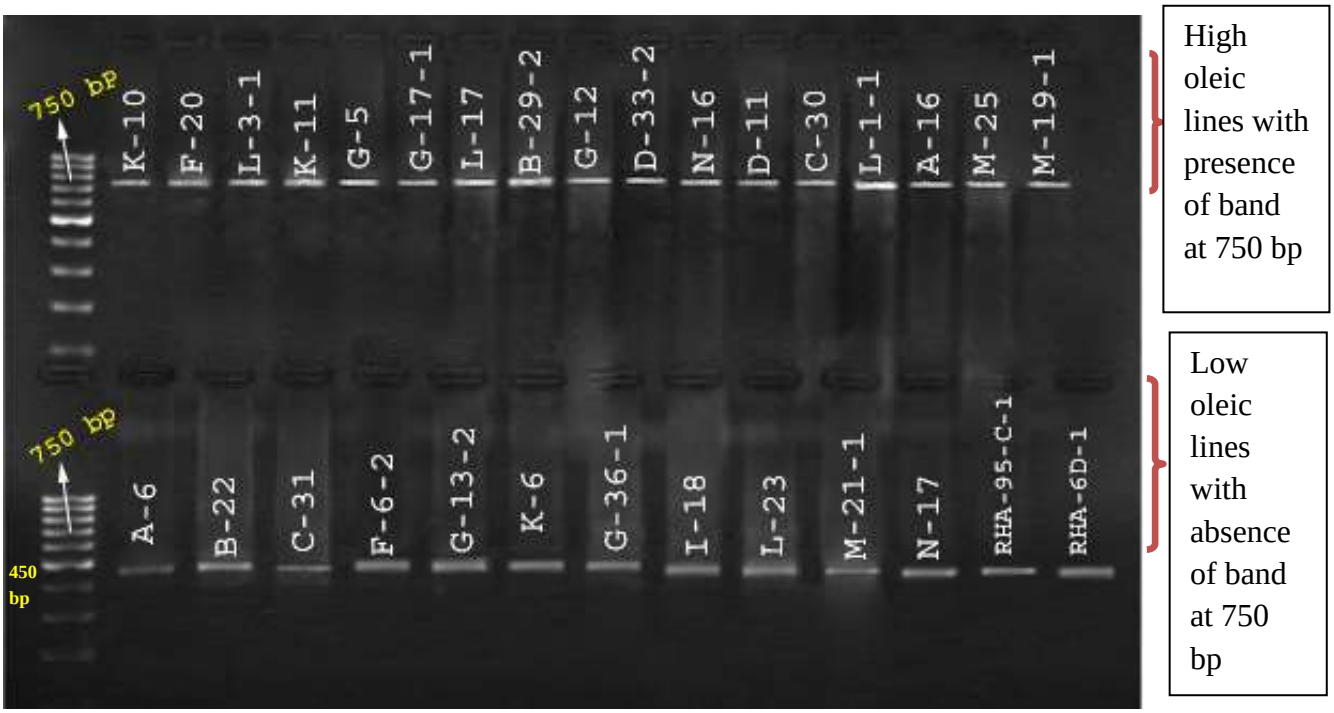

Fig 2. PCR amplification of High oleic and Low oleic lines for ORS 762 marker