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Evaluation of SSR and INDEL markers associated with high and low oleic acid content in sunflower (*Helianthus annuus* L.) genotypes

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Abstract
Sunflower is one of the most important oilseed crops due to high oxidative stability of its oil with high oleic acid content. The fatty acid composition of sunflower oil is: palmitic acid (SFA): 5-8 per cent, stearic acid (SFA) 4-6 per cent, oleic acid (MUFA) omega-9 (18:1): 25-30 per cent, lenoleic acid (PUFA) omega-6 (18:2): 60-72 per cent. The mutation was associated with Oleoyl-phosphatidylcholine desaturase (*ODS*) duplications that led to silencing of the *ODS* gene. As a result of silencing of the *ODS* gene resulted in accumulation of high oleic acid in seed. In this study two parents i.e 103A (female), 64NB (Male) and RSFH-1 (Hybrid) were evaluated during 2015-16 at Main agricultural research station, UAS, Raichur. For the purpose of genotyping the sunflower lines for high oleic acid content. We have used molecular markers that is associated with high oleic acid trait, i.e SSR and INDEL HO PCR specific fragment marker were chosen, isolated DNA, carried out PCR separately using five SSR and 6 INDEL markers and sequencing of all three genotypes. The results showed that high oleic containing parent and hybrid expressed a specific SSR band at 807bp for (103A) and 749 bp (RSFH-1) and in case of INDELs, PCR specific fragments was obtained at 618bp for (103A), 766bp for (RSFH-1), 1500bp for (103A) and another 1500bp length for (RSFH-1). The results of this work allowed for validation of one SSR (N1-3F/ N1-3R) out of five and two INDEL (F4/ R1 and F4/ R2) DNA markers out of six in sunflower genotypes for high oleic acid trait identification.

Keywords: *Helianthus annuus* L., Oleic acid, SSR, INDEL, Desaturation, *FAD2-1*.

Introduction
Sunflower is one of the most important oil crops in the world. It is produced in the world generally for human and non-food purposes (cosmetics, paints, etc.) due to the oil and fatty acid composition of the seed being adapted to these uses. It holds second position in world in edible oil manufacturing followed by soybean oil and found to be rich in minerals like magnesium, iron, copper, calcium, zinc, sodium, potassium, phosphorus, selenium and manganese and grouped among prominent plant oils for human diet due to its nutritional values (Skoric *et al.* 2008). It is nutritionally important because the proportion of oleic acid and linoleic acid content which determine the proportion of polyunsaturated fatty acid. There is genetic variation for the fatty acid composition in sunflower oil (Cumminis *et al.* 1967) [5]. Sunflower oil contains high level of unsaturated fatty acids (88 %); linoleic acid (48-74%), oleic acid (14-40 %) and also saturated fatty acids; palmitic acid (4-9 %) and stearic acid (1-7 %) (Nagarathna *et al.* 2011) [10]. In order to reach this aim, Sunflower lines and hybrids which have high oleic acid content in their seeds have been obtained by selection programs from HO (High oleic) Pervenet mutant by chemical mutagenesis (Soldatov 1976). The mean content of oleic acid of the seeds from Pervenet population is higher than 65% whereas this content in normal LO varieties is about 20% (Berville *et al.* 2009) [4]. Because of the interest in oleic acid and also the agronomic performance of HO varieties carrying the Pervenets mutation compared with the LO varieties, these varieties are widely used in the world. High oleic acid (MUFA) sunflower is usually defined as the oil having more than 60 per cent of oleic acid (Pecureanu-Joita *et al* 2005) [11]. In high oleic sunflower, *ol* gene is chemically induced. Oleic acid is correlated with reduced expression of seed-specific oleoyl-phosphatidyl choline desaturase (*FAD 2-1*) in developing seed of sunflower (Zhou *et al.* 2013) [16]. The use of molecular markers has become most power full tool for the genetic and breeding studies and it is rapid, cheaper and simple when suitable markers were developed (Varshney *et al.* 2005) [14]. The aims of this study is to characterize sunflower genotypes with high oleic acid content by DNA markers and evaluate the effectivity of marker types developed by (Berville *et al.* 2009) [4] and insilico analysis of *FAD 2-1* gene in sunflower using SSRs and INDEL markers. The conservation and divergence of the sequences from the selected lines and identifing the

homoloues and evolutionary relationship in genome, SSR and INDEL marker specific sequence.

Materials and Methods

Plant materials

The materials for the study comprised of CMS line (103A) high oleic acid, restorer line (64 NB) low oleic acid and hybrid (RSFH-1/ high oleic acid) obtained from Head, AICRP on sunflower, Main Agricultural Research Station, Raichur, Karnataka. The experiment was laid out at Main Agricultural Research Station, Raichur. Parents were sown during summer 2014-15 (Date of sowing (DOS): 29-1-15) and derived cross (RSFH-1) was sown during *kharif* 2015-16 (Date of sowing (DOS): 3-10-15). The Randomized Block Design (RBD) was followed with two replications with a plot size of 3 m x 1.2 m (two rows of three meter length) and each treatment having a row length of 3 m length with inter row spacing of 60 x 30 cm. One week staggered planting was done to CMS and restorer line to synchronize flowering and pollination. Both female and male parent were covered with cloth bags before opening of the flowers to avoid cross pollination through honey bees and wind. The crossing was effected artificially by collecting pollen separately in plastic bags and hand pollination was practiced till all the florets are fertilized. CMS line and restorer line were crossed to get seeds. Good seed setting was observed and after field maturity of the crop, all the heads were harvested separately, seeds were cleaned, dried, packed separately and stored for further studies. Observations were recorded for all morphological traits at appropriate stage.

Oil content (%): Properly cleaned twelve grams seeds of each entry were oven dried at 70ºc for three hours determined for their oil content with the help of NMR (Nuclear magnetic resonance) spectrometer available at MARS, Raichur, Karnataka. This instrument gives the oil content in terms of percentage.

Fatty acid estimation: The seed samples of each entry were analyzed for their fatty acid profile with the help of Gas chromatography available at Indian Institute of Oil seed Research, Hyderabad. The sunflower fatty acid profile includes estimation of various proportion of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) expressed in terms of percentages. The oleic acid proportion in sunflower genotypes varies from 30 to 90 per cent and generally the genotypes have been classified into three classes *viz.,* low oleic (10-29%), Mid oleic (30-59%) and High oleic (60-90%) (Pecureanu-Joita *et al* 2005) [11].

Gas liquid chromatography: Oil from seed was extracted in hexane on soxhlet apparatus (Extraction unit, E-816, Buchi). Methyl esters were obtained by a two-step catalytic process according to the slightly modified method of (Ghadge *et al.* 2005) [9]. Oil (100-150 mg) was treated with two per cent sulphuric acid in methanol (5 ml) for 2 hr at 60 ˚C. After the reaction, the mixture was allowed to settle for an hour and methanol-water mixture that separated at the top was removed. The second step product at the bottom was transesterified using two ml of 13 per cent methanolic KOH for 30 minutes at 55 ˚C. The organic phase was extracted with hexane and washed with water till it reaches to neutral pH. The hexane was dried over anhydrous sodium sulphate and concentrated with nitrogen to get methyl esters. Fatty acid composition was determined using an Agilent 7860A gas chromatograph (GC) equipped with a flame ionization detector (FID) and an auto sampler. Peak separation was performed on a DB-225 capillary column (Diameter-250 µm, Length-30 m, film thickness-0.25 µm) from Agilent Technology. The carrier gas was nitrogen set to a constant gas flow of 1.2 ml/min at 160 ˚C initial temperature. Sample of 0.2 µl was injected at a 20:1 split ratio into the column with the following temperature conditions: 160 ˚C for 2 min; raised from 150 to 220 ˚C at 6 ˚C /min. Both inlet and detector were set to 230 ˚C. Fatty acid composition was determined by identifying and calculating relative peak areas percent by GC post run analysis EZChrom elite compact software.

DNA isolation, PCR amplification, Electrophoresis

The procedure for DNA extraction, PCR amplification and electrophoresis. In brief, DNA was extracted from leaves by CTAB method (Doyle and Doyle, 1990) [6]. FAD2-1 SSR and insertion–deletion (INDEL) markers were screened for polymorphisms among three confectionery genotypes (103A, RSFH-1 and R-64NB). Touchdown PCR was used to reduce non-specific amplification. Temperature cycling for EST-SSR markers and Indel markers was carried using Eppendorf thermal cycler gradient version 2.30-31-09. Briefly, for For FAD2-1 SSR markers (N1-3F, ORS-311, ORS-339, ORS-371 and ORS-488). PCR amplification was carried out using 20 µl reaction mixture containing 1 × PCR: 94 °C for 4 min, 1cycle of 94°C for 50 s, 58 °C for 50 s, 72°C for 1 min. There were 35 cycles. However, the final extension was 2 min at 72 °C. Buffer, 0.2 µM dNTP's, 0.5 pmol of each primer (forward & reverse) and 0.2-0.5. LD-PCR analyses for FAD2-1 insertion–deletion (INDEL) markers in pairs (F3/F4, F3/F6, F4/R1, F4/R2, F5/R1, F5/R2) the initial denaturation step was performed at 94 °C for 4 min, followed by 11 cycles of 94°C for 20 s, 60 °C for 45 s, and 68 °C for 3.5–6.5 min. The extension time was increased by 10 s in each of the remaining 24 cycles with a final extension at 68 °C for 20 min. Amplified PCR products were controlled by 2 % agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by gel imaging system Vilber Lourmat Quantum.

Results and Discussion

Sunflower (*Helianthus annuus* L.) has become an important oil seed crop in the world and it is a potential source of healthy oil and protein. High oleic acid (MUFA) sunflower is usually defined as having a minimum of 60 per cent oleic acid and maximum of 80-90 per cent. The oil has a very neutral taste and provides excellent stability without hydrogenation. An intake of omega 6, omega 3 and omega 9 (Oleic acid) in the ratio of 5 to 10 has been recommended by world health organization (WHO/FAO. 2003) [15], they help in diminishing the cholesterol leading to reduction in heart diseases. Since there is lot variability for oleic acid proportion in genotypes from 30 per cent to 90 per cent and knowing its nutritional importance in the present scenario of human health. However some of the mutant lines in the proportion of oleic acid content varies with the environment condition. Development of high oleic acid hybrids need to be given prime importance in sunflower heterosis breeding programme. The importance of *FAD2* in modifying the composition of seed oils draws attention of many investigators to manipulate *FAD2* gene expression. Most of the studies conducted to reduce the expression of *FAD2* gene has showed increasing the oleic acid content, in sunflower high oleic mutant *Ol*, silencing of *FAD2* in rapeseed, and developing and introducing sense

suppression DNA construct into cotton to reduce the activity of *FAD2*. Molecular markers can be designed based on a homologous region and highly conserved gene family. *FAD-2* gene family is required for synthesis of PUFA in plants. Soybean, flax, sunflower, safflower and canola express at least one additional *FAD2* gene(s), which is tightly regulated during seed development. Hence, *Arabidopsis* has single copy of *FAD2* (Fatemeh *et al.* 2014) [8]. SSR marker specific sequence analysis of parents (103A, 64NB) and its cross (RSFH-1) were studied, the female parent of 807 bp, hybrid of 749 bp and male parent of 495 bp sequences obtained many hits in the *Helianthus annuus* L. cultivar RHA 345 with 99 per cent query coverage. Inheritance pattern of the gene responsible for the expression of high oleic acid (*Ol*) as observed with SSR marker (N1-3F) confirmed that DNA fragment amplified by these markers inherited at least one allele from the high oleic acid female parent (103A). An investigation was envisaged to evaluate parents and hybrid with molecular diversity and with a view to identify best high oleic acid parents and hybrid. Results obtained from the present investigation are furnished under following headings.

Oleic acid content, SSR, INDEL marker analysis

In this study among three genotypes, CMS line 103A and Hybrid RSFH-1 (Figure 1) exhibited high oleic acid content of 79.30 % and 78.64 % respectively and restorer line R-64NB exhibited low oleic acid content of 26.47 %. The pervenet mutation was labeled by the polymorphism of the SSR and INDEL locus located on delta12- desaturase gene (Berville *et al.* 2009) [4]. High oleic parents and hybrid were characterized at molecular level using SSR and INDEL markers. According to DNA fragment analysis for SSR (N1-3F/ N1-3R) high oleic containing Parent (103A) and hybrid (RSFH-1) expressed a specific SSR band at 807 bp and 749 bp length respectively (Figure 2). Parental lines 103A (High oleic), 64NB (Low oleic) and derived cross RSFH-1(High oleic) were analyzed for polymorphism using gene specific six INDEL markers. Out of six INDEL markers screened, two markers (F4/R1, F4/R2) revealed a dominant polymorphism between high and low oleic acid genotypes and were linked in coupling phase to the presence of the *Ol* allele determining high oleic acid content (Figure 3 and 4).

Polymorphic bands was obtained by primer F4/R1, were as in female amplification was at 618 base pair, in hybrid band obtained at 706 base pair and in case of male band was expressed at 300 base pair, which is low oleic. Primer F4/R2 produced amplicons of 1500 and 800 base pair in female and hybrid but it was not amplified in male parent, which is low oleic. Polymorphic bands were obtained in female at 670 base pair and hybrid at 766 base pair and 715 base pair in case of male using F5/R1 primer. Primer F5/R2 expressed 990 base pair in both female and male, 764 base pair in case of hybrid. However, monomorphic DNA amplification was observed with markers F3/R4 and F3/R6 for both high and low oleic acid genotypes (Figure 3) The primer F3/R4 amplified the product of 600 base pair for parents and hybrid, similarly primer F3/R6 also expressed monomorphic bands at 700 base pair in parents and hybrid. These two markers were not polymorphic and could not differentiate between high and low oleic acid genotypes shown in (Figure 4)

Conclusions

In order to assess the polymorphism between high and low oleic acid among 14 parental lines, 49 crosses and check hybrid RSFH-1, five SSR primers *viz.,* N1-3F, ORS-311, ORS-371, ORS-339 and ORS-448 were used, among five SSR primers, only one primer N1-3F showed polymorphism between high and low oleic acid genotypes. High oleic parents and hybrid were characterized at molecular level using SSR and INDEL markers. According to DNA fragment analysis for SSR (N1-3F/ N1-3R) high oleic containing Parent (103A) and hybrid (RSFH-1) expressed a specific SSR band at 807 bp and 749 bp length respectively. The amplicons obtained for high oleic was at 749 bp length presented in Table 10, this indicates the presence of high oleic acid. The high oleic specific band at 749 bp was absent in 31 mid oleic and 11 low oleic acid crosses. Both the parental species and derived crosses were analyzed for polymorphism using five SSR markers, (Berville *et al.* 2009) [4] reported similar results by using the primer N1-3F to derive useful information about polymorphism, genetic relatedness and diversity.

Similar work was carried out by (Ebrahimi *et al.* 2008) [7] by studying an sunflower F_2 mapping population comprising 115 F_2 individuals developed from a cross between R285 (high oleic acid) and R023 (low oleic acid) inbred lines. Three hundred eighty six SSR markers which have been previously mapped in sunflower were used (275 HA and 111 ORS series). Eighty two polymorphic SSR markers (21.24 %) between both parental inbred lines were identified. similar studies was carried out by (Atitaya Singchai *et al.* 2013) [2] using two inbreds in sunflower, 5A and PI 649855 representing low and high oleic acid content respectively for identifying oleic acid content trait by using 37 SSR markers and reported that 10 SSR primers showed polymorphism between high and low oleic acid lines, similarly (Ebrahimi *et al.* 2008) [7] used SSR markers to study high oleic character in cultivated sunflower. Furthermore, AFLP (Amplified Fragment Length Polymorphism) and RFLP (Restriction Fragment Length Polymorphism) mapping in this species were used. Different QTLs for oleic acid (OA) and stearic acid (SA) content were detected by (Pérez- Vich *et al.*2002) [12]. In order to assess the polymorphism between high and low oleic acid among parents and hybrid RSFH-1, five SSR primers *viz.,* N1-3F, ORS-311, ORS-371, ORS-339 and ORS-448 were used, among five SSR primers, only one primer N1-3F showed polymorphism between high and low oleic acid genotypes. CMS line 103 A and RSFH-1 found to be high oleic acid which has exhibited band at 749 bp, this indicates the presence of high oleic acid and restorer line R-64NB exhibited low oleic. The high oleic specific band at 749 bp was absent in restorer line R-64NB. Both the parental species and hybrid were analyzed for polymorphism using five SSR markers, (Berville *et al.* 2009) [4] reported similar results by using the primer N1-3F to derive useful information about polymorphism, genetic relatedness and diversity.

INDEL marker specific sequence analysis of parents (103A, 64NB) and its cross (RSFH-1) were studied, using six INDEL markers for high oleic acid content. INDEL based six primers using 618 bp (103A) blast search was performed with the query sequence and it formed two clusters in that one of them is U-box like protein family group another cluster is tyrosine aminotransferase gene. Using 766 bp (RSFH-1) blast search was performed it does not formed any clusters because of less conservation of sequences. Using 670 bp (103A), 706 bp (RSFH-1) and 466 bp (103A) blast search was performed, phylogenetic analysis formed a two clusters in that one of them is RHA-345 family group another cluster is *FAD-2-1* gene. The inheritance pattern of the gene responsible for the expression of high oleic acid (*Ol*) as observed with dominant INDEL markers (F3/R4, F3/R6, F4/R1, F4/R2, F5/R1 and

F5/R2) confirmed that each of the DNA fragments amplified by these markers inherited at least one allele from the high oleic acid female parent (103A).

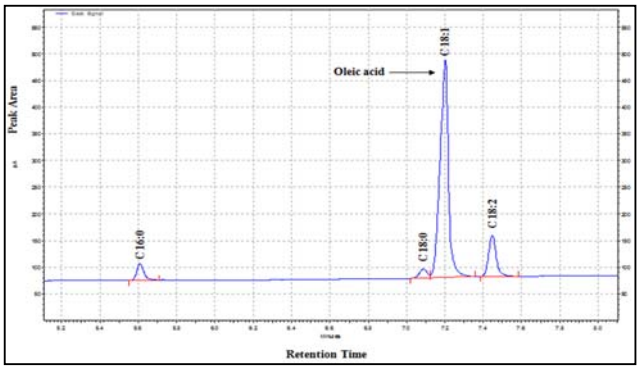

Fig 1: The chromatogram depicting the fatty acid methyl esters with their respective peak areas and retention time of high oleic hybrid RSFH-1, (C18:1 = 78.64%)

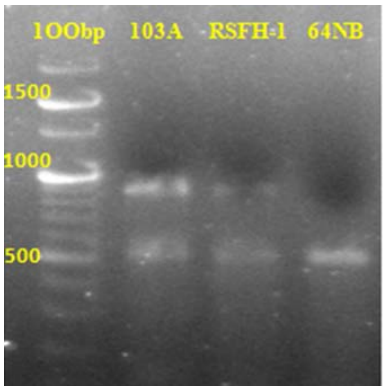

Fig 2: PCR amplicon patterns for high oleic (103A and RSFH-1) and low oleic (64NB) genotypes, generated by marker N1-3F/ N1-3R

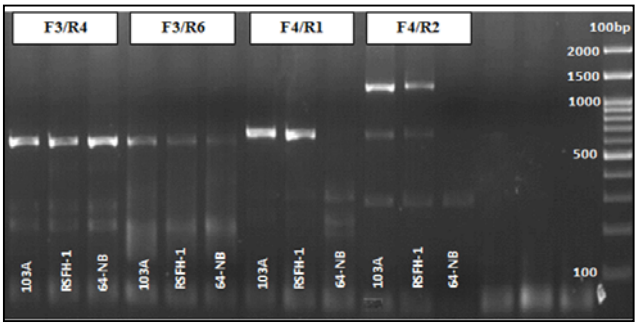

Fig 3: PCR amplification of parents (103A, 64-NB) and hybrid (RSFH-1) generated by INDEL markers

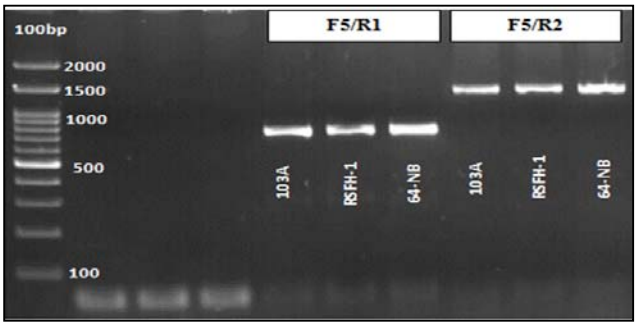

Fig 4: PCR amplification of parents (103A, 64-NB) and hybrid (RSFH-1) generated by INDEL markers

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