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Marker-assisted selection for fast-track breeding of high oleic lines in safflower (*Carthamus tinctorious* L.)

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ABSTRACT

Safflower is grown primarily for edible oil rich in unsaturated fatty acids. Due to higher stability and longer shelf life, the oil with high oleic acid content is preferred for food and other industrial applications. Safflower cultivars with high oleic acid content (>70%) have been developed and commercially successful. Incorporation of the high oleic trait through conventional breeding techniques has been a slow process due to recessive inheritance and difficulties associated with phenotyping by biochemical methods. DNA based marker-assisted selection (MAS) for high oleic trait would help speed up the breeding efforts in safflower. In this study, a set of high oleic varieties were found to carry the same mutation in the *fatty acid desaturase 2-1* gene, *CtFAD2-1*, which is presumed to be the '*ol*' allele associated with high oleic acid content in safflower. Genotypic assays namely Kompetitive Allele Specific PCR (KASP®) and the Amplifluor™ SNPs Genotyping System (Amplifluor®) were designed for the prediction of high oleic trait based on the mutation in the *CtFAD2-1*. The assays were thoroughly validated in segregating populations derived from crosses between low and high oleic parents. Through marker-assisted backcrossing scheme, the high oleic allele, '*ol*' from the exotic variety, Montola-2000 was incorporated into the background of popular Indian linoleic type variety, Bhima and a set of promising high oleic lines (75.2%–81.8%) were developed. The MAS-derived lines showed consistent expression of high oleic acid content over seasons and comparable seed/oil yield performance with the local check varieties. The genotypic assays reported in this study were robust, non-destructive, co-dominant and accurately predicted high oleic trait in segregating populations; thus, recommended for fast-track breeding of high oleic cultivars in safflower.

1. Introduction

Safflower (*Carthamus tinctorious* L.) is a globally important edible oil seed crop adapted to semi-arid environments. Traditional safflower oil is predominantly composed of four fatty acids namely C16:0 palmitic (~6%), C18:0 stearic (~3%), C18:1 oleic (~11%) and C18:2 linoleic (~78%) (Applewhite, 1966). Safflower oil is considered as premium quality edible oil due to high amount of linoleic acid (polyunsaturated fatty acid, PUFA) content. Edible oil rich in PUFA is considered good for human health but is prone to oxidation leading to poor stability and short shelf life. The hydrogenation process is generally employed to improve the stability of stored safflower oil; but the process results in the formation of trans-fats, which are detrimental to health. In contrast, edible oil with innate high oleic acid (monounsaturated fatty acid, MUFA) content is stable; hence, has extended shelf life making it attractive to the food industry. Plant breeding methods have been successfully employed in developing cultivars of several oilseed crops rich in oleic acid content (Jonnala et al., 2005; Škorić et al., 2007; Lee et al., 2018). In safflower, Horowitz and Winter (1957) and Knowles and Mutwakill (1963) identified high oleic (~70%) mutant plants in safflower accessions introduced from India. Subsequently, Knowles et al. (1965) developed a genetic stock named UC-1 with high oleic content (78.3%), which kick-started the breeding work. To date, several high oleic safflower cultivars have been bred and released for commercial cultivation worldwide (Bergman et al., 2005; Muñoz-Valenzuela et al., 2007; Montoya-Coronado, 2008; Anjani and Yadav, 2017).

Genetically, high oleic trait in safflower is simply inherited and controlled by a partially recessive allele, '*ol*' at a single locus '*OL*'

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(Knowles and Mutwakill, 1963; Knowles and Hill, 1964; Hamdan et al., 2009). Hamdan et al. (2012) reported that microsomal *oleoylphosphatidylcholine desaturase FAD2-1* gene underlies the '*OL*' locus. Guan et al. (2012) isolated cDNA sequence, *CtFAD2-1*, which putatively encoded a *delta-12 fatty acid desaturase* (*FAD2-1*) in normal safflower with high linoleic acid content. A variant, *CtFAD2-1'* was found with a deletion of cytosine (C) at the position +603 bp of *CtFAD2-1* in high oleic safflower genotype. This being non-sense mutation caused deactivation of *CtFAD2-1* enzyme. It is known that *FAD2* enzyme is responsible for converting oleic acid into linoleic acid during lipid biosynthesis (Okuley et al., 1994). When *CtFAD2-1* was defective it eventually resulted in high oleic content in safflower; therefore, the variant, *CtFAD2-1'* was presumed to be '*ol*' allele (Liu et al., 2013). A molecular marker assay was developed to predict the '*ol*' allele using the DNA sequence variation between large introns situated in the 5' UTR of *CtFAD2-1* and *CtFAD2-1'* (Liu et al., 2013).

Marker-assisted selection (MAS) is an effective tool in plant breeding, especially when the target phenotypic traits are laborious or expensive to measure (Collard and Mackill, 2008). Traditionally, phenotype with high oleic trait is selected based on biochemical profiling using Gas Chromatography (GC), which is a quantitative and accurate method but destructive, time consuming and requires the crop to be grown to maturity for analysis. Molecular markers provide an option of genotypic selection, which is simple, quick, non-destructive and the assay can be performed at any stage of the plant; hence, would reduce significantly the time, cost and other resources required for a breeding programme to develop cultivars. Particularly, marker-assisted backcrossing (MABC) is effective for transferring a recessive trait like high oleic content because heterozygous plants carrying recessive allele can be selected before flowering and backcrossing with the recurrent parent can be made in the same generation without the need for selfing to produce homozygous progeny, which is difficult by conventional methods. Improvement of high oleic trait through MABC has been well demonstrated in peanut (Chu et al., 2011; Mienie and Pretorius, 2013; Janila et al., 2016).

Molecular marker assay developed by Liu et al. (2013) for high oleic trait in safflower is a multiplex assay involving a combination of primer pairs that produced an amplicon of 315-bp from *CtFAD2-1'* intron (specific to high oleic genotypes), 603-bp from *CtFAD2-1* intron (specific to high linoleic genotypes) and 198-bp from *CtKASII* gene (positive control to check for successful PCR amplification in all the samples). This is a gel based assay and not amenable for high throughput application. SNP based genotyping assays such as Kompetitive Allele Specific PCR (KASP®) (Semagn et al., 2014; Shi et al., 2015) or Amplifluor™ SNPs Genotyping System (Amplifluor®) (Jatayev et al., 2017) are robust and breeder-friendly for practicing MAS in large scale breeding programmes.

Low cost and high throughput molecular marker assay for predicting high oleic trait is required in safflower to rapidly develop high oleic cultivars. In this study, our objectives were to design/validate SNP genotyping assays for prediction of high oleic trait based on the functional mutation in *CtFAD2-1* of high oleic safflower genotypes and to demonstrate the use of genotypic assays in MABC scheme to incorporate the '*ol*' allele in the background of a popular safflower cultivar in a fast-track manner.

2. Materials and methods

2.1. Plant material

A panel of 12 safflower varieties comprising of six low oleic types (A-1, Bhima, PBNS-12, NARI-57, Centennial and Ciano-Lin) and six high oleic types (Montola-2000, Oleic Leed, Quilantan-97, Ciano-OL, CC-1469 and CCC-B4) were used. The exotic varieties were obtained from United States Department of Agriculture (USDA), USA and Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias (INIFAP), Sonora, Mexico through ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India. Fatty acid composition of the selected varieties is given in Table 1. A series of segregating populations including F_2, BC_1, BC_2 and BC_3 generations were developed from the cross between low oleic variety, Bhima and high oleic variety, Montola-2000. Additionally, an F_2 population produced from the cross between A-1 (a low oleic variety from India, 17.4%) and EC-755673-1 (a high oleic line selected from an exotic variety from Mexico, 73.4%) was used for validation of inheritance of the high oleic trait. Furthermore, a set of 24 breeding lines developed from the ongoing safflower breeding programme were also used for the validation of genotypic assays. Details of segregating populations are provided in the later section.

2.2. Designing of genotypic assays for 'ol' allele (foreground selection)

2.2.1. Partial sequencing of CtFAD2-1 gene

Based on the published sequences of normal *CtFAD2-1* (HM165274.1) and mutant *CtFAD2-1* alleles (Guan et al., 2012), a primer pair named as CtFAD2-1_OL_New-F: ACACCGTGGGCTTCATAGTC and CtFAD2-1-OL-New-R: GACCCGTCGTAGTGAGGCAA was designed using Primer3 software (Rozen and Skaletsky, 2000). This primer pair was expected to produce an amplicon size of about 520 bp covering the specific mutation in *CtFAD2-1* in polymerase chain reaction (PCR).

DNA was extracted from leaf samples following the procedure described by Doyle and Doyle (1987). The PCR was performed following the standard protocol in a thermal cycler (SimpliAmp, Applied Biosystems, USA). The PCR products were partially sequenced using standard Sanger Sequencing protocol through outsourcing to a service provider [Bioserve Biotechnologies (India) Private Limited, Hyderabad, India]. The sequence chromatograms were analyzed for target mutation (deletion of C) and the alignments were performed against the standard reference *CtFAD2-1* sequences using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al., 2018).

2.2.2. KASP genotyping assay

The KASP assay for the targeted SNP was designed at LGC Genomics, UK. Details of KASP assay are provided by Semagn et al. (2014). The KASP assay involved three components: KASP assay mix, KASP master mix and template DNA. The KASP assay mix contained two different, allele specific, competing forward primers with unique tail sequences at the 5′ end (allele-1 tail had FAM-labelled oligo sequence and allele-2 tail had HEX-labelled oligo sequence) and one common reverse primer. The KASP master mix contained FAM and HEX specific FRET (Fluorescence Resonance Energy Transfer) cassette, ROX passive reference dye, KASP *Taq* DNA polymerase (specially modified for allele-specific PCR), dNTPs and $MgCl_2$ in an optimized buffer solution.

Two allele-specific forward primers and one common reverse primer were designed. Flanking sequences (50 bp each upstream and downstream) of the SNP position were used for designing primers. By using these primers, KASP assay was performed in a 96-well PCR plate (opaque) containing a final reaction volume of 10 μL. The PCR mixture contained 5 μL of 5 ng/μL of genomic DNA, 0.14 μL of assay mixture (12 μM each allele-specific forward primer and 30 μM reverse primer) and 5 μL of 2X master mix. Two no-template-controls (NTCs) were included in the genotyping plate to ensure better clustering and to improve confidence in the validity of the genotyping results. Reaction mixture of 10 μL was dispensed in the 96-well black plate and the plate was sealed with an optically-clear seal using a heat-based plate sealer. The PCR was carried out using the thermal cycler (SimpliAmp, Applied Biosystems, USA) with KASP thermal cycling program [94 °C at 15 min (initial activation); 10 touchdown cycles of 94 °C for 20 s and 61−55 °C for 60 s (dropping 0.6 °C per cycle); and finally 26 cycles at 94 °C for 20 s and 55 °C at 60 s]. After the completion of PCR, the plate was read with FRET-capable plate reader (Victor X3, PerkinElmer) with the relevant filter sets for fluorescence detection of reactions. Fluorescence readings from the plate reader were analysed using KlusterCaller™ Version

Table 1
Fatty acid composition of the safflower varieties used in this study.

Cultivar	Origin	Accession Identity Number[a]	Oil content (%)	Fatty acid composition (%)			Reference
				Oleic	Linoleic	Palmitic + Stearic	
A-1	India	–	26.5	15.6	76.4	8.0	Kadirvel et al. (2017)
Bhima	India	–	30.0	16.2	75.5	8.3	Kadirvel et al. (2017)
PBNS-12	India	–	28.0	17.1	74.4	8.6	Kadirvel (unpublished)
NARI-57	India	–	37.6	13.4	75.9	10.7	Kadirvel et al. (2017)
Centennial	USDA	EC-736516 (PI 538779)	44.1	10.8	79.6	7.7	Bergman et al. (2001)
Montola-2000	USDA	EC-736515 (PI 538025)	38.3	80.8	12.3	5.6	Bergman et al. (2000)
Oleic Leed	USDA	EC-736514 (PI 560177)	39.0	76.1	16.0	5.5	Urie et al. (1979)
Ciano-Lin	Mexico	EC-755668	41.5	–	79.6	–	Montoya-Coronado (2008)
Quilantan-97	Mexico	EC-755661	–	78.9	14.4	6.7	Kadirvel et al. (2017)
Ciano-OL	Mexico	EC-755659	37.4	75.0	–	–	Montoya-Coronado (2008)
CC-1469	Mexico	EC-755666	37.8	79.2	14.0	6.8	Kadirvel et al. (2017)
CCC-B4	Mexico	EC-755671	37.7	72.6	20.3	7.0	Kadirvel et al. (2017)

[a] EC series: ICAR-NBPGR accession number; PI series: USDA accession number.

3.4.1.36 software (LGC Genomics, UK) to visualize and record the allelic status of each genotype.

2.2.3. Amplifluor assay

The primers for Amplifluor assay were designed following the protocol reported by Jatayev et al. (2017). Three gene specific primers for *CtFAD2-1* were designed as below.

Forward 1: 5'GAAGGTGACCAAGTTCATGCTCAGGCGAAACGGTTGTAGGG3'

Forward 2: 5'GAAGGTCGGAGTCAACGGATTCAGGCGAAACGGTTGTAGGT3'

Common Reverse: 5'CAGCCTGTTCGCCACTCTCA3'

The forward 1 primer targeted the normal *CtFAD2-1* allele (*OL*) and the forward 2 primer targeted the mutant allele (*ol*). The reverse primer was common. The tail sequences are underlined.

Amplifluor assay was performed as per the instruction manual on Amplifluor SNPs Genotyping System published by EMD Millipore Corporation. The assay was performed in 96-well black PCR plate with final reaction volume of 10 µL. The PCR mixture contained 8 µL of Amplifluor master mix and 2 µL of template DNA (~5 ng/µL). Master mix was prepared according to the protocol mentioned in the manual.

The Amplifluor master mix contained 0.8 µL of dNTP mix (2.5 mM each), 0.5 µL of Amplifluor SNP FAM primer (20X), 0.5 µL of Amplifluor SNP JOE primer (20X), 0.5 µL of 'specific primer mix' (20X) (prepared from three unlabelled allele specific primers: forward 1, forward 2 and common reverse), 1 µL of PCR buffer (10X), 4.6 µL of water and 0.1 µL of Platinum® *Taq* DNA polymerase (Invitrogen). The 'specific primer mix' (20X) contained 0.5 µM of forward primer 1, 0.5 µM of forward primer 2 and 7.5 µM of reverse primer.

The following PCR profile was used. One cycle of initial denaturation step at 95 °C for 15 min; 10 cycles of denaturation at 95 °C for 20 s followed by annealing and extension at 65 °C for 1 min (drop 0.6 °C per cycle); 26 cycles of denaturation at 94 °C for 20 s followed by annealing and extension at 59 °C for 1 min; hold at 4 °C. After the completion of PCR, the plate was read with FRET-capable plate reader and the fluorescence readings were analysed with KlusterCaller™ software (LGC Genomics, UK) to visualize and record the allelic status of each genotype as in the case of KASP.

2.2.4. Scoring of CtFAD2-1 alleles in genotyping assays

The florescence data obtained from the plate reader were analysed and viewed graphically using 'KlusterCaller™' Version 3.4.1.36 (LGC Genomics, UK). Detected signals were plotted as a graph, with samples of the same genotype clustering together. The FAM and HEX data were plotted on the *x*- and *y*-axes respectively. Inclusion of a passive reference dye (ROX) allowed the data to be normalised by dividing FAM and HEX values by the passive reference value for that particular well, thus removing the variable of liquid volume. The samples carrying high FAM signals and plotted closer to *x*-axis were considered homozygous for the FAM allele (normal *CtFAD2-1* allele, *OLOL*), the samples carrying high HEX signals and plotted closer to *y*-axis were considered homozygous for HEX allele (mutant *CtFAD2-1* allele, *olol*) and the samples carrying more or less equal proportion of FAM and HEX signals and plotted in the middle of the graph were considered heterozygous (*OLol*).

2.3. Development of segregating populations and validation of 'ol' allele specific genotypic assays

2.3.1. Choice of parents

Bhima, the Indian variety and Montola-2000, the exotic variety imported from USA were used as parents in the crossing programme. Bhima (S-4) is a low oleic variety developed at Dry Farming Research Station (Solapur), Mahatma Phule Krishi Vidyapeeth, Rahuri (Maharashtra State), India and released for cultivation in 1982. Montola-2000 is a high oil and high oleic variety, which was developed by the Montana Agricultural Experiment Station and North Dakota Agricultural Experiment Station, USA (Table 1).

2.3.2. Development of F_2/BC_1F_1 populations

Bhima was used as recurrent parent (RP) and Montola-2000 was used as donor parent (DP) for high oleic trait in backcrossing programme. Montola-2000 (♂) was crossed with Bhima (♀) and F_1 was produced during October 2012-March 2013. The F_1 plant was grown in the field, selfed to produce F_2 population and simultaneously the F_1 pollen was used to pollinate the emasculated flowers of Bhima to produce BC_1F_1 population during October 2013-March 2014. The crossing work was done by hand as per the procedure described by Mündel and Bergman (2009).

2.3.3. Co-segregation analysis

A set of 65 F_2 and 71 BC_1F_1 plants were grown in the field during October 2014-March 2015. The F_2 and BC_1F_1 plants were genotyped using *CtFAD2-1* gene specific marker assays. Selfed seeds of F_2 and BC_1F_1 plants were collected. About 20–100 seeds per family (F_3 and BC_1F_2) were analyzed for fatty acid composition in GC as described in Kadirvel et al. (2017) and the procedure is briefly provided below.

Oil from safflower seed was extracted in hexane on soxhlet apparatus (Extraction unit, E-816, Buchi). Composition of fatty acids in the oil was analyzed by GC (Agilent 7890 B) equipped with a flame ionization detector (FID) after derivatization to fatty acid methyl esters. The oil (100−150 mg) was treated with 2 mL of 13% methanolic KOH for 50 min at 55 °C. The organic phase was extracted with hexane and washed with water till it reached neutral pH. The hexane was dried over anhydrous sodium sulphate and concentrated with nitrogen to get methyl esters.

Peak separation was performed on a DB-225 capillary column (50%

Cyanopropylphenyl and 50% Dimethylpolysiloxane, diameter 250 μm, length 30 m and film thickness 0.25 μm) from Agilent Technologies. The samples (0.1 μL) were injected in split mode (split ratio 1:30). The initial oven temperature was set at 160 °C for 2 min, raised to 220 °C (at a rate of 6 °C/min) and held at 220 °C for 10 min. Both inlet and detector were set to 230 °C. The carrier gas was nitrogen set to a constant flow rate of 1.2 mL/min. Peak identification was performed by comparing the relative retention times with those of a commercial standard mixture of FAME (Supelco 37 Component FAME Mix). The amount of individual fatty acid was determined by calculating relative percent peak areas using the EZChrom elite compact software.

2.4. Marker-assisted backcrossing of 'ol' allele (foreground selection)

2.4.1. Development of BC_2F_1 population

A single BC_1F_1 plant, which carried *OLol* alleles (heterozygous condition) and phenotypically resembled the recurrent parent (Bhima) was crossed back to Bhima. Pollen from the selected BC_1F_1 plant was used to pollinate the emasculated flowers of Bhima to produce BC_2F_1 seeds. The BC_1F_1 plant was also selfed continuously up to F_6 generation to obtain a BC_1 derived high oleic lines.

2.4.2. Development of BC_3F_1 population

A subset of 35 BC_2F_1 plants was grown in the field during October 2015-Febraury 2016 and were genotyped by KASP assay to select heterozygous BC_2F_1 plants. The selected BC_2F_1 plant was backcrossed to

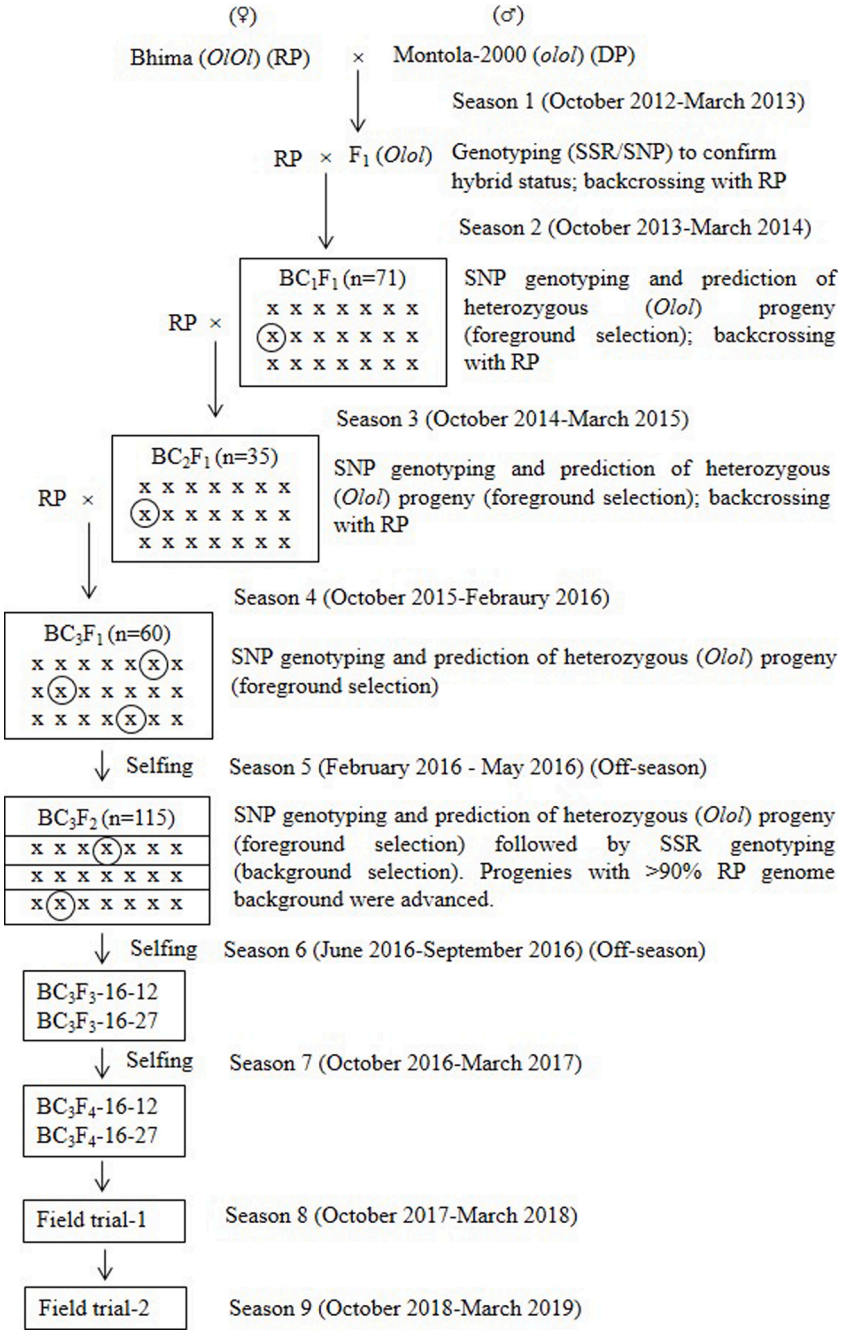

Fig. 1. A schematic diagram of marker-assisted backcrossing (MABC) procedure adopted for transferring high oleic allele '*ol*' in safflower. RP-Recurrent Parent and DP-Donor Parent.

Bhima to produce BC_3F_1 seeds. The BC_2F_1 plant was also selfed continuously up to F_6 generation to obtain BC_2 derived high oleic lines.

2.4.3. Development of BC_3F_2 families

A subset of 60 BC_3F_1 plants were grown during February 2016 to May 2016 (off-season) and genotyped. The BC_3F_1 plants that were heterozygous (*OLol*) were identified and the BC_3F_2 seeds were harvested. The BC_3F_2 seeds from a set of 10 BC_3F_1 heterozygous plants were grown familywise (20 plants each) in an off-season cultivation facility during June 2016-September 2016 and BC_3F_3 seeds were produced. A set of 115 BC_3F_2 plants were genotyped using KASP assay and plants carrying homozygous *ol* alleles were selected. The BC_3F_3 seeds (100 seeds/progeny) were analysed for fatty acid composition using GC. A schematic diagram of MABC procedure adopted in this study is presented in Fig. 1.

2.5. SSR analysis of high oleic BC_3F_2 selections (background genotyping)

The parents, Bhima and Montola-2000 were analysed for polymorphism at 1107 SSR loci in high resolution (3%) agarose gel electrophoresis. Primer sequences were obtained from different sources namely Mayerhofer et al. (2010), Yamini et al. (2013), Lee et al. (2014) and Usha Kiran et al. (2019). SSR genotyping was performed following the procedure described in Kadirvel et al. (2017). The PCR products were resolved in agarose gel electrophoresis (3%) and documented using ALPHA IMAGER gel documentation system (M/s Alpha Innotech). By parental survey, a set of polymorphic SSR loci between Bhima and Montola-2000 was shortlisted for genotyping of BC_3F_2 plants, which were positive for *ol* alleles in KASP assay. The SSR alleles of BC_3F_2 plants were compared with Bhima and Montola-2000 and the proportion of Bhima alleles in the BC_3F_2 plants was determined.

2.6. Field evaluation of high oleic varietal lines developed by MABC for seed yield, oil content and fatty acid composition

A set of six high oleic lines (BC_1-F_6-39-3−3, BC_2-F_6-38-1−7, BC_2-F_6-38-9−4, BC_2-F_6-38-14−5, BC_3-F_4-16-12 and BC_3-F_4-16-27) was evaluated for seed yield, seed weight, oil content and fatty acid composition along with four check varieties namely A-1, Bhima, PBNS-12 and NARI-57 during October 2017-April 2018. The fatty acid composition of the varietal lines was further confirmed in another trial during October 2018-April 2019. The field trials were conducted at a research farm of ICAR-IIOR located at ICRISAT, India. Alpha Lattice Design was followed with two replications. Seed oil content (%) was estimated with nuclear magnetic resonance (NMR) spectroscopy using 20 g of pooled seed samples from each plot (Yadav and Murthy, 2016).

2.7. Data analysis

Chi-square test was used to check if segregation of *CtFAD2-1* alleles in F_2, $BC_1F_{1:2}$, $BC_2F_{1:2}$ and BC_3F_2 progenies followed classical Mendelian ratio. Chi-square (χ^2) value was calculated as per the standard formula $\chi^2 = \sum (O\text{-}E)^2/E$, where O = observed frequency of low oleic/high oleic plants and E = expected frequency of low oleic/high oleic plants. The computed χ^2 value was compared with the tabular value at n-1 degrees of freedom, where 'n' is the total number of genotypic/phenotypic categories at 5% level of significance.

Linear regression analysis was used to confirm the co-segregation of *CtFAD2-1* allelic data of F_2, BC_1F_1, BC_2F_1 and BC_3F_2 plants with oleic content data (%) of the corresponding F_3, BC_1F_2, BC_2F_2 and BC_3F_3 seeds using regression function as implicated in the data analysis option available in the MS Excel. Data from field trial were subjected to analysis of variance (ANOVA) and mean comparison (LSD at 5% level of significance) using Plant Breeding Tools (PBTools) v 1.3 software (IRRI, 2013).

3. Results and discussion

3.1. Detection of 'ol' allele in high oleic safflower varieties

Alignment of the *CtFAD2-1* gene sequences (partial) showed the presence of target 'InDel' in high oleic safflower varieties used in this study (Fig. 2) indicating that these varieties carried the high oleic allele '*ol*'. Liu et al. (2013) also detected the *'ol'* allele in a different set of exotic safflower varieties including S-317, S-517, LeSaf-486, CW99-OL and Ciano-OL. These observations suggest that the high oleic varieties of safflower shared the same allele and possibly the '*ol*' allele has been exploited for improvement of high oleic acid content trait in safflower. It is also important to note that no other allele for high oleic acid content has been reported at the 'sequence level' in safflower to date.

3.2. Inheritance of high oleic allele 'ol' in safflower

Mendelian segregation of '*ol*' allele was observed in segregating populations [n = 65 (F_2 plants) and 71 (BC_1F_1 plants] derived from the cross between Bhima and Montola-2000 (Table 2). Genotypes of segregants (homozygous-Bhima type allele, homozygous-Montola-2000 type allele, heterozygous) were assigned based on '*CtFAD2-1* sequencing assay'. The actual oleic acid content of the progenies (estimated in GC) also indicated the Mendelian segregation. The oleic acid content values of $F_{2:3}$ progenies ranged from 15.01% to 84.1% and three-fourths of the progenies had oleic acid content values of less than 60 %. These phenotypic values clearly indicated the genotypic status of F_2 progenies (*OLOL*:*OLol*:*olol*) as expected in case of a monogenic recessive trait. Similarly, the oleic acid content of $BC_1F_{1:2}$ progenies ranged from 14.84% to 50.69% reflecting only two genotype categories (*OLOL*;*OLol*) as expected in a backcross population. The χ^2-values of F_2 (1:2:1) and BC_1F_1 (1:1) genotypic classes support monogenic segregation of '*ol*' allele. The χ^2-value of BC_1F_1 population was slightly higher than the expected indicating the deviation from 1:1 ratio, which could perhaps be due to 'selfed progenies' in the BC_1F_1 population, though diligent care was taken while backcrossing, selfing could not be ruled out. High R^2 values of $F_{2:3}$ (0.87) and $BC_1F_{1:2}$ (0.77) populations indicate major effect of '*ol*' allele on oleic acid content in safflower.

The 'KASP assay' also produced the same genotypic data of '*OL*' locus in the segregating populations as that of '*CtFAD2-1* sequencing' assay suggesting that it could reliably replace *CtFAD2-1* sequencing based assay for prediction of high oleic allele, '*ol*' in safflower. Subsequently, an additional F_2 population produced from the cross between A-1 and EC-755673-1 was genotyped by KASP, which also showed Mendelian inheritance of '*ol*' allele (n = 89; progenies with *OLOL*: 23, *Olol*: 45, *olol*: 21; 1:2:1 ratio; $\chi^2 = 0.1010$). The KASP profiles of progenies of segregating populations along with the parental varieties of safflower are presented in Fig. 3.

Monogenic segregation of high oleic trait in safflower has been well documented (Hamdan et al., 2009). As the evidence suggests that '*ol*' allele at the major locus '*OL*' is the primary source for high oleic trait in safflower, which in homozygous recessive condition (*olol*) produced more than 60% of oleic acid content; usually more than 75%. In addition to '*ol*' allele, possible existence of a different allele 'ol^1' at the same '*OL*' locus has been suggested, which in homozygous condition (ol^1ol^1) produced between 35 and 50% oleic acid content (Knowles and Hill, 1964). Furthermore, Knowles (1972) postulated the involvement of minor genes in producing high oleic content in safflower. Hamdan et al. (2012) reported a minor QTL associated with high oleic trait in safflower, which supported the prediction of Knowles (1972). However, the predicted new allele (ol^1) and QTL associated with high oleic acid content in safflower have not yet been investigated further.

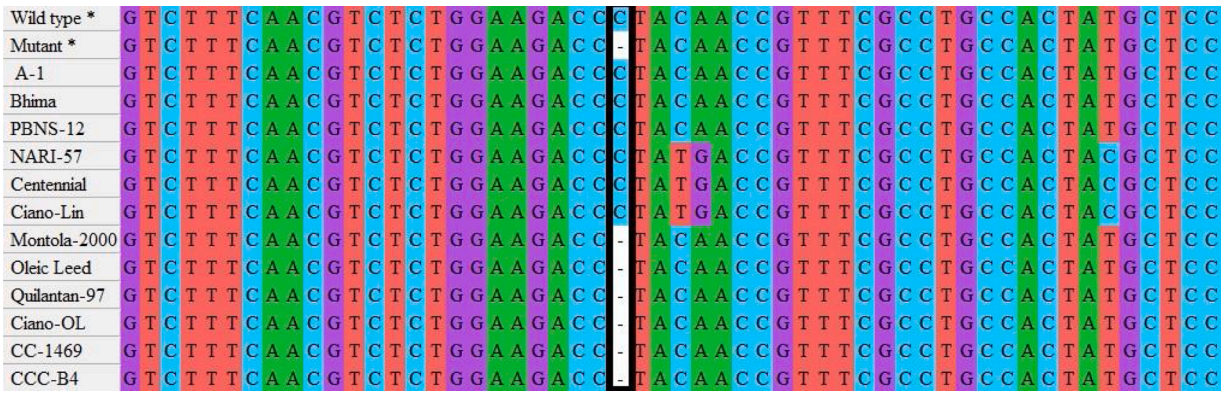

Fig. 2. Alignment of *CtFAD2-1* gene sequences (partial) of the safflower varieties. Presence of target mutation (InDel) in the high oleic varieties is highlighted. The wild (HM165274.1) and mutant sequences of *CtFAD2-1* published by Guan et al. (2012) were used as reference sequences.

Table 2
Co-segregation analysis of '*ol*' allele with high oleic acid content in different segregating populations of safflower.

	Genotypic groups in different populations based on KASP assay							
	$F_{2:3}$ (n = 65)			$BC_1F_{1:2}$ (n = 71)		$BC_3F_{2:3}$ (n = 115)		
	OLOL	*OLol*	*olol*	*OLOL*	*OLol*	*OLOL*	*OLol*	*olol*
Sample size (n)	17	32	16	46	25	29	63	23
Mean (%)[a]	18.88	39.46	79.75	18.23	36.90	18.91	36.72	71.23
Range (%)[a]	15.01–26.14	21.13–58.73	61.11–84.74	14.84–25.50	24.11–50.69	14.83–28.27	24.52–49.02	61.05–77.54
Median (%)[a]	17.85	38.76	81.95	17.92	36.12	18.79	36.18	71.93
SD	2.95	8.58	6.25	1.96	7.70	2.82	5.45	4.73
F value	405.18			234.62		836.29		
p value	<0.0001			<0.0001		<0.0001		
R^2 value	0.87			0.77		0.88		
r value	0.93			0.88		0.94		
χ^2 value	0.0461 (1:2:1)			6.212 (1:1)		1.678 (1:2:1)		

[a] Mean, Range and Median values are oleic acid content (%) as estimated by Gas Chromatography.

3.3. Co-segregation of 'InDel' mutation ('ol' allele) with high oleic acid content in safflower

Based on parental values, Bhima (*OLOL*) (19%) and Montola-2000 (*olol*) (81 %), oleic content of $F_{2:3}$ progenies were predicted genotypically as <20% for Bhima type allele-homozygous (*OLOL*), >70% for Montola-2000 type allele-homozygous (*olol*) and ~35% for heterozygous (*OLol*) plants. The predicted oleic values of $F_{2:3}$ progenies by genotyping were compared with actual oleic values obtained in GC, which matched well. High correlation ($r = 0.93$) between genotype and oleic acid content values supports co-segregation. The oleic acid content values of the genotypic group '*OLOL*' ranged from 15.01% to 26.14%, '*OLol*' from 21.13% to 58.73% and '*olol*' from 61.11% to 84.74%. It was interesting to note that the range of oleic acid content values was substantially higher within the genotypic groups. It is not clear if the environmental noise or the involvement of minor genes contributed for the variation in oleic acid content values within the groups. Especially, the range of oleic acid content values was high in case of heterozygous progenies. The phenotypic value of F_2 heterozygous progeny is expected to deviate from the predicted value due to sampling bias when F_3 seeds are taken for oleic estimation. The F_3 seed lot from the heterozygous progeny would contain mixture of genotypes in 1:2:1 ratio; bias is possible while sampling seeds that would lead to large variation in oleic acid content value of the family. Nevertheless, co-segregation of the '*ol*' allele and high oleic acid content was clearly evident from the results. Strong association of genotypic data of F_2 plants at '*OL*' locus with actual oleic values of corresponding F_3 seeds (n = 55; $r = 0.81$, $F = 107.3$, $p = 2.42E^{-14}$) was also observed in the additional segregating population of the cross between A-1 and EC-755673-1. The mean values of oleic acid content of *OLOL* (n = 10), *OLol* (n = 30) and *olol* (n = 13) genotype groups in this population were 24.2%, 39.7% and 78.7%, respectively and were comparable with the F_2 population of the cross between Bhima and Montola-2000, which strongly supported monogenic inheritance of high oleic trait in safflower. High level of marker-trait correlation is absolutely essential to practice MAS more confidently in breeding programmes (Cobb et al., 2019). Being a gene specific marker, very high level of correlation between marker and oleic acid content could be achieved in this case.

3.4. MAS for high oleic trait in safflower

In this study, three genotypic assays namely (1) partial sequencing of *CtFAD2-1* gene based assay, (2) KASP and (3) Amplifluor were found to be effective for prediction of high oleic trait in safflower. Both partial sequencing of *CtFAD2-1* gene based assay and KASP produced perfectly comparable results in early segregating populations ($F_{2:3}$/$BC_1F_{1:2}$). Subsequently, the MABC programme was continued with the KASP assay. Later on, KASP and Amplifluor assays were compared in a panel of 24 safflower breeding lines representing different allelic status (*OLOL, OLol* and *olol*) and found that both assays correctly predicted the genotypes (Fig. 4). Overall, high oleic trait in safflower was accurately predictable using the assays described in this study. However, the choice of marker assay depends on availability of resources. Obviously, partial sequencing of *CtFAD2-1* gene based assay would be costlier. The KASP assay was dependent on the oligos supplied by LGC Genomics, UK while the Amplifluor assay could be performed with the custom-made oligos; therefore, it provides scope for reduction in genotyping costs and turnaround time.

With the use of genotyping assay, it was possible to pick up a heterozygous progeny for making next cross at every stage of the backcross

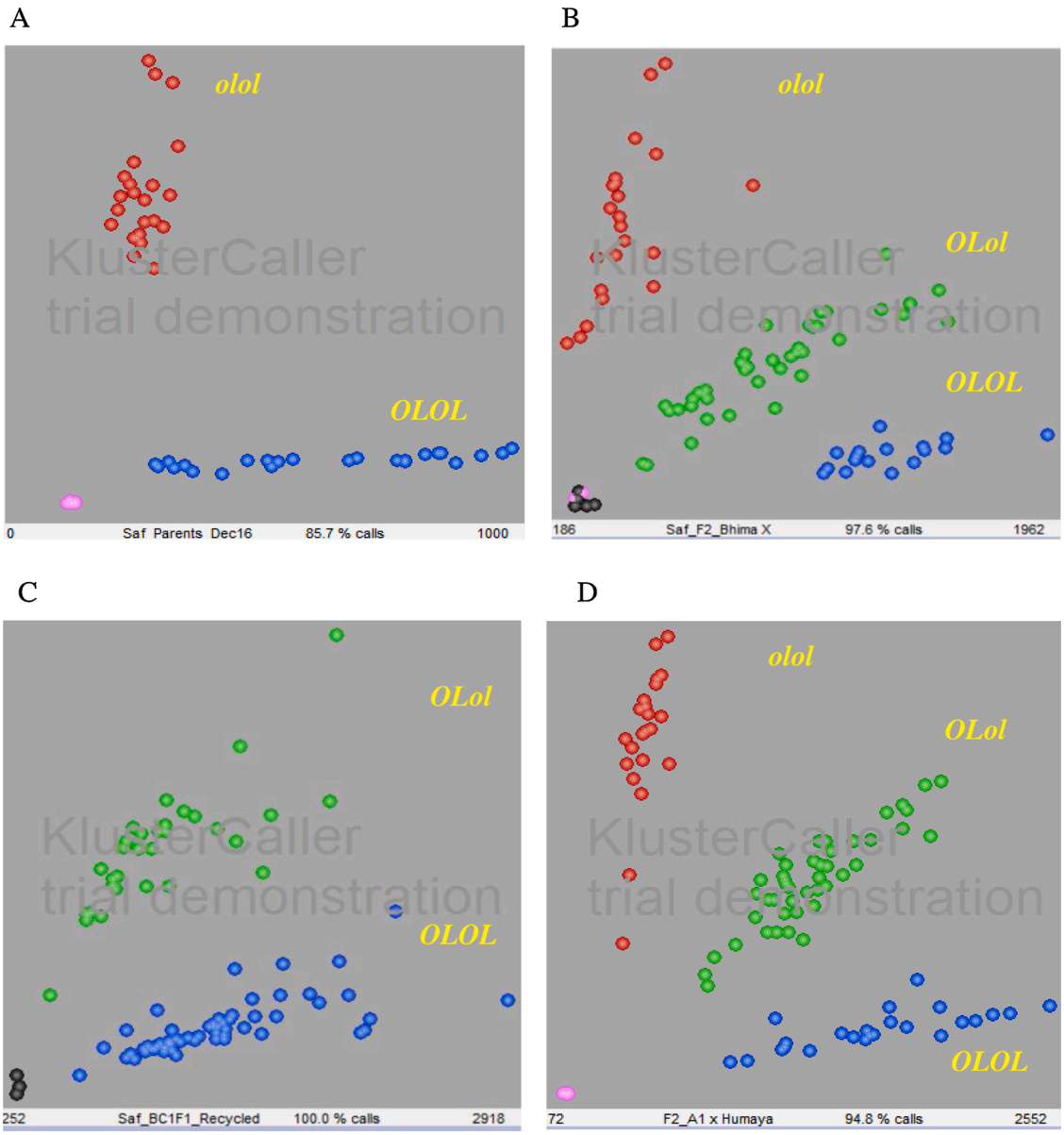

Fig. 3. KASP genotype profile of (A) safflower varieties (assay was repeated four times for each sample), (B) F_2 population of the cross between Bhima and Montola-2000, (C) BC_1F_1 population of the cross between Bhima and Montola-2000 and (D) F_2 population of the cross between A-1 and EC-755673-1.

programme. A spectacular advantage of MAS is that it enables the breeder to predict heterozygous progeny before flowering and effect the backcrossing immediately, which saves time and resources required for a breeding programme. Conventionally, in case of a recessive trait like high oleic acid content of safflower, finding high oleic progenies in BC_1F_1 population is not possible; therefore, one round of selfing is required to produce BC_1F_2 families, which need to be further analysed for fatty acid profiles using biochemical methods. The homozygous BC_1F_2 seeds are required to be grown again for effecting backcrossing so eventually it requires two seasons to make every backcross, which can be avoided by using MAS and thus, the breeding could be fast-tracked.

By adopting MAS, the backcrossing programme was continued up to BC_3 generation. At BC_3 stage, a set of 35 BC_3F_2 progenies were identified as high oleic types by foreground selection of '*ol*' alleles in homozygous condition (*olol*). The SSR genotyping using a set of 33 polymorphic SSR markers revealed that percentage of the recurrent parent (Bhima) alleles in the BC_3F_2 progenies ranged from 52% to 94% (Fig. 5). Two progenies namely BC_3F_2-16-12 (91%) and BC_3F_2-16-27 (94%) had the highest proportion of recurrent parent type SSR alleles suggesting that about 90% of genome of the recurrent parent could have possibly been recovered. More number of SSR markers would have been desirable for reliable assessment of recurrent parent genome recovery in the MAS-derived lines. It was difficult to find more SSR markers as the parents, Bhima and Montola 2000, exhibited low level of polymorphism (~3% in agarose gel electrophoresis). Nevertheless, SSR genotyping was useful to assess the genome status of MAS-derived lines. Finally, a set of six high oleic lines derived from the MABC programme at different stages including BC_1, BC_2 and BC_3 were shortlisted for field evaluation.

3.5. Agronomic performance of high oleic safflower lines derived by MAS

Seed or oil yield performance of high oleic lines was comparable with the local check varieties (Table 3). Test weight (g) and oil content (%) showed significant differences among the lines as well as the check varieties. The BC_1 derived line (BC_1F_6-39-3-3) showed clear differences in terms of 100-seed weight (3.93 g) and oil content (35.51%) compared with the recurrent parent, Bhima (5.24 g; 31.62%). As expected, BC_3 derived lines did not differ from Bhima. The backcrossing programme

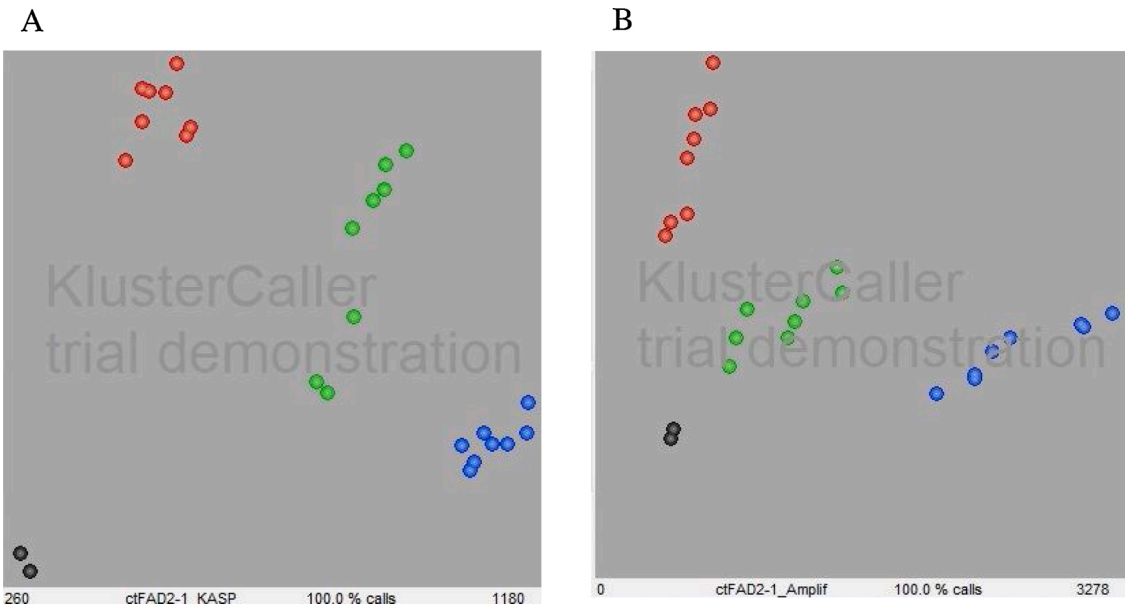

Fig. 4. Comparison of (A) KASP® and (B) Amplifluor® profiles in a panel of 24 safflower breeding lines representing *OLOL*, *OLol* and *olol* genotypic groups.

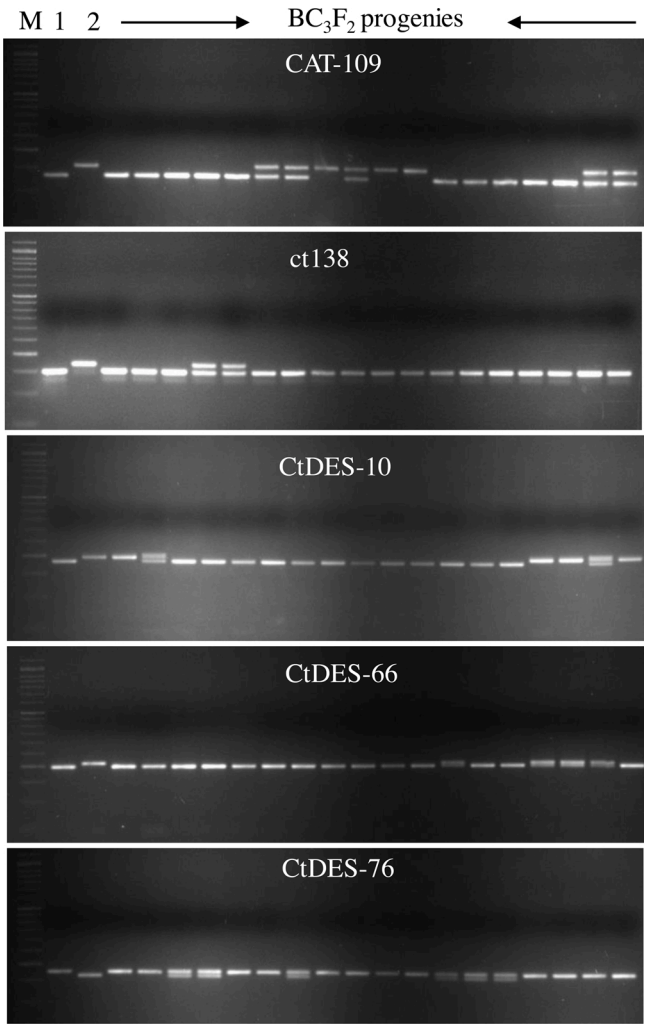

Fig. 5. Background genotyping of selected BC_3F_2 progenies of the cross between Bhima and Montola-2000 using SSR markers. M-Standard DNA marker (100 bp ladder), Lane 1: Bhima, Lane 2: Montola-2000 and Lane 3-20: BC_2F_2 progenies. The SSR markers used for genotyping are indicated on the gel image.

Table 3
Agronomic performance of high oleic safflower lines developed by MAS.

Line	Seed yield (g/plant)	100-seed weight (g)	Seed oil content (%)	Oil yield (g/plant)
BC_1F_6-39-3−3	28.10	3.93	35.51	10.00
BC_2F_6-38-1−7	30.70	5.06	33.56	10.31
BC_2F_6-38-9−4	32.70	5.17	33.61	10.96
BC_2F_6-38-14−15	31.65	5.24	34.12	10.81
BC_3F_4-16-12	30.20	4.79	33.72	10.19
BC_3F_4-16-27	30.00	4.82	32.79	9.84
A-1	32.50	5.30	29.72	8.94
Bhima	34.50	5.24	31.62	10.26
PBNS-12	34.40	5.08	29.56	10.43
NARI-57	21.95	3.30	35.69	6.49
F value	NS	20.39**	13.12**	NS
$LSD_{0.05}$	–	0.41	1.66	–

was intended for improving oleic acid content of the Indian safflower variety Bhima, which is adapted to local conditions. However, selection from early backcross population was helpful to identify a high oleic progeny with high oil content as well. This is expected because alleles for high oil content from Montola-2000 might be available in the early generations of backcross population, which would get eliminated by the successive backcrosses when foreground selection for high oleic allele is rigorously exercised. Average oleic acid content over two seasons ranged from 75.23% to 81.79% in the MAS-derived lines and from 17.98% to 22.64% in the Indian check varieties (Table 4). Expression of high oleic trait was consistent and stable over seasons. Morphological features and seed type of one of the MAS-derived elite high oleic lines, BC_3F_4-16-27 is shown in Fig. 6.

4. Conclusions

A set of high oleic safflower varieties (from USA and Mexico) were found to carry the same mutation in the *fatty acid desaturase* gene (*CtFAD2-1*, conventionally designated as '*ol*' allele), which is known to affect the oil quality in safflower.

Monogenic recessive inheritance of high oleic trait in safflower was

Table 4
Fatty acid composition of high oleic safflower lines developed by MAS across two seasons.

Line	Season (2017−18)				Season (2018−19)			
	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)
BC_1F_6-39-3−3	4.74	2.14	77.98	15.49	4.83	1.33	78.83	14.94
BC_2F_6-38-1−7	4.94	1.94	72.15	20.98	4.85	1.37	78.30	15.43
BC_2F_6-38-9−4	4.43	1.86	84.72	8.99	5.12	1.51	78.74	14.58
BC_2F_6-38-14−15	4.76	1.83	82.17	11.24	4.73	1.23	81.41	12.58
BC_3F_4-16-12	4.87	1.80	79.45	13.88	5.48	1.90	74.78	17.84
BC_3F_4-16-27	5.05	2.28	80.57	12.11	4.70	1.40	80.72	13.16
A-1	6.02	3.09	23.13	67.77	6.35	1.92	22.15	70.00
Bhima	5.59	2.10	26.16	66.17	6.09	1.81	19.10	72.96
PBNS-12	6.29	2.98	21.52	69.22	6.23	1.95	20.88	70.88
NARI-57	7.59	2.86	17.98	71.59	8.22	1.96	17.97	71.86
F value	26.25**	3.5*	46.32**	41.90**	23.71**	NS	124.85**	119.24**
$LSD_{0.05}$	0.53	0.76	12.39	12.48	0.64	–	7.70	7.58

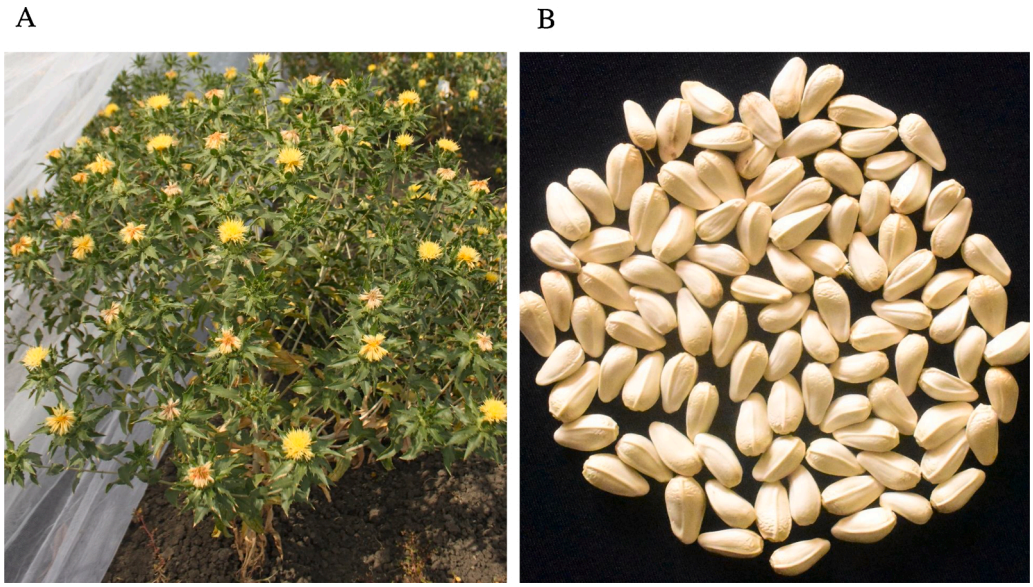

Fig. 6. (A) Morphological features and (B) seed type of the MAS-derived elite high oleic safflower line, BC_3F_4-16-27.

confirmed using segregating populations produced from the crosses involving low oleic (A-1, Bhima) and high oleic (Montola-2000, EC-755673-1) parents of safflower.

DNA marker based genotypic assays namely KASP® and Amplifluor® were found to be effective for prediction of high oleic trait accurately in safflower. The assays were simple, robust, non-destructive, co-dominant type for predicting heterozygotes and amenable for high throughput application in plant breeding programmes.

The high oleic allele, '*ol*' from the exotic variety, Montola-2000 was incorporated into the background of popular Indian linoleic type variety, Bhima through MABC scheme. A set of MAS-derived lines with high oleic acid content ranging from 75.2% to 81.8% were developed, which showed consistent expression of high oleic acid content over seasons and comparable seed/oil yield performance with the local check varieties.

CRediT authorship contribution statement

Palchamy Kadirvel: Conceptualization, Supervision, Writing - original draft. **Cheelam Veerraju:** Methodology. **Senapathy Senthilvel:** Methodology, Writing - review & editing. **Praduman Yadav:** Methodology. **Betha Usha Kiran:** Methodology. **Mobeen Shaik:** Methodology. **Ranjan Shaw:** Methodology. **Selvaraj Velu Mani:** Methodology. **Yarabapani Rushwanth Reddy:** Methodology. **Manmode Darpan Mohanrao:** Methodology. **N. Mukta:** Validation, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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