Mol Breeding (2012) 30:1279–1293
DOI 10.1007/s11032-012-9714-y

Mapping of major and modifying genes for high oleic acid content in safflower

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Received: 18 October 2011 / Accepted: 11 February 2012 / Published online: 28 February 2012

Abstract Oils with high oleic acid content are in great demand because they have optimal properties for food and non-food uses. Two different levels of high oleic acid content (>75 and >84%) have been reported in safflower (*Carthamus tinctorius* L.). The trait is mainly controlled by partially recessive alleles at a major gene *Ol*, but the highest levels have been attributed to modifying genes. The objectives of this research were to map the *Ol* locus and modifying genes involved in oleic acid content of safflower seeds and to determine the nature of *Ol* through a candidate gene approach. Two F2 mapping populations from the nuclear male-sterile line CL-1 and the high oleic acid lines CR-6 (>75% oleic acid) and CR-9 (>84%) were developed and phenotyped for oleic acid content at the F2 and F3 seed level. A genetic linkage map comprising 15 linkage groups and 116 random amplified polymorphic DNA, simple sequence repeat (SSR), and sequence-characterized amplified regions marker loci was constructed for the CL-1 × CR-9 population. The *Ol* gene was mapped to linkage group (LG) T3 tightly linked to the SSR marker ct365, which was confirmed in the CL-1 × CR-6 population. Additionally, a quantitative trait locus with a minor effect on increasing oleic acid content was identified on LG T2. The candidate gene approach indicated that an oleoyl-phosphatidylcholine desaturase *FAD2-1* locus underlies the *Ol* gene. Both the genetic information and the markers developed in this research will contribute to marker-assisted selection for high oleic acid content in safflower.

Keywords *Carthamus tinctorius* · Molecular markers · Modifying genes · Linkage map · Oleic acid · QTL analysis · Safflower

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Introduction

Safflower (*Carthamus tinctorius* L.) has been cultivated since antiquity for its flowers, used for flavouring and colouring foods and for making dyes, and for its seed oil (Dajue and Mündel 1996). Nowadays safflower is an underutilized minor oilseed crop with great potential in many areas of the world because of its agronomic performance and seed oil quality (Knowles 1989). Conventional safflower oil has a high linoleic acid content (>70%), which is a unique

trait amongst oilseed crops. Additionally, safflower germplasm producing high oleic acid oil has been identified (Knowles 1989). High oleic acid oils are highly appreciated for food and non-food applications because they combine a hypocholesterolemic effect (Mensink and Katan 1989) with a much greater oxidative stability than oils with greater polyunsaturation levels (Yodice 1990).

High oleic acid safflower was initially reported as having oleic acid content between 64 and 83% of the total fatty acids, which was produced by partially recessive alleles *ol* at a single locus *Ol* (Knowles and Hill 1964). Another different allele ol^1 at the same locus produced, in homozygous condition, between 35 and 50% oleic acid, compared to 10–15% in safflower plants carrying the wild-type alleles *Ol* (Knowles and Hill 1964). Fernández-Martínez et al. (1993) identified safflower germplasm with higher oleic acid content (86–91%) than that previously reported by Knowles and Hill (1964). Hamdan et al. (2009a) demonstrated that these very high oleic acid levels were produced by the recessive alleles *ol* in combination with modifying genes with a positive effect on oleic acid content. The role of modifying genes in increasing oleic acid content beyond the limits produced by the *ol* alleles had been previously suggested by Knowles (1972). Hamdan et al. (2009a) also identified an effect of modifying genes on reducing oleic acid content below the expected levels in germplasm carrying the *ol* alleles. A similar negative effect of modifying genes on oleic acid content has been reported in sunflower (Urie 1985; Fernández-Martínez et al. 1989).

In the fatty acid biosynthetic pathway, the microsomal enzyme oleoyl-phosphatidylcholine desaturase (FAD2) catalyses the desaturation step from oleic acid (18:1) to linoleic acid (18:2) (Okuley et al. 1994). Whereas only a single copy of the *FAD2* gene has been identified in plants such as *Arabidopsis* and maize (Okuley et al. 1994; Beló et al. 2008), the genome of the oil crops sunflower, soybean, and canola contains multiple copies of the gene (Scheffler et al. 1997; Martínez-Rivas et al. 2001; Schlueter et al. 2007). From these multiple copies, a highly expressed or seed-specific microsomal FAD2 (*FAD2-1*) has been demonstrated to underlie the elevated oleic acid levels in the seeds of the oil crops mentioned (Pérez-Vich et al. 2002; Schuppert et al. 2006; Hu et al. 2006; Falentin et al. 2007; Lacombe et al. 2009; Pham et al. 2010, 2011). Recently, three different *FAD2* genes (*FAD2-1*, *FAD2-2*, and *FAD2-3*) have been identified in safflower (Guan et al. 2011a, b). From them, *FAD2-1* is strongly expressed in developing seeds and has a high similarity to sunflower *FAD2-1*. Additionally, Guan et al. (2011a) determined that the *FAD2-1* transcript levels in a high oleic acid safflower genotype were significantly lower than in standard (high linoleic) genotypes during seed development. Accordingly, an alteration of the function of the enzyme encoded by safflower *FAD2-1* is a priori hypothesized as the factor underlying the high oleic acid trait in this crop.

Unlike most oilseed crops, research on the development of molecular markers for trait selection has been scarce in safflower. The use of molecular markers in this crop has mainly focused on the evaluation of genetic diversity (Yazdi-Samadi et al. 2001; Ravikumar et al. 2005; Sehgal and Raina 2005; Johnson et al. 2007; Yang et al. 2007; Amini et al. 2008) and the taxonomy of the genus *Carthamus* (Vilatersana et al. 2005; Chapman and Burke 2007). Nevertheless, SCAR (sequence-characterized amplified regions) markers have been developed linked to the *Li*, *Ms*, *HSya*, and *Tph2* genes determining very high linoleic acid content, nuclear male sterility, accumulation of hydroxysafflor yellow A, and high gamma-tocopherol content, respectively (Hamdan et al. 2008; Zhang et al. 2009; García-Moreno et al. 2011). Moreover, the recent development of safflower SSR (simple sequence repeats) and RFLP (restriction fragment length polymorphism) markers (Chapman et al. 2009; Mayerhofer et al. 2010; Hamdan et al. 2011) and a first genetic linkage map (Mayerhofer et al. 2010) has established the foundation for genomic studies in this species and supplied a critical mass of sequence-based DNA markers for safflower molecular trait breeding.

The objectives of this research were to map the *Ol* locus and modifying genes controlling high oleic acid content in safflower seeds and to determine the nature of *Ol* through a candidate gene approach.

Materials and methods

Plant material, phenotypic analyses, and DNA extraction

CR-6 is a safflower line with high oleic acid content (75–83%) isolated from the US Department of

Agriculture (USDA) germplasm accession PI 560177 (www.ars-grin.gov/npgs/). CR-9 is a safflower line with very high oleic acid content (84–88%) developed from the USDA germplasm accession PI 401479. CL-1 is a nuclear male-sterile (NMS) safflower line with conventional fatty acid profile (oleic acid content from 14 to 22%) isolated from the USDA-ARS germplasm accession PI 560161. The lines CR-6 and CR-9 carry the recessive alleles *ol* for high oleic acid content at the *Ol* gene. The different levels of oleic acid in both lines are determined by modifying genes with a small positive effect on the trait (Hamdan et al. 2009a). Male sterility in CL-1 is controlled by recessive alleles at a single gene *Ms* (Heaton and Knowles 1982).

Heads of male sterile plants of CL-1 were bagged before flowering and pollinated with fresh pollen of CR-6 and CR-9 plants. F1 plants from the CL-1 × CR-6 and the CL-1 × CR-9 crosses were grown and self-fertilized by bagging the heads before the beginning of flowering. One population of 119 F2 seeds from the cross CL-1 × CR-6 and another population of 116 F2 seeds from the cross CL-1 × CR-9 were used for the molecular analyses. These populations were analysed for fatty acid composition by the half-seed technique, as described below. The F2 half-seeds were germinated and the corresponding plants were grown in the field in 2005 and self-fertilized as described above to produce the F3 seed. Since *ms* alleles determining NMS were segregating in these populations, male fertility/sterility was recorded for each F2 plant. Additionally, male-sterile plants were testcrossed with plants of CR-6 (for the CL-1 × CR-6 cross) or CR-9 (for the CL-1 × CR-9 cross) in order to determine their genotype for oleic acid content. From 24 to 48 F3 seeds from each male-fertile F2 plant and from 24 to 48 testcross seeds from each male-sterile F2 plant were analysed to determine presence or absence of segregation for oleic acid content. F2 plants were classified as *OlOl* if they had a low oleic acid content in their respective F2 half-seed and also showed uniformly low oleic acid levels in F3 or testcross seeds, *Olol* if their F3 or testcross seeds segregated for low and high oleic acid content, and *olol* if both the F2 half-seed and the F3 or testcross seeds showed high oleic acid content. Male-fertile F2 plants not producing a minimum of 24 F3 seeds for fatty acid analyses (46 out of 97 in the CL-1 × CR-6 population, and 13 out of 79 in the CL-1 × CR-9 population) were classified as *Ol_* if oleic acid content in the F2 half-seed was <30% and *olol* if oleic acid content in the F2 half-seed was >75%. For the *Ms* gene, F2 plants were classified as *Ms_* if they were male-fertile, and *msms* if they were male-sterile.

The fatty acid composition of the oil in half-seeds was analyzed by simultaneous extraction and methylation of the fatty acids (Garcés and Mancha 1993) followed by gas–liquid chromatography (GLC) using a Perkin-Elmer Autosystem gas–liquid chromatograph (Perkin-Elmer Corporation, Norwalk, CT, USA). A 2-m-long column packed with 3% SP-2310/2% SP- 2300 on Chromosorb WAW (Supelco Inc., Bellefonte, PA, USA) was used. The oven, injector, and flame ionization detector were held at 198, 275, and 250°C, respectively.

For DNA extraction, ten fully expanded leaves were cut from individual F2 plants from the CL-1 × CR-6 and CL-1 × CR-9 populations and frozen at −80°C. The leaf tissue was lyophilised and ground to a fine powder in a laboratory mill. DNA was isolated from ground leaf tissue from each F2 plant using a modified version of the protocol described by Rogers and Bendich (1985). DNA was also isolated from five plants of the CL-1, CR-6, and CR-9 parental lines.

Molecular analysis of the CL-1 × CR-9 population

The CL-1 × CR-9 population was used to map the *Ol* gene and to identify molecular markers linked to modifying genes affecting its expression. A genetic linkage map from this population was constructed using random amplified polymorphic DNA (RAPD), SCAR, and SSR markers. For RAPD analysis, 122 RAPD primers (Operon Technologies, Alameda, CA, USA; complete kits G, H, L, M, and S, and two to six primers from kits A, B, F, J, and AA) and 313 pairwise combinations of these RAPD primers were surveyed in two replicate samples of the parental lines CL-1 and CR-9 and four F2 individuals. PCR mixture composition and reaction conditions were as described by Hamdan et al. (2008). Eighty primers revealing intense and clearly scorable polymorphic bands were chosen for the analysis of the CL-1 × CR-9 F2 population. Additionally, five SCAR markers developed in a previous study by Hamdan et al. (2008) linked to the *Li* gene determining very high linoleic acid content were screened in the parental lines CL-1

and CR-9, according to the protocols reported by Hamdan et al. (2008). Three of them (IASCA-39, IASCA-42, and IASCA-45) were polymorphic and mapped in the CL-1 × CR-9 population.

For SSR analysis, 109 genomic safflower SSR primers developed by Hamdan et al. (2011) (prefix CAT), 104 EST (expressed sequence tag)-based SSR primers reported by Chapman et al. (2009) (prefixes CT1 and EL), 112 EST-based and genomic safflower SSR primers developed and mapped by Mayerhofer et al. (2010) (prefixes ct, gd, and VL), and two EST-based *Carthamus maculosa* SSR primers (prefix cm) were screened in two replicate samples of the parental lines and four F2 individuals. The PCR reaction mixture (30 µl) contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen/Life Technologies, Carlsbad, CA, USA), 0.3 µM of primers, 0.7 U of *Taq* DNA polymerase (BioTaq™ DNA Polymerase, Bioline, London, UK), and 50 ng of template DNA. To reduce non-specific amplification, touchdown PCR was used with an initial denaturation at 94°C for 2 min, followed by one cycle of 94°C for 30 s, final annealing temperature (T_A) +10°C for 30 s, and 72°C for 30 s. The annealing temperature was decreased by 1°C per cycle during each of the nine following cycles, at which time the products were amplified for 32 cycles at 94°C for 30 s, T_A for 30 s, and 72°C for 30 s with a final extension of 20 min at 72°C. Final annealing temperatures varied from 46 to 62°C. Amplified products were separated on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1 × TBE buffer with ethidium bromide incorporated in the gel. A total of 60 SSR primers revealing clear polymorphisms were genotyped in the CL-1 × CR-9 F2 population.

For all RAPD, SSR, and SCAR loci, Chi-squared analyses were carried out on each locus to detect deviations from the expected Mendelian ratios for co-dominant (1:2:1) or dominant (3:1) markers. A RAPD–SCAR–SSR linkage map was constructed using the software MAPMAKER/EXP version 3.0b (Whitehead Institute, Cambridge, MA, USA) (Lander et al. 1987). Two-point analysis was used to identify linkage groups (LGs) at a LOD score of 4 and a maximum recombination frequency of 0.40. Three-point and multi-point analyses were used to determine the order and interval distances between the markers in each LG. The Haldane mapping function was used to compute the map distances in centiMorgans (cM) from the recombination fractions. Multiple loci detected by a single probe were coded with the probe name plus the suffix "a", "b" or "c". Linkage group maps were drawn using the MapChart software (Voorrips 2002). Nomenclature for the linkage groups followed, when possible, that of Mayerhofer et al. (2010). The *Ol* and *Ms* genes were mapped in the genetic linkage map using their segregation data. The genotypes for the *Ol* and *Ms* genes were inferred from oleic acid and male-sterility phenotypes, respectively, as described above. Two-point analysis was used to group the marker loci, *Ol*, and *Ms* with a LOD threshold of 6 and a maximum recombination fraction of 0.4 as linkage criteria. The significance of each marker's association with the phenotypic trait was determined by one-way analysis of variance (ANOVA) using the statistical package IBM SPSS Statistics version 19.0 (IBM Corp., Armonk, NY, USA), with marker genotypes being classes.

Composite interval mapping (CIM) (Zeng 1994) was used to scan the genome for quantitative trait loci (QTL) affecting oleic acid content, in order to strengthen and corroborate the *Ol* mapping results, evaluate the existence of additional QTL, and estimate the interaction and global effect of all the detected QTL. Phenotypic data consisted of the oleic acid content of each F2 half-seed and also the oleic acid content of the corresponding F2:3 family in the case of male-fertile F2 plants (average oleic acid content from 24–48 F3 seeds from self-fertilized F2 plants). Since minor or modifying genes are not detectable when the major gen *Ol* is segregating in the population, QTL analyses were also performed on a high oleic F3 subpopulation (F3 seeds classified as *olol*) to scan the genome for minor QTL without the highly significant effect of the *Ol* gene. This subpopulation contained F2:3 families with uniformly high oleic acid content coming only from *olol* F2 genotypes, and F2:3 families segregating for low and high oleic acid coming only from *Olol* genotypes. For the latter, only the phenotypic data of the high oleic acid class were used, since the analysis of F3 seeds from these *Olol* F2 genotypes revealed clear bimodal distributions in which the high oleic acid class could be clearly identified. The analyses were carried out by using the average oleic acid content, the minimum oleic acid content, and the maximum oleic acid content within

the high oleic acid class (*olol* F3 genotypes) in each *Olol* and *olol* F2:3 family.

CIM was performed first with the marker closest to a highly significant oleic acid QTL, detected by initial simple interval mapping analyses, as a co-factor. If new QTL were found, the whole genome was re-scanned including as co-factor the marker locus closest to the new QTL. Genome-wide threshold values ($\alpha = 0.05$) for declaring the presence of QTL were estimated from 1,000 permutations of each phenotypic trait (Churchill and Doerge 1994). The thresholds of the LOD score were 2.66 for the F2 oleic acid content, 2.63 for the average F3 oleic acid content (whole population), 2.52 for the average F3 oleic acid content in the high oleic acid class (*Olol* + *olol* subpopulation), 2.64 for the minimum F3 oleic acid content in the high oleic acid class (*Olol* + *olol* subpopulation), and 2.72 for the maximum F3 oleic acid content in the high oleic acid class (*Olol* + *olol* subpopulation). Estimates of QTL positions were obtained at the point where the LOD score assumes its maximum in the region under consideration. One-LOD support limits for the position of each QTL were also calculated (Bohn et al. 1996). The proportion of phenotypic variance explained by each individual QTL was calculated as the square of the partial correlation coefficient (R^2). Estimates of the additive (a_i) and dominance (d_i) effects were computed following Falconer (1989). The software package used to perform CIM was PLABQTL 1.2 (Utz and Melchinger 1996).

Finally, those RAPD fragments identified by CIM as associated with oleic acid QTL were transformed into more consistent SCAR markers. RAPD fragments amplified from the wild-type (CL-1) or the very high oleic acid parental line (CR-9) were separated on a 1.5% agarose gel before being excised and purified by means of the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The purified products were ligated in a T/A vector (pCR2.1) and cloned with the TOPO-TA cloning kit (Invitrogen/Life Technologies) as described by the manufacturer. Transformed *E. coli* were plated on selective media containing ampicillin and X-gal. Five white colonies per marker were picked from the plate and cultured overnight. The QIAprep Spin Miniprep Kit (Qiagen) was used for plasmid DNA extraction. PCR with M13 forward and reverse vector primers was performed to confirm the presence and size of the insert. Sequencing in both forward and reverse orientations of the cloned fragments (three clones per marker) was carried out at GATC Biotechnology (Konstanz, Germany) using the M13 forward and reverse sequencing primers. Sequence analysis was conducted with the aid of the software Vector NTI Advance 10.3.0 (Invitrogen/Life Technologies). The consensus sequence for the three clones sequenced for each RAPD fragment was used to design the SCAR markers. A first set of primer pairs (F1 and R1) was designed containing the original 10 bases of the RAPD primer plus the next 8–15 internal bases. A second set of primer pairs (F2 and R2) located more internally was designed with the software Vector NTI Advance 10.3.0. SCAR-PCR amplifications were carried out as described by Hamdan et al. (2008), adjusting annealing temperatures according to the primer-specific T_m. SCAR markers were genotyped in the F2 CL-1 × CR-9 individuals, mapped, and CIM analyses were re-run as described above. This final QTL analysis with the new SCAR markers included in the map is the one described in the Results section.

Molecular analysis of the CL-1 × CR-6 population

The CL-1 × CR-6 population was used to confirm the effect of the *Ol* gene and its map position. All SCAR and SSR markers linked to *Ol* in the CL-1 × CR-9 population were screened for polymorphisms in duplicated samples of the parental lines and four F2 individuals of the CL-1 × CR-6 population. PCR mixture composition and reaction conditions were as described above. Since two SCAR markers flanking the *Ol* gene were not polymorphic, their corresponding RAPD markers were tested for polymorphisms. RAPD reactions were also as described above. Three SCAR, three SSR, and one RAPD primers revealing polymorphisms were genotyped in the CL-1 × CR-6 F_2 population. Linkage analysis was run with MAPMAKER using segregation data for RAPD, SCAR, and SSR marker loci, as well as for the *Ol* and *Ms* loci. The genotypes for the *Ol* and *Ms* genes were inferred from oleic acid and male-sterility phenotypes, respectively, as described above. Two-point analysis was used to group the SCAR and SSR marker loci, *Ol*, and *Ms* with a LOD threshold of 3 and a maximum recombination fraction of 0.40. Multi-point analysis

was used to determine the order and interval distances between the markers. Recombination fractions were converted to cM using the Haldane mapping function. The significance of each marker's association with the phenotypic trait was determined by one-way ANOVA as described above.

Candidate gene analysis

The *FAD2-1* gene has been shown to be the candidate gene for high oleic acid content in other oilseed crops, since mutations in this gene disrupt the synthesis of linoleic acid and cause a build-up of oleic acid in the seeds (Pérez-Vich et al. 2002; Schuppert et al. 2006; Lacombe et al. 2009; Hu et al. 2006; Falentin et al. 2007). Twenty primers (10 forward and 10 reverse; Table 1) based on the safflower *FAD2-1* sequence (GenBank accesion no. HM165274) were designed and screened for polymorphisms in the parental lines CL-1, CR-6, and CR-9. Since no polymorphisms were found, other parental lines from mapping populations other than CL-1 × CR-9 and CL-1 × CR-6 from our safflower breeding program were screened for polymorphisms. Parental lines from an F2 population segregating for palmitic acid content (CL-1 × CR-50; Hamdan et al. 2009b) showed a robust one-band co-dominant polymorphism for HM165274-based primers and accordingly this population (145 F2 individuals) was used to map the *FAD2-1* gene. All polymorphic SSR and SCAR markers from the LG in which the *Ol* gene was mapped were also genotyped in the CL-1 × CR-50 population.

Since the three mapping populations CL-1 × CR-9, CL-1 × CR-6, and CL-1 × CR-50 shared CL-1 as a parent, identical alleles among the three populations could be identified. A consensus map for the three populations was made using the common markers mapped in these populations as anchor loci, i.e. a common data file for the three populations was constructed in which markers segregating in only one population were coded as missing data in the others. Two-point analysis was used to group all marker loci with a LOD threshold of 6 and a maximum recombination fraction of 0.40. Three-point and multi-point analyses were used to determine the order and interval distances between the markers. Recombination fractions were converted to cM using the Haldane mapping function.

Results

Phenotypic segregations

Oleic acid content in F2 seeds from the cross CL-1 × CR-6 followed a bimodal distribution, with a low–intermediate oleic acid class ranging from 12.9 to 33.3% ($n = 94$) and a high oleic acid class ranging from 77.7 to 81.5% ($n = 25$), which fitted the expected 3:1 (low–intermediate:high oleic acid content) ratio ($\chi^2 = 1.01$, $p = 0.31$) corresponding to the segregation of the recessive *ol* alleles. Ninety-seven F2 plants were male-fertile and 22 F2 plants were male-sterile, which fitted the expected 3:1 (male-fertile:male-sterile) ratio ($\chi^2 = 2.69$, $p = 0.10$) for the segregation of the recessive *ms* alleles. The analysis of the F3 seed generation (Fig. 1) uncovered the three genotypic classes *OlOl*, with uniformly low oleic acid content ($n = 10$), *Olol*, segregating for oleic acid content ($n = 27$), and *olol*, with uniformly high oleic acid content ($n = 14$), which did not differ significantly from an 1:2:1 (*OlOl*:*Olol*:*olol*) ratio ($\chi^2 = 0.80$, $p = 0.67$).

The analysis of F2 seeds from the cross CL-1 × CR-9 also revealed a bimodal distribution with 88 individuals with low–intermediate oleic acid content (10.4–30.9%) and 28 individuals with high oleic acid content (82.5–87.2%), which fitted a 3:1 ratio ($\chi^2 = 0.05$, $p = 0.83$). Seventy-nine F2 plants were male-fertile and 37 F2 plants were male-sterile, which fitted the expected 3:1 ratio ($\chi^2 = 2.94$, $p = 0.09$). The analysis of the F3 seed generation (Fig. 1) allowed the classification of F2 individuals within genotypic classes *OlOl*, with uniformly low oleic acid content ($n = 15$), *Olol*, segregating for oleic acid content ($n = 35$), and *olol*, with uniformly high oleic acid content ($n = 16$), which fitted a 1:2:1 (*OlOl*:*Olol*:*olol*) ratio ($\chi^2 = 0.27$, $p = 0.87$).

Oleic acid content in F3 seeds classified as *olol*, including F3 seeds from both *olol* and *Olol* F2 genotypes, ranged from 58.1 to 83.8% in the cross CL-1 × CR-6 and from 59.4 to 88.9% in the cross CL-1 × CR-9. Oleic acid content in the parents grown in the same environment ranged from 78.7 to 83.4% in CR-6 and from 84.0 to 88.9% in CR-9.

CL-1 × CR-9 linkage map construction

A preliminary RAPD–SCAR–SSR map was constructed with 172 RAPD, SCAR, and SSR polymorphic

Table 1 Primer sequences and their melting temperature (T_m) for SCAR (prefix IASCA) markers linked to oleic acid QTL and HM165274-based safflower *FAD2-1* sequences (prefix CAR_FAD2-1)

Marker name	RAPD fragment	Primer	Primer sequence (5′ to 3′)	T_m
IASCA-73	$OPG2_{1730}$	F1	GGCACTGAGGGAGATAGATACATA	59
		R1	GGCACTGAGGCAACCAATAT	60
		F2	GTTTTTTAGTTTCGGATTGG	54
		R2	GCAACAGATGCATTATGTCT	54
IASCA-74	$OPL4\text{-}OPB5_{150}$	F1	TGCGCCCTTCCTTTAACG	62
		R1	GACTGCACACACTCATCTCTCGC	65
		F2	ACAAGATTTAACCAACAATGGG	58
		R2	GGTTTTTTTCGGCAAATGAA	60
CAR_FAD2-1		F96	CGGAGAGGAAGAAGCACT	55
		F97	GCAAGATGGGAGGAGGAGGGTGTATG	70
		F98	AGCCATTCCTTCCCACTGTTTCAA	67
		F99	CCTTCGTCCTCTACTACCTTGCCACC	68
		F100	GTAGCCCACGAATGCGG	61
		F101	GTCATCGGCGCCACCACTCG	72
		F102	AGCCTGTTCGCCACTCTCACTCTCGG	73
		F103	TGTCGTTCATCCTTTACCGTGTTGCG	71
		F104	TCGGAATGGGACTGGCTGAGAGGAG	72
		F105	TGGTGCACCATCTGTTCTCG	63
		R106	GTCCCTTCCATTCACACTAACAA	60
		R107	AGCACACCACATCACAACTTTACTTA	61
		R108	CCAGCAACCCCTTCACCGCCCT	74.
		R109	GTCGCCAACGCTCCTCTCAG	66
		R110	ATACACACAGACCACCCAGCTCACGC	71
		R111	GGGAGCATAGTGGCAGGCGAAACGG	75
		R112	GGGAGTTTTGATCTGGGCTTTGGGACA	73
		R113	GTAAAAAGGAGTGGACTATGAAGCCC	63
		R114	AGGGGTTGGGAGACGGTGGATGTAGG	73
		R115	AGGAATGGCTTGCTTGATGTCACTG	68

The original RAPD fragments (primer name_fragment size) from the SCARs are indicated. The *underlined* nucleotides are derived from RAPD primers

markers. After removal of closely linked RAPD loci, the final CL-1 × CR-9 linkage map comprised 116 marker loci. Of these, 47 were RAPD, 60 were SSR, four were SCAR developed by Hamdan et al. (2008) linked to the *Li* gene (IASCA-39, IASCA-42, IASCA-44, and IASCA-45), three were SCAR newly developed in this study presumably linked to oleic acid QTL (IASCA-73, IASCA-75, and IASCA-76), one was a sunflower InDel marker (prefix IN), and one was a phenotypic marker corresponding to the *Ms* gene determining nuclear male sterility mapped as a Mendelian trait. Of the 60 SSR included in the map, 26 of them had been mapped previously by Mayerhofer et al. (2010) whereas 34 of them were mapped for the first time in this study. The linkage map spanned a distance of 816.4 cM with an average marker interval of 7.8 cM (Fig. 2). The loci coalesced into 15 linkage groups, of which eight could be matched to those described by Mayerhofer et al. (2010) based on at least two shared marker loci (Fig. 2), and four were tentatively matched to those of Mayerhofer et al. (2010) based on one marker locus (Fig. 2). The remaining three LGs which did not show any shared marker loci were named as LGs Tx, Ty, and Tz. Linkage group T4a of Mayerhofer et al. (2010) was split into two LGs in our map (LG T4a_I and LG T4a_II) and LGs T10a and T10b of Mayerhofer et al. (2010) were also maintained as two independent LGs in our map (Fig. 2). The linkage groups ranged in genetic length from 5.1 to 192.5 cM and comprised 2–23 marker loci (Fig. 2). 99.7% of the mapped genome was within 20 cM of the

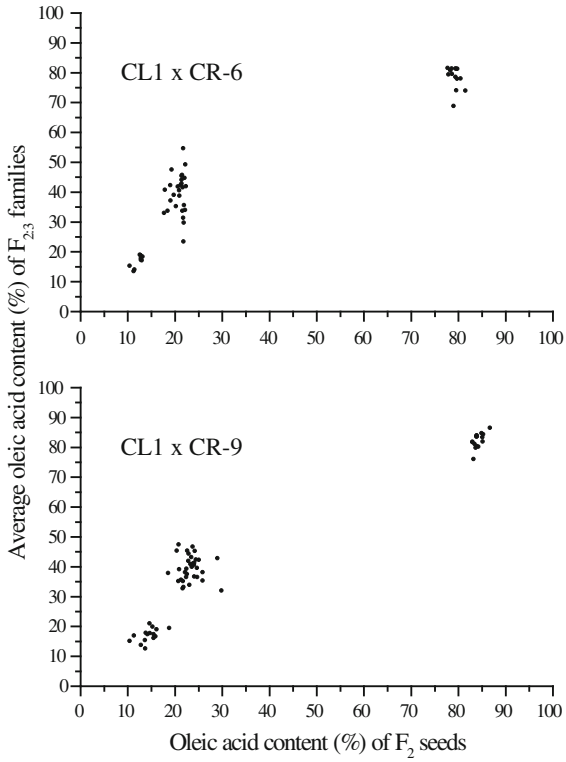

Fig. 1 Oleic acid content (% of total fatty acids) in individual F2 half-seeds and their corresponding F2 plants (F3 seeds averaged) in a population of 51 male-fertile F2 plants from the cross between the nuclear male-sterile safflower line CL-1, with wild-type oleic acid content, and the line CR-6, with high oleic acid content (>75%), and in a population of 66 male-fertile F2 plants from the cross CL-1 × CR-9, with very high oleic acid content (>84%)

nearest marker. None of the RAPD, SSR, or SCAR loci deviated significantly from the expected segregation ratios ($p < 0.001$).

Ol mapping in the CL-1 × CR-9 and the CL-1 × CR-6 populations

The *Ol* gene was mapped to LG T3 in the CL-1 × CR-9 population (Fig. 3a); the *Ms* gene was also mapped to the same LG (Fig. 3a). Initially, the *Ol* gene was flanked by the SCAR marker IASCA-45 developed by Hamdan et al. (2008) and the RAPD fragments OPG2 and OPL4-OPB5 of approximately 1,730 and 150 bp, respectively. The $OPG2_{1730}$ and $OPL4\text{-}OPB5_{150}$ RAPD fragments were transformed into the SCAR markers IASCA-73 and IASCA-74, respectively (Table 1). IASCA-74 (F1/R1 and F2/R2) was not polymorphic in the CL-1 × CR-9 population. IASCA-73 gave a co-dominant amplification pattern. The final LG T3 containing the *Ol* gene spanned 192.5 cM and contained 24 loci, including the phenotypic loci *Ol* and *Ms*, five SCAR, one sunflower InDel, eight RAPD, and eight SSR marker loci (Fig. 3a). From these SSR, three of them were previously mapped to LG T3 by Mayerhofer et al. (2010), one of them (ct309) was mapped by the same authors to LG T2, and the other four SSR loci were mapped for the first time in the present study (Fig. 3a). The *Ol* locus mapped between the SSR marker ct365 and the SCAR marker IASCA-73, at genetic distances of 0.4 and 39.1 cM, respectively. The *Ms* locus mapped between the SSR markers CAT83 (8.3 cM) and EL375341 (12.1 cM), and the genetic distance between *Ms* and *Ol* was 65.3 cM (Fig. 3a). ANOVA analyses with genotypes at the ct365 marker locus used as classes revealed significant ($p < 0.0001$) differences between the marker class means for oleic acid content at the F2 and the F3 generations (Table 2).

Once the *Ol* gene was mapped in the CL-1 × CR-9 population, all SCAR and SSR markers from the *Ol* linkage group (LG T3) were tested for polymorphisms in the CL-1 × CR-6 population. The SCAR markers IASCA-39, IASCA-42, and IASCA-45, and the SSR markers CAT26, ct365, and ct599 were polymorphic and genotyped in the CL-1 × CR-6 population. Since the SCAR markers IASCA-73 and IASCA-74 linked to the *Ol* gene were not polymorphic, their corresponding RAPD markers OPG2 and OPL4-OPB5 were tested for polymorphisms. A polymorphic 150-bp band from the OPL4-OPB5 RAPD marker was genotyped in CL-1 × CR-6. The *Ol* locus mapped between the SSR marker loci ct365 and ct599 at genetic distances of 2.2 and 57 cM (Fig. 3b). The *Ms* locus was also mapped to this LG and was flanked by the SCAR marker locus IASCA-39 and the SSR marker locus CAT26. The *Ol* locus mapped 63.8 cM from the *Ms* locus (Fig. 3b). ANOVA analyses based on F2 and F3 data also revealed a significant association ($p < 0.0001$) of the markers flanking the *Ol* gene with oleic acid content. Results for marker ct365 are shown in Table 2.

QTL analyses in the CL-1 × CR-9 population

One major QTL (*Ol3.1*) affecting F2 and F3 oleic acid content was found on LG T3, explaining 99.4 and

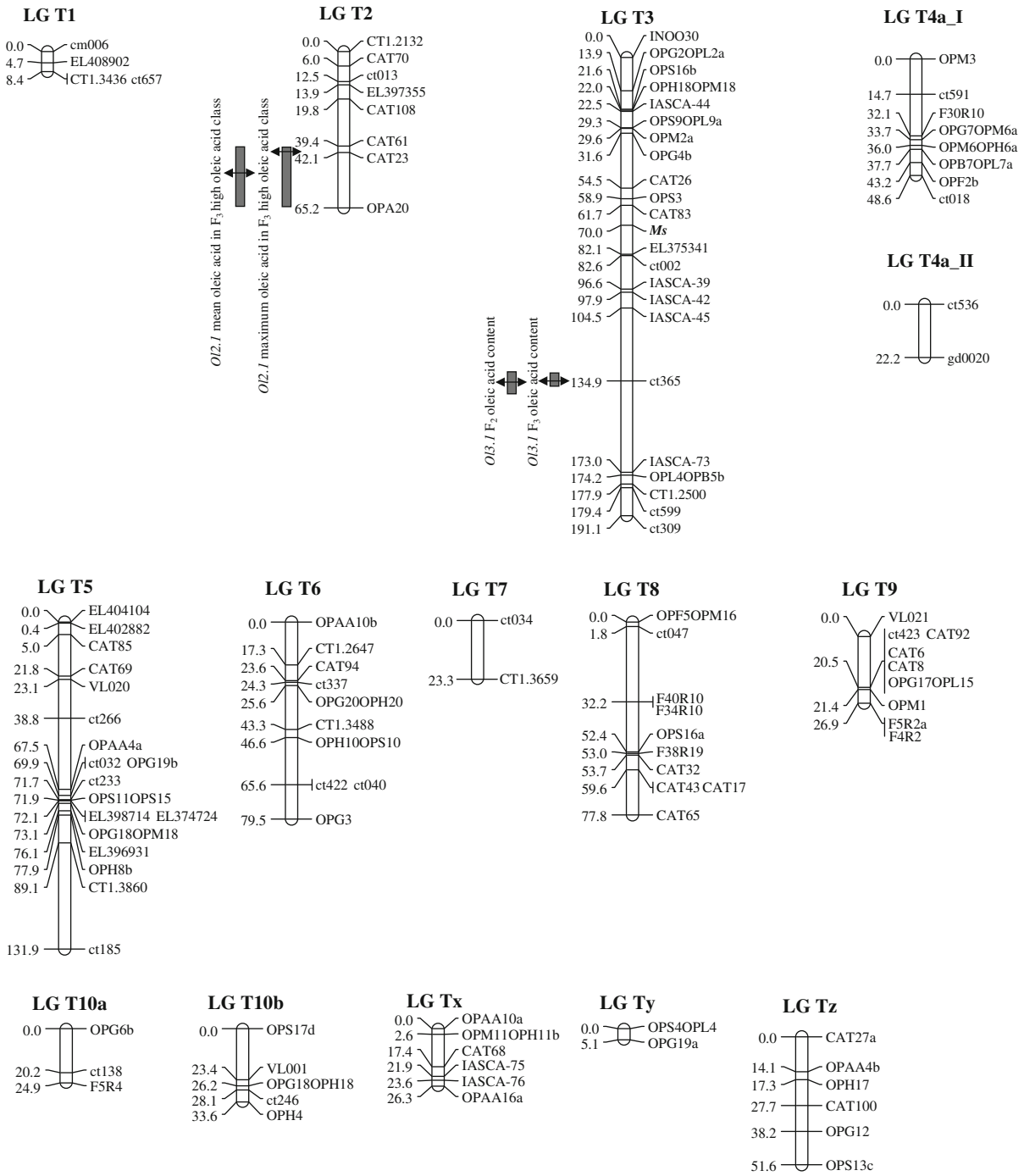

Fig. 2 Genetic linkage map of the CL-1 × CR-9 population. Linkage groups (*LG*) T1–T10 follow the nomenclature of Mayerhofer et al. (2010). Genetic distances are given in centiMorgans (Haldane) on the *left* of each LG. QTL locations for F2 and F3 seed oleic acid content (complete population), and for the mean and maximum oleic acid content of the high oleic acid class in the F3 (subpopulation *Olol* + *olol*) are indicated by *arrows* at the point where the LOD score reaches its maximum and by *boxes* corresponding to 1-LOD support intervals. The QTL names and their associated traits, as indicated in Table 3, are shown at the *left* of the QTL boxes. The position of the *Ms* gene determining nuclear male sterility mapped as Mendelian trait is also indicated in LG T3. Marker loci are labeled as follows: prefixes OP and F are RAPD marker loci, prefixes CAT, CT1, EL, ct, gd, VL, and cm are SSR marker loci, prefix IASCA are SCAR marker loci, and prefix IN are InDel marker loci

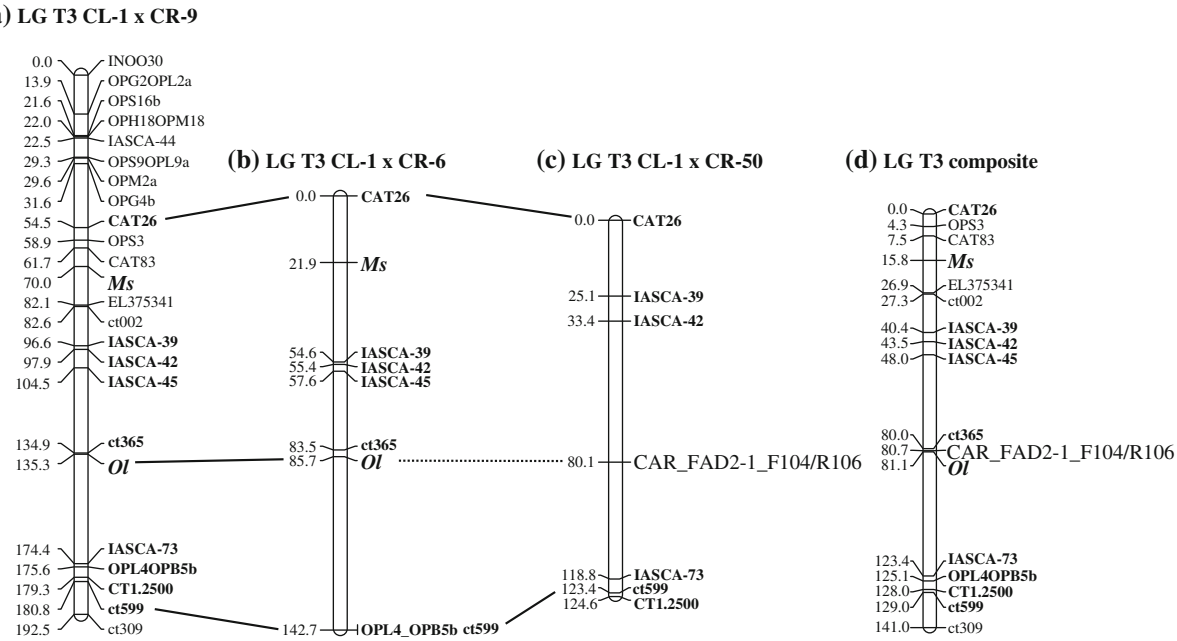

Fig. 3 Genetic mapping of the *Ol* gene for high oleic acid content, the *Ms* gene for nuclear male sterility, and the *FAD2-1* (microsomal oleoyl phosphatidylcholine desaturase) gene. **a** Genetic mapping of *Ol* and *Ms* in CL-1 × CR-9; **b** genetic mapping of *Ol* and *Ms* in CL-1 × CR-6; **c** genetic mapping of *FAD2-1* in CL-1 × CR-50; **d** consensus map for the three CL-1 × CR-9, CL-1 × CR-6, and CL-1 × CR-50 LG T3 maps. The cumulative distances in centiMorgans (Haldane) are shown at the *left* of each map. SSR and SCAR marker loci shared by at least two of the populations are highlighted in *bold*

Table 2 Association between the most significant marker loci close to oleic acid QTL on LG T3 (QTL underlying the *Ol* gene) and on LG T2 and oleic acid content (% of the total fatty acids) determined by variance analysis in the CL-1 × CR-9 and CL-1 × CR-6 populations

Marker, generation and trait	LG	No. of individuals within each marker class			Mean ± SD for oleic acid within each marker class			ANOVA analysis	
		A	H	B	A	H	B	*F*	*p*
CL-1 × CR-9 cross									
Complete population									
ct365_F2 Total mean oleic acid	T3	28	60	28	13.8 ± 1.71	23.0 ± 2.71	84.2 ± 1.21	8992.0	<0.0001
ct365_F3 Total mean oleic acid	T3	14	36	16	17.2 ± 2.31	38.9 ± 5.72	82.3 ± 2.49	827.5	<0.0001
Subpopulation Olol + olol									
CAT23_F3 Mean high oleic acid class	T2	16	22	10	81.0 ± 2.9	82.9 ± 1.4	83.6 ± 2.2	5.2	0.009
CAT23_F3 Maximum high oleic acid class	T2	16	22	10	84.8 ± 1.2	86.2 ± 1.4	86.4 ± 1.7	5.6	0.007
CL-1 × CR-6 cross									
ct365_F2 Total mean oleic acid	T3	32	59	23	14.8 ± 11.9	20.8 ± 2.8	76.4 ± 14.1	370.0	<0.0001
ct365_ F3 Total mean oleic acid	T3	10	26	13	17.0 ± 2.0	39.1 ± 6.1	78.8 ± 3.6	486.0	<0.0001

Mean oleic acid ± standard deviation (SD) are presented in different genotypic classes: *A* homozygous for the allele derived from CL-1, *B* homozygous for the allele derived from CR-9 or CR-6, *H* heterozygous

LG linkage group

Table 3 QTL affecting oleic acid content in the F2 and F3 generations from the cross CL-1 × CR-9

Generation and trait	LG	QTL	Absolute position on LG (cM)	One-LOD support interval (cM)	Left–right locus[a]	LOD score	R^2 (%)	Gene effects[b]	
								a	*d*
Complete population									
F2 oleic acid	T3	*Ol3.1*	135	134-136	ct365-IASCA-73	132.2	99.4	35.3**	-26.1**
F3 oleic acid	T3	*Ol3.1*	136	132-139	ct365-IASCA-73	47.3	96.4	33.2**	-11.1**
Subpopulation Olol + olol									
F3 oleic acid–Mean high oleic acid class	T2	*Ol2.1*	57	39-65	CAT23-OPA20	2.5	20.9	1.6*	0.6
F3 oleic acid–Maximum high oleic acid class	T2	*Ol2.1*	40	39-65	CAT61-CAT23	3.5	27.5	0.8**	0.3

LG linkage group

** Significant at the 0.01; * 0.05 probability level

[a] Loci flanking the likelihood peak for a putative QTL. The IASCA prefix denotes SCAR marker loci, the CAT and ct prefixes denotes SSR marker loci, and the OP prefix denotes RAPD marker loci

[b] *a* = additive effect: a positive sign means an increase of the mean value of the trait due to CR-9 alleles; *d* = dominant effect: a positive sign means dominance for higher values of the trait

96.3% of the phenotypic variation in the F2 and F3 oleic acid concentrations, respectively (Table 3). Its position corresponded to that of the *Ol* gene (Table 3; Figs. 2, 3a). The CR-9 allele at this QTL increased oleic acid content (Table 3). The *Ol3.1* QTL also showed a significant effect on palmitic acid, stearic acid, and linoleic acid, which was directly related to the correlation found between oleic acid and these other three fatty acids (Table 4).

As expected, no effect of the *Ol* major QTL on LG T3 was detected when the subpopulation of *Olol* and *olol* F2 plants was analysed using the phenotypic data of mean and maximum oleic acid content in F3 seeds of the high oleic acid class of each *Olol* and *olol* F2:3 family. However, a QTL with a minor effect (*Ol2.1*) on oleic acid content was identified on LG T2 (peak LOD 2.54 for the mean oleic acid content of the high oleic acid class and 3.49 for the maximum oleic acid content of the high oleic acid class) (Table 3; Fig. 2). This QTL explained 20.9% of the variation for the mean oleic acid content of the high oleic acid class, and 27.5% of the variation for maximum oleic acid content of the high oleic acid class (Table 3). The CR-9 allele at this QTL increased oleic acid content (Table 3). The mean oleic acid content in the high oleic acid class from plants with the CL-1 allele on the *Ol2.1* closest marker (CAT23) was 81.0%, whereas the mean oleic acid content from plants with the CR-9 allele was 83.6% (Table 2). No QTL with significant effect on oleic acid content were found when the QTL analyses were carried out using the minimum oleic acid content within the high oleic acid class.

Table 4 Correlation coefficients between palmitic acid, stearic acid, oleic acid, and linoleic acid content in the F2 generation from the cross CL-1 × CR-9

	Correlation coefficients		
	Stearic acid	Oleic acid	Linoleic acid
Palmitic acid	0.23*	−0.84**	0.84**
Stearic acid		−0.46**	0.46**
Oleic acid			−0.99**

** Significant at the 0.01; * 0.05 probability level

Candidate gene mapping

Markers based on the safflower *FAD2-1* sequence (GenBank accesion no. HM165274) were not polymorphic among the parental lines CL-1, CR-6, and CR-9. A primer combination (CAR_FAD2-1_F104 and CAR_FAD2-1_R106, Table 1) showed a one-locus co-dominant polymorphism between the parental lines CL-1 and CR-50 from an F2 population of 145

individuals segregating for palmitic acid content (Hamdan et al. 2009b). This primer combination, together with all SSR and SCAR markers from LG T3 showing polymorphism, was genotyped in the CL-1 × CR-50 population. Using a LOD threshold of 4.8 and a maximum recombination frequency of 0.45, a linkage map comprising six SSR marker loci and the CAR_FAD2-1_F104/R106 marker locus was constructed (Fig. 3c). The CAR_FAD2-1_F104/R106 marker locus mapped between markers IASCA-42 and IASCA-73 (Fig. 3c).

The CL-1 × CR-9, CL-1 × CR-6, and CL-1 × CR-50 populations shared four SSR markers and four SCAR markers on LG T3 (Fig. 3), which allowed the construction of a composite map (Fig. 3d). This map was constructed including all markers in the region between the SSR marker loci CAT26 and ct309 (Fig. 3d). The consensus map contained 17 marker loci and spanned 142.6 cM (Fig. 3d). *Ol* mapped to a similar position in consensus and individual maps (Fig. 3). The CAR_FAD2-1_F104/R106 marker locus was located 0.4 cM from the *Ol* gene (Fig. 3d).

Discussion

Previous genetic studies indicated that high oleic acid content in safflower is mainly controlled by partially recessive alleles at the *Ol* gene (Knowles and Hill 1964; Hamdan et al. 2009a). This gene is present in a homozygous recessive condition in the safflower lines CR-6, with high oleic acid content (>75%), and CR-9, with very high oleic acid content (>84%), and has been mapped to LG T3 of the safflower genetic map. QTL analyses confirmed the position of the *Ol* gene on LG T3 and showed no evidence of additional major factors controlling the high oleic acid trait.

The *Ol* gene has been mapped tightly linked to an oleoyl-phosphatidyl choline desaturase *FAD2-1* locus. Three FAD2 family members (*FAD2-1*, *FAD2-2*, and *FAD2-3*) have been reported in safflower (Gene Bank accessions nos. HM165274, HQ179940, and HQ179941). From the existing FAD2 family members in other oilseed crops, *FAD2-1* is strongly expressed in developing seeds, whereas *FAD2-2*, and *FAD2-3* are weakly expressed in developing seeds and exhibit more a "housekeeping" pattern of expression (Hongtrakul et al. 1998; Martínez-Rivas et al. 2001). This pattern of expression has been recently confirmed for safflower *FAD2-1* and *FAD2-2*, but not for *FAD2-3*, which also shows a strong seed expression and does not belong to any of the "seed-type" or "housekeeping-type" plant FAD2 desaturases (Guan et al. 2011b). *FAD2-1* plays an essential role in linoleic acid synthesis in developing seeds, and, when mutated, disrupts the synthesis of linoleic acid, leading to a build-up of oleic acid in the seeds, as has been shown in different oilseed crops such as sunflower, canola, and soybean (Pérez-Vich et al. 2002; Schuppert et al. 2006; Hu et al. 2006; Falentin et al. 2007; Lacombe et al. 2009; Pham et al. 2010). Additionally, Guan et al. (2011a) have determined that the expression of *FAD2-1* in high oleic acid safflower genotypes is significantly lower than in standard (high linoleic) genotypes during seed development, and, when comparing the *FAD2-1* sequence between high oleic and standard genotypes, these authors found differences that might be related to an altered protein function. Results from this study and recent evidence on the role of *FAD2-1* in safflower (Guan et al., 2011a) strongly support that the *FAD2-1* gene is the locus underlying the major *Ol* gene in safflower.

Since alleles determining high oleic acid content are partially recessive, the heterozygote is not clearly distinguishable from the wild-type homozygote. This fact makes it difficult to select plants carrying the *ol* allele in backcross programs focused on the high oleic acid trait. The use of marker-assisted selection (MAS) can contribute to overcoming such a limitation. Molecular markers developed in this research will greatly support introgression of *ol* alleles into elite lines. *FAD2-1*-based markers are highly predictive for the phenotype and optimal for selection. Additionally, the linkage map in which the *Ol* gene has been integrated, with robust SCAR and SSR markers flanking the gene, is an efficient tool for selecting against donor parent alleles around this gene.

In this study, the *Ms* gene controlling nuclear male sterility was also mapped to LG T3 at a genetic distance to *Ol* of 63.8 cM in the CL-1 × CR-6 population and 65.3 cM in the CL-1 × CR-9 population. Linkage between the *Ms* and *Ol* genes was not detected previously in classical genetic studies (Hamdan et al. 2009a) because the genetic distance between both loci was beyond the 50 cM expected from independent assortment. Previous studies by Hamdan et al. (2008) mapped the *Ms* gene 31.9–27.4 cM downstream of the SCAR markers IASCA-45,

IASCA-42, and IASCA-39. In this study, the map position of the *Ms* gene in relation to these SCAR markers has been confirmed in two new mapping populations, and four new SSR markers (CAT26, CAT83, EL375341, and ct002) linked to the *Ms* gene have been identified. The major use of the *Ms* gene controlling nuclear male sterility in safflower breeding is the production of F1 hybrid seed, the development of testers for inbred line evaluation, and as a parent for genetic studies because of the tedious and time-consuming manual emasculation of safflower flowers (Singh and Nimbkar 2007). The use of MAS for the identification of lines carrying the male-sterile or male-fertile alleles would allow early removal of fertile types in crossing blocks and also avoid progeny testing in backcrossing programs, which will increase the efficiency of breeding procedures using the *Ms* gene for nuclear male sterility.

Besides the *Ms* gene, Hamdan et al. (2008) also reported the map position of the *Li* gene, determining very high linoleic acid content in safflower. This gene was linked to *Ms*, and was located in the IASCA-39-*Ms* interval. According to the results of the present study, the *Ol* and *Li* genes are expected to be located in the same linkage group at an estimated genetic distance of around 45–50 cM. In a classical genetic study, Futehally and Knowles (1981) found that the major recessive allele *li* controlling very high linoleic acid content was at a different locus from the *ol* allele governing high oleic acid levels, which has been demonstrated in this study using a molecular approach. However, Futehally and Knowles (1981) reported that the *Ol* and the *Li* genes were segregating independently. Similarly to the above discussion on the the *Ms–Ol* linkage, the genetic distance between *Ol* and *Li* on LG T3 is sufficiently large for the *Ol–Li* linkage to be detected using only phenotypic data on oleic acid and linoleic acid content.

In addition to the major locus *Ol*, Knowles (1972) postulated the possible existence of modifying genes affecting high oleic acid content in safflower. Modifying genes are defined as genes having no known effect except to intensify or diminish the expression of a major gene (Briggs and Knowles 1967). Hamdan et al. (2009a) determined that the very high oleic acid content in the safflower line CR-9 was produced by the combination of the *ol* alleles and modifying genes with a small positive effect on oleic acid content. These genes with a minor effect are difficult to characterize, since they are masked by the large effect of major genes. Pérez-Vich et al. (2004) demonstrated that the highly significant effect of macromutations significantly reduces the power of QTL analysis to identify QTL with smaller effects. To avoid this masking effect, these authors proposed the study of populations segregating only for genes with a minor effect, independently of the macromutation or major gene controlling the trait. Another approach is the one followed in the present research, i.e. to analyze the variation produced by modifying genes in a subpopulation that includes only those phenotypes that are fixed for the major gene and accordingly are expected to segregate only for modifying genes. This approach has allowed the identification and mapping of a modifying gene on LG T2 of the CL-1 × CR-9 population with an effect of further increasing oleic acid content in individuals homozygous for the *ol* allele. Despite Hamdan et al. (2009a) also describing modifying genes having an effect on lowering oleic acid content, we have not identified modifying genes affecting negatively oleic acid levels. The complex role of modifying genes has also been described in sunflower, where their number and mode of action on oleic acid expression is still a matter of controversy (Fernández-Martínez et al. 2004).

The key components required for an efficient system for molecular breeding are the identification and characterization of suitable genetic markers and the development of a reference genetic map using both an internationally accepted linkage group nomenclature system and publicly available markers, useful for cross-referencing maps and mapped gene locations. Although safflower is lagging behind other oilseed crops in terms of development of these necessary molecular breeding tools, the recent development of sequence-based markers (Chapman et al. 2009; Mayerhofer et al. 2010; Hamdan et al. 2011) and a first genetic linkage map for this species (Mayerhofer et al. 2010) has laid the basis for future genomic studies in this species. Since the safflower map from Mayerhofer et al. (2010) comprised 116 marker loci and was not saturated due to a limited polymorphism found within their mapping populations, the next short-term strategy for improving molecular breeding tools in safflower might be to add new mapped loci to this reference map. In this study, a total of 67 sequence-based markers (60 SSR and seven SCAR) have been mapped, 41 of them (34 SSR and 7

SCAR) being mapped for the first time, which increases the marker density of the safflower genetic map and improves its coverage.

Oils rich in oleic acid have important market niches for both food and non-food uses. Safflower cultivars with high oleic acid content are already available in the market and most breeding companies include high oleic acid as a major target trait in their breeding programs. The present research has shed light on the genetic basis underlying high oleic acid phenotypes, including the dominant role of the *FAD2-1* gene and the involvement of at least one modifying gene that contributes to a further increase of oleic acid content. Additionally, molecular markers for selecting for high oleic acid content have been developed, which will contribute to facilitating selection for this trait within breeding programs in which MAS is routinely used.

Acknowledgments Y.A.S. Hamdan was the recipient of a grant from the Agencia Española de Cooperación Internacional (AECI). The research was funded by Junta de Andalucía support program to research groups (PAI-AGR-118).

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