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ORIGINAL PAPER

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Molecular analysis of the high stearic acid content in sunflower mutant CAS-14

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Abstract Increasing the stearic acid content to improve sunflower (*Helianthus annuus* L.) oil quality is a desirable breeding objective for food-processing applications. CAS-14 is a sunflower mutant line with a high stearic acid content in its seed oil (>35% vs. <6% in currently grown sunflower hybrids), which is controlled by the Es3 gene. However, the expression of the high stearic acid character in CAS-14 is strongly influenced by temperature during seed maturation and it is not uniform along the seed. The objectives of this study were (1) to identify PCR-based molecular markers linked to the Es3 gene from CAS-14, (2) to map this gene on the sunflower genetic map, and (3) to characterize the interaction between CAS-14 and CAS-3, a sunflower high stearic acid (about 26%) mutant line with the Es1 and Es2 genes determining this trait. Two F_2 mapping populations were developed from crosses between CAS-14 and P21, a nuclear male sterile line with the Ms_{11} gene controlling this character, and between CAS-14 and CAS-3. One hundred and thirty-three individuals from P21×CAS-14, and 164 individuals from CAS-3×CAS-14 were phenotyped in F_2 and F_3 seed generations for fatty acid composition using gas–liquid chromatography, and they were then genotyped with microsatellite [simple sequence repeat (SSR)] and insertion–deletion (INDEL) markers. Bulk segregant analysis in the P21×CAS-14 population identified two markers on LG 8 putatively linked to Es3. A large linkage group was identified using additional markers mapping to LG 8. Es3 mapped to the distal half of LG 8 and was flanked by the SSR markers ORS243 and ORS1161 at genetic distances of 0.5, and 3.9 cM, respectively. The Ms_{11} gene was also mapped to LG 8 and genetic distance between this gene and Es3 was found to be 7.4 cM. In the CAS-3×CAS-14 population, two QTLs were identified on LG 1 and LG 8, which underlie the Es1 gene from CAS-3 and the Es3 gene from CAS-14, respectively. A significant epistatic interaction between these two QTLs was found. Results from this study provided a basis for determining CAS-14 efficient breeding strategies.

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Introduction

The value and utility of an oilseed crop for both nutritional and industrial purposes primarily depends upon the fatty acid composition of the seed oil. Sunflower (*Helianthus annuus* L.) mutant lines with a high stearic acid content (>25%, vs. <6% in currently grown sunflower hybrids) in its seed oil have been developed (Osorio et al. 1995; Fernández-Moya et al. 2002). The seed oil of these mutant lines is characterized by a high viscosity, forming a semisolid fat at room temperature. Consequently, this high stearic acid oil is demanded for producing a wide range of margarine and spread products without need for chemical transformations such as hydrogenation, which produces trans fatty acids that are expected to raise the risk of coronary heart disease (Katan 1998). Within these mutant lines, CAS-3 exhibited a high stearic acid content of 26% (Osorio et al. 1995), whereas CAS-14 showed a very high stearic acid content of 37% (Fernández-Moya et al. 2002).

CAS-3 has been characterized in great detail both under a genetic and a molecular point of view. Genetic studies concluded the presence of partially recessive alleles at the two independent loci Es1 and Es2 controlling the high stearic acid content in the seed oil of this mutant line (Pérez-Vich et al. 1999). Both loci exhibited a differential additive contribution to the high

stearic acid content of CAS-3 seeds, with *es*1 alleles contributing about 18.8% in homozygous condition, and *es*2 alleles contributing about 9.0% in homozygous condition (Pérez-Vich et al. 2004a). Candidate-gene and QTL analyses demonstrated that Es1 co-segregates with a stearoyl–acyl carrier protein (ACP) desaturase locus (SAD17A) and underlies the major QTL affecting the concentration of stearic acid in CAS-3. This QTL has been named *st1-SAD17A* and is located on linkage group (LG) 1 of the sunflower genetic map (Pérez-Vich et al. 2002). Later QTL studies carried out in populations derived from CAS-3 in which Es1 was not segregating, detected the existence of other three minor QTLS on LG 3, LG 11, and LG 13 of the sunflower genetic map associated to an increased stearic acid content (Pérez-Vich et al. 2004b). These QTL were named *st2.1* (LG 3), *st2.2* (LG 11), and *st2.3* (LG 13). The *st2.2* QTL on LG 11 co-located to other stearate desaturase locus (SAD6). Within these studies, molecular markers tightly linked to the QTL determining high stearic acid content were also identified (Pérez-Vich et al. 2002, 2004b). These studies have established the basis for breeding for high stearic acid content in sunflower using CAS-3 as the stearic acid source, using both classical and molecular marker breeding technologies.

Genetic characterization of CAS-14 has been carried out recently. Pérez-Vich et al. (2006) reported that the very high stearic acid content in this line is a recessive character determined by a single gene that segregates independently of the Es1 gene with a major effect on stearic acid content in CAS-3. This new gene was named Es3. Molecular characterization of CAS-14 has not been carried out. Contrarily to CAS-3, the high stearic acid content in the CAS-14 mutant is strongly influenced by the temperature during seed maturation, with average values ranging from 14% at 30/20°C (day/night) to 37% at 39/24°C (Fernández-Moya et al. 2002). Additionally, the high stearic acid content is not uniformly expressed in CAS-14 seeds, which exhibit a strong longitudinal gradient starting from the embryo (10%) up to the end of the cotyledon (35%) (Fernández-Moya et al. 2003), which represents a serious drawback for phenotypic selection (Velasco et al. 2004). The use of molecular-marker technologies would allow an efficient selection regardless of the environmental and positional effects. This paper reports the mapping of the very high stearic acid gene Es3 from CAS-14, the identification of PCR-based molecular markers linked to this gene, and the molecular characterization of the interaction between CAS-3 and CAS-14.

Materials and methods

Plant materials, mapping populations, and phenotypic evaluation

Three sunflower lines were used to generate the two F_2 mapping populations employed in this study: (1) CAS-14, a very high stearic acid mutant obtained by Fernández-Moya et al. (2002), (2) CAS-3, a high stearic acid mutant line obtained by Osorio et al. (1995), and (3) P21, a low stearic, nuclear-male sterile line released by the USDA and the Texas Agricultural Experiment Station in 1970 as a reselection from their 1968 released P21 *ms* (Jan 1992). Fatty acid composition of these parental lines is given in Table 1. The resulting F_2 populations from the crosses P21×CAS-14 (133 individuals), and CAS-3×CAS-14 (164 individuals), and the phenotypic segregation for stearic acid content, have been previously described by Pérez-Vich et al. (2006). The P21×CAS-14 population segregated for the Es3 gene and the Ms_{11} gene, which determines nuclear male sterility in P21. The CAS-3×CAS-14 population segregated for both the Es1 and the Es3 genes determining a high stearic acid content in sunflower seed oil (Pérez-Vich et al. 2006). The fatty acid composition of F_2 half-seeds was determined by gas–liquid chromatography as described by Pérez-Vich et al. (2006). F_2 half-seeds from P21×CAS-14, and from CAS-3×CAS-14 were planted. F_2 plants were grown and self-pollinated at the experimental farm of the Instituto de Agricultura Sostenible at Córdoba (Spain). In the P21×CAS-14 cross, about 25% of the F_2 plants were sterile due to the segregation of the Ms_{11} gene from P21. F_3 seeds from each individual fertile F_2 plant were obtained. Twenty-four to 48 F_3 half-seeds were analyzed for fatty acid composition from

Table 1 Fatty acid composition of the seed oil from the sunflower lines P21, CAS-14 and CAS-3, and the F_2 and the F_3 seed generations (average value of 24–48 F_3 seeds from F_2 individual plants) from crosses P21×CAS-14 and CAS-3×CAS-14

Parent or cross	Number of individuals	Fatty acid (% of the total oil fatty acids)[a]							
		16:0		18:0		18:1		18:2	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
P21	46	5.4	4.1–6.0	6.6	4.9–8.9	31.7	25.9–39.7	56.4	47.7–63.3
CAS-14	46	9.2	8.0–11.1	40.5	33.5–45.7	7.0	4.3–20.4	43.3	32.4–48.7
CAS-3	46	6.0	5.0–7.0	26.7	23.1–30.4	14.4	10.7–21.3	52.9	45.9–59.2
F_2 (P21×CAS-14)	133	5.9	4.6–8.9	9.6	2.4–41.4	40.5	9.6–61.6	44.1	28.4–61.0
F_3 (P21×CAS-14)	95	7.8	5.8–11.4	16.1	3.6–40.7	20.9	4.1–49.6	55.0	39.1–71.8
F_2 (CAS-3×CAS-14)	164	5.7	4.1–8.0	10.7	1.9–31.4	38.0	12.2–64.0	45.6	24.0–59.9
F_3 (CAS-3×CAS-14)	153	7.0	5.3–10.7	16.9	4.4–41.3	24.0	4.2–43.3	52.0	42.3–64.7

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, and 18:2 = linoleic acid

each F_2 plant. For the molecular marker analyses, two fully expanded leaves were cut from each of the F_2 plants from populations P21×CAS-14 and CAS-3×–CAS-14 and frozen at -80°C. The leaf tissue was lyophilised and ground to a fine powder in a laboratory mill. DNA was isolated as described in Pérez-Vich et al. (2005).

Since the P21×CAS-14 population was also segregating for the Ms_{11} gene, F_2 plants from this population were also phenotyped for male sterility according to Pérez-Vich et al. (2005).

P21×CAS-14 molecular analysis

Molecular markers putatively linked to the Es3 gene in the P21×CAS-14 population were identified by bulk segregant analysis (BSA; Michelmore et al. 1991). Two bulks were constructed by pooling aliquots (20 µl) of DNA from two sets of individuals with contrasting genotypes. The low-stearic acid bulk was made up from five F_2 individuals classified as *Es3Es3*, and the high-stearic acid bulk was constructed from seven individuals classified as *es3es3*. Homozygosity of F_2 individuals included in the bulks was verified through the analysis of their respective F_3 seeds. Two replicate samples of each bulk and the parental lines were screened with a genome-wide framework of 95 sunflower previously mapped simple sequence repeats (SSRs) (Tang et al. 2003), identified by ORS prefixes. PCRs were performed as described by Pérez-Vich et al. (2004b), and the amplification products were resolved by electrophoresis on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1× TBE buffer with ethidium bromide incorporated in the gel.

Linkage between Es3 and the SSR markers polymorphic between the low-stearic acid and the high-stearic acid bulks was verified by genotyping these SSR markers on the 133 F_2 individuals from P21×CAS-14. The significance of each marker's association with the stearic acid content was determined by one-way analysis of variance (ANOVA) using the statistical package SPSS v 12.0 (SPSS for Windows; SPSS Inc., Chicago, IL, USA), with marker genotypes being classes. Additionally, linkage of these markers and Es3 was also verified by running a preliminary linkage analysis with MAPMAKER/EXP v 3.0b (Whitehead Institute, Cambridge, MA, USA; Lander et al. 1987) using segregation data from the markers and Es3. The genotypes for the Es3 gene were inferred from stearic acid phenotypes in F_2 and F_3 seeds. F_2 plants were classified as *Es3Es3* if their F_3 seeds had a uniform low stearic acid content (< 7%), *Es3es3* if their F_3 seeds segregated for stearic acid content [i.e., they had both high (> 20%) and low (< 7%) stearic acid values], and *es3es3* if their F_3 seeds showed a uniform high stearic acid content (> 20%). Only F_2 individuals that could be assigned unequivocally to one of the three possible genetic classes, based on the phenotypic analysis of the F_3 families (95 of a total of 133), were used for genetic mapping. Linkage was considered significant if the LOD score was > 6.0. For consideration of the positions of the SSR marker loci relative to the target locus Es3, linkage distances were calculated as two-point data.

Once the Es3 LG location was identified, all public SSR markers known to map to the same LG (Tang et al. 2002, 2003), excluding those already used for BSA, were screened for polymorphisms between the parental lines P21 and CAS-14 in order to construct a complete genetic map including the Es3 gene. SSR marker analyses were performed as described above. The SSR polymorphic markers were then genotyped in the 133 F_2 individuals from P21×CAS-14, and a linkage map including Es3 was constructed with MAPMAKER. The genotypes for the Es3 gene were deduced as described above, and mapped accordingly. Two-point analysis was used to group all SSR marker loci and Es3 at a LOD score of 4 and a maximum recombination frequency of 0.35. Three-point and multi-point analyses were used to determine the order and interval distances between the markers. The Kosambi mapping function was used to compute the map distances in centiMorgans. The LG maps were drawn using the MapChart software (Voorrips 2002). For all the marker loci mapped chi-square analyses were carried out on each locus to detect deviations from the expected Mendelian ratios for codominant (1:2:1) or dominant (3:1) markers. The significance of each marker's association with the palmitic, stearic, oleic, and linoleic acid content was determined by one-way ANOVA as described above.

CAS-3×CAS-14 molecular analysis

The CAS-3×CAS-14 population was analyzed in order to determine the effect and interaction among all genes and QTL described affecting increased levels of stearic acid in sunflower (Pérez-Vich et al. 2002, 2004b, and this study). SSR and INDEL (insertion–deletion polymorphisms) markers from those LGs in which these genes and QTL are located were selected and tested for polymorphisms between CAS-3 and CAS-14. Polymorphic markers were then genotyped in the F_2 individuals. SSRs were assayed as described above. SSR primers from several sources were used, and they are identified by the prefixes MS (Paniego et al. 2002), ORS (Tang et al. 2002), and CRT (Yu et al. 2003). INDEL analyses were performed as described by Yu et al. (2003), and they are identified by ZVG prefixes. SSR–INDEL linkage maps were constructed using MAPMAKER. Two-point analysis was used to identify LGs at a LOD score of 3.5 and a maximum recombination frequency of 0.30. Three-point and multi-point analyses were used to determine the order and interval distances between the markers in each LG. One locus initially unlinked was mapped using the "near" command by increasing the recombination default. Statistical analyses were performed in two steps: (1) to determine the significance of

each marker's association with the stearic acid content, a one-way ANOVA was carried out as described above and (2) to test for epistasis, two-factor ANOVA was performed on pairs of the most significant markers. Due to the small genome coverage, which will be explained in the results section, interval-mapping analyses were not performed.

Results

Phenotypic segregation for stearic acid content

Table 1 gives the mean and the range for fatty acid composition of the parental lines (P21, CAS-14, and CAS-3), the F_2 mapping populations, and their corresponding F_3 (average value of 24–48 F_3 seeds from F_2 individual plants). Significant correlations among traits were observed in these populations (Table 2). The P21×CAS-14 population segregated for the macromutation Es3 that determines the very high stearic acid content in CAS-14 (Pérez-Vich et al. 2006). This population also segregated for Ms_{11} (Pérez-Vich et al. 2006), which determines nuclear male sterility in P21 (Jan 1992). The CAS-3×CAS-14 population segregated for the two macromutations Es1 and Es3, which determine the high and very high stearic acid phenotype in CAS-3 and CAS-14, respectively (Pérez-Vich et al. 2006). Phenotypic segregation for the Es2 gene in the CAS-3×–CAS-14 cross could not be discerned, probably because of a masking effect of the Es1 ad Es3 macromutations or because the *es2* allele might also be present in the CAS-14 mutant (Pérez-Vich et al. 2006).

Table 2 Coefficients of correlation between palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) in the F_2 and the F_3 generations from crosses between P-21 and CAS-14 and CAS-3 and CAS-14

Generation and cross	Coefficients of correlation			
		18:0	18:1	18:2
F_2 (P-21×CAS-3)	16:0	0.90**	−0.85**	0.19*
	18:0		−0.82**	NS
	18:1			−0.59**
F_3 (P-21×CAS-14)	16:0	0.78**	−0.82**	−0.23*
	18:0		−0.76**	−0.59**
	18:1			NS
F_2 (CAS-3×CAS-14)	16:0	0.40**	−0.35**	NS
	18:0		−0.66**	NS
	18:1			−0.80**
F_3 (CAS-3×CAS-14)	16:0	0.59**	−0.72**	NS
	18:0		−0.79**	−0.29**
	18:1			−0.35**

NS not significant ($P > 0.05$)
*Significant at the 0.05 probability level
**Significant at the 0.01 probability level

Molecular analysis of the P21×CAS-14 population

From 95 SSRs assayed, eight failed to amplify the parental lines P21 and CAS-14. Thirty-three out of 87 markers (38%) that produced amplification products were polymorphic between P21 and CAS-14. Two markers from LG 8 (ORS70 and ORS1161) were also polymorphic between the low stearic acid bulk and the high stearic acid bulk (Fig. 1). The P21 allele was that present at the low stearic acid bulk, and the CAS-14 allele only amplified in the high stearic acid bulk (Fig. 1). These results indicated that Es3 might reside on LG 8. Linkage of ORS70 and ORS1161 with Es3 was verified by genotyping these markers on 133 individuals from the mapping population used to construct the bulks. ANOVA analyses revealed clear significant differences between the marker class means for the stearic acid content (Table 3). Significant differences were also observed between the marker class means for the fatty acids palmitic and oleic. Additionally, a preliminary linkage analysis was run using segregation data from Es3, ORS70, and ORS1161. Two-point analysis showed ORS70 and ORS1161 to be 5.8 and 4.6 cM, respectively, from Es3. These data confirmed linkage of ORS70 and ORS1161 with Es3.

All ORS–SSR markers known to map to LG 8 (Tang et al. 2002, 2003), excluding those already used for BSA, were screened for polymorphisms between P21 and CAS-14 to construct a complete linkage map of LG 8 including Es3. Five codominant (ORS328, ORS312, ORS536, ORS243, and ORS898) and one dominant (ORS780) marker loci were then genotyped on the 133 F_2 individuals from the P21×CAS-14 population. Additionally, the Ms_{11} gene, which has already been mapped to LG 8 of the sunflower genetic map (Pérez-Vich et al. 2005), was also included in the linkage analysis. The genotypes for the Ms_{11} gene were inferred from male fertility phenotypes in the F_2 plants from P21×CAS-14, and mapped accordingly. Male-fertile F_2 plants were scored as $Ms_{11}\_$, whereas male-sterile F_2 plants were scored as $ms_{11}ms_{11}$. None of the SSR marker loci deviated significantly from the expected segregation ratios ($P > 0.05$).

Linkage analysis was performed, being all markers grouped together with the exception of ORS328, which remained unlinked. LG 8 comprised nine marker loci,

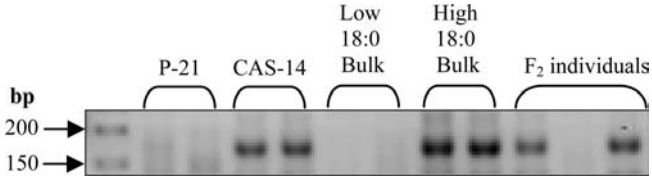

Fig. 1 Amplification products of the SSR marker ORS70. Replicate samples of the low stearic acid parental line P21, the high stearic acid parental line CAS-14, the low stearic acid (low-18:0) bulk, the high stearic acid (high-18:0) bulk, and three F_2 individuals are shown

Table 3 Association between marker loci polymorphic between the high stearic and low stearic acid bulks and fatty acid composition determined by variance analysis in the P21×CAS-14 population

Marker	Fatty acid	Number of individuals[a]				Mean (%) ± SD within each marker class				ANOVA analysis		
		A	H	B	C	A	H	B	C	*F*	*P*	R^{2b}
ORS70[c]	F_2-16:0	24			107	5.6 ± 0.4			5.9 ± 1.0	2.8	0.095	0.02
	F_2-18:0	24			107	4.5 ± 1.0			10.8 ± 10.5	8.5	0.004	0.06
	F_2-18:1	24			107	45.0 ± 5.9			39.3 ± 14.1	3.7	0.057	0.03
	F_2-18:2	24			107	44.9 ± 6.0			43.9 ± 7.7	0.3	0.556	0.00
ORS1161	F_2-16:0	29	70	28		5.7 ± 0.6	5.4 ± 0.4	7.3 ± 0.9		96.6	< 0.001	0.61
	F_2-18:0	29	70	28		5.8 ± 5.8	5.2 ± 2.5	25.3 ± 9.5		141.7	< 0.001	0.70
	F_2-18:1	29	70	28		43.3 ± 8.5	45.9 ± 8.4	22.1 ± 11.2		70.5	< 0.001	0.53
	F_2-18:2	29	70	28		45.3 ± 6.2	43.5 ± 7.7	45.3 ± 6.9		0.9	0.406	0.01
	F_3-16:0	7	61	21		7.4 ± 1.7	7.4 ± 0.7	9.5 ± 1.3		37.6	< 0.001	0.47
	F_3-18:0	7	61	21		13.7 ± 12.8	11.4 ± 4.6	31.6 ± 8.1		77.7	< 0.001	0.64
	F_3-18:1	7	61	21		25.3 ± 10.5	23.7 ± 6.7	9.0 ± 5.0		39.7	< 0.001	0.48
	F_3-18:2	7	61	21		53.6 ± 6.4	57.5 ± 6.4	49.7 ± 6.0		11.9	< 0.001	0.22

Mean fatty acid percentages ± standard deviation (SD) are presented in different genotype classes: A = homozygous with respect to the allele derived from P21, B = homozygous with respect to the allele derived from CAS-14, H = heterozygous, and C = H or B

[a] The number of individuals within each marker class

[b] Coefficient of determination

[c] F_3-ORS70 data were not included due to the scarcity of individuals in one marker class

including the Es3 and the Ms_{11} genes (Fig. 2), and was 50.5 cM long, with an average marker interval of 6.3 cM (Fig. 2). The locus order for the SSR markers and the reference linkage maps (Tang et al. 2002, 2003) was identical. Taking into account these reference maps, our LG 8 map span the distal half region of this LG. The Es3 gene mapped 17.5 cM downstream from the upper end of LG 8, between markers ORS243 and ORS1161. The ORS243 and the ORS1161 markers were 0.5 cM proximal and 3.9 cM distal, respectively, of the Es3 locus (Fig. 2). Ms_{11} mapped between the ORS70 and the ORS536 markers (Fig. 2). ORS70 was 3.3 cM proximal of Ms_{11}, and ORS536 was 4.5 cM distal of this gene. Genetic distance between Ms_{11} and Es3, based on two-point data, was 7.4 cM.

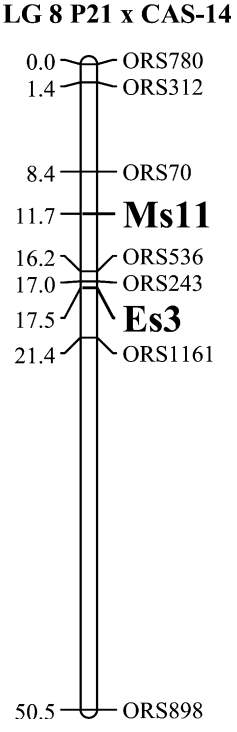

Fig. 2 Molecular map of the sunflower linkage group (LG) 8 containing the Es3 gene determining very high stearic acid content in CAS-14, and the Ms_{11} gene for nuclear male sterility. The ORS prefix denotes SSR marker loci. The cumulative distances in centiMorgans (Kosambi) are shown at the left of the map

ANOVA analyses revealed all the 7 ORS markers on LG 8 having significant association ($P < 0.01$) with stearic acid content, based both on F_2 and F_3 data. Markers ORS243 and ORS536 explained the highest proportion of the phenotypic variability for this fatty acid, with R^2 values of 0.80, and 0.81 for the F_2, and 0.80, and 0.79 for the F_3, respectively (Table 4). The mean stearic acid content in the plants with the CAS-14 allele on markers ORS243, and ORS536 was 26.7% (20 individuals), and 26.9% (27 individuals), respectively, based on F_2 data, and 34.0% (14 individuals), and 33.3% (21 individuals), respectively, based on F_3 data. With regard to the relationship between stearic acid and other fatty acids, palmitic acid and oleic acid were the most significantly correlated with stearic acid (Table 2). In ANOVA analyses, markers associated with stearic acid were also in some extent associated to palmitic and oleic acids (Table 4).

Molecular analysis of the CAS-3×CAS-14 population

A total of 130 SSR and 30 INDEL markers from those LG in which QTL or genes associated to increased stearic acid content have been reported in sunflower were assayed in the parental lines CAS-3 and CAS-14 in order to identify polymorphisms. The selected LG were

Table 4 Association between Es3 closest marker loci and fatty acid composition determined by variance analysis in the P21×CAS-14 population

Marker	Fatty acid	Number of individuals[a]			Mean (%) ± SD within each marker class			ANOVA analysis		
		A	H	B	A	H	B	*F*	*P*	R^{2b}
ORS536	F_2-16:0	26	79	27	5.6 ± 0.4	5.4 ± 0.5	7.4 ± 0.7	138.7	< 0.001	0.68
	F_2-18:0	26	79	27	4.6 ± 1.3	5.3 ± 3.2	26.9 ± 7.7	272.6	< 0.001	0.81
	F_2-18:1	26	79	27	44.1 ± 6.2	46.1 ± 8.7	20.3 ± 8.6	101.5	< 0.001	0.61
	F_2-18:2	26	79	27	45.6 ± 6.3	43.1 ± 7.7	45.4 ± 6.9	1.7	0.19	0.02
	F_3-16:0	3	69	21	6.1 ± 0.2	7.4 ± 0.9	9.5 ± 1.2	46.3	< 0.001	0.51
	F_3-18:0	3	69	21	5.1 ± 1.1	11.5 ± 4.4	33.3 ± 6.1	173.5	< 0.001	0.79
	F_3-18:1	3	69	21	38.2 ± 10.6	24.1 ± 6.5	7.6 ± 2.0	74.8	< 0.001	0.62
	F_3-18:2	3	69	21	50.5 ± 11.1	56.9 ± 6.5	49.3 ± 5.8	11.7	< 0.001	0.21
ORS243	F_2-16:0	27	55	20	5.6. ± 0.4	5.4 ± 0.6	7.4 ± 0.8	87.1	< 0.001	0.64
	F_2-18:0	27	55	20	4.6 ± 1.3	5.5 ± 3.7	26.7 ± 7.3	201.5	< 0.001	0.80
	F_2-18:1	27	55	20	45.0 ± 5.7	45.1 ± 8.8	18.7 ± 7.3	91.5	< 0.001	0.65
	F_2-18:2	27	55	20	44.8 ± 5.9	44.0 ± 7.5	47.2 ± 5.0	1.73	0.18	0.03
	F_3-16:0	5	47	14	6.4 ± 0.4	7.5 ± 0.9	9.7 ± 1.2	35.4	< 0.001	0.53
	F_3-18:0	5	47	14	4.6 ± 1.1	11.8 ± 4.7	34.0 ± 6.4	123.2	< 0.001	0.80
	F_3-18:1	5	47	14	35.5 ± 8.5	23.7 ± 6.0	7.7 ± 2.3	60.3	< 0.001	0.66
	F_3-18:2	5	47	14	53.5 ± 9.0	56.9 ± 6.5	48.6 ± 6.5	8.5	0.001	0.21

Mean fatty acid percentages ± SD are presented in different genotype classes: A = homozygous with respect to the allele derived from P21, B = homozygous with respect to the allele derived from CAS-14, and H = heterozygous

[a]The number of individuals within each marker class

[b]Coefficient of determination

LG 1 for the Es1 gene (Pérez-Vich et al. 2002), LG 3, LG 11, LG 13, and LG 7 for the *st2.1*, *st2.2*, *st2.3*, and *st2.4* QTL, respectively (Pérez-Vich et al. 2002, 2004b), and LG 8 for the Es3 gene (this study). From these 160 markers, only 30 (18.7%) were polymorphic between CAS-3 and CAS-14. This low level of polymorphism is due to the fact that CAS-3 and CAS-14 were obtained after mutagenesis of the same parental line RDF-1-532 (Osorio et al. 1995; Fernández-Moya et al. 2002), and therefore they have a close genetic background. The 30 polymorphic markers were then genotyped in the CAS-3×CAS-14 progeny (164 individuals). From these markers, 19 were mapped (Fig. 3), the remaining 11 either showed a strong segregation distortion and/or were unlinked to the previous markers. Only LG 8 had a good coverage (Fig. 3). Additional polymorphic markers within these LG were not found.

Probe-by-probe analyses were carried out for each of the measured traits. Two major QTLs were found associated to stearic acid content, one on LG 8 linked to marker ORS243, and the another on LG 1, below marker ZVG1 (Table 5). No significant associations

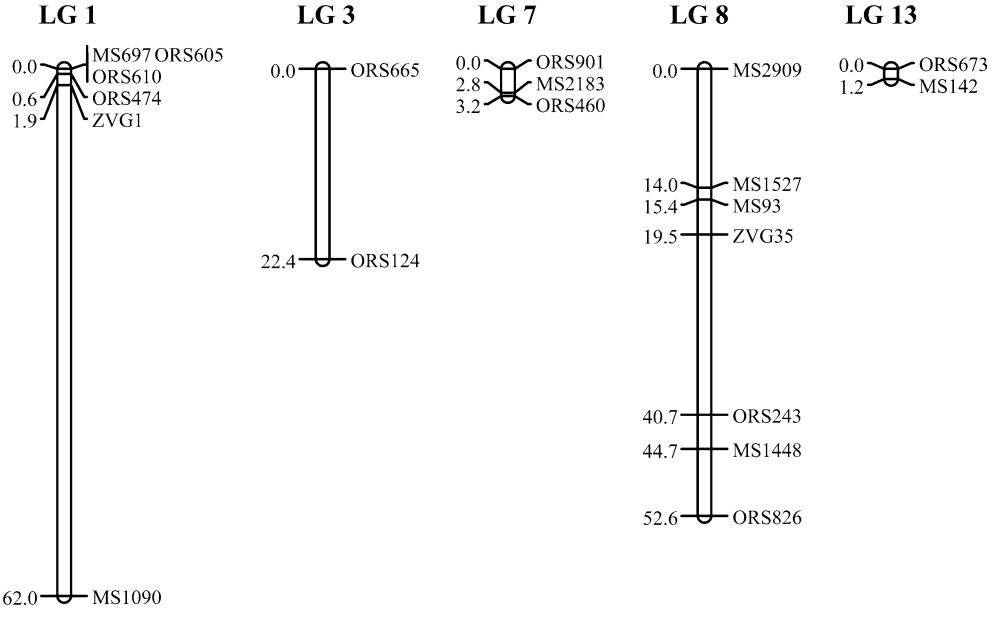

Fig. 3 Mapped marker loci in the CAS-3×CAS-14 population. The ORS prefix denotes public SSR marker loci. The MS prefix denotes proprietary SSR marker loci. The ZVG prefix denotes INDEL marker loci. The cumulative distances in centiMorgans (Kosambi) are shown at the left of the map

Table 5 Association between the most significant marker loci on LG 8 (ORS243), and on LG 1 (ZVG1) and fatty acid composition determined by variance analysis in the CAS-3×CAS-14 population

Marker	Fatty acid	Number of individuals[a]			Mean (%) ± SD within each marker class			ANOVA analysis		
		A	H	B	A	H	B	*F*	*P*	R^{2b}
ORS243 (LG 8)	F_2-16:0	33	79	22	5.7 ± 0.7	5.6 ± 0.6	6.3 ± 0.9	7.4	0.001	0.10
	F_2-18:0	33	79	22	9.0 ± 6.0	9.8 ± 4.5	16.9 ± 7.5	16.9	< 0.001	0.21
	F_2-18:1	33	79	22	38.9 ± 9.3	39.5 ± 9.9	29.6 ± 10.5	8.8	< 0.001	0.12
	F_2-18:2	33	79	22	46.4 ± 6.9	45.1 ± 7.8	47.2 ± 7.8	0.7	0.466	0.01
	F_3-16:0	29	75	19	6.7 ± 0.6	7.0 ± 0.7	8.1 ± 1.1	22.0	< 0.001	0.27
	F_3-18:0	29	75	19	11.0 ± 6.0	16.5 ± 4.3	29.3 ± 7.2	70.8	< 0.001	0.54
	F_3-18:1	29	75	19	28.6 ± 8.5	24.6 ± 6.2	12.9 ± 6.2	31.7	< 0.001	0.35
	F_3-18:2	29	75	19	53.7 ± 4.8	51.9 ± 4.8	49.7 ± 4.7	4.0	0.021	0.06
ZVG1 (LG 1)	F_2-16:0	36	77	43	5.8 ± 0.7	5.7 ± 0.7	5.7 ± 0.8	0.06	0.944	0.001
	F_2-18:0	36	77	43	14.2 ± 6.0	10.5 ± 4.9	8.4 ± 6.3	10.7	< 0.001	0.12
	F_2-18:1	36	77	43	33.4 ± 10.4	38.4 ± 9.1	41.0 ± 10.9	5.9	0.003	0.072
	F_2-18:2	36	77	43	46.7 ± 7.8	45.4 ± 7.7	44.8 ± 7.5	0.6	0.552	0.008
	F_3-16:0	35	70	40	7.0 ± 0.8	7.0 ± 0.8	7.0 ± 0.9	0.01	0.990	0.000
	F_3-18:0	35	70	40	18.1 ± 5.3	17.4 ± 7.1	14.8 ± 8.6	2.3	0.100	0.03
	F_3-18:1	35	70	40	21.7 ± 6.9	23.4 ± 7.5	27.3 ± 9.2	5.1	0.007	0.07
	F_3-18:2	35	70	40	53.2 ± 4.8	52.2 ± 4.8	50.9 ± 4.9	2.2	0.118	0.03
ORS243 × ZVG1	F_2-18:0							5.3	0.001	0.11 [c]
	F_3-18:0							9.7	< 0.001	0.12 [c]

Mean fatty acid percentages ± SD are presented in different genotype classes: A = homozygous with respect to the allele derived from CAS-3, B = homozygous with respect to the allele derived from CAS-14, H = heterozygous. The effect on stearic acid content of the interaction between ORS243 and ZVG1 is also shown

[a]The number of individuals within each marker class

[b]Coefficient of determination

[c]Total R^2 of the model with ORS243, ZVG1, and ORS243×ZVG1 is 0.40 for F_2-18:0, and 0.67 for F_3-18:0

($P < 0.01$) between stearic acid and the marker loci mapped on LG other than 1 or 8 were found. QTL effects were recessive for the QTL on LG 8, or partially recessive for the QTL on LG 1, with opposite effects. On LG 8, high stearic acid corresponds to the allele from CAS-14, and on LG 1 high stearic acid corresponds to the allele from CAS-3 (Table 5). Significant associations between the QTL on LG 8 and palmitic and oleic acid were also found. The QTL on LG 1 only showed a significant association to oleic acid. Taking into account previous molecular studies on CAS-3 (Pérez-Vich et al. 2002), the QTL on LG 1 detected in this study corresponds to the *st1-SAD17A* QTL that underlies the Es1 gene controlling increased stearic acid content in CAS-3, which is located about 20 cM distal of ZVG1. Similarly, and taking into account the results from the P21×CAS-14 population in this study, the QTL on LG 8 corresponds to the Es3 gene controlling high stearic acid content in CAS-14, which is located 0.5 cM distal of ORS243 (Fig. 3).

Two-way ANOVA models were run for the stearic acid content on the F_2 and the F_3. Markers with the highest effect on the single marker analysis (ORS243 from LG 8 and ZVG1 from LG 1) were chosen as factors in the model. The ANOVA table indicated interaction between ORS243 and ZVG1 (Table 5). The effects-plot (Fig. 4) showed that interaction arose when ORS243 had the CAS-14 allele (equivalent to a genotype "*es3es3*"). Here, the average stearic acid content when the allele at ZVG1 was that from CAS-3 (equivalent to a genotype "*es1es1es3es3*") was 17.3%, whereas the average stearic acid content was 22.9% when the allele at ZVG1 was that from CAS-14 (equivalent to a genotype "*Es1Es1es3es3*"), based on F_2 data (Fig. 4a). The effect was more evident and also detected for the ZVG1 heterozygous class in the F_3 (Fig. 4b). Thus, the highest stearic acid content is achieved when both loci at LG 8 and at LG 1 carry the CAS-14 allele. On the other hand, when the ZVG1 locus had the allele from CAS-3 (equivalent to a genotype "*es1es1*") an increase of the average stearic acid content was observed when ORS243 had the CAS-14 allele, both in the F_2 and the F_3 generations (Fig. 4). Thus, the F_2 and F_3 average stearic acid content was 12.8% and 15.2%, respectively, when both ZVG1 and ORS243 had the CAS-3 allele (equivalent to a "*es1es1Es3Es3*" genotype), and 17.3%, and 21.3%, respectively, when ZVG1 had the CAS-3 allele and ORS243 had the CAS-14 allele (equivalent to a genotype "*es1es1es3es3*") (Fig. 4).

Discussion

The Es3 gene determines a very high stearic acid phenotype in sunflower. In this study, the Es3 gene has been mapped to LG 8 of the sunflower genetic map. Previous studies in this oilseed crop have described the existence of QTL determining increased stearic acid content on

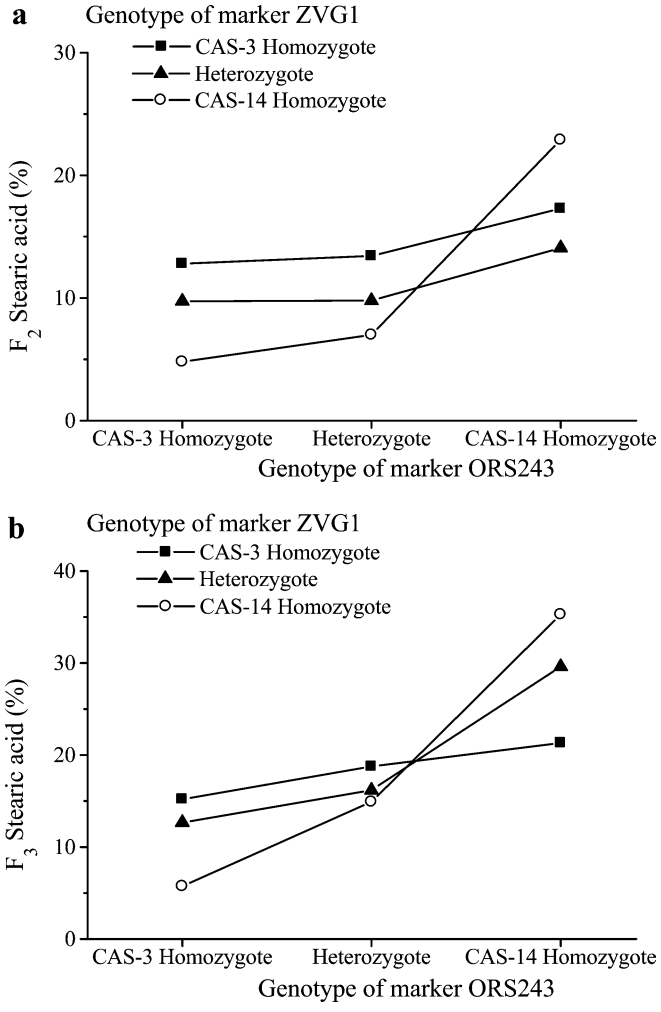

Fig. 4 **a**, **b** The interlocus effects between ORS243 and ZVG1 marker loci. Values shown are the mean stearic acid content for CAS-3×CAS-14 F_2 or F_3 progeny of a particular ORS243 genotype in the ZVG1 background. **a** F_2 progeny. **b** F_3 progeny

LG 1, LG 3, LG 7, LG 11, and LG 13 (Pérez-Vich et al. 2002, 2004b). Although these QTL were mapped using a proprietary RFLP marker system, LG nomenclature follows Berry et al. (1997), which is similar to the LG nomenclature of the public sunflower linkage maps (Tang et al. 2002; Yu et al. 2003) used in this study. Hence, as the map positions of the stearic acid QTL are comparable, it can be concluded that Es3 is different from all the previously known stearic acid QTL.

Es3 pleiotropically affected the levels of other seed oil fatty acids. This effect was not unexpected because seed oil fatty acids are formed by means of enzymatically controlled carbon chain elongations and desaturations (Somerville et al. 2000). Therefore a change in one fatty acid will generally affect the levels of the others, resulting in significant correlations among traits. This effect has also been observed for the Es1 gene controlling high stearic acid content in the sunflower mutant CAS-3 (Pérez-Vich et al. 2002).

Apart from the Es3 gene, the Ms_{11} gene determining nuclear male sterility in the sunflower line P21 has also been mapped to LG 8, and marker ORS536 was 4.5 cM distal of this gene. This position corresponds to the Ms_{11} map position reported by Pérez-Vich et al. (2005). Additionally, in a classical genetic study to determine inheritance of the very high stearic acid content in CAS-14 using P21 as a standard line, Pérez-Vich et al. (2006) described the genetic linkage between Es3 and Ms_{11}. The frequency of recombination between Es3 and Ms_{11} was estimated to be 0.09 ± 0.02. In the present research, using a molecular approach, we have determined Es3 to be 7.4 cM from Ms_{11}, which confirms these previous results.

The Es3 gene is an outstanding target for molecular-assisted breeding programs, since its expression is strongly influenced by temperature during seed maturation (Fernández-Moya et al. 2002) and it is not uniform along the seed (Fernández-Moya et al. 2003). Molecular markers closely linked to Es3 have been identified. The SSR markers ORS243, and ORS536 are 0.5, and 1.3 cM proximal, respectively, of Es3, and the ORS1161 marker is 3.9 cM distal of Es3. The fact that the markers flank the gene makes them more reliable than a single marker. These markers are co-dominant. Since the Es3 gene is recessive, they can therefore be used to identify and select *Es3es3* heterozygotes from *Es3Es3* homozygotes in a backcross program without the need for progeny testing.

Expression of the very high stearic acid content in CAS-14 is very different from that in CAS-3 seeds, which neither shows a seed gradient nor a strong temperature effect (Pérez-Vich et al. 1998; Martínez-Force et al. 1998). Through a candidate-gene strategy, Pérez-Vich et al. (2002) demonstrated that Es1 cosegregated with a stearate desaturase locus (SAD17A). Fernández-Moya et al. (2003) reported that the very high stearic acid phenotype in CAS-14 might be associated to a regulatory thermosensitive element that determines a lower expression of the stearate desaturase at high temperatures. According to these authors, the Es3 gene might not correspond to a stearate desaturase locus, as it is the case of Es1. The fact that none of the three stearate desaturase loci mapped in sunflower (Pérez-Vich et al. 2002, 2004b) is located on LG 8 of the sunflower genetic map support this hypothesis. Further studies are required to determine the role of Es3 in the fatty acid biosynthetic pathway. Mapping the Es3 gene on the sunflower genetic map has opened up different strategies to determine the nature of this gene, such as a candidate-gene approach or a map-based cloning strategy.

Results from this research have significant implications for breeding for high stearic acid content in sunflower using the CAS-3 and the CAS-14 mutants as the high stearic acid sources. Since Es1 from CAS-3 and Es3 from CAS-14 segregate independently, the *es1es1es3es3* combination is obtained from crosses between these lines. This genotype would be expected to have a transgresive stearic acid phenotype higher than that

found in the parental lines due to an additive action between *es1* and *es3*, as it has been reported for the *es1* and the e*s2* alleles in sunflower (Pérez-Vich et al. 1999, 2004a), and for the same trait in soybean [*Glycine max* (L.) Merr.] (Bubeck et al. 1989; Rahman et al. 1997). This was not observed in classical genetic studies (Pérez-Vich et al. 2006), where it was found that the maximum potential to accumulate stearic acid conferred by *es3* alleles was not further increased by an additive action of the *es1* alleles. Such an observation has been confirmed in the present study (Fig. 4). Moreover, interaction between the Es1 QTL at LG 1and the Es3 QTL at LG 8 has shown that the *es1* allele not only does not increase the stearic acid content, but it is detrimental to achieve the maximum potential to accumulate stearic acid conferred by the *es3* allele. In other words, the maximum potential of CAS-14 is expressed when it is not combined with CAS-3. However, the present study has also indicated that the combination *es1es1es3es3* (CAS-3 + CAS-14) expresses a higher stearic acid value than that of the *es1es1Es3Es3* genotype (CAS-3). Even though the maximum stearic acid content produced by the *es1es1es3es3* genotype is lower than that of *Es1Es1es3es3*, the former genotypic combination might play a role in attenuating the seed gradient and environmental instability of CAS-14.

In summary, Es3 was mapped to LG 8 and tagged with three closely linked microtatellite markers, ORS243, ORS536, and ORS1161. Additionally, an interaction between the Es3 gene from CAS-14 and the Es1 gene from CAS-3 has been determined. The information from this study will facilitate defining optimal strategies for molecular breeding for high stearic acid content in sunflower using both CAS-3 and CAS-14 genetic sources, thus contributing to develop novel sunflower lines with a seed oil more suitable for the food industry.

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