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Genetic control of high stearic acid content in the seed oil of the sunflower mutant CAS-3

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Abstract A sunflower mutant, CAS-3, with about 25% stearic acid (C18:0) in the seed oil was recently isolated after a chemical-mutagen treatment of RDF-1-532 seeds (8% C18:0). To study the inheritance of the high C18:0 content, CAS-3 was reciprocally crossed to RDF-1–532 and HA-89 (5% C18:0). Significant reciprocal-cross differences were found in one of the two crosses, indicating possible maternal effects. In the CAS-3 and RDF-1–532 crosses, the segregation patterns of the F_1, BC_1, and F_2 populations fitted a one-locus (designated *Es1*) model with two alleles (*Es1*, *es1*) and with partial dominance of low over high C18:0 content. Segregation patterns in the CAS-3 and HA-89 crosses indicated the presence of a second independent locus (designated *Es2*) with two alleles (*Es2*, *es2*), also with partial dominance of low over high C18:0 content. From these results, the proposed genotypes (C18:0 content) of each parent were as follows: CAS-3 (25.0% C18:0) =*es1es1es2es2*; RDF-1–532 (8.0% C18:0) =*Es1Es1es2es2*; and HA-89 (4.6% C18:0) =*Es1Es1Es2Es2*. The relationship between the proposed genotypes and their C18:0 content indicates that the *Es1* locus has a greater effect on the C18:0 content than the *Es2* locus. Apparently, the mutagenic treatment caused a mutation of *Es1* to *es1* in RDF-1–532.

Key words *Helianthus annuus* · Sunflower mutant · Stearic acid · Oil quality · Genetic control

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Introduction

The quality of sunflower seed oil, both for food and non-food applications, is mainly determined by its constituent fatty acids. Standard sunflower oil contains approximately 11% of the saturated palmitic (C16:0) and stearic (C18:0) fatty acids, the rest being the unsaturated oleic (C18:1) and linoleic (C18:2) acids (Dorrell and Vick 1997). Because of this fatty acid composition, sunflower oil is liquid at room temperature.

The use of mutagenesis produced sunflower lines with higher concentrations of C16:0 (>25%; Ivanov et al. 1988; Osorio et al. 1995; Fernández-Martínez et al. 1997) and C18:0 (>22%; Osorio et al. 1995). The higher saturated level of the oils from these mutants will increase the utility and improve the quality of sunflower oil for specific edible purposes. First, this type of oil requires no chemical transformations such as hydrogenation or transesterification in order to obtain solid or semi-solid fats. Such oil-transformations have been related to cardiovascular diseases (Kritchevsky et al. 1995; Ascherio and Willet 1997). Second, the oil from these mutants will show higher stability than the oil from currently available cultivars, as described in high C18:0 lines in soybean (Lui and White 1992), and will find many applications in the field of solid and semi-solid fats, such as the manufacture of margarine, shortenings for baking, or fats for deep frying.

A requisite for the commercial use of the new oils is the incorporation of the modified biosynthetic pathway into sunflower hybrids with a good agronomic performance. This requires previous knowledge on the genetic behavior of the trait. Previous studies on the genetics of the seed-oil quality in sunflower have been focused on the study of the high C18:1 trait. These studies demonstrated that the genetic control of the high C18:1 trait is mainly gametophytic, dominant, and determined by a low number of genes (Fick 1984; Urie 1985; Miller et al. 1987; Fernández-Martínez et al. 1989). Such characteristics have permitted the rapid development of sunflower hybrids with a very high C18:1 content (90%) (Fern-

ández-Martínez et al. 1993). Recently, Pérez-Vich et al. (1999) explained the inheritance of the high C16:0 trait in sunflower on the basis of recessive alleles at three loci, *P1*, *P2* and *P3*, in such a way that the genotypes with high C16:0 levels were homozygous for the recessive allele *p1* and for at least one of the other two recessive alleles, *p2* or *p3*.

The inheritance of the high C18:0 trait in sunflower remains unexplained to-date. In different mutants of other oilcrops, the genetic control of high C18:0 content was found to be simply inherited. In soybean, it is controlled by multiple recessive alleles at a single locus (Graef et al. 1985; Bubeck et al. 1989), or by recessive alleles at two different loci (Rahman et al. 1997). In safflower and maize, the high C18:0 trait has been reported to be controlled by a single gene (Ladd and Knowles 1970; Jellum and Widstrom 1983).

The objective of the present study was to determine the genetic control of the high C18:0 content in the sunflower mutant line CAS-3, with a view to designing efficient strategies to incorporate this trait into commercial hybrids.

Materials and methods

Plant material

The lines used in this study were the high-C18:0 mutant, CAS-3, obtained by Osorio et al. (1995) after chemical mutagenesis, its parental line RDF-1–532, and the inbred line HA-89, widely used for the development of commercial hybrids (Fernández-Martínez et al. 1993). The fatty acid composition of these lines is shown in Table 1.

Genetic study

Half-seeds of the line CAS-3, and its parental line RDF-1–532, breeding true for either high or low C18:0 content, were planted in a greenhouse in December 1995. At flowering each head was covered with a paper bag to avoid contamination with external pollen. Plants of the mutant line CAS-3 were reciprocally crossed with plants of RDF-1–532. Crossing was done by emasculating immature flower buds of the female parent followed by pollination of their stigmas with pollen from the male parent. The parents and F_1 half-seeds from each of the eight crosses obtained were planted in the field in Spring 1996. F_1 plants were self-pollinated to obtain the F_2 seed and also backcrossed to both parents to obtain the BC_1F_1 seed. Reciprocal crosses between the two parents were repeated.

Individual F_1 and parent seeds obtained in the field in 1996 were analyzed for fatty acid composition by gas-liquid chromatography (GLC) in 24 replications of a randomized complete-block design. Each replicate included one seed from each parent and the reciprocal F_1s. An evaluation of the fatty acid composition at the F_1 plant level was made by averaging the GLC analyses of the F_2 seeds from each F_1 individual plant. The F_2 generation was evaluated though the analysis of the fatty acid composition of 140 F_2 seeds from two reciprocal F_1 plants. A total of 190 BC_1F_1 seeds from the backcrosses to both parents were also analyzed.

The mutant line CAS-3 was reciprocally crossed with the inbred line HA-89 in September 1994. F_1 half-seeds were analyzed by GLC. A total of 20 F_1 plants were transplanted into the field in Spring 1995. F_1 plants were self-pollinated and backcrossed to both parents, and reciprocal crosses between the two parents were also made. Ninety six half-seeds from each of four backcrosses, and about 120 F_2 half-seeds from each of five F_1 plants were analyzed by GLC. A total of 21 F_2 half-seeds, representing all the classes for C18:0 concentration detected in this generation, were selected, germinated, and transplanted into the field in Spring 1996 to obtain the F_3 seed. For the evaluation of the F_3 generation, a preliminary screening on 12 F_3 half-seeds from each of the 21 F_2 plants was done in order to identify the presence or absence of segregation for C18:0 content. After that, about 96 additional seeds from each of the segregating F_3 populations were further analyzed.

The seeds of BC_1F_1, F_2 and F_3 generations were divided into phenotypic classes on the basis of the C18:0 content of the parents grown in the same environment. The classes consisted of phenotypes with C18:0 values (1) equal to the parent with the least C18:0 content, (2) equal to the parent with the greatest C18:0 content, and (3) intermediate to the parents. The proportions observed in each phenotypic class were compared to those expected on the basis of appropriate genetic hypotheses. The goodness of fit to tested ratios was measured by the chi-square statistic.

Fatty acid analyses

Fatty acid methyl esters were obtained as described by Garcés and Mancha (1993), then analyzed on a Perkin-Elmer Autosystem gas-liquid chromatograph (Perkin-Elmer Corporation, Norwalk, Conn.) with a 2-m-long column packed with 3% SP-2310/2% SP-2300 on Chromosorb WAW (Supelco Inc., Bellefonte, Pa.). The oven, injector and flame ionisation detector were held at 190, 275 and 250 C, respectively.

Results and discussion

Crosses between RDF-1–532 and CAS-3

Table 2 shows the C18:0 content of RDF-1–532, CAS-3, and the F_1 seeds and F_1 plants (F_2 seeds averaged) from their reciprocal cross. CAS-3 had a C18:0 content of about 25%, compared with 8% in the line RDF-1–532, and 4.6% in the line HA-89.

The average C18:0 content of the F_1 seeds was significantly different from both parents (Table 2) and lower than the midparent value (16.7%), indicating a partial dominance of low over high C18:0 levels. No significant

Table 1 Fatty acid composition of the seed oil from sunflower lines CAS-3, RDF-1–532, and HA-89. Fatty acids are expressed as mean value±standard deviation

Line	Fatty acid (%)[a]			
	C16:0	C18:0	C18:1	C18:2
CAS-3	7.3±0.4	25.0±1.8	15.0±2.6	52.6±2.7
RDF-1–532	8.6±0.6	8.0±0.9	23.1±3.7	60.2±3.9
HA-89	5.7±0.5	4.6±0.3	26.4±6.5	63.2±6.4

[a] C16:0=palmitic acid, C18:0=stearic acid, C18:1=oleic acid, C18:2=linoleic acid

Table 2 Mean stearic acid content at the seed or plant level of the parents RDF-1–532 and CAS-3 or HA-89 and CAS-3, and of their reciprocal F_1s

Level	Parent or cross	C18:0 (%)	Parent or cross	C18:0 (%)
Parent seed	RDF-1–532	8.0a[a]	HA-89	4.6a
	CAS-3	25.4c	CAS-3	25.0d
F_1 seed	F_1(RDF-1–532×CAS-3)	10.5b	F_1(HA-89×CAS-3)	8.0b
	F_1(CAS-3×RDF-1–532)	10.5b	F_1(CAS-3×HA-89)	9.7c
F_1 plant (F_2 seeds averaged)	F_1(RDF-1–532×CAS-3)	14.1a	F_1(HA-89×CAS-3)	11.8a
	F_1(CAS-3×RDF-1–532)	13.9a	F_1(HA-89×CAS-3)	11.1a
			F_1(HA-89×CAS-3)	11.4a
			F_1(CAS-3×HA-89)	11.8a
			F_1(CAS-3×HA-89)	12.5a

[a] Means followed by the same letter within each column, for F_1 seeds and parents or F_1 plants, are not significantly different at P=0.05 according to the LSD test

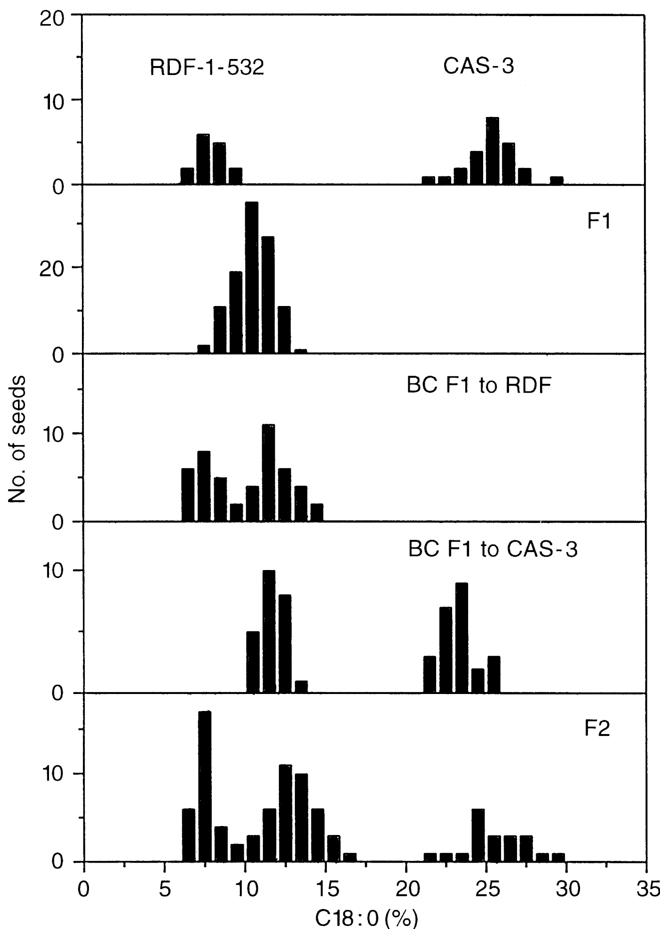

Fig. 1 Distribution of stearic acid content in individual seeds of the parental lines RDF-1–532 and CAS-3, in F_1 and F_2 seeds from their cross, and in BC_1F_1 to RDF-1–532 and to CAS-3 seeds

reciprocal differences in the F_1 seeds or in F_1 plants (F_2 seeds averaged) were observed (Table 2), indicating that the C18:0 content was controlled by the genotype of the embryo (not affected by the genotype of the maternal parent), and the absence of cytoplasmic effects in this cross.

The C18:0 content of individual F_2 seeds ranged from 6.5 to 29.0% (Fig. 1), showing a discontinuous distribution. Three C18:0 classes, consisting of seeds with 6.5–10.0% (low), 10.0–21.0% (intermediate) and 21.0–29.0% (high), respectively, were separated. These classes showed a C18:0 range similar to that observed for the low C18:0 parent, the F_1 seed, and the high C18:0 parent, respectively (Fig. 1). The observed frequencies in each class fitted satisfactorily a 1:2:1 (low:intermediate:high) ratio in the two F_2 populations analyzed (Table 3). The data of the BC_1 seeds also satisfactorily fitted a 1:1 (low:intermediate) ratio in the backcross to the low C18:0 parent, and a 1:1 (intermediate:high) ratio in the backcross to the high C18:0 parent (Table 3). These results indicated that the high C18:0 content was controlled by a recessive allele at a single locus. The recessive allele in CAS-3 was designated *es1*.

Crosses between HA-89 and CAS-3

The analysis of F_1 half-seeds from reciprocal crosses between HA-89 and CAS-3 confirmed the partial dominance of low over high C18:0 content (Table 2). In contrast to the cross between CAS-3 and RDF-1–532, the reciprocal F_1 seeds from the cross between HA-89 and CAS-3 differed significantly for the C18:0 content (Table 2), indicating the existence of a slight maternal effect. However, the differences between the C18:0 values of the reciprocal F_1s seeds were much smaller than those between the F_1 seed and each of the parents (Table 2). Therefore, it was concluded that the genetic control of the high C18:0 content in crosses between HA-89 and CAS-3 was mainly embryonic, with a slight maternal influence present. There were no significant reciprocal differences for the C18:0 content in the F_2 seeds on F_1 plants (Table 2), indicating the absence of cytoplasmic effects.

The different results obtained from reciprocal crosses of CAS-3 with RDF-1–532 and HA-89 suggested that the maternal influence for C18:0 content depended on the parental lines used for the cross, as reported in soybean for the unsaturated fatty acids C18:1 and C18:2 (Miller et al. 1996).

The C18:0 content of the F_2 seeds showed a continuous and asymmetric distribution (Fig. 2), with the phenotypic classes not as evident as in the F_2 generation from

Table 3 Number of seeds having a different stearic acid content and chi-square analyses in the F_2 and BC_1F_1 seeds from crosses between RDF-1–532 and CAS-3

Generation	No. of seeds with % C18:0 content			Chi-square value (P)[a]
	<10.0	10.0–21.0	>21.0	
F_2 (RDF-1–532×CAS-3)	26	44	20	0.84 (0.65)
F_2 (CAS-3×RDF-1–532)	14	21	12	0.70 (0.70)
Pooled	40	65	32	1.29 (0.52)
Heterogeneity				0.25 (0.75–0.50)
BC_1F_1[RDF-1–532×(RDF-1–532×CAS-3)]	22	26		0.33 (0.56)
BC_1F_1[(RDF-1–532 x CAS-3)×RDF-1–532]	24	24		0.00 (0.99)
Pooled	46	50		0.17 (0.68)
Heterogeneity				0.16 (0.75–0.50)
BC_1F_1[CAS-3×(RDF-1–532×CAS-3)]		24	24	0.00 (0.99)
BC_1F_1[(RDF-1–532×CAS-3)×CAS-3]		27	19	1.39 (0.24)
Pooled		51	43	0.87 (0.35)
Heterogeneity				0.52 (0.50–0.25)

[a] Ratios tested: F_2 generation =1:2:1; and BC_1F_1 generations =1:1

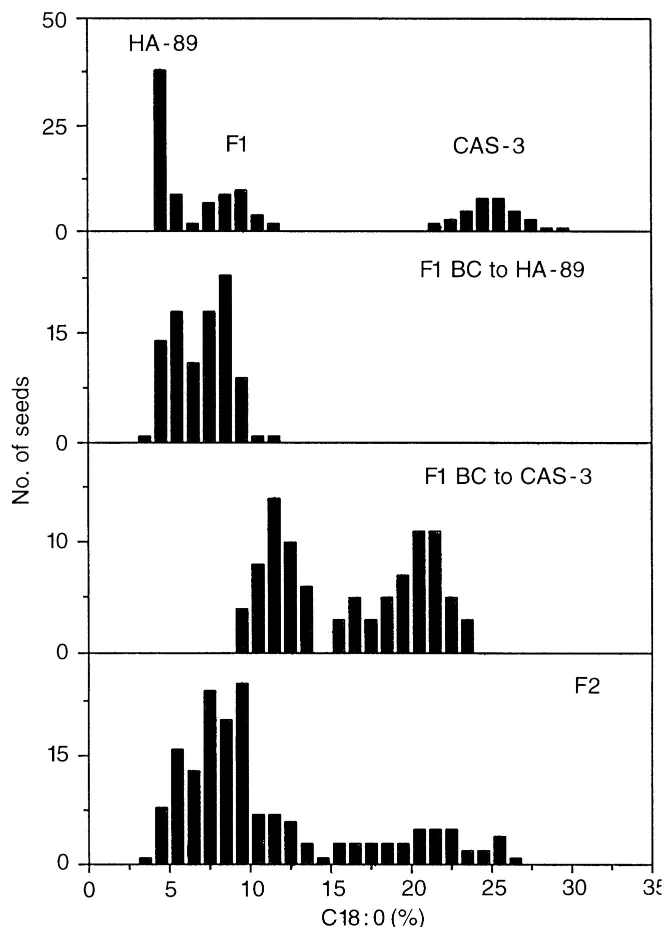

Fig. 2 Distribution of stearic acid content in individual seeds of the parental lines HA-89 and CAS-3, in F_1 and F_2 seeds from their cross, and in BC_1F_1 to HA-89 and to CAS-3 seeds

the cross between CAS-3 and RDF-1–532. Therefore, the phenotypic classes were defined on the basis of the C18:0 content of the parents grown in the same environment. According to this criterion, three classes (low:intermediate:high) were identified. In all the F_2 populations analysed (Table 4), the observed numbers for the three classes satisfactorily fitted a phenotypic ratio of 1:14:1 (low:intermediate:high). These data indicated that only 1 out of 16 F_2 seeds recovered the genotype of each parent, suggesting the presence of two major genes responsible for the high C18:0 content in CAS-3. These results were additionally supported by the 1:3 (low: intermediate) segregation found in the backcrosses to the low C18:0 parent HA-89, and the 3:1 (intermediate: high) ratio observed in the backcrosses with the high C18:0 parent CAS-3 (Table 4). The second locus detected in this cross was designated *Es2*.

Taking into account these results, the proposed genotypes for the three lines used in this study were *es1es1es2es2* for the mutant line CAS-3, *Es1Es1es2es2* for the original parental line RDF-1–532, and *Es1Es1Es2Es2* for the line HA-89. This model implies that the influence on the C18:0 content of the *es2* allele would be lower than that of the *es1* allele, as demonstrated by the inheritance pattern of crosses between RDF-1–532 and CAS-3, and between HA-89 and CAS-3, as well as by the C18:0 levels of the parents involved in each cross.

As a further confirmation of the two-locus model, a progeny test was conducted on crosses between HA-89 and CAS-3 by analyzing the F_3 seeds from each of 21 F_2 plants. These F_2 plants were selected on the basis of the C18:0 content of the corresponding F_2 half-seeds, which covered the whole range of C18:0 levels observed in the F_2 populations. Table 5 shows the results obtained in the progeny test. Seven groups of F_2 plants were arranged according to the mean value and range of variation of the C18:0 content (Fig. 3).

F_3 seeds of F_2 plants from groups I and VII (Table 5) did not segregate for C18:0 content, all the F_3 seeds analyzed having C18:0 values below 6.5% (group I) and above 20.0% (group VII; Fig. 3). Both F_2 genotypes were identified respectively as the homozygotes *Es1Es1Es2Es2* (group I) and *es1es1es2es2* (group VII). The F_3 seeds of group II showed a continuous distribution for C18:0 content (Fig. 3), ranging from 3.2% to 9.5%. This was interpreted as the result of the segregation of the *Es2* locus, the *Es1* locus being in a homozy-

Table 4 Number of seeds having a different stearic acid content and chi-square analyses in the F_2 and BC_1F_1 seeds from crosses between HA-89 and CAS-3

Generation	No. of seeds with % C18:0 content			Chi-square value (P)[a]
	<5.5	5.5–22.0	>22.0	
F_2 (HA-89×CAS-3)	8	111	11	1.08 (0.58)
F_2 (HA-89×CAS-3)	15	141	14	3.28 (0.19)
F_2 (HA-89×CAS-3)	17	142	14	5.06 (0.08)
F_2 (CAS-3×HA-89)	5	85	6	0.18 (0.91)
F_2 (CAS-3×HA-89)	2	83	8	3.36 (0.19)
Pooled	47	562	53	4.54 (0.10)
Heterogeneity				8.40 (0.10–0.05)
BC_1F_1(HA-89×(HA-89×CAS-3))	21	75		0.50 (0.48)
BC_1F_1((HA-89×CAS-3)×HA-89)	31	62		3.44 (0.06)
Pooled	52	137		0.64 (0.42)
Heterogeneity				3.30 (0.10–0.05)
BC_1F_1(CAS-3×(HA-89×CAS-3))		76	19	1.27 (0.26)
BC_1F_1((HA-89×CAS-3)×CAS-3)		78	16	3.19 (0.07)
Pooled		154	35	4.23 (0.04)
Heterogeneity				0.23 (0.75–0.50)

[a] Ratios tested: F_2 generation =1:14:1; BC_1F_1 to HA-89 generation =1:3; and BC_1F_1 to CAS-3 generation =3:1

Table 5 Number of F_3 seeds having a different stearic acid content in the analysis of 21 F_3 populations from the cross HA-89×CAS-3

F_3 population	% C18:0 content in F_2 half-seed	% C18:0 content in F_3 seeds		No. F_3 seeds with % C18:0 content		Type of segregation and chi-square value (P)[a]
		Mean	Range			
Group I						
F3–1	4.6	3.7	2.7–5.5			No segregation
F3–2	5.5	4.8	3.3–6.5			No segregation
F3–3	5.9	4.3	2.7–6.0			No segregation
Group II						
F3–4	6.4	6.4	3.2–9.5			Continuous
Group III				<10.0	>13.0	
F3–5	7.0	8.4	3.8–19.7	74	17	2.09 (0.15)
F3–6	7.2	8.8	3.3–20.0	70	25	0.06 (0.81)
F3–7	8.0	9.4	3.1–20.2	70	26	0.01 (0.90)
Group IV				<22.0	>22.0	
F3–8	8.7	11.8	5.0–26.9	86	10	2.84 (0.09)
F3–9	9.9	10.7	4.6–23.0	92	3	1.55 (0.21)
F3–10	11.2	10.1	4.2–25.0	90	5	0.16 (0.69)
F3–11	11.8	12.3	4.2–24.6	32	3	0.32 (0.57)
F3–12	12.8	12.2	3.8–23.7	43	5	1.42 (0.23)
Group V				<22.0	>22.0	
F3–13	17.6	18.1	8.5–26.4	49	10	2.23 (0.14)
Group VI						
F3–14	18.0	19.9	13.4–25.7			Continuous
F3–15	18.2	19.1	15.1–25.3			Continuous
F3–16	19.6	21.8	14.2–26.8			Continuous
F3–17	20.8	21.2	17.9–24.8			Continuous
F3–18	22.0	20.3	14.8–26.1			Continuous
Group VII						
F3–19	22.9	23.2	20.1–25.5			No segregation
F3–20	24.0	25.4	21.1–27.3			No segregation
F3–21	27.2	27.0	22.8–31.8			No segregation

[a] Ratios tested: Group III and V =3:1; Group IV =15:1

gous dominant state (F_2 genotype *Es1Es1Es2es2*). The C18:0 content of the F_3 seeds from F_2 plants in group III ranged from 3.1 to 20.0%. The higher C18:0 levels of this group did not reach those of the CAS-3 line, suggesting that the F_2 genotype was *Es1es1Es2Es2*.

The range for C18:0 content in the F_3 seeds of F_2 plants from Group IV was similar to that observed in the F_2 segregation of crosses between HA-89 and CAS-3 (Fig. 2). Therefore, the F_2 genotype of this group was the double heterozygote *Es1es1Es2es2*. F_3 seeds of F_2 plants from group V showed a range for C18:0 content from 8.5 to 26.4%. The absence of low C18:0 values (<8%) in this segregation indicated that the *Es2* gene was in a homozygous recessive state. Therefore, the F_2 genotype of

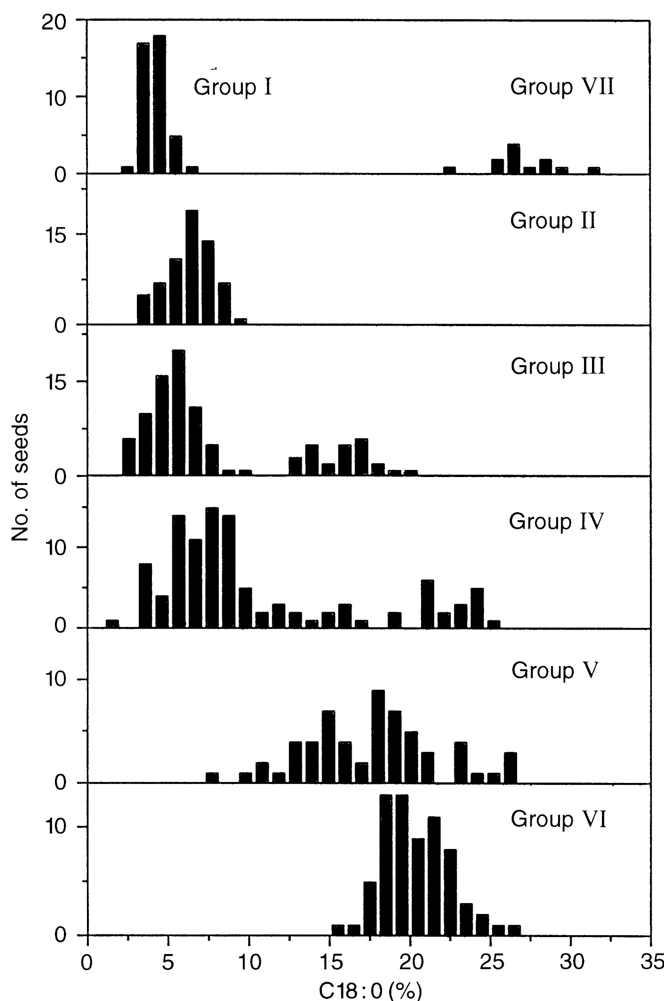

Fig. 3 Distribution of stearic acid content in individual seeds of the F_3 populations from the cross between HA-89 and CAS-3

this last group was *Es1es1es2es2*. Finally, the C18:0 content of F_3 seeds from F_2 plants of group VI ranged from 13.4 to 27.0%, which was interpreted in terms of the segregation of the *Es2* gene, the *Es1* gene being in a homozygous recessive state (F_2 genotype *es1es1Es2es2*). Some plants from this group that showed a smaller C18:0 range of variation, such as F3–17 (see Table 5), could also have the F_2 genotypic configuration *Es2Es2es1es1*.

Similar genetic systems with two genes, but both acting in an additive equal manner, have been proposed for the control of the erucic acid content in the three amphidiploid species of *Brassica*, *B. napus* (Harvey and Downey 1964), *B. juncea* (Kirk and Hurlstone 1983) and *B. carinata* (Getinet et al. 1997), as well as for the control of polyunsaturated fatty acid biosynthesis in flax (Green 1986).

The different genetic ratios obtained in the segregating generations from the crosses RDF-1–532×CAS-3 and HA-89×CAS-3, together with the high C18:0 content of the parental line RDF-1–532 (8%) as compared with the control line HA-89 (5%), suggested that RDF-1–532, from which the mutant CAS-3 originated, already carried the recessive allele *es2* involved in the control of the high C18:0 trait, as mentioned previously. Accordingly, the mutagenic treatment only induced a single recessive mutation in the wild dominant allele *Es1*, resulting in the mutant allele *es1* which, therefore, was the only allele segregating in crosses between RDF-1–532 and CAS-3. Similar conclusions have been reported by Pérez-Vich et al. (1999) in the inheritance studies on the high C16:0 sunflower mutant CAS-5, and demonstrate that the genetic background of the original parental line may be one of the main factors in obtaining new phenotypes for the fatty acid composition of the seed oil after a mutagenic treatment.

Because of the small number of loci involved in the genetic control of the high C18:0 trait in the mutant line CAS-3, a successful transfer of this trait into breeding lines can be performed in a few generations. Some of the conclusions of this study will be very helpful in this process. First, it is necessary to take into account the existence of a partial maternal effect on C18:0 content, which depends on the parents involved in the cross. Second, the high C18:0 content is a recessive trait, which indicates the need for seed production under isolation. And last, but not least, the presence of the *es2* allele in heterozygous genotypes in a backcross program can not be easily distinguished because of its small phenotypic contribution to the total C18:0 content. Consequently, we suggest that the F_2 seeds from each backcross generation should be split, and only those embryo portions containing a similarly high C18:0 content to the high C18:0 parent grown under the same conditions should be selected for additional backcrosses.

In conclusion, the results of this study clarify the genetic control of the high C18:0 content in the sunflower mutant line CAS-3 and establish the basis for effective breeding strategies for the development of cultivars producing an oil high in C18:0.

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