

MOLECULAR MARKERS ASSOCIATED WITH SUNFLOWER OLEIC AND STEARIC ACID CONCENTRATIONS IN A HIGH STEARIC X HIGH OLEIC CROSS

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Summary: The combination of seed oil phenotypes of sunflower with high oleic acid (C18:1), which imparts a higher oxidative stability to the oil and health benefits, and high stearic acid (C18:0), which gives greater solidity, would result in a novel oil quality of great value for the food industry. A high C18:0 line (CAS-3) and a high C18:1 line (HAOL-9) were crossed in order to obtain an F₂ population segregating for both traits. RFLP and AFLP markers were used to construct a linkage map from this population for the identification of QTL associated with high C18:0 and high C18:1 content in sunflower seed oil. Major QTL controlling the level of C18:0 and C18:1 were found on linkage groups 1 and 14, respectively. These QTL explained 78.0% and 84.5% of the phenotypic variance observed for C18:0 and C18:1 in this F₂ population. Moreover, candidate genes from the fatty acid biosynthetic pathway were also mapped. The major loci detected by the $\Delta 9$ -stearate desaturase (U91340) and $\Delta 12$ -oleate desaturase (U91341) gene probes coincided with the QTL peaks for C18:0 and C18:1 on linkage groups 1 and 14. This result strongly suggests that these genes were mutated, giving rise to give elevated levels of C18:0 and C18:1 in the seed oil of CAS-3 and HAOL-9 respectively. A FatB thioesterase gene (AF036565) was also mapped, but no association was found between this marker and the levels of C18:0 and C18:1. Due the fact that we are dealing with a biochemical pathway, most QTL had significant effects on the level of more than one fatty acid.

INTRODUCTION

The seed oil of normal cultivated sunflower has a characteristically high proportion of oleic (C18:1) and linoleic (C18:2) acids, which together account for about 90% of the total fatty acid composition. The remaining 10% corresponds to the saturated fatty acids palmitic (C16:0) and stearic (C18:0). Sunflower lines with modified fatty acid profiles in the seed (high C16:0, high C18:0, high C18:1) have been developed through induced mutagenesis (Soldatov, 1976; Ivanov, 1988, Osorio, 1995). These seed oils are in demand due to their improved nutritional and/or technological properties (Kinney, 1999).

The sunflower lines CAS-3 (high C18:0; Osorio, 1995), with the high C18:0 alleles *es1* and *es2* (Pérez-Vich et al., 1999), and HAOL-9 (high C18:1), carrying the high C18:1 *Ol* alleles (Fernández-Martínez et al., 1989), were crossed to study the feasibility of combining these two traits in a single line (Pérez-Vich et al., 2000). The study of the BC₁, F₂, and F₃ populations of this cross, suggested the existence of a genetic linkage between the alleles *Es2* and *Ol*. No recombination was found between these two alleles, which resulted in the absence of high C18:0 (>20%)/high C18:1 (>50%) phenotypes.

The aim of this study was to construct a genetic linkage map, comprising both RFLP (Restriction Fragment Length Polymorphism) and AFLP (Amplified Fragment Length Polymorphism) markers, in order to identify QTL controlling the levels of C18:0 and C18:1 in an F₂ population from this cross and therefore obtain a better understanding of the nature of the genetic relationship between these two traits. Dehmer and Friedt (1998) have already reported the development of a RAPD marker for high oleic acid content in sunflower, but this marker was not used in this study. However, a number of sunflower genes, for enzymes involved in the fatty acid biosynthetic pathway in seeds, have now been cloned and their polymorphism studied within cultivated sunflower (Kabbaj, 1996; Hongtrakul et al., 1998a, 1998b; Lacombe et al., 1999). The cDNA clones of the Δ^9 -stearate desaturase (Hongtrakul et al., 1998a), the Δ^{12} -oleate desaturase (Hongtrakul et al., 1998b), and a FatB thioesterase were also mapped as potential candidate genes for high C18:0 and high C18:1 QTL.

MATERIALS AND METHODS

The C18:0 and C18:1 F₂ segregating population used in this study (named F2-34) was derived from a cross between the high C18:0 line CAS-3, and the high C18:1 line HAOL-9 (Pérez-Vich et al., 2000). Fatty acid composition was determined for each of 139 F₂ half-seeds by gas-liquid chromatography (Pérez-Vich et al., 2000). Both the C18:0 and C18:1 showed a two-gene segregation in this population. F₂ half-seeds were grown to obtain the F₃ seeds. F₃ families from each individual F₂ plant were obtained in the field at Venado Tuerto (Argentina).

Tissue from 15 plants of each F₃ family was bulked prior to DNA extraction in order to recreate the F₂ genotype. The procedures of DNA isolation, digestion, Southern blotting, and hybridization were carried out as reported by Berry et al. (1994, 1995). Additionally, cDNA clones for the fatty acid biosynthesis enzymes Δ^9 -stearate desaturase [SAD-17

(U91340), Hongtrakul et al., 1998a], $\Delta 12$ -oleate desaturase [OLD-7 (U91341) Hongtrakul et al., 1998b] and FatB thioesterase (AF036565) were used as RFLP probes. AFLP analyses were carried out by Keygene N.V., Wageningen, NL. The segregation of alleles at the RFLP marker loci was checked against the expected ratios for codominant (1:2:1) or dominant (3:1) markers in an F₂ population using a Chi-squared test. An RFLP-AFLP linkage map was constructed with MAPMAKER V 3.0 (Lander et al., 1987), using the Kosambi centiMorgan function. For QTL analysis by interval mapping, using the computer program MAPMAKER/QTL V 1.1 (Lander and Botstein, 1989), a sub-set of codominant marker loci was selected to avoid problems associated with marker clustering. A LOD threshold of 3.0 was used to identify QTL regions affecting the concentrations of the different fatty acids. The data set and the unlinked loci were also analysed using a one-way ANOVA.

RESULTS AND DISCUSSION

Linkage map

In total, the 74 RFLP polymorphic probes detected 85 loci, of which 74 were codominantly scored. Keygene ran a twelve *EcoRI/MseI* primer combinations on 134 individuals, which revealed a total of 202 loci. All the AFLP loci were scored codominantly based on signal intensity differences between homozygotes and heterozygotes, using Keygene's proprietary software system; however most loci contained a variable number of dominant scores (i.e. C's and D's). The 85 RFLP and the 202 AFLP loci were arranged into 17 linkage groups (LGs) at a LOD of 3.0 and a maximum distance of 35 cM, and they covered a total of 1493 cM of the sunflower genome. The linkage group nomenclature is according to Berry et al. (1997). Three markers were found to be unlinked. The majority of probes segregated normally, but there were some regions of distorted segregation on LGs 2, 14 and 16. In general, the RFLP and AFLP data sets were complementary, in that, both marker systems revealed polymorphisms in regions of the genome where the other one did not. However, acute clustering of AFLP loci was seen on LGs 1, 3, 5, 8, 9, 10, 11, 13, 16 and 17.

For the QTL analysis, a sub-set of 131 loci was selected, which included loci detected by the SAD-17, OLD-7 (Fig. 1) and FatB cDNA probes. The average distance between adjacent markers was 10.7 cM, although on LG 2, 4, 10, 13 and 16 there were intervals of greater than 30 cM, which could not be reduced due to the lack of polymorphic markers in these regions. LG 6 had the lowest number of mapped loci, covering only 18 cM, but the overall length of the other 16 linkage groups ranged from 68 cM to 119 cM.

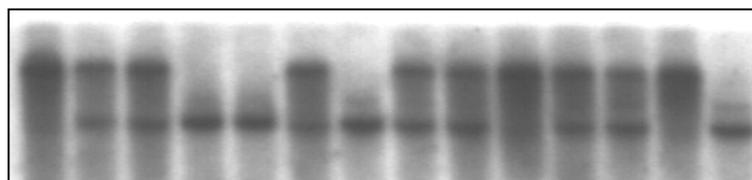


Figure 1.- Southern analysis of the ³²P-labelled OLD-7 probe hybridized to *HindIII* digested DNA from 14 F₂ individuals from the cross HAOL-9 x CAS-3. Upper band: allele from HAOL-9, lower band: allele from CAS-3.

QTL analysis

Three putative high C18:0 QTL from the CAS-3 parent, controlling the level of C18:0, were identified on LGs 1, 14 and 16. The main high C18:0 QTL (peak LOD 40.1) (Fig. 2) was detected on LG 1 and it explained 78.0 % of the phenotypic variation for this trait. The gene action at this QTL was essentially recessive (Table 1). The main locus detected by the SAD-17 cDNA maps very close to the peak of this QTL (Fig. 2). Since C18:0 is desaturated to C18:1 by the Δ^9 -stearate desaturase (Ohlrogge and Browse, 1995), it is not surprising to find a coincidence between this major QTL and a SAD-17 locus. Significant associations between the C18:0 concentration and other markers flanking SAD-17 were also found. The hypothesis of multiple high C18:0 QTL in this region is unlikely because the LOD score of a multi-locus model, including the three highest LOD peaks, was similar to that of the model for a single QTL (i.e. 43.28 versus 40.13).

A second region, associated with the C18:0 concentration, was detected in LG 16. However, five of the nine markers on this LG showed distorted segregation at the 1% level due to an almost complete absence of CAS-3 alleles. Therefore, the association between this region and the C18:0 content is an artifact of this gross distortion. Finally, a third high C18:0 QTL was located on LG 14, in the same position as the major QTL for C18:1 in this population. This QTL also has a significant effect on the level of palmitic acid (Table 1). This is explained by the fact that we are trying to map different components in the same biosynthetic pathway, in which adjacent steps are correlated (e.g. the linear correlation coefficient between C18:0 and C18:1 in this segregating population was -0.65)

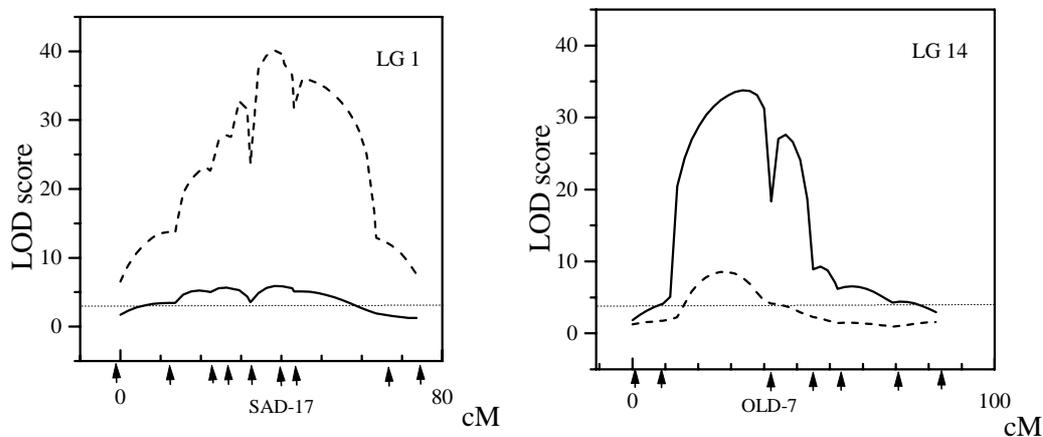


Figure 2.- LOD profiles for loci controlling stearic acid (---) and oleic acid (—) content on linkage groups 1 and 14 of sunflower. The X axis shows the marker loci, represented by black arrows. The dashed line indicates a LOD score threshold of 3.0.

Two major high C18:1 QTL (LOD threshold 3.0) controlling the C18:1 concentration were identified on LGs 1, and 14 (Fig. 2). As in the case of C18:0, the high C18:1 QTL on LG 1 is due to the negative correlation between C18:0 and C18:1. The peak LOD score for the major high C18:1 QTL on LG 14 was 33.8 and this QTL explained 83.9% of the phenotypic variation in C18:1 content. The close proximity of the LOD peak to the OLD-7 marker locus (Fig. 2) points to a dominant mutation in the Δ^{12} -oleate desaturase in HAOL-9. An alteration in the regulatory sequences of this gene has been previously reported for the explanation of the high C18:1 mutation in sunflower (Hongtrakul et al., 1998b). This major

QTL for C18:1 on LG 14 is probably one of the *Ol* genes controlling the high C18:1 trait previously described in high C18:1 sunflower lines (Fick, 1984; Urie, 1985; Miller et al., 1987; Fernández-Martínez et al., 1989).

ANOVA analysis confirmed the major QTL locations for C18:0 and C18:1, with the most significant marker loci being SAD-17 and OLD-7 on LGs 1 and 14 respectively (Table 1). No association between the three unlinked markers and the C18:0 or C18:1 contents was found.

Table 1.- ANOVA statistics for the SAD-17 and OLD-7 loci showing their significant effects ($P < 0.05$) on the levels of C16:0, C18:0, C18:1 and C18:2 in the F_2 population of sunflower cross HAOL-9 x CAS-3

Locus	Number of individuals ^a			Mean (m) C16:0 and F value ^a				Mean C18:0 (m) and F value ^a			
	n(A)	n(H)	n(B)	m(A)	m(H)	m(B)	F	m(A)	m(H)	m(B)	F
OLD7	16	64	28	6.27	5.34	5.07	6.18	12.84	9.06	8.27	3.91
SAD17	21	57	31	6.63	5.10	5.12	30.92	17.52	8.36	5.69	139.52
				Mean (m) C18:1 and F value ^a				Mean (m) C18:2 and F value ^a			
				m(A)	m(H)	m(B)	F	m(A)	m(H)	m(B)	F
OLD7	16	64	28	43.46	75.03	83.58	27.89	37.04	9.72	2.50	33.88
SAD17	21	57	31	54.68	77.04	76.89	14.39	20.37	8.77	11.65	4.51

^a The A allele correspond to the line CAS-3, and the B allele to the line HAOL-9

A minor QTL for C18:1 from CAS-3 (peak LOD 2.39) was detected on LG 8. Pérez-Vich et al. (2000) suggested the presence of high C18:1 alleles in some genotypes of CAS-3, as reported previously for other low C18:1 lines (Fernández-Martínez et al., 1989). This QTL and a second one in LG 2 were also identified when the major high C18:1 QTL on LG 14 and 1 were fixed. These minor QTL could be C18:1 modifier loci, previously reported by Urie, (1985), Miller et al. (1987) and Fernández-Martínez et al. (1989). A multi-locus model including the effects of QTL on LGs 1, 2, 8, and 14 explained 93.3% of the variance for C18:1.

General conclusions

Two major QTLs, one controlling high C18:0 and one high C18:1 content, have been identified on LGs 1 and 14 respectively. The QTL locations correspond closely with the map positions for a $\Delta 9$ -stearate desaturase and a $\Delta 12$ -oleate desaturase gene, on these LGs. It is therefore probable, given the biochemical role that these enzymes play in lipid biosynthesis, that these are the candidate genes for the major QTL identified in this study. Unfortunately, due to the limited population size, the small genetic effect of *Es2* and its partially recessive gene action, it was not possible to identify a minor high C18:0 QTL for this locus. Therefore this study could not prove or disprove the hypothesis of a genetic linkage between an *Ol* locus and *Es2*. Although the C18:0 and C18:1 levels in sunflower seed oil can be easily assayed by gas-liquid chromatography, the use of PCR markers derived from these genes (Hongtrakul et al., 1998a, 1998b), will greatly improve breeding efficiency for these traits, especially for the recessive high C18:0 QTL. Moreover, genotypic selection will be independent from the environment, which may have effects on the fatty acid composition of seed oil.

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REFERENCES

- Berry, S.T., Allen, R.J., Barnes, S.R., and Caligari, P.D.S. (1994) Molecular analysis of *Helianthus annuus* L. 1. Restriction length polymorphism between inbred lines of cultivated sunflower. *Theor. Appl. Genet.* 89: 435-441.
- Berry, S.T., Leon, A.J., Hanfrey, C.C., Challis, P., Burkholz, A., Barnes, S.R., Rufener, G. K., Lee, M., and Caligari, P.D.S. (1995) Molecular marker analysis of *Helianthus annuus* L. 2. Construction of an RFLP linkage map for cultivated sunflower. *Theor. Appl. Genet.* 91: 195-199.
- Berry, S.T., Leon, A.J., Peerbolte, R., Challis, C., Livini, C., Jones, R., and Feingold, S. (1997) Presentation of the Advanta sunflower RFLP linkage map for public research. In "Proc. 19TH Sunflower Res. Workshop", Fargo, USA, January 9-10, pp 113-118.
- Dehmer, K. J. and Friedt, W. (1998) Development of molecular makers for high oleic acid content in sunflower. *Industrial Crops and Products* 7: 311-315.
- Fernández-Martínez, J., Jimenez, A., Dominguez, J., García, J.M., Garcés, R., and Mancha M. (1989) Genetic analysis of the high oleic acid content in cultivated sunflower (*Helianthus annuus* L.). *Euphytica* 41: 39-51.
- Fick , G.N. (1984) Inheritance of high oleic acid content in the seed oil of sunflower. In " Proc. 6th Sunflower Research Forum" , National Sunflower Assoc., Bismark, ND, pp 9.
- Hongtrakul, V., Slabaugh, M.B., and Knapp, S. (1998a) DFLP, SSCP, and SSR markers for -9-stearoyl-acyl carrier protein desaturases strongly expressed in developing seeds of sunflower: intron lengths are polymorphic among elite inbred lines. *Molecular Breeding* 4: 195- 203.
- Hongtrakul, V., Slabaugh, M.B., and Knapp, S. (1998b) A seed specific -12 oleate desaturase gene is duplicated, rearranged, and weakly expressed in high oleic acid sunflower lines. *Crop Sci.* 38: 1245-1249.
- Ivanov, P., Petakov, D., Nikolova, V., and Petchev, E. (1988) Sunflower breeding for high palmitic acid content in the oil. In " Proc. 12TH Int. Sunflower Conf." Int. Sunflower Assoc., Toowoomba, Australia. pp 463-465
- Kabbaj, A., Vervoort, V., Abbott, A.G., Tersac M., and Bervillé, A. (1996) Expression d'une stéarate et d'une oléate désaturases chez le tournesol normal et à haute teneur en acide oléique, clonage de fragments génomiques et variabilité chez quelques *Helianthus* OCL 3: 452-458.
- Kinney, A.J. (1999) New and improved oils from genetically-modified oilseed plants. *Lipid Technol. Newsletter*, April 1999, pp 36-39
- Lacombe, S., Lambert, P., Cellier, F., Casse, F., and Bervillé, A. (1999) RFLP profiles in low oleic sunflower using SDI-, a stearoyl-ACP, and an oleoyl-PC desaturases c DNAs. *Helia* 22: 19-28.
- Lander, E., Green, P., Abrahanson, J., Barlow, A., Daley, M., Lincoln, S., and Newburg, L. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1: 174-181.
- Lander, E. S., Botstein, D. (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* 121: 185-199.
- Miller, J.F., Zimmerman, D.C., and Vick, B.A. (1987) Genetic control of high oleic acid content in sunflower oil. *Crop Sci.* 27: 923-926.
- Ohlrogge, J.B. and Browse, J. (1995) Lipid biosynthesis. *Plant Cell* 7: 957-970.
- Osorio, J., Fernández-Martínez, J.M., Mancha, M. and Garcés, R. (1995) Mutant sunflowers with high concentration of saturated fatty acids in the oil. *Crop Sci.* 35: 739-742.
- Pérez-Vich, B., Garcés, R. and Fernández-Martínez, J.M. (1999). Genetic control of high stearic acid content in the seed oil of sunflower mutant CAS-3. *Theor Appl Genet*: 99: 663-669.
- Pérez-Vich, B., R. Garcés, and Fernández-Martínez, J.M. (2000) Genetic relationships between loci controlling the high stearic and the high oleic acid traits in sunflower. *Crop Sci.* (In press).
- Soldatov, K.I. (1976) Chemical mutagenesis in sunflower breeding.. In

" Proc. 7th Int. Sunflower Conf., Krasnodar" , USSR. June 27-July 3,
1976. Int. Sunflower Assoc., Vlaardingen, The Netherlands, pp 352-357
Urie, A.L. (1985) Inheritance of high oleic acid in sunflower. Crop Sci. 25: 986-989.