The Pervenets mutation in sunflower knocks out the wild microsomal oleate desaturase gene and leads to high oleic acid content in the seed oil

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ABSTRACT

Mutations in the seed oil pathways are of interest to release cultivars with modified fatty acid composition without transgenic methods. In sunflower, Pervenets mutation has been widely used to release sunflower cultivars with about 90% of oleic acid content in the seed oil without the exact mechanism of the mutation being understood. Here, we report new features of the Pervenets mutation organization and function as we have revealed the part of the microsomal oleate desaturase which is duplicated and we point out the expression of siRNA homologous to the oleate desaturase cDNA in the developing embryo at the stage when the oleic acid is synthesized. This new fact infers that the oleate desaturase underwent post transcriptional gene silencing (PTGS), which explains all the difficulties faced by breeders in stabilizing the oleic acid content in cultivars whatever the environmental conditions.

Key words: gene silencing – mutation – oleic acid content – PTGS – seed oil – siRNA

INTRODUCTION

Sunflower oil is naturally rich in linoleic acid (55-70%), and, consequently, poor in oleic acid (20-25%). Classic varieties are qualified as low oleic (LO). Until the 1970s, mutagenesis programs were conducted in order to produce varieties with an increased oleic acid content (OAC) compared to the classic (LO) varieties. The Pervenets sunflower population was obtained by chemical mutagenesis. It displayed OAC in seed oil around 65% (Soldatov, 1976). New varieties with OAC higher than 83% (HO for high oleic) varieties were then derived from the Pervenets population through breeding programs. This fatty acid composition modification of oil is located specifically in embryo tissues (Garcés et al., 1989). Due to the increased health interest of oleic acid and the similar agronomic performance of the HO compared to the classic varieties, HO varieties are now widely used in the world covering about 1.2 million ha (Collectif, 2004). In these varieties, the microsomal oleate-desaturase (MOD)-mRNA accumulation is reduced compared to the classic genotypes, leading to a decrease in MOD activity in the seeds during lipid reserve elaboration steps (Garcés and Mancha, 1989; 1991; Kabbaj et al., 1996a,b,c; Hongtrakul et al., 1998b). Using a candidate gene approach in diversity analysis, linkage disequilibrium was reported between the Pervenets mutation and an HO-specific MOD allele. Genetic studies performed on F_2 and recombinant inbred lines (RI Lines) populations revealed that this linkage disequilibrium is due to a closely genetic linkage between the Pervenets mutation and the HO-specific MOD allele (Hongtrakul et al., 1998b; Lacombe et al., 2001; Lacombe and Bervillé, 2001). However, these approaches could not determine whether the HO-specific MOD allele carries or is genetically linked to the Pervenets mutation. Consequently, the nature of the mutation is still unknown. Recently, Schuppert et al. (2006) studied the HO-specific MOD allele using PCR based approaches to identified molecular markers of the Pervenets mutation. However, no PCR codominant markers were found between wild type and mutant oilseed inbred lines due to a lack of DNA polymorphism in the region tested.

Recently, Lacombe et al. (2001), in studying RI lines progenies, showed that half the families carrying the Pervenets duplication were not as high oleic as expected. They therefore suggested that a genetic factor masked the Pervenets mutation effect and that it segregated in the progenies. The factor as a suppressor was called olesup. Crosses between a RI line carrying an efficient *olesup* allele and an HO line led to a low oleic F_1 progeny, whereas crosses between a RI line without an efficient *olesup* and a high oleic line led to a high oleic F_1 progeny (Lacombe and Bervillé unpublished; Y. Demurin, pers com).

Post transcriptional gene silencing (PTGS) is a way to regulate gene expression when expressed sequences are repeated. It is not widely spread (Baulcombe, 2004) and it is associated with 21 to 25 bp RNA molecules homologous to the gene under knock out (Hamilton and Baulcombe, 1999; Della-Vedova et al., 2005). Their presence points to the PTGS mechanism.

In this work, we present the organization of the Pervenets specific MOD allele in two different parts. The first part is present in both Pervenets and classic genotypes and carries a classic MOD gene (MOD-Cs). The second section is specific in Pervenets genotypes and carries a duplication of the MOD gene (MOD-Per). We also showed that the Pervenets mutation acts in *trans* to induce MOD mRNA under- or no-accumulation. We propose that the Pervenets mutation corresponds to MOD duplication and induces gene silencing on the normal MOD gene. Moreover, this work allowed us to identify co-dominant SSR in the intron of the MOD gene and other PCR markers corresponding to the Pervenets mutation. Such molecular markers may represent advantageous and useful tools in breeding programs. Finally, we characterized siRNA in Pervenets embryos that are absent in classic sunflower and it sustains PTGS. Moreover, this mechanism is known to be suppressed, and, consequently, it questions whether OAC variation in high oleic hybrids is due to the environment or to a genetic suppressor as we revealed *olesup* alleles.

MATERIALS AND METHODS

Plant Material

The RI lines segregating population was produced as described by Lacombe et al. (2001). The classic line 83HR4 (INRA) as the female parent was crossed with the HO line RHA345 (USDA-Fargo). One F_1 plant was self-fertilized to produce the F_2 progenies composed of 390 plants. Further generations were obtained by selfing ten individuals from each progeny, but only one producing many seeds was kept for the next generation. Because of inbreeding depression and self incompatibility, the F_6 generation was composed of 174 lines. Five seeds of the F_6 generation were analysed and put in Jiffy pots and then transferred to the field, but only one (plant 2 of each line, or, if lacking, plant 3) was retained for inheritance analysis. Half a cotyledon from each seed was analyzed for oil composition before germination, and each plant was further genotyped with the MOD cDNA as a probe (RFLP) and with different PCR primer pairs.

For mRNA and siRNA accumulation studies in immature seeds, self progenies and controlled crosses were obtained under a protective paper bag set up a few days before flowering. Each plant from the LO and HO lines was numbered and studied separately for all steps. Crosses were performed by transferring pollen from the male to the female under a paper bag to prevent illicit fertilization.

Total RNA was extracted using TriReagent (Sigma) according to the manufacturer's instructions from immature seeds at 10 to 15 days after pollination. For OD mRNA accumulation studies, northern blots were performed according to Sambrook et al. (1989) and northern blots were probed with 32P random priming labelled OD-cDNA (U91341). For siRNA detection, northern blots were made as described previously (Herr et al., 2005). T7-OD riboprobe was generated by T7 transcription (Promega) of a 1176 bp fragment that was amplified with Forward OD primer carrying a T7 extension (5'ataatacgactcactatagggtcgctaacccgttcgttctc3') and a reverse OD primer (5'tctaaaacacacccaacacg3')

RESULTS

The MOD-HO allele carries the common part of the MOD-LO allele on a sequenced fragment of 13.5kb. It carries the common 5.5kb *Eco*RI fragment, as predicted (Fig. 1). One single intron of 1684nt was detected between nt 83 and 1767 in the putative 5' untranslated region, 29nt before the ATG. A 16nt repeats of a $^{5'}$ATT$^{3'}$ SSR motif was revealed in the intron between nt 784 and 832. A *Hind*III site is present in the intron. It enables a 2.1kb *Hind*III fragment carrying only 83nt similar to the MOD sequence. This fragment was never revealed in RFLP profiles of HO or classic genotypes probably due to the small size of the MOD sequence on this fragment. No other *Hind*III site was detected in the rest of the putative gene or in the 4.3kb sequenced on its 3' side. This suggests that the HO-specific insertion is beyond this sequenced region on the 3'side on the common fragment.

A primer pair was selected to amplify the SSR locus located in the intron (Table 1). Polymorphism of the putative MOD was evaluated and three different alleles with 14, 15 and 17 TTA were found. These three alleles occurred in both the HO and classic genotypes showing that no linkage disequilibrium exists between the Pervenets mutation and a specific SSR allele despite the tight genetic linkage between the two loci. This suggests that natural variation at the SSR locus has occurred since the Pervenets mutation event.

To isolate parts of the HO-specific fragment, long PCR experiments were performed on DNA from the RHA345 HO and the 83HR4 classic lines using the F_c-a primer in combination with primers designed for the whole MOD cDNA sequence in both orientations (F1 to F8 and R1 to R8, Table 1). PCR products

were detected with the F_c-a primer in combination with R1, R2, R3, R4, R5, R6 and R7 whereas for the classic line no PCR product was detected with any of the primer sets.

The 4kb F_c-a_R7 PCR fragment from the HO line was cloned and sequenced. The organisation of this fragment is, from 5' to 3': 1) - 1219nt overlapping with the previously sequenced MOD region as expected according to the F_c primer position; 2) - 1357nt without any similarity to database sequences or to previously sequenced regions; 3) - 1487nt identical to the putative MOD gene between nt1447 and nt2421 corresponding to 239nt of intron and the following 1248nt of the exon 2. The 5' extremity of the putative gene was not detected. This MOD sequence and the putative gene had the same orientation. No *Eco*RI or *Hind*III site was detected in the F_c-a_R7 fragment. This agrees with the physical map of the OD-HO allele established from RFLP profiles (Fig. 1).

Table 1. List of primer pairs used in pairwise combination and size of fragments.

F_R	5'-F_ 3'	5'-R_ 3'	Expected_observed
1_8	accctaagcctctgtcgctc	tctaaaacacacccaacacg	most of cDNA
8-4	agatgatgaagggaaaggag	gccatagcaacacgataaag	3.974 kb across insertion
Fc-a_7	caaaccaccacccactaac	ggttctgggtctgggtctggtt	902 bp
Fc-b_1	gagaagagggaggtgtgaag	agcggttatggtgaggtcag	880 bp
Fc-b_1	gagaagagggaggtgtgaag	acaaagcccacagtgtcgtc	1247 bp
Fc-b_4	agaagagggaggtgtgaag	gccatagcaacacgataaag	1608 bp
1f_1r -	ttggagttcggtttatttat	ttagtaaacgagcctgaac	240 Ssr-ha-diapc MOD*RHA345*
1f_1r -	ttggagttcggtttatttat	ttagtaaacgagcctgaac	237 Ssr-ha-diapc-MOD*83HR4*

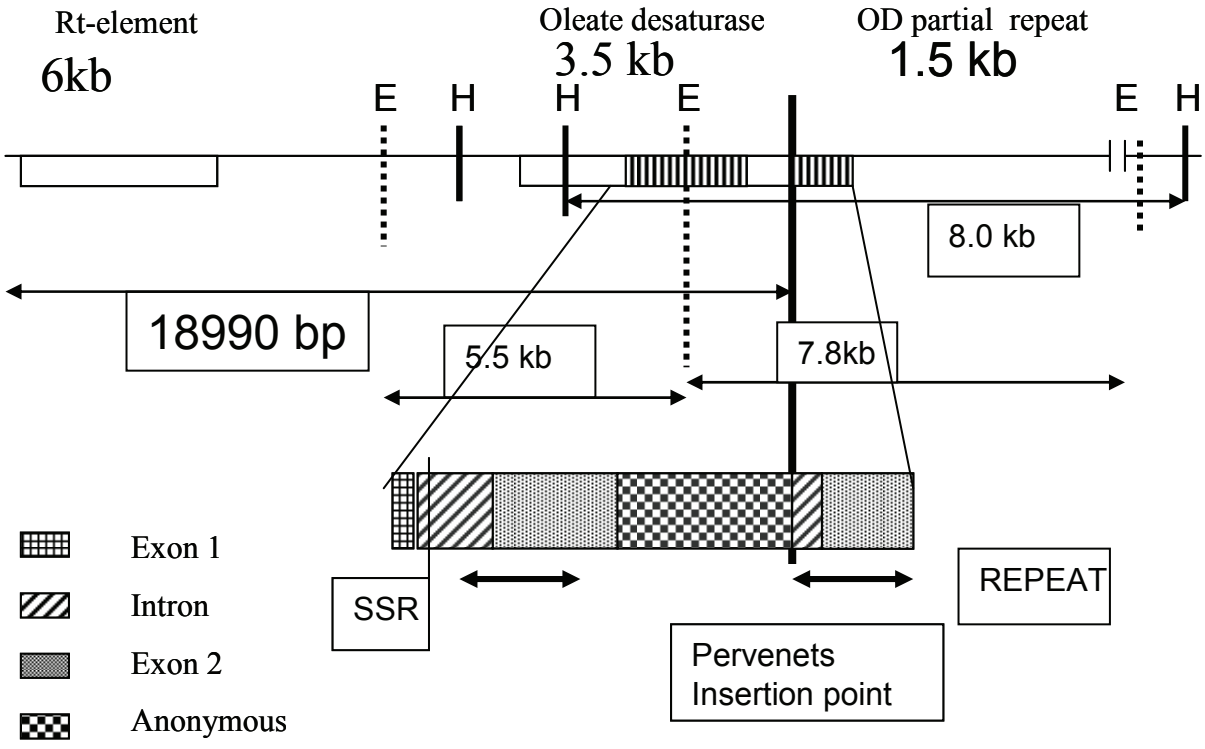

Fig. 1. A. Organisation of a microsomal MOD region in classic sunflower. Some features for regulation of the expression according to Lacombe and Bervillé (2001) and Bervillé et al. (2004). **B:** Organisation of the Pervenets mutation. Physical maps of the OD-Per region carrying an MOD gene and an extra fragment of 7.8kb carrying MOD-like sequences. E: *Eco*RI, H: *Hind*III site.

PCR amplification products of the expected size for all the HO genotypes tested were obtained with F_c-b designed on the F_c-a _R7 fragment in order to amplify an 880, 902, 1247, or a 1608bp fragment, in combination with R1, R2 or R4, respectively. These primer sets are suitable for the routine PCR technique according to the size of amplification products. They led to no amplification for the classic genotypes. An F2_R2 primer set used as a PCR control generated amplification at the expected size for

all the HO and classic genotypes. Consequently, the absence of PCR amplifications in classic genotypes with F_c-b / R1, R2 or R4 was not due to PCR failure, but, indeed, to the absence of these PCR fragments. Therefore, linkage disequilibrium exists between the Pervenets mutation and these HO-specific PCR fragments generated by F_c-b_R1, _R2 or _R4 primer sets.

The Pervenets mutation leads to an absence of, or a weak, MOD-mRNA accumulation in embryos (Fig. 2). No hybridisation signal was detected in HO embryos resulting from selfing or crosses. Moreover, results obtained in the hybrid embryos reveal that the mutation is dominant.

We selected primer pairs to amplify a 240pb PCR fragment carrying the microsatellite repeat in the MOD intron to map with Pervenets mutation. The use of these primers revealed polymorphism between the RHA345 HO line (17 TTA repeats) and the 83HR4 classic line (16 TTA repeats). For 174 RI lines studied, eighty-two display the 16-TTA allele and ninety-two RI lines carried the 17-TTA allele that fits the 1:1 ratio for the two SSR alleles (χ^2 P>0.1).

In the F_6 RI lines population, we then compared the segregation of the SSR polymorphism with the 7.8kb *Eco*RI Pervenets-specific fragment from the RHA345 line and with the PCR-specific 872bp fragment. This is in agreement with a tight genetic linkage between the Pervenets mutation and OD-HO allele (Table 2). PCR tests were set up to detect the Pervenets insertion. PCR assays included positive and negative controls Pervenets mutation segregation studies in RI lines

The positive control was provided by the 880bp PCR fragment amplified across the insertion point. The negative control was as previously the F2_R2 MOD primer pair wise combination. In 174 F_6 RI lines, eighty-two RI lines carry the MOD-SSR allele of the classic parent, and ninety-two lines carry the MOD-SSR from the HO parent. Seventy-eight RI lines displayed the HO-specific 7.8kb *Eco*RI and fourteen RI lines carried a shortened insertion (Table 2).

Table 2. Number of RI lines in each HO or LO class according to the MOD-Cs or MOD-Per alleles at the MOD- locus. Six plants were heterozygous and excluded from further analysis; Sup0, non expressed form assumed for the suppressor of silencing; *Supole*, expressed form of the suppressor of silencing; *excluded from further analysis; Sup0, non expressed form of the suppressor

174 RI lines	LO (OAC<50%)	HO (OAC>50%)
5.5kb *Eco*RI 8kb *Hind*III	125	35
SSR 237	82	0
SSR 240	43 (6*)	35
*Hind*III 13kb		
PCR OD-Per 872bp	0	88
Sup	Sup0 (ND)	*Supole* 43

SiRNA were revealed specifically in RHA345 lines in ten-days-old embryos, whereas they were absent in the classic line 83HR4 (Fig. 2).

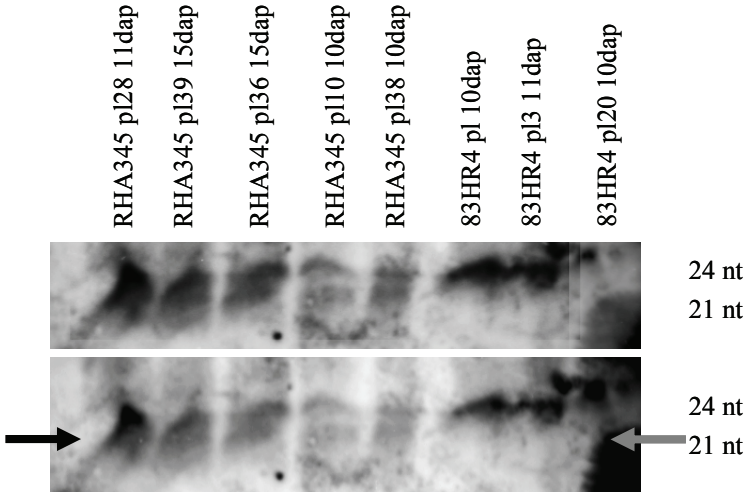

Fig. 2. Hybridization pattern with oleate desaturase as a riboprobe of total RNA fraction from immature (10 DAP) embryos harvested on different plants of lines RHA345 and 83HR4 showing (black arrow) and not showing (grey arrow) SiRNA at 21nt size, respectively.

DISCUSSION

Here we present evidence that the Pervenets mutation leading to the HO phenotype does not directly modify the MOD gene sequence but corresponds to MOD duplications. For other plant species, the mutations leading to an increase in OAC that have been characterised so far, directly affect MOD genes (Jung et al., 2000a,b; Patel et al., 2004; Okuley et al., 1994). The common MOD region is the only one detected in classic genotypes but other oleate desaturase do exist according to Martinez-Rivas et al. (2003). However, we showed that MOD-mRNA did not accumulate in segregating HO seeds whereas it accumulated in classic seeds confirming results already reported (Kabbaj et al*.,* 1996a,b,c; Hongtrakul et al*.,* 1998a,b). Moreover, the Pervenets mutation acts in *trans* to prevent MOD mRNA accumulation. A mutation in the MOD gene could not explain this dominance behaviour. Considering this result and the fact that the Pervenets mutation is associated with MOD duplications, we propose that the duplication induces a gene-silencing on the normal MOD gene leading to mRNA under accumulation.

Detection of MOD siRNA in the Pervenets mutant confirms the gene silencing mechanism. In Eukaryotes, gene silencing is a process that affects gene expression through sequence specific interactions. It involves 21nt and 24nt small interfering RNA (siRNA) produced from double strand RNA resulting from transcription of antisense or hairpin RNA and can act as dominant or semi dominant (Baulcombe, 2004). Strategies based on gene silencing against MOD genes have already been reported in crops to obtain transgenic plants with increased OAC. High OAC soybean and rapeseed were obtained through antisense and co-suppression mediated down-regulation of MOD (Kinney, 1996; Stoutjesdijk et al., 2000). Cotton transformed with an MOD inverted repeat construct showed high OAC (Liu et al., 2002). For the Pervenets mutant, the duplicated fragment is partially sequenced. Thus, we cannot predict whether antisense or hairpin RNAs is involved in the process. In sunflower, the Pervenets mutation associated with a gene silencing against the MOD gene may represent a new example of non-transgenic induced gene silencing in plants.

Molecular markers linked to the Pervenets mutation would represent advantageous and useful tools in breeding programs for rapid and early screening of genotypes carrying the mutation. PCR-based molecular markers linked to the Pervenets mutation were first co-dominant molecular markers.

The combination of these PCR-based molecular markers with the ones developed on the mutation itself would allow the determination of the homozygous or heterozygous status of the Pervenets mutation locus. Schuppert et al. (2006) have identified the same region and developed similar PCR tools for marker-assisted selection. However, some features did not fit our results. We cannot identify a 4.2kb *Eco*RI fragment on any autoradiogram. Probably, this fragment results from sequence concatenation of PCR products. Because several oleate desaturase genes do exist in sunflower (Martinez-Rivas et al., 2001), PCR may have produced a fragment from other MOD genes generating some confusion in the resulting sequence. We have also recognized some difficulties by primer extension upwards the insertion site. Apparently, the fragment obtained did not belong to the Pervenets locus since we obtained the same fragments for classic and Pervenets sunflower.

We provide evidence in the RI lines families that the Pervenets insertion can still be rearranged. All features for those RI lines converged, proving that the insertion shortened. Our results suggest that some RI lines may have lost duplicated MOD sequences. In fact, all these lines display low OAC in the seed oil, but the olesup suppressor in segregation may also be the cause of the LO phenotype. Genetic analyses are planned on these progenies to verify whether it may be due to the suppressor activity or to the loss of the duplication, which cancels silencing mechanisms.

REFERENCES

Baulcombe, D. 2004. RNA silencing in plants. Nature 431:356-363.

Bervillé, A., S. Lacombe, S. Veillet, C. Granier, S. Léger, and P. Jouve. 2004. Method of selecting sunflower genotypes with high oleic acid content in seed oil. November 02 2005. EP 1591536A1.

Collectif, 2004-2005. Collectif. De la production à la consommation, 2004-2005. PROLEA, Statistiques des Oléagineux & Protéagineux & Huiles & Protéines Végétales.

Della-Vedova, C.B., R. Lorbiecke, H. Kirsch, M.B. Schulte, K. Scheets, L.M. Borchert, B.E. Scheffler, U. Wienand, K.C. Cone, and J.A. Birchler. 2005. The dominant inhibitory chalcone synthase allele *C2-Idf (Inhibitor diffuse)* from *Zea mays* (L.) acts via an endogenous RNA silencing mechanism. Genetics 170:1989-2002.

Garcés, R, and M. Mancha. 1989. Oleate desaturation in seeds of two genotypes of sunflower. Phytochem. 28:2593-2595.

Garcés, R., and M. Mancha. 1991. In vitro oleate desaturase in developing sunflower seeds. Phytochem. 30:2127-2130.

Garcés, R., J.M. Garcia, and M. Mancha. 1989. Lipid characterization in seeds of a high oleic-acid sunflower mutant. Phytochem. 28:2597-2600.

Hamilton, A.J., and D.C. Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:886.

Herr, A.J., M.B. Jensen, T. Dalmay, and D.C. Baulcombe. 2005. RNA polymerase IV directs silencing of endogenous DNA. Science 308:118-120.

Hongtrakul, V., M.B. Slabaugh, and S.J. Knapp. 1998b. A seed specific Δ12 microsomal oleate desaturase gene is duplicated rearranged and weakly expressed in high oleic acid sunflower lines. Crop Sci. 38:1245-1249.

Jung, S, G. Powell, K. Moore, and A. Abbott. 2000b. The high oleate trait in the cultivated peanut *Arachis hypogaea* L. II. Molecular basis and genetics of the trait. Mol. Gen. Genet. 263:806-811.

Jung, S., D. Swift, E. Sengoku, M. Patel, F. Teule, G. Powell, K. Moore, and A. Abbott. 2000a. The high oleate trait in the cultivated peanut *Arachis hypogaea* L. I. Isolation and characterization of two genes encoding microsomal oleoyl-PC desaturases. Mol. Gen. Genet. 263:796-805.

Kabbaj, A., V. Vervoort, A.G. Abbott, M. Tersac, and A. Bervillé. 1996a. Expression of stearate and oleate desaturases in sunflower and high oleic sunflower: Cloning of genome fragments and variability in different *Helianthus*. OCL-Oleagineux Corps Gras Lipides 3:452-458.

Kabbaj, A., V. Vervoort, A.G. Abbott, M. Tersac, and A. Bervillé. 1996b. Expression of stearate, oleate and linoleate genes in sunflower with normal and high oleic contents. Helia 19-25:1-17.

Kabbaj, A, V. Vervoort, A.G. Abbott, M. Tersac, and A. Bervillé. 1996c. Expression d'une stéarate et d'une oléate desaturases chez le tournesol normal et à haute teneur en acide oléique clonage de fragments génomiques et variabilité chez quelques *Helianthus*. Oléagineux Corps Gras Lipides 3:452-458.

Kinney, A.J. 1996. Development of genetically engineered soybean oils for food applications. J. Food Lipids 3:273-292.

Lacombe, S., and A. Bervillé. 2001. A dominant mutation for high oleic acid content of sunflower (*Helianthus annuus* L.) oil is genetically linked to one oleate desaturase RFLP locus. Mol. Breed. 8:129-137.

Lacombe, S., F. Kaan, S. Léger, and A. Bervillé. 2001. An oleate desaturase and a suppressor loci direct high oleic acid content of sunflower (*Helianthus annuus* L.) oil in the Pervenets mutant C. R. Acad Sci. Paris, Série III 324:839-845.

Liu, Q., S. Singh, and A.G. Green. 2002. High-stearic and high-oleic cottonseed oils Produced by hairpin RNA-mediated post-transcriptional gene silencing. Plant Physiol. 129:1732-17430.

Martinez-Rivas, J.M., P. Sperling, W. Luhs, and E. Heinz. 2001. Spatial and temporal regulation of three different microsomal oleate desaturase genes (FAD2) from normal-type and high-oleic varieties of sunflower (*Helianthus annuus* L.). Mol. Breed. 8:159-168.

Martínez-Rivas, J.M., A. Sánchez-García, M.D. Sicardo, M.T. García-Díaz, and M. Mancha. 2003. Oxygen-independent temperature regulation of the microsomal oleate desaturase (FAD2) activity in developing sunflower (*Helianthus annuus*) seeds. Physiol. Plant. 117:179-185.

Okuley, J., J. Lightner, K. Feldmann, N. Yadav, E. Lark, and J. Browse. 1994. Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid-synthesis. Plant Cell 6:147-158.

Patel, M., S. Jung, K. Moore, G. Powell, C. Ainsworth, and A. Abbott. 2004. High-oleate peanut mutants result from a MITE insertion into the FAD2 gene. Theor. Appl. Genet. 108:1492-1502.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Second edition. Cold Spring Harbor Laboratory Press.

Schuppert, G.F., S. Tang, M.B. Slabaugh, and S. Knapp. 2006. the sunflower high-oleic mutant Ol carries variable tandem repreats of FAD2-1, a seed specific oleoyl-Phospatidyl choline desaturase. Mol Breed. 17:241-256.

Soldatov, K.I. 1976. Chemical mutagenesis in sunflower breeding. p. 352-357. In: Proc. 7th Int. Sunflower Conf.

Stoutjesdijk, P.A., C. Hurlstone, S.P. Singh, and A.G. Green. 2000. High-oleic Australian *Brassica napus* and *B. juncea* varieties produced by co-suppression of endogenous Δ12-desaturases. Biochem. Soc. Trans. 28:938 –940.