PROBLEMS AND GOALS IN STUDYING OIL COMPOSITION VARIATION IN SUNFLOWER

Séverine Lacombe* & André Bervillé

INRA, UR-GAP, 2 place P Viala 34060 Montpellier cedex 1 France
Lacombes@ensam.inra.fr, Berville@ensam.inra.fr

Summary: Plants present a large variability for seed oil composition that ensures a wide range of uses. Moreover, seed oil is modified by environmental factors. In different plants species, variation in seed oil composition has been generated by mutagenesis and transgene technologies. Understanding this variability is crucial to unravelling the oil seed modifying pathways in seeds. In this presentation, we summarise the phenotypes for oil mutants obtained in sunflower and develop in detail what has been obtained, what has been done and what is still not understood about the pathways of oil synthesis and modification in sunflower. Focusing on the Pervenets source, we compiled the data from literature about the effect of environmental factors on oil composition, genetic studies of seed oil composition and what has been reported more recently using molecular approaches. From this, we propose a simple model to explain the molecular origin of the high oleate (HO) mutation derived from Pervenets source.

INTRODUCTION

In plant, oil is made of triacylglycerol (TAG), each fatty acid esterifying one of the three alcohol functions. Fatty acids are synthetized by Fatty Acid Synthetase complex. They are first accumulated as saturated fatty acids (16:0: palmitic acid, 18:0: stearic acid). Several articles reviewed the basis for variation in different biosynthesis pathways (Ohlrogge *et al* 1991, Arondel *et al* 1992, Ohlrogge et Browse 1995, Browse 1997), focusing the light on desaturase enzymes. All desaturation of fatty acids after the production of oleoyl-ACP occurs most likely as phospholipid substrates. Three major desaturases are involved in fatty acid biosynthesis pathway in plant. The stearoyl-ACP desaturase or Δ9-desaturase catalyses the first desaturation of stearic acid (18:0) to oleic acid (18:1). The oleoyl-PC desaturase or Δ12-desaturase catalyses the second desaturation of oleic acid (18:1) to linoleic acid (18:2). The linoleoyl-PC desaturase or Δ15-desaturase catalyses the third desaturation of linoleic acid (18:2) to linolenic acid (18:3). Degradation of fatty acids occurs when they are as TAG. Lipase activity releases free fatty acids, which are toxic. Lipoxigenases also degrade fatty acids leading to production of lipid backbones used in secondary metabolism.

The activity of enzymes involved in seed oil composition is stage dependent. Genes that encode these enzymes are sequentially expressed according to development stages. Although they are expressed in the embryos, some maternal effects are sometimes observed. This has major consequences in the inheritance of oil composition.

Seed oils are utilised for dietary and industrial purposes, due to the large variation in fatty acid composition of different seed species. Moreover, each species displays a large variability in oil composition either naturally occurring (use of variability in breeding programs), or artificially induced by mutagenesis. Environmental conditions such as temperature, latitude, drought, radiation, and maturing stage / harvest date may also affect oil composition.

To unravel the biosynthesis and modifying pathways, mutants affecting seed oil fatty acid composition, are of great value (Table 1).

Table 1: Interests of mutants.

Mutant reveals a step in a pathway (function or component).
Mutants can be combined (transgression).
Mutant interacts with the environment (temperature, light, X rays).
Mutant reveals organ expression of genes in time and space.
Mutant leads to novel oil composition.

Mutant may provide directly new oil composition for breeders but interestingly they may reveal steps in pathway involved in fatty acid composition that is important for physiologists. A lot of mutants in different species, both natural and induced have been studied (Table 2). Studies of these mutants have significantly contributed to our knowledge of lipid synthesis and modification pathways.

Table 2: Some of natural variation, mutants and transgenes with altered fatty acid content.

Species	Mutagens, origin of variation	Main alteration Decreased / increased	References
Peanut	chemical	18:2 /18:1	Jeong *et al* 1998
Soybean	Chemical and natural	18:3 / 18:2 18:2 / 18:1 18:1+2+3 / 16:0, 18:0	Nickell *et al* 1993
Rapeseed, Canola	Chemical and natural	Polyunsaturated decreased 18:3 / 18:1+2 18:2 / 18:1+3	Auld *et al* 1992
Arabidopsis	Mutagens,	Low unsaturated 18:2 / 18:1	Ohlrogge *et al* 1991. Arondel *et al* 1992.
sunflower	Chemical	18:2 / 18:1, 18:1+2 / 18:0 18:1+2 / 16:0	Soldatov 1976 Osario *et al* 1995 Garcés *et al* 1992.
Maize	Natural chemical	temperature effect 18:2 / 18:1	Berberich *et al* 1998 Patent WO9201367
Sunflower, Soybean, Canola	Transformant overexpression or antisens	HO, 18:0 18/2	See Ivy *et al* 1998 for recent review

We would stress the diversity of the species, and the number of possible mutations leading to modifications in fatty acid composition. Recently, transgenics have been obtained (Ivy *et al* 1998) and their study has also increased our knowledge of the structure and regulation of seed oil modifying pathways. Mutations exist in different species leading to the same phenotype in the oil. For example, high oleic peanut, high oleic soybean, high oleic sunflower and high oleic transgenic Canola have been obtained, but they display various features (Table 3).

Table 3: Comparison of high oleic mutants from different species.

HO mutant	Δ-12 activity	Δ12-transcript	Mode of inheritance	References
Peanut	No	Yes	Two loci, recessive	Jeong *et al* 1995 Jung *et al* 2000
Soybean	No	Yes	One locus, recessive	Nickell *et al* 1993
Sunflower	No	No	One locus, dominant	Soldatov 1976
Transgenic Canola	No	No	Dominant	Patent Hitz & Kinney

We can wonder whether the same phenotype for oil content in different species or cultivars may be due to an equivalent mutation or to different mutations in different species? The answer is not obvious. For each species, we have to consider which enzyme activity is absent, in which organ and at which stage, knowing that there are a lot of ways to block an enzyme activity. Information obtained by the studies of all these different mutants can be combined to bring some general knowledge about basic metabolism pathways of plant lipids. However, mutants in each species can bring some knowledge specific of the species. So, each mutant requires specific studies at biochemical, genetic and molecular levels that represent a comprehensive work. Such approaches are developed on sunflower for high oleic, high stearic and high palmitic mutants and most of the results have been documented in posters (B2, B3, F29, F31, F33, Sp3, It17, Sp1, Us15), and have been discussed in the workshop session.

Seed oil composition in sunflower

Sunflower seed oil contains saturated fatty acids (palmitic and stearic acid), mono-unsaturated fatty acid (oleic acid) and poly-unsaturated fatty acids (mostly linoleic and traces of linolenic fatty acids). In Normal or Linoleic sunflower (LO), seed oil composition is characterized by a majority of 18:2, however, significant variation in linoleic acid and oleic acid content exists due to the overall plant genotype and environmental factors during plant growth.

Environment effects on sunflower seed oil composition

Studies performed on the effect of environment factors like temperature and drought stress on sunflower seed oil composition are summarised in Table 4.

Table 4: Main factors influencing oil composition variation in LO sunflower.

Factors (as increasing)	Lines / hybrids	Oil yield	Variation of fatty acid content in seed oil	References
Temperature	Peredovick	reduced	18:2 / 18:1	Harris *et al* 1978
Temperature	Sunfola 68-2 derived from Peredovick	Not checked	18:2 / 18:1	Silver *et al* 1984
Temperature	Wide ranges	High LO stable	Recessive gene	Simpson *et al* 1989
Temperature	RHA274		18:2 / 18:1	Garcés *et al* 1992
Temperature	Albena /	stable	18:2 / 18:1	Champollivier & Merien 1996
Temperature	Santiago LO Olbaril Platon	3 ranges of temperature	18:2 / 18:1 High oleic stable	Lagravère *et al* 2000
Temperature	Select		18:2 / 18:1	Piva *et al* 2000
Temperature	Various backgrounds	Reduced 1 % / °C	18:2 / 18:1	Triboi *et al* 2000
Temperature	Various background	A gene controlling stability	F1 not homogenous at high temperature	Velasco *et al* 2000
Drought	UD12 HO UD87 LO	reduced	18:2 / 18:1 18:2 / 18:1	Baldini *et al* 2000

All the studies, concerning the increase at medium temperature, in a range of 15 °C to 20 °C, revealed an enhancement of oleic acid content and concomitantly, a reduction of linoleic acid content. Concerning increases seen at higher temperature, in a range of 26 °C to 30 °C, the effect is not so ubiquitous and depends on the genotypes used in the studies. So, temperature affects the fatty acid composition in seed oil but the modifications are not stable and depend on the range of temperatures and on the genotypes. In order to obtain stable modification of fatty acid composition in sunflower seed oil, mutagenesis has been developed.

Mutagenised sunflower with different fatty acid oil composition of storage oil

Fatty acids contained in sunflower seed oil are very important in food and chemical industries. In order to modify proportions of the different fatty acids, mutageneses have been performed. Different teams have obtained several mutants (Table 5). Different mutagens and methods have been used to develop these mutants but little is known about the molecular nature of these mutations. Studies have been performed to unravel these mutations and some of these mutants are still under study in the different groups, and used in breeding programmes.

Table 5: Code, mutagens, main variation and references of different Oil sunflower mutants.

Code	Main trait	Origin	Mutagens	references	Last references
Pervenets	High Oleic 75-92 %*	VNIIMK8931	DMS	Soldatov 1976	Lacombe et Bervillé, this review
654-IWS	High oleic 75-92 %	Peredovick	NEC	Ivanov *et al* 1981	Petakov *et al* 1999 unpublished
275-HP	High palmitic Poor repeats	Zrya	Gamma rays	Ivanov et Ivanova 1985	
CAS-3	High stearic 50 %	RDF1-532	EMS	Osario *et al* 1995	Perez-Vich *et al.* 2000
CAS-5	High palmitic 25 %	BSD2-691	X rayx	Osario *et al* 1995	Perez-Vich *et al.* 2000
LP LS	Low Palmitic Low stearic Sum= 8 % instead of 15 %	RHA274-LP1 HA821-LS1 RHA274-LS2	NMU or EMS	Miller and Vick 1999	-

*In average % major fatty acids in TAG.

Among all these mutant traits, the High Oleic trait derived from Pervenets is the most widely used and studied in sunflower.

GENETIC APPROACH OF THE HIGH OLEIC PHENOTYPE FROM PERVENETS

Present situation:

Until now, all the commercial hybrids and most of the inbred lines having the HO trait from companies and public institutes were derived from the Pervenets source (see below).

Practical problems for breeders

There are: 1) To produce high oleic hybrids, as fast as possible, with the higher oleic acid level. 2) = To eliminate the unfavourable traits present from Pervenets population such as some disease susceptibility (Phomopsis and mildew susceptibility), low oil content and poor genetic background. Several Posters deal with these aspects (Jocic *et al* 2000 Yu12, Uhart *et al* 2000 Ar40+41). Moreover, variation for HO level between fixed HO lines appeared as a strange feature, Triboi *et al* (2000 F52).

Genetic study of the HO trait (Table 6)

Many genetic approaches have been developed to study the HO mutation derived from Pervenets (Table 6).

The main features in this table are: - the various behaviours of HO trait acting either as dominant, recessive or intermediary, - the different number of genes deduced by the authors, and - occasionally reported maternal effects.

Miller (1992) proposed an explanation of the differences between HO x LO crosses: the HO parents in different studies are not equivalent. Either HO parents have been self directly from Pervenets or were used as oleic segregant after crossing to low oleic lines, which means they may carry transgression factor for HO content (Table 6). Also, Fernàndez-Martinez *et al* (1987) suggested that there are several loci controlling the HO content. There is variability between HO lines according to the alleles at these different loci. This should explain the variability of the HO content in different crosses. However, these arguments are not necessarily valid because it may not be justifiable to increase the number of loci, unless a clear knowledge of the effect of each locus is ascertained.

Table 6: Summary of the genetic studies dealing with the HO trait in sunflower.

Oleic Source, All Pervenets	Non oleic line	Level for Oleic measure	Method for Oleic measure	Dominant / recessive / intermediary	Maternal effect Yes/ No	One major gene modifiers	references
Pervenets		F2 or equivalent		Partially dominant	Not checked	1M	Fick 1984
Pervenets	P21	F2 or equivalent	F2 half seed cotyledon	Dominant	No	1M + modifiers	Urie 1985
Pervenets	HA89	F2 or equivalent	Not clear Several seeds separately?	Intermediary	No	1M + modifiers	Miller *et al* 1987
Pervenets				Dominant	Not checked	1M	Schmidt *et al* 1989
Pervenets	Cms HA89	F2 or equivalent	F2 Half seed	Dominant	Not checked	3M additive + modifiers	Fernàndez-Martinez 1989
Pervenets AO-P-1	Cms HA89	F2 or equivalent	F2 half seed analyses and then Bulks of 10 seeds	Dominant	No	3M additive + modifiers	Fernàndez-Martinez *et al* 1990
Pervenets 6 different HO line	6 different LO lines	F2 or equivalent	Not said	Dominant but also sometimes recessive	Not checked	3 hypotheses increasing gene numbers	Demurin & Skoric 1996
Pervenets HAOL9	ROL71	F2 or equivalent	F1 not checked F2 = Mix of 15 F3 seeds	Dominant but also sometimes recessive	Not checked	1M + modifiers not clear	Dehmer and Friedt 1999
Pervenets R 978	HA89	F2 or equivalent	F1 not checked F2 mixture of F3 seeds?	Partially dominant	Not checked	2 interacting genes	Fernandez *et al* 1999
Pervenets 7 different HO lines	3 different LO lines	F1	F1, 10 seeds per head together	Dominant	Reciprocal effect	Not addressed	Vares *et al* 2000
Pervenets	High stearic mutant	F2 or equivalent	F2 half seed	1 Dominant	Not checked	Major QTL (85 % EV)	Pérez-Vich *et al* 2000
Pervenets HO line from Monsanto	LO line from Monsanto	F2	F2 half seed	1 Dominant	Not checked	1 locus = Δ12 RFLP	Lacombe *et al* 2000
Pervenets Different HO lines	Different LO lines	F1 HO x LO F1 LO x HO	F1 seed	Dominant	No effect	Not addressed	Lacombe *et al* 2000
Pervenets HAOl9	HA89	F2 or equivalent	F1 half seed segregate	Complex some maybe dominant	No	Five genes	Velasco *et al* 2000
Pervenets RHA345	83HR4	RILs	Seed analyses	Dominant (1:1)	Not checked	1M + modifiers	Kaan *et al* In preparation

Moreover, several questions and comments can be made:

1) *How many mutations are in Pervenets?* The first question deals with the genetics of this HO phenotype and whether there is only one mutation in Pervenets or several mutations, all leading to a HO phenotype. This has never been considered.

2) *Which generation to use for measuring HO:* Due to sampling F3 seeds, the mean of the HO measurement may be not equivalent to the F2 seed content. According to the HO measurement on F2 or F3 seeds, the ratio HO / LO may change and therefore the final genetic explanation will change.

3) *Environment modifies HO content in offspring's:* Oil composition in LO sunflower is clearly affected by the environment and the genetic background (unidentified genes) (Table 4). Is the introduction of the HO mutation enough to mask all these effects? Probably not and therefore in the HO sunflower and in HO x LO crosses, several effects pile up making difficult to determine which are due to the HO mutation and which are due to other genes. This effect cannot be determined in F2 progenies, which are unique, and with an oil composition determined on the mother F1 parent, which is selfed.

4) Genotype effects affect HO content: The HO trait is seed-specific (developmentally regulated) and requires analysis of a part of each seed for oil composition before sowing. All factors affecting the fertilization process will interfere with the inheritance pattern, as shown by Lagravère *et al* (1998) and Lagravère (1999). Depending on self-fertilizing ability of F1 and F2 plants, progenies may be more or less distorted for the ratio HO / LO. All these factors certainly accumulate in different progenies, creating complex and variable segregation patterns for the HO trait in various environments.

It is likely that these factors combine to determine the HO level and should explain such different results obtained in all the studies on this trait. All these factors which affect the HO level are not due to the Pervenets mutation. However, when the HO and LO lines are crossed with LO lines, these factors from LO lines can modulate the HO content and consequently, modify the HO / LO ratio. The modifier genes affecting the HO content, introduced to explain such distortions are independent of the Pervenets mutation.

Our genetic approach of the HO trait from Pervenets

All the points developed above should be considered for an academic genetic study.

1) Genetic studies cannot directly determine how many mutations occurred in Pervenets. Crosses between HO lines may answer this question. In the case of two different mutations in two different loci, LO individuals should appear in the F2 offspring by recombination of two wild alleles, at each of the two loci. Such crosses have never been performed systematically.

2) The HO content in offspring's must be examined in F1, F2 and back-crossed populations in order to determine seed by seed, the HO / LO segregation pattern.

3) To detect environmental effects, one approach is to study recombinant inbred lines (RILs), which require several years for construction and multiplication. In the end however, these lines enable experiments to be done with the same genotypes in different environments.

4) To check genotype and cytoplasm effects, di-allele crosses are required and on our knowledge, were never performed before the study by Vares *et al* (2000, F29).

Since there is no basis to define the range and number of oleic classes, the HO content in a F2 segregating population appears a complex trait. A quantitative analysis may be performed. In many cases such an approach is efficient but here several facts argue against this solution. The HO trait is almost dominant and in an F2 population the segregation is non-equilibrated making difficult to detect weak QTLs for variation in oleic levels. The quantitative approach developed by Pérez-Vich *et al* (2000) has revealed that the HO trait from Pervenets is due to a major effect at one locus (about 80 %) plus likely other minor effects. These minor effects are difficult to detect. It would be better to perform it on RILs. Moreover, RILs allow the estimation of environmental effect and enable us to check the oleic content on only homozygous offspring's.

Taking into account all these points the classical genetic approach of the HO phenotype found in Pervenets will be difficult to perform.

MOLECULAR APPROACHES STUDYING OF THE HIGH OLEIC PERVENETS

Furthermore, since the classical genetics cannot answer the key questions quickly, we naturally turned to the molecular approach to study the HO trait from Pervenets.

A simple model

A simple model for HO accumulation in the Pervenets mutant was considered.

We logically supposed that oleic accumulation could be due to 1) either an excess of activity of the Δ9-desaturase catalysing the desaturation of stearic acid to oleic acid. This excess could be due to an over accumulation of the transcript leading to an excess of the Δ9-desaturase enzyme, leading to an excess of the oleic product, or 2) to a lack of the Δ12-desaturase activity catalysing the desaturation of oleic acid to linoleic acid. Many possibilities exist to prevent enzyme activity such as point mutations, or effects on transcript accumulation, (deletion to no transcript and post-transcriptional events).

Two desaturase genes possibly involved

Consequently what about the Δ9-desaturase and the Δ12-desaturase genes in sunflower? For these genes, we were interested in determining the transcript patterns in time and space in normal oleate sunflower, the transcript profiles in HO plants and any obvious differences between them.

For this purpose, we used molecular tools available that consisted of heterologous or consensus sequences -from other species such as Arabidopsis or Peanut cDNA sequences - to detect those of Sunflower. This was performed by Kabbaj *et al*. (1995, 1996ab), and Abbott *et al* (1998) making available, the two cDNA sequences corresponding to Δ9- and Δ12-desaturase genes. Recently similar works have been reported by Hongtrakul *et al* 1998 with more or less similar conclusions.

Studies of desaturase transcript accumulation

Several methods can be used to evaluate gene expression. Methods based on reverse transcriptase PCR are difficult to quantify and require internal standards. The Northern method is heavy to handle. Using Northern hybridization analysis, Kabbaj *et al* (1996) and Teulé (1996) have shown that the timing of desaturase transcript accumulation in seed is between ten to twenty days after fertilisation. Lacombe *et al* (2000, F33) have studied Δ9- and Δ12- transcript accumulation in embryos resulting from selfing of 6 LO and 12 HO lines. The Δ9-desaturase cDNA sequence used as a probe on Northern blots did not reveal any difference with respect to intensity and transcript size, between HO and LO embryos. A clear dissymetry occurred between HO and LO embryos for the Δ12-transcript accumulation. The Δ12-desaturase sequence used as a probe on Northern blots, revealed none or a weak accumulation of the transcript in HO in comparison to LO embryos. Enzyme amount and thus enzyme activity are positively correlated to the amount of transcript. Consequently, we detected a breakdown in the Δ12-transcript accumulation, and therefore a lack in enzyme activity. These results suggest that the HO mutation leads to the reduction of Δ12- transcript, which could explain the HO trait.

Moreover, hybrid immature seeds resulting from reciprocal crosses HO x LO, and LO x HO, displayed the same reduction of Δ12- transcript (Lacombe *et al* 2000 F33). Thus, the mutation leading to this loss of transcript acts in trans on the normal Δ12-desaturase gene to prevent transcript accumulation in the heterozygotes. This dominance for reduction in transcript accumulation is quite unusual. This confirms the phenotypic dominance of the HO

trait in LO x HO and HO x LO hybrids, and must be explained by the mode of action of this mutation.

RFLP differences between HO and LO lines or hybrids

According to the model for HO mutation in Pervenets concerning the Δ9- and Δ12-desaturase activities, RFLP studies were performed on HO and LO genotypes. With the Δ9-desaturase cDNA as a probe, Lacombe *et al*. (1998) revealed several polymorphisms but none of them was correlated with the HO status of plants. With the Δ12-desaturase as a probe, Lacombe *et al* (1998) have reported fewer Δ12-RFLPs than those detected with Δ9-, but one with *Eco*RI and one with *Hind*III RFLPs were correlated (P< 0.1%) with the HO status of plants (3 HO hybrids: Platon, Sarah, Olbaril in comparison with 17 LO hybrids). This suggests a correlation between the Δ12 RFLP and the mutation.

Hongtrakul *et al* (1998) by comparing two isogenic HO and LO couples described the same RFLPs, but the number of lines was insufficient to check whether this result was significant or not.

Lacombe *et al* (2000 F31) have shown through a diversity analysis on 180 genotypes, that the Δ12-RFLPs were found in all 96 HO genotypes but absent in 84 LO genotypes. This Δ12-RFLP was also present in Pervenets (carrying the HO mutation) but absent in VNIIMK 8931 (before the mutagenesis). This established a strong correlation between the presence of the Δ12-RFLP and the HO trait. Moreover, Lacombe *et al* (2000 F31) have found the same difference in 22 commercial HO hybrids from 6 different companies by comparison to 13 LO hybrids (P<0.1%). Consequently, all the breeders used the same source of HO, which has been derived from Pervenets.

These results reveal that the HO trait has appeared in a unique source (Pervenets). They suggest that the HO mutation is either in the Δ12-RFLP region or strongly linked to this region. All the HO genotypes derived from Pervenets through several back-crosses. They do carry the HO mutation in a larger fragment from Pervenets than the gene responsible for the HO mutation. Depending on the size of this fragment, the measure of the distance between the Δ12-RFLP and the HO mutation would be more or less important. Nevertheless, the Δ12-RFLP and the HO mutation are statistically linked and we wonder whether this depends on their genetic linkage.

Co-segregation of the Δ12-RFLP and the HO level

The linkage between the HO mutation and the Δ12-RFLP region was checked in a F2 segregating population for the HO and LO traits (Lacombe *et al* 2000 F31). Oil composition was determined in 107 F2 half seeds before sowing and RFLP analyses were performed on DNAs from mature leaves of the corresponding plants. Co-segregation was established since all the plants with HO content lower than 65 % did not carry the *Hind*III HO-specific RFLP whereas those with more than 65 % carry it.

This sustains that the statistic correlation is due to tight genetic linkage (r^2 =0.8372). However, the accuracy of the linkage measure on 107 F2 progenies is insufficient to determine whether the Δ12-RFLP and the HO mutation coincide in a same locus or are tightly linked.

The mutation is not in the cDNA

Other studies showed that the HO mutation did not affected the translated sequence (not shown), leading to the conclusion that the mutation must be close (few Kb) from the coding sequence.

In conclusion we can summarise the main characteristics of both the HO mutation and the HO trait. According to all the points developped, we can suggest which trails should be followed to improve the knowledge on the HO trait.

Table 7: differences in the knowledge dealing with Pervenets HO mutation and variation in HO content.

Trait	Dominant Recessive	Genetic studies on Pervenets	RFLP Pervenets / LO	Expression Pervenets / LO
High oleic mutation	Dominant trait See Table 6 for references	Single dominant gene See Table 6 for references	2 RFLPs *Eco*RI and *Hind*III others are known Lacombe *et al* 1998, Hongtrakul *et al* 1998	Reduced Δ12-desaturase transcript accumulation
High oleic content	Not well understood	Multifactorial Pérez-Vich *et al* 2000	Not well defined Studied in RILs	Not characterized yet

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