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Oil Composition Variations

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ABSTRACT

This chapter deals with the range of fatty acid composition and variation, the nature of mutations, and environmental factors that may modify oil composition. Sunflower oil contains fatty acids and minor compounds such as tocopherols and phytosterols. Current knowledge of fatty acid, tocopherol and phytosterol synthesis and signalling pathways in model plants is discussed. Each major fatty acid variation, eventual mutants, and their roles are unravelled as to how they function in the pathway. The role that eventual abiotic stresses play is developed and discussed. Specific attention is given to oil modifications brought about by mutants with economic importance to the industry (oil rich in palmitic or stearic acid) or to prevent heart diseases (oil rich in oleic acid or well balanced in polyunsaturated fatty acids). Variation in minor compounds observed in sunflower oil are also discussed and the possibility to breed sunflower for these compounds.

Keywords: fatty acids; mutants; oil composition; oleic acid; palmitic acid; stearic acid

8.1 Introduction

This chapter deals with the range of variation in composition observed in sunflower oils including the oil phenotypes and the nature of the mutation which are revealed as well as the environmental factors that may modify oil composition in this crop.

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The standard reference for the oil composition in sunflower is the FAO's Codex Standards for Fats and Oils Derived from Edible Fats and Oils. Table 8-1 shows a variable range due to differences in cultivars and environmental influences in comparison to some other crop oils.

Traditional sunflower oil composition has a wide range of variation for plant seed oils. It is the main oil (48%) consumed in Europe. However, the trend is now to favor oil with high oleic (HO) acid content (HOAC) to prevent heart diseases. This HO oil broadly mimics olive oil, which is a fruit oil, although the cost is much lower. Other types of oil also exist for sunflower, and this range of variation will be examined and the potential to grow this crop with many oil compositions will be described.

Table 8-1 Average percent composition of sunflower oil in comparison to some other crude seed and fruit oils.

Fatty acid C: /others	Traditional Sunflower	Rapeseed/ Canola	Soybean	Safflower	Olive virgin	High oleic Sunflower
Palmitic acid 16:0	5–8	2.5–7.0	8.0–13.5	5.3–8.0	7.5–20	2.6–5.0
Stearic acid 18:0	2–7	0.8–3.0	2.0–5.4	1.9–2.9	0.5–5.0	2.9–6.2
Oleic acid 18:1	14–40	51.0–70.0	17.7–28.0	8.4–21.3	55.0–83.0	75–90.7
Linoleic acid 18:2	48–74	15.0–30.0	49.8–59.0	67.8–83.2	3.5–21.0	2.1–17
Linolenic 18:3	0.1–0.8	5.0–14.0	5.0–11.0	<0.1	<0.9	< 0.3
20:0	< 0.5	0.1–4.5	0.3–0.6	0.2–0.4	<0.6	< 0.5
22:0	0.3–1.5	<2.0	<0.7	<1.0	<0.2	0.5–1.6
Tocopherols mg/kg	440–1520	430–2680	600–3370	240–670	<4.5	450–1120
Phytosterols mg/kg	2400–5000	4500–11300	1800–4500	2100–4600	<1000	1700–5200

8.1.1 Main Features of Lipids

Oil is composed of several lipids, the major component being triacylglycerols (TAG), but tocopherols, sterols, phosphatides, and waxes are present in small amounts and all are stored in the seeds. In the plant, their roles are to protect the embryo from oxidative stress and to release available energy to facilitate germination.

Oil composition has consequences on the uses of the oil, effects on health, and prevention of heart diseases. For industrial purposes, the main source of fat is animals. To compete with animal fats that are inexpensive, plant fats should have technological advantages and specificities in composition

(Bessoule and Moreau 2004). For end products such as coffee and chocolate, the lipid composition affects the quality (taste and flavor) of the final product after cooking.

Historically, comprehensive examinations of oil diversity have been performed to enhance knowledge about the diversity of fatty acids and other compounds, but also to provide new compounds to industries. Biotechnology has modified oil composition since the mid-1970s, first in *Brassica*. Thereafter, applications spread rapidly to other oil crops such as soybean, sunflower, and cotton.

8.1.2 Fatty Acids

The structure of fatty acid synthase (FAS) in animals and fungi has been unravelled as a 262 kDa multipeptide functional complex. It releases saturated fatty acids, either C16:0 or C18:0, that are further processed in metabolism. However, animal metabolism is unable to synthesize C18:2 (linoleic acid) and C18:3 (linolenic acid), and as a consequence these fatty acids need to be found in the diet.

The FAS complex in plants is composed of eight polypeptides and is localized exclusively in plastids. Its complex structure is probably responsible for the lack of mutants that interrupt only one step in fatty acid synthesis. Mutations affecting FAS usually have complex effects.

Brown et al. (2006) have purified the fatty acid biosynthetic enzymes in *Arabidopsis thaliana* type-II FAS in which separate enzymes catalyze sequential reactions. Genes encoding all of the plant FAS components have been identified and the structure of a number of the individual proteins determined. Kachroo et al. (2003) have identified a plastidial fatty acid signalling pathway in the *Arabidopsis ssi2/fab2* mutant involved in defense mechanisms against the necrotrophic pathogen *Botrytis cinerea*. The mutation encodes stearoyl-acyl carrier protein desaturase (S-ACP-DES), and results in the reduction of oleic acid (18:1) levels in the mutant plants and also leads to the constitutive activation of NPR1-dependent and -independent defense responses. The alteration of the prokaryotic fatty acid signalling pathway in plastids required several mutations in fatty acid biosynthetic pathways that cause an increase in the levels of 18:1 in specific compartments of the cell. A loss-of-function mutation in the soluble chloroplastic enzyme glycerol-3-phosphate acyltransferase (*ACT1*) completely reverses salicylic acid and jasmonate-mediated phenotypes in *ssi2*. In conclusion, fatty acid signalling plays an essential role in the regulation of *ssi2*-mediated defense.

Most saturated and unsaturated fatty acids are synthesized for building membranes and to allow cellular functions. There are two main categories of plants according to composition of membrane lipids. One is due to a majority of "palmitic" acid, whereas the other is due to a majority of

"linolenic" acid (Mongrand et al. 1998). Membrane lipid composition is modulated by the environmental temperature, where "palmitic" membranes are typical of tropical species and "linolenic" membranes are more northern. This means that deep constraints limit the variation range for lipid membranes to enable biological functions.

Desaturation of fatty acids occurrs sequentially from C18:0, C18:1, C18:2, to C18:3 or from C16:0 to C16:1 and so on. For seed oils these steps occur in the developing embryo. Elongases add the acetate motif to fatty acids to lengthen them to C:20, C:22, and higher (Table 8-1). All these steps that are detailed in many reviews enhance the diversity of storage lipids (Roscoe et al. 2001).

Storage lipids are much more variable in composition and in quantity than structural lipids. Humans have consumed many plant oils that are not in use now, but some are still utilized for health or body care (e.g., *Camellina*, *Oenothera*, flax). Fruit oils (olive) and seed oils (oil crops) are not stored in the same structure. In the seed, oil droplets are surrounded by a membrane made up of a phospholipid monolayer embedded with oleosin proteins (Cummins et al. 1993). In fruit oil, this membrane is absent and the oil droplet floats in the tissue.

8.1.3 The Role of Mutations to Unravel Fatty Acid Biosynthesis Pathways

A mutation interrupts one biochemical pathway and causes the accumulation of the compound before the block. Thus, mutations have been found very useful to enhance knowledge about biosynthetic pathways. Practically, a mutant releases a specific oil composition that could be of value in the market. However, biochemical pathways are complex networks and one single mutation may not have the expected effect on one compound only. Consequently, to unravel a mutation mechanism and to determine which enzyme is involved may be more or less difficult. Regulation of a biochemical pathway is generally due to a feedback effect by the end product on one of the initial enzymes. Thus, a mutation may lead to deregulation of the pathway with complex effects on the accumulated products. From a genetic point of view, this is called as epistasis, but it does not suggest any mechanism. The biochemical mechanism of several mutations have now been unravelled in model and cultivated plants. Sobrino et al. (2003) reviewed several experiments performed in Cordoba to modify sunflower oil composition that will be detailed further.

8.1.4 Types of Mutation

Most frequently, a mutation is a change in the coding DNA sequence (exon) of an enzyme that induces one amino acid deletion, insertion, or substitution that makes the enzyme no longer functional. If the mutation is in a non-coding sequence (regulation sites, intron) and affects the transcription ability, the transcript of the enzyme will be absent, and therefore the enzyme will not be synthesized. The mutation may also delete a part of the entire gene and this will have the same consequence. All these mutations are recessive since this change does not affect the function of any normal gene in a diploid heterozygous plant, and is called a *cis* effect.

In some cases, the mutation may affect a regulatory peptide encoded by another gene. The regulatory peptide is modified, and the mutation may affect several functions (syndrome effect), as it has been reported for transcription factors (Schmidt et al. 1992), and will behave as dominant. Such a mutation has *trans* effect. A heterozygous plant for the mutation may be more or less affected depending on the effect of the normal allele, which competes with the mutated one.

Due to insufficient natural variation for oil composition in crop plants, breeders have used artificial mutagenesis, either physical (γ- or X-rays) or chemical (a series of compounds: ethylmethane sulfonate, EMS; diethyl sulphate, DES; N-nitroso-N-methylurea, NMU) to induce mutations. Mutations are random and the treatment enhances their rates of appearance. However, a mutagen may favor some base changes or DNA rearrangements such as deletions or insertions. They may also activate silenced transposon or retrotransposon sequences. As a result, the exact type of mutation cannot be predicted according to the agent and a rigorous study is required to determine the exact changes for a given induced mutation.

In the past few years, fatty acid pathways have been modified using transgenic strategies. We can distinguish when the insertion of an extra copy of a desaturase is in the same direction, because in most cases this causes overexpression of the gene. The mutation is, therefore, partially dominant but also its effect largely depends on the cultivar's genetic background. This, in most cases, will enhance the level of the next product. In another situation, a transgene can knock out a gene by insertion of another (extra) copy in the reverse (or same) direction elsewhere in the genome. This causes post-transcriptional gene silencing (PTGS) and the absence of the transcript. The level of the product before the blocked step will be enhanced. The insertion has a *trans* effect on all copies of the wild gene, and the mutation will be dominant.

8.1.5 Differences between Seed and Fruit Oils

When made by the embryo, oil composition is due to both the paternal and maternal genomes. The genetics of the trait has to be studied on the seed oil (phenotype) from the seed before planting and not from the seed harvested in the next generation. Thus, it is required to genotype each seed before sowing to correlate the phenotype with the molecular markers. This is true for oil in the embryo, including both seed oil and the fleshy fruit (pericarp) oil. Pericarp and seed oil of one fruit do not have the same composition, as has been demonstrated for olive (H. Sommerlatte, pers. comm.). Fleshy pericarp oil is due to the maternal genome only, whereas seed oil includes the effect of the genes from the pollen. Consequently, on a sunflower plant, each seed harvested on an F_1 hybrid plant may have a different oil composition, whereas on an olive tree, all the fleshy pericarps will have the same oil composition, but not the shell and embryo oil.

In the next section, we will examine which genetic events may have led to the diversity of sunflower oils.

8.2 Mutations Leading to One Main Fatty Acid Accumulated in Oil

8.2.1 Palmitic Acid

Enhancement of palmitic acid is required for some industrial applications whereas other applications require reducing both saturated fatty acids, palmitic and stearic. Thus breeding has followed both these paths.

8.2.2.1 Palmitic Acid in Other Species

High palmitic acid content has been obtained in leaf tissues in *Arabidopsis* (Browse et al. 1989). An increased level of palmitic acid in the oil has also been obtained in soybean. Two mutant soybean lines with palmitic acid contents of >18% were developed by treatment of 'A1937' seeds with NMU and "Elgin" seeds with EMS. The mutant lines, A1937NMU-85 and ElginEMS-421, were crossed to determine their genetic relationship for elevated palmitic acid content (Fehr et al. 1991). The inheritance of the trait was controlled by two loci.

In *Brassica napus*, an induced mutant from European winter oilseed rape with increased palmitic acid content was phenotypically characterized and genetically analyzed (Schnurbusch et al. 2000). The mutant showed a palmitic acid content of 9.2% compared to 4.5% in the parental cultivar. In contrast, the oleic acid content decreased from 61.6% to 44.2%, whereas the linoleic and linolenic acid contents increased. The mutant plants grew poorly

and their seed oil content was only 31.2% compared to 42.8% in the parental cultivar. The inheritance of the mutant was oligogenic and was determined by at least four genes. In the F_2 generation, palmitic acid content was negatively correlated with oil content.

Wilson et al. (2001) have produced soybean cultivars with oil that varied from less than 4% to about 35% palmitic acid, compared to about 11% palmitic acid in typical cultivars. A number of recessive alleles associated with these phenotypes have been described that represent different mutations at the *Fap* loci. These metabolic studies narrowed the identification of fap_1, fap_2, and fap_{nc} alleles to the genes that encode or regulate 3-keto-acyl-ACP synthetase II, 166:0-ACP thioesterase, 186:0-ACP desaturase, or 186:1-ACP thioesterase enzymes. This hypothesis was strongly supported by Northern blot assays that revealed a significant reduction in the accumulation of transcripts corresponding to the 166:0-ACP thioesterase in germplasm homozygous for the fap_{nc} allele.

8.2.2.2 Palmitic Acid in Sunflower

Pérez-Vich et al. (2000) have screened and studied sunflower genotypes with increased levels of palmitic acid (C16:0) in the seed oil. Several mutants were studied: CAS-5 displays more than 25% of the total oil fatty acids as C16:0, whereas the parental line BSD-2-691 displays 5.4% C16:0. The segregation fit a model of two alleles at one locus with partial dominance for the low content. To determine the inheritance of the high C16:0 content in the sunflower mutant line, the mutant was reciprocally crossed with standard sunflower line HA-89 (5.7% C16:0) and with its parental line, and F_1, F_2 and BC_1F_1 seeds were obtained from the crosses of CAS-5 with those lines. The cross with HA-89 revealed a segregation that fit a ratio of 19:38:7 for low (<7.5%), middle (7.5–15%), and high (>25%) C16:0 content, respectively. This segregation was explained on the basis of three loci (P1, P2, and P3) each having two alleles showing partial dominance for low content. Two of the loci revealed diversity present in the sunflower lines.

In contrast, low palmitic acid content has also been targeted. Miller and Vick (1999) have determined the mode of inheritance of low stearic and low palmitic acid content found in three sunflower mutant lines treated with two mutagens, NMU and EMS. Two lines, HA 821 LS-1 and RHA 274 LS-2, displayed lower stearic acid content (4.1% and 2.0%), compared to 4.7% for their respective parental lines. Segregation ratios of F_2 and testcross progenies indicated that the low stearic acid content in HA 821 LS-1 was controlled by one gene, designated *fas1*, with additive gene action. The low stearic acid content in RHA 274 LS-2 was controlled by two genes with additive gene action. The first gene was designated *fas2*, and the second gene was temporarily designated *fasx*. The allele *fap1* was identified in RHA 274 LP-1

to control low palmitic acid content with additive gene action. In practice, palmitic acid content and stearic acid content are frequently inversely correlated.

8.2.2.3 Palmitoleic Acid

Salas et al. (2007) have induced a mutation (a high-palmitoleic acid sunflower mutant) accumulating up to 20% of *n*-7 fatty acids. This line produces oil with a complex TAG composition, containing species that have not been previously identified in sunflower. In this regard, palmitoleic acid was esterified in an unexpected way in the three positions of the TAG molecules. The polar glycerolipid composition of the mutant was also studied, in order to identify and quantify the changes in membrane lipids imposed by the sunflower enzymatic machinery during the accumulation of the unusual *n*-7 fatty acids. The high-palmitoleic mutant accumulated important quantities of *n*-7 fatty acids in the polar lipid fraction, especially in the phosphatidylcholine lipid class. However, the total polar lipid content of these lines was not affected. On the other hand, the mutations responsible for the *n*-7 lipid accumulation induced an important decrease in the oil yield of the new mutant.

8.2.2 Stearic Acid

8.2.2.1 Stearic Acid in Other Species

Lightner et al. (1994) obtained a high stearic level in *Arabidopsis* leaves, and reduced growth of the plants. The fatty acid composition of corn oil has also been modified for stearic acid content (Jellum and Widstrom 1983). The inheritance of stearic acid was studied in crosses between standard inbred lines with approximately 2% stearic acid and three strains of an introduced genotype (PI 175334) with unusually high stearic acid of about 10%. Results from single kernel oil analyses of the parents, F_1, F_2, BC_1, and BC_2 generations strongly suggested the involvement of a major single gene recessive for high stearic acid in these crosses. Transgressive segregation for high stearic acid indicated the presence of one or more modifying genes of minor influence on stearic acid.

8.2.2.2 Stearic Acid in Sunflower

Pérez-Vich et al. (2004) have mapped quantitative trait loci (QTL) conferring increased C18:0 content in CAS-20 in an F_2 mapping population developed from a cross between HA-89 (wild type low C18:0) and CAS-20. A genetic linkage map of 17 linkage groups (LGs) comprising 80 RFLP and 19 SSR

marker loci from this population was used to identify QTLs controlling fatty acid composition. Three QTLs affecting C18:0 content were identified on LG 3, LG 11, and LG 13, with all alleles for increased C18:0 content inherited from CAS-20. These QTLs jointly explained 43.6% of the C18:0 phenotypic variation. On the basis of positional information, the QTL on LG 11 was suggested to be a *SAD6* locus. The results presented show that increased C18:0 content in sunflower seed oil is not a simple trait, and the markers flanking these QTLs constitute a powerful tool for plant breeding programs.

Pérez-Vich et al. (2006) have also studied the inheritance of high stearic acid content in the sunflower mutant CAS-3 and CAS-14. In contrast to CAS-3, high stearic acid expression in CAS-14 seeds is temperature-dependent and not uniformly distributed in the seed. The trait in CAS-3 has been found to be governed by two genes, *Es*1 and *Es*2. To study the inheritance of high stearic acid content in CAS-14 and CAS-3, crosses were made with P21, a nuclear male sterile (NMS) line with a wild type fatty acid profile. The genetic analysis included the evaluation of the F_1, F_2, F_3, BC_1F_1, and BC_1F_2 seed generations. Crosses between P21 and CAS-14 revealed that the high stearic acid trait was controlled by a single recessive gene designated *Es*3. The analysis of the F_3 and BC_1F_2 (to P21) generations demonstrated a repulsion-phase linkage between the *Es*3 and the *Ms* loci, the latter conferring the NMS trait. The frequency of recombination between *Es*3 and *Ms* was estimated to be 0.09. Crosses between CAS-3 and CAS-14 demonstrated that both lines possess alleles for high stearic acid content at different loci, as transgressive segregants with low stearic acid content were observed in all generations. Genetic recombination of *es*1 and *es*3 alleles did not result in an increment of the maximum stearic acid content in the seeds compared with the maximum levels produced by the *es*3 alleles alone. Further studies of CAS-14 have been done with the *Ol* mutation (high oleic).

Competition assays carried out with CAS-5, a mutant with a higher content of palmitic acid in the seed oil, indicated that a modified FatA-type thioesterase is involved in the mutant phenotype (Martínez-Force et al. 2000).

8.2.3 Oleic Acid

8.2.3.1 Oleic Acid in Other Species

Del Río-Celestino and De Haro-Bailón (2007) have studied the inheritance of high oleic acid content in the seed oil of mutant Ethiopian mustard lines, obtained by mutagenesis. Oleic acid segregation indicated control of accumulation by two segregating genetic systems, one acting on chain elongation from C18:1 to C22:1 and a *fad2* gene involving desaturation from C18:1 to linoleic acid (C18:2). In addition, C18:1 was influenced by one

additional locus (tentatively named *OL*) involved in control of desaturation of C18:1 to form C18:2. Transgressive recombinants were obtained from the cross L-1630×L-25X-1, with about a three-fold increase of the C18:1 content over that of the parents (> 64%) and free of C22:1 content, which represents a high potential for commercial exploitation. Other studies by Schierholt and Becker (2000) with winter oilseed rape mutant lines have also concluded that the high oleic trait is dependent on the *fad2* locus.

8.2.3.1.1 Cotton

Liu et al. (2002) have obtained high-oleic and high-stearic cotton seed oils using gene silencing. They have applied hpRNA-mediated PTGS in cotton to down-regulate key fatty acid desaturase genes and develop nutritionally improved high-oleic (HO) and high-stearic (HS) cottonseed oils. Silencing of the *ghFAD2-1* Δ12-desaturase gene raised oleic acid content from 13% to 78% and silencing of the *ghSAD-1* Δ9-desaturase gene substantially increased stearic acid from the normal level of 2% to as high as 40%. Additionally, palmitic acid was significantly lowered from 26% to 15% in both HO and HS lines. Intercrossing the HS and HO lines resulted in a wide range of unique intermediate combinations of palmitic, stearic, oleic, and linoleic contents.

8.2.3.1.2 Peanut

Jung et al. (2000a, b) have studied a peanut (*Arachis hypogea*) mutant that displays shrunken seeds with poor seed oil but with a high oleate content. Apparently, the mutation was spontaneous. Detailed studies have revealed that the high oleic trait is due to two changes in two oleate desaturases. One mutation causes lower activity of the oleate desaturase and a second mutation decreases transcript accumulation of the oleate desaturase. Two oleate desaturase genes are explained by the allopolyploid origin of *A. hypogea*. However, for this species the mutations have a deep genetic load and the trait has not exploited for the crop.

8.2.3.2 Oleic Acid in Sunflower

Two high-oleic mutations have been reported in sunflower (Soldatov et al. 1976; Ivanov and Giorgev 1981). The source studied by Petakov et al. (2000) has not been released and has not been as intensely studied as the Pervenets source of Nikolova et al. (2001).

The detailed story of the mutation has been obtained after long discussions with Soldatov both in France and at Krasnodar in Russia (Lacombe et al. 2002). The main feature of the story is that Soldatov screened the mutation in a heterozygous plant and therefore the mutation should act as dominant. Soldatov has named the population with high oleic acid

content (HOAC) as Pervenets, and we will further refer to the mutation as the Pervenets mutation. Other researchers have named the mutation *Ol*, which is a generic term. The *Ol* term is not adopted here because a dominant mutation does not affect a structural gene (as *Ol* for oleate), but a regulatory gene, which remains unknown.

8.2.3.2.1 Biochemical Changes Induced by the Pervenets Mutation

Oil enzyme modifications have been investigated by Garcés et al. (1989) and Garcés and Mancha (1991) by comparing traditional and Pervenets sources (Sarmiento et al. 1994, 1998). Desaturases are very hydrophobic membrane-bound enzymes, and comparisons of in vitro to in vivo activities may be questionable, such as comparisons of temperature effects. However, the results published by this team appear solid and reliable. They attributed the reduced conversion of oleic to linoleic acid to a deficiency in enzymatic activity corresponding to an oleoyl-phosphatidyl choline (PC) desaturase. Apparently, the modification is manifested only at this stage, but there is evidence that other oleoyl-phosphatidyl choline desaturase enzymes also exist in sunflower (Martínez-Rivas et al. 2001). Fatty acids to be desaturated should be attached to PC or to an acyl carrier protein (ACP). In contrast, the in vitro temperature effect detected on the desaturase activity was not as effective in vivo (Garcés et al. 1992; Sarmiento et al. 1999).

Other desaturase activities appeared unmodified between the traditional sunflower and the Pervenets sources. This has targeted a further molecular approach by Kabbaj et al. (1995, 1996a) on the desaturase steps before and after the presumed blockage point, involving namely the stearoyl-ACP- and oleoyl-PC-desaturases.

Several biosynthetic pathway routes may lead to oleic acid. Researchers have hypothesized that the block in the pathway by Pervenets was compensated by an alternative route. In this respect, all genes of the alternate pathways become candidates as modifiers of the high oleic acid trait. However, their direct involvement in the trait may be difficult to pinpoint (Perez-Vich et al. 2006).

8.2.3.2.2 Genetic Analysis of the HOAC Trait

Many genetic analyses have been performed on the inheritance of the HOAC trait in many genetic backgrounds. In most cases, we have already seen that one is expecting a single mutation. However, for high stearic and palmitic acid levels, we have discovered that the diversity in sunflower revealed a second locus interfering with the mutation (epistatic effect). Genetic studies on the Pervenets mutation revealed that one to five genes could interfere with the HOAC trait. Therefore, the methodologies used to reveal loci must be examined.

Since the progenies of a cross between a low oleic and a high oleic line segregated as continuous for oleic acid content (as a quantitative trait) depending on the population size and the oleic acid distribution of F_2 plants, and considering the dominance of the HO trait, authors have made two (3HO:1LO), three (1HO:2Mid:1LO), or more classes for oleic acid content. This implies a number of loci. All the studies have been reviewed in Lacombe and Bervillé (2000).

However, by the year 2000, individuals that carry the Pervenets mutation could not be identified. Thus, the studies dealt unambiguously with the inheritance of the HOAC trait. Authors tried to determine the locus that produced the main effect, which should be the locus at which the mutation has occurred, in comparison to the effect of loci with other minor effects, also called modifiers.

Lacombe and Bervillé (2001) have shown for the first time that the HOAC in half-cotyledons and a rearrangement in an oleate desaturase cosegregated (genetically linked) in an F_2 population of a cross between a LO with a HO line. Only the kernels that carried the Pervenets rearrangements displayed HOAC as high as 90%, compared to 83% in the female parent. However, many kernels without the Pervenets mutation displayed a range of variation for oleic acid content from 15 to 50%, although the LO maternal line was fixed at 28% oleic acid.

Only one QTL approach to the inheritance of HOAC has been published (Perez-Vich et al. 2004). It followed both the stearic and the oleic acid content in F_2 progenies. Each trait, high stearic and high oleic acid content displayed QTLs in the regions of a stearoyl- and oleoyl- desaturase, respectively. Other minor QTLs have been supposed corresponding to alternate pathways (thioesterase acyl carrier protein) (Pérez-Vich et al. 2004, 2006).

It is clear from all these studies that all HO lines carry the same Pervenets mutation. In contrast, other types of factors also act on the oleic acid content, such as the genetic backgrounds and modifier genes that have not yet been elucidated.

8.2.3.2.3 Expression Induced Changes by Pervenets Mutation

Since the enzymatic function of the oleate desaturase has been shown to be lacking in HOAC sunflower, Kabbaj et al. (1996b) studied whether the corresponding transcript is present or not. The oleate desaturase was first cloned in *Arabidopsis* by Arondel et al. (1992) and later cloned in other plant species. Unfortunately, the sunflower transcript did not display enough homology with *Arabidopsis* to be cloned directly. A cDNA library was constructed from developing sunflower embryos and probed by stearate desaturase cDNA and oleate desaturase cDNA from diverse species. Only with a stearate desaturase cDNA and a partial oleate desaturase clone from

olive, a partial cDNA for the stearate desaturase and complete cDNA for the oleate desaturase were isolated in sunflower.

Further studies in sunflower revealed expression of these genes in the developing embryos between 10 to 20 days after fertilization. Kabbaj et al. (1996a, b) demonstrated with the stearate desaturase transcript as control that the oleate desaturase transcript was lacking in HOAC sunflower, whereas it was present in the control.

Hongtrakul et al. (1998a, b) showed by RT-PCR that the oleate desaturase transcript was less expressed in HOAC sunflower than in traditional sunflower and they also used the stearate desaturase transcript level in both types as control. The absence of the transcript explains the absence of the protein. However, this does not indicate whether the gene is present or not. To look for the presence of the gene researchers have used RFLP, the most available technique at this time.

8.2.3.2.4 DNA Changes Correlated with the Pervenets Mutation

Lacombe et al. (1998) and Hongtrakul et al. (1998b) reported an RFLP between traditional and HOAC lines carrying the Pervenets mutation using oleate desaturase cDNA as a probe. Signals have been interpreted as a duplication of the oleate desaturase gene by Hongtrakul et al. (1998b), whereas Lacombe et al. (2000) did not postulate a complete duplication of the oleate gene.

Southern blots of genomic DNA from traditional sunflower restricted separately with *Eco*RI, *Hind*III, *Bam*HI, or *Sac*I, and hybridized with the oleate desaturase cDNA as a probe displayed RFLP profiles (Fig. 8-1). Only one fragment was clearly hybridized, suggesting that only one gene is present in the sunflower genome. We cannot exclude that several copies could exist. In contrast, all HOAC Pervenets lines displayed an RFLP profile that was different from traditional sunflower (Fig. 8-1b). With *Eco*RI, an extra fragment of 8 kb (Pervenets insertion) appeared in addition to the wild *Eco*RI 5.85 kb fragment and was strongly hybridized with the probe. This means that it should carry a sequence homologous to the oleate desaturase. With *Hind*III, the wild 8.0 kb fragment disappeared and was replaced by a 16 kb fragment. With *Sac*I and *Bam*HI, the wild fragments disappeared and were replaced by another lengthened fragment. The double restriction *Eco*RI and *Hind*III revealed a 2.1 kb fragment and the 8 kb Pervenets insertion. Taken all together, these facts suggest that an insertion of 8 kb occurred on the 8.7 *Hind*III fragment in the region of the *Eco*RI site.

Lacombe et al. (1999) attempted to clone genomic fragments from an HOAC line that hybridized with the oleate desaturase probe corresponding to the Pervenets RFLP. Unfortunately, they did not succeed in identifying hybridizing clones that displayed the oleate desaturase rearrangement. They

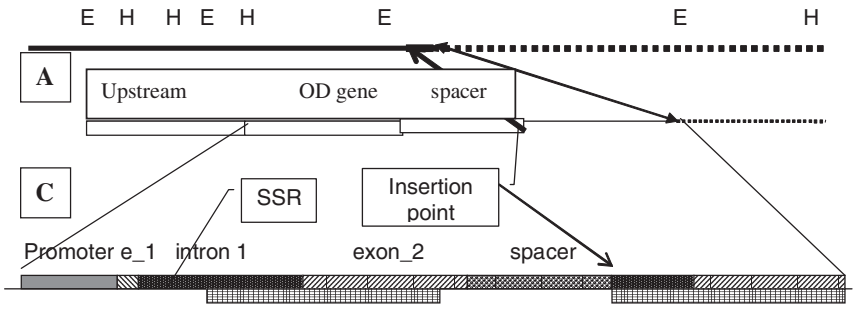

Figure 8-1 Organization of the oleate-desaturase region and details on the Pervenets mutation.

A: Thick line: region sequenced (17,770 bp); E, H: restriction site for *Eco*RI and *Hind*III, respectively. Thick bar marked by the thick and light arrows pinpoints the Oleate desaturase repeated sequence. Dotted line marks the unknown sequence with deduced restriction sites.

B: Scheme for the location of the *OD* gene. The line indicates the oleate-desaturase repeat. Dotted line indicates unknown sequence.

C: Enlargement for the *OD* gene, the spacer region and the oleate-desaturase repeat portion.

The promoter region, exon 1(e_1), intron 1 with the SSR-OD1, exon 2 and spacer are represented by boxes with motives and the bar below indicate the regions with the direct repeat.

also used several library constructions without success. However, they cloned an oleate desaturase gene belonging to a microsomal oleate desaturase (according to the targeted sequence) that displays all the features of a functional gene (Lacombe et al. 2002). The microsomal oleate desaturase (MOD) gene displayed a 1,683 bp intron carrying an SSR motif (ATT) repeated 17 times in RHA 345 (HA-INRA-OD1). The motif is repeated 16 times in 83HR4, allowing the origin of the oleate desaturase allele in each RIL to be determined. Moreover, with the hypothesis that Pervenets insertion is very close to the oleate desaturase sequence, recombination between the SSR locus and Pervenets was not expected.

Lacombe et al. (2002) turned to a PCR approach hypothesizing that the distance between the wild oleate desaturase sequence and the oleate desaturase sequence on the Pervenets insertion could be determined by PCR. Forward and reverse primers designed on the oleate desaturase cDNA were used in pairwise combinations to anchor the wild gene (F) and the Pervenets sequence R. The reverse primers were designed assuming the insert was in the same direction or in the opposite direction of the wild oleate desaturase gene. Long PCRs were run and the amplification products were hybridized (Southern blots) with the full oleate desaturase cDNA. A 4.3 kb fragment was revealed and cloned into a plasmid. It was sequenced

and the following features were revealed: 1) the end of the oleate desaturase coding sequence; 2) a spacer sequence with no hit in the databases; 3) the end of intron 1 of the *MOD* gene sequence; and 4) the exon2 *MOD* sequence was interrupted at the end of the R7 primer designed on the cDNA sequence. These sequences are included in the 18,990 bp fragment entirely sequenced and registered as EF469194 in GENBANK (Lacombe et al. 2009).

Further primer pairs were designed in the spacer region and the repeated *MOD* region to obtain a specific fragment of the Pervenets insertion (Bervillé et al. 2004, 2005, patent applied). These specific Pervenets fragments were verified to be specific to HOAC lines derived from the Pervenets population. The final organization of the Pervenets mutation is displayed in Fig. 8.1.

Further studies have been developed using a set of recombinant inbred lines (RILs) constructed from 83HR4 and RHA 345, which are two restorer lines used to avoid the Pet1 cytoplasm and the cytoplasmic male sterility in the progenies. Two hundred and fifty F_2 plants were produced and Lacombe et al. (2002) recovered 174 F_6 RILs. Five seeds per F_6 family were phenotyped on half a cotyledon (GC) to determine the oleic acid content and then sown in the field. Only, one plant per family was retained to study the segregation of the oleic acid content. All plants were genotyped for the following fragments: RFLP *Eco*RI LO:5.85 kb, Pervenets 5.85 kb + 8 kb and *Hind*III traditional (LO) 8.7 kb and Pervenets (HO) 16 kb; SSR-INRA-OD1 (LO 237 bp / Pervenets 240 bp); and one Pervenets-specific fragment, F: Fc-a_7 (caaaccaccacccactaac) and R: R-(ggttctgggtctgggtctggtt) of 902 bp. The control was the fragment F2-(tcgctaacccgttcgttctc) R2- (caaagcccacagtgtcgtc) of 173 bp designed on the *MOD* cDNA.

With these markers we attributed clearly which RIL carried the Pervenets mutation as judged by the Pervenets specific insertion fragment. Moreover, linkage disequilibrium between the SSR-INRA-MOD1 locus and the Pervenets insertion prevents any recombination in this region. As previously, the oleic acid content was determined on half a cotyledon. The results from crossing phenotypes and marker analyses, not yet published, can be summarized as follows:

1) 125 RILs were obtained with an oleic acid content of < 50% and 35 with oleic acid content of > 50%
2) The SSR analysis showed that the ratio of the lines carrying the 237 bp (82 lines) versus the lines carrying the 240 bp allele (78) was in agreement with 1:1, as expected (χ^2 P <0.6),

   All the lines carrying the 237 bp allele were LO with an oleic acid content < 50%.

   Out of the 78 lines carrying the 240 bp allele, 35 were with oleic acid content > 50% whereas 43 were with oleic acid content < 50%. The ratio 43/35 fits an 1:1 ratio, suggesting that one independent locus controls

the HOAC in the RIL carrying Pervenets. The Pervenets mutation is not sufficient for a high oleic acid content.

3) RFLP 5.85 *Eco*RI and RFLP 8.0 *Hind*III: 82 RILs displayed both the 5.85 kb *Eco*RI and the 8.0 *Hind*III fragments. However, 14 RILs displayed unusual RFLPs, 10 RILs displayed an extra 5.0 kb *Eco*RI instead of 8 kb, and 4 RILs gave a 13 kb *Hind*III fragment instead of a 16 kb fragment.
4) PCR Pervenets specific fragment: 78 RILs displayed a 902 bp fragment as expected. All lines displayed the 173 bp MOD fragment.

Skoric et al. (1996) reported unstable expression of the *Ol* gene for HOAC in one progeny, but they recovered the HOAC trait in subsequent progenies. Considered all together, the data suggested another independent locus that enables the Pervenets mutation for HOAC.

8.2.3.2.5 A Model as the Pervenets Mutation Functions

The three main features of the Pervenets mutation are: 1) its expression is mainly dominant over the traditional allele; 2) it corresponds to the duplication of part of intron and exon 2 of the *MOD* gene; and 3) the absence of the *MOD* transcript suggests a silencing mechanism as post-transcriptional gene silencing.

Up to now, all mutations that modify fatty acid levels have been found to be recessive against the wild type gene. In a few cases, it may behave as codominant. Any cases of regulation by silencing have been found for fatty acid metabolism in plants. However, in cotton using transgene methods, Liu et al. (2002) caused a total knockout of oleate desaturase leading to HOAC oil in this species. This points out that a silencing model is conceivable and functioning in plants. Moreover, a dominant suppressor of silencing has been found in rice (Kusaka et al. 1993).

Sunflower breeders have frequently experienced the unexpected behavior of the Pervenets mutation. According to breeders, the Pervenets mutation seems to disappear or to be unstable. In the RIL family, a genetic factor independent of the *MOD* locus with the Pervenets mutation directs the oleic acid level. Because it behaves as a suppressor of the Pervenets mutation (Lacombe and Bervillé 2001), we have called it "olesup" . We have shown that in RILs carrying both the Pervenets mutation and "olesup"— in RILs we cannot say whether it is dominant or recessive—the *MOD* transcript level is restored. This fact means that "olesup" could act directly on the silencing mechanism (Lacombe et al. 2009).

A suppressor explains perfectly that the Pervenets mutation may "disappear" (the plant's oil composition is indistinguishable from traditional sunflower) or be unstable when it segregates. We also have observed some rearrangements in the Pervenets mutation. In this case, each RIL family loses the Pervenets mutation and reverts to a traditional

sunflower. However, our molecular tools show that it remains as an insertion of approximately 1 kb instead of the 8 kb, but the fragment is not detectable due to its yet unknown sequence, however, it lengthens the 8.0 kb *Hind*III fragment to 9.0 kb.

Gene silencing in plants is environmentally susceptible and the variation of oleic acid content in commercial hybrids is not surprising. But we can predict that oleic acid variation is not due to the oleate desaturase but probably to a silencing mechanism that affects the final transcript stability.

8.2.3.2.6 Other Molecular and Biochemical Studies on the Pervenets Mutation

Martínez-Rivas et al. (2001) have characterized three oleate desaturase cDNAs. Two genes are constitutively expressed and one is embryo specific. The latter probably corresponded to *MOD* whereas the two others correspond to other loci. Only the *MOD* gene is affected by the Pervenets mutation, and consequently most of the lipid pattern is normal in HO sunflower as shown by Lagravère (1999) and Lagravère et al. (2000). To reconcile the results from Martínez-Rivas et al. (2001) with those of Lacombe et al. (2001) on the number of *MOD* genes in sunflower, we propose that the 5.85 kb *Eco*RI fragment carrying the *MOD* gene could be duplicated at three loci. The *MOD* locus expressed in the embryo is not affected by the Pervenets mutation as we have proposed that it is silenced.

Lagravère et al. (2004) proposed a new hypothesis based on biochemical differences found between traditional and HO sunflowers. They suggest that two different oleate desaturases could function in sunflower seeds to explain the remaining linoleic acid in HO sunflower.

Merrien et al. (2005) have studied environmental factors that modify the oleic acid content in HO sunflower oil. They measured variations in the coldest temperature during the time of oil deposition and desaturation. Temperature may also affect the intensity of gene silencing on fatty acid composition. More studies are, therefore, required to unravel whether breeding HO sunflower has to be done by targeting either the oleate desaturase or the silencing suppressors. However, in this review we show that most data favor the second hypothesis.

Schuppert et al. (2006) have studied in detail the *MOD* region using RT-PCR strategy. They have found most of the features already presented for the Pervenets mutation organization. However, some details seem different and in their map, the 5.85 kb *Eco*RI fragment is by 4 kb. An explanation of the difference is that they did not clone the genomic fragment and they concatenated the sequences of the PCR products. Because of the other loci revealed by Martínez-Rivas et al. (2001), Schuppert et al. (2006) may have concatenated fragments from different loci. There are many sequence repeats in the *MOD* region and they may have shifted a sequence to the wrong

position (Bervillé et al. 2004). However, broadly, there is a good fit between the two maps in the *MOD* region. Bervillé et al. (2004, 2005) have found the ATT SSR in the intron of the *MOD*. The SSR polymorphisms (four alleles) are very useful to map the locus and to follow linkage disequilibrium with the Pervenets insertion. Tang et al. (2006) have shown that the ORS1180 is tightly linked to the Pervenets locus, but there are still many recombination events that are worrisome for breeding.

8.2.4 Linoleic and Linolenic Acids

8.2.4.1 Linoleic and Linolenic Acids in Other Species

Linoleic acid content is an alternative to oleic acid content as we have shown for sunflower and other crops. High linoleic acid oil for industry could be of value, but when over 70% in seed oil, plant growth seems affected.

In soybean, Wang (2006) has found one plant containing zero linolenic acid content with the half-seed method in the strain 0358. It was the first report of a zero linolenic acid soybean. The material will be very useful for breeding cultivated varieties with low linolenic acid content and especially for studying gene action for linolenic acid in soybean by the aid of biotechnology.

Poneleit and Alexander (1965) have studied the inheritance of linoleic and oleic acids in maize. Their results suggest that desaturation of stearic acid to oleic acid is under simple Mendelian control. High linoleic acid content is recessive to low linoleic acid.

When linolenic acid content is too high in the oil, it becomes easily oxidized, and it is unstable. Moreover, the ratio C18:3 to C18:2 and C18:1 in the diet may cause health disorders. This is the case in soybean and rapeseed oils. Silva-Gesteira et al. (2003) have studied the inheritance and the nature and magnitude of gene effects on soybean seed linolenic acid level. They crossed an accession BARC-12 (low linolenic acid content) with a commercial Brazilian cultivar CAC-1 (normal linolenic acid content). Means and variances of F_1, F_2, and F_3 generations have been studied and results demonstrate that linolenic acid content in soybean is under the genetic control of a small number of genes.

Scarth and Tang (2006) have modified *Brassica* oil using conventional and transgenic approaches. The conventional approach to fatty acid modification has explored natural or induced mutations occurring in the same plant species or close relatives within the *Brassica* genus. These mutations have been shown to be associated with a few enzymes in the biosynthetic pathway of the fatty acids. Several types of *Brassica* oil with significantly altered levels of the long chain fatty acid, viz., erucic acid (C22:1) and medium chain fatty acids such as oleic acid (C18:1) and linolenic acid

(C18:3) have been developed for different end uses through conventional breeding. When the necessary genetic variation is not available within *Brassica* species, gene transfer by genetic transformation has been applied, as this approach is not restricted by the sexual incompatibility barrier across species. The fatty acids targeted by the transgenic approach included fatty acids with various carbon chain lengths ranging from C8 to C22, with different numbers of double bonds, and with various functional groups such as epoxy and hydroxy fatty acids. A commercial specialty oil with high level of a novel fatty acid, lauric acid (C12:0) was produced as a result of the transfer of a *FatB* thioesterase gene from a distantly related plant species that produces seed oil with high level of this unusual fatty acid. Considerable progress has been achieved in altering the relative levels of the fatty acids found in *Brassica* oils for increased health and economic benefits and in developing *Brassica* oils, which contain other unusual fatty acids, mainly through genetic transformation. Although the use of natural or induced mutations in the fatty acid biosynthesis within *Brassica* remains a valid option for oil modification, the transgenic approach will play an increasingly important role in the development of *Brassica* oils with altered novel fatty acid composition.

8.2.4.2 Linoleic and Linolenic Acids in Sunflower

In sunflower, as in most Asteraceae seed oils (*Carthamus*, *Vernonia*,) linolenic acid exists as a trace. Since it is a common feature to the whole taxon, this suggests that this function has been lost when the Asteraceae merged. In contrast, the whole plant tissues display common linolenic acid content in membranes and leaf tissues. The FAD3 enzyme is, therefore, not expressed in the seed whereas it is highly expressed in other tissue. Since some programs would seek to enhance linolenic acid content in sunflower, the underlying mechanism has to be unravelled to start these programs.

In high-oleic sunflower some RILs display a very low oleic level (10–12%) but at this moment we do not know if the trait is heritable to breed high-linoleic sunflower (Lacombe et al. 2002). Another interesting feature is the mid-oleic level of RIL families that did not carry the Pervenets mutation. Up to now, we have no explanation for such a high level of oleic acid content in traditional sunflower.

8.2.5 Wild Annual Sunflowers for Improving Oil Content and Quality in Cultivated Sunflower

We have shown through this review that sunflower oil has the potential to be improved for nutritional and industrial purposes through selection and breeding. The diversity in cultivated sunflower has been broadened by

mutations. Seiler (2007) focused on genes from wild *Helianthus* species, resulting in a continuous improvement in agronomic traits. Interest in using wild species in breeding programs has increased, but concerns about the introduction of low oil concentration and quality from the wild species persist. Two annual desert species, *Helianthus anomalus* Blake and *H. deserticola* Heiser, are excellent candidates for increasing oil concentration and enhancing quality based on their adaptation to desert environments. Seiler (2005) reported that the only *H. deserticola* population collected had an average oil concentration of 33%, whereas the two populations of *H. anomalus* had an oil concentration of 43 and 46%, the highest concentration recorded in any wild sunflower species. A linoleic acid concentration of 54% in *H. deserticola* was more typical for a desert environment. The linoleic fatty acid concentration in the oil of *H. anomalus* populations was uncharacteristically high for an Asteraceae and a desert environment, approaching 70%. Further research will be needed to determine the inheritance of the fatty acids and oil concentration in annual *Helianthus* species to determine whether the traits are transferable to cultivated sunflower.

8.3 Tocopherols

Tocopherols prevent oil from becoming rancid (Table 8-1). Sunflower oil is the richest oil for α-tocopherol (vitamin E). Several tocopherols are found in the seeds and their antioxidant property is inversely correlated with their vitamin E activity: α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol and the vitamin E activity is decreased 10-fold at each step.

Hunter and Cahoon (2007) have defined strategies to enhance vitamin E in oilseeds. They provided a recent review of tocopherol and tocotrienol biosynthesis, focusing on branch points and metabolic engineering to enhance and alter vitamin E content and composition in oilseed crops.

Moreover, a few elements of their biosynthetic pathway are known. α-tocopherol is largely predominant in sunflower oil (95%). Demurin et al. (1996) have characterized the genetic variability of tocopherol composition in sunflower seeds developed as isogenic lines in different backgrounds. They named the genes *Tph1* and *Tph2*, which undergo natural variation. They have shown that '*tph1*' (mutated allele) reduces α- and enhances β-tocopherol. The '*tph2*' allele reduces α-tocopherol and β-tocopherol, but enhances γ-tocopherol with a trace of δ-tocopherol. Together, *tph1+tph2* release an equilibrated set of the four compounds.

Garcia-Moreno et al. (2006) have mapped the *Tph1* locus by bulked segregant analysis on LG 1, enabling further marker-assisted selection and positional cloning. They performed genetic and molecular analysis of high γ-tocopherol content in sunflower. Four sources of high γ-tocopherol content

(> 85%) have been developed. The first studies have concluded that the trait in both lines was determined by recessive alleles at the *Tph2* locus. Bulked segregant analysis identified two simple sequence repeat (SSR) markers on LG 8 linked to *Tph2*. A large linkage group was constructed by genotyping additional markers. *Tph2* mapped closely to linked PCR-based markers. The location of the *Tph2* gene on the sunflower genetic map will be useful for marker-assisted selection and further characterization of tocopherol biosynthesis in sunflower seeds.

Tang et al. (2006) have shown that the Ty3/gypsy-like retrotransposon knockout of a 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase produces novel tocopherol (vitamin E) profiles in sunflower. The *m* (*Tph1*) mutation partially disrupted the synthesis of α-tocopherol in sunflower seeds and disrupted a methyltransferase activity necessary for the synthesis of α- and γ-tocopherol. It corresponded to a nonlethal knockout mutation of MT-1 caused by the insertion of a 5.2-kb Ty3/gypsy-like retrotransposon in exon 1. MT-1 and *m* cosegregated and mapped to LG 1. MT-1 was not transcribed in mutant homozygotes (*m/m*). They isolated two 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (MPBQ/MSBQ-MT) paralogs from sunflower (MT-1 and MT-2) and uncovered a cryptic codominant mutation (*d*).The *m* locus was epistatic to the *d* locus, that is, the *d* locus had no effect in *m*[+] *m*[+] and *m*[+] *m* individuals, but significantly increased γ-tocopherol percentages in *m/m* individuals. MT-2 and *d* cosegregated, MT-2 alleles isolated from mutant homozygotes (*d d*) carried a 30-bp insertion at the start of the 5'-UTR, and MT-2 was more strongly transcribed in seeds and leaves of wild type (*d*[+]/*d*[+]) than mutant (*d/d*) homozygotes (transcripts were 2.2- to 5.0-fold more abundant in the former than the latter). The double mutant (*m/m//d/d*) was nonlethal and produced 24-45% α- and 55-74% β-tocopherol.

8.4 Phytosterols

The sterol biosynthesis pathway produces a large set of phytosterols (sitosterol, campesterol), which are structurally similar to cholesterol and act in the intestine to lower cholesterol absorption. Campesterol also serves as a precursor to the brassinosteroid class of phytohormones. In plants, phytosterols have a structural role in membrane fluidity (Table 8-1). All their functions are not clear; however, in *Arabidopsis*, mutants have helped reveal a role for sterols in plant embryogenesis (Schrick et al. 2002).

Crude sunflower seed oil contains sterols up to 300 mg/100 g of oil, in which β-sitosterol is predominant (60%) and is followed by stigmasterol and campesterol (10% each). A natural variation is observed according to seed lots, but it is still not possible to explain if the variation is due to the genetic background or to environmental factors (Philips et al. 2005).

We have shown that many oil types can be produced by plant species and that sunflower may produce a wide range of oils. We did not focus on the commercial consequences of releasing so many cultivars. Regulations and markets may limit their distribution.

Acknowledgements

Thanks are due to Stéphane Dussert, IRD-Montpellier and Brady Vick, USDA-Fargo, for valuable suggestions and improving the English .

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