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# POLYMORPHISM IN *Helianthus* AND EXPRESSION OF STEARATE, OLEATE AND LINOLEATE DESATURASE GENES IN SUNFLOWER WITH NORMAL AND HIGH OLEIC CONTENTS

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SUMMARY

The steady state levels of sunflower transcripts, detected with heterologous probes to either the Δ9, the Δ12 or the Δ15 desaturase, was determined in embryos and vegetative tissues at different stages. The presence of Δ15 gene transcript was limited to the leaves. Transcripts of Δ9 and the Δ12 genes were detected in embryos between 12 and 20 DAP, and few as traces in the other tissues examined. The accumulation of Δ9 gene transcript precedes that of the Δ12 gene. The levels of mRNA accumulation in CANP3, a line with normal levels of oleic acid, and a high oleic acid line (HOC) were compared after standardization of signal intensities. Δ9 mRNA accumulation was slightly higher in HOC than in CANP3. In contrast, Δ12 mRNA accumulation was significantly reduced in HOC than in CANP3. The HOC mutation is correlated with a reduction in the steady state level of a putative seed specific Δ12 gene. We also observed that the Δ9 and Δ12 mRNA accumulate to higher levels in developing seeds maintained at 26°C day/22°C night than at 20°C day/18°C night.

From a genomic library 2 and 4 clones carrying homologous sequences to the Δ9 and the Δ12 desaturases, respectively, were used for amplification with primers unique to each desaturase, this allowed us to a clear identification for each desaturase. Moreover such a Δ9 desaturase fragment was amplified in 12 different wild species covering most of the *Helianthus genus*. Sequence variability revealed a mixture of two Δ9 sequences in all the species of *Helianthus*.

**Key words: Sunflower, high oleic mutant, oil quality, oleoyl-PC desaturase, seed triacylglycerides, stearoyl-ACP desaturase.**

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Abbreviations: STG seed triacylglycerol; ACP acyl carrier protein; HOC high oleic line, PC phosphatidylcholine; DAP day after pollination

## INTRODUCTION

The quality of seed triacylglycerol (STG) lipids depends upon the relative amounts of three fatty acid species, saturated (16:0, 18:0), monounsaturated (18:1) and polyunsaturated (18:2, 18:3). Three enzymes are involved in the sequential desaturation of fatty acids in developing seeds: **(i)** The stearoyl-ACP desaturase (=Δ9 desaturase), like the other desaturases, is nuclear encoded. It is located in the stroma of the proplastid and introduces the first double bond in the fatty acid chain between the 9 and 10 carbons leading to the oleoyl-ACP (Ohlrogge et al., 1979, McKeon and Stumpf, 1982). cDNA sequences encoding the enzyme have been isolated from many species, for a recent review see Kabbaj et al., (1995). Sequence comparisons show a high level of similarity in size and primary structure of the deduced protein. **(ii)** The oleoyl-PC desaturase (= Δ12 desaturase) is most likely found in the microsomal fraction and is responsible for desaturation of 18:1-phophatidylcholine (PC), leading to linoleyl-PC (Slack et al., 1979). In higher plants, only two cDNA sequences encoding the Δ12 desaturase, one from *A. thaliana* (Okuley et al., 1994) and *A. hypogaea* (Albert Abbott, unpublished results) are available. **(iii)** The linoley-PC desaturase (= Δ15 desaturase) is found in the endoplasmic reticulum or in the plastids and controls the desaturation of 18:2-PC producing linolenyl-PC. Several clones for the microsomal or plastidic forms of the Δ15 desaturase have been isolated from *B. napus* (Arondel et al., 1992) and *A. thaliana* (Yadav et al., 1993; Iba et al., 1993; Watahiki and Yamamoto, 1994; Gibson et al., 1994).

Sunflower is one of the major crops for oil production. The STG linoleic acid content is the main component (50-70%) with an average od 20-25% of STG oleic acid whereas saturated fatty acids are mainly STG stearic acid (5-8%) and STG palmitic acid (3-6%). The relative seed triacylglycerol fatty acid content has been reported to be influenced by temperature (Trémolieres et al., 1982; Silver et al., 1984; Rochester and Silver, 1983; Alonso, 1988). Several biochemical studies have shown that the seed oleate desaturase enzyme activity decreases *in vitro* as the temperature increases (Garcés and Mancha, 1991) and it has been noted by breeders that the higher the average temperature, the higher STG oleic acid content. Since the environment affects the oleic acid composition of seeds, the stability of STG fatty acid content has been targeted as a major breeding objective.

In recent years, sunflower varieties, producing oil high in oleic acid, have been developed from a high oleic acid mutant "Pervenets" obtained through seed mutagenesis of the population "Peredovic" (Soldatov, 1976). These varieties produce oils which are 80% oleic acid and 6-10% linoleic acid. Genetically, the high oleate trait corresponds to a major genetic factor influenced by modifiers which act independently of enviromental conditions (Fick, 1984). The mutant displays a low level of STG oleic acid desaturase activity in comparison with the normal variety as a control (Garcés and Mancha, 1991) whereas the leaf membrane

enzyme activity is normal (Garcés et al., 1989, Brady Vick, personal communication). In contrast to similar mutant of soybean, the high oleic acid trait in sunflower mutant is not affected by low temperature (Kinney, 1994).

Our objectives are to study the expression of the desaturase genes of sunflower. In this report, we present a Northern hybridization analysis of desaturase transcripts using heterologous probes for the Δ9, the Δ12, and Δ15 desaturases. We have examined RNA samples of vegetative tissues and immature embryos of plants grown under two different temperatures (20°C day/18°C night or 26°C day/22°C night) and on two lines differing by the STG oleic acid content. Moreover, we have cloned and characterised a 700bp DNA fragment of a Δ9 desaturase gene from a sunflower cDNA library (Kabbaj et al., 1995). From a genomic library clones corresponding to either the Δ9 or the Δ12 desaturases were characterised. Primers enabling to amplify a piece of Δ9 cDNA were used for 12 annual (sunflower) or perennial (Jerusalem artichoke) *Helianthus* species.

## MATERIALS AND METHODS

### Plant material and growth conditions
### Gene expression experiments

The CANP3 sunflower (*Helianthus annuus* L.) line derived from the Russian population "Armavir 9345" (designated as normal variety) and the high oleic acid variety issued from 'Pervenets', designated by HOC, provided by Cargill (France), were used in the experiments. Seeds were germinated aseptically between two pieces of filter paper imbibed with water in the dark at 20°C during 36 hours. Cotyledon, hypocotyl and root differentiating tissues were obtained from 10 day-old seedlings; seeds were germinated and grown in large sand supplemented with nutritive solution 2g/l "4% $NO_3$, 11% $NH_4$, 11% $P_2O_5$ and 15% $K_2O$". The three parts of the seedlings were separated and frozen in liquid nitrogen. Plants were grown in a greenhouse until the beginning of flowering, they were then transferred to controlled environment cabinets with 12 hr photoperiod at 20°C day/18°C night or 26°C day/22°C night. Developing seeds were picked from hand-pollinated capitulum at 12, 16, 20 or 28 days after pollination (DAP). The embryos, 3 to 5 g fresh weigh, were harvested and used immediately for RNA extraction.

### cDNA library

The cDNA libraries were prepared from poly (A) + mRNA of 12 or 20 DAP embryos of CANP3 grown either with 20°C for 12h day and 18°C night or 26°C for 12h day and 22°C night.

**Genomic library**

The genomic library was prepared from the sunflower variety "Albena". Fresh tissues from 10-day-old plants were harvested to prepare DNA.

**Phenetic study**

The plant material was provided by H.Serieys (INRA-Montpellier) who maintains the *Helianthus* collection. Eleven species (number/code) and two *H. annuus* (a wild and the line HA89) were chosen. They cover 3 sections of the genus (29): seven belong to sect. *Helianthus*, *H. argophyllus* 92/H01, *H. paradoxus* 206/H08, *H. neglectus* 222/H09, *H. annuus* 494//H19 & HA89/H28, *H. petiolaris fallax* 739/H23, *H. debilis tardiflorus* 837/H252; two belong to sect. *Ciliares*, series *Ciliares*, *H. arizonensis* 203/C07, series *Pumili*, *H. pumilus* 227/C10; four belong to sect. Atrotubentes, series Corona-solis, *H. decapetalus* 100/D02, *H. tuberosus* 571/D21, *H. divaricatus* 839/D26, series *Divaricati*, *H. occidentalis plantagineus* 231/D11.

**Probes**

We used the following probes: 1) the 1.05kb *Eco*R1/*Bam*H1 fragment of the 18 S rRNA gene from sunflower (Choumane and Heizmann, 1988); 2) the 1.5kb cDNA, pcΔ9-1(plasmid cDNA, -Δ9 desaturase), the Δ9 desaturase from *Arachis hypogea* (Tate et al., 1995) the 1.3kb cDNA, pcΔ12 (plasmid, cDNA, -Δ12 desaturase), the Δ12 desaturase from *A. hypogea* (A. Abbott, unpublished results); 4) the 1.3kb cDNA, clone pBNDES3, the microsomal omega 3-linoleoyl desaturase (Δ15) from *Brassica napus* (Arondel et al., 1992); 5) the 0.5kb partial cDNA of a methionine-rich albumine from sunflower, clone A7, (Kabbaj and Bervillé, in preparation).

**Primers**

On the Δ9 cDNA partial sequence we have defined two primers allowing to obtain a piece of a Δ9 desaturase gene. Other primers were defined allowing to amplify a piece of Δ12 desaturase.

**Molecular techniques and methods**

DNA preparation from leaves, DNA restriction, gel electrophoresis, and Southern's transfers were performed according to Gentzbittel et al., (1995). The total RNA fraction was obtained according to Teyssendier de la Serve et al., (1986). From each preparation 20µg of total RNA were loaded onto 1.2% agarose formaldehyde gels and size fractionated by electrophoresis at 80 V/cm for 5 h. Formaldehyde was removed by washing the gel in ultra pure water and RNAs were transferred overnight to nylon membranes (Biohylon Z+, Bioprobes, France) by capillary action.

### Hybridization and autoradiography

Prehybridisations were performed for 6 h in 6x SSC, 5x Denhardt's solution and 0.5% SDS, at 55°C for heterologous probes or at 65°C for homologous probes. cDNA probes were labelled using the random priming method using (alfa-$^{32}$P)-dCTP (3000Ci/mmol), denatured and added to the prehybridisation solution. After 18 h, filters were washed twice with 2xSSC, 0.1% SDS for 30 min, at 55°C for heterologous probes or at 65°C for homologous probes. A third washing was performed only for the homologous probes with 0.2 SSC, 0.1% SDS at 65°C for 30 min. Filters were autoradiographed at -80°C using X-ray films. For sequential hybridization, filters were immersed in a boiling solution with 0.1% SDS and allowed to cool to room temperature.

Autoradiographic hybridisation signals were quantified by densitometry (Ultroscan XL, Pharmacia LKB). To determine the linear response level of the film, a southern transfer of a cloned sequence was hybridized with the same clone and an exposure series were obtained for the film (FUJI MEDICAL X-RAY RX). The quantified signal intensities showed a linear response for absorbance values ranging from 0.16 to 2.64. In this interval, the signal intensity should be proportional to the amounts of radioactivity hybridized. The quantitation of each mRNA hybridization signal was performed by using the complementary cDNA and the 18 S rDNA sunflower gene as probes to hybridise the same blot. We express the quantity of desaturase mRNA as a ratio "mRNA signal/18 S rRNA signal" thereby, internally standardizing all sample loading.

## RESULTS

### Δ15 desaturase mRNA expression

The Δ15 cDNA from *Brassica napus* was used as a probe in northern blot hybridization's of total RNA from immature seeds 12 and 20 DAP and from leaves (Figure 1). With this probe, no transcript were detected in seed RNAs isolated from any stage whereas a hybridizing mRNA of 1.6kb was evident for leaves. As a positive control, the northern blot was stripped of hybridization signal and rehybridized with a partial cDNA of an albumine methionine-rich protein "A7", specifically expressed in immature seeds (Figure 1).

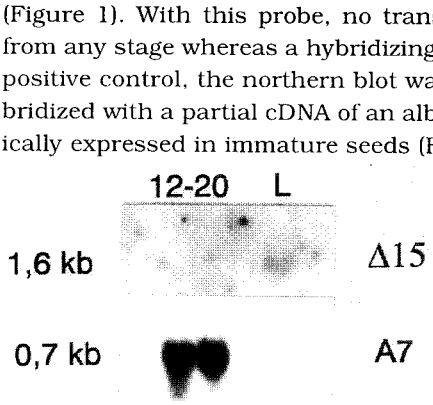


*Figure 1:*
*Northern blot analysis of total RNA from 12 or 20 DAP immature embryos or from leaves (L), sequentially hybridized with the Δ15 desaturase cDNA from Brassica napus and the albumine cDNA (A7) from sunflower, as probes.*

### Δ9 and Δ12 desaturase mRNAs expression in normal sunflower

Two other heterologous probes <<pcΔ9-1 and pcΔ12>> were sequentially hybridized onto northern transfers of total RNA prepared from the line CANP3 at a series of development stages from different tissues: leaves of adult plants; heads without mature seeds; cotyledons; hypocotyls and roots from 10-day-old plants; 12, 16, 20 and 28 DAP immature seeds developed at 26/22°C from CANP3 or HOC; dry seeds; and seeds soaked in water for 36 hours.

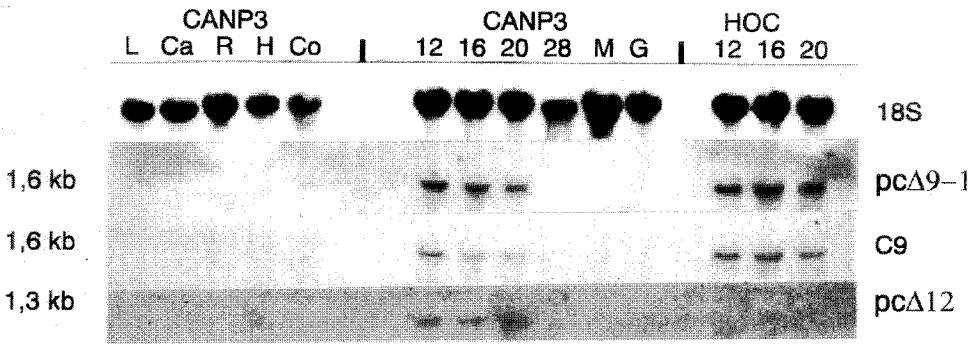


*Figure 2: Northern blot analyses of total RNA from different organs and stages, hybridized with the Δ9 and the Δ12 desaturase cDNAs from Arachys hypogea, (pcΔ9-1 and pcΔ12, respectively), the Δ9 partial cDNA from sunflower (C9), and the 18 S rRNA gene from sunflower, as probes. Total RNA was prepared from L: leaves; Ca: Capitulum without seeds; R: roots; H: hypocotyls; Co: cotyledons; 12, 16, 20 and 28 DAP immature embryos developed at 26°C day/22°C night from CANP3 or HOC; M: mature seeds and G: germinated seeds.*

Table 1: Influence of developmental stage in the Δ9 and Δ12 mRNA accumulation. Hybridization signal intensities in CANP3 line obtained with the 18 S rRNA gene, the pcΔ9-1 and the pcΔ12 probes at different developmental seed stages. Intensities are in arbitrary units.

| | Seeds at different developmental stages | | | | |
|---|---|---|---|---|---|
| Probes | 12 DAP | 16 DAP | 20 DAP | 28 DAP | Dry seeds |
| 18 S | 2.11 | 2.76 | 2.11 | 1.73 | 2.87 |
| pc Δ9-1 | 0.59 | 0.61 | 0.42 | 0 | 0 |
| **Δ9/18 S** | **0.28** | **0.22** | **0.20** | **0** | **0** |
| 18 S | 1.87 | 1.89 | 1.52 | 1.26 | 2.41 |
| pcΔ12 | 0.20 | 0.22 | 0.39 | 0 | 0 |
| **Δ12/18 S** | **0.11** | **0.12** | **0.26** | **0** | **0** |

The probes from peanut, pcΔ9-1 and pcΔ12, detected poly $(A)^+$ mRNA transcripts of 1.6kb and 1.3kb, respectively in embryos (Figure 2). The hybridization sig-

nals of immature seed RNAs were present at 12, 16, and 20 DAP and almost absent for all the other stages or tissues. For quantification and comparison of signals for each desaturase probe, all hybridizations were internally standardized by expressing signal intensity for the desaturase transcript in proportion to the signal obtained with the 18 S rRNA gene, as a probe. The maximal transcript level occurred as early as 12 DAP for the Δ9 desaturase gene and disappeared between 20 and 28 DAP whereas the Δ12 desaturase transcript was maximal at 20 DAP and disappeared between 20 and 28 DAP (Table 1).

### Comparison of the Δ9 and Δ12 mRNA accumulation levels between the normal CANP3 and the high oleic HOC lines

Comparisons of the expression of the Δ9 and Δ12 desaturase genes in CANP3 and HOC lines were performed at 12, 16 and 20 DAP and standardized with the intensity of signals obtained with the 18 S rRNA gene, as a probe. The standardized values of signals corresponding to the Δ9 desaturase were for the sum of the 3 stages, slightly higher in HOC (0.77) in comparison with CANP3 (0.70).

Table 2: Comparison of the Δ9 and Δ12 mRNA accumulation levels between a normal and a high oleic line.
Hybridization signal intensities obtained with the 18 S rRNA gene, the pcΔ9-1 and the pcΔ12 probes. Comparison of the ratio pcΔ9-1/18 S and pcΔ12/18 S between CANP3 and HOC immature embryos 12, 16 and 20 DAP. Plants were maintained at 26°C day/22°C night. Intensities are in arbitrary units.

| Probes | CANP3 | | | | HOC | | | |
|---|---|---|---|---|---|---|---|---|
| | 12 DAP | 16 DAP | 20 DAP | Sum | 12 DAP | 16 DAP | 20 DAP | Sum |
| 18 S | 2.11 | 2.76 | 2.11 | | 2.02 | 2.80 | 2.26 | |
| pcΔ9-1 | 0.59 | 0.61 | 0.42 | | 0.62 | 0.66 | 0.52 | |
| **Δ9/18 S** | **0.28** | **0.22** | **0.20** | **0.70** | **0.31** | **0.23** | **0.23** | **0.77** |
| 18 S | 1.87 | 1.89 | 1.52 | | 1.67 | 1.76 | 1.69 | |
| pcΔ12 | 0.20 | 0.22 | 0.39 | | 0.02 | 0.05 | 0.08 | |
| **Δ12/18 S** | **0.11** | **0.12** | **0.26** | **0.49** | **0.01** | **0.03** | **0.05** | **0.09** |

The Δ12 mRNA was weakly hybridized in HOC. The signal intensities obtained were too low and occurred below the linear response level of the film. We consider that standardized values of signals corresponding to the Δ12 desaturase were for the sum of the 3 stages, significantly higher in CANP3 in comparison with HOC (Table 2).

### Effect of temperature on the Δ9 and Δ12 mRNA accumulation levels

Northern blots of total RNA from embryos harvested 12 or 20 DAP from CANP3 or HOC plants grown either at 20°C day/18°C night or at 26°C day/22°C night were sequentially hybridized with the peanut pcΔ9-1 and the 18 S rRNA

probes (not shown). We observed for CANP3, that the level of Δ9 desaturase transcript is higher at 26/22°C (0.634, 0.430) than at 20/18°C (0.371, 0.389) for both 12 and 20 DAP stages, respectively (Table 3). Furthermore, the signal intensity value obtained for 12 DAP immature seeds, developed at 26/22°C occurred over the linear response level of the film and was then underestimated. We consider then that the Δ9 mRNA accumulation in 12 DAP developing seeds increased at least twofold between 20/18°C and 26/22°C. We observed the same range of activity for HOC at 20 DAP with a difference at 26/22°C (0.536) in comparison with 20/18°C (0.289).

Table 3: Effect of temeperature on the Δ9 and Δ12 mRNA accumulation levels. Hybridization signal intensities obtained with the 18 S rRNA gene, the pcΔ9-1 and the pcΔ12 probes. Comparison between 26°C and 20°C of the ratio pcΔ9-1 /18S and pcΔ12/18S at 12 and 20 DAP for CANP3 and at 20 DAP for HOC. Intensities are in arbitrary units.

| | CANP3 | | | | HOC | |
|---|---|---|---|---|---|---|
| | 12 DAP | | 20 DAP | | 20 DAP | |
| Probes | 26/22°C | 20/18°C | 26/22°C | 20/18°C | 26/22°C | 20/18°C |
| 18 S | 1.80 | 1.84 | 1.34 | 1.36 | 1.42 | 1.34 |
| pcΔ9-1 | 3.80 | 2.13 | 1.91 | 1.75 | 2.50 | 1.09 |
| Δ9/18 S | 2.11 | 1.16 | 1.42 | 1.29 | 1.76 | 0.81 |
| 18 S | 1.00 | 0.92 | 0.97 | 1.00 | 0.99 | 0.88 |
| pcΔ12 | 0.99 | 0.82 | 1.90 | 1.48 | 0.51 | 0.45 |
| Δ12/18 S | 0.99 | 0.90 | 1.96 | 1.48 | 0.51 | 0.51 |

Replicate RNA samples to those of the experiment above were sequentially hybridized with the peanut pcΔ12 and the 18 S rRNA probe (not shown). We observed for CANP3 that the level of Δ12 desaturase transcript is higher at 26/22°C (0.99, 1.96) than at 20/18°C (0.89, 1.48) for both 12 and 20 DAP stages, respectively (Table 3). We observed a lower activity for HOC at 20 DAP with no difference at 26/22°C (0.51) in comparison with 20/18°C (0.51), respectively.

**Estimation of variation between experiments**

In order to establish which variations were significant, we have compared treatments in the two different northern experiments presented in Table 2. and 3 with the desaturase probes as well as four additional cDNA probes of grain specific genes (unpublished results). Excluding the Δ12 hybridisation's, there is a maximal variation of 18% from one experiment to the other. Only those variations much greater than 18% were considered significant while those differences which were much smaller, may be significant but will need further testing. The Δ12 variation was not included because the level of exposure in Table 2 was below the quantitative exposure value of 0.16 for this film. However the magni-

tude of difference between the CANP3 (0.248) and HOC lines (0.047) is so great in both experiments, we feel this is clearly significant.

### Isolation of a partial cDNA encoding the carboxyterminal region of the sunflower Δ9 desaturase

A cDNA library prepared from poly(A) + mRNA of 12 DAP embryos was screened with the pcΔ9-1 probe. Only one clone was selected. The cDNA was about 700bp, it was purified and subcloned in pGEM-T for automatic sequencing. The partial amino acid deduced sequence shows a high homology with Δ9 desaturase carboxyterminal regions from other plants.

To verify the results obtained with the heterologous probe, the sunflower cDNA was used as a probe for northern blot hybridization at high stringency. It hybridized to poly(A) + mRNA of approximately 1.6kb, the same size as that revealed by pcΔ9-1 probe from peanut and exhibited the same tissue specific mRNA accumulation (Figure 2).

```
H. annuus     LAYXVSGRPYDRFASHYDPYAPIYSNRXRLLIYVSDXSVFAVTYLLYH
Glycine max   ..FN.......S.....H.........E........VAL.S...S..R
Glycine max   ..LN.........C.....G....D.EK.Q..I..AG.L..V.G.FR
R. communis   ..FN.........C.....G....E.E..Q..I..AG.L..V.G.FR
A. thaliana   ..FN.......G..C.FF.N....ND.E..Q..L..AGIL..CFG..R


H. annuus     IATLKGLGWVVCVYGVPLLIVNGFLVTITYLQHTHASL
Glycine max   V......V.LL.........................FA.
Glycine max   L.MA...A...........V......L..F.....PA.
R. communis   L.MA...A...........V......L..F.....PA.
A. thaliana   Y.AAQ.MASMI.L.........A...L........P..
```

*Figure 3: Alignment of the Δ9 sequences from two genomic sunflower clones and from 11 species. Only variable sites are represented by A, T, C. or G, and double nucleotides are Y (T+C), W (A+T), M (A+C), R (A+G), S (C+G), K (G+T).*

### Isolation of sunflower gDNA clones encoding a Δ9 or a Δ12 desaturase region

The genomic library was screened with the pcΔ9-1 and the pcΔ12 probe. Five clones hybridizing the pcΔ9-1 and ten clones hybridizing the pcΔ12 were isolated. With the couple of primers unique to the Δ9 desaturase, we amplified a 350bp fragment which were sequenced for two of the 5 clones (Figure 3). PCR amplification of a coding region of the Δ12 desaturase was performed on 3 of the 10 clones. The amplification product was sequenced and found of 268bp. The sequence aligns and matches 76.6% with the clone FAD-2 from *Glycine max* encoding a microsomal Δ12 desaturase (Figure 4).

*Figure 4: Alignment of the Δ12 sequences from sunflower, soybean and castor bean. Common amino acids are represented by points and those different by their respective conventional letters.*

```
          1.....50.....100
LG9.5  ..T.TR..T..M.....CA...C..C.C.C....W.....A.TW.....C..C.....Y.....G..Y.....K..T..Y.....
LG9.2  ..T.TG..T..A.....YA...Y..C.C.C....W.....A TW.....M..C.....Y.....G..Y.....K..T..Y.....
  h25  ..G.YR..T..M.....CA...A..C.C.C....T.....M.TW.....M..C.....Y.....K..Y.....K..T..Y.....
  h23  ..T.YR..T..M.....CA...A..Y.C.C....W.....R.YW.....M..C.....Y.....K..Y.....K..T..Y.....
  h09  ..T.YG..Y..M.....CW...A..Y.C.C....W.....M.TW.....M..C.....Y.....K..Y.....T..T..Y.....
  h08  ..T.YG..T..A.....CA...A..C.C.C....G.....A.AT.....C..Y.....T.....G..Y.....T..T..Y.....
  h01  ..G.NG..T..A.....CA...A..C.C.C....W.....M.NW.....Y..C.....Y.....K..Y.....T..T..T.....
  d26  ..T.YR..Y..M.....YA...A..C.C.C....W.....A.TW.....M..Y.....Y.....K..Y.....K..W..Y.....
  d21  ..T.YR..Y..M.....CA...A..C.C.C....R.....M.NW.....M..Y.....Y.....K..Y.....K..T..Y.....
  d11  ..G.TA..T..A.....CA...A..C.C.S....A.....A.TA.....C..C.....T.....G..C.....T..T..C.....
  d02  ..K.TR..Y..M.....CA...A..C.Y.C....A.....A.TA.....C..Y.....Y.....G..Y.....K..T..Y.....
  c10  ..T.YR..Y..N.....CA...A..C.C.C....N.....M.YW.....M..Y.....Y.....K..Y.....W..T..Y.....
  c07  ..N.YR..Y..A.....CAY..A..C.C.C....W.....N.YW.....M..C.....Y.....K..C.....W..T..Y.....

        101.....150.....213
LG9.5  .T.....C.Y.....Y..Y.....Y..Y..TR....Y..C..CC....C..A.CT.....C...A.G..C.....Y..Y
LG9.2  .T.....Y.Y.N.....Y..Y.....Y..Y..TR....C..C..MC....C..A.CT.....M...A.G..C.....Y..Y
  h25  .C.....Y.Y.....Y..Y.....Y..Y..YR....Y..C..WC....C..A.CT.....M...A.R..C.....Y..Y
  h23  .C.....C.Y.....Y..Y.....Y..T..YR....Y..C..RM....C..A.CW.....A...A.A..C.....Y..Y
  h09   C.....C.Y.....Y..Y.....T..T..YR....C..C..AM....C..R.CW.....M...A.R..C.....Y..Y
  h08  .Y.....C.Y.....Y..Y.....Y..T..TA....C..C..AC....C..A.CT.....C...A.R..C.....Y..T
  h01  .C.....C.Y.....Y..Y.....Y..Y..TG....Y..C..MA....Y..R.CT.....A...A.AN.C.....N..T
  d26  .Y.....C.Y.....Y..Y.....Y..T..YR....Y..C..MM....C..W.CT.....M...A.R..N.....Y..Y
  d21  .C.....C.Y.....Y..Y.....Y..T..YR....Y..C..NN....C..R.CT.....M...A.G..C.....Y..Y
  d11  .C.....C.T.....C..T.....T..T..TA....C..Y..AA....C..A.YT.....C...A.G..Y.....C..T
  d02  .C.....C.Y.....Y..Y.....Y..T..TR....C..C..AA....C..W.CT.....M...A.G..C.....Y..Y
  c10  .C.....C.Y.....Y..Y.....Y..Y..TR....Y..C..MC....Y..R.CT.....M...A.R..C.....Y..Y
  c07  .Y.....C.Y.....Y..Y.....T..Y..YR....Y..C..RC....Y..R.CT.....M...M.R..C.....Y..Y
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**Sequence comparison of Δ9 desaturase fragments in sunflower and wild *Helianthus* species**

We observed that for the two sunflower clones a mixture of two nucleotides was the rule for about 25 sites on both strands. We suggest that these clones carry a redundancy of the sequence which will be documentend elsewhere. We sequenced the amplification products in each species and clearly identified the same region of the Δ9 desaturase. We also observed the same ambiguities for all the species except for *H. occidentalis*. We computed the genetic distances according to Kimura taking into account the mixture of sequences and we constructed a phenetic tree (Figure 5). Surprisingly the two main branches correspond to the annual and the perennial forms except for *H. neglectus* improperly placed in the perennial forms. Moreover, among the perennial, the *Ciliares* and the *Atrorubentes* are also clearly shared.

## DISCUSSION

The Δ15 cDNA isolated from *Brassica napus* seeds corresponding to a microsomal desaturase detects a transcript in sunflower leaves but nothing in seeds. These results are consistent with the known spacial distribution of linolenic acid in sunflower where only trace amounts are present in seeds while in the leaves, linolenic acid represents 71% of the fatty acids of membrane lipids (Garcés et al., 1989). Therefore this gene appears to be tissue specifically regulated.

The mRNAs corresponding to the Δ9 and the Δ12 desaturases were detected as traces or not detected in the vegetative tissues assayed, however, in contrast to Δ15, the vegetative tissues do contain significant levels of oleic and linoleic acids. Detectable transcripts were accumulated in immature seeds between 12 and 20 DAP. Consequently, the corresponding genes are highly regulated at the transcription and/or RNA turnover levels. They exhibit significant transcript accumulation during the midmaturation stage of seed development and are undetectable during most of the plant life cycle. These genes may be seed specific with the possibility of a second form of each gene expressed in the vegetative tis-

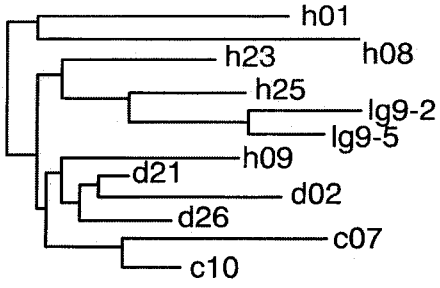


*Figure 5: Dendrogram built on the Kimura's distances using the NJ method, between the Δ9 sequences for 13 accessions of Helianthus.*

sues. Genes encoding for isoforms of each enzyme may differ sufficiently in sequence to prevent any cros-hybridization. A similar situation has been reported for *B. napus* (Slocombe et al., 1992) where a cDNA for the Δ9 desaturase, isolated from the seeds did not detect any mRNA in the leaves. However, for *R. communis* and *A. hypogaea*, cDNA for the Δ9 desaturase, isolated from the seeds hybridized mRNA from both seeds and leaves. In these two cases, the mRNA accumulation levels were significantly higher in immature seeds wich could be explained by the presence of a single gene that exhibits a much higher mRNA transcription or stability in seeds than in other tissues (Shanklin and Sommerville, 1991; Tate et al., 1995). No similar information is available on the tissue specific expression of the Δ12 desaturase gene in other species.

The mRNAs corresponding to the Δ9 and the Δ12 desaturases were mainly accumulated in immature embryos between 12 and 20 DAP and were as traces at 28 DAP and in dry seeds. This message accumulation during seed maturation is correlated with the synthesis of STG that begins at 12 DAP and reaches a maximum at 25 DAP (Garcés and Mancha, 1989). The Δ9 and the Δ12 desaturase mRNAs are sequentially accumulated as are their fatty acid products 18:1 and 18:2 respectively (Rochester and Silver, 1983). The maximum of the Δ12 mRNA accumulation occurs at 20 DAP, in correlation with the maximum of the Δ12 enzymatic activity (Garcés and Mancha, 1991). This suggests that the primary control of desaturase activity may be at the transcription level and is time dependent.

We observed a significant reduction in the level of Δ12 desaturase transcript in HOC. In this line, 18:1 represents more than 80% of the STG. The high oleic acid trait is associated with a loss of the Δ12 desaturase activity during the period of storage oil synthesis. The trait is seed specific since the normal and HOC lines displayed the same fatty acid composition of leaf membrane lipids (Garcés et al., 1989; B. Vick, personal communication). Mutants with high oleic acid content have been identified in many species (Kinney, 1994). The cause of high oleic content is still unknown except for sunflower and peanut where the mutation is correlated to a loss of oleate desaturase enzymatic activity in maturing seeds (Garcés et al., 1991; Ray et al., 1993). Our results supports the hypothesis that transcript level is directly correlated with enzyme activity and that a primary control of fatty acid desaturation can be exerted through modulation of the mRNA levels.

Furthermore, at 6 DAP the incorporation of fatty acids occurs in polar lipids to build cellular membranes. At this stage in normal and HOC varieties, the 18:2 content is similar (40%) (Garcés et al., 1989). The maximal activity of the Δ12 desaturase in HOC falls in the maximal incorporation of 18:2 in polar lipids at 10 DAP (Garcés and Mancha, 1991). This observation is consistent with the hypothesis that two genes encode for the Δ12 desaturase activity. One gene is expressed in very young seeds leading to 18:2 incorporated in polar lipids. This gene should

be expressed at the same rate in normal and HOC lines and likely constituvely expressed in all plant tissues. The second gene is induced in the seeds only for storage oil biosynthesis. This gene's expression, revealed by the pcΔ12 clone, is reduced or absent in HOC.

In maturing normal sunflower seeds, an augmentation in environmental temperature causes an increase of 18:1 content and a correlative decrease of 18:2 content (Trémolieres et al., 1982, Rochester and Silver, 1983; Silver et al., 1984). We observed that: 1) for CANP3 line the Δ9 and the Δ12 mRNA accumulation levels were higher in seeds grown at 26°/22°C than in those developed at 20/18°C, through increased synthesis or increased message stability at high temperature; 2) the increase was higher for the Δ9 mRNA than for the Δ12 mRNA; 3) the degree to which mRNA increased from 20/18°C to 26/22°C was significant at the stage of maximum mRNA accumulation, 12 DAP for the Δ9 mRNA and 20 DAP for the Δ12 mRNA. This is consistent with the kinetics of message accumulation in the grain for each of these genes. Furthermore, Garcés and Mancha (1991) have reported that the *in vitro* Δ12 desaturase activity reached a maximum at 20°C, and decreased with increasing temperature from 20 to 46°C. Consequently, the increase of oleic acid in response to higher temperature could be due to both the increase of Δ9 mRNA accumulation and the lowering of the Δ12 desaturase activity. The effect of temperature on the Δ12 desaturase activity may be due to additional physical factors which directly influence enzyme activity, the increase in Δ12 message steady state levels may be a cellular attempt to compensate the reduction of enzymatic activity associated with the higher growth temperature.

We observed for HOC, that the Δ9 mRNA accumulation level was higher in 20 DAP seeds grown at 26°/22°C. However, the 18:1 content of the mutant STG did not change significantly with temperature (Garcés et al., 1989). It is possible that in the HOC line, the unusual high level of 18:1 in maturing seeds negatively effects earlier steps in the synthesis pathway reducing the Δ9 desaturase levels. Therefore, increased Δ9 message levels at the higher temperature would not significantly increase 18:1 levels. The level of Δ12 mRNA accumulation did not vary with temperature. This level is probably too minimal to exhibit any significant temperature effect.

The ambiguities in the Δ9 sequences suggest two genes. One gene should be seed specific and the other expressed in all the other tissues. Such a situation has been found in canola (Slocombe et al., 1992) where the expression level of the two genes were different.

There is no ambiguity in the Δ12 sequences, this suggest that the three clones might correspond to the same gene for the microsomal form. It is likely that the chloroplastic Δ12 desaturase form is not detectable with this probe (Falcone et al., 1994). However the three clones display different restriction patterns (not shown) suggesting that they correspond to different copies. They could correspond to isoforms expressed either specifically in the embryos or constitutively in all tissues.

## CONCLUSIONS

Our study in sunflower is the first one that correlates the HOC mutation to a decreased transcript level of a putative oleate desaturase seed specific gene. This appears not to be the case in the peanut where the transcript levels are the same for both the normal and high oleate varieties (Abbott and Powell unpublished data). The regulation by temperature of the Δ9 and Δ12 desaturase activities seems oto be complex involving both genetic and physical factors. Consequently, in order to control the relative fatty acid content in sunflower seeds grown at different temperatures, it is of interest to study the direct effect of temperature on the enzymatic activities and its interaction with gene expression (Murphy, 1994; Bidney and Scelonge, 1995).

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## REFERENCES

Alonso, L., 1988. Genetic studies of the high oleic character in sunflower and its behaviour at different temperatures. In Proc. 12th Int. Sunflower Conference (Novi Sad) II: p454-p461.

Arondel, V., Lemieux, B., Hang, I., Gibson, S., Goodman, H.M., Sommerville, C.R., 1992. Map-based cloning of a gene controlling Omega-3 fatty acid desaturation in *Arabidopsis*. Science 258: 1353-1355.

Bidney, D., Scelonge, C., 1995. Sunflower biotechnology. In G. Seiler, eds, Present state and future for sunflower breeding, Publisher, City, Chapter 11, In press.

Choumane, W., Heizmann, P., 1988. Structure and variability of nuclear ribosomal genes in the genus *Helianthus*. Theor. Appl. Genet., 76: 481-489.

Falcone, D.L., Gibson, S., Lemieux, B., Somerville, C.R., 1994. Identification of a gene that complements an *Arabidopsis* mutant deficient in chloroplast w-6 desaturase activity. Plant Physiol., 106: 1453-1459.

Fick, G.N., 1984. Heritability of oil content in sunflower. Crop Sci., 15: 77-78.

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

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

Garcés, R., Mancha, M., 1991. *In vitro* oleate desaturase in developing sunflower seeds. Phytochemistry, 30 (7): 2127-2130.

Gentzbittel, L., Zhang, G., Vear, F., Bervillé, A., Nicolas, P., 1995. Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.). Theor. Appl. Genet., 90: 1079-1086.

Gibson, S., Arondel, V., Iba, K., Sommerville, C.R., 1994. Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from *Arabidopsis thaliana*. Plant Physiol., 106: 1615-1620.

Iba, K., Gibson, S., Nishiuchi, T., Fuse, T., Nishimura, M., Arondel, V., Hugly, S., Somerville, C.R., 1993. A gene encoding a chloroplast omega-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the fad7 mutant of *Arabidopsis thaliana*. J. Biol. Chem., 268: 24099-24105.

Kabbaj, A., 1995. Cloning of developmentally regulated genes in sunflower *(Helianthus annus* L.) and expression analysis of storage protein and fatty acid desaturase genes in normal and high oleic lines. Ph. D. Université Montpellier II.

Kabbaj, A., Abbott, A., Vervoort, V., Bervillé, A., 1995. Expression of stearate, oleate and linoleate desaturase genes in sunflower with normal and high oleic contents. 3 rd European sunflower Biotechnology Conference. Giessen 30. Oct.-2. Nov. 1995.

Kinney, A.J., 1994. Genetic modification of the storage lipids of plants. Plant Biotechnology Current Opinion in Biotechnology, 5: 144-151.

McKeon, T.A., Stumpf, P.K., 1982. Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-carrier protein thioesterase from maturing seeds of safflower. J. Biol. Chem., 257: 12141-12147.

Murphy, D.J., 1994. Biogenesis, function and biotechnology of plant storage lipids. Prog. Lipid Res. 33, N° 1/2: 71-85.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., 1994. Arabidopsis FAD2 Gene encodes the enzyme that is essential for polyunsatured lipid synthesis. The plant Cell 6: 147-158.

Ray, T.K., Holly, S.P., Knauft, D.A., Abbott, A.G., Powell, G.L. 1993. The primary defect in developing seed from the high oleate variety of peanut (*Arachis hypogaea*) is the absence of Δ12-desaturase activity. Plant Science, 91: 15-21.

Rochester, C., Silver, J., 1983. Unsaturated fatty acid synthesis in sunflower seeds in response to hight temperature. Plant cell reports, 2: 229-231.

Schilling, E., Heiser, C., 1981. Infrageneric classification of *Helianthus* (Compositae). Taxon, 30: 393-403.

Shanklin, J., Sommerville, C.R., 1991. Stearoyl-acyl-carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc. Natl. Acad. Sci. USA, 88: 2510-2514.

Silver Jenny, G., Rochester, C.P., Bishop, D.G., Harris Hazel, C., 1984. Unsaturated fatty acid synthesis during the development of isolated sunflower (*Helianthus annuus* L.) seeds. J. Exp. Botany, 35: (159) 1507-1514.

Slack, C.R., Roughan, P.G., Browse, J., 1979. Evidence for an oleoyl phosphatidylcholine desaturase in microsomal preparations from cotyledons of safflower seeds. Biochem. J., 179: 649-656.

Slocombe, S.P., Cummins, I., Jarvis, R.P., Murphy, D.J., 1992. Nucleotide sequence and temporal regulation of a seed specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol. Biol., 20: 151-155.

Soldatov, K.I., 1976. Chemical mutagenesis in sunflower breedings. In Proc 7th Int. Sunflower Conf., Krasnodar Russia, p352-p357.

Tate, P.L., Kochert, G., Abott, A.G., 1995. Isolation and characterization of a cDNA encoding the stearoyl-acyl-carrier protein desaturase in the peanut (*Arachis hypogaea*). In press.

Teyssendier de la Serve B, Jouanneau JP (1979). Preferential incorporation of exogenous cytokinin, N6-benzylademine into 18 S and 25 S ribosomal DNA of tabacco cells in suspension culture. Biochimie, 61: 913-922.

Trémolieres, A., Dubacq, J.P., Drapier, D., (1982). Unsaturated fatty acids in maturating seeds of sunflower and rape. Regulation by temperature and light intensity. Phytochemistry, 21: 41-45.

Watahiki Masaaki, K.. Yamamoto Kotaro, T. (1994). A new isozyme of plastid omega-3 fatty acid desaturase in *Arabidopsis thaliana*. Plant Physiol., 105: 1451-1452.

Yadav, N.S., Wierzbicki, A., Aegerter, M., Caster, C.S,, Pérez-Grau, L., Kinney, A.J., Hitz, W.D., Russel Booth, J., Scweiger, B., Stecca, K.L., Allen, S.M., Blackwell, M., Reiter, R.S., Carlosn, T.J., Russell, S.H., Feldmann, K.A., Pierce, J., Browse, J. 1993. Cloning of higher omega-3 fatty acid desaturases. Plant Physiol., 103: 467-476.

## POLIMORFISMOS EN HELIANTHUS Y EXPRESIÓN DE LOS GENES DE LAS DESATURASAS DE ESTEARTO, OLEATO Y LINOLEATO EN GIRASOL CON CONTENIDOS DE ÁCIDO OLEICO ALTOS Y NORMALES

RESUMEN

El estado de los niveles constantes de los productos de transcripción en girasol detectados con sondas para les desaturasas Δ9, Δ12 o Δ15, fue determinado en embriones y tejidos vegetativos en diferentes estados. La presencia de producto de transcripción del gen Δ15 estuvo limitado a las hojas. Las transcripciones de los genes Δ9 y Δ12 fueron detectados en embriones entre 12 y 20 dias después de siembra (DAP) y pocos como trazas en los otros tejidos examinados. La acumulación de la trancripción del gen Δ9 precede a la del Δ12. Los nivelas de acumulación de ARNm en CANP3, una línea con niveles normales de ácido oleico y una linea con alto ácido oleico (HOC) fueron comparados después de la estandarización de las intensidades señaladas. La acumulación de ARNm Δ9 fue ligeramente más alta en HOC que en CANP3. En contraste, la acumulación de ARNm Δ12 fue significativamente más reducida en HOC que en CANP3. La mutación HOC está correlacionada con una seducción en los niveles constantes de una semilla putativa especifica del gen Δ12. Nosotros observamos también que el ARNm de Δ9 y Δ12 se acumuló hasta niveles más altos en semillas en desarrollo mantenidas a 26° C dia/18° C noche.

A partir de una libreria genómica los clones 2 y 4 acarreando las secuencias homólogas a las desaturasas Δ9 y Δ12 respectivamente, fueron utilizadas para amplificación con "primas" únicos a cada desaturasa, lo que nos permitió una identificació clara de cada desaturasa. Además el fragmento de desaturasa Δ9 fue amplificado en 12 especies silvestres diferentes cubriendo la mayor parte de *Helinahtus*. La variabilidad de la secuencia reveló una mezcla de las dos secuencias Δ9 en todas las especies de *Helianthus*.

## POLYMORPHISME CHEZ LES *Helianthus* ET EXPRESSION DES GÈNES DE STÉARATE, OLÉATE ET LINOLÉATE DESATURASES DE TOURNESOLS À TENEUR NORMALE OU ÉLEVÉE EN ACIDE OLÉIQUE

RÉSUMÉ

Le niveau d'expression des transcrits, détecté par sondes hétérologues de la Δ9, la Δ12 ou la Δ15 desaturase a été déterminé dans les embryons et les tissus végétatifs à divers stades. La présence du transcrit du gène de la Δ15 était limité aux feuilles. Les transcrits des gènes de la Δ9 et Δ12 ont été détectés dans les embryons entre 10 et 20 jours après la pollinisation, et à l'état de traces dans les autres tissus étudiés. L'accumulation du transcrit du gène de la Δ9 précède celui du gène de la Δ12. Les niveaux de mRNA chez CANP3, lignée à teneur normale en acide oléique et chez une lignée à teneur élevée en acide oleique (HOC) ont été comparés après standardisation de l'intensité des signaux. L'accumulation du mRNA de la Δ9 est légèrement supérieure chez HOC que chez CANP3. La mutation HOC est corrélée à une

réduction du niveau d'expression d'un gène putatif de la Δ12, spécifique de la graine. On a aussi observé que le mRNA de la Δ9 et de la Δ12 s'accumule à des niveaux supérieurs dans les graines en cours de développement maintenues à 26°C le jour et 22°C la nuit qu'à 20°C de jour et 18°C de nuit.

A partir d'une banque génomique 2 et 4 clones portant respectivement des séquences homologues des Δ9 et Δ12 desaturases, ont été utilisés pour l'amplification avec des amorces uniques pour chaque desaturase, ceci nous a permis d'identifier clairement chacune des desaturases. De plus, un fragment de la Δ9 desaturase a été amplifié chez 12 espèces sauvages distinctes représentatives des *Helianthus*. La variabilité des séquences a révélé un mélange de deux séquences pour la Δ9, chez toutes les espèces d'*Helianthus* testées.