

## Expression of stearate, oleate and linoleate desaturase genes in sunflower with normal and high oleic contents

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### INTRODUCTION

Three enzymes are involved in the sequential desaturation of fatty acids in developing seeds: (i) The  $\Delta 9$  stearoyl-ACP desaturase, like the other desaturases, is nuclear encoded. It is located in the stroma of the plastid and introduces the first double bond in the fatty acid chain leading to the oleoyl-ACP (Ohlrogge et al., 1979). cDNA sequences encoding the enzyme have been isolated from many species (Tate et al., 1995). Sequence comparisons show a high level of similarity in size and primary structure of the deduced protein. (ii) The  $\Delta 12$  oleoyl-PC desaturase is most likely found in the microsomal fraction and is responsible for desaturation of 18:1-phosphatidylcholine (PC), leading to linoleyl-PC (Slack et al., 1979). In higher plants, only two cDNA sequences encoding the  $\Delta 12$  desaturase, one from *A. thaliana* (Okuley et al., 1994) and *A. hypogaea* (Albert Abbott, unpublished results) are available. (iii) The  $\Delta 15$  linoleyl-PC 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  $\Delta 15$  desaturase have been isolated from *B. napus* (Aronèl 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 of 20-25% of STG oleic acid whereas saturated fatty acids are mainly STG stearic acid (5-8%) and STG palmitic acid (3-6%). 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 targetted 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 obtained through seed mutagenesis « 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 environmental 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).

Unfortunately, nothing is known of the genes involved in STG fatty acid metabolism in sunflower making it difficult to manipulate the high oleic trait. Molecular markers tightly linked to quantitative trait loci (QTL) for oleic acid content should allow breeders to avoid monitoring oil quality thus facilitating improvement of other traits, however, the molecular mapping of QTLs for high oleic content is expensive and will not answer whether changes in STG oleic acid are due to changes in other desaturase genes.

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 stearoyl-ACP, the oleoyl-PC, and linoleoyl-PC desaturases.

## MATERIALS AND METHODS

The CANP3 sunflower (*Helianthus annuus L.*) line derived from the Russian population "Armavir 9345" (designated as normal variety) and the high oleic acid variety (designated by HOC), provided by Cargill (France), were used in the experiments. 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 weight, were harvested and used immediately for RNA extraction.

The total RNA fraction was obtained according to Teyssendier de la Serve et al., (1986). The cDNA libraries were prepared from poly (A)<sup>+</sup> mRNA of 12 and 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 (Kabbaj and Bervillé, in preparation).

We used the following probes: 1) the 1.05kb *EcoRI/BamHI* fragment of the 18 S rRNA gene from sunflower (Choumane and Heizmann, 1988); 2) the 1.5 kb cDNA, pc $\Delta$ 9-1 (plasmid, cDNA,  $\Delta$ -9 desaturase), the stearoyl-ACP desaturase from *Arachis hypogea* (Tate et al., 1995); 3) the 1.3 kb cDNA, pc $\Delta$ 12 (plasmid, cDNA,  $\Delta$ -12 desaturase), the oleoyl-PC desaturase from *A. hypogea* (A Abbott, unpublished results); 4) the 1.3 kb cDNA, clone pBNDES3, the microsomal omega 3-linoleoyl desaturase ( $\Delta$ 15) from *Brassica napus* (Arondel et al., 1992); 5) the 1.1 kb cDNA, clone pF2b, the oleoyl-PC desaturase from *Arabidopsis thaliana* (Okuley et al., 1994); 6) the 0.5 kb partial cDNA of a methionine-rich albumine from sunflower, clone A7, (Kabbaj and Bervillé, in preparation).

Autoradiographic hybridization signals were quantified by densitometry (Ultrosan 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 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 hybridize the same blot. We express the quantity of desaturase RNA as a ratio "mRNA signal/18 S rRNA signal" thereby, internally standardizing all sample loadings.

## RESULTS

Three heterologous probes « pF2b, pc $\Delta$ 9-1 and pc $\Delta$ 12 » 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.

The  $\Delta$ 12 probe from *Arabidopsis* (pF2b) did not exhibit detectable hybridization to RNAs from seeds or other tissues at any stages in the experimental conditions used. The other probes from peanut, pc $\Delta$ 9-1 and the pc $\Delta$ 12, detected poly (A)<sup>+</sup> mRNA transcripts of 1.6 kb and 1.3 kb, respectively in immature embryos. The hybridization signals of immature seed RNAs were present at 12, 16, and 20 DAP and absent for all the other

stages or tissues. The maximal transcript level occurred as early as 12 DAP for the stearoyl-ACP desaturase gene and disappeared between 20 and 28 DAP whereas the oleoyl-PC desaturase transcript was maximal at 20 DAP and disappeared between 20 and 28 DAP (Table I).

Comparisons of the expression of the stearoyl-ACP and oleoyl-PC 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  $\Delta 12$  mRNA was weakly hybridized in HOC. The signal intensities obtained were too low and occurred below the linear response level of the film.

Northern blots of total RNA from immature 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 pc $\Delta 9$ -1 and the 18 S rRNA probes. We observed for CANP3, that the level of stearoyl-ACP desaturase transcript is higher at 26/22°C than at 20/18°C for both 12 and 20 DAP stages, respectively (Table III). We consider then that the  $\Delta 9$  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 (1.76) in comparison with 20/18°C (0.81).

Replicate RNA samples to those of the experiment above were sequentially hybridized with the peanut pc $\Delta 12$  and the 18 S rRNA probe (Fig. 3). We observed for CANP3 that the level of oleoyl-PC desaturase transcript is higher at 26/22°C than at 20/18°C for both 12 and 20 DAP stages, respectively (Table III). 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. A cDNA library prepared from poly(A)<sup>+</sup> mRNA of 12 DAP embryos was screened with the pc $\Delta 9$ -1 probe. Only one clone was selected. The cDNA was about 700 bp, it was purified and subcloned in pGEM-T for automatic sequencing. The partial amino acid deduced sequence shows a high homology with  $\Delta 9$  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)<sup>+</sup> mRNA of approximately 1.6 kb, the same size as that revealed by pc $\Delta 9$ -1 probe from peanut and exhibiting the same tissue specific mRNA accumulation (Fig. 2).

#### DISCUSSION

The mRNAs corresponding to the  $\Delta 9$  and the  $\Delta 12$  desaturases were not detected in all the vegetative tissues assayed, however, in contrast to  $\Delta 15$ , the vegetative tissues do contain significant levels of oleic and linoleic acids. Detectable transcripts were limited to immature seeds between 12 and 20 DAP. Consequently, the corresponding genes are highly regulated at the transcriptional and/or RNA turnover levels. They exhibit significant transcript accumulation during the mid-maturation 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 vegetative tissues. Genes encoding for isoforms of each enzyme may differ sufficiently in sequence to prevent any cross-hybridization. A similar situation has been reported for *B. napus* (Slocombe et al., 1992) where a cDNA for stearoyl-ACP desaturase, isolated from seeds did not detect any mRNA in the leaves. However, for *R. communis* and *A. hypogaea*, cDNA for stearoyl-ACP desaturases isolated from seeds hybridized both mRNA from seeds and leaves with a higher mRNA accumulation in immature seeds (Shanklin and Sommerville, 1991; Tate et al., 1995). This 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. No similar information is available on the tissue specific expression of the  $\Delta 12$  desaturase gene in other species.

The mRNAs corresponding to the  $\Delta 9$  and the  $\Delta 12$  desaturases were accumulated in immature embryos between 12 and 20 DAP and were absent 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  $\Delta 9$  and the  $\Delta 12$  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  $\Delta 12$  mRNA accumulation occurs at 20 DAP, in correlation with the maximum of the  $\Delta 12$  enzymatic activity (Garcés and Mancha, 1991). This suggests that the primary control of desaturase activity may be at the transcriptional level and is time dependent.

We observed a significant reduction in the level of  $\Delta 12$  desaturase transcript in HOC. In this line, C18:1 represents more than 80% of the STG. The high oleic acid trait is associated with a loss of  $\Delta 12$  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). This evidence 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 mRNA level.

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 (Silver et al., 1984). We observed that: 1) for CANP3 line the  $\Delta 9$  and the  $\Delta 12$  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  $\Delta 9$  mRNA than for the  $\Delta 12$  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  $\Delta 9$  mRNA and 20 DAP for  $\Delta 12$  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*  $\Delta 12$  desaturase activity reached a maximum at 20°C, and decreased with increasing temperature from 20 to 45°C. Consequently, the increase of oleic acid in response to higher temperature could be due to both the increase of  $\Delta 9$  mRNA accumulation and the lowering of the  $\Delta 12$  desaturase activity.

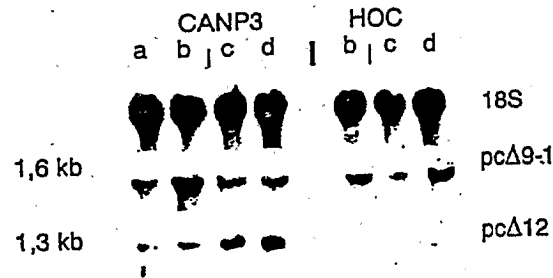
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**Figure 1:** Northern blot analyses of total RNA from different organs and stages, hybridized with the  $\Delta 9$  and the  $\Delta 12$  desaturase cDNAs from *Arachis hypogea*, (pc $\Delta 9$ -1 and pc $\Delta 12$ , respectively), the  $\Delta 9$  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.



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	
pc $\Delta 9$ -1	0.59	0.61	0.42		0.62	0.66	0.52	
$\Delta 9/18$ 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	
pc $\Delta 12$	0.20	0.22	0.39		0.02	0.05	0.08	
$\Delta 12/18$ S	0.11	0.12	0.26	0.49	0.01	0.03	0.05	0.09

**Table I** Comparison of the  $\Delta 9$  and  $\Delta 12$  mRNA accumulation levels between a normal and a high oleic line. Hybridization signal intensities obtained with the 18 S rRNA gene, the pc $\Delta 9$ -1 and the pc $\Delta 12$  probes. Comparison of the ratio pc $\Delta 9$ -1/18 S and pc $\Delta 12$ /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.

**Table II** Effect of temperature on the  $\Delta 9$  and  $\Delta 12$  mRNA accumulation. Hybridization signal intensities obtained with the 18 S rRNA gene, the pc $\Delta 9$ -1 and the pc $\Delta 12$  probes. Comparison between 26°C and 20°C of the ratio pc $\Delta 9$ -1/18 S and pc $\Delta 12$ /18 S at 12 and 20 DAP for CANP3 and at 20 DAP for HOC. Intensities are in arbitrary units.

Probes	CANP3				HOC	
	12 DAP		20 DAP		20 DAP	
	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
pc $\Delta 9$ -1	3.80	2.13	1.91	1.75	2.50	1.09
$\Delta 9/18$ 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
pc $\Delta 12$	0.99	0.82	1.90	1.48	0.51	0.45
$\Delta 12/18$ S	0.99	0.89	1.96	1.48	0.51	0.51