ANALYSIS OF DESATURASE TRANSCRIPT ACCUMULATION IN NORMAL AND IN HIGH OLEIC OIL SUNFLOWER DEVELOPMENT SEEDS

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ABSTRACT:

A population, *Pervenets*, has been obtained by chemical mutagenesis on the population *VNIIMK 8931* (Soldatov 1976). Genotypes developed from *Pervenets* population produce seeds with a High Oleic acid content (HO) compared to the seeds produced by normal genotype (LO: Linoleic). To unravel the mechanism(s) of this mutation, we studied the transcript accumulation of genes coding for enzymes involving in the oleic acid pathway synthesis in immature seeds. To check the possible effect of genetic background, different LO and HO inbred lines were used. Concerning the Δ9-desaturase transcript accumulation, no significant difference was observed between LO and HO embryos. In contrast, concerning the Δ12-desaturase transcript accumulation, no transcript accumulation was observed in any of the HO embryos whereas, this transcript accumulated in all LO embryos at critical stages of lipid reserve synthesis. The study of HO x LO or LO x HO crosses revealed a high oleic acid content in each hybrid seed. The same strong reduction in accumulation of Δ12-desaturase transcript was observed in hybrid embryos and in HO embryos resulting from selfing. Thus, the HO phenotype seems to be correlated to a strong reduction in accumulation of Δ12-desaturase transcript in seeds during critical stages of lipid reserve synthesis. Moreover, the expression of the HO character behaves as dominant in HO x LO hybrids. The results of these studies permitted us to suggest mechanisms explaining the oleic acid accumulation in HO mutant seeds.

RESUME:

Une population, *Pervenets*, a été obtenue par mutagenèse chimique sur la population *VNIIMK 8931* (Soldatov, 1976). Les génotypes développés à partir de la population *Pervenets* produisent des graines à Haute teneur en acide Oléique (HO) par rapport aux graines produites par les génotypes normaux (LO: Linoléique). Pour éclaircir le ou les mécanismes de cette mutation, nous avons étudié l'accumulation d'ARNm des gènes codant pour les enzymes intervenant dans la chaîne de biosynthèse de l'acide oléique. Pour vérifier l'effet possible du fond génétique, différentes lignées HO et LO ont été utilisées. En ce qui concerne l'accumulation des ARNm de la Δ9-désaturase, aucune différence significative a été observée entre les embryons LO et HO. En revanche, concernant l'accumulation des ARNm de la Δ12-désaturase, aucune accumulation a été observée dans les embryons HO alors que cet ARNm est accumulé dans tous les embryons LO aux stades critiques de l'élaboration des réserves lipidiques. L'étude des croisements soit HO x LO soit LO x HO a révélé de fortes teneurs en acide oléique dans les graines hybrides. Nous avons observé la même forte réduction dans l'accumulation des ARNm de Δ12 désaturase dans les embryons hybrides que dans les embryons HO résultant de l'autofécondation. Le phénotype HO semble donc être dû à une forte réduction dans l'accumulation des ARNm de Δ12-désaturase dans les graines pendant les stades critiques d'élaboration des réserves lipidiques. De plus, l'expression du caractère HO se comporte comme dominant dans les hybrides HO x LO. Les résultats de ces études nous permettent de suggérer des mécanismes pour expliquer l'accumulation d'acide oléique dans les graines des mutants HO.

INTRODUCTION:

Sunflower is one of the most cultivated oilseed crops in the world. It is mainly used for the oil production because of the high quantity of oil in the seeds (about 50 %). This oil is made of triacylglycerol that contain 10 to 14 % of saturated fatty acid (palmitic and stearic acids), 20 % of monoinsaturated fatty acid (oleic acid) and 70 % of polyinsaturated fatty acid (mainly linoleic acid). The desaturation of these fatty acids (Brown et al., 1998) from stearic acid requires 3 enzymes:

- The stearoyl-ACP-desaturase (Δ9-desaturase) which catalyses the first desaturation of the stearoyl-ACP in oleoyl-ACP (Mc Keon et Stumpf, 1982),
- The oleoyl-phosphatidyl-choline-desaturase (Δ12-desaturase) which catalyses the second desaturation of the oleoyl-PC in linoleoyl-PC (Slack et al., 1979; Stymme and Appelqvist, 1980),
- The linoleoyl-phosphatidyl-choline-desaturase (Δ15-desaturase) which catalyses the third desaturation of the linoleoyl-PC in linolenoyl-PC (Stymme and Appelqvist, 1980).

To enlarge the uses of sunflower oil, the mutagenesis has been used to modify the proportion of the different fatty acids. So, a population, *Pervenets*, leading to an oil with high oleic acid content in seeds has been obtained by chemical mutagenesis on the population *VNIIMK 8931* (Soldatov 1976). However, we ignored whether different mutational events have occurred in this population. «High Oleic» (HO) genotypes with an oleic acid content in seeds up to 80 % and a normal lipid composition of membranes (Garcés et al., 1989), were developed from this population. Several genetic studies has been made (Fernàndez-Martinez et al., 1989; Miller et al., 1987; Urie, 1985) to understand this HO phenotype but the mechanism(s) leading to this phenotype are still unknown. However, the hypotheses explaining oleic acid accumulation in seeds focus on the 2 enzymes responsible for the oleic acid accumulation: Δ9- and Δ12-desaturases (Kabbaj et al., 1996 a; 1996 b). The oleic acid accumulation in seeds could be due to an increase of the Δ9-desaturase activity or a decrease of the Δ12-desaturase activity. Mutations leading to a modification of enzymatic activities could occur at several levels: deletion or duplication of desaturase genes, the level of transcription of these genes, the accumulation of desaturases gene transcript…. To unravel the mechanism of this mutation, a Northern analysis of the accumulation of the Δ9- and the Δ12-desaturase transcript was carried out in several HO, LO (Linoleic) and hybrids immature embryos. The results of these studies permit us to suggest mechanisms explaining the oleic acid accumulation in HO mutants.

MATERIAL AND METHODS:

Material:

Several HO and LO genotypes (hybrids and inbred lines) were grown in the greenhouse or in the field to produce seeds resulting from selfing or crosses (Table 1). Seeds were collected on these heads at several developmental levels: 12-, 16- and 24- Days After Flowering (DAF) in the case of selfing, or Days After Pollination (DAP) in the case of crosses.

Methods:

Measure of oleic acid content in the resulting seeds:

The oleic acid content in mature seeds resulting from selfing and crosses was determined either by GC or by refractometry (Goss, 1978).

Northern analysis:

Total RNAs were extracted from 200 µg of immature embryos using « RNA Instra Pure System » protocol (Eurogentec). An equal amount of RNA (15 µg) was denatured with formaldehyde and formamide. The denatured RNAs were electrophoresed in a 1.2 % agarose-

15 % formaldehyde gel and the gel was photographed to demonstrate equal loading of RNA. The gel was transferred to a Nylon membrane to obtain Northern blots (Sambrook et al., 1989).

The Δ9-desaturase cDNA (Kabbaj et al., 1996 a, 1996 b) and the Δ12-desaturase cDNA (Abbott et al. unpublished) were used to produce labelled probes with [^{32} P] α-dCTP by random primer reaction, as described by Feinberg and Vogelstein (1983). Different length of exposure for membranes onto films was performed depending on the level of radioactive spots on the membranes.

					transcript	
Genotype	Parents phenotype	Source	Embryos production	Oleic acid content	Δ9	Δ12
Santiago	LO	Novartis	hybrid selfing	38 %	+	+
Trisun 870	HO	Mycogen	hybrid selfing	91 %	+	-
Olbaril	HO	Pioneer	hybrid selfing	84 %	+	-
HOC 97	HO	Monsanto	lines selfing	84 %	+	-
HOC B	HO	Monsanto	lines selfing	86 %	+	-
HOC 98	HO	Monsanto	lines selfing	85 %	+	-
HOC 500K	HO	Monsanto	lines selfing	85 %	+	-
Ha Ol 9	HO	CSIC	lines selfing	86 %	+	-
BD 70080	LO	Monsanto	lines selfing	36 %	+	+
BE 78078	HO	Monsanto	lines selfing	85 %	+	-
BD 70032	LO	Monsanto	lines selfing	35 %	+	+
BE 73201.1	HO	Monsanto	lines selfing	84 %	+	-
BE 73201.2	HO	Monsanto	lines selfing	83 %	+	-
BE 73201.4	HO	Monsanto	lines selfing	86 %	+	-
BE 73201.5	HO	Monsanto	lines selfing	85 %	+	-
90 R 19	LO	INRA	lines selfing	23 %	+	+
63 B	LO	INRA	lines selfing	29 %	+	+
83 HR 4	LO	INRA	lines selfing	25 %	+	+
83 HR 4 x HOC	LO x HO	INRA	lines crosses	61 %	+	-
Ha 342 x RHA 345	HO x HO	INRA	lines crosses	77 %	+	-
Ha 342 x OPA 2	HO x HO	INRA	lines crosses	81 %	+	-
63 A x HOC B	LO x HO	INRA	lines crosses	78 %	+	-
HOC A x 63 B	HO x LO	INRA	lines crosses	88 %	+	-

Table 1: Genotypes used to obtain embryos by selfing or crosses and oleic acid content of the seeds generated (in % of the total seed oil). The presence (+) or absence (-) of desaturase transcript accumulation is noted.

RESULTS:

Oleic acid content of the seeds:

The oleic acid content of the seeds resulting from selfing or crosses is shown in Table 1. The range of variation for seeds resulting from LO and HO lines selfing was from 23 to 38 % and 83 to 91 %, respectively. The content in oleic acid for seeds resulting from crosses between HO and LO lines ranges between 61 to 88%. The sense of the cross does not seem to influence greatly the fatty acid composition of the resulting seeds.

Accumulation of the Δ9-desaturase transcript

We verified by staining RNA that equivalent amounts were loaded in each lane (Fig. 1B). The accumulation of Δ9-desaturase transcript in immature embryos resulting from selfing of HO or LO genotypes was revealed by the hybridization of Northern blots by the Δ9-desaturase cDNA used as a probe (Fig. 1A, Table 1). A 1.6 kb transcript was revealed in each lane corresponding to the 12- and 16- DAF embryos. No significant intensity difference was shown between all HO embryos and LO embryos in all genotypes examined.

The same protocol was performed to reveal the Δ9-desaturase transcript accumulation in immature embryos resulting from crosses between HO x LO or LO x HO lines. A 1.6 kb transcript was revealed in each lane corresponding to the 12- and 16- DAP embryos. The

intensity of these bands was comparable to the intensity of the bands revealed in Northern blots of RNA of embryos resulting from HO or LO selfing (Fig. 1A, Table 1).

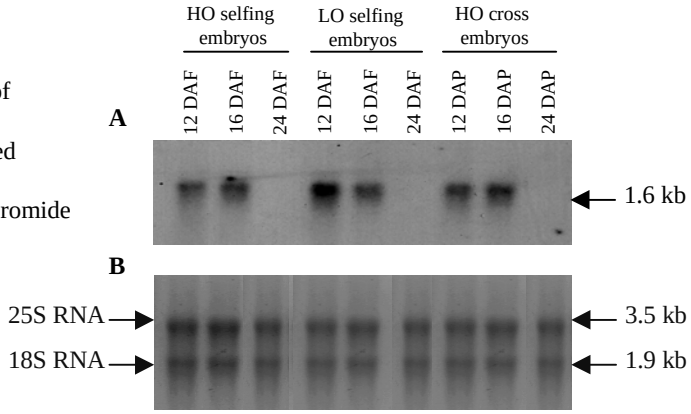

Fig 1: Autoradiography film of a Northern blot of total RNA from HO selfing, LO selfing and HO cross (HO x LO or LO x HO) embryos hybridized by the Δ9-desaturase cDNAused as a probe (**A**). Photograph of the Northern gel after Ethidium Bromide coloration (**B**).

Accumulation of Δ12-desaturase transcript

The accumulation of Δ12-desaturase transcript in immature embryos resulting from selfing of HO or LO genotypes was revealed by the hybridization of Northern blots by the Δ12-desaturase cDNA used as a probe (Fig. 2, Table 1).

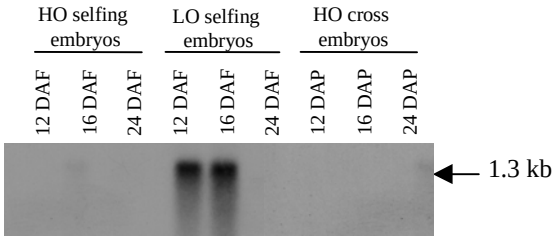

Fig 2: Autoradiography film of a Northern blot of total RNA from HO selfing, LO selfing and HO cross (HO x LO or LO x HO) embryos hybridized by the Δ12-desaturase cDNA used as a probe.

A 1.3 kb transcript was revealed in each lane corresponding to the 12- and 16- DAF LO embryos. However, no hybridization signal was revealed for HO embryos.

The same protocol was performed for the embryos resulting from HO x LO or LO x HO crosses. No hybridization signal was revealed in any of these HO embryos (Fig. 2, Table 1).

DISCUSSION:

The measure of the oleic acid content in the seeds revealed that all the seeds resulting from LO genoypes selfing have a Low Oleic content (between 23 % and 38 %). Moreover, all the seeds resulting from HO genotypes selfing or from crosses have a High Oleic content (between 83 % and 91 %) (Table 1). So, the study of accumulations of both desaturases transcript in these immature embryos reflects what happened during the constitution of linoleic lipid reserve in the case of LO embryos or what happened during the constitution of high oleic lipid reserve in the case of HO embryos.

The study of the Δ9-desaturase transcript accumulation in immature embryos revealed a transcript accumulation at 12- and 16- DAF or DAP. We revealed no difference in Δ9-desaturase transcript accumulation between LO and HO embryos resulting from selfing or crosses. These results agree with those of Kabbaj et al. (1996 a; 1996 b) on one LO and one HO lines. They have revealed a Δ9-desaturase transcript accumulation at 1.6 kb at the stages 12-, 16- and 20 DAF embryos resulting from selfing of *CANP 3* LO inbred line and *HOC* HO inbred line using heterologous probe. Hongstrakul et al. (1998) have shown a Δ9-desaturase transcript accumulation in 3 LO and 3 HO embryos using RT-PCR but with this method, it is difficult to determine the quantity of transcript accumulation. Thus, according to our results on a relative high number of different genotypes (6 LO, 14 HO and 5 hybrid genotypes), the

High Oleic phenotype did not appeared to be correlated to an over accumulation of the Δ9-desaturase transcript in HO embryos during the stages of lipid reserve biosynthesis.

On the other hand, the study of the Δ12-desaturase transcript accumulation revealed a specific transcript accumulation at 12- and 16- DAP only in LO embryos. This result agreed those of Kabbaj et al. (1996 a; 1996 b). They have revealed a Δ12-desaturase transcript accumulation at 1.3 kb only in LO embryos at 12-, 16- and 20 DAF and no significant Δ12-desaturase transcript accumulation in HO embryos. Using RT-PCR, Hongstrakul et al. (1998) have shown a drastically reduced signal in HO embryos, but this method is not quantitative. Thus, it is difficult to say if this signal correspond to a significant Δ12-desaturase transcript accumulation in HO embryos. So, our results clearly show that in all genotypes tested, the High Oleic phenotype is correlated to a strong reduction in the level of Δ12-desaturase transcript accumulation in HO embryos during lipid reserve biosynthesis.

The study of hybrid embryos resulting from crosses between HO and LO lines revealed some elements on the possible mechanisms leading to high oleic accumulation. Indeed, the measure of the oleic acid content in mature hybrid seeds showed high oleic content whatever the sense of the cross (Table 1). This high oleic phenotype appears consequently to be dominant without major maternal effect. These resulted agreed those of Varés et al., 2000. Moreover, the study of both desaturases transcript accumulation in immature hybrid embryos revealed the same results as the study on HO embryos resulting from selfing: the same Δ9-desaturase transcript accumulation as in LO embryos resulting from selfing and an under-accumulation or no-accumulation of Δ12-desaturase transcript. The expression of the mutation leading to this strong reduction in the level of Δ12-desaturase transcript accumulation behaves therefore as dominant. Reciprocal crosses indicate no major maternal effects.

The strong reduction in the level of Δ12-desaturase transcript accumulation was observed for all the HO genotypes studied so far. Consequently it is likely that all the genotypes shared the same mechanism for the HO trait. It may be due either to a weak or an absence of transcription or to a very weak stability of the transcript after synthesis. Several mechanisms may explain this (Table 2).

	Hypothesis 1	Hypothesis 2	Hypothesis 3	Hypothesis 4
Type of mutation	Deletion of a seed specific Δ12-desaturase gene	Mutation on a cis-regulatory element	Mutation on a trans-regulatory element	Mutation on a element involving in transcript stability
Consequence	No transcription	No transcription	No transcription	No-accumulation of the transcript
Behaviour of the mutant	Recessive	Recessive	Dominant	Dominant
Method to study the hypothesis	Study of the number of Δ12-desaturase gene	Study of the organization of the Δ12-desaturase genes	QTL analysis	QTL analysis

Table 2: Description of hypotheses of mutation leading to the HO phenotype

Mutation leading either to the deletion of the Δ12-desaturase gene responsible of the transcript accumulation in the HO seeds (Hypothesis 1) or to mutation in the cis-regulatory sequence leading to an inactivation of expression in the HO seeds (Hypothesis 2) may lead to an absence of Δ12-desaturase transcript in HO seeds. However, the expression of these mutations would behave as recessive (Table 2) which is not the case. To emphasise or not the impossibility of hypothesis 1 and 2, the Δ12-desaturase genes organisation in HO and LO genotypes could be studied by an RFLP method. Thus, the number and the organization of Δ12-desaturase genes could be compared between HO and LO genotypes (Lacombe et al., 2000).

Some other hypotheses concerning mutations on a trans-regulatory element of the seed specific Δ12-desaturase transcription (Hypothesis 3) or on an element stepping in the Δ12-desaturase transcript stability in HO seeds (Hypothesis 4) could be postulated. These mutations could lead to HO trait behaving as dominant. A QTL analysis on a segregating population for the HO trait could permit to determine the loci involved (Lacombe et al., 2000). The study of these loci could lead to identify genes potentially involved in the HO accumulation. Thus, the involvement of a trans-regulatory element or of an element stepping in transcript stability could be revealed.

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