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CLONING AND mRNA TRANSCRIPTION ANALYSIS OF FIVE DEVELOPMENTALLY REGULATED cDNA FAMILIES IN SUNFLOWER IMMATURE EMBRYOS

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SUMMARY

In order to isolate and to analyse the expression of developmentally regulated genes in sunflower, cDNA libraries were constructed using mRNA from 12 or 20 day-old seeds and then differentially hybridized with mRNA from dry seeds and leaves. cDNA encoding major storage proteins (helianthinin and albumin Met-rich), proteinase inhibitor and two other cDNA with unknown function were isolated.

The steady state levels of the transcripts, detected with each cDNA, were determined in immature seeds and vegetative tissues at different stages. The accumulation of the five mRNAs was tissue-specific, limited to the mid-maturation stage of seed development. The albumin Met-rich mRNA accumulation occurred relatively later than the accumulation of both the helianthinin mRNA and the previously studied HaG5 albumin mRNA in sunflower. This indicates that the mRNA synthesis of the two storage protein classes and of different precursors within one protein class is not synchronous. These observations were correlated to the deposit of the different storage protein precursors and are in agreement with a primary control of storage protein gene expression at the level of mRNA transcription. The accumulation of helianthinin transcript was higher in the CANP3 normal variety in comparison with the HOC « high oil and oleic » line. The levels of the albumin Met-rich mRNA were in contrast similar in both lines. This could be in relation with the protein redistribution that is known to accompany the increase of seed oil content in sunflower and agrees with the fact that low oil varieties have higher helianthinin content than high oil varieties. We suggest that the albumin Met-rich fraction is not involved in this protein redistribution.

Key words: Albumin, cDNA, development, helianthinin, proteinase inhibitor, sunflower.

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INTRODUCTION

Seed maturation is a complex process involving spacial and temporal regulated expression of specific genes. During this developmental process, seed increases in volume due to both cell expansion and storage synthesis. Storage proteins and lipids are used as nitrogen and carbon sources during germination initiating the subsequent development of mature plant. The structure and expression of genes specifically or preferentially expressed during seed maturation have been studied in many plant species. The best known genes are encoding storage proteins (Pernollet, 1985). Furthermore genes encoding lectins, oleosins, proteinase inhibitors and fatty acid desaturases, have also shown seed specific expression (Wilkins and Raikhel, 1989; Slocombe et al., 1992; Choi et al., 1993; Murphy, 1994; Kabbaj, 1995). In sunflower, two major classes of storage proteins have been studied, the globulins 11 S (*helianthinins*) and the albumins 2S. They together represent 85 % of total proteins stored in the seeds (Sosulski et al., 1969).

The helianthinins are soluble in 1M NaCl and particularly rich in Arg, Phe, Gln and Asp amino-acids. They consist of hexameric holoproteins of 300 kDa. Each intermediary subunit ($\alpha\beta$) includes a large (α: 42-39 kDa), acidic polypeptide and a smaller (β: 27-23 kDa), basic polypeptide linked by disulfide bonds (Dalgalarrondo *et al.*, 1984). The cDNA cloning and mRNA expression analysis of helianthinin genes have already been reported (Allen *et al.*, 1985). Transcripts accumulate to maximum levels approximately between 12 and 15 days after flowering (DAF) and disappear in mature seeds.

The albumins are soluble in water and consists of a unique polypeptide chain of 10 to 18 kDa (Kortt and Caldwell, 1990). In all the studied species except sunflower, the albumin precursors are cleaved at a conserved site into two subunits (9 and 3 kDa) linked by a disulfide bridge (Sharief and Li, 1982; Crouch *et al.*, 1983; Altenback *et al.*, 1987). The molecular weight and amino-acid composition of albumins in sunflower permit to distinguish at least two fractions. The major fraction includes albumins of « 18-14 kDa », rich in Cys 5-8 % and Lys 7-10 % (Kortt and Caldwell, 1990). The second fraction consists of albumins of 10 kDa, rich in Cys 8 % and Met 16 % and containing less Lys (4 %) than the major fraction. This Met-rich fraction represents 37 % of total albumins (Youle et Huang, 1981) and similar albumins have been identified only in Brazil nut (Altenbach *et al.*, 1987). The cloning of two albumin cDNAs, HaG5 and SFA8 clones, from both fractions has been reported (Allen *et al.*, 1987, Kortt *et al.*, 1991). Transcript for the HaG5 clone was detected in the seeds as early as 5 DAF, it reached maximum levels at 12 DAF and then decreased as the seed matured (Allen *et al.*, 1987).

The relative albumin and helianthinin seed content changes between varieties and in response to environmental conditions. Low oil varieties have higher contents of helianthinins than high oil varieties. Plant water supply also affects the quality of seed protein complex. With the increase of soil moistening the helianthinin seed

content drops and the relative quantity of albumin increases (Borodulina et Suprinova, 1976).

As a first step towards the investigation of the regulatory mechanisms involved in the developmental expression of genes in maturing seeds, we report here the cloning and mRNA transcription analyses of five different seed specific cDNAs.

MATERIALS AND METHODS

Plant materials

The CANP3 sunflower (*Helianthus annuus* L.) line derived from the Russian population « Armavir 9345 », further designated as normal variety, and the higher oil and oleic variety provided by Cargill (France), further designated as HOC, were used in the experiments. Plants were grown in a greenhouse until the beginning of flowering, they were then transfered 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 6, 12, 16, 20 or 28 DAP. The embryos, 2 g fresh weight at 6 DAP, and 3 to 5 g fresh weight at the other stages, were dissected from achenes and used immediately for RNA extraction.

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 on coarse sand supplemented with nutritive solution 2g/l "4% NO_3, 11% NH_4, 11% P_2O_5 and 15% K_2O". All parts of the seedlings were separated and frozen in liquid nitrogen.

Probes

We used -1) The 1,05kb fragment of the 18S rRNA gene from sunflower (Choumane and Heizmann, 1988). -2) The cDNA pHa2 corresponding to a helianthinin gene (Allen and Thomas, 1985).

Total RNA isolation and northen-blot hybridization

Total RNA was isolated from developing sunflower seeds, seedlings and germinating seeds (Teyssendier and Jouanneau, 1979). Twenty µg of denatured total RNA were loaded onto 1.2% agarose formaldehyde gels and size fractionnated by electrophoresis at 80 V/cm for 5 h. Formaldehyde was removed by washing the gel with distilled water. RNA was then transfered to nylon membranes by capillarity overnight (Biohylon Z+, Bioprobe) (Sambrook *et al.*, 1989).

Prehybridizations were carried out at 65°C in 6X SSC, 5X Denhardt's solution and 0.5% SDS for 6 hours. cDNA probes were labelled by the random priming method, denatured and added to the prehybridization solution. After 18 h of hybridization, filters were washed twice at 65°C with 2X SSC, 0.1% SDS for 30 min

followed by a third washing with 0.2X SSC, 0.1% SDS at 65°C for 30 min. Filters were autoradiographied at -80°C. For sequential hybridization with different probes, filters were immersed in a solution of boiling 0.1% SDS and allowed to cool to room temperature.

Autoradiographic hybridization 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 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 rRNA sunflower gene as probes to hybridize the same blot. We expressed the quantity of a specific mRNA as a ratio « mRNA signal/18 S rRNA signal » thereby, internally standardizing all sample loadings.

Estimation of experiment to experiment variation

In order to establish which variations were significant, we have compared treatments in two different northern experiments with the five probes described in this paper as well as with the Δ9 and Δ12 desaturase probes (Kabbaj, 1995). We observed a maximal variation of 25% from one experiment to the other. Only those variations much greater than 25% were then considered significant those differences which were much smaller, may be significant but will need further testing.

Construction and differential screening of the cDNA libraries

Poly(A)+ mRNA was purified from total RNA of developing CANP3 seeds at 12 and 20 DAP using HybondTm-mAP-messenger affinity paper according to the supplier's instructions (Amersham). Double-stranded cDNA was synthesized from 5+ mRNA using a cDNA synthesis kit (Riboclone cDNA synthesis system, Promega). The 3' end labelling of the double stranded-cDNA using Klenow polymerase showed a size for the synthetized cDNA ranging from 0.2 to 3 kb. EcoRI adaptors were added to the cDNA for ligation into the λgt10/EcoRI arms. Vectors were packaged *in vitro* using commercial extracts (Promega). Recombinant bacteriophages were selected using *E.coli* K12 C600 Hfl strain.

For differential screening of the libraries, Mo-MLV reverse transcriptase (Bethesda Research Laboratories), oligodT primers and 70 µCi of (α-32P) dCTP were used to synthesize radiolabelled first strand cDNA using 3µg poly $(A)^+$µRNA. The libraries were sequentially screened with radiolabelled first strand cDNA from 12 and 20 DAP embryos, dry seeds, and leaves. Hybridization was carried out as described above, except that oligodA (12) "0.5mg/ml" was added to the prehybridization solution. Phage plaques that showed specific hybridization with 12 or 20 DAP embryos probes were isolated.

RESULTS

Isolation and analysis of cDNA expressed during the mid-maturation stage of the seed

We constructed cDNA libraries in λgt10 using poly(A)+mRNA from 12 and 20 day-old embryos. About 10 000 Pfu from each library were screened by differential hybridization using as negative probes poly(A)$^+$mRNA from dry seeds and leaves. We selected 20 clones that hybridized specifically to 12 or 20 day-old embryos probes. Inserts were amplified from selected clones by PCR using λgt10 complementary primers flanking the *Eco*RI site with the following conditions: 92°C for 2 min then 25 cycles (92°C 1 min, 50°C 1 min, 72°C 1 min 30 sec) and finishing 72°C for 3 min. Subjected to southern-blot analysis, amplified cDNA showed size from 0.4 to 1.4 kbp. By cross-hybridation analysis, inserts were grouped into 5 cDNA families, each family consists of 1 to 5 cDNA carrying related sequences. The longest cDNA representative of each cDNA family, designated A8.1, A7.2, A2, C8.1 and C3, were used for sequence comparison homology to possibly identify their respective encoded proteins.

-- In order to identify cDNA encoding for helianthinin precursors, we performed hybridization of a southern blot carrying the various isolated cDNAs with the helianthinin pHa2 cDNA clone (Allen *et al.*, 1985). The cDNA A8.1 and two other related cDNAs were revealed.

-- The complete nucleotide sequence of the A7.2 cDNA was determined (Fig. 1). The cDNA is 0,6 kb in length and shows an open reading frame of 424 nucleotides. The encoded polypeptide exhibits 141 amino acids corresponding to a molecular weight of 15,993 kDa. The 3' untranslated region contains two putative polyadenylation signals. The deduced amino acid sequence shows 91.5 % homology with the SFA8 albumin Met-rich amino acid sequence already isolated from sunflower seeds (Kortt *et al.*, 1991). The SFA8 and A7.2 albumin precursors are similar in length and show 12 amino acid substitutions, most of them are conservative (Fig. 2).

-- The complete nucleotide sequence of the C3 cDNA was determined (Fig. 3). The insert has a length of 353 nucleotides which shows significant nucleotide sequence homology with cDNA sequences encoding probable proteinase inhibitors from many species (Stiekman *et al.*, 1988; Choi *et al.*, 1993). We could not identify any methionine initiating codon in the C3 nucleotide sequence suggesting that the insert is lacking the 5' end nucleotides. The search in the three probable ORF of putative coding regions enable us to identify a partial aminoacid sequence of 63 residues. A putative polyadenylation signal is located at the position 301, 39 nucleotides upstream the poly(A) tail. The deduced amino acid sequence shows 57 %, 51.5 %, and 47 % homology sequence with probable proteinase inhibitors from *Glycine max*, *Arabidopsis thaliana* and *Solanum tuberosum* (Fig. 4). The sequence homology is the lowest at the peptide 5' region which is strongly hydrophobic and could corresponds to a signal peptide for intramembrane transport.

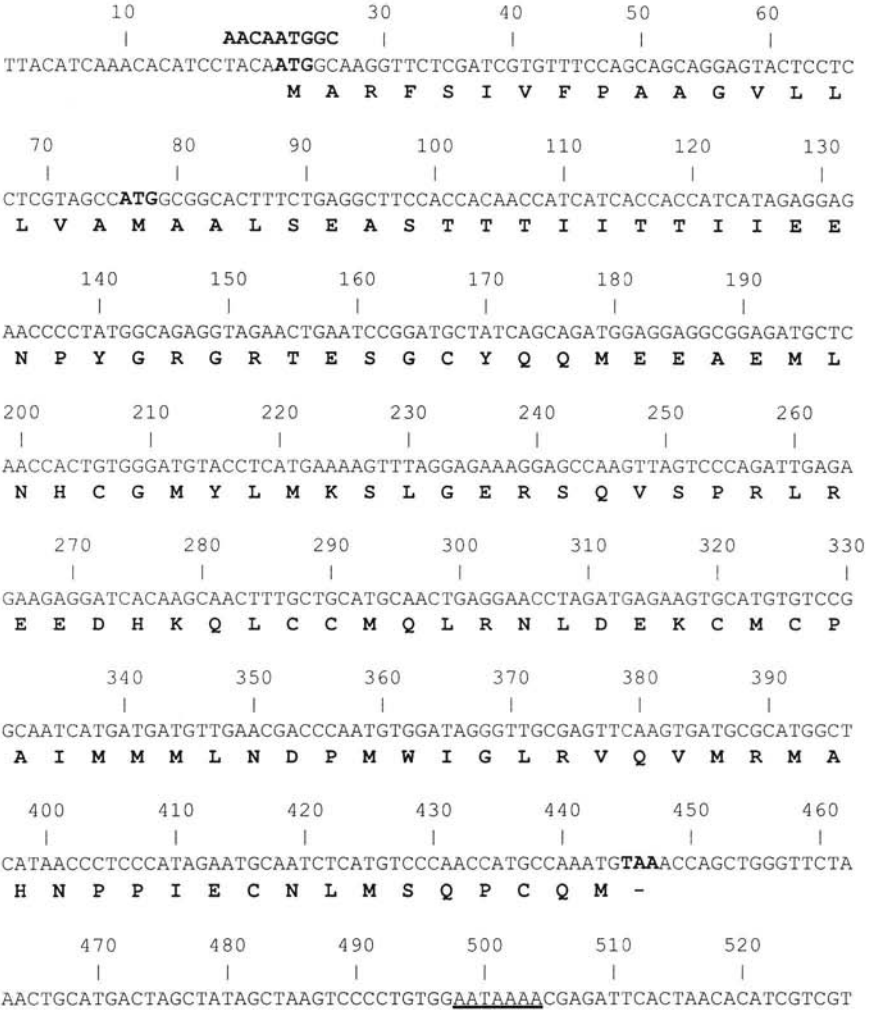

Figure 1: Nucleotide sequence of the cDNA A7.2 and deduced amino acid sequence The putative methionine initiation and stop codons are indicated in bold. The putative polyadenylation signals are underlined.

The cleavage site of the putative signal peptide could be predicted according to Von Heijne (1986) between the residues Gly-16 and Arg-17 leading to a 47 aminoacid mature protein. All the Cys residues of the mature protein are found in similar positions in the three other peptides compared except for *Arabidopsis thaliana* peptide which is lacking the last Cys residue. This suggests the establishement of similar types of disulphide bridges in all the polypeptides.

-- The two cDNAs C8.1 and A2 partial sequences (not shown) did not show significant nucleotide sequence homology with any known genes.

Tissue-specific and temporal mRNA expression analysis

The A8.1, A7, A2, C8.1 and C3 cDNA were labelled *in vitro* and separately hybridized to northern-blots containing total RNA from adult leaves, mature capitulum without seeds, the cotyledone leaves, hypocotyl and root expanding tissues from 10-day-old plants, mature seeds, germinated seeds for 36 h, and immature embryos at 6, 12, 16, 20 and 28 DAP. The autoradiograms (Fig. 5) show that each cDNA reacted with a single mRNA. The transcripts are 1.8, 1.4, 1.1, 0.6 and 0.4 kb long for the cDNA A8.1, C8.1, A2, A7 and C3, respectively. They are accumulated in the seeds between 12 and 20 DAP. The transcripts corresponding to the C8.1, A2 and C3 cDNAs disappeared at 28 DAP whereas those corresponding to the A8.1 and A7 cDNAs were still present but highly reduced in intensity. Furthermore all these transcripts were absent at 6 DAP, in mature seeds and in germinating seeds as well as in other tissues and organs, except for A7 homologous transcript also expressed in the capitulum at the maturity stage.

Variation in intensity of mRNA accumulation along seed growth

For quantitation and comparison of hybridized mRNA signals, all hybridizations were internally standardized by expressing the signal intensity for each mRNA obtained at any seed developmental stage in proportion to the signal obtained with the 18S rRNA gene (Table 1). We could in that way display the accumulation kinetics of each mRNA during seed maturation.

The mRNA accumulation revealed with the C3 cDNA showed a peak at 12 DAP, then the level was reduced by 37 % between 12 and 16 DAP and did not vary significantly between 16 and 20 DAP. The mRNA accumulation revealed with the A8.1 cDNA did not vary significantly between 12 and 20 DAP, but it was reduced by 80 % between 20 and 28 DAP. The mRNA accumulation revealed with the A7 cDNA was late. It increased by 43 % between 12 and 20 DAP. It was drastically reduced, by 93 % between 20 and 28 DAP. The levels of mRNA accumulation revealed with the A2 or C8.1 cDNA at 12 DAP were, respectively, only 43 % and 47 % of the maximal mRNA accumulations which were displayed from 16 to 20 DAP.

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                                   ↓                ↓
A7.2  - MARFSIVFPAAGVLLLVAMAALSEASTTTIITTIIEENPYGRGRTESGCY -50
SFA8  -         A           PV                              -50
A7.2  - QQMEEAEMLNHCGMYLMKSLGERSQVSPRLREEDHKQLCCMQLRNLDEKC -100
SFA8  -                     N          M              K    -100
A7.2  - MCPAIMMMLNDPMWIGLRVQVMRMAHNPPIECNLMSQPCQM -141
SFA8  -            E    RM D   S    L               -141
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Figure 2: Alignment of the amino acid sequence of the cDNA A7.2 with one of the SFA8 clone. The arrows show the putative proteolytic cleavage sites. Identical amino acids are indicated by a blank whereas amino acid substitutions are indicated by the amino acid abbreviations.

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        10         20         30         40         50         60
         |          |          |          |          |          |
CTTTCTCATCCTTCTCCTCATCTTGGTTCCATTTAAAGAGGCGGAGGGGAGGAACTGTGAG
  F  L  I  L  L  L  I  L  V  P  F  K  E  A  E  G  R  N  C  E
        70         80         90        100        110        120
         |          |          |          |          |          |
TCACAGAGCCATAAGTTCGAAGGCAGGTGCATGAGCAACCATAACTGCGGTCTCGTTTGC
 S  Q  S  H  K  F  E  G  R  C  M  S  N  H  N  C  G  L  V  C
       130        140        150        160        170        180
         |          |          |          |          |          |
AGAAACGAAGGCTTTACCAGTGGCGTTTGTCGTGGTGCTCGTGGACGATGCTTTTGCACC
 R  N  E  G  F  T  S  G  V  C  R  G  A  R  G  R  C  F  C  T
       190        200        210        220        230        240
         |          |          |          |          |          |
AAAACTTGTTAATTACTTCTTACTTCACTTCACTTCACTTCAATCTAATAACTAGTGGAG
 K  T  C  -
       250        260        270        280        290        300
         |          |          |          |          |          |
TGGAGTGGATCCACGCTCGATCGCTGGATGTTATGTTATCACTAGTTAGTTGTTTGTACA
       310        320        330        340        350
         |          |          |          |          |
ATAAATTTGATTGATTCCTATGGACTTGTCTTTCTTTATATTTAAAAAAAAAA
```

Figure 3: Nucleotide sequence of the cDNA C3 and deduced amino acid sequence. The putative stop codon is indicated in bold. The putative polyadenylation signal is underlined.

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                                              ↓
H.annuus                FLILLLILVPFKE-----AEGRNCESQSHKFEGRCMSNHNC
G.max         MEMRKSCGFF  L   VFASQVVVQT---   V      G H L NRD
A.thaliana    MKLSMRLISAV  MFM F ATGMGPVTV- A T      R K T V AS
S.tuberosum   M----RFFAT FL AMLV AT MGPMRI  A H   L   R K P TRDS

H.annuus      GLVCRNEGFTSGVCRGARGRCFCTKTC
G.max         A        SG R KRS -     RI
A.thaliana    AN  H    VG N   F R     R--
S.tuberosum   AS  ET R SG N H F R       P
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Figure 4: Alignment of the sunflower amino acid sequence of the cDNA C3 with those of the probable proteinase inhibitors from three other species. The arrow shows the putative proteolytic cleavage sites. Identical amino acids are indicated by a blank whearas amino acid substitutions are indicated by the abridged amino acid. (-) are used to obtain the best alignement between the compared sequences

Table 1: Influence of developmental stage in the mRNA accumulation. Hybridization signal intensities in CANP3 line obtained with the 18S rRNA gene and the A8.1, A7, C3, C8.1 or A2 cDNA probes at different developmental seed stages. Intensities are in arbitrary units

Probe	Seeds at different developmental stages					
	6 DAP	12 DAP	16 DAP	20 DAP	28 DAP	dry seeds
18S	1.25	1.82	1.79	1.57	1.30	1.59
A7	0	1.69	2.38	2.31	0.11	0
A7/18S	0	0.93	1.33	1.47	0.08	0
18S	1.25	1.99	2.08	1.99	1.21	2.19
A8.1	0	2.11	2.54	2.31	0.28	0
A8.1/18S	0	1.06	1.22	1.16	0.23	0
18S	1.06	2.41	2.03	1.58	1.50	1.60
C3	0	2.11	1.12	0.87	0	0
C3/18S	0	0.87	0.55	0.55	0	0
18S	1.06	2.41	2.03	1.58	1.50	1.60
C8.1	0	0.89	1.58	1.18	0	0
C8.1/18S	0	0.37	0.78	0.75	0	0
18S	0.69	1.52	1.06	1.14	0.60	0.82
A2	0	0.72	1.16	1.23	0	0
A2/18S	0	0.47	1.09	1.08	0	0

Comparison of the mRNA accumulation levels between CANP3 and HOC lines

Northern blot signals of each of the 5 cDNA used separately as probes were compared between CANP3 and HOC at 12, 16 and 20 DAP using standardized signals (Fig.5, Table 2). The A7 and C8.1 homologous mRNA kinetics and accumulation levels were similar in the two lines. The A8.1 and A2 homologous mRNA accumulations were significantly reduced in HOC at 20 DAP. The levels of mRNA accumulation were estimated in HOC at only 63% and 50% of those observed for CANP3, respectively, for A8.1 and A2 cDNAs for the sum of the three stages. Conversely, the C3 homologous mRNA accumulation was reduced in CANP3 in comparison with HOC, by 47 % and 36 %, respectively, at 16 and 20 DAP.

DISCUSSION

The cDNA libraries were constructed from mRNA isolated at 12 or 20 DAP. At these sunflower seed developmental stages, there occurs a massive synthesis and accumulation of oil and storage proteins (Roberston *et al.*, 1978; This *et al.*, 1988). Two of the studied cDNAs, A8.1 and A7.2, encode helianthinin and albumin Met-rich precursors, respectively. The A7.2 amino acid deduced sequence shows 91.5%

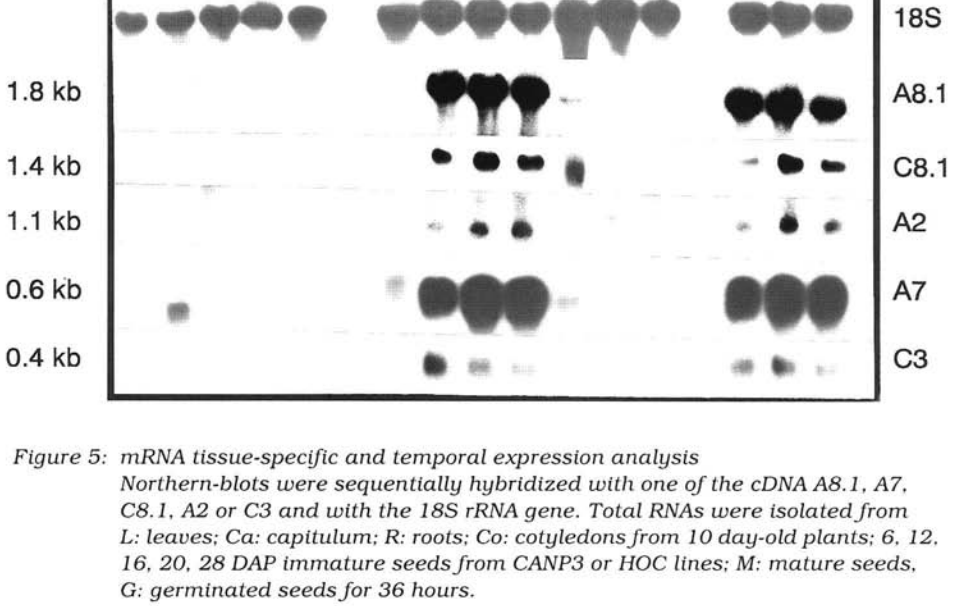

Figure 5: mRNA tissue-specific and temporal expression analysis
Northern-blots were sequentially hybridized with one of the cDNA A8.1, A7, C8.1, A2 or C3 and with the 18S rRNA gene. Total RNAs were isolated from L: leaves; Ca: capitulum; R: roots; Co: cotyledons from 10 day-old plants; 6, 12, 16, 20, 28 DAP immature seeds from CANP3 or HOC lines; M: mature seeds, G: germinated seeds for 36 hours.

homology with the previously described SFA8 albumin precursor (Kortt *et al.*, 1991). The divergence between SFA8 and A7.2 amino acid deduced sequences indicates that both are part of a gene family with a minimum of two members. This is consistent with other observations on seed storage albumins that seem to be encoded by small multigenic families (Crouch *et al.*, 1983; Ericson *et al.*, 1986). A third cDNA, C3, encodes an undescribed probable proteinase inhibitor. As yet, the physiological function of the proteinase inhibitors is not clear. They appear to be involved in plant defence mechanisms against insects (Green and Ryan 1972; Ryan, 1973). Furthermore, they accumulate specifically in storage organs, tubers and seeds, of many species and are supposed to have some additional function as sulphur-rich storage proteins (Odani *et al.*, 1983). Two other cDNAs, with unidentified functions, were also cloned.

Each cDNA, representing the five families, hybridized one mRNA on northern-blots. All these mRNAs were specifically expressed in the immature seeds between 12 and 20 DAP whereas they were absent in all the vegetative tissues. 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 repressed during most of the plant

Table 2: Comparison of the mRNA accumulation levels between a normal line CANP3 and a high oil and oleic line HOC. Hybridization signal intensities obtained with the 18S rRNA gene and the A8.1, A7, C3, C8.1 or A2 cDNA probes. Plants were maintained at 26°C day/18°C night. Intensities are in arbitrary units

Probe	CANP3			HOC		
	12 DAP	16 DAP	20 DAP	12 DAP	16 DAP	20 DAP
18S	1.82	1.79	1.57	1.69	1.59	1.52
A7	1.69	2.38	2.31	1.54	2.18	2.17
A7/18S	0.93	1.33	1.47	0.91	1.37	1.43
18S	1.99	2.08	1.99	2.19	2.05	1.91
A8.1	2.11	2.54	2.31	2.15	2.09	1.40
A8.1/18S	1.06	1.22	1.16	0.98	1.02	0.73
18S	2.41	2.03	1.58	1.66	1.71	1.17
C3	2.11	1.12	0.87	1.41	1.80	1.01
C3/18S	0.87	0.55	0.55	0.85	1.05	0.86
18S	2.41	2.03	1.58	1.66	1.71	1.17
C8.1	0.89	1.58	1.18	0.46	1.44	0.80
C8.1/18S	0.37	0.78	0.75	0.28	0.84	0.68
18S	1.52	1.06	1.14	1.35	1.45	1.56
A2	0.72	1.16	1.23	0.47	1.60	0.84
A2/18S	0.47	1.09	1.08	0.35	1.10	0.54

life. All the cDNAs exhibit similar expression time frame during seed maturation since they were all not expressed in immature seeds at 6 DAP, they all appeared between 6 and 12 DAP and were absent in the mature quiescent seeds.

In contrast to the other transcripts, those corresponding to the helianthinin and the Met-rich albumin were still present at 28 DAP. The persistence of the seed storage protein mRNAs in the late seed maturation stage and in some cases in mature seeds has been reported for many species (Mori *et al.*, 1978; Larohe-Raynal and Delseny, 1986). However, it has been suggested that the production of storage protein mRNA is restricted to the phase of protein accumulation and that the subsequent mRNA levels are due to a relative long-life of these mRNAs (Gatehouse *et al.*, 1982). In soybean, the half-lives of legumine mRNAs was estimated at approximatively 4-6 days (Goldberg *et al.*, 1981). On the other hand, the expression of storage protein genes is usually undetected at 6 DAP, although for pea, some storage protein precursors have been detected immunologically (Domoney *et al.*, 1980). The northern-blot hybridization analysis may not be sensitive enough to detect storage protein mRNA at this stage.

The mRNA accumulation kinetics analysis over the seed development revealed that each mRNA accumulation pattern was specific. We suggest that the modula-

tions reflect the order of the encoded protein function or deposit. The unidentified proteins encoded by the cDNAs C8.1 and A2 may have a function related to either minor storage protein or to other storage compounds accumulations. In the case of the helianthinin and the albumin Met-rich mRNAs, we observed that the accumulation kinetics were not synchronous. We also noted that the A7 albumin Met-rich mRNA accumulation occurred later than the HaG5 homologous albumin mRNA wich was detected in immature seeds as early as 5 DAP and accumulated at the maximum level at 12 DAP (Allen *et al.*, 1987). However, two different albumin fractions have been identified in sunflower: (1) the major 18-14 kDa fraction is similar to the albumin of other plant species, it includes the HaG5 deduced polypeptide and accumulates earlier than the helianthinines (Youle et Huang, 1981; Allen *et al.*, 1987). (2) The 10 kDa Met-rich fraction has been found only in sunflower and Brazil nut (Kortt *et al.* 1990; Altenbach *et al.*, 1987) and it includes the A7 deduced polypeptide and have been shown to be accumulated between 18 and 20 DAF, later than the 18-14 kDa albumin fraction and the helianthinins (This *et al.*, 1988). This concordance between the mRNA accumulation kinetics and the deposit of their corresponding protein precursors is in agreement with a primary control of storage protein gene expression at the level of mRNA transcription.

Relationships between oil content and storage protein mRNA accumulation

We observed that the A8.1 helianthinin mRNA accumulation levels were significantly lower in HOC in comparison with CANP3, suggesting that the HOC seed contains less helianthinin than the CANP3 seed. The studied lines exhibit variable oil content, 45% and 36%, respectively, for HOC and CANP3 (unpublished data). It has been reported by Borodulina and Suprinova (Borodulina and Suprinova, 1976) that the increase of sunflower seed oil content induces an increase of albumin and a relative decrease of helianthinin seed contents. Furthermore a positive correlation has been established between the oil and the lysine seed contents (Borodulina and Suprinova, 1976). We suggest that the high mol weight albumin fraction which is also the most Lys-rich is the only one that increases with seed oil content increase. The albumin low mol weight Met-rich could be stable whatever the oil content. We observed in the high oil variety HOC a lower level of the helianthinin mRNA in comparison with the CANP3 low oil variety. However, for the albumin Met-rich mRNA, the accumulation levels were similar in both lines.

REFERENCES

Allen, R.D., Nessler, C.L., Thomas, T.L., 1985. Developmental expression of sunflower 11S storage protein genes. Plant Mol. Biol., 5: 165-173.

Allen, R.D., Cohen, E.A., Von der Haar, R.A., Orth, K.A., Ma, D.P., Nessler, C.L. and Thomas, T.L., 1987a. Expression of embryo specific genes in sunflower. In: Davidson EH, Firtel R (eds) UCLA Symposium on molecular approaches to developmental biology. Alan R. Liss, New York, p415.

Allen, R.D., Cohen, E.A., Von der Haar, R.A., Adams, C.A., Ma, D.P., Nessler, C.L., and Thomas, T.L., 1987b. Sequence and expression of a gene encoding an albumin storage protein in sunflower. Mol. Gen. Genet., 210: 211-218.

Altenbach, B.S., Pearson, K. W., Leung, F. W. and Sun, S. M., 1987. Cloning and sequence analysis of a cDNA encoding a Brazil nut protein exceptionally rich in methionine. Plant Mol. Biol., 8: 239-250.

Boroludina, A.A. and Suprunova, L.V., 1976. Genetic and ecological factors effecting a qualitative change of the sunflower seed protein complexe content. In Proc. 7th Int. Sunflower Conf. p428-432.

Choi, Y., Choi, Y.D. and Lee, J.S., 1993. Nucleotide sequence of a cDNA encoding a low molecular weight sulfur-rich protein in soybean seeds. Plant Physiol., 101: 699-700.

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

Crouch, M.L., Tenbarge, K.M., Simon, A.E. and Ferle, R., 1983. cDNA clones for *Brassica napus* seed storage proteins: evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. J. Mol. & Appl. Genetics, 2: 273-283.

Dalgalarrondo, M., Raymond, J. and Azanza, J.L., 1984. Sunflower seed proteins: characterization and subunit composition of the globulin fraction. J. Exp. Bot., 35: 1618-1628.

Domoney, C., Davies, D.R. and Casey, R., 1980. The initiation of legumin synthesis in immature embryos of *Pisum sativum* L. grown in vivo and *in vitro*. Planta, 149: 454-460.

Ericson, M.L., Rödin, J., Lenman, M., Glimelius, K., Josefsson, L.G. and Rask, L., 1986. Structure of the rapesseed 1,7 storage protein, napin, and its precursor. J. Biol. Chem., 261: 14 516-14 581.

Gatehouse, J.A., Evans, I.M., Brown, D., Croy, R.R.D. and Boulter, D., 1982. Control of storage protein synthesis during seed development in pea (*Pisum sativum* L). Biochem. J., 208: 119-127.

Goldberg, R.B., Hoschek, G., Ditta, G.S. and Breidenbach R.W., 1981. Developmental regulation of cloned superabundant embryo mRNAs in soybean. Dev. Biol. 83: 218-231.

Green, T.R. and Ryan, C.A., 1972. Wound inducible proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science, 175: 776-777.

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

Kortt, A.A. and Caldwell, J.B., 1990. Low molecular weight albumins from sunflower seed: Identification of a methionine-rich albumin. Phytochemistry, 29: 2805-2810.

Kortt, A.A., Caldwell, J.B., Lilley, G.G. and Higgins, T.J.V., 1991. Amino acid and complementary DNA sequences of a methionine-rich 2S protein of sunflower (*Helianthus annuus* L.). Eur. J. Biochem., 195: 329-334.

Laroche-Raynal, M. and Delseny, M., 1986 Identification and characterization of the mRNA for major storage proteins from radish. Eur. J. Biochem. 157: 321-327.

Mori, T., Wakabayashi, Y. and Takagi, S., 1978. Occurence of mRNA for storage protein in dry soybean seeds. J. Biochem., 84: 1103-1111.

Mossé, J. and Baudet, J., 1972. Biochemical aspects of quality and content of lysine in sunflower seed proteins. In: Proc. 5th Int. Sunflower Conf., Clermont-Ferrand, p 437-440.

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

Odani, S., Koide, T., Ono, T. and Ohnishi, K., 1983. Structural relationship between barley (*Hordeum vulgare*) trypsin inhibitor and castor bean (*Ricinus communis*) storage protein. Biochem. J., 213: 543-545.

Pernollet, J-C., 1985. Biosynthesis and accumulation of storage proteins in seeds. Physiol. Veg., 23: 45-59.

Robertson, J.A., Chapman, G.W. and Wilson, R.L., 1978. Relation of days after flowering to chemical composition and physiological maturity of sunflower seed. JOACS N° 2 p126 .

Ryan C.A., 1973. Proteolytic enzymes and their inhibitors in plants. Annu. Rev. Plant Physiol., 24: 173-196.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. Molecular cloning. A laboratory manual. Second edition. Cold Spring Harbor Laboratory Press.

Sharief, F.S. and Li S.S.L., 1982. Aminoacid sequence of small and large subunits of seed protein from *Ricinus communis*. J. Biol. Chem., 257: 14753-14759.

Slocombe, S.P., Cummins, I., Jarvis, R.P. and 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.

Sosulski, F.W. and Kabal, A., 1969. Isolated proteins from rapeseed, flax and sunflower meals. Can. Inst. Food Technol. J., 2: 28-32.

Stiekema, W.J., Heidekamp, F., Dirkse, W.G., Van Beckum, J., De Haan, P., Ten Bosch, C. and Louwerse, J.D., 1988. Molecular cloning and analysis of four potato tuber mRNAs. Plant Mol. Biol., 11: 255-269.

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

This, P., Goffner, D., Raynal, M., Chartier, Y., and Delseny, M., 1988. Characterization of major storage proteins of sunflower and their accumulation. Plant Physiol. Biochem., 26 (2): 125-132.

Von Heijne G., 1986: A new method for predicting signal sequence cleavage sites. Nuc. Acid Res., 14: 4683-4690.

Wilkins, T.A. and Raikhel, N.V., 1989. Expression of rice lectin is governed by two temporally and spacially regulated mRNA in developing embryos. The Plant Cell 1: 541-549.

Youle, R.J. and Huang, A.H.C., 1981. Occurence of low molecular weight and high cystein containing albumin storage proteins in oil seeds of diverse species. Am. J. Bot., 68: 44-48.

ANÁLISIS DE LA TRANSCRIPCIÓN DE mRNA Y CLONAJE DE CINCO FAMILIAS cDNA REGULADAS CON EL DESARROLLO EN EMBRIONES INMADUROS DE GIRASOL

RESUMEN

Para aislar y analizar la expresión de genes reguladores del desarrollo en girasol, bibliotecas de ADNc fueron construidas utilizando ARN mensajero (ARNm) de semillas de 12 a 20 días y entonces hibridadas diferencialmente con ARNm de semillas secas y hojas. Fueron aisladas ADNc codificando proteínas de reserve mayores (heliantina y albumina Met-rich) inhibidor de la proteína y otras dos ADNc de función desconocida.

Los niveles estables de los transcritos, detectados con cada ADNc, fueron determinados en semillas inmaduras y tejidos vegetativos en estados diferentes. La acumulación de los cinco ARNm fueron específicos con los tejidos, limitados al estado de desarrollo de semilla de media maduración. La acumulación de albúmina Met-rich en ARN ocurrió relativamente más tarde que la acumulación de ambos el ARNm heliantina y la previamente estudiada en girasol ARNm HaG5 albúmina. Esto indica que la síntesis de ARNm de las dos clases de proteínas de reserva y de diferentes precursores dentro de una clase de proteína no está sincronizada. Estas observaciones estuvieron correlacionadas con el depósito de los distintos precursores de proteínas de reserva y están de acuerdo con un control primario de la expresión génica de la proteína de reserva al nivel de la transcripción de ARNm. La acumulación del transcripto de heliantina fué más alta en la variedad normal CANP3 en comparación con línea Hoc "alto aceite y oleico". Los niveles de ARNm albúmina Met-rich estuvieron en contraste similar en ambas líneas. Esto podría estar en relación con la redistribución de proteína que es conocido de acompañar el incremento de aceite de la semilla en girasol y está de acuerdo con el hecho que las variedades de bajo aceite tienen más contenido de heliantina que las variedades alto aceite. Nosotros sugerimos que la fracción Met-rich de albúmina no está implicada en la redistribución de esta proteína.

CLONAGE ET ÉTUDE DE LA TRANSCRIPTION DE L'ARNm DE 5 FAMILLES DE cDNA RÉULÉS PAR LE STADE DE DÉVELOPPEMENT, CHEZ LES EMBRYONS IMMATURES DE TOURNESOL

RÉSUMÉ

Pour isoler et analyser l'expression de gènes régulés par le développement chez le tournesol, des banques d'ADN complémentaires ont été construites en utilisant des ARNm, préparés de graines immatures de 12 et 20 jours, et ensuite différentiellement hybridées avec des ARNm de graines mures et de feuilles. Des ADNc correspondants aux protéines de réserve majeures (hélianthinine et albumine riche ne méthionine), un inhibiteur de protéase et deux autres ADNc à fonction non déterminée ont été isolés.

L'accumulation des transcrits détectés avec chaque ADNc a été déterminée dans les graines immatures et dans les tissus végétatifs à différents stades. L'accumulation des cinq ADNc est tissu spécifique, limitée au stade de maturation moyen du développement de la graine. L'accumulation de l'ARNm de l'albumine riche en méthionine se produit relativement plus tard que pour celui de l'Hélianthinine et de l'albumine HaG5. Ceci indique que la synthèse des ARNm des deux classes de protéines de réserve et des précurseurs d'une classe, n'est pas synchrone. L'accumulation se produit dans l'ordre de dépôt des protéines de réserve et suggère donc un contrôle primaire de l'expression du gène au niveau de la transcription. L'accumulation du transcrit de l'hélianthinine est plus élevée dans CANP3 que dans la variété HOC à haute teneur en huile et à haute teneur en acide oléique. Les niveaux du transcrit de l'albumine riche en méthionine sont semblables dans les deux lignées. Ceci pourrait être en relation avec la redistribution des protéines qui est connue pour accompagner l'augmentation de la teneur en huile chez le tournesol. C'est en accord avec le fait que les variétés à faible teneur en huile contiennent plus d'hélianthinine que les variétés riches en huile qui en ont peu. On peut alors penser que cette redistribution n'affecte pas la fraction d'albumine riche ne méthionine.