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Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs

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Abstract

We have cloned and sequenced three different cDNAs from sunflower seed-stored mRNA. Sequence similarities and response to heat-shock identified one of the cDNAs as a low-molecular-weight heat-shock protein (lmw-HSP). The other two clones showed significant sequence similarity to the cotton and carrot late-embryogenesis-abundant (Lea) proteins D-113 and Emb-1, respectively. The three cDNAs showed similar expression patterns during zygotic embryo development, as well as in vegetative tissues of 3-day-old seedlings in response to stress. Maximal accumulation of all three mRNAs was detected in dry seeds and during embryo mid-maturation stage, in the absence of exogenous stress. In seedlings, mRNAs accumulated to lower levels in response to osmotic stress and exogenous abscisic acid (ABA) treatments. A differential time course of response to osmotic stress was observed: lmw-HSP mRNA accumulation was induced earlier than that of Lea mRNAs. The coordinate accumulation of Lea and lmw-HSP transcripts during embryo development and in response to stress and ABA suggests the existence of common regulatory elements for Lea and lmw-HSP genes, and supports the notion that HSPs might have alternative functions in the plant cell.

Introduction

During seed development, plant embryos dry out and adapt to a changing osmotic environment without losing viability. Dry seeds remain viable even after long periods of storage indicating that damage induced in embryos by desiccation is either prevented during development, or (and) is readily repaired upon germination. To investigate the basis of desiccation tolerance at the molecular level, a lot of attention is being paid to genes encoding transcripts accumulating in developing embryos. These include a family of conserved genes expressed abundantly during late embryogenesis, defined as Lea-class genes. Their transcripts accumulate in dry seeds and disappear shortly upon germination [1]. In some cases, Lea mRNA accumulation is also induced in vegeta-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X59699 (Ha ds10), X59700 (Ha ds11) and X59701 (Ha hsp17.6).

tive tissues in response to ABA and osmotic stress (reviewed in [2]). Recently, Lea mRNAs have been reported to be induced by drastic desiccation in leaves of poikilohydric ('resurrection') plants [3]. Based on their peculiar amino acid composition and predicted amphiphilic α-helical secondary structure, roles in cellular osmoprotection have been suggested for several Lea proteins [1, 4]. Other transcripts that accumulate to high levels in dry seed include those of genes involved in the biosynthesis of osmolites [5] and others with unknown functions [5, 6]. The roles played by seed-stored transcripts are probably not essential for germination, although perhaps the encoded proteins are necessary for ensuring the survival of the mature embryo under desiccation.

Sunflower is an excellent system to study gene expression during embryo development. Gram quantities of large, stage-specific embryos are easily obtained from a few plants. To identify genes expressed in late embryogenesis, we started by isolating and characterizing cDNA clones from mRNA stored in dry seed. We report here the nucleotide sequence of three different clones and their mRNA expression patterns during zygotic embryo development and in response to ABA, osmotic and heat-shock stress. The proteins deduced for two clones show large similarities with Lea proteins of other plant species. A third clone, Ha hsp17.6, was identified tentatively as a lmw-HSP. The observation of similar Lea and lmw-HSP mRNA expression patterns indicates that both gene families respond to common regulatory signals during development and in response to stress. This observation also suggests specific functional roles for lmw-HSPs during plant zygotic embryo development and in recovery or protection from various stresses.

Materials and methods

Plant materials and stress treatments

Mature dry seeds, collected from field-grown sunflower (*Helianthus annuus* L. cv. Sunweed), were provided by Rhône-Poulenc Agrochimie (Sevilla) are stored at room temperature. For embryo analysis, plants were grown under controlled environment conditions: 16-hour day cycles; day temperature 25 °C; night temperature 18 °C; and 80% relative humidity. Embryos were dissected from achenes at the indicated times, frozen immediately in liquid nitrogen, and stored at −80 °C. Seeds were germinated aseptically in the dark at 21 °C. Seeds were laid in Petri dishes, between two pieces of filter paper, imbibed with sterile distilled water. Treatments with mannitol (0.3 M) or ABA (0.1 mM) were initiated, 3 days after imbibition, by transferring germinated seedlings to dishes with filters soaked with ABA or mannitol aqueous solutions. For heat-shock treatments, Petri dishes with germinated seedlings were transferred, for 2 h and 30 min, to incubators at 37 °C or 42 °C. Immediately after stress treatments, whole seedlings were frozen in liquid nitrogen and stored at −80 °C.

Isolation of RNA and poly(A)$^+$ mRNA; northern blot hybridizations

Total RNA was purified from frozen dry seed, embryos and seedling tissues using the method described by Williamson *et al.* [7]. Polyadenylated mRNA was isolated from total RNA by two rounds of oligo dT-cellulose (Pharmacia, Type 7) chromatography, using standard procedures [8]. Samples were quantified by UV-light absorption at 260 and 280 nm. 20 μg denatured total RNA (or 0.5 μg poly(A)$^+$ mRNA) were loaded on 1% agarose-formaldehyde gels. After electrophoresis, formaldehyde was removed from the gel by three washings with distilled water at 65 °C, and RNA transferred [8] overnight to Hybond-N filters. Prehybridizations (4 h) and hybridizations (16 h) were carried out, at 44 °C, in 50% formamide, 5× SSPE, 5× Denhardt's solution, and 0.1% SDS. Probes (labelled to specific activities of 1.5×10^9 cpm/μg) were added to a final concentration of 1.6×10^6 cpm/ml. Filters were washed at 44 °C, first with 150 ml 6× SSPE, 0.1 SDS (15 min); followed by 1× SSPE, 0.1% SDS

(150 ml twice, 15 min). Finally, filters were washed in 1 × SSPE, 0.1% SDS, for 15 min at 65 °C. Wet filters were exposed at −80 °C for autoradiography, using intensifying screens (Dupont, Hi-Plus). For sequential hybridization with the different probes, filters were re-used following the procedures recommended in Amersham's 'Membrane transfer and detection methods' user manual.

Construction and differential screening of a dry-seed cDNA library

Poly(A)$^+$ mRNA (5 μg) purified from dry seed was used as a template for double-stranded cDNA synthesis, performed with a kit from Pharmacia. After addition of *Eco* RI-*Not* I linkers, the cDNA was fractionated by Sephacryl S-300 spun-column chromatography, ligated to λgt11/*Eco* RI arms and packaged *in vitro*, using commercial extracts (Boehringer). The library was plated on Y1088 *Escherichia coli* cells and analysed by differential screening with ^{32}P-labelled single-stranded cDNAs, synthesized from 0.5 μg poly(A)$^+$ RNA, isolated from dry seeds and 3-day-old seedlings. Phage plaques hybridizing preferentially to the dry seed probe were purified [8].

DNA sequencing and computer analysis

The cDNA inserts in recombinant phages were excised with *Eco* RI or *Not* I and subcloned in pBluescript SK(+). Overlapping exonuclease III deletions were constructed using the Pharmacia (LKB) double-stranded nested deletion kit. The complete cDNA nucleotide sequences were determined on both strands of DNA, using single- or double-stranded [9] templates and the Sequenase version 2.0 kit (USB Biochemicals). Sequence analysis was performed on a VAX computer using the UWGCG software package [10]. Amino acid sequence comparisons were carried out with the programs FASTA and BESTFIT.

Recombinant DNA techniques

Radioactive probes were prepared from purified restriction fragments by the random hexamer labelling method, using α-^{32}P-dCTP (Amersham, 3000 Ci/mmol) and Boehringer's random-primed DNA labelling kit. The DNA fragments used as probes include the whole isolated sunflower cDNAs (see Results for details); a 900 bp *Xho* I/*Sal* I fragment from the coding region of sunflower helianthinin HaG3-D1 gene [11]; the G *Eco* RI fragment from a radish 18S rRNA gene [12]; and the complete kα1 human α-tubulin cDNA [13]. Isolation of plasmid and phage DNA, preparation of DNA fragments, ligations, phage growth and other general molecular cloning techniques were essentially as described by Maniatis *et al.* [8].

Results

Isolation of sunflower cDNA clones for dry-seed-stored mRNAs

We constructed a cDNA library in λgt11, using poly(A)$^+$ RNA purified from sunflower dry seed. About 10000 pfu were screened by differential hybridization (see Materials and methods). We selected three independent clones that hybridized preferentially to a dry-seed cDNA probe: Ha ds10, Ha ds11, and Ha hsp17.6 (Table 1). On the basis of its sequence (Figs. 1 and 2) and heat-shock response (Fig. 4), the clone Ha hsp17.6 was tentatively identified as a lmw-HSP and named accordingly. To estimate the multiplicity of genes homologous to the cloned cDNAs, total sunflower DNA was analysed by Southern blot hybridization, using the different cDNA inserts as probes (data not shown). Ha ds10, Ha ds11 and hsp17.6 were found to be members of small, conserved gene families (Table 1).

Nucleotide sequence of cDNAs and deduced amino acid sequences

The DNA sequences of the three cDNA inserts and their corresponding predicted amino acid se-

Table 1. Characteristics of cDNA clones and predicted proteins.

cDNA clone[1]	Length (bp)	mRNA length[2] (nt)	Coding region (aa)	Predicted protein MW[3]	Isoelectric point[3]	Number of loci[4]
Ha ds10	493	560	92	10052	8.0	3–5
Ha ds11	891	910	104	11075	8.0	1–3
Ha hsp17.6	697	800	153	17564	7.0	1–3

[1] Numbers in designations refer to the molecular weight of their predicted proteins, 'ds' to their accumulation in dry seed, and 'Ha' to *Helianthus annuus*; [2] Estimated from electrophoretic mobility in northern hybridizations; [3] Calculated from the amino acid composition; [4] Estimated by Southern hybridizations under high-stringency conditions [8].

quences are shown in Figure 1. The most likely translation initiation codons are indicated. The nucleotides in their vicinity show similarities to plant initiation consensus sequences [14]. For Ha ds11 and hsp17.6, putative polyadenylation signals (underlined) and poly(A) tracts were identified in their 3′ flanking region (panels B and C).

The length of cDNA inserts was compared to the estimated size of mRNAs detected in northern experiments (Table 1). From these data it is clear that Ha ds11 is essentially a full-length cDNA clone; Ha hsp17.6 lacks at least part of its 5′ untranslated sequences. On the other hand, Ha ds10 could be truncated in its 3′ flanking sequences: it is 67 bp shorter than homologous mRNA and does not show a poly(A) tract or typical polyadenylation signals. Similar 3′-truncated cDNAs have been isolated from seed-stored poly(A)$^+$ RNA [15].

Only one plausible open reading frame (ORF) was found in the sequences of Ha ds10 and hsp17.6 (Fig. 1, panels A and C). In Ha ds11, three overlapping ORFs of similar length were observed: the one shown in Fig. 1 (panel B), and two more on the complementary strand. The transcribed strand was determined by hybridization of seed RNA with riboprobes prepared from either strand of the cDNA (data not shown). The proteins predicted for Ha ds10 and ds11 are very hydrophilic and, although different in sequence, share a very characteristic composition, found in plant Lea proteins of class I [1]. In both sequences, the most prevalent amino acid is glycine (16.3% and 9.6%, respectively). There is also an unusually high abundance of other hydroxylated, acidic and basic amino acids: Ser + Thr + Tyr, 13% and 19.2%; Glu + Asp, 21.7% and 12.5%; Lys + Arg, 18.6% and 16% (first values for ds10, second for ds11). The putative Ha ds10 and ds11 proteins do not show signs of a signal peptide and are probably cytoplasmic. In the predicted amino acid sequence for Ha hsp17.6, the motif GVLTV, characteristic of α-crystallins and several lmw-HSPs [16] is found in a hydrophobic domain in the carboxy terminal region. The predicted proteins are supported by extensive amino acid sequence similarities to other plant proteins (Fig. 2, see below). Their estimated isoelectric points and other characteristics are given in Table 1.

Homology to other proteins

Database searches revealed convincing similarities between the predicted Ha ds10, ds11 and hsp17.6 proteins and previously published sequences: Ha ds10 protein is very similar (75% identical, most changes conservative) to carrot emb1 [17], which is expressed during early somatic embryogenesis (Fig. 2A). Other similar

Fig. 1. Nucleotide and predicted amino acid sequence of sunflower cDNAs. On each sequence (mRNA strand), the putative initiation codons and nucleotides matching the plant initiation consensus are underlined as well as (in panels B and C) possible polyadenylation signals. Predicted stop codons are shown in bold face. In panel C, the motif GVLTV is underlined.

A Ha ds10

```
   1  TTAACAACTTAAGTTAAAAGAGTGAACAAAATGGCATCACAACAGGGACAACAAACACGC   60
                                  M  A  S  Q  Q  G  Q  Q  T  R

  61  AAAATTCCGGAACAGGAGAAGAAGGATCTCGACCAACGAGCAGCTAAAGGCGAGACCGTT  120
      K  I  P  E  Q  E  K  K  D  L  D  Q  R  A  A  K  G  E  T  V

 121  GTTCCGGGTGGTACTCGTGGCAAATCTCTTGAGGCTCAAGAACGTCTTGCTGAAGGCGG  180
      V  P  G  G  T  R  G  K  S  L  E  A  Q  E  R  L  A  E  G  R

 181  ACGAAGGGAGGACAAACGAGGAAAGACCAGCTGGGAACTGAAGGGTACAAGGAGATGGGG  240
      T  K  G  G  Q  T  R  K  D  Q  L  G  T  E  G  Y  K  E  M  G

 241  AAAAAGGGCGGTCAGACCACCGGTGACAAGTCGGCTGGTGAGCGAGAGGAGGAGGAGGAG  300
      K  K  G  G  Q  T  T  G  D  K  S  A  G  E  R  E  E  E  E  E

 301  GAGGACTAGATAGTCAATAGTGGTGTGATGGTGTTTGCATGTACGATGATGTTAATTTCC  360
      E  D

 361  ATGTTTTATATATGTGTATGTACCTGTAGTATGGTTTAGCTCGTGTTTCATGTTTTGTTG  420
 421  GTCGTTTTGGTATCTTCTTTAGTGCATGTACGACTAGTAGTCCTATGATGATGTGATGTG  480
 481  ATTTGCATATGTTG                                                494
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B Ha ds11

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   1  TTTTGATCCTAATCTTTTCTACACATATTGCGAAATCATGTTTGTAAATAATCAAGCATT    60
  61  TGGCTCAGAAGATAAAAGTTTAGAAAAAAGTAATGTAGAGAAAAGCCATAAAAAATATGT   120
 121  TCTAGAGAGATTTGTTTTGCAGAGAGTCGATAATGTCGATGCAATTCTTCATTAATGACT   180
 181  TGGCCTGCTCATTTTTTTCAATTTCCCATTGATGAACAAGCTCCGTTTTAAAACCTACTT   240
 241  CTACTTGTACTTGCGCACCTCTTGCTCGAGTAAAGTCTCTGTACAGCTTTGTCACTTCGT   300
 301  CAAGACACTCTGAAGCTGCGATGTGAACTTGGGCAATCTTTATTATGGATATACAATCGA   360

 361  TCACCTTAGGTGACACAGAATGATCGACGAACCTTTTCAAAGTGAAACAAGATGCAGTCA   420
                                                    M  Q  S

 421  GGCAAAAATGCAGCGGCGTCCGCCAAGGAGACCGCAGCGAACGTGGCGGCATCCGCAAAA   480
      G  K  N  A  A  A  S  A  K  E  T  A  A  N  V  A  A  S  A  K

 481  GCCGGTATGGAAAAGACCAAAGCAAGTCTCCAAGAGAAGGGTGAAAAGATGACTGCTCAT   540
      A  G  M  E  K  T  K  A  S  L  Q  E  K  G  E  K  M  T  A  H

 541  GACCCTATGCAGAAGGAAATGGCTAGAGAGAAGAAGGAAGAGAGGAAACACGAGGCGGAG   600
      D  P  M  Q  K  E  M  A  R  E  K  K  E  E  R  K  H  E  A  E

 601  TATGAGAAGCAGGCCGCGAAAGAGCACAACGCCGCGCAGAAACAGACCACCGGAATAGGC   660
      Y  E  K  Q  A  A  K  E  H  N  A  A  Q  K  Q  T  T  G  I  G

 661  ACCGGAACTCATAGCTACACGACCACCAATGTAACCGGCCATCGCACAGGCACCGGCGGG   720
      T  G  T  H  S  Y  T  T  T  N  V  T  G  H  R  T  G  T  G  G

 721  ATTTAAACTTAGCGGACGTAAAGATGTGTTCCTTGATGGTTTTTTTTTTTAATGGAACTA   780
      I

 781  TGCTTTATTTGCTTGTGAGTTGCTGTTTTCCGAGTGTTGTATAGTTTGCTTATGATGTAA   840
 841  TAAGGCACTACTTGTCATCAATAATACCTTTAAACCTTTTAAGGATAAAAA            891
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C Ha hsp17.6

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   1  AAAACATTCACCATGTCAATCATTCCAAGCTTTTTCACCAGCAAAAGAAGCAACATATTC    60
                M  S  I  I  P  S  F  F  T  S  K  R  S  N  I  F

  61  GACCCATTCTCCCTAGACACATGGGACCCGTTCCAAGGGATCATCTCCACCGAACCCGCG   120
      D  P  F  S  L  D  T  W  D  P  F  Q  G  I  I  S  T  E  P  A

 121  CGGGAAACCGCAGCGATCGTGAACGCGAGAATCGACTGGAAAGAGACGCCCGAGGCGCAC   180
      R  E  T  A  A  I  V  N  A  R  I  D  W  K  E  T  P  E  A  H

 181  GTGTTAAAAGCCGACTTACCCGGGATGAAGAAGGAAGAGGTGAAGGTGGAAGTGGAAGAC   240
      V  L  K  A  D  L  P  G  M  K  K  E  E  V  K  V  E  V  E  D

 241  GGGAGGGTGTTGCAGATAAGTGGAGAGAGGTGTAGAGAGCAGGAGGAGAAGGATGACACG   300
      G  R  V  L  Q  I  S  G  E  R  C  R  E  Q  E  E  K  D  D  T

 301  TGGCATAGGGTTGAGAGGAGTAGTGGGAAGTTTATTAGGCGGTTTCGGTTGCCGGAGAAT   360
      W  H  R  V  E  R  S  S  G  K  F  I  R  R  F  R  L  P  E  N

 361  GCGAAGATGGATGAGGTGAAGGCGATGATGGAGAACGGTGTGTTGACGGTGGTTGTGCCT   420
      A  K  M  D  E  V  K  A  M  M  E  N  G  V  L  T  V  V  V  P

 421  AAGGAGGAGGAGGAGAAGAAGCCGATGGTGAAGGCTATTGATATTTCTGGGTAGGTTTGT   480
      K  E  E  E  E  K  K  P  M  V  K  A  I  D  I  S  G

 481  GTCAGGGGTAGGGGTGCTCCACTTTTCATCATTCACCGTGACGCCCCGACCCGCTTATGT   540
 541  GAGGCTGGGAGCTTTGTTTTGCATGTTGTAATGAAATAAACGTAATAGGTTCGTGGTGTT   600
 601  TTGAGGTGGGGTTTGTATGTATGTATTATGTATATATGTATGTTTGCTTGAATAAAGGT   660
 661  TGTGTTATATTTGTATCTAAAAAAAAAAAAAAAAAA                          697
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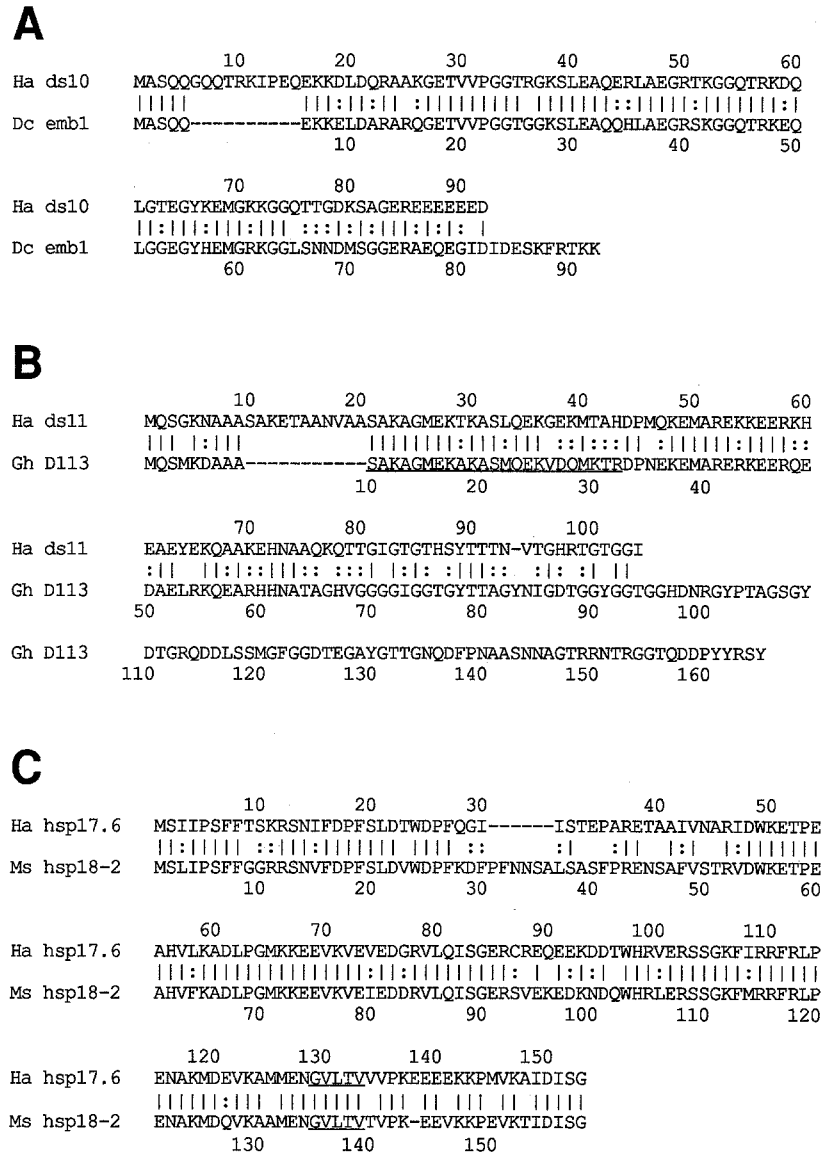

Fig. 2. Alignment of predicted amino acid sequences to other plant embryogenesis proteins. Identical amino acids are indicated by bars, conservative changes by colons. Gaps, introduced to optimize the alignments, are shown by hyphens (one per amino acid). In panel B, on the D113 sequence, 23 amino acids predicted to adopt an amphiphilic α-helix are underlined. In panel C, the heat-shock GVLTV motif is underlined.

proteins include the wheat Em [18] and the radish p8B6 [15] Lea proteins (data not shown). Although all these proteins are very similar in length (92, 92, 93 and 83 amino acids, respectively), for optimal alignment with the sunflower sequences, a gap of 10 amino acids had to be introduced. Ha ds10 was also similar (66.3% identical) to the larger (120 amino acids) cotton Lea D19 [4].

Although somewhat smaller, Ha ds11 was found to be homologous (55% identical) to the cotton Lea D-113 protein. Similarity was higher in sequences near the amino terminus. In particular, we noticed that a region in D-113 (amino acids 10–32), with potential amphiphilic α-helical structure [4], is very conserved in Ha ds11 (amino acids 21–43): 69.6% identity, 95.6% similarity, (Fig. 2, panel B).

Ha hsp17.6 was very similar, at both the nucleotide and amino acid level, to several members of the plant lmw-HSP gene superfamily [19]. Highest homology (75.7% amino acid identity) was found to a recently reported alfalfa cDNA clone [20], which is expressed during somatic embryogenesis and in response to osmotic and heat-shock stress (Fig. 2, panel C). Interestingly, Ha hsp17.6 mRNA showed a similar expression pattern during zygotic embryogenesis and also accumulated in response to osmotic stress (see below).

mRNA expression during zygotic embryo development and accumulation in dry seeds

Total RNA was prepared from staged embryos, mature dry seeds and 3-day-old seedlings. Accumulation of mRNAs homologous to Ha ds10, ds11, and hsp17.6, was investigated by northern analysis. The amount of RNA in each sample was controlled with hybridization to radish 18S rRNA. The developmental stage of analysed embryos was checked by hybridization with a helianthinin, HaG3, probe [11]. Helianthinins (11S globulins) are the major seed storage proteins in sunflower. As reported earlier [21], helianthinin mRNAs began to accumulate by 7 days after flowering, peaking by mid-maturation (12 days after flowering) and disappearing from mature dry seeds. Helianthinin mRNA was absent from 3-day-old germinated seedlings (Fig. 3). The investigated sunflower mRNAs showed similar patterns of accumulation: comparable to helianthinin, their mRNAs were detectable early in development (by 7 days after flowering). The mRNAs accumulated through maturation, peaking around 12 days after flowering, (more clearly observed for Ha ds10 and hsp17.6). All three mRNAs were essentially absent from 3-day-old germinated seedlings. Unlike helianthinin, Ha ds10, ds11 and hsp17.6 mRNAs accumulated to high levels in mature dry seed (Fig. 3). A similar expression pattern has been observed with a different (Ha hsp17.9), partially sequenced, sunflower lmw-HSP cDNA (data not shown).

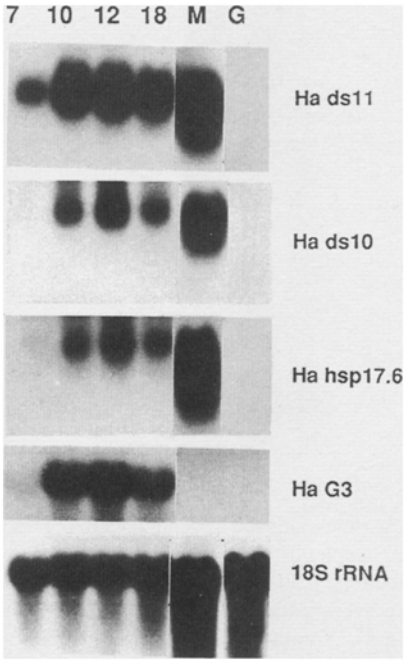

Fig. 3. Developmental expression of sunflower Lea (ds) and lmw-HSP mRNAs. Total RNA, purified from embryos 7, 10, 12 and 18 days after flowering, mature dry seeds (M) and 3-day-old seedlings (G/2), was analysed by northern hybridization. Probes used are indicated to the right of each panel. The same nylon filter was hybridized sequentially with all the probes. Helianthinin mRNAs, and 18S rRNA, are much abundant than Ha ds10, ds11 and hsp17.6. Thus, exposures to detect them were considerably shorter, under comparable hybridization conditions (see Methods): Ha ds10 and ds11, 24 h; hsp17.6, 48 h; 18S rRNA, 7 h; HaG3, 1 h.

Accumulation of mRNAs in response to heat-shock, ABA and osmotic stress

Three-day-old seedlings, germinated and cultivated in the dark at 21 °C, were subjected to heat-shock, at two different temperatures. Total mRNA was analysed by northern hybridization using α-^{32}P-labelled probes prepared from each cDNA (Fig. 4). We found that heat-shock induced the accumulation of the different homologous mRNAs: higher levels were observed for Ha hsp17.6. Induction of mRNA accumulation, although at lower levels, was significant also for Ha ds10 mRNA (at 42 °C), and ds11 (at 37 °C).

Sunflower seedlings were treated with 0.3 M

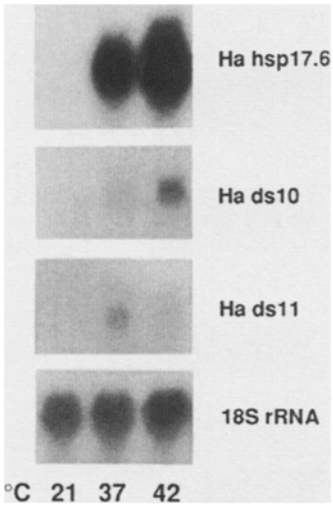

Fig. 4. Heat-shock-induced accumulation of transcripts. Total RNA samples from 3-day-old seedlings, grown at 21 °C and shocked for 2 h 30 min at 37 °C or 42 °C, were analysed by northern hybridization with the indicated probes (see legend to Fig. 3). Autoradiography exposure times: Hsp17.6, 16 h; ds10 and ds11, 48 h; and 18S RNA, 5 h.

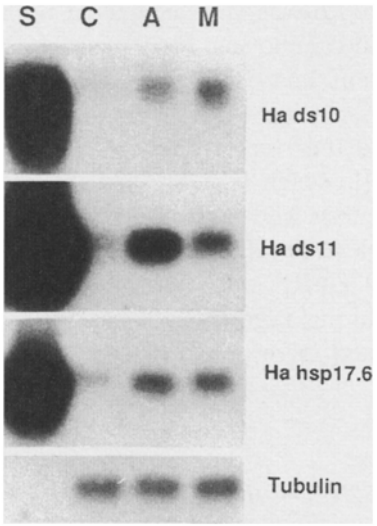

Fig. 5. Northern analysis of poly(A)$^+$ RNA extracted from dry mature seed (S), control seedlings (C), and seedlings exposed for 24 h to ABA (A) or mannitol (M). Each panel depicts hybridization of the same filter with a different cDNA probe. Hybridization to human α-tubulin was used as control. Autoradiography exposure times: Tubulin, 72 h; Ha ds10 and ds11, 24 h; Ha hsp17.6, 6 h.

mannitol or 0.1 mM ABA. The induction of accumulation of Ha ds10, ds11 and hsp17.6 mRNAs was investigated, 24 h after treatment, by northern analysis. Hybridization with an α-tubulin probe controlled the amount of RNA in each lane. Poly(A)$^+$ RNA prepared from dry seed, used for the cDNA library construction, was included to compare levels of mRNA accumulation. Unlike α-tubulin, mRNAs homologous to Ha ds10, ds11 and hsp17.6 accumulated in dry seed to very high levels. ABA and mannitol induced accumulation of Ha ds10, ds11 and hsp17.6 mRNAs in vegetative tissues of seedlings, although to much lower levels than in dry seeds (Fig. 5).

The time course of accumulation, in response to osmotic stress, was investigated for the different mRNAs. Total RNA, prepared at various times after the mannitol treatment, was analysed by northern hybridization with the cDNA probes. The RNA amount in each sample was controlled with hybridization to 18S rRNA probe. Maximal levels of Ha ds11 mRNA were detected 24 h after treatment. A similar response was observed for Ha ds10 (data not shown). In contrast, accumulation of Ha hsp17.6 showed an earlier maximum (by 4 h after treatment), followed by a secondary peak coincident with that of Ha ds11 (Fig. 6).

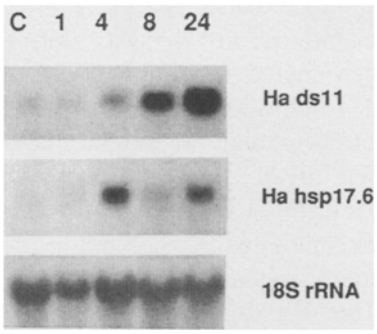

Fig. 6. Time course of Ha ds11 and hsp17.6 mRNA accumulation in response to osmotic stress. Changes in mRNA levels were investigated by northern hybridization with the probes indicated in each panel. Analysed total RNA was extracted from seedlings 1 h, 4 h, 8 h and 24 h after being subjected to osmotic stress with mannitol. C: RNA from control 4-day-old seedlings. Exposure times: 18S rRNA, 1 day; Ha ds11 and hsp17.6, 3 days.

Discussion

Based on high similarities with the amino acid and nucleotide sequences of other plant proteins and on the expression patterns of their mRNAs, we assume that we have isolated cDNAs for two sunflower Lea proteins, and one lmw-HSP. The coordinate accumulation of Lea (Ha ds10, ds11) and lmw-HSP transcripts during embryo development and in response to stress and ABA, suggests the existence of common regulatory elements for Lea and lmw-HSP genes, and it supports the notion that HSPs might have alternative functions in the plant cell.

Expression of Ha hsp17.6, ds10 and ds11 mRNAs during sunflower embryogenesis under normal conditions: accumulation in dry seeds

It has been suggested that the developmental expression of HSPs at normal growth temperature could be universal [22]. This suggestion is based mainly on studies carried out in animal systems (reviewed in [23]): in *Drosophila*, lmw-HSPs 22, 23, 26 and 27 are synthesized in imaginal discs during early development. In the absence of high-temperature stress, HSPs are also among the first products of zygotic gene activation in mice and *Xenopus*. In plants, the experimental evidence for developmental expression of HSPs is still scarce. It has been shown that a mRNA for a 90 kDa protein, similar to HSP70, was detected early in pea seed development, and related mRNAs could be detected at later stages [24]. Accumulation of lmw-HSP mRNAs during globular and (early) heart stages of somatic embryogenesis, has been reported in alfalfa [20] and carrot [25]. Our results with Ha hsp17.6 mRNA (Fig. 3) and recent data from other laboratories [26] demonstrate that the latter observation can be extended to plant zygotic embryogenesis. All this evidence is consistent with the idea that certain HSPs might play specific functions during plant embryo development, perhaps different from their suggested involvement in thermotolerance upon heat-shock stress [19, 22], and related with the control of cell differentiation [20, 23] and/or desiccation protection [19] at the latest stages of embryogenesis. Ha hsp17.6 is highly homologous to Ms hsp18.2 which is expressed during alfalfa somatic embryogenesis [20]. Both proteins could play similar functions during somatic and zygotic embryo development. The developmentally regulated plant lmw-HSPs could assist the correct folding or assembly of proteins during embryogenesis and seed desiccation.

Ha hsp17.6 is probably the first plant HSP cDNA that has been cloned from dry-seed-stored RNA. This result confirms early suggestions that HSP mRNAs are stored for long times in dry seed. In sorghum [27], wheat [28] and pea [29], dry-seed mRNAs could be translated *in vitro* to proteins undistinguishable from HSPs in two-dimensional electrophoresis. These proteins were also translated *in vivo*, during the first moments of germination, even in the presence of strong inhibitors of nuclear transcription [27, 28]. For the putative lmw-HSPs of wheat, similar results were obtained with plants grown under controlled temperature conditions, in the field, or subjected to short heat-shock treatments [28]. All these observations indicate that HSP mRNAs accumulate also during embryo desiccation by developmental rather than environmental regulation. As suggested for the hydrophobic Lea proteins [30], some lmw-HSPs could be also involved in desiccation protection and/or stress-induced damage repair during early germination [28].

We also would like to emphasize the similar distribution of lmw-HSP (Ha hsp17.6) and Lea (Ha ds10, ds11) transcripts during sunflower embryo maturation. This could suggest the existence of common regulatory elements in their developmental expression. The observed mRNA accumulation pattern, with peaks in mid maturation and in dry seeds, is similar to Lea-A genes of cotton [30]. In this pattern, the maturation abundance component is coincident with maximal levels of endogenous ABA in developing embryos. This might indicate an involvement of ABA, during early and mid-maturation, in the developmental regulation of sunflower ds and hsp mRNAs.

Expression of Lea and Hsp17.6 mRNAs in vegetative tissues of seedlings in response to ABA and stress

Induction to high levels of Ha hsp17.6 mRNA upon heat-shock stress confirmed its identity as a HSP by sequence analysis (Figs. 2 and 4). As observed in other lmw-HSPs from plants and *Drosophila* [16], the carboxy terminus of Ha hsp17.6 is very well conserved and contains the GVLTV amino acid motif which is also characteristic of vertebrate α-crystallins. It has been suggested that this sequence motif may have a functional role in protein aggregation upon stress [16]. We reproducibly observed the induction by heat-shock of Lea (Ha ds10, ds11) mRNAs, although to levels of accumulation much lower than Ha hsp17.6 (Fig. 4). We cannot exclude that what we detected is a partial desiccation response, but we consider this explanation unlikely: the heat-shock treatments were short (2 h 30 min) and performed under saturating humidity conditions. Furthermore, in plants of similar age, Ha ds10 and ds11 genes did not respond to more dramatic treatments with osmoticum after comparable exposure time to stress (Fig. 6, and data not shown).

The proteins predicted for ds11 and LE25 [31], a recently cloned cDNA in tomato, are the only known homologues to the cotton Lea D113 [4]. Both ds11 and LE25 are shorter, relatively diverged versions of D113 that include a predicted α-helical domain, conserved in the three proteins, that may be important for their suggested function as cellular osmoprotectants [4]. Although Ha ds11 mRNA is induced to low levels by both ABA and osmotic stress in vegetative tissues, its expression is mainly embryo-specific, as observed for D113 [4]. Similar expression patterns were detected for Ha ds10 mRNA, as reported for the homologous cDNAs from wheat [7], radish [15] and maize [32].

Accumulation of HSP mRNAs in response to osmotic stress has been reported for HSP70 in young maize seedlings [33]. We show similar results for hsp17.6 mRNA in sunflower. In both cases a response to exogenous ABA treatments was observed, suggesting that this hormone could mediate the osmotic-stress response of HSPs, as proposed for Lea genes [2]. The mRNA for alfalfa hsp18-2, a lmw-HSP showing the highest homology to Ha hsp17.6, is induced in cultured cells 2 h after osmotic stress treatments [20]. Interestingly, we also observed for Ha hsp17.6 mRNA a fast response to osmotic stress in whole seedlings. The kinetics of accumulation of HSP mRNAs is clearly different from that of Lea mRNAs (Fig. 6). This result could suggest that other signals besides ABA might be also involved in the response of HSP genes to osmotic stress. In plants, a particular set of HSP genes could be involved in the early events of responses to various stresses.

Preliminary results in our lab indicate that, unlike Lea (ds) genes, the osmotic-stress response of sunflower lmw-HSP mRNAs is not restricted to very young seedlings. Thus, the described cDNAs could be used as molecular probes to investigate gene response to various stresses at different developmental stages. Such probes could be also useful as genetic markers in studies of the association with drought tolerance in sunflower breeding programs. Our work currently focuses on the isolation and characterization of genomic sequences which will allow for further studies on the developmental and environmental expression regulation of sunflower Lea (ds) and lmw-HSP genes.

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