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Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications

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Abstract

We isolated and sequenced Ha hsp17.9, a DNA complementary (cDNA) of dry-seed stored mRNA that encodes a low-molecular-weight heat-shock protein (LMW HSP). Sequence analysis identified Ha hsp17.9, and the previously reported Ha hsp17.6, as cDNAs encoding proteins (HSP17.6 and HSP17.9) which belong to different families of cytoplasmic LMW HSPs. Using specific antibodies we observed differential expression of both proteins during zygotic embryogenesis under controlled environment, and a remarkable persistence of these LMW HSPs during germination. Immuno-blot analysis of HSP17.9 proteins in two-dimensional gels revealed that the polypeptides expressed in embryos were indistinguishable from LMW HSPs expressed in vegetative tissues in response to water deficit; but they appeared different from homologeous proteins expressed in response to thermal-stress. Tissue-print immunolocalization experiments showed that HSP17.9 and HSP17.6 were homogeneously distributed in every tissue of desiccation-tolerant dry seeds and young seedlings under non-stress conditions. These results demonstrate developmental regulation of specific, cytoplasmic, plant LMW HSPs, suggesting also their involvement in water-stress tolerance.

Introduction

Heat shock, as well as other stresses, induces the expression of groups of conserved genes that encode heat-shock proteins (HSPs). The stress-induced activation of HSP genes is a cellular response characteristic of most living organisms, both prokaryotic and eukaryotic [1]. The plant heat-shock response is characterized by the synthesis of multiple, abundant, low-molecular-weight (LMW) HSPs. These proteins, with molecular sizes between 15 and 28 kDa, belong to at least four gene families, two of which (class I and class II) encode polypeptides primarily located in the cytoplasm [2].

HSP genes are also expressed during normal development, in particular at definite stages of embryogenesis and gametogenesis. Considerable evidence for their developmental regulation has been gathered in animal systems [review in 3].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Z29554.

Similar observations in plants are still scarce [reviews in 4, 5], although there are examples showing expression of specific HSP genes during pollen development [6, 7], fungal sporulation [8], and in zygotic [9–11] as well as somatic embryogenesis [12, 13]. Some of these examples include LMW HSP genes [6, 7, 10, 12, 13], but in most cases only mRNA accumulation or *in vitro* translation data have been reported. Use of antibodies against cytoplasmic class I LMW HSPs [14] has confirmed *in vivo* accumulation of these proteins in mature seeds from field-grown plants [15]. The latter observation is strongly indicative of developmental expression of these proteins during plant zygotic embryogenesis, which had been suggested by earlier studies [10, 16–18].

To investigate the molecular basis of the desiccation tolerance of zygotic embryos, we have isolated and characterized cDNA clones from mRNA stored in sunflower mature (dry) seeds [10]. We found novel LMW HSP genes whose transcripts accumulate during embryogenesis [10], in response to ABA and osmotic stress in young seedlings [10], and in response to water stress in vegetative tissues of mature plants [19]. In a previous paper, using antibodies against the LMW HSPs encoded by seed-stored mRNAs, we demonstrated expression of homologeous HSPs in response to water stress. This expression was specific for lateral meristems in the root and stem of adult sunflower plants [19]. The water-stress response of specific LMW HSP genes could be distinguished from a heat-shock response by a distinct tissue specificity of the proteins induced by either stress [19].

In this paper, using specific antibodies, we provide direct evidence for developmental expression of a class II LMW HSP (HSP17.9), during plant zygotic embryogenesis and in early stages of germination. We also confirm the developmental expression of sunflower HSP17.6, a class I LMW HSP. Our experiments thus independently corroborate conclusions reached in a recent report by DeRocher and Vierling [20]. In addition, we compare developmental expression of HSP17.6 and HSP17.9 proteins with expression, in vegetative tissues, of homologeous polypeptides in response to stress. We find inspring similarities and differences between the two phenomena: the water stress-induced and developmentally expressed LMW HSPs could be very similar, if not identical, polypeptides; but only in seeds and in young seedlings is their expression widespread. The latter result contrasts with the narrow tissue specificity of LMW HSPs observed in stressed, adult, vegetative tissues [19]. We discuss possible protective and (or) repair functions for specific LMW HSPs during sygotic embryogenesis. These functions are deduced from a correlation between the observed protein expression patterns (this work), and the acquisition of embryo desiccation tolerance during seed development, or its loss early after imbibition of the mature seed (i.e., physiological observations reviewed in [21]).

Materials and methods

Plant material

Sunflower (*Helianthus annuus* L., cv. Sunweed, Rhône Poulenc) was grown under controlled environment: 18-h day cycles, day temperature 20 °C, night temperature 18 °C, 80% relative humidity. Mature seeds and staged embryos were collected, dissected and stored essentially as described [10]. Seeds were germinated either by placing them in water-soaked vermiculite or by laying them in Petri dishes between two pieces of filter paper, moistened with sterile distilled water. Seeds germinated in Petri dishes were kept in the dark at 25 °C. Seeds germinated in vermiculite were exposed to light after cotyledon expansion. Heat-shock and water-stress treatments of 4–6-week-old sunflower plants were performed as in Almoguera *et al.* [19].

Recombinant DNA techniques, RNA hybridizations and DNA sequencing

Construction and differential screening of a sunflower dry-seed cDNA library has been described in Almoguera and Jordano [10]. Conventional molecular cloning techniques were used for purification of plasmid and phage DNA, purification

and ligation of DNA fragments, and labelling of RNA and DNA probes [19, 22]. Helianthinin (Ha G3), 18S rRNA, and Ha hsp17.6 probes have been described earlier [10, 19]. The Ha hsp17.9 cDNA insert (described in this work), as well as its two internal *Eco* RI fragments, were excised with *Not* I (or *Eco* RI) and subcloned in pBluescript SK+. The 514 bp *Eco* RI fragment that contains the coding region and 5′ leader sequences was used to prepare Ha hsp17.9 DNA probes. Uniformly labelled RNA probes (riboprobes) complementary to hsp17.9 mRNAs were also used. The plasmid containing the 514 bp *Eco* RI fragment was linearized with *Xho* I and *in vitro* transcribed, using T3 RNA polymerase as described in Almoguera *et al.* [19]. RNA preparation and sequential hybridization of Hybond-N filters were performed as described previously [19]. The nucleotide sequence of both strands of Ha hsp17.9 cDNA was obtained by dideoxy sequencing, using Sequenase (United States Biochemicals) and single-stranded DNA templates. Sequence analysis was performed with the GCG (Madison, WI) software package. Sequence comparisons were carried out as described [10].

Protein analysis and immunodetection

Seedlings, three day after imbibition (and older), were dissected in two portions that contained the cotyledons, or the hypocotyl plus the radicle. Total protein from frozen embryos, mature seeds and seedlings, was extracted and analyzed by one-dimensional or two-dimensional gel electrophoresis and protein blotting essentially as reported [19]. To improve resolution of LMW HSPs, the SDS-PAGE gels were cast with 15% polyacrylamide. The production and purification of the HSP17.6 and HSP17.9 antibodies, as wel as their characterization, and the experimental conditions used for the immunodetection experiments have been described in detail [19].

Tissue printing

Mature dry seeds were soaked in distilled water for 2–4 h at room temperature. Once moistened, and after removal of the seed coat, hand-cut sections of the embryo were printed on nitrocellulose (B45, Schleicher & Schuell) by applying them with gentle pressure. Sections from the cotyledon, hypocotyl, and primary root of three-day-old seedlings, germinated in the dark (see Plant material), were directly printed without pre-soaking. Tissue prints were stained with Ponceau S (Sigma), and processed for immunodetection [19]. Microphotography was performed with a Nikon SMZ-10 stereomicroscope, using Kodacolor II (100 ASA) film.

Results

Isolation of Ha hsp17.9 cDNA and sequence analysis

Differential screening of a sunflower cDNA library [10], using dry-seed and three-day post-imbibition cDNA probes, allowed us to isolate a cDNA clone named Ha hsp17.9 (see below). This clone hybridized preferentially to the dry-seed probe (data not shown) and did not cross-hybridize [19] with the previously isolated sunflower cDNA Ha hsp17.6 [10]. Based on its sequence (Figs. 1 and 2) and heat-shock response [19], Ha hsp17.9 was tentatively identified as a LMW HSP and thus named accordingly (Ha, for *Helianthus annuus* L.).

The complete DNA sequence of Ha hsp17.9, and its corresponding predicted amino acid sequence is shown in Fig. 1. The length of the cDNA insert (824 bp) agrees quite well with the estimated size (850 nt) of mRNAs detected in northern hybridization experiments (Fig. 3). This indicates that Ha hsp17.9 is essentially a full-length clone. On the cDNA sequence, we identified a putative polyadenylation signal, as well as the most likely initiation codon (Fig. 1). Sequences in the vicinity of the latter show similarity to the plant translation initiation consensus [23]. The only plausible open reading frame (ORF) present in Ha hsp17.9 is flanked by a relatively short leader (24 nt) and a long trailer (312 nt). Translation of this ORF predicts an encoded polypep-

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1   ATCAGACATAAATTCTATAACCAATCGAAATGGACATCGATAGTTTGATGGGGTTCGATC   60
                              M  D  I  D  S  L  M  G  F  D  P

61  CGTTACTCCGCAACCTCCACTACATCCTCGAAGCCACTGACGACAACACCACTGGAAACA   120
      L  L  R  N  L  H  Y  I  L  E  A  T  D  D  N  T  T  G  N  K

121 AGTCCAACAACAGCGGTCCGTCTCGCGCTTACGTCCGCGACGCAAGAGCAATGGCGGCAA   180
      S  N  N  S  G  P  S  R  A  Y  V  R  D  A  R  A  M  A  A  T

181 CCCCTGCTGACGTCAAGGAGTGTCCTAACTCCTACGTGTTTATTGTTGATATGCCGGGGC   240
      P  A  D  V  K  E  C  P  N  S  Y  V  F  I  V  D  M  P  G  L

241 TGAAGTCAGGGGATATAAAGGTGCAGGTGGAACGCGACAACGTTTTGGTGATAAGCGGAA   300
      K  S  G  D  I  K  V  Q  V  E  R  D  N  V  L  V  I  S  G  K

301 AGAGGAACCGAGAGGAAGAGAAGGAAGGAGTCAAGTATGTGAGGATGGAGAGGCGGATGG   360
      R  N  R  E  E  E  K  E  G  V  K  Y  V  R  M  E  R  R  M  G

361 GGAAGTTCATGAAGAAGTTTGCATTGCCAGAGGATGCGAATACGGATAAGATATCGGCGA   420
      K  F  M  K  K  F  A  L  P  E  D  A  N  T  D  K  I  S  A  I

421 TTTGTCAAGACGGAGTGCTTACGGTGACTGTGGAGAAGCTGCCTCCGCCTGAGCCCAAGA   480
      C  Q  D  G  V  L  T  V  T  V  E  K  L  P  P  P  E  P  K  K

481 AGCCGAAGACGATTCAGGTGCAGGTGGCTTGAAGAATTCGTATGCGATATGGGAATGAAG   540
      P  K  T  I  Q  V  Q  V  A  *

541 GATGTTTGGTGTGTTATGAGTTAAGGGGTTTGAAGCATGTTTTGTTGTTTTGCTCTGTTT   600
601 ATAATGAATCTGATATCAGGTGCTCTGTTTGATACGATAATGAATCTGATATCAGTTTTT   660
661 TTTTTTTTTAACCAAGTACAATGTTCTTGATATTAGTAATAAACTGTAACATAAAACAAA   720
721 CAAACCGAAAATACAGTACAGAAAACACAAACCGCATAACCAAAGAAAAACCCAACGAAC   780
781 ACAATCCACAAACCAAACATTTAAACCATCAACACAACAAACAG                   824
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Fig. 1. Nucleotide and predicted amino acid sequence of Ha hsp17.9 cDNA. On the mRNA strand, nucleotides matching plant initiation and polyadenylation consensus are underlined. The predicted stop codon is shown in bold face on the amino acid sequence. The conserved LMW HSP motif GVLTV is underlined.

tide with a molecular mass of 17.9 kDa and an isoelectric point (pl) of 8.0. Its deduced amino acid sequence (Fig. 1) contains the characteristic motif GVLTV located within a conserved hydrophobic domain which is present in the carboxy-terminal region of LMW HSPs ([2, 24]; see below).

Dry-seed-stored LMW HSP mRNAs encode putative class I and class II cytoplasmic proteins

Recently, Vierling [2] classified plant LMW HSPs into at least four families, two of which (class I and class II) encode cytoplasmic proteins. Alignment, with minimal gaps, between the predicted amino acid sequences for sunflower HSP17.9, that of previously reported HSP17.6 [10], and other well-characterized plant LMW HSPs is shown in Fig. 2. Sunflower HSP17.6 was found to be highly homologeous (similarity 96.1%, identity 70.1%) to *Arabidopsis* HSP17.6 [25], as well as to other members of class I proteins (Fig. 2A and data not shown). Conversely, sunflower HSP17.9 showed the highest homology (similarity 95.0%; identity 63.5%) to proteins such as soybean HSP17.9D [24], belonging to class II (Fig. 2B). Thus the two sunflower LMW HSPs could be respectively assigned to these distinct protein families. As reported for other class I and class II LMW HSPs [2], their sequence conservation improved towards the carboxy-terminal portion of the protein (Fig. 2). Considerably less homology was found between the two sunflower LMW HSPs (similarity 55%, identity 35%), than between members of each class. This latter observation agrees with the previously reported specificity of antibodies prepared against each sunflower protein. The two antibodies did not cross-react [19]; and antibodies against pea HSP18.1 [14], another class I protein, detected Ha HSP17.6 but not Ha HSP17.9 [19].

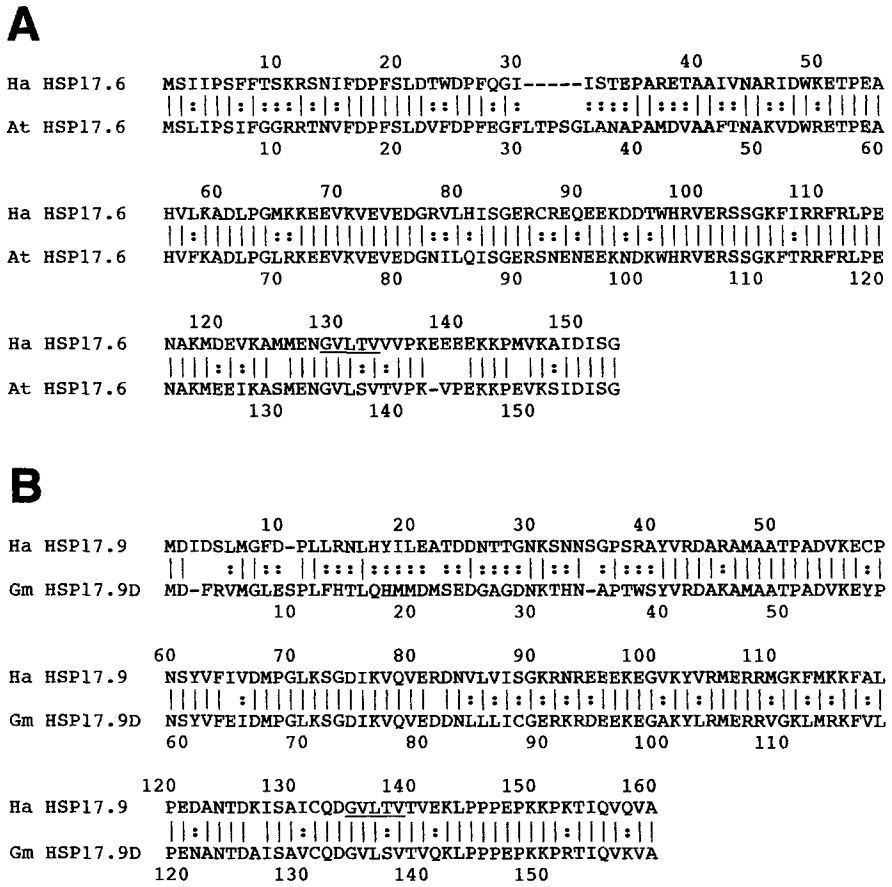

Fig. 2. Comparison of sunflower, seed-stored, heat shock proteins with canonical cytoplasmic LMW HSPs. A. Alignment between predicted amino acid sequences for sunflower HSP17.6 (X59701) and class I *Arabidopsis* HSP17.6 (X16076). B. Alignment between sunflower HSP17.9 and class II soybean HSP17.9D (X07159). Identical amino acids are indicated by bars, and conservative changes by colons. Gaps, introduced to optimize the alignments, are shown by hyphens (one per amino acid). The conserved GVLTV motif is underlined.

Expression of sunflower LMW HSPs during zygotic embryogenesis and their persistence in early stages of germination

We first investigated the accumulation pattern of Ha hsp17.9 mRNAs during zygotic embryogenesis. Total mRNA from seedlings or staged embryos, dissected from plants grown under controlled environment, was analyzed by northern blot experiments (Fig. 3A). Hybridization of the same filter with a seed-storage protein (Ha G3) and 18S rRNA probes was performed to verify the developmental stage of analyzed embryos, and the amount of RNA in each sample, respectively [10]. Ha hsp17.9 mRNAs were initially detected in embryos 8–10 days after flowering (DAF). The amount of accumulated hsp17.9 mRNAs increased during seed maturation (8–15 DAF), and reached its highest level in dry (25 DAF, and older) seeds. However, and unlike previously reported for Ha hsp17.6 mRNAs [10], we did not observe for HSP17.9 a secondary peak of accumulation coincident with maximal expression of Ha G3 during mid-maturation [26]. We also investigated the time course for disappearance of dry-seed accumulated LMW HSP mRNA during germination (Fig. 3B). Using cDNA probes, hsp17.6 [10] and hsp17.9 homologeous mRNAs

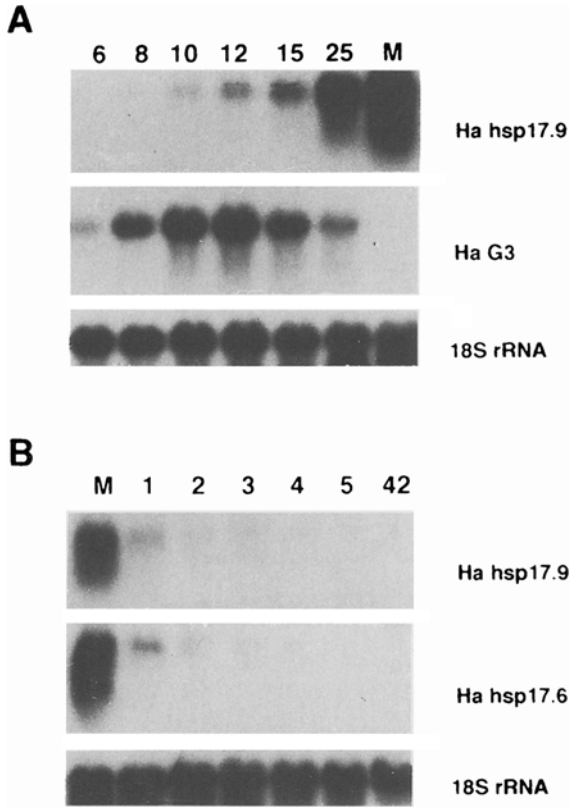

Fig. 3. A. Accumulation of Ha hsp17.9 mRNAs during zygotic embryogenesis. B. Disappearance of dry-seed-stored LMW HSP mRNAs during germination. In both cases total mRNA was analyzed by northern hybridization. Numbers correspond to either the age of staged embryos (A: 6, 8, 10, 12, 15, and 25 DAF) or, in B, to time (DPI) after imbibition of mature dry seeds (M). Probes used are indicated to the right of autoradiographs. In panel B, Ha hsp17.6 and Ha hsp17.9 mRNAs were detected using riboprobes. All other probes were labelled DNA. Each filter was sequentially hybridized with all probes (see Materials and methods).

were nearly undetectable 2 days post-imbibition (DPI). Most of dry seed-accumulated mRNAs was degraded during the first 1–2 days of germination. Similar results were observed with either sunflower cDNA probe (compare Ha hsp17.6 and Ha hsp17.9, Fig. 3B).

Because of complex post-transcriptional regulation of HSP genes [1, and data reviewed in 27], plant HSP mRNA accumulation does not always imply actual synthesis of the encoded heat-shock proteins [19]. The availability of characterized antibodies [19] against sunflower HSP17.6, and HSP17.9, allowed us to investigate the expression of these two proteins during embryogenesis and germination. Expression of class I (HSP17.6) and class II (HSP17.9) proteins during zygotic embryogenesis, and their accumulation to high levels in dry-seeds, was confirmed with immunoblot experiments. Total protein was extracted from staged embryos, or mature seeds, and analyzed by SDS-PAGE followed by western immunodetection (Fig. 4). We show that proteins that react with HSP17.9 antibodies appeared about 12 DAF. These proteins accumulated to higher levels later in development (12–25 DAF), once seed desiccation has started (Fig. 4A). This expression pattern was very similar to that observed for the corresponding mRNAs (Fig. 3A). On the other hand, proteins that reacted with HSP17.6 antibodies also accumulated to high levels in ma-

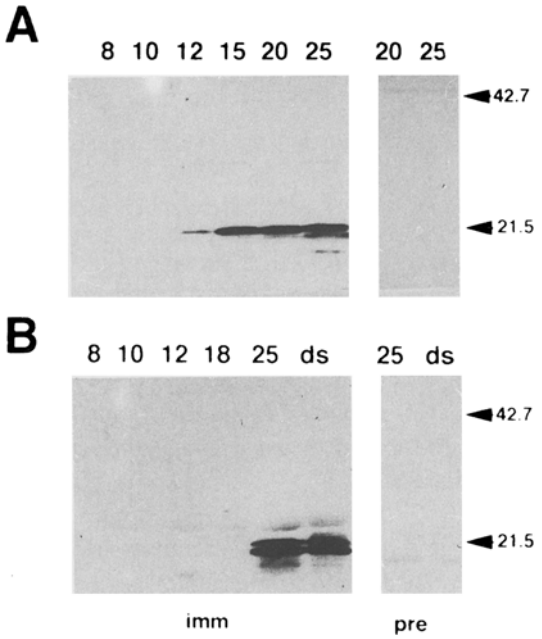

Fig. 4. Differential developmental expression of sunflower LMW HSPs. Protein extracts isolated from staged embryos, or mature dry seeds (ds), were separated by SDS-PAGE, blotted, and probed with HSP17.9 anti-β-galactosidase-free antisera (A) or with HSP17.6 purified antibodies (B). Reactions with the respective pre-immune antisera are shown to the right. Numbers correspond to embryo age (DAF). Ordinate, molecular size in kDa.

ture seeds (Fig. 4B); but, in contrast to HSP17.9 (Fig. 4A), HSP17.6 proteins did not appear until 25 DAF, about the stage when sunflower sygotic embryos desiccate faster in the seed [28]. Thus, for HSP17.6, the accumulation of homologeous mRNAs earlier (10–15 DAF) in embryogenesis [10] did not result in detectable LMW HSP synthesis before 25 DAF. This result agrees with other previous observations that suggest considerable post-transcriptional regulation of sunflower hsp17.6 genes [19].

We also used the HSP17.6 and HSP17.9 antibodies to investigate expression of LMW HSPs during germination of sunflower seeds. Figure 5 summarizes representative results of these experiments. In seedlings 3 DPI and older, dissection of the hypocothyl plus radicle showed that HSP17.9 proteins are expressed in these organs at high levels, even by 5 DPI (Fig. 5A). In cotyledons of germinating seeds, proteins immuno-reacting with HSP17.9 antibodies are detectable until 3 DPI, at levels comparable to those present in dry seeds (Fig. 5B). Similar results were obtained using

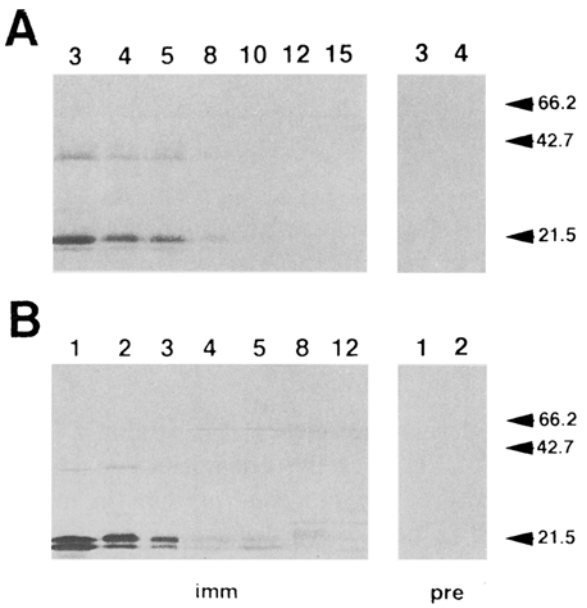

Fig. 5. Persistence of HSP17.9 during germination. Proteins extracted either from the hypocothyl plus radicle (A), or from cotyledons (B), of sunflower seedlings were analyzed, as described in the legend of Fig. 4. Numbers indicate days post imbibition of mature dry seeds.

HSP17.6 antibodies, and independently of illumination conditions during germination (data not shown). The apparent molecular weight for the major protein(s) recognized by each antibody (Figs. 4 and 5) resulted in values (data not shown) very similar, or identical, to those previously found for sunflower LMW HSPs synthesized in response to water stress, and detected using the same antibodies [19]. This observation prompted us to further investigate the relation between some of these homologeous polypeptides, as described below.

Class II LMW HSPs expressed during seed development in sunflower plants are indistinguishable from homologeous, water stress-inducible, vegetative proteins

Most plant LMW HSP genes belong to conserved multigene families with complex expression patterns, which are result of the intrinsic genetic variability of these families, and (or) of diverse post-translational modification of their encoded proteins [2, 24]. In a previous paper, we showed that using antibodies against seed-stored HSP17.9, we could distinguish between the immuno-reacting polypeptides that were expressed either in response to heat shock, or in response to water stress in sunflower plants. The heat-shock induced proteins were more acidic, and had lower apparent MW than the water-stress-induced proteins [19]. To analyze the relation of these polypeptides with HSP17.9 (class II) protein(s) expressed during embryo development and seed germination, we performed similar two-dimensional (2D) gel electrophoresis and immunoblot analyses. The result of these experiments is shown in Fig. 6. During early embryo development, a unique polypeptide (apparent MW 21.4 kDa; pI ca. 5.9) was detected in 2D gels using HSP17.9 antibodies (Fig. 6B). This polypeptide was also observed in protein extracts from older (25 DAF to dry seeds) embryos (data not shown). As demonstrated by mixing experiments such polypeptide (arrowed) was indistinguishable from the major protein that reacts with

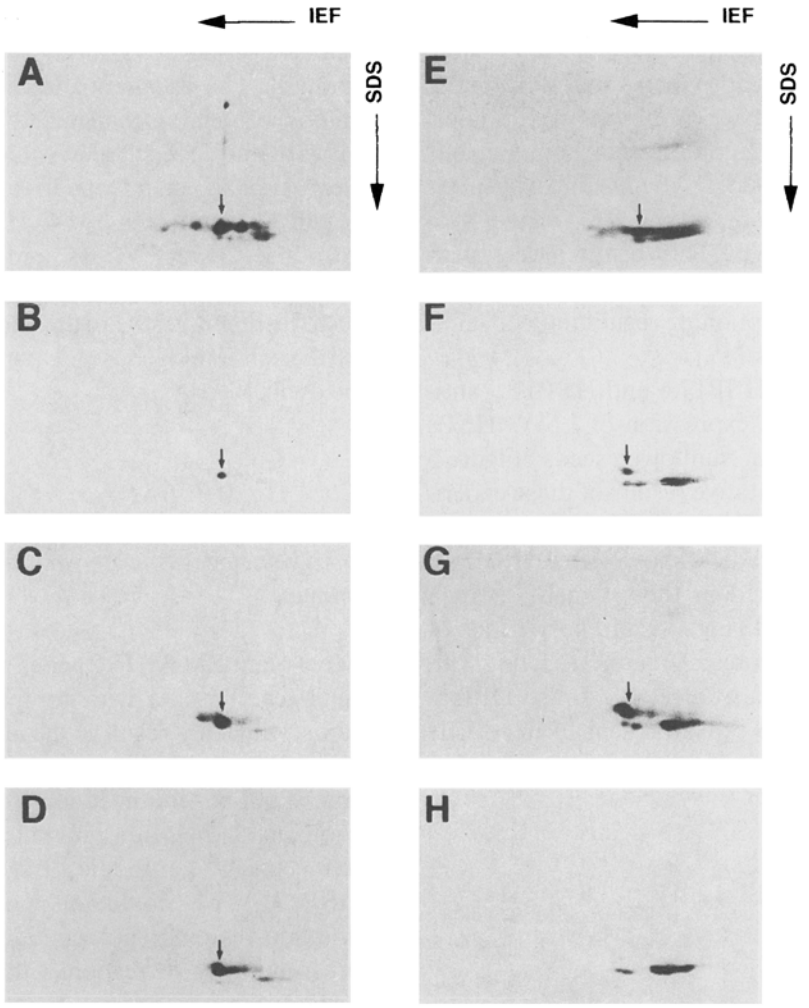

Fig. 6. 2D gel analysis of the different polypeptides, detected by HSP17.9 antibodies. Proteins in extracts from 3 DPI cotyledons (A), 12 DAF embryos (B), and water-stressed (C) or heat-shock-stressed (H) sunflower stems were resolved by denaturing IEF and SDS-PAGE electrophoresis. LMW HSPs were immuno-detected after transfer to nitrocellulose membranes. Small resolution, or migration, differences observed between the individual analyses were controled by mixing experiments. Protein-extract mixtures analyzed in the same gels were: 12 DAF embryo + water-stressed stem (D); 3 DPI cotyledons + heat-shock-stressed stem (E); 12 DAF embryo + heat-shock-stressed stem (F); and water-stressed stem + heat-shock-stressed stem (G).

the HSP17.9 antibody, and that is expressed in vegetative tissues of adult sunflower plants in response to water stress (Fig. 6C). In contrast, and in agreement with the results reported earlier [19], the heat-shock induced proteins (Fig. 6H) clearly differ from the seed- and water-stress-specific polypeptide(s); both in apparent MW (17.9 kDa), and in pI (ca. 5.0).

Although one-dimensional electrophoretical analyses of HSP17.9 proteins yielded similar results in developing seeds and in seedlings (Figs. 4A and 5), 2D gel analysis demonstrated a higher complexity of homologeous proteins in mature seeds (data not shown), and after germination (Fig. 6A). Mixing and immunodetection experiments, performed with protein extracts from cotyledons of 3 DPI seedlings, demonstrated the presence during germination of the same seed- (and water stress-) specific polypeptide (Fig. 6E). This protein was perhaps the most abundant

among other homologeous, HSP17.9 cross-reacting, polypeptides that were conspicuous after seed imbibition (Fig. 6A).

Sequence data predict that Ha hsp17.9 encodes a polypeptide with a pI of 8.0. Using antibodies against HSP17.9, we failed to detect homologeous proteins with this pI. The most abundant cross-reacting protein (arrowed in Fig. 6) was considerably more acidic (pI ca. 6). Other, less abundant, more basic, polypeptides were detected with pI reaching ca. 7.5, but only after imbibition (Fig. 6A). Differences between the predicted and apparent MW and pI of HSP17.9 (discrete increase of MW and acidity) might be due to post-translational modification of HSP17.9 during seed development.

Immunolocalization of developmentally expressed LMW HSPs

We have used antibodies against HSP17.6 and HSP17.9 to analyze the organ and tissue localization of these proteins in mature seeds. Tissue print immunostaining showed a similar generalized distribution of LMW HSP proteins recognized by either antibody. The observed LMW HSP distribution in the cotyledon, hypocothyl and radicle of dry seeds (representative results in Fig. 7B, E and G) was comparable in most cases to the histological staining of total protein obtained with Ponceau S. In the embryo, LMW HSPs were localized in the parenchyma, as well as in the pro-vascular tissues (Fig. 7G). These results are clearly distinct from the tissue-specific localization of homologeous, apparently identical polypeptides (see above) in the lateral meristems of water-stressed sunflower plants [19].

Localization of HSP17.6 and HSP17.9 cross-reacting proteins in tissues of 3 DPI seedlings matched the results obtained with mature seeds (representative results in Fig. 7H–O). Both antibodies recognized proteins located in every analyzed organ and tissue (radicle, 7J and 7K; hypocothyl, 7N and 7O; cotyledon, identical results as in 7E, data not shown). As observed for mature seeds, immunostaining of prints from seedling sections with either HSP17.9 (Fig. 7J and N), or HSP17.6 (Fig. 7K and O) antibodies gave results similar to those obtained with Ponceau S. Both antibodies detected LMW HSPs in parenchyma, as well as in provascular tissues. Comparable localization results were obtained with seedlings germinated either in the dark (Fig. 7), or exposed to the light (data not shown, see methods). Accumulation of pigments in light-grown seedlings resulted in 'colored' tissue prints from cotyledon and hypocothyl sections. Immunostaining of these prints was possible, but esthetically poorer results were obtained because of color interference with used detection procedures [19].

Discussion

Preliminary observations in our laboratory (10) and other groups [15–28, 29] indicated expression of LMW HSPs during zygotic embryogenesis and seed germination. The results presented in this paper demonstrate differential developmental expression of two distinct families of cytoplasmic proteins. In addition, we propose a functional relationship between this LMW HSP expression and desiccation tolerance.

Developmental expression of cytoplasmic LMW HSPs during sunflower zygotic embryogenesis

Sequence comparison of the predicted amino acid sequences for the proteins encoded by cDNAs Ha hsp17.6 and Ha hsp17.9 (this work) allowed us to assign these proteins (HSP17.6 and HSP17.9) to two distinct families (class I and class II, respectively) of cytoplasmic LMW HSPs [2]. Study of mRNA accumulation during embryogenesis did not reveal substantial differences between these two cDNAs. Ha hsp17.6 [10] and Ha hsp17.9 mRNAs (Fig. 3) appear quite early (8–10 DAF) and accumulate to become more abundant in mature seeds. However, protein expression patterns, determined using specific antibodies, were very different. HSP17.9 proteins

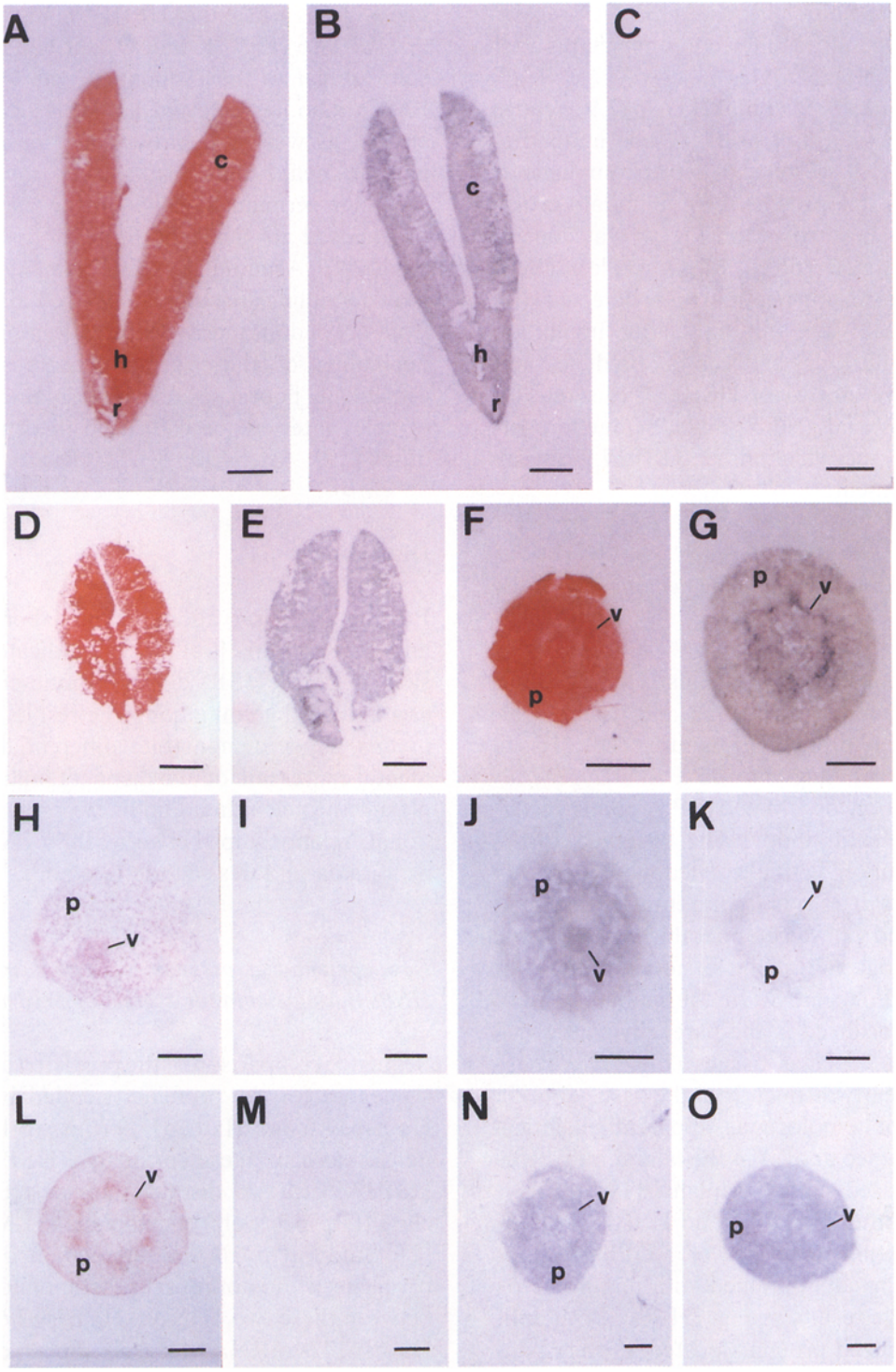

Fig. 7. Tissue-print immunolocalization of LMW HSPs in sunflower seeds and 4 DPI seedlings. Longitudinal sections (A–C), and cross sections (D–G) of mature seeds sections through the cotyledon (D and E); and sections between the hypocothyl and the radicle (F and G). Seedling cross sections (H–O): radicle (H–K); and hypocotyl (L–O). Prints in B, G, J, and N probed with HSP17.9 anti-β-galactosidase-free antisera. Prints in E, K, and O probed with purified HSP17.6 antibodies. Reactions with

were detected from 12 DAF (Fig. 4A), roughly in correspondence with mRNA accumulation data (Fig. 3). In contrast, HSP17.6 did not appear until 25 DAF (Fig. 4B). In these experiments we used identical protein samples from embryos dissected from plants grown under conditions that ensure homogeneous, low temperature in all organs [19]. The work by Hernandez and Vierling [15] provides evidence comparable to data presented here. In that study, using antibodies against pea HSP18.1 [14] and plants grown in a greenhouse, they found abundant accumulation of class I LMW HSPs in mature seeds, but not in developing pods. In their conditions, temperature differences between reproductive organs and leaves would not induce these proteins, unless the threshold temperature for HSP induction were much lower in seeds than in leaves. Thus they suggested that seed accumulation of these LMW HSPs could be developmentally regulated. Our results confirm their suggestions and provide, novel, stronger evidence for developmental expression of class II LMW HSPs. Differential expression of HSP17.6 and HSP17.9 leaves very little margin for alternative explanations to developmental expression of both proteins. If HSP17.6 and HSP17.9 were induced in seeds by temperature differences, those would be quite small in our controlled growth conditions (≤ 1–2 °C [19]). In addition, each class of LMW HSPs would have a different induction temperature, or their induction by minor temperature changes would be developmentally regulated. We consider the latter possibilities very unlikely. Our work thus independently cooroborates the recent demonstration of developmental control of Class I and Class II LMW HSPs during pea seed maturation by DeRocher and Vierling [20]. Furthermore, analysis of HSP17.9 proteins from embryos and comparison with homologeous proteins that accumulate in heat-shock- or water-stress-induced vegetative tissue, strengthens our conclusions. The HSP17.9 protein(s) expressed in developing seeds differ in their apparent MW and pI from homologeous peptides synthesized in response to heat shock in vegetative tissues. However, they are very similar, if not identical, to LMW HSPs produced in response to water stress in the same tissues (Fig. 6). Thus, expression of HSP17.9 proteins, at least, could be induced by the progressive water loss of the embryo during sunflower seed development [28].

Localization of HSP17.6 and HSP17.9 and their persistence during germination: involvement in desiccation tolerance?

Whereas seed-accumulated HSP17.6 and HSP17.9 mRNAs disappear shortly after imbibition, the proteins persist at high levels in the germinating seedling even after 3 DPI (Figs. 4 and 5). Similar disappearance patterns were observed for class I and class II LMW HSPs (Fig. 5). A recent immunological study by Kruse *et al.* [29] demonstrated accumulation of plastid-localized barley HSP26 in 2 DPI seedlings. They assumed that detected HSP26 was synthesized shortly after imbibition from mRNAs which were pre-formed in the embryo [29]. Because of the long life of LMW HSPs [15], it could be also assumed that proteins accumulated during seed development might endure early germination. In seedlings, HSP17.6 and HSP17.9 proteins were present in the cotyledons and in the embryo axis, being homogeneously distributed in most tissues, as was also observed in mature dry seeds (Fig. 7). To our knowledge, no similar observations have been reported before for LMW HSPs. Interestingly, high-molecular-weight HSPs detected in mung-bean seeds and seedlings show similar distribution [30]. The temporal and spatial expression patterns of HSP17.6 and HSP17.9 may be important in considering their possible functions. We would like to point out striking similarities between the data presented in this paper and work

(HSP17.9) preimmune antisera are shown in C, I, and M. For anatomical comparisons, prints in A, D, F, H and L were stained with Ponceau S. Abbreviations: c, cotyledon; h, hypocothyl; r, radicle; v, pro-vascular tissue, and p, parenchyma. Scale bars indicate 1 mm (A–E), and 0.5 mm (F–O).

on expression of late embryogenesis-abundant (lea) proteins in desiccation-tolerant seeds, seedlings, and resurrection plants. Like HSP17.6 and HSP17.9, lea proteins are cytoplasmic and mostly hydrophilic [review in 31]. As observed for lea proteins, HSP17.6 and HSP17.9 accumulate during seed maturation, being more abundant in desiccation-tolerant seeds. During germination, some lea proteins persist at high levels until 0.5 to 1 DPI [31]. A recent report showed that, in wheat seedlings, expression of lea proteins during early germination correlates with their ability to tolerate more than 90% of water loss [32]. In preliminary desiccation tolerance experiments performed in our lab, sunflower 2–3 DPI seedlings were able to withstand water losses of 80%; and to recover growth upon dehydration in almost 100% of the cases. In contrast, older (7 DPI) seedlings did not recover from similar treatments (data not shown). Thus, accumulation of high levels of specific LMW HSPs, in some instances distinguishable from proteins expressed in response to heat shock (Figs. 4, 5 and 6), could correlate with the desiccation tolerance of seeds and young seedlings. Differences in the tissue localization of apparently identical polypeptides (i.e., HSP17.9 proteins, Fig. 6) could also be important. These polypeptides show a widespread distribution, but only in desiccation-tolerant seeds and young seedlings (Fig. 7). This contrasts with the narrow tissue specificity observed in adult, water-stressed plants [19]. Again, the homogeneous distribution of these LMW HSPs in seeds and young seedlings is comparable to the observations made for some lea proteins in tissues of desiccation-tolerant resurrection plants [33], and in embryos of normal dicot plants [34].

Potential functions of sunflower LMW HSPs in seed development

Although the correlations outlined above are appealing, a functional involvement of specific LMW HSPs in desiccation tolerance, as hypothesized here, is based on circumstantial evidence only. Other roles would also be consistent with the expression patterns of these proteins. For example, expression of HSP17.9 during embryogenesis (Fig. 4A) parallels storage protein [26] and lipid [28] accumulation in developing sunflower seeds. However, expression of HSP17.6 (Fig. 4B) occurs well after deposition of most reserve substances in sunflower seeds [26, 28]. During germination, HSP17.9 and HSP17.6 disappear after the major storage proteins, which are degraded before 3 DPI (data not shown). The persistence of LMW HSPs beyond this stage could be related to their involvement in mobilization of lipid reserves. Even if most steps of deposition and mobilization of reserve substances take place within membrane-bound organelles (plastids, the vacuole, oil bodies etc.), a different subcellular localization of HSP17.6 and HSP17.9 could still be consistent with such role(s). As proposed for cytoplasmic HSPs of the 70 kDa family [35], HSP17.6 and HSP17.9 could be involved in assisting the translocation of structural proteins (or enzymes) between the cytoplasm and membranes in these organelles.

In animals, LMW HSPs have been proposed to maintain, or help to recover, cel integrity after stress [1]. Recent work has demonstrated *in vitro* a molecular chaperone activity for HSP27 [36], thus elucidating molecular mechanisms for its involvement in heat-shock tolerance, which has been demonstrated with HSP27 over-expression in transformed cells. In this system, it was suggested that HSP27 would interact with, and stabilize, actin filaments [37]. In plants, experimental work on HSP function is still far for similar refinement [reviewed in 2]. If plant LMW HSPs were also molecular chaperones, as inferred from their structural similarity with animal proteins [2, 24], such activity would be meaningful in any of the possible functional scenarios for HSP17.6 and HSP 17.9 discussed above. LMW HSPs present in dry seeds and young seedlings could be abundant (0.5–1.5% of total proteins [15]). This observation, as suggested for lea proteins with similar localization and abundance [34], would fit better functional hypotheses [2] involving interaction of plant LMW HSPs with abundant molecules (RNA or protein), rather than with

minor cellular components (i.e., enzymes). Future experiments, similar to those performed for animal proteins, will be necessary to analyze possible functions of these plant LMW HSPs.

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References

1. Lindquist S, Craig EA: The heat-shock proteins. Annu Rev Genet 22: 631–677 (1988).
2. Vierling E: The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42: 579–620 (1991).
3. Heikkila JJ: Heat shock gene expression and development. II. An overview of mammalian and avian developmental systems. Devel Genet 14: 87–91 (1993).
4. Winter J, Sinibaldi R: The expression of heat shock protein and cognate genes during plant development. In: Hightower L, Nover L (eds) Heat Shock and Development, pp. 85–105. Springer-Verlag, Berlin (1991).
5. Heikkila JJ: Heat shock gene expression and development. I. An overview of fungal, plant and poikilothermic animal developmental systems. Devel Genet 14: 1–5 (1993).
6. Bouchard RA: Characterization of expressed meiotic prophase repeat transcript clones of *Lilium*: meiosis-specific expression, relatedness and affinities to small heat shock protein genes. Genome 33: 68–79 (1990).
7. Atkinson BG, Raizada M, Bouchard RA, Frappier JRH, Walden DB: The independent stage-specific expression of the 18-kDa heat shock protein genes during microsporogenesis in *Zea mays* L. Devel Genet 14: 15–26 (1993).
8. Kurtz S, Rossi J, Petko L, Lindquist S: An ancient developmental induction: heat shock proteins induced in sporulation and oogenesis. Science 231: 1154–1157 (1986).
9. Domoney C, Ellis N, Turner L, Casey R: A developmentally regulated early-embryogenis protein in pea (*Pisum sativum* L.) is related to the heat-shock protein (HSP70) gene family. Planta 184: 350–355 (1991).
10. Almoguera C, Jordano J: Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat shock protein and lea mRNAs. Plant Mol Biol 19: 781–792 (1992).
11. Duck N, McCormick S, Winter J: Heat shock cognate gene expression in vegetative and reproductive organs of *Lycopersicon esculentum*. Proc Natl Acad Sci USA 86: 3674–3678 (1989).
12. Pitto J, Lo Schiavo F, Guiliano G, Terzi M: Analysis of the heat-shock protein pattern during somatic embryogenesis of carrot. Plant Mol Biol 2: 231–237 (1983).
13. Györgyey J, Gartner A, Németh K, Magyar Z, Hirt H, Herberle-Bors E, Dudits D: Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol 16: 999–1007 (1991).
14. DeRocher, AE, Helm, KE, Lauzon, LM, Vierling E: Expression of a conserved family of cytoplasmic low molecular weight heat shock proteins during heat stress and recovery. Plant Physiol 96: 1038–1047 (1991).
15. Hernandez LD, Vierling E: Expression of low molecular weight heat-shock proteins under field conditions. Plant Physiol 101: 1209–1216 (1993).
16. Howarth C: Heat shock proteins in *Sorghum bicolor* and Pennisetum americanum. II. Stored RNA in sorghum seed and its relationship to heat shock protein synthesis during germination. Plant Cell Envir 13: 57–64 (1990).
17. Helm KE, Abernethy RH: Heat shock proteins and their mRNAs in dry and early imbibing embryos of wheat. Plant Physiol 93: 1626–1633 (1990).
18. Vierling E, Sun A: Developmental expression of heat shock proteins in higher plants. In: Cherry J (ed) Environmental Stress in Plants. Biochemical and Physiological Mechanisms Associated with Environmental Stress Tolerance in Plants, pp. 343–354. Springer-Verlag, New York (1987).
19. Almoguera C, Coca MA, Jordano J: Tissue-specific expression of sunflower heat shock proteins in response to water stress. Plant J 4: 947–958 (1993).
20. DeRocher AE, Vierling E: Developmental control of small heat shock protein expression during pea seed maturation. Plant J 5: 93–102 (1994).
21. Bewley JD: Physiological aspects of dessication tolerance. Annu Rev Plant Physiol 30: 195–238 (1979).
22. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
23. Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA: Selection of AUG initiation codon differs in plants and animals. EMBO J 6: 43–48 (1987).
24. Raschke E, Baumann G, Schoeffl F: Nucleotide sequence analysis of soybean small heat shock protein genes belonging to two different multigene families. J Mol Biol 199: 549–557 (1988).

25. Helm KE, Vierling E: An *Arabidopsis* cDNA clone encoding a low molecular heat shock protein. Nucl Acids Res 17: 7995 (1989).
26. Allen RD, Nessler CL, Thomas TL: Developmental expression of sunflower 11S storage protein genes. Plant Mol Biol 5: 165–173 (1985).
27. Gallie DR: Post-transcriptional regulation of gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol 44: 77–105 (1993).
28. Robertson JA, Chapman Jr GW, Wilson Jr RL: Relation of days after flowering to chemical composition and physiological maturity of sunflower seed. J Am Oil Chem Soc 55: 266–269 (1978).
29. Kruse E, Liu Z, Kloppstech K: Expression of heat shock proteins during development of barley. Plant Mol Biol 23: 111–122 (1993).
30. Wang C, Lin BL: The disappearance of an hsc70 species in mung bean seed during germination: purification and characterization of the protein. Plant Mol Biol 21: 317–329 (1993).
31. Dure L III: The lea proteins of higher plants. In: Verma DPS (ed) Control of Plant Gene Expression, pp. 325–336. CRC Press, Boca Raton, FL (1993).
32. Ried JL, Walker-Simons MK: Group 3 late embryogenesis abundant proteins in desiccation-tolerant seedlings of wheat (*Triticum aestivum* L.). Plant Physiol 102: 125–131 (1993).
33. Schneider K, Wells B, Schmelzer E, Salamini F, Bartels D: Desiccation leads to the rapid accumulation of both cytosolic and chloroplastic proteins in the resurrection plant *Craterostigma plantagineum* Hochst. Planta 189: 120–131 (1993).
34. Roberts JK, DeSimone NA, Lingle WL, Dure L III: Cellular concentrations and uniformity of cell-type accumulation of two lea proteins in cotton embryos. Plant Cell 5: 769–780 (1993).
35. Chirico WJ, Waters MG, Blobel G: 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 332: 805–810 (1988).
36. Jakob U, Gaestel M, Engel K, Buchner J: Small heat shock proteins are molecular chaperones. J Biol Chem 268: 1517–1520 (1993).
37. Lavoie JN, Gingrasbreton G, Tanguay RM, Landry J: Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. J Biol Chem 268: 3420–3429 (1993).