A Plant Small Heat Shock Protein Gene Expressed during Zygotic Embryogenesis but Noninducible by Heat Stress*

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A small heat shock protein (sHSP) gene from sunflower, Ha hsp17.6 G1, showed expression patterns that differ from what is known for members of this gene family. The mRNAs of this gene accumulated in seeds during late desiccation stages of zygotic embryogenesis but not in response to heat shock in vegetative tissues. The failure to respond to heat shock was independent of the developmental stage after germination and shock temperature. Nuclear run-on analyses demonstrated that transcription from the Ha hsp17.6 G1 promoter is not induced by heat shock. This agrees with the presence, in this promoter, of sequences with little similarity to heat shock elements. Our results show an evolutionary divergence, in the regulation of plant sHSP genes, which has originated stress-responsive genes and nonresponsible members within this gene family. We discuss implications for mechanisms controlling the developmental regulation of sHSP genes in plants.

One of the characteristics of the plant heat shock response is the synthesis of a large number of different, but evolutionarily related, polypeptides of 17–30 kDa (the sHSPs). In contrast, animals express only one to four sHSPs upon heat shock. The diversification in plants of heat-inducible sHSP genes could be a consequence of sesility; because plants cannot move away from heat, they would have evolved a battery of specialized “stress genes,” the sHSPs. These are expressed in response to heat in all subcellular compartments and could allow plants to cope better with the stress conditions on site (for review, see Ref. 1). In animal and plant systems, heat shock genes encoding proteins of higher molecular weight, for example the HSP70s, have been shown to contain heat-inducible and non-inducible members (2, 3). In the case of plant sHSPs the evidence for the existence of genes that are not induced by heat shock is weak and indirect, as it is based on the detection in seeds of sHSP isoforms that are different from the heat shock-induced polypeptides (4, 5).

In addition to being part of the heat shock response, some plant sHSP genes have been shown to be expressed at normal growth temperatures during zygotic embryogenesis (4–7). Developmental regulation studies of plant sHSP genes are scarce. So far, only two plant sHSP promoters and 5’-flanking sequences have been reported to confer regulation to chimeric genes in maturing seeds: those from soybean Gm hsp17.3B (8) and sunflower Ha hsp17.7 G4 (9). Initial studies have pointed to common control elements between the heat shock response and activation during embryogenesis. For example, the functional implication of HSEs in both processes is supported by results of deletion analysis (8, 9). Other observations point to the involvement in embryos of distinct control elements, for example, the effect of abi3 mutations on sHSP accumulation in Arabidopsis seeds (10). It is also clear that not all plant sHSP genes are developmentally regulated during embryogenesis, at least for the most systematically analyzed sunflower (9) and Arabidopsis genes (10). Thus, there is no obvious explanation for the differential regulation of a subset of sHSP genes during embryogenesis, and such regulation might involve control elements that are common and/or distinct from those involved in the heat shock response.

In this work, we describe and analyze the genomic sequences of Ha hsp17.6 G1, a sunflower sHSP gene expressed during zygotic embryogenesis (6). By detailed analyses of gene-specific mRNA accumulation and transcription, we demonstrate that transcription from the Ha hsp17.6 G1 promoter is not induced in response to heat shock. Interestingly, such a unique expression pattern has been described in other animal genes that are structurally related (11) to the sHSPs, as for the eye lens-specific a-crystallins (12) and, more recently, for some o-crystallins (13). Thus, our observation provides strong evidence for a similar evolution of the regulation of a member of the sHSP superfamily in the plant kingdom. In addition, the structural and functional characteristics of the Ha hsp17.6 G1 promoter would support developmental control mechanisms, during plant embryogenesis, which are different from those involved in the heat shock response.

EXPERIMENTAL PROCEDURES

Binding and Electrophoretic Mobility Shift Assays—Two restriction fragments containing the HSE sequences of Ha hsp17.7 G4 and Ha hsp17.6 G1 were purified from 1.6% agarose gels and end labeled with Klenow and dATP (14). The Ha hsp17.6 G1 probe is a 175-nucleotide fragment containing the HSE sequences of HindIII sites at −126 and +50, and the Ha hsp17.7 G4 probe is a 287-nucleotide fragment that contains promoter sequences between −188 (EcORV) and +80 (9). This fragment was labeled at an EcoRI site from vector polylinker sequences.

Binding reactions were performed for 15 min, at 20 °C, in 10 mM Hepes, pH 7.9; 1.5 mM MgCl2; 0.05 mM EDTA; 120 mM NaCl; and 6% glycerol. Reactions included 1 ng of each labeled fragment, 1.8 µg of poly(dI-dC) (Pharmacia Biotech Inc.) and 2 µg of a protein extract, obtained from Escherichia coli BL21 cells expressing human HSF1 from plasmid pHu HSF1M (15). For competition experiments the binding reactions also included a 50-fold molar excess of the unlabeled fragments or of a synthetic, double-stranded, HSE oligonucleotide described by Hübner and Schöffl (16). The same molar excess of the 445-bp PutII fragment from pBluescript SK+ was used as negative control. Subsequent to binding of protein extracts, samples with labeled DNA were subjected to gel shift assays (17).
Plant Material, Stress Treatments, and Ribonuclease Protection Assays—Conditions for growth and heat shock treatments of whole sunflower plants and seedlings (Helianthus annuus L., cv Sunweed, Rhône Poulenc) have been described elsewhere in detail (4, 6, 18). Embryos were collected at different stages of zygotic embryogenesis under control growth conditions and analyzed as reported (6). Four days postinhibition (dpi) seedlings were treated with 100 μM abscisic acid for 24 h (6). RNase A protection assays were performed as described (19).

The RNA probes (riboprobes) were prepared by in vitro transcription with T3 RNA polymerase (19). In the case of Ha hsp17.6 G1, the 813 nucleotide riboprobe contains, in addition to 55 nucleotides from the vector (from the T3 promoter to Smal in the SK+ polylinker), the noncoding strand sequences between positions 127 and 1069 (19). Hybridizations were performed at 45 °C for 50 h in 1.3 ml of hybridization buffer. After prehybridization, hybridization, and washing under the described conditions (4, 6), filters were exposed for autoradiography.

RESULTS

Isolation of Ha hsp17.6 G1—The genomic library described in Coca et al. (9) was rescreened using as a probe the complete cDNA Ha hsp17.6 (6). A genomic clone that corresponded exactly to the transcribed sequences in the original cDNA was isolated and accordingly named Ha hsp17.6 G1. This gene encodes HSP17.6, a canonical class I sHSP that, as well as the corresponding mRNA, accumulates during zygotic embryogenesis in the absence of exogenous stress (4, 6). A 4.5-kilobase SacI fragment that contained the coding region was subcloned in plasmid SK+, and the nucleotide sequence of this region, as well as 1521 bp of 5′-flanking and 723 bp of 3′-flanking sequence, was determined on both strands of DNA (Fig. 1A and data not shown). Initiation of transcription in mRNAs from embryos was determined by primer extension using a synthetic primer from +144 to +120 in Ha hsp17.6 G1 (not shown) and confirmed by RNase A protection assays (Fig. 2; other analyses, in higher resolution gels, not shown). These two techniques detected two close transcription start sites at 35 and 43 nucleotides upstream from the initiation codon (indicated by arrows and numbers in Fig. 1A). The first of these two sites (site 1) conforms better to the consensus sequence for transcriptional initiation of plant genes (23) and is placed at a normal distance from the putative TATA box. Thus, site 1 was chosen to start numbering the hsp17.6 nucleotide sequence (Fig. 1A).

Expression Pattern of Ha hsp17.6 G1 during Zygotic Embryogenesis—RNase A protection assays with total RNA samples from staged embryos determined the accumulation patterns of these transcripts during normal zygotic embryogenesis, with messages appearing around 12 dpa and drastically increasing their abundance from 18 dpa, coincident with seed desiccation.
during late embryogenesis. Transcripts originating from the two transcription initiation sites accumulated to similar levels during late embryogenesis, indicating their functional equivalence (Fig. 2, 18 and 20 dpa). The mRNAs that accumulated during seed maturation disappeared during germination by 4 dpi (Fig. 2, seedlings, lane 1). Exogenous abscisic acid treatments of seedlings at this stage failed to induce the accumulation of the same transcripts (Fig. 2, seedlings; compare lanes 1 and 2); whereas such treatment induced accumulation of mRNAs from the homologous gene Ha hsp17.7 G4 (Fig. 2, seedlings; compare lanes 3 and 4).

Imperfect Heat Shock Elements in the Ha hsp17.6 G1 Promoter—Inspection of the sequences in the proximal promoter region of Ha hsp17.6 G1 revealed unusual characteristics when compared with the same regions of two homologous sHSP genes from sunflower, Ha hsp17.7 G4 and Ha hsp18.6 G2 (9, Fig. 1). Most noteworthy was the absence in the proximal promoter region of clearly defined arrays of potential HSEs as those described previously for Ha hsp17.7 G4, Ha hsp18.6 G2 (respectively, Fig. 1 B and C, Ref. 9), and for most plant sHSP genes (for review, see Ref. 3). These arrays normally comprise alternating nGAAn/nTTCn repeats, in which the G or C nucleotides situated at position 1 or 3 of the core repeats have been shown to be crucial for HSF binding and heat-induced activation (24, 25). A functional HSE is thought to require at least three of these perfect repeats in a proper promoter context (26). The nucleotide sequence of Ha hsp17.6 G1 showed only three alternating repeats (Fig. 1A), of which the one situated in the middle (TTT) does not conform to the consensus sequence for the core repeat at the crucial third position. A fourth imperfect repeat that fits the core consensus at the crucial first position (GAT) is placed upstream of the other three (Fig. 1A). This is a potentially correct spatial alignment, as functional HSEs can tolerate a 5-bp gap between repeats (27). The four core repeats that comprise the sole putative HSE array of Ha hsp17.6 G1 are placed at considerable distance (50 bp) upstream from the putative TATA box. This position would correspond better to that of the most 5′-distal of the two HSE arrays normally found in the proximal promoter region of other plant sHSP genes, including the Ha hsp17.7 G4 and Ha hsp18.6 G2 promoters (site II, Fig. 1, B and C, Refs. 3 and 9).

To investigate the binding potential of the putative HSE array in Ha hsp17.6 G1, we conducted mobility shift assays using hHSF1 (15) and two radiolabeled DNA fragments containing the HSE arrays of Ha hsp17.7 G4 or Ha hsp17.6 G1. Binding of hHSF1 to each fragment was compared by performing competition experiments using the same molar excesses of unlabeled fragments, including a positive control (the same fragment used as labeled probe) and a negative control (a DNA fragment from plasmid vector sequences). We detected binding of hHSF1 to a fragment containing the imperfect HSE array in Ha hsp17.6 G1 (Fig. 3, lane 2). The specificity of binding was demonstrated by competition experiments. Addition of vector DNA fragment did not affect the retarded band, whereas the same excess of either unlabeled Ha hsp17.6 G1 or Ha hsp17.7 G4 fragments affected detection of the complexes. The Ha hsp17.7 G4 fragment was the most efficient competitor (Fig. 3, lanes 3–5). This specificity was verified further by other experiments using various molar excesses of the same and other DNA fragments, including one oligonucleotide with a perfect synthetic HSE array (not shown). We also observed similar complexes using the labeled Ha hsp17.7 G4 probe, and these complexes could be competed efficiently by a 50-fold molar excess of the unlabeled Ha hsp17.7 G4 fragment (Fig. 3, lanes 7 and 10); but in contrast, the same excess of the unlabeled Ha hsp17.6 G1 fragment was as an inefficient competitor as the vector DNA fragment (Fig. 3, lanes 8 and 9). Thus, reverse competition experiments indicated that even if able to bind hHSF1 in vitro, the Ha hsp17.6 G1 sequences had a much lower affinity for this factor than the HSEs of Ha hsp17.7 G4. In conclusion, the promoter region of Ha hsp17.6 G1 contains a short and imperfect HSE array compared with other similar sHSP genes. This raised doubts over the heat induction of this promoter.

Ha hsp17.6 G1 mRNAs Do Not Accumulate in Response to Heat Shock—In previous studies, we detected by Northern hybridization the heat-induced accumulation of homologous sHSP mRNAs, using as probe the complete Ha hsp17.6 cDNA (6, 18). Because this probe is not gene-specific, we decided to investigate the heat stress expression patterns of the Ha hsp17.6 G1 mRNAs using the more sensitive technique of ribonuclease protection, which has been used successfully to investigate gene-specific expression patterns of plant sHSP genes (9, 28).

Heat-induced accumulation of the Ha hsp17.6 G1 mRNAs was investigated using total RNA samples from seedlings and plants (representative results in Figs. 4 and 5). The same RNA samples were hybridized to either Ha hsp17.7 G4 or to Ha hsp17.6 G1 riboprobes. Ha hsp17.7 G4 was used as a positive control for heat induction in sunflower (9). Messages from the
specific DNA, "Experimental Procedures"). Added as binding competitors at 50-fold molar excess (for details, see 1)
labeled

The RNA samples, processed as indicated in the legend of Fig. 2, were
Ha hsp17.6 G1

using

heat shock in various organs and at different developmental

samples from seeds (Fig. 4 A

Ha hsp17.6 G1

gene were detected only in a positive control

lanes 1–5) or in Ha hsp17.7 G4 (lanes 6–10) were subjected to mobility shift assays in agarose gels. Reactions
labeled + included hHSF1. Different unlabeled DNA fragments were
added as binding competitors at 50-fold molar excess (for details, see "Experimental Procedures"). SK, pBlueScript SK +; G1, Ha hsp17.6 G1; G4, Ha hsp17.7 G4; (−), no competitor added. The arrow indicates the specific DNA hHSF1 complexes mentioned in the text. The position of unbound DNA fragments is also marked (*, §).

FIG. 3. In vitro binding of hHSF-1 to the proximal promoter region of Ha hsp17.6 G1. Labeled DNA fragments that contain the putative HSEs in Ha hsp17.6 G1 (lanes 1–5) or in Ha hsp17.7 G4 (lanes 6–10) were subjected to mobility shift assays in agarose gels. Reactions labeled + included hHSF1. Different unlabeled DNA fragments were added as binding competitors at 50-fold molar excess (for details, see "Experimental Procedures"). SK, pBlueScript SK +; G1, Ha hsp17.6 G1; G4, Ha hsp17.7 G4; (−), no competitor added. The arrow indicates the specific DNA hHSF1 complexes mentioned in the text. The position of unbound DNA fragments is also marked (*, §).

FIG. 4. Ha hsp17.6 G1 mRNAs do not accumulate in response to heat shock in various organs and at different developmental stages. Total RNA samples were analyzed by RNase A protection, using Ha hsp17.6 G1 (panel A) or Ha hsp17.7 G4 riboprobes (panel B). The RNA samples, processed as indicated in the legend of Fig. 2, were from seeds (s; embryos at 20 dpi), seedlings at 14 dpi (lanes 1 and 2), and from leaves (lanes 3 and 4) or stems (lanes 5 and 6) of adult plants. Lanes 1, 3, and 5 correspond to RNAs from plant material at control (20 °C) growth temperature; and lanes 2, 4, and 6 are RNAs from seedlings or plants heat shocked for 2 h 30 min at 42 °C. The autoradiogram in panel A corresponds to an exposure of 4 days. Panel B depicts a shorter exposure of 20 h. Symbols are as in the legend of Fig. 2.

Ha hsp17.6 G1 gene were detected only in a positive control sample from seeds (Fig. 4A, lane s) but not in control or heat-stressed samples from seedlings and stems or leaves from adult plants (Fig. 4A, lanes 1–6). Only an almost undetectable signal was barely visible in the heat-stressed stem sample (Fig. 4A, lane 6). In contrast, the analysis of the same RNA samples with the Ha hsp17.7 G4 probe showed that messages from this gene accumulated in all organs after heat shock treatment (Fig. 4B, lanes 2, 4, and 6), which was evident even after shorter autoradiography exposures than those used for detection of Ha hsp17.6 G1 mRNAs (Fig. 4, compare panels A and B).

The observed lack of heat shock response of Ha hsp17.6 G1 could be dependent on temperature, as not all plant heat shock genes would optimally respond at the same temperature. We investigated this possibility by performing different heat shock treatments at various temperatures within natural growth conditions for sunflower. Heat-induced accumulation of Ha hsp17.6 G1 and Ha hsp17.7 G4 messages was investigated by RNase A protection as described above. The heat-induced accumulation of Ha hsp17.7 G4 messages was observed from 30 °C, reaching maximal levels at 40 °C and decreasing slightly at 45 °C (Fig. 5). The same RNA samples analyzed with the Ha hsp17.6 G1 riboprobe did not show heat shock-induced accumulation of Ha hsp17.6 G1 messages in any of the tested stress conditions (Fig. 5).

Nuclear Run-on Analysis of Ha hsp17.6 G1 Expression—the experiments described above established that the mRNAs of Ha hsp17.6 G1 do not accumulate in response to heat stress, in different organs and developmental stages, after germination of sunflower. Our observation could be explained by a peculiar transcriptional regulation of Ha hsp17.6 G1 in response to heat shock. To investigate this possibility, we carried out run-on transcription analyses with isolated nuclei from embryos at normal growth temperature and from control and heat-stressed seedlings. As a control of the transcriptional activity by RNA polymerase II, we used the detection of transcripts of sunflower polyubiquitin genes expressed in all of these experimental conditions (19). The transcripts of two heat-inducible genes, Ha hsp18.6 G2 and Ha hsp17.7 G4 (9), were used as a control for transcriptional activation by heat shock; and those of Ha hsp17.7 G4 served also as a control for transcriptional activation in embryos. The transcripts from Ha hsp17.6 G1, Ha hsp17.7 G4, and Ha hsp18.6 G2 were distinguished by hybridization with gene-specific probes. The specificity of probe hybridization was verified with Southern blot analysis using recombinant phage and sunflower genomic DNA (not shown).

Transcriptional run-on analysis confirmed the heat induction of Ha hsp17.7 G4 and Ha hsp18.6 G2 (Fig. 6, lanes 3 and 4, compare control and heat shock) but failed to detect heat shock-induced transcription from the Ha hsp17.6 G1 promoter (Fig. 6,
Fig. 6. Nuclear run-on transcriptional analysis of sHSP gene expression in sunflower seeds and seedlings. Representative hybridization of radiolabeled RNA synthesized by nuclei isolated from embryos and from seedlings growing at 20 °C (Control) or after a heat shock treatment. The three autoradiograms represent hybridization of membranes containing the same excess amount of different DNA fragments from the following sunflower genes (for details, see "Experimental Procedures"): Ha UbiS (lanes 1), Ha hsp17.6 G1 (lanes 2), Ha hsp18.6 G2 (lanes 3), and Ha hsp17.7 G4 (lanes 4). lane 2, compare control and heat shock). In embryos, however, this analysis detected transcriptional activity of Ha hsp17.6 G1 and Ha hsp17.7 G4 (Fig. 6, embryos, lanes 2 and 4), although it failed to detect significant transcription from the Ha hsp18.6 G2 promoter (Fig. 6, embryos, lane 3). In all cases the ubiquitin probe confirmed the transcriptional activity of RNA polymerase II in the isolated nuclei (Fig. 6, lane 1).

DISCUSSION

The finding of a developmentally regulated plant sHSP gene transcribed during zygotic embryogenesis, but which is not responsive to heat shock in vegetative tissues, demonstrates the evolution of the regulation in members of the sHSP gene superfamily in the plant kingdom. HSP families known to contain genes noninducible by heat shock include only those encoding large proteins, for example, the HSP70s. Some of these genes are developmentally regulated, whereas others show constitutive expression irrespective of development (i.e. Ref. 2). An accepted evolutionary scenario for the origin of constitutive HSP genes is gradual divergence from heat-inducible ancestors, as heat-induction is regarded as an ancient and well conserved trait. It is assumed that this divergence involved changes in crucial cis-elements for the heat shock response (i.e. the HSEs). The presence of relictic (low homology) HSEs is a trademark of plant constitutive HSP genes (for review, see Ref. 3 and references therein). This view fits nicely with the unusual structure and in vitro HSF binding characteristics of the HSE region in Ha hsp17.6 G1 compared with those of other plant sHSP genes, including two homologues from sunflower (Figs. 1 and 3). The putative HSE region in Ha hsp17.6 G1 (Fig. 1A) would not support either efficient HSF1 binding in vitro (Fig. 3) or heat shock-induced transcriptional activation (Fig. 6, heat shock panel). The HSEs in Ha hsp17.7 G4 show intermediate characteristics and still support heat shock induction (Figs. 1B and 6 and Ref. 9). This is indicative of less divergence from the features observed in Ha hsp18.6 G2 (Fig. 1C) and in other plant sHSP promoters (for review, see Ref. 3). Interestingly, there are precedents for a similar evolution of the regulation of α-crystallin genes in animals. These genes encode proteins (the A and B subunits of α-crystallins) expressed mostly in the eye lens. The α-crystallins are structurally related to the sHSPs and might have evolved from a common ancestor (11). Whereas most of the αB-crystallin promoters retain HSEs and heat inducibility, many αA-crystallin and some peculiar αB-crystallin promoters do not contain HSEs and are not heat-inducible (12, 13). Other work has indicated that a sHSP gene from chicken (hsp23) is not transcriptionally activated in response to heat shock in embryo cells (29). Our results show that an apparently parallel evolution has taken place in plants for other members of the sHSP superfamily, raising new questions on the origins of the differentially regulated genes.

The observation of efficient transcription from different plant sHSP promoters in seeds, regardless of the presence, or absence, of high homology HSEs puts some constraints to current models explaining the developmental regulation of these genes (9, 30). A general involvement of HSEs and of trans-acting factors similar to mammalian HSF1 does not fit the available data for the sunflower genes. Thus, transcription from the Ha hsp18.6 G2 promoter is not substantially active in zygotic embryos, despite its efficient heat induction in vegetative tissues, consistent with the presence of high homology HSEs at a proximal location (Figs. 1C and 6). In addition, Ha hsp17.6 G1 and Ha hsp17.7 G4 provide examples of transcriptional activation in embryos (Fig. 6), respectively, without and with recognizable HSEs (Fig. 1, A and B, and Fig. 3). In the case of Ha hsp17.7 G4, recent mutagenesis analyses of its HSEs determined that heat induction of this gene in vegetative tissues can be eliminated without effects on its developmental regulation during early seed maturation. However, these mutations reduced expression of Ha hsp17.7 G4 during later seed desiccation stages. In the case of Ha hsp17.6 G1, natural evolution produced much more diverged HSEs than in any of the tested Ha hsp17.7 G4 mutations; and yet Ha hsp17.6 G1, despite not responding to heat shock, is expressed efficiently during late embryogenesis. An involvement of HSEs in the developmental regulation of Ha hsp17.6 G1 during seed desiccation seems unlikely unless these elements would bind HSFs (or even different factors) with sequence specificity different from that of mammalian HSF1. The multiplicity and divergence of plant HSFs, still uncharacterized for their possible DNA binding and functional diversity (for review, see Ref. 31), could allow the specialization of some HSFs in developmental regulation, as observed similarly in animal systems (32).

Ha hsp17.6 G1 and Ha hsp17.7 G4 provide unique opportunities to investigate the molecular basis of developmental regulation of plant sHSP genes during zygotic embryogenesis. They constitute so far a unique pair of homologous genes, expressed with similar patterns in the same species, for which sequence information and preliminary characterization are available. Sequence analysis and expression data indicate that their developmental expression in seeds might be conferred by distinct mechanisms. Future work will elucidate these control mechanisms.

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