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An Imperfect Heat Shock Element and Different Upstream Sequences Are Required for the Seed-Specific Expression of a Small Heat Shock Protein Gene[1]

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Chimeric constructs containing the promoter and upstream sequences of *Ha hsp17.6 G1*, a small heat shock protein gene, reproduced in transgenic tobacco (*Nicotiana tabacum*) its unique seed-specific expression patterns previously reported in sunflower. These constructs did not respond to heat shock, but were expressed without exogenous stress during late zygotic embryogenesis coincident with seed desiccation. Site-directed mutagenesis of its distal and imperfect heat shock element strongly impaired in vitro heat shock transcription factor binding and transgene expression in seeds. Deletion analyses of upstream sequences indicated the contribution of additional cis-acting elements with either positive or negative effects on transgene expression. These results show differences in the transcriptional activation through the heat shock element of small heat shock protein gene promoters in seeds compared with the heat shock response. In addition, they suggest that heat shock transcription factors and other distinct trans-acting factors cooperate in the regulation of *Ha hsp17.6 G1* during seed desiccation.

Plant heat shock genes are not only expressed in response to heat stress, but also during zygotic embryogenesis and in other developmental stages in the absence of exogenous stress (for review, see Hightower and Nover, 1991; Schöffl et al., 1998). The regulation of heat shock gene expression during embryogenesis has been investigated for the class I small heat shock protein (sHSP) gene family that encodes cytoplasmic proteins (Waters, 1995). Studies of class I sHSP promoters showed that heat shock elements (HSEs), the cis-acting elements necessary for the heat shock response, were also involved in their regulation during zygotic embryogenesis (Coca et al., 1996; Prändl et al., 1995). Synthetic HSEs could even confer developmental regulation in plant seeds to a minimal cauliflower mosaic virus 35S promoter (Prändl and Schöffl, 1996). Site-directed mutagenesis of the sunflower *Ha hsp17.7 G4* promoter determined that HSEs are required for its developmental regulation, although only during the desiccation stages characteristic of late embryogenesis. This observation demonstrated the seed regulation of *Ha hsp 17.7 G4* by both HSE-dependent and -independent transcriptional activation mechanisms (Almoguera et al., 1998). That work also showed that the heat response of chimeric constructs containing the *Ha hsp17.7 G4* promoter and 5′-flanking sequences was abolished by point mutations that only partially affected their expression in embryos. This suggested possible differences in the HSE-mediated activation mechanism of the same sHSP promoter in response to heat stress or during development (for discussion, see Almoguera et al., 1998).

The effect of the Arabidopsis abi3 mutants on sHSP gene expression in seeds might indicate additional, although more indirect, evidence for such differences. The sHSPs expressed in seeds during embryogenesis did not accumulate to detectable amounts in the null mutant abi3-6, but the same mutation did not affect expression of these proteins in response to heat shock (Wehmeyer et al., 1996). The ABI3 gene encodes a transcription factor that regulates various seed-specific genes (Giraudat et al., 1992; Parcy et al., 1995). Thus, a possible inference from this observation would be that ABI3, together with heat shock factors (HSFs), are involved in transcriptional activation of at least some sHSP promoters in seeds. Such involvement would imply mechanisms that differ from the heat shock response.

We also have isolated and initially characterized in sunflower the mRNA accumulation patterns and seed-specific transcriptional activation of a peculiar plant sHSP gene, *Ha hsp17.6 G1*. The *Ha hsp17.6 G1* promoter is, to our knowledge, the sole example for a heat-stress-non-responsive member of the plant class I sHSP gene family. The presence of an imperfect HSE in the 5′-flanking region of *Ha hsp17.6 G1* posed an interesting interpretation dilemma. If that HSE were not functional, the promoter should be activated by mechanisms not involving HSFs. Alternatively, in the case of a functional HSE, the transcriptional activation of the *Ha hsp17.6 G1* promoter would require HSFs. In that case, activation should mechanistically differ from a typical heat shock response (for discussion, see Carranco et al., 1997). In the present work we found the answer to this dilemma by analyzing the expression effects of site-directed mutagenesis of the imperfect HSE. The HSE is indeed functional and is required for seed expression of *Ha hsp17.6 G1*. Additional deletion analyses of the 5′-flanking sequences identified other cis-acting elements with positive or negative effects on the promoter. These observations further define models for sHSP gene regulation during plant zygotic embryogenesis.

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MATERIALS AND METHODS

Site Directed Mutagenesis of the *Ha hsp17.6 G1* Promoter

We used a megaprimer PCR procedure, essentially as described by Almoguera et al. (1998), but with the following modifications. The megaprimer was a 206-bp DNA fragment that included the *Ha hsp17.6 G1* sequences between −109 and +49 (all positions given from the transcription initiation site [Carranco et al., 1997]), followed by the pBluescript SK sequences between *Hin*dIII (in the vector polylinker) and the SK (5′-TCTAGAACTAGTGGATC-3′) primer. The megaprimer was amplified after 30 cycles using an annealing temperature of 48°C and the SK and G1 mutagenic primers. The G1 mutagenic primer was: 5′-GTCCAtATAAGTACAtAATATTTCAtAACACTACTACG-3′, corresponding to the *Ha hsp17.6 G1* sequences (coding strand) between −109 and −72, with lowercase letters indicating the three nucleotide substitutions. The megaprimer and the KS (5′-CGAGGTCGACGGTATCG-3′) primer were used to amplify another 242-bp DNA fragment with the *Ha hsp17.6 G1* sequences between the *Hin*dIII sites at −126 and +49. The second PCR was for 30 cycles with annealing at 52°C. The 175-bp *Hin*dIII DNA fragment, including the mutations, was used to construct −1,486(m)::GUS (see below).

Electrophoretic Mobility Shift Assays

The conditions for DNA probe labeling and for binding and mobility shift assays in agarose gels using recombinant hHSF1 were essentially as described by Carranco et al. (1997). Binding reactions differed only in the amount of poly [dI.dC] used (4 μg/reaction) and in the presence of variable amounts of bacterial extracts containing hHSF1 (from 2–5 μg protein/reaction). The DNA probes were 175-bp *Hin*dIII DNA fragments that contained the wild-type and mutant *Ha hsp17.6 G1* sequences between −126 and +49.

Ha hsp17.6 G1::GUS Chimeric Constructs and Generation of Transgenic Plants

We constructed four *Ha hsp17.6 G1*::GUS chimeric translational fusions between position +121 of *Ha hsp17.6 G1* and the *Sma*I site in the polylinker of pBI 101.2. The *Ha hsp17.6 G1* junction sequence was in all cases an end-filled (with Klenow polymerase) *Sty*I site. The chimeric constructs −1,486::GUS, −533::GUS, and −126::GUS, respectively contained 5′-upstream sequences to the *Eco*RI (−1,486), *Xho*I (−533), and *Hin*dIII (−126) sites present in *Ha hsp17.6 G1* (Carranco et al., 1997). Each chimeric *Ha hsp17.6 G1*::GUS::nos cassette also contained different synthetic sequences placed immediately upstream of the *Ha hsp17.6 G1* sequences, and derived from the pBluescript SK polylinker coming from intermediate plasmids (details available upon request). To obtain −1,486(m)::GUS, the wild-type *Ha hsp17.6 G1* sequences between the *Hin*dIII sites at −126 and +49 were deleted and replaced by the mutant sequences in the 242-bp, *Hin*dIII digested PCR fragment (see above). The nucleotide sequence at the *Ha hsp17.6 G1*::GUS junction for all chimeric constructs, as well as the sequence and orientation of the PCR amplified fragment in −1,486(m)::GUS, was verified by dideoxy sequencing using the GUSIII primer. All DNA manipulations were carried out using previously described standard procedures (Coca et al., 1996; Sambrook et al., 1989).

The four *Ha hsp17.6 G1::GUS* translational fusions constructed in pBI 101 (see Fig. 2) were mobilized into transgenic tobacco (*Nicotianum tabacum*) with *Agrobacterium tumefaciens* using the standard leaf disc method of transformation (Horsch et al., 1985). A total of at least 10 independent primary transformants for each chimeric construct was obtained (actual numbers of analyzed plants indicated in the legends of Figs. 3–6). These plants were studied after their selection by Southern and PCR analysis (Coca et al., 1996; Almoguera et al., 1998). Such techniques showed the presence of an average of one to three copies of stable-integrated intact transgenes at different integration sites (data not shown).

Heat Stress Treatments

Transgenic and non-transgenic tobacco plants were subjected to control and heat shock treatments after clonal duplication of the individual plants, as previously described (Coca et al., 1996). For each original plant, three segregants were used in these experiments (see Fig. 5). Stem samples (a piece of approximately 5-cm length per clone) were collected from 4 cm below the apical meristem. Leaf samples included (per each clone) a complete leaf (without the petiole) removed from 5 cm below the apical meristem. For the assays with whole seedlings, we used the segregating progeny of the original transgenic plants (a pool of approximately 100 kanamycin-resistant seedlings per plant) and similar numbers of non-transgenic seedlings. The thermal stress treatments were also as described by Coca et al. (1996).

GUS Assays and Statistical Analysis of Data

Transgenic tobacco plants were produced and characterized for developmental and heat-induced GUS expression. GUS activity in seedling, leaf, stem, pollen, seed, and embryo samples from the transgenic tobacco plants was histochemically and/or fluorometrically assayed. The statistical distributions of values for plants transgenic for each chimeric construct were compared by analysis of variance (ANOVA) after logarithmic transformation of data. For a detailed description of these procedures, see Almoguera et al. (1998) and references therein.

RESULTS

Mutagenesis of the HSE in the *Ha hsp17.6 G1* Promoter: Effect on in Vitro HSF Binding

We previously demonstrated that the *Ha hsp17.6 G1* gene was not transcriptionally active in response to heat shock in sunflower, very likely because of the characteristics of its

only distal and imperfect HSE (Carranco et al., 1997). However, this HSE might be still involved in the developmental regulation of *Ha hsp17.6 G1* during late embryogenesis. We investigated this possibility by a mutagenesis approach analogous to that described for *Ha hsp17.7 G4*, another sunflower sHSP gene with a more complex HSE structure and expression pattern (Coca et al., 1996; Almoguera et al., 1998). The HSE sequences in the *Ha hsp17.6 G1* promoter (Carranco et al., 1997; Fig. 1) were altered by introducing three nucleotide substitutions (G–T) at a crucial position within the GAA core repeat (see "Materials and Methods" for details). These mutations were designed to severely impair binding of HSFs and subsequent promoter activation, as previously demonstrated for plant sHSP genes by Barros et al. (1992) and Almoguera et al. (1998).

Before performing functional analyses in transgenic plants, we verified in vitro the effect of these mutations. We analyzed binding of recombinant human HSF1 (hHSF1) to fragments of the *Ha hsp17.6 G1* promoter containing the HSE. The results of electrophoretic mobility shift assays are summarized in Figure 1. Using a fragment with unaltered HSE sequences, we detected the previously described specific hHSF1-DNA complex (Fig. 1; Carranco et al., 1997). This complex was observed with various amounts of hHSF1, and it could be abolished only by competition with DNA sequences containing the *Ha hsp17.6 G1* or more perfect HSEs (Carranco et al., 1997; data not shown). In contrast, using the fragment containing the mutant HSE, the same complex could not be detected, even with the highest amount of hHSF1 (Fig. 1). The faint, higher mobility bands observed with the mutant fragment upon long autoradiograph exposures likely represent minor nonspecific complexes, as they could be abolished by competition with excess nonspecific DNA fragments (data not shown).

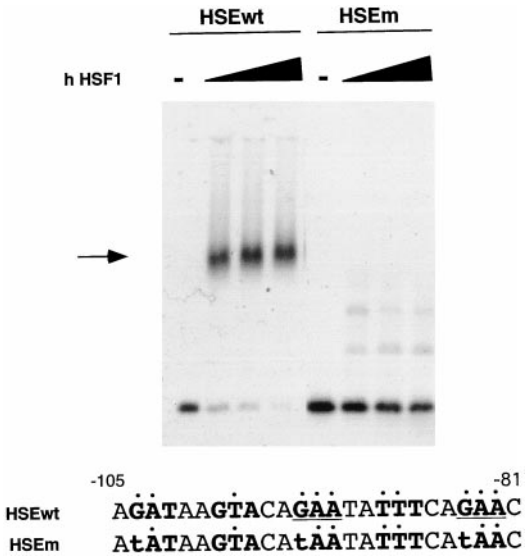

Figure 1. Electrophoretic mobility shift assays with *Ha hsp17.6 G1* probes. Top, Binding of recombinant hHSF1 to 175-bp end-labeled DNA fragments containing the wild-type (HSEwt, left lanes) or mutant (HSEm, right lanes) HSE. Binding reactions contained either no (−) or increasing amounts of hHSF1 (from left to right, 2, 4, or 5 μg, as indicated by the filled triangles for each probe). The arrow points to the specific hHSF::DNA complexes mentioned in the text. Bottom, Nucleotide sequences of the wild-type and mutant HSE. Dots on the sequence indicate agreement with GAA and TTC consensus core repeats. The two perfect (full consensus matching) core repeats are underlined. Mutations are indicated in lowercase. All sequence positions are given from the transcription initiation site of *Ha hsp17.6 G1*.

Expression Patterns of the Full-Length Ha hsp17.6 G1::GUS Construct in Transgenic Tobacco

Histochemical GUS assays in embryos dissected at different developmental stages from the −1,486::GUS transgenic plants (see Fig. 2) determined that in transgenic tobacco, the *Ha hsp17.6 G1* promoter directs the expression of this chimeric construct from 24 DPA (data not shown). The highest expression level was reached at 28 DPA (Fig. 3). A similar expression pattern was also observed in the seed endosperm (Fig. 3). These expression patterns essentially match the *Ha hsp17.6 G1* mRNA accumulation patterns coincident with late seed desiccation that were previously reported in sunflower (Carranco et al., 1997). The tobacco heterologous system thus at least reproduced the temporal expression patterns during zygotic embryogenesis of two different sunflower sHSP genes, *Ha hsp 17.6 G1* (Fig. 3) and *Ha hsp17.7 G4* (Coca et al., 1996; Almoguera et al., 1998).

Similar analyses of other tissues and organs from the −1,486::GUS transgenic plants cultivated under controlled conditions did not detect the expression of the transgene, with the exception of mature pollen grains (Figs. 3A and 6). This result was not an artifact of our GUS assay conditions (i.e. detection of similar endogenous enzyme activities in tobacco), as demonstrated by negative results using pollen from untransformed tobacco (Fig. 3B). A significant expression in pollen grains, observed both for G1::GUS (Fig. 3A) and G4::GUS (Coca et al., 1996) chimeric constructs, might reflect a natural activation of the corresponding sunflower promoters in this tissue or an ectopic expression in tobacco (for discussion, see Coca et al., 1996; for review, see Mascarenhas et al., 1996). We further analyzed the pollen expression of the G1::GUS constructs to investigate the specificity of the effect of mutations and deletions in *Ha hsp17.6 G1* regulatory sequences. These experiments, which were also used to further define the expression patterns of these genes in seeds, were carried out with more sensitive fluorometric assays using larger numbers of transgenic plants (see below).

Requirement of the Imperfect HSE for Efficient Seed Expression: Effects of Upstream Sequence Deletion

We investigated the possible involvement of the HSE in the developmental regulation of *Ha hsp17.6 G1* by comparing the expression patterns of the −1,486::GUS and −1,486(m)::GUS chimeric constructs in seeds of transgenic tobacco. The −1,486(m)::GUS construct differs only from −1,486::GUS in three point mutations at crucial positions within the HSE (Fig. 1). These mutations were incorporated in the context of a full-length promoter fusion that reproduced the developmental regulation of *Ha hsp 17.6 G1* in

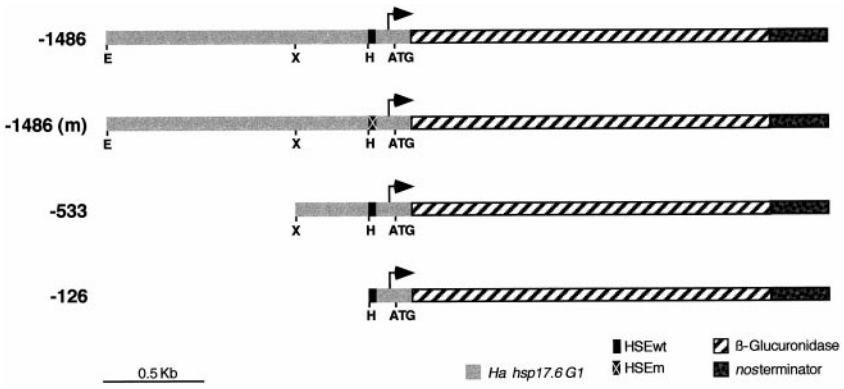

Figure 2. Maps of the *Ha hsp17.6 G1*::GUS chimeric constructs. The four translational fusions contain identical 5′-untranslated and coding sequences, as well as different upstream sequences from *Ha hsp17.6 G1* (both represented as gray boxes). The 5′-flanking ends denoted by numbers were also used for construct names: −1,486::GUS, −1,486(m)::GUS, −533::GUS, and −126::GUS. Numbers indicate the position from the *Ha hsp17.6 G1* transcription initiation site (depicted by arrows). The wild-type (HSEwt) or mutant (HSEm) HSE are indicated by small black boxes in each gene. Reference restriction sites are *Eco*RI (E), *Xho*I (X), and *Hin*dIII (H).

transgenic tobacco (Figs. 2 and 3). Fluorometric assays of GUS activity showed expression of −1,486::GUS in seeds from 20 to 28 DPA (Fig. 4). These assays also detected low expression at 16 DPA, but at levels not significantly distinct from those of non-transformed plants ($F = 3.399$, $P = 0.071$). These levels, an average of 31.13 ± 23.9 pmol methylumbelliferone (MU) mg^{-1} protein min^{-1}, were undetectable by histochemical assays (Fig. 3). Expression from the −1,486(m)::GUS gene was significantly reduced at 24 and 28 DPA ($F = 9.97$, $P = 0.002$ and $F = 18.79$, $P = 0.001$, respectively), although it was unaffected at 20 DPA ($F = 0.48$, $P = 0.49$). Histochemical GUS assays with dissected embryos and endosperm from the −1,486(m)::GUS plants did not detect GUS expression in samples from 16 to 28 DPA, confirming the more sensitive fluorometric assays (data not shown; Fig. 4A). These results revealed that the integrity of the HSE in the *Ha hsp17.6 G1* promoter is required for its developmental regulation during late zygotic embryogenesis.

The functional involvement of distal *Ha hsp17.6 G1* 5′-flanking upstream sequences was investigated by analyzing the effects of deletions with chimeric constructs

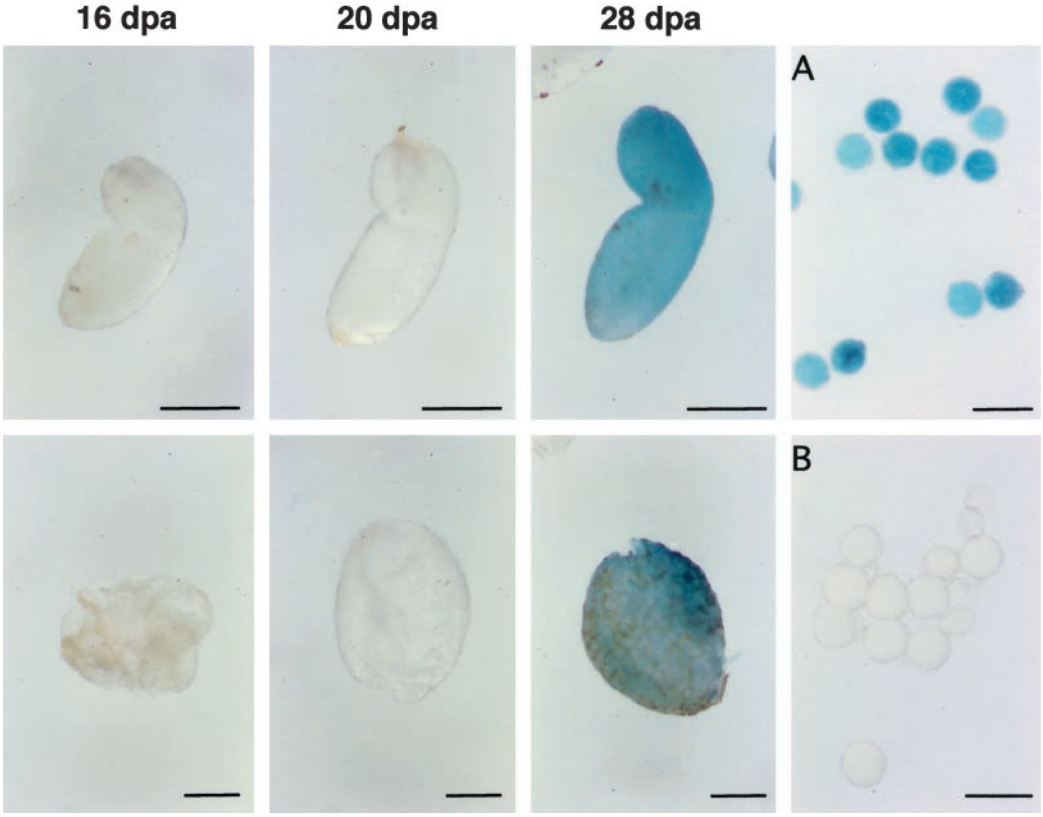

Figure 3. Histochemical localization of GUS activity in seeds and pollen of the −1,486::GUS transgenic plants. Left, Developmental stages at top correspond to either dissected embryos (top) or endosperm (bottom). Right, Pollen grains from −1,486::GUS (A) or non-transformed tobacco plants (B). A total of 12 different −1,486::GUS plants were analyzed. For seeds, samples were dissected from at least two different capsules per individual plant. Representative results are shown in each case. Histochemical reactions were for 15 h at 28°C. Scale bars correspond to either 300 μm (seed) or 40 μm (pollen).

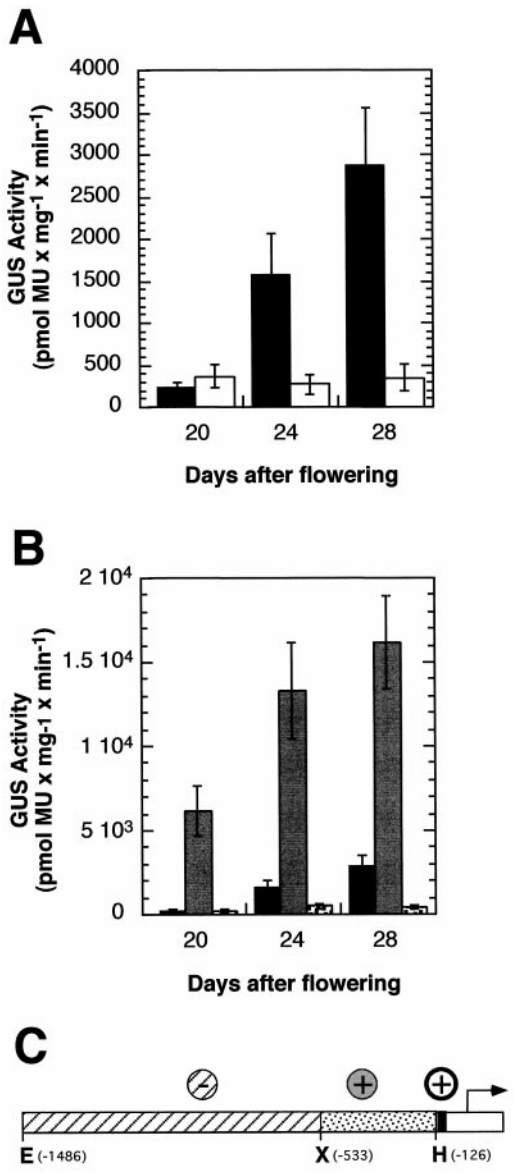

Figure 4. Fluorometric quantification of GUS activity during seed maturation in transgenic plants for the different *Ha hsp17.6 G1*::GUS chimeric constructs. A, Comparison between the expression patterns of the wild-type (■; −1,486::GUS) and mutant (□; −1,486(m)::GUS) constructs. B, Effect of the −533::GUS (▩) and −126::GUS (▨) deletions. GUS assays were performed with protein extracts prepared from whole seeds at each developmental stage. Activities are given in pmol 4-MU mg^{-1} protein min^{-1}. Individual GUS assays were performed in duplicate. The following numbers of primary transformants were analyzed per chimeric construct: −1,486::GUS, 12 plants; −1,486(m)::GUS, 13 plants; −533::GUS, 10 plants; and −126::GUS, 12 plants. Mean values and SES bars are represented. C, Summary of sequences functionally defined in this work by either mutation (HSE, solid black box) or deletion analyses (hatched and dotted boxes). We indicate the observed positive (+) and negative (−) effects on the *Ha hsp17.6 G1* promoter. Other symbols as in Figure 2.

−533::GUS and −126::GUS. Both promoter constructs contain the intact HSE and various lengths of *Ha hsp17.6 G1* upstream sequences (Fig. 2). The expression patterns in seeds of transgenic plants for −533::GUS or −126::GUS were compared with those of −1,486::GUS plants (Fig. 4B). Deletion of upstream sequences to −126 significantly affected GUS expression in seeds at 24 DPA ($F = 4.51$, $P = 0.037$) and 28 DPA ($F = 9.32$, $P = 0.003$), but not at 20 DPA ($F = 0.04$, $P = 0.84$). Thus, the effects of either the HSE mutation or this deletion were similar, both resulting in poor reporter gene expression levels during seed desiccation (see above; Fig. 4A).

These results demonstrated that the HSE by itself is not sufficient to activate the *Ha hsp17.6 G1* promoter from 24 DPA. In contrast, deletion of upstream sequences to −533 in chimeric construct −533::GUS resulted in substantially higher levels (5.6- to 25.8-fold) of reporter gene expression at 20 ($F = 38.99$, $P = 0.0001$), 24 ($F = 23.36$, $P = 0.001$), and 28 ($F = 13.45$, $P = 0.004$) DPA, compared with −1,486::GUS (Fig. 4B). In summary, we determined that distinct *Ha hsp17.6 G1* upstream sequences contain cis-acting elements involved in the temporal and quantitative regulation of seed expression during embryogenesis (Fig. 4C).

Lack of Heat Shock Response of G1::GUS Chimeric Constructs in Transgenic Tobacco

Previously reported gene-specific RNase A protection and nuclear run-on assays determined that in sunflower the *Ha hsp17.6 G1* mRNAs do not accumulate in response to heat shock, mainly because the promoter is transcriptionally inactive under heat stress, at least in seedlings (Carranco et al., 1997). We tested the heat shock response of the different G1::GUS chimeric constructs in transgenic tobacco. This heterologous system has been successfully used to reproduce the heat stress response of a different sHSP sunflower promoter, *Ha hsp17.7 G4*, which also contains imperfect, although more complex, proximal and distal HSEs (Coca et al., 1996). In experiments performed with whole plants or seedlings containing the −1,486::GUS construct, fluorometric assays revealed only insignificant levels of GUS activity under control or heat shock treatments. That activity was similar in magnitude to that in nontransgenic plants. Similar results were obtained in different organs and developmental stages after imbibition (Fig. 5).

The same results were observed with transgenic plants containing the −1,486(m), −533, or −126 chimeric constructs. Furthermore, these experiments showed that in vegetative tissues the −533 and −126 5′-deletions did not have any effect on either the basal or the heat-induced GUS activities. As previously observed (Coca et al., 1996), we were able to detect heat-shock-induced GUS activity from another chimeric construct with *Ha hsp17.7 G4* sequences, in stems of transgenic tobacco plants (Fig. 5, G4). However, in seedlings, we observed the heat-induced accumulation of the chimeric Ha hsp17.7 G4::GUS mRNA (data not shown). The latter is consistent with the reported accumulation of the *Ha hsp17.7 G4* mRNA in sunflower seedlings (Carranco et al., 1997). In contrast, the *Ha hsp17.6 G1* mRNAs did not accumulate in response to heat shock in either sunflower seedlings or different adult organs under various stress conditions (Carranco et al., 1997). Thus, the results in Figure 5 agree with the lack of heat-shock-

induced transcriptional activation of the *Ha hsp17.6 G1* promoter in seedlings and with the absence of heat-induced *Ha hsp17.6 G1* mRNA accumulation in other organs (Carranco et al., 1997).

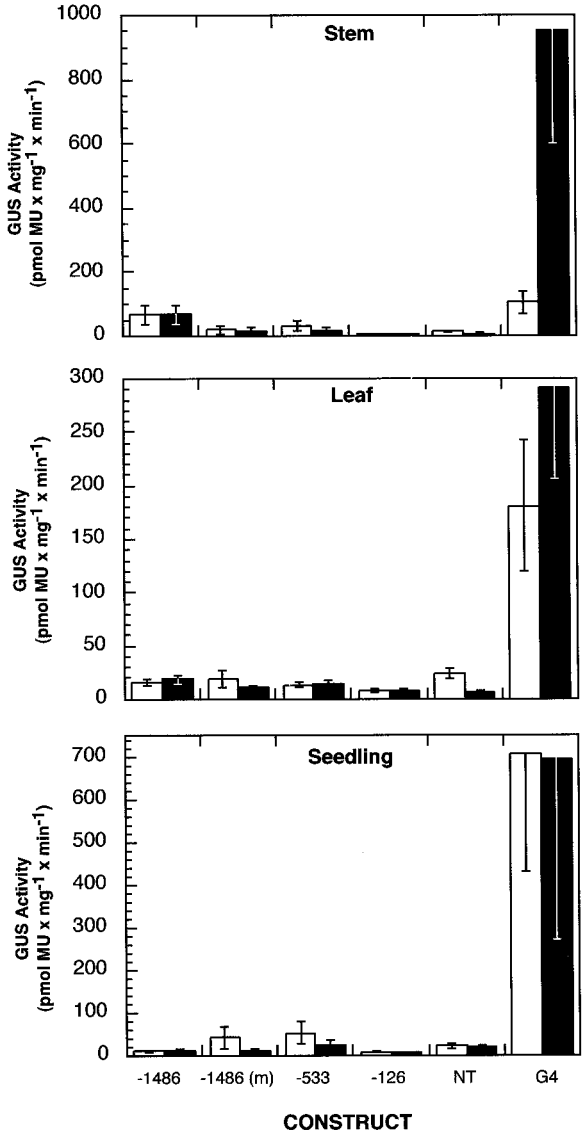

Figure 5. Absence of GUS activity in vegetative tissues of transgenic plants. Data in this figure correspond to the progeny of a subset of the original transgenic plants analyzed in Figures 3 and 4. These plants showed similar seed expression patterns as their parents (data not shown). We analyzed protein extracts from stems and leaves of adult plants (2 months post imbibition, top) or from whole seedlings at earlier developmental stages (20 d post imbibition, bottom). Samples from non-transgenic tobacco (NT) were used as a reference for basal levels of GUS activity. Plants containing a chimeric construct with the *Ha hsp17.7 G4* sequences between −1,132 and +163 (G4, Coca et al., 1996) were used as a positive control for heat induction in the stem samples. The following numbers of plants were used (for denominations see also Fig. 2): −1,486, −1,486(m), −533, and −126, five plants each; G4, three plants; and NT, six plants. Values for control (white bars), and heat shock induced (black bars) GUS activities are represented as indicated in the legend of Figure 4.

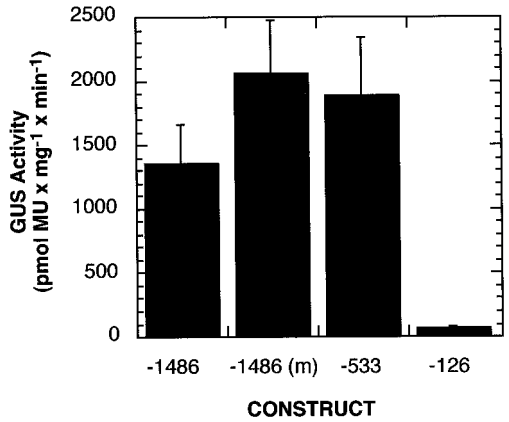

Figure 6. Quantification of GUS activity in pollen grains. GUS assays were performed with protein extracts prepared from pollen from the following numbers of independent transgenic plants for each chimeric construct: −1,486, 11; −1,486(m), 13; −533, 14; and −126, 11. GUS activities are represented as indicated in the legend of Figure 4. For chimeric construct denomination see Figure 2.

Distal Sequences of *Ha hsp17.6 G1* Promoter Show Different Specificity in Seeds and Pollen

The results in Figure 5 also suggested that the effects of the tested HSE mutation and 5′-flanking deletions were seed specific. For example, in the −533 deletion, we did not observe increased levels of GUS activity in different organs of corresponding transgenic plants. The specificity of the deletion and mutation effects was further verified by fluorometric quantification of GUS activities in pollen of the −1,486::GUS, −1,486(m)::GUS, −533::GUS, and −126::GUS plants (Fig. 6). The average GUS activity in pollen of the −1,486::GUS plants was 1,361 ± 296 pmol MU mg^{-1} min^{-1}. Compared with this value, only that for the −126::GUS plants was significantly reduced (62.8 ± 13.1 pmol MU mg^{-1} min^{-1}, $F = 5.77$, $P = 0.02$). This result confirmed the seed specificity of the negative and positive effects, respectively, observed for the HSE mutation and the −533 deletion. In contrast to results with the other chimeric constructs, the clear effect of the −126 deletion in pollen indicated that sequences between −126 and −533 contain positive cis-acting elements that might function not only in seeds but also in pollen (compare Figs. 4 and 6).

DISCUSSION

The HSE in the *Ha hsp17.6 G1* Promoter Is Required for Developmental Regulation in Seeds

The *Ha hsp17.6 G1* promoter contains a HSE, which, compared with those in other plant sHSP genes (including two sunflower promoters), has unique structural characteristics (Carranco et al., 1997). This element could be considered as a relic resembling similar regulatory sequences found in constitutive HSP genes from other families (i.e. HSP70; for review, see Gurley and Key, 1991). Among the structural characteristics of this HSE are its relative distal position from the TATA box and the presence of only five HSE core motifs, only two of which are perfect. The perfect

core motifs are adjacent to imperfect ones (Fig. 1). The second core repeat is the most imperfect and it could either be regarded as a five-nucleotide gap (Carranco et al., 1997) or as a very low homology core repeat with only one conserved position in the TTC sequence (Fig. 1).

The *Ha hsp 17.6 G1* HSE lacks other more proximal HSE core motifs present, for example, in other promoters as *Ha hsp17.7 G4* (Carranco et al., 1997). Despite this structure, previous results indicated that the *Ha hsp17.6 G1* HSE could be a functional regulatory element. Thus, we showed that in vitro it was able to bind recombinant hHSF1, although with lower affinity than the more extended, complex, and perfect HSEs of *Ha hsp17.7 G4* (Carranco et al., 1997; see also Fig. 1). By introducing three very specific nucleotide substitutions in the HSE of *Ha hsp17.6 G1* (Fig. 1; Barros et al., 1992; Almoguera et al., 1998), we have been able now to abolish the in vitro binding of hHSF1 (Fig. 1). The same mutations drastically impaired expression from the *Ha hsp17.6 G1* promoter in desiccating seeds (Fig. 4A). These results demonstrate the necessity of the HSE for the regulation of *Ha hsp17.6 G1* during late embryogenesis.

The peculiar architecture of the HSE in *Ha hsp17.6 G1*, and perhaps of other imperfect HSEs as those in *Ha hsp17.7 G4* (Coca et al., 1996), might contribute to differences in an HSF-mediated transcription activation mechanism. The HSE in *Ha hsp17.6 G1* has a distal location compared with more proximal elements located in other plant sHSP genes, including different sunflower promoters (Gurley and Key, 1991; Carranco et al., 1997). The fact that this HSE is functional during embryogenesis (Fig. 4A) and its failure to support heat shock induction (Carranco et al., 1997; Fig. 5) might reflect differences in the effect of distance from the HSE to the initiation site. Heat shock induction in vegetative tissues appears to be more dependent on the presence of more proximal HSEs in sunflower (Carranco et al., 1997; Almoguera et al., 1998) and other plant sHSP promoters (Gurley and Key, 1991; Marrs and Sinibaldi, 1997, and refs. therein). In contrast, distal HSEs are required for (this work, Fig. 4A) or substantially contribute to (Almoguera et al., 1998) developmental regulation. Another interesting possibility is that, as observed in yeast, the imperfect structure of HSEs could influence the conformation of DNA-bound HSF(s) and subsequent promoter activation (Santoro et al., 1998).

Our observations support models explaining the activation in seeds of the *Ha hsp17.6 G1* promoter with participation of HSF(s). As previously proposed for other sHSP gene promoters, such HSF(s) would have a crucial role in promoter activation (Prändl et al., 1995; Coca et al., 1996; Prändl and Schöffl, 1996; Almoguera et al., 1998). In the case of *Ha hsp17.6 G1*, the involved HSFs might differ in their sequence specificity from those involved in the heat shock response of other plant sHSP genes (previously discussed by Carranco et al. [1997]). However, based on the results reported here (Figs. 2–6), we propose that the promoter context (the structure of HSE and its functional interaction with other cis-elements) is perhaps the most crucial factor for promoter activation by HSFs during zygotic embryogenesis, at least for *Ha hsp17.6 G1*.

Additional cis-Acting Elements Contribute to the Developmental Regulation of *Ha hsp17.6 G1*

The effects of 5′-flanking sequence deletions (Figs. 2 and 4B) indicated the existence of other cis-acting elements different from the HSE and located upstream of it. These elements have either positive or negative quantitative effects that modulate the seed-specific expression of the *Ha hsp17.6 G1* promoter (summarized in Fig. 4C). The HSE, although necessary for temporal and quantitative developmental regulation, is not sufficient for full induction of the *Ha hsp17.6 G1* promoter (see results for −126::GUS in Fig. 4B). We observed a synergism for promoter activation between the HSE and other positive cis-elements located between −126 and −533 (Fig. 4). This might indicate direct or indirect functional interaction(s) among proteins binding to these cis-elements.

We propose that the distal cis-acting elements and unidentified trans-acting factors that interact with them cooperate with HSFs in the developmental regulation of this promoter. A conceivable scenario for such hypothetical interaction is that the HSFs could reach only limiting concentrations in developing embryos. The interaction of such HSFs with the *Ha hsp17.6 G1* promoter could be facilitated by accessory, seed-specific factors that would bind to more distal promoter sequences. This accessory factor(s) could also facilitate other crucial interactions, as cooperative interactions between distally bound HSFs and TFIID at the TATAA sequence. In vegetative tissues the HSE would be too imperfect and distal to support heat induction of the promoter in absence of the seed-specific accessory factors. The trans-acting factor(s) with negative effects on *Ha hsp17.6 G1* promoter activation would balance the activity of those with positive effects on the same promoter. At the desiccation stages of embryogenesis, the action of positive factor(s) and cooperation with HSFs would be dominant. Earlier in embryogenesis, the negative factor(s) would repress the promoter. In other sHSP promoters efficiently expressed before desiccation (e.g. *Ha hsp 17.7 G4*; Coca et al., 1996; Almoguera et al., 1998), the negative factor(s) would not bind the promoter, or additional factors would compensate for their effects and allow promoter activation at these stages.

Our hypothesis could be extended to other plant sHSP promoters and help to explain the paradox of their differential transcriptional activation during embryogenesis (for discussion, see Carranco et al., 1997) despite the presence of functional HSEs (Carranco et al., 1997; this work, Fig. 4A). Crucial aspects of this hypothesis are that in addition to the HSE, other distinct cis-acting elements are also required, and that both work in concert in promoter activation. Because not all sHSP promoters are active during embryogenesis, the HSEs would not be sufficient for developmental regulation in the context of a natural promoter. However, out of context, even a multimerized HSE (20 copies of synthetic core sequences) was shown to activate transcription from a minimal cauliflower mosaic virus 35S promoter in seeds (Prändl and Schöffl, 1996). However, 5′-deletions of the *Gm hsp17.3B* promoter in its natural context revealed that the truncation to −237 position was

not active in developing seeds, nor was it heat inducible in leaves, despite the presence of nine perfect HSE core repeats (Prändl and Schöffl, 1996). These results agree with our observations of the requirement, but insufficiency, of HSE for the developmental regulation of *Ha hsp 17.6 G1*. Whereas additional cis-elements necessary for the developmental regulation of *Gm hsp17.3B* might include other distal HSE core repeats (Prändl and Schöffl, 1996), in *Ha hsp17.6 G1* the distal sequences do not include HSEs (Carranco et al., 1997; Figs. 2 and 4).

Inferences from the previously discussed observations with plant sHSP gene promoters would be also comparable to the regulation of the yeast HSP82 promoter during early meiotic induction (Szent-Gyorgyi, 1995). HSEs are also required for promoter activation and are even able to confer meiotic induction to a different promoter. However, not all yeast HSP promoters are meiotically induced, and this induction requires functional interaction between proteins binding the HSEs and an upstream repression sequence (URS1; Szent-Gyorgyi, 1995). The activation of the *Ha hsp 17.6 G1* promoter during embryogenesis differs, however, from the meiotic induction of HSP82. Regulation of *Ha hsp17.6 G1*, mediated by sequences between −533 and −1,486, would be seed specific, as these sequences are not involved in negative regulation in pollen or vegetative tissues (Figs. 5 and 6). In contrast, URS1 functions in yeast as both a vegetative repressor and a meiotic coactivator (Szent-Gyorgyi, 1995).

This work did no attempt to directly identify the transacting factors involved in the regulation of *Ha hsp17.6 G1* promoter. However, the characterization of the HSE as an imperfect but functional cis-acting element and a preliminary delimitation of other positive and negative cis-acting elements allowed us to further define models of developmental regulation of plant sHSP genes. Our results will also help the eventual isolation and characterization of these unknown factors.

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