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The Expression of Small Heat Shock Proteins in Seeds Responds to Discrete Developmental Signals and Suggests a General Protective Role in Desiccation Tolerance[1]

Nadja Wehmeyer and Elizabeth Vierling*

Department of Biochemistry (N.W., E.V.) and Department of Molecular and Cellular Biology (E.V.), University of Arizona, Tucson, Arizona 85721

To learn more about the function and regulation of small heat shock proteins (sHSPs) during seed development, we studied sHSP expression in wild-type and seed maturation mutants of Arabidopsis by western analysis and using an HSP17.4 promoter-driven β-glucuronidase (GUS) reporter gene in transgenic plants. In the absence of stress, GUS activity increases during development until the entire embryo is stained before desiccation. Heat-stressed embryos stained for GUS at all stages, including early stages that showed no detectable HSP17.4::GUS activity without heat. Examination of HSP17.4 expression in seeds of the transcriptional activator mutants *abi3-6*, *fus3-3* (AIMS no. CS8014/N8014), and *lec1-2* (AIMS no. CS2922/N2922) showed that protein and HSP17.4::GUS activity were highly reduced in *fus3-3* and *lec1-2* and undetectable in *abi3-6* seeds. In contrast, heat-stressed *abi3-6*, *fus3-3*, and *lec1-2* seeds stained for GUS activity throughout the embryo. These data indicate that there is distinct developmental and stress regulation of HSP17.4, and imply that ABI3 activates HSP17.4 transcription during development. Quantitation of sHSP protein in desiccation-intolerant seeds of *abi3-6*, *fus3-3*, *lec1-2*, and *line24* showed that all had <2% of wild-type HSP17.4 levels. In contrast, the desiccation-tolerant but embryo-defective mutants *emb266* (AIMS no. CS3049/N3049) and *lec2-1* (AIMS no. CS2728/N2728) had wild-type levels of HSP17.4. These data correlate a reduction in sHSPs with desiccation intolerance and suggest that sHSPs have a general protective role throughout the seed.

Virtually all organisms respond to high-temperature conditions with the synthesis of small heat shock proteins (sHSPs). These ubiquitous proteins have monomeric molecular masses of 15 to 42 kD, but assemble into oligomers of nine to over 30 subunits depending on the protein (Vierling, 1997). In plants, sHSPs are generally undetectable in vegetative tissues in the absence of stress, but are among the most abundant proteins synthesized in response to high temperature. The plant sHSPs can be divided into five nuclear-encoded gene families based on DNA sequence analysis, immunological cross-reactivity, and intracellular localization (Waters et al., 1996). There are two classes of sHSPs that localize to the cytosol (classes I and II), and distinct classes of organelle-localized sHSPs found in the endoplasmic reticulum, the mitochondrion, or the chloroplast. Although in vivo data are still lacking, recent experiments in vitro suggest that cytosolic sHSPs function as molecular chaperones by preventing the thermal aggregation of substrate proteins and facilitating their subsequent reactivation (Lee et al., 1995, 1997).

It is now well established that in addition to being synthesized in response to stress, sHSPs are also expressed during specific stages of plant development. Induction of sHSPs in the absence of stress has been seen in a variety of plant species at several different developmental stages (for review, see Waters et al., 1996). sHSP expression during seed development is the most extensively characterized example of this non-stress regulation. In Arabidopsis embryos, cytosolic class I sHSPs begin to accumulate at mid-maturation and are abundant throughout the late maturation program and in the dry seed (Wehmeyer et al., 1996). In pea, class I and class II sHSPs appear in embryos during reserve synthesis at mid-maturation and increase in abundance as the seed dehydrates (DeRocher and Vierling, 1994). A similar accumulation of class II sHSPs is seen in sunflower seeds, while the class I sHSPs accumulate later in the seed maturation program (Coca et al., 1994).

A study of tomato, *Nicotiana rustica*, maize, pea, and fava bean reported that the onset of sHSP accumulation occurred at different times after anthesis, however sHSP expression was always observed significantly before discernible seed desiccation (zur Nieden et al., 1995). During germination, the developmentally regulated sHSPs are relatively abundant for the first few days and then decline quickly (Coca et al., 1994; DeRocher and Vierling, 1994; zur Nieden et al., 1995; Wehmeyer et al., 1996). Generally, only a subset of the class I and class II sHSPs are developmentally regulated, suggesting that these sHSPs have distinct regulatory controls and possibly distinct functions during seed maturation as opposed to during heat stress. Supporting this idea, developing seeds can mount a full heat shock response with the expression of all of the heat-inducible class I and class II sHSPs (DeRocher and Vierling, 1994).

Control of sHSP expression in seeds in the absence of heat stress has been investigated using promoter/reporter constructs in transgenic plants. A comparison of heat inducibility and developmental β-glucuronidase (GUS) expression controlled by the *Gmhsp17.3B* promoter in trans-

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* Corresponding author; e-mail vierling@u.arizona.edu; fax 520–621–3709.

genic tobacco implied a co-localization of the promoter elements responsible for both types of control (Prändl and Schöffl, 1996). Thus, it was suggested that a heat shock element (HSE)/heat shock factor (HSF) complex could also be responsible for the developmental and the heat-inducible transcription of these genes. In contrast, investigation with the *Hahsp17.7* G4 promoter suggested that two distinct regulatory mechanisms were activated during seed maturation (Almoguera et al., 1998). At the onset of sHSP expression in the seed, regulation appeared to be independent of the HSE and, presumably, of the HSF. In the mature seed, however, the HSE was necessary for activation of the promoter, suggesting HSF regulation of sHSPs during seed maturation. There has been only a single study using sHSP promoter/reporter gene analysis in a homologous system, a study of HSP18.2 expression in Arabidopsis (Takahashi et al., 1992). Interestingly, HSP18.2, which is strongly heat regulated in vegetative tissues, is not expressed during seed development, providing additional evidence that HSE/HSF alone are insufficient for developmental regulation.

Due to the pattern of sHSP accumulation in the late part of seed maturation, we and others have hypothesized that sHSPs function in one or more processes characteristic of this developmental stage (Coca et al., 1994; DeRocher and Vierling, 1994; Wehmeyer et al., 1996). This is a unique time of development for the embryo, as a complete developmental arrest must be achieved, yet the embryo must remain viable. Late seed development is characterized by the acquisition of dormancy and desiccation tolerance. Dormancy, which sets certain environmental cues in the seed to prevent inopportune germination, has been extensively studied, but the regulatory pathways are just now being characterized, (for review, see Leung and Giraudat, 1998).

Desiccation tolerance allows the seed to be dried and stored for an extended period, yet retain viability (Ingram and Bartels, 1996). The molecular and biochemical events that govern these processes are incompletely defined, although LEA (late embryogenic abundant) proteins have been hypothesized to be involved (Finkelstein, 1993; Ingram and Bartels, 1996; Kermode, 1997). Several studies have speculated that sHSPs may function to protect cellular components during seed desiccation and/or during rehydration (Coca et al., 1994; DeRocher and Vierling, 1994; Alamillo et al., 1995; Wehmeyer et al., 1996). Supporting this, sHSPs that are developmentally regulated in sunflower seeds are also regulated in response to water stress (Almoguera and Jordano, 1992; Coca et al., 1996), and in the resurrection plant *Craterostigma plantagineum*, vegetative tissues express sHSPs in response to dehydration (Alamillo et al., 1995). However, in leaves of Arabidopsis, class I sHSPs are not detected in response to water stress (Wehmeyer et al., 1996).

Our analysis of sHSP levels in several seed maturation mutants of Arabidopsis supports the idea that sHSPs are not sufficient for, but could be necessary for, dormancy, and that sHSPs may be essential for desiccation tolerance. We found that sHSPs are produced at wild-type levels in mature seeds of several reduced dormancy mutants (*aba1*, *abi1*, *abi2*, *abi4*, and *abi5*), whereas a desiccation-intolerant null allele of abi3 (*abi3-6*) had undetectable levels of sHSPs in the mature seed (Wehmeyer et al., 1996). These data correlate a reduction in sHSP protein levels with the desiccation intolerance phenotype, and suggest that sHSPs may be among several factors required for desiccation tolerance. As ABI3 is believed to be a transcriptional activator (Giraudat et al., 1992), these data also implicated ABI3 in the regulation of sHSP gene expression in seeds. However, the *abi3-6* allele has pleiotropic effects on seed maturation, so failure to express sHSPs could be an indirect effect of this mutation.

To gain additional insight as to the function and regulation of sHSPs in seeds, we have examined sHSP gene transcription during seed development and heat stress using an Arabidopsis sHSP promoter/GUS fusion (AtHSP17.4::GUS) transformed into Arabidopsis. AtHSP17.4 is the most highly expressed sHSP gene during seed development in Arabidopsis (Wehmeyer et al., 1996). We then tested sHSP expression in mutants of other transcriptional activators required for seed development and desiccation tolerance, *fus3-3* (Keith et al., 1994; Luerssen et al., 1998) and *lec1-2* (West et al., 1994; Lotan et al., 1998), to determine if their effects were similar or different from that of *abi3*. Finally, we analyzed sHSP accumulation in an additional desiccation-intolerant mutant, *line24* (K. Yamagishi and J. Harada, personal communication), and two desiccation-tolerant mutants with severe defects in embryogenesis, *lec2-1* (Meinke et al., 1994) and *emb266* (Vernon and Meinke, 1995). This expanded analysis of seed development mutants further supports a function for sHSPs in desiccation tolerance, and provides direct evidence that ABI3 is required for transcriptional activation of HSP17.4 in seeds.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis Heynh. ecotype Nössen was used for in planta transformation and was the standard wild type for the development experiments. Seed development mutants used in this work are listed in Table I, which includes ecotype and information about phenotype. Homozygous seeds of the desiccation-intolerant mutants *abi3-6*, *lec1-2*, and *fus3-3* were maintained by propagation of green seeds, prior to desiccation, on plates (Haughn and Somerville, 1986). Mutants that could not be propagated as homozygotes due to more severe embryo defects (*line24, lec2-1*, and *emb266*) were maintained as heterozygotes. *Line24* was also desiccation intolerant, and germinated prior to desiccation. The presence of a T-DNA insert in *line24*, *lec2-1*, and *emb266* allowed selection for the mutation by resistance to kanamycin. For these recessive mutations the visible phenotype of the homozygous seed (detailed in Table I) was readily distinguishable from wild-type and heterozygous seeds. All plants were grown in a growth chamber on a 18°C/16°C, 16-h/8-h day/night cycle. Seeds were collected from 3- to 4-week-old plants. The light intensity was approximately 300 μmol m^{-2} s^{-1}.

Heat stress was imposed as previously described (Chen et al., 1990) and the experiment was performed identically

Table I. *Seed mutants analyzed for HSP17.4 expression*

Mutant	Ecotype	Desiccation Tolerance	Other Seed Phenotypes[a]	Reference	Gene Homology
fus 3-3	Columbia	Intolerant	Anthocyanin accumulation	Keith et al. (1994)	Transcription factor VP1 homology (Luerssen et al., 1998)
lec1-2	WS[b]	Intolerant	Arrested development anthocyanin accumulation	West et al. (1994)	Transcription factor HAP3 homology (Lotan et al., 1998)
abi3-6	Columbia	Intolerant	Green seed	Nambara et al. (1994)	Homology with VP1 (Giraudat et al., 1992)
lec2-1	WS	Tolerant	Arrested development anthocyanin accumulation	Meinke et al. (1994)	Unknown (na)
line24	WS	Intolerant	Arrested development	K. Yamagishi and J. Harada (personal communication)	Unknown (na)
emb266	WS	Tolerant	Embryo defective	Vernon and Meinke (1995)	Unknown (na)
abi3-1	Landsberg	Tolerant	Reduced dormancy	Koornneef et al. (1984)	Homology with VP1 (Giraudat et al., 1992)

[a] Both desiccation tolerant and intolerant plants have many other seed and embryo defective phenotypes that are not detailed here. [b] WS, Wassilewskija.

for both leaf and seed samples. The growth chamber temperature was increased at 4°C/h up to the 38°C stress temperature, which was maintained for 4 h, and then the temperature was decreased at the rate of 4°C/h back to 22°C. High humidity was maintained during the heat stress to prevent transpirational cooling. Samples were processed for protein isolation or measurement of GUS activity immediately after the chamber temperature had returned to 22°C.

The Arabidopsis seed development profile was established as previously described (Wehmeyer et al., 1996). Arabidopsis plants were grown until 2 weeks after the onset of flowering, after which opening flowers were tagged to denote the day of pollination. Siliques were removed at the indicated days after pollination (DAP), and either seeds or embryos were immediately processed for SDS-PAGE and western analysis or for histochemical staining of GUS activity, as described below.

Construction of HSP17.4 Promoter::GUS Fusion Vector

The gene for GUS (*uidA*) and the NOS terminator were cut from the pBI101.2 vector (CLONTECH, Palo Alto, CA) with *Eco*RI and *Bam*HI and ligated into the pPZP221 binary transformation vector (GenBank accession no. U10491) that carries the *aacC1* gene, which encodes gentamycin acetyltransferase, conferring gentamycin resistance for selection of transformed plants (Hajdukiewicz et al., 1994). Approximately 1,200 bp of the HSP17.4 promoter was removed from the genomic clone (accession no. X17293) (Takahashi and Komeda, 1989) with *Xba*I and *Bam*HI and ligated into the transformation vector at these sites. This fragment included 69 bp of the coding region of HSP17.4, creating a translational fusion with the GUS gene, which was verified by DNA sequencing.

Generation of Transgenic Plants and Histochemical Staining for GUS Activity

The *Agrobacterium tumefaciens* strain C58CIRif^R, containing the Ti plasmid pGV3101 (Van Larebeke et al., 1974), was transformed with HSP17.4::GUS in pPZP221. *A. tumefaciens* cells were transformed by electroporation (Mozo and Hooykaas, 1991). The transformation of Arabidopsis (ecotype Nössen) was performed with the vacuum infiltration method (Bechtold and Pelletier, 1998). Plants were grown to maturity, and seeds harvested from individual gentamycin-resistant plants were classified as independent transformants. These seeds were designated the T_1 population.

T_2 seeds from eight independent transformants were analyzed for GUS activity (Jefferson et al., 1987). Two lines with strong GUS activity and single inserts (based on segregation and Southern analysis, data not shown) were chosen for the experiments. GUS staining was performed at room temperature for 4 h to avoid inadvertent activation of the HSP17.4 promoter due to heat stress. If the tissue samples had already been subjected to a heat stress, then the incubation for GUS activity was conducted at 37°C for 30 min to 1 h, unless otherwise indicated. Leaf tissues of T_1 and T_2 plants were stained for GUS activity in a similar manner as the embryo. After 4 h of incubation with X-Gluc at room temperature, tissue was cleared of chlorophyll by repeated 10-min washes in 70% (v/v) ethanol. Quantitative GUS activity measurements utilized the GUS-Light reporter gene assay system from Tropix (Bedford, MA). The luminescence measurement was integrated over 5 s on a luminometer (TD20/20, Turner Designs, Sunnyvale, CA).

Crosses of HSP17.4::GUS Transgenics to Seed Transcriptional Activator Mutants

Flowers were emasculated by removal of anthers for the reduced dormancy mutant *abi3-1* (AIMS no. CS24/NW24) (Koornneef et al., 1984) and the desiccation-intolerant mutants *fus3-3*, *lec1-2*, and *abi3-6*. Pollen from a homozygous HSP17.4::GUS plant was used to pollinate the mutant flowers. Whether the cross was successful was immediately apparent in the F_1 seeds of the desiccation-intolerant mutants because of the loss of the recessive mutant phenotypes: *abi3-6* seeds do not lose chlorophyll and therefore appear green, *fus3-3* seeds accumulate anthocyanins, and

seeds from *lec1-2* accumulate anthocyanins and arrest in the early cotyledon stage of embryo development. These phenotypes reappear in Mendelian ratio in the F_1 self (F_2 seed). Only F_2 seeds with the appropriate mutant seed phenotypes were planted for eventual GUS activity analysis. GUS staining was performed as described above.

Seeds homozygous for *abi3-1* had no visual seed phenotype. Instead, *abi3-1* homozygous mutant seeds were recovered from the F_2 based on decreased sensitivity of germination on abscisic acid (ABA). F_2 seeds were germinated on 3 μM ABA, and seedlings were transferred to soil after 4 d. Leaf tissues from both F_1 and F_2 plants, as well as F_2 and F_3 embryos, were heat-stressed as described above.

Microscopy and Photography

All photographs were taken at 40× magnification with a camera (N50, Nikon, Tokyo) mounted on a dissecting microscope (WILD M32, Heerbrugg, Switzerland) utilizing Ektachrome 160T film. Images were scanned and processed with Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA).

Protein Electrophoresis and Western Analysis

Total leaf or seed protein was extracted in SDS sample buffer (60 mM Tris-HCl, pH 8.0, 60 mM dithiothreitol, 2.0% [w/v] SDS, 15% [w/v] Suc, 5 mM ϵ-amino-*N*-caproic acid, and 1 mM benzamidine) at a ratio of 1.0 mL per 0.1 g fresh weight of leaf tissue, or 1.0 mL per 0.05 g of dry seed, in a ground glass homogenizer. The protein concentration was measured using a Coomassie Blue dye binding assay (Ghosh et al., 1988). Samples were separated on 14% (w/v) acrylamide gels in the presence of SDS. Two-dimensional gel electrophoresis was performed as previously described (Wehmeyer et al., 1996). Protein samples were precipitated from SDS sample buffer in 5 volumes of 0.1 M ammonium acetate in methanol for 1 h on ice. The precipitate was washed four times with 80% (v/v) acetone, and then resuspended in sample buffer containing 9.5 M urea, 2% (v/v) NP-40, 5% (v/v) 2-mercaptoethanol, and 2% (v/v) ampholines (pH = 3.5–10 and pH = 5–7). Protein samples were analyzed on a two-dimensional gel unit (Mighty Small, Hoefer, San Francisco) following the manufacturer's protocol. Protein gels were processed for western analysis by electroblotting to nitrocellulose. Nitrocellulose was blocked in 1% (w/v) bovine serum albumin (A-3803, Sigma, St. Louis) in a low-salt buffer (0.05 M NaCl, 0.02 M Tris, and 0.1% [v/v] Triton). Western blots were incubated with AtHSP17.6 antiserum, which recognizes all Arabidopsis class I sHSPs including HSP17.4 (Wehmeyer et al., 1996). Antiserum was diluted 1:1,000 in 1% (w/v) bovine serum albumin in low-salt buffer for 2 h at room temperature, then washed four times 10 min each in low-salt buffer. Secondary antibody incubation consisted of a 1:2,500 dilution of donkey anti-rabbit Ig-conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) in low-salt buffer. Blots were washed again and visualized by chemiluminescent detection (ECL system, Amersham). After two-dimensional western analysis, HSP17.4 was quantified using the NIH Image program version 1.54 (National Institutes of Health, Bethesda, MD).

RESULTS

HSP17.4::GUS Activity during Seed Development

We chose a reporter gene strategy to examine HSP17.4 gene expression in developing embryos in more detail. Two Arabidopsis lines carrying a single homozygous insertion of an HSP17.4 promoter::GUS reporter gene were characterized extensively (see "Materials and Methods"). These experiments are the first (to our knowledge) to test an sHSP-promoter-driven reporter gene for developmental regulation in a homologous plant system. T_2 plants were examined to ensure that the reporter gene was regulated by heat stress in mature leaves, as would be predicted from previous studies of HSP17.4 protein accumulation (Wehmeyer et al., 1996). Three-week-old leaves were heat-stressed to 38°C for 4 h and then stained for GUS activity. As shown in Figure 1A for one transgenic line, neither line showed GUS activity in the control leaves, but exhibited high levels of fairly uniform staining throughout heat-stressed leaves. We concluded that the HSP17.4::GUS reporter gene was being expressed similarly to the endogenous gene.

Activity of the HSP17.4::GUS reporter gene was then examined during normal seed development (Fig. 1B). GUS activity was first seen at approximately 9 DAP, somewhat earlier than HSP17.4 protein, which was not detectable by western analysis until approximately 11 DAP (Wehmeyer et al., 1996). GUS activity staining is more sensitive than western analysis, and it is not surprising that HSP17.4 transcription occurs before measurable amounts of the corresponding protein accumulate. Significant GUS activity was first apparent in the cotyledons, and until around 12 DAP, GUS did not accumulate in the radicle. GUS levels increased throughout seed development until desiccation, and by the onset of desiccation (about 21 DAP), the entire embryo stained for GUS activity. During desiccation GUS activity decreased in the tip of the radicle and in the tips of the cotyledons, resulting in the staining seen in mature embryos (Fig. 1B; 28 DAP and dry seed). The same pattern of staining was observed in both HSP17.4::GUS reporter lines and was confirmed in two separate developmental time courses.

The fact that the entire embryo stains for GUS expression prior to desiccation suggests that the HSP17.4 protein is present throughout the seed at this point in development. The tip of the radicle in mature seeds did not stain for GUS, indicating that HSP17.4 is not actively transcribed in this meristematic region late in the desiccation program. However, the sHSP protein could still be present in the root meristem of mature seeds. To determine whether the shoot meristematic region also showed reduced HSP17.4::GUS activity, mature seeds were stained for GUS and sectioned. GUS staining was detected in the shoot meristem region (data not shown). Therefore, the decline in HSP17.4 promoter activity is not correlated simply with the meristematic nature of tissues.

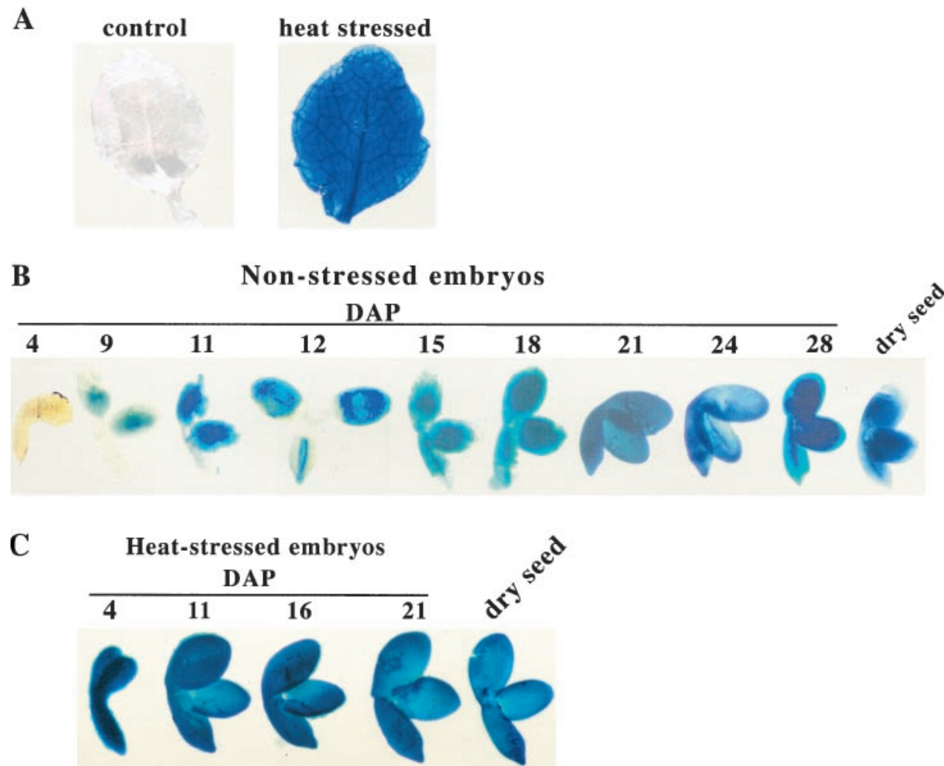

Figure 1. HSP17.4::GUS activity in heat-stressed leaves, developing embryos, and heat-stressed embryos of Arabidopsis. A, Arabidopsis leaves (T_3 generation) non-stressed (control) or heat-stressed. B, Arabidopsis embryos (T_3) stained for GUS activity during seed maturation. Seeds were collected at the designated times, and embryos were dissected away from the seed coat for staining. All non-stressed samples were stained with X-gluc for 4 h at room temperature, and heat-stressed leaves were stained for 30 min at 37°C. C, Arabidopsis seeds were heat-stressed for 4 h at 38°C, embryos were dissected from the seed coat at the corresponding time, and stained with X-gluc for 30 min at 37°C (4 h at room temperature yielded the same results).

Differential Regulation of the HSP17.4 Promoter by Heat Stress and Development

To examine the heat-stress regulation of HSP17.4 promoter during seed development, seeds from HSP17.4::GUS plants were collected at representative times during development, heat-stressed, and stained for GUS activity. Localization of GUS driven by the HSP17.4 promoter shows a different pattern of expression in heat-stressed embryos (Fig. 1C) compared with control embryos (Fig. 1B). The difference between heat stress and developmental expression is particularly dramatic earlier in development, when there is little or no GUS expression in the non-stressed embryo.

The intensity of HSP17.4 staining in heat-stressed embryos suggested that heat shock not only leads to uniform GUS expression throughout the embryo, but also results in a significant overall increase in HSP17.4 promoter activity. To quantify this difference, GUS was measured in mature embryos with and without heat stress (38°C, 4 h). A consistent 3- to 4-fold increase in GUS activity was detected for heat-stressed compared with non-heat-stressed HSP17.4::GUS seed (data not shown). These quantitative differences, along with the different spatial regulation of the HSP17.4 promoter during development compared with heat stress, suggest that the HSP17.4 promoter is regulated by distinct stress-mediated and developmental factors.

HSP17.4 Protein Expression in the Seed Transcriptional Activator Mutants *abi3-6*, *fus3-3*, and *lec1-2*

The regulation of HSP gene transcription by heat is known to involve HSF (Wu, 1995). While HSF may also be required for developmental regulation in seeds (Prändl and Schöffl, 1996), at least one other factor must be involved (Coca et al., 1996), based on the data presented above and the fact that only a subset of the class I sHSPs accumulate in mature embryos (Wehmeyer et al., 1996). We have previously shown that HSP17.4 protein is undetectable in mature seeds of a deletion allele of ABI3, *abi3-6* (Nambara et al., 1994), implicating ABI3 in HSP17.4 transcriptional activation (Wehmeyer et al., 1996). However, we could not rule out an indirect effect of the pleiotropic nature of this mutation, or a possible loss of HSP17.4 protein during the abnormal late stage of seed development in the *abi3-6* mutant. We therefore expanded our study to examine seeds carrying mutations in two other transcriptional activators that regulate seed development, LEC1 and FUS3 (Table I), and followed HSP17.4 accumulation throughout seed maturation.

HSP17.4 protein levels were examined by western analysis of homozygous seed from all three mutants, *abi3-6*, *lec1-2*, and *fus3-3*. HSP17.4 was found to accumulate in *lec1-2* and *fus3-3*, but was undetectable in *abi3-6* seed all through development (Fig. 2A). To confirm the identity of the polypeptides in the *lec1-2* and *fus3-3* mutants that reacted with the sHSP antibody, and to obtain a quantitative estimate of HSP17.4 reduction in all of the mutants, two-dimensional gel electrophoresis and western blotting were performed. To facilitate detection of HSP17.4 in the mutants, 10-fold more protein was analyzed and compared with that of the wild type. Both *fus3-3* and *lec1-2* contained a polypeptide that migrated identically to HSP17.4 in wild-type seeds (Fig. 3, A–C), albeit in a greatly reduced amount (1%–2% of wild type). Consistent with the SDS-PAGE data, no HSP17.4 protein was detected in *abi3-6* seed. Therefore, the absence of HSP17.4 in mature *abi3-6* seed appears to result from the complete inability to express sHSP, rather than just being a failure to maintain the protein in mature seed. The basic cross-reacting polypeptide detected in the two-dimensional western analysis of protein from *abi3-6* seeds was not detected consistently, and therefore we conclude it represents a non-specific reaction detected due to the high levels of protein analyzed. This basic polypeptide was also detected when high concentrations of wild-type seed protein were analyzed (data not shown), indicating that it is also not unique to *abi3-6* seed.

Localization of HSP17.4::GUS in Seed of Transcriptional Activator Mutants

To determine if the decrease in HSP17.4 in the transcriptional activator mutants is due to transcriptional or post-transcriptional control, we crossed *abi3-1* (a weak allele of ABI3, Table I), *abi3-6*, *fus3-3*, and *lec1-2* mutants to the HSP17.4::GUS plants and measured the induction of GUS. Figure 4A shows the localization of GUS activity in homozygous F_3 seed of HSP17.4::GUS crossed to *abi3-6*, *abi3-1*, *fus3-3*, and *lec1-2* compared with the wild type. After 4 h of staining, virtually no GUS activity was observed in F_3 seed from *abi3-6* crosses. Seeds from *fus3-3* and *lec1-2* plants had greatly reduced levels of GUS, and activity was localized only in the cotyledons. GUS staining of *abi3-6* seed for 24 h revealed a small additional increase (data not shown), but still much less than the levels of GUS seen in *fus3-3* and *lec1-2* after only 4 h. The level and localization of expression in the F_3 seed was consistent with observations in the F_2 generation, where we were able to compare GUS activity in wild type to *abi3-6*, *fus3-3*, or *lec1-2* from the

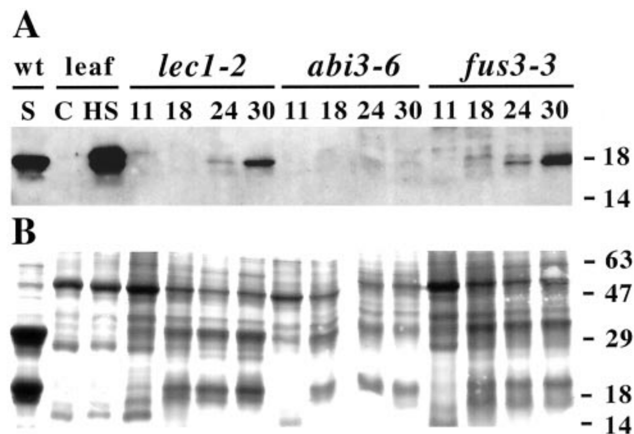

Figure 2. HSP17.4 accumulation in mutants of the seed transcriptional activators LEC1, ABI3, and FUS3. Seeds were collected from *abi3-6*, *lec1-2*, and *fus3-3* homozygous mutant plants during seed development at the indicated times (nos. above the lanes in DAP). For comparison, heat-stressed leaf (HS), control leaf (C), and wild-type seed (S) samples were also analyzed. A, Total seed proteins separated by SDS-PAGE and analyzed by western blotting with anti-HSP17.6 antibodies. B, Total protein profile of samples in A visualized on separate gels by staining with Coomassie Blue. Ten micrograms of total seed protein was loaded in each lane. Molecular mass markers are indicated on the right (in kD).

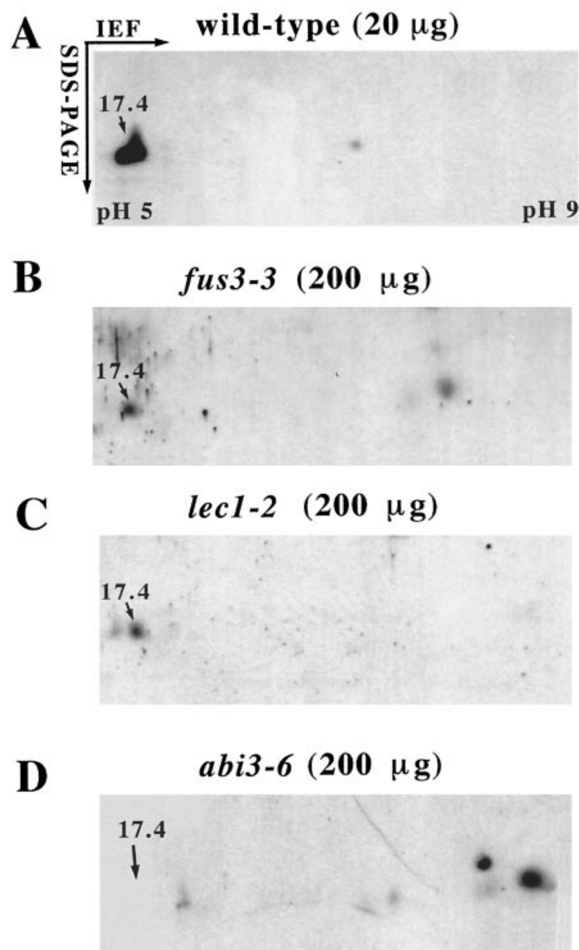

Figure 3. HSP17.4 accumulates to <2% of wild-type levels in dry seeds from transcriptional activator mutants (dry seed samples are equivalent to 30 DAP). Total seed proteins from wild type or the indicated mutants were separated by two-dimensional electrophoresis and analyzed by western blotting with HSP17.6 antibodies. A, Wild-type seed proteins (20 μg). B, *fus3-3* seed proteins (200 μg). C, *lec1-2* seed proteins (200 μg). D, *abi3-6* seed proteins (200 μg). Only a portion of the SDS-PAGE is shown, however, there were no other significant cross-reacting polypeptides. The position of HSP17.4 is indicated with an arrow.

same silique (data not shown). Although the total GUS activity also seemed to be reduced in the *abi3-1* seed, localization of HSP17.4::GUS expression was very close to the wild type in this mutant. These data suggest that the decrease in HSP17.4 accumulation in these mutants is due to decreased transcript levels.

In contrast to the differences in developmental control of GUS in the *abi3-6*, *abi3-1*, *fus3-3*, and *lec1-2* mutants compared with the wild type, heat stress strongly induced GUS expression throughout the mutant embryos, similar to what was observed in the wild type (Fig. 4B). These results support the independence of control of the promoter during heat stress versus development, as well as the hypothesis that ABI3 regulates HSP17.4 transcription during development.

HSP17.4 Levels in Other Seed Development Mutants

The fact that *abi3-6*, *fus3-3*, and *lec1-2* mutants are all desiccation intolerant (Table I) and have severely reduced or no HSP17.4 suggests that sHSPs may be involved in desiccation tolerance, as suggested previously (Wehmeyer et al., 1996). This correlation was further tested with three other mutants (Table I) with defects in late seed development: (a) *line24* (K. Yamagishi and J. Harada, personal communication), which is desiccation intolerant and embryo defective; (b) *lec2-1* (Meinke et al., 1994), which is desiccation tolerant and has many phenotypic similarities to *fus3-3* and *lec1-2*; and (c) *emb266* (Vernon and Meinke, 1995), which is desiccation tolerant and has embryo defects. These mutants were maintained as heterozygotes due to the lethal nature of the embryo defects. The mixed population seeds were planted, and heterozygotes (based on Kan[r]) were grown to flowering. From each plant, phenotypically wild-type and mutant seeds were collected and total seed proteins were extracted. Proteins were separated by two-dimensional gel electrophoresis for western analysis. Figure 5 presents the HSP17.4 accumulation in these three seed mutants (B, D, and F) compared with their phenotypically wild-type siblings (A, C, and E). The desiccation-intolerant seeds of *line24* had much reduced levels of HSP17.4, estimated as 2% of wild type, whereas desiccation-tolerant, embryo-defective *lec2-1* and *emb266* seeds had wild-type levels of HSP17.4. Thus, the level of sHSPs is correlated with the desiccation intolerance phenotype of these three mutants.

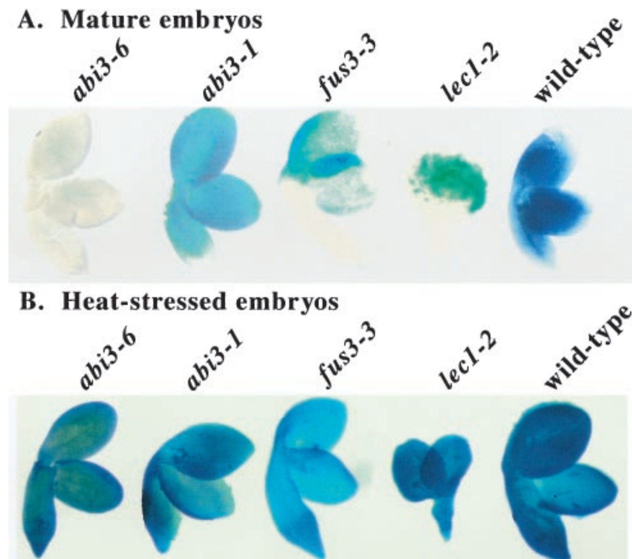

Figure 4. Comparison of developmental and heat regulation of HSP17.4::GUS in *abi3-6*, *fus3-3*, and *lec1-2* mutants. A, Mature (30 DAP) wild-type embryos or homozygous mutant embryos stained 4 h at room temperature for HSP17.4::GUS activity in the absence of heat. B, Embryos stained for GUS activity directly after a heat stress. Mutant embryos represent F_3 seeds from the appropriate cross. Embryos were dissected from the seed coat and stained as described in "Materials and Methods."

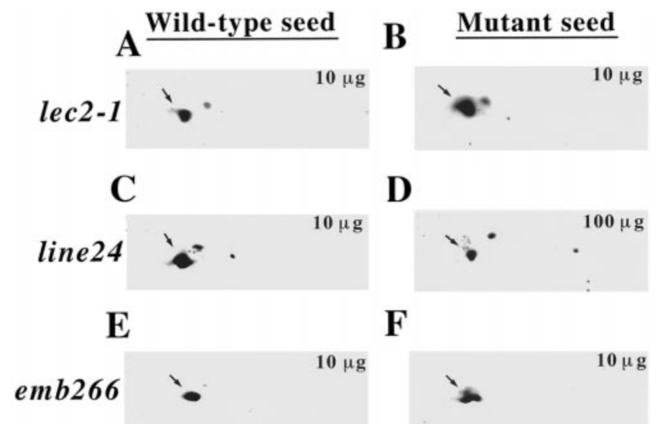

Figure 5. HSP17.4 levels are reduced only in desiccation-intolerant seed mutants, not in mutants with other defects in embryogenesis. Heterozygous mutant plants were grown to collect homozygous mutant or phenotypically wild-type seed, as described in "Materials and Methods." For desiccation-tolerant *lec2-1*: A, phenotypically wild-type seed; B, homozygous mutant. For desiccation-intolerant *line 24*: C, phenotypically wild-type seed; D, homozygous mutant. For desiccation-tolerant *emb266*: E, phenotypically wild-type seed; F, homozygous mutant. Total seed proteins (10 or 100 µg as indicated) were extracted from dry seeds, separated by two-dimensional electrophoresis, blotted to nitrocellulose, and probed with anti-HSP17.6 antibodies. Dry seed samples showed similar results to earlier time points (approximately 21 DAP) as analyzed by one-dimensional electrophoresis.

DISCUSSION

Our investigation of the temporal and spatial regulation of sHSP expression during seed development in both wild-type and mutant embryos provides new insight into the possible function of sHSPs in the embryo, and clearly demonstrates distinct control of sHSP gene transcription during development compared with heat stress. Assay of HSP17.4::GUS reporter gene transcription revealed that sHSP expression shows little tissue specificity, but instead spreads throughout the embryo during development until essentially all cells are stained in mature seeds prior to complete desiccation. This pattern of expression suggests a generalized protective role for the sHSPs rather than spe-

cialized roles in specific cell or tissue types. These data are consistent with the sHSP immunolocalization data of zur Nieden et al. (1995) in fava bean and pea. We cannot rule out that HSP17.4 protein accumulation in the embryo is different from the pattern of GUS activity due to the absence of transcriptional regulatory elements or to post-transcriptional control. However, as the temporal pattern of GUS activity closely parallels the observed sHSP protein accumulation (Wehmeyer et al., 1996), we believe tissue specificity is likely to be accurately reflected by this reporter gene analysis. We have been unable to investigate sHSP protein localization directly because of background reactivity of the HSP17.6 antibodies with other seed proteins.

The function of sHSPs during heat stress and in the seed maturation process is still unknown. The expression of HSP17.4 during seed maturation parallels the acquisition of dormancy and desiccation tolerance, and we have hypothesized that HSP17.4 may be important for one of these processes. Our previous work revealed that mutants with reduced seed dormancy generally had wild-type levels of HSP17.4 (Wehmeyer et al., 1996), implying that HSP17.4 is not sufficient, although it may still be necessary for dormancy. However, all of the desiccation-intolerant mutants we examined, *abi3-6*, *fus3-3*, *lec1-2*, and *line24*, had greatly reduced (1%–2% of wild type) or undetectable HSP17.4. Although the four desiccation-intolerant mutants have multiple defects, their major shared phenotype is desiccation intolerance, supporting a correlation between decreased HSP17.4 levels and the inability of the seed to survive desiccation.

In contrast, *lec2-1* seeds, which have many of the general pleiotropic embryo defects detected in *lec1-2* and *fus3-3*, have wild-type levels of HSP17.4 and are desiccation tolerant. In addition, *emb266* mutants, which are arrested in development before late seed maturation, accumulate HSP17.4 to approximately wild-type levels and survive desiccation. Thus, failure to express HSP17.4 is not a general consequence of aberrant embryo development. The observation that sHSPs, which are expressed as part of late seed maturation, accumulate in *lec2-1* and *emb266* adds to previous work showing that transcription of late maturation genes can still proceed in embryo-defective mutants (Yadegari et al., 1994; Devic et al., 1996). In total, these data correlate a desiccation intolerance phenotype with low levels of HSP17.4. However, these data do not suggest that sHSPs are the only component required to enable the seed to survive desiccation, as other aspects of the late seed maturation program may be defective in the desiccation-intolerant mutants. Given the complexity of the desiccation process, we conclude that while sHSPs may be necessary for desiccation tolerance, they are unlikely to be sufficient.

Previous studies have hypothesized that a glassy matrix state made up of soluble sugars serves to immobilize macromolecules, thus providing protection to cell membranes and proteins in the cytoplasm during seed desiccation (Bernal-Lugo and Leopold, 1998). Recent investigations have hypothesized that sugars may have a lesser role in this cytoplasmic matrix, due to the presence of more sugars in the seeds of desiccation-intolerant alleles of *abi3* than in those of the desiccation-tolerant *abi3-1* allele and the wild type (Wolkers et al., 1998). It was suggested that proteins specific to late embryo maturation may have an important role in this molecular packaging. HSP17.4 acting as a molecular chaperone may be a good candidate for functioning in the formation of a glassy matrix. However, much information is still needed to determine the function of sHSPs in seed development.

A few studies have utilized heat shock promoter reporter gene constructs to identify HSP gene regulatory elements (Prändl and Schöffl, 1996; Marrs and Sinibaldi, 1997; Almoguera et al., 1998), as well as to investigate tissue-specific localization of HSPs in seed development (Takahashi et al., 1992; Coca et al., 1994; Prändl et al., 1995). However, with only one exception (Takahashi et al., 1992), these studies have been conducted in heterologous systems and have not resolved the relative importance of the HSE in developmental regulation. Using a homologous reporter system in Arabidopsis, we found that HSP17.4::GUS expression in response to heat stress was always active throughout the entire embryo. The expression of HSP17.4::GUS in the heat-stressed embryo at 4 d after flowering, a time at which the HSP17.4 promoter is not active developmentally, was indistinguishable from heat-stressed embryos at other times during seed development. This implies a clear difference in regulation of the HSP17.4 promoter during heat stress versus during seed development.

The HSE and HSF are known to be required for the regulation of sHSP expression during heat stress (Wu, 1995), and Prändl and Schöffl (1996) provided evidence that HSF also plays a role in developmental regulation. However, our data and those of others (Almoguera et al., 1998) now directly demonstrate that other factors must be involved. Obvious candidates are transcriptional activators that control late seed maturation. Our data implicate ABI3 as an sHSP gene activator during development. HSP17.4 protein is undetectable in the deletion allele of *abi3* (*abi3-6*), whereas other likely null mutations in seed-specific transcriptional activators, *lec1-2* (a T-DNA insertion mutation) and *fus3-3* (a splice site mutation), still produce detectable levels of HSP17.4. In *abi3-6*XHSP17.4::GUS, we measured transcriptional activation of the HSP17.4 promoter in the absence of ABI3 gene product. These seeds exhibited extremely low GUS activity when stained under the same conditions as *fus3-3* and *lec1-2* crossed to HSP17.4::GUS. In contrast, heat-stressed embryos of *abi3-6*, *fus3-3*, and *lec1-2* crossed to HSP17.4::GUS stained for GUS activity throughout the entire embryo, providing further support for the independence of stress and developmental regulation. These data are consistent with the hypothesis that ABI3 is necessary for transcriptional regulation of HSP17.4 during seed development. We cannot rule out that ABI3 transcriptional control may be in conjunction with FUS3 or LEC1, as FUS3 and LEC1 have been shown to interact genetically with ABI3 (Parcy et al., 1997).

The DNA-binding elements recognized by these seed transcriptional activators have not been defined. Both ABI3 and FUS3 have a homologous B3 domain (Giraudat et al., 1992; Luerssen et al., 1998), which has recently been proposed to recognize a CACCTG motif (Kagaya et al., 1999). Examination of the HSP17.4 promoter reveals two of these

potential motifs that could be recognized by the B3 domain of ABI3. In comparison, Arabidopsis HSP18.2, which is not developmentally regulated (Takahashi et al., 1992), does not have similar CACCTG motifs. However, CACCTG motifs do not seem to be present in the *GmHSP17.3B* promoter, which was regulated during development in transgenic tobacco (Prändl and Schöffl, 1996). Additionally, 35S::ABI3 transgenic plants do not accumulate HSP17.4 in vegetative tissue without stress or with application of ABA (data not shown). These data are consistent with a trans-acting factor in addition to ABI3 being necessary for HSP17.4 expression. A better understanding of ABI3 transcriptional activation will be required in order to define fully any role in sHSP gene regulation.

Class I sHSPs from plants have been shown to have molecular chaperone activity in vitro (Lee et al., 1995). In vivo, HSP17.4 may have a similar role during the later part of seed development, i.e. preventing the irreversible aggregation of other proteins during desiccation and/or assisting in the refolding of denatured proteins during imbibition. The heat-induced expression of sHSPs is well established (Vierling, 1997). Clearly, plants have evolved new modes of regulation for these proteins to take advantage of their function during development.

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