Plant Physiol. (1996) 112: 747–757

Synthesis of Small Heat-Shock Proteins Is Part of the Developmental Program of Late Seed Maturation[1]

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Small heat-shock proteins (sHSPs) accumulate in plants in response to high-temperature stress. Specific sHSPs, the cytosolic class I and class II proteins, are also expressed in the absence of stress in maturing seeds of several species, and a role for these proteins in desiccation tolerance, dormancy, or germination has been hypothesized. We demonstrate that class I sHSPs are expressed during Arabidopsis seed development in a pattern similar to that previously observed in other species: they are first detected during mid-maturation, are most abundant in dry seeds, and decline rapidly during germination. Although the class I sHSP family in Arabidopsis appears to consist of four genes, expression of a single gene, *Athsp17.4*, accounts for the majority of sHSPs in maturing seeds. sHSP levels were also examined in seeds of several Arabidopsis mutants with reduced sensitivity to abscisic acid inhibition, including *aba1*, *abi1* and *abi2*, *abi3-1*, *abi3-6*, *abi4*, and *abi5-1*. The *abi3-1* mutant has 10-fold reduced levels of sHSPs; sHSPs are undetectable in the *abi3-6* mutant. All other mutants were indistinguishable from wild type. These results suggest that sHSP expression in seeds is regulated by the ABI3 response pathway and wild-type levels of sHSPs are not sufficient for seed dormancy and not necessary for desiccation tolerance. However, roles in either process cannot be ruled out. In total the data indicate that the expression of sHSPs in seeds is part of the normal developmental program of late seed maturation and the presence of sHSPs has adaptive significance for plant reproduction.

In response to heat and other stresses plants synthesize numerous sHSPs ranging in size from 15 to 30 kD (Vierling, 1991; Waters et al., 1996). Up to 30 unique sHSPs have been shown to accumulate in some plants in response to heat stress, compared with one or a few sHSPs seen in other eukaryotes. Plant sHSPs are localized to different cellular compartments and make up at least five evolutionarily distinct protein classes: class I cytosolic, class II cytosolic, chloroplast-localized, ER-localized, and mitochondria-localized (for a review, see Waters et al., 1996). Because of their abundance and diversity, it has been hypothesized that sHSPs have an important role in adaptation to and/or recovery from stress. Numerous studies have correlated sHSP expression with the development of thermotolerance (for a review, see Vierling, 1991). Lee et al. (1995b) have also shown that Arabidopsis plants, which were engineered to overexpress an active heat-shock transcription factor, constitutively express class I cytosolic sHSPs and show higher basal thermotolerance. However, none of these experiments demonstrated directly that sHSPs are required for thermotolerance and their mode of action has not been defined. Recent in vitro studies have shown that cytosolic class I and class II sHSPs have molecular chaperone activity (Lee et al., 1995a), which could account for a protective effect at high temperatures. However, overall, the function of sHSPs in vivo remains undefined.

In vegetative tissues sHSPs are not constitutively expressed but can accumulate to high levels in response to temperature stress (Chen et al., 1990; DeRocher et al., 1991; Hsieh et al., 1992). Both class I and II sHSPs are also expressed during an intriguing array of developmental processes, indicating that they may have important roles in the absence of stress (zur Nieden et al., 1995; for a review, see Waters et al., 1996). Investigating developmental regulation of the sHSPs may provide new insight into the function of these ubiquitous proteins. Developmental regulation has been most extensively characterized during seed development and germination. Expression of both class I and class II sHSP mRNAs and/or proteins has been documented in maturing embryos of pea (Vierling and Sun, 1989; DeRocher and Vierling, 1994), wheat (Helm and Aber- nathy, 1990), sunflower (Almoguera and Jordano, 1992; Coca et al., 1994), alfalfa (Howarth, 1990), and a variety of legumes (Hernandez and Vierling, 1993). In pea embryos class I and class II sHSPs appear 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 seed, whereas the sunflower class I sHSPs accumulate during late seed maturation (Coca et al., 1994). In pea embryos sHSPs are relatively abundant in the cotyledon during the stage of development when the seeds are acquiring dormancy and desiccation tolerance. In axes of developing pea

[1] Research supported by U.S. Department of Agriculture-National Research Initiative Competitive Grants Program grant no. 93–021–43 and State of Arizona HATCH funds to E.V. N.W. received partial support from the Flinn Foundation (Phoenix, AZ) through an Interdisciplinary Genetics Training Grant.

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Abbreviations: *aba*, ABA-reduced; *abi*, ABA-insensitive; DAI, days after initiation of imbibition; DAP, days after pollination; HS, heat-stressed; LEA, late embryogenesis abundant; sHSPs, small heat-shock proteins; WS, Wassilewskija.

seeds class I sHSPs are first detected at approximately the time of seed abscission from the pericarp, which coincides with the onset of desiccation. The protein as well as the mRNA persist in both dry pea and sunflower seed, and the protein is detected during germination for 2 to 3 d after the emergence of the radicle in pea seedlings (DeRocher and Vierling, 1994) and 2 to 4 d afterward in sunflower seedlings (Coca et al., 1994). These data have led to the general hypothesis that class I sHSPs may facilitate either the acquisition of desiccation tolerance or dormancy during seed development, or they function in the rehydration of embryos during germination.

To address further the role of class I sHSPs during seed development we are using Arabidopsis as a model because it is well defined genetically. Three Arabidopsis class I sHSP genes have been cloned: *Athsp17.4*, *Athsp18.2* (Takahashi and Komeda, 1989), and *Athsp17.6* (Helm and Vierling, 1989). *Athsp17.4* and *Athsp17.6* are very similar, with 90.3% identity at the amino acid level and 80.5% nucleotide identity. *Athsp18.2* is more divergent, with 82.7 and 85.9% amino acid identity and 66.0 and 70.8% nucleotide identity to *Athsp17.4* and *Athsp17.6*, respectively. As seen in other plant species, Arabidopsis class I sHSPs are not expressed in vegetative tissue during normal conditions, but mRNA levels are dramatically increased during heat stress (Takahashi and Komeda, 1989; E. Vierling, unpublished results).

Seed development is also well defined in Arabidopsis and a number of mutations affecting seed development have been described. Seed development is complete about 3 weeks after flowering in Arabidopsis. On the first 3 to 4 DAP general embryogenesis involving cell division and elongation of the embryo to the globular to heart stage occurs (Meinke, 1994). By approximately 10 to 11 DAP embryonic ABA reaches its peak and the seed begins to acquire dormancy and desiccation tolerance (Koornneef et al., 1989; Meurs et al., 1992; Giraudat et al., 1994; Koornneef and Karssen, 1994). Reserve synthesis is first detected at this time and defines the mid-maturation stage of development. Complete maturation involves loss of chlorophyll, decrease in water content, and acquisition of maximal seed dormancy and desiccation tolerance (Finkelstein and Somerville, 1990; Nambara et al., 1992; Ooms et al., 1993).

Mutations in the ABA synthesis pathway and in the ABA response pathway of Arabidopsis that result in aberrant seed development, primarily affecting mid-maturation/maturation, have been isolated (Koornneef et al., 1982, 1984; Finkelstein, 1994). The seed phenotype of an ABA synthetic mutant (*aba1*) is mainly a reduction in dormancy, as are two of the response mutants (ABA-insensitive) *abi1* and *abi2*. All three of these mutants have additional vegetative phenotypes, including wilty leaves due to increased water loss (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Patel et al., 1994). ABA response mutants that have more seed-specific phenotypes, *abi3*, *abi4*, and *abi5*, have also been identified and characterized (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Finkelstein, 1994). Weak alleles at all three loci result in reduced sensitivity to ABA inhibition of germination. *abi3* mutants are nondormant, as seen in the seeds of the *abi3-1* allele. Seeds carrying strong alleles of *abi3*, such as *abi3-6*, an internal deletion mutant (Nambara et al., 1994), are desiccation-intolerant, fail to lose chlorophyll, have reduced storage proteins, and can germinate viviparously (Nambara et al., 1992, 1994; Ooms et al., 1993). Genetic data indicate that *abi1* and *abi2* regulate seed ABA sensitivity from the same signal transduction pathway, whereas *abi3*, *abi4*, and *abi5* seem to function in a distinct parallel pathway (Finkelstein and Somerville, 1990; Finkelstein, 1994).

In this work the Arabidopsis class I sHSP gene and protein family were further characterized. To determine whether sHSPs have a possible role in Arabidopsis seed development or germination, the accumulation of class I sHSPs was examined in developing seeds and their persistence was measured in germinating seedlings. To distinguish the possible role of class I sHSPs during seed development or germination, the protein profiles of *aba* and *abi* mutant seeds were examined. Results provide new information about the possible role of sHSPs in seeds and have implications concerning sHSP gene regulation during development.

MATERIALS AND METHODS

Plant Growth and Stress Treatments

Arabidopsis thaliana (ecotypes Nössen, Columbia, Landsberg, or WS) were grown in soil in a growth chamber on a 18/14°C 16-/8-h day/night cycle. Light intensity was approximately 300 μmol m^{-2} s^{-1}. Plants were watered with one-quarter-strength Hoagland solution. Intact plants were HS as previously described (Chen et al., 1990). The growth chamber temperature was increased at 4°C/h until the desired stress temperature (32–40°C) was attained, maintained at the stress temperature for 4 h, and then decreased at 4°C/h until the chamber temperature returned to 22°C. High humidity was maintained during the heat stress to prevent transpirational cooling. Protein samples were collected at the end of the stress, after the chamber temperature had returned to 22°C.

Antibody Production

Antibodies against AtHSP17.6 were generated using antigen synthesized as a fusion protein in *Escherichia coli* as described previously, with slight modifications (Vierling et al., 1989). The fusion protein construct was created by inserting a *Sau*IIIA fragment of *Athsp17.6* into the pET3b expression vector. The fusion construct included 1.5 kD of the phage T7 gene 10 capsid protein fused to 133 carboxyl-terminal residues (from amino acid 24 to 156, 14.9 kD) of AtHSP17.6 (Helm and Vierling, 1989) for a total fusion protein size of 16.4 kD. The fusion protein was overexpressed in *E. coli*, total bacterial protein extracts were separated by SDS-PAGE, and the fusion protein was electroeluted. The purified protein was injected into New Zealand White female rabbits to generate antibodies. Preimmune serum was taken before any inoculations were given.

Protein Electrophoresis and Western Analysis

Protein was extracted in SDS sample buffer containing 60 mM Tris-HCl, pH 8.0, 60 mM DTT, 2.0% SDS, 15% Suc, 5 mM ε-amino-*N*-caproic acid, and 1 mM benzamidine (1 mL 0.1 g^{-1} fresh weight leaf tissue and 1 mL 0.05 g^{-1} dry seed) using a ground glass homogenizer. Protein concentration was measured using a Coomassie blue dye-binding assay (Ghosh et al., 1988). Samples were separated on 12.5% acrylamide or 10 to 16% acrylamide gradient gels in the presence of SDS. For two-dimensional gel electrophoresis, 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% acetone and then resuspended in IEF sample buffer containing 9.5 M urea, 2% Nonidet P-40 (Sigma), 5% 2-mercaptoethanol, and 2% ampholines. The protein samples were analyzed (Mighty Small-2D-gel unit, Hoefer Scientific, San Francisco, CA) using a modified procedure previously described (DeRocher and Vierling, 1994). Protein gels were either stained with Coomassie blue or processed for western analysis by electroblotting proteins to nitrocellulose. Nitrocellulose was blocked in 5% dry milk, incubated with AtHSP17.6 antibodies diluted 1:1000 in 5% dry milk, washed, incubated in a 1:2500 dilution of donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham), washed, and visualized by chemiluminescent detection (ECL system, Amersham).

DNA Isolation and Southern Hybridization

Genomic DNA from Arabidopsis (ecotype Columbia) was isolated by phenol extraction and cesium chloride banding (Murray and Thompson, 1980). Overnight enzymatic digestion of 4 μg of genomic DNA was carried out with *Bam*HI, *Eco*RI, and *Xho*I. DNA digests were separated on a 0.8% agarose gel in Tris-borate-EDTA followed by capillary transfer to Nytran (Schleicher & Schuell). The DNA was fixed to the Nytran by UV cross-linking. Blots were hybridized and washed under high-stringency conditions as follows: hybridization was done at 65°C in 5× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate), 5× Denhardt's solution, 0.5% SDS, and 0.1% BLOTTO (powdered milk, Lucerne, Oakland, CA), and washes were done at 65°C three times for 15 min in 0.2× SSC and 0.1% SDS. A lower stringency hybridization followed the same protocol, with hybridization and wash temperatures at 55°C. DNA fragments used as hybridization probes consisted of the entire cDNA insert for *Athsp17.4*, *Athsp18.2* (Takahashi and Komeda, 1989), and *Athsp17.6* (Helm and Vierling, 1989). Inserts were labeled with [α-^{32}P]dATP (Dupont NEN, 3000 Ci $mmol^{-1}$) by the random primer method (Feinberg and Vogelstein, 1983). Specific activities attained were 1×10^9 to 2×10^9 cpm μg^{-1} DNA.

In Vitro Transcription and Translation

For in vitro transcription of the sHSP cDNAs, plasmids were linearized with *Xba*I such that RNA in the sense direction could be synthesized using T7 RNA polymerase. In vitro translation was performed with reticulocyte lysate as described by the supplier (Promega) using [^{35}S]Met (Dupont NEN, 1165 Ci $mmol^{-1}$) at 0.5 μCi μL^{-1} translation mixture. The [^{35}S]Met-labeled in vitro translation products were analyzed by either SDS-PAGE on 10 to 16% polyacrylamide gradient gels or two-dimensional gel electrophoresis. Proteins were visualized by autoradiography.

Seed Development and Germination Samples

Seed Development

Arabidopsis plants (ecotype Nössen) were grown to flowering under the conditions described above. Opening flowers were tagged with different color ties to denote the day of pollination. At the onset of flowering siliques produce a significant number of aborted embryos; therefore, flower tagging was initiated 10 d to 2 weeks after the onset of flowering. Siliques were removed 4 to 24 DAP and proteins were extracted immediately.

Germination

Arabidopsis seeds were soaked in a Petri dish on wet filter paper under the growth chamber conditions described above at 100% RH. Seeds/seedlings were collected 1 to 8 DAI, frozen, and lyophilized. Total proteins were extracted from dried material in SDS-sample buffer (0.05 mL sample buffer/mg dried material), and separated by SDS-PAGE. Under the conditions used seeds were still imbibing 1 DAI, whereas by 2 DAI most seed coats had split and some radicles had emerged. By 3 DAI radicles had emerged and some green cotyledons were visible, and by 4 DAI the cotyledons of most of the seedlings had emerged, some of which were unfolded. At 5 DAI all of the germinated seedlings had unfolded cotyledons, and by 10 DAI the first true leaves were visible.

ABA Biosynthetic and Insensitive Mutants

The mutants used in this investigation were previously isolated and characterized based on their ability to germinate on ABA (Koornneef et al., 1982, 1984; Finkelstein, 1994). All of the seeds carrying these mutations result in plants with reduced sensitivity to ABA inhibition of germination. The mutant lines and more specific phenotypes are summarized in Table I. Plants were grown to seed under the same conditions as described above, except *aba1*, *abi1*, and *abi1 abi2* were grown at 22°C in continuous fluorescent light (100–150 μE m^{-2} s^{-1}) (Finkelstein and Somerville, 1990). The homozygous seeds of *abi3-6* (Nambara et al., 1994) were removed from the plant before desiccation, germinated on plates to the four-leaf stage, and then transferred to soil.

RESULTS

Arabidopsis Class I Proteins Are Encoded by Single-Copy Genes

It was important to determine the complexity of the class I gene family in Arabidopsis to investigate expression of

Table I. *Characteristics of Arabidopsis mutants used in this study*

Mutant[a]	Ecotype of Wild-Type Parent	Mutagen	Phenotype[b]	Tissue Specificity of Phenotype	Ref.
aba1	Landsberg	Ethyl methanesulfonate	Reduced dormancy; wilty leaves	Seed and leaf	Koornneef et al., 1982
abi1	Landsberg	Ethyl methanesulfonate	Reduced dormancy; wilty leaves	Seed and leaf	Koornneef et al., 1984
abi2	Landsberg	Ethyl methanesulfonate	Reduced dormancy; wilty leaves	Seed and leaf	Koornneef et al., 1984
abi3-1	Landsberg	Ethyl methanesulfonate	Reduced dormancy	Seed	Koornneef et al., 1984
abi3-6	Columbia	Fast neutron irradiation	Desiccation-intolerant	Seed	Nambara et al., 1994
abi4	Columbia	γ-radiation	Reduced sensitivity to ABA inhibition	Seed	Finkelstein, 1994
abi5	WS	T-DNA insertion	Reduced sensitivity to ABA inhibition	Seed	Finkelstein, 1994

[a] Double mutant of *abi1* and *abi2* and *abi3-1* were also analyzed. [b] The reduced dormancy phenotype is greater for *aba1*, *abi1*, and *abi2* compared with *abi3-1*, *abi4*, and *abi5-1*.

class I sHSPs in Arabidopsis seeds. The three Arabidopsis genes that had been previously cloned, *Athsp17.4*, *Athsp18.2* (Takahashi and Komeda, 1989), and *Athsp17.6* (Helm and Vierling, 1989), were used in Southern analysis at both high and low stringency to investigate the complexity of the class I gene family. Southern analysis of *Athsp17.4* and *Athsp18.2* had been previously conducted using high-stringency conditions and indicated that these genes were single-copy genes; no cross-hybridizing bands were seen on the Southern blots (Takahashi and Komeda, 1989). *Athsp17.6* had not previously been analyzed. Our data for *Athsp18.2* under high-stringency conditions (not shown) were comparable to those of Takahashi and Komeda (1989). However, with the Southern blot analysis of *Athsp17.4* (Fig. 1) a minor cross-hybridizing band was detected that had not been seen in the earlier work, probably because of slightly different stringency conditions. Comparison of the *Athsp17.4* and *Athsp17.6* blots (Fig. 1) indicated that the minor band on the *Athsp17.4* blots corresponds to the major band on the *Athsp17.6* blot and vice versa. This is consistent with the fact that the cDNAs for these genes are 80% identical. Lower stringency hybridization (hybridization and washes at 55°C) with *Athsp17.6* resulted in one additional band that did not superimpose with *Athsp17.4* or *Athsp18.2*. An equivalently reduced stringency hybridization with either *Athsp17.4* or *Athsp18.2* did not result in the appearance of additional bands (data not shown). These data suggest that the Arabidopsis class I sHSP family consists of a minimum of four genes and that each of these genes are a single copy, signifying that there are no related genes with greater than 80% identity at the nucleotide level.

Specific Class I sHSPs Accumulate in Dry Seeds

The anti-AtHSP17.6 antibodies were tested to determine whether they would specifically detect polypeptides induced by heat in Arabidopsis leaves. Figure 2A represents a western analysis of protein from leaves HS at temperatures between 32 and 40°C or control leaves (22°C). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with AtHSP17.6 antibodies. The antibodies detected two distinct bands at approximately 18 kD in leaves subjected to relatively mild stress (32°C). Both bands accumulated proportionally with increasing temperature and reached a maximum at 38°C. These proteins were not present in leaves grown at 22°C (Fig. 2A) and did not react with preimmune serum (data not shown).

To determine whether class I sHSPs are present in Arabidopsis seeds matured in the absence of heat stress, as has been found for other plant species (DeRocher and Vierling, 1994; zur Nieden et al., 1995), the same Athsp17.6 antibodies were used for western analysis of total seed protein. Arabidopsis plants used for the collection of these seeds

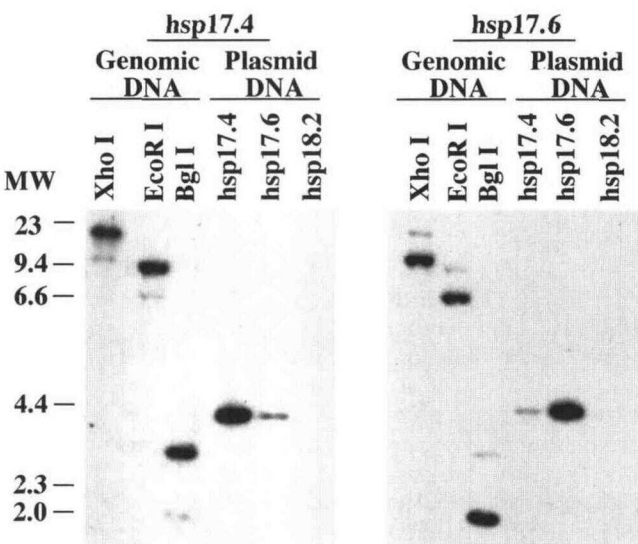

Figure 1. The Arabidopsis class I sHSPs AtHSP17.4 and AtHSP17.6 are encoded by single-copy genes. Total Arabidopsis genomic DNA (4 μg/sample) was digested with the indicated restriction enzymes and processed for Southern blot analysis. The nylon filters were hybridized under high-stringency conditions (see "Materials and Methods"). The full-length cDNAs were used to synthesize the radiolabeled probes. Plasmid DNA (5×10^{-3} ng, equivalent to a single-copy gene in 4 μg of Arabidopsis genomic DNA) containing each full-length cDNA insert was linearized and run for comparison. Molecular size (MW) markers, values in kb, are indicated at the left.

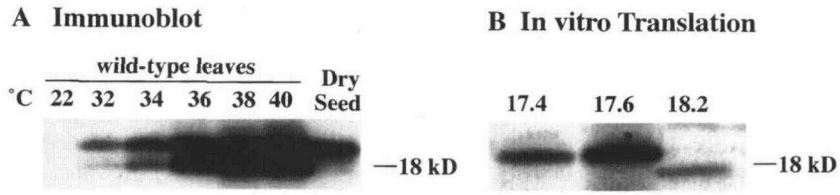

Figure 2. Class I sHSPs accumulate in leaves during heat stress and specific class I sHSPs are detected in dry seeds matured in the absence of stress. A, Western analysis of proteins from HS leaves or nonstressed dry seed using AtHSP17.6 antibodies. Intact plants were HS to maximum temperatures of 32 to 40°C as indicated above each lane. Protein was isolated from leaves at the end of the stress treatment and compared with the control leaf (22°C) and seed proteins. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, probed with AtHSP17.6 antibodies, and visualized by chemiluminescence. Only the portion of the gel containing the class I sHSPs is shown. B, In vitro transcription/translation products of *Athsp17.4*, *Athsp17.6*, and *Athsp18.2* cDNAs. ^{35}S-labeled in vitro translation products of transcripts synthesized from the three class I genes were added to either HS leaf or seed protein samples and separated by SDS-PAGE. The in vitro translation products were visualized by autoradiography. Only the portion of the autoradiogram containing the class I sHSPs is shown.

were grown at 18/14°C (day/night) to ensure that the class I sHSPs could not have accumulated because of heat stress. As shown in Figure 2A class I sHSPs are also present in dry seeds, but seeds accumulate only the slower migrating of the two bands seen in leaves. Although in leaves the slower migrating band is more abundant at all temperatures than the faster migrating band, both bands are detected at all temperatures between 32 and 40°C. The amount of the slowly migrating protein detected in the seed is similar to that seen in leaves at 36°C. Thus, these data indicate that expression of the class I sHSPs is differentially regulated during development; only a specific subset of class I sHSPs accumulate in the seed.

Experiments were performed to identify which of the three cloned Arabidopsis class I sHSP genes encode the developmentally regulated protein(s) in Arabidopsis seeds. In vitro transcripts produced from the *Athsp17.4*, *Athsp17.6*, and *Athsp18.2* cDNAs were used to synthesize ^{35}S-labeled in vitro translation products and were combined with leaf or seed proteins. The combined samples were separated by SDS-PAGE and transferred to nitrocellulose to examine co-migration of the in vitro translation products with the leaf and the seed immunoreactive polypeptides. The autoradiograph of the ^{35}S-labeled proteins is shown in Figure 2B. The endogenous class I proteins from seed and HS leaf samples were visualized by incubation with AtHSP17.6 antibodies and chemiluminescent detection, as shown in Figure 2A. The western blot and autoradiograph were then superimposed to compare the migration of the in vitro translation products to the immunoreactive class I sHSPs (not shown). The AtHSP17.4 and AtHSP17.6 translation products both co-migrated with the upper immunoreactive band from HS leaves, above the 18-kD marker, and corresponded to the only immunoreactive band seen in seed samples. A higher apparent molecular mass compared with that predicted from the amino acid sequence of AtHSP17.4 and AtHSP17.6 has also been observed for the related class I sHSPs from pea (DeRocher et al., 1991). The AtHSP18.2 translation product co-migrated with the faster migrating immunoreactive band in HS leaves, near the 18-kD marker (Fig. 2B). These data indicate that either AtHSP17.4 or AtHSP17.6, or both, are developmentally regulated in the seed, whereas AtHSP18.2 is found only in HS leaves.

AtHSP17.4 Is the Predominant sHSP in Seeds

Because the AtHSP17.4 and AtHSP17.6 gene products co-migrated on SDS-PAGE, two-dimensional gel electrophoresis was used to distinguish between AtHSP17.4 or AtHSP17.6 accumulation in the seed. Figure 3A is a western blot analysis of Arabidopsis seed and HS leaf proteins separated by two-dimensional gel electrophoresis and probed with the AtHSP17.6 antibodies. Two polypeptides were detected in the dry seed, with the predominant protein being the most acidic, whereas four major cross-reacting proteins were detected in the HS leaf sample. Total protein from seeds and HS leaves were also combined, separated by two-dimensional gel electrophoresis, and then transferred to nitrocellulose and reacted with AtHSP17.6 antibodies. The two most acidic proteins seen in the HS leaf sample were found to co-migrate with the two proteins detected in the seed (data not shown). Proteins from seed samples were combined with each of the in vitro translation products of class I sHSPs detected in seeds (AtHSP17.4 and AtHSP17.6) and separated by two-dimensional gel electrophoresis. The autoradiographs shown in Figure 3B are of AtHSP17.4 and AtHSP17.6; these were superimposed on the western blot of Arabidopsis seeds (Fig. 3A). The autoradiograph of AtHSP17.4 matched the predominant and most acidic polypeptide detected on two-dimensional western blots of seeds, whereas AtHSP17.6 matched the polypeptide that appears slightly more basic. These results strongly support the conclusion that the two acidic spots represent unique products of the *Athsp17.4* and *Athsp17.6* genes and are not the result of posttranslational modifications. Thus, AtHSP17.4 is the predominant class I sHSP in Arabidopsis seeds and appears to be the most acidic (Fig. 3B).

The HS leaf samples separated by two-dimensional gel electrophoresis possess two polypeptides that are not seen in seed samples and are slightly basic and faster migrating than the acidic polypeptides. The one-dimensional SDS-

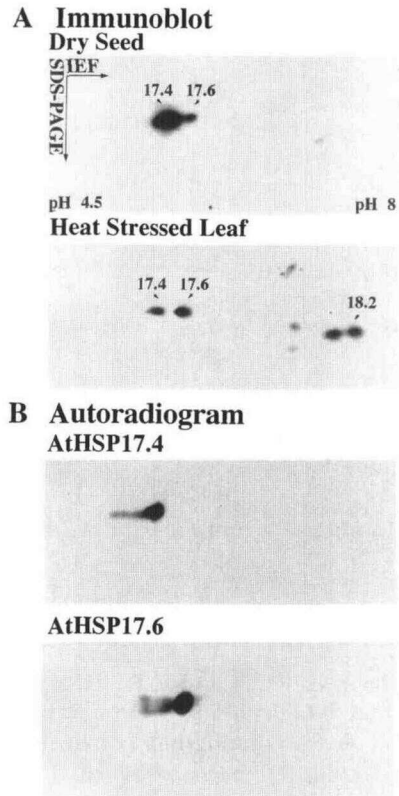

Figure 3. The two acidic class I sHSPs are present in dry seeds; AtHSP17.4 is the predominant polypeptide. A, Western analysis of two-dimensional gel separation of HS leaf (bottom) or seed proteins (top). Total seed or HS leaf protein (15 μg) was separated by IEF, followed by SDS-PAGE, blotted to nitrocellulose, and reacted with AtHSP17.6 antibodies. Arrowheads designate the specific class I sHSP as labeled (see text for details). Only a portion of the SDS-PAGE is shown; however, there were no other cross-reacting polypeptides. B, Autoradiography of *Athsp17.4* and *Athsp17.6* in vitro translation products separated by two-dimensional gel electrophoresis. ^{35}S- labeled proteins from in vitro transcription/translation of the *Athsp17.4* and *Athsp17.6* cDNAs were separated by IEF, followed by SDS-PAGE, and blotted onto nitrocellulose. The ^{35}S-labeled proteins were visualized by autoradiography.

PAGE data predicted that the in vitro translation product of AtHSP18.2 would co-migrate with one of the more basic proteins. When the autoradiograph of the in vitro translation product of AtHSP18.2 was superimposed with the western blot analysis of HS leaves separated by two-dimensional gel electrophoresis, AtHSP18.2 was the most basic polypeptide detected in HS leaf samples (data not shown). In total these data are consistent with the calculated pIs of Arabidopsis class I sHSPs: AtHSP17.4 = 5.06, AtHSP17.6 = 5.24, and AtHSP18.2 = 7.69. A fourth polypeptide that cross-reacts with the class I antibody does not correspond with the in vitro translation products of the three cDNAs, suggesting posttranslational modification or the presence of another gene that has not yet been identified. The low-stringency Southern blot analysis using *Athsp17.6* as a probe has revealed a possible fourth class I sHSP gene that could encode this protein.

Timing of Class I sHSP Expression during Seed Development and Germination

The temporal pattern of sHSP accumulation and the decline in developing and germinating seeds has led to the hypothesis that sHSPs function in the acquisition of dormancy or desiccation tolerance or in seedling germination (DeRocher and Vierling, 1994). Initially the accumulation of class I sHSPs during Arabidopsis seed development was examined to determine whether it was similar to the pattern of sHSP expression seen in developing pea seeds. Siliques were collected from tagged flowers from 4 to 24 DAP and from mature seeds, and the proteins were subjected to western analysis as shown in Figure 4A. The class I sHSPs were first detected 12 DAP and their level increased until approximately 18 to 20 DAP. Mature seeds had class I sHSP levels similar to that seen in HS leaves at 36°C. The total protein profile in developing siliques is shown in Figure 4B. Between 4 and 6 DAP the siliques had developed to their fully expanded size. Before mid-maturation of seed development the green siliques with seeds had a total protein profile characteristic of the vegetative tissue, with Rubisco being the predominant protein. Between 11 and 12 DAP, approximately the time that class I sHSPs were first detected, seed storage proteins were also first detected and Rubisco levels began to decline. It should be noted that class I sHSPs were not detected in siliques, from which seeds had been removed, at several stages during development (data not shown). Thus, the class I sHSPs detected in total silique protein in these experiments is assumed to be present only in the seeds. The accumulation of the class I sHSPs during mid-maturation in Arabidopsis seed development parallels observations of sHSP accumulation in pea seeds (DeRocher and Vierling, 1994).

The presence of class I sHSPs in germinating seedlings was measured to determine whether and when the sHSPs could function during germination. Germinating seedlings were collected 1 to 8 DAI. Figure 5 is a representation of the western blot and the stained gel of proteins from these seedlings. Arabidopsis class I sHSPs persisted in the seed and seedling through approximately 4 DAI (Fig. 5A). This is very reminiscent of the pattern seen in pea seed (DeRocher and Vierling, 1994) and in other species (zur Nieden et al., 1995). Because the class I sHSPs are present during both seed development and germination, their function may be important in either or both processes.

In developing pea seeds the class I sHSPs accumulate in both the cotyledons and embryonic axes (DeRocher and Vierling, 1994). To determine whether the seedlings of Arabidopsis retained class I sHSPs in these tissues, proteins were extracted from the cotyledons and roots of germinating seedlings (2.5 DAI). Western analysis of these samples revealed that both tissues had similar levels of class I sHSPs relative to total protein (data not shown). This implies that the localization of class I sHSPs in Arabidopsis seeds is similar to pea, although the timing of the accumulation in specific tissues in developing Arabidopsis seeds has not been investigated.

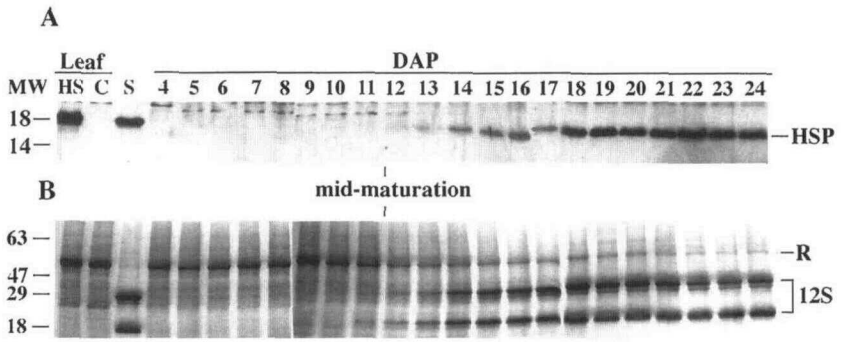

Figure 4. Timing of sHSP accumulation during Arabidopsis seed development. Immediately after opening, flowers were tagged, and seeds were collected 4 to 24 DAP. Proteins were extracted and 20 μg of total protein per lane was separated by SDS-PAGE. A, Class I sHSPs were visualized with AtHSP17.6 antibodies (only the portion of the western blot containing the class I sHSPs is shown). For comparison, HS leaf (HS), control leaf (C), and wild-type seed samples (S) were also analyzed. The position of class I sHSPs is indicated (HSP). The seed development samples were run on three separate gels; the discontinuity in the position of the HSP bands between 16 and 17 DAP was due to aberrant migration at the bottom edge of one gel. B, The total protein profile of samples in A was visualized on separate gels by staining with Coomassie blue. The positions of Rubisco (R) and the 12S seed storage (12S) proteins are indicated. Mid-maturation is indicated at 11/12 DAP. Molecular mass (MW) markers in kD are on the left.

sHSP Expression in Seeds of ABA-Deficient and -Insensitive Mutants

Class I sHSP levels were monitored in Arabidopsis seed mutants to distinguish the possible role of class I sHSPs in seed maturation or germination. Several mutants with reduced dormancy have been found in two different categories: *aba* and *abi*. The *aba1*, *abi1*, *abi2*, *abi3-1*, *abi3-6*, *abi4*, and *abi5-1* mutants were examined because, as described in the introduction, they exhibit phenotypes in late seed maturation that are correlated with the timing of sHSP accumulation. Western analysis of proteins in the seeds of these mutants is presented in Figure 6 and reveals that wild-type levels of class I sHSPs were detected in *aba1*, *abi1*, *abi2*, and the double mutant *abi1 abi2*. All of these mutants have reduced seed dormancy and a wilty vegetative phenotype. Among those mutants with seed-specific phenotypes (*abi3-1*, *abi4*, *abi3-6*, *abi5-1*) only seeds carrying an *abi3* mutation showed reduced levels of class I sHSPs (Fig. 6, lanes i3-1, i3-6, 1/3, and 2/3). Seeds carrying the *abi3-1* mutation showed reduced levels of class I sHSPs, and in seeds carrying the deletion mutation *abi3-6* class I sHSPs were undetectable. Wild-type levels of class I sHSPs are

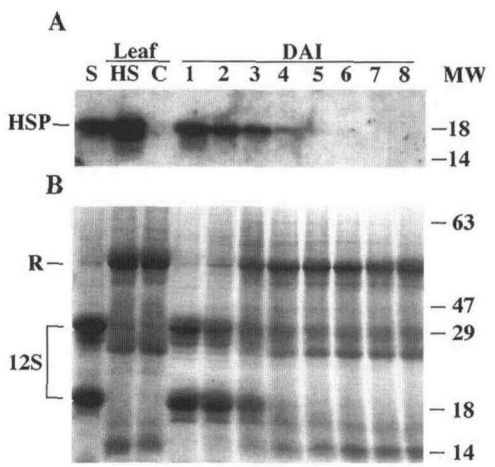

Figure 5. The sHSPs decline during germination. Arabidopsis seeds were soaked in Petri dishes on wet filter paper and seeds/seedlings were collected 1 to 8 DAI. Proteins were extracted and 20 μg of total protein per lane was separated by SDS-PAGE. A, Western blot probed with AtHSP17.6 antibodies. HSP designates the class I sHSPs that accumulate in HS seed and leaf samples. For comparison, HS leaf (HS), control leaf (C), and wild-type seed samples (S) were also analyzed. B, Total protein profiles were analyzed by staining an identical gel with Coomassie blue. The positions of Rubisco (R) and the 12S seed storage (12S) proteins are indicated. Molecular mass (MW) markers in kD are on the right.

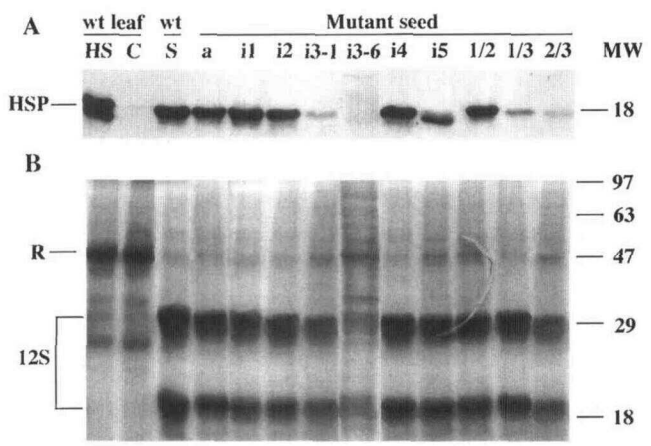

Figure 6. Seeds of *abi* mutants at the *abi3* locus have reduced levels of class I sHSPs. A, Proteins were extracted from wild-type seed (S), HS leaf (HS), control leaf (C), and seeds with reduced dormancy phenotype *aba1* (a) *abi1* (i1), *abi2* (i2), *abi3-1* (i3-1), *abi3-6* (i3-6), *abi4* (i4), *abi5-1* (i5), *abi1 abi2* (1/2), *abi1 abi3-1*(1/3), and *abi2 abi3-1* (2/3). Western blots were probed with AtHSP17.6 antibodies. B, Total protein profiles were analyzed by staining an identical gel with Coomassie blue. The positions of Rubisco (R) and the 12S seed storage (12S) proteins are indicated. Molecular mass (MW) markers in kD are on the right.

present in *abi4* and *abi5-1* seeds. It should be noted that in the *abi5* seed the major class I sHSP migrates faster than in the other mutants. This difference was determined to be an allelic variation specific to the WS ecotype background of the *abi5* mutation (Table I); i.e. when western analysis of *abi5* seeds was directly compared with WS wild-type seeds the same pattern of class I sHSPs was observed. Additionally, class I sHSPs from HS leaves of *abi5* plants have a pattern of class I sHSPs similar to that seen in *abi5* seeds (data not shown).

Two-dimensional gel electrophoresis was used to determine whether the levels of AtHSP17.4 or AtHSP17.6 were differentially affected by the *abi3-1* mutation. Western analysis of *abi3-1* seeds (Fig. 7) revealed two polypeptides that co-migrate with HSP17.4 and HSP17.6 from wild-type seeds. The relative ratios of HSP17.4 and HSP17.6 are similar to those seen in wild-type seeds. However, approximately 10 times more total *abi3-1* seed protein was loaded to obtain a similar signal to wild type. Therefore, the seeds of *abi3-1* produce approximately 10-fold less class I sHSPs than wild type.

To ascertain that the reduction of class I sHSP in *abi3* seed is specific to development, both seed and leaf samples of *abi3-1* and *abi3-6* were HS at 40°C. The western blots of the proteins separated by SDS-PAGE resulted in a class I sHSP profile nearly identical with wild-type HS leaves (data not shown). These results indicate that the *abi3* mutation specifically affects the developmental regulation of class I sHSPs and not the overall production of class I sHSPs.

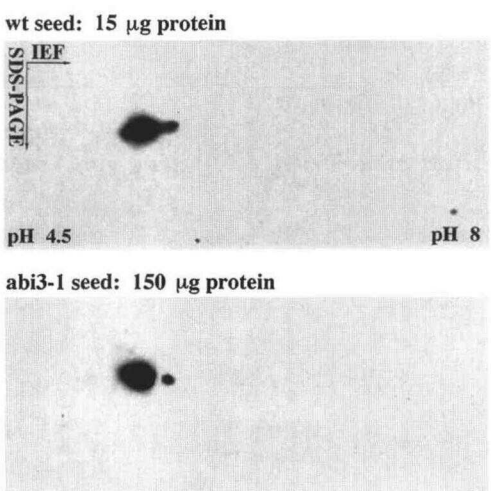

Figure 7. sHSP levels in *abi3-1* are 10-fold reduced, but AtHSP17.4 and 17.6 are present in the same relative stoichiometry as in the wild type. Protein was extracted from *abi3-1* seeds and 150 μg of total protein was separated by two-dimensional gel electrophoresis and then processed for western blot analysis with AtHSP17.6 antibodies. Results were compared with western blot analysis of two-dimensional gel electrophoresis of 15 μg of total protein from wild-type seed (wt), which gave a comparable signal. Only a portion of the SDS-PAGE is shown; however, there were no other cross-reacting polypeptides.

DISCUSSION

Three class I sHSP genes from Arabidopsis have been previously cloned and sequenced, *Athsp17.4*, *Athsp17.6*, and *Athsp18.2* (Helm and Vierling, 1989; Takahashi and Komeda, 1989). Data from Takahashi and Komeda (1989) and data presented here indicate that these are all single-copy genes. Evidence for a fourth class I gene with less than 80% identity to *Athsp17.6* was obtained by reduced stringency Southern blot analysis. Polyclonal antibodies raised against AtHSP17.6 detected four major heat-induced proteins, and three of these were shown to correspond to gene products of the isolated class I genes. It is not unexpected that the AtHSP17.6 antibodies show broader cross-reactivity than does the *Athsp17.6* gene in nucleic acid hybridization studies. We assume that the fourth polypeptide detected by these antibodies corresponds to the product of a fourth gene, but we cannot rule out the possibility that it represents a posttranslational modification of one of the other class I sHSPs. In total these data indicate that Arabidopsis has a family of class I sHSP genes, probably consisting of four members. Class I sHSPs are typically encoded by moderate to large gene families in plants (Vierling, 1991).

The functional significance of differential regulation of class I sHSPs in seeds is unclear. The Arabidopsis class I sHSPs are greater than 80% identical on the amino acid level and greater than 66% identical at the nucleotide level. There are insufficient data to identify any unique characteristics of the developmentally regulated proteins. In Arabidopsis the two proteins expressed in seeds (AtHSP17.4 and AtHSP17.6) share the greatest similarity (90% identical) and are the most acidic forms of the Arabidopsis class I proteins. However, developmental regulation is not restricted to acidic isoforms; both acidic and more neutral class I sHSPs are present in mature pea seeds (DeRocher and Vierling, 1994). The potential functional differences among sHSP isoforms requires further investigation.

Takahashi et al. (1992) generated transgenic Arabidopsis plants expressing the GUS reporter gene under control of the 5′ promoter of the *Athsp18.2* gene, and they saw no expression of the reporter in developing seeds. This result is consistent with that from our western blot analyses, which demonstrated that AtHSP18.2 is not present in seeds. It is interesting to note that both the *Athsp17.4* gene, which is highly expressed in seeds, and the *Athsp18.2* gene have heat-shock consensus elements within their 5′ promoter regions (Takahashi and Komeda, 1989; Takahashi et al., 1992). The fact that these genes show such extreme differences in seed-specific expression strongly argues that other promoter elements must influence their regulation during development.

As previously detected in pea (DeRocher and Vierling, 1994), in sunflower (Almoguera et al., 1993), and more recently in other species (zur Nieden et al., 1995), the class I sHSPs accumulate in Arabidopsis seeds at mid-maturation and decline during germination. The similarity of sHSP regulation in such diverse species supports the conclusion that there is a selective advantage to this pattern

of sHSP accumulation. Although mid-maturation is still many days before noticeable desiccation of the seed, the acquisition of desiccation tolerance and dormancy are manifested at the mid-maturation stage of development (Meurs et al., 1992; Ooms et al., 1993; Giraudat et al., 1994; Koornneef and Karssen, 1994). The correlation of sHSP expression with the development of desiccation tolerance and dormancy suggests a possible role for the sHSPs in either or both of these processes, as has been previously hypothesized (Almoguera and Jordano, 1992; DeRocher and Vierling, 1994). A similar pattern of expression is seen for the LEA genes (Parcy et al., 1994), which have also been hypothesized to be important in these aspects of seed maturation. It is interesting that several LEA genes are also expressed under dehydrating conditions (water stress, osmotic stress, and cold stress) in vegetative tissues (Skriver and Mundy, 1990), and Almoguera et al. (1993) reported that seed sHSPs are expressed in response to dehydration stress in sunflower leaves. However, previous studies have shown that sHSP expression is not a general phenomenon during water stress (for a review, see Vierling, 1991), and we have found that Arabidopsis leaves that were water stressed for 10 d did not accumulate detectable levels of class I sHSPs (N. Wehmeyer, unpublished observations). Thus, there is not a simple relationship between low tissue water content and sHSP expression. It is not surprising that the response of vegetative tissues to desiccation would be different from the process of desiccation in seeds, and a specialized role for sHSPs related to seed desiccation is still consistent with these data.

Understanding how sHSPs function in seeds would be facilitated by information concerning the localization of their expression in seed tissues. In developing pea seeds the class I sHSPs accumulated in both the cotyledons and embryonic axis, but localization within these organs was not reported (DeRocher and Vierling, 1994). Coca et al. (1994) concluded from immune localization by tissue printing that sHSPs were uniformly distributed throughout the seed. zur Nieden et al. (1995) found intracellular localization of developmentally expressed sHSPs to be mainly in the nuclei for *Lycopersicon esculentum*, *Vicia faba*, and *Zea mays*. Immunostaining was detected in a variety of tissues in these seeds, but a uniform distribution throughout the seed was not apparent. We found that the class I sHSPs could be detected in the cotyledon and root of germinating Arabidopsis seedlings. This suggests that the proteins were present in both the cotyledon and in the embryonic axis of the mature seed, although we cannot strictly rule out that these sHSPs were newly synthesized during early imbibition/germination. Very recently, in situ hybridization studies with *Athsp17.6* confirmed that transcripts of this gene are found in both cotyledons and axes (Prändl et al., 1995). The same study also showed that expression was most prominent in the vascular cords of the cotyledons and the procambium of the axis. Whether AtHSP17.4, which we showed is the most predominant gene product in the seed, has a similar expression pattern to *Athsp17.6* has not been investigated. Considerable information remains to be gathered concerning the localization of sHSP expression during seed development.

To investigate further the relationship of sHSP expression to the development of seed dormancy and desiccation tolerance, we examined the level of class I sHSPs in several Arabidopsis seed mutants. The mutants tested in this study, which included *aba1* and mutants at five *abi* loci (Table I), were all originally isolated by screening for reduced seed dormancy or the ability to germinate in the presence of ABA (Koornneef et al., 1982, 1984; Finkelstein, 1994). We found that mutations at the *abi3* locus strongly reduced levels of class I sHSPs compared with wild-type seeds, whereas all other mutants had essentially wild-type sHSP levels. Because seeds of *aba1*, *abi1*, *abi2*, and *abi1 abi2* display a greater reduction in dormancy than *abi3-1* (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Finkelstein, 1994), but do not show reduced levels of class I sHSPs, it can be surmised that, if class I sHSPs are necessary for the acquisition of dormancy, they are not sufficient.

The five *abi* mutants we examined are believed to define two ABA response pathways, with *abi1* and *abi2* in the pathway that affects both seed and vegetative phenotypes and *abi3*, *abi4* and *abi5* in another pathway that is specific to seeds (Finkelstein and Somerville, 1990; Finkelstein, 1994). Our results indicate that only the latter pathway mediates sHSP expression. The presence of apparently wild-type levels of sHSPs in *abi4* compared with severely reduced levels in *abi3* is similar to what has been observed regarding the expression of several LEA genes in these mutants (R.R. Finkelstein, unpublished data). Presence of wild-type levels of sHSPs in both *abi4* and *abi5-1* could be due to the weak nature of these alleles and is not inconsistent with the conclusion that the seed-specific response pathway modulates sHSP expression. The *abi3* locus is well characterized and affects a variety of seed processes, including the accumulation of seed storage proteins (Finkelstein and Somerville, 1990; Nambara et al., 1992). However, the reduction of sHSPs in *abi3-1* seeds is much greater than the reduction in seed storage proteins, indicating that the sHSP phenotype is not merely a consequence of an overall decrease in seed protein production. The seeds of *abi3-1* are desiccation-tolerant. Therefore, if class I sHSPs are required for desiccation tolerance, as we and others have hypothesized, apparently wild-type levels of sHSPs are not necessary for this function.

The desiccation-intolerant *abi3-6* seeds do not appear to produce class I sHSPs. The absence of sHSPs in the strong *abi3* allele correlates with the lack of desiccation tolerance of this allele and supports a role for sHSPs in desiccation tolerance. However, because *abi3* mutants are pleiotropically defective in many aspects of late seed maturation, it is difficult to causally relate specific defects with the desiccation-intolerant phenotype. Null sHSP mutants or transgenic sHSP antisense plants (with <10% wild-type levels of sHSPs) would facilitate a critical test of the hypothesis that sHSPs are necessary for desiccation tolerance. We conclude that sHSPs are part of the normal program of late seed maturation and are in some way controlled by the seed-specific ABA response pathway. We do not rule out a role for sHSPs in desiccation tolerance.

Nothing is known about the molecular nature of the *abi4* and *abi5* gene products. In contrast, the *abi3* gene has been cloned and the ABI3 protein was found to have homology to the maize transcriptional activator VP1 (Giraudat et al., 1992; Nambara et al., 1995). Several seed-specific mRNAs are decreased in *abi3* mutants, including those encoding certain seed storage proteins, proteins that may function in the maturation process, and LEA proteins. The fact that ABI3 is present prior to sHSP expression in seeds, along with the observed decrease in sHSPs in *abi3* seeds, suggests that ABI3 may be directly or indirectly involved in the regulation of *Athsp17.4* and *Athsp17.6* expression.

abi3 has also recently been described as a heterochronic mutation (Nambara et al., 1995), because developing seeds of strong alleles appear to directly enter a germination program, bypassing the maturation program. This aberrant timing of expression of the germination program is observed in at least two other mutants, *fus3* and *lec1* (Meinke, 1992; Keith et al., 1994), which are desiccation-intolerant but can be rescued during development. Similar to *abi3* the *fus3* and *lec1* defects do not appear to affect vegetative growth. Studies of *fus3* indicate that it may also encode a transcriptional activator important for proper expression of the late seed maturation program and that *fus3* regulates a subset of late maturation genes distinct from those regulated by *abi3* (Bäumlein et al., 1994; Nambara et al., 1995). It will be of interest to measure sHSP expression in these mutants to test further whether sHSPs are specifically regulated by ABI3, as opposed to affected indirectly by the absence of the late seed maturation program.

Recent in vitro work has implicated the plant class I sHSPs as molecular chaperones that both prevent aggregation of denatured proteins and assist in the refolding of denatured proteins (Lee et al., 1995a). In vivo this function could be envisioned to prevent the aggregation of other proteins in the dehydrating seed; it could also be important during germination, because sHSPs could act as molecular chaperones by assisting in the refolding of other proteins upon water uptake during germination. Further investigations of the molecular mechanism of sHSP action, including identification of potential substrates within the seed, will be necessary to understand the significance of sHSP expression to plant development. The fact that sHSPs accumulate in such diverse species during seed maturation indicates that this process is evolutionarily significant, and determining its function warrants additional investigation.

ACKNOWLEDGMENTS

We would like to thank Dr. Y. Komeda for *Athsp17.4* and *Athsp18.2* cDNAs, Dr. P. McCourt for homozygous *abi3-6* seed, and Drs. G. Lee and R. Azpiroz for critical review of the manuscript.

Received February 14, 1996; accepted June 22, 1996.

LITERATURE CITED

Almoguera C, Coca MA, Jordano J (1993) Tissue-specific expression of sunflower heat shock proteins in response to water stress. Plant J **4:** 947–958

Almoguera C, Jordano J (1992) Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock proteins and Lea mRNAs. Plant Mol Biol **19:** 781–792

Bäumlein H, Miséra S, Luerssen H, Kölle K, Horstmann C, Wobus U, Müller AJ (1994) The FUS3 gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. Plant J **6:** 379–387

Chen Q, Lauzon LM, DeRocher AE, Vierling E (1990) Accumulation, stability, and localization of a major chloroplast heat-shock protein. J Cell Biol **110:** 1873–1883

Coca MA, Almoguera C, Jordano J (1994) Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. Plant Mol Biol **25:** 479–492

DeRocher AE, Helm KW, Lauzon LM, Vierling E (1991) Expression of a conserved family of cytoplasmic low molecular weight heat shock proteins during heat stress and recovery. Plant Physiol **96:** 1038–1047

DeRocher AE, Vierling E (1994) Developmental control of small heat shock protein expression during pea seed maturation. Plant J **5:** 93–102

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Finkelstein RR (1994) Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. Plant J **5:** 765–771

Finkelstein RR, Somerville CR (1990) Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol **94:** 1172–1179

Ghosh S, Gepstein S, Heikkila JJ, Dumbroff EB (1988) Use of a scanning densitometer or ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissues. Anal Biochem **169:** 227–233

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis* ABI3 gene by positional cloning. Plant Cell **4:** 1251–1261

Giraudat J, Parcy F, Bertauche N, Gosti F, Leung J, Morris P-C, Bouvier-Durand M, Vartanian N (1994) Current advances in abscisic acid action and signalling. Plant Mol Biol **26:** 1557–1577

Helm KW, Abernathy RH (1990) Heat shock proteins and their mRNAs in dry and early imbibing embryos of wheat. Plant Physiol **93:** 1626–1633

Helm KW, Vierling E (1989) An *Arabidopsis thaliana* cDNA clone encoding a low molecular weight heat shock protein. Nucleic Acids Res **17:** 7995

Hernandez LD, Vierling E (1993) Expression of low molecular weight heat-shock proteins under field conditions. Plant Physiol **101:** 1209–1216

Howarth C (1990) Heat shock proteins in *Sorghum bicolor* and *Pennisetum americanum*. II. Stored RNA in sorghum seed and its relationship to heat shock protein synthesis during germination. Plant Cell Environ **13:** 57–64

Hsieh M-H, Chen J-T, Jinn T-L, Chen Y-M, Lin C-Y (1992) A class of soybean low molecular weight heat shock proteins. Immunological study and quantitation. Plant Physiol **99:** 1279–1284

Keith K, Kraml M, Dengler NG, McCourt P (1994) *fusca3*: a heterochronic mutation affecting later embryo development in *Arabidopsis*. Plant Cell **6:** 589–600

Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol **90:** 463–469

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLVC, Karssen CM (1982) The isolation of abscisic acid (ABA)-deficient mutants by selection of induced revertants in non-germinating gibberellin-sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet **61:** 385–393

Koornneef M, Karssen CM (1994) Seed dormancy and germination. *In* EM Meyerowitz, CR Somerville, eds, Arabidopsis. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 313–334

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid insensitive mutants of *Arabidopsis thaliana*. Physiol Plant **61:** 377–383

Lee GJ, Pokala N, Vierling E (1995a) Structure and *in vitro* molecular chaperone activity of cytosolic small heat shock proteins from pea. J Biol Chem **270:** 10432–10438

Lee JH, Hübel A, Schöffl F (1995b) Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic *Arabidopsis*. Plant J **8:** 603–612

Meinke DW (1992) A homoeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. Science **258:** 1647–1650

Meinke DW (1994) Seed development in *Arabidopsis thaliana*. *In* EM Meyerowitz, CR Somerville, eds, Arabidopsis. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 253–295

Meurs C, Basra AS, Karssen CM, van Loon LC (1992) Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana*. Plant Physiol **98:** 1484–1493

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res **8:** 4321–4329

Nambara E, Keith K, McCourt P, Naito S (1994) Isolation of an internal deletion mutant of the *Arabidopsis thaliana ABI3* gene. Plant Cell Physiol **35:** 509–513

Nambara E, Keith K, McCourt P, Naito S (1995) A regulatory role for the ABI3 gene in the establishment of embryo maturation in *Arabidopsis thaliana*. Development **121:** 629–636

Nambara E, Naito S, McCourt P (1992) A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. Plant J **2:** 435–441

Ooms JJJ, Lèon-Kloosterziel KM, Bartels D, Koornneef M, Karssen CM (1993) Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*. Plant Physiol **102:** 1185–1191

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J (1994) Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. Plant Cell **6:** 1567–1582

Patel A, Bang N, Finkelstein R (1994) Comparison of ABA- and ABI-regulated gene expression in ABA-insensitive (*abi*) mutants of *Arabidopsis thaliana*. Plant Cell Physiol **35:** 969–973

Prändl R, Kloske E, Schöffl F (1995) Developmental regulation and tissue-specific differences of heat shock gene expression in transgenic tobacco and *Arabidopsis* plants. Plant Mol Biol **28:** 73–82

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512

Takahashi T, Komeda Y (1989) Characterization of two genes encoding small heat-shock proteins in *Arabidopsis thaliana*. Mol Gen Genet **219:** 365–372

Takahashi T, Naito S, Komeda Y (1992) The *Arabidopsis* HSP18.2 promoter/GUS gene fusion in transgenic *Arabidopsis* plants: a powerful tool for the isolation of regulatory mutants of the heat-shock response. Plant J **2:** 751–761

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol **42:** 579–620

Vierling E, Harris LM, Chen Q (1989) The major LMW HSP in chloroplasts shows antigenic conservation among diverse higher plant species. Mol Cell Biol **9:** 461–468

Vierling E, Sun A (1989) Developmental expression of heat shock proteins in higher plants. *In* J Cherry, ed, Environmental Stress in Plants. Springer-Verlag, Berlin, pp 343–354

Waters ER (1995) The molecular evolution of the small heat-shock proteins in plants. Genetics **141:** 785–795

Waters ER, Lee GJ, Vierling E (1996) Evolution, structure and function of the small heat shock proteins in plants. J Exp Biol **47:** 325–338

zur Nieden U, Neumann D, Bucka A, Nover L (1995) Tissue-specific localization of heat-stress proteins during embryo development. Planta **196:** 530–538