Plant Physiol. (1993) 101: 1209–1216

# Expression of Low Molecular Weight Heat-Shock Proteins under Field Conditions[1]

**Lorraine D. Hernandez[2] and Elizabeth Vierling***

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721

**Heat-shock proteins (HSPs) are known to be expressed in plants experiencing high-temperature stress. We have examined the expression of class I cytoplasmic low molecular weight (LMW) HSPs and find that these HSPs also frequently accumulate in seeds, seed pods, and flowers during a normal growing season. We first examined the expression of class I cytoplasmic LMW HSPs by western blot analysis in a range of seed samples from both commercially grown and wild legumes. LMW HSPs were present in all seed samples, indicating that these HSPs are regularly expressed in these tissues. To examine more specifically conditions under which LMW HSPs were produced during an average growing season, additional studies of *Medicago sativa* were carried out during the fall season in Tucson, AZ. Plants were irrigated to avoid conditions of water stress, and canopy temperature was monitored throughout the study period. LMW HSP expression in leaves, flowers, and developing seed pods was analyzed by western blotting. Results show that in the field HSPs are frequently produced in flowers and seed pods, even in plants that show no HSP expression in leaves. Parallel greenhouse studies indicate that HSP expression in seeds is in part developmentally regulated. In total our data suggest a more widespread occurrence of HSPs in optimal growth environments and emphasize their potential role during reproduction.**

In response to high-temperature stress, plants and other organisms synthesize a discrete set of proteins known as HSPs that are hypothesized to prevent and/or repair stress-induced damage (Lindquist and Craig, 1988). In higher plants, LMW HSPs with molecular masses between 15,000 and 30,000 D are the major proteins synthesized during heat stress and can accumulate to more than 1% of total leaf cell protein (Mansfield and Key, 1987; DeRocher et al., 1991; Hsieh et al., 1992). Four gene families of LMW HSPs have been characterized in plants (for review, see Vierling, 1991). The class I and class II families encode cytoplasmic proteins, and the other two families encode endomembrane- and chloroplast-localized proteins. Although there is good evidence that HSPs in the HSP90, HSP70, and HSP60 classes act as "molecular chaperones" (Gething and Sambrook, 1992), the function of the LMW HSPs is unknown. However, the evolutionary conservation of the LMW HSPs and their presence in three different cell compartments suggest that they are critical to plant survival.

Few investigators have examined the expression of HSPs in plants grown under field conditions. Kimpel and Key (1985) showed that mRNAs encoding class I and II LMW HSPs accumulated in leaf tissues of soybeans experiencing high temperatures in the field (at least 38°C air temperatures). The mRNA levels were highest in leaves from nonirrigated plants, presumably correlated with higher leaf temperatures. In a study of dry-land cotton, total leaf protein profiles from plants grown in irrigated and nonirrigated plots were examined (Burke et al., 1985). Based on comigration with HSPs identified in leaves in laboratory experiments, Burke et al. concluded that high molecular weight and LMW HSPs accumulated in the nonirrigated plants in which canopy temperatures reached 40°C. Using growth chamber conditions designed to simulate a day of high-temperature stress in the field, Chen et al. (1990) and DeRocher et al. (1991) demonstrated that both chloroplast and class I cytoplasmic LMW HSPs accumulated in direct proportion to leaf temperature in *Pisum sativum*. These studies provide good evidence that HSPs are expressed in the natural environment but have been limited to an examination of vegetative tissues and primarily to instances of severe stress.

Although LMW HSPs have been found only under stress conditions in vegetative tissues, recent observations have suggested that specific LMW HSPs are also expressed in developing seeds in the absence of stress. LMW HSP mRNAs have been shown to be present in commercially produced *P. sativum* seeds (Vierling and Sun, 1987), in growth chamber-grown *Triticum aestivum* and *Helianthus annus* seeds (Helm and Abernethy, 1990; Almoguera and Jordano, 1992), and in *Sorghum bicolor* and *Pennisetum americanum* seeds (Howarth, 1990). Initial results indicate that the mRNAs are also translated during seed development; the LMW HSP proteins are detectable in seeds of *T. aestivum* and *P. sativum* (Helm and Abernethy, 1990; Helm et al., 1991). Thus, LMW HSPs may play a role in seed development in addition to their role during temperature stress.

We were interested in investigating further the extent to

[1] Supported by U.S. Department of Agriculture Competitive Grants Office grant 88–37264–3914, U.S. Department of Agriculture Southwest Consortium grant 88–34186–3340, and State of Arizona Hatch funds. L.D.H. was supported in part by grants from the Howard Hughes Medical Institute and the National Science Foundation to the University of Arizona for undergraduate biology research.

[2] Present address: University of California San Francisco School of Medicine, Department of Biochemistry and Biophysics, San Francisco, CA 94143.

* Corresponding author; fax 1–602–621–3709.

Abbreviations: DAP, days after pollination; HSP, heat-shock protein; LMW, low molecular weight.

which LMW HSPs are expressed in different plant organs during growth in a field environment. We first tested for the presence of class I cytoplasmic LMW HSPs in seeds from both commercially grown and wild legumes using western blot analysis. Specific LMW HSPs were detected in the seeds of all species. In further studies of *Medicago sativa*, LMW HSP expression in leaves, flowers, and developing seed pods was examined under field conditions. Results show that the LMW HSPs are expressed in flowers and seeds at times when they are absent in leaves. These data demonstrate that different plant organs respond differently to environmental conditions and emphasize that the functions of the LMW HSPs are not restricted to conditions of severe temperature stress.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Pisum sativum* L. (cv Little Marvel) and *Glycine max* were obtained from Carolina Biological Supply (Burlington, NC; *G. max*, catalog No. 15–8442). *Vigna unguiculata* cv CB5 (cowpea) seeds (a gift of Dr. A. Hall, University of California, Riverside, CA) were harvested from plants grown in the Imperial Valley, California, and were not subjected to heating or drying after harvesting. *Phaseolus acutifolius* (tepary bean) seeds (a gift of Dr. R. Robichaux, University of Arizona) were produced commercially by Pima Indians on the Gila River Reservation, Arizona. *Acacia constricta* Benth. (white-thorn acacia) seeds were harvested in October 1990 from dried pods of wild plants growing in the vicinity of Tucson, AZ. *Medicago sativa* (CUF101) seeds were produced under greenhouse conditions in Tucson, AZ. They were harvested between March and April and not further treated (a gift of Dr. T. McCoy, now at Montana State University). Seeds utilized showed a minimum of 80% germination (alfalfa) or 75% germination (all others) when planted in soil.

### Plant Growth and Heat-Stress Treatments

*P. sativum*, *G. max*, *V. unguiculata*, *P. acutifolius*, *M. sativa*, and *A. constricta* seeds were planted in soil and grown in a growth chamber with approximately 300 $\mu$mol $m^{-2}$ $s^{-1}$ PAR under a 16-h, 22°C day/8-h, 18°C night cycle. Plants (2 to 3 weeks old) were subjected to a 4-h, 40°C heat-stress treatment according to the procedure of Chen et al. (1990), in which the temperature was increased gradually (4°C $h^{-1}$) to the maximum temperature and maintained at the maximum for 4 h. Treatments were carried out during the day cycle in the lighted growth chamber. Leaf samples for protein analysis were isolated before the stress treatment and directly after the 4 h of maximal stress.

### Growth Conditions for *M. sativa* Field and Greenhouse Experiments

Field plots of *M. sativa* CUF101 (Lehman et al., 1983) were maintained on the Campbell Avenue Farm facility of the University of Arizona Field Experiment Station. *M. sativa* CUF101 is the major, nondormant cultivar used in commercial alfalfa production in Arizona and is adapted to year-round growth and harvest. All plants were derived from two original clones designated CUF101–3 and CUF101–10 and were transplanted to the field approximately 1 year before the experiment. Plant spacing in the field was 0.9 m between rows and 0.45 m between individual plants. The field was cut back in early September, and all measurements were made after plants had regenerated full vegetative crowns. Plants were watered by flood irrigation a minimum of once per week. Whole plant temperature was monitored using two stationary IR thermometers (15° view angle; Everest Interscience, Fullerton, CA). Plant temperature along with wet and dry bulb air temperatures were recorded with a CR10 data logger (Campbell Scientific, Inc., Logan, UT) at 10-min intervals throughout the sampling period, with the exception of 3 d during which the recording equipment was not operational. Air temperatures during the sampling period were average for this season based on comparisons of monthly maximum, minimum, and average temperatures during a 5-year period as recorded by AZMET, the meteorological station of the University of Arizona Campus Agricultural Station.

Several *M. sativa* plants of the same clonal origin (CUF101–3 or CUF101–10) were removed from the field and maintained in a greenhouse at the same farm facility. Maximum and minimum daily temperatures were recorded at two positions in the greenhouse in the vicinity of the plants on the day of sampling.

*M. sativa* leaf, flower, and pod surface temperatures were measured directly before samples were harvested for protein analysis using an IR thermometer with a spot detection size of 0.46 $cm^2$ (Everest Interscience). For leaf samples, fully expanded leaves were used. Leaf temperatures as determined with the hand-held thermometer were in good agreement with the plant temperatures recorded by the stationary IR monitors. Flower temperatures were approximated by focusing the thermometer on freshly pollinated flowers of large racemes. Open, pollinated flowers were tagged on the day after pollination to obtain pod samples at different developmental stages. Pod temperature at all stages of development were similarly measured. Three to six leaves, flowers, or pods from one or more plants were measured and used to derive an average temperature for that day. The SD for these measurements was approximately ±1.0°C. Samples were obtained between 2 and 3 PM, which for the field samples was typically close to the time of day when air temperature was at a maximum.

### Protein Sample Preparation

For one-dimensional SDS-PAGE, total proteins were extracted by grinding tissue directly in SDS-PAGE sample buffer (60 mM Tris-HCl, 60 mM DTT, 2% SDS, 15% Suc, 5 mM $\epsilon$-amino-*n*-caproic acid, 1 mM benzamidine [pH 8.0]) using 10 $\mu$L $mg^{-1}$ of tissue. For *A. constricta* the same buffer was further supplemented with 0.275% diethyldithiocarbamic acid and 4% polyvinylpolypyrrolidone (insoluble, mol wt 240,000). After grinding, samples were boiled for 3 min, and insoluble debris was removed by centrifugation (5 min, 12,000*g*). The protein-containing supernatant was stored at −20°C until use. Protein concentration was determined after

acetone precipitation using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

For two-dimensional gel analysis protein samples were prepared in the same way and then acetone precipitated, with the exception of the *A. constricta* samples. For these samples, protein was extracted in 4% SDS, 5% 2-mercaptoethanol, 5% Suc, and 6 mg $mL^{-1}$ polyvinylpolypyrrolidone (20 $\mu L$ $mg^{-1}$ of tissue) and processed as for SDS-PAGE. Samples were then precipitated with 5 volumes of cold acetone before gel analysis.

Samples from field- and greenhouse-grown *M. sativa* were removed from the plant, placed in plastic bags, and stored on ice until protein extractions were performed. All protein extractions were performed within 90 min of harvest. Flowers sampled showed evidence of pollination the same day or were ready to open the following day.

### HSP Antiserum

Rabbit antiserum used for the detection of LMW HSPs was prepared against the carboxyl-terminal 15.3-kD *P. sativum* PsHSP18.1, which had been overexpressed as a fusion protein in *Escherichia coli* (DeRocher et al., 1991). The PsHSP18.1 antiserum reacts strongly with five members of the class I cytoplasmic LMW HSP gene family in pea (DeRocher et al., 1991).

### Gel Electrophoresis and Immunoblotting

Protein samples were analyzed by SDS-PAGE on continuous 12.5% acrylamide gels or on 10 to 16% gradient gels in the buffer system of Laemmli (1970). Either the gels were stained or proteins were electrophoretically transferred to nitrocellulose for immunodetection reactions.

Two-dimensional gel analysis was performed basically according to the method of O'Farrell (1975). Acetone-precipitated protein samples were air dried and resuspended in 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 0.2% 3.5 to 10 ampholines, and 1.8% 5 to 7 ampholines.

Proteins were transferred to nitrocellulose from either one- or two-dimensional gels and were reacted with crude PsHSP18.1 antiserum (1:500 dilution) as described previously (Vierling et al., 1989). Immunoblots were subsequently reacted with $^{125}$I-protein A (ICN Radiochemicals, Irvine, CA; 3000 Ci $mmol^{-1}$) and visualized by autoradiography with one or two intensifying screens.

## RESULTS

### Detection of HSPs in Seeds of Several Legumes

To determine whether HSP expression in seeds, as observed in *P. sativum* (Helm et al., 1991), is a general phenomenon in legumes, five other species of cultivated or wild legumes were examined: *G. max, V. unguiculata, P. acutifolius, A. constricta,* and *M. sativa*. To compare immunoreactive proteins in seeds with immunoreactive proteins induced during heat stress, leaf samples from nonstressed and heat-stressed plants of each species were also examined.

Figure 1 (A-C) shows the results of one-dimensional analyses of leaf and seed protein samples from the six legume species. For all species, antiserum against *P. sativum* PsHSP18.1 was used for detection of HSPs. PsHSP18.1 antiserum reacted with one or more protein bands at a $M_r$ of approximately 18,000 in all samples prepared from heat-stressed leaves (lanes 5). No reaction was obtained with proteins of this size in either the control leaf samples (lanes 4) or in leaf samples reacted with preimmune serum (lanes 7 and 8). In all of the seed samples (lanes 6), PsHSP18.1 antiserum reacted with proteins of the same $M_r$ as seen in leaves. In *G. max, V. unguiculata, P. acutifolius,* and *M. sativa,* one or more additional proteins of lower $M_r$ reacted with the antiserum. Preimmune controls showed no reaction with seed proteins of the same sizes (lanes 9).

Two-dimensional immunoblot analysis (Fig. 1, D and E) was used to confirm comigration of HSPs in leaves and seeds from the different species. In heat-stressed *P. sativum* leaves, the antiserum detects five major polypeptides in the $M_r$ 18,000 region, as reported previously (DeRocher et al., 1991). All five of these are also present in the seed sample. In the other legume species, the heterologous antiserum detects multiple polypeptides of approximately $M_r$ 18,000 in the heat-stressed leaf samples. These proteins most likely represent members of the class I LMW HSP gene family in each species. In all species except *G. max,* there was good correspondence between most of the immunoreactive proteins in seeds and the major HSPs detected in leaves. Comigration of the leaf HSPs and seed polypeptides was demonstrated unambiguously by combining the two samples on a single gel, which was then blotted to nitrocellulose and reacted with the PsHSP18.1 antiserum (not shown).

The *G. max, V. unguiculata, P. acutifolius,* and *M. sativa* seed samples contained additional proteins that reacted with the antiserum but that were not present in the heat-stressed leaf samples or in the preimmune controls. These polypeptides were detected in both the one-dimensional and two-dimensional gel analyses. It is possible that these cross-reacting seed proteins are additional HSPs not expressed in leaves. However, those proteins that are significantly larger than $M_r$ 18,000, as in the *G. max* sample (lane 6), are most likely unrelated proteins that react nonspecifically with the antiserum. Although the immunoreactive polypeptides of $M_r$ approximately 18,000 may be LMW HSPs uniquely expressed in seeds, another possibility is that they are partial proteolytic products of the major LMW HSPs seen in both leaves and seeds. Two lines of evidence suggest that the latter explanation is invalid. First, the seed pattern was reproducible (two or more replications per species), which is inconsistent with the variation that would be expected from sample to sample if proteolysis were occurring. Second, tests performed in an attempt to allow proteolysis during sample preparation failed to change the protein pattern. For these tests, seeds of *M. sativa* were ground in 60 mM Tris-HCl, 60 mM DTT (pH 8.0) or in 60 mM Tris-HCl, 5 mM DTT, 0.2% SDS (pH 8.0) and allowed to incubate at room temperature for 60 min. Samples were taken before, during, and after the incubation and analyzed by western blotting. All samples showed an identical pattern of cross-reacting bands (not shown). Therefore, we conclude that these smaller proteins are not proteolytic products produced during sample preparation.

In total, these results demonstrate that the class I LMW

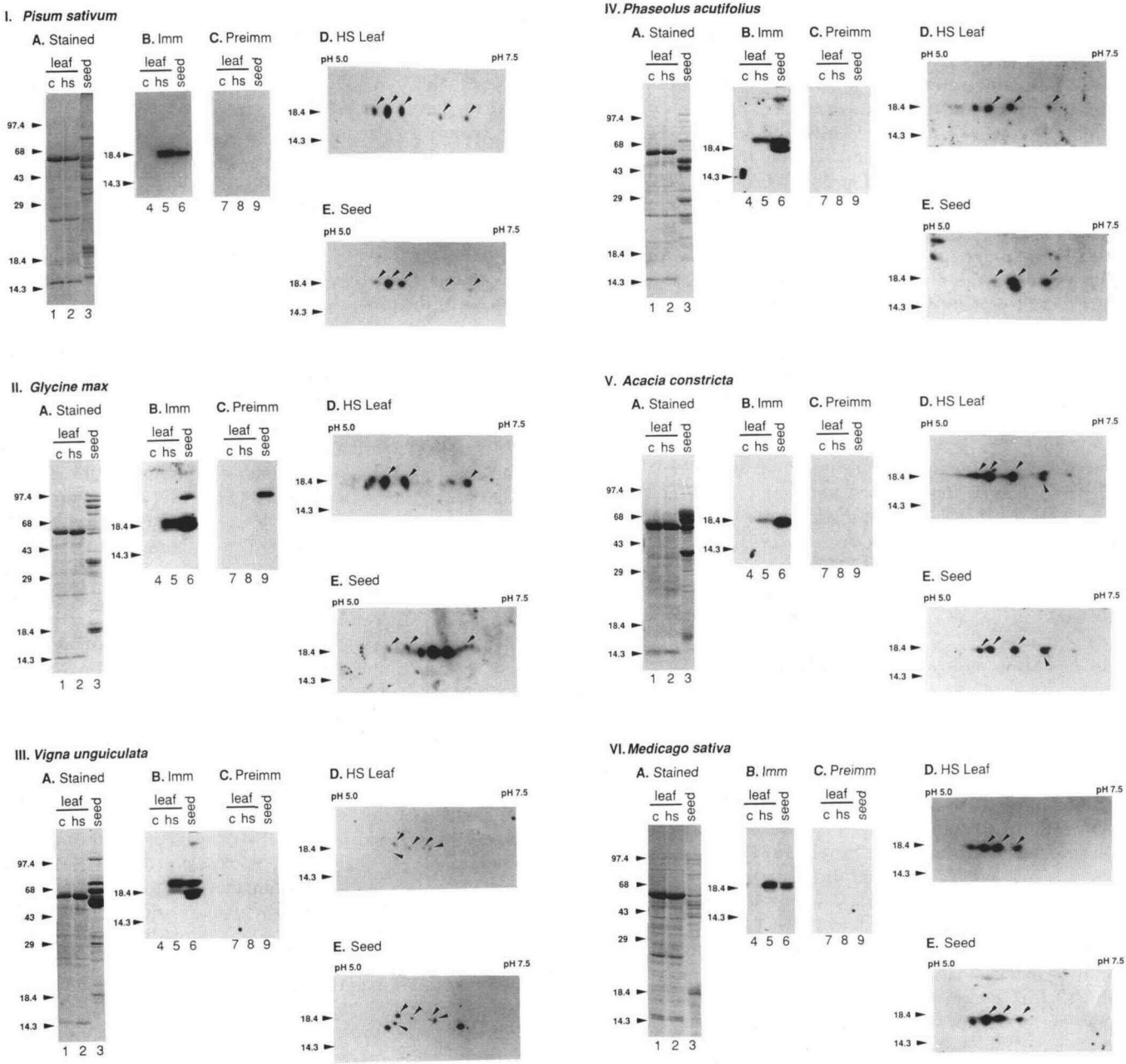


**Figure 1.** Detection of class I LMW HSPs in mature seeds and heat-stressed leaves of several legume species. Species names are given above each set of panels. A to C, SDS-PAGE and immunoblot analysis of proteins from control leaf (lanes 1, 4, and 7), heat-shocked leaf (lanes 2, 5, and 8), and seed (lanes 3, 6, and 9) samples from the indicated species. A, Coomassie blue-stained samples. B, Samples reacted with PsHSP18.1 antiserum. C, Samples reacted with preimmune serum. Approximate mol wt, expressed in thousands, is indicated at the left of each panel. D and E, Two-dimensional immunoblot analysis of the heat-shocked leaf and seed samples from each species as labeled. Major HSPs detected in both seeds and leaves are indicated by arrowheads. One-dimensional gel analysis was performed on 10 to 16% gradient gels with 45 $\mu$g of protein per lane. For two-dimensional gel analysis and immunoblotting, 250 $\mu$g of protein were used per gel, and second-dimension gels were 12.5% acrylamide. Only the lower half of the two-dimensional gel is shown. Immunoblots were processed as described in "Materials and Methods."

HSPs are not normally present in leaves unless they are heat stressed and that certain HSPs are present in seeds matured in their normal growth environment.

### *M. sativa* Leaf, Flower, and Pod Temperatures

To investigate further HSP expression in different plant organs during a normal growing season, experiments in which plant temperature was monitored were performed with *M. sativa*. *M. sativa* is grown in the Tucson area, and cultivars adapted to the southern Arizona environment have been developed (Lehman et al., 1983). To obtain samples throughout a representative growth cycle, plants were cut back to initiate new growth. Sampling was begun 2 weeks after cutting and continued for approximately 2 months (mid-September to mid-November). In the field, plant and air temperatures were monitored continuously. Leaf, flower, and pod samples for protein analysis were collected once weekly from both field and greenhouse plants.

In the field the maximum daily air temperature (dry bulb) varied from 20.5 to 39.2°C during the course of the sampling period, including 8 d of more than 35°C and an additional 8 d of between 33 and 35°C. Despite these high air temperatures, plant temperature, as measured with the stationary IR thermometers, never exceeded 31.3°C. The relationship between plant and air temperature is illustrated in Figure 2, in which the highest daily plant temperature is plotted as a function of the corresponding air temperature. Plant temperature was always less than air temperature, and at higher air temperatures the difference between plant and air temperatures increased. The regression analysis in Figure 2 shows that plant temperature increased approximately 0.64°C for every 1.0°C increase in air temperature ($r^2$ = 0.908). This relationship is not unexpected for plants adapted to their environment and growing under well-watered conditions and low humidity. Ability to maintain temperatures well below air temperatures also indicates that the plants were not water stressed during the experiment (Idso et al., 1981).

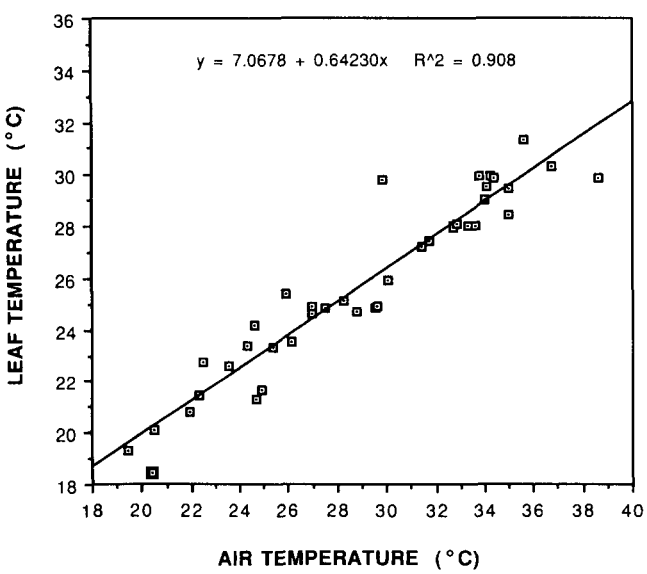


**Figure 2.** Comparison of *M. sativa* leaf and air temperature. The daily high plant temperature is plotted as a function of the corresponding air temperature for all days during the experiment. A simple regression of the relationship between leaf and air temperature is shown.

**Table I.** *Average temperature differences between different plant organs*

| Organs | Field[a] | Greenhouse[b] |
|---|---|---|
| | °C | °C |
| Flower-leaf | 2.92 ± 1.32 | 2.26 ± 1.42 |
| Pod[c]-leaf | 2.59 ± 0.83 | 2.68 ± 1.29 |

[a] ±SD, $n$ = 7. [b] ±SD, $n$ = 9 (flower-leaf) or $n$ = 8 (pod-leaf). [c] Includes temperatures of pods of different ages.

Plants maintained in the greenhouse experienced significantly lower air temperatures than those in the field. The maximum air temperature on a day of tissue sampling was 33.3°C, recorded on two occasions. On all other sampling days the maximum air temperature was 30°C or less, and at the time tissue samples were taken the temperature was several degrees below 30°C.

Estimates of leaf, flower, and pod temperature were obtained directly before harvesting samples for protein analysis. The highest temperatures recorded in the field were 25.6 ± 0.4°C for leaves, 28.6 ± 1.4°C for flowers, and 29.2 ± 1.1°C for pods. In the greenhouse the highest temperatures were 23.8 ± 0.8°C for leaves, 24.6 ± 0.9°C for flowers, and 25.0 ± 0.6°C for pods. We noted that leaf temperature was always lower than flower or pod temperature. This relationship was quantified by determining the difference between the flower or pod temperature and the leaf temperature for each day. On average, the difference between leaf temperature and the temperature of flowers or pods was approximately 2.5°C whether estimated in the field or in the greenhouse (Table I). There was no significant difference between flower and pod temperatures.

### HSP Expression in *M. sativa* Leaf, Flower, and Pod Tissues

The level of LMW HSPs in alfalfa leaves, flowers, and pods was examined by SDS-PAGE and immunoblotting with *P. sativum* HSP18.1 antibodies. In the field, leaf temperatures at the time of sampling ranged from 20.0 to 25.6°C, and LMW HSPs were not detected, as shown for several samples in Figure 3A. Leaf temperatures in the greenhouse were lower than in the field, and HSPs were also not detected in these samples (not shown). These results are consistent with previous observations showing that the threshold for class I LMW HSP synthesis is 29 to 30°C in pea leaves (DeRocher et al., 1991). Results obtained with flower samples from the same plants were quite different; LMW HSPs were present in all flowers harvested throughout the field sampling period (Fig. 3A). The relative amount of HSPs in the flowers appeared to correlate with tissue temperature (with the exception of the sample in lane 11), although flower temperatures were only 22.5 to 28.6°C, which is below the threshold temperature for HSP synthesis in leaves. That HSPs are not always expressed in flowers was confirmed by analysis of

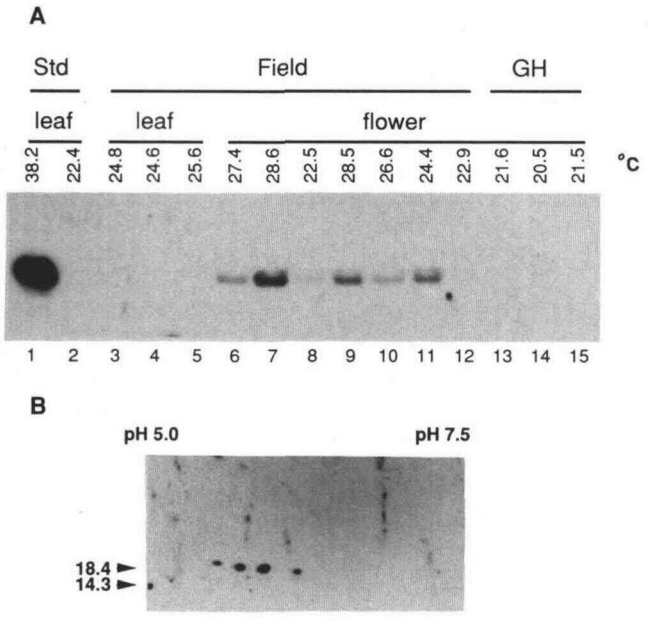


**Figure 3.** Detection of class I LMW HSPs in leaves and flowers of *M. sativa*. A, SDS-PAGE and immunoblotting. Lanes 1 and 2, Standard (Std) *M. sativa* leaf samples treated at control or heat-shock temperatures as in Figure 1. Lanes 3 to 5, Leaf samples from field plants taken on 3 different weeks of the growing season. Lanes 6 to 12, Flower samples from field plants taken on 7 consecutive weeks of the growing season. Samples in lanes 6, 7, and 8 were taken at the same time as leaf samples in lanes 3, 4, and 5, respectively. Lanes 13 to 15, Flower samples from greenhouse-grown (GH) plants. Samples in lanes 13, 14, and 15 were taken on the same day as samples in lanes 6, 7, and 12, respectively. Protein extracts were analyzed by 12.5% SDS-PAGE and reacted with *P. sativum* HSP18.1 antiserum as described in "Materials and Methods." Equal protein (45 $\mu$g) was loaded in each lane. Average temperature of the organ at the time of harvest is shown above each lane. B, Two-dimensional immunoblot analysis of a representative flower sample. Mol wt, expressed in thousands, is indicated on the left.

greenhouse samples, as shown in Figure 3. Six other greenhouse flower samples showed no evidence of HSPs (not shown). Two-dimensional electrophoresis and immunoblotting confirmed that the HSPs in flower samples were the same as those identified in heat-shocked alfalfa leaves (Fig. 3B). These results demonstrate that expression of HSPs can vary between organs of the same plant.

Results from samples of developing pods or mature seeds from the field and greenhouse suggest that LMW HSP expression is controlled by both development and the environment in these organs. In samples developing in the greenhouse, HSPs were not detected in younger pod samples but accumulated later in development and were present in dry seeds (Fig. 4). Two additional greenhouse experiments with tagged pods confirmed the absence of HSPs before 29 DAP, and mature seeds collected from three independent greenhouse harvests showed the same accumulation of HSPs (not shown). Leaf samples taken in parallel to the pod samples showed no HSP expression (not shown). Two $M_r$ classes of immunoreactive polypeptides were detected in a majority of the seed samples but only the larger $M_r$ band has been positively identified as an HSP, as discussed above.

In samples harvested from the field, HSPs were also always detected in mature dry seeds and in pods 29 DAP or older (Fig. 4). However, high levels of HSP expression were also occasionally observed in younger pods, as shown in Figure 4 for a sample harvested 22 DAP. The higher temperature of this sample suggests that in this case HSP expression is a temperature effect. The temperature data and greenhouse study results support the conclusion that class I LMW HSPs are normally expressed during the later stages of seed development even in the absence of stress. The fact that these samples were taken from the same plants as leaf and flower samples shown in Figure 3 further emphasizes that HSP expression can vary between organs of the same plant.

### HSP Expression during Temperature Stress in the Field

In addition to examining well-watered *M. sativa* plants that maintained leaf temperatures well below air temperatures, we also examined leaves, flowers, and pods on 2 d when plants were experiencing heat stress due to water deficit. The temperature differential between leaves and flowers and pods seen in well-watered plants was not seen in the water-stressed plants. Figure 5 shows the results of western analysis of one set of samples. HSP levels were the highest in pod (20 DAP) and flower samples, and HSPs were present, but at low levels, in leaves. These results are consistent with the data obtained with the well-watered plants in that HSP

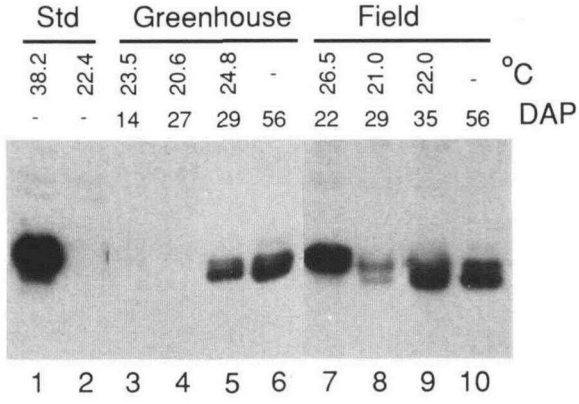


**Figure 4.** LMW HSPs are present in developing seed pods or mature seeds from greenhouse- and field-grown *M. sativa*. Lanes 1 and 2, Standard (Std) samples from leaves treated at heat-shock or control temperatures. Lanes 3 to 5, Samples of whole pods from greenhouse plants harvested at the indicated DAP. Lane 6, Mature, dry seeds from greenhouse-grown plants. Lanes 7 to 9, Samples of whole pods harvested from field-grown plants at the indicated DAP. Lane 10, Mature, dry seeds from greenhouse-grown plants. Temperatures of pods directly before harvest are shown. No temperature is reported for the dry seed samples in lanes 6 and 10 because mature pods were removed from plants and stored at room temperature until fully dry. Equal protein samples (45 $\mu$g) were separated by SDS-PAGE (12.5% gels) and analyzed by immunoblotting with the *P. sativum* HSP18.1 antibodies.

expression varies among organs on the same plant and that young pods at higher temperatures express HSPs.

## DISCUSSION

This is the first study to examine accumulation of a defined class of HSPs under field conditions in a variety of plants as well as in different plant organs. In our survey of different legumes we found that specific LMW HSPs were present in seeds from all species. The samples tested were obtained from crop plants grown under standard production conditions and from a wild species grown in its natural habitat, and the results imply that severe stress conditions are not necessary for expression of HSPs in seeds. The *V. unguiculata* and *A. constricta* seeds matured when air temperatures often may have exceeded 37°C. *P. acutifolius* seeds are typically harvested in the summer, and our samples were most likely exposed to air temperatures up to 38°C. The *M. sativa* seeds used in the first part of this study were matured under greenhouse conditions in which temperatures reached 34°C. We do not know the exact growth and post-harvest conditions for the commercially obtained *P. sativum* and *G. max* seeds. The actual seed temperatures during maturation are not known for any of the samples. Thus, it is possible that the LMW HSPs detected in these seeds were produced in response to high temperatures usually experienced by these plants during seed maturation. However, our data obtained under conditions in which HSPs accumulated in *M. sativa* seeds, along with our observations of HSP accumulation in developing *P. sativum* (Helm et al., 1991) and results from other laboratories (Helm and Abernethy, 1990; Almoguera and Jordano, 1992), suggest that accumulation of LMW HSPs is a component of the normal program of seed development. We conclude that at least some of the LMW HSPs observed in these seeds were produced in response to a developmental, rather than to a temperature, signal. We are currently performing controlled growth experiments with *P. sativum* to examine further the developmental regulation of HSP synthesis.

Class I LMW HSPs have been cloned and sequenced from several plant species and are members of large gene families (Vierling, 1991). Therefore, it was not surprising to find that the *P. sativum* PsHSP18.1 antibodies reacted with multiple polypeptides in each of the species tested. We assume that the immunoreactive proteins detected in heat-stressed leaves of the different legumes are primarily class I LMW HSPs; these polypeptides were not present in control leaves, and there is no evidence that the antibodies cross-react with other classes of HSPs (DeRocher et al., 1991). The pattern and number of HSPs detected were extremely variable between species, consistent with a lower degree of conservation of the LMW HSPs compared with other HSPs. Not all of the HSPs detected in leaves were seen in the seeds of every species, indicating that expression of these HSPs is not coordinately regulated under all conditions. In some species additional immunoreactive proteins with a lower $M_r$ were observed in seeds, but it is not possible to conclude that these are seed-specific HSPs as opposed to proteins that react nonspecifically with the antibodies. We have not tried to quantify the abundance of the HSPs relative to other seed proteins. However, previous experiments have shown that class I LMW HSPs can accumulate to 1 to 1.5% of total protein in vegetative tissues of *P. sativum* and *G. max* under conditions of heat stress similar to those used here (DeRocher et al., 1991; Hsieh, et al., 1992). Therefore, these HSPs appear to be present at comparable levels (within an order of magnitude) in legume seeds.

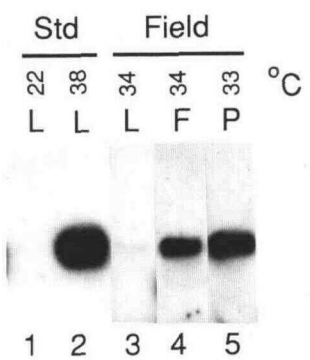


**Figure 5.** HSP levels in leaves, flowers, and pods of water-stressed plants. Lanes 1 and 2, Standard (Std) leaf (L) control and heat-shock samples. Lanes 3, 4, and 5, Leaf (L), flower (F), and seed pod (P) samples from field plants. Average temperatures measured directly before harvest are shown above each lane. Equal protein samples (45 μg) were analyzed as for Figure 4.

Results of our field and greenhouse studies of *M. sativa* not only support the idea that LMW HSP synthesis is developmentally regulated in seeds but also show that reproductive structures frequently express LMW HSPs under conditions that do not elicit HSP accumulation in leaves. Under both well-watered and water-stress conditions, HSPs were present in flowers and seed pods at higher levels than in leaves. We have not attempted to determine whether HSPs are expressed in all parts of the flower or pod or are restricted to specific tissues of these organs. Our data emphasize the cell-autonomous nature of the heat-shock response; there is no evidence that the signal to synthesize HSPs is transmitted between different parts of the same plant.

In most cases in which water is nonlimiting, leaves are capable of thermoregulating by transpirational cooling. Our data show that under well-watered conditions *M. sativa* leaves maintained leaf temperatures at or below 30°C even when air temperatures were more than 35°C. Flowers and developing seed pods have less ability to cool by transpiration, and they were indeed warmer than surrounding leaves. However, although flowers and pods were at higher temperatures than leaves under well-watered conditions, the temperatures at which HSPs were detected in these structures were in many cases lower than temperatures demonstrated to elicit HSP expression in leaves (DeRocher et al., 1991). Also, leaves, flowers, and pods from water-stressed plants had similar temperatures, but flowers and pods had higher levels of HSPs. As already discussed, expression at low temperature in seeds or pods could be explained in part by developmental regulation at later stages of maturation. Expression in younger pods and flowers at temperatures of less than 29°C is more difficult to reconcile. It is possible that measurement of surface temperature inaccurately reflects the

temperature throughout the organ and that the internal temperature of the organs was higher. However, a very large temperature differential seems unlikely.

It is also possible that the flowers or pods reached higher temperatures at a time of day that was not sampled in our study. Because HSPs are stable proteins (Chen et al., 1990; DeRocher et al., 1991), the level of HSPs will depend not just on the temperature of the tissues at the time of sampling but also on the temperature during the rest of the day or on previous days. HSP synthesis is known to recur during daily periods of high temperature (Howarth, 1991). However, from our continuous recording of whole plant temperature, we know that our daily sampling period corresponded closely with the time of highest daily leaf temperatures and that plants were sampled on days corresponding to the highest temperatures recorded during the experiment. Another possibility is that different tissues may show some difference in the temperature of HSP induction. Although there is no evidence from plants or other systems for differences in temperature threshold between organs, a rigorous test of the threshold temperature of HSP induction in flower and pod tissues remains to be performed.

In summary, our results indicate that expression of HSPs in the field occurs more commonly than has been previously demonstrated, even in plants grown in their optimal natural environment or under standard cultivation conditions. HSP expression was frequently observed in reproductive structures, suggesting that HSPs may be particularly important for reproductive success. Our results also add to the growing body of data indicating that LMW HSPs are expressed during development in the absence of stress and demonstrate that the proteins, not only the mRNAs, are synthesized. How these proteins function and the adaptive significance of their expression during development remains to be determined.

## ACKNOWLEDGMENTS

We thank Dr. T. McCoy for advice concerning working with alfalfa and use of his alfalfa fields, Drs. D. Fangmeier, D. Garrot, and S. Huisman for the field IR thermometers and continuous recording equipment, Dr. M. Donoghue for identification of the wild *Acacia* species, Drs. R. Robichaux and A. Hall for gifts of legume seeds, Ms. L. Lauzon for photographic work, and the Campbell Avenue farm and greenhouse crews for plant maintenance. Drs. J. Kimpel and D. DellaPenna provided helpful critiques during preparation of the manuscript.

Received October 7, 1992; accepted December 11, 1992.


## LITERATURE CITED

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

**Burke JJ, Hatfield JL, Klein R, Mullet JE** (1985) Accumulation of heat shock proteins in field-grown cotton. Plant Physiol **78:** 394–398

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

**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

**Gething M-J, Sambrook J** (1992) Protein folding in the cell. Nature **355:** 33–45

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

**Helm KW, DeRocher A, Lauzon L, Vierling E** (1991) Expression of low molecular weight heat shock proteins during seed development (Abstr). J Cell Biochem **14E:** 301

**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

**Howarth CJ** (1991) Molecular responses of plants to an increased incidence of heat shock. Plant Cell Environ **14:** 831–841

**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. Plant Physiol **99:** 1279–1284

**Idso SB, Reginato RJ, Reicosky DC, Hatfield JL** (1981) Determining soil-induced plant water potential depressions in alfalfa by means of infrared thermometry. Agron J **73:** 826–830

**Kimpel JA, Key JL** (1985) Presence of heat shock mRNAs in field grown soybeans. Plant Physiol **79:** 672–678

**Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

**Lehman WF, Nielson MW, Marbel VL, Stanford EH** (1983) Registration of CUF 101 alfalfa. Crop Sci **23:** 398

**Lindquist SL, Craig E** (1988) The heat shock proteins. Annu Rev Genet **22:** 631–677

**Mansfield MA, Key JL** (1987) Synthesis of the low molecular weight heat shock proteins in plants. Plant Physiol **84:** 1007–1017

**O'Farrell PH** (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **10:** 4007–4021

**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 low-molecular weight heat shock protein in chloroplasts shows antigenic conservation among diverse higher plant species. Mol Cell Biol **9:** 461–468

**Vierling E, Sun A** (1987) Developmental expression of heat shock proteins in higher plants. *In* J Cherry, ed, Environmental Stress in Plants. Springer-Verlag, Berlin, pp 343–354