

THE METHIONINE-RICH LOW-MOLECULAR-WEIGHT CHLOROPLAST HEAT-SHOCK PROTEIN: EVOLUTIONARY CONSERVATION AND ACCUMULATION IN RELATION TO THERMOTOLERANCE¹

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The evolutionary conservation of the low-molecular-weight chloroplast-localized heat-shock protein (LMW chlP_{Hsp}) in vascular plants was examined using immunological methods. An antibody (Ab_{met}) specific to the LMW chlP_{Hsp} was produced using a synthetic 28-residue peptide containing the most conserved elements of its unique "methionine-rich domain" as an antigen. This antibody detected a heat-inducible low-molecular-weight chloroplast protein in plants of six divergent Anthophyta species, including C₃, C₄, CAM, monocot, and dicot species. Ab_{met} also detected a LMW chlP_{Hsp} in species from the Divisions Psilotophyta, Equisetophyta, Polypodiophyta, and Ginkgophyta. A preliminary examination of the relationship between accumulation of the LMW chlP_{Hsp} and habitat was also conducted. Seven Anthophyta species originating from both warm- and cool-temperature habitats were grown at 28°C and then heat stressed at 40°C. A positive qualitative relationship between the accumulation of the LMW chlP_{Hsp} and organismal thermotolerance in these species was observed; similar results were obtained separately with four nonAnthophyta species. The strong evolutionary conservation of this LMW Hsp and its localization to the chloroplast, and the correlation between production of this protein and plant thermotolerance, suggest that the LMW chlP_{Hsp} plays an important role in adaptation to heat stress.

Key words: chloroplast; evolution; heat-shock protein (Hsp); heat stress; photosynthesis; thermotolerance.

The super family of low-molecular-weight heat-shock proteins (LMW Hsps) exhibits the greatest genetic and biochemical variation among plant Hsp gene families (Vierling, 1991). Molecular and biochemical analyses have demonstrated the existence of five classes of LMW-Hsp families in plants (Waters, 1995). Two of these classes encode cytoplasmic proteins, one encodes a protein localized to the mitochondria, one appears to encode an endomembrane protein, and another encodes a protein localized to the chloroplast. Various lines of *in vitro* evidence (e.g., Jinn et al., 1989; Lee, Pokala, and Vierling, 1995), as well as results from *in vivo* studies with LMW-Hsp mutants (e.g., Loomis and Wheeler, 1982; Landry et al., 1989), have indicated an association between LMW Hsps and organismal thermotolerance. Inference from nonplant species, in conjunction with molecular-evolutionary studies and analysis of protein dynamics of LMW Hsps in plants, strongly suggests an important role of LMW Hsps in plant stress physiology (Vierling, 1991;

Howarth and Ougham, 1993; O'Connell, 1994). However, the evolutionary and ecological significance of these proteins has yet to be elucidated (Coleman, Heckathorn, and Hallberg, 1995).

The nuclear-encoded LMW Hsp that localizes to the chloroplast (LMW chlP_{Hsp}) exhibits a high degree of conservation in three consensus regions (Vierling, 1991; Waters, 1995). Consensus regions I and II of the LMW chlP_{Hsp} reside near the carboxyl-terminal end of the protein and exhibit homology to consensus regions found in the other families of LMW Hsps and the "α-crystallin domain" (Caspers, Leunissen, and de Jong, 1995). Consensus region III, known as the "methionine-rich domain" (Chen and Vierling, 1991), is the most conserved region within the predicted amino acid sequences of the LMW chlP_{Hsp} in ten species of angiosperms analyzed by Waters (1995), and is found in dicotyledons and monocotyledons, which are thought to have diverged 230 million years ago (Martin et al., 1993). The methionine-rich domain has been suggested to form an amphipathic α-helix tertiary structure, with one face of the helix consisting of methionine and other hydrophobic residues, while the opposite face consists entirely of strongly hydrophilic residues that have 100% identity in the LMW chlP_{Hsp} of five plant species examined by Chen and Vierling (1991).

The occurrence of LMW chlP_{Hsps} in whole-tissue extracts and isolated chloroplasts has been studied only in mesic agronomic species of Anthophyta using antibodies specific to LMW Hsps (Vierling, Harris, and Chen, 1989; Chen et al., 1990) and *in vivo* or *in vitro* radiolabeling (e.g., Glaczinski and Kloppstech, 1988; Clarke and Critchley, 1994). Immuno-analysis of whole-tissue ex-

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tracts from several Anthophyta species was conducted using polyclonal antiserum raised against a fusion-protein construct containing consensus regions I and II and a partial segment of consensus region III (Vierling, Harris, Chen, 1989). Due to the conserved homology in consensus regions I and II, this antiserum could contain antibodies to several families of LMW Hsp. Thus, the antigenic conservation of the methionine-rich domain of the LMW chlpHsp within Anthophyta remains uncertain. Also, the evolutionary conservation of this protein among thermotolerant wild Anthophyta species has not been studied, and conservation among species from other Divisions of higher plants has not been investigated.

Since the methionine-rich domain is unique to the family of LMW chlpHsps (Waters, 1995), we designed an antibody raised against a synthetic peptide that contained the most conserved elements in consensus region III from the predicted amino-acid sequence of seven species. The primary objective of this study was to use this antibody to determine the antigenic conservation of this methionine-rich domain (and by inference, the LMW chlpHsp) in evolutionarily diverse terrestrial plant taxa. We investigated species belonging to six divisions within Kingdom Plantae, ranging from the descendants of the earliest terrestrial vascular species to the main groups in Division Anthophyta.

The second objective was to conduct a preliminary examination to determine whether a correlation between accumulation of the LMW chlpHsp and interspecific thermotolerance exists. Several species from warm- and cool-temperature habitats were grown and heat stressed under the same conditions, and the relative accumulation of LMW chlpHsp was compared among these species. For the purposes of this comparison, organismal thermotolerance was estimated by qualitative differences in maximum growing-season temperature of the habitat from which each species originated, and hence refers to inherent, rather than induced thermotolerance.

Variation in Hsp production among closely related animal species from thermally contrasting habitats has been observed even when these species are grown and heat stressed under identical conditions (e.g., Bosch et al., 1988; Sanders et al., 1991; Ulmasov et al., 1992; White, Hightower, and Schultz, 1994; Gehring and Wehner, 1995). This variation in Hsp production is often correlated with organismal thermotolerance, suggesting that Hsp production is an important adaptation to heat stress and that variation in the Hsp response could be related to the different environments in which species have evolved. Previous studies have shown that there are likely to be trade-offs (i.e., both benefits and costs) associated with Hsp production (Werner-Washburne et al., 1989; Krebs and Loeschcke, 1994; Heckathorn et al., 1996a, b), and it has been suggested that interspecific variation in Hsp production may result in part because natural selection has acted towards maximizing the ratio of benefits to costs of the Hsp response (Coleman, Heckathorn, and Hallberg, 1995).

For these reasons, interspecific variation in production of the LMW chlpHsp is likely when different species are heat-stressed under the same conditions. However, we did not assume a priori that a relationship between production of the LMW chlpHsp and whole-plant thermotolerance/

habitat would be observed. Several notable studies have failed to detect any correlation between Hsp production and thermotolerance among closely related genotypes in plants (O'Connell, 1994). On the other hand, if either a positive or negative correlation is observed between whole-plant thermotolerance and accumulation of LMW chlpHsp in response to heat stress, then an important contribution of the LMW chlpHsp to organismal thermotolerance would be indicated (i.e., fulfilling a protective function if a positive correlation, or a repair function if negative). If no correlation is observed, then an important adaptive role for the LMW chlpHsp cannot be excluded as a possibility, since a lack of significant correlation could indicate that the importance of the LMW chlpHsp to whole-plant performance is not robust enough to be detected across relatively unrelated species or that comparison of Hsp production under species-specific native growth and heat-stress conditions is necessary for detection of a thermotolerance-Hsp relationship.

MATERIALS AND METHODS

Plant material and growth conditions—Five-year-old specimens of *Ferocactus wislizenii* Britt. and Rose were obtained from Alta Greenhouse, Inc. (Mansfield, Ohio, USA). Seeds of *Pisum sativum* L. cv. Super Snappy were obtained from W. Altee Burpee and Co. (Warminster, Pennsylvania, USA), and seeds of *Zea mays* L. cv. LH74 x LH163 were obtained from Holden's Foundation Seeds, Inc. (Stanton, Minnesota, USA). Young vegetative adults of *Equisetum arvense* L. were obtained from a local field population near Syracuse, New York, USA. Adult *Psilotum nudum* (L.) Beauv. plants were kindly provided by James Kramer of the University of Illinois, Urbana-Champaign, Illinois, USA. Mature individuals of *Chlorophytum comosum* Voss were obtained from the greenhouses of the State University of New York College of Environmental Sciences and Forestry (Syracuse). Specimens of *Nymphaea capensis* N. cv. Charles Thomas were donated by Virgil Downs and Frank Spangler (Mansfield). Specimens of *Platyserium bifurcatum* Cav. and *Senecio serpens* K. were donated by Kingwood Gardens (Mansfield). Saplings of *Pinus aristata* Engelm and *Ginkgo biloba* L. were obtained from Musser Forests, Inc. (Indiana, Pennsylvania, USA). *Pseudotsuga menziesii* var. *menziesii* (Mirbel) Franco cuttings came from the Department of Biology, Syracuse University. Seeds of *Chenopodium album* L. were obtained from a local population. Saplings of *Populus deltoides* Marsh var. *deltoides* were obtained from the Institute for Ecosystem Science (Millbrook, New York, USA), but were originally collected from Mississippi, USA. These species were chosen for study because they encompass a wide range of organismal thermotolerance and evolutionary divergence. The phylogenetic relations of these species are indicated in Table 1.

All plants were grown in a greenhouse for at least 2 mo before use. Plants were placed in growth chambers 7 d before use under 28°C/14-h days at 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetic photon flux density and 20°C/10-h nights. Depending on the species and experiment, plants were heat stressed by gradually increasing the temperature from 28°C to either 35°, 38°, 40°, 43°, or 45°C (see figure legends) over a period of 2 h and then maintained at elevated temperature for 8 h. Leaf tissue was harvested after a 12-h recovery period at 20°C. To compare interspecific accumulation of LMW chlpHsp, plants were heat stressed at 40°C for 8 h and then harvested immediately. All experiments were independently duplicated.

Whole-tissue and chloroplast preparation—Leaf tissue samples (or chlorenchyma for ferocactus) from both non-heat-stressed and heat-stressed plants ($N = 3$ for each species per treatment) were harvested, ground in liquid N_2 , and then extracted in buffer containing 100 mmol/L

TABLE 1. Study species and their phylogenies, photosynthetic pathways, habitats/origins, thermotolerance, and relative LMW chlHsp levels.

Species	Division/ Class ^a	Photosynthetic pathway	Native habitat/collection site (climate, ecosystem, local, etc.)	Thermotolerance (estimated, relative)	LMW chlHsp levels (monomers ^b /dimers ^c)
<i>Psilotum nudum</i> (whisk fern)	Psilotophyta	C ₃	tropical-to-warm-temperate moist forests; forest understory; global ^{d,e,f}	moderate	++/ constitutive
<i>Equisetum arvense</i> (horsetail)	Equisetophyta	C ₃	warm-temperate/cool season-to-arctic; moist open sites; global; collected in New York, USA ^{d,e,f}	low	+/+
<i>Platycerium bifurcatum</i> (staghorn fern)	Polypodiophyta	C ₃	tropical/subtropical forests; forest understory; New Guinea and Australia ^{d,g}	moderate	+++ / +
<i>Ginkgo biloba</i> (maiden hair tree)	Ginkgophyta	C ₃	temperate montane forests; SE China ^{e,f}	low	+/+
<i>Pinus aristata</i> (bristlecone pine)	Coniferophyta	C ₃	cool-temperate dry montane/subalpine forests; Rocky Mtns., SW USA ^{f,h}	low	+/
<i>Pseudotsuga menziesii</i> (Douglas fir)	Coniferophyta	C ₃	cool-temperate moist coastal forests; NW North America ^{f,h}	low	none detected
<i>Chenopodium album</i> (lamb's quarter)	Anthophyta dicot	C ₃	temperate/mostly cool season; open disturbed sites; European origin; alien, collected in New York, USA ⁱ	low	+/
<i>Chlorophytum comosum</i> (spider plant)	Anthophyta monocot	C ₃	subtropical moist cool forests; understory; S. Africa; optimal growth temp.: 16°–21°C ^g	low	+/
<i>Ferocactus wislizenii</i> (barrel cactus)	Anthophyta dicot	CAM	warm deserts; sonoran desert, SW USA ^j	high ^k	+++ / +
<i>Nymphaea capensis</i> (blue water lily)	Anthophyta dicot	C ₃	tropical/subtropical S. Africa; warm, sunny, still, aquatic habitats; growth temp. >21°C ^g	moderate	+++ / +
<i>Pisum sativum</i> (pea)	Anthophyta dicot	C ₃	cool moist Mediterranean/SW Asia origin ^l ; cool season commercial cultivar ^m	low	+/
<i>Populus deltoides</i> (eastern cottonwood)	Anthophyta dicot	C ₃	warm-to-cool-temperate forests/fields of E. USA; moist, often open sites; collected in Mississippi, USA ⁿ	moderate	++ /
<i>Senecio serpens</i> (chalkstick cactus)	Anthophyta dicot	C ₃ /CAM	warm deserts; S. Africa ^g	high	+++ /
<i>Zea mays</i> (corn)	Anthophyta monocot	C ₄	tropical/subtropical origin; Central America ^l ; drought-tolerant commercial cultivar ^o	moderate ^p	++ /

^a Phylogeny following *Flora of North America* (1993).

^b Indicates relative accumulation of monomeric LMW chlHsp based on Fig. 6 (and personal observations for *N. campensis*, *P. menziesii*, and *E. arvense*).

^c Indicates detection of apparent dimeric LMW chlHsp in at least some experiments.

^d Tryon and Tryon (1982).

^e Scagel et al. (1984).

^f *Flora of North America* (1993).

^g Everett (1980).

^h Barbour and Billings (1988).

ⁱ Clapham, Tutin, and Warburg (1962).

^j Gibson and Nobel (1986).

^k See Smith, Didden-Zopf, and Nobel (1984) for data on whole-plant thermotolerance.

^l Sauer (1993).

^m W. Atlee Burpee & Co.

ⁿ *Silvics of Forest Trees of the United States* (1965).

^o Holden's Foundations Seeds.

^p See Heckathorn et al. (1996a) for data on photosynthetic thermotolerance.

Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS; w/v), 1% dithiothreitol (DTT; w/v), 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF), 5 μmol/L leupeptin, 5 mmol/L ε-amino caproic acid, 1% ascorbate (w/v), and 3 mmol/L Na₂EDTA (reagents from Sigma Chemical Co., St Louis, Missouri, USA). Depending on the species, 1–6% (w/v) polyvinylpyrrolidone (PVP) and/or 20–50 mmol/L sodium tetraborate was used to remove phenolics. Samples were boiled for 3 min and then centrifuged at 14 000 g for 6 min. The supernatant was collected and stored at –20° C.

Chloroplasts from both non-heat-stressed and heat-stressed plants of each species were isolated by Percoll step-gradient centrifugation using a protocol modified from Gegenheimer (1990). Leaf tissue was homogenized using a Waring blender or a mortar and pestle in chloroplast buffer (CB) consisting of 0.33 mol/L sorbitol, 10 mmol/L MgCl₂, 10 mmol/L MnCl₂, 1% DTT (w/v), 1% ascorbate (w/v), 3 mmol/L EDTA, and 50 mmol/L HEPES/KOH buffer (pH 7.9). Depending on the spe-

cies, 1–6% PVP (w/v) and/or 20–50 mmol/L sodium tetraborate was included. The homogenate was filtered through a nylon mesh membrane. Homogenates were centrifuged at 150 g for 10 min. The supernatant was poured off, and the pellet was suspended in CB.

Chloroplast suspensions were layered on the Percoll gradients and centrifuged for 15 min in a swinging-bucket rotor at 7000–11 000 g, depending on the species. Percoll solution consisted of 1% Ficoll 400 (w/v), 0.1% bovine serum albumin (BSA; w/v), and 2–4% polyethylene glycol 8000 (w/v), all dissolved in Percoll. Percoll step-gradients contained 85, 65, 45, 25, and 15% steps of Percoll solution dissolved in CB. After centrifugation, the lower, most rapidly sedimenting green band in the Percoll gradient was collected, protease-treated, and isolated as described by Marshall et al. (1990). Thermolysis was used at 40 μg/mL with 1.0 mmol/L CaCl₂ and trypsin was used at 20 μg/mL. Chloroplasts were pelleted, lysed by suspension in the extraction buffer used for whole-plant preparations, frozen in liquid N₂, and then boiled

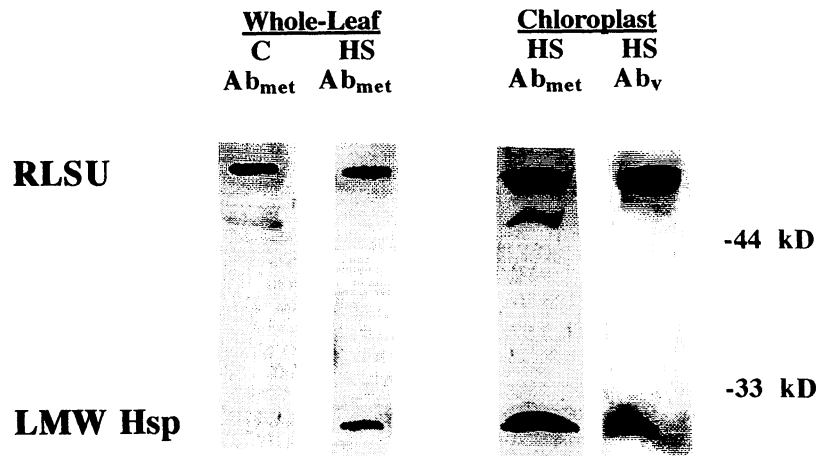


Fig. 1. Immunoblot of whole-leaf and isolated-chloroplast extracts from control (C; 28° C) and heat-stressed (HS; 38° C) *Pisum sativum* (pea) plants. Extracts were fractionated by SDS-PAGE and then proteins were transferred electrophoretically to PVDF membranes. Individual lanes of the membrane were probed with either Ab_{met} or Ab_v, for the LMW chlHsp, and then antiserum to rubisco large subunit (RLSU). Equal amounts of total protein (60 µg) were loaded per lane. The locations of molecular mass standards are indicated.

for 3 min. Isolated chloroplast extracts were stored at -20° C and centrifuged for 2 min at 14 000 g before use.

Antiserum production and immunoblotting—The most highly conserved amino-acid sequence from consensus region III (the methionine-rich domain) was derived from a sequence alignment (DNA Star software) using predicted amino-acid sequences from *Pisum sativum*, *Zea mays*, *Arabidopsis thaliana*, *Glycine max*, *Triticum aestivum*, *Petunia hybrida*, and *Solanum tuberosum*. An unconjugated synthetic oligopeptide, NH₂-Pro-Phe-Gly-Leu-Leu-Asp-Pro-Met-Ser-Pro-Met-Arg-Thr-Met-Arg-Gln-Met-Leu-Asp-Thr-Met-Arg-Met-Phe-Glu-Asp-Thr-COOH, was prepared as an immunogen at Bio-Synthesis, Inc. (Lewisville, Texas, USA).

Polyclonal antiserum (Ab_{met}) against the oligopeptide was generated in guinea pigs. Injection of the antigen and maintenance of the guinea pigs were carried out by Pocono Rabbit Farm & Laboratory, Inc. (Candens, Pennsylvania, USA). Fatty material was removed from the serum by centrifugation (9000 g for 8 min). Whole-serum fraction was used in subsequent steps. Antiserum (Ab_v) against the chlHsp22 of *P. sativum* COOH-fusion protein was kindly provided by Dr. E. Vierling (University of Arizona, Tucson, Arizona, USA) and was used as a positive control for antigen detection; gelatin was used as a negative control. Protein-antibody complexes were detected using Protein-A-conjugated alkaline-phosphatase (Cappel, Organon Teknika Corp., Durham, North Carolina, USA) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Aliquots of each sample containing equal amounts of total protein (determined in duplicate by BioRad Bradford assay; Bradford, 1976) were fractionated on 15% or 4–20% SDS-PAGE gels (Laemmli, 1970) and electrophoretically transferred to PVDF filters (Towbin, Staehelin, and Gordon, 1979). After transfer, blots were stained with 0.1% Ponceau-S to ensure transfer. Blots were incubated in nonfat dry milk for 2 h and then incubated with antiserum for 14 h at 4° C. Protein-antibody complexes were detected as described above. Molecular mass standards were included on all gels (either Kaleidoscope Prestained or SDS-PAGE standards; BioRad, Hercules, California, USA).

The relative amounts of protein-antibody complexes were estimated using a desktop scanner (Scanjet Ilcx, Hewlett Packard, Palo Alto, California, USA) and NIH imaging software (ver. 1.53). Serial dilutions of extracts from *Ferocactus wislizenii*, the most thermotolerant species examined and the species in which LMW chlHsp accumulation was greatest, were carried out to insure that LMW chlHsp content of sam-

ples remained within the linear range of the protein content–optical density relationship.

The LMW chlHsp is thought to be primarily localized to the stroma (Chen et al., 1990; Osteryoung and Vierling, 1994). To confirm that chloroplast preparations were enriched in intact chloroplasts, whole-leaf and chloroplast samples were evaluated for their relative content of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) large subunit (RLSU) using antiserum graciously provided by Dr. R. Zielinski (University of Illinois, Urbana, Illinois, USA). Finally, to insure that isolated chloroplasts were free of mitochondrial contamination, whole-leaf and chloroplast samples were assayed immunologically for their relative content of either α-ketoglutarate dehydrogenase (α-KGDH) or mitochondrial chaperonin 60 (cpn60) (McMullin and Hallberg, 1988), depending on availability.

RESULTS

Ab_{met} antiserum, but not pre-immune serum, recognized the purified oligopeptide antigen when it was vacuum-bound to PVDF membranes (not shown), indicating that Ab_{met} should recognize consensus region III of the LMW chlHsp present in plant tissue extracts. Similar results were obtained with Ab_v antiserum. Neither antiserum immunoreacted with gelatin.

As a first step in determining whether Ab_{met} detects a chloroplast Hsp in plant tissue samples, whole-leaf and chloroplast extracts from nonstressed and heat-stressed pea plants were assayed with both Ab_{met} and Ab_v, since it has been established that Ab_v identifies a LMW chlHsp in pea (Vierling et al., 1989; Chen et al., 1990). Ab_{met} reacted with a protein of ~21 kD in samples from heat-stressed plants, but not in unstressed plants (Fig. 1). The amount of LMW chlHsp detected in chloroplast preparations with Ab_{met} was much greater than that detected in whole-plant extracts containing equal amounts of total protein. Comparable increases in the content of rubisco large subunit (RLSU), a chloroplast-encoded stromal protein (Hall and Rao, 1994), confirmed that chloroplast preparations were enriched in intact chloroplasts. These results, and the fact that Ab_v and Ab_{met} reacted with an Hsp of the same apparent mass, confirm

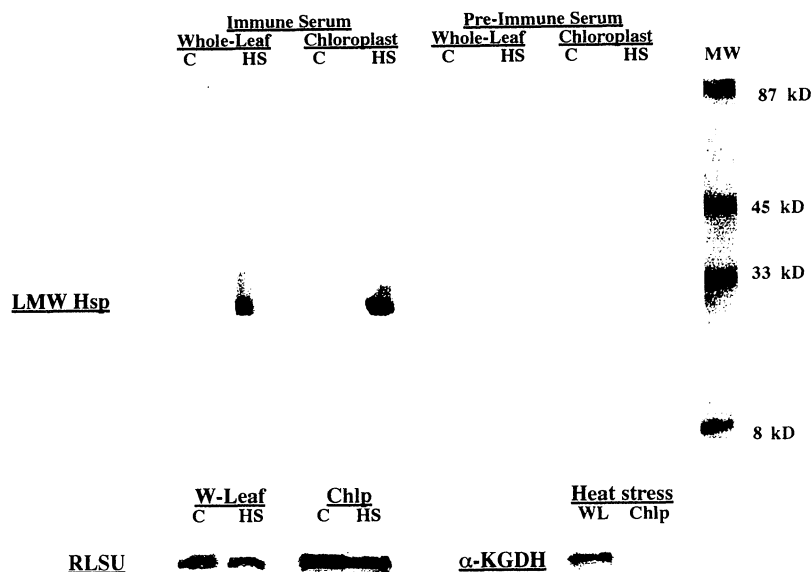


Fig. 2. Immunoblot of whole-leaf (WL) and isolated-chloroplast (Chlp) extracts from control (C; 28° C) and heat-stressed (HS; 43° C) *Ferocactus wislizenii* plants. Extracts were fractionated by SDS-PAGE and then proteins were transferred electrophoretically to PVDF membranes. Individual lanes of the membrane were probed with either immune or pre-immune Ab_{met}, for the LMW chlHsp, and on a companion gel, probed with antisera to either rubisco large subunit (RLSU) or α-ketoglutarate dehydrogenase (α-KGDH). Equal amounts of total protein (40 μg) were loaded per lane. The locations of molecular mass standards are indicated.

that Ab_{met} identifies a LMW chlHsp in pea, and indicate that this protein is predominately, if not exclusively, localized in chloroplasts.

To determine whether Ab_{met} reacted with a similar Hsp in other species, whole-leaf extracts of nonstressed and heat-stressed plants of other Anthophyta species were screened using Ab_{met} and Ab_v. Occasional differences in specificity between the two antibodies were observed. These included detection of different-sized proteins by

the two antisera (e.g., in equisetum and ferocactus) and detection of multiple LMW Hsps by Ab_v (in corn). Therefore, to ascertain whether Ab_{met} reacts specifically with a LMW chlHsp in species other than pea, we examined whole-leaf extracts and chloroplast preparations from nonstressed and heat-stressed plants from three species of dicots (ferocactus, a CAM species, senecio, a C₃/CAM species, and water lily, a C₃ species) and two species of monocots (corn, a C₄ species, and spider plant, a C₃) (Table 1).

For example, in ferocactus, immune Ab_{met} antiserum reacted with a LMW protein in heat-stressed plants, but not in control plants; pre-immune serum did not detect a LMW protein in either heat-stressed or control plants (Fig. 2). As with pea, the amount of LMW Hsp detected was greater in chloroplast vs. chlorenchyma (“whole-leaf”) samples, indicating that this LMW Hsp is predominately localized to the chloroplast. Increases in the content of rubisco in chloroplast, compared with whole-leaf, samples confirmed that chloroplasts purified from this species were largely intact. The absence of detectable levels of α-KGDH in chloroplast vs. whole-leaf samples indicates that the chloroplast preparations were essentially free of mitochondrial contamination.

In senecio, corn, spider plant, and water lily, Ab_{met} also reacted with a single, 21–30 kD, chloroplast protein in heat-stressed, but not in unstressed, plants (Fig. 3). As with pea and ferocactus, the relative content of the large subunit of rubisco and either α-KGDH or cpn60 in whole-leaf extracts vs. isolated chloroplasts was determined in the remaining species in order to insure that chloroplasts had not ruptured and resealed during the isolation process and that no significant mitochondrial contamination was present in chloroplast samples. In all species, chloroplast samples were enriched in intact chloro-

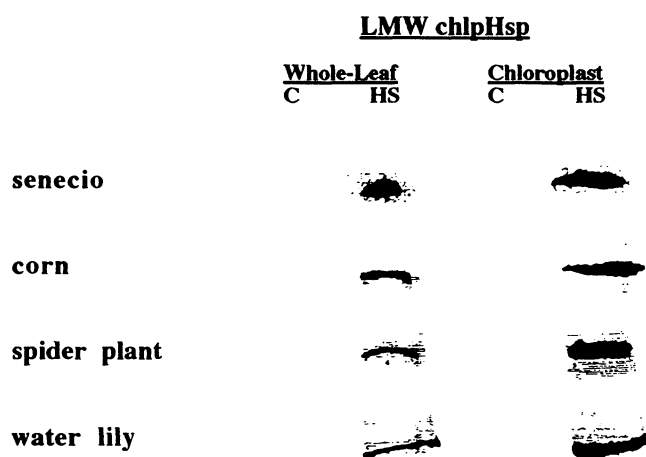


Fig. 3. Immunoblots of whole-leaf and isolated-chloroplast extracts from control (C; 28° C) and heat-stressed (HS; 38° C) *Senecio serpens* (senecio), *Zea mays* (corn), *Chlorophytum comosum* (spider plant), and *Nymphaea capensis* (water lily) plants. Extracts were fractionated by SDS-PAGE and then proteins were transferred electrophoretically to PVDF membranes. Blots were probed with Ab_{met}, for the LMW chlHsp. Within each species, equal amounts of total protein were loaded per lane (60 μg for spider plant and 40 μg for the remaining species). The apparent molecular mass of the LMW chlHsp was between 21 and 30 kD for all species (standards not shown).

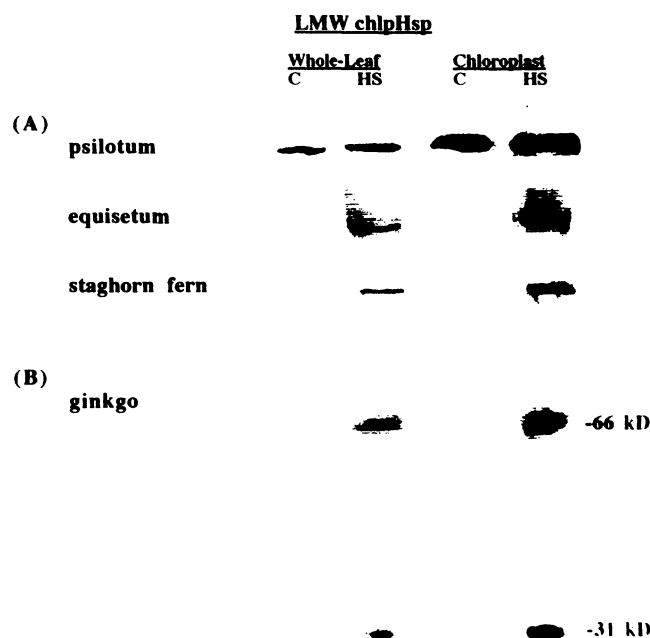


Fig. 4. Immunoblots of whole-leaf and isolated-chloroplast extracts from control (C; 28° C) and heat-stressed (HS; 38° C) (A) *Psilotum nudum* (psilotum), *Equisetum arvense* (equisetum), *Platycerium bifurcatum* (staghorn fern), and (B) *Ginkgo biloba* (ginkgo) plants. Extracts were fractionated by SDS-PAGE and then proteins were transferred electrophoretically to PVDF membranes. Blots were probed with Ab_{met} , for the LMW chlHsp. Within each species, equal amounts of total protein were loaded per lane (60 μ g for ginkgo and equisetum; 40 μ g for the remaining species). The locations of molecular mass standards are indicated for ginkgo; for the remaining species, the apparent molecular mass of the LMW chlHsp was between 21 and 30 kD (standards not shown).

plants and no mitochondrial contamination was detected in chloroplast preparations (not shown).

To determine the distribution of the LMW chlHsp among vascular plants, we examined species from four other divisions in Kingdom Plantae: Psilophyta, Equisetophyta, Polypodiophyta, and Ginkgophyta (Table 1). In psilotum, Ab_{met} reacted with a LMW chloroplast protein that was expressed in both unstressed and heat-stressed plants (Fig. 4a). Further, on an equal protein basis, there was no significant difference in LMW chlHsp levels between unstressed and heat-stressed plants. In equisetum, staghorn fern, and ginkgo, Ab_{met} reacted with a single LMW chlHsp (~21–30 kD) that was produced only in heat-stressed plants (Fig. 4A, B).

Interestingly, in ginkgo Ab_{met} also reacted with a protein roughly twice the size of the LMW chlHsp (Fig. 4B), indicating that dimerization of the LMW chlHsp probably occurred in this species. We also observed apparent dimerization of the LMW chlHsp in other situations as well. For example, in ferocactus, apparent dimerization of the LMW chlHsp increased with the total protein content of samples (Fig. 5) and was accentuated under severe (43° C) vs. moderate (38° C) heat-stress temperatures in staghorn fern (not shown). The influence of total protein content of samples and heat-stress temperature was taken into consideration when quantifying LMW chlHsp production among species and treatments.

In order to directly compare accumulation of the LMW

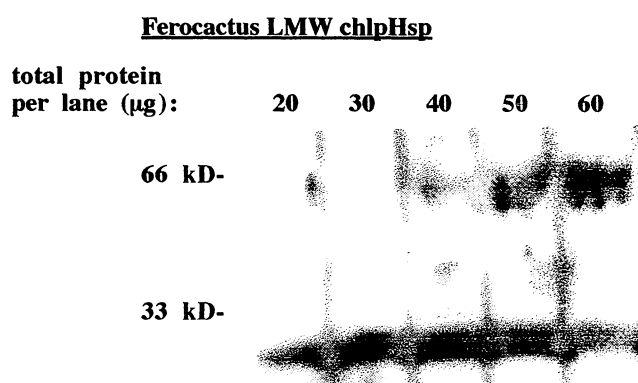


Fig. 5. Immunoblot of isolated-chloroplast extracts from heat-stressed (43° C) *Ferocactus wislizenii* plants. Extracts were fractionated by SDS-PAGE and then proteins were transferred electrophoretically to PVDF membranes. Blots were probed with Ab_{met} , for the LMW chlHsp. Different amounts of total protein were loaded per lane as indicated (20–60 μ g). The locations of molecular mass standards are also indicated. The mean optical integrated (density \times area) densities of the upper and lower bands in each lane, relative to the upper band at 20 μ g, are as follows: upper band—0.1, 1.1, 40.8, 108.4, and 178.3 for 20–60 μ g lanes, respectively; lower band—64.9, 131.5, 144.4, 128.3, and 94.5 for the comparable lanes.

chlHsp among species, Ab_{met} was used to assay an immunoblot containing equal protein from whole-leaf extracts of unstressed and heat-stressed plants of the following species: ferocactus, senecio, corn, cottonwood, lamb's quarter, spider plant, and pea (Fig. 6A). When grown and heat shocked under identical conditions that minimized dimerization (40° C and 40 μ g protein), ferocactus accumulated more of the LMW chlHsp than other species examined. Senecio, corn, and cottonwood accumulated intermediate amounts of LMW chlHsp. Levels of the LMW chlHsp were very low in lamb's quarter, spider plant, and pea.

A similar immunoblot was assayed containing the non-Anthophyta species, psilotum, staghorn fern, ginkgo, and bristlecone pine (Fig. 6B). When grown and heat stressed under similar conditions, staghorn fern accumulated more LMW chlHsp than did psilotum, ginkgo, or bristlecone pine. As before, psilotum produced LMW chlHsp constitutively and accumulation of this protein was not affected greatly by heat stress in this species. The LMW chlHsp was not detected with Ab_{met} in bristlecone pine in Fig. 6B or in Douglas-fir (not shown), although, additional analyses (not shown) of whole-plant extracts from bristlecone pine with both Ab_{met} and Ab_v suggest that this species accumulates very low levels of LMW chlHsp.

To determine whether relative differences in LMW chlHsp levels among species might change if each species was heat shocked at temperatures more closely reflecting maximum temperatures for their respective native habitats, we heat shocked a representative thermosensitive and thermotolerant species (spider plant vs. ferocactus, respectively) at temperatures other than 40° C (as in Fig. 6A, B) and monitored accumulation of LMW chlHsp. Accumulation of LMW chlHsp was reduced in spider plant at 36° C, relative to 40° C (Fig. 6C), and 44° C was lethal for this species. Production of LMW chlHsp in pea is also greatest at ~40°–43° C, relative to lower and higher temperatures (Chen et al., 1990; Oster-

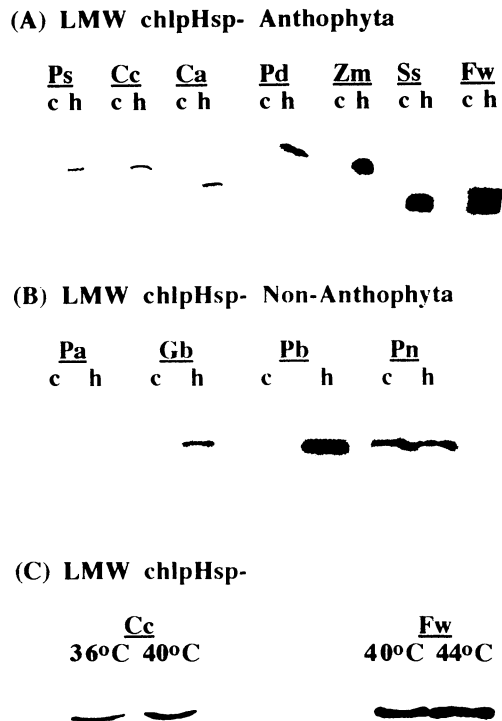


Fig. 6. Immunoblot of whole-leaf extracts from control (c; 28°C) and heat-stressed (h; 40°C) plants of (A) seven different Anthophyta species: *Pisum sativum* (Ps; pea), *Chlorophytum comosum* (Cc; spider plant), *Chenopodium album* (Ca; lamb's quarters), *Populus deltoides* (Pd; cottonwood), *Zea mays* (Zm; corn), *Senecio serpens* (Ss; senecio), and *Ferocactus wislizenii* (Fw; ferocactus); and (B) four different non-Anthophyta species: *Pinus aristata* (Pa; bristlecone pine; Coniferophyta), *Ginkgo biloba* (Gb; ginkgo; Ginkgophyta), *Platycerium bifurcatum* (Pb; staghorn fern; Polypodiophyta), and *Psilotum nudum* (Pn; psilotum; Psilotophyta). (C) Immunoblots of whole-leaf extracts from *C. comosum* (Cc; spider plant) heat shocked at 36°C and 40°C, and *F. wislizenii* (Fw; ferocactus) heat shocked at 40°C and 44°C. Extracts were fractionated by SDS-PAGE and then proteins were transferred electrophoretically to PVDF membranes. Blots were probed with Ab_{met} for the LMW chlP Hsp. Equal amounts of total protein (40 µg) were loaded per lane.

young and Vierling, 1994). In ferocactus, accumulation of LMW chlP Hsp increased at 44°C vs. 40°C (Fig. 6C; similar results were obtained for senecio). These results suggest that the relative differences among species in accumulation of LMW chlP Hsp observed in Fig. 6A and B may be somewhat conservative; that is, maximum accumulation of LMW chlP Hsp occurs at temperatures > 40°C for thermotolerant species, but accumulation is probably greatest at temperatures close to 40°C in the thermosensitive species that we examined.

DISCUSSION

We have provided evidence that the "methionine-rich domain" of the LMW chlP Hsp is antigenically conserved in six evolutionarily divergent Anthophyta species, including monocot, dicot, C₃, C₄, and CAM species. We also demonstrate the apparent evolutionary conservation of the LMW chlP Hsp in single representative species of four other divisions in Kingdom Plantae (Psilotophyta, Equisetophyta, Polypodiophyta, and Ginkgophyta); two of these divisions, Psilotophyta and Equisetophyta, have

fossil records dating back to the Silurian and Devonian periods (Scagel et al., 1984). Evolutionary conservation of the methionine-rich domain in a chloroplast Hsp strongly suggests that not only are selective pressures acting to conserve the methionine-rich domain, but also its localization to the chloroplast. Although the function of the LMW chlP Hsp is unknown (Vierling, 1991; Osteryoung and Vierling, 1994), the conservation of its localization and of consensus region III implies an important functional role for this protein in the chloroplast during or after heat stress.

We observed a qualitative relationship among diverse species between the accumulation of the LMW chlP Hsp and organismal thermotolerance, as defined by habitat (Fig. 6, Table 1), despite the fact that these species may vary in their antigenicity to our antibody. This indicates that variation in Hsp levels among species is attributable primarily to real differences in Hsp accumulation, rather than to differences in antigenicity. Others have used similar immunological methods to compare Hsp production among divergent species (e.g., Ulmasov et al., 1992; Hernandez and Vierling, 1993; White, Hightower, and Schultz, 1994; Gehring and Wehner, 1995; Wisniewski et al., 1996).

In Anthophyta species, accumulation was relatively greatest in senecio and ferocactus, species originating from desert habitats. Ferocactus, the species in which accumulation was most pronounced, is among the most thermotolerant of vascular plants (Smith, Didden-Zopf, and Nobel, 1984; Gibson and Nobel, 1986). The corn and cottonwood plants used in this study, which originated from thermally variable warm-temperate climates, accumulated moderate amounts of LMW chlP Hsp. In contrast, lamb's quarter, spider plant, and pea, which are adapted to somewhat cooler habitats, accumulated much smaller amounts of this protein. For the nonAnthophyta species examined, relative accumulation of the LMW chlP Hsp was greatest in staghorn fern, moderate in psilotum (although expression was constitutive and largely unresponsive to heat stress), low in ginkgo, and undetectable (or barely so) in bristlecone pine. Of these four species, staghorn fern and psilotum are probably the most thermotolerant. These results indicate that the association of LMW chlP Hsp accumulation with thermotolerance may be widespread among and within divisions of Plantae.

The positive qualitative relationship between LMW chlP Hsp accumulation and thermotolerance suggests that the LMW chlP Hsp plays an important, though unidentified, role in adaptation to heat stress. In the Anthophyta species examined, analysis of production of a chloroplast Hsp70, a chaperonin that is involved in protein import into the chloroplast (Marshall et al., 1990; Wang, Goffreda and Leustek, 1993), shows that Hsp70 accumulation increases significantly with heat stress in thermosensitive species, but not in thermotolerant species (Downs et al., unpublished data). Together, these observations suggest that the LMW chlP Hsp may function in thermoprotection, rather than repair, while the reverse may be true for chlP Hsp70. Lee, Pokala, and Vierling (1995) also concluded from in vitro experiments that the LMW chlP Hsp probably functions in thermoprotection, rather than repair.

Despite the fact that the LMW chlP Hsp has been ex-

tensively studied, the function and organismal importance of this Hsp have yet to be elucidated, though several studies have provided indirect evidence that chloroplast Hsps are of benefit to photosynthesis during or after heat stress (e.g., Stapel, Kruse, and Kloppstech, 1993; Clarke and Critchley, 1994; Heckathorn et al., 1996a). Recently, genetic evidence has been obtained for a positive relationship between accumulation of the LMW chlHsp and thermotolerance among genotypes of *Zea mays* (Frova and Gorla, 1993) and *Agrostis palustris* (Park et al., 1996).

Finally, we had observed apparent dimerization of the LMW chlHsp in some species and under certain conditions. It is known that LMW Hsps tend to aggregate into multimeric structures under native conditions, and dimerization bands have been observed for the LMW chlHsp (Osteryoung and Vierling, 1994), as well as other LMW Hsps in plants (e.g., Alamillo et al., 1995). Since consensus regions I and II are the common element among all the members of the LMW Hsps, it has been suggested by various workers that dimerization and higher order aggregation of LMW Hsps may be attributable to regions I and II, collectively called the "heat-shock domain" (Casper, Leunissen, and de Jong, 1995; Waters, 1995). It is worth noting that in this study, the species in which apparent dimerization of the LMW chlHsp was particularly noticeable are nonAnthophyta species and/or species that accumulate high levels of LMW chlHsp (Table 1). We had also obtained preliminary results indicating that dimerization increased with temperature in *P. bifurcatum*, which is consistent with observations of temperature sensitivity of dimerization in pea (Osteryoung and Vierling, 1994). Awareness of potential dimerization in LMW Hsps is thus necessary for accurate determinations of LMW Hsp production.

In summary, we have provided evidence that the LMW chlHsp is produced in a wide range of species and that it is evolutionarily conserved in taxa that have diverged over 400 million years ago. We also suggest that an ecologically important relationship appears to exist between accumulation of this Hsp and organismal thermotolerance among evolutionarily divergent species, thereby extending the results of Frova and Gorla (1993) and Park et al. (1996) discussed above. Several studies with animal species have provided evidence (often immunological, as in the present study) for an important relationship between Hsps and the environments that organisms are able to inhabit (e.g., Bosch et al., 1988; Sanders et al., 1991; Ulmasov et al., 1992; White, Hightower, and Schultz, 1994; Gehring and Wehner, 1995). Prior to the evolutionary emergence of complex multicellular organisms, metabolic responses such as synthesis of heat-shock proteins may have been a prime adaptation to heat stress. With the advent of alternative adaptive responses to heat stress in higher plants (e.g., morphological and gross physiological adaptations to either decrease heat gain or increase heat loss), the relative importance of heat-shock proteins may have decreased (O'Connell, 1994; Coleman, Heckathorn, and Hallberg, 1995). Our findings suggest that the production of Hsps, in this case the LMW chlHsp, may still be an important determinant of organismal thermotolerance in vascular plants.

LITERATURE CITED

- ALAMILLO, J., C. ALMOGUERA, D. BARTELS, AND J. JORDANO. 1995. Constitutive expression of small heat shock proteins in vegetative tissues of the resurrection plant *Craterostigma plantagineum*. *Plant Molecular Biology* 29: 1093–1099.
- BARBOUR, M. G., AND W. D. BILLINGS. 1988. North American terrestrial vegetation. Cambridge University Press, Cambridge.
- BOSCH, T. C. G., S. M. KRYLOW, H. R. BODE, AND R. E. STEELE. 1988. Thermotolerance and synthesis of heat shock proteins: these responses are present in *Hydra attenuata* but absent in *Hydra oligactis*. *Proceedings of the National Academy of Sciences, USA* 85: 7927–7931.
- BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- CASPERS, G. J., J. A. M. LEUNISSEN, AND W. W. DE JONG. 1995. The expanding small heat-shock protein family, and structure predictions of the conserved "α-crystallin domain." *Journal of Molecular Evolution* 40: 238–248.
- CHEN, Q., L. M. LAUZON, A. E. DEROCHE, AND E. VIERLING. 1990. Accumulation, stability, and localization of a major chloroplast heat-shock protein. *Journal of Cell Biology* 110: 1873–1883.
- , AND E. VIERLING. 1991. Analysis of conserved domains identifies a unique structural feature of a chloroplast heat shock protein. *Molecular and General Genetics* 226: 425–431.
- CLAPHAM, A. R., T. G. TUTIN, AND E. F. WARBURG. 1962. Flora of the British Isles, 2d ed. Cambridge University Press, London.
- CLARKE, A. K., AND C. CRITCHLEY. 1994. Characterization of chloroplast heat shock proteins in young leaves of *C₄* monocotyledons. *Physiologia Plantarum* 92: 118–130.
- COLEMAN, J. S., S. A. HECKATHORN, AND R. L. HALLBERG. 1995. Heat-shock proteins and thermotolerance: linking molecular and ecological perspectives. *Trends in Ecology and Evolution* 10: 305–306.
- EVERETT, T. H. 1980. The New York Botanical Garden illustrated encyclopedia of horticulture. Garland Publishing, New York, NY.
- Flora of North America north of Mexico. 1993. Oxford University Press, Oxford.
- FROVA, C., AND M. S. GORLA. 1993. Quantitative expression of maize HSPs: genetic dissection and association with thermotolerance. *Theoretical and Applied Genetics* 86: 213–220.
- GEGENHEIMER, P. 1990. Preparation of extracts from plants. In M. P. Deutscher [ed.], Guide to protein purification, Methods in Enzymology, vol. 182, 174–193. Academic Press, San Diego, CA.
- GEHRING, W. J., AND R. WEHNER. 1995. Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proceedings of the National Academy of Sciences, USA* 92: 2994–2998.
- GIBSON, A. C., AND P. S. NOBEL. 1986. The cactus primer. Harvard University Press, Cambridge.
- GLACZINSKI, H., AND K. KLOPPSTECHE. 1988. Temperature-dependent binding to the thylakoid membranes of nuclear-coded chloroplast heat-shock proteins. *European Journal of Biochemistry* 173: 579–583.
- HALL, D. O., AND K. K. RAO. 1994. Photosynthesis. Cambridge University Press, Cambridge.
- HECKATHORN, S. A., G. J. POELLER, J. S. COLEMAN, AND R. L. HALLBERG. 1996a. Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. *Oecologia* 105: 413–418.
- , ———, ———, AND ———. 1996b. Nitrogen availability and vegetative development influence the response of ribulose 1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, and heat-shock protein content to heat stress in *Zea mays* L. *International Journal of Plant Sciences* 157: 546–553.
- HERNANDEZ, L. D., AND E. VIERLING. 1993. Expression of low molecular weight heat-shock proteins under field conditions. *Plant Physiology* 101: 1209–1216.
- HOWARTH, C. J., AND H. J. OUGHAM. 1993. Gene expression under temperature stress. *New Phytologist* 125: 1–26.
- JINN, T. L., Y. C. YEH, Y. M. CHEN, AND C. Y. LIN. 1989. Stabilization of soluble proteins *in vitro* by heat shock proteins-enriched ammonium sulfate fraction from soybean seedlings. *Plant Cell Physiology* 30: 463–469.

- KREBS, R. A., AND V. LOESCHKE. 1994. Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. *Functional Ecology* 8: 730–737.
- LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- LANDRY, J., P. CHRÉTIEN, H. LAMBERT, E. HICKEY, AND L. A. WEBER. 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *Journal of Cell Biology* 109: 7–15.
- LEE, G. J., N. POKALA, AND E. VIERLING. 1995. Structure and *in vitro* molecular chaperone activity of cytosolic small heat shock proteins from pea. *Journal of Biological Chemistry* 270: 10432–10438.
- LOOMIS, W. F., AND S. A. WHEELER. 1982. Chromatin-associated heat shock proteins of *Dictyostelium*. *Developmental Biology* 90: 412–418.
- MARSHALL, J. S., A. E. DEROCHE, K. KEEGSTRA, AND E. VIERLING. 1990. Identification of heat shock protein hsp70 homologues in chloroplasts. *Proceedings of the National Academy of Sciences, USA* 87: 374–378.
- MARTIN, W., D. LYDIATE, H. BRINKMANN, G. FORKMAN, H. SAEDLER, AND R. CERFF. 1993. Molecular phylogenies in angiosperm evolution. *Molecular Biology and Evolution* 10: 140–162.
- MCMULLIN, T.W., AND R. L. HALLBERG. 1988. A highly evolutionarily conserved mitochondrial protein is structurally related to the protein encoded by the *Escherichia coli* groEL gene. *Molecular and Cellular Biology* 8: 371–380.
- O'CONNELL, M. A. 1994. Heat shock proteins and thermotolerance. In A. S. Basra [ed.], *Stressed-induced gene expression in plants*, 163–183. Harwood, Chur, Switzerland.
- OSTERYOUNG, K. W., AND E. VIERLING. 1994. Dynamics of small heat shock protein distribution within the chloroplasts of higher plants. *Journal of Biological Chemistry* 269: 28676–28682.
- PARK, S. Y., R. SHIVAJI, J. V. KRANS, AND D. S. LUTHE. 1996. Heat-shock response in heat-tolerant and nontolerant variants of *Agrostis palustris* Huds. *Plant Physiology* 111: 515–524.
- SANDERS, B. M., C. HOPE, V. M. PASCOE, AND L. S. MARTIN. 1991. Characterization of the stress protein response in two species of *Collisella* limpets with different temperature tolerances. *Physiological Zoology* 64: 1471–1489.
- SAUER, J. D. 1993. *Historical Geography of crop plants: a selected roster*. CRC Press, Boca Raton, FL.
- SCAGEL, R. F., R. J. BANDONI, J. R. MAZE, G. E. ROUSE, W. B. SCHOFIELD, AND J. R. STEIN. 1984. *Plants, an evolutionary survey*. Wadsworth, Belmont, CA.
- Silvics of forest trees of the United States. 1965. United States Department of Agriculture handbook No. 271.
- SMITH, S. D., B. DIDDEN-ZOPFY, AND P. S. NOBEL. 1984. High-temperature responses of North American cacti. *Ecology* 65: 643–651.
- STAPEL, D., E. KRUSE, AND K. KLOPPSTECH. 1993. The protective effect of heat shock proteins against photoinhibition under heat shock in barley (*Hordeum vulgare*). *Journal of Photochemistry and Photobiology B21*: 211–218.
- TOWBIN, H., T. STAHELIN, AND J. GORDON. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* 76: 4350–4354.
- TRYON, R. M., AND A. F. TRYON. 1982. *Ferns and allied plants*. Springer-Verlag, New York, NY.
- ULMASOV, K. A., S. SHAMMAKOV, K. KARAEV, AND M. B. EVGEN'EV. 1992. Heat shock proteins and thermoresistance in lizards. *Proceedings of the National Academy of Sciences, USA* 89: 1666–1670.
- VIERLING, E., L. M. HARRIS, AND Q. CHEN. 1989. The major low-molecular-weight heat shock protein in chloroplasts shows antigenic conservation among diverse higher plant species. *Molecular and Cellular Biology* 9: 461–468.
- VIERLING, E. 1991. The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 42: 579–620.
- WANG, H., M. GOFFREDA, AND T. LEUSTEK. 1993. Characteristics of an Hsp70 homolog localized in higher plant chloroplasts that is similar to DnaK, the Hsp70 of prokaryotes. *Plant Physiology* 102: 843–850.
- WATERS, E. R. 1995. The molecular evolution of the small heat-shock proteins in plants. *Genetics* 141: 785–795.
- WERNER-WASHBURNE, M., J. BECKER, J. KOSIC-SMITHERS, AND E. A. CRAIG. 1989. Yeast hsp70 levels vary in response to the physiological status of the cell. *Journal of Bacteriology* 171: 2680–2688.
- WHITE, C. N., L. E. HIGHTOWER, AND R. J. SCHULTZ. 1994. Variation in heat-shock proteins among species of desert fishes (Poeciliidae, *Poeciliopsis*). *Molecular Biology and Evolution* 11: 106–119.
- WISNIEWSKI, M., T. J. CLOSE, T. ARTLIP, AND R. ARORA. 1996. Seasonal patterns of dehydrins and 70-kDa heat-shock proteins in bark tissues of eight species of woody plants. *Physiologia Plantarum* 96: 496–505.