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Tissue-Type-Specific Heat-Shock Response and Immunolocalization of Class I Low-Molecular-Weight Heat-Shock Proteins in Soybean[1]

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A monospecific polyclonal antibody was used to study the tissue-type specificity and intracellular localization of class I low-molecular-weight (LMW) heat-shock proteins (HSPs) in soybean (*Glycine max*) under different heat-shock regimes. In etiolated soybean seedlings, the root meristematic regions contained the highest levels of LMW HSP. No tissue-type-specific expression of class I LMW HSP was detected using the tissue-printing method. In immunolocalization studies of seedlings treated with HS (40°C for 2 h) the class I LMW HSPs were found in the aggregated granular structures, which were distributed randomly in the cytoplasm and in the nucleus. When the heat shock was released, the granular structures disappeared and the class I LMW HSPs became distributed homogeneously in the cytoplasm. When the seedlings were then given a more severe heat shock following the initial 40°C → 28°C treatment, a large proportion of the class I LMW HSPs that originally localized in the cytoplasm were translocated into the nucleus and nucleolus. Class I LMW HSPs may assist in the resolubilization of proteins denatured or aggregated by heat and may also participate in the restoration of organellar function after heat shock.

The induction of HSP synthesis is a universal response to HS that occurs in all organisms ranging from bacteria to humans (Schlesinger et al., 1982; Vierling, 1991). We reported previously that when soybean (*Glycine max*) seedlings were shifted from a normal growth temperature of 28°C to a 40°C HS temperature, there was a dramatic change in protein biosynthesis; HSPs were rapidly synthesized and normal protein synthesis was substantially reduced (Key et al., 1981, 1985). Following a HS treatment, the seedlings become more tolerant to a nonpermissive or lethal temperature (e.g. 45°C for 2 h) (Lin et al., 1984). Although the physiological functions of HSPs have not been clearly established, the acquisition of thermotolerance is correlated with de novo synthesis and accumulation of HSPs (Lin et al., 1984). Under HS conditions, the level of expression of the class I LMW HSPs in soybean can reach more than 1% of the total cellular proteins (Hsieh et al., 1992), and this accumulation is correlated with the acquisition of thermotolerance.

One possible function of HSPs may be to stabilize cellular proteins in their soluble form. This can be demonstrated in vitro by an HSP-enriched fraction (Jinn et al., 1989). These fractions are exchangeable among soybean, mung bean (*Vigna radiata* L.), and rice (*Oryza sativa* L.) in providing such protein thermostabilization (Jinn et al., 1993). Depletion of the 15- to 18-kD HSPs in the HSP-enriched fraction in soybean resulted in the loss of thermostabilizing ability; this protective ability was restored by adding the 280-kD purified class I LMW HSP complex (Jinn et al., 1995). The protection provided by these class I LMW HSPs is effective mainly for high-salt-extractable proteins, which are probably membrane-associated (Jinn et al., 1993). A molecular chaperone function for LMW HSPs has also been shown in mammalian systems (Jakob et al., 1993) and in pea (*Pisum sativum* L.) (Lee et al., 1995).

The dramatic response of cells to HS is extensively documented by biochemical and molecular biological studies, as well as by investigation of cell ultrastructure (Nover et al., 1989; Nover, 1990). The most significant change in the ultrastructure of all organisms under HS occurs within the nucleolus, where the granular components are broken down, the fibrillar components became more prominent, and the degree of vacuolization is increased (Simard and Bernhard, 1967; Heine et al., 1971; Fransolet et al., 1979; Neumann et al., 1984; Chen et al., 1988; Mansfield et al., 1988). These observations are strongly correlated with the inhibition of rRNA processing and ribosome assembly that is rapidly initiated after the beginning of HS (Arrigo et al., 1980; Neumann et al., 1984; Hadjiolov, 1985; Bell et al., 1988). Structural changes in the cytoplasm, mitochondrion, chloroplast, ER, and dictyosome have also been reported after HS (Heine et al., 1971; Skogqvist, 1974; Schnepf and Schmitt, 1981; Craig, 1985; Welch and Suhan, 1985).

HS also induces formation of hsgs, or cytoplasmic aggregates, in cultured cells of tomato and other plant tissues, as well as in yeast (Nover et al., 1989; Nover, 1990; Parsell et al., 1994). In our early studies using cell-fractionation procedures, some LMW HSPs were preferentially localized in

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Abbreviations: HS, heat shock; hsg(s), HS granule(s); HSP(s), heat-shock protein(s); LMW, low-molecular-weight; RNP, ribonucleoprotein particles; TTC, 2,3,5-triphenyltetrazolium chloride.

cellular organelles such as the nucleus, mitochondrion, plasma membrane, and ribosome. This localization occurred in a temperature-dependent fashion, and the HSPs relocalized as a soluble form in the cytoplasm after the HS was ended (Lin et al., 1984). In addition, isolated mitochondria with associated HSPs are functional in oxidative phosphorylation under HS, whereas control mitochondria without HSPs are not (Chou et al., 1989).

These phenomena indicate that the association of HSPs with organelles during HS provides protection from damage. However, cellular fractionation methods disrupt the native state of cellular organization, which may result in the loss or gain of proteins from sensitive structures, producing nonspecific results. Therefore, analyses using immunological methods, combined with light- and electron-microscopic techniques, should provide more definitive information on the in situ localization of HSPs.

In this study we used tissue printing and light- and electron-microscopic immunolocalization to examine tissue-type specificity of the expression of class I LMW HSPs and their intracellular localization. Heat damage in root tissue using the TTC test and the accumulation of class I LMW HSPs in different growth sections of young seedlings under different HS regimes were also studied.

MATERIALS AND METHODS

Soybean (*Glycine max* cv Taita Kaohsiung no. 8) seeds were surface-sterilized in 10% chlorine bleach for 10 min, rinsed thoroughly in water, and then germinated in a roll of moist paper towels at 28°C in a dark growth chamber (Lin et al., 1984). For growth analysis seedlings were incubated with shaking in 1 mM potassium phosphate buffer (pH 6.0) containing 1% Suc at the indicated temperatures after varying periods of treatment, and replanted in a roll of moist paper.

SDS-PAGE, Western-Blot Analysis, and Quantitative Estimation of Class I LMW HSPs

One-dimensional SDS-PAGE was performed according to the method of Laemmli (1970) using 13.75% (w/v) polyacrylamide gels. Proteins were transferred from polyacrylamide gels to PVDF membranes (Immobilon, Millipore) with Tris/Gly electroblotting buffer for immunoblotting according to the method of Towbin et al. (1979). Protein bands cross-reacting with the LMW HSP antibodies (Hsieh et al., 1992) were identified by reaction with alkaline phosphatase conjugated with goat anti-rabbit IgG (Bio-Rad). Bound antibodies were visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium according to the manufacturer's specifications (Bio-Rad). The amount of protein was quantified by scanning the PVDF membranes after immunoblot with a densitometer (model SI, Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Molecular Dynamics). An internal standard contained 1.34 μg of class I LMW HSPs per 100 μg of total protein isolated from 2-d-old seedlings that had undergone HS at 40°C for 3 h (Hsieh et al., 1992).

Tissue Printing

Two-day-old seedlings without cotyledons were freshly cut by hand-section and blotted onto nitrocellulose membrane (Schleicher & Schuell) for 30 s, and the prints were immediately dried with warm air according to the method of Cassab and Varner (1987). The nitrocellulose membrane blots were used for western-blot analysis using goat anti-rabbit IgG conjugated with alkaline phosphatase secondary antibody as described above, or they were used for northern-blot analysis (as described by McClure and Guilfoyle [1989]) to study the expression of HS-regulated genes using soybean cDNA pCE53 (a class I LMW HSP cDNA clone) as a probe according to the method of Czarnecka et al. (1985). The nitrocellulose membranes were prehybridized for 4 h in buffer containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 7.0), 5× Denhardt's solution, 100 μg/mL yeast RNA, and 0.1% SDS. ^{32}P-Labeled probe was added to fresh hybridization buffer, and blots were hybridized for 24 h at 42°C and washed as described by Kimpel and Key (1985).

Light-Microscopic Immunocytochemistry

Two-day-old soybean hypocotyls were cut into 1-cm-long segments and fixed in 75% ethanol for 2 d at 4°C. Segments were cross-sectioned into 100- to 200-μm thicknesses using a hand-sectioning method, and these sections were further fixed in 75% ethanol for 2 h at room temperature. After 1 h of rehydration in TBS (20 mM Tris-HCl, pH 7.5, and 500 mM NaCl), sections were incubated with 0.5% BSA in TBS for 1 h to minimize nonspecific binding. Sections were incubated with antibodies raised against class I LMW HSPs (Hsieh et al., 1992), diluted 1:100 in TBSTB (TBS + 0.05% Tween 20 + 0.5% BSA) at room temperature for 1 h, and washed 10 times (5 min each) in TBST (TBS + 0.05% Tween 20). Sections were then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (dilution 1:1000 in TBSTB) for 1 h at room temperature, and rinsed extensively 10 times (5 min each) in TBST. Color development was done according to the manufacturer's specifications (Bio-Rad).

Immunogold Localization of Class I LMW HSPs in Soybean Seedlings

Root tips (1–2 mm) were excised from 2-d-old seedlings and fixed in 0.5% glutaraldehyde and 3.2% paraformaldehyde in 100 mM potassium phosphate buffer (pH 7.2) on ice under low vacuum for 2 h, and then washed three times with potassium phosphate buffer (10 min each). The specimens were dehydrated through an ethanol series, embedded with London Resin White (London Resin Co., London, UK) in gelatin capsules, and polymerized at 60°C for 36 h with an N_2 gas flush. Thin sections (pale gold-silver reflectance, 60–150 nm) of the specimens were prepared using a glass knife with an ultramicrotome (MT2-B, Sorvall) and then placed on Formvar-coated gold grids.

Section staining was performed by floating the grids, section-side down, on droplets of the immunolabeling and washing solutions placed on strips of Parafilm (American

National Can, Greenwich, CT), according to the method of Bendayan (1983) and Doman and Trelease (1985). Sections were blocked with a solution containing TBS with 2% BSA, 2.5% lamb serum, 0.25% cold-water-fish-skin gelatin (Sigma), 20 mM Gly, and 0.02% NaN_3 for at least 4 h. Grids were then floated on drops of immune serum or preimmune serum (1:10 dilution with 0.5× blocking solution), and incubated overnight at 4°C in a moistened chamber. They were washed 10 times with a 0.1× blocking solution using care not to wet the reverse side of the grid, placed on a drop (20 μL) of gold-labeled goat anti-rabbit antibodies (GAR G15, Janssen, New Brunswick, NJ) at a 1:25 dilution with 0.5× blocking solution, and incubated for 1 h at room temperature. Sections were rinsed 10 times sequentially with 0.1× blocking solution, 1% glutaraldehyde, and distilled water for 10 min, and were then air-dried. After staining with 4% aqueous uranyl acetate (5 min) and lead citrate (5 min), the sections were examined on an electron microscope (EM 10A, Zeiss) at 60 kV.

TTC Assay

Two-day-old seedlings (with cotyledons removed) were incubated overnight in 50 mM potassium phosphate buffer (pH 7.4) with 0.6% TTC in small assay tubes (16 × 125 mm) without shaking under a slight vacuum in the dark (Towhill and Mazur, 1974).

RESULTS

Tissue-Type-Specific HS Response

To determine whether different tissues in the soybean seedlings responded differently to HS, we localized the class I LMW HSPs by tissue printing and hand-sectioning methods (Fig. 1). Expression of the class I LMW HSPs during HS was not limited to specific cell types, since all cells examined, from the shoot apex to the root tip through the longitudinal sections (Fig. 1A) and cross-sections of the hypocotyl (Fig. 1B), gave positive signals. All parenchyma cells, including the epidermal layers to the middle region of cortex and the cells in the central part of the stele (vascular tissues), produced HSPs in response to HS. The lower panels in Figure 1A show cross-sections of the mature zone of the hypocotyls from three different seedlings, which display the highest apparent accumulation of the class I LMW HSPs in response to HS in the cambial cells. Expression of mRNAs coding for the class I LMW HSPs detected by hybridization with the cDNA probe pCE53, which contains sequences shared by class I LMW HSPs (Czarnecka et al., 1985), correlated with the accumulation of the corresponding LMW HSPs (Fig. 1C). Again, there was no tissue-type-specific HS response. Although there was no qualitative tissue-type-specific expression of the class I LMW HSPs, there were quantitative differences in protein levels in different soybean sections. Table I shows that the different growth sections of 3-d-old seedlings accumulated different levels of the class I LMW HSPs, as measured by quantitative western-blot analysis. The meristematic region of the root tip accumulated the highest levels of the class I LMW HSPs (3.12 μg HSPs/100 μg total protein), whereas the lowest levels were found in the mature region of the hypocotyls (1.25 μg HSPs/100 μg total protein).

HSP Synthesis and Acquisition of Thermotolerance

In an earlier study (Lin et al., 1984), the acquisition of thermotolerance was monitored by measuring growth as an increase in the length of the seedlings following HS treatments. Here we used a different measurement, TTC staining, to assess cell viability following different HS treatments (Fig. 2). TTC is normally colorless, but viable cells are able to reduce the compound to form deep-red, insoluble formazan.

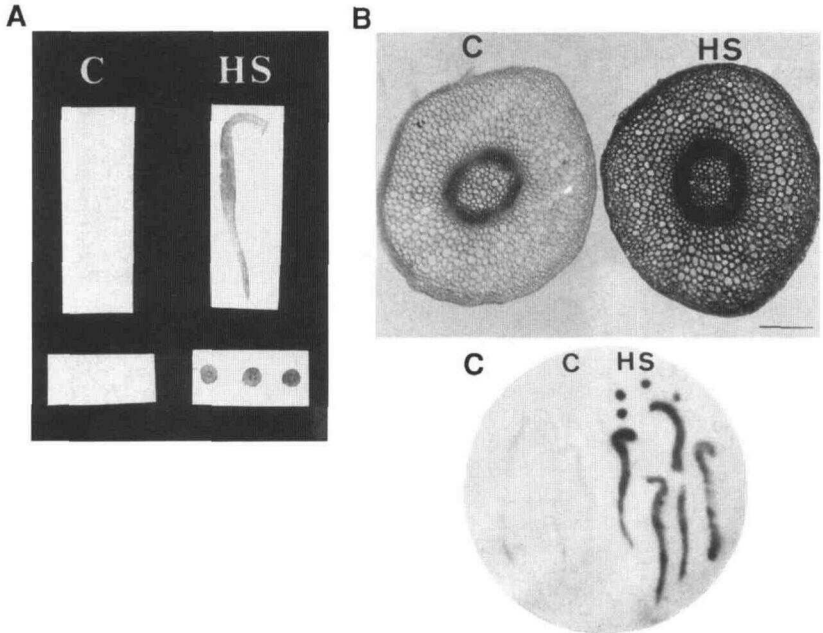

Figure 1. Tissue-type-specific expression of the class I LMW HSPs and mRNAs. Two-day-old soybean seedlings were treated at 28°C for control treatments (C) or at 40°C for 2 h for HS treatments (HS). A, The longitudinal sections and cross-sections of seedlings were printed onto the nitrocellulose membrane, as described in "Materials and Methods." B, The cross-sections of hypocotyls were incubated with the class I LMW HSP antibody, followed by color development, as described in "Materials and Methods." C, The sections from A were incubated with the class I LMW HSP antibodies or probed with the pCE53 clone, a class I LMW HSP cDNA clone. Bar = 0.5 mm.

Table I. *Quantitative estimation of the class I LMW HSPs synthesized in different sections of 3-d-old etiolated soybean seedlings treated at 40°C for 3 h*

The protein samples (25 μg) were separated by SDS-PAGE and blotted onto PVDF membranes. The amount of protein was quantified by scanning the membrane with a densitometer using ImageQuant software. Each value is expressed as the mean ± SE of two separate experiments.

Section	HSPs
	μg 100 μg^{-1} total proteins
Root	
Meristematic region	3.12 ± 0.19
Elongation region	2.63 ± 0.11
Mature region	2.37 ± 0.12
Shoot	
Meristematic region	1.83 ± 0.14
Elongation region	1.33 ± 0.23
Mature region	1.25 ± 0.07
Plumule	1.91 ± 0.28
Internal standard[a]	1.34 ± 0.17

[a] Internal standard was from 2-d-old seedlings heat-shocked at 40°C for 3 h and contained 1.34 μg LMW HSPs 100 μg^{-1} total protein, as determined by Hsieh et al. (1992).

For assessing the results of HS, TTC staining has an advantage over growth-rate measurement in that it indicates where in the root the cells have died. When exposed to TTC, control seedlings also exposed to nonlethal temperatures (40°C, 2 h) showed uniform deep-red staining (Fig. 2A, 1 and 2). The TTC staining clearly revealed the ability of a pretreatment at 40°C to protect against a subsequent lethal HS (45°C); a 2-h pretreatment at 40°C gave better thermotolerance than the shorter treatments (Fig. 2A, 3–6). The most responsive region in the primary root of 2-d-old soybean seedlings subjected to the HS treatment was the root tips (Fig. 2A, 4), a region where the class I LMW HSPs quickly accumulate to a relatively higher level than in the mature region. The results of exposing seedlings to 45°C without pretreatment are shown in Figure 2B. Cortical cells of the mature region in primary roots showed greater injury from a relatively short lethal treatment (30 min) at 45°C (indicated by arrowheads) than cells of the stele (vascular cylinder) in the root, which remained active in reducing the TTC (as did the other tissues of the elongating and meristematic regions). Figure 2C, 1, shows in more detail the root-tip region represented in Figure 2A, 3, and 2B, 4 (indicated by circles), including a longitudinal section of the tip (Fig. 2C, 2). The meristematic cells in the root tip were killed by treatment at 45°C for 2 h, but the root cap cells that surrounded the root tip were still able to reduce the colorless tetrazolium salt to the deep-red, insoluble formazan, indicating that the root cap cells were at least partially metabolically active after the treatment.

The presence of the class I LMW HSPs during recovery was studied by western-blot analysis. Seedlings were given a 40°C, 3-h treatment and then harvested immediately (Fig. 3, lane 0) or at daily intervals for 4 d during recovery at 28°C (Fig. 3, lanes 1–4). The amount of class I LMW HSPs present in soybean seedlings after a 1-, 2-, 3-, and 4-d recovery from HS treatment (40°C for 3 h) relative to the amount present at the end of the 40°C, 3-h HS, was quantified as 81, 68, 58, and 47%, respectively, based on scanning of the western blot with a densitometer equipped with ImageQuant software. These results indicate that the half-life of class I LMW HSPs is at least 3 d.

Data presented in Figure 4 demonstrate the effects of HS treatments on the rapidly growing, etiolated 2-d-old seedlings; results of treatments of 4-d-old seedlings are shown (Fig. 4A). In treatment 1 seedlings received a 45°C, 2-h treatment on d 2, which was lethal by d 4; in treatment 2 seedlings given a 40°C, 2-h HS at d 2 showed normal growth at d 4. In treatment 3 seedlings were treated at 40°C for 2 h, 28°C for 1 d, 45°C for 2 h, and 28°C for 1 d; cells in seedlings that experienced the first HS at d 2, and thus contained approximately 80% of the level of LMW HSPs

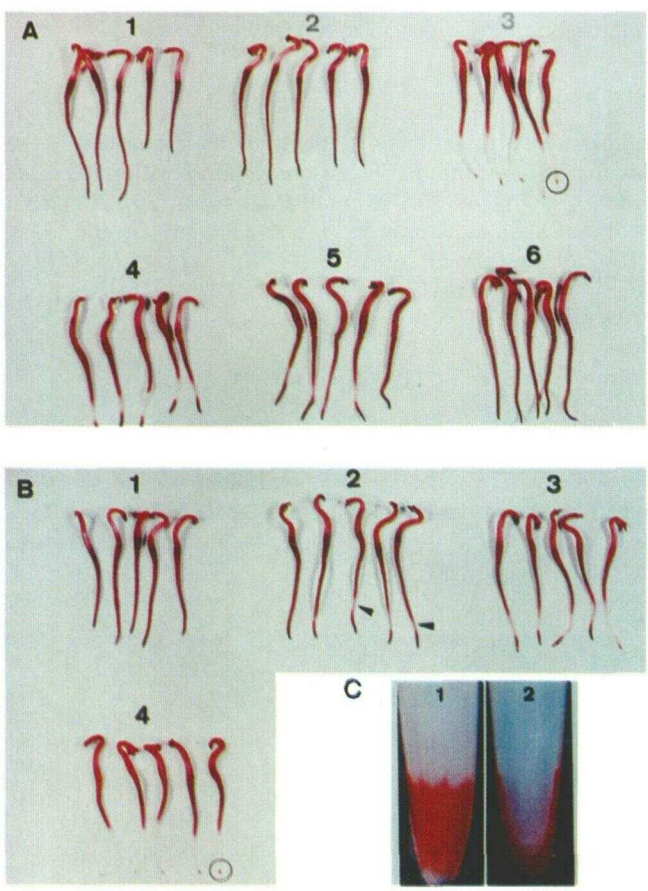

Figure 2. Soybean seedling response to HS as visualized by TTC staining. A, Two-day-old soybean seedlings were pretreated at 40°C for different time intervals and then shifted to 45°C for 2 h. They were then stained with TTC solution and treated as follows: treatment 1 and 2, 28°C (2 h) and 40°C (2 h), respectively, as controls; treatment 3, 40°C (15 min) → 45°C (2 h); treatment 4, 40°C (30 min) → 45°C (2 h); treatment 5, 40°C (1 h) → 45°C (2 h); and treatment 6, 40°C (2 h) → 45°C (2 h). B, Nonpretreated seedlings were subjected to 45°C for different time intervals, followed by TTC staining: treatment 1, 45°C for 15 min; treatment 2, 45°C for 30 min; treatment 3, 45°C for 1 h; and treatment 4, 45°C for 2 h. C, An enlargement of a root tip (1) from the seedling, such as the ones circled in A treatment 3 and B treatment 4, and the corresponding longitudinal section (2).

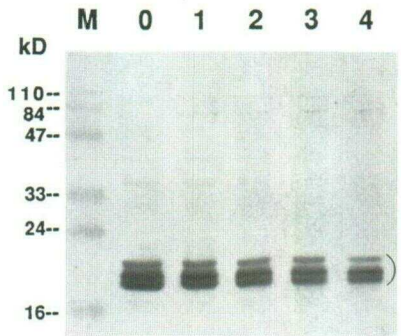

Figure 3. Half-life of the class I LMW HSPs. Two-day-old soybean seedlings were treated at 40°C for 3 h (lane 0) and replanted in the growth chamber at 28°C for 1 (1), 2 (2), 3 (3), and 4 d (4). The total protein of each sample was extracted and subjected to SDS-PAGE followed by western-blot analysis with the class I LMW HSP antibody. The class I LMW HSPs are indicated by the arch. Protein extracted from an equal amount of tissue was loaded in each lane. The amount of protein was quantified by scanning the blot with a densitometer using the ImageQuant software, as described in "Materials and Methods."

present in cells immediately following the 40°C HS, remained viable (indicated by an arrowhead). New cells, which were formed by division after the initial 40°C HS, were killed by the 45°C treatment (indicated by an arch) at d 3. In treatment 4 seedlings were given a treatment regime of 40°C for 2 h, 28°C for 1 d, 40°C for 2 h, 45°C for 2 h, and 28°C for 1 d. Following this treatment, newly formed cells were viable after the 45°C treatment at d 3, suggesting that the second 40°C HS allowed the new cells to become thermotolerant and thus survive the otherwise lethal 45°C treatment. Western analyses were done to correlate HSP synthesis and the acquisition of thermotolerance by these tissues. Figure 4B shows results for the roots and shoots of seedlings treated as in Figure 4A. Only cells that experienced the 40°C HS-induced synthesis of HSPs (e.g. lanes 2–4 under "shoot") or the cells in the root tip that received the 40°C HS treatments (e.g. lane 4 under "root") were thermotolerant to the subsequent lethal treatment (45°C for 2 h), whereas cells from the root tip that did not experience the 40°C HS and did not accumulate HSPs (e.g. lane 3 under "root") were killed by the 45°C, 2-h treatment.

Immunolocalization of the Class I LMW HSPs in Soybean Seedlings

Studies of the intracellular localization of the class I LMW HSPs may aid in understanding their physiological functions when cells are under HS. We localized the class I LMW HSPs at the electron microscope level using conventional methods. In soybean at least two obvious changes were observed in the immunolocalization of the class I LMW HSPs during HS treatment; one was the immunogold accumulation in the nuclei and the other was the electron-dense regions, which were insoluble denatured/aggregated protein structures induced by HS. Figure 5 shows sections of the root apical cells under varying HS treatments, and detection of class I LMW HSPs with immunogold labeling. Control cells (28°C) had no obvious immunogold labeling, only some nonspecific background signals, especially in the region of the middle lamella (Fig. 5A). After HS treatment (40°C for 2 h) many gold particles were found in the nuclei and in the electron-dense regions of the cytoplasm (Fig. 5B). The electron-dense regions that contain hsgs were similar to those seen in tomato suspension cultures and other plant species reported by Nover et al. (1989) and Parsell et al. (1994). As cells recovered from HS treatment for 3 h at 28°C, the aggregated protein structures disaggregated and the immunogold particles redistributed uniformly in both the cytoplasm and the nucleus (Fig. 5C). If the seedlings were given a severe HS (45°C for 30 min) following the 40°C pretreatment, as shown in Figure 5C (cells were in the thermotolerant condition), more compact denatured/aggregated protein structures were reformed in the cytoplasm (Fig. 5D). The immunogold particles were again localized to the nucleus.

We also examined the nucleus, which is sensitive to HS (Chen et al., 1988; Mansfield et al., 1988). The localization of class I LMW HSPs in HS and thermotolerant cells was

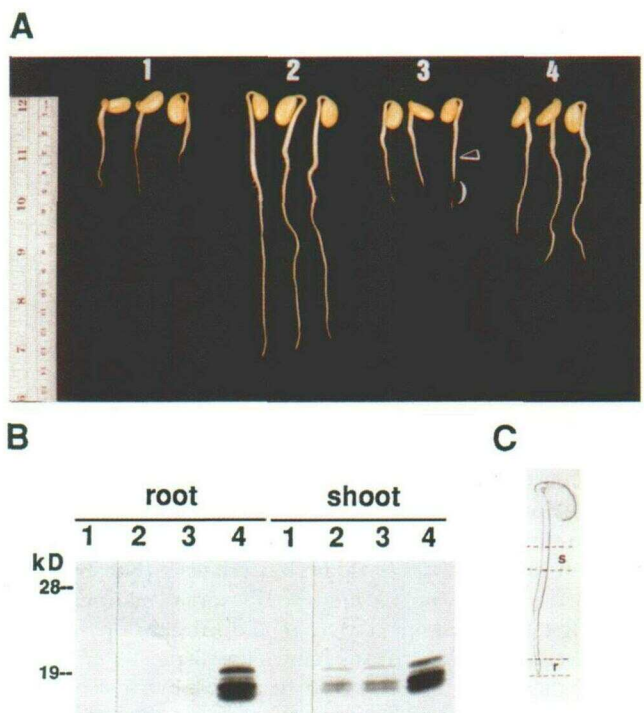

Figure 4. Acquisition of thermotolerance in relation to HSP synthesis. A, Two-day-old soybean seedlings were treated as follows: treatment 1, 45°C (2 h) → 28°C (1 d); treatment 2, 40°C (2 h) → 28°C (1 d); treatment 3, 40°C (2 h) → 28°C (1 d) → 45°C (2 h); and treatment 4, 40°C (2 h) → 28°C (1 d) → 40°C (2 h) → 45°C (2 h). Photographs show 4-d-old seedlings after 1 additional d at 28°C following the end of the last treatment. The arrowhead indicates that the tissue had acquired thermotolerance, and the arch indicates that the tissue was dead. B, Immunoblot of class I LMW HSPs in roots and shoots of 3-d-old seedlings treated as in A. C, Protein samples were extracted from 1-cm segments from the mature zone of the shoot (s) and from 0.5-cm sections from the root tip (r) immediately after the treatments noted in B. Total soluble protein extracted from an equal amount of tissue was loaded in each lane.

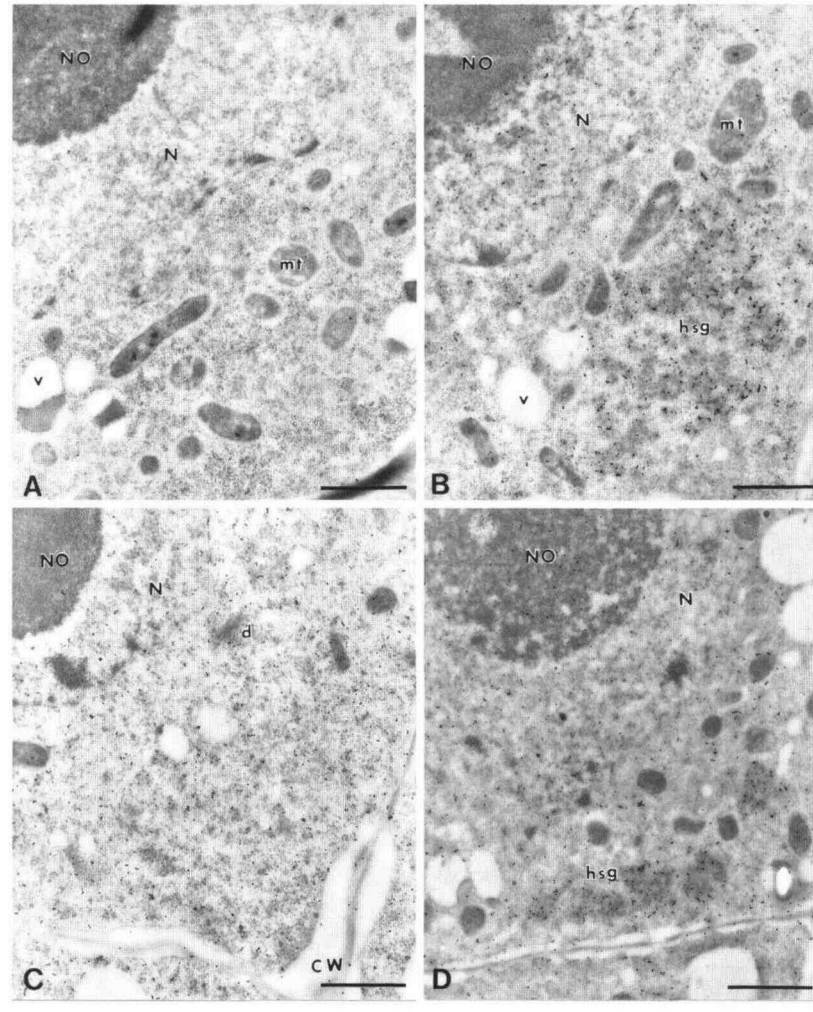

Figure 5. Immunolocalization of the class I LMW HSPs in soybean root tips (1- to 2-mm long). The root tips were fixed in glutaraldehyde (0.5%)/paraformaldehyde (3.2%) and embedded in London Resin White. After incubation with the anti-class I LMW HSP antibodies, localization of HSPs was visualized by reaction with secondary antibodies conjugated with 15-nm gold particles. Treatments were as follows: 28°C for 2 h as a control (A); 40°C for 2 h as HS (B); 40°C (2 h) → 28°C (3 h) as cells recovered from HS (C); 40°C (2 h) → 28°C (3 h) → 45°C (30 min) as cells acquired thermotolerance (D). CW, Cell wall; d, dictyosome; mt, mitochondrion; N, nucleus; NO, nucleolus; v, vacuole. Bar = 1 μm.

examined using immunogold labeling. In Figure 6A (40°C for 2 h) many gold particles specifically localized in the HS-induced dispersed region of the nucleolus. This region has previously been identified to contain the unprocessed preribosomal RNP, the processing of which is the most heat-sensitive step of ribosome biosynthesis (Nover et al., 1989). The formation of mature ribosomal subunits was immediately and completely stopped during the onset of HS (Nover et al., 1989). Figure 6B shows the dispersed morphology of the nucleolus recovered to a more normal densely packed structure as in the control cells, and the gold particles dispersed from the nucleolus to the nucleoplasm and cytoplasm when HS was ended. Figure 6C shows that a subsequent severe HS (45°C for 30 min) following the treatment at 40°C (2 h) → 28°C (3 h) resulted in the thermotolerant condition. The class I LMW HSPs became relocalized to the unprocessed preribosomal RNP of the nucleolus, similar to the results shown in Figure 6A, as well as to the nucleoplasmic region of the nucleus, similar to the results shown in the Figure 6B. Figure 6D shows that immunogold localization in the nucleolus in the acquired thermotolerant condition had results very similar to those shown in Figure 6A, except that there was much less immunogold labeling in the nucleoplasm.

Since the HSP24 (Collier et al., 1988) and HSP70 (Lim et al., 1984; Clark and Brown, 1986) in vertebrate cells were associated with elements of the cytoskeleton, we used soybean anti-tubulin antibodies to determine microtubule localization under control and HS treatments. Figure 7, A and B, shows that the immunogold particles were localized at microtubules near the cell walls, but no gold was found in the denatured/aggregated protein structures. In Figure 7C (40°C for 2 h) the denatured/aggregated protein structures were labeled by anti-class I LMW HSP antibodies, but no immunogold particles were localized at microtubules (indicated by arrowheads). Figure 7D shows a higher magnification of the same treatment shown in Figure 7C. In general, the basic size of the hsgs (indicated by circles) appeared to be 35 to 45 nm in diameter using the 15-nm particles of immunogold as the standard (Fig. 7D).

We used three serial sections to observe the morphology and distribution of the insoluble denatured/aggregated protein structures in the cytoplasm. There were no fixed shapes for the denatured/aggregated protein structures. They were huge cytoplasmic structures (according to the 60- to 150-nm thickness of each section) that may be larger than mitochondria (data not shown). We also examined the random distribution of the denatured/aggregated protein structures

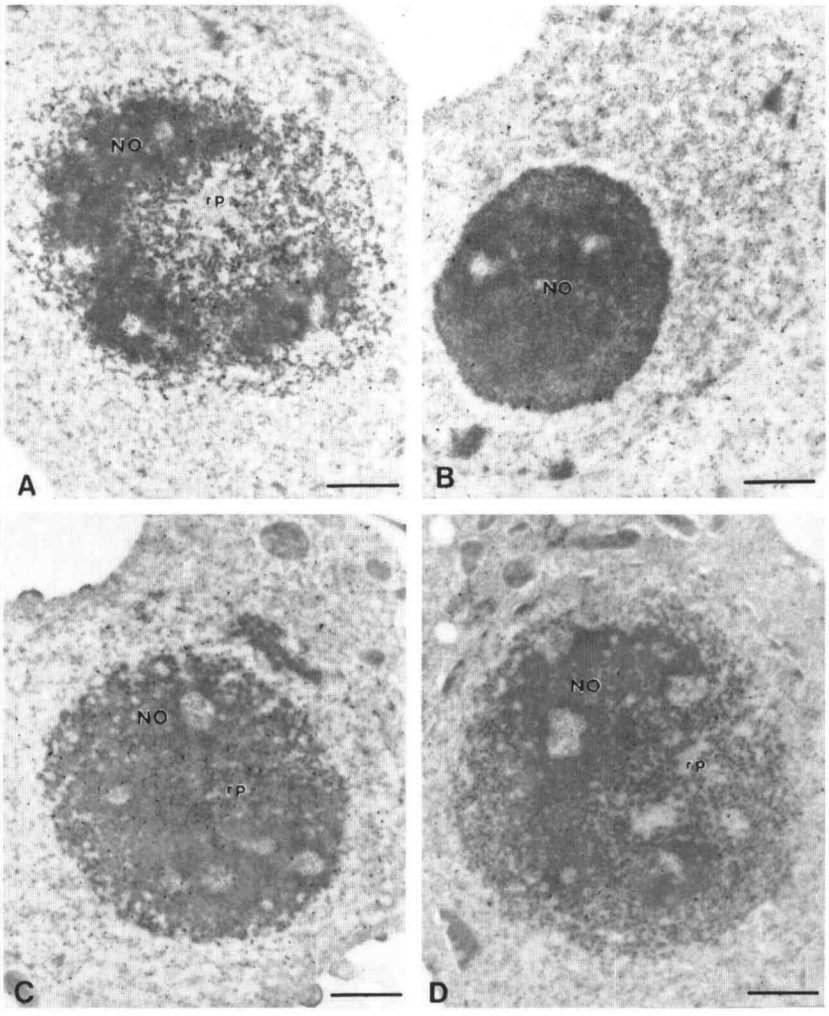

Figure 6. Immunolocalization of the class I LMW HSPs in the nucleus. Treatments were as follows: 40°C (2 h) as HS (A); 40°C (2 h) → 28°C (3 h) as cells recovered from HS (B); 40°C (2 h) → 28°C (3 h) → 45°C (30 min) (C); 40°C (2 h) → 45°C (30 min) as cells acquired thermotolerance (D). NO, Nucleolus; rp, ribonucleoprotein particle. Bar = 1 μm. (Magnification ×13,500.)

(which contained the hsgs) in the cytoplasm. This was different from the distribution of the small HSP24 of chicken embryo fibroblast cells (Collier et al., 1988) and the HSP27 of *Drosophila melanogaster* (Duband et al., 1987), which were preferentially associated with the perinuclear region (data not shown). Root cap cells seemed to be more resistant than other cells to HS. Serial sections (Fig. 8) show that root cap cells exposed to HS specifically accumulate a very large amount of the class I LMW HSPs and certain huge denatured/aggregated protein structures, a phenomenon that may account for the higher tolerance of the root cap to HS (also shown in Fig. 2C).

DISCUSSION

In this study no tissue-type-specific expression patterns for the class I LMW HSPs in soybean seedlings were found. Tissues with high metabolic and mitotic activities, such as the meristematic regions of roots, shoots, and the cambium cells in the vascular tissues, were more sensitive to HS and responded by synthesizing higher levels of HSPs. The TTC staining method can ascertain and visualize differential viability of cells of root or shoot segments representative of different growth stages in response to HS. In 2-d-old etiolated soybean seedlings the root tip was the most responsive to HS and responded by synthesizing HSPs quickly and to higher levels than other regions of the root. The mature region of the root was much more easily damaged by the HS, particularly in the cortical cells, which were highly vacuolated and contained smaller amounts of HSPs. In contrast, the root-cap cells were the most tolerant of a severe HS at 45°C for 2 h based on TTC reduction (Fig. 2A, 3; 2B, 4; 2C, 1; and 2C, 2). The half-life of class I LMW HSPs in young, etiolated soybean seedlings was estimated to be more than 3 d, which was longer than that of the pea HSP18.1 (38 ± 8 h) shown by DeRocher et al. (1991), and that of the pea chloroplast HSP21 (52 ± 13 h) shown by Chen et al. (1990). The longer half-life of the class I LMW HSPs in soybean seedlings may contribute to a greater duration of thermotolerance than in other plant species that have HSPs with a shorter half-life. This long half-life of HSPs is probably associated with the need to repair the damaged cells and/or to provide adaptive thermotolerance in the event of further HS. The fact that cells that have not experienced a HS (i.e. cells formed by division after a HS treatment administered to seedlings 24 h earlier) do not survive a lethal HS (45°C for 2 h) provides additional circumstantial evidence that HSPs are essential for the acquisition of thermotolerance (Fig. 4A, treatment 4).

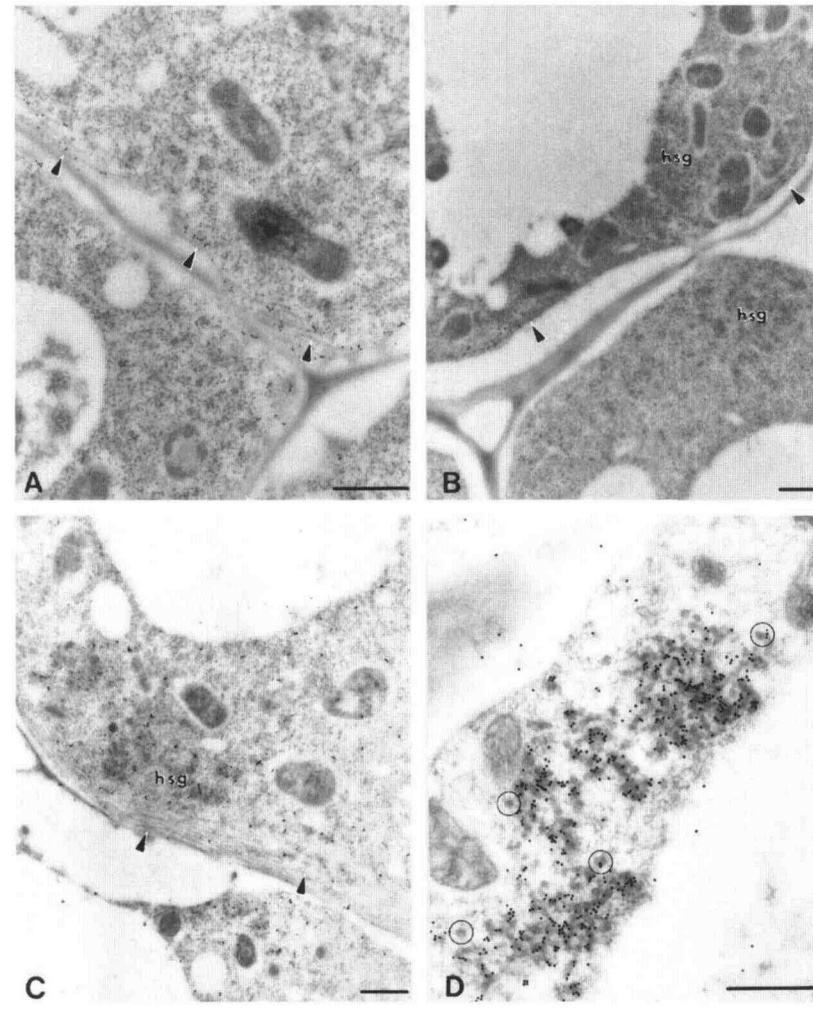

Figure 7. Immunolocalization using soybean anti-tubulin antibodies and class I LMW HSP antibodies. Seedlings from 28°C (2 h) control (A) and 40°C (2 h) HS (B) were treated with anti-tubulin antibodies. C and D, Seedlings were treated at 40°C for 2 h and anti-class I LMW HSP antibodies were used. Arrowheads show the microtubules of the cytoskeleton around the cell wall. hsgs that aggregated in the electron-dense region shown by a circle have a globular shape in size from 35 to 45 nm (using the 15-nm gold particles as standard). Bar = 0.5 μm.

The 15- to 18-kD class I LMW HSPs of soybean, which exist in a soluble form in the cytoplasm after HS (Lin et al., 1984), can be enriched in an ammonium sulfate fraction of 70 to 100% saturation (Jinn et al., 1989). We identified a higher-order structural complex with a molecular mass of 280 kD, which was composed of at least 15 polypeptides of the 15- to 18-kD class I LMW HSPs in soybean (Jinn et al., 1995). Although rice, pea, and mung bean synthesize different numbers of the class I LMW HSPs with differences in pI and molecular mass, they all form similar-sized molecular structures ranging from 270 to 310 kD. Nover (1990) reported that HSPs were a part of smaller precursor particles that aggregated during HS into very large aggregate structures of about 40 nm in size (i.e. hsgs); these structures were composed of 5 to 10% HSP70 and 50 to 80% LMW HSPs. The purified hsgs isolated from soybean seedlings by Mansfield and Key (1988) had a density of 1.20 to 1.21 g/cm^3, a density typical of RNP; no high-molecular-weight HSPs were associated with these particles. The molecular mass of the hsg in soybean could be calculated by using the equation $4/3\ \pi r^3$ = mass/density, where the average density of hsg is 1.205 g/cm^3 (Mansfield and Key, 1988) and the diameter was estimated to be around 40 nm (shown in Fig. 7D). The fact that the hsgs are not homogeneous in size may be due to their aggregation into multiple hsg units. A molecular mass of approximately 24.3 MD was calculated for the hsgs in soybean. If the hsg is composed of only a class I LMW HSP complex, it would contain 80 to 90 subunits of the class I LMW HSP complex.

According to Nover et al. (1989), hsg formation is the consequence of HS; however, formation of the class I LMW HSP complex (280 kD) is not the consequence of HS, since it forms when HSPs are induced by arsenite or amino acid analogs at 28°C (Jinn et al., 1995). The electron-dense (insoluble, denatured/aggregated protein) regions containing hsgs in the cytoplasm that form during HS disaggregate into the 280-kD class I LMW HSP complexes when seedlings are relieved of the HS (Fig. 5C). This aggregation and disaggregation is similar to the activity of HSP104 in yeast, which mediates the resolubilization of heat-inactive proteins from insoluble aggregates (Parsell et al., 1994).

Chou et al. (1989) demonstrated that isolated mitochondria with associated HSPs were functional in oxidative phosphorylation under thermal stress. Additionally, some plant LMW HSPs localized in the endomembrane system (Helm et al., 1993) and mitochondria (Cooper and Ho, 1987; Lenne and Douce, 1994), suggesting that the association of HSPs with organelles provided protection from heat dam-

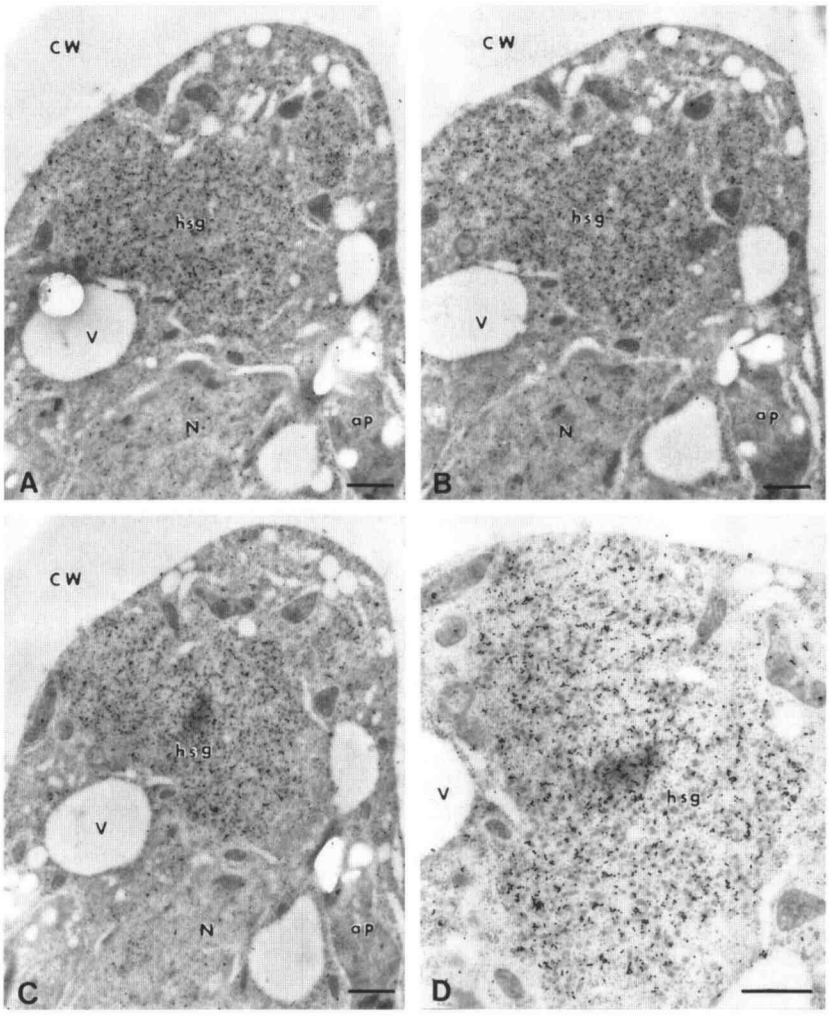

Figure 8. Localization of the class I LMW HSPs in the root-cap cells of 2-d-old seedlings that received a HS at 40°C for 2 h, using three serial sections (A, B, and C) to demonstrate the huge structure of the hsg aggregates in the electron-dense region. D, Magnified version of C. ap, Amyloplast; other abbreviations are as in Figure 5. Bar = 1 μm.

age. Some of the denatured/aggregated protein structures were surrounded by mitochondria, as shown in Figure 5, B and D, Figure 7, B, C, and D, and Figure 8D. These observations were correlated with the disaggregation of the aggregated protein structures in the cytoplasm that might be mediated by a supply of energy from the mitochondria, and with the observation that relocalization of the HSPs in vivo to the cytoplasm was energy-dependent (NaF or anaerobiosis inhibited relocalization; C.Y. Lin, unpublished results). It should be noted, however, that the in vitro chaperone activity of the class I LMW HSPs was reported to be energy-independent by Lee et al. (1995).

Early investigations in *D. melanogaster* and mammalian systems demonstrated that ribosome synthesis, a typical housekeeping function of cells, was immediately blocked by HS. The first step of ribosome biosynthesis was the assembly of ribosomal proteins to the nascent pre-rRNA in the nucleolus (Nover et al., 1989). Lin et al. (1984) demonstrated that during a 40°C HS treatment some HSPs were stably associated with the purified nuclei. In this study, the class I LMW HSPs were shown to localize in the nuclei and nucleolus specifically in association with RNP (Fig. 6, A–D), confirming earlier results using the biochemical cell-fractionation method. These data suggest that these HSPs may participate in the recovery of nuclear/nucleolar functions from heat damage. Although our previous data using cell fractionation indicated an association between LMW HSPs and the plasma membrane during HS, we did not observe localization of the class I LMW HSPs in the membrane in this study. The LMW HSPs shown previously to be associated with the membrane may belong to a class other than the class I LMW HSPs or may be an artifact of cell fractionation.

Although the physiological function of HSPs remains unclear, we showed previously that the synthesis and accumulation of LMW HSPs and their cellular localization are strongly correlated with the acquisition of thermotolerance in soybean seedlings (Lin et al., 1984). With the studies presented here we may be able to establish a potentially important role for this class of HSPs in the protection of plants from thermal damage.

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