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Localization of Small Heat Shock Proteins to the Higher Plant Endomembrane System

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Three related gene families of low-molecular-weight (LMW) heat shock proteins (HSPs) have been characterized in plants. We describe a fourth LMW HSP family, represented by PsHSP22.7 from *Pisum sativum* and GmHSP22.0 from *Glycine max*, and demonstrate that this family of proteins is endomembrane localized. PsHSP22.7 and GmHSP22.0 are 76.7% identical at the amino acid level. Both proteins have amino-terminal signal peptides and carboxyl-terminal sequences characteristic of endoplasmic reticulum (ER) retention signals. The two proteins closely resemble class I cytoplasmic LMW HSPs, suggesting that they evolved from the cytoplasmic proteins through the addition of the signal peptide and ER retention motif. The endomembrane localization of these proteins was confirmed by cell fractionation. The polypeptide product of PsHSP22.7 mRNA was processed to a smaller-M_r form by canine pancreatic microsomes; in vivo, GmHSP22.0 polysomal mRNA was found to be predominantly membrane bound. In vitro-processed PsHSP22.7 corresponded in mass and pI to one of two proteins detected in ER fractions from heat-stressed plants by using anti-PsHSP22.7 antibodies. Like other LMW HSPs, PsHSP22.7 was observed in higher-molecular-weight structures with apparent masses of between 80 and 240 kDa. The results reported here indicate that members of this new class of LMW HSPs are most likely resident ER proteins and may be similar in function to related LMW HSPs in the cytoplasm. Along with the HSP90 and HSP70 classes of HSPs, this is the third category of HSPs localized to the ER.

Most eukaryotic cells respond to high temperature and certain other stresses with the production of heat shock proteins (HSPs), which appear to be required for cell survival (26). Four major classes of HSPs, HSP90, HSP70, HSP60, and low-molecular-weight (LMW) HSPs (16 to 30 kDa), have been identified in eukaryotes (26). The role of the LMW HSPs is the least well understood. These HSPs have a conserved carboxyl-terminal domain (~70 amino acids), termed the heat shock domain, also found in the alpha-crystallin eye lens proteins. Although their functions remain obscure, LMW HSPs have been proposed either to maintain cell structure during stress (26), to have an unknown enzymatic activity (12), or to sequester translationally inactive mRNA during stress (34). Inability to demonstrate a function for the single LMW HSP of *Saccharomyces cerevisiae* has also led to the proposal that these HSPs are the nonessential products of ancient viral or "selfish" DNA (45).

LMW HSPs in both plants and animals have been observed complexed in higher-molecular-weight aggregates. Two general size classes of the aggregates have been observed: a 200- to 500-kDa form and a larger particle of greater than 1 MDa (31). Neither the exact composition nor the functional significance of these aggregates is well understood. However, Lindquist and Craig (26) proposed that the conserved carboxyl-terminal domain is important for promoting the formation of the high-molecular-weight aggregates of these HSPs. This hypothesis is based on the homology of the carboxyl-terminal heat shock domain with the alpha-crystallins, which form highly ordered, tightly packed aggregates in the eye lens.

Higher plants synthesize an unusually large number of nuclear-encoded LMW HSPs (30, 31). This complexity is due not to expression of functionally redundant genes but to the presence of distinct gene families which have been conserved since before the divergence of monocots and dicots (>150 × 10^6 years ago) (46). Of the three well-characterized LMW HSP gene families, two encode HSPs which reside primarily in the cytoplasm and a third encodes chloroplast-localized HSPs (31, 46). While proteins from all of the gene families possess the heat shock domain, genes from within each family encode characteristic amino-terminal regions, and the amino-terminal regions encoded by different gene families share little homology (10, 46). It is therefore plausible that the heat shock domain allows the formation of LMW HSP aggregates (26) and that the divergent amino termini confer either different substrate specificities or distinct functions.

The conservation of these families suggests that LMW HSPs are important components of multiple plant cell compartments. Furthermore, cloning studies indicated that there are additional gene families (42, 46), and cell fractionation experiments suggested that specific LMW HSPs are located in other cell compartments, including the endomembrane system (14, 25, 43).

We have characterized homologous genes from pea (*Pisum sativum*; PsHSP22.7) and soybean (*Glycine max*; GmHSP22.0) that represent a fourth family of plant LMW HSPs (46). We establish that the proteins encoded by these genes are localized in the endomembrane system, most probably in the endoplasmic reticulum (ER). The significance of this finding in relation to determining LMW HSP function is discussed.

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MATERIALS AND METHODS

Gene isolation and analysis. A cDNA encoding soybean GmHSP22.0 was initially isolated by Schöffl and Key (42) and designated pFS2033. The pFS2033 cDNA contained an incomplete coding region. This cDNA was used to screen a λ 1059 library of soybean (*G. max* L. cv. 'Corsoy') genomic DNA (29). The λ 1059-2033 clone contained a complete open reading frame (579 bp) which corresponded to the pFS2033 cDNA where the clones overlapped. The λ 1059-2033 coding region was subcloned and sequenced by the chemical cleavage method (27). The coding region was uninterrupted by introns. The *P. sativum* PsHSP22.7 cDNA was isolated from a cDNA library prepared in λ gt10 with poly(A)$^+$ RNA from pea leaves which had been heat stressed for 2 h at 39°C (48). The library was screened at reduced stringency with a cDNA homologous to *G. max* cDNA pFS2033. PsHSP22.7 was identified and subcloned into Bluescript (Stratagene), and the sequence was determined by dideoxy sequencing (41) using Sequenase (United States Biochemical, Cleveland, Ohio). After the completion of this study, Knack et al. (22) reported the deduced amino acid sequence of a pea LMW HSP cDNA, HSP26-22, encoding a protein identical to PsHSP22.7. They did not identify it as an endomembrane protein.

In vitro transcription, translation, and processing. Transcription of PsHSP22.7 in vitro was performed by using T3 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in accordance with the manufacturer's directions. RNA isolations and hybrid selections were done as previously described (19). In vitro translations were performed with either wheat germ (38) (Fig. 2, lanes 1 and 2) or rabbit reticulocyte lysate (Bethesda Research Laboratories/Life Technologies, Inc., Gaithersburg, Md.) (Fig. 2, lanes 3 to 6) translation systems supplemented with 0.75 μCi of ^{35}S-methionine μl^{-1} (1,200 Ci mmol^{-1}; NEN Research Products, Wilmington, Del.). Cotranslational processing was stimulated by the addition of 2 μl of canine pancreatic microsomes (Promega Corp., Madison, Wis.) to 15-μl reticulocyte translation reaction mixtures prior to the addition of RNA. For protease digestions, completed translation reaction mixtures were supplemented with 0.1 μg of trypsin μl^{-1} (Sigma, St. Louis, Mo.) and 0.1% Triton X-100 (where indicated) and maintained at 4°C for 30 min. All reaction mixtures were solubilized by boiling in Laemmli sample buffer (24). Translation products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10 to 16% gradient gels and visualized by fluorography.

Polysomal RNA analysis. Free and membrane-bound polysomes were isolated (1) from 40°C heat-stressed soybean seedlings. RNA was extracted (5) from the polysomal fractions, and an equal percentage of each fraction was assayed for LMW HSP mRNAs by Northern (RNA) analysis (13) with the [^{32}P]ATP random primer-labeled cDNA probes indicated.

Antiserum preparation. PsHSP22.7 fusion protein was synthesized in *Escherichia coli* by using the T7 expression system (44). DNA encoding the carboxyl-terminal 65 amino acids of PsHSP22.7 was subcloned into the expression vector pET3a, and the fusion protein was synthesized in *E. coli* BL21(DE3). The fusion protein was isolated from whole cell extracts by SDS-PAGE and electroelution and was used to generate antibodies in New Zealand White rabbits. Serum was collected 7 to 10 days after booster injections and stored at −20°C. Rabbit antiserum against PsHSP18.1 was available in the laboratory prior to this study (16), and rabbit antiserum against protein disulfide isomerase (PDI) from *Medicago sativa* was kindly provided by Basil Shorrosh (Noble Foundation, Ardmor, Okla.).

Plant material and heat stress treatments. Peas (*P. sativum* L. cv. 'Little Marvel') were germinated and grown in darkness between water-saturated paper towels at 21°C for 4 days. In experiments dealing with whole plants, peas were grown from seed in 2-in. (ca. 5-cm) pots containing vermiculite (four seeds per pot) for 12 to 14 days in a growth chamber under an 8-h dark (18°C)/16-h light (22°C) cycle. Plants were watered for the first 5 days with tap water and subsequently with quarter-strength Hoagland's solution as needed. Heat stress was administered to the dark-grown seedlings by placing the paper towels containing seedlings in covered trays and incubating the seeds at 37°C for 3 h. In experiments addressing the organ-specific accumulation and native structure of the LMW HSPs, heat stresses that more closely emulate what plants experience in nature were used (8). Growth chamber temperatures were increased at 4°C h^{-1} to a maximum of 38 or 40°C and maintained at the maximum temperature for 4 h. When potted seedlings were subjected to these heat stresses, 100% relative humidity was maintained throughout the stress period to minimize transpirational cooling of leaves. Plants were harvested and analyzed immediately after the end of the stress period.

Protein electrophoresis and immunoblotting. Prior to electrophoresis, samples were prepared by using Laemmli (24) sample buffer and quantified by using a Coomassie blue dye binding assay (21). Proteins were separated by SDS-PAGE (12.5% acrylamide or 10 to 16% gradient gels) or by the two-dimensional gel system of O'Farrell (35), in which case proteins were acetone precipitated from Laemmli sample buffer and resuspended in O'Farrell buffer A prior to isoelectric focusing. Radiolabeled in vitro translation products were visualized by fluorography. Gels of root or leaf protein samples were analyzed for LMW HSPs by immunoblotting. Proteins were transferred to nitrocellulose, and immunoreactive proteins were detected by using antisera at a dilution of 1:1,000 and ^{125}I-protein A (>30 mCi mg^{-1}; ICN Biomedicals, Inc., Irvine, Calif.) as described previously (47). Alternatively, immunoreactive proteins were visualized by chemiluminescence detection. Following incubation in the primary antisera, blots were incubated for 30 to 60 min in a 1:2,500 dilution of horseradish peroxidase-linked donkey anti-rabbit immunoglobulin G (Amersham International, Amersham, United Kingdom). After four washes in low-salt buffer (47), blots were immersed in a 1:1 mixture of enhanced chemiluminescence detection reagents (Amersham) diluted with 1 volume of water. Membranes were drained, wrapped in plastic, and exposed to X-ray film (XAR-5; Eastman Kodak Inc., Rochester, N.Y.) for 10 to 120 s. Relative levels of immunoreactive proteins in leaves and roots were estimated by laser densitometry of fluorograms.

Organelle isolation and fractionation. For the results shown in Fig. 6, microsomal preparations from 2.0 g of roots from heat-stressed pea seedlings were isolated by centrifugation on a 12/16/60% sucrose step gradient according to the method of Chrispeels (11), using solutions containing 2 mM EDTA. Sepharose 4B chromatography was not performed. Clarified homogenate, material from the top of the gradient, and material from the 16/60% interface were designated total, cytosolic, and microsomal fractions, respectively. Trypsin digestions were performed on aliquots of microsomal fractions for 90 min as for the in vitro transcription/translation experiments.

In the analyses shown in Fig. 7, microsomal fractions were isolated from 0.25 g of heat-stressed (37°C, 3 h) roots by using 5 ml of buffer as for Fig. 6 except that all solutions were supplemented with 10 mg of bovine serum albumin (BSA; Sigma catalog no. A-3803) ml^{-1}. Microsomal preparations were separated on 20 to 60% continuous sucrose gradients as described previously (11) except that the solutions used for the gradients were supplemented with 0.1 mg of BSA ml^{-1}. Cytochrome *c* reductase (CCR) assays were performed as described by Bowles and Kauss (6). Inosine diphosphatase (IDPase) activity was monitored as described by Chrispeels (11). IDPase reaction mixtures were supplemented with 0.03% Triton X-100. Vanadate-sensitive ATPase assays were performed as previously described (7).

Analysis of LMW HSP aggregates. Following a 38°C, 4-h heat stress, roots, excluding tips, were excised from 4-day-old seedlings and immediately homogenized in 3 volumes of ice-cold buffer (100 mM Tris-HCl, [pH 8.0], 25 mM KCl, 10 mM dithiothreitol, 5 mM ε-amino-*n*-caproic acid, 2 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.05 mg of BSA ml^{-1}, 0.5 μg each of aprotinin and leupeptin ml^{-1}, 0.01% [wt/vol] sodium azide, 6% [wt/vol] sucrose), using a chilled glass homogenizer. Homogenates were clarified by filtration through acid-washed sand and centrifugation at 2,000 × *g* for 5 min at 4°C. Where noted, organelles were disrupted either by supplementation of this buffer with 1% (vol/vol) Nonidet P-40 or by sonication of 1.5 ml of clarified homogenates. Sonication was administered with a W-225 (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) sonicator fitted with a Tapered Microtip for 30 min at a power level of 5 and pulse mode setting of 25%. During sonication, samples were kept in 15-ml Corex tubes immersed in ice water. Debris was pelleted from 1 ml of all homogenates by ultracentrifugation at 541,000 × *g* for 30 min at 4°C, using a TLA 100.3 rotor in a TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). These ultraclarified supernatants were immediately fractionated further by sucrose density gradient centrifugation or nondenaturing gel electrophoresis.

To determine the sedimentation characteristics of LMW HSP-containing aggregates, 1-ml samples of each of the ultraclarified supernatants described above were loaded onto 12-ml 17 to 34% sucrose gradients. The buffer composition of these gradients was identical to that of the initial homogenization buffer except that phenylmethylsulfonyl fluoride was omitted. Gradients were spun in an SW40 rotor (Beckman) at 40,000 rpm for 21 h at 4°C. Following centrifugation, gradients were fractionated from the bottom into 1-ml fractions, immediately mixed with 333 μl of 4× Laemmli sample buffer (24), boiled, and stored at −20°C. The sedimentation of LMW HSPs in the sucrose gradients was determined by SDS-PAGE and immunoblot analysis of aliquots of the SDS-denatured gradient fractions.

To examine the LMW HSP aggregates observed in the sucrose gradients at greater resolution, native gel analysis was performed. Samples (10 to 50 μl, approximately 2.5 to 12.5 μg of protein) of the sonicated ultraclarified supernatants were separated on 4.0 to 22.5% acrylamide (0.0 to 12.5% glycerol) gradient gels prepared with the buffer system of Laemmli (24) but without SDS. Since Nonidet P-40 adversely affects the quality of these gels, detergent-treated samples were not analyzed. Samples were loaded onto gels precooled to 4°C and were electrophoresed at 100 V for 48 h at 4°C. Under these conditions, proteins and protein complexes migrate until reaching their gel pore exclusion limit. This method allows the determination of apparent molecular mass by using a single gel (2). Following electrophoresis, LMW HSPs were detected by immunoblot analysis, and parallel gels were stained with Coomassie brilliant blue.

Nucleotide sequence accession numbers. The sequences reported have been assigned EMBL accession number X63198 (GmHSP22.0) and GenBank accession number M33898 (PsHSP22.7).

RESULTS

Analysis and expression of PsHSP22.7 and GmHSP22.0 genes. The amino acid sequences of PsHSP22.7 from pea (46) and GmHSP22.0 from soybean, as determined from corresponding DNA sequences, share 76.7% identity and 89% similarity (Fig. 1A). In contrast, the amino acid sequence similarity between PsHSP22.7 or GmHSP22.0 and members of the three other gene families of LMW HSPs is 72.5% or less (Table 1), indicating that PsHSP22.7 and GmHSP22.0 represent a distinct LMW HSP gene family. PsHSP22.7 and GmHSP22.0 proteins are most similar to the class I cytoplasmic HSPs of plants, and alignment of the PsHSP22.7 and PsHSP18.1 sequences and hydropathy profiles emphasize this structural relationship (Fig. 1B and C). The region of the hydropathy profile from residues 110 to 167 of PsHSP22.7 is typical of all eukaryotic LMW HSPs (26) and classifies PsHSP22.7 and its *G. max* homolog as members of the superfamily of eukaryotic LMW HSP genes.

The pattern of PsHSP22.7 and GmHSP22.0 mRNA expression is characteristic of plant LMW HSPs. Northern analysis using the PsHSP22.7 cDNA to probe total RNA isolated from pea leaves or roots prior to stress or following a 4-h stress at 38°C confirmed that PsHSP22.7 mRNA accumulation is heat induced (not shown). Heat induction of GmHSP22.0 mRNA was demonstrated in a previous study (42).

Cotranslational processing of PsHSP22.7 by canine pancreatic microsomes in vitro. The greatest differences between PsHSP22.7 and the cytoplasmic protein PsHSP18.1 are amino- and carboxyl-terminal extensions of PsHSP22.7 (Fig. 1B and C). The PsHSP22.7 and GmHSP22.0 amino-terminal extensions are characteristic in sequence composition and hydropathic character to signal peptides (50). The possibility that the amino terminus of PsHSP22.7 functions as a signal peptide was tested in vitro. Translation of RNA hybrid selected with the PsHSP22.7 cDNA from poly(A)$^+$ RNA isolated from heat-stressed pea leaves produced a polypeptide that migrated at approximately 24 kDa on SDS-PAGE (Fig. 2, lane 2). Translation of in vitro-transcribed PsHSP22.7 RNA produced an identical polypeptide (Fig. 2, lane 3), confirming that the PsHSP22.7 cDNA contains the entire protein coding region. To determine whether PsHSP22.7 can be cotranslationally processed and imported into the ER, PsHSP22.7 RNA was translated in rabbit reticulocyte lysates supplemented with ER-derived canine pancreatic microsomes (51). In the presence of microsomes, the primary translation products were processed to a single protein of about 20 kDa (Fig. 2, lane 4). This value is in close agreement with the calculated mass of PsHSP22.7 following removal of the proposed signal peptide at Gly-28, as predicted by the −1,−3 rule (49). The processed translation product was protected from trypsin digestion, and protection was lost when the microsomes were lysed with detergent (Fig. 2, lanes 5 and 6). These results demonstrate that PsHSP22.7 was translocated into the microsomes and suggest that the protein is located in the endomembrane system in vivo.

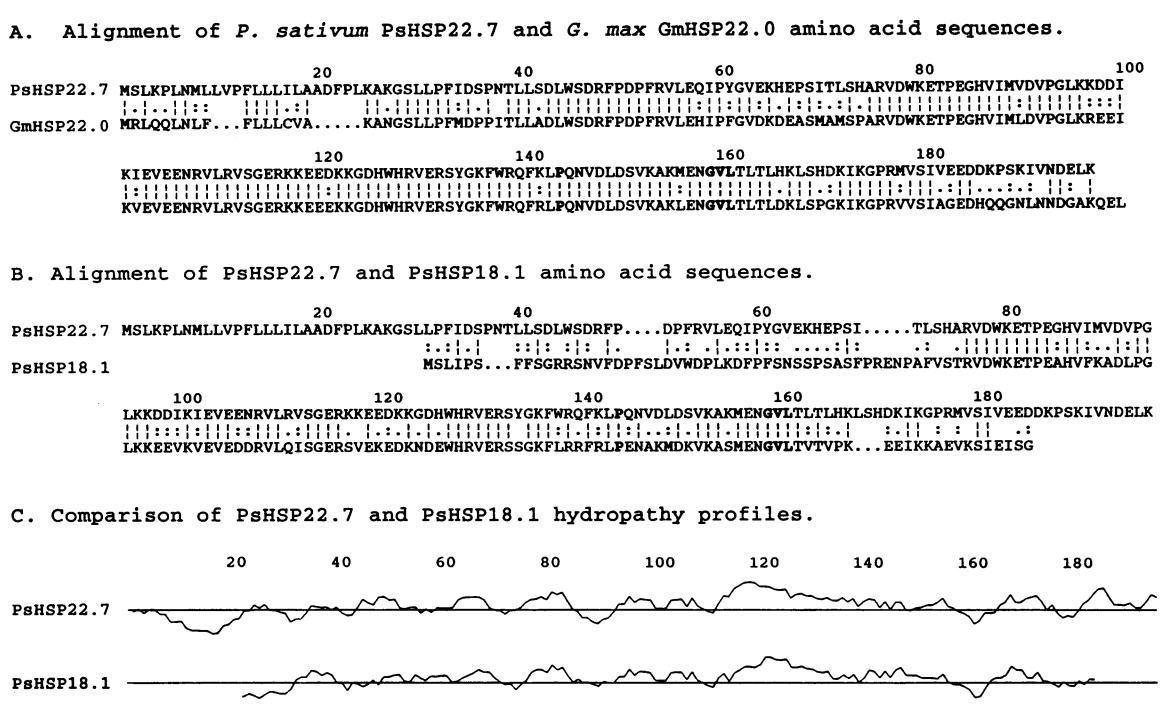

FIG. 1. (A and B) Comparisons of the primary structure of PsHSP22.7 with those of GmHSP22.0 and PsHSP18.1. Shown is optimal alignment of PsHSP22.7 with GmHSP22.0 (A) and PsHSP18.1 (B), using the Wisconsin Genetics Computer Group software tool GAP (17), with program parameters set at default values. Solid lines represent identical residues, colons show conservative replacements, and periods indicate weakly conserved replacements (17). Dots within a sequence indicate gaps inserted to optimize alignment. Pro-144 and Gly-159–Val-160–Leu-161 (boldface) are conserved in almost all eukaryotic LMW HSPs (26). (C) Hydropathy profiles of PsHSP22.7 and PsHSP18.1 as generated by the Genetic Computer Group program using the method of Kyte and Doolittle (23), with positive (hydrophilic) and negative (hydrophobic) values plotted above and below the center lines, respectively.

Expression of the PsHSP22.7-encoded protein. To study PsHSP22.7 in vivo, rabbit antiserum against a PsHSP22.7 fusion protein was produced. Anti-PsHSP22.7 reacted with two proteins of 20 and 21 kDa present in total protein extracts from heat-stressed pea seedlings but was not detectable in seedlings grown at 21°C (Fig. 3A). The 20- and 21-kDa anti-PsHSP22.7 immunoreactive proteins had pIs of approximately 6.8 and 7.0 (Fig. 3B). Southern analysis of pea genomic DNA showed that there are probably two closely related genes which hybridize to the PsHSP22.7 cDNA (not shown), suggesting that the antiserum raised against the PsHSP22.7 fusion protein reacts to all members of this LMW HSP family in pea. The antiserum did not detect proteins of the related PsHSP18.1 gene family, which migrate between 18 and 20 kDa and are more acidic (16).

Comigration of in vivo-synthesized PsHSP22.7 with the in vitro-processed protein was examined by two-dimensional gel electrophoresis. The processed in vitro transcription/translation product of the PsHSP22.7 cDNA comigrated with the 20-kDa, pI 7.0 polypeptide (Fig. 3C). The major unprocessed form of the in vitro-transcribed and -translated PsHSP22.7 migrated at about 24 kDa, with a pI of approximately 7.2 (Fig. 3D). These results are consistent with the hypothesis that PsHSP22.7 is processed in the ER in vivo.

On a total protein basis, the two PsHSP22.7 immunoreactive proteins are enriched approximately eightfold in the roots of 40°C heat-stressed seedlings compared with the levels observed in leaves of the same plants (Fig. 4). The relative level of PDI, a 65-kDa protein localized to the lumen of the ER (18), is similarly elevated in roots, suggesting that the abundance of PsHSP22.7 could be related to the abundance of its target organelle system. However, the level of PsHSP18.1, a cytoplasmic HSP, is also higher in roots, although not as high as the level of either PsHSP22.7 or PDI.

TABLE 1. Amino acid sequence comparison of PsHSP22.7 and GmHSP22.0 with other representative LMW HSPs

HSP	% Amino acid similarity (identity) with[a]:					
	Class I cytoplasmic		Class II cytoplasmic		Chloroplast localized	
	PsHSP18.1 (M3389)[b]	GmHSP17.5-E (M11395)	PsHSP17.7 (M33901)	GmHSP17.9-D (X07159)	PsHSP21 (X07187)	GmHSP21.0 (X07188)
PsHSP22.7	72.5 (48.3)	70.6 (47.1)	57.7 (35.3)	59.7 (32.7)	58.5 (31.1)	54.0 (29.9)
GmHSP22.0	71.8 (53.0)	69.9 (50.3)	63.7 (36.9)	62.3 (34.0)	60.6 (31.3)	58.8 (27.3)

[a] Determined with the Wisconsin Genetics Computer Group program GAP (17), using the default parameters for protein sequence alignments. Data are from reference 46.

[b] Accession numbers are given in parentheses.

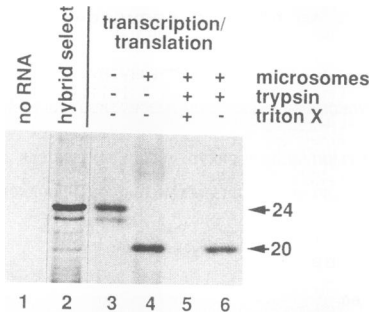

FIG. 2. In vitro transcription, translation, and cotranslational processing of PsHSP22.7. Lanes: 1, translation reaction with no added RNA; 2, translation of RNA hybrid selected with the PsHSP22.7 cDNA and poly(A)$^+$ RNA isolated from seedlings heat stressed for 4 h at 38°C; 3, in vitro transcription and translation of the PsHSP22.7 cDNA; 4, translation of PsHSP22.7 mRNA supplemented with canine pancreatic microsomes; 5 and 6, trypsin digestion of microsome-supplemented translation reactions of PsHSP22.7 transcripts in the presence (lane 5) and absence (lane 6) of 0.1% Triton X-100. Positions of molecular weight markers (in kilodaltons) are indicated at the right.

Thus, the elevated relative levels of these proteins in roots could also be a result of their dilution in leaves by the major leaf protein, ribulose bisphosphate carboxylase. The root-specific enrichment of these two HSPs is in contrast to chloroplast-localized PsHSP21, which is greatly enriched in leaves (46). We took advantage of this organ-specific enrichment in subsequent experiments addressing the subcellular localization and native structure of PsHSP22.7.

Endomembrane localization of PsHSP22.7 and GmHSP22.0 in vivo. Further evidence indicating that PsHSP22.7 and GmHSP22.0 are targeted to the endomembrane system by signal peptides was obtained by comparing the abundance of GmHSP22.0 mRNA associated with free and membrane-bound polysomes from heat-stressed soybean seedlings (Fig. 5). The majority of GmHSP22.0 polysomal mRNA was in the membrane-bound fraction, as expected. In contrast, mRNAs encoding members of the other three LMW HSP gene families from *G. max* listed in Table 1 were detected primarily in the unbound fraction (Fig. 5).

To demonstrate that PsHSP22.7 is endomembrane localized in vivo, microsomal preparations isolated from heat-stressed pea seedlings were treated with trypsin and assayed for the presence of the HSPs by Western immunoblot analysis. Relative levels of immunoreactive proteins were estimated by measuring ^{125}I bound to excised bands and normalizing the abundance of immunoreactive proteins from each fraction to the highest value measured on the same blot. Results for five replicates indicated that HSPs which reacted with anti-PsHSP22.7 were enriched an average of 2.4-fold in the microsomal fractions and were not detectable among cytosolic proteins (Fig. 6A, lanes 2 and 3). Both of the HSPs that reacted with anti-PsHSP22.7 were protected from trypsin digestion and became susceptible to protease digestion only in the presence of detergent (Fig. 6A, lanes 4 and 5). In contrast, PsHSP18.1 immunoreactive proteins were present in both cytosolic and microsomal fractions (Fig. 6B, lanes 2 and 3). The microsome-associated PsHSP18.1 immunoreactive proteins were susceptible to trypsin digestion, indicating that they were bound to the outside of the microsomes (Fig. 6B, lanes 4 and 5). The two immunoreactive forms of PsHSP22.7 are therefore membrane bound and reside in the endomembrane system.

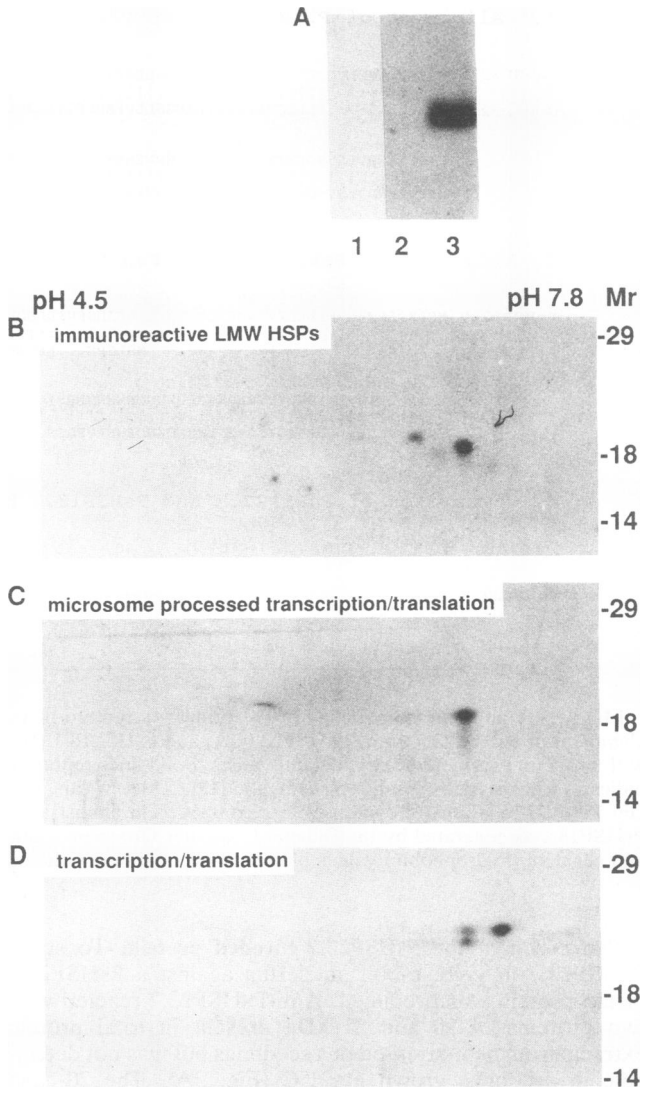

FIG. 3. Correspondence of in vitro-processed and in vivo-synthesized PsHSP22.7. (A) Specificity of anti-PsHSP22.7 antiserum. Lanes: 1, total protein (25 μg) from heat-stressed roots reacted with preimmune serum; 2 and 3, total protein (25 μg) from control (lane 2) and heat-stressed (lane 3) roots reacted with anti-PsHSP22.7. (B) Total proteins (150 μg) from heat-stressed pea roots reacted with anti-PsHSP22.7 antiserum. (C) Canine pancreatic microsome-processed PsHSP22.7 transcription/translation products. (D) Unprocessed PsHSP22.7 transcription/translation products. M_rs are indicated in thousands.

Localization of PsHSP22.7 to the ER. To localize PsHSP22.7 more precisely, microsomal preparations from heat-stressed pea seedlings were fractionated by centrifugation in isopycnic sucrose gradients in the presence or absence of $MgCl_2$. Gradient fractions were analyzed for anti-PsHSP22.7-reactive proteins and enzymes characteristic of ER, Golgi complexes, and plasma membranes. When endomembrane components are isolated in the presence of Mg^{2+}, ribosomes remain associated with the ER, causing the density of the ER to be greater than when isolations are performed without Mg^{2+} (11). The majority of PsHSP22.7 banded at a mean density of 1.14 g ml^{-1} in the absence of Mg^{2+} and at 1.19 g ml^{-1} when extraction solutions contained

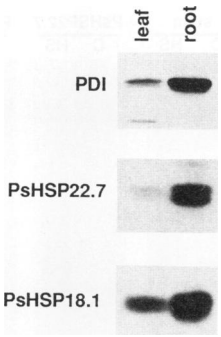

FIG. 4. Enrichment of PDI, PsHSP22.7, and PsHSP18.1 in roots of heat-stressed seedlings. Immunoblot analysis of proteins from 40°C heat-stressed pea seedlings was performed with anti-PDI from alfalfa, anti-PsHSP22.7, or anti-PsHSP18.1. Total proteins were extracted from leaves and roots immediately following heat stress, and 20 μg was subjected to SDS-PAGE and immunodetection by chemiluminescence.

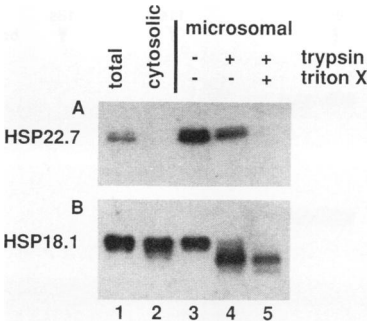

FIG. 6. Endomembrane localization of PsHSP22.7. Immunoblot analysis of proteins from 37°C heat-stressed roots was performed with anti-PsHSP22.7 (A) or anti-PsHSP18.1 (B). SDS-PAGE and immunoblot analysis were performed by using 15 (A) or 5 (B) μg of protein from each fraction. Treatments are as described in the text.

Mg^{2+} (Fig. 7). Maximum activity of the ER-localized enzyme CCR (6) corresponded with these values closely, suggesting that PsHSP22.7 is retained in the ER. The activity of IDPase, a Golgi complex-associated enzyme (15), was greatest at a density of 1.14 g ml^{-1} and was not affected by the presence of Mg^{2+}. Maximum activity of vanadate-sensitive ATPase, a plasma membrane marker (7), banded at 1.21 g ml^{-1} in both EDTA- and Mg^{2+}-supplemented membrane preparations. The sedimentation behaviors of these marker enzyme activities were identical in preparations from control and 37°C heat-stressed tissues (not shown). When microsomal preparations were lysed with detergent prior to fractionation, all detectable PsHSP22.7 and most marker enzyme activity was found at the top of the gradient (not shown), demonstrating that the density at which PsHSP22.7 banded was dependent on the presence of intact membranes. Thus, PsHSP22.7 is located in the endomembrane system and is probably a resident ER protein in heat-stressed plants.

To investigate the possibility that PsHSP22.7 is secreted during recovery from heat stress, pea seedlings were incubated at 22°C for 16 h following a 38°C heat stress before fractionation of endomembrane components. If PsHSP22.7 was secreted, either it would disappear from the endomembrane system or its location and distribution within the gradients would change. However, neither the location nor the distribution of PsHSP22.7 in the gradients changed after recovery from heat stress (not shown). The maximal density was increased from 1.14 to 1.19 g ml^{-1} as a result of extraction in the presence of $MgCl_2$, and this shift paralleled that of CCR activity. This result again suggests that PsHSP22.7 is a resident ER protein.

Native structure of PsHSP22.7. A distinguishing feature of eukaryotic LMW HSPs is their tendency to form oligomeric aggregates (31). Although the composition and structure of these aggregates are not well understood, it is believed that they represent functional forms of the LMW HSPs in vivo. It was therefore of interest to determine whether PsHSP22.7 is also found in high-molecular-weight aggregates in the ER. For comparison, the native structure of cytoplasmic PsHSP18.1 was examined simultaneously. Luminal ER proteins were released from total tissue homogenates either by treatment with nonionic detergent or by sonication. The homogenates were then subjected to sucrose gradient cen-

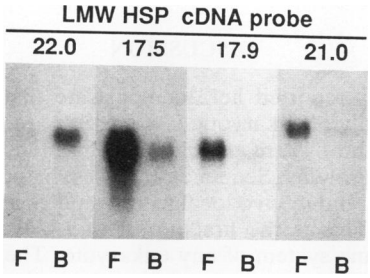

FIG. 5. Distribution of soybean LMW HSP mRNAs between free and membrane-bound polysomes. Northern blots of RNA from free (lanes F) and membrane-bound (lanes B) polysomes from heat-stressed soybean seedlings were probed for GmHSP22.0, GmHSP17.5-E (cytoplasmic class I), GmHSP17.9-D (cytoplasmic class II), and GmHSP21.0 (chloroplast localized) (46).

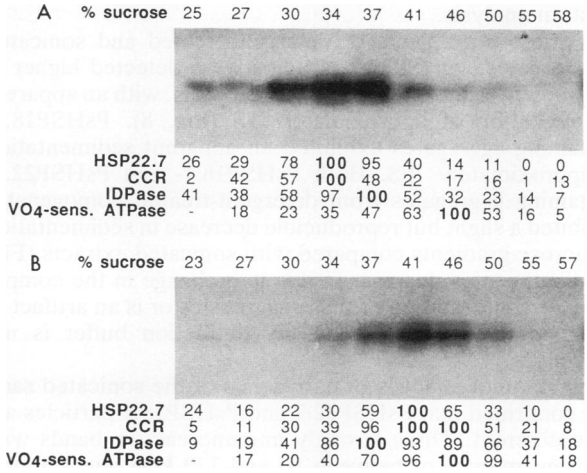

FIG. 7. Localization of PsHSP22.7 to the ER by subcellular fractionation. Microsomal fractions were separated on 20 to 60% isopycnic sucrose gradients, using solutions supplemented with 2 mM EDTA (A) or 1.5 mM $MgCl_2$ (B). Gradient fractions (1 ml of each) were assayed for PsHSP22.7 by SDS-PAGE and immunoblot analysis with anti-PsHSP22.7 antiserum. Sucrose concentrations are shown above each panel. The relative activity of PsHSP22.7 (as determined by counting ^{125}I on excised bands with use of liquid scintillation spectroscopy) and the relative activities of CCR, IDPase, and vanadate-sensitive ATPase are shown below each panel. The results shown are representative of three replications.

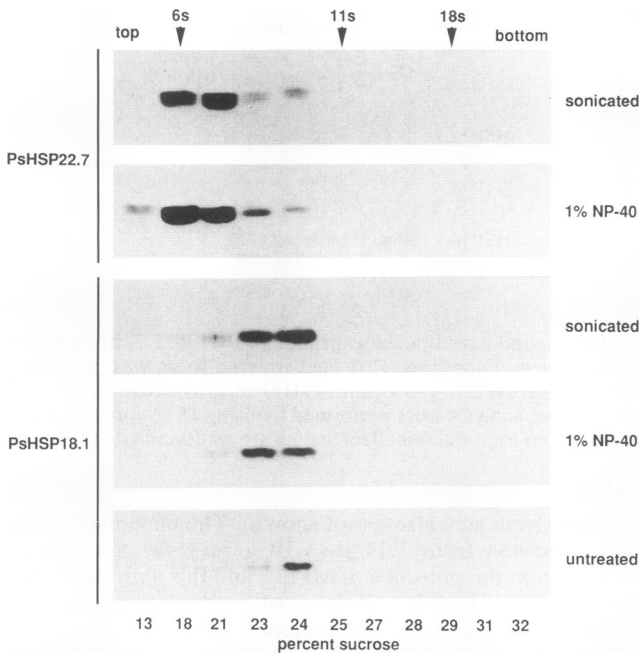

FIG. 8. Sucrose density gradient separation of PsHSP22.7- and PsHSP18.1-containing aggregates. For blots probed with anti-PsHSP22.7, 250 μl of each gradient fraction was precipitated with 0.1 M ammonium acetate in methanol, resuspended in 50 μl of 1× Laemmli (24) sample buffer, and separated by SDS-PAGE. For blots probed with anti-PsHSP18.1, 50 μl was loaded directly onto identical gels. Immunoreactive proteins were detected by chemiluminescence. Approximate sedimentation coefficients indicated at the top were determined previously (9). The sucrose concentration of each fraction, determined from parallel gradients, is given at the bottom. NP-40, Nonidet P-40.

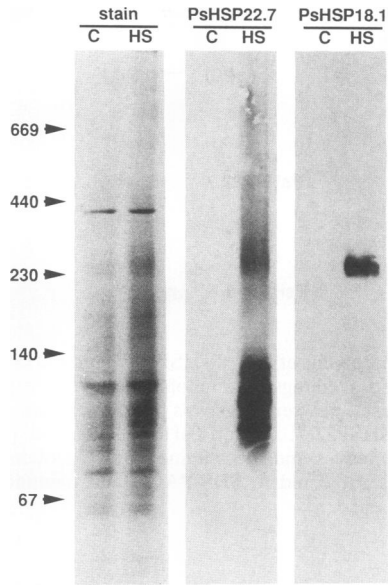

FIG. 9. Pore exclusion native gel analysis of PsHSP22.7- and PsHSP18.1-containing aggregates. Coomassie blue-stained lanes were loaded with 50 μl of sonicated extracts. Lanes probed with anti-PsHSP22.7 and anti-PsHSP18.1 were loaded with 25 and 10 μl, respectively, of the same extracts. Indicated molecular masses (in kilodaltons) represent, in ascending order, the migration of BSA, lactate dehydrogenase, catalase, ferritin, and thyroglobulin.

trifugation or pore exclusion native gel electrophoresis and Western analysis.

In sucrose gradients of detergent-treated and sonicated homogenates, PsHSP22.7 proteins were detected higher in the gradient than were PsHSP18.1 proteins, with an apparent sedimentation of approximately 7S (Fig. 8). PsHSP18.1-containing aggregates exhibited an apparent sedimentation of approximately 10S. Both PsHSP18.1- and PsHSP22.7-containing aggregates from detergent-treated homogenates exhibited a slight but reproducible decrease in sedimentation in sucrose gradients compared with sonicated extracts (Fig. 8). Whether this decrease is due to a change in the composition or conformation of these aggregates or is an artifact of having detergent present in the extraction buffer is not known.

Immunoblot analysis of native gels of the sonicated samples confirmed that PsHSP22.7 and PsHSP18.1 particles are quite different. Three strongly immunoreactive bands with apparent masses of between 80 and 120 kDa and a fourth band with an apparent migration of 240 kDa were detected with the anti-PsHSP22.7 antiserum (Fig. 9). Immunoblot analysis of native gels with the anti-PsHSP18.1 antiserum revealed only a single band migrating at an apparent mass of 240 kDa (Fig. 9).

It is not likely that the observed differences between PsHSP22.7- and PsHSP18.1-containing particles are a result of selective degradation of the PsHSP22.7 particles during extraction. Lysis of microsomal vesicles by two independent techniques yielded particles with almost identical sedimentation on sucrose gradients. It is also unlikely that these lysis techniques, which were required to release PsHSP22.7 from the microsomes, disrupted the PsHSP22.7 particles. The sedimentation behavior of the cytoplasmic PsHSP18.1 particles from sonicated and detergent-treated preparations was nearly identical to that of the untreated extracts. Immunoblots of native gels comparing untreated and sonicated extracts verified that sonication had no effect on the size of PsHSP18.1-containing particles (not shown). Thus, the PsHSP22.7-containing particles are probably not degraded or dissociated by the extraction process. With the possible exception of PsHSP22.7 from detergent-treated extracts (Fig. 8), free LMW HSPs were not detected either in sucrose gradients or on the immunoblots of native gels. Further, less than 20% of the immunoreactive proteins were detected in the pellets of these extracts (not shown). Taken together, these results show that the majority of the ER-localized and cytoplasmic class I LMW HSPs in these tissues are in 80- to 240-kDa aggregates.

DISCUSSION

The results reported here demonstrate that members of the superfamily of eukaryotic LMW HSPs are present in the endomembrane system of higher plants and are probably resident ER proteins. Sequence analyses, in vitro processing experiments, and in vivo localization studies all support this conclusion. This is the first report of LMW HSPs in the endomembrane system of any eukaryote. Their amino acid sequence similarity to each other and to other plant LMW HSPs demonstrates that PsHSP22.7 and GmHSP22.0 are members of a fourth LMW HSP gene family. Observation of LMW HSPs of similar mass cofractionating with endomembrane components from monocots such as maize (14) and barley (43) suggests that this gene family is represented

throughout the plant kingdom. Given the conservation of the heat shock response, it is possible that similar LMW HSPs exist in the ER of other eukaryotes but have gone unobserved. Alternatively, ER-localized LMW HSPs may have evolved after the divergence of plants from other eukaryotes. Representatives from three of the four major families of HSPs have now been found in the endomembrane system: an HSP70-related protein called BiP (28), an HSP90-related protein (26), and these LMW HSPs.

The carboxyl-terminal sequences of PsHSP22.7 and GmHSP22.0, NDELK and KQEL, respectively, are good candidates for ER retention signals. Pelham (37) has demonstrated that KDELK is a functional ER retention signal in COS cells and suggests that the carboxyl-terminal lysine is enzymatically removed by carboxypeptidases specific for carboxyl-terminal basic residues, leaving the characteristic KDEL- retention signal. The C terminus of PsHSP22.7, NDELK, could be similarly processed to NDEL. Although NDEL and KQEL have not been reported to function as such, they resemble known carboxyl-terminal ER retention signals such as KDEL, HDEL (36), and KNEL (3). These sequences, in combination with the localization data, strongly suggest that PsHSP22.7 and GmHSP22.0 are resident ER proteins.

A common feature of eukaryotic LMW HSPs is their tendency to assemble into higher-molecular-weight structures. There appear to be two general types of these structures: a class of very large aggregates called heat shock granules (HSGs) with masses of 1 MDa or more and a 200- to 700-kDa form termed pre-HSGs by Nover et al. (33). HSGs are proposed to form by assembly of pre-HSGs under certain stress conditions (34). The exact composition of these structures and the role that they play in LMW HSP function are not known. Our results show that, like other eukaryotic LMW HSPs, both PsHSP22.7 and PsHSP18.1 are present in higher-molecular-weight structures (7S to 10S, 80 to 240 kDa) in heat-stressed pea seedlings. The relatively low masses of these particles classify them as pre-HSGs. Even as pre-HSGs, the particles observed here are smaller than the 15S to 16S reported for tomato, *Drosophila melanogaster*, and vertebrates (33). Pre-HSGs of 180 and 200 kDa have been observed in chicken fibroblasts (12) and pea chloroplasts (9), respectively, however. PsHSP22.7 and PsHSP18.1 were not detectable as free monomers, suggesting that some assembly into higher-order structures is important for function. Further, under the conditions used in this study, relatively low levels of these proteins were detected in the pellets of the native extracts, indicating that the large HSGs either are present at very low levels or are absent in these tissues. Studies in which HSGs have been described typically used tissues which had been abruptly stressed at potentially nonphysiological high temperatures (4, 12, 34, 40). In contrast, the heat stresses used in our experiments involved moderate maximum temperatures (38°C) reached over a period of 4 h, more closely mimicking what a plant might experience in nature. It is therefore possible that heat stress conditions required for formation of HSGs are more severe than those used here. It is also possible that the formation of HSGs is limited to tissues other than the root tissues used in these studies. Previous results showed that in plants, HSGs are abundant in cell suspension cultures and meristematic cells, such as those found in root tips (33).

The large differences observed in apparent native molecular size between PsHSP22.7 and PsHSP18.1 were unexpected. The remarkable sequence and hydropathic similarities between the two classes of proteins suggested that their native structures might also be similar. Instead, PsHSP22.7 was associated with smaller particles than was PsHSP18.1. Analysis of PsHSP22.7 on native gels revealed a complex group of bands with apparent masses of between 80 and 120 kDa and a minor band migrating at 240 kDa. In contrast, the size of the PsHSP18.1 particles was more consistent with the size of LMW HSP particles described previously (33); it was found to migrate exclusively as one band of 240 kDa. There are several possible explanations for the observed size differences between the cytoplasmic and ER LMW HSP particles. It could be that the ionic environments in the ER and cytoplasm are sufficiently different that the ER-localized LMW HSPs are prevented from forming aggregates as large as those found in the cytoplasm. Alternatively, it is possible that the aggregates found in the ER and cytoplasm are stoichiometrically equivalent with respect to LMW HSPs but are bound to substrates of different mass or number, leading to altered sedimentation and migration characteristics. Whether these findings indicate that the function of LMW HSPs in the ER differs from that of the class I cytoplasmic LMW HSPs cannot be ascertained until more is known about the composition of the particles in both compartments.

The functions of LMW HSPs are unknown. Their conservation and presence in three plant cell compartments (cytoplasm, chloroplast, and endomembrane system) argue that they serve an important role in survival of high-temperature stress. Because the amino acid sequence and hydropathy profile of PsHSP22.7 closely resemble those of the class I cytoplasmic LMW HSPs, we suggest that the two families of LMW HSPs have analogous functions in their respective compartments. Nover et al. (34) suggested that the LMW HSPs might sequester and protect mRNA during heat stress. The lack of RNA in the ER rules out this function for the ER-localized LMW HSPs. The two primary functions of the ER are the export of endomembrane and secretory proteins and the synthesis of lipids, suggesting that ER-localized LMW HSPs are involved in the protection or repair of these processes in response to heat-induced damage. It could be that ER-localized LMW HSPs play a role as molecular chaperones, ensuring the proper folding or oligomerization of other ER proteins (reviewed in reference 20). Such a function would be distinct from that of BiP, as the LMW HSPs appear to be present exclusively in heat-stressed tissues whereas BiP is a normal (though stress-inducible) component of the ER that is required for survival at normal growth temperatures (32, 39). Alternatively, ER-localized LMW HSPs might be important in stabilizing ER membranes themselves. LMW HSPs have been observed in association with membranes in plant cell homogenates (25), but the nature of this association is not understood.

The development of detailed hypotheses of ER-localized LMW HSP function awaits a better understanding of the effects of heat stress on the ER. Since the ER-localized LMW HSPs are physically separated from their counterparts in other cell compartments, investigation of the ER-localized HSPs provides a simplified model system for determining the functions of LMW HSPs in eukaryotes.

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