The Major Low-Molecular-Weight Heat Shock Protein in Chloroplasts Shows Antigenic Conservation among Diverse Higher Plant Species

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Several plant species are known to synthesize low-molecular-weight nucleus-encoded heat shock proteins (HSPs) which localize to chloroplasts. DNA sequence analysis of chloroplast HSP cDNAs from pea (*Pisum sativum*) and soybean (*Glycine max*) has shown that the carboxyl-terminal halves of these proteins are homologous to low-molecular-weight HSPs from a wide range of eucaryotes (E. Vierling, R. T. Nagao, A. E. DeRocher, and L. M. Harris, EMBO J. 7:575–581, 1988). We used a pea cDNA to construct fusion proteins containing either the carboxyl-terminal heat shock domain or the amino-terminal domain of the chloroplast HSP. The fusion proteins were overexpressed in *Escherichia coli* and used to produce chloroplast HSP-specific polyclonal antibodies. The carboxyl-terminal antibodies recognized chloroplast HSP precursor proteins from pea and from three divergent plant species, *Arabidopsis thaliana*, petunia (*Petunia hybrida*), and maize (*Zea mays*). The amino-terminal antibodies recognized effectively only the pea precursor. When intact plants of each species were subjected to a heat stress regime mimicking field growth conditions, significant levels of the mature forms of the chloroplast HSPs accumulated in pea, *A. thaliana*, and maize. The levels of accumulated HSPs remained unchanged for 12 h following the stress treatment. We conclude that the synthesis of chloroplast-localized HSPs is an important component of the stress response in all higher plants and that chloroplast HSPs from dicotyledonous and monocotyledonous plants have a conserved carboxyl-terminal domain.

The cellular response to high-temperature stress, or heat shock (HS), has been conserved through the evolution of procaryotic and eucaryotic organisms (3, 10). HS treatment causes major changes in gene expression, with a primary effect being new or increased transcription of specific genes encoding several heat shock proteins (HSPs). HSPs appear to be important for survival during or following stress and are also involved in basic cellular processes which occur in the absence of stress.

We and others have shown that, in addition to HSPs typical of eucaryotes, higher plants and algae also synthesize nucleus-encoded HSPs which localize to chloroplasts (7, 17, 19–21). Major chloroplast HSPs have been identified in soybean (*Glycine max*) (20), pea (*Pisum sativum*) (7, 20), maize (*Zea mays*) (20), bean (*Phaseolus vulgaris*) (17), petunia (*Petunia hybrida*) (19), and *Chlamydomonas* spp. (7). They range in size from 21 to 24 kilodaltons (kDa) in the different species. For pea chloroplast HSP21 and soybean chloroplast HSP22, cDNAs have been isolated and shown to be 79% identical in the protein-coding sequence (21). The homology of the pea and soybean chloroplast HSPs to low-molecular-weight (low-MW) chloroplast HSPs of nonleguminous plants has not been investigated. As is typical of nucleus-encoded chloroplast proteins (15), the pea and soybean chloroplast HSPs are synthesized as precursor proteins which are processed by the removal of 5 to 6.5 kDa during import into isolated chloroplasts (21). Pea chloroplast HSP21 mRNA is undetectable in control leaf tissues but increases to 0.75% of the total poly(A)⁺ RNA during 2 h of HS treatment at 39°C (21). Induction of chloroplast HSP mRNA is therefore the most dramatic example of environmental regulation of a chloroplast protein gene known to date.

Analysis of the derived amino acid sequence of soybean

HSP22 and pea HSP21 has shown that the carboxyl-terminal halves of the mature proteins are homologous to the low-MW cytoplasmic HSPs of plants and other organisms and also to the alpha lens crystallins (21). Thus, these chloroplast HSPs belong to one of the major classes of HSPs in eucaryotes. We have proposed that these proteins participate in functions similar to those performed by the low-MW cytoplasmic HSPs to which they are homologous (19, 21). Unfortunately, the function of these low-MW HSPs is still unknown.

As organisms which are unable to avoid temperature fluctuations in the environment, plants may frequently experience conditions which induce HSP expression. Very little data are available concerning HSP expression in plants growing under natural conditions, and measurements of HSP accumulation or stability have been limited, owing to a lack of specific antibodies for positively identifying the HSPs (2, 6, 14). HSP mRNAs are known to be expressed in soybeans under high-temperature conditions in the field (6). Proteins which appear to comigrate with HSPs on one-dimensional electrophoresis have been observed to accumulate in field-grown, heat-stressed cotton (2). Laboratory studies of etiolated soybean seedlings have indicated that low-MW HSPs accumulate to high levels and are stable for several hours following stress (9, 12, 14). The accumulation of chloroplast HSPs has not been studied.

The facts that chloroplast HSPs are found in diverse plant species and that chloroplast HSP mRNA is abundant during stress suggest that these proteins are a major component of the HS response in plants. To investigate further the relationship between chloroplast HSPs of various plant species and to examine chloroplast HSP accumulation and stability during stress, we have prepared monospecific polyclonal antibodies against pea chloroplast HSP21. We report here the production and characterization of these antibodies and

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FIG. 1. Pea HSP21 cDNA segments used for fusion protein construction. The pea HSP21 cDNA with relevant restriction enzyme sites is shown at the top of the diagram. The HSP21-coding sequence is indicated below the corresponding portion of the cDNA. The solid line represents the mature protein, and the dotted line represents the transit peptide segment. The amino- and carboxyl-terminal regions used for construction of the fusion proteins are shown as described in the text. The sizes of the DNA fragments in base pairs (bp) are indicated by the bar at the bottom of the diagram. aa, Amino acids.

their use in demonstrating antigenic similarities among major low-MW HSPs of peas, *Arabidopsis thaliana*, petunia, and maize. We have also used the antibodies to monitor HSP accumulation during an HS regimen that mimics high-temperature stress encountered under field growth conditions. We demonstrate that the mature forms of the chloroplast HSPs in pea, *A. thaliana*, and maize accumulate dramatically and are stable for at least 12 h following the stress.

MATERIALS AND METHODS

Fusion protein constructs. The pATH series of expression plasmids (16) was utilized for the production of fusion proteins in *Escherichia coli*. In these plasmids the desired protein-coding region is ligated in-frame with the *E. coli* TrpE protein via a multicloning site inserted at the codon corresponding to Ile-321 of TrpE.

A diagram of the pea HSP21 cDNA and segments of the coding region used for construction of the fusion protein plasmids is shown in Fig. 1. The amino-terminal construct included 53 amino acids from Gln-50 to Met-102 contained in a 162-base-pair *AluI*-*SphI* fragment of the cDNA. This fragment contains only that portion of HSP21 which is not homologous to other low-MW HSPs (21). The first 46 to 47 amino acids of HSP21 were not included in the amino-terminal construct because they are predicted to encode the transit peptide which is removed from the protein during import into chloroplasts. The final fusion protein included approximately 35,670 daltons (Da) of TrpE, the 5,772-Da HSP21 fragment, and an estimated 1,540 Da following the insertion, for a total predicted molecular mass of 42,982 Da.

The carboxyl-terminal construct was made by inserting into the pATH vector a fragment of the pea HSP21 cDNA from the *SphI* site (at Met-102) to the *EcoRI* linker at the 3' end of the cDNA (3' to the termination codon of HSP21) (Fig. 1). This fragment encodes 131 amino acids from Met-102 through the terminal Gln-232 (15,374 Da) and includes the entire segment of HSP21 which has been shown to be homologous to other low-MW eucaryotic HSPs (21). The total predicted molecular weight (MW) of the carboxyl-terminal fusion protein is 51,046.

Both plasmid constructs were transformed into *E. coli* JM83, and the plasmid structure was verified by restriction enzyme digestion.

Antigen production and purification. Overproduction of the fusion proteins was achieved by a modification of the method described by Spindler et al. (16). *E. coli* JM83 strains carrying the recombinant plasmids were grown overnight in M9 medium plus 1% Casamino Acids, 20 μ g of tryptophan ml^{-1} , and 100 μ g of ampicillin ml^{-1} . The overnight cultures were diluted 1:10 in M9 plus Casamino Acids plus ampicillin and grown at 30°C with a high degree of aeration (250-ml culture in a 2-liter flask). After 1.5 h, 5 μ g of indoleacrylic acid ml^{-1} (1 mg ml^{-1} stock in 100% ethanol) was added, and cultures were grown for an additional 3.5 h at 30°C. Fusion protein levels were assessed in whole bacterial cell extracts by sodium dodecyl sulfate (SDS) gel electrophoresis. The amino-terminal fusion polypeptide migrated at 44.5 kDa, close to the predicted molecular size (42,982) (data not shown). In contrast, the carboxyl-terminal fusion polypeptide migrated anomalously at 46 kDa, 5 kDa below the predicted size (data not shown).

For purification of the fusion proteins, an insoluble bacterial protein fraction was prepared essentially as described by Goedel et al. (4). Cells from 1 liter of culture were pelleted, washed once with 25 ml of 10 mM Tris hydrochloride (pH 7.5), and suspended in 10 ml of 0.2 M Tris hydrochloride (pH 7.6) (at 4°C)-0.2 M KCl-0.01 M magnesium acetate-0.3 mM dithiothreitol-5% glycerol. The cells were lysed by passage through a French press at 4,000 lb/in². The insoluble fraction containing the fusion protein was pelleted at 27,000 $\times g$ for 30 min, and the supernatant was discarded. The pellet was suspended in 60 mM Tris hydrochloride (pH 8.0)-60 mM dithiothreitol-2% lithium dodecyl sulfate-5.0 mM ϵ -aminocaproic acid-1.0 mM benzamide-15% sucrose (gel sample buffer), using 10 ml liter⁻¹ of the original culture. The sample was incubated at 60°C for 15 min, and insoluble material was removed by centrifugation at 12,000 $\times g$ for 5 min.

The fusion proteins were further purified by electrophoresis on preparative SDS-polyacrylamide (10%) gels with the buffer system of Laemmli (8). The fusion proteins were visualized by soaking gels in 4.0 M sodium acetate for 30 min at room temperature. The fusion proteins, which produced the major protein band, were excised and electroeluted into 10 mM Tris-acetate (pH 8.6)-0.1% SDS with an ISCO electroeluter. The isolated fusion proteins were estimated to be 90% pure by analytical SDS gel electrophoresis and used as antigens without further purification. Approximately 3.0 mg of purified fusion protein was recovered per liter of starting culture.

Antibody production. Antibodies were generated in New Zealand White female rabbits. Animals were given a primary injection of 200 to 400 μ g of fusion protein emulsified in Freund complete adjuvant, followed at monthly intervals with booster injections of 50 to 100 μ g of fusion protein in Freund incomplete adjuvant. Animals were bled from the ear 7 to 9 days following booster injections. Cellular material was removed from the serum by centrifugation (12,000 $\times g$ for 10 min), and the whole serum fraction was used for all immunological experiments. Serum obtained after the second booster injection showed a strong reaction against the antigen at a 1:500 dilution in Western blot (immunoblot) analysis.

Plant material and growth conditions. Peas (*P. sativum* cv. 'Little Marvel'), petunias (*P. hybrida* cv. 'Mitchell diploid'), *A. thaliana* (Columbia wild type), and maize (*Z. mays*,

inbred line B73; Pioneer Hybrid International, Inc.) were grown from seeds in lighted growth chambers (350 microeinsteins $m^{-2} s^{-1}$) with a 16-h day. The temperature cycle was 22°C for day and 18°C for night. Plants were grown as follows prior to the experiments: peas for 8 to 10 days, maize for 14 days, petunias for 19 days, and *A. thaliana* for 16 days.

RNA isolation and in vitro translation. Total RNA was isolated from leaf tissues of all plant species, and the poly(A)⁺ RNA fraction was prepared as previously described (20). HS treatments were performed by shaking whole seedlings in water-filled beakers in controlled temperature baths under room light. The following treatment temperatures and times were identified for the maximal production of HSP mRNAs: peas, 39°C for 2 h; petunias, 39°C for 2 h; *A. thaliana*, 37°C for 90 min; and maize, 38°C for 90 min. Plants used for preparing control RNA samples were incubated, with shaking, in water at 23°C for the same time periods as those used in the HS treatments.

In vitro translation of poly(A)⁺ RNA (0.1 μ g of RNA 10 μ l of translation mixture⁻¹) was performed with reticulocyte lysates as described by the supplier (Bethesda Research Laboratories, Inc.) and with [³⁵S]methionine (>1,000 Ci mmol⁻¹; 40.0 TBq mmol⁻¹; New England Nuclear Corp.) at 0.75 μ Ci μ l of translation mixture⁻¹. Incorporation into hot trichloroacetic acid-precipitable radioactivity was determined by a filter paper disk assay (11). Either translation mixtures were used directly for immunoprecipitation or aliquots were dissociated in gel sample buffer (see above) by being boiled for 1 min.

Immunoprecipitation. Immunoprecipitation from reticulocyte in vitro translation reactions was performed as described by Anderson and Blobel (1). Briefly, lysates were brought to 4% SDS, boiled for 1 min, diluted with an equal volume of water, and subsequently diluted with 4 volumes of 2.5% Triton X-100–190 mM NaCl–6 mM EDTA–50 mM Tris hydrochloride (pH 7.5)–1.0 mM aminocaproic acid–5 mM benzamidine. For each sample, 10 μ l of translation mixture was processed and incubated overnight with 1.5 μ l of preimmune or immune serum. Antigen-antibody complexes were recovered with 20 μ l of Staph A cells (Sigma Chemical Co.), washed as described previously (1), and dissociated by being boiled for 1 min in 50 μ l of gel sample buffer. Samples were analyzed on SDS-polyacrylamide (10 to 16%) gradient gels in the buffer system of Laemmli (8). Gels were processed for fluorography.

Leaf protein samples. Samples of total leaf proteins were prepared from leaves of all plant species by grinding tissues directly in gel sample buffer in a Kontes ground-glass homogenizer. Samples were boiled for 3 min, and insoluble debris was removed by centrifugation in a Microfuge (12,000 $\times$ g) for 5 min. Protein content was determined with a protein assay kit (Bio-Rad Laboratories).

Immunoblotting. Proteins (approximately 100 μ g lane⁻¹) were separated on 12.5% polyacrylamide–SDS gels and blotted to nitrocellulose (18). After transfer, blots were stained with 0.2% Ponceau S (Sigma) in H₂O to check transfer and to locate MW standards. Nonspecific protein-binding sites were blocked by incubation in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.15 M NaCl–0.1% Triton X-100 (low-salt buffer) with 5% nonfat dry milk for 1 h. Blots were incubated with whole serum at a 1:500 dilution in low-salt buffer with 1% milk (approximately 1.0 ml of solution 5.0 cm² of nitrocellulose⁻¹). Blots were washed three times with low-salt buffer plus 5% milk and incubated for 1 h in low-salt buffer plus 1% milk and containing 1.0 μ Ci

of [¹²⁵I]protein A (46.6 μ Ci μ g⁻¹; ICN Radiochemicals) for each 150 cm² of nitrocellulose. Blots were washed three times with 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–1.0 M NaCl–0.1% Triton X-100, dried, and exposed to X-ray film with intensifying screens.

In vitro import of proteins into isolated chloroplasts. Import of in vitro translation products into isolated, intact pea chloroplasts was performed as described previously (20). After import, chloroplasts were protease treated and intact chloroplasts were reisolated (20).

RESULTS

Detection of chloroplast HSP precursor proteins in peas and other plant species. We characterized the specificity of the pea HSP21 antibodies and used them to determine if other plant species synthesize HSPs which are antigenically related and thus would be predicted to localize to chloroplasts. For these experiments we chose species representative of two families of dicotyledonous plants, the Brassicaceae (*A. thaliana*) and Solanaceae (petunia), and one species of monocotyledonous plant (maize). Poly(A)⁺ RNA from heat-shocked leaves of each species was translated in vitro, and aliquots were immunoprecipitated with antiserum specific for either the amino-terminal or the carboxyl-terminal fusion proteins. Aliquots of the in vitro translation mixtures were also used for precipitation with preimmune sera. Two polypeptides were precipitated from the pea translation products with either antiserum: a minor species of 27.5 kDa and a major species of 26 kDa (Fig. 2A). These polypeptides correspond in molecular mass to the previously characterized precursor polypeptides of the mature 21-kDa pea chloroplast HSP (21).

In the other species tested, proteins with molecular masses similar to those of pea HSPs were immunoprecipitated by the carboxyl-terminal antiserum (Fig. 2). The major protein immunoprecipitated from the *A. thaliana* translation mixtures was approximately 25.5 kDa, with a minor band at 18.0 kDa (Fig. 2B). In petunias (Fig. 2C), the major precipitated protein was approximately 30 kDa. This polypeptide corresponds in size to the precursor protein previously identified by hybridization-selection experiments with a petunia cDNA homologous to pea HSP21 (19; Q. Chen, unpublished data). Minor polypeptides at 24 and 18 kDa were also present in the petunia immunoprecipitates. In maize (Fig. 2D), two major polypeptides of 29 and 27.5 kDa were precipitated. We suggest that both of these polypeptides are precursors to the mature maize chloroplast HSP, as appears to be the case for the two forms of the pea precursor (21). Interestingly, the 27.5-kDa polypeptide from maize comigrated with one of the major HSPs identified by comparison of the translation products of control and HS poly(A)⁺ RNA.

Although the carboxyl-terminal antiserum produced strong reactions in all species, the amino-terminal antiserum reacted strongly only with the pea HSPs (Fig. 2). In *A. thaliana* and petunia, polypeptides corresponding to the major polypeptides precipitated with the carboxyl-terminal antiserum were present as minor bands in the immunoprecipitates when amino-terminal antiserum was used. In maize, there was no detectable reaction with the amino-terminal antiserum.

In all species, the immunoprecipitated proteins appeared to comigrate with polypeptides present in the translation products of HS but not control poly(A)⁺ RNA. However, to confirm that the mRNAs for these proteins were absent from

FIG. 2. Immunoprecipitation of chloroplast HSPs. Lanes 1 and 2 contain total translation products of control or HS RNA, respectively. Lanes 3 contain the immunoprecipitate (I) obtained with the carboxyl-terminal antiserum (C), and lanes 4 contain the corresponding preimmune sample (PI). Lanes 5 contain the immunoprecipitate obtained with the amino-terminal antiserum (A), and lanes 6 contain the corresponding preimmune sample. All immunoprecipitations were performed with the translation products of HS poly(A)⁺ RNA as described in the text. (A) Peas. (B) *A. thaliana*. (C) Petunias. (D) Maize. Positions of MW markers (in thousands) are indicated to the right.

control samples, translation products of control poly(A)⁺ RNA were also immunoprecipitated with carboxyl-terminal antiserum or preimmune serum (Fig. 3). No polypeptides were precipitated from control samples in any of the species. These data substantiate the interpretation from Fig. 2 that the immunoprecipitated proteins represent products of mRNAs which accumulate during HS treatment.

Accumulation of chloroplast HSPs. We were interested in determining the MWs of the mature chloroplast HSPs in these species and in investigating whether these HSPs are present at detectable levels in leaves prior to heat treatment and to what level they accumulate when intact plants are subjected to heat stress. For these studies we developed an HS regimen to simulate a day of temperature stress under field growth conditions. Plants of each species were placed in a growth chamber maintained at maximum humidity with a vaporizer. The growth chamber temperature was programmed to increase linearly to 38°C, remain at 38°C for 4 h, and then return to 22°C (Fig. 4). The high humidity was used to limit transpirational cooling of the leaves. Measurements of pea leaf temperatures with an infrared thermometer (Everest Interscience) showed that they followed air temperatures under these conditions (data not shown). Measurements of the leaf temperatures of the other plant species were not taken. Pea, *A. thaliana*, and petunia plants subjected to this type of stress regimen showed no visible damage during the stress, nor did signs of damage appear during a subsequent week of growth and observation. Maize plants developed some bleached and necrotic areas along the leaf margins, particularly in leaves that were undergoing rapid expansion during the stress period.

Samples of total leaf proteins were prepared at four times during the treatment: before the stress, when the temperature reached 38°C, after 4 h at 38°C, and immediately after the temperature had returned to 22°C. Proteins (100-μg samples) were separated by SDS gel electrophoresis and analyzed by Western blotting. Analysis at all times indicated that samples taken immediately after the temperature had returned to 22°C (and were thus exposed to high-temperature stress for the longest time) contained maximal levels of the HSPs (data not shown).

Leaf protein samples prepared prior to stress were compared with samples taken at the time of maximal stress (Fig. 5). When the carboxyl-terminal antiserum was used, a major low-MW polypeptide was labeled in the pea, *A. thaliana*, and maize HS samples but not in the control samples (Fig. 5A). These low-MW polypeptides were the only proteins which showed no reaction with preimmune serum (Fig. 5B) and were present only in the HS samples. No polypeptides which specifically reacted with the antiserum were present in the petunia samples.

The polypeptide in the pea HS sample migrated at 21 kDa, in agreement with the predicted molecular mass of the mature chloroplast HSP determined previously from both sequence analysis of the cDNA clone and studies of *in vitro* import into isolated chloroplasts (21). There was no detectable 21-kDa protein in the control leaf samples.

In *A. thaliana* the small HSP was also approximately 21 kDa. In comparison with the molecular mass of the primary translation product from *A. thaliana* (25.5 kDa), 21 kDa is a reasonable size for a mature protein from which the transit

FIG. 3. Immunoprecipitation of translation products of control poly(A)⁺ RNA. Lanes 1, 4, 7, and 10 contain total in vitro translation products of poly(A)⁺ RNA isolated from control leaves. Lanes 2, 5, 8, and 11 contain immunoprecipitates (I) prepared from these translation mixtures with the carboxyl-terminal antiserum. Lanes 3, 6, 9, and 12 contain the corresponding preimmune samples (PI). (A) Peas. (B) *A. thaliana*. (C) Petunias. (D) Maize.

peptide has been removed during import into chloroplasts (15).

The maize HSP was approximately 24 kDa. This size is in agreement with previous data from chloroplast import experiments (21). The differences in molecular mass between the mature 24-kDa HSP and the primary translation products at 29 and 27.5 kDa are also within the typical range for differences between precursor and mature forms of chloroplast proteins. We suggest that both precursor forms are processed to the same mature, 24-kDa form, as appears to be the case for the two precursor forms of HSP21 in peas (21).

The amino-terminal antibody was used to detect antigens on identical blots (Fig. 5C). This antibody detected pea HSP21 and *A. thaliana* HSP21 but did not recognize the maize or petunia proteins. The inability to detect maize HSP24 with this antibody is in agreement with the results of the immunoprecipitation experiments. The apparent difference in the reactivity of the amino- and carboxyl-terminal antisera seen in the immunoprecipitation experiments with *A. thaliana* (Fig. 2) was not found in the Western blot analysis. We suggest that the amino-terminal antibody preferentially recognized the more denatured protein on Western blots and not the partially native protein in immunoprecipitates because the antibodies were raised against a denatured, SDS-solubilized protein.

It is clear from the Western blot analysis that these HSPs accumulated significantly during heat stress. When gels identical to those used for Western blot analysis were stained with Coomassie blue, no HS-induced change in low-MW polypeptides was detectable (Fig. 5D). No changes in the total protein profile were observed at all at this level of resolution. The lack of significant changes in the total protein profile determined by one-dimensional gel electrophoresis

FIG. 4. Temperature regimen used to subject intact plants to heat stress. The growth chamber temperature was programmed as shown.

analysis is in agreement with previous data for heat-shocked soybean seedlings (9, 14).

The fact that polypeptides identical in size to the HSPs identified by immunoprecipitation in the in vitro translation mixtures were not detected in vivo by Western blot analysis suggests that the proteins underwent proteolytic processing. Such processing is typical of chloroplast-localized proteins synthesized in the cytoplasm (15) and provides further evidence that the pea HSP antibodies reacted with the corresponding chloroplast-localized HSP from the other plant species and not with a highly related cytoplasm-localized HSP.

Chloroplast localization of the 21-kDa HSP in *A. thaliana*.

As indicated above, previous experiments had shown that when total in vitro translation products of HS poly(A)⁺ RNA from maize are incubated with isolated pea chloroplasts, a 24-kDa protein is recovered in the soluble chloroplast protein fraction (20). These data support the conclusion that the 24-kDa polypeptide identified here on Western blots of total maize protein is localized to chloroplasts. To obtain evidence that the 21-kDa protein in *A. thaliana* also is localized to chloroplasts, we performed similar experiments. In vitro translation products of control or HS poly(A)⁺ RNA from *A. thaliana* were used for in vitro import into isolated intact pea chloroplasts. Proteins recovered in the soluble fraction of the chloroplasts were analyzed directly by SDS gel electrophoresis or immunoprecipitated with HSP21 carboxyl-terminal antiserum (Fig. 6). A comparison of proteins imported from control versus HS translation products showed that a 21-kDa protein was present in the HS sample (lane 4) and absent from the control sample (lane 3). An abundant polypeptide of approximately 100 kDa was also increased in the HS import sample. The 21-kDa polypeptide was specifically immunoprecipitated by the pea HSP21 antiserum only from the HS sample (lane 6 versus lane 8). We conclude that this is the same polypeptide seen by Western blot analysis and that this protein is localized to chloroplasts.

Stability of chloroplast HSPs. Previous studies have suggested that HSPs in plants and other organisms are stable for several hours following removal of the heat stress (9, 14). We tested the stability of the chloroplast HSPs by preparing samples of total leaf proteins 12 h after the plants had been returned to normal growth temperatures. The samples were analyzed on Western blots with the carboxyl-terminal antibodies. In comparison to samples prepared immediately

FIG. 5. Accumulation of chloroplast HSPs. Total leaf protein samples were prepared from leaves before stress (lanes 1, 3, 5, and 7) or at 8 p.m. following stress (lanes 2, 4, 6, and 8) (see Fig. 4 for the stress regimen). Samples were from petunias (lanes 1 and 2), maize (lanes 3 and 4), *A. thaliana* (lanes 5 and 6), and peas (lanes 7 and 8). (A) Autoradiogram of a Western blot reacted with carboxyl-terminal antiserum. Arrowheads indicate the 24-kDa maize HSP in lane 4 (top arrowhead) and the 21-kDa HSP in *A. thaliana* (lane 6) and peas (lane 8) (bottom arrowhead). (B) Autoradiogram of a Western blot reacted with preimmune serum from the rabbit in which the carboxyl-terminal antiserum was produced. (C) Autoradiogram of a Western blot reacted with amino-terminal antiserum. The arrowhead indicates the 21-kDa HSP in *A. thaliana* (lane 6) and peas (lane 8). (D) Coomassie blue-stained gel. Approximately 100 μ g of protein was loaded in each gel lane. Positions of MW markers (in thousands) are shown at the right.

following the stress, samples prepared the next morning showed little or no decrease in antigen (Fig. 7).

DISCUSSION

We have shown that three divergent plant species, *A. thaliana*, petunia, and maize, all contain a low-MW HSP which is antigenically related to the chloroplast-localized 21-kDa HSP of pea. We conclude that these HSPs are also chloroplast proteins on the basis of (i) their antigenic similarity to pea HSP21, (ii) evidence that the HSPs are synthesized as precursor proteins of a higher MW than the form which accumulates in vivo, and (iii) the demonstration that the HSPs which accumulate in *A. thaliana* and maize correspond in MW to major HSPs imported into chloroplasts in vitro. Low-MW chloroplast HSPs have now been identified in seven plant species, those reported in this study and also soybean (19, 20), *P. vulgaris* (17), and the green alga *Chlamydomonas* (7). Synthesis of these proteins is clearly a fundamental response of plants to temperature stress.

The different plant species show an interesting pattern of cross-reactivity with the carboxyl-terminal and amino-terminal antisera. The carboxyl-terminal fusion protein in-

cluded the entire region of HSP21, referred to as the heat shock domain, which is homologous to low-MW HSPs of plants and other eucaryotes (21). The carboxyl-terminal antiserum reacts strongly with in vitro translation products of all four species. The amino-terminal antiserum reacts strongly only with translation products of peas, in which the amount of HSP precipitated appears identical to that obtained with the carboxyl-terminal antiserum. Failure of the amino-terminal antiserum to precipitate the heterologous HSPs suggests that the amino-terminal domain is not as highly conserved as the carboxyl-terminal heat shock domain.

There is no obvious cross-reactivity of the carboxyl-terminal antiserum with major low-MW cytoplasmic HSPs, which also contain the carboxyl-terminal heat shock domain. Thus, this domain is more highly conserved between chloroplast HSPs of different species than it is between low-MW HSPs within a species. How this may reflect differences in the functions of chloroplast and cytoplasmic HSPs remains to be determined.

Our data show that neither the chloroplast HSPs nor their mRNAs are present prior to heat stress. Pea HSP21 is not

FIG. 6. Identification of the major low-MW chloroplast HSP from *A. thaliana*. Lanes: 1 and 2, total in vitro translation products of control (C) or HS (HS) poly(A)⁺ RNA prepared from *A. thaliana* leaves; 3 and 4, proteins recovered in the soluble fraction of the chloroplast following in vitro import of translation products (shown in lanes 1 and 2) into isolated intact pea chloroplasts; 5 and 6, preimmune (PI) and immune (I) precipitates of proteins from lane 4; 7 and 8, preimmune and immune precipitates of proteins from lane 3. Numbers are MW markers (in thousands).

detected on Western blots when 100 μ g of total leaf protein from control leaves is used. In contrast, we have been able to detect pea HSP21 by using as little as 0.5 μ g of total leaf protein from heat-stressed leaves (Q. Chen, unpublished data). Therefore, HSP21 accumulates at least 200-fold during the stress treatment. Surprisingly, we have been unable to detect the accumulation of a low-MW HSP in petunias by Western blot analysis. This inability may be the result of a combination of weak reactivity and low levels of accumulation. In comparison with pea HSP21, the apparent lower levels of HSPs in *A. thaliana* and maize are most likely the result of decreased reactivity of the antiserum with heterologous species rather than lower levels of HSP accumulation. The immunoprecipitation results indicate that chloroplast HSP mRNA is abundant in both species. It will be necessary to generate antibodies against the HSPs of each species to investigate further their accumulation and abundance during stress.

Levels of chloroplast HSPs remain essentially unchanged during the first 12 h following stress. From previous experiments it has been estimated that some HSPs in plants are stable for at least 24 h following stress (9, 14). This long period of stability suggests that chloroplast HSPs are necessary not only during stress but also for recovery processes.

It has only recently been recognized that representatives of several major classes of HSPs are localized to both chloroplasts and mitochondria. Yeast mitochondria have been reported to contain proteins homologous to HSP70 (22) and GroEL, an HSP in *E. coli* (13). GroEL has also been identified in mitochondria from a wide range of other organisms, including humans and maize (13). In addition to the

FIG. 7. Stability of chloroplast HSPs. Shown is an autoradiogram of a Western blot of total leaf proteins from control (C) leaves (lanes 1, 4, and 7), leaves sampled at 8 p.m. following heat stress (HS) (lanes 2, 5, and 8), or leaves sampled at 8 a.m. on the day following heat stress (R) (lanes 3, 6, and 9) reacted with carboxyl-terminal antiserum. (A) Peas. (B) *A. thaliana*. (C) Maize. MW markers and the positions of the pea and *A. thaliana* HSPs (21 kDa) and the maize HSP (24 kDa) are indicated (in thousands).

low-MW HSPs identified here, chloroplasts also contain a GroEL homolog (5). In chloroplasts this protein is constitutive and participates in the assembly of ribulose biphosphate carboxylase holoenzyme (5). Our data show that chloroplasts of many different plant species contain a major HSP with homology to low-MW cytoplasmic HSPs. The discovery of HSPs localized to chloroplasts and mitochondria extends the range of processes in which HSPs function in eucaryotic cells.

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