Members of the 70-kD heat-shock protein (Hsp70) family are important cellular factors that are thought to mediate protein folding and assembly. A chloroplast-localized Hsp70 homolog (Chsp70) was recently identified based on its similarity to DnaK, the Hsp70 homolog of Escherichia coli (D. Amir-Shapira, T. Leustek, B. Dalie, H. Weissbach, N. Brot [1990] Proc Natl Acad Sci USA 87: 1749–1752). To learn more about the function of Chsp70, we purified the protein from Spinacia oleracea chloroplasts by ATP-agarose affinity chromatography. A single, 75,000-D protein was isolated which becomes phosphorylated on a threonine residue when incubated with [γ-32P]ATP and 10 mM Ca2+, a property similar to DnaK. Chloroplast fractionation and immunoblot analysis showed that Chsp70 is a soluble stromal protein. Chsp70-specific antiserum was used to clone a partial cDNA that shows greater homology with Hsp70 from prokaryotes than with cytoplasmic Hsp70 from eukaryotes. The antiserum and cDNA were used to study Chsp70 expression. Following heat shock of spinach seedlings at 37°C, Chsp70 synthesis increases 12-fold, the level of Chsp70 mRNA increases 5-fold, and the level of Chsp70 protein increases less than 2-fold. Chsp70 is constitutively expressed in all spinach seedlings.

Here we report the use of the Ca2+-dependent phosphorylation activity to purify chloroplast Hsp70 (Chsp70) from spinach (Spinacia oleracea). Antibodies raised against Chsp70 were used to study its subchloroplast localization and expression and to clone its cDNA.

**MATERIALS AND METHODS**

**General Methods**

The Laemmli (1970) procedure was used for SDS-PAGE. The Bio-Rad dye-binding assay was used for protein quantitation with a BSA standard. Immunoblot analysis was carried out as described by Harlow and Lane (1988) using Immobilon-P transfer membranes. The Bio-Rad dye-binding assay was used for protein quantitation with a BSA standard. Immunoblot analysis was carried out as described by Harlow and Lane (1988) using Immobilon-P transfer membranes.

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ion-P membrane (Millipore, Inc.). All procedures for nucleic acid manipulation were carried out as described by Sambrook et al. (1989). An LKB Ultroscan XL laser densitometer was used to quantitate protein and RNA bands on autoradiographs.

**Purification of Chsp70**

Chsp70 was purified by ATP-agarose affinity chromatography using a modification of the protocol described by Welch and Feramisco (1985). Spinach (Spinacia oleracea) was purchased from a local market, and intact chloroplasts were isolated by centrifugation through a Suc cushion (Setsuko and Bogorad, 1980). Chloroplasts were lysed in a glass dounce in buffer A (50 mM Hepes-KOH [pH 7.5], 10 mM NaCl, 5 mM β-mercaptoethanol, 1 mM MgCl₂, 1 mM benzamidine, 1 mM PMSF) supplemented with 1% (v/v) NP-40.

The lysate was centrifuged (12,000g, 20 min) to remove insoluble material, and the supernatant was batch adsorbed to ATP-agarose (5 mL of hydrated volume) by mixing for 2 h on ice. The agarose was then loaded into a chromatography column and washed with 25 mL of buffer A containing 1% (v/v) NP-40, followed by 50 mL of buffer A, and then 30 mL of buffer A containing 0.5 mM NaCl. Proteins were eluted with 50 mL of buffer A containing ATP in a linear gradient from 0 to 10 mM. Fractions containing the major protein peak were pooled, and the proteins were precipitated with ammonium sulfate (80% saturation). This step concentrates the protein and removes ATP. The proteins were dissolved in 10 mM Hepes-KOH (pH 7.5) with 10 mM NaCl and then dialyzed against the same buffer.

**In Vitro Phosphorylation and Phosphoamino Acid Analysis**

The reaction mixture used to assay in vitro phosphorylation of Chsp70 contained, in a final volume of 50 μL, 50 mM Mes-KOH (pH 6.0), 2.5 μM [γ-³²P]ATP (40 Ci mmol⁻¹), 0.2 μg of Chsp70, and divalent cations or EGTA as detailed in the figure legends. The reaction mixture was incubated for 30 min at 25°C, and then phosphorylation was analyzed by SDS-PAGE and autoradiography. Phosphoamino acid analysis of Chsp70 phosphorylated in vitro in the presence of 10 mM CaCl₂ was carried out by high-voltage thin-layer electrophoresis using the technique described by Boyle et al. (1991).

**Preparation and Use of Antisera**

Polyclonal antiserum against Chsp70 was generated in a guinea pig. Approximately 50 μg of Chsp70, run on an SDS gel, was used for immunization. The preparation and injection of antigen embedded in polyacrylamide and extraction and preparation of the antiserum were carried out as described by Harlow and Lane (1988). For immunoblot analysis, Chsp70 antiserum was used at a concentration of 1:2000 (v/v). Rabbit antiserum against the GroEL protein of *Escherichia coli* was used to detect Cpn60 on immunoblots at a dilution of 1:1000 (v/v) (Leustek et al., 1989). Rabbit antiserum against the 18-kD polypeptide of PSI was kindly provided by Dr. Nathan Nelson (Roche Institute of Molecular Biology, Nutley, NJ) and was used at a dilution of 1:1000 (v/v). Horseradish-peroxidase-linked goat anti-guinea pig or goat anti-rabbit antibodies (Sigma) were used to detect immune complexes according to the manufacturer's instructions.

**Chloroplast Fractionation and Protease Treatment**

Chsp70 localization experiments were carried out using chloroplasts isolated by Percoll step-gradient centrifugation (Orozco et al., 1986). Osmotic lysis was carried out by resuspension of intact chloroplasts (10 μg of Chl) in 100 μL of buffer containing 25 mM Hepes-KOH (pH 7.5), 1 mM benzamidine, and 1 mM PMSF, followed by incubation on ice for 10 min. The lysate was centrifuged at 16,000g for 15 min, and the supernatant and pellet were separated. The pellet was resuspended in 100 μL of the same buffer and centrifuged as before. The pooled supernatants and pellet were brought to 200 μL. A volume of whole chloroplasts equal to 10 μg of protein and equivalent volumes of supernatant or pellet fractions were analyzed by immunoblotting.

Protease treatment of chloroplasts was carried out as described by Marshall et al. (1990) with some modifications. The reactions were carried out in a final volume of 2 mL containing intact chloroplasts equivalent to 0.2 mg of Chl. Trypsin was used at 15 μg mL⁻¹, and thermolysin was used at 30 μg mL⁻¹ with 0.5 mM CaCl₂. Protease treatments were also conducted in the presence of 1% (v/v) NP-40. Intact chloroplasts were repurified from reactions without NP-40. Samples containing 1 μg of Chl were analyzed by immunoblotting.

**Cloning and Analysis of Chsp70 cDNA**

A spinach cDNA expression library was constructed in λZAP (Stratagene, Inc.) using the procedure recommended by the supplier. RNA was extracted from 3-week-old spinach seedlings (Chomczynski and Sacchi, 1987), and poly(A)⁺ RNA was purified on an oligo(dT) column for library construction. The cDNA library was screened with Chsp70 antiserum as described by Sambrook et al. (1989). Individual immunopositive plaques were isolated, and the cDNA was excised in vivo into pBluescript SK(−) as recommended by the supplier. Twelve clones were isolated and found to be of a single cDNA species by mapping for restriction endonuclease sites. Restriction fragments from the longest clone, pHSW1, were subcloned into pUC18 for sequence analysis. The dideoxy-chain termination method was used for sequence analysis (Sanger et al., 1977) using M13 forward/reverse primers and Sequenase (United States Biochemical). The pHSW1 cDNA sequence of both strands was determined using overlapping subclones.

**Analysis of Chsp70 Expression**

Chsp70 synthesis was analyzed by labeling spinach seedlings with [³⁵S]met and immunoprecipitating the protein. Seeds were germinated aseptically for 3 weeks at 18°C with illumination. Whole seedlings were temperature treated in capped test tubes containing a small amount of water. The tubes were submerged in a water bath at the temperatures...
and for the times indicated in the figure legends. Following temperature treatment, seedling shoots (severed 5 mm below the mesocotyl) were radioactively labeled by absorption of 10 μL of [7-35S]met (30 μCi μL−1, 600 Ci mmol−1; New England Nuclear, Inc., cell-labeling grade) through the cut surface. Absorption of the radioactive fluid required approximately 10 min, after which the shoots were placed into 1.5-mL microcentrifuge tubes with the cut end immersed in 100 μL of sterile water. Labeling was for 1 h at the prelabeling temperature.

For immunoprecipitation, labeled seedlings were lysed in IP buffer (50 mM Hepes-KOH [pH 7.5], 150 mM NaCl, 1% [v/v], NP-40, 1 mM benzamidine, and 1 mM PMSF) using a small dounce. The lysate was microcentrifuged, and the supernatant was used to measure radioactivity incorporated into protein by precipitation with 10% (w/v) TCA. A volume of supernatant equivalent to 1 × 10^3 TCA-precipitable counts was added to 100 μL of IP buffer containing 10% (v/v) Chsp70 antisem. The mixture was incubated with mixing for 1 h at 4°C. Immune complexes were collected by addition of 100 μL of a 10% (v/v) suspension of protein A-Sepharose (Pharmacia, Inc.) prepared in IP buffer, and the incubation continued for an additional 1 h at 4°C. Protein A-Sepharose was recovered by brief centrifugation and washed three times with IP buffer. The final pellet was suspended in SDS-PAGE sample buffer and heated for 5 min in a boiling water bath to release the immune complexes. The proteins were analyzed by SDS-PAGE and autoradiography.

Chsp70 mRNA levels, after heat shock, were measured by RNA blot analysis. Total RNA was isolated from temperature-treated seedlings (Chomczynski and Sacchi, 1987). Forty micrograms of RNA were separated on a formaldehyde agarose gel (Sambrook et al., 1989) and blotted onto nylon membrane (Zeta Probe; Bio-Rad). An 831-bp EcoRI-BglII fragment from pZme9019 (Altman et al., 1984) was used to detect Chsp70 mRNA. An 1180-bp EcoRI-BglII fragment from the 3' end of the cDNA insert of pHSW1 was used to determine the phosphorylation and the phosphorylated amino acid. When EGTA was added to a reaction with [γ-32P]ATP, Chsp70 phosphorylation was inhibited (Fig. 2A, lane 3). The purified protein also cross-reacts with polyclonal antiserum against DnaK and shows an isoelectric point of approximately pH 5.4, as does DnaK. An amino acid sequence obtained from an internal peptide of the protein was found to be homologous with a conserved region of all Hsp70 proteins (data not shown). These characteristics, particularly the divalent cation requirement for phosphorylation, indicate that the purified protein is probably identical with Chsp70; the DnaK-like protein previously identified in crude lysates of spinach chloroplasts (Amir-Shapira et al., 1990).

Antiserum against Chsp70 was used to determine the abundance of the protein in chloroplasts. Figure 1C shows that 10 μg of total chloroplast protein contains the same amount of antigen as 0.1 μg of purified Chsp70 (lane 1 versus lane 2). This indicates that Chsp70 makes up slightly less than 1% of the total chloroplast protein.

Purified Chsp70 was used to investigate the Ca^{2+} requirement for phosphorylation and to determine the phosphorylated amino acid. When EGTA was added to a reaction with [γ-32P]ATP, Chsp70 phosphorylation was inhibited (Fig. 2A, lane 1 versus lane 2). This is probably due to chelation of Ca^{2+}, present as a contaminant in the reaction mixture. Titration of Ca^{2+} into the reaction from 0.02 to 100 mM (lanes 3–8) showed that the optimal concentration is 10 mM (lane 6). The phosphorylated amino acid was determined by analysis of Chsp70 phosphorylated in vitro with 10 mM Ca^{2+}. The result shown in Figure 2B indicates that phosphorylation occurs exclusively on Thr. The Ca^{2+} requirement for Chsp70 phosphorylation and the phosphorylated amino acid are identical with that reported for DnaK and indicates the biochemical similarity of Chsp70 and DnaK.
Figure 2. Characteristics of Chsp70 phosphorylation. A, Ca\(^{2+}\) optimum for Chsp70 phosphorylation was determined by analysis of in vitro phosphorylated protein. Chsp70 (0.2 \(\mu\)g) incubated with \(\gamma^{32P}\)ATP and 0.5 mM EGTA (lane 1), no added divalent cation (lane 2), or the following concentrations of CaCl\(_2\): 0.02 mM (lane 3), 0.1 mM (lane 4), 1 mM (lane 5), 10 mM (lane 6), 50 mM (lane 7), 100 mM (lane 8). B, Chsp70 phosphorylated amino acid was determined by two-dimensional thin-layer electrophoresis of protein labeled in the presence of 10 mM CaCl\(_2\). The circled areas indicate the positions of the phosphoamino acid standards phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y).

Subchloroplast Localization of Chsp70

Chsp70 was localized in chloroplasts by osmotic lysis and separation of the soluble and particulate proteins by centrifugation. Immunoblot analysis of the fractions showed that Chsp70 is found primarily in the supernatant with a minor amount associated with the pellet (Fig. 3A, lanes 1–3). To control for the fractionation procedure, the distribution of Cpn60, a soluble chloroplast protein, and the 18-kD polypeptide of PSI, a chloroplast membrane protein, were also analyzed by immunoblotting. Cpn60 fractionates completely with the supernatant (lanes 4–6), whereas PSI fractionates completely with the pellet (lanes 7–9). Therefore, the portion of Chsp70 found in the pellet (lane 3) probably reflects its association with a particulate component within chloroplasts and is not due to incomplete separation of the supernatant and pellet during fractionation. These results indicate that Chsp70 is primarily, although not completely, a soluble chloroplast protein.

To distinguish between Chsp70 localization in the stroma or intermembrane space, intact chloroplasts were treated with the proteases thermolysin and trypsin. Thermolysin is unable to penetrate the outer chloroplast membrane and can only degrade surface proteins (Marshall et al., 1990). Trypsin penetrates the outer chloroplast membrane and is able to degrade proteins localized within the envelope (Marshall et al., 1990). As a control for protease activity, simultaneous treatment of chloroplasts with NP-40 and protease exposes internal proteins to degradation. The results of this experiment are shown in Figure 3B. Chsp70 from untreated chloroplasts is shown in lane 1. Treatments with either protease in combination with NP-40 (lanes 2 and 3), thermolysin (lane 4), or trypsin (lane 6) did not result in degradation of Chsp70. Treatment with both proteases in combination with NP-40 (lanes 3 and 5) resulted in degradation of Chsp70. This experiment, together with the chloroplast fractionation shown in Figure 3A, indicates that Chsp70 is a stromal protein.

Cloning and Analysis of a cDNA Encoding Chsp70

Chsp70 antisera was used to screen a cDNA expression library constructed in XZAP with poly(A) \(^+\) RNA isolated from spinach seedlings. Restriction enzyme analysis of 12 immunopositive clones indicated that they were all of a single cDNA species. The clone with the longest insert, 2040 bp, named pH50W1, was chosen for sequence analysis. pH50W1 was determined to be a partial clone, missing approximately 300 bp from its 5' terminus; therefore, its sequence is not

Figure 3. Localization of Chsp70 within chloroplasts. A, Distribution of Chsp70 in supernatant and pellet fractions from osmotically disrupted chloroplasts compared with that of Cpn60, a known soluble chloroplast protein, or the 18-kD polypeptide of PSI, a known chloroplast membrane protein. Chloroplast fractions were analyzed by immunoblotting with polyclonal antisera against Chsp70 (lanes 1–3), polyclonal antisera against Cpn60 (lanes 4–6), or polyclonal antisera against PSI (lanes 7–9). Antibody reactions with whole chloroplast proteins are shown in lanes 1, 4, and 7; chloroplast equivalents of soluble proteins are shown in lanes 2, 5, and 8; and chloroplast equivalents of the pellet fraction are shown in lanes 3, 6, and 9. We believe that the doublet in lane 4 results from distortion of the Cpn60 band because of its comigration in the gel with a membrane protein that is separated from Cpn60 by the fractionation procedure. B, The localization of Chsp70 carried out by treatment of chloroplasts with combinations of proteases and the nonionic detergent NP-40 followed by immunoblot analysis. Lane 1, Untreated chloroplasts; lane 2, chloroplasts treated with NP-40; lane 3, NP-40 and thermolysin; lane 4, thermolysin; lane 5, NP-40 and trypsin; lane 6, trypsin.
Figure 4. Comparison of Hsp70 homologous proteins. The deduced amino acid sequences of all plant Hsp70s in GenBank as of November 1992 and two human and one yeast sequence were analyzed by pairwise alignment using the Genetics Computer Group, Inc., program PileUp. The length of horizontal lines is proportional to the similarity between the sequences. The names of the species of origin and the name of the gene, mRNA, or protein are shown to the right. The dendrogram can be divided into four groups of sequences delineated on the right by the brackets and labeled A, B, C, and D. The GenBank accession numbers for each sequence in the histogram from top to bottom are as follows:

- Petunia (HSP70), X13301;
- Tomato (HSCI), X54029;
- Spinach (SCE70), X61491;
- Maize (HSP70), X03697;
- Tobacco (HSP70), X63106;
- Chlamydomonas (HSP70), M76725;
- Human (HSP70), M11717;
- Tobacco (BLP4), X66874;
- Tobacco (BLP5), X66873;
- Maize (BIP), X60057;
- Human (BIP), M59449;
- Spinach (Chsp70), X62240;
- Pea (CSS1), M75518;
- Cryptomonas, M76547;
- Porphyra, X62240;
- Synechocystis, M57518;
- Pavlova, X59555;
- Pea (PHSP1), X54739;
- Bean, X66874;
- E. coli (DNAK), K01298;
- Yeast (SSC1), M27229;

Expression of Chsp70

When spinach seedlings grown at 18°C are exposed to 30 or 37°C for 4 h, there is an increase in the synthesis of several heat-shock proteins (Fig. 5, lane 1 versus lanes 2 and 3). Seedlings heat shocked at 40°C did not incorporate L-[35S]-Met (data not shown). Immunoprecipitation of Chsp70 from these samples and laser densitometric quantitation of the autoradiographs showed that its synthesis increases 5.5-fold after heat shock at 30°C (lane 4 versus lane 5) and 12.6-fold at 37°C (lane 4 versus lane 6). Chsp70 was identified by its specific immunoprecipitation with immune serum. The other radioactive proteins present in lanes 4 through 6 were found to bind to protein A-Sepharose used in the immunoprecipitation procedure and, therefore, are unrelated to Chsp70 (not shown).

The synthesis of many heat-shock proteins is controlled primarily at the transcriptional level (Nover, 1991). To determine whether Chsp70 mRNA accumulates after heat shock, we used a DNA fragment from pHSW1 as a probe on RNA

Figure 5. Analysis of proteins synthesized by heat-shocked spinach seedlings. Heat-shock proteins were analyzed by pulse labeling with L-[35S]-Met and immunoprecipitating of Chsp70. Spinach seedlings were labeled at 18°C (lanes 1 and 4), 30°C (lanes 2 and 3), or 37°C (lanes 3 and 6). The 30 and 37°C treatments were given for a total of 4 h. Whole-cell extracts prepared from the seedlings were analyzed by SDS-PAGE and autoradiography (lanes 1–3) or by immunoprecipitation with Chsp70-specific antisera (lanes 4–6). Protein molecular mass standards are shown on the left in kD.
Figure 6. Analysis of Chsp70 mRNA expression. Chsp70 mRNA levels were compared to that of the Rubisco-LS after heat shock. RNA was isolated from spinach seedlings grown at 18°C (lanes 1 and 3) or treated for 4 h at 37°C (lanes 2 and 4). RNA samples were analyzed by hybridization with a Chsp70-specific cDNA probe (lanes 1 and 2) or a Rubisco-specific DNA probe (lanes 3 and 4). RNA size standards are shown on the left in kb. The more narrow width of the band shown in lane 3 is the result of inadvertent trimming of half the lane during processing of the blot. Notice that the intensity of the signals in lanes 3 and 4 are similar.

Chsp70 was measured in the same extracts by immunoblotting. The results, presented in Figure 7A, show that Chsp70 synthesis is elevated for up to 25 h of heat shock at 37°C with a peak of synthesis after 2.5 h (lanes 1–6). The results presented in Figure 7B show that the level of Chsp70 increases only slightly during the same time (lanes 1 and 2 versus lanes 3–6). The slight increase in Chsp70 level after heat shock was observed in three independent experiments. These results indicate that, although Chsp70 synthesis is inducible by heat shock, it does not accumulate significantly above the level expressed under normal growth conditions. The slight degradation of Chsp70 visible in Figure 7A appears to be limited to the labeled protein, because the same extracts show only a single Chsp70 band in the immunoblot shown in Figure 7B. The reason for this degradation is unknown, and it has occurred to varying extents in different experiments despite the addition of protease inhibitors to the immunoprecipitation buffer.

Because Chsp70 is localized within chloroplasts, it was of interest to determine its pattern of expression in photosynthetic and nonphotosynthetic tissues. Figure 8 shows that Chsp70 is present in all spinach tissues, including seeds (lane 1), green seedlings (lane 2), etiolated seedlings (lane 3), florets (lane 4), mature leaves (lane 5), mature stems (lane 6), and mature roots (lanes 7 and 8). Notice that 5 times more protein (lane 7 versus 8) was required to detect Chsp70 in the root extract. Results of this experiment indicate that the level of Chsp70 is generally lower in nonphotosynthetic tissues with the exception of spinach seeds (lane 1), which contain levels of Chsp70 equal to that of photosynthetic tissues. The immunoreactive proteins visible below the Chsp70 band in lanes 3 and 6 may be degradation products or possibly other Hsp70 proteins.

DISCUSSION

Chsp70 was previously identified by its ability to undergo autophosphorylation in vitro with [γ-32P]ATP and Ca2+. Using purified Chsp70, we found...
that phosphorylation is optimal at 10 mM Ca\textsuperscript{2+} and is inhibited by Mg\textsuperscript{2+}. Thr is the phosphate-acceptor amino acid. DnaK and Hsp70 homologs localized in the major, membrane-bound organelles of eukaryotic cells are autophosphorylated under similar conditions (Cegielska and Georgopoulos, 1989; Leustek et al., 1992; Miemyk et al., 1992). McCarty and Walker (1991) reported that DnaK is autophosphorylated on Thr\textsuperscript{191} in vitro. This residue, positioned in the ATP-binding cleft of the protein (Flaherty et al., 1990), is conserved among all known Hsp70 proteins. Mutation of this site destroys the ATPase activity of DnaK, suggesting that Thr\textsuperscript{191} is functionally important (McCarty and Walker, 1991). ATPase activity is thought to be important for Hsp70 function (Liberek et al., 1991). However, the significance of Ca\textsuperscript{2+}-dependent autophosphorylation at this site is unclear. DnaK has never been shown to be phosphorylated in vivo on Thr\textsuperscript{191}, and the in vitro reaction conditions, similar to the conditions for Chsp70 phosphorylation, are distinctly nonphysiological. Both DnaK and Chsp70 require high levels of Ca\textsuperscript{2+}, low pH, and low levels of Mg\textsuperscript{2+} (Leustek et al., 1992, and this study).

In this report we show that Chsp70 is the major chloroplast Hsp70 homolog of spinach and can be purified by ATP-agarose affinity chromatography. This result is surprising because spinach is known to contain at least one other Hsp70 homolog, SCE70, localized in the envelope (Ko et al., 1992), and pea chloroplasts contain at least three Hsp70 homologs, one localized in the envelope and two in the stroma (Marshall et al., 1990). Possibly, SCE70 does not co-purify with Chsp70 under the conditions of our experiment, or it may be a low-abundance protein relative to Chsp70.

The sequence relatedness of Chsp70 with Hsp70 from cyanobacteria is consistent with the idea that an endosymbiotic cyanobacteria is the progenitor of chloroplasts. The sequence similarity of mitochondrial Hsp70 with prokaryotic Hsp70 has previously been noted (Craig et al., 1989). It is interesting that Chsp70 shows much lower homology with SCE70, which may have originated from cytosolic Hsp70 (Ko et al., 1992).

Here we show that Chsp70, a major chloroplast protein under normal growth conditions, is synthesized more rapidly after heat shock, although its total concentration increases only slightly. The small increase in Chsp70 level after heat shock, despite the significant increase in synthesis, may be due to its high basal level in unstressed spinach. Alternatively, the turnover rate of Chsp70 may increase after heat shock, and increased synthesis may be required to maintain the level of Chsp70. However, results of experiments to measure Chsp70 turnover do not support this hypothesis (results not shown). A third possibility is that newly synthesized Chsp70 may fail to enter chloroplasts after heat shock and may be degraded in the cytoplasm. The observation that labeled Chsp70 from heat-shocked seedlings migrates as a doublet on SDS gels and in some cases appears as a smear (Figs. 6 and 8) lends credence to this hypothesis. However, others have found that the ability of chloroplasts to import some proteins is not impaired after heat shock (Vierling and Key, 1985).

The presence of Chsp70 in all spinach tissues indicates that this protein is not strictly chloroplast localized and is probably localized in a wide variety of plastid types. Chsp70 is approximately 5-fold less abundant in spinach roots than in leaves, a result similar to that observed for Hsp21, another plastid-localized heat-shock protein (Chen et al., 1990). This probably reflects the lower number of plastids in roots. In contrast, the high level of Chsp70 in seeds probably reflects the large number of plastids present in the dormant embryo. The high level of Chsp70 in spinach florets is an interesting observation that is corroborated in the literature. Recently, Domoney et al. (1991) cloned a partial cDNA for a protein expressed rapidly during early embryogenesis in pea. The partial cDNA is identical with CSS1 (Marshall et al., 1992).

In our experiment the spinach florets likely contained high concentrations of developing embryos.

It is possible to speculate concerning the function of Chsp70 in plastids from studies of other Hsp70s. For example, Hsp70 localized in the mitochondrial matrix, and ER lumen are required for translocation of precursor proteins into these organelles (Kang et al., 1990; Baker and Schatz, 1991). Similarly, Chsp70 may be involved in translocation of proteins into plastids or protein targeting to membranes within plastids. Recently, Yalovsky et al. (1992) reported that chloroplast Hsp70 is involved in the integration of a thylakoid membrane protein. However, this result is controversial and has not yet been corroborated. The ER form of Hsp70, known as BiP (binding protein), is thought to mediate the assembly of multimeric secretory and membrane proteins (Gething and Sambrook, 1992). Chsp70 could function similarly in plastids, perhaps in conjunction with Cpn60, another heat-shock protein involved in the assembly of Rubisco (Gething and Sambrook, 1992; Langer et al., 1992). Hsp70 is also known to function in protecting cells against heat stress (Li et al., 1991). DnaK was shown to protect RNA polymerase from heat denaturation in vitro and to mediate the renaturation of heat-denatured RNA polymerase (Skowrya et al., 1990). Chsp70 may play a similar thermoprotective role in plastids. Such a function could be of major technological importance, because chloroplasts are particularly heat sensitive (Nover, 1991). To date, numerous cellular processes have been identified that require Hsp70. Many of these processes also occur in plastids, suggesting that Chsp70 is likely to be an important factor in plastid function.

ACKNOWLEDGMENTS

We wish to thank Dr. Nathan Nelson for PSI antiserum and Drs. Peter Day and Alexander Enyedi for their helpful suggestions concerning the manuscript.

Received February 12, 1993; accepted March 31, 1993.
Copyright Clearance Center: 0032-0889/93/102/0843/08.
The GenBank accession number for the sequence reported in this article is M99565.

LITERATURE CITED
