Accumulation, Stability, and Localization of a Major Chloroplast Heat-Shock Protein

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Abstract. Diverse higher plant species synthesize low molecular weight (LMW) heat shock proteins (HSPs) which localize to chloroplasts. These proteins are homologous to LMW HSPs found in the cytoplasm of all eukaryotes, a class of HSPs whose molecular mode of action is not understood. To obtain basic information concerning the role of chloroplast HSPs, we examined the accumulation, stability, tissue specificity, and intra-chloroplast localization of HSP21, the major LMW chloroplast HSP in pea. Intact pea plants were subjected to heat stress conditions which would be encountered in the natural environment and HSP21 mRNA and protein levels were measured in leaves and roots. HSP21 was not detected in leaves or roots before stress, but the mature, 21-kD protein accumulated in direct proportion to temperature and HSP21 mRNA levels in both tissues. All of the HSP21 in leaves was localized to chloroplasts; there was no evidence for its transport into other organelles. In chloroplast fractionation experiments, >80% of HSP21 was recovered in the soluble chloroplast protein fraction. The half-life of HSP21 at control temperatures was 52 ± 12 h, suggesting the protein's function is critical during recovery as well as during stress. We hypothesize that HSP21 functions in a catalytic fashion in both photosynthetic and nonphotosynthetic plastids.

ALL organisms produce heat-shock proteins (HSPs)[1] in response to elevated temperatures and certain other stresses (Lindquist and Craig, 1988). Physiological and genetic data indicate that the production of HSPs during stress is essential for cell survival or recovery from stress. There are four major classes of HSPs in eukaryotes, HSP90, HSP70, HSP60 (or GroE), and low molecular weight (LMW) HSPs (15–30 kD). Proteins with homology to the first three classes are also synthesized by *Escherichia coli* in response to heat stress. Although HSPs were first identified by their elevated expression during stress, in many cells it has been found that HSPs or highly homologous proteins are expressed constitutively, or under cell cycle or developmental control (Lindquist and Craig, 1988). Furthermore, HSP60 and distinct homologues of HSP70 are present in semi-autonomous organelles, the mitochondria, and chloroplasts (Hemmingsen et al., 1988; Craig et al., 1989; Marshall et al., 1990). Chloroplasts also contain specific LMW HSPs (Kloppstech et al., 1985; Vierling et al., 1986). Clearly, HSPs are critical to multiple functions in eukaryotes.

LMW, chloroplast-localized HSPs have been identified in diverse higher plant species, including both dicots and monocots (Kloppstech et al., 1985; Vierling et al., 1986, 1989). Each species produces a single, major LMW chloroplast HSP between 21 and 24 kD. These proteins are antigenically related and their corresponding cDNAs cross-hybridize. The proteins are nuclear encoded and are synthesized as

1. *Abbreviations used in this paper:* FNR, ferredoxin NADP reductase; HSP, heat-shock protein; LMW, low molecular weight.

precursor polypeptides ∼5 kD larger than the mature protein. The mRNA encoding the LMW chloroplast HSP has not been detected in unstressed leaf tissues, but it rapidly accumulates during heat stress to an estimated 0.75% of the poly(A)RNA after 2 h at 38°C (Vierling et al., 1986). This is the most dramatic environmental regulation of any chloroplast protein studied to date. Using specific antibodies, the LMW chloroplast HSP was also shown to be undetectable in control tissues, although significant protein accumulated during stress (Vierling et al., 1989).

The amino acid sequences of LMW chloroplast HSPs have been derived from the DNA sequence of cDNA clones from pea (*Pisum sativum*) (Vierling et al., 1988), soybean (*Glycine max*) (Vierling et al., 1988), *Arabidopsis thaliana* (Vierling, E., unpublished data), and petunia (*Petunia hybrida*) (Chen, Q., unpublished data). The carboxy-terminal domain of the mature protein (∼100 amino acids) is homologous to a domain found in LMW cytoplasmic HSPs and in the alpha-crystallins, identifying these chloroplast HSPs as members of a eukaryotic superfamily of LMW HSPs (Lindquist and Craig, 1988; Vierling et al., 1988). The amino-terminal transit peptide which is removed during import into chloroplasts has characteristics typical of transit peptides from other nuclear-encoded chloroplast proteins (von Heijne et al., 1989). We have hypothesized that the chloroplast HSP arose from a duplication of a nuclear gene for a cytoplasmic protein which subsequently acquired amino-terminal sequences sufficient to direct it to the chloroplast (Vierling et al., 1988).

The homology of the LMW chloroplast and cytoplasmic HSPs suggests that these proteins perform similar functions in different cellular compartments. Similar to other HSPs, some studies have correlated the presence or absence of LMW cytoplasmic HSPs with cellular thermotolerance or thermosensitivity, respectively (Lindquist and Craig, 1988; Landry et al., 1989). In yeast (Rossi and Lindquist, 1989), plants (Nover et al., 1989), and other eukaryotes (Arrigo, 1987; Arrigo et al., 1988; Collier et al., 1988). LMW cytoplasmic HSPs are found in 10-20-S particles, composed primarily of the HSP, but no biochemical activity has been ascribed to these particles. Nover et al. (1989) reported that LMW cytoplasmic HSPs are associated in vivo with specific mRNAs and hypothesized that they function in translational control during heat stress. It has also been proposed that LMW HSPs perform a structural role, based on their homology with the alpha-crystallins, structural proteins of the eye lens (Lindquist and Craig, 1988). Schuster et al. (1988) correlated the production of HSPs in *Chlamydomonas* with decreased heat damage to photosystem II. They reported that a LMW chloroplast HSP was localized to thylakoid membranes, and suggested that it interacts directly with photosystem II components to prevent or repair heat-induced damage. Clearly, considerable further work is required to resolve the functional roles of both chloroplast and cytoplasmic LMW HSPs.

To understand under what conditions and in which tissues the LMW chloroplast HSP functions, we have examined the expression of chloroplast HSP21 in young, intact pea plants under a series of stress treatments mimicking temperature conditions in the natural environment. Using antibodies specific to HSP21 we find that the protein cannot be detected in control tissues, but accumulates in both leaves and roots in proportion to temperature and mRNA levels. In both organ types the protein is highly stable after stress, having a half-life of ∼52 h. We also show that HSP21 is found in the soluble fraction of the chloroplast and shows no apparent association with thylakoid membranes. These studies provide some of the first data addressing the importance of HSPs to plant growth in their normal environment and have implications with regard to LMW chloroplast HSP function.

Materials and Methods

Plant Growth and Stress Conditions

Pea plants (*Pisum sativum*, cv. "Little Marvel") were grown in a controlled environment growth chamber for 9 d under a 22°C day/18°C night regime with a 16-h daylength. Light intensity was 240 μE m^{-2} s^{-1}. Plants were grown in vermiculite and watered with quarter-strength Hoagland's solution. During stress experiments the growth chamber humidity was maintained at saturation with a vaporizer. Leaf temperature was measured using an infrared thermometer (Everest Interscience, Inc., Fullerton, CA) with a measuring field of 0.18 inches squared focused on the second pair of expanded leaves. Root temperature was estimated by measuring the temperature of the vermiculite. After the stress treatments, plants were grown for an additional 7 d under the 22°C day/18°C night regime.

Isolation of Total Leaf and Root Protein

Total protein samples were prepared from both leaves and roots as described previously (Vierling et al., 1989). Briefly, tissue was ground in SDS gel sample buffer (60 mM Tris-HCl, pH 8.0, 60 mM DTT, 2% SDS, 5.0 mM ε-aminocaproic acid, 1.0 mM benzamidine, 15% sucrose), the samples were boiled, and insoluble debris removed by centrifugation (5 min, 12,000 g).

The second pair of expanded leaves from the 9-d-old peas, or their morphological equivalent on subsequent days, were used for all protein samples. Root samples were taken from three individual seedlings. Protein samples were prepared from the following timepoints: (a) before the temperature increase, (b) when the temperature had increased to half maximum, (c) at the beginning, middle, and end of the 4-h maximum temperature period (three samples), (d) at the midpoint of the temperature decrease, (e) after the plants had returned to control temperatures, and (f) once each day during the 7 d after the stress. For protein determinations, samples were precipitated with 4 vol of 100% acetone and resuspended in 0.1 N NaOH. Protein content was then determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

Chloroplast Isolation and Fractionation

Intact pea chloroplasts were isolated on Percoll gradients as described by Vierling et al. (1986). Chloroplasts were protease treated (100 μg ml^{-1} of thermolysin for 30 min at 4°C) and intact organelles reisolated by pelleting through 40% Percoll containing a mixture of protease inhibitors: 5.0 mM ε-aminocaproic acid, 1.0 mM benzamidine, pepstatin A (0.1 mg/ml), and antipain, aprotinin, chymostatin, leupeptin (0.5 μg/ml). Chloroplasts were hypotonically lysed in buffer containing 10 mM Hepes, pH 8.0, 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 150 mM NaCl, and the above protease inhibitors (0.5 ml buffer mg^{-1} chlorophyll). Membranes were pelleted for 10 min at 12,000 g and the supernatant removed. The membranes were then resuspended in the same buffer and recentrifuged. The supernatant was combined with the first supernatant and designated as the soluble protein fraction. The membrane fraction was further washed two times in 0.5 mM EDTA (20 ml mg^{-1} chlorophyll) at room temperature for 20 min. The pooled EDTA washes comprised the membrane wash fraction. For electrophoretic analysis, the soluble and membrane wash fractions were mixed with twofold concentrated gel sample buffer or were concentrated by TCA precipitation before resuspension in gel sample buffer. The membrane fraction was solubilized directly in gel sample buffer.

Determination of Total Chlorophyll and Chlorophyll to Protein Ratios

For all samples, chlorophyll was measured in the 80% acetone extracts according to Arnon (1949). Total chlorophyll per milligram leaf fresh weight was determined by weighing leaves directly after their removal from the plant and then grinding the leaves in 80% acetone. Total leaf protein was measured as described above, using a known fresh weight of leaf tissue. By this method the leaf chlorophyll to protein ratio was determined to be 1 mg chlorophyll per 27.6 ± 2.6 (SD) mg protein. Total leaf chlorophyll was also determined by acetone extraction of leaf samples which had been solubilized in gel sample buffer but not boiled. Protein was then determined on these samples as described above. Both methods gave similar chlorophyll to protein ratios. To determine the total chlorophyll per chloroplast, intact chloroplasts were counted using a hemacytometer and a known number of chloroplasts were lysed directly in 80% acetone. An equal number of chloroplasts were acetone precipitated for protein determination. Alternatively, chloroplasts were solubilized in gel sample buffer and chlorophyll determined before boiling. Total protein was determined in the SDS extracts as described above. Both methods gave chlorophyll to protein estimates of 1 mg chlorophyll per 11.14 ± 1.09 (SD) mg protein.

Gel Electrophoresis and Immunoblotting

Leaf, root, or chloroplast protein samples were separated on 12.5% (unless otherwise noted) polyacrylamide SDS gels and blotted to nitrocellulose. Blots were reacted with antiserum and ^{125}I-protein A (46.6 μCi μg^{-1}; ICN Radiochemicals, Irvine, CA) as described previously (Vierling et al., 1989). Rabbit antiserum against pea chloroplast HSP21 was obtained using a fusion protein comprised of the carboxy-terminal (Met_{102} to Gln_{232}) segment of HSP21 fused to the *E. coli* TrpE protein as the antigen. Characterization of these antibodies has been described in detail (Vierling et al., 1989). Rabbit anti-HSP70 antibodies were a gift from Dr. L. Nover and were produced using purified tomato HSP70 as described by Neumann et al. (1987). Rabbit antibodies against spinach chloroplast ATPase and spinach ferredoxin NADP reductase (FNR) were gifts from Dr. A. Jagendorf and M. Caffrey, respectively.

Results from the immunoblot analyses were quantified by aligning the nitrocellulose filters with the autoradiograms and excising the ^{125}I-labeled bands. Excised portions were counted in a scintillation counter. Background

levels were estimated by excising an equal sized segment from a nonreacting portion of the gel lane. Samples from different immunoblots were normalized relative to each other by re-analyzing representative samples from each experiment on the same blot.

RNA Extraction and Northern Analysis

To obtain RNA samples in parallel to the protein samples, total leaf RNA was extracted from the second pair of expanded leaves of three plants, and total root RNA was prepared from three plants. Samples were taken at the same timepoints during the stress treatment as the protein samples, and twice on the first day after the stress treatment. The method of RNA preparation has been described (Vierling and Key, 1985). To remove DNA, RNA was precipitated with 2.0 M LiCl. RNA was quantified by absorbance at 260 nm.

Samples of RNA (10 μg) were separated on 1.5% agarose formaldehyde gels and blotted to Nytran membrane (Schleicher & Schuell, Inc., Keene, NH). Electrophoresis, blotting and hybridization conditions were as outlined by Vierling and Key (1985). Hybridization probes were labeled by the random primer method according to Feinberg and Vogelstein (1983) using ^{32}P-dATP (3,000 Ci/mmol; ICN Biomedicals, Inc., Irvine, CA). Chloroplast HSP21 mRNA was detected with a probe made using the pea HSP21 cDNA (Vierling et al., 1988). HSP70 mRNA was detected with a cDNA that was isolated from a pea lambda gt10 cDNA library using a previously described soybean HSP70 cDNA to probe the library (Nagao et al., 1986).

Results

Leaf Temperatures during Stress Treatments

To determine under what conditions chloroplast HSP21 functions, we examined HSP21 expression in intact plants subjected to temperatures typical of a field growth environment (Ehrler, 1973; Gardner et al., 1981; Gates, 1968). The imposed heat stress treatments involved a gradual increase (4°C/h) to a maximum temperature, a 4-h period at the maximum temperature, and a gradual decrease returning to the
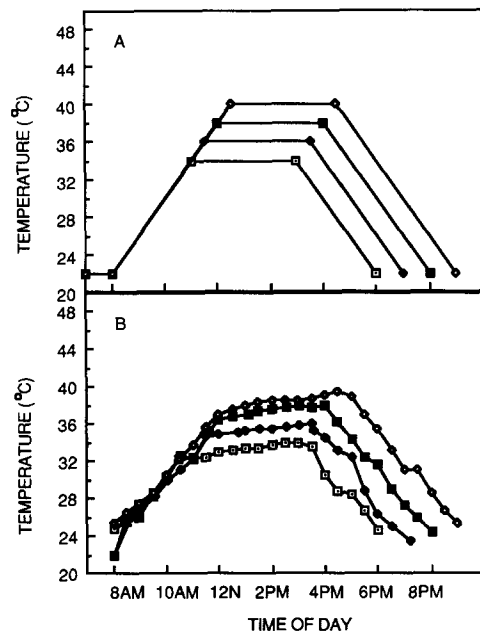

Figure 1. Heat stress conditions and leaf temperatures. (A) Programmed change in growth chamber temperature during the day of heat stress. Separate sets of plants were used for experiments at the different maximum temperatures (34, 36, 38, and 40°C). (B) Temperature of the second pair of expanded leaves during the different stress treatments. 34°C, □; 36°C, ◆; 38°C, ■; 40°C, ◇.

growth temperature (Fig. 1 A). Four stress conditions were used which differed by 2°C in the maximum stress temperature (34, 36, 38, or 40°C). We had previously shown that a 38°C treatment of this type caused HSP21 to accumulate (Vierling et al., 1989).

Many studies have shown that the rate of HSP synthesis in plants and other organisms is proportional to cell or tissue temperature, and that the rate of HSP synthesis can vary dramatically over a small temperature range (Nagao et al., 1986). Therefore, to determine the relationship between stress temperature and the production and stability of HSP21 in intact plants, it was necessary to be able to control and measure leaf temperature accurately. Controlling leaf temperature in intact plants is complicated by the fact that leaf temperature can be significantly different from air temperature depending on factors such as humidity, plant water status, and irradiance (Gates, 1968). We found that when growth chamber humidity was maintained at high levels to prevent transpirational cooling of the leaves, leaf temperatures were similar to air temperatures. Leaf temperatures during the different stress treatments, as measured using an infrared thermometer, are shown in Fig. 1 B. Repeated measurements over the stress timecourse gave error estimates of ±0.5°C. Leaf temperatures lagged behind the air temperature, reaching the highest levels during the second 2 h of the maximum stress. Plants subjected to 40°C never registered 40°C leaf temperatures, but rather remained at 39°C. Leaf temperature returned to normal (22–24°C) in parallel to the reduction of the growth chamber temperature. On days after the stress treatment, the growth chamber was maintained at a constant 22°C during the day, and leaf temperatures did not rise above 24°C. It should be noted that none of the temperature treatments caused visible damage to the plants as observed during the subsequent week of growth.

Accumulation of Chloroplast HSP21 in Leaves

To determine the relationship between leaf temperature and the accumulation of HSP21, total leaf proteins were extracted before, during, and after the maximum temperature stress as indicated in Materials and Methods. Total leaf proteins were also extracted from the treated plants once each day for 7 d after the day of stress treatment. Examination of the abundant leaf proteins by one-dimensional gel electrophoresis showed no significant changes during or after the stress treatment (Fig. 2). When the samples were analyzed on immunoblots probed with HSP21 antiserum, HSP21 could not be detected in leaves before the heat stress, but was seen to accumulate during the period of maximum temperature stress (Fig. 2). The protein continued to accumulate as the plants returned to normal temperature and reached the highest levels at the end of the stress day. The maximum level of HSP21 and the number of days HSP21 could be detected after the stress were both proportional to the maximum leaf temperature. We have not tested the possibility that the response to temperature shows diurnal variation. However, HSP21 was not present in leaves at any time during the day unless the plants were heat-stressed (not shown). The 26-kD precursor form of HSP21 did not accumulate during any of the treatments.

Fig. 3 graphically shows the pattern of HSP21 accumulation during the day of stress (A) and the subsequent decline
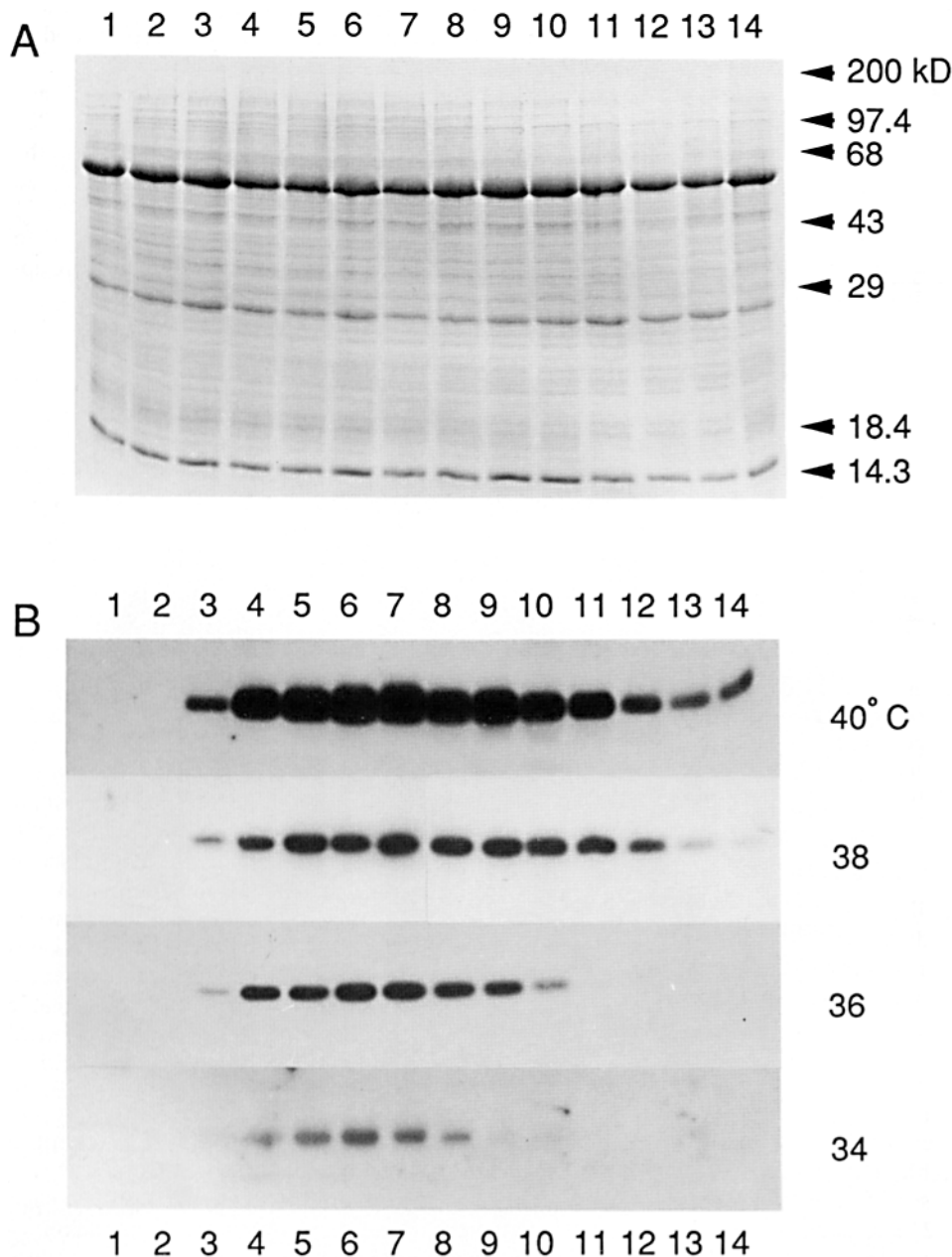
Figure 2. Accumulation of HSP21 in heat stressed leaf tissue. (*A*) Coomassie blue–stained gel of total leaf proteins from a 38°C stress experiment. Leaf proteins were prepared throughout the day of stress and once each day for 7 d after the stress. 40 μg of total leaf protein were loaded per lane. Numbers at right indicate molecular mass in kilodaltons. (*B*) Autoradiograms of sections of immunoblots reacted with HSP21 antiserum. Immunoblots of total leaf proteins were prepared from gels essentially identical to that shown in *A*, using 40 μg protein samples from plants subjected to the 34, 36, 38, or 40°C stress treatments diagrammed in Fig. 1. The maximum temperature stress for each sample is indicated at the right. Lanes *1*, Control leaves before stress treatment; lanes *2*, midpoint of increase to maximum temperature; lanes *3*, beginning of maximum temperature; lanes *4*, 2 h of maximum temperature; lanes *5*, end of maximum temperature; lanes *6*, midpoint of decrease to control temperature; lanes *7*, end of stress treatment (return to normal temperature); lanes *8–14*, leaves from 1, 2, 3, 4, 5, 6, or 7 d after the stress treatment, respectively.

during the week after the stress (*B*). The production of HSP21 was quantitatively assessed by determining the radioactivity of the 21-kD protein band identified on the immunoblots. The validity of this method for estimating the relative levels of HSP21 was tested by determining the relationship between the amount of HSP21 loaded on the gel and the cpm of ^{125}I recovered. The relationship of ^{125}I cpm μg^{-1} total protein was found to be linear between 0.5 and 50 μg of total leaf protein (not shown). We estimate that the error in these measurements is ±10%. In all four temperature treatments, the highest rate of accumulation occurred during the first 2 h at the maximum temperature. The rate of accumulation declined sharply in the second 2 h of stress and essentially no additional protein accumulated after leaf temperature had returned to 24°C. It is likely that the amount of protein made at 40°C was only slightly higher than the level in the 38°C

treatment because the leaves only reached 39°C and also because 40°C is close to the lethal temperature for these plants.

HSP21 Expression in Roots

Chloroplast proteins which are required solely for photosynthesis are not typically produced by roots, although plant root tissues contain plastids which are derived from the same precursor organelles and have the same genome as chloroplasts. Root plastids carry out other processes which occur in chloroplasts such as RNA and protein synthesis and steps in amino acid and fatty acid metabolism (Mullet, 1988). We hypothesized that if HSP21 were involved only in photosynthetic processes it would not accumulate in root tissues, and conversely, if HSP21 were essential for processes which occur in all plastids, the protein would accumulate in roots.
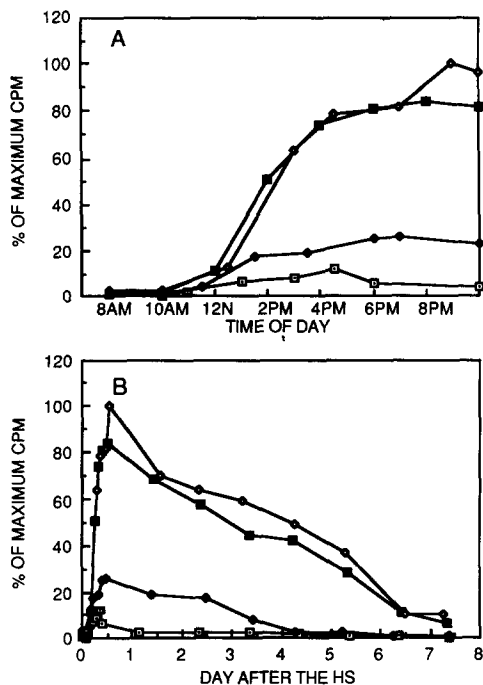
Figure 3. Quantification of HSP21 during heat stress and recovery in leaves. All values are expressed as a percentage of the maximum ^{125}I cpm obtained in the 40°C stress experiment. (*A*) Levels of HSP21 during the day of stress at different maximum temperatures. (*B*) Levels of HSP21 from the stress day through 7 d after the stress. 34°C, □; 36°C, ♦; 38°C, ■; 40°C, ◊.

Root samples were prepared in parallel to the leaf samples to determine the temperature dependence and stability of HSP21 in root tissues. Root temperatures were estimated and shown to be similar to the growth chamber temperature (Fig. 4). Total root protein samples were tested for the presence of HSP21 on immunoblots and a similar pattern of HSP21 accumulation was seen as that found in leaves (Fig. 5). No significant change in the stained pattern of the abundant root proteins was observed during or after the stress treatment (not shown). HSP21 was not detectable in control root tissues, but accumulated during the stress period in proportion to the maximum temperature. The 21-kD, mature form of HSP21 accumulated in the root tissues, indicating that the protein was appropriately processed. At the higher temperatures, 38 and 40°C, the HSP21 antibodies also detected a low level of a polypeptide at ∼26 kD, the expected size of the
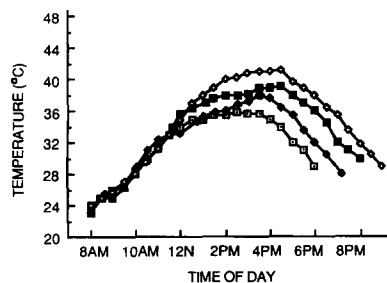

Figure 4. Root temperature during the stress treatments. Different sets of plants were used for each stress treatment. 34°C, □; 36°C, ♦; 38°C, ■; 40°C, ◊.

precursor protein (see Fig. 7). However, the possibility that this polypeptide reacts non-specifically with the antibodies has not been ruled out.

The level of HSP21 in the roots was quantified as shown in Fig. 6. As found in leaves, the maximum rate of HSP21 accumulation occurred during the first 2 h of stress and the protein no longer accumulated after the roots were returned to control temperatures. On days after the stress, HSP21 gradually declined, persisting longer in tissues stressed to higher temperatures. The production of HSP21 therefore appears to be regulated similarly in roots and leaves.

To estimate the relative amounts of HSP21 in roots and leaves, the maximum level of HSP21 at each stress temperature was compared in roots and leaves. Fig. 7 *A* shows that as a percentage of total cell protein, HSP21 accumulates in roots to only 22–30% of the level seen in leaves. The lower level of HSP21 accumulation in roots does not appear to be the result of a decreased response of roots to heat stress. When the same protein samples are reacted with anti-HSP70 antiserum, two molecular mass forms of HSP70 can be distinguished, both of which are more abundant in roots than in leaves. A polypeptide of ∼72 kD appears only after heat shock, and a 70-kD polypeptide was constitutively present and also increased during heat shock.

HSP21 mRNA Levels and HSP21 Stability

We were interested to determine if HSP21 accumulation was proportional to the amount of HSP21 mRNA and to estimate the half-life of the protein. HSP21 mRNA levels were examined by Northern analysis of total leaf and root RNA. Northern blots were probed with the HSP21 cDNA and a cDNA encoding HSP70 as shown in Fig. 8 for the 38°C treatment. The presence of equal quantities of intact RNA in all lanes was verified by probing blots with a cDNA which detected a constitutively expressed transcript (not shown). To enable direct comparison of mRNA levels in leaves and roots, equal quantities of total RNA from both tissues were analyzed on the same blot. HSP21 mRNA was barely detectable at 10 AM when the tissues had reached 30°C and it increased to maximum levels by noon. HSP21 mRNA levels remained high throughout the 38°C treatment and then declined to the point that mRNA was essentially absent the morning after the stress. A similar pattern of mRNA levels was observed for all temperature treatments and proportionally more RNA accumulated at 40°C and less at 36 and 34°C (not shown). HSP21 mRNA levels were not significantly different in leaf and root tissues when compared as a percentage of total mRNA. Similar results were obtained when poly(A)RNA samples were compared (not shown). Interestingly, HSP21 mRNA levels were equally high during the first and second 2-h periods of the stress treatment, although the rate of accumulation of the protein was significantly higher during the first 2 h of stress. These results suggest there is either increased turnover of HSP21 or decreased translation of HSP21 mRNA during the second 2 h of stress.

HSP70 mRNA showed a similar pattern of expression as the HSP21 mRNA (Fig. 8). The level of HSP70 mRNA was clearly higher in roots than in leaves, similar to what was observed for the protein. The HSP70 probe used is specific to a heat-induced mRNA and does not detect mRNA in control samples. It may encode the 72-kD HSP70 which appears
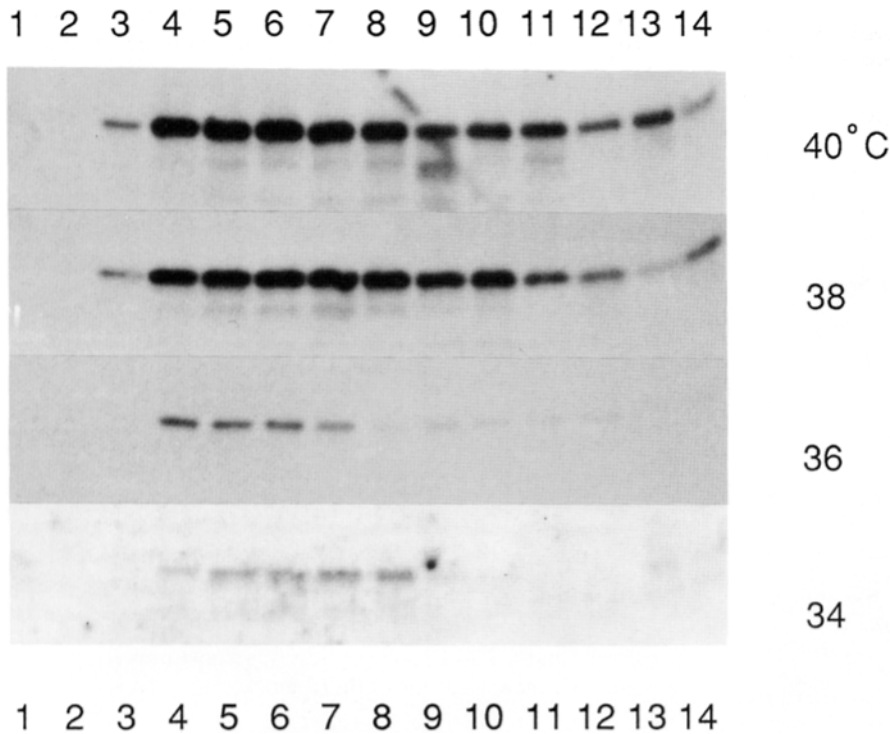
40°C

38

36

34

Figure 5. Accumulation of HSP21 in heat-stressed root tissue. Autoradiograms showing immunoblots of total root proteins reacted with HSP21 antibodies. Total root proteins were prepared from plants subjected to the 34, 36, 38, or 40°C stress treatments as diagrammed in Fig. 1. The maximum temperature stress for each sample set is shown at the right. 40 μg of total root protein were loaded per lane. Lane *1*, Control roots before stress treatment; lane *2*, midpoint of increase to maximum temperature; lane *3*, beginning of maximum temperature; lane *4*, 2 h of maximum temperature; lane *5*, end of maximum temperature; lane *6*, midpoint of decrease to control temperature; lane *7*, end of stress treatment (return to normal temperature); Lanes *8-14*, roots from 1, 2, 3, 4, 5, 6, or 7 d after the stress treatment, respectively.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

during heat stress. These results further support the observations at the protein level that heat stress differentially affects the expression of HSP70 and HSP21 in the two organs.

Given the extremely low levels of HSP21 mRNA on the day after the stress treatment (<1/100 of the maximum levels), we have assumed that new synthesis of HSP21 has essentially stopped at this time. Therefore, the decrease in

HSP21 during the following week can be used to estimate the half-life of the protein under normal temperature conditions. Using the data for 38°C from Fig. 3, the best fit exponential curve was derived for points from day one through seven after heat stress. The resulting half-life was estimated to be ∼52 h (SD 12.7 h). Similar estimates were derived using data from other temperatures. There was also no significant difference in the estimated protein half-life in roots.
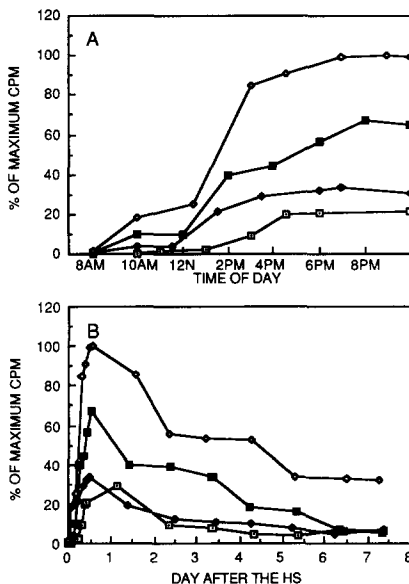
HSP21 Is Found Only in Chloroplasts in Leaves

In the above experiments, detection of HSP21 in the mature, processed form in leaf tissue, strongly indicates that the protein has been translocated across a membrane. In previous experiments, HSP21 has been shown to localize to chloroplasts in vivo (Vierling et al., 1986). However, none of these experiments rule out the possibility that HSP21 is transported into other cellular compartments, in particular the mitochondria. Transit peptides for chloroplast and mitochondrial proteins have similar characteristics and chloroplast transit peptides have been used to direct proteins to mitochondria in yeast (Hurt et al., 1986). To demonstrate that all of the HSP21 in leaves is localized within the chloroplast, we compared the amount of HSP21 in protein samples from chloroplasts and whole leaf tissue. Comparisons were made on the basis of sample chlorophyll content. Because all of the chlorophyll in a leaf is contained within the chloroplast, leaf and chloroplast samples containing equal amounts of chlorophyll will also contain approximately equal numbers of chloroplasts.

Chloroplasts were isolated from plants treated at 38°C for 4 h (4 PM timepoint in Fig. 1), protease treated, and reisolated. Total leaf protein samples were prepared from identically treated plants. Chloroplast and total leaf protein samples representing 2.0 μg of chlorophyll were analyzed on
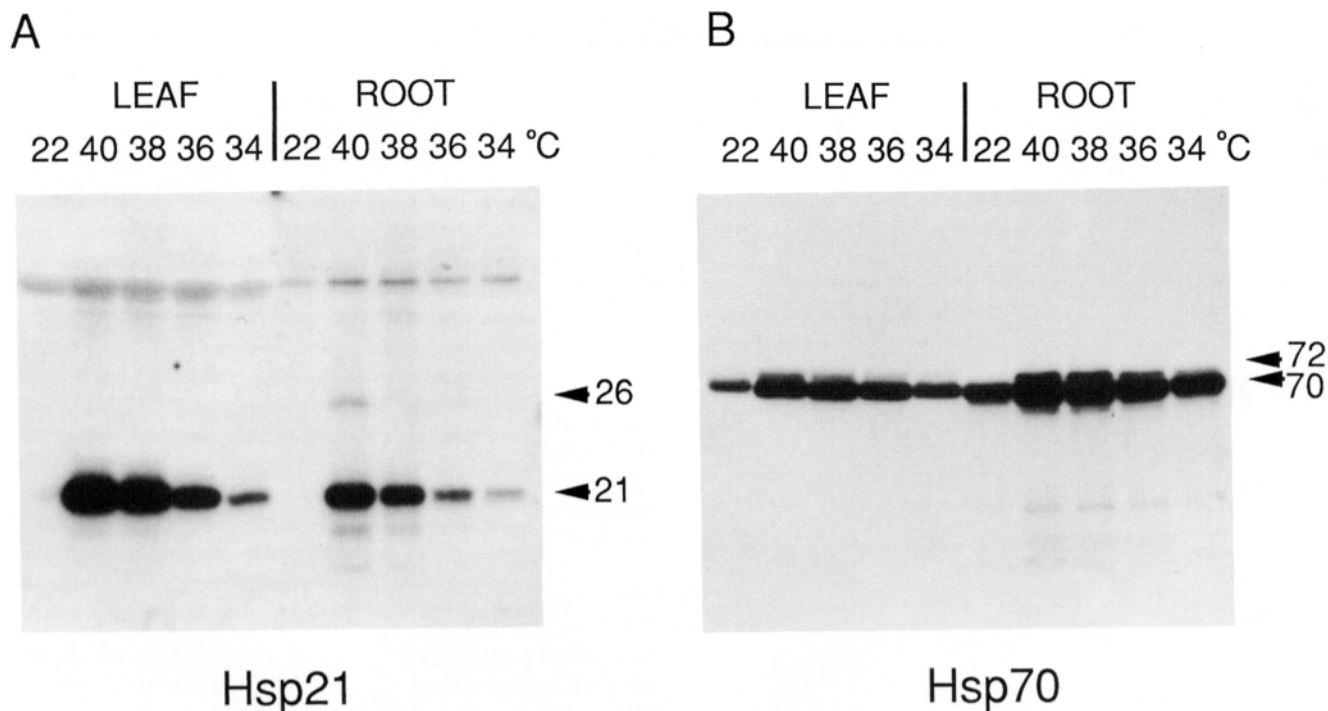

Figure 6. Quantification of HSP21 during heat stress and recovery in roots. All values are expressed as a percentage of the maximum ^{125}I cpm obtained in the 40°C stress experiment. (*A*) Levels of HSP21 during the day of stress at different maximum temperatures. (*B*) Levels of HSP21 from the stress day through 7 d after the stress. 34°C, □; 36°C, ◆; 38°C, ■; 40°C, ◇.

A

LEAF | ROOT
22 40 38 36 34 | 22 40 38 36 34 °C

◄26

◄21

Hsp21

B

LEAF | ROOT
22 40 38 36 34 | 22 40 38 36 34 °C

◄72
◄70

Hsp70

Figure 7. Relative levels of HSP21 and HSP70 in roots and leaves stressed to different maximum temperatures. 40 μg of protein from leaves or roots isolated at the point of maximum HSP21 accumulation for each temperature stress treatment were analyzed. (*A*) Autoradiogram of immunoblot reacted with HSP21 antibodies. (*B*) Autoradiogram of immunoblot reacted with HSP70 antibodies. Samples in *B* were separated on 7.5% polyacrylamide gels.

immunoblots which were reacted with HSP21 antibodies. Fig. 9 *A* shows that essentially equal quantities of HSP21 are detected in the leaf and chloroplast samples. Similar results were obtained in a duplicate experiment, and the ^{125}I cpm

of the excised reactive band was 5,227 ± 112 for the total leaf sample and 5,575 ± 172 for the chloroplast protein sample. When identical samples were probed with antibodies against spinach FNR (Fig. 9 *B*) or chloroplast ATPase (not shown),

A   LEAF

B   ROOT

◄ Hsp70

◄ Hsp21
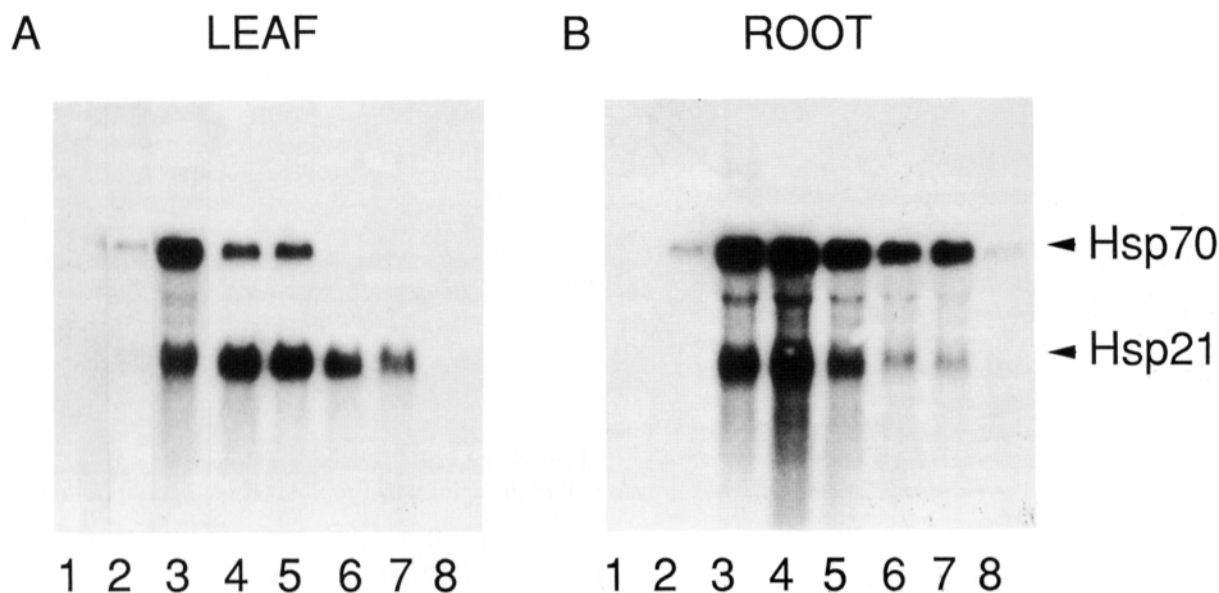
1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8

Figure 8. HSP21 mRNA expression in leaves and roots. Autoradiogram of Northern blot probed with HSP21 and HSP70 cDNAs. 10 μg of total RNA from leaves or roots subjected to the 38°C stress treatment were loaded in each lane. RNA was isolated from leaves and roots at the following timepoints: Lanes *1*, before the temperature increase; lanes *2*, the midpoint of the temperature increase; lanes *3*, *4*, and *5*, the beginning, middle, or end of the maximum temperature period, respectively; lanes *6*, the middle of the temperature decrease; lanes *7*, when plants had returned to normal temperature, and lanes *8*, at 8 AM on the morning after the stress.
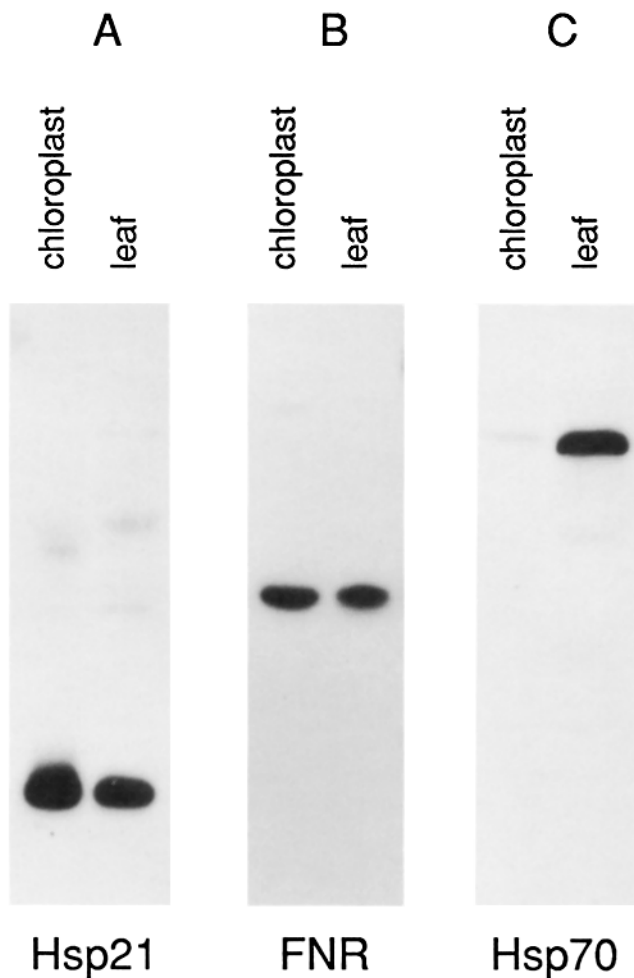
A   B   C

Hsp21   FNR   Hsp70

Figure 9. HSP21 in leaves is found only in chloroplasts. Total chloroplast protein or total leaf protein samples containing 2 μg of chlorophyll were analyzed by electrophoresis and immunoblotting. (*A*) Autoradiogram of samples reacted with HSP21 antibodies. (*B*) Autoradiogram of samples reacted with FNR antibodies. (*C*) Autoradiogram of samples reacted with HSP70 antibodies. Samples in *C* were separated on 7.5% polyacrylamide gels.

equal quantities of these proteins were also detected in both samples. As a control to demonstrate that cytoplasmic proteins are not present in the chloroplast sample, the same samples were reacted with HSP70 antibodies (Fig. 9 *C*). The major polypeptides which reacted with the HSP70 antibodies in leaves were completely absent from the chloroplast sample. A minor polypeptide in the chloroplast sample which reacted with the HSP70 antibodies was of a higher molecular weight than the major leaf HSP70s. This polypeptide has recently been shown to be a specific chloroplast-localized HSP70 homologue (Marshall et al., 1990). We conclude that all the HSP21 is localized to the chloroplast.

HSP21 Is Found in the Soluble Fraction of the Chloroplast

To understand the function of HSP21 it is important to determine where the protein is localized within the chloroplast. Glaezinski and Kloppstech (1988) have reported that the major pea chloroplast HSP binds to thylakoids at temperatures

above 38°C, while we have consistently observed the protein in the soluble chloroplast fraction (Vierling et al., 1986, 1989). We have performed several fractionation experiments to reinvestigate HSP21 localization.

Intact chloroplasts were isolated from plants which had been treated for 4 h at 38°C (4 PM timepoint, Fig. 1). They were then lysed and separated into soluble, membrane, and membrane wash fractions. Aliquots from each fraction, representing protein from an equal quantity of chloroplasts, were analyzed by electrophoresis and immunoblotting. Fig. 10 shows identical samples stained with Coomassie blue or reacted with anti–HSP21, anti–chloroplast ATPase, or anti–FNR antibodies. Under these lysis and fractionation conditions, ∼89% of the HSP21 was recovered in the soluble protein fraction (9586 cpm), 8% in the membrane fraction (879 cpm), and 3% in the membrane wash fraction (349 cpm). The majority of the chloroplast ATPase, a peripheral membrane protein, was retained on the thylakoids. FNR, which is functionally associated with thylakoid components, was found distributed between the three fractions consistent with previous observations, showing that a percentage of FNR is released during osmotic shock and the remaining percentage consists of loosely and tightly bound membrane pools of enzyme (Matthijs et al., 1986). These data indicate that HSP21 is either a soluble protein, or bound to the membrane more loosely than ATPase, or the loosely bound pool of FNR. Additional membrane washes using excess EDTA removed a greater percentage of the chloroplast ATPase, but had little effect on the remaining HSP21. We suggest that the residual membrane–associated HSP21 represents protein trapped in thylakoid vesicles or cross-linked to membrane components during lysis.

To investigate further whether HSP21 may form some type of association with membranes, we have tried other chloroplast lysis conditions in an attempt to preserve such associations. Chloroplasts were lysed in 10 mM Hepes, pH 8.0, 1.0 mM 2-mercaptoethanol at a range of $MgCl_2$, and NaCl concentrations including: (*a*) 5 mM $MgCl_2$, 0 mM NaCl, (*b*) 5 mM $MgCl_2$, 250 mM NaCl, (*c*) 5 mM $MgCl_2$, 500 mM NaCl, (*d*) 10 mM $MgCl_2$, 500 mM NaCl, (*e*) 20 mM $MgCl_2$, 500 mM NaCl, and (*f*) 0.75 mM EDTA, 500 mM NaCl. Chloroplasts were also lysed in unbuffered H_2O or 5 mM $MgCl_2$. Under all these conditions >80% of the HSP21 was recovered in the soluble protein fraction of the chloroplast (not shown). The same distribution of HSP21 was also observed when chloroplasts were isolated during the stress recovery period, from plants treated at 40°C, or when plants were subjected to an abrupt heat stress treatment of 38 or 40°C for 2 h (not shown).

Discussion

The synthesis of LMW HSPs which localize to chloroplasts is a recently recognized component of the heat-shock response in higher plants and *Chlamydomonas* (Kloppstech et al., 1985; Vierling et al., 1986). These LMW HSPs are homologous to LMW cytoplasmic HSPs of plants and other eukaryotes (Vierling et al., 1988). As initial steps toward determining the function of the LMW chloroplast HSPs, we have examined the conditions which elicit their synthesis, the organ specificity of their expression, and their localization within the chloroplast. In our studies, intact plants and
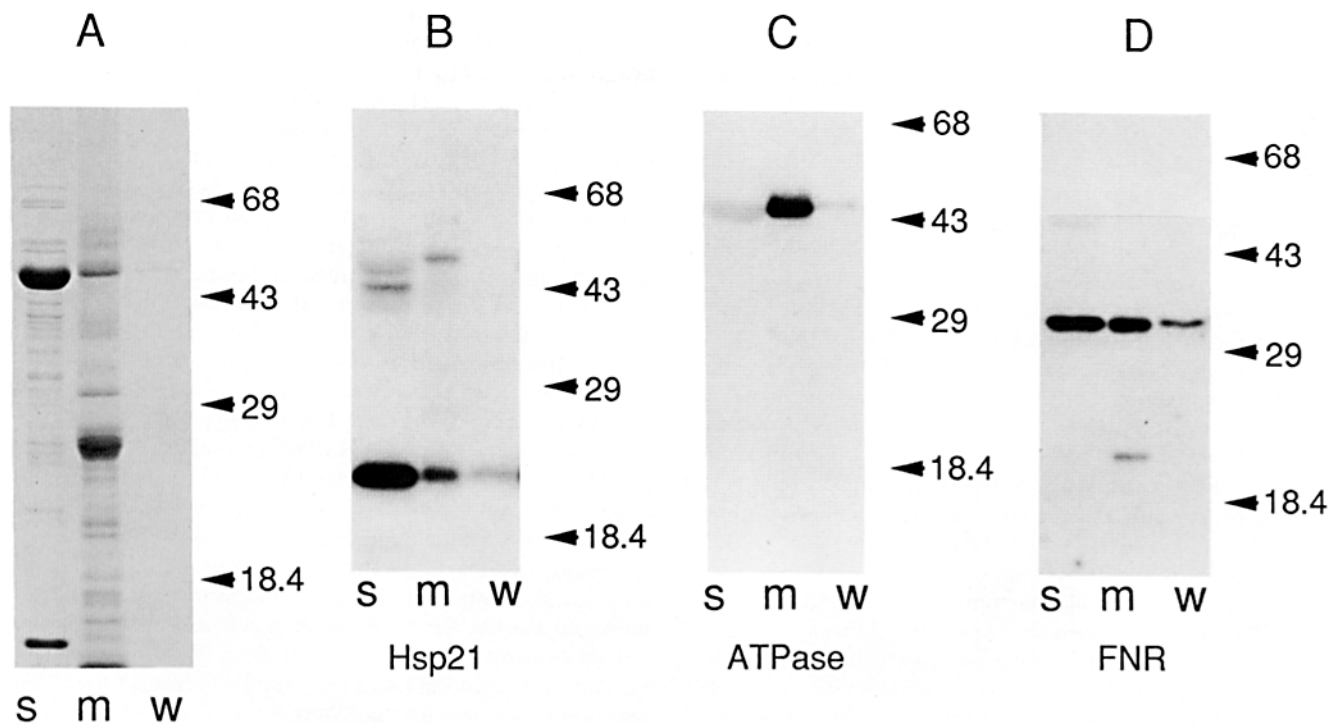
A   B   C   D

◀68

◀43

◀29

◀18.4

s m w

◀68

◀43

◀29

◀18.4

s   m   w

Hsp21

◀68

◀43

◀29

◀18.4

s   m   w

ATPase

◀68

◀43

◀29

◀18.4

s   m   w

FNR

Figure 10. HSP21 is localized to the soluble fraction of the chloroplast. Chloroplasts were isolated from plants stressed at 38°C and separated into soluble, membrane, and membrane wash fractions. Samples of each fraction representing an equal proportion of the chloroplasts (33 μg of soluble, 72 μg of membrane, and 4.8 μg of membrane wash protein) were analyzed by electrophoresis and immunoblotting. (*A*) Coomassie blue stained gel. (*B*) Autoradiogram of immunoblot reacted with HSP21 antibodies. (*C*) Autoradiogram of immunoblot reacted with chloroplast ATPase antibodies. (*D*) Autoradiogram of immunoblot reacted with FNR antibodies. *s*, Soluble fraction; *m*, membrane fraction; *w*, membrane wash fraction. Molecular mass in kilodaltons is shown at right of each section.

gradual temperature stress conditions were used, providing information relevant to the expression of chloroplast HSPs in the natural environment.

The rate of HSP synthesis in plants and other eukaryotes has been shown to be directly proportional to the temperature of the applied stress (Nagao et al., 1986; Lindquist and Craig, 1988). Our experiments showed that the accumulation of HSP21 in plant tissues was also proportional to temperature and that, in leaves, 10-fold more protein accumulated at 38°C than at 34°C. HSP21 was detected in as little as 0.5 μg of total protein from leaves stressed at 38°C, but was not detected in as much as 100 μg of total protein from controls. Therefore HSP21 accumulated >200-fold during stress. In leaves, all of this protein accumulated in its mature processed form in the chloroplasts. Field measurements of plant temperatures (Ehrler, 1973; Gardner et al., 1981) indicate that the types of temperature changes used in our experiments are similar to what may be experienced by plants growing in the natural environment, although temperatures >35°C represent extreme stress conditions.

Currently, purified HSP21 is not available to use as a standard for quantitation of HSP21. However, the amount of HSP21 which accumulates per chloroplast can be approximated if it is assumed that the immunoblotting technique can detect 0.05–0.1 ng of antigen. This is the lower limit of detection generally reported for immunoblotting methods (Oberfelder, 1989). Assuming this level of sensitivity, combined with the observation that HSP21 was seen in as little as 0.5 μg of leaf protein from 38°C stressed leaves, it can be estimated that the protein accumulated to 0.01–0.02% of the total

leaf protein. This estimate is consistent with our inability to detect HSP21 on Coomassie blue stained gels of heat stressed leaf or chloroplast proteins. We have measured the amount of protein in chloroplasts to be 40% of the total leaf protein and determined that an individual pea chloroplast contains on the average 11.14×10^{-6} μg of protein and 1.0×10^{-6} μg of chlorophyll. If HSP21 is equally distributed among leaf chloroplasts, the amount of HSP21 would be ~1 × 10⁵ molecules per chloroplast at 38°C and 1 × 10⁴ molecules per chloroplast at 34°C. These HSP21 levels are low relative to other chloroplast components, such as photosystems I and II (each ~1 × 10⁶ per pea chloroplast). We suggest that HSP21 is not abundant enough to have a structural role in the chloroplast, and is more likely to have a catalytic role in stress protection or recovery.

Our data indicate that HSP21 is not found in control tissues although we cannot rule out that HSP21 is present at 10–100 molecules per chloroplast. LMW cytoplasmic HSPs are constitutively present in mammalian cells, and also increase during heat stress (Arrigo et al., 1988). In *Drosophila* and yeast, LMW HSPs are not constitutively expressed, but are expressed during development or at specific growth phases in the absence of heat stress (Arrigo, 1987; Rossi and Lindquist, 1989). In contrast, other major classes of HSPs, HSP90, HSP60, and HSP70 or HSP70 homologues, are constitutive proteins in most cells, in addition to increasing during stress treatments. We have not yet identified conditions other than stress which lead to the production of chloroplast HSP21. Furthermore, there is no evidence that distinct proteins with homology to HSP21 are present in the chloroplast

under normal growth conditions. Therefore, we conclude that the major function of HSP21 is specifically required by organelles subjected to stress.

The stability of HSPs after heat stress has not been extensively examined. Pulse-labeling of etiolated soybean seedlings showed that LMW cytoplasmic HSPs were still present 24 h after the stress treatment (Nagao et al., 1986). We had previously shown HSP21 was still abundant 12 h after heat stress (Vierling et al., 1989). In mammals, HSP28 remains at elevated levels at least 12 h after stress (Arrigo et al., 1988). However, these studies did not examine later timepoints after the stress nor determine the half-life of these HSPs. We were able to estimate the half-life of HSP21 without invasive treatment of the plant tissues. These half-life measurements describe how the protein behaves at normal leaf temperatures; the half-life of HSP21 during the stress period was not estimated and may be different. The relatively long half-life of HSP21 at control temperatures suggests HSP21 function may be as critical during the recovery period as it is during the stress period.

Although HSP21 mRNA had previously been shown to be induced in etiolated soybean hypocotyls (Vierling et al., 1986) and in etiolated pea leaves (Kloppstech et al., 1985), the present study provides the first data showing HSP21 is synthesized in tissues which contain nonphotosynthetic plastids. Relative to temperature and mRNA levels, the pattern of accumulation and stability of HSP21 in root tissues was very similar to that seen in leaves, indicating the basic mechanisms controlling HSP21 production are similar in both tissues. Only the mature, 21-kD form of the protein accumulated to significant levels in roots, suggesting that the protein was transported into root plastids and correctly processed. In cell fractionation experiments HSP21 is significantly enriched in the root plastid fraction (Chen, Q., unpublished data). The presence of HSP21 in roots argues that the protein is essential to processes which occur in both photosynthetic and nonphotosynthetic plastids. This does not rule out a role for HSP21 in protecting photosynthesis; HSP21 may protect more than one plastid function.

Although the relative pattern of HSP21 accumulation and the protein's half-life were similar in roots and leaves, the amount of HSP21 in roots was 22–30% of that in leaves. This is not the result of a less vigorous response of roots to heat stress; HSP70 is synthesized to higher levels in roots than in leaves. The difference in the relative expression of HSP21 and HSP70 suggests there is some degree of tissue-specific control of the heat shock response in these two organs. There are 50–150 chloroplasts per leaf mesophyll cell (Mullet, 1988), and in pea, chloroplasts contain ~40% of total leaf protein. In contrast, there are only 10–30 plastids per root cell (Juniper and Clowes, 1965). The amount of total cell protein present in root plastids has not been estimated, but is likely to be significantly <40%. Thus, it is not surprising that roots produced lower levels of HSP21, although the actual amount of HSP21 per plastid may be similar to what is found in leaves. Whether this decreased expression is controlled transcriptionally or posttranscriptionally remains to be investigated.

Glaczinski and Kloppstech (1988) studied the localization of HSP21 synthesized in vitro and transported into chloroplasts isolated from heat-stressed pea plants. When plants had been treated for 2 h at 38°C or above, all the imported

HSP21 associated with the thylakoids. Using chloroplasts from plants treated at temperatures below 38°C, they found HSP21 in the soluble fraction. In contrast, our data indicate that HSP21 is a soluble component of the chloroplast stroma at 38°C and 40°C. It is possible that we have not reproduced the stress conditions, or chloroplast fractionation conditions, under which these workers observed thylakoid association. Alternatively, protein imported in vitro may behave differently from in vivo synthesized HSP21. In Chlamydomonas a major chloroplast HSP has been reported to be associated primarily with thylakoids and to bind and protect components of photosystem II (Schuster et al., 1988). However, comparison of the Chlamydomonas HSP sequence (Grimm et al., 1989) with pea HSP21 indicates these proteins have limited homology. Thus, it is possible that the Chlamydomonas thylakoid associated HSP is functionally distinct from pea HSP21. We conclude that under most stress conditions which would be experienced by plants, HSP21 is not tightly associated with thylakoids.

Although LMW HSPs have been observed in all eukaryotes during heat stress, and are thought to be essential for thermotolerance, their molecular mechanism of action has not yet been determined. The homology of the LMW cytoplasmic and chloroplast HSPs clearly points to a similar function for these two groups of proteins. We have also found that HSP21 is complexed into a 200-kD particle in the chloroplast stroma (Chen, Q., unpublished data), providing further evidence for functional homology with the cytoplasmic LMW HSPs which form similar particles (Arrigo, 1987; Arrigo et al., 1988; Collier et al., 1988; Rossi and Lindquist, 1989; Nover et al., 1989). It is interesting to note that the HSP90, HSP70, and HSP60 classes of proteins have all been implicated in functions related to protein assembly or the alteration/maintenance of specific protein conformations (Lindquist and Craig, 1988). Perhaps the LMW HSPs have a similar function. Because chloroplasts contain only a single major LMW HSP they provide a simple model system for studies of the function of this ubiquitous class of HSPs.

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