

NUCLEAR-ENCODED CHLOROPLAST PROTEINS ACCUMULATE IN THE CYTOSOL DURING SEVERE HEAT STRESS

Scott A. Heckathorn,¹ Craig A. Downs, and James S. Coleman²

Department of Biology, Syracuse University, 130 College Place, Syracuse, New York 13244, U.S.A.

Localization of proteins to chloroplasts is thought to be unaffected by heat stress, and this idea has influenced the interpretation of heat stress effects on photosynthesis. Here we show that a nuclear-encoded plastid heat-shock protein (Hsp) accumulated in the cytosol of plants that were grown at 27°C and then heat-stressed at 43°C but accumulated in the chloroplast during heat stress at 37°C. Immunological evidence indicated that other nuclear-encoded chloroplast proteins also accumulated in the cytosol at 43°C but accumulated in the chloroplast at 37°C. Radiolabeling detached leaves with ³⁵S-met confirmed that newly synthesized, nuclear- and chloroplast-encoded proteins accumulated in the chloroplast at 36°C, while at 46°C the chloroplast-encoded proteins accumulated in the chloroplast, but the nuclear-encoded proteins (including Hsps) accumulated in the cytosol. Cytosolic accumulation of nuclear-encoded chloroplast proteins was readily reversible (e.g., within 1–3 h) on return to normal growth temperatures and was observed in evolutionarily and functionally diverse Anthophyta species (C₃, C₄, CAM, monocot, and dicot). These results are the first solid evidence that newly synthesized nuclear-encoded chloroplast proteins accumulate in the cytosol (i.e., localization is disrupted) during severe, but not moderate, heat stress. This has important implications for understanding the effects of heat stress on photosynthesis and may be relevant to other stresses or to proteins localized to other organelles.

Introduction

Many chloroplast proteins are coded for in the nucleus, synthesized on cytosolic ribosomes, and then imported into chloroplasts where a targeting/transit sequence is cleaved off (Keegstra et al. 1995; Schatz and Dobberstein 1996). The *in vivo* localization of proteins to chloroplasts did not appear to be affected by moderate heat stress (i.e., <40°C) in pea (Vierling and Key 1985) or by severe heat stress (50°C) in heat-acclimated bean plants (Süss and Yordanov 1986), and *in vitro* import of proteins at 25°C into chloroplasts isolated from pea plants stressed at 45°C was observed (Vierling et al. 1986). These results indicate that localization of nuclear-encoded chloroplast proteins is not affected by heat stress.

However, apparent accumulation of the pre-import isoforms of nuclear-encoded mitochondrial (Debel et al. 1994, 1997) and chloroplast proteins (Halle et al. 1990; Wang et al. 1993) has been observed during heat stress in whole-leaf, -seedling, or -cell extracts at ≤40°C, indicating that these proteins were accumulating in the cytosol and, therefore, that localization of these proteins was impaired. The possibility of impaired localization during heat stress was discussed in two of these articles (Wang et al. 1993; Debel et al. 1994) but dismissed as unlikely in one (Wang et al. 1993), given the results of earlier studies (Vierling and Key 1985; Vierling et al. 1986). We obtained additional evidence hinting of impaired localization in the course of characterizing an antibody specific to a small nuclear-encoded chloroplast heat-shock protein (Hsp;

Downs et al. 1997). Specifically, we observed that in plants grown at 27°C and then heat-stressed at 43°C, the protein with which the antibody reacted accumulated in the chloroplast in some species, as expected; however, in other species, the reactive protein was not localized to the chloroplast (unpublished results). This indicated to us the possibility that localization of this chloroplast Hsp to the plastid was impaired in certain species in our preliminary experiments.

To investigate whether localization of nuclear-encoded proteins to chloroplasts may be disrupted under certain circumstances during heat stress, we first examined localization of the methionine-rich low-molecular-weight chloroplast heat-shock protein (lmw chl p Hsp) in plants shifted from 27°C to either 37°C or 43°C (here referred to as moderate and severe heat stress, respectively). This small Hsp, ranging in mass from ca. 21–24 kD (Vierling 1991), depending on species, is the same protein discovered by Vierling (Vierling et al. 1986; see Downs et al. 1997). The lmw chl p Hsp is an ideal protein with which to address this problem because it is a nuclear-encoded plastid-localized Hsp that is synthesized only in response to heat stress (i.e., heat-induced), thereby eliminating the need to separate effects of heat stress on newly synthesized protein from effects on previously existing protein (Vierling et al. 1986; Chen et al. 1990; Vierling 1991; Heckathorn et al. 1996a, 1996b; Downs et al. 1997). In addition, the lmw chl p Hsp is very heat responsive, thermostabile, and long-lived. At the same time that we studied lmw chl p Hsp localization, we also examined whether localization of certain other nuclear-encoded chloroplast proteins was disrupted during heat stress.

Material and Methods

Plants and Growth Conditions

Seeds of *Lycopersicon esculentum* Mill. cv. Condine Red (tomato) were provided by the C. M. Rick Tomato Genetics Resource Center (University of California, Davis, Calif.,

¹Author for correspondence and reprints; present address: Department of Biology, University of Charleston, 58 Coming Street, Charleston, South Carolina 29424, U.S.A.; fax 803-953-5453; E-mail heckathorns@cofc.edu.

²Present address: Desert Research Institute, 7010 Dandini Blvd., Reno, Nevada 89506, U.S.A.

Manuscript received May 1997; revised manuscript received September 1997.

U.S.A.), and seeds of *Zea mays* L. cv. LH74×LH163 (corn) were obtained from Holden's Foundation Seeds (Stanton, Minn., U.S.A.). Adult specimens of *Senecio serpens* K. (chalkstick cactus) were donated by Kingwood Gardens (Mansfield, Ohio, U.S.A.).

All plants were grown in pots in a greenhouse for at least 6 wk before use. Plants were placed in growth chambers 7 d before use under 27°C/14-h d at ca. 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density and 20°C/10-h nights. Depending on the species and experiment, plants were heat stressed by gradually increasing temperature from 27°C to either 36°C, 37°C, 42°C, 43°C, or 46°C (see figure legends) over a period of 2 h (beginning at 7:00 AM, 1 h postdawn) and then maintained at elevated temperature for 8 h. Leaf tissue was harvested at this time, rather than earlier, in order to maximize cytosolic levels of chloroplast proteins. In a separate experiment, tomato plants were heat stressed at 43°C for 6 h (at which time tissue was harvested) and then returned to 27°C (during which time tissue was again harvested).

Whole-Tissue and Chloroplast Preparation

Leaf tissue samples from both nonheat-stressed and heat-stressed plants ($n = 3$ for each species per treatment) were harvested, ground in liquid N_2 , and then extracted in buffer containing 100 mM Tris-HCl (pH 8.0), 2% SDS (w/v), 0.1% DTT (w/v), 1 mM PMSF, 5 μM leupeptin, 5 mM ϵ -amino caproic acid, 1% ascorbate (w/v), and 3 mM Na_2EDTA . Depending on the species, 2%–4% (w/v) of both PVP and PVPP was used to remove phenolics. Samples were boiled for 3 min and then centrifuged at $14,000 \times g$ for 5 min. The supernatant was collected and stored at -20°C .

Chloroplasts were isolated by Percoll step-gradient centrifugation using a protocol modified from Gegenheimer (1990) as in Downs et al. (1997). Leaf tissue was homogenized using a Waring blender or a mortar and pestle in chloroplast buffer (CB) consisting of 0.33 M sorbitol, 10 mM MgCl_2 , 10 mM MnCl_2 , 0.1% DTT (w/v), 1% ascorbate (w/v), 3 mM EDTA, and 50 mM HEPES/KOH buffer (pH 7.9). Depending on the species, 1–6% PVP (w/v) was included. The homogenate was filtered through a nylon mesh membrane. Homogenates were centrifuged at $1500 \times g$ for 10 min. The supernatant was poured off and the pellet was suspended in CB.

Chloroplast suspensions were layered on the Percoll gradients and centrifuged for 15 min in a swinging-bucket rotor at 7000 – $11,000 \times g$, depending on the species. Percoll solution consisted of 1% Ficoll 400 (w/v), 0.1% BSA (w/v), and 2%–4% polyethylene glycol 8000 (w/v), all dissolved in Percoll. Percoll step-gradients contained 85%, 65%, 45%, and 25% steps of Percoll solution dissolved in CB. After centrifugation, the lower, most rapidly sedimenting green band in the Percoll gradient was collected and protease-treated with trypsin ($20 \mu\text{g mL}^{-1}$) and isolated as described by Marshall et al. (1990). Visual microscopic inspection of samples from both upper and lower green bands indicated that intact chloroplasts were largely confined to the lower band and that ca. 80%–90% of chloroplasts in this band were intact; this was confirmed by immunoblot analysis of the content of representative chloroplast stromal proteins (see results). Isolated chloroplasts were pelleted, lysed by suspension in the extraction buffer used for whole-plant preparations, frozen in liquid N_2 , and then boiled for 3 min. Isolated chloroplast extracts were stored at -20°C and centrifuged for 2 min at $14,000 \times g$ before use.

Electrophoresis and Immunoblotting

Aliquots of each sample containing equal amounts of total protein (determined by either BioRad Bradford assay [Bradford 1976] or following the protocol of Ghosh et al. 1988) were fractionated on 12.5% SDS-PAGE gels (Laemmli 1970) and electrophoretically transferred to PVDF membranes (Towbin et al. 1979). For all experiments save one, 80 μg of total protein per sample was loaded per lane, in order to facilitate resolution of both pre- and post-import isoforms of nuclear-encoded chloroplast proteins. Following transfer of proteins to membranes, blots were incubated in nonfat dry milk for 2 h and then incubated with antiserum for 12 h at 4°C . Antiserum specific to the chloroplast lmw Hsp was produced as described in Downs et al. (1997); all other antibodies were provided by other researchers (see acknowledgments). Protein-antibody complexes were detected using secondary antibodies conjugated to alkaline-phosphatase, followed by reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Molecular mass standards were included on all gels.

In Vivo Radiolabeling of Proteins

Lycopersicon esculentum plants were grown in pots as above. Detached leaves (cut under water) were placed (petiole downward) in glass culture tubes (12×75 mm) containing 50 μCi of ^{35}S -met in 0.5 mL of distilled water (specific activity = 1209 Ci/mmol; Trans ^{35}S -label, ICN Pharmaceuticals, Irvine, Calif., U.S.A.). The culture tubes with leaves were placed in test-tube racks in water baths maintained at either 36° or 46°C . Because of isotope and time limitations (this experiment was conducted in duplicate at the Universities of Illinois and Wisconsin, not in our lab at Syracuse), we chose to heat stress leaves in this experiment at 46°C , rather than at 43°C as in all other experiments, to insure that localization of proteins was disrupted at the high-temperature treatment. The water baths were covered with plastic wrap to permit light penetration (ambient room lighting), yet facilitate heat and isotope retention (this also decreased transpiration and thus isotope uptake to some extent).

Leaves were heat-stressed for 8 h, at which time chloroplasts were isolated and whole-leaf and chloroplast proteins were extracted. Whole-leaf and chloroplast proteins (60 μg per lane) were fractionated by SDS-PAGE (12.5% gels) and then transferred electrophoretically to PVDF membranes. Incorporation of radioactivity into proteins was determined by autoradiography (Biomax-MR film, Eastman Kodak Co., Rochester, N.Y., U.S.A.). Following autoradiography, individual lanes of the same PVDF membrane were then used to ascertain the position of specific proteins (as in Martino-Catt et al. 1993). The position of the DnaK-like heat-shock protein 70 (Hsp70), ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) large and small subunits (RLSU and RSSU, respectively), chaperonin 60 (Cpn60), the 33-kD oxygen-evolving-complex protein of Photosystem II (OEC33), and the low-molecular-weight heat-shock protein (lmw Hsp) was determined by immunoreaction with antibodies to these proteins.

Results and Discussion

In *Lycopersicon esculentum* (tomato), a C_3 dicot, the lmw Hsp (ca. 24 kD) accumulated in chloroplasts of plants grown at 27°C and then heat-stressed at 37°C ; however, this protein did not accumulate in the chlo-

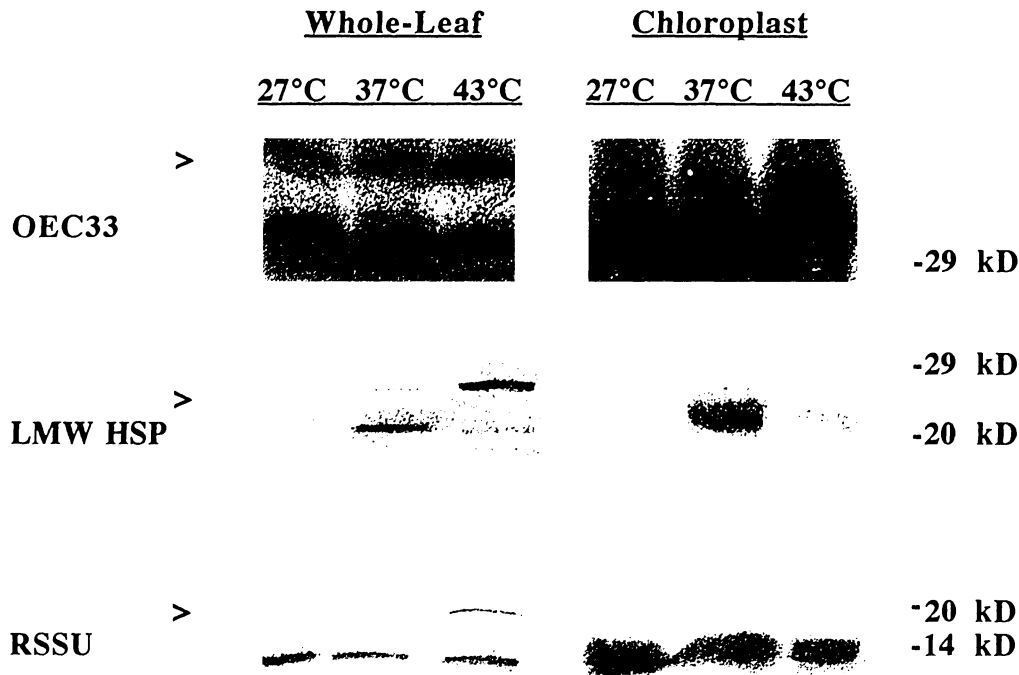


Fig. 1 Immunoblot analysis of localization of the 33-kD oxygen-evolving-complex protein (OEC33), the low-molecular-weight heat-shock protein (*LMW HSP*), and rubisco small subunit (*RSSU*) in leaves of the C_3 dicot, *Lycopersicon esculentum* (tomato). Equal amounts of total whole-leaf and chloroplast protein from plants subjected to moderate (37°C) or severe (43°C) heat stress for 8 h, or from unstressed controls (27°C), were analyzed for content of *OEC33*, *LMW HSP*, and *RSSU*. *OEC33*, the *LMW HSP*, and *RSSU* are all nuclear-encoded; pre-import isoforms of these proteins are indicated with arrows (>). The positions of molecular mass markers are indicated. The relative content of *RSSU*, a stromal protein, in whole-leaf and chloroplast samples was determined, in part, to insure that chloroplast samples were enriched for intact chloroplasts.

roplast at 43°C (fig. 1). The content per unit total protein of *RSSU* (fig. 1), a nuclear-encoded chloroplast stromal protein, and *RLSU* (not shown), a chloroplast-encoded stromal protein (Schmidt and Mishkind 1983; Vierling and Key 1985), was greater in isolated-chloroplast compared with whole-leaf samples; this indicates that chloroplast samples were enriched for intact chloroplasts. Thus, the absence of *lmw Hsp* in chloroplast samples at 43°C was not attributable to ruptured plastids. In support of this, the *lmw chl p Hsp* band on immunoblots from plants stressed at 43°C was shifted several kD higher than the band in plants stressed at 37°C, indicating that the larger pre-import isoform of the *lmw chl p Hsp* was accumulating in the cytosol at 43°C, but the smaller post-import isoform was accumulating at 37°C. Because the *lmw chl p Hsp* is nuclear-encoded, has a transit sequence that is removed following import into the chloroplast, and is produced only in response to heat, these results indicate that localization of this protein to chloroplasts was impaired during heat stress at 43°C but not at 37°C.

Since targeting and import of most nuclear-encoded chloroplast proteins is achieved by similar mechanisms or utilizing common import systems (Keegstra et al. 1995; Schatz and Dobberstein 1996), we hypothesized that localization of many or most other chloroplast proteins, if they are still being synthesized, might be impaired during severe heat stress if localization of the

lmw chl p Hsp is disrupted. To test this, we first examined the relative content of two additional nuclear-encoded chloroplast proteins in whole-leaf and chloroplast samples from control and heat-stressed *L. esculentum* plants (fig. 1). For both the 33-kD protein of the oxygen-evolving-complex (*OEC33*; Ghanotakis and Yocum 1990) and *RSSU*, accumulation of apparent pre-import isoforms of these proteins was observed at 43°C, relative to 27°C or 37°C. That is, using antibodies specific to either *OEC33* or *RSSU*, we observed accumulation of proteins in whole-leaf samples at 43°C, relative to lower temperatures, that were 4–8 kD larger than the proteins detected in isolated chloroplast samples (note that for *OEC33*, the ratio of pre-import to post-import protein content increased with temperature as well). Accumulation of the pre-import isoform of *OEC33* at 27°C was unexpected but may have resulted from the large amount of total protein loaded per gel lane (80 µg), enabling visualization of the pre-import isoform at control temperatures, or may indicate that the leaves used in our analyses were mildly stressed in some way.

These above results are consistent with the prediction that localization of most newly synthesized nuclear-encoded chloroplast proteins may be impaired during severe heat stress. To confirm our immunological data, we pulse-labeled newly synthesized proteins during heat stress with ^{35}S -met and then ascertained

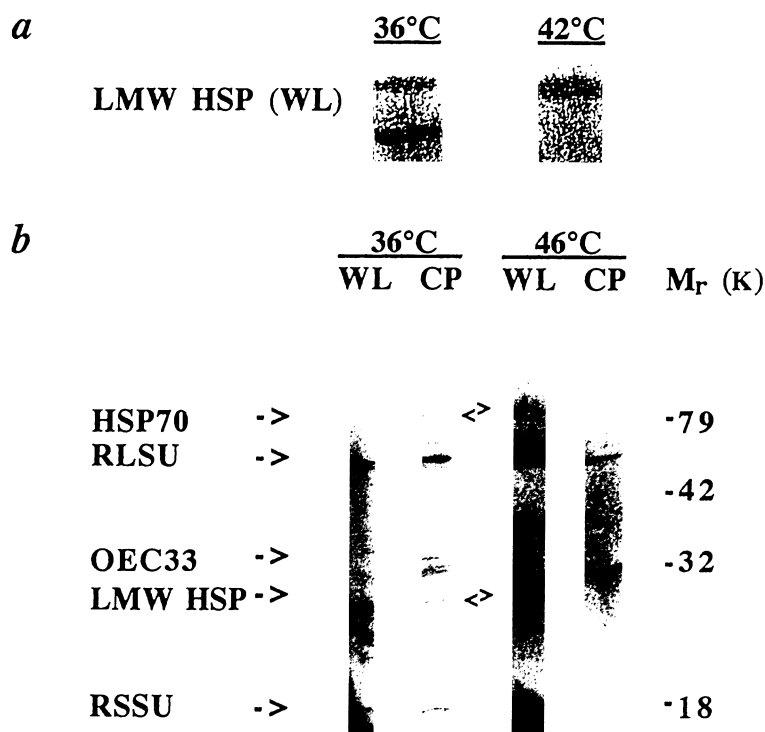


Fig. 2 Localization of newly synthesized chloroplast proteins during moderate (36°C) or severe (42° or 46°C) heat stress in leaves of the C_3 dicot, *Lycopersicon esculentum* (tomato). *a*, Immunoblot analysis of the content of the LMW HSP in equal amounts of whole-leaf (WL) protein extracted from intact plants. *b*, Autoradiogram of WL and chloroplast (CP) proteins from detached leaves radiolabeled during heat stress with ^{35}S -met. The positions of the DnaK-like heat-shock protein 70 (HSP70), rubisco large subunit (RLSU), the 33-kD oxygen-evolving-complex protein (OEC33), the low-molecular-weight heat-shock protein (LMW HSP), and rubisco small subunit (RSSU), determined by immunoblotting, are indicated. The PSII reaction-center protein, D1, is assumed to be located just below OEC33, and chaperonin 60 is assumed to be located in between HSP70 and RLSU. The positions of apparent pre- and post-import isoforms of HSP70 and LMW HSP are indicated with small arrows (>). The positions of molecular mass (kD) markers are also indicated.

whether newly synthesized nuclear-encoded plastid proteins accumulated in chloroplasts during moderate, compared to, severe stress. We utilized *L. esculentum* for the radiolabeling experiment, working with plants that were slightly older (10 wk vs. 8 wk) and grown under somewhat sunnier light regimes in the greenhouse (summer vs. winter) before transfer to growth chambers. Nevertheless, as in the first experiment, we obtained immunological evidence indicating that only the pre-import isoform of the lmw chl p Hsp (upper higher-kD band; fig. 2a) accumulated in whole-leaf samples during severe heat stress in *L. esculentum* but that both pre- and post-import isoforms of this protein accumulated during moderate heat stress in this experiment.

Patterns of incorporation of ^{35}S -met after 8 h into newly synthesized chloroplast proteins in detached leaves were consistent with our immunological data. For protein bands that comigrated with the nuclear-encoded proteins, DnaK-like plastid Hsp70, OEC33, the lmw Hsp, and RSSU, radioactivity per unit total protein was greater in chloroplast samples than in whole-leaf samples at 36°C (fig. 2b). At 46°C, the reverse was observed: for the Hsp70- and lmw Hsp-bands, radioactivity was greater in whole-leaf than in chloroplast samples (OEC33 and RSSU bands were

not detected in either whole-leaf or chloroplast samples at 46°C; others have also found synthesis of these proteins to be relatively thermosensitive; Vierling and Key 1985; Süß and Yordanov 1986). The Hsp70 and lmw Hsp bands were shifted up (relative to RLSU and D1/D2; see below) to higher molecular mass positions (ca. 4–6 kD for each) in whole-leaf compared to chloroplast samples, indicating that these bands represent pre- and post-import isoforms of these proteins (pre- and post-import isoforms were also observed in these positions on the immunoblot; not shown). A similar pattern was observed for the two bands at ca. 60–64 kD (just above RLSU), which are likely to be the two major isoforms of the nuclear-encoded heat-responsive plastid Cpn60 (Vierling 1991; Gatenby and Viitanen 1994), although we could not confirm the location of Cpn60 on this particular immunoblot.

In contrast to Hsp70 and the lmw Hsp, accumulation of ^{35}S -met into a protein band that comigrated with chloroplast-encoded RLSU was greater in chloroplast, compared with whole-leaf, samples at both 36°C and 46°C; similar results were observed for a band at ca. 31 kD that is probably the abundant and rapidly turned-over, chloroplast-encoded D1/D2 proteins of PSII (Süß and Yordanov 1986; Ghanotakis and Yocum 1990). The RLSU and D1/D2 data indicate that

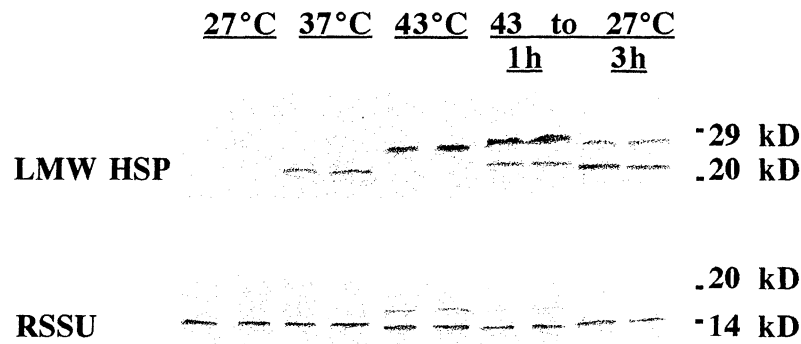


Fig. 3 Immunoblot analysis of localization of the low-molecular-weight chloroplast heat-shock protein (*LMW HSP*) and rubisco small subunit (*RSSU*) in leaves of *Lycopersicon esculentum* (tomato). Equal amounts of total whole-leaf protein from control plants (27°C), plants subjected to moderate (37°C) or severe (43°C) heat stress for 8 h, or from plants stressed at 43°C for 8 h and then transferred to 27°C for either 1 h or 3 h, were analyzed for content of *LMW HSP* and *RSSU*. The positions of molecular mass markers are indicated.

chloroplasts were intact and that chloroplast protein metabolism was not completely disrupted at 46°C. Results from the radiolabeling experiment confirm that newly synthesized nuclear-encoded proteins accumulate in the cytoplasm during severe, but not moderate, heat stress.

To determine if disrupted localization of nuclear-encoded chloroplast proteins was permanent or temporary, whole-leaf samples from tomato plants either experiencing or recovering from heat stress were analyzed for the content of two representative nuclear-encoded proteins, the lmw chl p Hsp and *RSSU* (fig. 3). As expected, at 27°C, lmw chl p Hsp was not detected, but constitutively expressed *RSSU* was detected. At 37°C, lmw chl p Hsp was detected and *RSSU* levels and molecular mass remained unchanged relative to 27°C. At 43°C, the molecular mass of the lmw chl p Hsp was shifted several kD higher relative to 37°C, and a second *RSSU* isoform of slightly greater molecular mass was detected. Following 1 h (for *RSSU*) or 3 h (for lmw Hsp) of post-heat stress recovery at 27°C, levels of the higher molecular mass isoforms of the two proteins decreased. These results

indicate that efficient localization of proteins to the chloroplast was resuming within 1–3 h after heat stress and that impairment of localization was temporary and not associated with irreversible damage to the chloroplast protein import apparatus.

To ascertain if localization of nuclear-encoded chloroplast proteins is impaired at very high temperatures in other evolutionarily and functionally diverse species, we then examined localization of the lmw chl p Hsp during moderate and severe heat stress in *Senecio serpens*, a C_3 /CAM dicot, and *Zea mays*, a C_4 monocot. In both of these species, the post-import isoform of the lmw chl p Hsp accumulated in the chloroplast at 37°C, but the pre-import isoform accumulated in the cytosol at 43°C (fig. 4). That is, at 37°C, the protein that accumulated in whole-leaf samples comigrated with the protein that accumulated in isolated chloroplast samples, and chloroplast samples contained more lmw Hsp per unit total protein than did whole-leaf samples. However, at 43°C, the protein that accumulated in whole-leaf samples was several kD greater in mass and the lmw Hsp was not detected in chloroplast samples, even though these chloroplasts were assayed

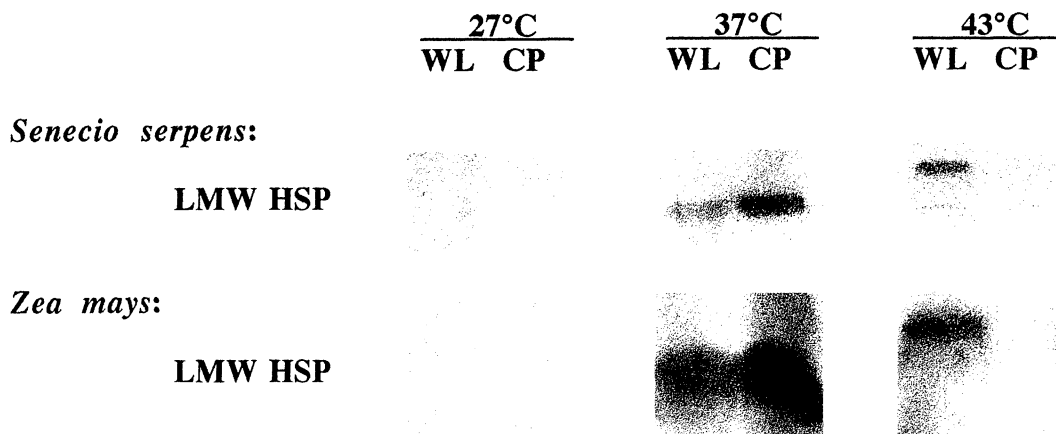


Fig. 4 Immunoblot analysis of localization of the plastid low-molecular-weight heat-shock protein (*LMW HSP*) in leaves of the C_3 /CAM dicot, *Senecio serpens* K. (chalkstick cactus), and the C_4 monocot, *Zea mays* L. cv. LH74xLH163 (corn). Equal amounts of total whole-leaf (WL) and chloroplast (CP) protein from plants subjected to moderate (37°C) or severe (43°C) heat stress for 8 h, or from unstressed controls (27°C), were analyzed for content of *LMW HSP*.

for RLSU content and were determined to be intact (not shown).

Collectively, the results of this study indicate that (1) newly synthesized nuclear-encoded chloroplast proteins accumulate in the cytosol during severe (but not moderate) heat stress (i.e., localization is impaired); (2) this effect is readily reversible on return to lower temperatures; and (3) impaired localization occurs in evolutionarily and functionally diverse species (C_3 , C_4 , CAM, monocot, and dicot Anthophyta species). These results are consistent with previous studies, which observed efficient *in vivo* localization of RSSU in pea at temperatures less than 40°C (Vierling and Key 1985) and *in vitro* import of proteins at 25°C into chloroplasts isolated from plants heat stressed at 45°C (analogous to our recovery experiment; Vierling et al. 1986).

At present, it is not clear why localization of proteins to the chloroplast is impaired during severe heat stress; e.g., whether targeting or routing to the plastid, transport across the plastid outer envelope, or turnover of imported proteins is affected during heat stress. It seems unlikely that impaired localization would result strictly from rapid degradation of new proteins after transport to the chloroplast, rather than disrupted targeting or import. The data for RLSU and D1/D2 (chloroplast-encoded proteins that accumulate during severe heat stress) indicate that indiscriminate degradation of proteins within chloroplasts is not occurring; hence, degradation would need to be selective for nuclear-encoded proteins. It is known that imported RSSU can be selectively degraded in the chloroplast to regulate stoichiometric accumulation of this protein relative to chloroplast-encoded RLSU (and probably occurs for other proteins also; Schmidt and Mishkind 1983), and that damaged proteins imported into the endoplasmic reticulum are exported back to the cytoplasm to be degraded, which may apply to chloroplasts as well (Hiller et al. 1996). However, degradation of imported proteins either within the chloroplast or following export would likely need to be very rapid to account for the complete absence of newly synthesized lmw Hsp (figs. 1–4) and Hsp70 (fig. 2) in chloroplasts from plants subjected to severe heat stress. This scenario seems unlikely since (1) Hsps presumably fulfill adaptive protection or repair roles in response to heat stress; (2) Hsps are assumed to be thermostable and long-lived (the lmw chl p Hsp has a half-life of ca. 52 h [Chen et al. 1990], similar to other lmw Hsps [DeRocher et al. 1991]); and (3) previous studies have provided no evidence that nuclear-encoded chloroplast proteins are any more susceptible to heat-induced damage or degradation within the chloroplast than are chloroplast-encoded proteins (Berry and Björkman 1980; Süß and Yordanov 1986).

Impaired localization of nuclear-encoded proteins to the chloroplast has important implications for understanding the effects of severe heat stress on photosynthesis and other chloroplast functions. For example, impaired localization may prevent replace-

ment within the chloroplast of nuclear-encoded proteins lost through normal turnover or heat-related damage, thereby delaying post-heat stress recovery of photosynthesis. In support of this possibility, we have observed that in *Zea mays*, a decrease in net photosynthesis during heat stress and recovery of photosynthesis from heat stress were more closely correlated with whole-leaf levels of nuclear-encoded chloroplast proteins (OEC33, 23, and 16) than with chloroplast-encoded proteins (RLSU and D1) or cytoplasmically localized photosynthetic proteins (phosphoenolpyruvate carboxylase; Heckathorn et al. 1997). If disrupted localization extends to chloroplast Hsps, as in this study, then chloroplast processes are deprived of the protective or repair functions that are thought to be associated with Hsps. For example, we have previously observed a relationship between Photosystem II function during and after heat stress and accumulation of the lmw chl p Hsp in *Zea mays* (Heckathorn et al. 1996a). We have also observed a relationship between production of the lmw chl p Hsp and organismal thermotolerance among diverse species (Downs et al. 1997), and a genetic correlation between whole-plant thermotolerance and synthesis of this protein has been observed among genotypes of *Agrostis palustris* Huds. (Park et al. 1996). Results from the present study might also explain why apparent accumulation of the pre-import isoform of RSSU and chl p Hsp70 has been observed in leaves during heat stress (Halle et al. 1990; Wang et al. 1993).

Since translocation of proteins across the membranes of other cell organelles occurs via systems very similar to those of the chloroplast (Schatz and Dobberstein 1996), localization of proteins to other cell compartments may also be impaired at high temperatures. To our knowledge, this has not been studied. In addition, if impaired localization during severe heat stress results from disruption of targeting, routing, or translocation of proteins, then we postulate that other stresses that damage cellular proteins (e.g., heavy metal stress, anaerobiosis, drought stress, etc.) may also impact localization of proteins to chloroplasts or other organelles.

The temperatures at which plants were heat-stressed in this study and the magnitude of the temperature increases relative to prestress conditions are both well within the range that plants often experience under field conditions (Larcher 1995; Sharkey 1996). For example, it is common for even arctic plants to experience rapid increases in leaf temperatures exceeding 15°C during cloud flecks or on sunny days, and plants in tropical and cool- and warm-temperate habitats sometimes encounter growing conditions under which tissue temperatures exceed those in this study (43°C and 46°C). Therefore, impaired localization of nuclear-encoded proteins to chloroplasts is not likely to be restricted to laboratory situations but may occur in field-grown plants subjected to acute heat stress. We anticipate that the extent of impaired localization will

depend on preheat stress conditions (e.g., are plants acclimated or not) and be species specific. For example, species adapted to hot environments may possess mechanisms to maintain localization at higher temperatures relative to species from cool habitats. We have observed efficient localization of proteins to chloroplasts at temperatures of 43°C and 50°C in *Ferocactus wislizenii* (fish-hook cactus; Downs et al. 1997, and unpublished results), an extremely thermotolerant species native to the Sonoran desert, U.S.A. (Smith et al. 1984). This indicates that thermostable localization may be a trait on which natural selection has acted or can act.

Acknowledgments

We thank T. D. Sharkey and D. R. Ort for use of their labs for the radiolabeling work; D. R. Ort, T. D. Sharkey, J. K. Bryan, R. L. Hallberg, S. Perry, S. J. McNaughton, J. Belote, B. Micallef, and the anonymous reviewers for advice on radiolabeling and valuable comments on the manuscript; and E. Camm and A. Eastmann, R. Zielinski, S. Hemmingsen, and T. Leustek for providing OEC33, rubisco, Cpn60, and Hsp70 antibodies, respectively. This research was funded by grants from the National Science Foundation and the Andrew W. Mellon Foundation (to J. S. Coleman) and Sigma-Xi (to C. A. Downs).

Literature Cited

- Berry J, O Björkman 1980 Photosynthetic response and adaptation to temperature in higher plants. *Annu Rev Plant Physiol* 31:491–543.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Chen Q, LM Lauzon, AE DeRocher, E Vierling 1990 Accumulation, stability, and localization of a major chloroplast heat-shock protein. *J Cell Biol* 110:1873–1883.
- Debel K, G Knack, K Kloppstech 1994 Accumulation of plastid HSP 23 of *Chenopodium rubrum* is controlled post-translationally by light. *Plant J* 6:79–85.
- Debel K, WD Sierralta, HP Braun, UK Schmitz, K Kloppstech 1997 The 23-kDa light-stress-regulated heat-shock protein of *Chenopodium rubrum* L. is located in the mitochondria. *Planta* 201:326–333.
- DeRocher AE, KW Helm, LM Lauzon, E Vierling 1991 Expression of a conserved family of cytoplasmic low molecular weight heat shock proteins during heat stress and recovery. *Plant Physiol* 96:1038–1047.
- Downs CA, SA Heckathorn, JK Byran, JS Coleman 1997 The methionine-rich low-molecular-weight chloroplast heat-shock protein: evolutionary conservation and accumulation in relation to thermotolerance. *Am J Bot* (in press).
- Gatenby AA, PV Viitanen 1994 Structural and functional aspects of chaperonin-mediated protein folding. *Annu Rev Plant Physiol Plant Mol Biol* 45:469–491.
- Gegenheimer P 1990 Preparation of extracts from plants. Pages 174–193 in MP Deutscher, ed. *Guide to protein purification: methods in enzymology*, Vol 182. Academic Press, San Diego, Calif.
- Ghanotakis DF, CF Yocum 1990 Photosystem II and the oxygen-evolving complex. *Annu Rev Plant Physiol Plant Mol Biol* 41:255–276.
- Ghosh S, S Gepstein, JJ Heikkila, EB Dumbroff 1988 Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissues. *Anal Biochem* 169:227–233.
- Halle JR, S Ghosh, EB Dumbroff, JJ Heikkila 1990 Effect of elevated temperatures on heat shock protein and ribulose-1,5-bisphosphate carboxylase gene expression in *Brassica napus*. *Biochem Cell Biol* 68:609–615.
- Heckathorn SA, JS Coleman, RL Hallberg 1997 Recovery of net CO₂ assimilation after heat stress is correlated with recovery of levels of oxygen-evolving-complex proteins in *Zea mays* L. *Photosynthetica* (in press).
- Heckathorn SA, GJ Poeller, JS Coleman, RL Hallberg 1996a Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. *Oecologia* 105:413–418.
- 1996b Nitrogen availability and vegetative development influence the response of ribulose 1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, and heat-shock protein content to heat stress in *Zea mays* L. *Int J Plant Sci* 157:546–553.
- Hiller MM, A Finger, M Schweiger, DH Wolf 1996 ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* 273:1725–1728.
- Keegstra K, B Bruce, M Hurley, HM Li, S Perry 1995 Targeting of proteins into chloroplasts. *Physiol Plant* 93:157–162.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Larcher W 1995 *Plant Physiological Ecology*. Springer-Verlag, Berlin. 506 pp.
- Marshall JS, AE DeRocher, K Keegstra, E Vierling 1990 Identification of heat shock protein hsp70 homologues in chloroplasts. *Proc Natl Acad Sci USA* 87:374–378.
- Martino-Catt SJ, TL Jones, DR Ort 1993 *In vivo* pulse labeling of proteins in attached leaves with radioactive amino acids. *Anal Biochem* 211:188–196.
- Park SY, R Shivaji, JV Krans, DS Luthe 1996 Heat-shock response in heat-tolerant and nontolerant variants of *Agrostis palustris* Huds. *Plant Physiol* 111:515–524.
- Schatz G, B Dobberstein 1996 Common principles of protein translocation across membranes. *Science* 271:1519–1526.
- Schmidt GW, ML Mishkind 1983 Rapid degradation of unassembled ribulose 1,5-bisphosphate carboxylase small subunits in chloroplasts. *Proc Natl Acad Sci USA* 80:2632–2636.
- Sharkey TD 1996 Isoprene synthesis by plants and animals. *Endeavor* 1:74–78.
- Smith SD, B Didden-Zopf, PS Nobel 1984 High-temperature responses of North American cacti. *Ecology* 65:643–651.
- Süss KH, IT Yordanov 1986 Biosynthetic cause of *in vivo* acquired thermotolerance of photosynthetic light reactions and metabolic responses of chloroplasts to heat stress. *Plant Physiol* 81:192–199.
- Towbin H, T Staehelin, J Gordon 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354.
- Vierling E 1991 The roles of heat shock proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* 42:579–620.
- Vierling E, JL Key 1985 Ribulose 1,5-bisphosphate carboxylase synthesis during heat stress. *Plant Physiol* 78:155–162.
- Vierling E, ML Mishkind, GW Schmidt, JL Key 1986 Specific heat shock proteins are transported into chloroplasts. *Proc Natl Acad Sci USA* 83:361–365.
- Wang H, M Goffreda, T Leustek 1993 Characteristics of an Hsp70 homolog localized in higher plant chloroplasts that is similar to DnaK, the Hsp70 of prokaryotes. *Plant Physiol* 102:843–850.