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Association of 70-Kilodalton Heat-Shock Cognate Proteins with Acclimation to Cold[1]

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ABSTRACT

Exposure of young spinach seedlings (*Spinacia oleracea* L. cv Bloomsdale) to 5°C leads to an increase in the synthesis of several 79-kilodalton proteins that are present in leaf tissue grown at 20°C. Protein sequence analyses and immunological cross-reactivity indicate that this group of proteins belongs to the 70-kilodalton heat-shock family. Steady-state transcript levels and protein synthesis are increased two- to threefold within 1 day, but immunoblot analyses suggest that the steady-state concentration of this protein group in leaf tissue only gradually accumulates at low temperature. It is proposed that the increased synthesis of several members of the 70-kilodalton heat-shock family could result from an influence of low temperature on protein folding and/or assembly processes.

Plants are poikilotherms and must be able to adjust to changes in ambient temperature to ensure survival. In keeping with this need to adjust to a changing environment, polar, temperate, and alpine perennial plants follow a cyclical rhythm of growth and dormancy. In these plants, the cessation of vegetative growth and onset of dormancy is a prerequisite for the induction of tolerance to the stresses imposed by freezing (35). Collectively, the set of biochemical and physiological events that augments a plant's tolerance to low temperature stress is termed cold acclimation (35). In hardy plants, cold acclimation performs two major functions: (a) the adjustment of cellular metabolism and function to the kinetic and biophysical constraints imposed by reduced temperature, and (b) the induction of freezing tolerance (11).

At low nonfreezing temperatures that promote cold acclimation, many plant species exhibit subtle changes in gene expression (11, 15) that appear to be different from the responses induced by other types of environmental stresses (30). For example, unlike the heat-shock response (3), plants exposed to low temperature continue synthesis of the vast majority of housekeeping proteins while simultaneously inducing or upregulating the synthesis of selected proteins (12). The purpose of this increased expression of certain proteins by plants in response to low temperature is not understood. Do these proteins serve in the adjustment of metabolic functions to kinetic restraints imposed by low nonfreezing temperatures, or do they play a role in freezing tolerance mechanisms? Here we describe the identification of a group of proteins belonging to the 70-kD heat-shock family that may be involved with a metabolic adjustment of plants to low nonfreezing temperature during cold acclimation.

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MATERIALS AND METHODS

Plant Material

Spinach seedlings (*Spinacia oleracea* L. cv Bloomsdale) were grown from seed in a controlled environment as previously described (12). Cold acclimation and deacclimation treatments were conducted with a 12-h photoperiod as previously described (12).

Protein Purification

Protein was extracted from etiolated hypocotyl-cotyledon tissue grown at 5°C for 4 weeks. Frozen tissue was ground in a dry-ice-cooled mortar. Forty grams of tissue was homogenized in a Polytron blender with 100 mL of 80% (v/v) distilled phenol buffered with 120 mM Tris-HCl (pH 6.8), 50 mM EDTA, 100 mM KCl, 2% (v/v) Triton X-100, 5% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and an additional 100 mL of the preceding buffer (based on ref. 13). The total protein extract was centrifuged at 15,000*g* for 5 min. The aqueous phase was filtered through glass wool to remove cellular debris. Protein was precipitated from the phenol with 5 volumes of −20°C acetone with 1% (v/v) 2-mercaptoethanol for 2 h at −20°C and pelleted at 15,000*g* for 10 min. The protein pellet was vacuum dried, dissolved in 5 mL of buffer solution (1 mM Tris-HCl, pH 8.0, 8 M urea, 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic-acid, 5% [v/v] glycerol) and centrifuged at 11,000*g* for 5 min to remove undissolved material. The supernatant was stored at −20°C. Protein content was determined by the Bradford method (5).

Protein extracts containing 35 to 180 mg of protein were fractionated by free-solution-isoelectric focusing in a Rotofor

apparatus (Bio-Rad) in a pH gradient containing 2% ampholytes (0.8% pH 5–7, 0.8% pH 4–6, and 0.4% pH 3–10). The ampholyte solution was prefocused for 1 h at 12 W constant power with cooling at 10°C to establish the pH gradient. Ampholytes were added to the protein sample to make a 2% ampholyte concentration. The protein-ampholyte solution was loaded into the middle of the pH gradient (approximately pH 6.5). Proteins were focused for 5 h at 12 W constant power. Coolant at 10°C was circulated through the focusing cell to minimize heat buildup. After focusing, the fractions were collected and pH values determined. SDS-PAGE analyses revealed that free-solution-isoelectric focused fractions closely approximated analytical two-dimensional separations (12) for spinach cold acclimation proteins, which facilitated identification of fractions containing CAP79.[2]

Fractions most enriched for CAP79 were selected for further purification. The amount of CAP79 present in selected fractions was determined by densitometry following SDS-PAGE and Coomassie blue staining. Prior to CNBr cleavage, protein was precipitated from CAP79-enriched fractions with 5 volumes of −20°C acetone with 1% 2-mercaptoethanol for 2 h followed by centrifugation at 11,000*g*. Coprecipitated urea was removed by washing with 10 mL of methanol. After centrifugation, the supernatant was discarded and the protein pellet dried under vacuum. CAP79 was further purified by SDS-PAGE fractionation. Following electroblotting to PVDF and staining with Coomassie blue, the discrete CAP79 band was excised and the protein cleaved with CNBr in 70% formic acid (23) overnight in an Eppendorf tube. The cleavage solution was lyophilized to remove the formic acid, and the dried residue dissolved in sterile distilled water and lyophilized twice before final SDS-PAGE fractionation and electroblotting to PVDF. Gas-phase sequencing was performed on amido black-stained bands excised from the PVDF membrane. Polyclonal antibodies against CAP79 were prepared using protein purified as described for sequencing with only slight modification. After resolution of CAP79 by preparative SDS-PAGE, the gel was stained with Coomassie blue. The visualized band (about 66 μg) was excised and the gel slice homogenized in PBS and injected into Balb/c mice. The final antiserum titer was 1:6400 as determined by western blot analyses.

Chemiluminescent Quantitation of Protein Blots

Protein extracts were fractionated by SDS-PAGE and electroblotted onto PVDF membrane. Blots were probed with an anti-CAP79 mouse polyclonal antisera overnight at 4°C. Antigen-antibody complexes were visualized with an alkaline phosphatase-conjugated anti-mouse second antibody in the presence of Lumi-Phos 530 (31). Light produced from the chemiluminescence reaction, catalyzed by alkaline phosphatase, was recorded on x-ray film. Film exposures ranged from 1 to 10 min. Lumigraphs were scanned using a Molecular Dynamics Computing Densitometer model 300. Each blot for CAP79 quantitation was separated between the 60- and 70-kD region and the upper half was used for CAP79 immunoblots. The lower half was probed with a rabbit antiserum reactive against the large subunit of Rubisco. All determinations of CAP79 are standardized internally on the same protein blot against the signal for Rubisco.

Amplification and Cloning of Genomic DNA Sequences

Spinach genomic DNA was purified from spinach leaves using the method of Dellaporta *et al.* (7) with modifications. Synthetic primers were constructed using HSP70 sequences from maize and *Arabidopsis* (28, 37). Genomic DNA was amplified using *Taq* polymerase according to the directions of Perkin-Elmer/Cetus. The 5′ oligo TC(ACTG) TC(ACTG) CG(CT) GCT CG(CT) TTC GAG GAG and the 3′ oligo (AGC)GT CAT (GA)AC (GA)CC ACC (ATG)GC (AG)GT CTC were used to amplify a discrete 350-base pair region of genomic DNA (spin350) that should contain the region of the gene encoding for the sequenced peptide. The DNA was denatured for 3 min at 94°C and amplified during 35 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 50°C, and extension for 3 min at 72°C. The PCR products were then incubated at 72°C for 7 min to complete extension of all products. PCR products were excised from a 2% LMP agarose (BRL) gel, solubilized in 10 mM Tris, 1 mM EDTA, and 0.5% SDS at 65°C for 20 min, purified by phenol/chloroform extraction and ethanol precipitation, and subjected to an additional round of 25 cycles of PCR amplification. After gel purification, PCR products were made blunt ended with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase (24). After phenol extraction and ethanol precipitation, the PCR products were cloned into *Eco*RV-digested Bluescript plasmid (Stratagene). Templates were purified and sequenced by the automated dideoxynucleotide method (DuPont).

RNA Purification and Northern Analysis

RNA was extracted from spinach leaves using the phenol extraction LiCl precipitation method (1). Poly(A^+) RNA was purified using oligo(dT)-cellulose for northern blot analyses as described (24). Equal amounts of poly(A^+) RNA from each time point of the cold-acclimation regimen were separated on a 1.2% formaldehyde agarose gel (24). The gel was stained with ethidium bromide, photographed, and pressure blotted onto Hybond-N (Amersham) nylon membrane. The RNA was fixed and hybridized to a random-primed [α-^{32}P]dCTP-labeled spin350 probe (24). RNA blots were stripped and standardized against the message for actin using an *Arabidopsis* cDNA. For RNA abundance determinations, laser densitometric analyses of blots using the spin350 probe were normalized as the ratio of the signal with that obtained using actin. Actin expression appears to be invariant at the temperatures and durations used in this study.

RESULTS

Isolation and Identification of CAP79 Proteins

Early studies involving two-dimensional gel analyses of *in vivo* labeled proteins in spinach seedlings undergoing cold

[2] Abbreviations: CAP79, cold acclimation protein 79; CNBr, cyanogen bromide; PCR, polymerase chain reaction; PVDF, polyvinyldifluoride; HSP70, 70-kD heat-shock protein; BiP, binding immunoglobulin protein.

acclimation at 5°C revealed about a threefold increase in the synthesis of CAP79 with an isoelectric point of 5.3 (12). Using preparative free-solution-isoelectric focusing and SDS gel electrophoresis, we were able to prepare adequate amounts of pure CAP79 from cold-acclimated hypocotyl tissue for use in protein sequencing and antibody production. Increased synthesis of CAP79 upon exposure to 5°C was confirmed by SDS gel analysis of immunoprecipitated protein from nondenatured cell lysates prepared from leaf tissues that were pulse-labeled with [^{35}S]methionine (Fig. 1). Greater radiolabel incorporation in CAP79 was observed during the first 2 d of exposure to 5°C, but by 7 d the level of incorporation returned to that seen in plants kept at 20°C. The decline in synthesis after the initial increase at low temperature contrasts with previous *in vivo* labeling data, which showed peak synthesis at 7 d (12). This discrepancy could result from differences in experimental design: (a) two-dimensional PAGE analyses of total *in vivo* labeled products *versus* immunoprecipitation of a specific labeled product and/or (b) variation in the culture and age of the plants. The antibody used in the immunoprecipitation reactions was prepared against denatured CAP79. It is fortunate that this antibody immunoprecipates native CAP79, but its efficiency in this reaction and its ability to recognize all members of the CAP79 complex remains uncharacterized. Also, in the previous report (12), plants were younger and grown *in vitro*, but in this study the plants were older and grown *ex vitro* in a soil medium. Differences in age and cultural conditions could contribute to the disparity with the previous work (12). Two-dimensional gel analyses of CAP79 immunoprecipitates of *in vivo* radiolabeled proteins detected the synthesis of three isoforms in leaves of plants grown at 20°C (Fig. 1B), and five isoforms from leaves exposed to 5°C for 1 d (Fig. 1C).

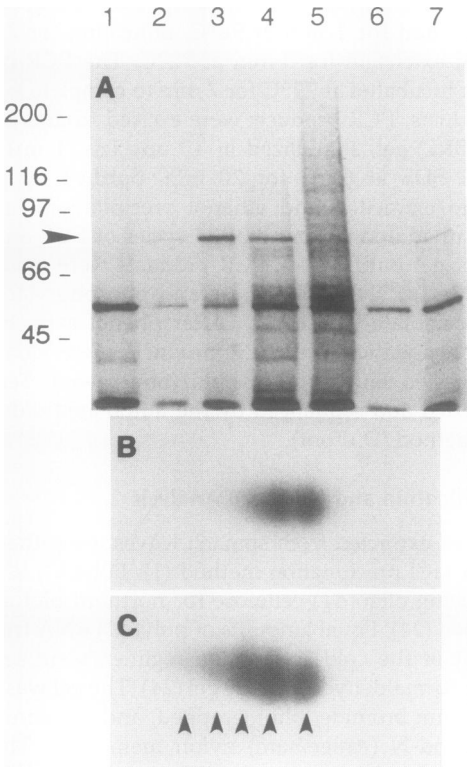

Figure 1. Immunoprecipitation of *in vivo* labeled CAP79. Leaf tissue was radiolabeled (12) with [^{35}S]methionine for 24 h, and cell lysates were prepared in 50 mM Mops, pH 7.5, 10 mM $MgCl_2$, 2.5 mM DTT, 1 mM EDTA, 1 mM methionine, and 0.05% Triton X-100. CAP79 was immunoprecipitated from 10^6 TCA-insoluble counts with 10 μL anti-CAP79 antiserum. Immune complexes were dissolved and the proteins resolved by SDS-PAGE and visualized by fluorography. A, Lane 1, nonimmune serum; lane 2, nonacclimated; lane 3, cold-acclimated 1 d; lane 4, cold-acclimated 2 d; lane 5, cold-acclimated 7 d; lane 6, deacclimated 1 d; lane 7, deacclimated 7 d. Arrow shows the CAP79 band. B and C, Two-dimensional gel analyses of immunoprecipitates from nonacclimated and 1-d cold-acclimated tissue. The panels are oriented with the acidic end on the left. The arrowheads in C denote the five isoforms.

Attempts at Edman gas-phase sequencing from the NH_2 terminus of CAP79 purified from analytical two-dimensional gel electroblots failed because of N-terminal blockage. Compositional analyses of small amounts of the purified protein from the two-dimensional gel electroblots revealed the presence of 18 methionine residues (14). Partial cleavage of purified CAP79 by CNBr produced two large peptides that were resolved from smaller peptides by SDS-PAGE (Fig. 2A). Gas-phase sequencing demonstrated that these two peptides had identical NH_2 termini. Homology searches of the National Biomedical Research Foundation and GenBank databases revealed CAP79's relationship to HSP70s (Fig. 2B). The greatest similarity was found with a *Petunia* HSP70 where 31 of 32 residues were identical to CAP79/30 (36). The sequence homology of the two CAP79 CNBr fragments with a previously described maize HSP70 sequence begins at methionine residue 330 and runs through residue 362 (28). This region is a highly conserved domain in HSP70s (26) and defines the high degree of homology of CAP79 within this heat-shock family. Because the purified CAP79 is composed of several isoforms, it is possible that both CNBr peptides were products from the same polypeptide or from different isoforms. The generation of 30- and 40-kD partial CNBr digest peptides having the same NH_2 terminus and identical amino acid sequence can be explained by a 10-kD truncation of the carboxyl terminus at one of several methionine target sites (28). Generation of a 40-kD CNBr peptide beginning at residue 330 suggests a molecular mass greater than 70 kD in keeping with our estimated size of 79 kD for CAP79. HSP70s generally vary from 633 to 666 residues in length (26). If CAP79 were a classical heat-inducible HSP70, the longest expected fragment beginning at residue 330 and extending to the carboxyl terminus would be about 320 residues, or roughly 35 kD. To account for the extra 5 kD of the 40-kD fragment would require roughly an extra 45 residues, extending the protein to about 700 residues in length. This would suggest a protein strikingly similar in length to an *Arabidopsis* heat-shock cognate protein (HSC70) that was reported to be 76 kD and approximately 700 residues in length (37).

CAP79 is not a typical heat-inducible HSP70 (18, 20), because it is present in nonheat-shocked and cold-acclimated tissues, and its synthesis is not strongly responsive to heat-shock treatment (12). Western blot analysis, with the polyclonal mouse antiserum raised against the purified CAP79

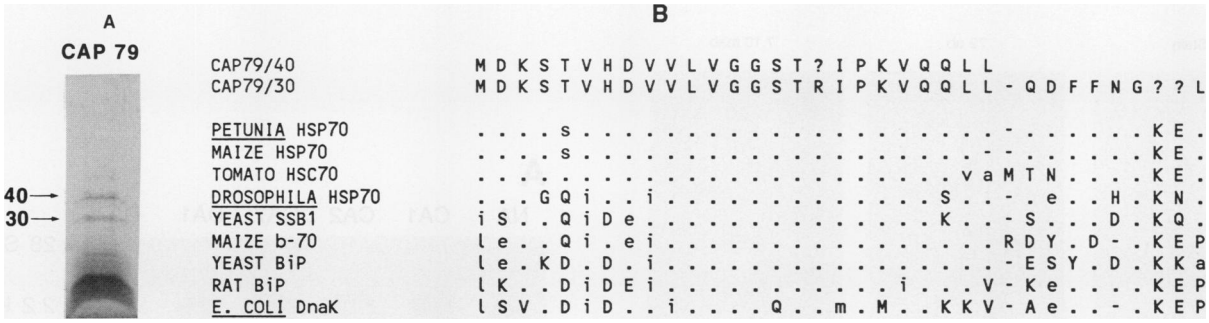

Figure 2. Homology of CAP79 CNBr fragments with HSP70s. A, The partial CNBr cleavage peptides resolved by SDS-PAGE used for gas-phase amino acid sequencing. B, The homology of the CAP79 with HSP70s. The amino acid sequences are indicated by the one-letter code. Residues identical to CAP79 sequences are marked by dots, homologous substitutions by lowercase letters, and nonhomologous substitutions by uppercase letters. The HSP70 sequences compared were maize b-70 (9), *Petunia* (36), maize (28), tomato (26), *Drosophila* (19), yeast SSB1 (32), yeast BiP (27), rat BiP (25), and *E. coli* DnaK (2).

and a rat monoclonal antibody (7.10, produced against a *Drosophila* HSP70 that cross-reacts with a wide range of heat-shock cognates of various organisms) (34), provides definitive evidence to support the assignment of spinach CAP79 as a member of the HSP70 family. Both antibodies identified a family of proteins of between 70 and 80 kD on western blots of two-dimensional gels (Fig. 3A). Close inspection of the CAP79 reactivity on these blots suggests it represents a closely migrating constellation of HSC70s. The *Drosophila* monoclonal antibody also shows reactivity with two additional proteins of 76 kD that the CAP79 antiserum does not. Experiments were conducted to determine whether the CAP79 complex was responsive to heat shock. When spinach leaf tissue was heat shocked for 3 h over a temperature range known to elicit the heat-shock response (33), only moderate changes were observed in the concentration of the CAP79 band, as judged by immunoblotting (Fig. 3B). The 1.5-fold increase at 35°C and virtually no change at higher temperatures agrees with previous conclusions (12) that this complex is not strongly induced by heat shock. The moderate increase at 35°C in CAP79 abundance may result from cross-reactivity to unique heat shock-induced isoforms, or by the heat shock-induced synthesis of the constitutive CAP79 isoforms. Further two-dimensional analyses should resolve this uncertainty.

Analysis of CAP79 during Cold Acclimation

Synthetic oligonucleotides were prepared using DNA sequence information from HSP70 sequences of maize and *Arabidopsis* (28, 37) that would likely bracket the region of the gene(s) corresponding to the known protein sequence (Fig. 2B). These oligonucleotides were used to amplify spinach genomic DNA by PCR. Of the three amplified products obtained from this procedure, a 350-base pair fragment (spin350) was determined through sequence analysis to belong to an HSP70 gene. Although it is not identical to any previously reported HSP70 gene, spin350 was most similar to b-70 (9) and showed homology to other BiPs (25, 27, 29). This fragment was found to have homology with the protein sequence determined for CAP79, but it also was not identical. Northern blot analyses of poly(A^+) RNA isolated from 2-d cold-acclimated leaves showed a single region that hybridized with the spin350 probe. Under the northern blot conditions, the spin350 probe is likely to hybridize to the RNAs of most or all members of the HSP70 family. Therefore, these data provide a view of the total pool of transcript for the family and not that for a single member.

The spin350 probe and the anti-CAP79 antiserum were used to determine the changes in steady-state RNA abundance and protein levels in leaf tissue during cold acclimation/deacclimation by northern and western blot analyses (Figs. 4, A and B, 5). CAP79 isoforms are expressed in nonacclimated leaves; however, when the tissue undergoes cold acclimation, the pool increases. Northern analysis (Fig. 4A) indicated a two- to threefold increase in message at 1 d of 5°C exposure, but the level returned to that found in 25°C-grown plants by 7 d of low-temperature exposure. There is a slight increase in message upon return to 25°C from 5°C. In agreement with these results, increased RNA levels for low molecular mass HSPs have also been found in chilled soybean (22). In contrast, immunoblot analysis using the anti-CAP79 mouse antiserum (Figs. 4B, 5) indicated that the abundance of the CAP79 complex showed a modest accumulation in response to cold acclimation or 1 d of deacclimation. Extensive efforts to determine the effect of low-temperature exposure on the abundance of the protein complex by densitometry and chemiluminescence techniques indicated slightly less than a doubling over the 7-d 5°C exposure. Together, the data show that RNA and protein synthesis increased upon exposure to 5°C, and this response was followed by a slower accumulation of protein. As the duration of cold acclimation was prolonged, RNA and CAP79 synthesis declined at the same time that the abundance of the CAP79 complex reached its maximal level.

Comparative Aspects of CAP79 during Cold Acclimation

Western blot analyses using the mouse CAP79 antiserum showed cross-reactive proteins for several plant species tested, and an increased abundance for many of these after low-temperature treatment (Fig. 6). Similarly, two species of insects and maize, subjected to cold shock, synthesize heat-shock proteins during recovery at a nonheat-shock tempera-

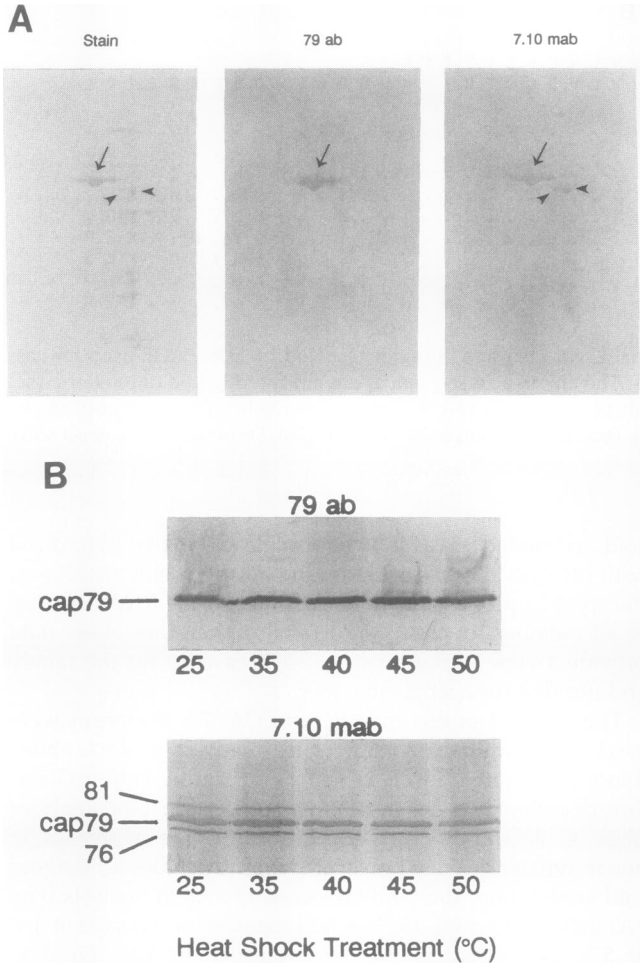

Figure 3. Western blot analyses of spinach proteins using *Drosophila* HSP70 monoclonal and anti-CAP79 mouse antiserum. A, Two-dimensional western blots of CAP79. Spinach CAP79 was partially purified by free-solution-isoelectric focusing and subjected to two-dimensional gel electrophoresis. Electroblots of gels were stained for total protein (Coomassie blue) or probed with *Drosophila* HSP70 monoclonal (7.10) or anti-CAP79 antiserum (79 ab). B, Western blot of heat-shocked spinach leaf tissue proteins. Plants were grown at room temperature for 4 weeks following germination, and then placed in closed test tubes and heated in a water bath for 3 h. Proteins were extracted and SDS-PAGE was performed as previously described.

ture (21, 38, 39). These results suggest that many organisms may accumulate members of the 70-kD heat-shock family upon exposure to low, nonfreezing temperature. In chilling-sensitive species like tomato and pepper, accumulation of members of this family may be a manifestation of low-temperature injury as cells become unable to function properly at low temperature. In chilling-insensitive species like spinach, *Petunia*, and broccoli, the accumulation of CAP79 and other HSC70s may be more a reflection of an adjustment of metabolism than a response to cell injury, because exposure to low nonfreezing temperatures is not inherently deleterious.

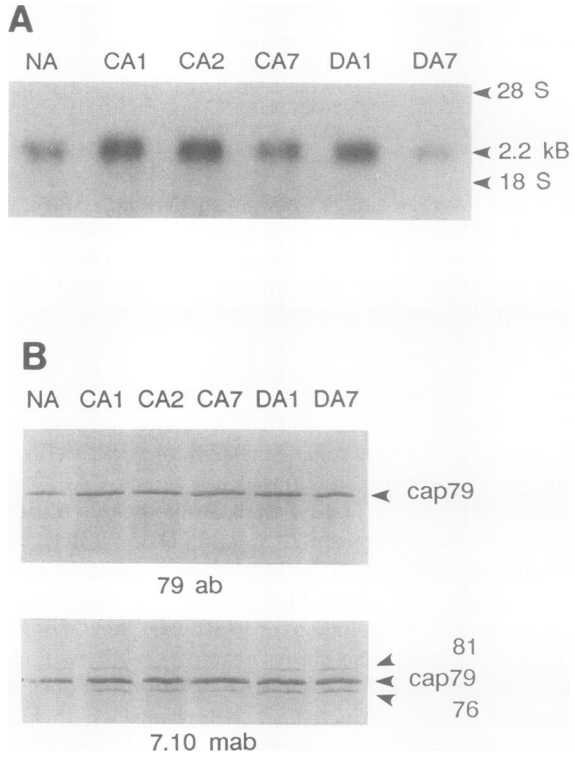

Figure 4. Northern (A) and western (B) blot of spinach leaf tissues during acclimation/deacclimation treatment. Lanes are nonacclimated, NA; cold acclimated 1, 2, and 7 d are marked CA1, CA2, and CA7, respectively; deacclimated 1 and 7 d are marked DA1 and DA7, respectively. RNA was extracted using the LiCl precipitation method (1) from spinach leaves held for 2 d at 5°C. Poly(A^+) RNA was extracted using oligo(dT)-cellulose as described in Maniatis *et al.* (24). A 1.2% formaldehyde gel was used to separate samples containing 1 μg of poly(A^+) RNA. The gel was pressure blotted to Hybond N (Amersham) nylon membrane and then baked *in vacuo* at 80°C for 2 h. The membrane was prehybridized at 45°C for 6 h in 50% formamide, 6 × SSC, 0.5% SDS, 40 mM Pipes (pH 6.5), 0.02% PVP, 0.02% Ficoll, 100 μg/mL fish DNA. Membranes were hybridized overnight at 45°C with 5 × 10^6 cpm/mL probe in prehybridization solution. Filters were washed twice with 2 × SSC, 0.2% SDS at room temperature, twice with 0.5 × SSC, 0.5% SDS at 55°C, and twice with 1 × SSC, 0.2% SDS at 55°C, then exposed to film (XAR5, Kodak). Films were scanned with a EC Apparatus Corporation densitometer. The membranes were then stripped and rehybridized with an *Arabidopsis* actin cDNA probe. Protein extracts were run at 10 μg/lane on 10% SDS-PAGE and transferred to PVDF membrane and immunoreacted against anti-CAP79 mouse antiserum (1:5000 dilution), then exposed to alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G and substrate to permit visualization.

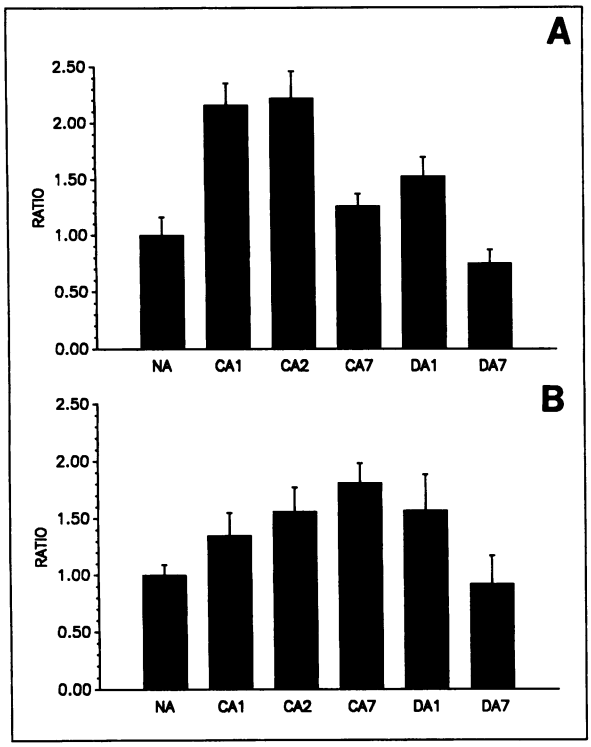

Figure 5. Densitometric analyses of CAP79 RNA and protein levels in leaf tissue during cold acclimation of spinach. A, RNA content. B, Protein content. RNA blots were probed with a PCR product spin350, stripped, and reprobed with an *Arabidopsis* actin cDNA. Abundance was determined as the ratio of CAP79 to actin signals obtained by transmission laser densitometry normalized against the signal for nonacclimated tissue. Means and SE values were calculated from several blots; NA, $n = 5$; all others, $n = 7$. Protein abundance was determined as the ratio of CAP79 to Rubisco normalized against that of nonacclimated tissue. SDS-PAGE-fractionated spinach proteins were blotted to PVDF membranes, and the membranes were separated between 60 and 70 kD as judged by molecular mass standards. Chemiluminescence detection on x-ray film was employed to visualize antigen-antibody complexes involving antibodies reactive against CAP79 or Rubisco. Chemiluminescence signals were quantitated by transmission laser densitometry. Means and SE values were calculated from several blots; DA7, $n = 4$; all others, $n = 8$.

DISCUSSION

HSP70s are found in organisms as diverse as *Escherichia coli*, yeast, *Drosophila*, *Arabidopsis*, and humans (26). Various functions have been attributed to heat-shock proteins, such as posttranslational assembly of oligomeric proteins, protein translocation, eukaryotic mitochondrial DNA replication, lysosomal degradation, protein folding, and protein transport (6, 8, 17). Their universal presence in living organisms clearly indicates a central function in cells. The data reported here support the hypothesis that the elevated synthesis of several CAP79 isoforms represents an adjustment of cellular metabolism to the biophysical constraints experienced during cold acclimation. The identification of heat-shock cognates that appear to be up-regulated during cold acclimation raises important questions concerning the metabolic changes that occur during this process. The proposed involvement of heat-shock cognates in protein folding, oligomeric assembly, and transport (4, 6, 8, 17) is a case in point. During cold acclimation, the up-regulation of a protein(s) that participates in any of these processes could facilitate formation of active complexes and provide an adaptive advantage not only for plants, but for any organism that experiences the inhibitory effects of low, nonfreezing and/or freezing temperatures. A potentially important function of CAP79s in cold acclimation could be to play a role in renaturation of low-temperature denatured proteins. It is clear that a number of metabolically important oligomeric enzymes undergo dissociation or are otherwise denatured upon exposure to low, nonfreezing temperatures in chilling-sensitive plants, as well as in other mesophiles (11). Increased synthesis of certain CAP79 isoforms, or comparable heat-shock cognates may be a response to the presence of denatured cold-sensitive enzymes at low, nonfreezing temperatures. It is interesting that we have noted that as the duration of low-temperature exposure increases, immunoprecipitation of native CAP79 from *in vivo* radiolabeled tissues shows increasing contamination by other radiolabeled proteins (Fig. 1). This result was observed in two sets of immunoprecipitations, in spite of extensive washing, and leads us to believe the phenomena is real. This could be a manifestation of a more than transient association between CAP79 and other cellular proteins at low temperature, and may indicate that some aspects of protein metabolism (folding and assembly) are slowed or impaired (10). In support of this idea is the finding of a kinetic partitioning of a slow-folding nonnative maltose-binding protein with the *E. coli* chaperone SecB at 5°C (16). At 25°C, the folding of this protein is more rapid, and this in turn reduces the opportunity for association with SecB, which is necessary for proper targeting. It has already been demonstrated in other systems that some proteins show an extended interaction with HSP70s, especially when normal maturation is impaired by amino acid analogs (4).

Why does low temperature appear to alter the RNA level and protein synthesis of HSP70s? Members of the HSP70 family could play a role both in chilling and under more severe low-temperature stress conditions when freezing may occur. It is conceivable that CAP79 may assist in refolding of

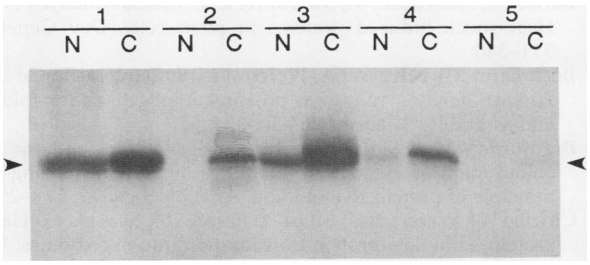

Figure 6. Accumulation of proteins during cold acclimation that cross-react with CAP79 antiserum. N, Grown at 25°C; C, 2 d at 5°C. 1, *Phaseolus vulgaris*; 2, *Capsicum annuum*; 3, *Lycopersicon esculentum*; 4, *Petunia hybrida*; 5, *Brassica oleracea*. Samples were loaded at 25 μg of protein/lane.

freeze/thaw denatured proteins following return to warmer temperatures in the same manner as heat-shock proteins seem to do following heat denaturation (10). In freezing-tolerant plants exposed to subzero temperatures, the extracellular solution freezes, causing cellular desiccation, increased solute concentration, ionic strength changes, and pH shifts, all of which can favor protein denaturation (11, 35). Consequently, freezing-tolerant organisms must have the ability to safeguard against or minimize protein denaturation during a freeze/thaw cycle. A mechanism that avoids aggregation and favors folding and renaturation at low temperature would be advantageous in restoring the proper conformation and function of proteins upon thawing and return to favorable temperatures. Thus, the accumulation of HSP70s at low temperature may not only be indicative of a stress condition but may also have adaptive value as well.

A surprising finding in this work was that CAP79 protein abundance showed smaller increases during low-temperature exposure than expected considering the extent of the increases in mRNA level and protein synthesis observed in this and previous studies (13). The reason for the disparity is not clear. One possibility is that current synthesis is small relative to the total pool abundance. If this were the case, then moderate changes in synthesis might require long durations to alter abundance of the total pool, as seems to be indicated in Figure 5. Another explanation is that temperature affects protein turnover in such a fashion as to maintain a somewhat uniform abundance. Additional studies will be required to clarify this disparity.

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