Plant Physiol. (1994) 105: 181–190

Abscisic Acid-Induced Heat Tolerance in *Bromus inermis* Leyss Cell-Suspension Cultures[1]

Heat-Stable, Abscisic Acid-Responsive Polypeptides in Combination with Sucrose Confer Enhanced Thermostability

Albert J. Robertson, Masaya Ishikawa[2], Lawrence V. Gusta*, and S. L. MacKenzie

Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada; and National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan S7N 0W9, Canada

Increased heat tolerance is most often associated with the synthesis of heat-shock proteins following pre-exposure to a nonlethal heat treatment. In this study, a bromegrass (*Bromus inermis* Leyss cv Manchar) cell suspension cultured in a medium containing 75 μM abscisic acid (ABA) without prior heat treatment had a 87% survival rate, as determined by regrowth analysis, following exposure to 42.5°C for 120 min. In contrast, less than 1% of the control cells survived this heat treatment. The heat tolerance provided by treatment with 75 μM ABA was first evidenced after 4 d of culture and reached a maximum tolerance after 11 d of culture. Preincubation with sucrose partially increased the heat tolerance of control cells and rendered ABA-treated cells tolerant to 45°C for 120 min (a completely lethal heat treatment for control cells). Comparative two-dimensional polyacrylamide gel electrophoresis of cellular protein isolated from heat-tolerant cells identified 43 ABA-responsive proteins of which 26 were heat stable (did not coagulate and remained soluble after 30 min at 90°C). Eight heat-stable, ABA-responsive proteins ranging from 23 to 45 kD had similar N-terminal sequences. The ABA-responsive (43–20 kD), but none of the control heat-stable, proteins cross-reacted to varying degrees with a polyclonal antibody directed against a conserved, lysine-rich dehydrin sequence. A group of 20- to 30-kD heat-stable, ABA-responsive proteins cross-reacted with both the anti-dehydrin antibody and an antibody directed against a cold-responsive winter wheat protein (Wcs 120). In ABA-treated cells, there was a positive correlation between heat- and pH-induced coagulation of a cell-free homogenate and the heat tolerance of these cells. At 50°C, control homogenates coagulated after 8 min, whereas cellular fractions from ABA-treated cells showed only marginal coagulation after 15 min. In protection assays, addition of heat-stable, ABA-responsive polypeptides to control fractions reduced the heat-induced coagulation of cell-free homogenates. Sucrose (8%) alone and control, heat-stable fractions enhanced the thermostability of control fractions, but the most protection was conferred by ABA-responsive, heat-stable proteins in combination with sucrose. These data suggest that stress-tolerance mechanisms may develop as a result of cooperative interactions between stress proteins and cell osmolytes, e.g. sucrose. Hypotheses are discussed implicating the role of these proteins and osmolytes in preventing coagulation and denaturation of cellular proteins and membranes.

The development of heat tolerance and the concomitant production of Hsps is usually associated with preconditioning cells at an elevated, but nonlethal, temperature (Nagao et al., 1990). Many experiments have correlated increased thermotolerance with the increased synthesis or accumulation of Hsps, but Hsps or homologous forms of Hsps are also present in nonstressed cells (Vierling, 1991). In recent years Hsps have been shown to function in normal cell processes as molecular chaperones, assisting in the folding of newly synthesized proteins and the assembly of functional protein complexes (Vierling, 1991; Craig et al., 1993). The Hsp 70 family normally interacts transiently during the synthesis and folding of cellular proteins, but under stress conditions a more stable association occurs (Beckmann et al., 1990), suggesting that Hsps interact with cellular proteins to confer thermal stability. Heat-inducible proteins isolated from thermophilic archaebacterium show chaperonin activity and homology to cytosolic Hsp 60 (Craig et al., 1993). One of these chaperonins, purified from *Thermus thermophilus*, protected other cellular proteins from heat denaturation if heat treatment occurred in the presence of the chaperonin (Taguchi and Yoshida, 1993). Recently, another protein related to Hsp 60, t-complex polypeptide-1, was implicated in tubulin (Yaffe et al., 1992) and actin (Gao et al., 1992) biogenesis.

Deletion mutation of the Hsp 104 gene in yeast demonstrated that a functional Hsp 104 gene product was necessary for full expression of thermotolerance (Sanchez and Linquist, 1990). Although increased levels of cellular Hsps are correlated with increased thermotolerance, a yeast mutant defective in induction of Hsp 70, 82, and 104 genes developed thermotolerance (Smith and Yaffe, 1991). These contradictory observations demonstrate that the assumed protective

[1] This research is supported by National Sciences and Engineering Research Council of Canada grants to L.V.G.

[2] Present address: Department of Genetic Resources, National Institute of Agrobiological Resources, Kannodai, Tsukuba, Japan 305.

* Corresponding author; fax 1–306–966–5015.

Abbreviations: Hsp, heat-shock protein; pI, isoelectric point; PVDF, polyvinylidene difluoride; RAB, responsive to ABA; TTC, triphenyltetrazolium chloride.

role of Hsps may not be entirely correct and that increased levels of Hsps are not always necessary for increased thermotolerance. Hsp synthesis may be increased during heat stress because of an increased frequency of unfolded polypeptide chain substrates (Beckmann et al., 1990). Alternatively, cellular processes or proteins other than Hsps may function in protecting cellular structures against heat injury.

Exogenous ABA (Bonham-Smith et al., 1988), heavy metals, or a dehydration stress also induce thermotolerance in maize seedlings (Bonham-Smith et al., 1987). Although dehydration stress, ABA treatment, and wounding appeared to induce the Hsp 70 gene (Heikkila et al., 1984), a study by Bray (1991) demonstrated that elevated levels of ABA were not required for the induction of Hsps. ABA, proposed as a common mediator of plant stress responses (Quarrie and Jones, 1977), is associated with cold (Chen et al., 1983), salt (LaRosa et al., 1985), drought (Innes et al., 1984), and heat tolerance (Hiron and Wright, 1973). These plant stresses all impose some degree of osmotic stress, and changes in gene expression associated with osmotic or dehydration stresses can also be induced by exogenous ABA (Skriver and Mundy, 1990).

Temperature stresses produce protein conformational changes resulting in denaturation, polypeptide chain unfolding, and coagulation. Levitt (1980) reviewed substantial evidence demonstrating a relationship between cellular heat tolerance and protein thermostability. A number of possible mechanisms were postulated to link these phenomena, such as increased cell solute concentrations, changes in protein hydrophobic bonding arrangements, and the possible role of disulfide linkages. Evidence for a relationship among freezing, drought, and heat stresses was also reviewed (Levitt, 1980); a number of factors such as dehydration and high sugar content were often correlated with increased tolerance. A family of proteins called dehydrins (Close et al., 1989) accumulate in a wide range of plant species in response to dehydration stresses and ABA treatment. Dehydrin-like proteins also accumulate in osmotically stressed cyanobacteria (Close and Lammers, 1993), indicating that they have been evolutionarily conserved. A novel property of some ABA-responsive proteins (Jacobsen and Shaw, 1989) and dehydrins (Close et al., 1989) is their heat stability, or their resistance to heat-induced coagulation. Some Hsps also show heat stability (Jinn et al., 1989), and heat-stable proteins exist in cells under nonstressed conditions. Minton et al. (1982) proposed that heat-stable proteins may nonspecifically confer heat tolerance to heat-sensitive proteins.

Our purpose in this work was to test the hypothesis that ABA-responsive, heat-stable proteins may function in a more specific fashion than uninduced heat-stable proteins to confer increased stability against heat-induced coagulation of proteins and subcellular fractions. Stabilizing and protecting sensitive proteins, subcellular structures, and membranes against the denaturing stresses imposed by dehydration, heat, or high salt concentrations would implicate a possible role for ABA- and dehydration-responsive proteins in conferring increased tolerance to the stresses imposed by heat, drought, freezing, and salinity. The protective role of the cellular osmolyte Suc, alone and in combination with the ABA-responsive, heat-stable proteins, was also investigated, since cellular solutes have been correlated with increased stress-tolerance mechanisms.

In these investigations a bromegrass cell-suspension culture was used as a model to study the effect of ABA and Suc on cellular heat tolerance and to compare the heat stability of subcellular fractions isolated from control and ABA-treated cells. These cells were also used to identify, isolate, and characterize heat-stable, ABA-responsive proteins and to test the effects of these proteins on conferring increased stability to thermal-sensitive subcellular structures and proteins in vitro.

MATERIALS AND METHODS

Cell Cultures and Viability Assays

Nonembryogenic bromegrass (*Bromus inermis* Leyss cv Manchar) suspension cultures derived from mesocotyl tissue were subcultured weekly in Erickson's medium (Ishikawa et al., 1990) at 25°C. Cells were cultured for specific periods in 75 μM commercially available (Calbiochem) racemic (±)-ABA, which was dissolved initially in DMSO prior to filter sterilization (0.22-μm filters, Nalgene). A final DMSO concentration of 0.05% in the culture medium did not affect growth or stress tolerance. After 7 d, heat tolerance was determined on cultures that were washed with 250 mL of sterile distilled H_2O to remove Suc and other growth factors. Heat tolerance was determined by incubating 0.40 g fresh weight of cells at 42.5°C for up to 160 min, with cells collected at 20-min intervals for viability assessment. Viability was assessed by regrowth, TTC reduction, and fluorescein diacetate staining (Chen and Gusta, 1983). In regrowth assays, 0.15 to 0.20 g fresh weight of stressed cells were transferred to fresh Erickson's medium and increases in fresh weight were determined after 10 d at 25°C.

Protein Isolation

After 7 d, control and ABA-treated bromegrass cells were harvested by filtration on Miracloth and washed with distilled H_2O (20 mL/g fresh weight). Three grams of washed cells were resuspended in 6 mL/g fresh weight of 62.5 mM Tris, pH 6.8, containing 6 μL of 1.0 M PMSF and were disrupted in a motorized ground glass homogenizer until cell breakage approached 100%, as determined by phase-contrast microscopy. To facilitate breakage, the cells were rapidly frozen and thawed in liquid nitrogen prior to homogenization. The 13,000*g* (20 min) supernatant fraction was collected and assayed for protein by a modified Lowry method (Peterson, 1977) following TCA precipitation.

Supernatant (13,000*g*) fractions (1.5 mL at 1.5–2.5 mg/mL) from treated and control cells were held at 90°C for 30 min. Following heat treatment, coagulated proteins were sedimented at 10,000*g* for 10 min. Proteins remaining in the supernatant were either stored in liquid nitrogen for electrophoretic analyses or subjected to $(NH_4)_2SO_4$ precipitation for further purification. $(NH_4)_2SO_4$ was added slowly to 70% saturation to the heat-stable protein supernatant with stirring. The precipitate was collected by centrifugation at 13,000*g* for 10 min. The sediment was resuspended in a small volume of 62 mM Tris, pH 6.8, and desalted either by Sephadex G-50

chromatography or by dialysis overnight at 4°C against 62.5 mM Tris, pH 6.8. These heat-stable protein fractions were used in heat-induced coagulation protection assays.

Sample Preparation, Electrophoresis, and Gel Analyses

Protein solvation, IEF, and two-dimensional electrophoresis were carried out as described by O'Farrell (1975) with the following modifications. Nuclease solution (0.1 mg/mL DNase I, 50 μg/mL RNase A, 0.05 M Tris [pH 7.0]), 5 mM $MgCl_2$) was added to each sample (1 μL/10 mg protein) and incubated for 10 min on ice, followed by a 5-min incubation at 37°C. Nuclease-treated samples were frozen in liquid nitrogen, freeze-dried, and dissolved in sample dilution buffer containing 9.5 M urea, 2% LKB ampholines (1.0% pH range 5–8 and 1.0% pH 3.5–10), 5% (w/v) mercaptoethanol, and 8% (w/v) Nonidet P-40 at a final protein concentration of 5 μg/μL. Prior to IEF, samples were preheated to 37°C for 10 min to assist protein solvation. Second-dimension SDS gels (10% polyacrylamide) were silver stained (Merrill and Goldman, 1984).

The apparent molecular mass of unknown proteins was estimated using a Pharmacia low mol wt calibration kit containing six standard proteins ranging from 94 to 14 kD. The apparent pI of unknown proteins was estimated as described by O'Farrell (1975). A minimum of four two-dimensional gels were analyzed for each treatment. Only consistent observations of well-defined polypeptides are reported. Spots were identified as numbered polypeptides, with the number placed adjacent to the spot. Matching spots having the same pI and molecular mass values were assigned the same numbers.

Protein Sequencing and Western Blotting

Proteins from SDS-PAGE gels were electroblotted for sequencing and western blotting as described by Aebersold et al. (1986). Proteins were transferred to PVDF membranes (Immobilon, pore size 0.45 μm) using a Biotrans semidry electrophoretic transfer unit (Gelman Sciences, Ann Arbor, MI) and the manufacturer's protocol. Protein sequencing was performed using an Applied Biosystems, Inc. model 471A sequencer equipped with a model MG5 microgradient pump and a Blott cartridge for PVDF-type membranes. Data were acquired and analyzed using an Applied Biosystems Inc. model 610A data system. Protein sequence data bases were compared using the TFASTA program.

In western blot analyses, a Protoblot Western Blot AP System kit purchased from Promega was used according to manufacturer's specifications. Two polyclonal antibodies, both of which recognize late embryogenic abundant D-11 family proteins, one produced against a synthetic dehydrin peptide (courtesy of T.J. Close) and the other against a cold-induced protein from Norstar winter wheat (courtesy of F. Sarhan), were used to probe western blots of bromegrass heat-stable protein fractions. Preimmune serum was also assessed for nonspecific binding to bromegrass protein isolated from heat-stable fractions by the same methods.

Heat-Induced Coagulation and Protection Assays

A Beckman DU-65 spectrophotometer equipped with a kinetics "soft-Pac" module was used to monitor protein denaturation and coagulation. The temperature of the cuvette was controlled by a circulating temperature bath connected to a Peltier temperature-controlling accessory in the spectrophotometer. The sample cuvette (1 mL) and buffer (62 mM Tris, pH 6.8) were pre-equilibrated to the desired temperature (50°C) prior to addition of total cell protein samples (2.0 mg/mL). Spectrophotometric measurements were initiated when the samples reached the desired temperature, which was monitored continuously. The increase in apparent A_{600} over time (20 min) was used as a measure of turbidity (Minton et al., 1982). In protection assays up to 400 μg of fractionated heat-stable protein isolated from control and ABA-treated cells were added to the control 13,000*g* supernatant fractions in the presence and absence of 8% Suc.

RESULTS

Effect of Exogenous ABA and Suc Addition on the Heat Tolerance of Bromegrass Suspension-Culture Cells

Exogenous ABA (75 μM ABA for 7 d at 25°C) increased the tolerance of a bromegrass cell culture to a heat stress of 42.5°C for 120 min, relative to the untreated control. ABA-treated cells retained 87% viability as determined by regrowth, whereas the viability in the control cells decreased to less than 1% (Table I). The fluorescein diacetate viability assay indicated that ABA-treated cells retained 93% viability, whereas viability in the control cells decreased to 25%. Addition of Suc (3%) 30 min prior to the heat stress to bromegrass cells cultured for 6 d increased the survival of nontreated cells from 25 to 72% when stressed at 42°C and from 2 to 45% when stressed at 45°C. Suc addition increased survival of ABA-treated cells above that of the ABA-treated cells without Suc.

A time-course experiment was also performed to determine the effect of culture time with and without ABA on the tolerance of cells subjected to a heat treatment of 45°C for 2 h. The survival of nontreated cells initially declined, showing minimum survival after 6.5 d, but increased after 10.5 to 14 d of culture to a maximum survival rate of 35% (Fig. 1). The percentage of survival (TTC tests) of heat-stressed, ABA-treated cells steadily increased with culture time, reaching maximum levels after 10.5 to 11 d of culture.

Table I. *The effect of 75 μM ABA for 7 d at 25°C and Suc added just prior to the heat stress on the heat tolerance of bromegrass cells*

Cells (0.4 g fresh weight) were heat stressed at 42.5°C for 2 h in distilled H_2O prior to viability assays.

Viability Assay	Percent Viability			
	Control	Control + Suc	ABA	ABA + Suc
Regrowth	<1		87	
Fluorescein diacetate	25 (2)[a]	72 (45)	93 (93)	100 (100)

[a] Numbers in parentheses, After heat treatment at 45° for 2 h.

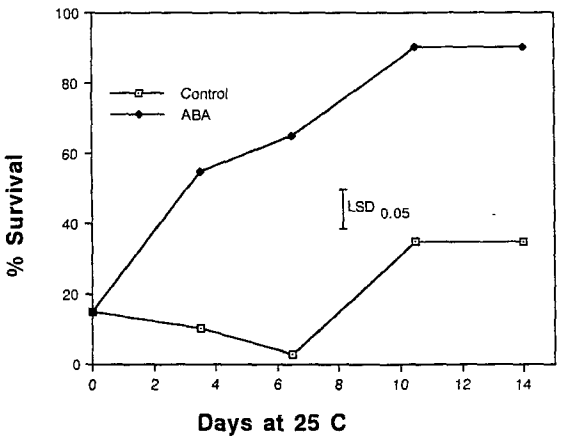

Figure 1. Effect of culture period, in the presence and absence of 75 μM ABA, on the heat tolerance of bromegrass suspension cultures. Cultures were initiated with 1 g fresh weight and incubated in the dark at 25°C for the indicated times prior to heat-stress treatments. Cell viability was determined by TTC reduction following incubation at 45°C for 2 h. LSDs, P = 0.05, were calculated from pooled SDs (n = 10).

Identification and Characterization of Proteins Responsive to Exogenous ABA Treatment

Electrophoretic analyses were performed to investigate protein changes occurring during ABA treatment and the induction of heat tolerance. Comparative two-dimensional analyses of total cell protein fractions detected 43 ABA-responsive polypeptides that were either newly synthesized or increased over control levels after 7 d of ABA treatment (Fig. 2; Table II). The most abundant RAB polypeptides had apparent pI values between 5.5 and 7.2 and molecular masses between 20 and 45 kD (spots 6–24, Fig. 2B). The samples analyzed in Figure 2, A and B, were subjected to 90°C for 30 min prior to electrophoresis, resulting in the identification of a unique set of 25 ABA-responsive, heat-stable polypeptides (Fig. 2D, cf. solid arrowheads). These polypeptides were not detected in the control heat-stable fraction of control samples (Fig. 2C). Some of the RAB polypeptides (Fig. 2D, spots 35–42) were not detected in ABA-treated total cell fractions (Fig. 2B), presumably because of low abundance. Conversely, some of the RAB proteins were coagulated by the heat treatment (spots 11–14, 18, 20–23, 26–30, and 100; Table II; Fig. 2). RAB proteins that coagulated after boiling were not characterized in these studies. Although the majority of the RAB heat-stable proteins identified were boiling stable as previously described in other analyses (Jacobsen and Shaw, 1989; Lin et al., 1990), this treatment often generated artifacts in both the isoelectric (charge heterogeneity) and molecular mass (extensive smearing) dimensions in two-dimensional gel analyses. In some cases, abundant protein species were detected that were not identified in nonheated fractions, and the two-dimensional profiles were not reproducible. More reproducible two-dimensional patterns were obtained by heating at 90°C for 30 min. Two-dimensional separations of control heat-stable fractions, which represented 2% of the total control protein fraction, identified 14 (Fig. 2C, spots 25,

Table II. *Analyses of ABA-responsive spots identified in total cell protein fractions (Fig. 2)*

Spots were characterized by their molecular mass (kD), apparent pI, and heat stability. A polypeptide spot was defined as heat stable (+) if no coagulation occurred after incubation at 90°C for 30 min. Polypeptides that precipitated were designated −.

Spot No.	kD	pI	Heat Stability
1	67	7.24, 7.00[a]	+
2	52	6.66, 6.48	+
3	51	6.65, 6.58	+
4	42	7.20	?
5	43	6.93	?
6	43	6.73	+
7	44	6.53	+
8	43	6.20	+
8.1	43	6.10	+
9	45	6.17	+
10↑	45	6.10	+
11↑	39	7.20	−
12	37	6.10	−
13	36	6.15	−
14	29	7.25	−
15	27	6.13	+[c]
16	21	6.66	+
17	20	6.28	+
18	26	6.04	−
19↑	27	5.68	+
20	36	7.25	−
21↑	24	5.78	−
22	23	5.78	−
23↑	24	5.50	−
24↑	23	5.80	+
25↑	17	5.68	+[c]
26	19	5.20	−
27↑	32	5.25	−
28↑	31	5.16	−
29↑	63	4.90	−
30	36	6.15	−
31↑	32	4.45	+
32	26	5.95	+[c]
33↑	27	6.05	+[c]
35	50	5.03	+[d]
36	52	4.91	+[d]
37	21	4.20	+[d]
38	20	4.20	+[d]
39	90	4.98	+[d]
40	112	4.50	+[d]
41	22	6.14	+[d]
42	20	6.15	+[d]
100↑	37	6.60	−

[a] Spots with satellites are presented with the more acidic isoelectric species on the right. [b] Spots with increased intensity in ABA-treated compared to control protein fractions are followed by an ↑. All other spots were not detected by silver staining control protein fractions. [c] Heat-stable polypeptides that do not increase in intensity in heat-stable fractions. [d] ABA-responsive spots not detected in total cell protein fractions, only in heat-stable fractions.

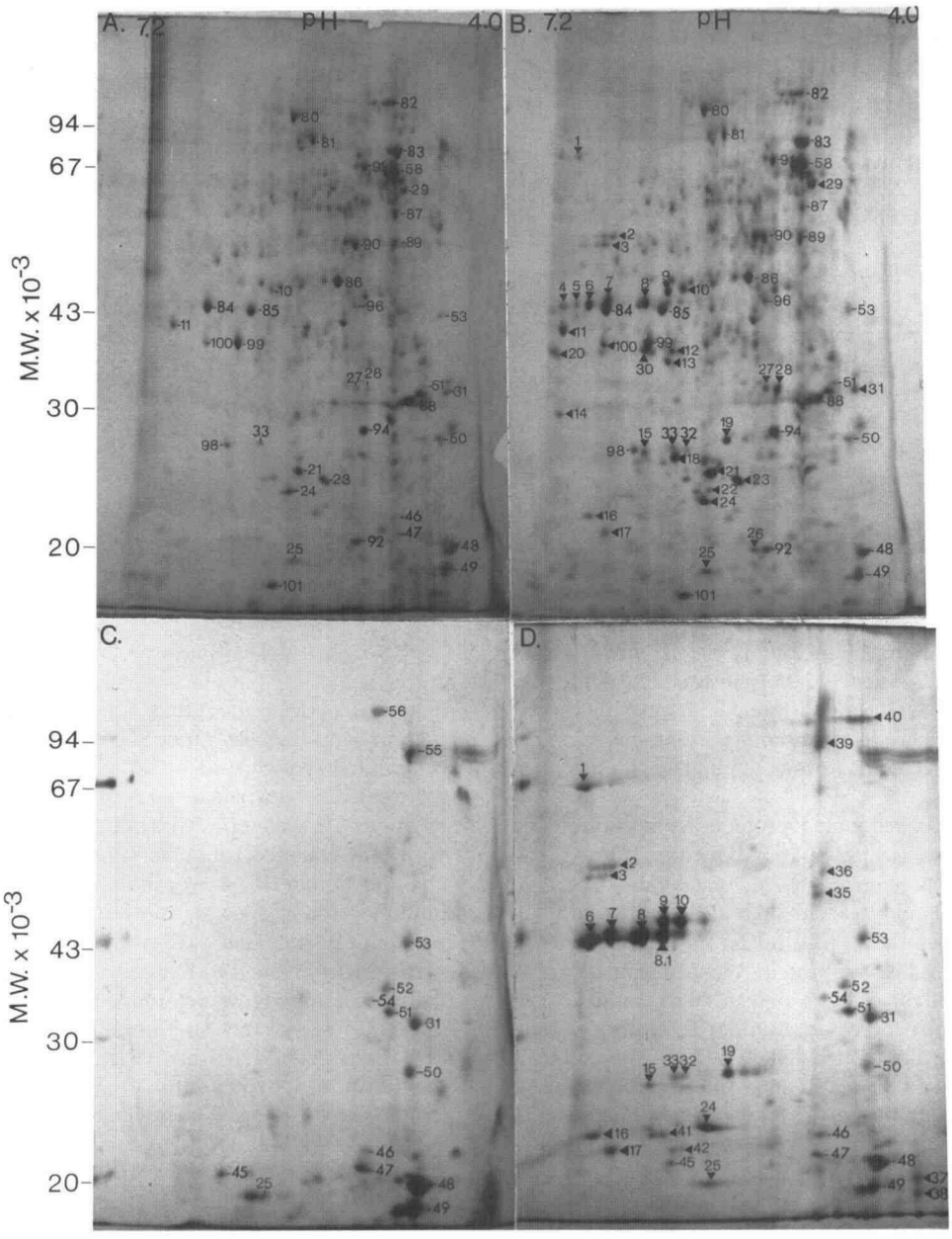

Figure 2. Silver-stained, two-dimensional gels of total cell and heat-stable protein fractions from control and ABA-treated cultures. pH gradients were from pH 7.2 on the left to 4.0 on the right of each panel. IEF gels A and B were loaded with 100 μg of protein and C and D were loaded with 50 μg of protein. A, Control total cell protein (100 μg). B, Total cell protein from ABA-treated cells (100 μg). C, Control cell heat-stable proteins that did not coagulate and precipitate after incubation at 90°C for 30 min. D, ABA-responsive, heat-stable proteins. Polypeptide spots responsive to ABA treatment are designated by solid triangles. The characteristics of 43 ABA-responsive polypeptide spots (B and D) are summarized in Table II. Several major spots (48, 49, 58, 80–92, 94, 96, 99, and 101) were common to both ABA-treated and nontreated fractions (A and B). Five of the seven most intense 42- to 45-kD heat-stable ABA-responsive polypeptide spots (D) occupy overlapping coordinates with ABA-responsive spots 6 to 10 (B) in total cell fractions. In heat-stable fractions spot 8 overlapped with an isoelectric satellite species and a new spot (8.1) between spots 85 and 9 was detected.

31, and 45–56) major heat-stable polypeptides. Heat-stable proteins that accumulated during ABA treatment (Fig. 2D) represented 4% of the total cell protein fraction. Heat-stable proteins common to both ABA and control fractions were spots 25, 31, and 45 to 54. In contrast to the 42- to 45-kD heat-stable RAB polypeptides, another subset (spots 2, 3, 15, 24, 25, 32, and 33, Fig. 2, B and D) was not enriched following in vitro heat treatments, and two of these were evident in the control fraction (Fig. 2A, spots 24 and 33).

Heat-stable, RAB proteins separated by two-dimensional SDS-PAGE (Fig. 2D) were electroblotted onto PVDF membranes, and selected polypeptides (spots 1–3, 6–10, 19, and 24) were subjected to N-terminal sequencing by automated Edman degradation. Sequences were obtained only for polypeptides 6 to 10, 19, and 24 (Table III). Spots 6 to 10 all have similar molecular masses but varying pI values (Table II; Fig. 2D) and similar N-terminal sequences (Table III). Eleven of the first 12 residues had the same sequence, with some variability in the identity of the first-cycle residues (Table III). Spot 19 (27.5 kD) and spot 24 (23 kD) had N-terminal sequences identical with polypeptides 6 to 10 except for Glu residues at positions 5 and 8. The best score using comparisons of the N-terminal sequence in Table III with those in the GenBank/EMBL sequence data base was 42.9% identity in a 14-amino acid overlap with mouse microtubule-associated protein 1B. No significant homology in these N-terminal sequences was found for any other previously characterized plant protein.

In Figure 3, heat-fractionated proteins from control and ABA-treated cultures were separated by one-dimensional SDS-PAGE and characterized by silver staining (Fig. 3A) prior to western blot analyses with an anti-dehydrin antibody (courtesy of T. Close) (Fig. 3B) and an antibody produced against a cold-induced wheat protein, Wcs120 (courtesy of F. Sarhan, Fig. 3C). Silver staining detected four major bands either unique to or of increased abundance in protein fractions isolated from ABA-treated cells (Fig. 3A, lanes 3 and 4). The most intense band, in the 42- to 43-kD region, formed a doublet (Fig. 3A). Duplicate gels were electroblotted onto PVDF membranes for amino-terminal sequencing and western blot analyses. Protein sequencing of the 42- to 43-kD band resulted in sequences identical with those obtained for spots 6 to 10 (Fig. 2D; Table III) with one exception. In spots

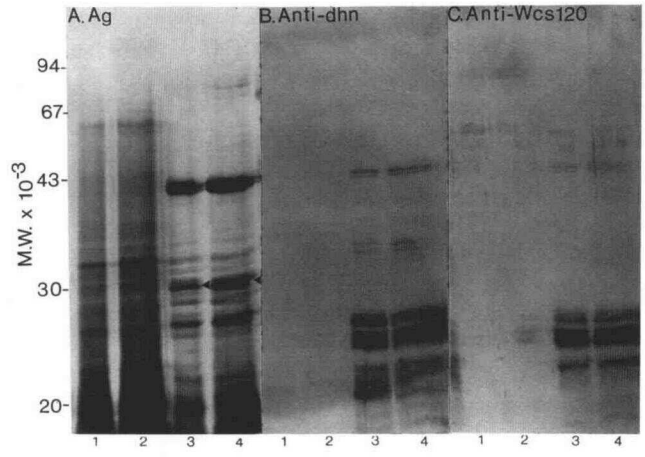

Figure 3. SDS-PAGE of heat-stable proteins isolated from control (lane 1, 25 μg; lane 2, 50 μg) and 75-μM ABA-treated (lane 3, 25 μg; lane 4, 50 μg) bromegrass cells. The proteins in A were silver (Ag) stained. In B and C, the proteins were transferred to PVDF membranes and probed with anti-dehydrin (B) and Wcs120 (C) antibodies.

6 to 8.1, cycle 1 identified Glu instead of Lys. A 30-kD protein band (Fig. 3A, lanes 3 and 4, cf. arrowhead) gave an N-terminal sequence identical with the 43-kD band except for residue 5, where Asp was replaced by Glu, and residue 8, where Gln was replaced by Glu. This sequence is identical with that obtained for spots 19 and 24 in two-dimensional analyses (Table III). Lower molecular mass bands contained multiple protein species, and reliable sequence information could not be obtained.

A protein in the 43-kD band cross-reacted with the anti-dehydrin antibody as determined by western blot analyses (Fig. 3B, lanes 3 and 4). Two additional bands (34 and 36 kD) and six bands between 20 and 28 kD also cross-reacted with the anti-dehydrin antibody (Fig. 3B, lanes 3 and 4). Four bands (Fig. 3C, lanes 3 and 4) between 22 and 27 kD bound the anti-Wcs120 antibody. All of the proteins showing antigenicity with the anti-dehydrin and anti-Wcs120 proteins were ABA induced (Fig. 3, B and C, lanes 3 and 4). No significant reaction occurred in control heat-stable protein

Table III. *N-terminal amino acid sequence of eight bromegrass ABA-responsive heat-stable polypeptides identified in Figure 2B*

Spot No.	Molecular Mass	pI	N-Terminal Amino Acid Sequence														
			1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	kD																
6	43	6.73	E	T	T	L	D	D	A	Q	V	A	P	G	L	E	E
7	44	6.53	E	T	T	L	D	D	A	Q	V	A	P	G	K	E	E
8	43	6.20		T	T	L	D	D	A	Q	V	A	P	G			
8.1	43	6.10		T	T	L	D	D	A	Q	V	A	P	G	K		
9	45	6.10	K	T	T	L	D	D	A	Q	V	A	P	G		E	
10	45	6.10	K	T	T	L	D	D	A	Q	V	A	P	G		E	
19	27.5	5.68	K	T	T	L	E	D	A	E	V	A	P	G	K	E	E
24	23	5.80	K	T	T	L	E	D	A	E	V	A	P	G	K	E	E

[a] Cycle number.

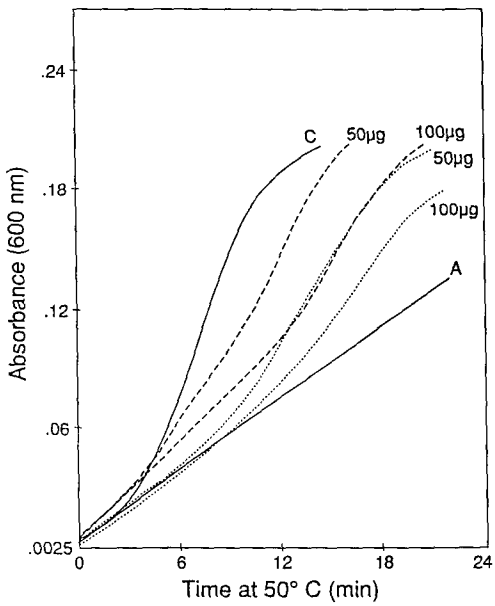

Figure 4. A_{600} as a measure of turbidity during incubation of 13,000*g* supernatant fractions from control (curve C) and ABA-treated (curve A) bromegrass cell homogenates (1.0 mg/mL in 62.5 mM Tris, pH 6.8) at 50°C. Fifty and 100 µg of heat-stable protein fractions from control (----) and ABA-treated (· · · · · ·) cells were mixed with 1 mg of the control supernatant fraction in a total volume of 1 mL.

fractions (Fig. 3, B and C, lanes 1 and 2) with either immune or preimmune serum, nor did preimmune serum show detectable reaction with ABA-responsive, heat-stable proteins (data not shown). It is interesting that a major heat-stable RAB 30-kD protein (Fig. 3A, lanes 3 and 4, cf. arrow), with an N-terminal sequence similar to the 43-kD protein, did not cross-react with the anti-dehydrin antibody (Fig. 3B, lanes 3 and 4), whereas a number of lower molecular mass proteins did.

Effect of ABA Treatments on the in Vitro Stability of Cell-Free Fractions to Heat- and pH-Induced Coagulation

Eighty-seven percent of ABA-treated bromegrass suspension cells survived a 2-h incubation at 42.5°C compared with 1% for control cells (Table I). Cell-free fractions (13,000*g* supernatants) isolated from both control and ABA-treated cells were subjected to thermal protein denaturation analyses in vitro at 50°C (Fig. 4) to investigate possible differences in protein stability. In the control samples, coagulation initiated after 3 min and was complete by 15 min (Fig. 4, curve C), whereas there was a gradual increase in A_{600} with time in the extract from ABA-treated cells (Fig. 4, curve A) but no evidence of coagulation after 20 min. Addition of 50 and 100 µg (5 and 10% of the total protein, respectively) of control heat-stable protein fractions (Fig. 2C) decreased the rate of coagulation of control fractions in a concentration-dependent manner (Fig. 4). However, addition of 50 µg (5%) of ABA-induced, heat-stable protein fractions was more effective at decreasing the rate of coagulation of control fractions than were 100 µg (10%) of control heat-stable protein (Fig. 4). Addition of 100 µg of ABA-induced, heat-stable proteins prevented coagulation for up to 12 min (Fig. 4), but addition of more than 100-µg amounts of heat-stable proteins showed no further increase in protection. Resistance to acidic conditions was also examined by titrating control fractions with 5 N HCl (Fig. 5). Control fractions began coagulation at $[H^+]$ of 0.50 M, with the majority of the proteins completely coagulated at 0.70 M. A portion of the ABA fraction coagulated at $[H^+]$ between 0.48 and 0.55 M; however, the majority of the ABA cell-free fraction remained resistant to $[H^+]$ greater than 0.90 M (Fig. 5).

Effect of RAB and Control Heat-Stable Proteins in the Presence and Absence of Suc on the Heat-Induced Coagulation of Control Total Cell Protein

The possibility that heat-stable proteins can confer increased heat stability to total cell protein fractions was tested. The heat-stable proteins from both the untreated controls and ABA-treated cells were precipitated with 70% $(NH_4)_2SO_4$ saturation, collected by centrifugation at 13,000*g*, and desalted by dialysis or gel filtration (Sephadex G-50). Silver-stained two-dimensional separations of the $(NH_4)_2SO_4$ precipitate from the RAB heat-stable fraction (not shown) identified spots 6 to 10, 19, and 24 as the major polypeptide constituents with minor amounts of spot 31 and 51 (Fig. 2D). Polypeptide spots 31 and 51 were common to both the control and RAB heat-stable proteins (Fig. 2C). Figure 6 shows the effect of adding equal amounts of heat-stable fractions from control and ABA-treated cells on the heat stability of 13,000*g* supernatant fractions isolated from control cells. Both heat-stable protein fractions conferred a protective effect against heat-induced coagulation of control 13,000*g* supernatant fractions; however, the ABA-induced, heat-stable fraction conferred a greater degree of protection than the control heat-stable fraction.

Figure 6 also demonstrates the effect of Suc added to a final concentration of 8% (w/v) on the heat stability of control

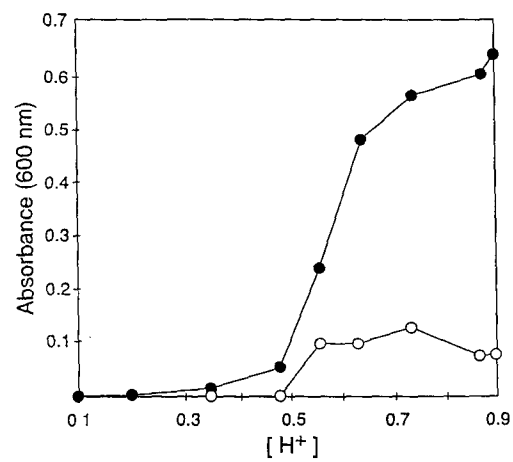

Figure 5. Increase in turbidity at 600 nm in cell fractions (2.0 mg/mL in 62.5 mM Tris, pH 6.6) isolated from control and ABA-treated bromegrass cultures during titration with 5 N HCl. The *x* axis denotes the concentration of H^+ in eq/L.

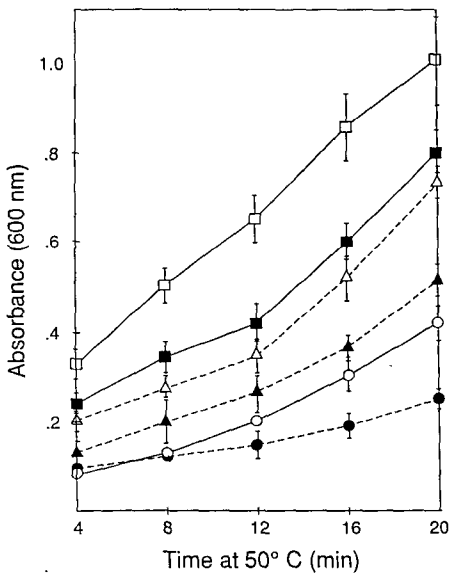

Figure 6. Control supernatant fractions (2 mg protein) were mixed in a total volume of 1 mL with equal amounts (400 μg) of heat-stable protein partially purified from control and ABA-treated cells. Effect of partially purified heat-stable proteins and Suc on heat-induced coagulation of 13,000g supernatants from control bromegrass cell homogenates (2 mg/mL). □, Control; ■, 8% Suc; △, heat-stable control protein; ▲, heat-stable control protein and 8% Suc; ○, heat-stable ABA-responsive protein; ●, heat-stable ABA-responsive protein plus 8% Suc. Values presented represent means ± SE (n = 3).

fractions. Suc alone showed significant protection against heat-induced denaturation, but more importantly, Suc enhanced the protective effects of both sets of heat-stable proteins. The protective effect was most pronounced in the RAB heat-stable fraction plus Suc treatment. The control fraction was most protected by the addition of ABA heat-stable protein and 8% Suc. After a 20-min incubation at 50°C, the A of the control fraction increased to 1.0 versus 0.25 for the control fraction plus ABA-induced heat-stable protein and 8% Suc.

DISCUSSION

Our results indicate that ABA treatment at 25°C is capable of increasing the heat tolerance of bromegrass suspension-culture cells without prior exposure to elevated temperatures or heat-shock conditions. ABA has also been reported to increase the freezing tolerance of these cells at nonacclimating temperature (Chen and Gusta, 1983). These results suggest that ABA simultaneously regulates the development of tolerance to more than one stress in this bromegrass suspension culture, in the absence of a prior hardening period. Addition of Suc prior to a heat stress increased the heat tolerance of both control and ABA-treated cells (Table I). In freezing studies, Suc addition to ABA-treated cells 30 min prior to freezing and before full expression of ABA-induced freezing tolerance enhanced freezing tolerance but had little or no effect on control cells (Reaney, 1989; Ishikawa et al., 1990). Tanino et al. (1990) demonstrated, using the same line of bromegrass cells as in our experiments, that ABA enhanced the uptake of exogenous Suc. Solute concentration increases during stress acclimation have led to the hypothesis that these compounds protect and stabilize cellular structures against the physical effects of the stress and the denaturing forces of water removal (Yancey et al., 1982; Steponkus, 1984). Sugar hydroxyl groups may functionally replace water bound to membranes and proteins to confer structural stability (Crowe et al., 1984).

Two-dimensional gel analysis of cellular proteins isolated from ABA-treated cells resolved 43 RAB proteins and identified a set of 26 heat-stable RAB proteins (Table II; Fig. 2D). Conversely, 15 RAB proteins were characterized that did not show heat stability but coagulated following a heat treatment. A number of RAB and stress proteins resistant to heat-induced coagulation (boiling stable) have been identified in barley aleurone cells (Jacobsen and Shaw, 1989), in dehydrated barley and corn seedlings (Close et al., 1989), and in the in vitro translation products of low-temperature-responsive mRNAs isolated from *Arabidopsis* and wheat (Lin et al., 1990). At present, however, the function of these proteins has not been elucidated.

The 42- to 45-kD polypeptides (spots 6–10 and 19 and 24) formed the most abundant set of heat-stable polypeptides in the cell fractions isolated from ABA-treated cells (Fig. 2D). Protein sequencing of these RAB polypeptides (spots 6–10, 19 and 24) showed almost identical N-terminal residues in the first 14 positions (Table III), implying that these polypeptides are closely related and may be encoded by the same gene or gene family. Computer data base searches did not reveal extensive homology with other plant proteins, but some similarity was found with mouse microtubule-associated proteins. It is of interest that microtubule-associated proteins are required in the cold stabilization of mammalian microtubules reassembled in DMSO or glycerol-containing solutions (Pajot-Augy, 1993). Polypeptides 6 to 10 have similar molecular masses but varying pI values, suggesting the possibility of posttranslational modification such as phosphorylation, which has been observed for other RAB proteins (Goday et al., 1988). Spots 19 and 24 may be proteolytic fragments of spots 6 to 10 or may result from posttranslational processing, since they share N-terminal sequences with spots 6 to 10, inclusively. One-dimensional separation (Fig. 3) of RAB heat-stable proteins identified a 43-kD protein, with an N-terminal sequence equivalent to spots 6 to 10. The 43-kD, a 36- and 34-kD, and six low molecular mass bands (20–28 kD) cross-reacted with an anti-dehydrin antibody, indicating that these polypeptides are immunologically related to other plant dehydration-responsive proteins (Close and Lammers, 1993). A 30-kD band with an N-terminal sequence showing identity with spot 24 and similar to the 43-kD band did not show antigenicity to anti-dehydrin. It is possible that the RAB heat-stable 30-kD peptide is an N-terminal proteolytic fragment of the 43-kD protein, whereas some of the lower molecular mass polypeptides binding the anti-dehydrin antibody are C-terminal fragments. These observations are consistent with the consensus dehydrin sequences occurring at the 3′ end of dehydration-responsive proteins (Close et al., 1989). Immunoblot analyses of two-dimensional separations with anti-dehydrin antibodies showed cross-reaction with polypeptides having pI values

between 5.6 and 7.2 and molecular masses of 18 to 28, 34 to 36, 43, and 67 kD (data not shown). Cross-reaction with polypeptides in the region of spots 6 to 10 (Fig. 2D) was weak relative to the amount of protein staining in this region. An antibody recognizing a cold-responsive protein from Norstar winter wheat (Houde et al., 1992) also cross-reacted with four low molecular mass (22–26 kD) heat-stable, ABA-responsive protein bands. Some of these proteins were also recognized by the anti-dehydrin antibodies, indicating that bromegrass ABA-responsive proteins share sequence homologies with both dehydration and cold-responsive proteins.

A set of polypeptides (spots 2, 3, 15, 24, 32, and 33, Fig. 2D) isolated from ABA-treated cells did not increase in intensity relative to the 42- to 45-kD set (spots 6–10) following a 90°C heat treatment. For example, spots 2 and 3, which did not sequence (Fig. 2, B and D), were present but not enriched in the heat-stable protein fractions. It is highly unlikely that more than one RAB polypeptide with the same isoelectric satellite species, pI, and molecular mass (Table II; Fig. 2, B and D) occupies the same spot. One possible explanation is that polypeptides 2 and 3 are not in themselves heat stable but acquire thermostability in total cell fractions isolated from ABA-treated cells. Experiments designed to test the stability of subcellular fractions (13,000*g* supernatants) isolated from ABA-treated cells showed dramatic increases in resistance to both heat (Fig. 4) and pH (Fig. 5) denaturation in vitro compared with control subcellular fractions. The mechanism by which increased stability to heat- and pH-induced denaturation and coagulation is conferred to cellular fractions isolated from ABA-treated cells is not understood.

However, a possible role of the heat-stable RAB proteins could be to confer conformational or heat stability to sensitive proteins and membranes. To test this possibility a protection assay was developed using a control subcellular fraction (13,000*g* supernatant). Stability to heat-induced denaturation and coagulation was monitored by measuring turbidity at 600 nm as a function of time. Control and heat-stable RAB polypeptides (spots 6–10, 19, and 24, Fig. 2D) were isolated and added to control cell fractions. The heat-stable RAB polypeptides conferred a greater protective effect against heat-induced coagulation than control heat-stable proteins (Figs. 4 and 6). The finding that heat-stable proteins confer stability to control cell fractions is consistent with the data of Minton et al. (1982). Since results of this type appear to be nonspecific, Minton et al. (1982) offered an explanation on the basis of the excluded volume theory. This theory predicts that, as the concentration of protein increases in a fixed volume, the equilibrium will be shifted toward the self-association of each protein species, rendering each protein species less susceptible to denaturation.

Our results show that a set of heat-stable RAB proteins (spots 6–10, 19, and 24, Fig. 2D; Table II) increases the thermostability of control cell protein over that conferred by an equal amount of control heat-stable proteins or what would be accounted for by the excluded volume hypothesis. In fact, 100 μg (10%) of control heat-stable proteins were no more effective than 50 μg of heat-stable, ABA-responsive protein (Fig. 4). These observations suggest that the heat-stable RAB proteins may play a unique or specific role in conferring increased stability to heat-induced coagulation of proteins and membranes.

Jinn et al. (1989) reported that $(NH_4)_2SO_4$-fractionated, low mol wt Hsps that were heat stable conferred in vitro heat tolerance to soluble thermosensitive proteins. Similarly, Hincha et al. (1990) demonstrated that a boiling-stable protein fraction, isolated from cold-acclimated cabbage, conferred stability during a freeze-thaw cycle against membrane rupture of nonhardy spinach thylakoid membranes. Recently, a cold-regulated, boiling-stable polypeptide identified in *Arabidopsis thaliana* cryoprotected lactate dehydrogenase in an in vitro assay system (Lin and Thomashow, 1992). However, there are no reports of the effects of these proteins in combination with other protein protectants. We found that Suc dramatically increased the freezing tolerance of bromegrass cells 4 d after ABA treatment, whereas it had little or no effect on control cells (Ishikawa et al., 1990). Thus, Suc could only interact in a cryoprotective fashion in bromegrass cells after induction and expression of ABA-responsive factors, which includes the RAB proteins.

Suc (8%) increased the effectiveness of heat-stable proteins in conferring thermostability to heat-induced coagulation in vitro. Suc, in combination with RAB heat-stable proteins, was the most effective in conferring heat stability (Fig. 6). These observations imply that Suc and selected RAB proteins may function in concert to protect sensitive proteins and membranes against heat-induced denaturation. The possibility that solutes such as Suc and defined sets of RAB proteins complement each other in vitro to counteract membrane and protein-denaturing stresses such as heat warrants further investigation. For example, expression of three desiccation-responsive proteins from the desiccation-tolerant plant *Craterostigma plantagineum* in transgenic tobacco was not sufficient in itself to increase the drought tolerance of tobacco (Iturriaga et al., 1992). However, Blackman et al. (1992) provided evidence that seed maturation proteins and sugars must both accumulate for the development of desiccation tolerance in soybean seeds.

On the basis of amino acid sequence consideration, Baker et al. (1988) proposed that RAB, heat-stable, dehydration-responsive late embryogenic-abundant proteins may have "solvation" properties and confer desiccation tolerance. Theoretically, the heat-stable, RAB proteins characterized here could undergo noncovalent protein-protein or protein-lipid domain interactions with other proteins, macromolecular structures (ribosomes), or membranes to prevent heat- or stress-induced inactivation, denaturation, or coagulation. It is proposed here that the role of these unique proteins is to function in combination with cell osmolytes, such as Suc, to prevent denaturation and coagulation of cellular proteins and membranes under stressful conditions. It remains to be experimentally tested whether heat-stable RAB proteins, some of which are dehydrin-like and osmolytes, function to prevent coagulation and denaturation by masking hydrophobic interactions, preventing disulfide bond formation, and/or stabilizing cellular structures by volume exclusion.

ACKNOWLEDGMENTS

The technical assistance of Rosanne Woloschuk is gratefully acknowledged. We thank Don Tenaschuk for assistance with protein sequencing, Dr. S. Hemmingsen for searching sequence data bases at the National Research Council (Plant Biotechnology Institute,

University of Saskatchewan), and Dr. S.K. Bhadula (Biochemistry Department, University of Saskatchewan) for repeating and confirming protein denaturation experiments. We are indebted to Dr. T. Close (University of California, Riverside) for supplying us with antidehydrin antibodies and to Dr. F. Sarhan (Université du Québec a Montrèal) for supplying Wcs120 antibodies.

Received September 9, 1993; accepted January 17, 1994.
Copyright Clearance Center: 0032-0889/94/105/0181/10.

LITERATURE CITED

Aebersold RH, Teplow DB, Hood LE, Kent SBH (1986) Electroblotting onto activated glass: high efficiency preparation of proteins from analytical sodium dodecyl sulfate polyacrylamide gels for direct sequence analysis. J Biol Chem **261:** 4229–4238

Baker J, Steele C, Dure L III (1988) Sequence and characterization of 6 *Lea* proteins and their genes from cotton. Plant Mol Biol **11:** 277–291

Beckmann RD, Mizzen LA, Welch WJ (1990) Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science **248:** 851–855

Blackman SA, Obenderf RL, Leopold AC (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiol **100:** 225–230

Bonham-Smith PC, Kapoor M, Bewley JD (1987) Establishment of thermotolerance in maize by exposure to stresses other than a heat shock does not require heat shock protein synthesis. Plant Physiol **85:** 575–580

Bonham-Smith PC, Kapoor M, Bewley JD (1988) Exogenous application of abscisic acid or triadimefon affects the recovery of *Zea mays* seedlings from heat shock. Physiol Plant **73:** 27–30

Bray EA (1991) Wild-type levels of abscisic acid are not required for heat shock protein accumulation in tomato. Plant Physiol **97:** 817–820

Chen H-H, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol **71:** 362–365

Chen THH, Gusta LV (1983) Abscisic acid-induced freezing resistance in cultured plant cells. Plant Physiol **73:** 71–75

Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. Plant Mol Biol **13:** 95–108

Close TJ, Lammers PJ (1993) An osmotic stress protein of Cyanobacteria is immunologically related to plant dehydrins. Plant Physiol **101:** 773–779

Craig EA, Gambill BD, Nelson RJ (1993) Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol Rev **57:** 402–414

Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science **233:** 701–703

Gao Y, Thomas JO, Chow RL, Lee GH, Cowan NJ (1992) A cytoplasmic chaperonin that catalyzes β-actin folding. Cell **69:** 1043–1050

Goday A, Sanchez-Martinez D, Gomez J, Puigdomenech P, Pages M (1988) Gene expression in developing *Zea mays* embryos. Regulation by abscisic acid of a highly phosphorylated 23 to 25 kD group of proteins. Plant Physiol **88:** 564–569

Heikkila JJ, Papp JET, Schultz GA, Bewley JD (1984) Induction of heat shock protein messenger RNA in maize mesocotyls by water stress, abscisic acid, and wounding. Plant Physiol **76:** 270–274

Hincha DK, Heber U, Schmitt JM (1990) Proteins from frost-hardy leaves protect thylakoids against mechanical freeze-thaw damage *in vitro*. Planta **180:** 416–419

Hiron RWP, Wright STC (1973) The role of endogenous abscisic acid in the response of plants to stress. J Exp Bot **24:** 769–781

Houde M, Dhindsa RS, Sarhan F (1992) A molecular marker to select for freezing tolerance in Gramineae. Mol Gen Genet **234:** 43–48

Innes P, Blackwell RD, Quarrie SA (1984) Some effects of genetic variation in drought-induced abscisic acid accumulation on the yield and water use of spring wheat. J Agric Sci **102:** 341–351

Ishikawa M, Robertson AJ, Gusta LV (1990) Effect of temperature, light, nutrients and dehardening on abscisic acid induced cold hardiness in *Bromus inermis* Leyss suspension cultured cells. Plant Cell Physiol **31:** 51–59

Iturriaga G, Schneider K, Salamini F, Bartels D (1992) Expression of desiccation-related protein from the resurrection plant *Craterostigma plantagineum* in transgenic tobacco. Plant Mol Biol **20:** 555–558

Jacobsen JV, Shaw DC (1989) Heat-stable proteins and abscisic acid action in barley aleurone cells. Plant Physiol **91:** 1520–1526

Jinn TL, Yeh YC, Chen YM, Lin CY (1989) Stabilization of soluble proteins *in vitro* by heat shock protein-enriched ammonium sulfate fraction from soybean seedlings. Plant Cell Physiol **30:** 463–469

LaRosa PC, Handa AK, Hasegawa PM, Bressan RA (1985) Abscisic acid accelerates adaptation of cultured tobacco cells to salt. Plant Physiol **79:** 138–142

Levitt J (1980) High-temperature stress. *In* Responses of Plants to Environmental Stresses, Ed 2, Vol 1: Chilling, Freezing and High Temperature Stresses. Academic Press, New York, pp 347–445

Lin C, Guo WW, Everson E, Thomashow MF (1990) Cold acclimation in *Arabidopsis* and wheat: a response associated with expression of related genes encoding "boiling-stable" polypeptides. Plant Physiol **94:** 1078–1083

Lin C, Thomashow MF (1992) A cold-regulated *Arabidopsis* gene encodes a polypeptide having potent cryoprotective activity. Biochem Biophys Res Commun **183:** 1103–1108

Merrill CR, Goldman D (1984) Detection of polypeptides in two-dimensional gels using silver staining. *In* JE Celis, R Bravo, eds, Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications. Academic Press, New York, pp 93–109

Minton K, Karmin P, Hahn G, Minton A (1982) Nonspecific stabilization of stress-susceptible proteins by stress-resistant proteins: a model for the biological role of heat shock proteins. Proc Natl Acad Sci USA **79:** 7107–7111

Nagao RT, Kimpel JA, Key JL (1990) Molecular and cellular biology of the heat shock response. Adv Genet **28:** 235–266

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021

Pajot-Augy E (1993) Comparative effects of cryosolvents on tubulin association, thermal stability, and binding of microtubule-associated proteins. Cryobiology **30:** 286–298

Peterson GL (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal Biochem **83:** 346–356

Quarrie SA, Jones HG (1977) Effects of abscisic acid and water stress on development and morphology of wheat. J Exp Bot **28:** 192–203

Reaney MJT (1989) Factors affecting abscisic acid induced freezing resistance in cell suspension cultures. PhD thesis, University of Saskatchewan, Saskatoon, Canada

Sanchez Y, Lindquist SL (1990) Hsp 104 required for induced thermotolerance. Science **248:** 1112–1115

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512

Smith BJ, Yaffe MP (1991) Uncoupling thermotolerance from the induction of heat shock proteins Proc Natl Acad Sci USA **88:** 11091–11094

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Taguchi H, Yoshida M (1993) Chaperonin from *Thermus thermophilus* can protect several enzymes from irreversible heat denaturation by capturing denaturation intermediate. J Biol Chem **268:** 5371–5375

Tanino KK, Chen THH, Fuchigami LH, Wesier CJ (1990) Metabolic alterations associated with abscisic acid-induced frost hardiness in bromegrass suspension culture cells. Plant Cell Physiol **31:** 505–511

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol **42:** 579–620

Yaffe MB, Farr GW, Miklos D, Horinch AL, Sternlicht ML, Sternlicht H (1992) TCP1 complex is a molecular chaperone in tubulin biogenesis. Nature **358:** 245–248

Yancey PH, Clark ME, Hand, SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science **217:** 1214–1222